THE BIOTRANSFORMATION OF PHENOLIC POLLUTANTS USING POLYPHENOL OXIDASE

THESIS

Submitted in fulfilment of the
requirements for the degree of
DOCTOR OF PHILOSOPHY
of Rhodes University

by

Aileen Boshoff

Department of Biochemistry and Microbiology

April 2001

ABSTRACT

The potential of using mushroom polyphenol oxidase (EC 1.14.18.1) as a biocatalyst for the biotransformation of phenols to produce catechols in an aqueous medium was investigated. Polyphenol oxidase is characterised by two distinct reactions *i.e.*, the *ortho*-hydroxylation of phenols to catechols (cresolase activity) and the subsequent oxidation of catechols to *ortho*-quinones (catecholase activity). In order to facilitate the development of a process to produce catechols, the accumulation of catechol as a true intermediate product released in the reaction system needed to be investigated, as its release had been disputed due to the oxidation of catechols to *o*-quinones. Using LC-MS, catechol products were successfully identified as true intermediate products formed during biocatalytic reactions in water.

Manipulation of the biocatalytic system resulted in the formation of a highly efficient process to produce catechols. A system was developed in which 100% conversion of the phenolic substrate to catechol was achieved using borate buffer and ascorbate. This method was based on the formation of a borate-ascorbate complex for the reduction of *o*-quinone to catechol and was used for the synthesis of 4-chlorocatechol and 4-methoxycatechol, as these catechols are not available commercially. Diethyl ether was used to extract the catechols, and 98 - 100% recovery was obtained. Michaelis-Menton kinetics were used to obtain apparent K_M and V_{max} values with respect to chosen phenolic substrates during reactions carried out in an aqueous medium.

The use of the biocatalyst was extended in the development of a process for the synthesis of catechols as products by immobilisation of polyphenol oxidase, to facilitate its re-use and removal from the reaction system. Polyphenol oxidase was successfully immobilised on a range of synthetic membranes and incorporated within polyurethane foam and an organogel. A novel mathematical model was developed and used to determine reaction rate constants for the two activities of the enzyme, and application of the model facilitated the identification of membrane supports which favoured catechol accumulation. The use of conditions of low concentration of phenols, with polyphenol oxidase immobilised on hydrophilic membranes, were found to be optimal for catechol production. Nylon and polyurethane were the most effective immobilisation supports, resulting in 22.97% and 66.9% 4-methylcatechol accumulation respectively, from 1.25mM *p*-cresol.

Non-immobilised and nylon-immobilised polyphenol oxidase were used to synthesise catechol products from phenolic substrates present in two locally produced petrochemical industry effluents. Polyphenol oxidase was able to function in the chemical environment of the effluents, with 100% conversion of *p*-cresol and 75 - 100% conversion of phenol in the effluents diluted using the borate-ascorbate system. Immobilisation of polyphenol oxidase on nylon membranes increased the productivity of the enzyme, allowing for more economical application of the enzyme. Catechol products were successfully extracted from the effluents using diethyl ether. This study has demonstrated an effective process to produce and extract catechol products from both synthetic and industrial effluents using immobilised polyphenol oxidase.

TABLE OF CONTENTS

CONTENTS **PAGE**

CHAPTER 1

INTRODUCTION

CHAPTER 2

CHARACTERISATION AND KINETIC STUDY OF NON-IMMOBILISED POLYPHENOL OXIDASE-CATALYSED REACTION WITH PHENOLS

CHAPTER 3

IMMOBILISED POLYPHENOL OXIDASE-CATALYSED REACTION WITH PHENOLS

CHAPTER 4
APPLICATION OF POLYPHENOL OXIDASE IN THE TREATMENT OF PHENOLIC INDUSTRIAL EFFLUENTS

CHAPTER 5

LIST OF ABBREVIATIONS

ESI	electrospray ionization
GC-MS	gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
kDa	kilo Dalton
LC-MS	liquid chromatography-mass spectrometry
L-DOPA	L-β-3,4-dihydroxyphenylalanine
MTBH	3-methyl-2-benzothiazolinone
NADH	nicotinamide adenine dinucleotide
PEG	polyethylene glycol
PPO	polyphenol oxidase
PVPP	polyvinylpolypyrrolidone
PTFE	polytetrafluoroethylene
UV	ultraviolet

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

Appendix A: Experimental data used as the basis for formulating a mathematical model of the process.

Appendix B: Data generated using the mathematical model.

LIST OF PRESENTATIONS

The following is a list of publications, reports and conference proceedings resulting from the work in this thesis.

PUBLICATIONS

Boshoff, A., Edwards, W., Leukes, W.D., Rose, P.D., Burton, S.G. 1998. Immobilisation of polyphenol oxidase on nylon and polyethersulfone membranes: Effect on product formation. *Desalination.* **115**: 307-312.

Burton, S.G., Boshoff, A., Edwards, W., Rose, P.D. 1998. Biotransformation of phenols using immobilised polyphenol oxidase. *J. Mol. Catal. (B:Enzymatic)* **5**: 411-416.

Boshoff, A., Burton, M.H., Burton, S.G. 2000. Optimisation of catechol production by membrane-immobilised polyphenol oxidase - a modelling approach. (Submitted to Biotechnol. Bioeng., December 2000).

REPORTS

Burton, S.G., Boshoff, A., Edwards, W., Leukes, W.D., Russell, A.K., Russell, I., Ryan, D., Rose, P.D., Jacobs, E.P. 1998. Membrane-based Biotechnological Systems for the Treatment of Organic Pollutants. WRC project No. K6/687. *Water Research Commission*, Pretoria, South Africa.

INTERNATIONAL CONFERENCE PROCEEDINGS

Edwards, W., Boshoff, A., Leukes, W.D., Jacobs, E.P., Sanderson, R.D., Saayman, H.M., Burton, S.G., Rose, P.D. 1996. Continuous treatment of phenol-polluted waters in a novel capillary membrane bioreactor. International Congress on Membranes and Membrane Processes. ICOM '96, Yokohama, Japan.

Burton, S.G., Rose, P.D., Boshoff, A., Edwards, W. 1997. Biotransformation of phenols using immobilised polyphenol oxidase. Gordon Research Conference on Environmentally Benign Organic Synthesis. Oxford, U.K.

Burton, S.G., Rose, P.D., Boshoff, A., Edwards, W. 1997. Biotransformation of phenols and the influence of method of immobilisation on polyphenol oxidase activity. Biotrans '97. Montpellier, France.

LOCAL CONFERENCE PROCEEDINGS

Edwards, W., Boshoff, A., Leukes, W.D., Burton, S.G., Rose, P.D., Jacobs, E.P., Sanderson, R.D. 1996. A comparison between two parallel transverse-flow membrane bioreactor modules in terms of bioremediation efficiency using immobilised tyrosinase. 2nd African Water Conference. Gallagher Estate. Midrand.

Boshoff, A., Edwards, W., Leukes, W.D., Jacobs, E.P., Burton, S.G., Rose, P.D. 1997. Application of membrane bioreactors for the biotransformation of organic pollutants in water. Biotech SA '97 and 14th SASBMB, Grahamstown.

Boshoff, A., Edwards, W., Burton, S.G. 1997. Application of membrane-immobilised polyphenol oxidase for the biotransformation of phenolic pollutants. WISA-MTD '97. 2nd Water Institute of South Africa - Membrane Technology Division Workshop. Badplaas.

Burton, S.G., Leukes, W.D., Edwards, W., Boshoff, A., Russell, I., Russell, A.K., Rose, P.D., Jacobs, E.P., Sanderson, R.D. 1997. Biotransformations using membrane technology. WISA-MTD '97. 2nd Water Institute of South Africa - Membrane Technology Division Workshop. Badplaas.

Boshoff, A., Edwards, W., Burton, S.G. 1998. Application of membrane-immobilised polyphenol oxidase for the biotransformation of phenolic pollutants. WISA '98. Cape Town.

Boshoff, A., Burton, S.G. 1998. The effect of polyphenol oxidase immobilisation on the biotransformation of phenolic pollutants. 2nd FASBMB and 15th SASBMB. Potchefstroom.

Boshoff, A., Burton, M.H.and Burton, S.G. 1999. The effects of membrane immobilization on the activity of polyphenol oxidase. 3rd WISA - MTD Workshop. Drakensville Resort. Drakensberg.

Boshoff, A., Burton, M.H.and Burton, S.G. 2000. The effects of membrane immobilization on the activity of polyphenol oxidase. Biotech SA. BioY2K. Grahamstown.

ACKNOWLEDGEMENTS

I would like to acknowledge the supervision, guidance and commitment of Dr. Stephanie Burton throughout the course of the project. At last we can produce catechols!

I would also like to acknowledge the following :- Prof. Mike Burton for his invaluable assistance with the formulation of the mathematical model used in this study.

Prof. Alan Russell for facilitating my trip to the University of Pittsburgh in America and the National Research Foundation (NRF) for financial assistance. Dr. Keith LeJeune and Dr. Erbeldinger for their willing assistance in the formation of the polyurethane foams.

Friends, colleagues and staff members of the Department of Biochemistry and Microbiology for their encouragement and assistance over the years. A special thank-you to Debbie Bodley and Helen Skilling for sharing their HPLC expertise and for their friendship. Edith Antunes for her guidance and assistance with LC-MS and Dr. Koteschwar for his assistance with the formation of the organogel and his organic chemistry expertise. Fritha Hennessy for her efficient proof-reading of the thesis.

Special thanks are due to my family and boyfriend, Pieter du Plessis, for their support and confidence in me and to my mother for proof-reading the thesis.

The financial assistance of Rhodes University, the Water Research Commission (WRC) and the National Research Foundation (NRF) is gratefully acknowledged.

CHAPTER 1

INTRODUCTION

This thesis reports on a study of the biotransformation of phenolic pollutants to produce catechols using mushroom polyphenol oxidase. Numerous investigations have been conducted into the effectiveness of polyphenol oxidase for the bioremediation of phenolic pollutants in waste waters as an alternative to conventional treatment methods. Less attention has been paid to the potential of the polyphenol oxidase reaction as a biotransformation system for the production of catechols. The effects of immobilisation on the activity of polyphenol oxidase were explored as well as reaction conditions resulting in maximum catechol production. This chapter focuses on the properties of polyphenol oxidase, and summarises previous and current research on the biocatalytic activities and applications of the enzyme.

1.1 POLYPHENOL OXIDASE

1.1.1 Overview

Polyphenol oxidase (PPO; EC 1.14.18.1), also known as tyrosinase and phenolase, is a copper-containing monooxygenase. It is well known that polyphenol oxidase catalyses two reactions (Figure 1.1): the *ortho*-hydroxylation of phenols to catechols (cresolase activity) and the subsequent oxidation of catechols to *ortho*-quinones (catecholase activity), both using molecular oxygen (Sánchez-Ferrer *et al.*, 1995).

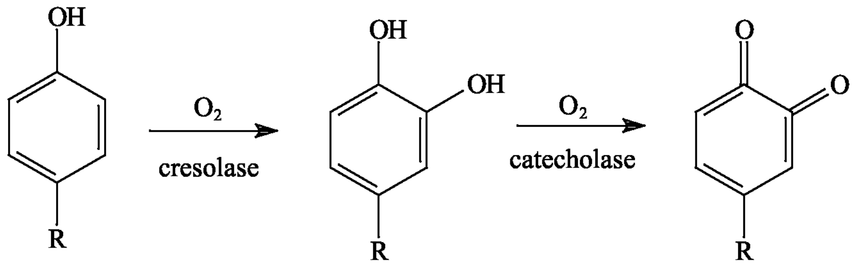


Figure 1.1: Reactions catalysed by polyphenol oxidase.

The *o*-hydroxylation activity of polyphenol oxidase distinguishes it from other phenol-oxidising enzymes such as laccase and peroxidase (Sánchez-Ferrer *et al.*, 1995). It is an unusual monooxygenase in that it does not require a reducing cofactor such as NADH (Aitken *et al.*, 1994). The *ortho*-quinones are unstable, reactive species which may polymerize, oxidize other substrates or undergo nucleophilic attack by various substances including water, proteins or polyphenols (Cheynier and Moutounet, 1992).

Polyphenol oxidase from *Agaricus bisporus* was discovered by Schoenbein in 1856 and later first prepared and partially purified by Kelin and Mann in 1938 (Jolivet *et al.,*1998). It was also the first enzyme recognized as being able to incorporate molecular oxygen into organic molecules and one of the first examples of suicide enzyme inactivation (Zawistowski *et al.*, 1991). It is widely distributed in living systems but the fact that it is not ubiquitous suggests that it is unlikely to play a vital role in metabolism (Sánchez-Ferrer *et al.*, 1995; Martinez and Whitaker, 1995).

Mushroom polyphenol oxidase is unusual in that it is a heterotetramer consisting of two "heavy" chains of 43-45 kDa, which contain the catalytic sites, and two "light" chains of 13-14 kDa (Jolivet *et al.*, 1998). Bouchilloux *et al.* (1963) prepared and studied four isoenzymes, α, β, γ and δ, of which the most common is the β-isoenzyme. The isoenzymes exhibit differences in their ratios of cresolase and catecholase activities (Burton, 1994).

1.1.2 Sources of polyphenol oxidase

Polyphenol oxidase is widely distributed throughout the phylogenetic scale from bacteria, fungi and plants to animals, including invertebrates and mammals. However, it is not ubiquitous and in some instances it is undetectable due to the presence of endogenous inhibitors or because it is in a latent form (Robb, 1984; Sánchez-Ferrer *et al.*, 1995). Polyphenol oxidase is of central importance in such processes as melanization in animals, browning in plants and cuticle formation in insects (Cabanes *et al.*, 1988; Ros *et al.*, 1994). The polyphenol oxidase in *Agaricus bisporus* is the main enzyme involved in the economically detrimental phenomenon of enzymatic browning of mushrooms and prevention of this browning reaction has long been a challenge to food scientists (Kermasha *et al.*, 1993a; Ricquebourg *et al.*, 1996; Jolivet *et al.*, 1998). Large differences in the properties and compartmentalization of polyphenol oxidase are evident across the phylogenetic scale (Longa *et al.*, 1996). The enzyme substrate specificity also differs with the source of the enzyme (Burton, 1994) and appears to become narrower with a rise in the phylogenetic scale (Robb, 1984).

1.1.2.1 Plant polyphenol oxidases

Although polyphenol oxidase occurs widely in plants, and is not restricted to any particular part of the plant, its function in intact plants is not fully understood (Robb, 1995; Van Gelder *et al.*,1997). In higher plants, polyphenol oxidase has been shown to protect the plant from infections by viruses, bacteria and fungi as well as mechanical damage by forming a scab of melanin (Zawistowski *et al.*, 1991; Van Gelder *et al.*, 1997). Of the polyphenol oxidases isolated from a great number of fruits and vegetables, the enzymes from grapes, apples and potato continue to receive the most attention due to their contribution to the quality of wine, juice and processed potatoes (Zawistowski *et al.*, 1991).

In most fruits and vegetables, polyphenol oxidase is responsible for enzymatic browning by the oxidation of endogenous phenolic compounds, following bruising, cutting or other cellular damage. Although the browning products are thought to be safe for human consumption, they result in the development of unpleasant colours and flavours, and a decrease in nutritional value (Kermasha *et al.*, 1993a; Weemaes *et al.*, 1999). Control of enzymatic browning, using inhibitors, temperature or pressure to inactivate polyphenol oxidase, is thus very important to food manufacturing industries (Martinez and Whitaker, 1995). However, browning is sometimes desirable as it improves the sensory properties of products such as raisins and tea (Martinez and Whitaker, 1995; Halder *et al.*, 1998).

Polyphenol oxidase exists in multiple and interconvertible forms, showing differences with regard to development, subcellular location and plant source (Angleton and Flurkey, 1984; Martinez and Whitaker, 1995; Robb, 1995). In some sources, the enzymes have been shown to exist in a latent state, requiring activation by proteolysis, fatty acids, denaturants and detergents (Angleton and Flurkey, 1984; Moore and Flurkey, 1990). For example, latent grape polyphenol oxidase was shown to be activated by polyglucans (Jiménez and García-Carmona, 1993). In plants, the predicted molecular weights of the pro forms of plant polyphenol oxidases are 68-73 kDa, these are later processed to 58-68 kDa mature proteins, and proteolysis of the mature proteins yields monomeric active forms of 40-45 kDa (Martinez and Whitaker, 1995; Van Gelder *et al.*, 1997).

1.1.2.2 Microbial polyphenol oxidases

Of a number of microbes which have been found to produce polyphenol oxidase, the most studied sources are *Streptomyces glaucescens* and *Neurospora crassa* (Burton, 1994), while production of the enzyme from *Trametes versicolor* is also being studied (Lacki and Duvnjak, 1997). However, the enzyme occurs very rarely in bacteria (Robb, 1984; Martinez and Whitaker, 1995). The physiological role of polyphenol oxidase in fungi also appears to be protective (Van Gelder *et al.,* 1997).

The polyphenol oxidase from *Agaricus bisporus* has been found in both the latent and active forms in ratios of approximately 3:20 (Van Gelder *et al.,* 1997). Activation can be effected by detergents such as SDS or protease treatment (Angelton and Flurkey, 1984; Burton and Duncan, 1995b; Van Gelder *et al.,* 1997). Jiménez-Atiénzar *et al.* (1991) demonstrated activation of mushroom polyphenol oxidase using polyamines which induced a conformational change.

Neurospora crassa polyphenol oxidase is a 46 kDa monomer which is formed upon proteolytic processing of a 75 kDa precursor and the complete coding sequence of the precursor has been elucidated (Van Gelder *et al.,* 1997). The polyphenol oxidase of the mould *Aspergillus oryzae* consists of a monomeric enzyme of 67 kDa after activation (Van Gelder *et al.,* 1997). The polyphenol oxidase obtained from *Streptomyces glaucescens* is the smallest reported at 30.9 kDa, and shares 24% similarity with *Neurospora crassa* (Burton, 1994; Martinez and Whitaker, 1995).

1.1.2.3 Animal polyphenol oxidases

In insects, polyphenol oxidase is involved in sclerotization of the exoskelton, resulting in protection against other organisms by encapsulation in melanin (Andersen, 1992; Van Gelder *et al.*, 1997). Hardening of the cuticle of insects and crustaceans is due to quinone-tanning of the outer layers of protein by a quinoid product which is produced via the oxidation of *N*-acetyldopamine, catalysed by polyphenol oxidase (Sizer, 1953; Suguraman and Nelson, 1998). Quinone tanning of collagenous protein or mucoproteins may be a general phenomenon in the hardening of the cuticle or holdfast organs among many different kinds of invertebrates (Sizer, 1953; Yamamoto *et al.*, 1990). Insect polyphenol oxidase is produced in an inactive form that can be activated by certain detergents (Sizer, 1953). In arthropods, the enzyme is usually found in the blood in an inactive form and in the cuticle (Robb, 1984).

In higher animals, polyphenol oxidase is confined mainly to the skin, hair, feathers and eyes, where it is responsible for the biosynthesis of pigments, but it may occur in interior tissue such as parts of the brain, in which its biological function is unknown (Robb, 1984; Longa *et al.*, 1996; Espín *et al.*, 1997). Mammalian polyphenol oxidase is present in small amounts where it occurs membrane-bound, in melanocytes (Burton, 1993). The enzyme catalyses the first step in the synthesis of melanin (Figure 1.2), *i.e.*, the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and its subsequent oxidation to dopaquinone (Ferrari *et al.*, 1997), the following conversions are spontaneous chemical reactions resulting from the reactivity of the *o*-quinones.

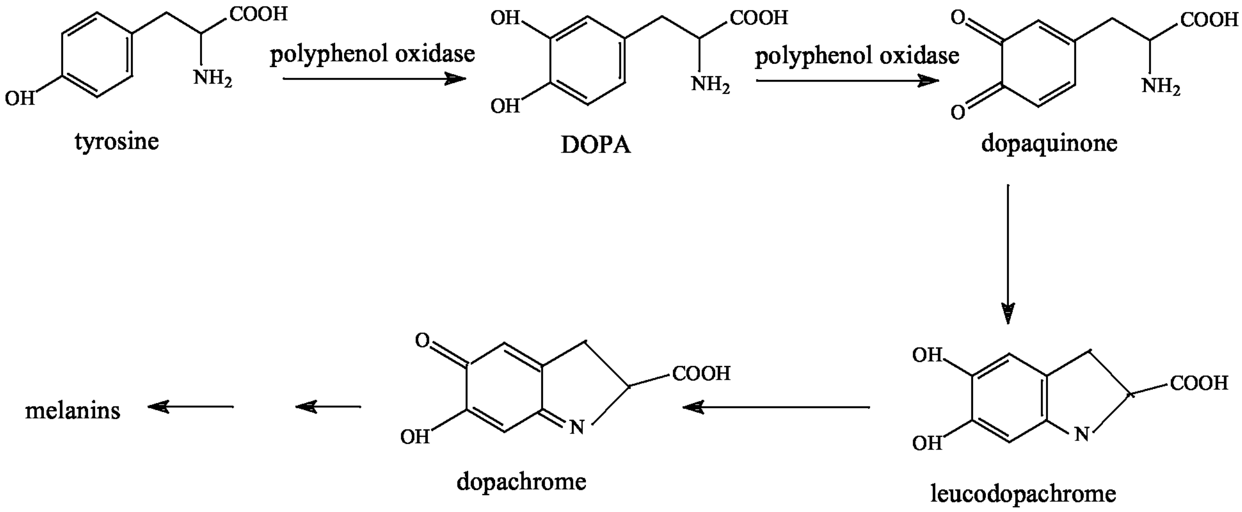


Figure 1.2: Schematic diagram of the general pathway for the formation of melanins.

1.1.3 Reaction mechanism of polyphenol oxidase

The antiferromagnetically coupled dinuclear copper active site of polyphenol oxidase, which shows a close structural relationship to that of hemocyanin (Robb, 1984; Longa *et al.*, 1996), exists in either the bicupric (met) or bicuprous (deoxy) states with oxytyrosinase being formed by the reaction of dioxygen with the deoxy form, or hydrogen peroxide with the met form (Conrad *et al.*, 1994). The dinuclear copper ions each form ligands with three nitrogen atoms from histidine side chains in the protein (Jolivet *et al.*, 1998; Neves *et al.*, 1999).

Polyphenol oxidase is characterised by two different activities, cresolase activity and catecholase activity, with the cresolase activity coupled to the catecholase activity (Ros *et al.*, 1994). Studies have demonstrated that the catecholase activity is generally much faster than the cresolase activity (Krol and Bolton, 1997). 'Resting' (met) tyrosinase cannot hydroxylate monophenols; it must first be reduced to deoxytyrosinase which then binds oxygen, resulting in the oxy, active form, but both resting and oxy-tyrosinase can oxidize *o*-diphenols to *o*-quinones (Jolivet *et al.*, 1998). The next step in the catalytic cycle involves the binding of phenol or catechol to one of the copper ions in the active site of oxy-tyrosinase, followed by proton transfer from the phenolic hydroxy group to the copper bound peroxide (Krol and Bolton, 1997). Oxidation of the phenol is accomplished by attack of the copper-coordinated hydroperoxide at the carbon-hydrogen bond *ortho* to the phenolic hydroxy substituent, resulting in a bidentate bound catechol (Krol and Bolton, 1997; Jolivet *et al.*, 1998). Electron transfer from the catechol to the two copper ions results in *o*-quinone formation and the deoxy active site combines with oxygen forming oxytyrosinase, thus completing the catalytic cycle (Conrad *et al.*, 1994; Krol and Bolton, 1997). The reaction mechanisms of both the cresolase and catecholase activities of polyphenol oxidase are shown in Figure 1.3.

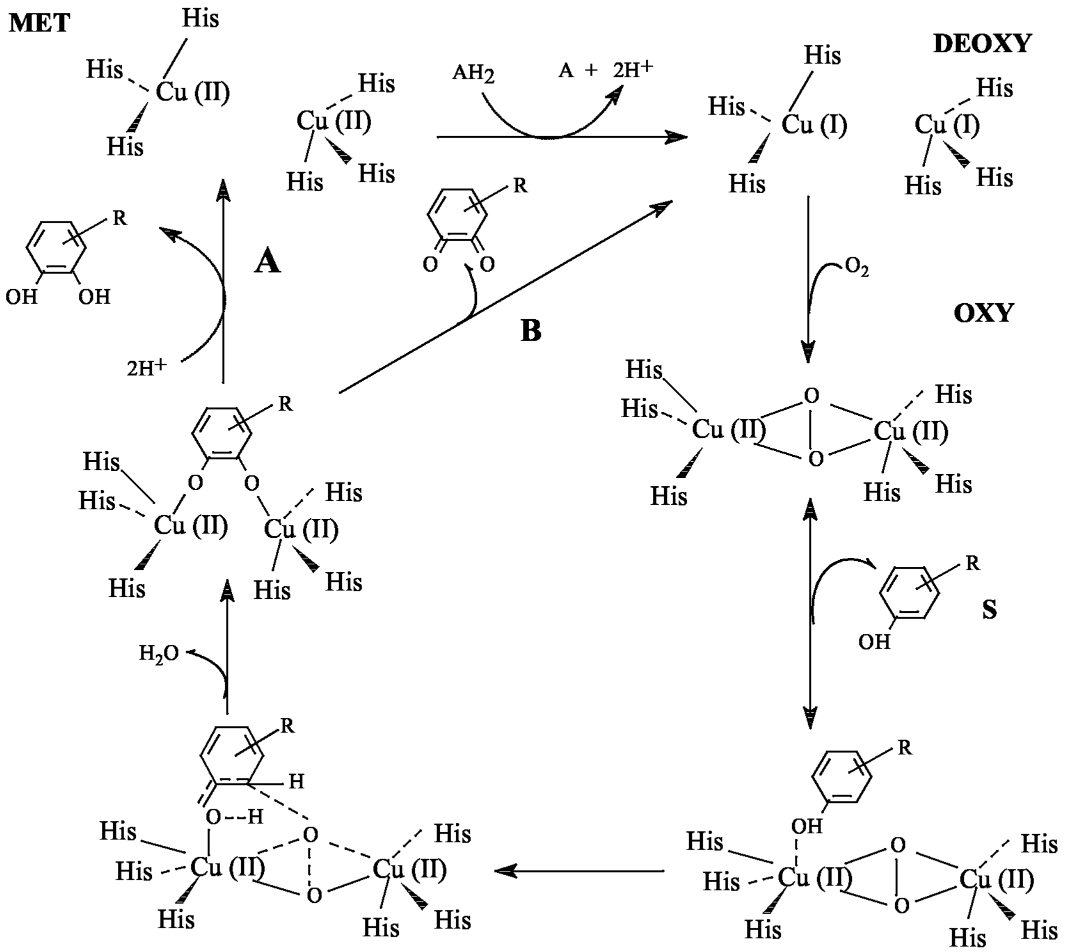


Figure 1.3: Reaction mechanism of polyphenol oxidase showing the cresolase activity (pathway **A**) and the cresolase-catecholase coupled activities (pathway **B**). The enzyme-bound product, which resulted from *o*-hydroxylation of monophenol substrate **S**, can be released as *o*-diphenol or *o*-quinone (Jolivet *et al.*, 1998).

According to Krol and Bolton (1997), the effect of substituents at the 2 and 6 position on the mechanism of polyphenol oxidase-catalysed oxidation has not been well studied. The results of Krol and Bolton (1997) show that the presence of these substituents led to a change in the mechanism and type of quinoid formed, including quinone methides. For example, the reaction of alkyl phenols with polyphenol oxidase yields *o*-quinones, which readily isomerise to form quinone methides (Figure 1.4).

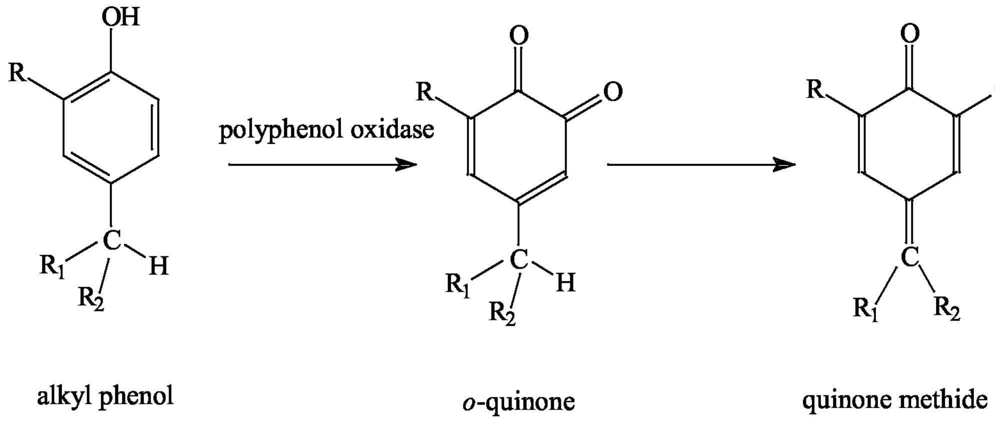


Figure 1.4: Quinone methide formation by oxidation of alkyl phenols, by polyphenol oxidase, to *o*-quinones and isomerization to quinone methide.

1.1.4 The lag phase of cresolase activity

The *o*-hydroxylation of monophenols (cresolase activity) shows a characteristic initial lag period which was found to complicate kinetic studies (Osaki, 1963; Krol and Bolton, 1997; Jolivet *et al.*, 1998). Most of the enzyme is in the 'resting' or met form, with only a small proportion being present as oxytyrosinase, the form required for the *o*-hydroxylation of monophenols. The lag period is the time needed to convert the met form into active oxytyrosinase (Naish-Byfield *et al.*, 1994). The length of the lag period depends on factors such as the nature and concentration of the enzyme, substrate, oxygen concentration, temperature and pH (Jolivet *et al.*, 1998). The lag phase was found to decrease with increasing enzyme concentration and decreasing pH; changing the pH from 6.8 to pH 5 resulted in the lag phase disappearing (Duckworth and Coleman, 1970). The lag phase can also be shortened or eliminated by adding low concentrations of a reducing agent such as L-DOPA (Ros *et al.*, 1993), NADH or ascorbate, which act indirectly by reducing *o*-quinone to *o*-diphenol (Robb, 1984; Jolivet *et al.*, 1998). The addition of ferrous ions was also found to shorten the lag period (Ros *et al.,* 1993).

1.1.5 Suicide inactivation of polyphenol oxidase

Polyphenol oxidase was one of the earliest examples of enzymes found to undergo suicide inactivation which occurs during the oxidation of catechol to *o*-quinone (Asimov and Dawson, 1950). This inactivation has been interpreted as being due to direct attack of an *o*-quinone on a histidine residue near the active centre or attack of a copper-bound hydroxyl radical formed by the Cu(I)-peroxide complex (Sánchez-Ferrer *et al.*, 1995). However, inactivation was found to still occur when the *o*-quinones were removed by adding reducing agents (Golan-Goldhirsh and Whitaker, 1985).

1.1.6 Assay methods for polyphenol oxidase activity

There are a number of different methods available to assay polyphenol oxidase activity, but all are complicated by the two different activities of the enzyme occurring simultaneously. A popular method is to follow the consumption of oxygen either manometrically or using an oxygen electrode (Jolivet *et al.*, 1998). Absorbance-based methods are complicated by the fact that the *o*-quinones are reactive and can undergo side reactions resulting in non-linear absorbance responses. This can be overcome by recycling the *o*-quinones back to *o*-diphenol or by trapping the *o*-quinones using 3-methyl-2-benzothiazolinone hydrazone (MBTH) (Pifferi and Baldassari, 1973; Rodríguez-López *et al.*, 1994; Espín *et al.*, 1997), 2-nitro-5-thiobenzoic acid (Jolivet *et al.*, 1998), cysteine (Gauillard *et al.*, 1993) or argitine (Espín *et al.*, 1999) to form stable coloured compounds. Other methods to assay for enzyme activity make use of L-tyrosine or L-DOPA as substrates, as in the method of Gardner and Cadman (1990) where the conversion of L-DOPA to dopachrome is monitored at pH 6. Specific enzyme inhibitors can also used, in the presence of laccases or peroxidases, as these enzymes may oxidise the same substrates as polyphenol oxidase (Rescigno *et al.*, 1997).

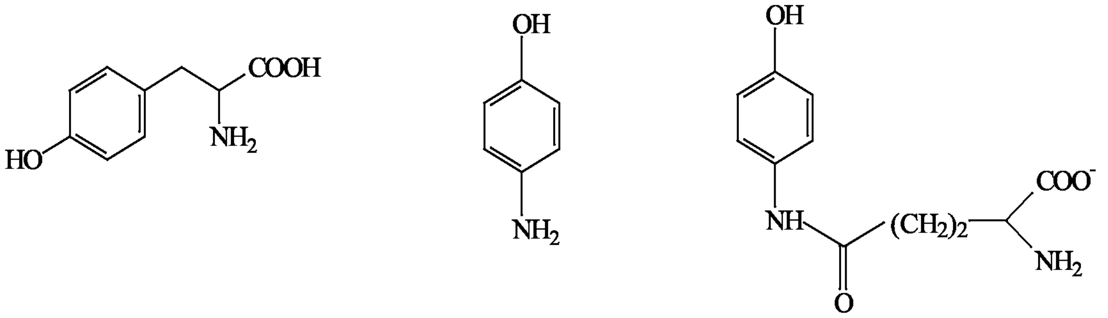
1.1.7 Substrates of polyphenol oxidase

Polyphenol oxidases utilise a wide range of substrates. Polyphenol oxidases from plant sources utilise the widest range of substrates, and amphibian and insect polyphenol oxidases also show a broad substrate specificity, but the mammalian enzyme is optimally active with DOPA and its analogues (Robb, 1984; Burton, 1994). The highest reaction rates were obtained with those phenolic compounds which had a *para*-substituent which were electron donating (Passi and Nazzaro-Porro,1981). Wada *et al.* (1995) also found that *p*-substituted substrates were more reactive than *o*- or *m*-substituted phenols.

The oxidation by polyphenol oxidase of monophenols and *o*-diphenols with simple chemical structures have been widely studied, but polyphenols with complex structures such as flavanoids have been attracting attention recently due to their antioxidant and anticarcinogenic properties (Jiménez and García-Carmona, 1999b). Direct oxidation of fisetin (Jiménez *et al.*, 1998), myricetin (Jiménez and García-Carmona, 1999a) and quercetin (Jiménez and García-Carmona, 1999b) was demonstrated using polyphenol oxidase. Polyphenol oxidase from *Trametes versicolor* was investigated by Lacki and Duvnjak (1997) for its ability to reduce the levels of sinapine in canola meal, as this compound is responsible for the bitter taste of the meal.

A well studied application of polyphenol oxidase was in the production of tea, where the enzyme is responsible for the theaflavin content associated with the colour and flavour of the tea (Halder, 1998; Zawistowski *et al.*, 1991). Immobilised polyphenol oxidase was used to decrease catechin in wines thereby increasing the stability of the wine (Zamorani *et al.*, 1993). Polyphenol oxidase was also capable of hydroxylating and oxidising tyrosine residues in proteins secreted by marine invertebrates, such as barnacles and mussels, for the formation of naturally occurring adhesives (Yamamoto *et al.*, 1990). The effect of polyphenol oxidase in deodorization was investigated by Negishi and Ozawa (1997) where thiol compounds, such as 2-mercaptoethanol, which produce bad odours, were reacted with *o*-quinones to form odourless conjugates.

Evidence for the catalytic activity of polyphenol oxidase on arylamines and *o*-aminophenols was given by Toussaint and Lerch (1987). The oxidation of neurotoxins, which destroy catecholamine neurons, by polyphenol oxidase, was demonstrated by Morrison and Cohen (1983). As a result of the reactivity of *o*-quinones generated by the action of polyphenol oxidase on 4-methylcatechol and catechol, Schved and Kahn (1992) noted a synergistic effect of the *o*-quinones where non-enzymatic oxidation of D,L-DOPA gave the corresponding dopaquinone product. Common endogenous substrates of mushroom polyphenol oxidase are L-tyrosine, *p*-aminophenol and its condensation product with glutamate (Figure 1.5) (Van Gelder *et al.,* 1997).



L-tyrosine *p*-aminophenol γ - L (+)-glutaminyl- 4-hydroxybenzene

Figure 1.5: Structures of common endogenous substrates of polyphenol oxidase.

1.1.8 Inhibitors of polyphenol oxidase

Inhibitors of polyphenol oxidase fall into three main groups: i) small molecules or ions that bind to the copper in the active site, ii) aromatic inhibitors which compete for phenolic substrates in binding to the active site and iii) compounds which reduce or oxidise the copper ions (Burton, 1994).

Inhibitors of polyphenol oxidase may be important for controlling enzymatic browning (Kermasha *et al.*, 1993a; Weemaes *et al.*, 1999). Sulphur dioxide and its derivatives are powerful inhibitors of enzymatic browning of mushrooms during storage and processing (Zawistowski *et al.*, 1991; Czapski, 1994). However, due to the health hazards associated with these compounds, alternative compounds and methods are being sought, such as using thermal or high-pressure processing to inactivate polyphenol oxidase (Czapski, 1994; Weemaes *et al.*, 1999). It has been proposed by Fayad *et al.* (1997) that β-cyclodextrin can be used to control enzymatic browning by forming inclusion complexes with substrates of polyphenol oxidase. The addition of amino acids to foods is an alternative method to prevent browning as they chelate with the copper in the active site, and also react with *o*-quinones (Kahn, 1985).

Cyanide and azide ions coordinate with Cu(I) to form a stable non-reactive complex in the active site (Healey and Strothkamp, 1981; Streffer *et al.*, 1998). Similarily, tropolone, mimosine, pyridinones and vanillyl compounds have copper-chelating properties (Burton, 1994; Streffer *et al.*, 1998).

The ability of phenolic substrates to act as competitive inhibitors requires electron acceptor groups on the substrates (Passi and Nazzaro-Porro, 1981). Conrad *et al.* (1994) determined that binding of the inhibitor was facilitated by interactions of the aromatic ring with hydrophobic groups in the active site. Competitive inhibition by aromatic acids, such as benzoic acid, and *p*-nitrophenol is strongly pH-dependent, with inhibitor binding favoured at lower pHs (Conrad *et al.*, 1994). Hydroxylation and methylation of the benzene ring of aromatic acids decreased their inhibitory effect (Kermasha *et al.*, 1993b) and substitution of both *ortho* positions of benzoic acid with a methyl group sterically prevented binding (Conrad *et al.*, 1994).

Sulfite ions and mercaptoethanol reduce the copper in the active site and react with *o*-quinone products (Burton, 1994). Naish-Byfield *et al.* (1994) demonstrated that thiol compounds inhibit the enzyme by reducing copper in the active site, and they are also capable of reacting with the *o*-quinone products. Many studies concerning the effect of ascorbic acid on the activity of polyphenol oxidase have been carried out, with contradictory results. It has been suggested that it affects specific structures in the enzyme leading to inhibition, but it has also been used as a reducing agent for *o*-quinones, with no apparent effect on the active site (Ros *et al.*, 1996).

1.2 APPLICATIONS OF POLYPHENOL OXIDASE

1.2.1 Biocatalysis

The field of biocatalysis is rapidly expanding and the number of biocatalytic applications is growing as it offers the prospect of clean industrial processing with a shift from waste management to pollution prevention at the source. Biocatalytic transformations carried out using partially purified enzymes or whole-cells are used for the production of a wide variety of chemicals, from bulk to fine chemicals (Schulze and Wubbolts, 1999). The present selection, design and operating environment of biocatalysts in industry is largely made on a case-by-case basis (Bull *et al.*, 1999). The number of novel and valuable biotechnology products can be effectively increased by the discovery of new biocatalysts with novel and improved activities, and by the ability to scale-up bioprocesses in a reproducible manner (Cheetham, 1998). Continued studies of biodiversity should reveal new and useful enzyme structure-activity relationships for the design of future biocatalysts (Bull *et al.*, 1999).

The attraction in using enzymes as catalysts lies in their cleanliness when compared to most chemical catalysts, particularly toxic metal catalysts (Bull *et al.*, 1999) and the chemoselectivity, regioselectivity and stereoselectivity of these catalysts (Schulze and Wubbolts, 1999). Other potential advantages of enzymes are their high specificity, high activity under mild environmental conditions, high turnover number (Klibanov, 1983) and their biodegradable nature. Enzymes are required to perform under operational conditions that are quite different from their natural environments. Various strategies have been developed to increase the operational stability of enzymes, including protein engineering, derivatisation, crystallization, medium engineering and immobilisation techniques (Bull *et al.*, 1999).

An advantage of using polyphenol oxidase as a biocatalyst is that crude enzyme extracts are as effective as purified commercial enzyme preparations, and no expensive cofactors are required (Davis and Burns; 1990; Wada *et al.*, 1993). The activity of polyphenol oxidase has been studied in aqueous media as well as in organic solvents or emulsions of water and organic solvents. The enzyme has also been immobilised on a number of different matrices for the removal of phenols in water. An important practical application of polyphenol oxidase capitalises on the activity of immobilised polyphenol oxidase, in aqueous and organic media, for the development of biosensors (see section 1.2.6).

1.2.1.1 Enzyme immobilisation

Another important aspect of biocatalysis is enzyme immobilisation. A considerable amount of research has been conducted into enzyme immobilisation due to the versatility of immobilised biocatalysts for the synthesis of chemical products varying from milligram quantities of high value fine chemicals, to large scale bulk chemicals. It is generally believed that immobilisation results in improved stability of the enzyme to physical and chemical stress, although in certain instances there is a decrease in stability (Scouten *et al.*, 1995). Immobilisation of proteases was found to prevent autolysis and unfolding which improved resistance to inactivation (Klibanov, 1983). Immobilisation allows for recovery and reuse of the catalyst, resulting in decreased cost of the enzyme (Prazeres and Cabral, 1994; Kragl *et al.*, 1996). Other advantages of immobilisation include uniformity of conversions (Messing, 1985), simplification of product recovery (Holland, 1998) and flexibility of reactor design (Hayashi and Ikada, 1990).

There are five general techniques for immobilising enzymes (Figure 1.6), although many immobilisation procedures involve a combination of these techniques. The choice of technique depends upon the application, cost of the enzyme and effects of immobilisation on the activity of the enzyme.

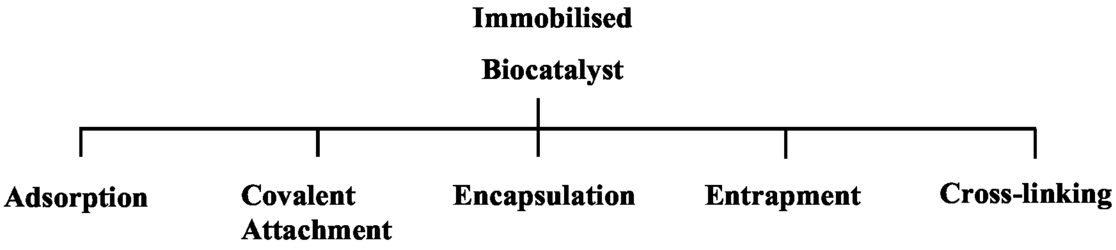


Figure 1.6: Five different techniques of enzyme immobilisation (Messing, 1985).

Many studies have shown that some properties of immobilised enzymes, including catalytic activity, differ from that of the free enzyme in solution (Clark, 1994). The Michaelis-Menten constant, K_M, could be changed by more than one order of magnitude when compared to the soluble enzyme (Emnéus and Gorton, 1993). The activity of the immobilised enzyme may be influenced by the surface size, porosity, hydrophilicity of the support and the immobilisation technique (Bahulekar *et al.*, 1991). The reactivity of an immobilised enzyme may also be subject to diffusional limitations and steric hindrances of substrate and product (Klibanov, 1983), and these factors may influence K_M values. The immobilisation procedure should be optimized to ensure that the K_M value of the immobilised enzyme should be close to that of the soluble enzyme.

1.2.1.2 Membrane bioreactors

The development of membranes in which enzymes are attached, immobilised or contained, is an important application of membrane technology (Prenosil and Hediger, 1988; Bryjak *et al.*, 1996). This technology has been used in biosensor design and bioreactor systems (Ulbricht and Papra, 1997), and could be combined with biological treatment of waste waters, as first reported in 1969 (Brindle and Stephenson, 1996). There are two main enzyme immobilisation techniques used in membrane reactors: enzymes are bound chemically or physically, using various immobilisation techniques, on or in the membrane, or enzymes are used in the soluble form and enclosed by the membrane (Bryjak *et al.*, 1996). The choice of immobilisation technique depends upon the reaction conditions, stability and cost of the enzyme. One of the most important advantages is the possibility of a continuous process leading potentially to an increase in productivity (Prazeres and Cabral, 1994). The disadvantages can include a decrease in performance during operation due to enzyme leakage, shear forces leading to inactivation, enzyme inhibition which can be increased due to the gradual accumulation of some molecular species on the membrane, and the loss of mass transfer efficiency due to fouling (Prazeres and Cabral, 1994).

1.2.2 Biocatalysis in organic media

The use of organic solvents in biocatalytic reactions has expanded the range and efficiency of practical applications of biocatalysis (Khmelnitsky and Rich, 1999). The advantages of using organic solvents include increased solubility of hydrophobic substrates (Yang and Russell, 1995), formation of new products (Vazquez-Duhalt, *et al.*, 1992), favourable shifts of reaction equilibria

(Khmelnitsky and Rich, 1999), higher dissolved oxygen levels (Sariaslani and Rosazza, 1984) and increased thermal stability of the enzyme (Díaz-García and Valencia-González, 1995). The production of speciality polymers that are difficult to produce chemically is an expanding field of interest in applications of biocatalysis in organic solvents (Khmelnitsky and Rich, 1999).

The instability of polyphenol oxidase-generated *o*-quinones in water causes rapid polymerization, resulting in enzyme inactivation, which has led to the development of catalysis in organic media (Kazandjian and Klibanov, 1985; Estrada *et al.*, 1993). However, a layer of essential water around the enzyme is required for its catalytic function in organic solvents and the amount of water needed seems to depend on the enzyme (Zaks and Russell, 1988; Yang and Robb, 1994; Bell *et al.*, 1995). Yang and Robb (1993b) identified three salt hydrates which were efficient water donors to dry polyphenol oxidase in chloroform or toluene. In further studies on *o*-quinone stability in organic media, Doddema (1988) concluded that the hydroxylation of hydrophobic compounds with relatively stable *o*-quinones could be best carried out in organic solvents, but for more hydrophilic substrates, the hydrophilic *o*-quinones were found to accumulate in the aqueous layer around the enzyme, and an aqueous system could be used with continual reduction of the product.

Yang and Robb (1993a) compared the activity of polyphenol oxidase in an aqueous environment, in chloroform and in reverse micelles and found that the enzyme was most stable in chloroform. The enzymatic oxidation of phenols in water was found by Kazandjian and Klibanov (1985) to result in negligible yields as a result of rapid inactivation and polymerization of *o*-quinones, while in chloroform the enzyme was shown to function vigorously with little loss in catalytic activity and the quantitative yield of *o*-quinones was reduced to catechols using ascorbate. A kinetic study of the activity of mushroom polyphenol oxidase in chloroform was carried out by Burton *et al.* (1993) and Burton and Duncan (1995a) resulting in detailed kinetic data relating to optimized reaction conditions. Polyphenol oxidase-coated glass beads in butanol were able to oxidize catechol resulting in *o*-quinone accumulation with no further polymerization to melanins (Jacobsohn *et al.*, 1993).

The inactivation of the catecholase activity of polyphenol oxidase was investigated by Warrington and Saville (1999) with the enzyme immobilised on glass beads, and the study revealed that the inactivation of the enzyme in toluene, chloroform, butanol and amyl acetate was not affected by the *o*-quinone concentration in the medium. The addition of hydrophilic solvents was found to alter the activity of polyphenol oxidase with certain substrates. Using catechin and vanillin as model substrates, Tse *et al.* (1997) optimized the activity of mushroom polyphenol oxidase in chloroform and demonstrated that the presence of methanol had an activating effect on catechin while methanol had an inhibitory effect when vanillin was used as the substrate.

1.2.3 Biocatalysis in aqueous media

An important advantage of using polyphenol oxidase, as a biocatalyst in aqueous media, is the broad range of substrates transformed by the enzyme (Burton, 1994). The rapid polymerization of polyphenol oxidase-catalysed *o*-quinones could be overcome by the addition of a reducing agent (Kazandjian and Klibanov, 1985; Burton, 1994). Polyphenol oxidase has been used for the production of a variety of catechols, with L-DOPA production being one the most important applications of the enzyme as it is used for the treatment of Parkinson's disease (Pras *et al.*, 1989; Pialis *et al.*, 1996; Pialis and Saville, 1998).

An important application of polyphenol oxidase is the bioremediation of phenol-containing water, which takes advantage of the polymerization of *o*-quinones to form insoluble precipitates. A number of studies have also been carried out on the use of polyphenol oxidase to remove phenols from waste water (Atlow *et al.*, 1984) and to decontaminate polluted soils (Claus and Filip, 1990; Park *et al.*, 1999).

Due to the advantages of using immobilised enzymes as biocatalysts, polyphenol oxidase has been successfully immobilised on numerous supports. Polyphenol oxidase was immobilised on magnetite (Wada *et al.*, 1992), cation exchange resins (Wada *et al.*, 1993) and in gelatin gels (Crecchio *et al.,* 1995) for phenol removal. Using chitosan and polyphenol oxidase, Payne and Sun (1994) were able to remove an hydroxylated contaminant which was produced during the industrial production of penicillin V.

Patel *et al.* (1994) were able to increase the accessibility of the amine groups in the chitosan by using it in a gel form rather than as flakes, and further demonstrated the usefulness of chitosan to remove polymerization storage inhibitors, such as *tert*-butylcatechol, using polyphenol oxidase to produce *o*-quinones. The immobilisation of polyphenol oxidase was found to further enhance the removal of phenols, using a combination treatment with chitosan, as it allowed for enzyme reuse (Wada *et al.*, 1993), reduction in the amount of chitosan needed (Wada *et al.*, 1995) and increased enzyme stability with decreased inactivation (Sun and Payne, 1996). Edwards *et al.* (1999a and b) immobilised polyphenol oxidase on two morphologically different capillary membranes for the bioremediation of synthetic and industrial effluents, and a downstream chitosan column or chitosan gel was included to remove the *o*-quinones.

Polyphenol oxidase has also been used for the development of environmentally benign polymers. Payne *et al.* (1996) demonstrated the potential of using polyphenol oxidase to enzymatically modify chitosan. Lenhart *et al.* (1998) and Kumar *et al.* (2000) found that the enzyme could be used to modify thin films of chitosan polymer by producing *ortho*-quinones from phenols which were then able to covalently bond with amine groups in the chitosan. It was also demonstrated by Shao *et al.* (1999) that polyphenol oxidase was able to enzymatically modify the surface of the synthetic polymer polyhydroxystyrene, which is beneficial for the subsequent attachment of macromolecules to the polymer (Figure 1.7).

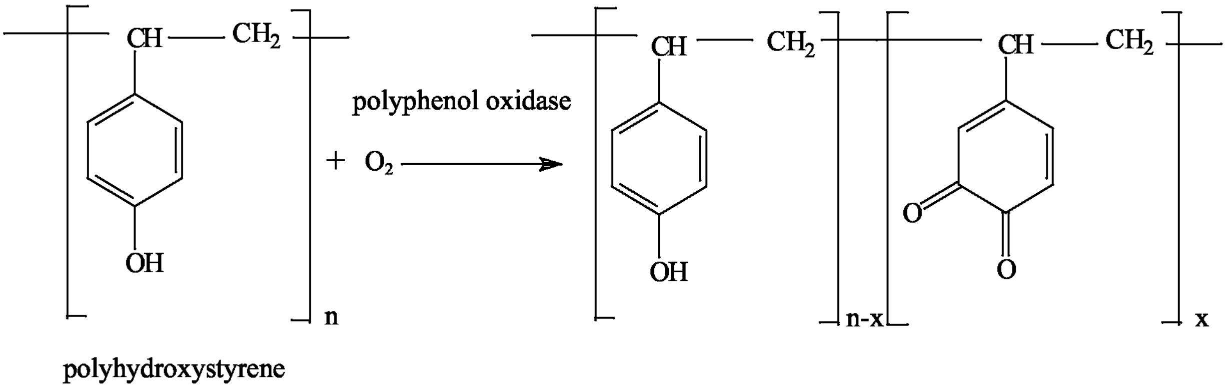


Figure 1.7: Modification of the synthetic polymer polyhydroxystyrene by polyphenol oxidase.

1.2.4 Catechol production

The regioselective *ortho*-hydroxylation of phenols, yielding catechols (*ortho*-diphenols), which is the reaction catalysed by polyphenol oxidase, is difficult to perform by conventional organic methods (Kazandjian and Klibanov, 1985). Thus, a biocatalytic route is an appealing alternative method. This section deals with the usefulness of catechols, conventional methods of catechol production and biocatalytic methods using enzymes from micro-organisms.

1.2.4.1 Uses of catechol

Catechols are industrially important chemicals used as the starting compound in the manufacture of pharmaceuticals, flavours, fragrances, insecticides and anti-oxidants (Latkar and Chakrabarti, 1994; Kakinuma *et al.*, 2000). Other uses of catechol include photography and a polymerization inhibitor for the storage of polymeric materials (Chae and Yoo, 1997). Bacterial enzymes from the 3-oxoadipate pathway were shown by Blasco *et al.* (1995) to be able to utilize 4-chlorocatechol for the production of an antibiotic known as protoanemonin (Figure 1.8).

Catechol derivatives in nature play a role in a variety of biological processes, such as neurotransmission, hormonal action, browning of plant products, siderophore formation, melanization and sclerotization (Suguraman *et al.*, 1990; Barnes and Ishimaru, 1999). *Para*-substituted catechols, including adrenalin (epinephrine) and the anti Parkinson's disease drug L-DOPA, are physiologically important compounds (Held *et al.*, 1998). Catechols carrying a substituent at the 3-position are used for the synthesis of pharmaceutical compounds such as barbatusol (blood pressure lowering drug) and taxodione (anti carcinoma drug), they are also present in many natural compounds with adverse or beneficial effects to humans, such as urushiol found in poison ivy (Held *et al.*, 1999).

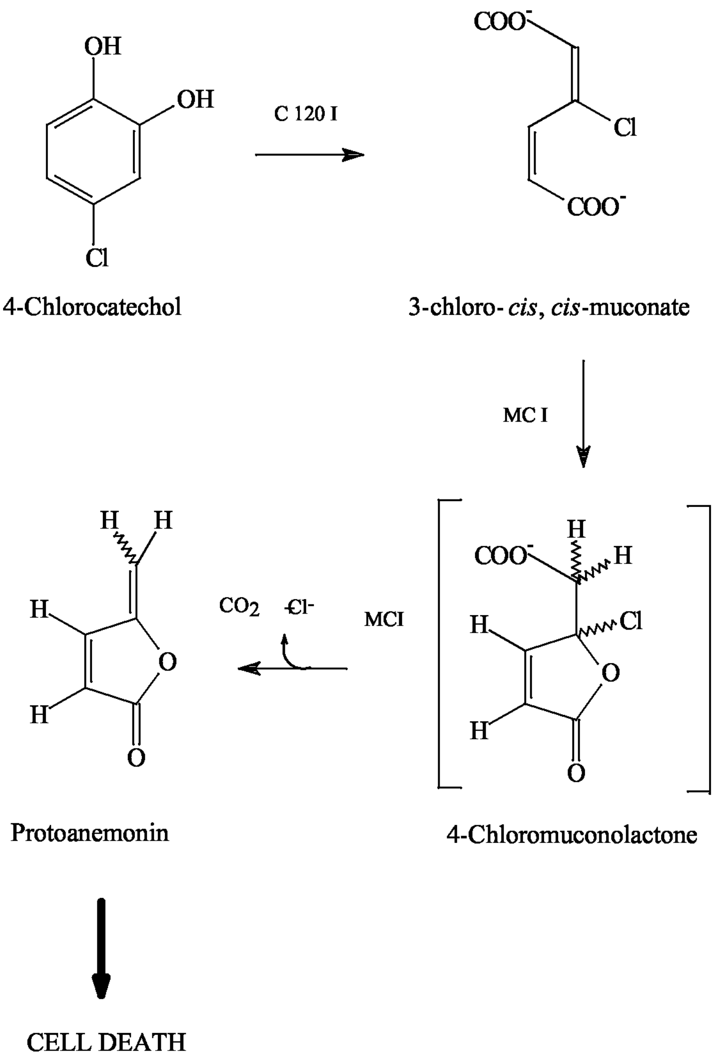


Figure 1.8: Enzymes from the oxoadipate pathway are responsible for 4-chlorocatechol metabolism with the enzymes involved as follows: C120 I, catechol 1,2-dioxygenase type I and MCI, muconate cycloisomerase (Blasco *et al.*, 1995).

1.2.4.2 Catechol production using chemical methods

Regioselective hydroxylation of aromatic compounds to catechols, using organic chemical methods, is difficult and tedious with low yields due to product instability (Doddema, 1988; Held *et al.*, 1998). Catechol is produced commercially by several processes (Figure 1.9), using copper catalysts at high temperatures (Liu *et al.*, 1997; Chae and Yoo, 1997).

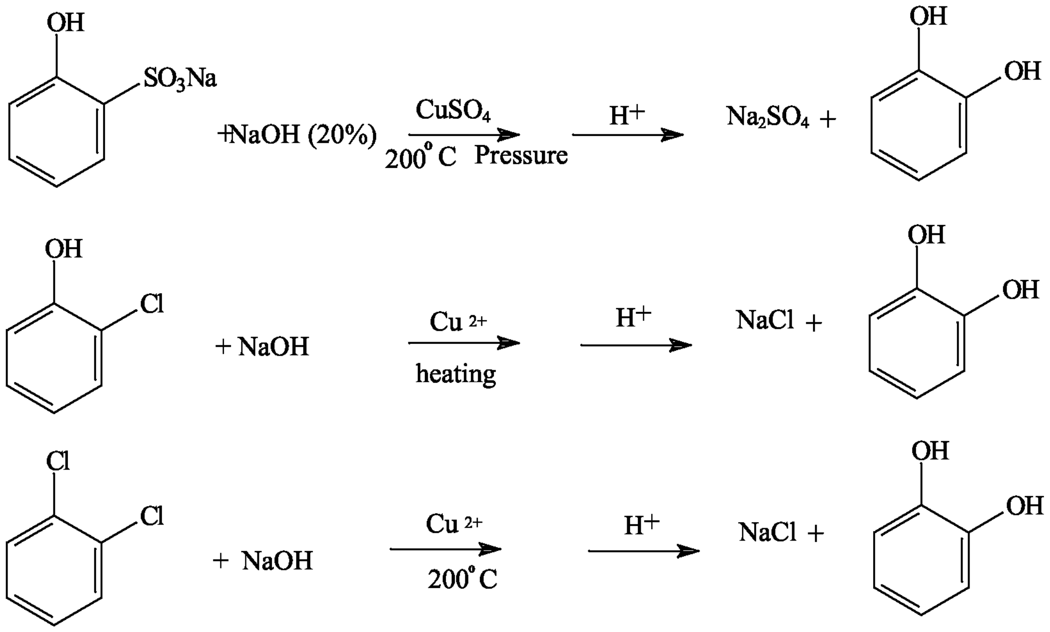


Figure 1.9: Industrial processes used for catechol production (Liu *et al.*, 1997).

These methods have failings such as low conversion rates, and the need for high temperatures and pressures, and they result in serious environmental pollution (Liu *et al.*, 1997). One of the chemical methods for the hydroxylation of phenols is the classical Elbs reaction which uses potassium persulfate in an alkaline media, but *ortho*-hydroxylated products occur only when the *para* position is blocked, and in low yields (Held *et al.*, 1998).

The use of copper complexes for the *ortho*-hydroxylation of phenols has been reported and the main limitation is the lack of selectivity resulting in the formation of many products and low yields of catechol (Casella *et al.*, 1991; Chioccara *et al.*, 1991; Maumy and Capdevielle, 1996; Neves *et al.*, 1999). Metal substituted aluminophosphate molecular sieves are capable of hydroxylating phenols (Dai *et al.*, 1996), but high concentrations of hydrogen peroxide and high temperatures are required.

Environmentally unacceptable processes need to be replaced by pollution-free catalysts. A number of chemical catalysts have been investigated, these include immobilised and non-immobilised transition metal complexes and porphyrin complexes, but the disadvantages are low phenol conversion and complicated preparation methods (Liu *et al.*, 1997).

1.2.4.3 Catechol production using enzymes from micro-organisms

By utilizing enzymes involved in natural product biosynthesis, viable and environmentally acceptable methods are being sought for catechol production (Kakinuma *et al.*, 2000). The enzymes involved in hydroxylation and oxidation of aromatic compounds by micro-organisms are peroxidases, oxidases and oxygenases (Sikkema and de Bont, 1991). Differences exist in the way prokaryotic (bacteria) and eukaryotic (fungi and yeasts) organisms hydroxylate aromatic compounds (Sariaslani and Rosazza, 1984).

In most bacteria dioxygenase enzymes introduce both atoms of molecular oxygen into aromatic substrates, whereas most fungi use monooxygenases to catalyse the introduction of a single atom of molecular oxygen into aromatic substrates (Sariaslani and Rosazza, 1984; Sikkema and de Bont, 1991). Microbial degradation of aromatic compounds yield catechol derivatives as central pathway intermediates, but most of these methods rely on the generation of mutants that are capable of producing catechol but are also defective in metabolising catechol (Chae and Yoo, 1997; Schmid *et al.*, 1998).

A number of micro-organisms, such as *Gliocladium aeliquescens* and *Aspergillus ochraceous*, have been used for the synthesis of L-DOPA from L-tyrosine (Figure 1.10) by using N-blocked tyrosine to prevent further metabolism of the substrate and product (Sariaslani and Rosazza, 1984). *Escherichia coli* and *Erwinia herbicola* have also been used for the microbial production of L-DOPA (Pialis *et al.*, 1996).

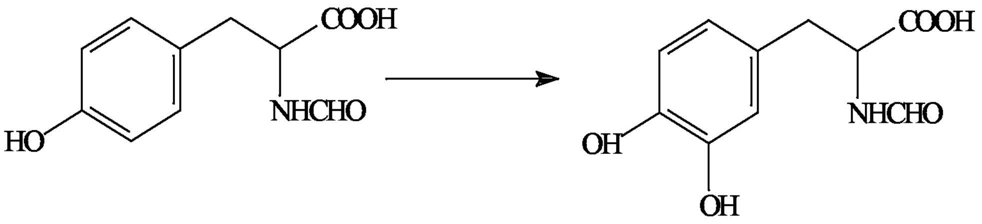


Figure 1.10: Enzymatic hydroxylation of L-tyrosine to L-DOPA.

Catechol oestrogens, associated with a range of biological effects, could be produced by the hydroxylation of phenolic steroids using enzymes from *Aspergillus alliaceus* (Sariaslani and Rosazza, 1984). A strain of *Pseudomonas fluorescens* produced chlorocatechol as the main intermediate during 3-chlorobenzoate co-metabolism, but chlorocatechol had undergone oxidative polymerization yielding black pigments (Fava *et al.*, 1993).

Fluorocatechol was produced using fluorobenzene in a two-step oxidation using benzene dioxygenase and dehydrogenase from *Pseudomonas putida* ML2 (Figure 1.11). Due to its toxicity fluorocatechol was not further degraded but was adsorbed onto activated carbon (Lye and Woodley, 1999).

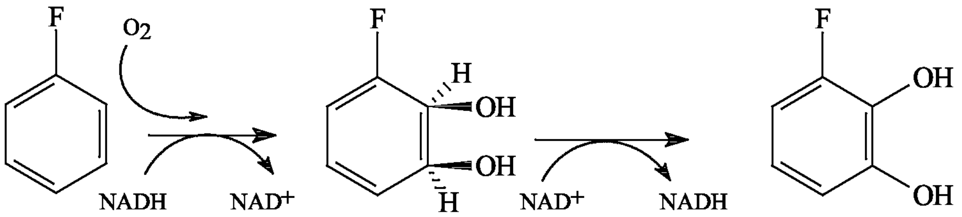


Figure 1.11: Oxidation of fluorobenzene to fluorocatechol using *Pseudomonas putida* ML2 (Lye and Woodley, 1999).

Intact cells of the styrene-degrader *Rhodococcus rhodochrous* NCIMB 13259 were used to produce 3-methylcatechol and 3-ethylcatechol from toluene and ethylbenzene respectively, 3-fluorocatechol was used as an inhibitor of catechol oxygenase (Warhurst *et al.*, 1994).

A mutant *Pseudomonas putida* was isolated by Chae and Yoo (1997) which produced catechol in a medium containing benzoic acid. A recombinant biocatalyst, *E. coli* JM109 pHBP461 hydroxylated *ortho*-substituted phenols to their corresponding 3-substituted catechols via the heterologous expression of 2-hydroxybiphenyl-3-monooxygenase (HbpA) from *Pseudomonas azelaica* HBP1 (Schmid *et al.*, 1998). A disadvantage in using whole cells for catechol production was the toxicity of the products on the bacteria, and an *in situ* product removal strategy was required to maintain biocatalytic activity (Schmid *et al.*, 1998; Held *et al.*, 1999). A chemo-enzymatic route to produce catechol from D-glucose, which is a renewable resource, was demonstrated by Kakinuma *et al.* (2000).

1.2.5 Treatment of phenolic pollutants

Due to the close structural similarity of the natural substrates of oxidative enzymes, such as polyphenol oxidase, to phenolic pollutants, the enzyme is capable of reacting with phenols. Phenols are common pollutants in many different industries and effective methods for their elimination or recovery have been sought. Conventional treatment methods comprise physical and chemical treatment of the pollutants. Biological treatment systems for the bioremediation of phenolic pollutants involve the use of living organisms, such as bacteria and fungi, or the use of enzymes isolated from these organisms, to decontaminate polluted soil or water (Bogan and Lamar, 1996).

1.2.5.1 Sources of phenolic pollutants

Phenolic compounds in the environment arise from natural or man-made sources, with examples of both being harmful to natural ecosystems (Shuttleworth and Bollag, 1986; Geißler and Schöler, 1994; Martirani *et al.*, 1996*)*. Many natural phenols, such as ferulic acid, originate from natural lignin decomposition, but various processes such as the production of olives, olive oil and wine also result in the generation of phenolic waste waters (Pérez *et al.*, 1992; Greco *et al.*, 1999). Phenols are present in the waste waters from various industries such as coal-petroleum conversion, resins and plastics production, petroleum refining, production of textiles, dyes and organic chemicals, pulp and paper processing, soap and detergent production, paving and roofing manufacture and ore mining (Klibanov *et al.*, 1980; Atlow *et al.*, 1984; Lee *et al.*, 1996). Many phenols have been declared hazardous pollutants and thus, removal of these chemicals from industrial aqueous effluents, by biological, chemical or physical treatment processes, is of great practical significance (Klibanov *et al.*, 1980; Aitken *et al.*, 1994).

1.2.5.2 Chemical and physical treatment methods

In the treatment of phenolic waste waters, the choice of method depends on phenol concentrations and economics (Alejandre *et al.*, 1998a). Conventional processes for the removal of phenols from industrial waste waters include solvent extraction, adsorption on activated carbon, ozonation, steam distillation, chemical oxidation, electrochemical techniques, photo-catalytic oxidation, processes in supercritical conditions or incineration (Mantzavinos *et al.*, 1996; Alejandre *et al.*, 1998b). All of these methods suffer from serious drawbacks such as high cost, incompleteness of removal due to limited phenol concentration ranges, formation of hazardous by-products and low efficiency (Klibanov *et al.*, 1980; Atlow *et al.*, 1984). This has led to the need to find alternative treatment methods. Nonionic polymeric resins have been increasingly favoured as an alternative to activated carbon because of easier regeneration (Juang *et al.*, 1999).

Supported liquid membrane processes, using a polypropylene support and aromatic solvents, have been used for phenol recovery (Arana *et al.*, 1999). Yun *et al.* (1992) used a membrane-based solvent extraction system for the removal of organic pollutants from a synthetic high strength aqueous waste stream. Ceramic membranes have also been evaluated for the treatment of petrochemical waste water (Lahiere and Goodboy, 1993). Abiotic catalysts, such as birnessite or kaolinite, were evaluated by Pal *et al.* (1994) in the removal of phenolic compounds through coupling reactions. Huh *et al.* (2000) studied the sorption of organic contaminants from an aqueous solution using organoclays. The oxidation of dilute solutions of organic pollutants was carried out using air over a copper oxide catalyst supported on γ-alumina (Alejandre *et al.*, 1998a) and TiO_2 immobilised on solid support substrates (Byrne *et al.*, 1998), however high temperatures were required to produce the catalyst and there was loss of activity during the reaction process.

1.2.5.3 Microbial degradation

Micro-organisms were isolated as early as 1908 to degrade phenols (Collins and Daugulis, 1997). During microbial degradation, metabolic assimilation and possibly oxidation is carried out by multi-enzyme pathways in the organism (Nicell *et al.*, 1992). The metabolic pathway for phenol degradation typically occurs by means of a catechol intermediate before ring cleavage through an *ortho*- or *meta*-oxidation (Wang and Loh, 1999). Microbial degradation of phenols is achieved mainly by using activated sludge and aerated lagoons, but these methods require large capacity plants and is time consuming due to the toxic effect of the phenols (Davis and Burns, 1990; Siddique *et al.*, 1993; Lee *et al.*, 1996). For example, the biodegradation rates of chlorophenols are influenced by the position and nature of the substituents on the benzene ring (Parker *et al.*, 1994) and also upon the age and acclimatization of micro-organisms in the sludge (Annachhatre and Gheewala, 1996). During acclimatization in the presence of phenols, bacteria, yeasts and fungi produce inducible enzymes, providing mechanisms to deal with phenolic compounds. This induction mechanism was also found to occur in a eukaryotic algae which was capable of catabolising phenol (Semple and Cain, 1995).

Collins and Daugulis (1997) were able to overcome the toxic effects of the phenol on *Pseudomonas putida* by using a two-phase organic-aqueous system to degrade phenol in which phenol was partitioned into the aqueous phase at a rate determined by the metabolism of the phenol by the micro-organism. Use of an integrated chemical-biological process was successful in decreasing phenolic concentrations in waste water generated during olive oil production (Mantzavinos *et al.*, 1996). The preliminary removal of oil and control of pollutant loads resulted in a better quality effluent during the treatment of petrochemical waste (Rebhun and Galil, 1994).

Treatment costs could be reduced and biological treatment methods improved by restricting or removing toxic or inhibitory compounds at an early stage during treatment (Aitken *et al.*, 1994). Due to the toxicity of the inorganic components of a waste water, such as high salt concentrations, an extractive membrane bioreactor was used to remove the phenolic pollutants which were then subjected to a biological treatment process (Livingston, 1993a and b; Brooks and Livingston, 1994). Much of the research concerning bioremediation of phenolics has centred on *Phanerochaete chrysosporium,* which is capable of degrading lignin as well as a broad spectrum of recalcitrant aromatic pollutants (Dhawale *et al.*, 1992; Venkatadri *et al.*, 1992; Rothschild *et al.*, 1995; Bogan and Lamar, 1996) but many other fungi and micro-organisms are being evaluated for their pollutant-degrading abilities.

1.2.6 Biosensors

Due to the needs, in environmental and clinical analyses, for sensitive and rapid measurements preferably without needing technically sophisticated devices, the development of analytical techniques that incorporate a biological sensing component *i.e.* biosensors, is a rapidly expanding area of bioanalytical chemistry (Coche-Guérente *et al.*, 1999a; Abel *et al.*, 1999; Nistor and Emnéus, 1999). Biosensors are becoming very important tools for environmental monitoring, medicine, food quality control and research (Scouten *et al.*, 1995). They offer the possibility of monitoring a single analyte in complex and undefined media (Scheper *et al.*, 1996). The biological components of a biosensor can be classified into two groups: the catalytic group comprising enzymes, microbial, plant or animal cells, and the non-catalytic group consisting of antibodies, receptors or nucleic acids (Russell, 1998).

Enzymes are by far the most commonly used biological components and immobilisation of the enzymes allows for reuse and simplification of the analytical device (Scouten *et al.*, 1995). To increase the sensitivity and operational stability of the enzyme biosensor, reseachers have used additives such as polyethylene glycol, gelatin, histidine, cationic antibiotics, polymers and DNA (Chen *et al.*, 1997; Dantoni *et al.*, 1998; Akyilmaz and Dinçkaya, 1999) and incorporated a substrate recycling step using electrochemical means or co-immobilised enzymes (Lisdat and Wollenberger, 1998; Coche-Guérente *et al.*, 1999a and b).

Mushroom, banana and potato polyphenol oxidases have been used for the construction of biosensors (Ruzgas *et al.*, 1995) which have been used for a wide variety of applications, with the largest area of research being the detection of toxins and pollutants in the environment. These biosensors can also be used to monitor the rancidification process in olive oils (Campanella *et al.*, 1999). Other interesting applications include the monitoring of biological metabolites. A highly sensitive polyphenol oxidase-based chemically amplified biosensor, based on substrate recycling of polyphenolic compounds produced from salicylic acid derivatives has been developed for the determination of *Escherichia coli* density (Hasebe *et al.*, 1997).

1.2.5.1 Environmental monitoring

Analytical methods for environmental pollutant monitoring purposes should be rapid, inexpensive and capable of being used *in situ* (Campanella *et al.*, 1993), but many standard methods to determine phenolic concentrations do not easily allow continuous on-site monitoring, require extraction steps or pre-concentration and are time consuming (Besombes *et al.*, 1995; Li *et al.*, 1998; Nistor *et al.*, 1999). This has led to the rapid development of biosensors as a supplementary technique for environmental analysis as they are portable and allow for fast screening and on-site monitoring (Nistor *et al.*, 1999; Nistor and Emnéus, 1999).

Numerous biosensors have been proposed for phenol analysis using polyphenol oxidase-incorporated biosensors. Due to the fact that biosensors mimic biological systems (Smit and

Rechnitz, 1993), polyphenol oxidase-based biosensors are based on two modes of action, firstly the correlation between toxicity of a compound and a decrease in enzyme activity (inhibition) and secondly the fact that certain phenolic compounds are specific substrates of polyphenol oxidase (Tatsuma and Oyama, 1996; Streffer *et al.*, 1998; Nistor and Emnéus, 1999).

A wide range of polyphenol oxidase-biosensors have been developed, with amperometric detection of phenols being the most widely used detection system (Campanella *et al.*, 1993; Besombes *et al.*, 1996; Desprez and Labbé, 1996). Quantification of phenols in aqueous systems as well as in organic solvents has been possible using polyphenol oxidase immobilised onto a wide variety of matrices. Organic-phase biosensors allow for the detection of water-insoluble substrates with improved thermal stability of the enzyme and prevention of polymerization of *o*-quinones (Wang and Lin, 1993; Wang *et al.*, 1993; Cosnier *et al.*, 1998).

A biosensor was developed by Russell and Burton (1999) which was based on the reaction of membrane-immobilised polyphenol oxidase with phenols to produce *o*-quinones which react with a colour reagent, 3-methyl-2-benzothiazolinone (MBTH), to produce colour intensities which are proportional to the concentration of the phenol, thus allowing for detection and quantification of phenols in water.

Polyphenol oxidase is a widely distributed enzyme exhibiting interesting biochemical characteristics, including the ability to catalyse two distinct reactions. Polyphenol oxidase utilises a broad range of substrates present in organic and aqueous environments, leading to its application in a wide range of fields. Immobilisation of polyphenol oxidase has extended its application to environmental monitoring. Polyphenol oxidase has further potential uses in biotransformation reactions for fine chemical production.

1.3 OBJECTIVES

The main aims of this study were to investigate the potential of using non-immobilised and immobilised mushroom polyphenol oxidase, as a biocatalyst, for the biotransformation of phenolic pollutants. Mushroom polyphenol oxidase, extracted from *Agaricus bisporus*, has been shown to be a convenient source of the enzyme with a relatively simple extraction procedure.
Biological hydroxylations, using sustainable or recyclable resourses, provide opportunities for fine chemical production due to regio- and enantioselectivity of the reactions. Polyphenol oxidase is an enzyme which catalyses hydroxylation reactions yielding catechols. Catechols are industrially important chemicals, and in view of their usefulness, and the fact that there are few effective methods for their synthesis by conventional methods, polyphenol oxidase was used to explore the potential of producing catechols from phenols. This investigation would also provide a novel opportunity to produce catechols not available commercially.

A biocatalytic system would need to be developed for the biotransformation reaction using model phenolic pollutants as substrates. As a result of the complexity of the reaction products in water, analytical techniques would need to be investigated to separate and characterise the products of polyphenol oxidase-catalysed reaction with phenols. Characterisation of the reaction products of the polyphenol oxidase-catalysed reaction with phenols has received insufficient attention and this study would have the potential to further characterise and identify the reaction products. The effects of manipulating the biocatalytic reaction system on catechol yields and the subsequent extraction of the catechols would provide a means of identifying characteristics which would enhance the efficiency of the biotransformation process.

Due to the numerous advantages of using an immobilised biocatalyst, polyphenol oxidase would be immobilised on various support materials using a range of immobilisation techniques. The wide range of immobilisation matrices used in this study would provide a novel opportunity to determine the characteristics of the membrane support would that enhance catechol production. The effects of different supports on enzyme activity and catechol production would be investigated with the aid of mathematical modelling, to determine reaction rate constants and conditions that would increase catechol production.

Phenolic effluents provide a substrate resource for the production of catechol products. The final objective would be to use the optimised aqueous biocatalytic system for the production and isolation of catechols from phenolic effluents produced by the local petrochemical industry, as catechol products have not been previously isolated from these industrial effluents.

CHAPTER 2

CHARACTERISATION AND KINETIC STUDY OF NON-IMMOBILISED POLYPHENOL OXIDASE-CATALYSED REACTION WITH PHENOLS

2.1 INTRODUCTION

This chapter describes studies focussed on characterising the biocatalytic activity of non-immobilised mushroom polyphenol oxidase during the biotransformation of phenolic substrates in an aqueous medium. An important advantage in the use of mushroom polyphenol oxidase, for certain applications, is that partially purified extracts were as effective as purified enzyme (Wada *et al.*, 1993; Burton *et al.*, 1993).

Analytical techniques were developed to separate, identify and quantify the enzymic reaction products which would be used to facilitate a kinetic study of the enzyme. There are few reports of kinetic studies of polyphenol oxidase in aqueous media and such results would contribute to the understanding of the substrate specificity and catalytic efficiency of the enzyme as a biocatalyst. The reaction conditions resulting in catechol as opposed to quinone production were investigated to maximise catechol yields and to facilitate extraction and isolation of catechols from the reaction system.

2.1.1 Polyphenol oxidase extraction

Polyphenol oxidase has been extracted from many different sources including plants, animals and micro-organisms. Extraction of polyphenol oxidase from plants has been problematic due to mixing of the enzyme and the endogenous substrate during extraction, leading to inactivation and enzyme browning (Zawistowski *et al.*, 1991). Mushrooms are regarded as the best source of polyphenol oxidase (Zawistowski *et al.*, 1991), and several methods for mushroom polyphenol oxidase extraction have been published (Frieden and Ottesen, 1959; Bouchilloux *et al.*, 1963; Nelson and Mason, 1970).

Previous studies have shown that crude mushroom polyphenol oxidase is an effective biocatalyst for the hydroxylation and oxidation of phenols in chloroform (Burton *et al.*, 1993; Burton and

Duncan, 1995a). The extraction method of Burton (1993), used for this study, resulted in a partially purified freeze-dried extract of polyphenol oxidase. The procedure involved the homogenization of brown mushrooms in cold acetone, which solubilized the lipids and facilitated their removal by filtration. During homogenization endogenous phenolics react with proteins leading to enzymatic browning, and several methods were investigated to minimise phenolic-protein interactions, including the use of polyvinylpolypyrrolidone (PVPP) which acts as a phenol adsorbent and quinone scavenger (Zawistowski *et al.*, 1991). In this study PVPP was added to the homogenate during the initial extraction procedure, and ammonium sulphate fractionation was then carried out on the homogenate.

During the extraction procedure samples were assayed for polyphenol oxidase activity and protein concentration to determine the effectiveness of the extraction procedure. Polyphenol oxidase activity was measured by monitoring the formation of dopachrome from L-DOPA, using the assay procedure of Burton (1993), which is a modification of the method described by Gardner and Cadman (1990). Protein determinations were carried out using the Bradford method (Bradford, 1976). This method is based on an observed shift in the absorbance maximum when Coomassie Brilliant Blue G-250 reacts with protein.

2.1.2 Characterisation of the polyphenol oxidase-catalysed reaction with phenols

Little attention has been paid to characterising the reaction products from the enzyme-catalysed oxidation of phenolic pollutants using phenol oxidising enzymes such as polyphenol oxidase and horseradish peroxidase (Aitken *et al.*, 1994). Using RP-HPLC, Aitken *et al.* (1994) found that the nature and distribution of reaction products of enzymic oxidations differed according to the type of enzyme used, and polyphenol oxidase was the only enzyme that gave highly polar products *i.e.* product peaks eluting before the phenolic pollutant.

Polyphenol oxidase catalyses the *o*-hydroxylation of phenols to *o*-quinones via the intermediacy of catechol (Sayre and Nadkarni, 1994). According to Naish-Byfield and Riley (1992) and Sayre and Nadkarni (1994), the question of whether catechols were true intermediates was never resolved due to the oxidation of catechol to *o*-quinone without release of the catechol intermediate. Maumy and Capdevielle (1996) reported that although catechols have not been isolated in the transformation of monophenols using this enzymatic system, they are considered as the intermediate compound. Using polyphenol oxidase biomimetics (copper complexes), catechols were shown to be produced during the *ortho*-hydroxylation of phenols (Casella *et al.*, 1991; Chioccara *et al.*, 1991; Maumy and Capdevielle, 1996). The suitability of using polyphenol oxidase for catechol production is limited by the subsequent oxidation of the catechol to *o*-quinone (Held *et al.,* 1998).

One of the earliest reports of catechol production by polyphenol oxidase focussed on the non-enzymatic reduction of *o*-quinones in chloroform to produce catechols (Kazandjian and Klibanov, 1985). The reaction was performed in chloroform due to the rapid polymerization of *o*-quinones in water which caused enzyme inactivation. Alginate-entrapped plants cells of *Mucuna pruriens* were used to produce L-DOPA from L-tyrosine in an aqueous medium using ascorbic acid to reduce the quinones to catechols (Pras *et al.,*1989). The hydroxylation of monophenols by mushroom and mung bean (*Vigna mungo*) polyphenol oxidase, in the presence of L-ascorbic acid, was monitored by HPLC where the amount of hydroxylated product was calculated by the decrease of the substrate on the chromatograms (Takeuchi *et al.*, 1996). The effects of membrane pore size and glutaraldehyde concentration were studied during the production of L-DOPA, using polyphenol oxidase immobilised on chemically modified nylon-6,6 membranes (Pialis *et al.,* 1996). In further experiments by Pialis and Saville (1998) the effects of enzyme stability and oxygen partial pressure on L-DOPA production were investigated.

Reactions in an aqueous system are further complicated by the formation of additional intermediates which form as a result of the highly reactive *o*-quinones (Forsyth and Quesnel, 1957; Forsyth *et al.*, 1960). During the oxidation of higher than 1mM catechol concentrations by polyphenol oxidase in aqueous systems at physiological pH, paper chromatography was used to trap the intermediates, and three isomeric tetrahydroxydiphenyls and a quinone were identified (Dawson and Tarpley, 1990). The structures of these intermediates reveal that carbon-carbon and carbon-oxygen bonds are formed (Figures 2.1 and 2.2).

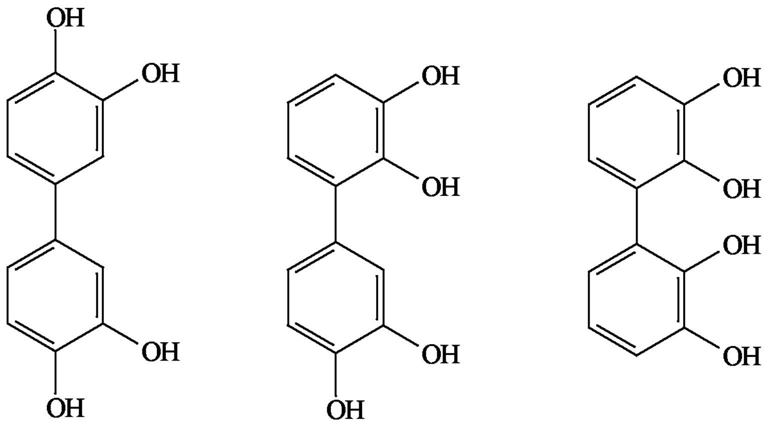


Figure 2.1: The structures of the three isomeric tetrahydroxydiphenyls (Dawson and Tarpley, 1990).

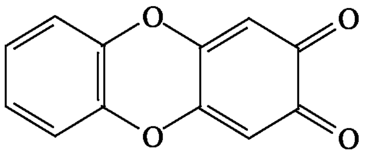


Figure 2.2: The quinone was structurally identified as diphenylenedioxide-2,3-quinone (Dawson and Tarpley, 1990).

In the present study, phenol, *p*-cresol, *m*-cresol, 4-methoxyphenol and 4-chlorophenol were chosen as model phenolic pollutants to react with polyphenol oxidase. RP-HPLC was used to separate the enzymic reaction products, and Liquid Chromatography - Mass Spectrometry (LC-MS) was used to confirm the identity of the catechol peaks and to identify catechol peaks of phenolic compounds not available commercially.

Mass spectrometry is playing an increasingly important role in biochemical research. Due to the development of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), its utility now extends beyond molecular weight determination to include molecular characterization of combinatorial libraries, drug discovery, determination of natural products and biopolymers (Siuzdak, 1996; Siuzdak and Lewis, 1998). Gas Chromatography - Mass

Spectrometry (GC-MS) has been used extensively for the analysis of organic compounds in water, but only 10-20% of organic compounds present in water are amenable to this method because of low volatility and/or thermal instability, leading to the use of LC-MS as a complementary technique (Games *et al.*, 1984). The utility of ESI lies in its ability to produce ionized molecules from a liquid solution by applying a strong electric field (4000 V) to a fine spray of the solution containing the analyte, thus providing a convenient mass detector for liquid chromatography (Siuzdak and Lewis, 1998).

Catechol, 4-methylcatechol and 3-methylcatechol are available commercially and these have been used to identify and quantify enzymatically produced catechol peaks in the HPLC chromatograms of reaction mixtures. 4-Chlorocatechol and 4-methoxycatechol were not available commercially for use as model compounds, making quantification of reaction products problematic, and therefore a method was needed to produce pure samples of 4-chlorocatechol and 4-methoxycatechol. Quantification of compounds by HPLC makes use of calibration curves consisting of peak areas of compounds versus concentrations, and thus in this study, 4-chlorocatechol and 4-methoxycatechol concentrations were required to correlate peak areas with concentrations in order to construct calibration curves. Several colorimetric methods exist for the detection of catechols, and the colorimetric method of García *et al.* (2000) was used to quantify 4-chlorocatechol and 4-methoxycatechol.

2.1.3 The effects of chitosan, gelatin and PEG on catechol production

The *o*-quinones produced by polyphenol oxidase may react with a free amino group in the active site of the enzyme resulting in inactivation (Wada *et al.,* 1993). However, polyphenol oxidase-generated *o*-quinone can be chemisorbed onto chitosan, removing it from solution (Sun *et al.*, 1992). Chitosan is an abundant, natural hydrophilic polymer produced as a byproduct of the shellfish industry. It is a polycationic polymer produced by the alkaline deacetylation of chitin (Crestini *et al.*, 1996). Reactive amine groups on the glucosamine units, in the chitosan polymer, are able to react with *o*-quinones (Sun *et al.*, 1992; Sun and Payne, 1996) (Figure 2.3). The addition of chitosan to the reaction medium was used to decrease the concentration of *o*-quinones in solution, resulting potentially in decreased enzyme inactivation.

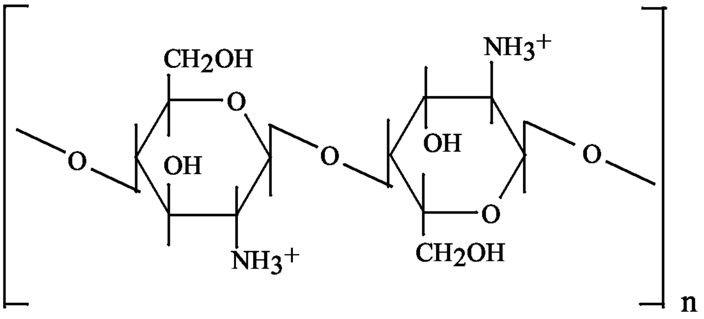


Figure 2.3 The chemical structure of chitosan (1- 4)-2-amino-2-deoxy-β-D-glucan.

In studies by Nakamoto and Machida (1992), using horseradish peroxidase to remove phenols from solution, it was found that the apparent inactivation of peroxidase was found to be caused mainly by adsorption of the enzyme to polymerized phenol. The addition of proteins (gelatin) or polyethylene glycol (PEG), a hydrophilic synthetic polymer, suppressed peroxidase adsorption resulting in significantly decreased enzyme inactivation which led to a reduction in the amount of enzyme required for the reaction. The amount of peroxidase required to achieve at least 95% removal of 10mM phenol was reduced 75-fold in the presence of PEG (Wu *et al.*, 1993). The addition of PEG allowed a 22-fold reduction in the amount of horseradish peroxidase required to achieve 99% removal of phenols from foundry waste (Cooper and Nicell, 1996). Studies have suggested that polyethylene glycol and gelatin combine with the polymerization products formed during the reaction, thereby protecting the enzyme (Wu *et al.*, 1998).

The aim of this study was to determine whether the addition of chitosan, gelatin or PEG would increase the production of catechols during the polyphenol oxidase-catalysed reaction with phenols. Attempts to use polyphenol oxidase as a biocatalyst in water have been unsuccessful because the instability of *o*-quinones leads to enzyme inactivation (Kazandjian and Klibanov, 1985). Thus, chitosan, gelatin and PEG 6000 were added to the reaction medium to evaluate their effects on increasing the production of catechol and decreasing enzyme inactivation.

2.1.4 The effects of buffers and ascorbate on polyphenol oxidase activity

In the course of the research described in Sections 2.1.2 and 2.1.3, the reactions were carried out in water as buffer salts interfered with LC-MS analyses. The reaction of polyphenol oxidase with phenols was also carried out in the presence of different buffers to determine their effects on the product profiles.

Owing to their reactivity, the enzymatically produced *o*-quinones were prone to undergo side-reactions leading to enzyme inactivation. The *o*-quinone product could be removed either by the addition of a reducing agent, such as ascorbate (ascorbic acid) or NADH to reduce the *o*-quinone back to catechol, or by trapping with compounds such as proline or 2-nitro-5-thiobenzoic acid (Jolivet *et al.*, 1998). The addition of ascorbate to the reaction medium could be exploited to reduce *o*-quinones to catechols, resulting in increased catechol yields (Figure 2.4).

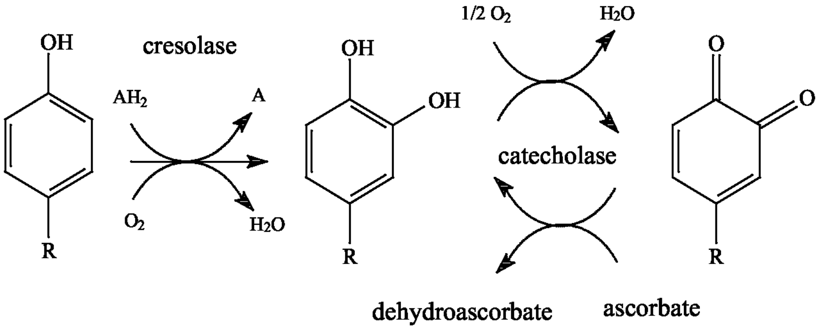


Figure 2.4: The *o*-quinone product, produced during the catecholase activity of polyphenol oxidase, is reduced back to catechol by ascorbate (Jolivet *et al.*, 1998).

Nylon-immobilised polyphenol oxidase has been used for the production of L-DOPA from tyrosine, with ascorbic acid added to the medium, at a concentration equal to the tyrosine concentration, to prevent the subsequent conversion of L-DOPA to melanin (Pialis *et al.*, 1996). Evidence for the prevention of melanin formation was inconclusive as its presence was not directly confirmed by assays and ascorbate may have been insufficient to prevent melanin formation (Pialis *et al.*, 1996). Low concentrations of ascorbic acid were used because it has been shown to inactivate polyphenol oxidase (Golan-Goldhirsh and Whitaker, 1984; Doddema, 1988).

Polyphenol oxidase has also been reported to utilise ascorbate as a substrate. Mushroom and frog epidermis polyphenol oxidases have been shown to exhibit ascorbate oxidase activity, the two adjacent hydroxyl groups of ascorbic acid make it similar to natural substrates of the enzyme (Ros *et al.*, 1995; Ros *et al.*, 1996) (Figure 2.5). Thus, in the present study ascorbate oxidase activity of polyphenol oxidase was assayed as the activity would result in decreased concentrations of ascorbate in the reaction mixture.

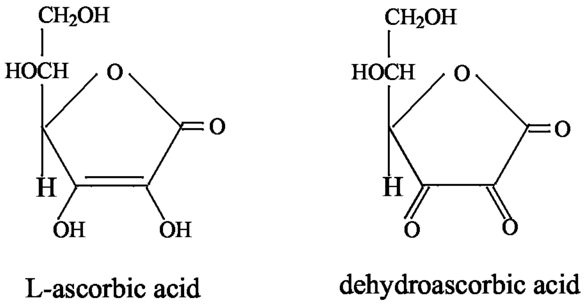


Figure 2.5: The structures of L-ascorbic acid and its oxidation product L-dehydroascorbic acid (Ros *et al.*, 1995).

Doddema (1988) used borate buffer to circumvent the inhibition of polyphenol oxidase by ascorbic acid, during the reaction with *p*-hydroxyphenylglycine methyl ester and *p*-hydroxyphenylhydantoin, resulting in high yields of catechol. In aqueous buffers, borate forms a complex with the hydroxyl groups of ascorbate, reducing the activity of free ascorbate in solution (Doddema, 1988). However, when all the ascorbate was oxidised the catechols rapidly oxidised to *o*-quinones which then polymerized.

In the present study, the ascorbate-borate complex was employed to increase the production of catechols, without leading to polyphenol oxidase inactivation by ascorbate, and the effects of changing borate and ascorbate concentrations were explored in order to prevent catechol oxidation. Catechol is also known to form a complex with borate under weakly basic conditions (Yasunobu and Norris, 1957; Barnes and Ishimaru, 1999) (Figure 2.6).

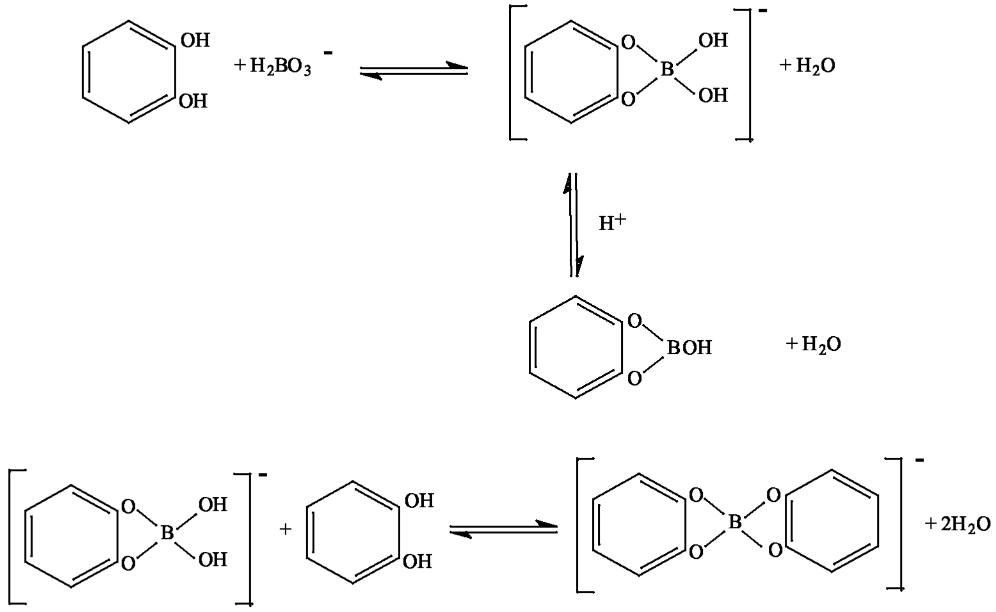


Figure 2.6: The structures of both monocatecholborate and dicatechol borate complexes (Yasunobu and Norris, 1957).

2.1.5 Catechol extraction and isolation

The objective of the work described in this section of the present study was to explore methods to extract and isolate catechol from the reaction system during the polyphenol oxidase-catalysed reaction with phenols.

Due to the structural similarity of catechol and phenol, extraction methods for phenol can usually be extended to catechols. Won and Prausnitz (1975) found that polar organic solvents are better solvents for phenol extraction than non-polar hydrocarbons. Phenol was best extracted from an aqueous phase at a pH below 7, with ketones and esters being particularly good solvents for phenol extraction (Amen-Chen *et al.*, 1997). Ethyl acetate was used for the extraction of wood tar phenols (Amen-Chen *et al.*, 1997).

Alternative biocatalytic systems, using bacterial whole cells, have been developed for the production of catechols, and an *in situ* recovery procedure for catechols was developed by using the solid adsorbent Amberlite XAD-4 to extract residual phenol and catechol from the medium

(Held *et al.*, 1998). Amberlite XAD resins have been recently evaluated for their ability to adsorb phenols, as an alternative to activated carbon, because the regeneration process in easier than that used for activated carbon (Juang *et al.,* 1999). Amberlite is a hydrophobic polystyrene-based resin that has been used for the adsorption of alkaloids from plants and the recovery of small molecules from fermentation broth (Held *et al.*, 1999). Product recovery was carried out by using acidified methanol to elute the compounds from the resin.

Numerous methods exist for extracting phenols and catechols but isolating catechol from residual phenol in the reaction system would be problematic. Possible methods to separate catechol from phenol are listed below:

- Lead acetate precipitation (Suguraman and Lipke, 1982),
- Adsorption on polyamide (Robinson, 1979),
- Flash chromatography, a frequently used adsorption chromatographic technique, has been used for the routine purification of organic compounds (Still *et al.*, 1978),
- Recrystallization of phenol and catechol mixtures from *n*-hexane (Held *et al.*, 1999),
- Preparative HPLC was used for the production of 3-substituted catechols (Schmid *et al.,* 1998),
- Dihydroxyboryl cellulose chromatography, which exploits the complex formed between catechol and borate for the isolation and concentration of catechols from biological samples (Suguraman and Lipke, 1982) and
- Boronate affinity chromatography was used for isolating catechol siderophores, (Barnes and Ishimaru, 1999). This chemistry was first exploited in the affinity chromatography of other *cis*-diols such as carbohydrates and ribonucleotides (Weith *et al.*, 1970).

2.1.6 Kinetic study using polyphenol oxidase as the biocatalyst

The reaction mechanism of polyphenol oxidase is complicated by the existence of a multi-subunit structure, with two active sites per molecule, and the requirement of oxygen for the reactions (Burton, 1993). The enzyme is also characterised by two distinct activities, the cresolase and catecholase activities. Monophenol *o*-hydroxylation (cresolase activity) is generally slower than the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase activity) and is the rate limiting step. Thus quantification of the *o*-quinone end-product is used to determine the cresolase reaction rate (Jolivet *et al.*, 1998). The reaction of polyphenol oxidase with monophenols is also characterised by an initial lag period which depends on factors such as enzyme and substrate concentrations. The lag period can be eliminated by the addition of *o*-diphenols.

An extensive kinetic study of the activity of polyphenol oxidase in chloroform was previously undertaken by Burton *et al.* (1993). During this study, the molar absorption coefficients (ϵ) of several phenolic compounds were determined using NMR spectroscopy to measure component concentrations in the reaction mixtures. Michaelis-Menten kinetics were used to determine apparent K_M and V_{max} values resulting in quantitative data concerning substrate specificities of polyphenol oxidase during the reaction with numerous phenolic compounds (Burton *et al.*, 1993). Apparent K_M and V_{max} values were measured because partially purified polyphenol oxidase was immobilised on glass beads.

In the present study, the kinetic parameters of mushroom polyphenol oxidase were determined during the reaction of various phenolic substrates in potassium phosphate buffer, in order to determine the substrate specificity of the enzyme in an aqueous environment. Polyphenol oxidase can utilise a wide range of substrates in an aqueous system and the activity of the enzyme is influenced by substituents of the phenolic substrates (Section 1.1.7). The calculation of kinetic parameters allows for the further correlation of polyphenol oxidase activity and substrate structure.

Due to the presence of a long lag phase during the reaction of polyphenol oxidase with *p*-cresol and 4-chlorophenol, 4-methylcatechol was added as a cofactor. Long and Alben (1968) also used 4-methylcatechol as a cofactor at a concentration that was 500 times less than the initial substrate concentrations. The molar absorption coefficients (ϵ) of the *o*-quinones were determined by relating UV-visible absorbances with HPLC-determined concentrations of *o*-quinone products which had been prepared by sodium iodate oxidation, a method used by Forsyth *et al.* (1960) to isolate *o*-quinones during their investigation into the enzymic oxidation of catechol by polyphenol oxidase. Michaelis-Menten kinetics were used to determine apparent K_M and V_{max} values for mushroom polyphenol oxidase with phenolic substrates in an aqueous system.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Brown mushrooms (*Agaricus bisporus*) were purchased from a local fruit and vegetable store. Polyvinylpolypyrrolidone(PVPP), ammonium sulphate, L-β-3,4-dihydroxyphenylalanine (L-DOPA), Bradford reagent, commercially prepared polyphenol oxidase, catechol, 4-methylcatechol, 3-methylcatechol, ascorbate, chitosan, gelatin, PEG 6000 and Amberlite™ XAD-4 resin were purchased from Sigma Chemical Co., USA. 4-Methoxyphenol, *p*-cresol and 4-chlorophenol were purchased from Aldrich Chemical Company, USA. Phenol and hexane were purchased from BDH Laboratory Supplies, England. *m*-Cresol was purchased from SAARChem Ltd., South Africa.

2.2.2 Extraction of polyphenol oxidase

Fresh mushrooms (1.25 kg) were homogenized in ice cold acetone (3.125 L) using a Waring blender. The homogenate was filtered through a Buchner funnel. The residual pulp was further dried using filter paper and then frozen with liquid nitrogen. Water was added to form a slurry followed by 50g PVPP per 500g mushrooms. The mixture was allowed to stand overnight at 4°C. Muslin was used to filter the solution and the filtrate was stood on ice, while nitrogen gas was bubbled gently through the solution for 2 - 3 hours, to remove residual acetone.

The extract was further purified using ammonium sulphate fractionation. Ammonium sulphate was added to achieve 40% saturation (22.6g/100mL). The solution was allowed to stand overnight at 4°C before being centrifuged at 10 000g for 10 minutes. The supernatant was brought to 52% ammonium sulphate saturation (7.35g/100mL) and allowed to stand overnight at 4°C before being centrifuged at 10 000g for 20 minutes. The resulting pellet was resuspended in water. Ammonium sulphate was added to the supernatant to achieve 60% saturation (4.8g/100mL) and it was allowed to stand overnight at 4°C and then centrifuged at 10 000g for 10 minutes. The pellet from the 52% saturated fraction was dialyzed against potassium phosphate buffer (50mM, pH 7) overnight at 4°C and then freeze-dried.

2.2.3 Measurement of polyphenol oxidase activity

The activity of polyphenol oxidase was determined by monitoring the production of dopachrome at 475nm in 3ml 10mM L-β-3,4-dihydroxyphenylalanine (L-DOPA) in potassium phosphate buffer (50mM, pH 6) (Burton *et al.*, 1993). One unit of enzyme activity is defined as the amount of biocatalyst that catalyses the formation of dopachrome from L-DOPA at a rate of 1 μmol.min^{-1} where the extinction coefficient (ϵ) is 3600M^{-1}min^{-1}. A Shimadzu UV-160A UV/VIS spectrophotometer was used for all spectrophotometric assays.

2.2.4 Protein determinations

The Bradford method of protein determination was used to quantify the protein concentrations in various extracts (Bradford, 1976). The analysis gives a linear response from 1 μg to 140μg protein using BSA as a standard protein and the standard curve is shown in Figure 2.7.

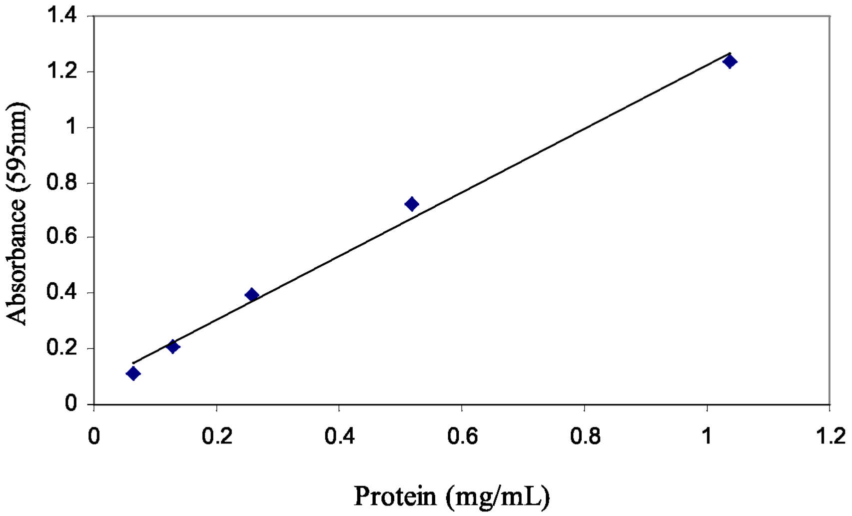


Figure 2.7: Standard curve for protein determinations using the Bradford method ($r^2 = 0.99$).

3mL of the Bradford reagent was added to 100μL protein solution. The absorbances were read at 595 nm, after 5 minutes, for the standard solutions and the unknown solutions.

2.2.5 Chromatographic analysis of polyphenol oxidase-catalysed conversion of phenols

The disappearance of the phenolic compounds and the appearance of the products was monitored by HPLC with UV detection (Beckman Instruments, Inc., San Ramon, USA) using reverse-phase 5µm C_{18} Spherisorb (250mm x 4.6mm) column with a mobile-phase consisting of water and acetonitrile (60:40) at a flow rate of 1mL.min^{-1}. Peaks were detected at 270nm and analysed with Beckman System Gold Chromatography Software.

2.2.6 LC-MS analysis

Catechols were identified using a Finnigan LCQ system, in negative ESI (Electrospray Ionisation) mode, with a reverse-phase 5µm C_{18} Spherisorb (250mm x 4.6mm) column and mobile-phase of water (0.1 % acetic acid) and acetonitrile (60:40) at a flow rate of 1mL.min^{-1}. The following settings, using an Electrospray Ionisation source, were employed to record the mass spectra, after tuning to give maximum sensitivity: capillary temperature 245 °C, capillary voltage 2.5 V, source voltage 4.7 kV, source current 100 µA, tube lens offset 17 V and the sheath gas was set at 85 arbitrary units. Commercially available catechols were dissolved in water and reactions of the phenols with polyphenol oxidase were conducted in water as non-volatile buffers cannot be used during LC-MS. Acetic acid is often used in LC-MS to aid ionisation of the analyte. Where catechols were available commercially, *i.e.* 3-methylcatechol, 4-methylcatechol and catechol, the molecular masses of these compounds were compared to the molecular masses of the catechol peaks obtained after reaction of the substrate with the enzyme.

2.2.7 The addition of chitosan, gelatin and PEG during the reaction of polyphenol oxidase-catalysed reaction with phenols

The reaction system consisted of 2 mM 4-methoxyphenol (25mL), 0.6U polyphenol oxidase and the addition of varying amounts of chitosan (0.3g, 0.5g, 0.7g and 1g), gelatin (0.5g, 0.75g and 1g) or PEG 6000 (0.5g, 1g, 1.5g and 1.75g). Experiments included experimental controls consisting of (a) substrate and enzyme; (b) substrate and chitosan; (c) substrate and gelatin and (d) substrate and PEG. The solutions were allowed to stir continuously at 25°C using an orbital shaker.

2.2.8 The effects of buffers on polyphenol oxidase activity

Solutions of phenol, *p*-cresol, *m*-cresol and 4-chlorophenol (20mL; 2.5mM) were prepared in 75mM borate buffer (pH 7.8), 50mM potassium phosphate buffer (pH 6.8) and 50mM sodium acetate buffer (pH 6.8). 1.4-1.9U polyphenol oxidase was added to the solutions which were allowed to stir continuously on an orbital shaker.

2.2.9 The effects of borate and ascorbate on polyphenol oxidase activity

Solutions of *p*-cresol and 4-chlorophenol (20mL; 2.5mM) were prepared in water and 75mM borate buffer with the addition of 1.3U and 1.8U polyphenol oxidase respectively to the solutions. The effect of ascorbate on polyphenol oxidase was determined by the addition of 12.5mM ascorbate to the phenol solutions prepared in water and borate buffer.

2.2.10 Measurement of ascorbate oxidase activity of polyphenol oxidase

Different ascorbate concentrations were used to determine the presence of ascorbate oxidase activity of polyphenol oxidase. Ascorbate (2mM, 4mM, 6mM and 12.5mM) was dissolved in 75mM borate buffer (pH 7.8) and 1.8U polyphenol oxidase was added to each ascorbate solution. The experimental control consisted of ascorbate in buffer with no enzyme present in solution. Samples were withdrawn every 30 minutes for 2 hours.

2.2.11 The effects of varying borate and ascorbate concentrations on catechol production

Solutions of phenol, *p*-cresol, *m*-cresol and 4-chlorophenol (20mL; 1-3.5mM) were prepared in borate buffers of varying concentrations, ranging from 25mM-100mM borate. Different concentrations of ascorbate were added to the substrate solutions prepared in borate buffers. 0.9-2.4U polyphenol oxidase was added to the reaction systems and allowed to stir continuously on an orbital shaker. Samples were withdrawn every 30 minutes for 2-3 hours.

2.2.12 The effect of pH on catechol extraction using organic solvents

Diethyl ether was used to extract catechol from the reaction medium containing 75mM borate buffer (pH 7.8) and 12.5mM ascorbate. The solution was extracted 2x using 50mL organic solvent and 50mL catechol solution in a separating funnel. The extractions were carried out at different pHs of the catechol solution using diethyl ether. Catechol dissolved in water was used as an experimental control to determine the effect of borate on the extraction.

2.2.13 Catechol extraction by adsorption on Amberlite XAD-4 resin

5g Amberlite XAD-4 resin was cleaned prior to use by washing in hot water followed by a wash with methanol and a final rinse with 15 volumes of distilled water. Different amounts of Amberlite were used to determine the amount needed for maximum catechol extraction. A solution of 1.5mM catechol and 0.5mM phenol (200mL) was prepared in 75mM borate buffer (pH 7.8) with 12.5mM ascorbate. 0.4g, 0.8g, 1.2g and 1.6g XAD-4 resin was added to 50mL solutions respectively, and samples were removed after 3 and 5 hours, and analysed by HPLC. The experiment was repeated using 8g, 12g, 16g and 20g XAD-4. The solution containing 20g XAD-4 resin was poured into a column and the catechol and phenol was eluted using methanol acidified to pH 2 using concentrated HCl, 5mL fractions were collected and analysed using HPLC.

2.2.14 Product purification by recrystallization from hexane

A solution of 50mM catechol and 25mM phenol (100mL; 75mM borate buffer) was acidified to pH 2 using 0.5M H_2SO_4. The solution was extracted with 200mL diethyl ether The organic layer was dried using Na_2SO_4 and then filtered through Whatman paper. The rotary evaporator was used to remove the organic solvent. The residue was re-dissolved in 100mL hexane and refluxed for 2 hours at 60°C. The solution was placed at 4°C overnight and then filtered, the resulting crystals were dissolved in water and analysed by HPLC.

The experiment was repeated using a significantly lower phenol concentration and polyphenol oxidase was added to produce catechol from the phenol substrate. Polyphenol oxidase (19U) was added to a solution of 2.7mM phenol (250mL; 75mM borate buffer (pH 7.8); 12.5mM ascorbate). The solution was placed on a Labcon shaker at 180 rpm, samples were removed every 30 minutes for 2 hours and analysed by HPLC. After 2 hours 1.98mM catechol and 0.72mM phenol were present in the solution which was acidified to pH 2 using 0.5M H_2SO_4. The solution was extracted into 500mL ethyl acetate The organic layer was dried using Na_2SO_4 and then filtered through Whatman paper. The rotary evaporator was used to remove the organic solvent. The residue was re-dissolved in 20mL hexane and refluxed for 2 hours at 60°C. The solution was placed at 4°C overnight and then filtered, the resulting crystals were dissolved in water and analysed by HPLC.

2.2.15 Isolation and extraction of 4-chlorocatechol and 4-methoxycatechol

Solutions of 3mM 4-methoxyphenol and 4-chlorophenol (300mL; 75 mM borate buffer (pH 7.8); 12.5mM ascorbate) were prepared, and 27U and 36U of polyphenol oxidase were added to each phenol respectively. The solutions were placed on a Labcon shaker at 180 rpm, samples were removed every hour and analysed by HPLC using the method in Section 2.2.6, except 0.1% acetic acid was present in the mobile phase.

The reaction was stopped when 4-methoxyphenol or 4-chlorophenol were no longer detected in the solutions. The solutions were then acidified to pH 2 using 0.5M H_2SO_4. 4-Methoxycatechol and 4-chlorocatechol were then extracted with 600mL diethyl ether. The organic layers were then dried using Na_2SO_4 and then filtered through Whatman paper. The rotary evaporator was used to remove the organic solvent. The resulting residue was then dissolved in water and analysed by HPLC.

2.2.16 Determination of catechol concentrations

Catechols were quantified according to the method of García *et al.* (2000). Catechol was prepared in 50% ethanol at various concentrations and used to construct a catechol standard curve (Figure 2.8). 4-chlorocatechol and 4-methoxycatechol samples were also dissolved in 50% ethanol. 2mL portions of 5% sodium molybdate in 50% ethanol were added to 10mL samples. After 15 minutes the absorbances were measured at 370nm with 2mL 50% ethanol being used as the blank.

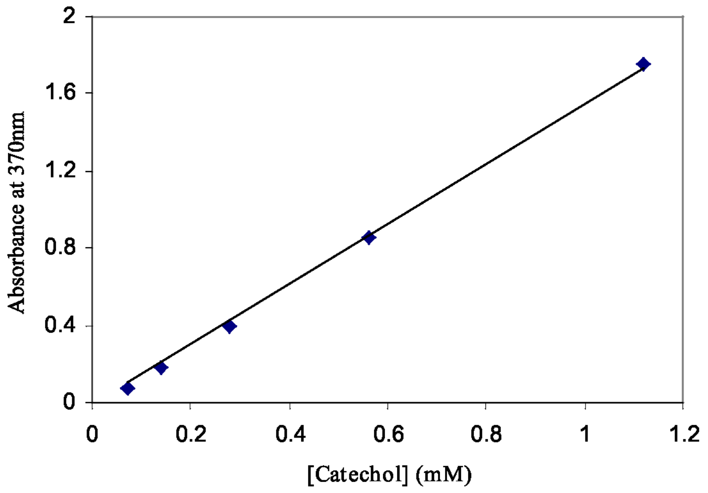


Figure 2.8: Standard curve for catechol determinations using the method of García *et al.* (2000) ($r^2 = 0.99$).

2.2.17 Determination of molar absorption coefficients of *o*-quinones

The wavelength of maximum absorbance of the *o*-quinone product was determined by measuring the UV spectrum (600-200nm) after the reaction of polyphenol oxidase with the phenolic substrates. The molar absorption coefficients of the *o*-quinones were determined by using sodium iodate as an oxidising agent to oxidise the catechols. 5mL portions of solutions of 1mM or 0.5mM catechol, 4-methylcatechol, 3-methylcatechol, 4-methoxycatechol and 4-chlorocatechol, dissolved in potassium phosphate buffer (50mM, pH 6) were allowed to stir continuously, after the addition of sodium iodate to give a final concentration of 0.5-2.5mM sodium iodate.

The wavelength of maximum absorbance of *o*-quinone products, generated by the reaction of polyphenol oxidase with phenol, *p*-cresol, 4-methoxyphenol and *m*-cresol, was determined to be 395nm, while the reaction with 4-chlorophenol resulted in the wavelength at maximum absorbance to be 400nm. Samples were withdrawn every 5 minutes and analysed by HPLC, according to the above method, and the absorbances were read at 395nm or 400nm. The molar absorption coefficients of the *o*-quinones were calculated by correlating the absorbance at 395nm or 400nm, with HPLC-determined concentrations of catechol substrate.

2.2.18 Determination of kinetic parameters of polyphenol oxidase in an aqueous medium

Initial reaction rates for polyphenol oxidase activity in potassium phosphate buffer (50mM; pH 6.8) were measured using phenol, *p*-cresol, *m*-cresol, 4-methoxycatechol and 4-chlorophenol as the substrates. Four different substrate concentrations were chosen, but due to the presence of a lag phase, when *p*-cresol and 4-chlorophenol were used, 4-methylcatechol was added at a concentration that was 2000x less than the substrate concentration. Reactions were carried out by adding polyphenol oxidase (1U) to substrate(2mL) and measuring the absorbances. Linear regression analysis of the results of each assay was used to determine the change in absorbance per minute. Using the molar absorption coefficients determined for each substrate, as described above, the concentration of product was determined per minute. Hanes plots were used to determine the kinetic parameters.

2.3 RESULTS AND DISCUSSION

2.3.1 Extraction of polyphenol oxidase

The purification table of polyphenol oxidase extracted from brown mushrooms is shown in Table 2.1. The highest specific activity of polyphenol oxidase was found in the 52% ammonium sulphate saturated pellet. The extraction procedure was repeated several times during the study. The protein content of the resulting freeze-dried powder was determined to be between 15 - 22% protein during different extraction procedures.

Table 2.1: Purification table for polyphenol oxidase extracted from brown mushrooms

Fraction	**Volume (mL)**	**Protein (mg/mL)**	**Activity (µmols/min/mL)**	**Specific activity (µmols/min/mg)**	**Total activity (µmols/min)**	**Total protein (mg)**	**Yield (%)**	**Fold purification**
Crude	1980	0.738	5.385	6.426	10662.3	1461.2	100	1
40% sup.	2120	0.589	1.623	2.756	3440.8	1248.7	32	0.4
40% pellet	128	0.465	3.816	8.207	488.5	59.5	5	1.3
52% sup.	2145	0.579	0.380	0.657	815.1	1241.9	8	0.1
52% pellet	210	0.828	16.44	19.855	3452.4	173.9	32	3.1
60% sup.	2140	0.423	0.055	0.130	117.7	905.2	1	0.02
60% pellet	72	2.559	9.752	2.740	702.1	184.3	7	0.4

2.3.2 Separation of products of polyphenol oxidase-catalysed reaction with phenols

RP-HPLC provided an effective method to separate the substrates and products of the polyphenol oxidase-catalysed reaction with phenols. Analysis of reaction mixtures revealed a number of peaks eluting before the phenolic substrate. The products formed were thus more polar than the phenolic substrate. Figure 2.9 shows an HPLC chromatogram of the polar products, *o*-quinone and 4-methylcatechol, formed during the reaction of polyphenol oxidase with *p*-cresol.

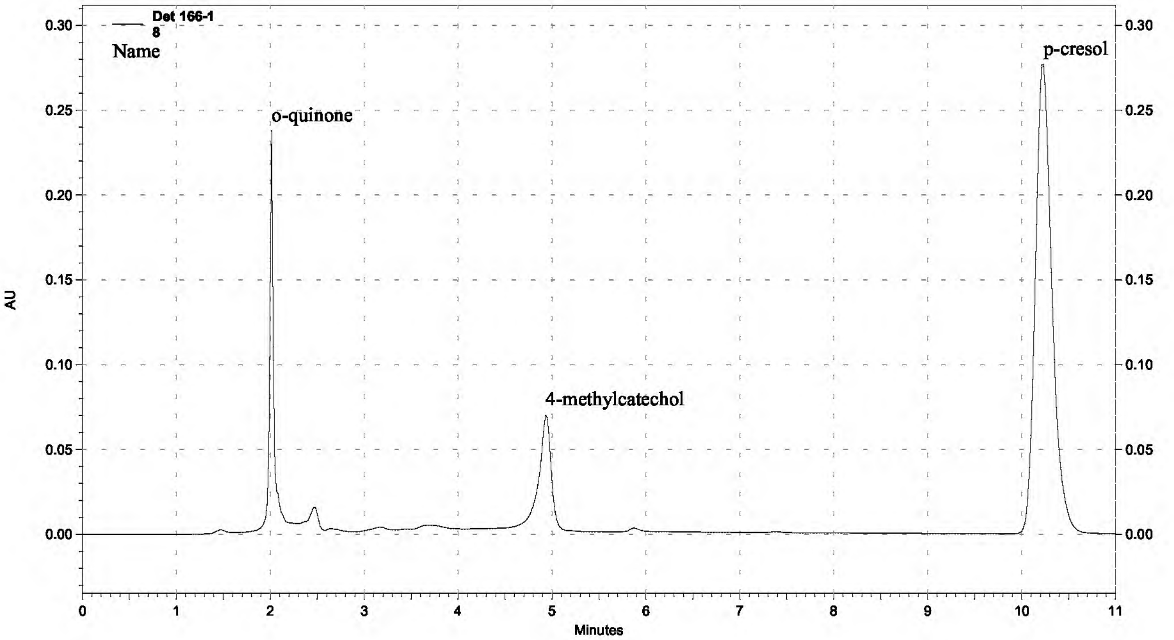
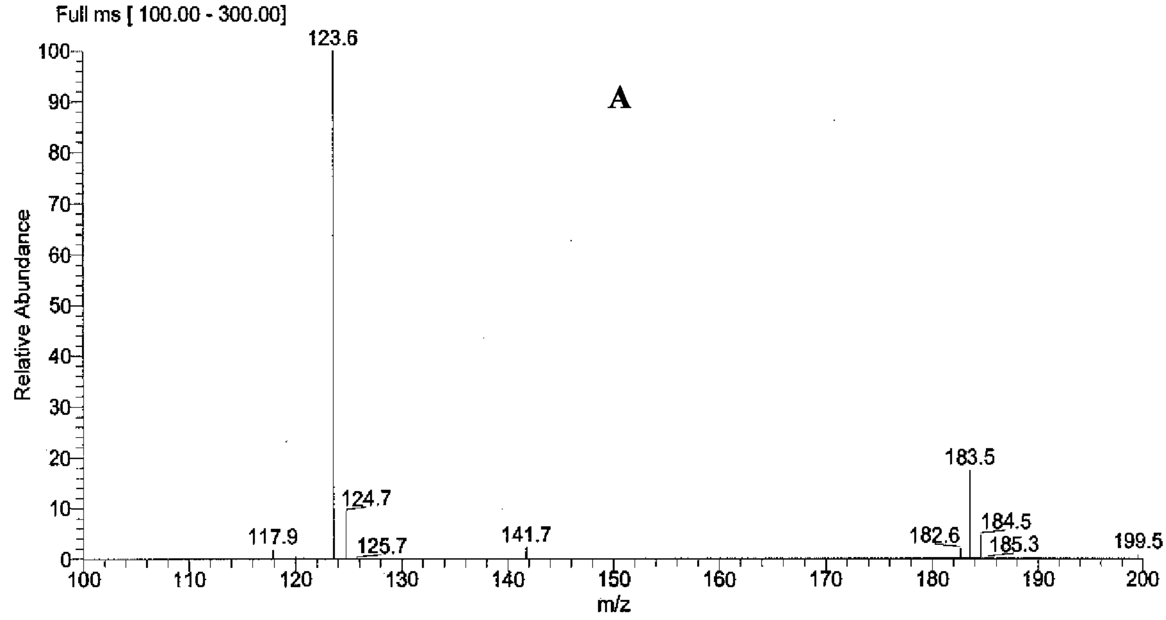


Figure 2.9: HPLC chromatogram of products generated by polyphenol oxidase-catalysed reaction with *p*-cresol.

2.3.3 LC-MS analysis

The catechol peaks of commercially available and enzymatically produced catechols were analysed by MS as they eluted from the RP-HPLC column. This allowed the analysis of the catechol peak without the need to extract catechol from the reaction solution. The mass spectra of the catechols generated by the polyphenol oxidase-catalysed reaction were compared with those of the commercially available catechols, where available, and are shown in Figures 2.10 to 2.13



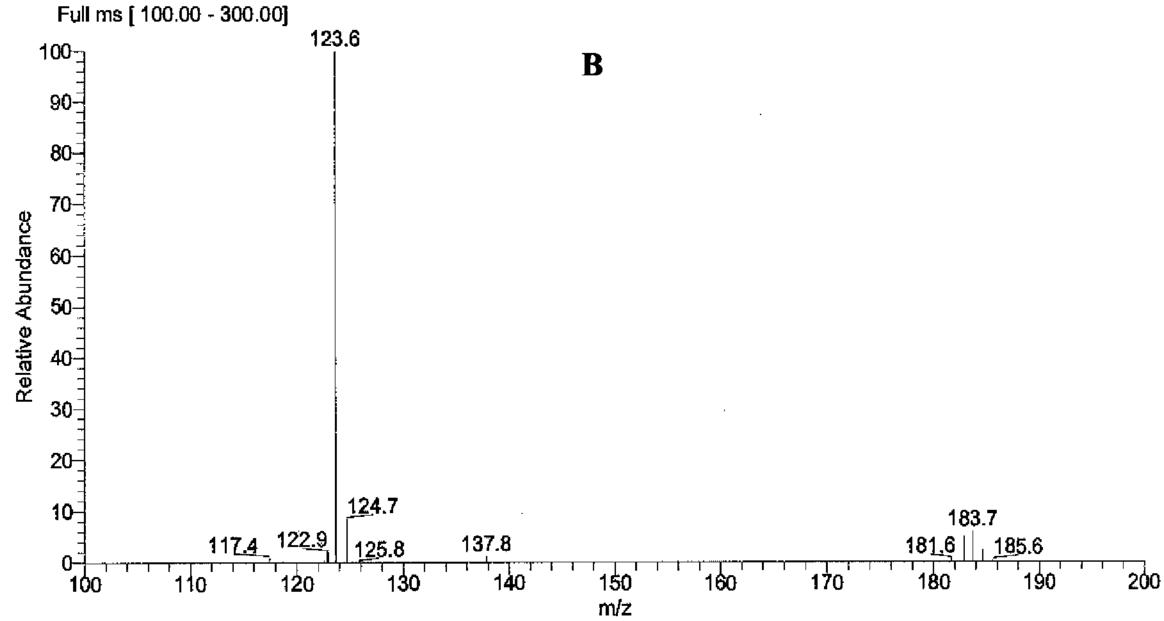


Figure 2.10: The mass spectra of 4-methylcatechol generated by the polyphenol oxidase-catalysed reaction with *p*-cresol (**A**) and commercially available 4-methylcatechol (**B**).

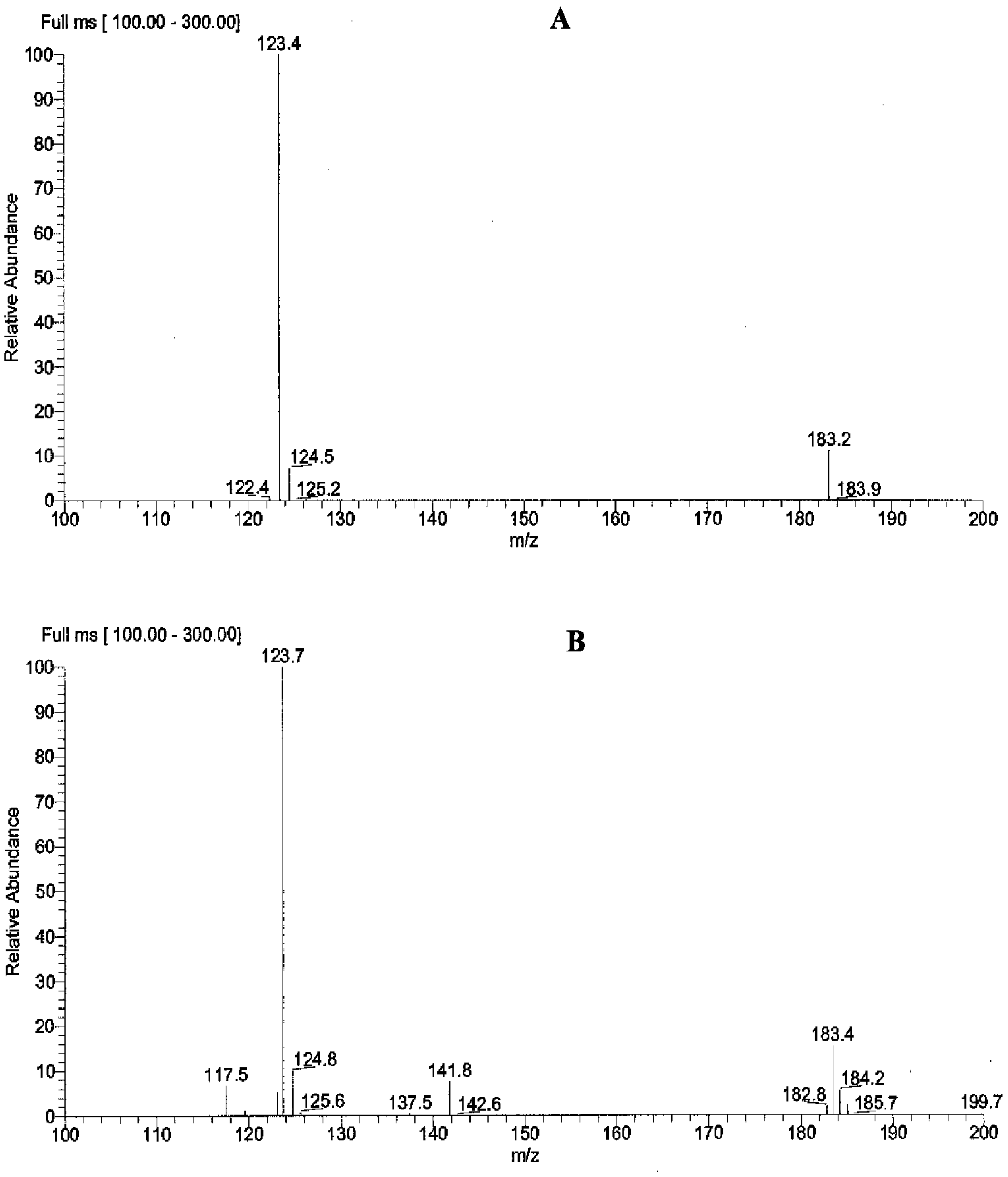


Figure 2.11: The mass spectra of commercially available 3-methylcatechol (**A**) and 3-methylcatechol generated by the polyphenol oxidase-catalysed reaction with *m*-cresol (**B**).

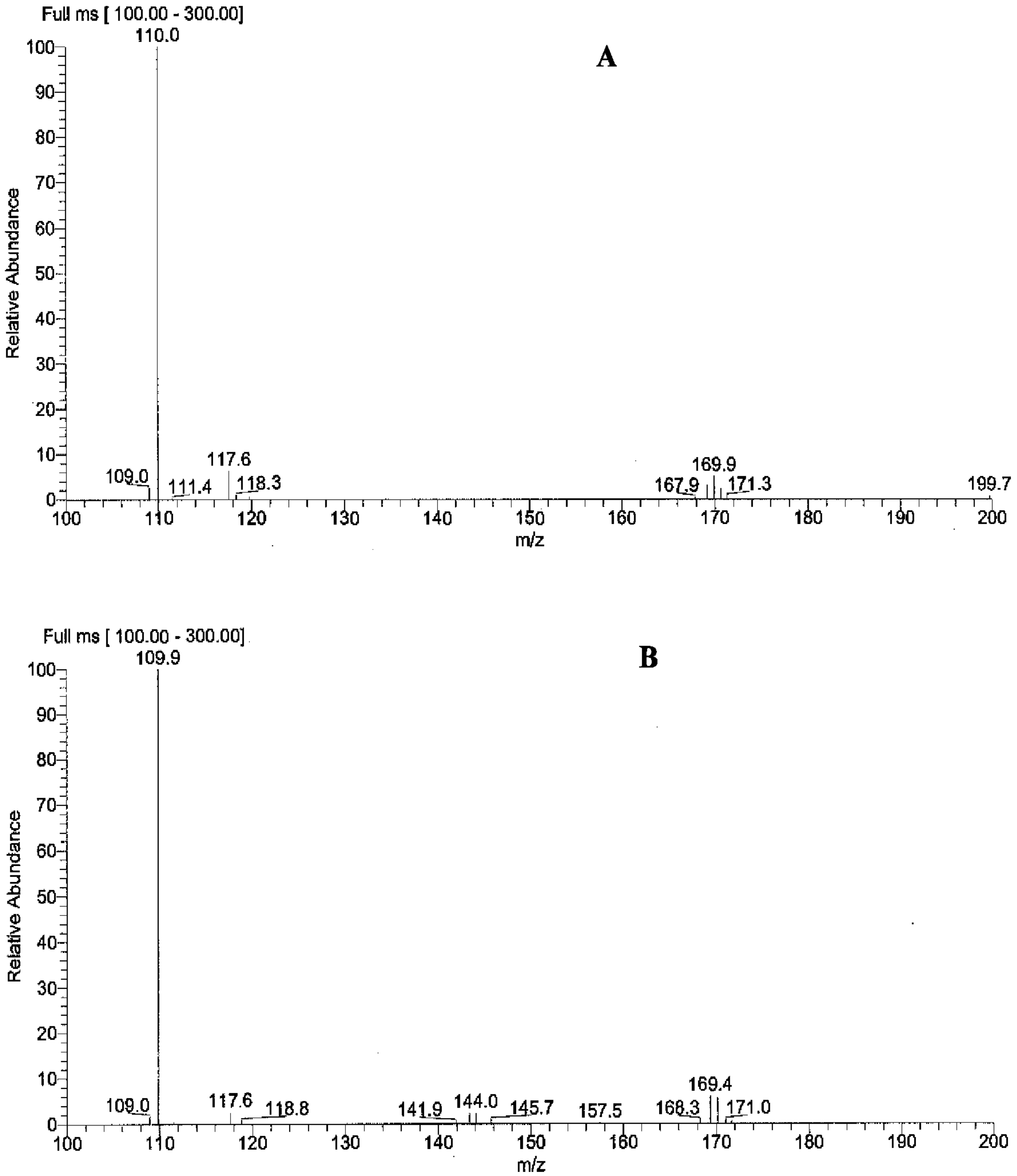


Figure 2.12: The mass spectra of commercially available catechol (**A**) and catechol generated by the polyphenol oxidase-catalysed reaction with phenol (**B**).

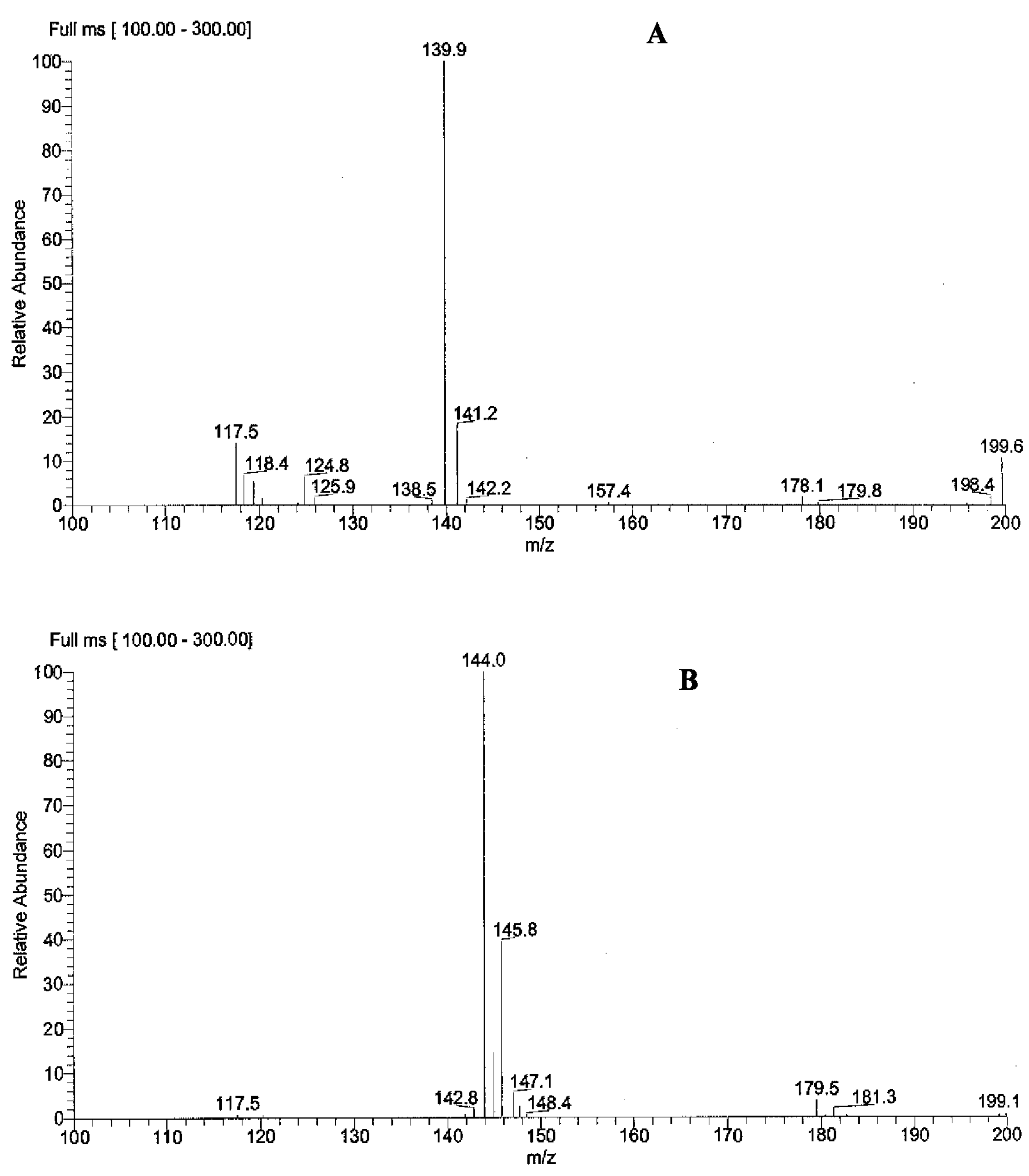


Figure 2.13: The mass spectra of 4-methoxycatechol generated by the polyphenol oxidase-catalysed reaction with 4-methoxyphenol (**A**) and 4-chlorocatechol generated by the polyphenol oxidase-catalysed reaction with 4-chlorophenol (**B**).

Using ESI it was possible to obtain the molecular weights of the catechols generated by the polyphenol oxidase-catalysed reactions with model phenolic compounds. The molecular weights of the catechols produced commercially and by polyphenol oxidase are tabulated in Table 2.2. Similar molecular masses were produced for the commercially available and enzymatically produced catechols which indicated that LC-MS could be used to accurately determine the molecular weights of catechols generated by polyphenol oxidase. The use of LC-MS to identify catechols generated by polyphenol oxidase had not been previously reported in the literature.

Table 2.2: Molecular masses of commercially available catechols and polyphenol oxidase-generated catechols, determined by LC-MS.

Substrates	**Corresponding catechols**	**Molecular masses of catechols**	**Molecular masses of commercially available catechols from LC-MS**	**Molecular masses of catechols after enzyme reaction from LC-MS**
m-cresol	3-methylcatechol	124.14	123.90	123.81
p-cresol	4-methylcatechol	124.14	123.87	123.83
phenol	catechol	110.12	109.70	109.70
4-chlorophenol	4-chlorocatechol	145.57	n/a	145.50
4-methoxyphenol	4-methoxycatechol	139.14	n/a	139.80

n/ a = not available commercially

2.3.4 The influence of chitosan, gelatin and PEG on the reaction of polyphenol oxidase-catalysed reaction with phenols

The effects of adding chitosan, gelatin and PEG to the reaction system during the polyphenol oxidase-catalysed reaction with phenols were monitored. The ability of chitosan, gelatin and PEG to remove *o*-quinones from solution was measured in terms of *o*-quinone HPLC peak areas. Substrate conversion and catechol concentrations were also monitored and compared to controls.

The addition of 12 – 40g/L chitosan to the polyphenol oxidase-catalysed reaction with 2mM 4-methoxyphenol was studied (Table 2.3). Control experiments of chitosan incubated with 2mM 4-methoxyphenol revealed that substrate adsorption did not occur. The use of 40g/L chitosan resulted in the highest adsorption, of 97.87% *o*-quinone. Amine groups in the chitosan polymer were able to react with *o*-quinones, removing them from solution (Sun and Payne, 1996). However, the removal of *o*-quinones from solution did not alter polyphenol oxidase activity, as 4-methoxyphenol transformation was similar to the control experiment in the absence of chitosan. This conclusion is in accordance with the results of Ikehata and Nicell (2000) who demonstrated that the presence of 400mg/L chitosan did not improve the transformation of 2mM phenol and was not able to protect polyphenol oxidase from inactivation.

The production of 4-methoxycatechol, by polyphenol oxidase, in the presence of chitosan was compared to the control experiment that contained no chitosan. In the presence of chitosan, there was nearly 100% adsorption of 4-methoxycatechol when compared to the control experiment. The decrease in the concentration of 4-methoxycatechol in the presence of chitosan could be attributed to reactive groups on the chitosan that are capable of binding 4-methoxycatechol.

The effect of PEG, over the concentration range of 20 – 70g/L, on the reaction with 2mM 4-methoxyphenol was studied and the results indicated that substrate adsorption did not occur. Significantly less *o*-quinone was adsorbed to PEG when compared to chitosan, with the addition of 70g/L PEG resulting in 49.38% *o*-quinone adsorption (Table 2.3). Ikehata and Nicell (2000) demonstrated that the use of 400mg/L PEG did not improve phenol transformation, and significantly higher concentrations of PEG were used in this study and the transformation of 4-methoxyphenol in the presence of PEG was similar to the control experiment without PEG. The inability of PEG to protect polyphenol oxidase could be attributed to differences in the nature of the products of polyphenol oxidase and peroxidase (Ikehata and Nicell, 2000). The peroxidase-catalysed polymerisation of phenols yields precipitating polymers and hydrogen bonding sites of PEG could interact with hydroxyl groups on these polymers thus reducing peroxidase inactivation by blocking hydrogen bonding sites (Nakamoto and Machida, 1992). In contrast, the polyphenol oxidase-catalysed reaction with phenols yields *o*-quinones that react to form soluble, low molecular weight polymers which may be unable to react with PEG (Ikehata and Nicell, 2000).

Adsorption of 4-methoxycatechol to PEG did not occur, demonstrated by the levels of 4-methoxycatechol in solution being similar to the control experiment in the absence of PEG.

The inclusion of 20 – 40g/L gelatin to the polyphenol oxidase-catalysed reaction with 2mM 4-methoxyphenol was studied, and the control experiments of gelatin incubated in substrate alone indicated that substrate adsorption to gelatin did not occur. The addition of 40g/L gelatin resulted in 94.58% *o*-quinone adsorption (Table 2.3). The presence of gelatin did not improve 4-methoxyphenol transformation, in that it was similar to the control experiment without gelatin. Thus gelatin was not able to reduce polyphenol oxidase inactivation. There was 29.22% - 43.10% adsorption of 4-methoxycatechol to the gelatin, which was considerably lower than the adsorption by chitosan.

Table 2.3: The influence of chitosan, PEG 6000 and gelatin on the adsorption of *o*-quinone and 4-methoxycatechol from solution during the reaction of polyphenol oxidase with 2mM 4-methoxyphenol.

Chitosan			**PEG 6000**			**Gelatin**		
g/L	**Quinone adsorption (%)**[a]	**4-Methoxy-catechol adsorption (%)**[b]	**g/L**	**Quinone adsorption (%)**[a]	**4-Methoxy-catechol adsorption (%)**[b]	**g/L**	**Quinone adsorption (%)**[a]	**4-Methoxy-catechol adsorption (%)**[b]
12	96.53	98.95	20	23.76	0	20	77.53	29.22
20	97.75	99.39	40	29.87	0	30	85.82	36.48
28	97.82	99.43	60	46.45	0	40	94.58	43.10
40	97.87	99.46	70	49.38	0			

[a]: Percentage quinone adsorption as compared to the control experiment after 3.5 hours

[b] : Percentage catechol adsorption as compared to the control experiment after 3.5 hours

2.3.5 The effect of buffers on phenol removal and catechol production

The effects of reacting polyphenol oxidase with phenols dissolved in various buffers at pH 6.8 were studied to determine the effects on the cresolase and catecholase activities of the enzyme, with the objective of determining conditions resulting in increased catechol production. Polyphenol oxidase has been reported to exhibit maximum catalytic activity around pH 7 and the enzyme is generally more active in slightly acidic buffers (Ikehata and Nicell, 2000).

Phenol conversion and catechol production were monitored during the biocatalytic activity of polyphenol oxidase with phenol (Figure 2.14), *p*-cresol (Figure 2.15), *m*-cresol (Figure 2.16) and 4-chlorophenol (Figure 2.17). The highest conversion of phenol, *p*-cresol and *m*-cresol was found to occur during reactions carried out in water, while the greatest 4-chlorophenol conversion was observed in sodium acetate buffer. During the reaction of 4-chlorophenol in borate buffer, no substrate conversion was observed, presumably due to inhibition of the cresolase activity of polyphenol oxidase by borate. Decreased *p*-cresol, *m*-cresol and phenol conversion was also observed in reactions carried out in borate buffer.

Catechol products were found to accumulate in solution at significantly higher concentrations in water than in buffer for each phenolic substrate. The reduced levels of catechol observed during reactions in borate buffer are presumably due to borate inhibition of the oxidation of catechols by polyphenol oxidase, as observed by Yasunobu and Norris (1967). The chemical structure of each phenolic substrate also influenced the amount of catechol produced, with the highest concentrations being produced using *p*-cresol and *m*-cresol as substrates. Reactions carried out in potassium phosphate and sodium acetate buffers did not allow catechols to accumulate in solution as they were rapidly oxidized to *o*-quinones, presumably as a result of the higher catecholase activity of polyphenol oxidase in these buffers.

These results indicated that water was a better reaction medium for catechol production, as the catecholase activity of polyphenol oxidase was lower in water than in potassium phosphate and sodium acetate buffers, which enabled catechol products to accumulate in solution.

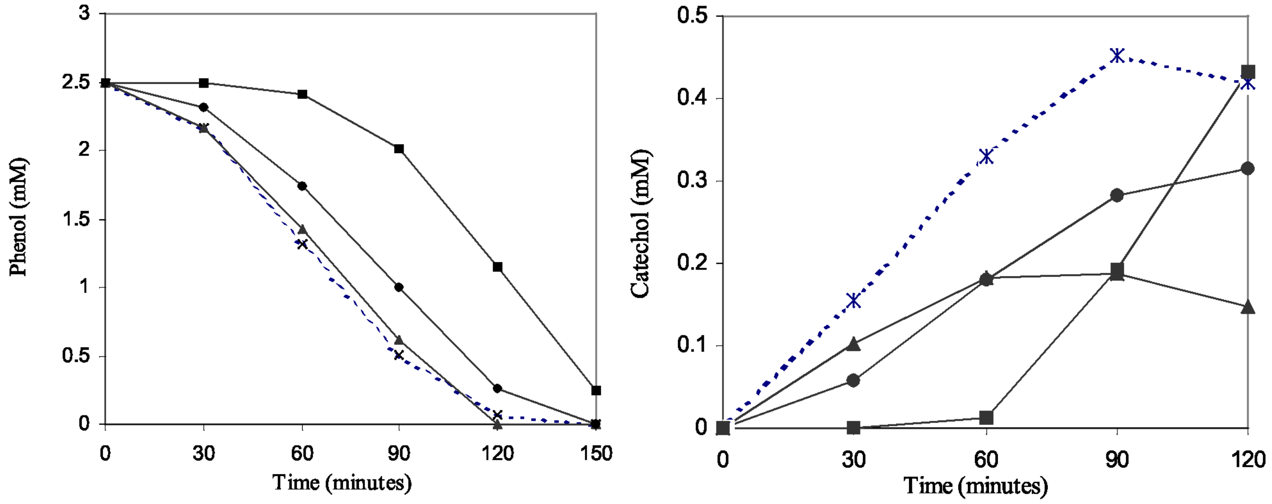


Figure 2.14: Reaction of 1.87U polyphenol oxidase with 2.5mM phenol demonstrating phenol conversion and catechol production in water (x); 75mM borate buffer (■); 50mM potassium phosphate buffer (▲) and 50mM sodium acetate buffer(●).

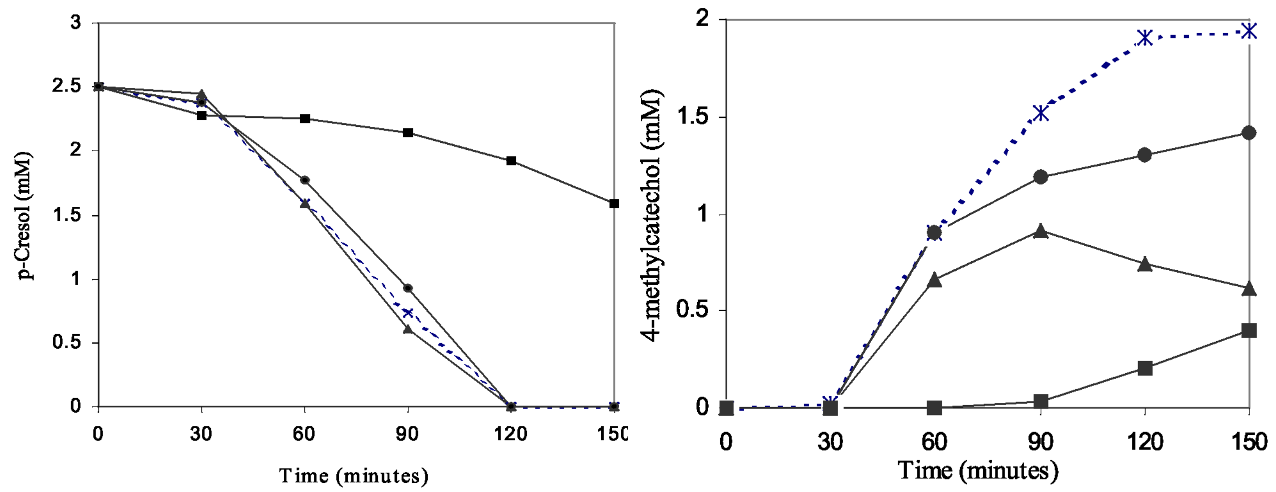


Figure 2.15: Reaction of 1.35U polyphenol oxidase with 3 mM *p*-cresol demonstrating *p*-cresol conversion and 4-methylcatechol production in water (x); 75mM borate buffer (■); 50mM potassium phosphate buffer (▲) and 50mM sodium acetate buffer(●).

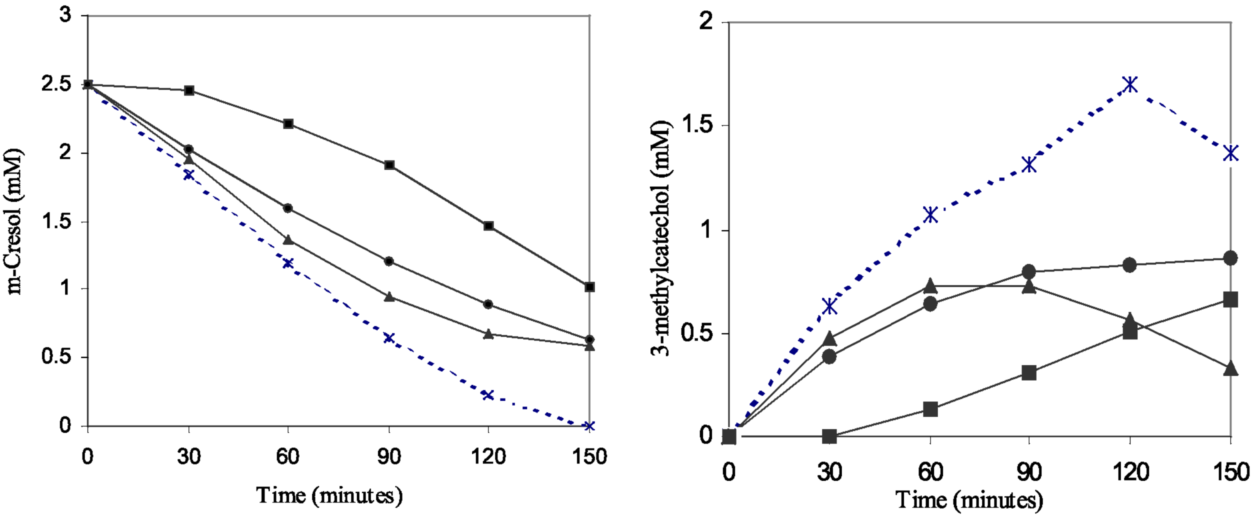


Figure 2.16: Reaction of 1.41U polyphenol oxidase with 2.5 mM *m*-cresol demonstrating *m*-cresol conversion and 3-methylcatechol production in water (x); 75mM borate buffer (■); 50mM potassium phosphate buffer (▲) and 50mM sodium acetate buffer(●).

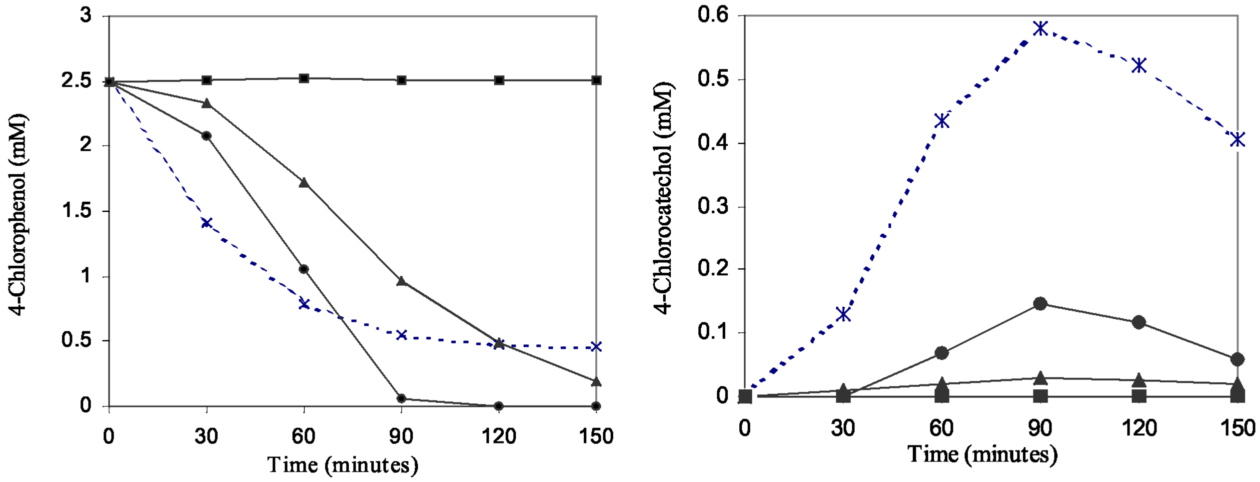


Figure 2.17: Reaction of 1.8U polyphenol oxidase with 2.5 mM 4-chlorophenol demonstrating 4-chlorophenol conversion and 4-chlorocatechol production in water (x); 75mM borate buffer (■); 50mM potassium phosphate buffer (▲) and 50mM sodium acetate buffer(●).

2.3.6 The effect of borate and ascorbate on polyphenol oxidase activity

The aim in this section of the study was to determine the effect of adding varying ascorbate concentrations to reaction media containing water and borate buffer by measuring substrate conversion by polyphenol oxidase. The activity of polyphenol oxidase in water and in 75mM borate buffer, in the presence and absence of ascorbate, during the reaction with *p*-cresol (Figure 2.18) and 4-chlorophenol (Figure 2.19) was monitored using HPLC. The reaction of *p*-cresol and 4-chlorophenol in 75mM borate buffer indicated inhibition of the cresolase activity of polyphenol oxidase. The addition of 12.5mM ascorbate resulted in enzyme inactivation after 60 minutes using *p*-cresol, and after 30 minutes using 4-chlorophenol, as the substrates. 5mM ascorbate caused 50% polyphenol oxidase inactivation after 130 minutes (Golan-Goldhirsh and Whitaker, 1984). Thus, the addition of ascorbate during the reaction of polyphenol oxidase in water decreased substrate conversion due to enzyme inactivation. However, the combination of borate and ascorbate in the reaction system led to a significant decrease in enzyme inactivation by ascorbate, resulting in more efficient substrate conversion.

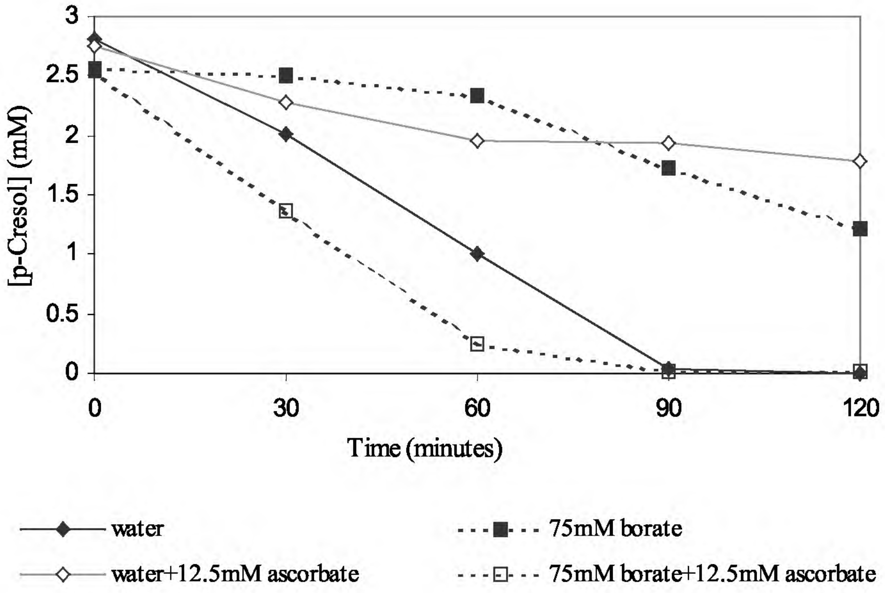


Figure 2.18: *p*-Cresol conversion by 1.3U polyphenol oxidase in the presence of ascorbate and borate.

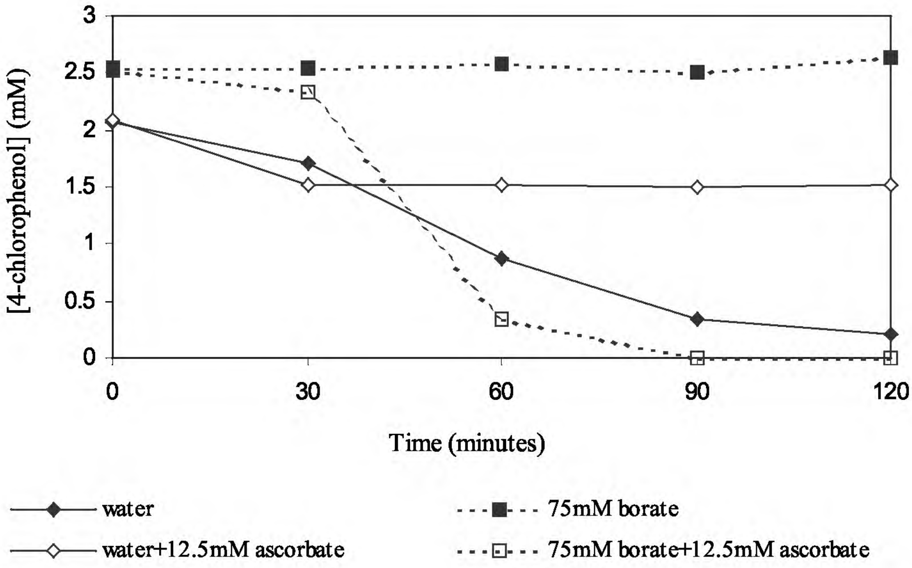


Figure 2.19: 4-Chlorophenol conversion by 1.8U polyphenol oxidase in the presence of ascorbate and borate.

Ascorbate oxidase activity of polyphenol oxidase was investigated to determine whether the enzyme was capable of utilising ascorbate as a substrate. After incubating polyphenol oxidase for 2 hours in different concentrations of ascorbate, dissolved in 75mM borate buffer, ascorbate oxidase activity was not detected. Thus, the decrease in ascorbate levels in solution could be attributed to its activity as a reducing agent for the reduction of *o*-quinones to catechols.

2.3.7 Optimisation of ascorbate and borate concentrations for catechol production

The borate concentrations were varied to determine the amount needed for the formation of the ascorbate-borate complex to minimise polyphenol oxidase inactivation and allow ascorbate to act as a reducing agent. The accumulation of catechol products during the reaction of varied amounts of polyphenol oxidase were monitored by HPLC, in water and in the presence of different concentrations of borate, ascorbate and mixtures of borate and ascorbate using *p*-cresol (Table 2.4), phenol (Table 2.5), *m*-cresol (Table 2.6) and 4-chlorophenol (Table 2.7) as substrates.

Reactions of polyphenol oxidase with *p*-cresol as the substrate, carried out in the presence of 75mM borate buffer or 12.5mM ascorbate, resulted in yields of 26.2% and 38.6% respectively, which are lower than the yields of 41.4% 4-methylcatechol produced in water (Table 2.4). The effect of borate buffer concentrations on 4-methylcatechol production were studied, and the addition of 12.5mM ascorbate to the *p*-cresol solutions in 25mM, 50mM and 75mM borate buffer, respectively, resulted in the yields of 4-methylcatechol increasing to 72%, 80% and 100%. Borate concentrations lower than 75mM appeared to be insufficient to form a complex with 12.5mM ascorbate and were unable to effectively reduce polyphenol oxidase inactivation by ascorbate. However, during the reaction of polyphenol oxidase with phenol, a yield of 26.8% catechol was observed in 12.5mM ascorbate, which is higher than the yield in water or 75mM borate buffer (Table 2.5). The addition of 12.5mM ascorbate to phenol in 50mM borate buffer increased the catechol yield to 77.7%. However, increasing the ascorbate concentration to 15mM and 18mM decreased catechol yields to 71.6% and 58.8% respectively. A similar trend to the reaction of *p*-cresol was observed during the reaction of phenol with 12.5mM ascorbate and 75mM borate buffer as 100% catechol was produced. Thus the results of the reaction of polyphenol oxidase with phenol and *p*-cresol indicate that ascorbate concentrations higher than 12.5mM ascorbate result in decreased catechol yields.

Reactions of polyphenol oxidase with *m*-cresol as the substrate, carried out in the presence of 75mM borate buffer or 12.5mM ascorbate, resulted in yields of 51% and 10.3% respectively, which are lower than the yields of 61.3% 3-methylcatechol produced in water (Table 2.6). This trend was similar to the reaction of polyphenol oxidase with *p*-cresol. However, the reaction of *m*-cresol in a solution of 75mM borate buffer containing 12.5mM ascorbate did not increase 3-methylcatechol yields to 100%, as in the case of the reactions with *p*-cresol and phenol, and increasing the borate buffer concentration to 100mM led to a further decrease in the yield of 3-methylcatechol to 22.1%. The addition of a reducing agent to a solution of *m*-cresol was unable to increase 3-methylcatechol yields.

In contrast, during the reaction of polyphenol oxidase with 4-chlorophenol, a yield of 24.9% 4-chlorocatechol was observed in 12.5mM ascorbate, which was higher than the yield in water or 75mM borate (Table 2.7). The reaction of 4-chlorophenol in a solution of 75mM borate buffer containing 12.5mM ascorbate increased 4-chlorocatechol yields to 100%. Increasing the borate concentration to 100mM resulted in decreased yields of 67.5% 4-chlorocatechol.

Table 2.4: 4-Methylcatechol yields obtained after the polyphenol oxidase-catalysed reaction using *p*-cresol in the presence of different borate and ascorbate concentrations.

[Initial *p*-cresol] (mM)	Borate buffer (mM)	Ascorbate (mM)	Units PPO (U)	Reaction time (minutes)	Final [4-methylcatechol] (mM)	% yield
1.16	0	0	1.20	120	0.66	41.4
2.75	0	12.5	1.80	120	1.06	38.6
1.69	25	6	0.90	120	1.05	62.1
1.69	25	12.5	0.90	120	1.22	72.0
1.31	50	12.5	1.20	120	1.09	80.0
2.56	75	0	1.80	120	0.67	26.2
3.06	75	12.5	1.20	180	3.04	99.4
2.6	75	12.5	1.50	165	2.6	100.00
3.06	75	15	1.20	180	3.03	99.0
2.66	100	12.5	1.50	180	2.57	96.6

Table 2.5: Catechol yields obtained after the polyphenol oxidase-catalysed reaction using phenol in the presence of different borate and ascorbate concentrations.

[Initial phenol] (mM)	Borate buffer (mM)	Ascorbate (mM)	Units PPO (U)	Reaction time (minutes)	Final [catechol] (mM)	% yield
2.92	0	0	1.64	150	0.28	9.6
2.13	0	12.5	1.95	120	0.57	26.8
2.53	50	12.5	1.64	150	1.97	77.7
2.54	50	15	1.64	150	1.82	71.6
2.53	50	18	1.64	150	1.49	58.8
2.30	75	0	1.95	120	0.13	5.7
2.60	75	12.5	1.64	150	2.34	89.9
3.02	75	12.5	2.40	120	3.02	100.0
2.62	75	15	1.64	150	2.33	88.9
2.62	75	18	1.64	150	2.2	84.0

Table 2.6: 3-Methylcatechol yields obtained after the polyphenol oxidase-catalysed reaction using *m*-cresol in the presence of different borate and ascorbate concentrations.

[Initial *m*-cresol] (mM)	Borate buffer (mM)	Ascorbate (mM)	Units PPO (U)	Reaction time (minutes)	Final [3-methylcatechol] (mM)	% yield
2.43	0	0	1.95	120	1.49	61.3
2.42	0	12.5	1.95	120	0.25	10.3
2.00	75	0	1.95	120	1.02	51.0
2.78	75	12.5	1.62	150	0.9	32.4
1.99	75	12.5	1.80	120	0.67	34.0
2.17	100	12.5	1.80	180	0.48	22.1

Table 2.7: 4-chlorocatechol yields obtained after the polyphenol oxidase-catalysed reaction using 4-chlorophenol in the presence of different borate and ascorbate concentrations.

[Initial 4-chlorophenol] (mM)	Borate buffer (mM)	Ascorbate (mM)	Units PPO (U)	Reaction time (minutes)	Final [4-chlorocatechol] (mM)	% yield
2.56	0	0	1.46	120	0.43	16.8
2.09	0	12.5	1.95	120	0.52	24.88
2.54	75	0	1.95	120	0.00	0.0
2.35	75	12.5	1.80	180	1.55	65.96
2.52	75	12.5	1.95	120	1.96	77.78
3.17	75	12.5	2.40	180	3.17	100
2.4	100	12.5	1.80	180	1.62	67.5

Polyphenol oxidase catalysed reactions with phenol, *p*-cresol and 4-chlorophenol carried out in 75mM borate buffer containing 12.5mM ascorbate resulted in 100% yield of catechol products; an exception was observed with *m*-cresol. Reactions in solutions of 75mM borate buffer and ascorbate concentrations higher than 12.5mM resulted in decreased catechol yields, presumably as a result of polyphenol oxidase inactivation by free ascorbate in solution. Thus, manipulation of borate and ascorbate concentrations can lead to the achievement of 100% yield of certain catechol products. For the reaction conditions used in this study, 75mM borate buffer was sufficient to complex with 12.5mM ascorbate minimising polyphenol oxidase inactivation, while allowing the ascorbate to act as a reducing agent. As a comparison with results of other researchers, after the reaction of polyphenol oxidase with *p*-cresol in chloroform, 71% yield of 4-methylcatechol was obtained using L-ascorbic acid to reduce *o*-quinones (Kazandjian and Klibanov, 1985) while 56 - 59% conversion of tyrosine to L-DOPA was evidenced after 170 hours of reaction between nylon-immobilised polyphenol oxidase and 2.5mM tyrosine and ascorbic acid (Pialis *et al.*, 1996).

2.3.8 Effect of pH on catechol extraction using organic solvents

There are numerous methods available to extract catechol from the reaction system after the reaction of polyphenol oxidase with phenolic substrates, and of these the use of diethyl ether for the extraction of catechol dissolved in 75mM borate buffer and 12.5mM ascorbate was investigated. Preliminary investigations revealed that ethyl acetate was just as effective as diethyl ether for the extraction of catechol products. Since the extraction process was complicated by the formation of a catechol-borate complex under weakly basic conditions, the effect of changing the pH of the catechol solution before extraction was determined in the presence of borate (Table 2.8). The concentration of catechol in the aqueous phase was measured by HPLC after each solvent extraction. 20.6% catechol was extracted into the organic solvent when catechol was dissolved in borate buffer, without changing the pH of the solution, but acidification resulted in nearly 100% extraction of catechol, as was observed with water as a solvent. The advantage of using organic solvents to extract the catechol product was the absence of ascorbate as a contaminant, as it remained in the aqueous phase. Thus, acidification of the reaction medium prior to the extraction of catechol products is necessary for optimal product extraction.

Table 2.8: The effect of acidification on the extraction of catechol dissolved in 75mM borate buffer (pH 7.8).

	water (no pH change)	**75mM borate buffer (pH 7.8)**	**75mM borate buffer (acidified to pH 5)**
[Initial catechol] (mM)	2.85	2.52	2.76
[Catechol] (mM) after first solvent extraction	0.41	2.31	0.46
[Catechol] (mM) after second solvent extraction	0.039	2.00	0.037
% catechol extracted	98.6	20.6	98.6

2.3.9 Catechol extraction by adsorption on Amberlite XAD-4 resin

The adsorption of catechol on Amberlite XAD-4 resin was an alternative method for extracting catechol from the reaction medium. Both catechol and phenol adsorb to the resin and differences in the adsorption of each compound to the resin were investigated by adding 0.4 - 1.6g XAD-4 resin to 50mL mixtures of 1.5mM catechol and 0.5mM phenol prepared in 75mM borate buffer (pH 7.8) with 12.5mM ascorbate. Phenol preferentially adsorbed to the Amberlite resin with 95% phenol adsorption and 27% catechol adsorption occurring after 3 hours with very little further adsorption after 5 hours (Table 2.9). Although using 0.4g resin per 50mL solution resulted in the highest amount of catechol being adsorbed per gram resin, only 12% catechol adsorption occurred. The experiment was repeated using more resin to increase the adsorption of catechol.

Table 2.9: Phenol and catechol adsorption to varying proportions of Amberlite XAD-4 resin[a] after 3 and 5 hours.

	3 hours			**5 hours**		
resin (g)	**% phenol adsorption**	**% catechol adsorption**	**µmoles catechol adsorbed/g resin**	**% phenol adsorption**	**% catechol adsorption**	**µmoles catechol adsorbed/g resin**
0.4	64	12	23	64	12	23
0.8	82	18	17	89	22	21
1.2	89	22	14	82	23	14
1.6	95	27	13	95	29	14

[a]: resin placed into 50mL of 1.5mM catechol and 0.5mM phenol in 75mM borate buffer (pH 7.8) with 12.5mM ascorbate.

Increasing the proportions of XAD-4 resin resulted in 100% adsorption of phenol within 1 hour while most catechol adsorption also occurred within 1 hour (Table 2.10). Using 20g XAD-4 resin resulted in the highest concentration of catechol adsorption of 1.42mM after 210 minutes. Although using 8g resin per 50mL solution resulted in the highest amount of catechol being adsorbed per gram resin, only 0.79mM catechol adsorption occurred. Thus a high ratio of resin to reaction volume is required to obtain efficient catechol adsorption.

Table 2.10: Catechol adsorption to varying proportions of Amberlite XAD-4 resin[a] after 60, 150 and 210 minutes.

	Concentration catechol adsorbed to XAD-4 resin (mM)			
Time (minutes)	**8g**	**12g**	**16g**	**20g**
60	0.83	1.13	1.01	1.19
150	0.75	0.86	1.03	1.18
210	0.79	1.05	1.11	1.42
Final µmoles catechol adsorbed/g resin	4.9	4.4	3.5	3.5

[a]: resin placed into 50mL of 1.5mM catechol and 0.5mM phenol in 75mM borate buffer (pH 7.8) with 12.5mM ascorbate.

The solution containing 20g XAD-4 resin was poured into a column and the adsorbed catechol and phenol were eluted using methanol acidified to pH 2 using concentrated HCl. Fractions (5mL) were collected from the column and analysed by HPLC. Most of the catechol appeared to elute in the first 5 fractions, but ascorbate and phenol were also present in the fractions. The subsequent fractions consisted of numerous contaminating peaks which could have been impurities present in the resin, or oxidised products.

These results demonstrate that the adsorption of catechol products to Amberlite XAD-4 resin is a viable approach for the extraction of catechols from solution as an alternative to the use of organic solvents. Even though a high ratio of resin to reaction volume is required for effective catechol removal, the resin could be reused. The extraction procedure could be further optimised by ensuring 100% phenol conversion by polyphenol oxidase before the addition of the resin. The subsequent elution of catechol products from the resin, using acidified methanol, successfully concentrated the catechol products, resulting in smaller volumes of product solution, and the catechol products could be further purified by solvent extraction using decreased volumes of solvent.

2.3.10 Product purification by recrystallization from hexane

Recrystallization of catechol from a mixture of phenol and catechol was investigated as a method to separate catechol from residual phenol. A mixture of 50mM catechol and 25mM phenol was prepared and acidified to pH 2, before extraction using diethyl ether. After removal of the organic solvent, the residue was re-dissolved in hexane and refluxed for 2 hours at 60°C. The resulting crystals were dissolved in water and analysed by HPLC. There was 98.5% catechol recovery with 0.07% phenol present as a contaminant.

The experiment was repeated using 2.7mM phenol, prepared in 75 mM borate buffer (pH 7.8) containing 12.5mM ascorbate, but 19U polyphenol oxidase was added to the phenol solution to produce catechol. After 2 hours 1.98mM catechol and 0.72mM phenol were extracted using diethyl ether and the residue was re-dissolved in hexane and refluxed. When these significantly lower catechol concentrations were used, only 40 - 60% catechol was recovered. This yield was similar to that reported by Held *et al.* (1999), using a whole cell biocatalyst for the production of 3-substituted catechols, where 59% overall yield was obtained. Product purification using hexane recrystallization is a successful method to remove phenol but in a process, to achieve significant yields of catechol products, it would be more beneficial to obtain 100% conversion of phenol.

2.3.11 Isolation and extraction of 4-chlorocatechol and 4-methoxycatechol

The methods developed as described above were used to produce pure samples of 4-chlorocatechol and 4-methoxycatechol required for the calculation of molar absorption coefficients, and hence for the determination of kinetic parameters of polyphenol oxidase. 4-Chlorophenol and 4-methoxyphenol were dissolved in 75mM borate buffer (pH 7.8) containing 12.5mM ascorbate as these were the reaction conditions found to result in 100% conversion of phenol to catechol products (Section 2.3.7).

The reaction volumes were increased from 20mL to 300mL of 3mM 4-chlorophenol and 4-methoxyphenol, and 36U and 27U of polyphenol oxidase were added to each phenol respectively. After 100% conversion of 4-chlorophenol and 4-methoxyphenol, diethyl ether was used to extract the catechol products, and 98 - 100% recovery of 4-chlorocatechol and 4-methoxycatechol was achieved.

This investigation has shown that in order to achieve efficient catechol production and high product purity, it was necessary to use sufficient polyphenol oxidase to ensure 100% conversion of the phenolic substrate. This minimised loss of catechol as it reduced the number of steps required to obtain pure catechol extracts.

The study demonstrated that the procedure could be scaled up in terms of higher initial substrate concentrations and volumes, but the borate and ascorbate concentrations would need to be increased if higher substrate concentrations were used. If larger volumes were used, Amberlite XAD-4 resin could be used to adsorb the catechol, residual phenols and ascorbate, and acidic methanol elution of the adsorbents would result in lower volume, higher concentration eluent, which would reduce the amount of solvent required to extract the catechol and residual phenol. This procedure potentially could be developed into a process for the enzymatic production of catechols by using borate and ascorbate in the reaction medium to obtain 100% conversion of phenolic substrates to catechol products using polyphenol oxidase.

2.3.12 Determination of catechol concentrations

4-Chlorocatechol and 4-methoxycatechol were not available commercially and could not be quantified by HPLC during reactions of polyphenol oxidase with 4-chlorophenol and 4-methoxyphenol respectively. Quantification of these catechols was required to calculate the molar absorption coefficients of *o*-quinones and hence to determine the kinetic parameters of polyphenol oxidase. The catechol peaks had been successfully identified using LC-MS, and a method developed to obtain high yields of pure 4-chlorocatechol and 4-methoxycatechol (Section 2.3.11).

Due to the small quantities of 4-chlorocatechol and 4-methoxycatechol obtained, it was difficult to accurately weigh the mass of each catechol, and a more accurate method to quantify these catechols was determined to be a colorimetric method. The colorimetric assay of Garcia *et al.* (2000) was used to quantify these catechols, using commercially available catechol as a standard compound. Thus the concentration of each 4-chlorocatechol and 4-methoxycatechol solution was measured, including the HPLC peak area. HPLC uses calibration curves of peak area against concentration to quantify peaks present on the chromatogram, and the peak area of a particular compound depends on the HPLC conditions used to perform the separation. The colorimetric method to quantify the catechols was used in conjunction with HPLC to obtain peak areas corresponding to concentrations. Calibration curves of 4-methoxyphenol (Figure 2.20) and 4-chlorophenol (Figure 2.21) were successfully constructed to quantify these compounds in further experiments.

This study has established that it is possible to identify novel products of polyphenol oxidase-reactions, to obtain pure fractions of these products, and to quantify the products colorimetrically and by HPLC.

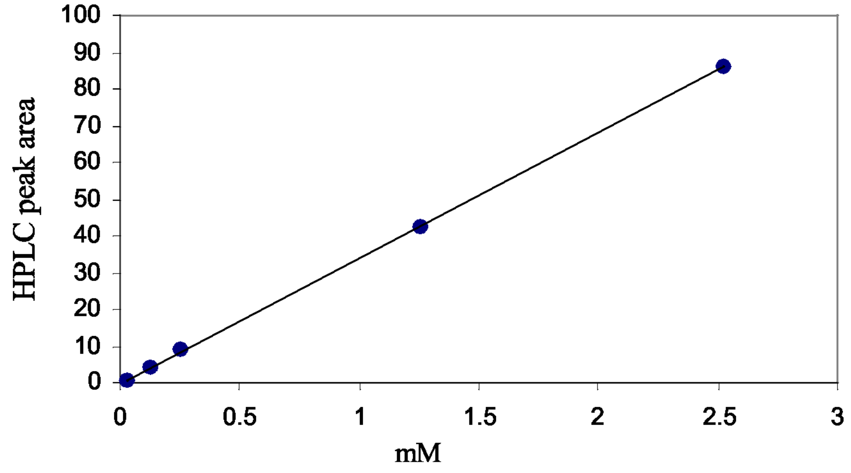


Figure 2.20: HPLC calibration curve of 4-methoxycatechol using a mobile phase of water-acetonitrile (60:40) ($r^2 = 0.99$).

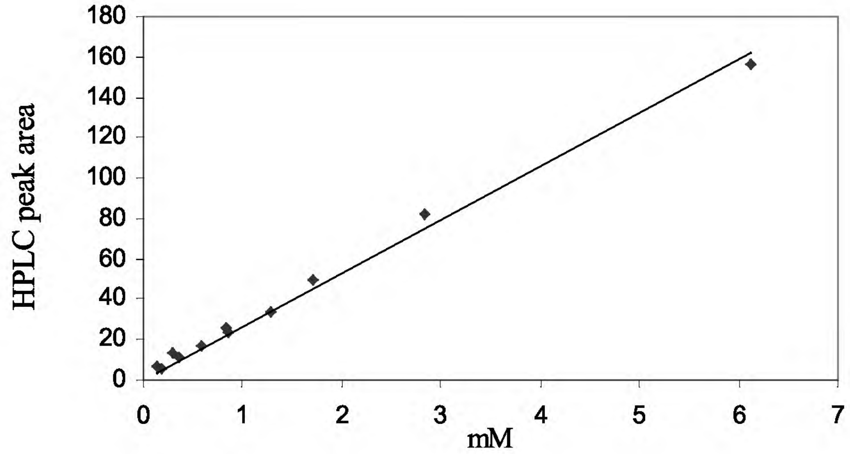


Figure 2.21: HPLC calibration curve of 4-chlorocatechol using a mobile phase of water-acetonitrile (60:40) ($r^2 = 0.99$).

2.3.13 Determination of molar absorption coefficients of *o*-quinones

o-Quinones are unstable compounds and, as a result, molar absorption coefficients are not generally available. However, these values were required to calculate kinetic parameters of polyphenol oxidase. Waite (1976) reported that several *o*-quinones are unstable above pH 6.8 and therefore, in this study, the oxidation of catechols to *o*-quinones was conducted in potassium phosphate buffer (50mM, pH 6).

Oxidation of the respective catechols to *o*-quinones was conducted by adding sodium iodate to the solutions. The conversion of each catechol was measured periodically by HPLC and *o*-quinone formation was monitored simultaneously by measuring the absorbances of each *o*-quinone at the maximum wavelength. The molar absorption coefficients were calculated by correlating UV-visible absorbances with HPLC-determined concentrations. The molar absorption coefficients obtained correspond to literature values where available (Table 2.11).

Table 2.11: Determination of molar absorption coefficients[a] of *o*-quinones.

Substrate	**λ_{max}[a] of *o*-quinone**	**Experimental values ∊ ($M^{-1}.cm^{-1}$)**	**Literature values ∊ ($M^{-1}.cm^{-1}$)**
p-cresol	395	1161 ± 76	1460 ± 100 (Burton *et al.*, 1993); 1230 (Long and Alben, 1968); 1010 and 1400 (Waite, 1976)
m-cresol	395	2016 ± 32	
phenol	395	1812 ± 303	
4-chlorophenol	400	3873 ± 416	3800 ± 300 (Burton *et al.*, 1993)
4-methoxyphenol	395	2184 ± 341	1800 ± 100 (Burton *et al.*, 1993)

[a] : At wavelength of maximum absorbance

2.3.14 Determination of kinetic parameters of polyphenol oxidase in an aqueous medium

The kinetic parameters of polyphenol oxidase were measured, during the reaction of various phenolic substrates in potassium phosphate buffer. Initial rates of reaction of polyphenol oxidase with the phenolic substrates were measured at the wavelengths of maximum absorbance of the *o*-quinone products. Linear regression analysis of the data from each assay was used to determine the initial rates in terms of change in UV absorbance per minute. For each substrate, different concentration ranges were chosen, depending on the linearity of the measured rates. The molar absorption coefficients, calculated as described in Section 2.3.12, were used to convert the initial rates of absorbance increase to increases in *o*-quinone concentration per minute. Using the initial reaction rate data obtained, apparent K_M , V_{max} and the catalytic efficiency V_{max}/K_M were calculated using Hanes plots (Figures 2.22 - 2.26).

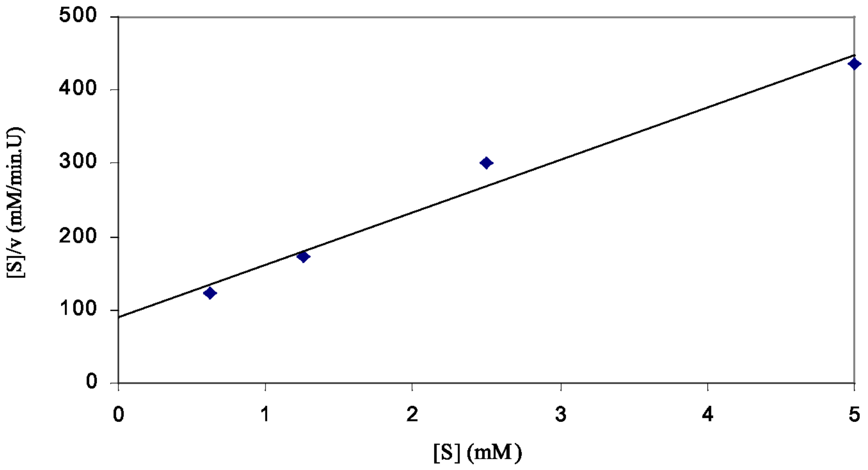


Figure 2.22: Hanes plot used to obtain K_M and V_{max} values for polyphenol oxidase conversion of phenol ($r^2 = 0.98$).

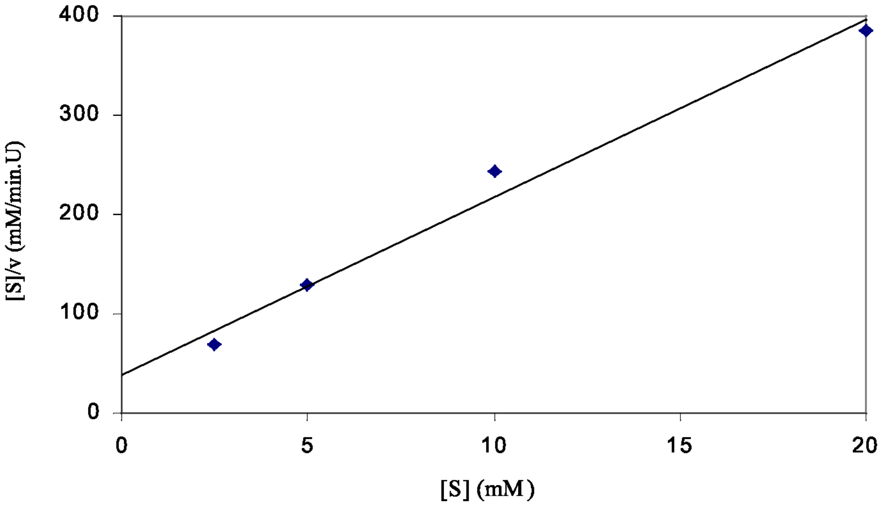


Figure 2.23: Hanes plot used to obtain K_M and V_{max} values for polyphenol oxidase conversion of *p*-cresol ($r^2 = 0.98$).

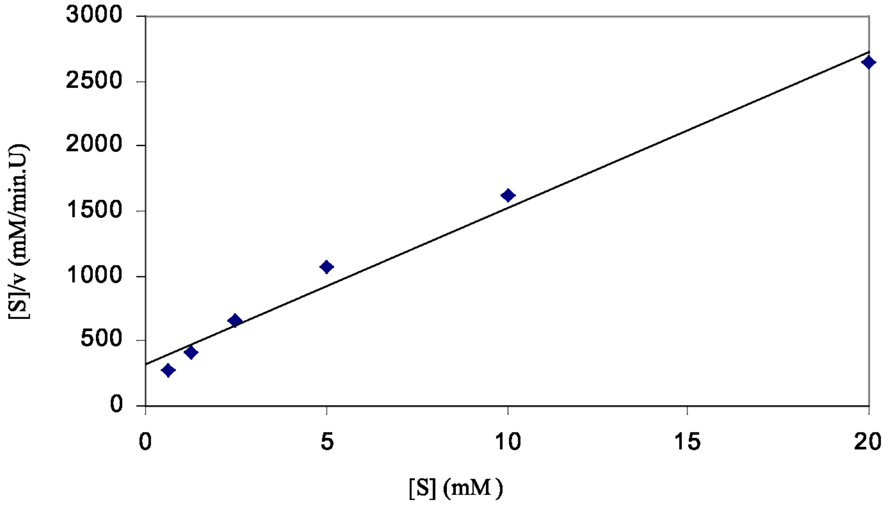


Figure 2.24: Hanes plot used to obtain K_M and V_{max} values for polyphenol oxidase conversion of m-cresol ($r^2 = 0.98$).

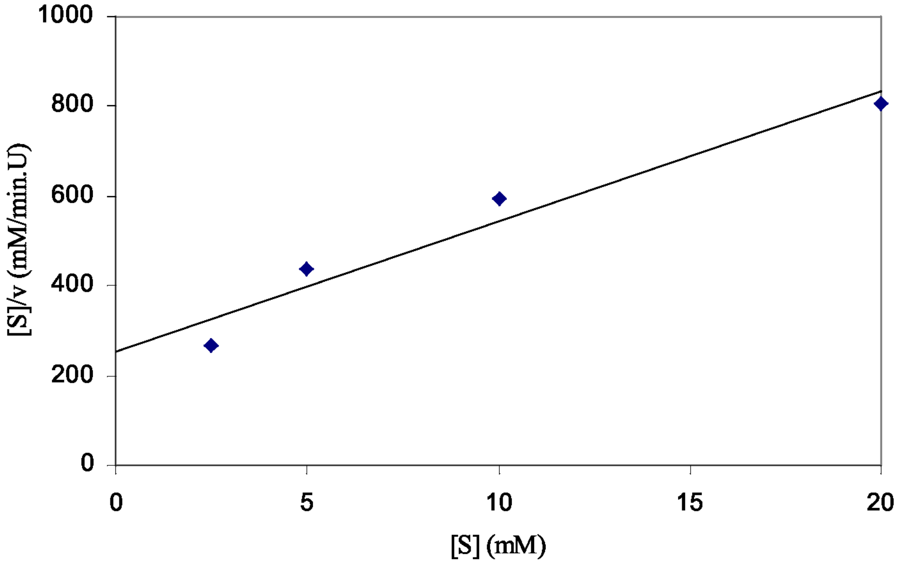


Figure 2.25: Hanes plot used to obtain K_M and V_{max} values for polyphenol oxidase conversion of 4-chlorophenol ($r^2 = 0.97$).

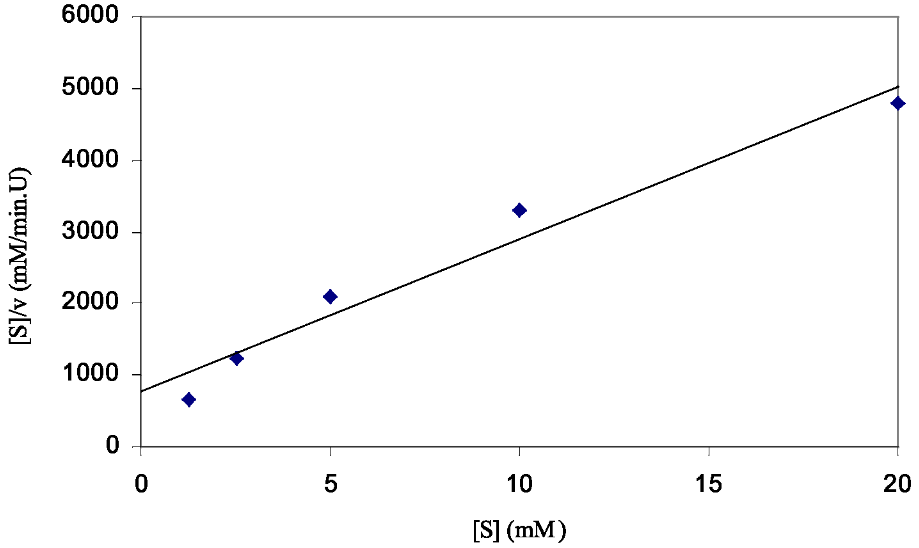


Figure 2.26: Hanes plot used to obtain K$_M$ and V$_{max}$ values for polyphenol oxidase conversion of 4-methoxyphenol ($r^2 = 0.95$).

A lag phase was observed in activity assays carried out using *p*-cresol and 4-chlorophenol as substrates. The presence of the lag phase resulted in initial reaction rates being similar for each substrate concentration used during the assay. However, the addition of a cofactor, 4-methylcatechol, allowed for the consistent measurement of kinetic parameters. The apparent K$_M$ values were calculated from the x-axis intercepts, while V$_{max}$ values were calculated from the y-axis intercepts on the Hanes plots. Results of the kinetic analysis of polyphenol oxidase reacting with the phenolic substrates are shown in Table 2.12.

Table 2.12: Apparent kinetic parameters determined for the reaction of polyphenol oxidase in potassium phosphate buffer.

Substrate	**Concentration range (mM)**	**4-Methylcatechol concentration (μM)**	**V_{max} ($mM.min^{-1}.U^{-1}$) (x 10^{2})**	**K_M (mM)**	**V_{max} / K_M ($min^{-1}.U^{-1}$) (x 10^{-3})**
phenol	0.625 - 5		1.21 ± 0.2	1.24	9.76
***p*-cresol**	2.5 - 20	1.25 - 10	3.5 ± 0.6	1.98	17.68
***m*-cresol**	0.625 - 20		0.77 ± 0.08	2.72	2.83
4-chlorophenol	1.25 - 20	0.625 - 10	0.41 ± 0.09	3.62	1.13
4-methoxyphenol	2.5 - 20		3.05 ± 0.6	4.42	6.90

The K_M value is generally accepted to indicate the ability of an enzyme to bind a substrate, while V_{max} / K_M (catalytic efficiency) demonstrates the ability of an enzyme to transform a substrate (Ryu and Dordick, 1989, cited by Burton *et al.*, 1993). The hydrophobicity of the substrates is likely to affect their reaction with the enzyme in an aqueous system, and Burton *et al.* (1993) noted a trend of increasing K_M values with increasing hydrophobicity during the reaction of polyphenol oxidase in chloroform. From Table 2.12, this trend was also apparent, with K_M values increasing with substrate hydrophobicity.

2.4 CONCLUSION

Polyphenol oxidase was successfully isolated from brown mushrooms (*Agaricus bisporus*) for use as a biocatalyst in the biotransformation of phenols. The resulting partially purified extract was stored as a freeze-dried powder with minimal loss in activity.

The enzymic reaction products generated during the polyphenol oxidase reaction with phenols in an aqueous system were successfully separated by HPLC and the catechol products identified using LC-MS. This study has revealed that catechols are products formed during the reaction of phenols with polyphenol oxidase without the addition of a reducing agent. Catechol products were found to accumulate as true intermediate products which were released into the reaction medium. This was an important outcome as catechols had not been isolated using this enzymatic system and previous researchers had relied on the addition of ascorbate to produce catechols. The biocatalytic system was manipulated to determine factors which would influence catechol yields.

Chitosan, PEG and gelatin were added to the reaction medium to determine their effects on the cresolase and catecholase activities of polyphenol oxidase. These compounds had no effect on the cresolase activity of polyphenol oxidase and were unable to decrease enzyme inactivation. These additives were able to successfully protect peroxidases but were unable to protect polyphenol oxidase. Alternative methods will need to be investigated to enhance the catalytic efficiency of polyphenol oxidase.

Biocatalytic reactions in water resulted in increased catechol production compared to reactions carried out in different buffers, as the catecholase activity of the enzyme was lower in water allowing catechol products to accumulate in solution. However, low yields of catechol (9.6%), 4-methylcatechol (41.4%) and 4-chlorocatechol(16.8%) were produced, even in water, due to their further oxidation to *o*-quinones.

The stable complex formed between ascorbate and borate was used to increase the production of catechol, as it minimised the inhibitory effect of ascorbate on polyphenol oxidase activity, and was able to function as a reducing agent to reduce *o*-quinone to catechol. By manipulating the borate and ascorbate concentrations, 100% yields of catechol, 4-methylcatechol and 4-chlorocatechol were achieved without *o*-quinones present as contaminants, but the highest yields of 3-methylcatechol (61.3%) were produced in water.

Using the borate-ascorbate system, two catechols not available commercially, 4-chlorocatechol and 4-methoxycatechol, were successfully produced as pure samples and characterised. The process involved 100% conversion of 4-chlorophenol and 4-methoxyphenol to their corresponding catechols by polyphenol oxidase, and the subsequent catechol extraction using diethyl ether to obtain 98 - 100% product recovery. Sufficient enzyme was used to obtain 100% phenol conversion which simplified the extraction procedure.

A comprehensive kinetic study of the conversion of a range of phenolic substrates in an aqueous medium was completed using polyphenol oxidase. Michaelis-Menten kinetics were used to calculate apparent K_M , V_{max} and V_{max} / K_M (catalytic efficiency) values with respect to each phenolic substrate. These values were not reported in the literature in such a thorough manner.

An efficient enzymatic process has been developed to produce catechols not available commercially. The optimal borate and ascorbate concentrations were determined to obtain 100% catechol yield, and an efficient extraction procedure was developed. This study has indicated that there is potential for scale-up of the catechol extraction procedure which would result in an environmentally benign method to produce catechols. In extending the present project later, the effect of glutathione, dithiothreitol and sodium bisulphate could be investigated to determine the most cost effective reducing agents for catechol production. Another important consideration would be recycling of the solvents used during the extraction procedure. The process could be developed further by an investigation pertaining to the immobilisation of polyphenol oxidase to facilitate its re-use as a biocatalyst.

CHAPTER 3

IMMOBILISED POLYPHENOL OXIDASE-CATALYSED REACTION WITH PHENOLS

3.1 INTRODUCTION

In this study a biotransformation system using immobilised polyphenol oxidase was investigated in which manipulation of the reaction conditions would allow production of catechols. In chapter 2, research was described on the use of non-immobilised polyphenol oxidase to characterise the reaction with phenols, whereas this study investigated the effects of immobilisation on the reaction. The development of a membrane bioreactor, using immobilised polyphenol oxidase for the production of catechol, was an important aspect of this work. In addition, a viable process using polyphenol oxidase would require that quinone production was minimised, to ensure minimal product inactivation of the biocatalyst.

The advantage of enzyme immobilisation is that it results in separation of the immobilised enzyme phase and the aqueous phase, allowing for separation of the enzyme from substrates and products (Klibanov, 1983). However, it also leads to alteration in the micro-environment of the enzyme and this can result in changes in the behaviour of the biocatalyst, which may be advantageous or disadvantageous in a biotransformation process. Another important advantage of immobilisation is that the immobilised biocatalyst can be retained and reused, facilitating a continuous production process, resulting in the economical utilization of the enzyme (Bahulekar *et al.*, 1991; Fernández-Romero *et al.*, 1993; Pialis *et al.*, 1996). In addition, immobilisation of enzymes may be useful for increasing the stability and activity of enzymes (Fernández-Lafuente *et al.*, 1999).

Thus, the aim of this investigation was to use immobilised polyphenol oxidase for the development of a continuous process to synthesize catechols. Polyphenol oxidase was immobilised on a range of supports using various immobilisation techniques, such as covalent coupling, cross-linking, adsorption or a combination of these techniques and allowed to react with several phenolic substrates. The influences of the hydrophobic or hydrophilic properties of the synthetic immobilisation supports were studied and mathematical modelling was used to predict which substrates and support materials would allow for maximum catechol accumulation to occur during the reaction of polyphenol oxidase with phenolic substrates.

3.1.1 Immobilisation of polyphenol oxidase on polyethersulphone capillary membranes

The objective of this part of the study was to use capillary membranes to develop high rate, high volume bioreactors for the application of polyphenol oxidase in such a way as to allow high yields of reaction products to be isolated and characterised. Initial experiments focussed on the adsorption of polyphenol oxidase on polyethersulphone capillary membranes.

Integration of the properties of synthetic membranes with biocatalysts, such as isolated enzymes, in order to carry out biocatalysed reactions, is one of the most advanced applications of membrane processes in biotechnology (Heath and Belfort, 1992), and this has, in turn, led to the development of membrane bioreactors (Brindle and Stephenson, 1996). The basic concept of membrane reactors is based on the separation of enzymes and products or substrates by a semipermeable membrane which created a selective barrier, although membranes may be used exclusively as a support for enzyme immobilisation (Prazeres and Cabral, 1994). Immobilisation of enzymes on synthetic micro- or ultrafiltration membranes makes it possible to integrate both biocatalysis and separation functions within one structure (Ulbricht and Papra, 1997). The criteria for selection of a membrane to be used in an enzyme bioreactor should include the molecular size of the enzyme, substrates and products, and the chemical nature of the membrane (Prazeres and Cabral, 1994). The membrane structure, symmetric or asymmetric, plays a role in the separation mechanism and hence in the application. Asymmetric membranes consist of a dense top layer (ultrafiltration layer) with a thickness of 0.1 to 0.5µm surrounded by a macroporous spongy layer, and symmetric membranes are 10 to 200µm thick with a uniform wall structure (Mulder, 1996).

There are two main enzyme immobilisation techniques used in membrane reactors: the membrane could be used simply as a barrier for the retention of soluble enzyme, or the membrane could be used as an immobilisation matrix where enzymes could be bound chemically or physically, using various immobilisation techniques, on or in the membrane (Bryjak *et al.*, 1996). Polyethersulphone (Figure 3.1), polysulphone and polyamide membranes lack reactive groups for enzyme immobilisation by covalent attachment (Gekas, 1986), which led to polyphenol oxidase being immobilised by adsorption, in this study.

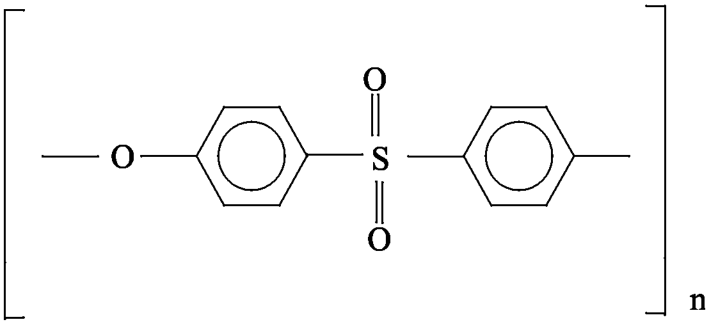


Figure 3.1: Chemical structure of polyethersulphone (Mulder, 1996).

Symmetric or asymmetric capillary membranes have been used for microfiltration or ultrafiltration purposes. The first reported use of capillary membranes as an immobilisation support for enzymes was by Rony (1971), as cited by Prazeres and Cabral (1994). The macroporous area of asymmetric membranes provides the surface for the immobilisation of enzymes (Gekas, 1986). The particular advantage of using capillary membranes is a surface area-to-volume ratio that is significantly higher than in the case of other immobilisation matrices, and a high flux across the membrane facilitates removal of enzymatic products. One of the most important advantages of membrane technology is the possibility of effective operation in a continuous process (as opposed to the treatment of small volumes in a batch system) which can lead to an increase in productivity (Prazeres and Cabral, 1994).

A collaborative study between researchers in our laboratory and the Institute of Polymer Science, Stellenbosch University, resulted in the development of novel (IPS 763) polyethersulphone capillary membranes with no external skin resulting in high trans-membrane flux (Burton *et al.*, 1998). These capillaries were used in the development of a novel transverse flow module (Figure 3.2) consisting of layers of capillary membranes arranged in a plane transverse to the direction of flow (Leukes *et al.*, 1995; Burton *et al.*, 1998). This arrangement of the capillary membranes was important to prevent a high-pressure drop over the layers of membranes and allowed for the application of feed steams either through the lumen or on the shell-side (outer surface) of the membranes in the bioreactor.

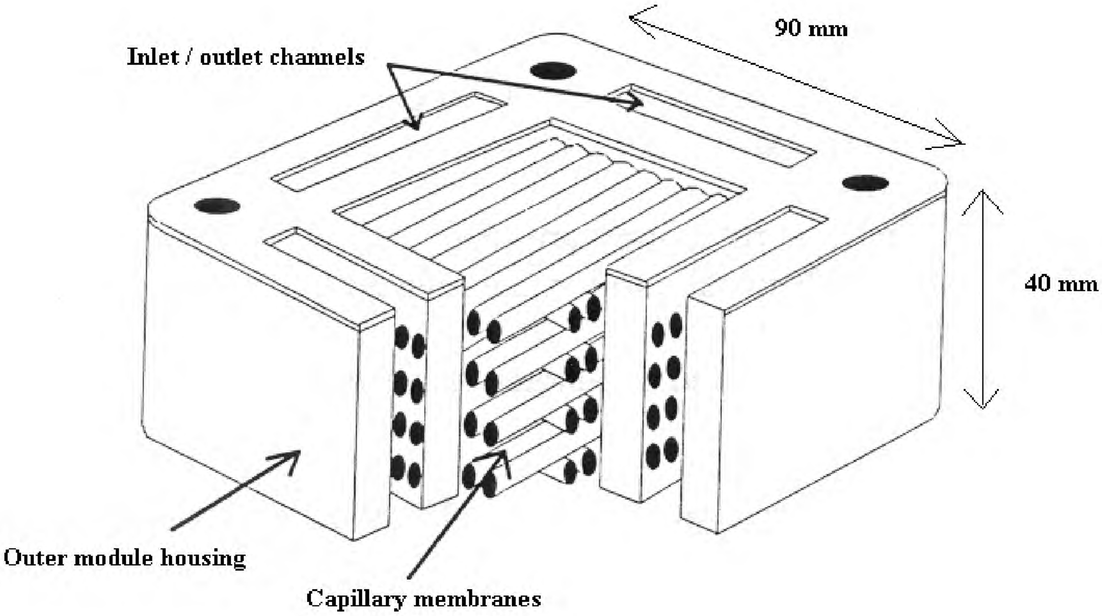


Figure 3.2: A transverse flow capillary membrane bioreactor module (Burton *et al.*, 1998).

Membrane module geometry needs to provide a well-defined feed and permeate flow across the membrane surface, which in turn leads to control of concentration polarisation. Application of the transverse flow capillary membrane bioreactor, using polyphenol oxidase immobilised on the membranes, resulted in significant phenolic pollutant removal as *o*-quinones, from certain industrial effluents (Edwards *et al.,*1999a and b). This study focussed on the biotransformation of phenol and *p*-cresol for the production of catechol products, using the same transverse flow capillary membrane bioreactor.

3.1.2 Immobilisation of polyphenol oxidase on flat-sheet membranes

In order to examine the influence of the hydrophobic or hydrophilic properties of synthetic immobilisation supports in the development of a bioreactor system for the synthesis of catechols, polyphenol oxidase was immobilised on a range of flat-sheet membranes, and allowed to react with several phenolic substrates. The effects of the nature of the immobilisation support, and reaction conditions on catechol production, were investigated using mathematical modelling.

An important consideration was to find an immobilisation method that would minimise chemical modification effects on the enzyme, and could be applied consistently to different membrane supports. Initial experiments focussed on immobilising polyphenol oxidase on nylon membranes to optimise the immobilisation procedure, which involved adsorption on to the membrane followed by cross-linking using glutaraldehyde. The effects of different immobilisation supports on the activity of polyphenol oxidase and on the enzyme-catalysed formation of catechol were then determined using various phenolic substrates.

An investigation into the reuse and stability of polyphenol oxidase immobilised on nylon membranes was carried out using L-DOPA as the substrate. Stability studies carried out by Pialis *et al.* (1996) using polyphenol oxidase covalently immobilised on nylon membranes indicated a 20% reduction in activity after 14 days under turnover conditions, and therefore it was important to identify any changes in the activity of polyphenol oxidase during its reuse. Another consideration was the storage of the immobilised biocatalyst between reactions and thus the effect of different storage buffers on the activity of polyphenol oxidase was also explored.

Since immobilised biocatalysts are not in the soluble state, conventional enzyme kinetic analysis of the reaction rates did not provide an adequate means of characterising the reactions, and therefore mathematical modelling of the data was used to determine reaction rate constants and to elucidate the effects of the nature of the immobilisation matrix on the kinetics of the process. Using this modelling approach, it was demonstrated that the polyphenol oxidase reaction system could be manipulated to produce catechols under conditions in which quinone formation, and hence product inactivation, were minimal. The extent of product inactivation was also investigated by comparing models which either included or excluded the effects of enzyme inhibition by the quinone. This provided a useful indicator of the extent to which product inactivation affected the polyphenol oxidase reaction under the experimental conditions applied.

3.1.3 Incorporation of polyphenol oxidase within polyurethane foam

The study of polyphenol oxidase immobilisation was extended to a preliminary investigation of the incorporation of the enzyme within an immobilisation matrix. The aims were to investigate whether polyphenol oxidase could be incorporated into polyurethane foams and to determine the effect of immobilisation on the enzyme activity. Immobilisation of polyphenol oxidase in polyurethane foams would involve a different technique in which the enzyme would be entrapped in the polymer by covalent linkage during polymer synthesis. Polyurethanes are widely used in foam manufacture with their major commercial application in cushions and foam-in-place insulating material (Daley and Daley, 1996), but they are finding application as an immobilisation matrix for a number of enzymes.

Phosphotriesterase (LeJeune and Russell, 1996) and organophosphorus hydrolase (LeJeune *et al.*, 1999) were successfully immobilised within polyurethane foams, using a Hypol prepolymer to synthesize the enzyme-linked polyurethane foams. Hypol is synthesized from the reaction of polyether (or polyester) polyol with polyisocyanates in the presence of cross-linking agents (LeJeune and Russell, 1996). To synthesize the foam, water was needed to initiate the reaction and shear forces were needed to disperse the prepolymer in water (LeJeune and Russell,1996). Water reacted with some of the isocyanate groups to form an unstable carbamic acid, which then degraded to form an amine and carbon dioxide was liberated (LeJeune and Russell,1996; Daley and Daley, 1996). As the polymer formed, it trapped the bubbles of carbon dioxide enabling the foam to rise (LeJeune and Russell,1996; Daley and Daley, 1996). Amines then reacted with the isocyanate groups resulting in urea-type linkages (LeJeune and Russell,1996). Enzymes also contain amine groups allowing multipoint covalent attachment of the enzymes into the polymer via the formation of urea linkages (LeJeune and Russell,1996). High enzyme loadings were possible without disrupting the polymer formation (LeJeune and Russell,1996). The reaction occurring during the incorporation of enzymes into polyurethane foams is shown in Figure 3.3.

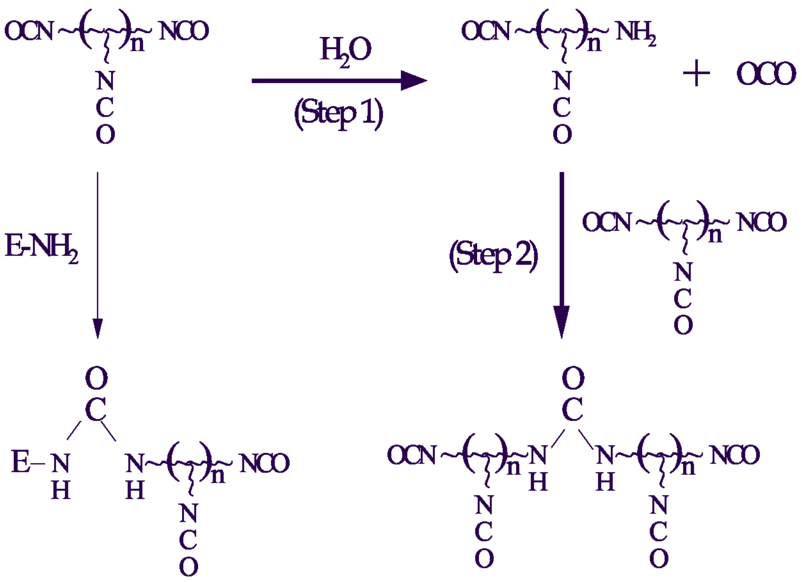


Figure 3.3: The two step reaction occurring during the formation of enzyme-polyurethane foams, step 1 - formation of amines and evolution of CO_2 and step 2 - urea linkages between isocyanate groups and amines.

During polymer formation, LeJeune and Russell (1996) added Pluronic surfactants which were incorporated into the foam via a terminal hydroxyl group, resulting in changes in the properties of the foam such as increasing water adsorbance, thereby significantly extending the catalytic lifetime of phosphotriesterase in polyurethane foams. Incorporation of organophosphorus hydrolase into polyurethane foams reduced the enzyme's sensitivity to pH changes and bleach-induced enzyme denaturation (LeJeune *et al.*, 1999).

The catalytic lifetime of polyphenol oxidase incorporated into the polyurethane foam and the effect of different storage buffers was examined. The action of various surfactants on the formation of the foam and physical properties of the foam, including substrate and product adsorption were investigated. Polyurethane-immobilised polyphenol oxidase was used for the conversion of *p*-cresol and *m*-cresol to 4-methylcatechol and 3-methylcatechol respectively.

3.1.4 Incorporation of polyphenol oxidase within an organogel

The study of polyphenol oxidase immobilisation was extended to a preliminary investigation of the incorporation of the enzyme into an organogel. Immobilisation of polyphenol oxidase in organogels would involve entrapment and cross-linking of the enzyme in the gel during its synthesis. Reverse micelles, obtained by the addition of small amounts of water to an organic solution of a surfactant, were transformed into viscous gels by solubilizing gelatin at 50°C and then cooling the mixture under continuous stirring to produce organogels (Crecchio *et al.*, 1995). Enzymes added during the cooling phase become entrapped in the gel.

Polyphenol oxidase immobilised in organogels was reported to show an increased stability to thermal and proteolytic treatments, and was used for the removal of aromatic compounds from aqueous suspensions (Crecchio *et al.*, 1995). Immobilisation of horseradish peroxidase by solubilizing the enzyme in a gelatin solution followed by cross-linking using glutaraldehyde resulted in the enzyme retaining 70 - 80% of its activity (Fadnavis and Koteshwar, 1999). In this study, polyphenol oxidase was immobilised in organogels by cross-linking the enzyme during gel formation to prevent leaching of the enzyme into the reaction medium. Gel-entrapped polyphenol oxidase was used for the conversion of *p*-cresol to 4-methylcatechol.

3.2 MATERIALS AND METHODS

3.2.1 Immobilisation of polyphenol oxidase in a transverse flow bioreactor module

The capillary membranes were pre-treated before enzyme immobilisation by wetting using Milli-Q water and washing with 0.1M NaOH for one hour. They were then rinsed with Milli-Q water, followed by a final rinse with 50mM potassium phosphate buffer (pH 6.8). Polyphenol oxidase was dissolved in 50mM potassium phosphate buffer (pH 6.8) and immobilised by recirculating the enzyme solution on the shell-side of the membranes. After immobilisation, residual enzyme was removed by rinsing the bioreactor with 50mM potassium phosphate buffer (pH 6.8). The enzyme solution was initially recirculated for 12 hours and the time was later reduced to 3 hours. The amount of enzyme immobilised on the membranes was calculated by determining the protein concentration in solution before and after immobilisation.

3.2.2 Bioreactor configuration and operation

Two different configurations of the transverse flow capillary membrane bioreactor were investigated with regard to comparison of yields of product, *viz.*, dead-end flow and a recycle system. Polyphenol oxidase was immobilised on the shell-side of the membranes and 10mM phenol or *p*-cresol solution was pumped through the lumen or on the shell-side of the membranes. A hydrostatic pressure was applied to the bioreactor to facilitate flux across the membrane. The bioreactor was operated at a gauge pressure of 20kPa which was maintained during the experiments in order to maintain an average feed flow rate of 70mL/hour. After addition of the phenolic compound, permeate fractions were collected every 30 minutes and analysed by HPLC (Section 3.2.16) To determine whether polyphenol oxidase was being washed out of the bioreactor, the absorbance of permeate fractions was monitored every 5 minutes at 400nm as *o*-quinone products adsorb at this wavelength.

3.2.3 Immobilisation of polyphenol oxidase on nylon membranes

Nylon membranes (MAGNA, 47mm, 0.45μm), obtained from Micron Separation Inc. (Westborough, MA, USA), were soaked overnight in distilled water. The immobilisation of polyphenol oxidase on nylon was performed according to the procedure of Pialis *et al.* (1996). Nylon membranes were placed in 50mL of 3M HCl for 10 minutes. The nylon membranes were then washed in 150mL of HPLC grade water and incubated in 30mL methylene chloride solution containing 1% (w/v) *N,N'* - dicyclohexylcarbodiimide (Sigma Chemical Co., St. Louis, USA) and 1% (w/v) 3,3' ,5,5' - tetramethylbenzidine (Sigma Chemical Co., St. Louis, USA) for 24 hours. Following the incubation procedure the membranes were washed with methylene chloride, acetone and finally water. The nylon membrane was activated by soaking in 0.1M sodium phosphate buffer (pH 8) containing 3% glutaraldehyde (25%, SAARChem Ltd., Krugersdorp, SA), for 2.5 hours at 4°C with gentle stirring. The membranes were then washed with 50mL 0.1M sodium phosphate buffer (pH 7), then placed in 3mL solution of 0.1M phosphate buffer (pH 7) containing 20.4U polyphenol oxidase. The membranes were incubated for 24 hours at room temperature and at 4°C during polyphenol oxidase immobilisation. A control was set up which contained the same number of units of polyphenol oxidase but to which no membrane was added. The membranes were then washed with 0.1M sodium phosphate buffer (pH 7) containing 9g/l

NaCl. To determine polyphenol oxidase uptake on the membranes, aliquots of enzyme solution were collected before and after incubation of the membrane in the polyphenol oxidase solution.

3.2.4 Optimised procedure for immobilisation of polyphenol oxidase on hydrophilic membranes

Nylon, cellulose acetate membranes (47mm, 0.45µm, Micron Separation Inc., Westborough, MA, USA), glass microfibre filters (GF/A, 47mm, Whatman, Kent, England) and nitrocellulose flat-sheet membranes (0.45µm, Amersham) were soaked overnight in distilled water.

Polyphenol oxidase was dissolved in 50mM phosphate buffer pH 6.8. Each membrane was then placed in a solution of polyphenol oxidase containing a sufficient number of enzyme units to ensure that 0.3U enzyme was immobilised onto each membrane. This was determined previously by placing the membranes into solutions containing varying concentrations of polyphenol oxidase and glutaraldehyde and determining the percentage of enzyme immobilised over different time periods. The membranes were left in the enzyme solution for 1 hour at 4°C. Glutaraldehyde was then added to give a final concentration of 1% glutaraldehyde, and a further 30 minutes was allowed for cross-linking. Membranes were removed, washed in 50mM phosphate buffer pH 6.8 and then used for biocatalytic reactions.

3.2.5 Optimised procedure for immobilisation of polyphenol oxidase on hydrophobic membranes

PTFE membranes (47mm, 0.2µm, Lida Manufacturing Corp., Kenosha, WI), were pre-wetted using ethanol. Polysulphone (47mm, MWCO 30 000, Sartorius, Goettingen, Germany), polypropylene and polycarbonate membranes (47mm, 0.2µm, Micron Separation Inc., Westborough, MA, USA) were rinsed in water. Each membrane was placed in a stirred cell (Spectrum, Houston, Texas) and a solution of 0.3U enzyme was placed in the reservoir, and positive pressure, using nitrogen gas, was applied to the enzyme solution. This was followed by 1 hour treatment with 5mL 1% glutaraldehyde solution in water.

3.2.6 Reaction of phenolic substrates with soluble and flat-sheet membrane-immobilised polyphenol oxidase

Four different phenolic compounds were used as substrates, *viz.*, phenol, *p*-cresol, *m*-cresol and 4-chlorophenol. The substrates were dissolved in double-distilled water. All reactions with soluble and immobilised polyphenol oxidase were carried out at 25°C in 20mL substrate solution at two different concentrations (1.25mM and 5mM). After the addition of soluble or immobilised biocatalyst the reaction mixture was allowed to stir continuously on an orbital shaker.

3.2.7 Substrate and product adsorption using flat-sheet membranes

Substrate and product adsorption experiments were carried out, using each of the five membranes, to determine the extent of substrate and product adsorption to the immobilisation supports. Each membrane, without polyphenol oxidase, was placed in 20mL 1.25mM *p*-cresol solution. To determine the extent of product adsorption, each membrane was placed in 20mL of 1.25mM *p*-cresol solution containing 0.9U non-immobilised polyphenol oxidase. Control experiments involved monitoring substrate and product concentrations during the reaction with 0.9U polyphenol oxidase. Samples were removed every hour for 3 hours and analysed by HPLC (Section 3.2.16).

3.2.8 Stability of polyphenol oxidase immobilised on nylon membranes

Polyphenol oxidase was immobilised on nylon membranes according to the method in Section 3.2.4. Membrane-immobilised polyphenol oxidase was placed in 20mL of 10mM L-DOPA dissolved in phosphate buffer (50mM, pH 6) and allowed to stir continuously on an orbital shaker. Samples were withdrawn every two minutes for 12 minutes and the absorbance was read at 475nm. Linear regression analysis of the results of each assay was used to determine the change in absorbance per minute. The activity of polyphenol oxidase was then calculated for each assay.

A short term study of the reusability was determined by placing 0.15U membrane-immobilised and non-immobilised polyphenol oxidase (0.15U) into 6 consecutive solutions of L-DOPA and the relative activity was calculated.

A longer term study of stability was conducted by placing the membrane-immobilised polyphenol oxidase into saline solutions or 50 mM potassium phosphate buffer, pH 6.8 and determining the relative activity over several days.

3.2.9 Polyurethane foam synthesis

The foams were synthesized by Dr. LeJeune and Dr. Erbeldinger at the Centre of Biotechnology and Bioengineering, University of Pittsburgh, according to the method of LeJeune and Russell (1996). During the synthesis of the polyurethane foam, 4 different surfactants were used, Pluronic L-62, F-68, P-65 and Emolgrade and one foam was synthesized containing no surfactant. 5mL of 50mM phosphate buffer pH 6 containing 1% (w/v) of surfactant was added to 5g of Hypol pre-polymer. The solution was rapidly mixed using a hand-held drill with a mixing device attached. The foams were placed overnight in a fume hood to facilitate the removal of residual water.

3.2.10 Substrate and product adsorption using polyurethane foam

Adsorption of the substrates to the foam was determined by placing 200mg of each foam into 20mL of 5mM phenol and 5mM *p*-cresol. Samples were withdrawn over a 90 minute period and analysed at 270nm using a Hewlett Packard HPLC, using a mobile phase of 40% acetonitrile : 60% water (1% glacial acetic acid). Adsorption of the products was determined in a similar way using 2.5mM catechol and 4-methylcatechol.

3.2.11 Polyphenol oxidase-polyurethane foam synthesis

Polyurethane foams were synthesized containing 70 mg of crude polyphenol oxidase and 10mg of commercial polyphenol oxidase with specific activities of 0.404U/mg and 6.9U/mg, respectively. The foams were synthesised according to the method in Section 3.2.9, with the incorporation of 3 surfactants *viz.*, Pluronic L-62, F-68, P-65. The effect of these surfactants on polyphenol oxidase activity was investigated.

3.2.12 Storage stability of polyphenol oxidase-polyurethane foam

The storage stability of dry polyphenol oxidase-polyurethane foams was examined by placing known quantities of the foam into vials and storing them at room temperature. Activity assays were carried out on the foams at regular intervals. Pieces of the foam were also placed in 2 different storage solutions, 50 mM potassium phosphate buffer pH 6.8 and saline, to investigate the reuse of the foams and conditions of storage.

3.2.13 Immobilisation of polyphenol oxidase in an organogel

The method of Fadnavis and Koteshwar (1999) was used to prepare the organogel for the immobilisation of polyphenol oxidase. 5g of gelatin was dissolved in 8.5mL water. 50mL of 0.3M sodium bis-diethylhexyl sulfosuccinate, dissolved in heptane, was added to the gelatin solution at 50°C. The solution was agitated until viscous then cooled to 5°C. 25mL was removed and 1mL of polyphenol oxidase, containing 24.95U, was added. The solution was allowed to stir for 5-10 minutes. The enzyme was cross-linked by adding 1mL glutaraldehyde (25%) to the solution and allowing it to stir for 10-15 minutes. The gel was poured into 3 petri dishes and dried overnight, then rinsed in hexane and washed extensively in water to remove traces of glutaraldehyde.

3.2.14 Reaction of phenolic substrates with polyurethane and organogel-immobilised polyphenol oxidase

The polyurethane foam synthesised using commercial polyphenol oxidase and Pluronic F-68 surfactant was allowed to react with *p*-cresol and *m*-cresol. 0.2302g and 0.226g pieces of foam were allowed to react with 30mL of 1.25mM and 5mM *p*-cresol respectively and 0.2218g and 0.2369g were allowed to react with1.25mM and 5mM *m*-cresol respectively. The solutions were allowed to stir continuously and samples were analysed every 30 minutes for 3 hours by HPLC. The freeze-dried organogel, containing polyphenol oxidase, was ground in liquid nitrogen using a mortar and pestle, and 0.35g of freeze-dried gel was placed into 10mL of 1.25mM and 5mM *p*-cresol and allowed to stir continuously on an orbital shaker. Samples were filtered before being analysed on the HPLC (Section 3.2.16).

3.2.15 Effectiveness of immobilisation

The activity of polyphenol oxidase immobilised on synthetic membranes and incorporated into polyurethane foam or an organogel was calculated using L-DOPA as the substrate. These assay methods were used regularly to monitor the activity of the immobilised biocatalyst.

Each type of synthetic membrane was placed in 30mL 10mM L-DOPA in phosphate buffer (50mM, pH 6). The membranes were allowed to stir continuously while samples were withdrawn every two minutes for 10 minutes and the absorbance was measured at 475nm. After 10 minutes the membranes were removed and further absorbance readings were taken to detect any residual enzyme activity in the L-DOPA solution.

200mg pieces of polyurethane foam were placed in 30mL 10mM L-DOPA in phosphate buffer (50mM, pH 6). The foams were allowed to stir continuously while samples were withdrawn every two minutes for 10 minutes and the absorbance was measured at 475nm.

200mg portions of freeze-dried gel were placed in 3mL 10mM L-DOPA in phosphate buffer (50mM, pH 6). The foams were allowed to stir continuously while samples were withdrawn every 10 minutes for 60 minutes and the absorbance was measured at 475nm.

3.2.16 Chromatographic analysis of polyphenol oxidase-catalysed conversion of phenols

The disappearance of the phenolic compounds and the appearance of the products was monitored by HPLC with UV detection (Beckman Instruments, Inc., San Ramon, USA) using reverse-phase 5µm C_{18} Spherisorb (250mm x 4.6mm) column with a mobile-phase consisting of water :acetonitrile (52:48) at a flow rate of 1mL.min^{-1}. Peaks were detected at 270nm and analysed with Beckman System Gold Chromatography Software.

3.3 RESULTS AND DISCUSSION

Initial experiments focussed on the immobilisation of polyphenol oxidase on polyethersulphone capillary membranes, in a transverse-flow bioreactor, because this system could be used to develop a continuous process for the production of catechols. The effects of different membrane supports were later investigated using flat-sheet membranes, as these provided a convenient method to perform numerous experiments using several substrates at two concentrations. The support materials were evaluated in terms of catechol production achieved using each membrane.

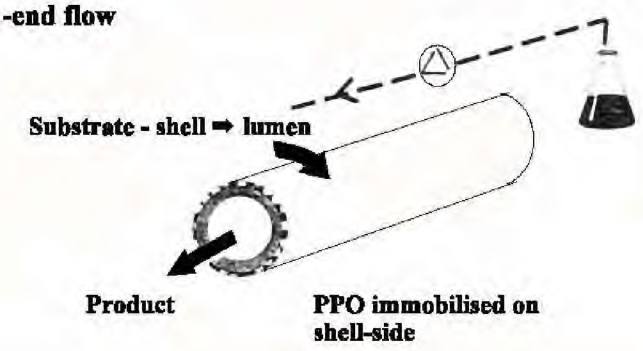
3.3.1 Modes of operation of the bioreactor module

The bioreactor was used in a dead-end or recycle configuration (Figure 3.4). In the recycle configuration, it was operated in either of two different ways: substrate was pumped through the lumen and a pressure was applied to force it through the lumen to the shell-side where it was allowed to react with immobilised polyphenol oxidase and permeate fractions were collected while the substrate was continuously recycled, or substrate was pumped through the shell-side and permeate fractions were collected from the lumen.

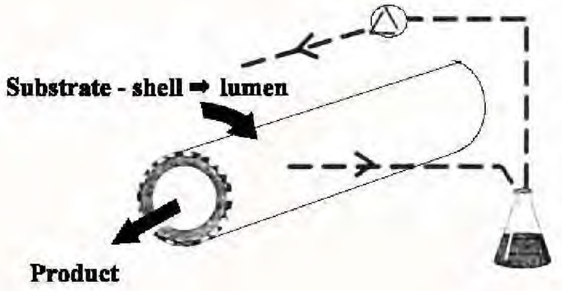
3.3.2 Immobilisation efficiency of polyphenol oxidase in a bioreactor module

Polyethersulphone capillary membranes in the transverse flow bioreactor (Figure 3.2) were used as an immobilisation support for polyphenol oxidase, with the macroporous layer on the shell-side of the capillary membrane providing the surface for the physical immobilisation. Adsorption of polyphenol oxidase to the capillary membranes is a simple procedure that would allow for facile regeneration of the capillary membranes. Immobilisation of polyphenol oxidase in the transverse flow capillary membrane bioreactor was carried out by recycling the polyphenol oxidase solution on the shell-side of the membranes (Figure 3.4). The concentration of polyphenol oxidase immobilised was determined by assaying the protein concentration before and after immobilisation. When polyphenol oxidase was immobilised over 12 hours, 55 - 65% of the protein was immobilised, whereas decreasing the time to 3 hours resulted in 40 - 60% of the protein being immobilised. For the purposes of this investigation it was concluded that 3 hours would ensure sufficiently effective enzyme immobilisation.

(1) Dead -end flow



(2) Recycle (shell ➡ lumen)



(3) Recycle (lumen ➡ shell)

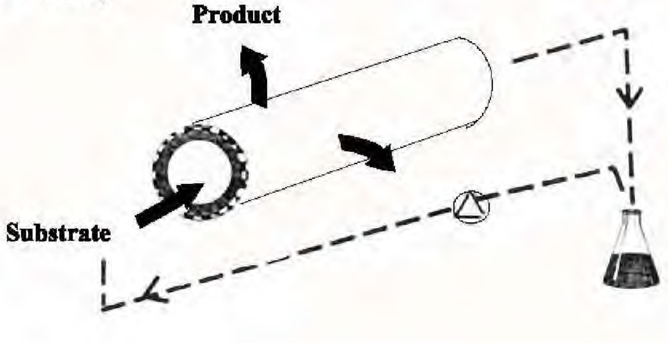


Figure 3.4: Reactor configurations of transverse flow capillary membrane bioreactors used for the reaction of phenolic compounds with polyphenol oxidase.

3.3.3 Bioreactor operation with phenolic compounds

The efficiency of the immobilisation procedure was evaluated during operation of the bioreactor with *p*-cresol. Leakage of polyphenol oxidase from the capillary membranes was monitored by measuring the absorbance of the permeate fractions as follows: polyphenol oxidase was immobilised on the shell-side of the membranes in the bioreactor, 10mM *p*-cresol was pumped through the lumen of the capillary membranes and permeate fractions were collected every 30 minutes for 3 hours. The absorbance of the permeate fractions was measured at 400nm, and consecutive readings were taken every 5 minutes over 40 minutes. An increase in absorbance in the permeate would be attributed to the continued production of *o*-quinones by the enzyme. The results indicate that polyphenol oxidase was not washed off the membrane, as the absorbance values decreased, presumably as the *o*-quinones polymerized (Figure 3.5).

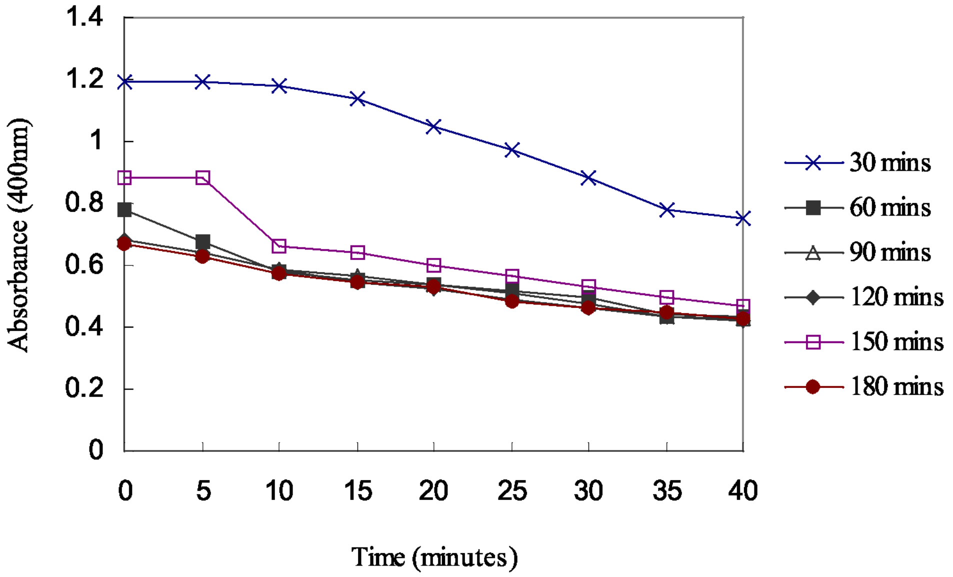


Figure 3.5: The absorbances at 400 nm of the permeate fractions, collected every 30 minutes for 3 hours, measured periodically after the reaction of *p*-cresol with immobilised polyphenol oxidase, time t=0 taken from time of collected fraction.

The polyethersulphone capillary membrane bioreactor was used in the recycle configurations (Figure 3.4), with 30.7U of polyphenol oxidase immobilised on the shell-side of the membranes in the bioreactor, 10mM *p*-cresol pumped through the shell-side of the capillary membranes and permeate fractions collected every 30 minutes for 3.5 hours from the lumen. Alternatively, 10mM *p*-cresol was pumped through the lumen and permeate fractions were collected every 30 minutes for 4 hours from the shell-side. The permeate fractions collected from the lumen (Figure 3.6) and the shell-side (Figure 3.7) of the bioreactor were analysed by HPLC to monitor the disappearance of substrate and the formation of products.

In the recycle configuration where *p*-cresol was recycled on the shell-side and permeate fractions collected from the lumen of the bioreactor, 28% of the initial 10mM *p*-cresol was removed after 3.5 hours, while a significantly higher percentage removal of 47% occurred after 4 hours when *p*-cresol was recycled through the lumen and permeate fractions collected from the shell-side of the bioreactor. The HPLC results indicated that 4-methylcatechol was not detected during the reaction.

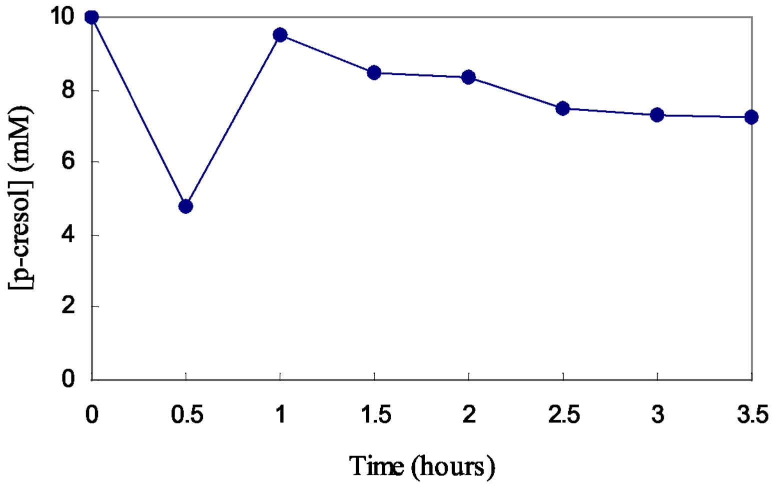


Figure 3.6: *p*-Cresol concentrations of permeate fractions collected from the lumen during the reaction of immobilised polyphenol oxidase with the bioreactor operated in a recycle configuration.

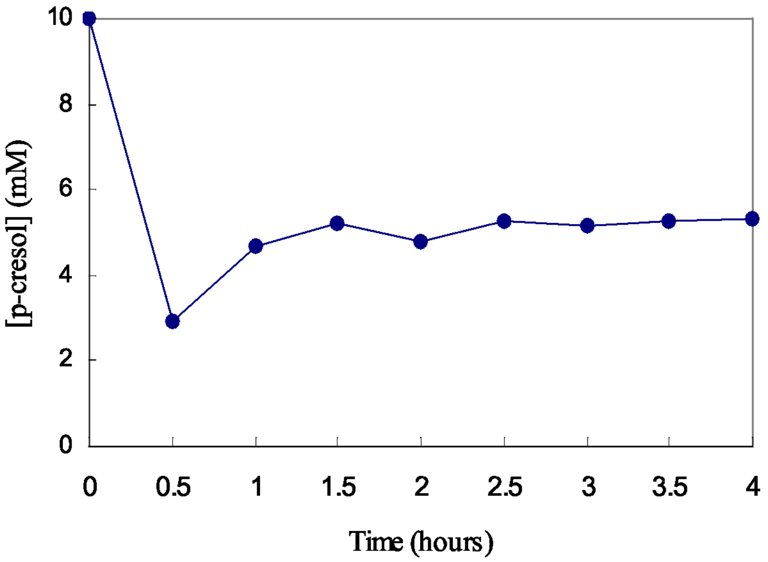


Figure 3.7: *p*-Cresol concentrations of permeate fractions collected from the shell-side during the reaction of immobilised polyphenol oxidase with the bioreactor operated in a recycle configuration.

Phenol was also used as a substrate for the reaction of polyphenol oxidase immobilised in the bioreactor, and phenol removal and catechol accumulation were again monitored. 36.85U of polyphenol oxidase was immobilised on the shell-side of the membranes in the bioreactor, 10mM phenol was pumped from the shell-side and fractions were collected from the lumen in a dead-end configuration. Permeate fractions were collected every 30 minutes for 7 hours and analysed by HPLC (Figure 3.8). 26% of the initial 10mM phenol was removed after 7 hours of operation of the bioreactor.

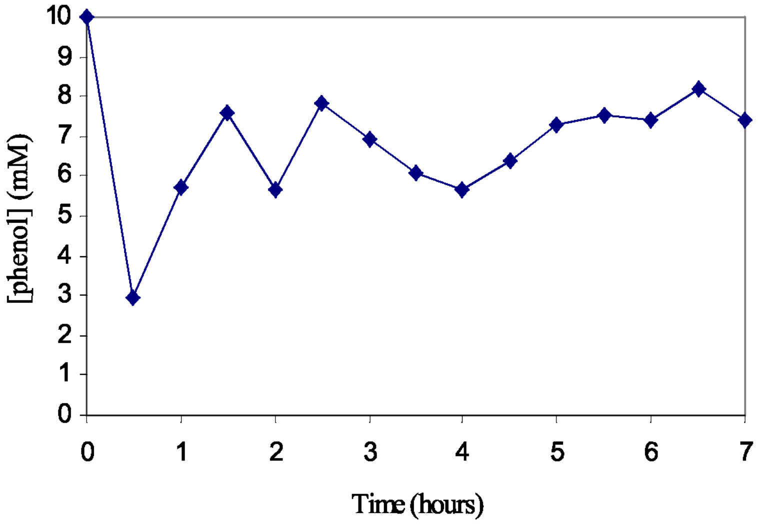


Figure 3.8: Phenol concentrations of permeate fractions collected during the reaction of immobilised polyphenol oxidase with the bioreactor operated in a dead-end configuration.

The bioreactor was also operated in recycle configurations where 35U of polyphenol oxidase was immobilised on the shell-side of the membranes in the bioreactor, 10mM phenol was pumped through the shell-side of the capillary membranes, and permeate fractions were collected from the lumen every 30 minutes for 7 hours and analysed by HPLC. Alternatively, 10mM phenol was passed through the lumen and permeate fractions were collected from the shell-side. The phenol concentrations in the permeate fractions collected from the lumen are shown in Figure 3.9 and permeate fractions collected from the shell-side are shown in Figure 3.10. 21% of the initial 10mM phenol was removed after 7 hours when permeate fractions were collected from the lumen, while 29% removal occurred after 8 hours when permeate fractions were collected from the shell-side of the bioreactor. Catechol product peaks were not detected by HPLC during the reaction.

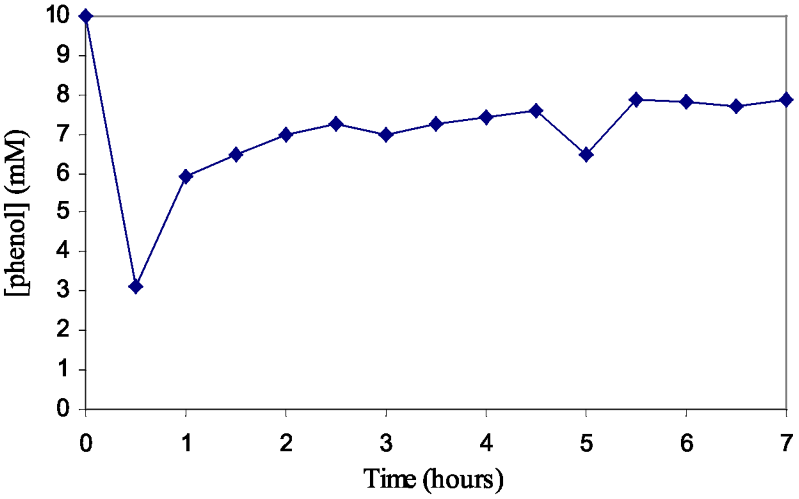


Figure 3.9: Phenol concentrations of permeate fractions collected from the lumen during the reaction of immobilised polyphenol oxidase with the bioreactor operated in a recycle configuration.

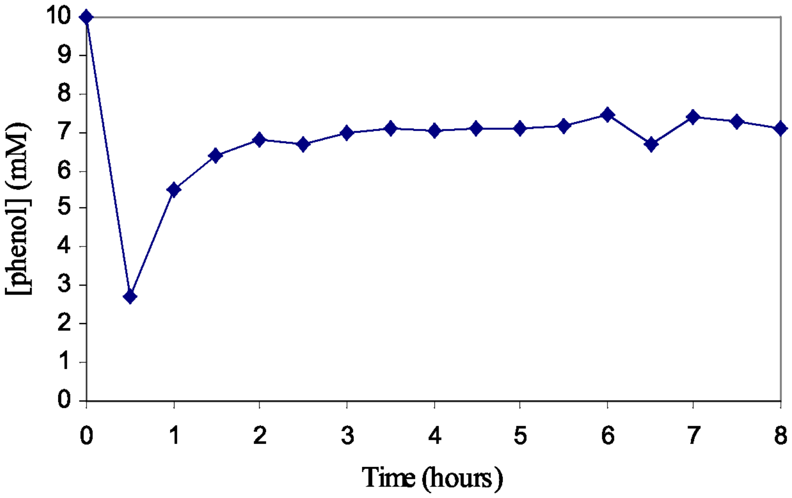


Figure 3.10: Phenol concentrations of permeate fractions collected from the shell-side during the reaction of immobilised polyphenol oxidase with the bioreactor operated in a recycle configuration.

When using both phenol and *p*-cresol as substrates, higher substrate removal occurred using the bioreactor in a recycle configuration with the substrate being recycled through the lumen and permeate fractions collected from the shell-side of the membranes in the bioreactor. This could be attributed to decreased inactivation of polyphenol oxidase by *o*-quinones as separation of the *o*-quinone products from the substrate was possible, with substrate being pumped through the lumen to the shell-side of the membranes. Significantly decreased enzyme inactivation was also demonstrated by Edwards *et al.* (1999b) during the reaction of membrane bioreactor-immobilised polyphenol oxidase with a synthetic effluent solution. Polymerization of the *o*-quinones in water was found to cause enzyme inactivation (Kazandjian and Klibanov, 1985). Flux across the membrane would result in the removal of *o*-quinones from the immediate environment surrounding the enzyme, resulting in a significantly lowered effect of suicide inactivation.

The highest rate of substrate removal was achieved using *p*-cresol as the substrate, but the lower rate of phenol removal could be attributed to the inhibitory nature of its reaction products. In order for the membrane bioreactor to be used efficiently for the treatment of phenols, increased removal of substrates should be achieved. Greater substrate conversion could be achieved by increasing the enzyme loading on the membranes or decreasing the initial substrate concentrations to reduce the possible effects of substrate inhibition.

3.3.4 Immobilisation of polyphenol oxidase on flat-sheet membranes

The results reported in Section 3.3.3 indicated that the use of hydrophobic polyethersulphone membranes resulted in significant substrate removal but were unsuitable for catechol production, as catechols were not detected by HPLC. The investigation into the immobilisation of polyphenol oxidase was therefore extended to a range of membranes with varied composition, in order to identify a more suitable immobilisation support which would enhance catechol production. Hydrophilic membranes (nylon, glass-fibre and nitrocellulose) and hydrophobic membranes (polysulphone and PTFE) were selected to test as immobilisation supports for polyphenol oxidase, and in this case, a flat sheet configuration was used due to ready availability of these membranes.

Initial immobilisation experiments involved immobilisation of polyphenol oxidase on nylon membranes, using the procedure of Pialis *et al.* (1996) which involved the covalent linkage of the enzyme to nylon membranes which had previously been activated using carbodiimide. The membranes were acid washed to increase the presence of amino and carboxyl functional groups on the surface of the membrane. In our hands, measurements of polyphenol oxidase activity before and after the immobilisation procedure revealed that the immobilisation was unsuccessful as the enzyme activity in solution was almost identical after the immobilisation procedure had been carried out. The immobilisation procedure was tedious and involved many chemical treatment steps and, in addition relied on the presence of carboxyl and amino groups present on the surface of the membrane. A simpler immobilisation procedure was sought that could be used for several types of immobilisation support

3.3.5 Effectiveness of polyphenol oxidase immobilisation on different membrane supports

A simple immobilisation method was developed which could be used for many different immobilisation supports, and which minimised chemical modification effects on the enzyme. For the sake of consistency and economy polyphenol oxidase was adsorbed on the membranes and then cross-linked using glutaraldehyde. The number of units of active enzyme immobilised was calculated by placing each membrane in L-DOPA solution, measuring the increase in absorbance due to dopaquinone production with time, and using linear regression to find the enzyme activity.

Different immobilisation procedures were developed, depending on whether the membranes were hydrophilic or hydrophobic. Polyphenol oxidase could not be immobilised on hydrophobic membranes by the passive adsorption procedure, as indicated by the detection of little or no polyphenol oxidase activity on the membranes using the L-DOPA assay. However, use of a stirred cell and application of positive pressure, using nitrogen gas, ensured that polyphenol oxidase was immobilised by dynamic adsorption into the pores of hydrophobic membranes, and no subsequent polyphenol oxidase leakage from the membrane was observed. The number of units of active polyphenol oxidase immobilised on each type of membrane was determined before reaction with substrate, to facilitate comparison of different immobilisation supports (Table 3.1). In order to determine the effect of immobilisation on polyphenol oxidase activity, the same number of units of polyphenol oxidase was used in a non-immobilised form. The immobilisation procedure resulted in approximately 30% of the original activity being retained after immobilisation.

Table 3.1: Units of active polyphenol oxidase immobilised on each type of membrane and non-immobilised polyphenol oxidase used for biocatalytic reactions, determined using L-DOPA as substrate.

	Units[a] (µmols.min.$^{-1}$)
Non-immobilised polyphenol oxidase	0.318∓0.03
Immobilised polyphenol oxidase	
nylon	0.309∓0.01
nitrocellulose	0.292∓0.01
glass-fibre	0.300∓0.03
PTFE	0.281∓0.04
polysulphone	0.308∓0.07

[a] : mean∓SD of 5 replicates

3.3.6 Biocatalytic conversion of phenol substrates by membrane-immobilised and non-immobilised polyphenol oxidase

Polyphenol oxidase in membrane-immobilised and non-immobilised forms, with equivalent amounts of activity, was used to convert phenol, *p*-cresol, *m*-cresol and 4-chlorophenol, over a period of 6 hours, using 1.25mM and 5mM starting concentrations of substrate. The properties of immobilised enzymes can be altered by both external diffusional constraints, resulting from the transport of substrates to the surface of the biocatalyst across a boundary layer of water, and internal diffusional constraints due to the fact that substrates must diffuse inside the immobilised enzyme particle (Klibanov, 1983). During the reactions of immobilised polyphenol oxidase the same diffusional limitations were applied to all the membranes, and these limitations were decreased by allowing the membranes to stir continuously, and by applying the same simple immobilisation procedure to the surface of each type of membrane. The concentrations of phenolic substrates and catechol intermediates were monitored and the resulting data (Appendix A) was used as the basis for formulating a mathematical model of the process, as described below (Boshoff *et al.,* 2000 - submitted for publication).

3.3.7 Modelling the biocatalytic reaction of membrane-immobilised and non-immobilised polyphenol oxidase

On the basis of the experimental data, the following model of the polyphenol oxidase reaction system was proposed. Let S denote the substrate, C the catechol, Q the quinone, E the enzyme and P the inactivated enzyme product.

$$E+S\xrightarrow{k_1}E+C$$

$$E+C\xrightarrow{k_2}E+Q$$

$$E+Q\xrightarrow{k_3}P$$

For the enzyme, let [E] denote the normalised enzyme concentration. In other words
[E]= (enzyme concentration)/(initial enzyme concentration).
Let:

$$s=[S],c=[C],q=[Q],p=[P],e=[E].$$

If s_0 denotes the initial substrate concentration, then:

$$s+c+q=s_0$$

and the reaction is governed by the following system of differential equations.

$$\frac{ds}{dt}=-k_1se$$

$$\frac{dc}{dt}=k_1se-k_2ce \tag{1}$$

$$\frac{de}{dt}=k_3qe$$

The reactions were run over a time period of 6 hours with 13 readings: X=[X(1), …, X(13)] for [S] and Y=[Y(1), …, Y(13)] for [C] being taken every 30 minutes. The last equation describes the inhibition of active polyphenol oxidase by the quinone product. This non-linear system has no explicit solution for

$$s = s(t), c = c(t), e = e(t)$$

and, furthermore, no readings of e=[E] were obtainable. Therefore, a data set for [E] was generated as follows.

If [E] were to diminish exponentially by d % over 6 hours then

$$e = \exp(-k_3 t) \text{ and } \frac{100-d}{100} = \exp(-k_3 6).$$

So the rate constant, k_3 which would achieve this would be given by

$$k_3 = \tfrac{1}{6}\log\left(\tfrac{100}{100-d}\right)$$

Given d, the corresponding 13 values, Z=[Z(1), …, Z(13)] for [E] could be computed for t=0, .5, …, 6.

Thus for each d we have the computed data set, Z, for e and the recorded data sets: X, Y for substrate and catechol. The problem was then to find the decay rate, d, and the values of k_1, k_2, k_3 such that the solution curves: s=s(t), c=c(t), e=e(t) to the system (1) fit the data set: [X, Y, Z] optimally. Specifically, the norm:

$$r = \sqrt{\sum_{i=1}^{13} (s(i) - X(i))^2 + (c(i) - Y(i))^2 + (e(i) - Z(i))^2}$$

of the residual vector should be minimized. It was therefore necessary to perform non-linear regression on the solution curves to the system (1). This was achieved with MATLAB programs which invoked the least squares, multiple non-linear regression function: NLR (Constantinides and Moustoufi, 1999). The function returns the vector K=[k_1, k_2, k_3] of parameters such that the residual vector, r, above is minimized.

Two MATLAB programs were written.

Program 1:

The first program solicited the decay rate *d* and used the vector $K=[\ k_1,\ k_2,\ k_3\ ]$, returned from the NLR function, to find solution curves using the MATLAB differential equation solver: ODE45. This is a 4-5 step Runge-Kutta integrator. The maximum point on the $c=c(t)$ curve was identified and the norm, *r*, of the residual vector was calculated. The decay rate, *d*, gives a measure of the inhibition of the enzyme. In particular, if $d = 0$, then there is no inhibition. An example of the output of the first program for the reaction of 1.25mM *p*-cresol with polyphenol oxidase immobilised on a nylon membrane is shown in Figure 3.11.

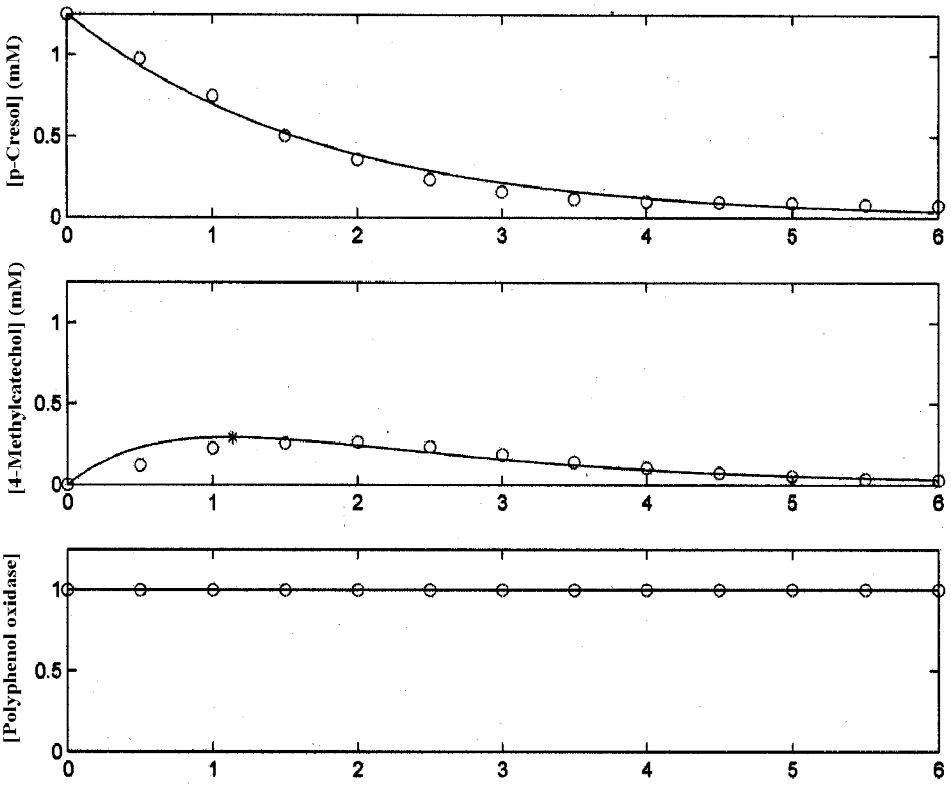


Figure 3.11: The reaction of *p*-cresol with polyphenol oxidase immobilised on a nylon membrane with (o) representing the experimental data and (-) representing the results obtained using the MATLAB differential equation solver: ODE45, $d = 0$, implying no polyphenol oxidase inactivation.

Program 2:

The second program ran the first program for *d* varying from 0 to 95 on the assumption of an exponential decrease of the enzyme and identified that value of *d* which produced the best fit in terms of a minimum value of *r*. Sample output of this program for the same reaction is shown in Figure 3.12.

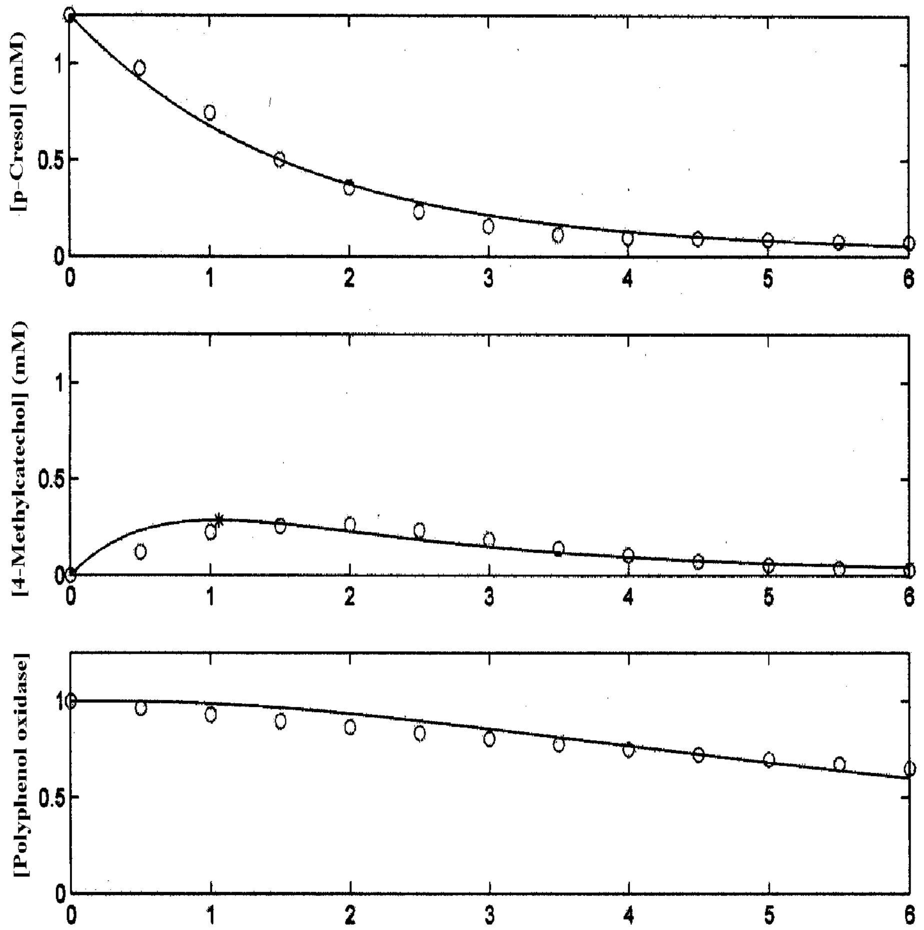


Figure 3.12: The reaction of *p*-cresol with polyphenol oxidase immobilised on a nylon membrane with (o) representing the experimental data and (-) representing the results obtained using the MATLAB differential equation solver: ODE45, assuming the possibility of progressive polyphenol oxidase inactivation.

Output obtained from Programs 1 and 2 of the mathematical model is shown in Appendix B.

The second program produced reaction rate constants, k_1 and k_2 for the reactions. These could be compared with the rate constants generated by the first program in the case where d=0, representing the case where there was no polyphenol oxidase inhibition. Finally, if the simple model

$$\frac{ds}{dt} = -k_1 s$$

is adopted, then the value of k_1 which produced the best fit to the data points X could be obtained and compared with the value obtained from Program 2.

3.3.8 Characterisation of the biocatalytic reaction, derived from the mathematical model

The output of the models is a series of graphs which can be compared with the experimental data (see Figures 3.11 and 3.12), and the closeness of the fit between the graph and the corresponding data then gives an indication of the accuracy of the model. In this study, the closeness of fit was quantified in terms of the statistic r as described above.

The reaction rate constants (k_1) for the first reaction step, under two different concentration conditions (1.25mM and 5mM), were obtained in three different ways:

1) assuming exponential decay of the substrate, and using experimental data to fit the simple model:

$$\frac{ds}{dt} = -k_1 s$$

2) using Program 1 which sets $d = 0$, implying no polyphenol oxidase inactivation,

3) using Program 2, assuming the possibility of progressive polyphenol oxidase inactivation

The reaction rate constants (k_2) for the second reaction step, under two different concentration conditions (1.25mM and 5mM), were obtained using steps 2) and 3) as mentioned above. Calculating the reaction rate constants (k_1) in three different ways presented a useful indication of the extent to which product inactivation of the enzyme was affecting the reaction system. Thus, for example, in a reaction where the rate constants, calculated using Program 1 (implying no inhibition) and those calculated using Program 2 (which includes the possibility of inhibition) were very similar and the r values were low, this demonstrates that the product inhibition was not present to a degree that would alter the experimental results *ie.*, it could be assumed to be negligible. During the reaction of polyphenol oxidase with phenolic substrates, the reaction may be preceded by a lag phase. However, the mathematical model was able to account for the lag phase as the model calculated a corresponding reaction rate constant based on the shape of the graph.

No values of k_1 and k_2 were calculated using Programs 1 and 2 in cases were catechol accumulation was negligible as the mathematical model relies on both phenol and catechol data. In the reactions using 1.25mM substrate concentrations, the rate constants k_1 and k_2, for each substrate are shown in Table 3.2.

Table 3.2: A comparison of the reaction rate constants k_1 and k_2, using 1.25mM substrate, determined in three different ways: assuming exponential decay of the substrate, using Program 1 (where $d = 0$, implying no enzyme inactivation) and using Program 2 (possibility of progressive enzyme inactivation).

	Reaction rate constant (k_1) [a]	**Reaction rate constant (k_1) using Program 1 [b]**	**Reaction rate constant (k_1) using Program 2 [b]**		**Reaction rate constant (k_2) using Program 1 [b]**	**Reaction rate constant (k_2) using Program 2 [b]**	**k_1/k_2 using Program 1**	**k_1/k_2 using Program 2**
1.25mM	k_1 (s^{-1})	k_1 (s^{-1})	k_1 (s^{-1})	%decay	k_2 (s^{-1})	k_2 (s^{-1})		
***p*-cresol**								
non-immobilised	0.64	0.56	0.56	0	0.21	0.21	2.67	2.05
nitrocellulose	0.6	-	-	-	-	-	-	-
nylon	0.6	0.59	0.61	35	1.29	1.41	0.46	0.43
glass-fibre	0.29	0.29	0.29	0	7.3	7.3	0.04	0.04
PTFE	0.22	-	-	-	-	-	-	-
polysulphone	0.24	0.22	0.29	50	13.31	428.1	0.02	0.0007
***m*-cresol**								
non-immobilised	0.43	0.5	0.56	80	0.26	0.38	1.92	1.47
nitrocellulose	0.09	-	-	-	-	-	-	-
nylon	0.07	0.07	0.08	30	1.59	2.23	0.04	0.04
glass-fibre	0.04	-	-	-	-	-	-	-
PTFE	0.03	0.03	0.03	10	31.87	649.89	0.0009	0.0001
polysulphone	0.33	-	-	-	-	-	-	-
phenol								
non-immobilised	0.49	0.47	0.46	5	0.99	1.05	0.48	0.44
nitrocellulose	0.06	-	-	-	-	-	-	-
nylon	0.07	0.07	0.07	10	19.11	31.09	0.004	0.002
glass-fibre	0.03	-	-	-	-	-	-	-
PTFE	0.03	-	-	-	-	-	-	-
polysulphone	0.13	0.13	0.14	25	26.43	649.93	0.005	0.0002
4-chlorophenol								
non-immobilised	0.11	0.11	0.11	0	9.43	-	-	-
nitrocellulose	0.15	-	-	-	-	-	-	-
nylon	0.14	-	-	-	-	-	-	-
glass-fibre	0.06	-	-	-	-	-	-	-
PTFE	0.08	-	-	-	-	-	-	-
polysulphone	0.29	-	-	-	-	-	-	-

[a]: assuming exponential decay of the substrate

[b]: no values were obtained using Program 1 and 2 in cases when catechol accumulation was negligible as the model relies on both phenol and catechol data

In the reactions using 1.25mM substrate concentrations, the rate constants k_1 were found to be very similar by all three methods of calculation, for each substrate (Table 3.2). For example, in the conversion of *p*-cresol, using polyphenol oxidase immobilised on nylon, k_1 values of $0.60s^{-1}$, $0.59s^{-1}$ and $0.61s^{-1}$ were obtained by the three methods of calculation. This suggests that polyphenol oxidase inactivation was not significant under these conditions. The highest reaction rate constants k_1 were found during the reaction of non-immobilised polyphenol oxidase with *p*-cresol, *m*-cresol and phenol. However, when 4-chlorophenol was used the highest reaction rate constant of $0.29s^{-1}$ was measured during the reaction of polyphenol oxidase immobilised on polysulphone membranes, which could be attributed to the fact that this is the least polar substrate, as evidenced by RP-HPLC. Thus, some hydrophobic interactions may enhance the affinity of the 4-chlorophenol for the hydrophobic polysulphone membrane, increasing its accessibility to the enzyme and hence increasing the reaction rate.

Conversely, with 1.25mM substrate, the reaction rate constants k_2 (Table 3.2) were lowest when the enzyme was not immobilised for all the substrates. Thus, in general, the catecholase activity was favoured by immobilisation, particularly in the case of the hydrophobic polysulphone membranes. Marked differences in the k_2 values, calculated using Programs 1 and 2, were observed for reactions in which polyphenol oxidase was immobilised on hydrophobic membranes. Such differences imply significant polyphenol oxidase inactivation occurring during the reaction. In the case of the reaction of phenol with polyphenol oxidase immobilised on polysulphone, the values were $k_2 = 26.43s^{-1}$ (using Program 1) and $k_2 = 649.93s^{-1}$ (using Program 2), and the corresponding enzyme inactivation was determined to be 25%. During the reactions of *p*-cresol and *m*-cresol the values of k_2 were lower than k_1 when the enzyme was not immobilised, favouring catechol accumulation, and higher when the enzyme was immobilised. When phenol and 4-chlorophenol were used as substrates, the values of k_2 were found to be higher than k_1 whether the enzyme was immobilised or non-immobilised indicating that the catecholase activity was favoured.

In the reactions using 5mM substrate concentrations, the rate constants k_1 and k_2, for each substrate are shown in Table 3.3.

Table 3.3: A comparison of the reaction rate constants k_1 and k_2, using 5mM substrate, determined in three different ways: assuming exponential decay of the substrate, using Program1 (where $d = 0$, implying no enzyme inactivation) and using Program 2 (possibility of progressive enzyme inactivation).

	Reaction rate constant (k_1) [a]	**Reaction rate constant (k_1) using Program 1 [b]**	**Reaction rate constant (k_1) using Program 2 [b]**		**Reaction rate constant (k_2) using Program 1 [b]**	**Reaction rate constant (k_2) using Program 2 [b]**	**k_1/k_2 using Program 1**	**k_1/k_2 using Program 2**
5mM substrate	k_1 (s^{-1})	k_1 (s^{-1})	k_1 (s^{-}	% decay	k_2 (s^{-1})	k_2 (s^{-1})		
***p*-cresol**								
non-immobilised	0.12	0.12	0.12	10	0.52	0.53	0.23	0.23
nitrocellulose	0.13	-	-	-	-	-	-	-
nylon	0.12	0.12	0.15	55	2.05	2.56	0.06	0.06
glass-fibre	0.09	0.09	0.09	15	4.67	4.91	0.02	0.02
PTFE	0.07	0.22	0.22	0	0.96	0.96	0.23	0.23
polysulphone	0.11	0.11	0.29	95	4.7	13.7	0.02	0.02
***m*-cresol**								
non-immobilised	0.07	0.07	0.13	90	0.65	1.16	0.11	0.11
nitrocellulose	0.06	0.06	0.07	60	19.09	574	0.003	0.0001
nylon	0.05	0.05	0.08	80	3.56	5.58	0.01	0.01
glass-fibre	0.03	-	-	-	-	-	-	-
PTFE	0.03	-	-	-	-	-	-	-
polysulphone	0.12	-	-	-	-	-	-	-
phenol								
non-immobilised	0.1	0.1	0.13	60	1.55	2.03	0.07	0.06
nitrocellulose	0.04	-	-	-	-	-	-	-
nylon	0.03	0.03	0.04	25	20.02	46.17	0.002	0.0009
glass-fibre	0.04	-	-	-	-	-	-	-
PTFE	0.03	-	-	-	-	-	-	-
polysulphone	0.08	0.08	0.22	95	16.97	63.1	0.005	0.004
4-chlorophenol								
non-immobilised	0.01	0.01	0.01	0	1.9	1.9	0.005	0.005
nitrocellulose	0.05	-	-	-	-	-	-	-
nylon	0.07	0.07	0.1	75	17.12	710	0.004	0.0001
glass-fibre	0.03	-	-	-	-	-	-	-
PTFE	0.03	-	-	-	-	-	-	-
polysulphone	0.11	-	-	-	-	-	-	-

[a]: assuming exponential decay of the substrate

[b]: no values were obtained using Program 1 and 2 in cases when catechol accumulation was negligible as the model relies on both phenol and catechol data

In the reactions using 5mM substrate concentration, the rate constants k_1 were found to be very similar for all three methods of calculation for each substrate (Table 3.3). Exceptions were observed in reactions where polyphenol oxidase was immobilised on PTFE and polysulphone membranes. For example in the conversion of *p*-cresol using polyphenol oxidase immobilised on PTFE membranes, a k_1 value of $0.22s^{-1}$ was calculated using both Programs 1 and 2. Again, no significant product inactivation was indicated. The highest reaction rate constants k_1 were obtained during the reaction of *p*-cresol, with $k_1 = 0.12s^{-1}$ using non-immobilised polyphenol oxidase, $k_1 = 0.13s^{-1}$ for polyphenol oxidase immobilised on nitrocellulose and $k_1 = 0.12s^{-1}$ using polyphenol oxidase immobilised on nylon membranes. The values of k_2 were always higher than k_1 when polyphenol oxidase was non-immobilised or immobilised using 5mM concentrations of each substrate. The catecholase activity of the enzyme was favoured using 5mM substrate concentrations under immobilised and non-immobilised conditions.

In a comparison of the results, reactions conducted using 1.25mM substrate concentrations resulted in k_1 values that were always higher than the corresponding values obtained using 5mM substrate concentrations. This same trend was not observed in the case of k_2 values. Measured values of k_2 which are higher than the corresponding k_1 for the same reaction, characterise reactions where the catecholase activity of the enzyme is higher than the cresolase activity. Thus, the ratio of k_1 / k_2 gives an indication of conditions under which catechol would accumulate. The ratio of k_1 / k_2 was measured using 1.25mM substrate concentrations and 5mM substrate concentrations. For example, a value of 2.67 was obtained for the ratio of k_1 / k_2 using 1.25 mM *p*-cresol and non-immobilised polyphenol oxidase, while 0.23 was obtained using 5mM substrate concentration under the same conditions. The k_1 / k_2 ratio was generally higher when 1.25 mM substrates were used, indicating that catechol accumulation would occur more at lower substrate concentrations. At higher substrate concentrations, k_1 / k_2 ratios were consistently low implying that catechols do not accumulate under these conditions. Thus the optimal conditions for catechol production would require low substrate concentrations.

Programs 1 and 2 were used to determine the maximum catechol and corresponding quinone concentration reached for a given reaction, and hence the maximum concentration of catechol achievable under the respective reaction conditions (Tables 3.4 and 3.5). The ratio of the catechol concentration to the quinone concentration ([catechol] / [quinone]) calculated as a high ratio would imply a high concentration of catechol had accumulated in solution. This ratio was used to investigate reaction conditions best suited to catechol production.

For each catechol, the highest concentration was produced when polyphenol oxidase was not immobilised. In spite of this result, there are many advantages to using an immobilised enzyme rather than a soluble one in biotransformation processes, and hence consideration was given to the membrane providing the best immobilisation conditions for catechol production.

Table 3.4: Using Program 1 (where $d = 0$, implying no polyphenol oxidase inactivation) and using Program 2 (possibility of progressive polyphenol oxidase inactivation), the maximum catechol and corresponding quinone concentrations were determined using 1.25mM substrate.

1.25mM substrate		**maximum [catechol] (mM) [a] no inactivation**	**corresponding [quinone] (mM) no inactivation**	**maximum [catechol] (mM) [a] inactivation**	**corresponding [quinone] (mM) inactivation**	**[catechol]/ [quinone] no inactivation**	**catechol]/ [quinone] inactivation**
***p*-cresol**	non-immobilised	0.692	0.296	-	-	2.334	-
	nylon	0.294	0.314	0.287	0.309	0.936	0.927
	glass-fibre	0.043	0.115	-	-	0.374	-
	polysulphone	0.019	0.067	0.001	0.005	0.291	0.168
***m*-cresol**	non-immobilised	0.615	0.305	0.553	0.328	2.018	1.684
	nylon	0.048	0.121	0.039	0.106	0.399	0.364
	PTFE	0.001	0.008	0	0.001	0.165	0.074
phenol	non-immobilised	0.3	0.303	0.288	0.305	0.99	0.942
	nylon	0.004	0.02	0.003	0.014	0.21	0.19
	polysulphone	0.006	0.026	0.0002	0.0034	0.222	0.08
4-chlorophenol	non-immobilised	0.013	0.05	-	-	0.265	-

[a]: catechol was not detected in all cases

Table 3.5: Using Program 1 (where *d* = 0, implying no polyphenol oxidase inactivation) and using Program 2 (possibility of progressive polyphenol oxidase inactivation), the maximum catechol and corresponding quinone concentrations were determined using 5mM substrate.

5mM substrate		**maximum [catechol] (mM) [a] no inactivation**	**corresponding [quinone] (mM) no inactivation**	**maximum [catechol] (mM) [a] inactivation**	**corresponding [quinone] (mM) inactivation**	**[catechol]/ [quinone] no inactivation**	**[catechol] / [quinone] inactivation**
***p*-cresol**	non-immobilised	0.722	1.029	0.724	1.046	0.701	0.692
	nylon	0.25	0.559	0.243	0.545	0.447	0.446
	glass-fibre	0.088	0.282	0.087	0.28	0.312	0.312
	PTFE	0.146	0.422	-	-	0.345	-
	polysulphone	0.104	0.32	0.097	0.302	0.324	0.32
***m*-cresol**	non-immobilised	0.414	0.787	0.427	0.781	0.526	0.546
	nitrocellulose	0.015	0.07	0.0007	0.01	0.211	0.065
	nylon	0.07	0.238	0.068	0.231	0.295	0.295
phenol	non-immobilised	0.269	0.584	0.257	0.56	0.46	0.459
	nylon	0.008	0.044	0.0038	0.023	0.182	0.162
	polysulphone	0.022	0.096	0.017	0.079	0.229	0.219
4-chlorophenol	non-immobilised	0.028	0.119	-	-	0.234	-
	nylon	0.019	0.084	0.0007	0.006	0.223	0.124

[a]: catechol was not detected in all cases

Based on the high [catechol]/[quinone] ratios (Tables 3.4 and 3.5), *p*-cresol and *m*-cresol are the substrates best suited for catechol production using this system. The immobilisation support resulting in observation of the highest [catechol]/[quinone] ratio was nylon, where values of 0.936 and 0.447 were obtained using 1.25mM and 5mM substrate concentrations respectively. However, when phenol was used as the substrate, polysulphone- immobilised polyphenol oxidase produced the highest [catechol]/[quinone] ratios of 0.222 and 0.229, for 1.25mM and 5mM substrate concentrations respectively, calculated on the basis of Program 1.

The maximum catechol concentration produced for each substrate concentration, under immobilised and non-immobilised reaction conditions, was calculated as a percentage and used for quantitative comparison of the immobilised biocatalysts (Table 3.6). Use of starting substrate concentrations of 1.25mM resulted in a higher percentage accumulation of catechol products than when the starting concentration was 5mM. The differences in the production of catechol products with substrate concentration could be attributed to enzyme inactivation at higher phenol concentrations. Efficient conversion of phenols occurred using polyphenol oxidase provided that the enzyme concentration increased with increasing phenol concentration as enzyme inactivation was evident at low enzyme concentrations (Atlow *et al.*, 1984) and in this study the enzyme concentration did not increase with increasing substrate concentration. Each substrate resulted in differences in the percentage accumulation of catechol products. 1.25mM *p*-cresol and *m*-cresol resulting in the highest percentage accumulation of catechol products with 54.84% and 49.16% 4-methylcatechol and 3-methylcatechol being produced under non-immobilised conditions respectively. Immobilisation of polyphenol oxidase resulted in decreased production of catechol products compared to non-immobilised enzyme. Use of nylon and nitrocellulose-immobilised polyphenol oxidase resulted in the highest percentage accumulation of catechol products under immobilised conditions.

Table 3.6: Maximum catechol produced using each substrate concentration under non-immobilised and immobilised reaction conditions.

	4-methylcatechol		**3-methylcatechol**		**catechol**		**4-chlorocatechol**	
	1.25mM *p*-cresol (%)^{a}	**5mM *p*-cresol (%) ^{a}**	**1.25mM *m*-cresol (%) ^{a}**	**5mM *m*-cresol (%) ^{a}**	**1.25mM phenol (%) ^{a}**	**5mM phenol (%) ^{a}**	**1.25mM 4-chlorophenol (%) ^{a}**	**5mM 4-chlorophenol (%) ^{a}**
non-immobilised	54.84	14.36	49.16	8.25	22.94	4.69	3.11	0.58
nylon	22.97	4.34	3.39	0.46	0	0	-	-
nitrocellulose	25.91	6.04	0	0	-	-	-	-
glass-fibre	4.33	1.39	-	-	-	-	-	-
polysulphone	0.01	0	0	-	0	0	-	-
PTFE	15.05	2.94	-	-	-	-	-	-

^{a} : calculated using maximum catechol concentration produced / substrate concentration

3.3.9 Substrate and product adsorption using flat-sheet membranes

To compare the activity of immobilised polyphenol oxidase to non-immobilised polyphenol oxidase, the effect of substrate and product adsorption on the immobilisation support needed to be assessed. *p*-Cresol was used to calculate the degree of substrate adsorption while the polyphenol oxidase-catalysed formation of 4-methylcatechol was used to determine the degree of product adsorption to the membranes. Table 3.7 shows the percentage substrate (*p*-cresol) and product (4-methylcatechol) adsorption to the immobilisation supports. In general, low levels of substrate and product adsorption were detected and this varied with the membrane type. 4.8% substrate and 3.9% product adsorption occurred using nylon membranes while no substrate adsorption occurred using glass-fibre membranes and no product adsorption occurred using nitrocellulose and PTFE membranes. However, 50% substrate and 19.6% product adsorption was detected using polysulphone membranes. The reaction rates calculated in Section 3.3.8 did not take substrate and product adsorption into account as it was assumed that the adsorbed substrate and catechol product were not irreversibly bound to the membranes and still accessible to the enzyme.

Table 3.7: Substrate and product adsorption on each of the immobilisation supports using 1.25mM *p*-cresol and polyphenol oxidase-catalysed formation of 4-methylcatechol from 1.25mM *p*-cresol.

Membrane type	**% substrate adsorption**	**% product adsorption**
nylon	4.8	3.9
nitrocellulose	11	0
glass-fibre	0	2.5
PTFE	3.3	0
polysulphone	50	19.6

3.3.10 Operational stability of polyphenol oxidase immobilised on nylon membranes

Characterisation of membrane supports, as derived from the mathematical model, revealed that nylon membranes were the most suitable support for catechol production and therefore these membranes were used for a stability study. Immobilisation of enzymes allows for ease of handling and reuse of the biocatalyst, whereas the difficulties involved in reusing non-immobilised enzymes meant that it was not possible to compare the stability of nylon-immobilised polyphenol oxidase with non-immobilised polyphenol oxidase. The stability of polyphenol oxidase immobilised on nylon was assessed by reusing the immobilised enzyme for 6 batch experiments. The relative activity of immobilised polyphenol oxidase was calculated after each run (Figure 3.13) and was found to have decreased to 20% after 6 batch experiments

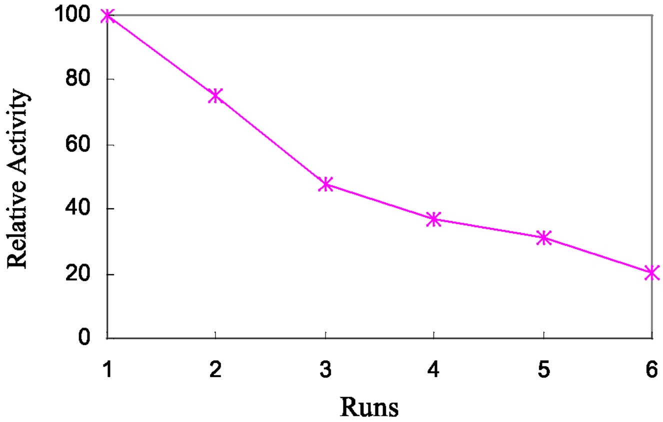


Figure 3.13: Activity of nylon-immobilised polyphenol oxidase after 6 batch experiments using L-DOPA as the substrate.

A long term stability study of polyphenol oxidase immobilised on nylon membranes was carried out by storing the immobilised biocatalyst in saline and potassium phosphate buffer and monitoring the activity of the enzyme using L-DOPA as the substrate. The relative activity of polyphenol oxidase was calculated over 11 days and is shown in Figure 3.14. The activity of the immobilised biocatalyst decreased sharply over the first 3 days. There was little difference in the activity of polyphenol oxidase stored in saline or buffer with approximately 90% loss in activity after 11 days. The freeze-dried enzyme powder maintained its activity, thus the enzyme should be stored in a freeze-fried form before being immobilised for use as a biocatalyst.

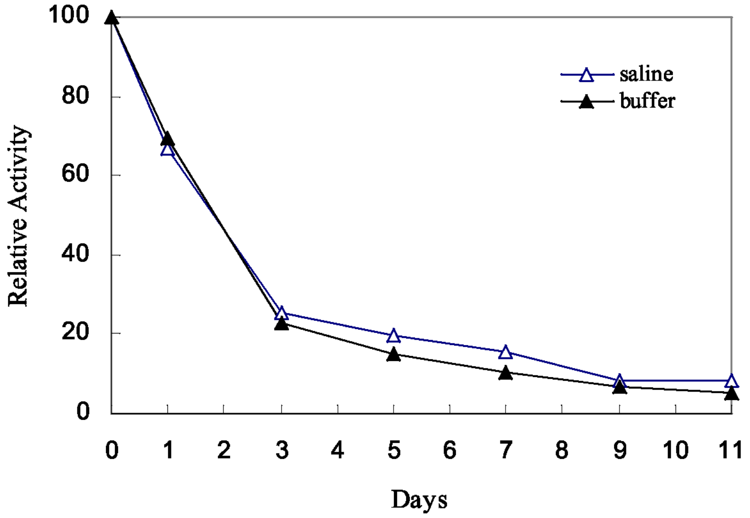


Figure 3.14: Activity of nylon-immobilised polyphenol oxidase, monitored over 11 days, whilst stored in saline and potassium phosphate buffer, using L-DOPA as the substrate.

3.3.11 Effect of incorporating different surfactants on polyurethane foam properties

According to the literature polyphenol oxidase has not been previously immobilised within polyurethane foams. A preliminary investigation was carried out on the activity of polyphenol oxidase within the foam. The foams were initially synthesised using Emolgrade and Pluronic L-62, P-65 and F-68 surfactants without the addition of enzyme. The effect of each surfactant on the properties of the foam was evaluated. The appearance of the foams differed and the hydrophilicity was determined by placing pieces of the foam into a solution of buffer (Table 3.8).

Table 3.8: Appearance of polyurethane foams using different surfactants.

	Appearance	**Density**
Pluronic F-68	large pores	sinks in buffer
Pluronic P-65	very large pores	sinks in buffer
Pluronic L-62	very dense, small pores	sinks in buffer
Emolgrade	small pores	floats in buffer
Potassium phosphate buffer	small pores	floats in buffer

Foams containing Emolgrade surfactant and potassium phosphate buffer exhibited hydrophobic properties and were not considered for subsequent experiments as mixing of the biocatalyst with the substrate would be problematic.

3.3.12 Substrate and product adsorption using polyurethane foam

The effect of the foams on the bulk substrate and product concentrations was evaluated by placing pieces of the foam into solutions of *p*-cresol, phenol, 4-methylcatechol and catechol and monitoring the changes in the concentrations of these compounds. Adsorption of substrate and product to the foam varied with the surfactant used to synthesise the foam (Table 3.9). Low levels of 4-methylcatechol and catechol were adsorbed on each of the foams and the highest adsorption, of 17.7% *p*-cresol, occurred using polyurethane foam containing Pluronic F-68.

Table 3.9: Substrate and product adsorption on polyurethane foams containing Pluronic L-62, F-68 and P-65 surfactants using 5mM substrate and 1.25mM product.

		Percentage Adsorption (%)		
		Pluronic P-65	**Pluronic L-62**	**Pluronic F-68**
Substrate	phenol	13.66	8.76	13.24
	p-cresol	13.37	12.81	17.7
Product	catechol	7.61	10.65	7.61
	4-methylcatechol	3.92	6.28	2.75

3.3.13 Immobilisation of polyphenol oxidase within polyurethane foam

The activity of crude polyphenol oxidase in polyurethane foams, synthesised containing Pluronic P-65, L-62 and F-68, was determined by placing 200mg pieces of the foams into 20mL 10mM L-DOPA and monitoring the appearance of dopachrome at 475nm (Figure 3.15).

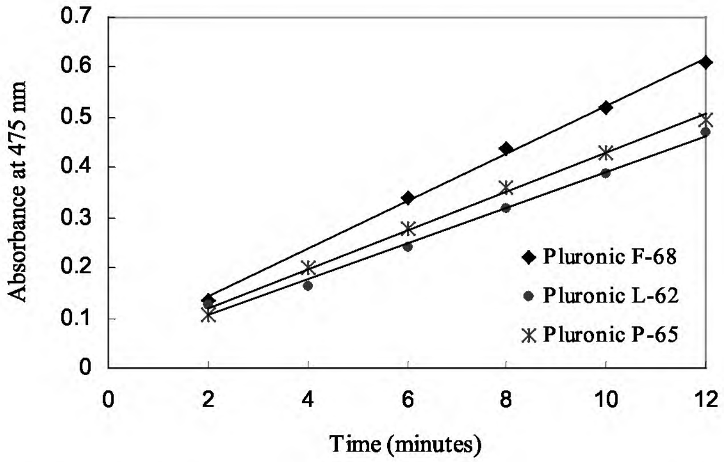


Figure 3.15: Monitoring formation of dopachrome from L-DOPA at 475nm catalysed by crude polyphenol oxidase immobilised in polyurethane foams.

The percentage of crude polyphenol oxidase activity in the foams was calculated by determining the number of units of active enzyme after immobilisation as a percentage of the number of units used to synthesise the foam (Table 3.10). The foam containing Pluronic F-68 retained the highest polyphenol oxidase activity of 33.2% and based on these results, the experiment was repeated using commercial polyphenol oxidase. The foam prepared using commercial polyphenol oxidase retained 35 % activity (Table 3.10).

Table 3.10: The activity of crude and commercially available polyphenol oxidase present in polyurethane foams synthesised in the presence of Pluronic F-68, L-62 and P-65 surfactants.

Polyphenol oxidase preparation	**Surfactant**	**ΔA/min**	**Units polyphenol oxidase (U) /g foam**	**Polyphenol oxidase activity (%)**
Crude	Pluronic F-68	0.0474	1.317	33.2
	Pluronic L-62	0.0355	0.986	25.1
	Pluronic P-65	0.0389	1.081	26.8
Commercial	Pluronic F-68	0.3616	10.045	35

3.3.14 Stability of polyphenol oxidase - polyurethane foam

The activity of polyphenol oxidase immobilised in polyurethane foams was assessed by reusing the immobilised enzyme for 6 runs, and the relative activity was calculated after each run (Figure 3.16). After 6 runs polyurethane-immobilised polyphenol oxidase had retained 37% activity.

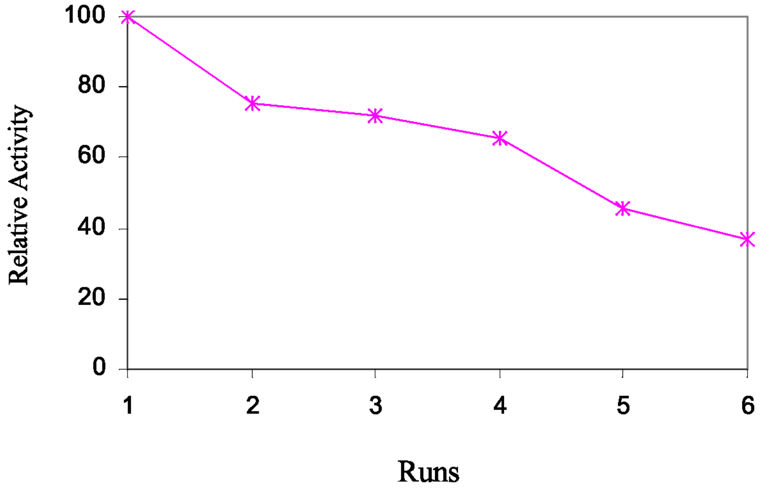


Figure 3.16: Activity of polyurethane-immobilised polyphenol oxidase after 6 consecutive batch reactions using 10mM L-DOPA as substrate.

The stability of polyurethane-immobilised polyphenol oxidase stored at room temperature was monitored by placing pieces of the foam into vials and measuring the activity of each piece of foam on different days over a 15 day period using L-DOPA as the substrate (Figure 3.17). After 15 days 65% polyphenol oxidase activity was still present in the polyurethane foam.

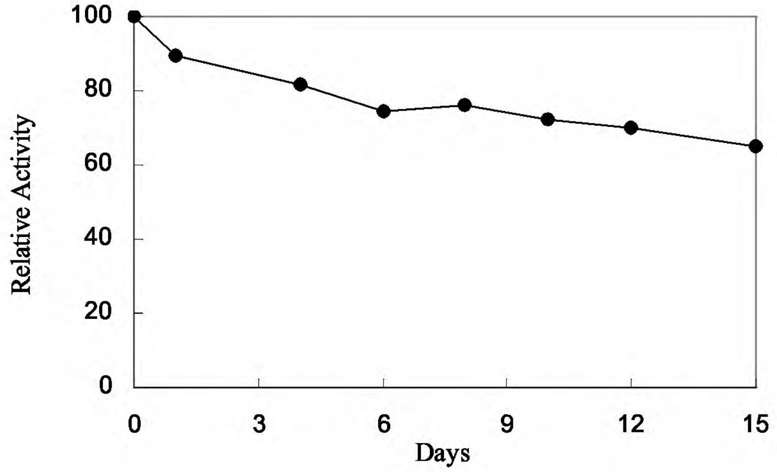


Figure 3.17: Activity of polyurethane-immobilised polyphenol oxidase over 15 days at room temperature using 10mM L-DOPA as substrate.

The effect of storing the immobilised biocatalyst in saline solution and potassium phosphate buffer at 4°C was investigated by periodically monitoring the enzyme activity of the same piece of foam using L-DOPA as the substrate. The relative activity of polyphenol oxidase was calculated over 11 days (Figure 3.18). The activity of the immobilised biocatalyst was slightly higher when stored in saline than buffer, and approximately 15% polyphenol oxidase activity was retained after 11 days.

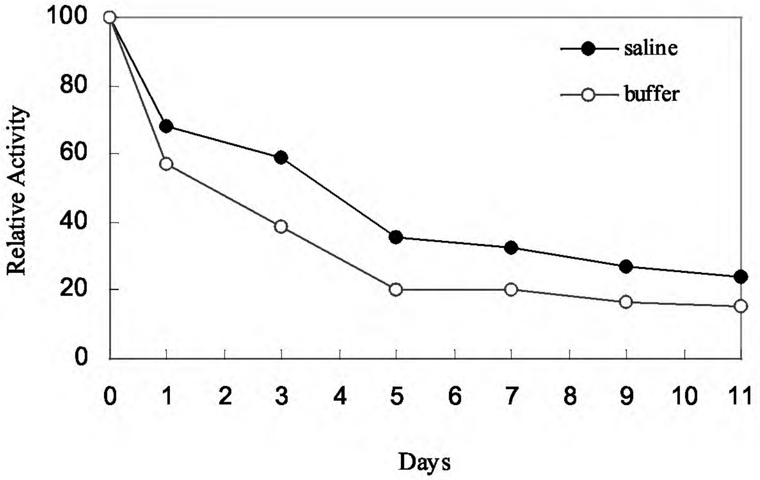


Figure 3.18: The activity of polyphenol oxidase immobilised in polyurethane foam and stored in saline and potassium phosphate buffers, using L-DOPA as the substrate.

3.3.15 Reaction of polyurethane-immobilised polyphenol oxidase with phenolic substrates

The polyurethane foam synthesised using commercial polyphenol oxidase and Pluronic F-68 surfactant was allowed to react with 1.25mM and 5mM *p*-cresol and *m*-cresol (Table 3.11). Using *p*-cresol as the substrate resulted in a higher percentage accumulation of catechol products than *m*-cresol, which is the same trend as was observed using polyphenol oxidase immobilised on synthetic membranes (Section3.3.8).

Table 3.11: Substrate converted and catechol produced after 3 hours during the reaction of polyurethane-immobilised polyphenol oxidase with *p*-cresol and *m*-cresol.

Substrate	Concentration (mM)	Substrate converted (mM)	Catechol produced (mM)	Catechol accumulation (%)[a]
***p*-cresol**	1.33	1.15	0.89	66.9
	5.11	1.87	0.92	18
***m*-cresol**	1.62	0.47	0.079	4.9
	6.25	1.54	0.15	2.4

[a] : catechol concentration / substrate concentration

3.3.16 Immobilisation of polyphenol oxidase in an organogel

Immobilisation of polyphenol oxidase in an organogel involved cross-linking the enzyme using glutaraldehyde and entrapping the enzyme during gel synthesis. The most significant problem was diffusional limitations due to the thickness of the gel. When the gel was poured into more plates, resulting in thinner gels, freeze-dried and crushed, the substrate was more accessible to the enzyme. 200mg of the crushed gel was placed into L-DOPA to determine the retained activity of the enzyme.

The retained activity in the gel was found to be very low, in that only 2.6 % of the original number of units of polyphenol oxidase were active. However, when the gel was placed in 1.45mM and 5.2mM *p*-cresol solutions (Figures 3.19 and 3.20) and allowed to react for 3 hours, significant substrate removal was observed, with 80 % removal of *p*-cresol being observed after 3 hours. Thus, the L-DOPA assay, used to determine polyphenol oxidase activity in the gel, did not appear to give an accurate indication of polyphenol oxidase activity. This may have ben due to diffusional limitations of the gel. The solution was highly coloured, indicating *o*-quinone formation but low levels of 4-methylcatechol were detected in solution, with 8.6% and 2.9% accumulation using 1.45mM and 5.2mM *p*-cresol solutions respectively. Polyphenol oxidase immobilised in an organogel resulted in the conversion of phenols, but was not suitable for 4-methylcatechol production. The organic environment of the enzyme in the organogel was not suited to catechol accumulation as catechols were rapidly oxidised to *o*-quinones.

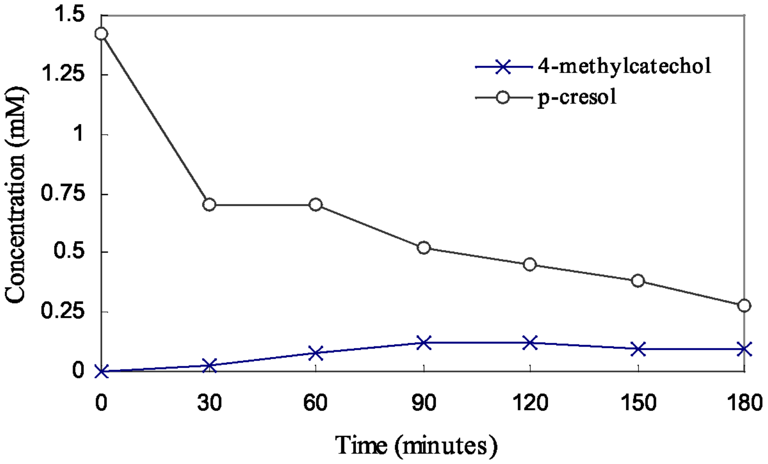


Figure 3.19: The utilisation of 1.45mM *p*-cresol and the formation of 4-methylcatechol over 3 hours using polyphenol oxidase immobilised in an organogel.

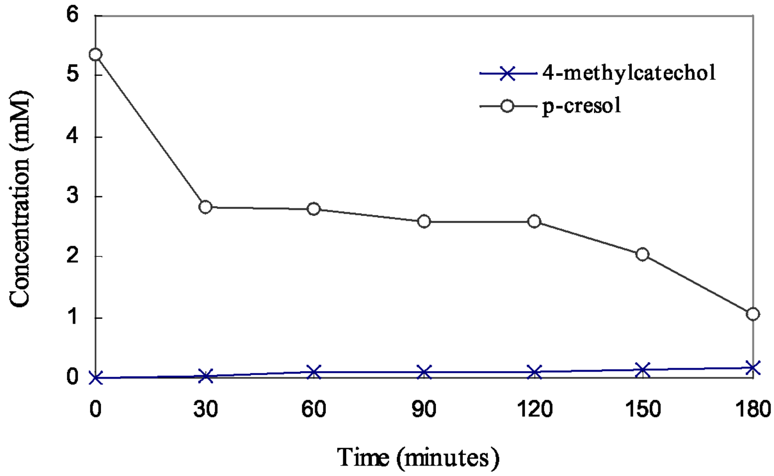


Figure 3.20: The utilisation of 1.45mM *p*-cresol and the formation of 4-methylcatechol over 3 hours using polyphenol oxidase immobilised in an organogel.

3.3.17 Comparison of polyphenol oxidase immobilisation supports for catechol production

The identification of a suitable polyphenol oxidase immobilisation support for the development of a process for catechol synthesis was the objective of this study. Polyphenol oxidase was immobilised on a variety of supports in order to determine the type of support that allowed catechols to accumulate in solution.

Reactions carried out using polyphenol oxidase immobilised on synthetic membranes, which resulted in the highest production of catechol products, were compared to polyurethane-immobilised reactions (Table 3.12). The substrates resulting in the highest production of catechol products, using polyphenol oxidase immobilised on synthetic membranes, were *p*-cresol and *m*-cresol, and greater accumulation of catechol products occurred using 1.25mM than 5mM substrate concentrations. The immobilisation supports were compared in terms of μmoles of 3-methyl- and 4-methylcatechol produced per unit of polyphenol oxidase. Polyurethane-immobilised polyphenol oxidase resulted in the highest number of μmoles of 4-methylcatechol being produced per unit enzyme of 63.57 and 67.31μmoles, using 1.33mM and 5.11mM *p*-cresol respectively. Nylon-immobilised polyphenol oxidase also resulted in significant 4-methylcatechol production of 18.71 μmoles per unit enzyme. The results indicate that a hydrophilic enzyme support enhances catechol accumulation.

Table 3.12: A comparison of various immobilisation supports in terms of μmoles of 4-methyl- and 3-methylcatechol produced per unit polyphenol oxidase.

Immobilisation support	**Substrate**		**Units PPO (U)**	**Reaction volume (mL)**	**Reaction time (hours)**	**Catechol product produced (mM)**	**μmoles catechol product produced /U PPO**
	Type	**[mM]**					
nylon	*p*-cresol	1.25	0.31	20	6	0.29	18.71
	m-cresol	1.25	0.31	20	6	0.04	2.58
nitrocellulose	*p*-cresol	1.25	0.29	20	6	0.32	22.07
	m-cresol	1.25	0.29	20	6	0	0.003
glass-fibre	*p*-cresol	1.25	0.3	20	6	0.054	3.6
polysulphone	*p*-cresol	1.25	0.31	20	6	0	0.008
	m-cresol	1.25	0.31	20	6	0	0.002
PTFE	*p*-cresol	1.25	0.28	20	6	0.19	13.57
polyurethane	*p*-cresol	1.33	0.42	30	3	0.89	**63.57**
	p-cresol	5.11	0.41	30	3	0.92	**67.31**
	m-cresol	1.62	0.4	30	3	0.079	5.93
	m-cresol	6.25	0.43	30	3	0.15	10.47

The influence of the immobilisation support on catechol production by polyphenol oxidase was demonstrated in this study, and the results highlight the need to choose the immobilisation support carefully for a particular biotransformation reaction. Hydrophobic supports appeared to increase the catecholase activity of polyphenol oxidase resulting in the rapid oxidation of catechol products to *o*-quinones, thus these supports would be better suited to a bioremediation approach resulting in the polymerization of *o*-quinones. For reactions carried out in water, each phenolic substrate reaction accumulated catechol products to a different extent, with the *p*-cresol and *m*-cresol reactions resulting in the highest accumulation of catechol products in solution. The concentration of the phenolic substrate also influenced the levels of catechol accumulation, with greater accumulation evident at 1.25mM than 5mM concentrations. This effect could be overcome by the addition of increased amounts of polyphenol oxidase to the reaction system.

3.4 CONCLUSION

The immobilisation of polyphenol oxidase was a further development in the process to produce catechols as it facilitates re-use and easy removal of the biocatalyst from the reaction system. Polyphenol oxidase was efficiently immobilised on a variety of synthetic membranes and incorporated into polyurethane foams and an organogel for the conversion of phenolic substrates. Reaction conditions and properties of the immobilisation support were shown to strongly influence catechol formation.

Polyphenol oxidase was effectively immobilised on a transverse-flow polyethersulphone capillary membrane bioreactor with potential for the development of a continuous process, as opposed to the treatment of small volumes in batch systems. Significant substrate removal was achieved with polyphenol oxidase immobilised on the shell-side of the bioreactor, with the substrate being recycled through the lumen and permeate fractions being collected from the shell-side. This configuration allowed for separation of the *o*-quinone products from the immediate environment surrounding the enzyme which resulted in a significantly lowered effect of suicide inactivation. However, 4-methylcatechol or catechol did not appear to accumulate in solution but were rapidly oxidised to *o*-quinone, which suggested that polyethersulphone was not an ideal polyphenol oxidase immobilisation support for catechol production. The hydrophobic nature of polyethersulphone appeared to influence the catecholase activity resulting in the rapid oxidation of catechol products to *o*-quinones.

The use of alternative immobilisation supports on the reaction of polyphenol oxidase with phenolic compounds was investigated. The influences of the hydrophilic or hydrophobic properties of the immobilisation supports were studied for the identification of the most suitable support for catechol accumulation. A range of hydrophilic supports (nylon, nitrocellulose and glass-fibre) and hydrophobic supports were evaluated with the aid of a novel mathematical model which was developed and used to determine the reaction rate constants for the cresolase and catecholase activities of the enzyme. The application of the mathematical model proved to be a useful aid in the interpretation of reaction kinetic data which could then be used to determine which substrates and support materials allowed for maximum catechol accumulation. The mathematical model was also able to take polyphenol oxidase inactivation into account and the extent of inactivation could be determined by comparing reaction rate constants. The results indicated that enzyme inactivation was not significant at low substrate concentrations. In the application of the system for catechol production, the results have shown that different concentrations of catechol were produced for the different phenolic substrates. Higher concentrations of catechol accumulated in solution using 1.25mM substrate concentrations than 5mM substrate concentrations, which could be attributed to enzyme inactivation at higher phenol concentrations. Nylon and nitrocellulose-immobilised polyphenol oxidase could be applied in the production of 4-methylcatechol with the percentage accumulation being 22.97% and 25.91%, respectively using 1.25mM *p*-cresol.

Polyphenol oxidase was successfully immobilised in polyurethane foams in the presence of various surfactants. The foam containing Pluronic F-68 resulted in the formation of a hydrophilic foam with the highest percentage polyphenol oxidase. The enzyme was also incorporated into an organogel. Polyurethane and gel-immobilised polyphenol oxidase were allowed to react with *p*-cresol as it was the substrate which resulted in the highest percentage accumulation of catechol products by immobilising polyphenol oxidase on synthetic membranes. Polyurethane-immobilised polyphenol oxidase resulted in 66.9% 4-methylcatechol accumulation using 1.25mM *p*-cresol. Significant substrate conversion occurred with polyphenol oxidase immobilised in an organogel, although minimal catechol accumulation occurred (8.6%) using 1.45mM *p*-cresol.

The operational stability of polyphenol oxidase after 6 consecutive batch experiments, and storage of the immobilised biocatalyst in buffer and saline over a period of 11 days, was higher for polyurethane-immobilised polyphenol oxidase than nylon-immobilised polyphenol oxidase. However, immobilisation of polyphenol oxidase did not increase the stability of the biocatalyst as the activity of the immobilised enzyme decreased significantly after the second day of storage.

An investigation of the effects of immobilisation on the production of catechol products using polyphenol oxidase has not been reported previously in the literature. The results of this study have allowed for the prediction and control of the reaction products, using immobilised polyphenol oxidase, by manipulating the enzyme immobilisation support and, type and concentration of substrate. The mathematical modelling approach used could later be extended to other immobilised biocatalyst systems for the interpretation of kinetic data. Nylon and polyurethane foam were shown to be the most effective immobilisation supports for the production of catechol products. Thus, the development of an efficient process will require the immobilisation of polyphenol oxidase on a transverse-flow bioreactor consisting of nylon membranes or polyurethane for the continuous production of catechol using low concentrations of phenolic substrates.

CHAPTER 4

APPLICATION OF POLYPHENOL OXIDASE IN THE TREATMENT OF PHENOLIC INDUSTRIAL EFFLUENTS

4.1 INTRODUCTION

The study was extended to an investigation of the conversion of phenolic pollutants found in industrial effluents, with the aim of achieving catechol production. The effective conversion of phenolic substrates by polyphenol oxidase has been demonstrated as described in Chapters 2 and 3, and reaction conditions which resulted in catechol accumulation could then be applied to the treatment of industrial effluents.

Phenol and its derivatives are common constituents of industrial residues in petroleum and other chemical industries. However, most phenols are toxic at the concentrations discharged into the effluents (Juang *et al.*, 1999). As a result of increasing concern for public health and the environmental impact of phenolic pollutants, limits have been set on the acceptable levels of specific pollutants. The European Community directive (80/778/EEC) sets the maximum concentration permitted for all phenols in aquatic environments at 0.5 μg/L, and 0.1 μg/L for individual phenols (Nistor *et al.*, 1999).

A great deal of research has been directed towards enzymatic processes, using oxidative enzymes such as polyphenol oxidase, peroxidase and laccase to remove phenolic contaminants from aqueous industrial streams (Sun *et al.*, 1992; Nicell *et al.*, 1993). A number of chemical or biological treatment processes resulted in complete mineralization of the pollutant, while the use of enzymes resulted in partial transformation of the parent substrate (Massey *et al.*, 1994). The advantages of enzymes over conventional chemical methods are high degree of specificity, broad concentration ranges, mild operating conditions and high reaction rates (Nicell *et al.*, 1993; Klibanov *et al.*, 1983; Martirani *et al.*, 1996).

The use of oxidoreductase enzymes, isolated from a number of micro-organisms, to catalyse the removal of phenolic pollutants, was first proposed in the mid-1970's (Klibanov *et al.*, 1980; Massey *et al.*, 1994). These enzymes have been shown to play an essential role in the biodegradation of many natural and xenobiotic compounds (Spain *et al.*, 1989). The basis of enzymatic treatments was that phenolic pollutants would be polymerized to form water-insoluble precipitates that could be removed by filtration (Klibanov *et al.*, 1983; Nicell *et al.*, 1992).

Early experiments focussed on the use of horseradish peroxidase to treat aqueous solutions of phenolic pollutants (Klibanov *et al.*, 1980; Claus and Filip, 1990; Buchanan and Nicell, 1997; Ghioureliotis and Nicell, 1999). However, it was found that a large quantity of peroxidase enzyme was required to achieve high removal efficiencies, due to inactivation at the active site by phenoxy radicals (Klibanov *et al.*, 1983; Wu *et al.*, 1993). The addition of proteins or hydrophilic synthetic polymers was found to significantly reduce enzyme inactivation (Nakamoto and Machida, 1992; Buchanan and Nicell, 1998; Wu *et al.*, 1998).

Polyphenol oxidase has an advantage over the peroxidase system in that it utilises molecular oxygen instead of costly hydrogen peroxide as an oxidant (Atlow *et al.*, 1984). In studies conducted by Atlow *et al.* (1984) polyphenol oxidase was found to catalyse nearly complete removal of phenols in the range from 0.01 to 1 g/L provided that the enzyme concentration increased with increasing phenol concentration as enzyme inactivation was evident at low enzyme concentrations.

Research has focussed on phenol removal efficiencies of polyphenol oxidase in solution or immobilised on various supports, and on the subsequent removal of *ortho*-quinones by precipitation or adsorption onto chitosan (Payne *et al.*, 1992; Wada *et al.*, 1993; Wada *et al.*, 1995; Edwards *et al*, 1999b). Chitosan is a biopolymer with a high amine content commercially produced by deacetylation in alkali of shrimp and crabshell chitin, which is a waste product of the shellfish industry (Crestini *et al.*, 1996). Sun *et al.* (1992) found that unless high phenol concentrations were used the products of the enzyme-catalysed reaction with phenols remained in solution and did not precipitate, resulting in a highly coloured effluent. A two-step approach was developed where polyphenol oxidase catalysed the oxidation of phenols to *o*-quinones, chitosan was then added which has amine groups to absorb the *o*-quinones (Sun *et al.,* 1992). The addition of chitosan removed the residual colour and it was found to stabilize the enzyme.

The oxidation of various substituted phenols by polyphenol oxidase was carried out in the presence of soil related adsorbents (Claus and Filip,1990). The oxidation products became covalently linked to soil organic matter and the enzyme was found to adsorb strongly to the adsorbents resulting in partial loss of activity. Park *et al.* (1999) demonstrated the enhanced removal of chlorinated phenols in the presence of humic constituents.

Many of these studies involved a bioremediation approach where the disappearance of the phenolic compound from the aqueous solution was monitored and very little attention was given to the biotransformation of these phenolic pollutants into useful products. Biotransformation is the conversion of one compound to another catalysed by whole cells or isolated enzymes with most of the products being speciality or high-value products (Zelder and Hauer, 2000). The phenolic pollutants in industrial effluents represent a recyclable resource for the biocatalytic production of industrially important catechols using polyphenol oxidase.

Two effluents produced by the local petrochemical industry were used during this study *viz*., cresylic and phenolic (also known as Black Product) industrial effluents. The effluents contained high concentrations of phenol and three cresol isomers. The concentrations of the components in each effluent may be variable due to upstream operating conditions. The HPLC method of Yoshikawa *et al.* (1986), based on the addition of β-cyclodextrin to the mobile phase, was used for the complete separation of all three structural isomers of cresol as *m*- and *p*-cresol were found to co-elute during initial experiments. Cyclodextrins have a doughnut-shaped sugar-ring structure and *p*-cresol is incorporated into the cavity of β-cyclodextrin by displacing water.

Initial experiments focussed on the reaction of non-immobilised polyphenol oxidase with phenols in industrial effluents to determine whether the enzyme was capable of functioning in the chemical environment of the effluent. High concentrations of phenol are known to inhibit polyphenol oxidase activity due to the inhibitory nature of its reaction products, and thus, dilution of the effluents was based on the phenol concentrations present in each effluent. The effluents were diluted with water and the pH adjusted to neutrality. This was assumed to be appropriate because the effective utilisation of phenolic compounds and production of catechols, catalysed by polyphenol oxidase in water, was previously demonstrated as described in Section 2.3.5.

In addition to the reaction of polyphenol oxidase in water, the effect of using the borate/ascorbate system was investigated. The reaction of polyphenol oxidase with phenolic substrates dissolved in 75mM borate buffer (pH 7.8) in the presence of 12.5mM ascorbate had resulted in significant yields of catechol being produced with the absence of *o*-quinone products (Section 2.3.7). Thus, the industrial effluents were diluted with the same reaction medium, with the aim of increasing the yield of catechol products. After completion of the reaction, the yield of catechol products was determined by extraction using diethyl ether.

Immobilised polyphenol oxidase was then investigated as a biocatalyst for the conversion of phenols in the effluents. The use of nylon membranes as the immobilisation support had been demonstrated as the most suitable synthetic membrane support for the production of catechols (Section 3.3.7). Thus, polyphenol oxidase was immobilised on nylon membranes and allowed to react with phenolic effluents diluted with water and with borate buffer.

4.2 MATERIALS AND METHODS

4.2.1 Reactions of non-immobilised polyphenol oxidase in effluents diluted with water

Initial experiments focussed on the reaction of non-immobilised polyphenol oxidase with each of the petrochemical effluents diluted with water. The phenolic content of these effluents was high and dilution of the effluents was necessary. The effluents were filtered using a 0.45 micron filter (Micron Separation Inc.), diluted in water and the pH was adjusted to neutrality using 0.1M NaOH. The cresylic effluent was diluted 25x and the phenolic effluent was diluted 10x. The experiments were carried out in duplicate with a control experiment which contained no enzyme. 6U polyphenol oxidase was reacted with 20ml of each effluent for 4 hours. The samples were allowed to stir continuously on an orbital shaker and samples were analysed by HPLC.

4.2.1.1 Chromatographic analysis of the reaction of polyphenol oxidase with the effluents diluted in water

The HPLC method of Yoshikawa *et al.* (1986) was used to separate cresol isomers. The method reported use of ß-cyclodextrin at an optimum concentration of 25g/L mobile phase to separate *p*-cresol and *m*-cresol, but ß-cyclodextrin was difficult to dissolve and 10g/L was found to be sufficient for complete separation of *p*-cresol and *m*-cresol. The disappearance of the phenolic compounds and the appearance of the products was monitored by HPLC with UV detection (Beckman Instruments, Inc., San Ramon, USA) using reverse-phase 5µm C_{18} Waters (250mm x 4.6mm) column with a mobile-phase of water:acetonitrile (85:15), containing 10g/L ß-cyclodextrin, at a flow rate of $1ml.min^{-1}$. Peaks were detected at 270nm and analysed with Beckman System Gold Chromatography Software.

4.2.2 Reaction of non-immobilised polyphenol oxidase with industrial effluents prepared in borate buffer

The cresylic effluent was diluted 30 and 50x respectively using 75mM borate buffer (pH 7.8) containing 12.5mM ascorbate. 19U polyphenol oxidase was added to 50mL diluted effluent. The phenolic effluent was diluted 4x using 75mM borate buffer (pH 7.8) containing 12.5mM ascorbate. 15U polyphenol oxidase was added to 50mL diluted effluent. The experiments were carried out in duplicate with a control experiment which contained no enzyme. The effluents were allowed to stir continuously on an orbital shaker (160rpm) and samples were removed and analysed by HPLC (Section 4.2.4).

4.2.3 Reaction of nylon-immobilised polyphenol oxidase with industrial effluents

Polyphenol oxidase was immobilised on nylon membranes to react with cresylic and phenolic effluents. The enzyme was immobilised on nylon membranes by placing each membrane in 5mL of 1.2U polyphenol oxidase for 1 hour at 4°C and then allowing 1.5% glutaraldehyde (25%) to react with the enzyme for 30 minutes. The units of active polyphenol oxidase immobilised on each membrane was determined by placing the membrane in 20mL 10mM L-DOPA solution and monitoring the absorbance at 475nm every 2 minutes for 12 minutes. The effluents were diluted in water and borate buffer before the addition of nylon-immobilised polyphenol oxidase.

4.2.3.1 Reaction of nylon-immobilised polyphenol oxidase with cresylic effluent

The cresylic effluent was diluted 50 and 75x respectively using water and 75mM borate buffer (pH 7.8) containing 12.5mM ascorbate. 20mL of effluent diluted in water was allowed to react with 2 nylon membranes containing 0.44U polyphenol oxidase per membrane. 20mL of effluent diluted in borate buffer was allowed to react with 2 nylon membranes containing 0.50U polyphenol oxidase per membrane. The effluents were allowed to stir continuously on an orbital shaker (150rpm) and samples were removed and analysed by HPLC (Section 4.2.4).

4.2.3.2 Reaction of nylon-immobilised polyphenol oxidase with phenolic effluent

The phenolic effluent was acidified to pH 2 using concentrated HCl and then filtered through 0.45 micron disposable filters. 15mL of acidified effluent was added to 15mL of water and the pH was adjusted to pH 7 using 0.5M NaOH. 10mL of acidified effluent was added to 10mL 75mM borate buffer (pH 7.8) and ascorbate was added to achieve 12.5mM ascorbate. 20mL of each effluent was allowed to react with 2 nylon membranes containing 0.56U polyphenol oxidase per membrane. The effluents were allowed to stir continuously on an orbital shaker (150rpm) and samples were removed and analysed by HPLC (Section 4.2.4).

4.2.4 Chromatographic analysis of the reaction of polyphenol oxidase with the effluents

The disappearance of the phenolic compounds and the appearance of the products was monitored by HPLC (LaChrom, Merck, Germany) with UV detection (L-7400) using reverse-phase 5µm C_{18} Waters (250mm x 4.6mm) column with a mobile-phase of water:acetonitrile (90:10), containing 10g/L ß-cyclodextrin, at a flow rate of $1 ml.min^{-1}$. Peaks were detected at 270nm and analysed using Peak Simple Software (version 1.59).

4.3 RESULTS AND DISCUSSION

4.3.1 Analysis of each industrial effluent

Two different petrochemical effluents were used, *i.e.* cresylic and phenolic industrial effluents. The effluents were analysed by HPLC (Section 4.2.1.1) and found to contain phenol, *o*-, — and *p*-cresol. The chemical analysis of the phenolic effluent is shown in Table 4.1 and the concentrations of the phenolic pollutants present in each effluent are listed in Table 4.2. The cresylic effluent contained low levels of settled solids and high concentrations of phenol and the isomers of cresol. The phenolic effluent was black and consisted of high concentrations of suspended and dissolved solids with a significant phenol concentration and low concentrations of the cresol isomers.

Table 4.1: Chemical analysis of phenolic industrial effluent expressed as mass %.

	Phenolic effluent
Compound	**Concentration (mass %)**
acetone	0.0047
acetonitrile	0.0418
methanol	0.0542
ethanol	0.1236
1-propanol	0.0186
iso-butanol	0.0024
n-butanol	0.0062
2-propanone	0.0236
Suspended solids	218.4 mg/L
Total dissolved solids	866 mg/mL

Table 4.2: Concentrations of phenolic compounds in the industrial effluents.

	Cresylic effluent	**Phenolic effluent**
Compound	**Concentration (mM)**	**Concentration (mM)**
phenol	87.1	11.7
p-cresol	25.2	0.8
m-cresol	26.9	0.5
o-cresol	81.1	0.3

4.3.2 Application of non-immobilised polyphenol oxidase in the treatment of phenolic effluents prepared in water

During the reactions of polyphenol oxidase with the effluent phenolics diluted in water, substrate selectivity and catechol accumulation were monitored. The inclusion of ß-cyclodextrin in the mobile phase facilitated the complete separation of the structural isomers of cresol by HPLC.

The reaction of polyphenol oxidase in the effluents resulted in decreases in the concentrations of the phenolic pollutants. The conversion of phenol and the cresol isomers, present in each effluent, by polyphenol oxidase is shown in Table 4.3.

Table 4.3: Conversion of phenolic pollutants present in diluted cresylic and phenolic effluents, by 6U polyphenol oxidase.

	Cresylic effluent		**Phenolic effluent**	
Phenolic pollutants	**Initial concentrations (mM)**	**Conversion (%)**	**Initial concentrations (mM)**	**Conversion (%)**
phenol	3.48	42.3	1.17	92.5
p-cresol	1.01	100	0.08	100
m-cresol	1.08	30.4	0.05	74.3
o-cresol	3.24	42.1	0.03	95.9

There was 100% removal of *p*-cresol in both effluents indicating a selectivity preference for *p*-cresol by polyphenol oxidase. This trend was also reported in Section 3.3.3 during the reaction of polyethersulphone capillary membrane-immobilised polyphenol oxidase with phenol and *p*-cresol. Significant *m*- and *o*-cresol removal occurred during the reaction with polyphenol oxidase. Greater phenol utilisation occurred in the phenolic effluent, where the initial concentrations of phenol were lower than in the cresylic effluent, which is to be expected since high concentrations of phenol inhibit polyphenol oxidase activity. In general, these results indicate that polyphenol oxidase was capable of reacting with the phenolic substrates present as pollutants in industrial effluents.

4-Methylcatechol was detected by HPLC during the reaction of polyphenol oxidase with the cresylic effluent (Figure 4.1). The concentration of 4-methylcatechol increased to 0.53mM during the first 30 minutes and then started to decrease due to further oxidation to *o*-quinone, until it was almost completely utilised after 4 hours. Catechol and 3-methylcatechol were not detected by HPLC, possibly due to their rapid oxidation to *o*-quinones, and catechol products were not detected during the reaction of polyphenol oxidase with the phenolic effluent.

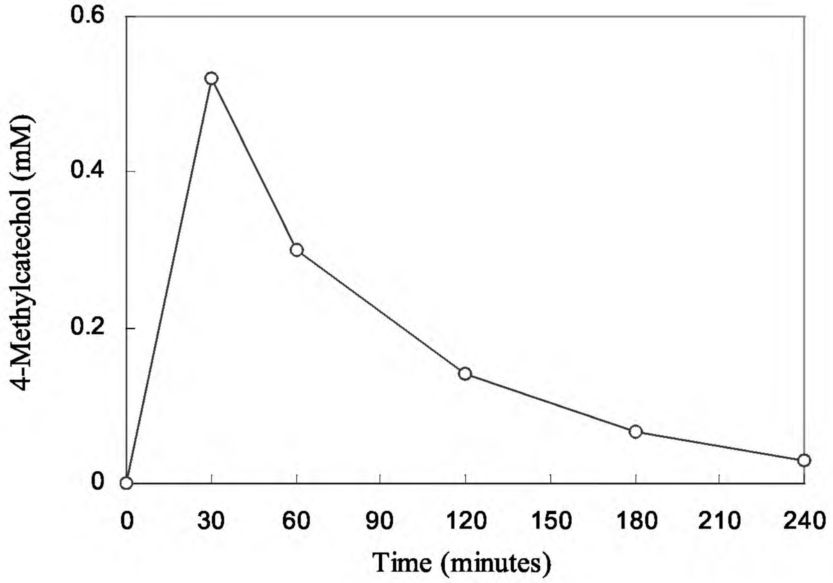


Figure 4.1: Production of 4-methylcatechol during the reaction of polyphenol oxidase with cresylic effluent.

4.3.3 Application of non-immobilised polyphenol oxidase in the treatment of phenolic effluents prepared in borate buffer

The effluents were diluted with 75mM borate buffer, with the addition of ascorbate, in an attempt to increase the yield of catechol products. The conversion of the phenolic pollutants in the effluents is shown in Table 4.4. There was 100% *p*-cresol conversion in the diluted cresylic (50x diluted) and phenolic effluents. 93% and 65.1% phenol conversion occurred in reactions using 1.78mM and 1.6mM initial phenol concentrations respectively, while 35.4% conversion occurred using a higher initial concentration of 3mM phenol. Increased concentrations of phenol appeared to inhibit polyphenol oxidase activity.

Table 4.4: Conversion of phenolic pollutants present in cresylic and phenolic effluents, diluted with the borate/ascorbate buffer system, by polyphenol oxidase.

	Cresylic effluent				**Phenolic effluent**	
	30x dilution		**50x dilution**		**4x dilution**	
Phenolic pollutants	**[Initial] (mM)**	**Conversion (%)**	**[Initial] (mM)**	**Conversion (%)**	**[Initial] (mM)**	**Conversion (%)**
phenol	3.0	35.4	1.6	65.1	1.78	93.0
p-cresol	0.9	96.3	0.5	100.0	0.05	100
m-cresol	0.8	14.0	0.5	26.7	0.03	100
o-cresol	2.6	18.3	1.4	22.7	0.01	100

The accumulation of catechol products and utilisation of ascorbate was monitored during the reactions of polyphenol oxidase with cresylic and phenolic effluents, ascorbate being required to maintain catechol concentrations during the reaction. During the reaction of polyphenol oxidase with cresylic effluent (diluted 30x in 75mM borate buffer containing 12.5mM ascorbate) (Figure 4.2), 0.82mM catechol and 0.9mM 4-methylcatechol was produced after 4.5 hours. The 4-methylcatechol concentration dropped to 0.4mM after 5.5 hours which corresponded to the rapid depletion of ascorbate in solution. During the reaction using cresylic effluent, diluted 50x (Figure 4.3), 1.07mM catechol and 0.49mM 4-methylcatechol was produced after 3 hours.

Catechol and 4-methylcatechol concentrations dropped to 0.8mM and 0.02mM respectively after 4 hours which corresponded to the complete depletion of ascorbate in solution. The levels of ascorbate in solution need to be increased or maintained in the reaction system to prevent a decrease in the concentration of catechol products.

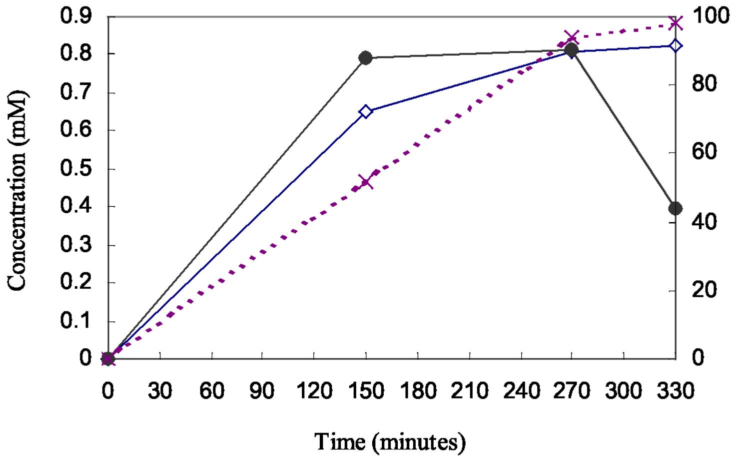


Figure 4.2: Production of catechol (◇), 4-methylcatechol (●) and utilisation of ascorbate (- -x- -), during the reaction of polyphenol oxidase with cresylic effluent, diluted 30x with the borate/ascorbate buffer system.

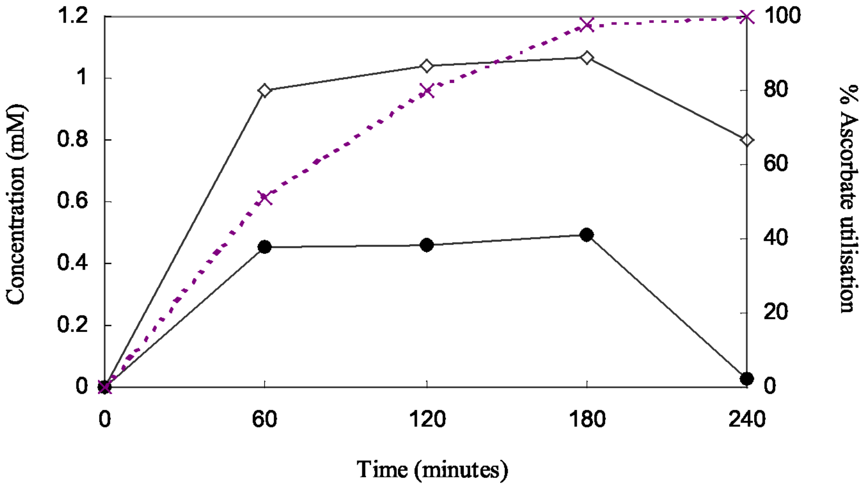


Figure 4.3: Production of catechol (◇), 4-methylcatechol (●) and utilisation of ascorbate (- -x- -), during the reaction of polyphenol oxidase with cresylic effluent, diluted 50x with the borate/ascorbate buffer system.

During the reaction of polyphenol oxidase with the phenolic effluent diluted with the borate/ascorbate buffer system (Figure 4.4), 1.6mM catechol was produced after 2 hours, indicating 96.7% conversion of phenol to catechol.

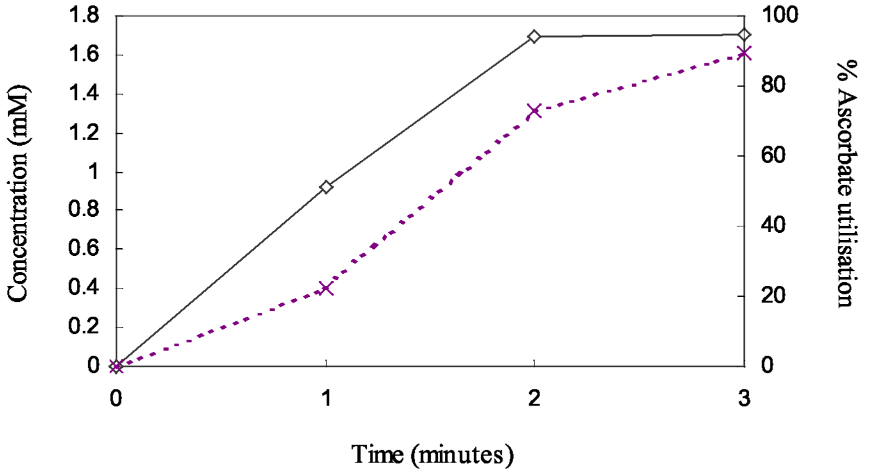


Figure 4.4: Production of catechol (◇) and utilisation of ascorbate (- -x- -), during the reaction of polyphenol oxidase with phenolic effluent, diluted 4x in 75mM borate buffer (pH 7.8) containing 12.5mM ascorbate.

75% conversion of phenol to catechol occurred in the 30x diluted cresylic effluent while 100% conversion occurred in the 50x diluted cresylic effluent, indicating that low phenol concentrations were better suited to catechol accumulation. Virtually 100% conversion of *p*-cresol to 4-methylcatechol occurred in the diluted effluents. Dilution of each effluent in the borate/ascorbate system was an effective way to enhance catechol production.

The effluent solutions were acidified and diethyl ether was used to extract the catechol products. During the extraction of catechol from the phenolic effluent, acidification of the solution resulted in precipitation of the suspended solids in the effluent, and filtration of the effluent resulted in the removal of all of the suspended solids. The presence of the suspended solids did not appear to affect the activity of polyphenol oxidase but interfered with the extraction procedure resulting in decreased catechol yields. In subsequent experiments, pre-treatment of the phenolic effluent by acidification and filtration to remove the suspended solids before the addition of polyphenol oxidase, resulted in catechol yields being increased from 59% to 86%.

4.3.4 Application of nylon-immobilised polyphenol oxidase in the treatment of cresylic and phenolic effluents

Polyphenol oxidase was immobilised on nylon membranes and allowed to react with cresylic and pre-treated phenolic effluents diluted in water and the borate/ascorbate buffer system. The conversion of the phenols present in the cresylic and phenolic effluents, diluted in water and the borate/ascorbate buffer system, is shown in Table 4.5. Between 10 and 20% greater substrate conversion occurred in water than in the borate buffer system. This could be due to ascorbate that had not formed a complex with borate. There was 100% removal of *p*-cresol in all the reactions.

Table 4.5: Conversion of phenolic pollutants in cresylic and phenolic effluents, diluted in the borate/ascorbate buffer system and water, by nylon-immobilised polyphenol oxidase.

	Cresylic effluent						**Phenolic effluent**		
	50x dilution			**75x dilution**			**4x dilution**		
Phenolic pollutant	**[Initial] (mM)**	**Conversion (%)**		**[Initial] (mM)**	**Conversion (%)**		**[Initial] (mM)**	**Conversion (%)**	
		water	**borate**		**water**	**borate**		**water**	**borate**
phenol	1.60	82.1	67.0	1.08	89.7	78.3	1.62	74	52
p-cresol	0.45	100	100	0.31	100.0	100	-	-	-
m-cresol	0.44	60.2	40.3	0.29	70.5	51.9	-	-	-
o-cresol	1.31	53.6	41.4	0.85	55.6	42.9	-	-	-

There was a 10-fold increase in the concentration of catechol produced in the cresylic effluent diluted in the borate/ascorbate buffer system, as compared to water, during the reaction of nylon-immobilised polyphenol oxidase (Figure 4.5). There was a 16-fold increase in the concentration of catechol produced in the phenolic effluent diluted in the borate buffer system, compared to water, during the reaction of nylon-immobilised polyphenol oxidase (Figure 4.6).

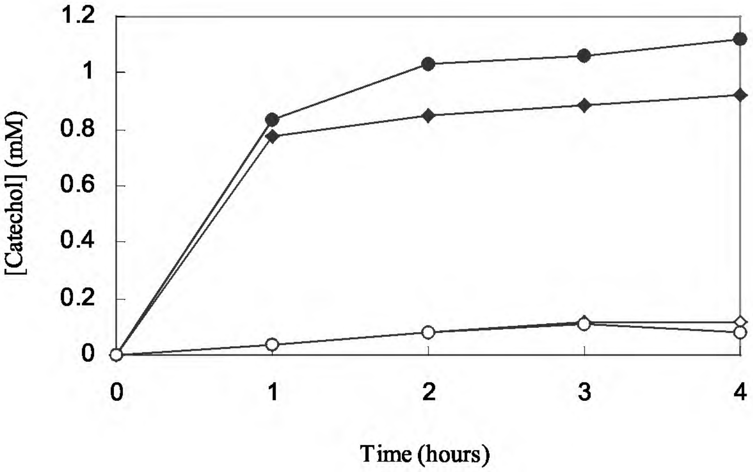


Figure 4.5: Catechol production during the reaction of nylon-immobilised polyphenol oxidase with cresylic effluent, diluted 50x (●) and 75x (◆) in 75mM borate buffer (pH 7.8) containing 12.5mM ascorbate and diluted 50x (◇) and 75x (○) in water.

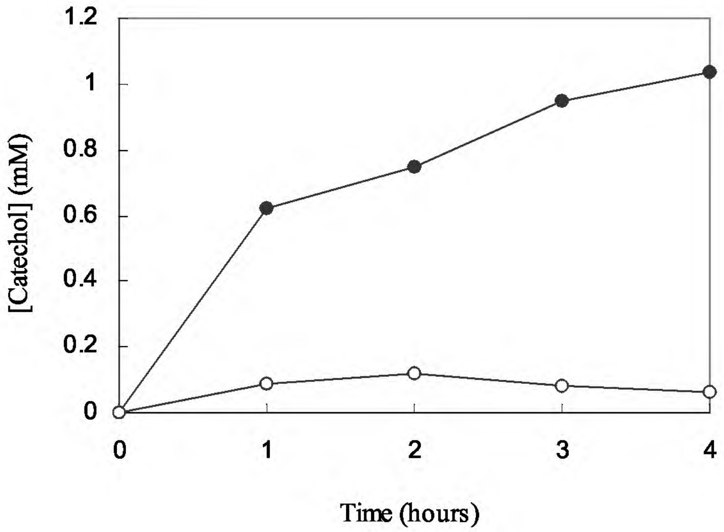


Figure 4.6: Catechol production during the reaction of nylon-immobilised polyphenol oxidase with phenolic effluent, diluted 4x in 75mM borate buffer (pH 7.8) containing 12.5mM ascorbate (●) and water (○).

Efficient phenolic substrate conversion occurred during the reactions of non-immobilised and nylon-immobilised polyphenol oxidase with the industrial effluents diluted in water and the borate buffer system. However, a significantly higher number of units of polyphenol oxidase were used for reactions when the enzyme was not immobilised. The number of µmoles of each phenolic substrate converted in the cresylic (Table 4.6) and phenolic effluents (Table 4.7) per unit polyphenol oxidase were calculated, demonstrating an increase in the number of µmoles of each phenolic substrate converted per unit immobilised polyphenol oxidase, as compared to non-immobilised enzyme. There was a 6-fold increase in the number of µmoles of phenol converted per unit of immobilised polyphenol oxidase in the cresylic diluted in water and borate buffer, and a 6-fold and 3-fold increase in the phenolic effluent diluted in water and borate buffer respectively.

Table 4.6: The number of µmoles of each phenolic substrate converted in the cresylic effluent, diluted in water and the borate buffer system, per unit of non-immobilised and nylon-immobilised polyphenol oxidase.

			Phenolic substrate			
			phenol	***p*-cresol**	***m*-cresol**	***o*-cresol**
			µmoles/ U PPO	**µmoles/ U PPO**	**µmoles/ U PPO**	**µmoles/ U PPO**
Non-immobilised	**Diluent**	**Dilution rate**				
	water	25	4.91	3.36	1.09	4.55
	borate	30	2.79	2.29	0.30	1.27
		50	2.74	1.31	0.36	0.84
Nylon-immobilised	**Diluent**	**Dilution rate**				
	water	50	29.83	10.19	5.99	15.97
		75	25.36	7.04	4.62	10.72
	borate	50	21.46	8.97	3.51	10.82
		75	14.70	6.20	3.05	7.31

Table 4.7: The number of µmoles of each phenolic substrate converted in the phenolic effluent, diluted in water and the borate buffer system, per unit of non-immobilised and nylon-immobilised polyphenol oxidase.

		Phenolic substrate			
		phenol	***p*-cresol**	***m*-cresol**	***o*-cresol**
		µmoles/ U PPO	**µmoles/ U PPO**	**µmoles/ U PPO**	**µmoles/ U PPO**
Non-immobilised	water	3.61	0.23	0.12	0.10
	borate	5.52	0.17	0.10	0.03
Nylon-immobilised	water	21.44	-	-	-
	borate	15.08	-	-	-

Productivity, expressed as µmoles of catechol or 4-methylcatechol produced in the cresylic and phenolic effluents per unit polyphenol oxidase, was calculated to compare non-immobilised and immobilised polyphenol oxidase (Table 4.8). In the cresylic effluent diluted in water, 0.11 µmoles of catechol were produced per unit non-immobilised polyphenol oxidase, while 2.58µmoles of catechol were produced per unit nylon-immobilised enzyme. Dilution of the cresylic effluent in the borate/ascorbate system resulted in 2.82µmoles of catechol being produced per unit non-immobilised polyphenol oxidase, while 18.53µmoles of catechol were produced per unit nylon-immobilised polyphenol oxidase. There was an approximately 20-fold and 9-fold increase in the productivity of immobilised polyphenol oxidase for catechol production, in the cresylic effluent diluted in water and borate buffer respectively. During the reaction of non-immobilised polyphenol oxidase with the phenolic effluent diluted in water, catechol was not detected while 1.14µmoles of catechol were produced per unit nylon-immobilised polyphenol oxidase. Dilution of the phenolic effluent in the borate/ascorbate system resulted in 5.33µmoles of catechol being produced per unit non-immobilised polyphenol oxidase, while 18.49µmoles of catechol were produced per unit nylon-immobilised polyphenol oxidase.

4-Methylcatechol was not detected during reactions with the phenolic effluent or during reactions of nylon-immobilised polyphenol oxidase with cresylic effluents. This could be due to the low initial *p*-cresol concentrations during these reactions. Immobilised polyphenol oxidase exhibited the highest productivity during the reactions of the effluents diluted with borate buffer. Thus immobilisation of the biocatalyst increased the economical application of polyphenol oxidase for catechol production as enzyme requirements were decreased.

Table 4.8: The number of µmoles of catechol and 4-methylcatechol produced after 4 hours in the cresylic and phenolic effluents diluted 50x and 4x respectively, in water and the borate buffer system, per unit of non-immobilised and nylon-immobilised polyphenol oxidase.

			catechol	**4-methylcatechol**
			µmoles/ U PPO	**µmoles/ U PPO**
Cresylic effluent	**non-immobilised**	water	0.11	-
		borate	2.82	1.29
	nylon-immobilised	water	2.58	-
		borate	18.53	-
Phenolic effluent	**non-immobilised**	water	-	-
		borate	5.33	-
	nylon-immobilised	water	1.14	-
		borate	18.49	-

4.4 CONCLUSION

The production and isolation of catechol products from industrial effluents using polyphenol oxidase has not been reported previously in the literature. Past research has concentrated on the removal of phenols by polymerization of *o*-quinones, but the addition of insufficient polyphenol oxidase to the effluents resulted in a highly coloured effluent due to the presence of free *o*-quinone in solution.

Polyphenol oxidase functioned in the presence of numerous non-phenolic compounds found in the petrochemical effluents, without the need to extract the phenolic substrates. Previous experiments with polyphenol oxidase involved the reaction of single substrate solutions, while the effluents consisted of a mixture of phenols. The enzyme effectively converted phenols present in the effluents to catechol products.

Initial experiments focussed on the reaction of non-immobilised polyphenol oxidase with each of the effluents diluted in water. There was 100% conversion of *p*-cresol which indicated a substrate selectivity for *p*-cresol by the enzyme. Significant phenol conversion also occurred, particularly at lower initial substrate concentrations. 52.5% conversion of *p*-cresol to 4-methylcatechol occurred after 30 minutes during the reaction of polyphenol oxidase with the cresylic effluent, but the concentration decreased rapidly due to further oxidation by polyphenol oxidase to *o*-quinone.

The cresylic and phenolic effluents were diluted in 75mM borate buffer, with the addition of 12.5mM ascorbate, to determine their effect on catechol yields. There was 75 - 100% conversion of phenol to catechol in the diluted cresylic effluents, 96.7% in the phenolic effluent, and almost 100% conversion of *p*-cresol to 4-methylcatechol in both effluents. A higher conversion of phenol to catechol occurred in the more dilute cresylic effluent, implying that low phenol concentrations were better for catechol accumulation. During the reaction of the diluted cresylic effluents with polyphenol oxidase, ascorbate was depleted before completion of the reaction, leading to a decrease in catechol and 4-methylcatechol. This could be attributed to insufficient ascorbate in solution as higher substrate concentrations were used in the cresylic than the phenolic effluent. Catechol was successfully extracted using diethyl ether, and pre-treatment of the phenolic effluent increased extracted catechol yields from 59% to 86%.

The effects of immobilisation on phenol conversion and catechol production were investigated by immobilising polyphenol oxidase on nylon membranes. Nylon-immobilised polyphenol oxidase was allowed to react with each effluent diluted in water and the borate/ascorbate system and substrate conversion and catechol production were compared to reactions using non-immobilised polyphenol oxidase. There was a 10-fold increase in catechol production during the reaction of immobilised polyphenol oxidase with the cresylic effluent diluted in the buffer system compared to water. During these reactions a more dilute cresylic effluent was used and the catechol concentrations did not decrease due to the presence of ascorbate in solution. Nylon-immobilised polyphenol oxidase exhibited superior productivity compared to non-immobilised polyphenol oxidase, with a 20-fold and 9-fold increase in catechol production per unit polyphenol oxidase during reactions carried out in the cresylic effluent diluted in water and borate buffer respectively.

During the reaction of polyphenol oxidase with the effluents diluted with water, the dilution rates of the effluents were based on the phenol concentrations measured in each effluent because of the inhibitory effect of its reaction products on polyphenol oxidase activity. To ensure the effective conversion of phenols and production of catechol products, the phenolic substrate concentrations in the effluents could not exceed approximately 4mM during reactions with the effluents diluted in borate buffer and ascorbate. The excess ascorbate concentration (12.5mM) used in this study was sufficient to reduce the *o*-quinones to catechols within 4 hours during reactions with approximately 4mM substrate concentrations, and ensured that the effluent did not change colour after the reaction with the enzyme. The borate buffer and ascorbate concentrations had been optimised for substrate concentrations of 3-4mM, and any increase in substrate concentration would need to be accompanied by a corresponding increase in both borate and ascorbate concentrations. Thus variations in the concentration of phenols in the effluents will need to be monitored regularly to maintain effective biocatalytic activity.

Pre-treatment of the phenolic effluent resulted in increased catechol yields, while no pre-treatment of the cresylic effluent was necessary. A process for the production and isolation of catechol in yields greater than 90% from industrial phenolic effluents is a definite viability using nylon-immobilised polyphenol oxidase with effluents diluted in 75mM borate buffer and ascorbate.

CHAPTER 5

CONCLUSION

Mushroom polyphenol oxidase, in immobilised and non-immobilised forms, has been demonstrated to be a suitable biocatalyst for the biotransformation of phenols and phenolic pollutants to produce catechols. The biocatalytic reaction conditions resulting in catechol accumulation were identified and optimised to maximise catechol production.

Regioselective hydroxylation of aromatic compounds is difficult to achieve and control, and investigations have expanded to use biocatalysis as an alternative to replace conventional chemical syntheses. Polyphenol oxidase was identified as a suitable biocatalyst for the hydroxylation of selected phenols to catechols, which are industrially important chemical products. However, the application of polyphenol oxidase for catechol production was hindered by the subsequent oxidation of catechol to *o*-quinone by the enzyme, and the contention that catechol was not a true intermediate product. Using LC-MS, catechol products were successfully identified as true intermediate products formed during biocatalytic reactions in water.

A process to produce catechols would require their accumulation in solution, and each substrate resulted in the formation of different concentrations of catechol products with the conversion of *p*-cresol to 4-methylcatechol resulting in the highest conversion of 50% in water. To increase the efficiency of the process would require decreased quinone production or reduction of the quinone by a reducing agent. The concentration of catechol products in solution was increased by the addition of ascorbate which reduced *o*-quinone to catechol, but ascorbate had an inhibitory effect on polyphenol oxidase activity. In aqueous media, borate formed a stable complex with the hydroxyl groups of ascorbate, which minimised the inhibitory effect of ascorbate on the enzyme by keeping free ascorbate concentrations low, while still allowing it to function as a reducing agent for any free quinone. This development greatly improved the feasibility of using polyphenol oxidase for catechol production because manipulation of borate and ascorbate concentrations resulted in 100% conversion of phenols to catechol products.

An effective method to extract catechol products from the reaction system included acidification of the reaction mixture to disrupt the complex formed between catechol and borate, followed by solvent extraction using diethyl ether. Use of the borate buffer and ascorbate reaction system resulted in the absence of *o*-quinone from the reaction medium, which simplified the extraction procedure. Thus, an efficient process to produce and extract catechol using polyphenol oxidase was developed , which resulted in 98 - 100% recovery of catechol products.

Two catechols not available commercially, namely 4-chlorocatechol and 4-methoxycatechol, were successfully produced as pure samples and their identities confirmed. The catechol products of each phenolic substrate were used to facilitate measurement of UV molar absorption coefficients of *o*-quinones, as some of these values were not available in the literature, and were used to enable a comprehensive kinetic study of the conversion of a range of substrates in an aqueous medium by polyphenol oxidase.

The polyphenol oxidase biocatalytic system was extended to the use of immobilised polyphenol oxidase to enhance the economic viability of the enzyme for catechol production. A viable process using polyphenol oxidase would require reduced reaction inactivation of the enzyme. Immobilisation of polyphenol oxidase in a transverse-flow capillary membrane bioreactor allowed for separation of the *o*-quinone products from the immediate environment surrounding the enzyme, which resulted in a significantly lowered degree of suicide inactivation.

The effects of the nature of the immobilisation support on enzyme activity and catechol production were investigated. Polyphenol oxidase was successfully immobilised on a range of synthetic hydrophobic or hydrophilic membranes, and incorporated within polyurethane foam and an organogel and allowed to react with selected phenolic substrates for the synthesis of catechols. The immobilisation of polyphenol oxidase on a wide range of synthetic membranes and its incorporation within polyurethane foam has not been reported previously in the literature. The influences of the hydrophobic or hydrophilic properties of the synthetic immobilisation supports were studied with the aid of a novel mathematical model used to predict which substrates and support materials would allow maximum catechol accumulation. The mathematical model was used to calculate reaction rate constants of the cresolase and catecholase activities of polyphenol oxidase during reaction with each phenolic substrate. The extent of polyphenol oxidase inactivation was also investigated using the mathematical model, and the results indicated that at low substrate concentrations, inactivation was not significant.

The biotransformation of phenols to catechols was strongly influenced by the immobilisation support and resulted in differing yields of catechols. A hydrophilic support favoured catechol accumulation, while a hydrophobic support influenced the catecholase activity of the enzyme resulting in oxidation of catechol products to *o*-quinone. Nylon and polyurethane-immobilised polyphenol oxidase resulted in the highest concentration of catechol products, with 22.97% and 66.9% 4-methylcatechol production respectively using 1.25mM *p*-cresol as the substrate. The mathematical modelling approach provided a means of determining kinetic parameters of the biocatalytic system as an alternative to conventional enzyme kinetic approaches.

Polyphenol oxidase was successfully applied in the production and isolation of 4-methylcatechol and catechol from two phenolic effluents produced by the petrochemical industry, *i.e.* cresylic and phenolic effluents. Non-immobilised and nylon-immobilised polyphenol oxidase were capable of effectively functioning in the chemical environment of the effluents, without the need for prior extraction of the phenols from the effluent. The effluents consisted of a mixture of phenol and three isomers of cresol, and 100% conversion of *p*-cresol occurred which indicated a substrate selectivity for *p*-cresol by the enzyme.

For the production of high yields of purified catechol products it was necessary to perform the reactions in a buffered medium containing ascorbate. The yields of 4-chlorophenol increased from 16.8% in water to 100% in borate buffer. Reactions in water are also complicated by the formation of highly coloured *o*-quinone products which are difficult to separate from the catechol products. Thus the expense of performing the reactions in a buffered medium containing ascorbate could be justified for the production of 4-methylcatechol which has a higher economic value than catechol, and in particular for the production of catechol products not available commercially, such as 4-chlorocatechol and 4-methoxycatechol.

Catechols were successfully extracted using diethyl ether or ethyl acetate, and pretreatment of the phenolic effluent to remove the suspended solids, increased catechol yields from 59% to 86%. A comprehensive cost analysis of the production and isolation of catechol products will need to be investigated to justify an expensive pre-treatment step.

In the application of the polyphenol oxidase biocatalytic system for the production of catechols from phenols, the results of this study have demonstrated the potential of achieving highly efficient catechol production as an alternative to chemical synthesis. Further, this method has been shown to be suitable for application in conversion of phenols present in complex industrial effluents.

Future experimentation could be extended to an investigation of alternative reducing agents, such as glutathione, dithiothreitol , sodium bisulphate or an electrochemical method to determine the most cost effective reducing agents for the development of an enzymatic process to produce catechols. However, the method will need to be manipulated to minimise polyphenol oxidase inactivation by these reducing agents, and the reaction medium should exclude buffer to decrease the cost of the process. Effective methods to recycle the organic solvents will also need to be investigated.

Immobilisation of polyphenol oxidase should be studied further in terms of the influence of hydrophilic supports on catechol production, in particular the effects of incorporating different surfactants during the formation of polyurethane foams on catechol accumulation, as this method resulted in the highest concentration of 4-methylcatechol accumulation. Immobilisation of polyphenol oxidase did not significantly increase the operational stability of the enzyme, and the enzyme is characterised by suicide inactivation and could not be used repeatedly due to loss of activity. Thus a cheaper and more readily available source of the enzyme could be investigated, particularly for the treatment of industrial effluents.

APPENDIX A

Experimental data used as the basis for formulating a mathematical model of the process (Section 3.3.6).

1 = non-immobilised polyphenol oxidase
2 = polyphenol oxidase immobilised on nitrocellulose
3 = polyphenol oxidase immobilised on nylon
4 = polyphenol oxidase immobilised on glass-fibre
5 = polyphenol oxidase immobilised on PTFE
6 = polyphenol oxidase immobilised on polysulphone

	Substrate	Concentration	Immobilisation method	Time	Substrate mM	Catechol mM	Quinone mM
1	p-cresol	1.25	1	0.0	1.25	0	0
	p-cresol	1.25	1	0.5	1.1271	0.0783	0.0446
	p-cresol	1.25	1	1.0	0.85375	0.26895	0.1273
	p-cresol	1.25	1	1.5	0.58155	0.4325	0.23595
	p-cresol	1.25	1	2.0	0.34365	0.562	0.34435
	p-cresol	1.25	1	2.5	0.1527	0.68095	0.41635
	p-cresol	1.25	1	3.0	0.0526	0.785	0.4124
	p-cresol	1.25	1	3.5	0	0.74015	0.50985
	p-cresol	1.25	1	4.0	0	0.68585	0.56415
	p-cresol	1.25	1	4.5	0	0.64285	0.60715
	p-cresol	1.25	1	5.0	0	0.5969	0.6531
	p-cresol	1.25	1	5.5	0	0.49495	0.75505
	p-cresol	1.25	1	6.0	0	0.45225	0.79775
2	p-cresol	5	1	0.0	5	0	0
	p-cresol	5	1	0.5	4.90885	0.06245	0.0287
	p-cresol	5	1	1.0	4.67725	0.26495	0.0578
	p-cresol	5	1	1.5	4.3304	0.43995	0.22965
	p-cresol	5	1	2.0	4.0943	0.55975	0.34595
	p-cresol	5	1	2.5	3.7049	0.6555	0.6396
	p-cresol	5	1	3.0	3.3809	0.713	0.9061
	p-cresol	5	1	3.5	3.2	0.80425	0.99575
	p-cresol	5	1	4.0	2.9461	0.814	1.2399
	p-cresol	5	1	4.5	2.7163	0.7335	1.5502
	p-cresol	5	1	5.0	2.7975	0.748	1.4545
	p-cresol	5	1	5.5	2.6999	0.64745	1.65265
	p-cresol	5	1	6.0	2.6745	0.6006	1.7249

	Substrate	**Concentration**	**Immobilisation method**	**Time**	**Substrate mM**	**Catechol mM**	**Quinone mM**
3	m-cresol	1.25	1	0.0	1.25	0	0
	m-cresol	1.25	1	0.5	0.9815	0.238079	0.030421
	m-cresol	1.25	1	1.0	0.734097	0.44815	0.067753
	m-cresol	1.25	1	1.5	0.560941	0.57775	0.111309
	m-cresol	1.25	1	2.0	0.43779	0.6408	0.17141
	m-cresol	1.25	1	2.5	0.355719	0.65505	0.239231
	m-cresol	1.25	1	3.0	0.308124	0.63649	0.305386
	m-cresol	1.25	1	3.5	0.285163	0.594339	0.370498
	m-cresol	1.25	1	4.0	0.276452	0.546911	0.426637
	m-cresol	1.25	1	4.5	0.27029	0.505877	0.473833
	m-cresol	1.25	1	5.0	0.259635	0.463658	0.526706
	m-cresol	1.25	1	5.5	0.254275	0.42985	0.565875
	m-cresol	1.25	1	6.0	0.230721	0.43495	0.584329
4	m-cresol	5	1	0.0	5	0	0
	m-cresol	5	1	0.5	4.915	0.229215	0
	m-cresol	5	1	1.0	4.484642	0.604846	0
	m-cresol	5	1	1.5	4.102875	0.775247	0.121878
	m-cresol	5	1	2.0	3.903679	0.732532	0.363789
	m-cresol	5	1	2.5	3.842755	0.604352	0.552893
	m-cresol	5	1	3.0	3.80829	0.468725	0.722985
	m-cresol	5	1	3.5	3.791173	0.399186	0.809642
	m-cresol	5	1	4.0	3.78405	0.35955	0.8564
	m-cresol	5	1	4.5	3.766635	0.299556	0.933809
	m-cresol	5	1	5.0	3.75	0.27165	0.97835
	m-cresol	5	1	5.5	3.75047	0.25275	0.99678
	m-cresol	5	1	6.0	3.671831	0.22548	1.102688
5	phenol	1.25	1	0.0	1.25	0	0
	phenol	1.25	1	0.5	1.185	0.032	0.033
	phenol	1.25	1	1.0	0.965	0.115	0.17
	phenol	1.25	1	1.5	0.725	0.185	0.34
	phenol	1.25	1	2.0	0.495	0.24	0.515
	phenol	1.25	1	2.5	0.302	0.245	0.703
	phenol	1.25	1	3.0	0.181	0.26	0.809
	phenol	1.25	1	3.5	0.115	0.235	0.9
	phenol	1.25	1	4.0	0.077	0.22	0.953
	phenol	1.25	1	4.5	0.056	0.19	1.004
	phenol	1.25	1	5.0	0.0455	0.175	1.0295
	phenol	1.25	1	5.5	0.046	0.115	1.089
	phenol	1.25	1	6.0	0.0385	0.08	1.1315
6	phenol	5	1	0.0	5	0	0
	phenol	5	1	0.5	4.87	0	0.13
	phenol	5	1	1.0	4.66	0.019	0.321
	phenol	5	1	1.5	4.34	0.108	0.552
	phenol	5	1	2.0	4.01	0.2	0.79
	phenol	5	1	2.5	3.67	0.26	1.07

	Substrate	Concentration	Immobilisation method	Time	Substrate mM	Catechol mM	Quinone mM
	phenol	5	1	3.0	3.41	0.33	1.26
	phenol	5	1	3.5	3.26	0.36	1.38
	phenol	5	1	4.0	3.18	0.23	1.59
	phenol	5	1	4.5	3.13	0.24	1.63
	phenol	5	1	5.0	3.05	0.24	1.71
	phenol	5	1	5.5	3.04	0.24	1.72
	phenol	5	1	6.0	3.04	0.31	1.65
7	cl-phenol	1.25	1	0.0	1.25	0	0
	cl-phenol	1.25	1	0.5	1.220532	0	0.029468
	cl-phenol	1.25	1	1.0	1.215975	0	0.034025
	cl-phenol	1.25	1	1.5	1.198809	0	0.051191
	cl-phenol	1.25	1	2.0	1.156508	0	0.093492
	cl-phenol	1.25	1	2.5	1.074153	0	0.175847
	cl-phenol	1.25	1	3.0	0.982193	0	0.267807
	cl-phenol	1.25	1	3.5	0.872658	0.026751	0.350591
	cl-phenol	1.25	1	4.0	0.782129	0.025479	0.442392
	cl-phenol	1.25	1	4.5	0.714692	0.024632	0.510676
	cl-phenol	1.25	1	5.0	0.67423	0.024017	0.551753
	cl-phenol	1.25	1	5.5	0.675252	0.023981	0.550767
	cl-phenol	1.25	1	6.0	0.529809	0.02362	0.696571
8	cl-phenol	5	1	0.0	5	0	0
	cl-phenol	5	1	0.5	4.933891	0	0.066109
	cl-phenol	5	1	1.0	4.879328	0	0.120672
	cl-phenol	5	1	1.5	4.89772	0	0.10228
	cl-phenol	5	1	2.0	4.895677	0	0.104323
	cl-phenol	5	1	2.5	4.893633	0	0.106367
	cl-phenol	5	1	3.0	4.914069	0	0.085931
	cl-phenol	5	1	3.5	4.914069	0	0.085931
	cl-phenol	5	1	4.0	4.852762	0	0.147238
	cl-phenol	5	1	4.5	4.77102	0	0.22898
	cl-phenol	5	1	5.0	4.709714	0.08108	0.209207
	cl-phenol	5	1	5.5	4.648407	0.099902	0.251691
	cl-phenol	5	1	6.0	4.607536	0.107141	0.285323
9	p-cresol	1.25	2	0.0	1.25	0	0
	p-cresol	1.25	2	0.5	1.027497	0.037278	0.185225
	p-cresol	1.25	2	1.0	0.81836	0.247835	0.183804
	p-cresol	1.25	2	1.5	0.61377	0.278298	0.357932
	p-cresol	1.25	2	2.0	0.445552	0.288328	0.51612
	p-cresol	1.25	2	2.5	0.254601	0.264165	0.731234
	p-cresol	1.25	2	3.0	0.103659	0.238245	0.908096
	p-cresol	1.25	2	3.5	0.018186	0.208402	1.023412
	p-cresol	1.25	2	4.0	0	0.167043	1.082957
	p-cresol	1.25	2	4.5	0	0.110831	1.139169
	p-cresol	1.25	2	5.0	0	0.106578	1.143422
	p-cresol	1.25	2	5.5	0	0.064525	1.185475
	p-cresol	1.25	2	6.0	0	0.048796	1.201204

	Substrate	**Concentration**	**Immobilisation method**	**Time**	**Substrate mM**	**Catechol mM**	**Quinone mM**
10	p-cresol	5	2	0.0	5	0	0
	p-cresol	5	2	0.5	4.623861	0.006378	0.369761
	p-cresol	5	2	1.0	4.370766	0.139271	0.489963
	p-cresol	5	2	1.5	4.190678	0.222708	0.586614
	p-cresol	5	2	2.0	3.830504	0.246528	0.922968
	p-cresol	5	2	2.5	3.699089	0.244893	1.056018
	p-cresol	5	2	3.0	3.460595	0.235647	1.303758
	p-cresol	5	2	3.5	3.217234	0.244544	1.538223
	p-cresol	5	2	4.0	2.969006	0.248486	1.782508
	p-cresol	5	2	4.5	2.696441	0.270881	2.032678
	p-cresol	5	2	5.0	2.45308	0.318957	2.227963
	p-cresol	5	2	5.5	2.341134	0.324798	2.334068
	p-cresol	5	2	6.0	2.141578	0.345437	2.512986
11	m-cresol	1.25	2	0.0	1.25	0	0
	m-cresol	1.25	2	0.5	1.115033	0.00643	0.128538
	m-cresol	1.25	2	1.0	1.061705	0.012791	0.175504
	m-cresol	1.25	2	1.5	1.027769	0.009508	0.212723
	m-cresol	1.25	2	2.0	0.984137	0.01031	0.255553
	m-cresol	1.25	2	2.5	0.950202	0.008857	0.290942
	m-cresol	1.25	2	3.0	0.896874	0.007772	0.345354
	m-cresol	1.25	2	3.5	0.887178	0.006741	0.356081
	m-cresol	1.25	2	4.0	0.877482	0.005924	0.366594
	m-cresol	1.25	2	4.5	0.85809	0.003965	0.387945
	m-cresol	1.25	2	5.0	0.829002	0.003236	0.417761
	m-cresol	1.25	2	5.5	0.819307	0.001586	0.429107
	m-cresol	1.25	2	6.0	0.809611	0.001586	0.438803
12	m-cresol	5	2	0.0	5	0	0
	m-cresol	5	2	0.5	4.613697	0.009817	0.376486
	m-cresol	5	2	1.0	4.402383	0.021932	0.575685
	m-cresol	5	2	1.5	4.367164	0.022205	0.610631
	m-cresol	5	2	2.0	4.281632	0.021008	0.69736
	m-cresol	5	2	2.5	4.186037	0.019033	0.79493
	m-cresol	5	2	3.0	4.130693	0.015511	0.853797
	m-cresol	5	2	3.5	4.065286	0.014324	0.92039
	m-cresol	5	2	4.0	3.899253	0.009516	1.09123
	m-cresol	5	2	4.5	3.939504	0.007041	1.053456
	m-cresol	5	2	5.0	3.853972	0.005551	1.140477
	m-cresol	5	2	5.5	3.76844	0	1.23156
	m-cresol	5	2	6.0	3.758377	0	1.241623
13	phenol	1.25	2	0.0	1.25	0	0
	phenol	1.25	2	0.5	1.14058	0	0.10942
	phenol	1.25	2	1.0	1.126382	0	0.123618
	phenol	1.25	2	1.5	1.097986	0	0.152014
	phenol	1.25	2	2.0	1.060124	0	0.189876
	phenol	1.25	2	2.5	1.041193	0	0.208807

	Substrate	Concentration	Immobilisation method	Time	Substrate mM	Catechol mM	Quinone mM
	phenol	1.25	2	3.0	1.022263	0	0.227737
	phenol	1.25	2	3.5	1.008065	0	0.241935
	phenol	1.25	2	4.0	0.984401	0	0.265599
	phenol	1.25	2	4.5	0.96547	0	0.28453
	phenol	1.25	2	5.0	0.941807	0	0.308193
	phenol	1.25	2	5.5	0.927609	0	0.322391
	phenol	1.25	2	6.0	0.913411	0	0.336589
14	phenol	5	2	0.0	5	0	0
	phenol	5	2	0.5	4.742221	0	0.257779
	phenol	5	2	1.0	4.691044	0	0.308956
	phenol	5	2	1.5	4.655015	0	0.344985
	phenol	5	2	2.0	4.618987	0	0.381013
	phenol	5	2	2.5	4.521517	0	0.478483
	phenol	5	2	3.0	4.475341	0	0.524659
	phenol	5	2	3.5	4.36746	0	0.63254
	phenol	5	2	4.0	4.341784	0	0.658216
	phenol	5	2	4.5	4.311078	0	0.688922
	phenol	5	2	5.0	4.285402	0	0.714598
	phenol	5	2	5.5	4.239226	0	0.760774
	phenol	5	2	6.0	3.967783	0	1.032217
15	cl-phenol	1.25	2	0.0	1.25	0	0
	cl-phenol	1.25	2	0.5	1.107496	0	0.142504
	cl-phenol	1.25	2	1.0	0.989886	0	0.260114
	cl-phenol	1.25	2	1.5	0.803669	0	0.446331
	cl-phenol	1.25	2	2.0	0.622354	0	0.627646
	cl-phenol	1.25	2	2.5	0.480241	0	0.769759
	cl-phenol	1.25	2	3.0	0.382233	0	0.867767
	cl-phenol	1.25	2	3.5	0.308727	0	0.941273
	cl-phenol	1.25	2	4.0	0.254822	0	0.995178
	cl-phenol	1.25	2	4.5	0.215619	0	1.034381
	cl-phenol	1.25	2	5.0	0.176415	0	1.073585
	cl-phenol	1.25	2	5.5	0.161714	0	1.088286
	cl-phenol	1.25	2	6.0	0.142112	0	1.107888
16	cl-phenol	5	2	0.0	5	0	0
	cl-phenol	5	2	0.5	4.808175	0	0.191825
	cl-phenol	5	2	1.0	4.652425	0	0.347575
	cl-phenol	5	2	1.5	4.406238	0	0.593762
	cl-phenol	5	2	2.0	4.044494	0	0.955506
	cl-phenol	5	2	2.5	3.778211	0	1.221789
	cl-phenol	5	2	3.0	3.612412	0	1.387588
	cl-phenol	5	2	3.5	3.481782	0	1.518218
	cl-phenol	5	2	4.0	3.441588	0	1.558412
	cl-phenol	5	2	4.5	3.341104	0	1.658896
	cl-phenol	5	2	5.0	3.33608	0	1.66392
	cl-phenol	5	2	5.5	3.290862	0	1.709138
	cl-phenol	5	2	6.0	3.250668	0	1.749332

	Substrate	Concentration	Immobilisation method	Time	Substrate mM	Catechol mM	Quinone mM
17	p-cresol	1.25	3	0.0	1.25	0	0
	p-cresol	1.25	3	0.5	0.976485	0.120463	0.153052
	p-cresol	1.25	3	1.0	0.74731	0.224462	0.278228
	p-cresol	1.25	3	1.5	0.503189	0.256828	0.489983
	p-cresol	1.25	3	2.0	0.358709	0.264316	0.626975
	p-cresol	1.25	3	2.5	0.234157	0.235215	0.780628
	p-cresol	1.25	3	3.0	0.159426	0.186184	0.90439
	p-cresol	1.25	3	3.5	0.114587	0.141508	0.993905
	p-cresol	1.25	3	4.0	0.099641	0.106336	1.044023
	p-cresol	1.25	3	4.5	0.094659	0.073272	1.082068
	p-cresol	1.25	3	5.0	0.089677	0.054193	1.10613
	p-cresol	1.25	3	5.5	0.079713	0.036051	1.134236
	p-cresol	1.25	3	6.0	0.074731	0.028431	1.146838
18	p-cresol	5	3	0.0	5	0	0
	p-cresol	5	3	0.5	4.602386	0.08958	0.308034
	p-cresol	5	3	1.0	4.336766	0.202118	0.461116
	p-cresol	5	3	1.5	4.055821	0.252062	0.692117
	p-cresol	5	3	2.0	3.769768	0.254691	0.975541
	p-cresol	5	3	2.5	3.514364	0.243519	1.242117
	p-cresol	5	3	3.0	3.320257	0.239741	1.440003
	p-cresol	5	3	3.5	3.15169	0.235305	1.613005
	p-cresol	5	3	4.0	2.983123	0.222325	1.794552
	p-cresol	5	3	4.5	2.875853	0.199489	1.924658
	p-cresol	5	3	5.0	2.72261	0.167782	2.109608
	p-cresol	5	3	5.5	2.727719	0.140673	2.131609
	p-cresol	5	3	6.0	2.651097	0.116687	2.232216
19	m-cresol	1.25	3	0.0	1.25	0	0
	m-cresol	1.25	3	0.5	1.209177	0.043595	-0.00277
	m-cresol	1.25	3	1.0	1.131666	0.059538	0.058796
	m-cresol	1.25	3	1.5	1.085159	0.06918	0.095661
	m-cresol	1.25	3	2.0	1.033485	0.062774	0.153741
	m-cresol	1.25	3	2.5	1.028317	0.052575	0.169108
	m-cresol	1.25	3	3.0	0.976643	0.043191	0.230165
	m-cresol	1.25	3	3.5	0.955974	0.039023	0.255004
	m-cresol	1.25	3	4.0	0.930136	0.035637	0.284227
	m-cresol	1.25	3	4.5	0.919802	0.032636	0.297563
	m-cresol	1.25	3	5.0	0.904299	0.032242	0.313459
	m-cresol	1.25	3	5.5	0.893964	0.030446	0.32559
	m-cresol	1.25	3	6.0	0.86296	0.028439	0.358601
20	m-cresol	5	3	0.0	5	0	0
	m-cresol	5	3	0.5	4.624985	0.052393	0.322622
	m-cresol	5	3	1.0	4.514627	0.074496	0.410877
	m-cresol	5	3	1.5	4.429351	0.09222	0.478429
	m-cresol	5	3	2.0	4.349091	0.092845	0.558064
	m-cresol	5	3	2.5	4.268831	0.084088	0.647081

	Substrate	**Concentration**	**Immobilisation method**	**Time**	**Substrate mM**	**Catechol mM**	**Quinone mM**
	m-cresol	5	3	3.0	4.143425	0.069493	0.787083
	m-cresol	5	3	3.5	4.133392	0.058649	0.807959
	m-cresol	5	3	4.0	3.992937	0.049265	0.957798
	m-cresol	5	3	4.5	3.992937	0.047807	0.959256
	m-cresol	5	3	5.0	3.902645	0.043636	1.053719
	m-cresol	5	3	5.5	3.867531	0.040091	1.092378
	m-cresol	5	3	6.0	3.807336	0.035921	1.156743
21	phenol	1.25	3	0.0	1.25	0	0
	phenol	1.25	3	0.5	1.165825	0.001571	0.082604
	phenol	1.25	3	1.0	1.139086	0.002706	0.108208
	phenol	1.25	3	1.5	1.096304	0.003433	0.150263
	phenol	1.25	3	2.0	1.08026	0.004073	0.165667
	phenol	1.25	3	2.5	1.03213	0.004364	0.213506
	phenol	1.25	3	3.0	0.994695	0.004364	0.250941
	phenol	1.25	3	3.5	0.973304	0.004073	0.272623
	phenol	1.25	3	4.0	0.951912	0.003782	0.294305
	phenol	1.25	3	4.5	0.935869	0.003782	0.310349
	phenol	1.25	3	5.0	0.919825	0.003491	0.326683
	phenol	1.25	3	5.5	0.90913	0.003491	0.337379
	phenol	1.25	3	6.0	0.887739	0.003491	0.35877
22	phenol	5	3	0.0	5	0	0
	phenol	5	3	0.5	4.827213	0.003269	0.169518
	phenol	5	3	1.0	4.793377	0.005528	0.201095
	phenol	5	3	1.5	4.669313	0.005543	0.325144
	phenol	5	3	2.0	4.646756	0.007273	0.34597
	phenol	5	3	2.5	4.584724	0.008304	0.406971
	phenol	5	3	3.0	4.517053	0.008728	0.474219
	phenol	5	3	3.5	4.443743	0.008364	0.547893
	phenol	5	3	4.0	4.404268	0.008364	0.587368
	phenol	5	3	4.5	4.353514	0.008364	0.638122
	phenol	5	3	5.0	4.229451	0.008105	0.762444
	phenol	5	3	5.5	4.212533	0.008146	0.779321
	phenol	5	3	6.0	4.161779	0.008146	0.830075
23	cl-phenol	1.25	3	0.0	1.25	0	0
	cl-phenol	1.25	3	0.5	1.070481	0	0.179519
	cl-phenol	1.25	3	1.0	1.020224	0	0.229776
	cl-phenol	1.25	3	1.5	0.949863	0	0.300137
	cl-phenol	1.25	3	2.0	0.884529	0	0.365471
	cl-phenol	1.25	3	2.5	0.809143	0	0.440857
	cl-phenol	1.25	3	3.0	0.763911	0	0.486089
	cl-phenol	1.25	3	3.5	0.703602	0	0.546398
	cl-phenol	1.25	3	4.0	0.678474	0	0.571526
	cl-phenol	1.25	3	4.5	0.6835	0	0.5665
	cl-phenol	1.25	3	5.0	0.663397	0	0.586603
	cl-phenol	1.25	3	5.5	0.653345	0	0.596655
	cl-phenol	1.25	3	6.0	0.653345	0	0.596655

	Substrate	Concentration	Immobilisation method	Time	Substrate mM	Catechol mM	Quinone mM
24	cl-phenol	5	3	0.0	5	0	0
	cl-phenol	5	3	0.5	4.367361	0.004332	0.628307
	cl-phenol	5	3	1.0	4.281923	0.005551	0.712525
	cl-phenol	5	3	1.5	4.236692	0.006905	0.756403
	cl-phenol	5	3	2.0	4.196486	0.008663	0.794851
	cl-phenol	5	3	2.5	4.111049	0.011644	0.877307
	cl-phenol	5	3	3.0	3.970328	0.015027	1.014645
	cl-phenol	5	3	3.5	3.874839	0.024108	1.101053
	cl-phenol	5	3	4.0	3.844685	0.033712	1.121603
	cl-phenol	5	3	4.5	3.799453	0.022741	1.177806
	cl-phenol	5	3	5.0	3.754222	0.024504	1.221274
	cl-phenol	5	3	5.5	3.583347	0.025317	1.391336
	cl-phenol	5	3	6.0	3.528064	0.025047	1.446889
25	p-cresol	1.25	4	0.0	1.25	0	0
	p-cresol	1.25	4	0.5	1.096068	0.011505	0.142428
	p-cresol	1.25	4	1.0	0.999498	0.023311	0.227191
	p-cresol	1.25	4	1.5	0.80153	0.031417	0.417053
	p-cresol	1.25	4	2.0	0.753245	0.049591	0.447164
	p-cresol	1.25	4	2.5	0.656675	0.041126	0.552199
	p-cresol	1.25	4	3.0	0.54562	0.03634	0.66804
	p-cresol	1.25	4	3.5	0.458707	0.038873	0.75242
	p-cresol	1.25	4	4.0	0.376622	0.02324	0.850138
	p-cresol	1.25	4	4.5	0.313852	0.018787	0.917361
	p-cresol	1.25	4	5.0	0.275224	0.013786	0.960989
	p-cresol	1.25	4	5.5	0.212454	0.009286	1.028261
	p-cresol	1.25	4	6.0	0.173826	0.007452	1.068722
26	p-cresol	5	4	0.0	5	0	0
	p-cresol	5	4	0.5	4.667883	0	0.332117
	p-cresol	5	4	1.0	4.507441	0.057337	0.435223
	p-cresol	5	4	1.5	4.27179	0.117082	0.611128
	p-cresol	5	4	2.0	4.14143	0.116238	0.742332
	p-cresol	5	4	2.5	4.041154	0.098241	0.860606
	p-cresol	5	4	3.0	3.830572	0.088058	1.08137
	p-cresol	5	4	3.5	3.650074	0.073798	1.276128
	p-cresol	5	4	4.0	3.514701	0.069051	1.416249
	p-cresol	5	4	4.5	3.354258	0.07075	1.574992
	p-cresol	5	4	5.0	3.208856	0.057845	1.733299
	p-cresol	5	4	5.5	3.093538	0.05462	1.851842
	p-cresol	5	4	6.0	2.97822	0.050038	1.971742
27	m-cresol	1.25	4	0.0	1.25	0	0
	m-cresol	1.25	4	0.5	1.197057	0	0.052943
	m-cresol	1.25	4	1.0	1.172727	0	0.077273
	m-cresol	1.25	4	1.5	1.148396	0	0.101604
	m-cresol	1.25	4	2.0	1.138664	0	0.111336
	m-cresol	1.25	4	2.5	1.124066	0	0.125934
	m-cresol	1.25	4	3.0	1.124066	0	0.125934

	Substrate	**Concentration**	**Immobilisation method**	**Time**	**Substrate mM**	**Catechol mM**	**Quinone mM**
	m-cresol	1.25	4	3.5	1.090003	0	0.159997
	m-cresol	1.25	4	4.0	1.075405	0	0.174595
	m-cresol	1.25	4	4.5	1.065673	0	0.184327
	m-cresol	1.25	4	5.0	1.060807	0	0.189193
	m-cresol	1.25	4	5.5	1.046208	0	0.203792
	m-cresol	1.25	4	6.0	1.017012	0	0.232988
28	m-cresol	5	4	0.0	5	0	0
	m-cresol	5	4	0.5	4.789827	0	0.210173
	m-cresol	5	4	1.0	4.714908	0	0.285092
	m-cresol	5	4	1.5	4.659967	0	0.340033
	m-cresol	5	4	2.0	4.63	0	0.37
	m-cresol	5	4	2.5	4.615016	0	0.384984
	m-cresol	5	4	3.0	4.590043	0	0.409957
	m-cresol	5	4	3.5	4.56507	0	0.43493
	m-cresol	5	4	4.0	4.470172	0	0.529828
	m-cresol	5	4	4.5	4.365285	0	0.634715
	m-cresol	5	4	5.0	4.295361	0	0.704639
	m-cresol	5	4	5.5	4.290366	0	0.709634
	m-cresol	5	4	6.0	4.25041	0	0.74959
29	phenol	1.25	4	0.0	1.25	0	0
	phenol	1.25	4	0.5	1.189727	0	0.060273
	phenol	1.25	4	1.0	1.174004	0	0.075996
	phenol	1.25	4	1.5	1.158281	0	0.091719
	phenol	1.25	4	2.0	1.137317	0	0.112683
	phenol	1.25	4	2.5	1.137317	0	0.112683
	phenol	1.25	4	3.0	1.121593	0	0.128407
	phenol	1.25	4	3.5	1.10587	0	0.14413
	phenol	1.25	4	4.0	1.100629	0	0.149371
	phenol	1.25	4	4.5	1.090147	0	0.159853
	phenol	1.25	4	5.0	1.084906	0	0.165094
	phenol	1.25	4	5.5	1.069182	0	0.180818
	phenol	1.25	4	6.0	1.048218	0	0.201782
30	phenol	5	4	0.0	5	0	0
	phenol	5	4	0.5	4.606578	0	0.393422
	phenol	5	4	1.0	4.505976	0	0.494024
	phenol	5	4	1.5	4.473924	0	0.526076
	phenol	5	4	2.0	4.43352	0	0.56648
	phenol	5	4	2.5	4.428536	0	0.571464
	phenol	5	4	3.0	4.36214	0	0.63786
	phenol	5	4	3.5	4.340054	0	0.659946
	phenol	5	4	4.0	4.317968	0	0.682032
	phenol	5	4	4.5	4.309616	0	0.690384
	phenol	5	4	5.0	4.30854	0	0.69146
	phenol	5	4	5.5	4.275411	0	0.724589
	phenol	5	4	6.0	4.259385	0	0.740615

	Substrate	Concentration	Immobilisation method	Time	Substrate mM	Catechol mM	Quinone mM
31	cl-phenol	1.25	4	0.0	1.25	0	0
	cl-phenol	1.25	4	0.5	1.214455	0	0.035545
	cl-phenol	1.25	4	1.0	1.194708	0	0.055292
	cl-phenol	1.25	4	1.5	1.174961	0	0.075039
	cl-phenol	1.25	4	2.0	1.135466	0	0.114534
	cl-phenol	1.25	4	2.5	1.115719	0	0.134281
	cl-phenol	1.25	4	3.0	1.076224	0	0.173776
	cl-phenol	1.25	4	3.5	1.026856	0	0.223144
	cl-phenol	1.25	4	4.0	0.972551	0	0.277449
	cl-phenol	1.25	4	4.5	0.952804	0	0.297196
	cl-phenol	1.25	4	5.0	0.893562	0	0.356438
	cl-phenol	1.25	4	5.5	0.859005	0	0.390995
	cl-phenol	1.25	4	6.0	0.839258	0	0.410742
32	cl-phenol	5	4	0.0	5	0	0
	cl-phenol	5	4	0.5	4.790259	0	0.209741
	cl-phenol	5	4	1.0	4.755583	0	0.244417
	cl-phenol	5	4	1.5	4.730814	0	0.269186
	cl-phenol	5	4	2.0	4.710999	0	0.289001
	cl-phenol	5	4	2.5	4.661462	0	0.338538
	cl-phenol	5	4	3.0	4.602017	0	0.397983
	cl-phenol	5	4	3.5	4.577248	0	0.422752
	cl-phenol	5	4	4.0	4.334516	0	0.665484
	cl-phenol	5	4	4.5	4.43359	0	0.56641
	cl-phenol	5	4	5.0	4.423683	0	0.576317
	cl-phenol	5	4	5.5	4.403868	0	0.596132
	cl-phenol	5	4	6.0	4.43359	0	0.56641
33	p-cresol	1.25	5	0.0	1.25	0	0
	p-cresol	1.25	5	0.5	1.15855	0.079431	0.012019
	p-cresol	1.25	5	1.0	1.049343	0.147416	0.053241
	p-cresol	1.25	5	1.5	0.944883	0.189563	0.115553
	p-cresol	1.25	5	2.0	0.864165	0.197799	0.188036
	p-cresol	1.25	5	2.5	0.764453	0.187464	0.298082
	p-cresol	1.25	5	3.0	0.678987	0.171962	0.399052
	p-cresol	1.25	5	3.5	0.598268	0.156621	0.495112
	p-cresol	1.25	5	4.0	0.536542	0.149354	0.564104
	p-cresol	1.25	5	4.5	0.441579	0.120932	0.687489
	p-cresol	1.25	5	5.0	0.375104	0.108498	0.766397
	p-cresol	1.25	5	5.5	0.327623	0.103169	0.819208
	p-cresol	1.25	5	6.0	0.284889	0.099616	0.865494
34	p-cresol	5	5	0.0	5	0	0
	p-cresol	5	5	0.5	4.797222	0.061589	0.141189
	p-cresol	5	5	1.0	4.640257	0.140574	0.219168
	p-cresol	5	5	1.5	4.483293	0.179241	0.337466
	p-cresol	5	5	2.0	4.409716	0.180893	0.409391
	p-cresol	5	5	2.5	4.208606	0.160073	0.631322
	p-cresol	5	5	3.0	4.017305	0.139252	0.843442
	p-cresol	5	5	3.5	3.963349	0.125702	0.910949

	Substrate	Concentration	Immobilisation method	Time	Substrate mM	Catechol mM	Quinone mM
	p-cresol	5	5	4.0	3.713187	0.093646	1.193167
	p-cresol	5	5	4.5	3.664136	0.108517	1.227347
	p-cresol	5	5	5.0	3.438499	0.104552	1.456949
	p-cresol	5	5	5.5	3.428689	0.1105	1.460811
	p-cresol	5	5	6.0	3.325681	0.102899	1.571419
35	m-cresol	1.25	5	0.0	1.25	0	0
	m-cresol	1.25	5	0.5	1.210058	0	0.039942
	m-cresol	1.25	5	1.0	1.185464	0	0.064536
	m-cresol	1.25	5	1.5	1.160869	0	0.089131
	m-cresol	1.25	5	2.0	1.151031	0	0.098969
	m-cresol	1.25	5	2.5	1.136274	0	0.113726
	m-cresol	1.25	5	3.0	1.136274	0	0.113726
	m-cresol	1.25	5	3.5	1.101842	0	0.148158
	m-cresol	1.25	5	4.0	1.087085	0.002341	0.160575
	m-cresol	1.25	5	4.5	1.077247	0.003901	0.168852
	m-cresol	1.25	5	5.0	1.072328	0.003121	0.174551
	m-cresol	1.25	5	5.5	1.057571	0.002341	0.190088
	m-cresol	1.25	5	6.0	1.028058	0.002341	0.219602
36	m-cresol	5	5	0.0	5	0	0
	m-cresol	5	5	0.5	4.815804	0	0.184196
	m-cresol	5	5	1.0	4.740479	0	0.259521
	m-cresol	5	5	1.5	4.68524	0	0.31476
	m-cresol	5	5	2.0	4.65511	0	0.34489
	m-cresol	5	5	2.5	4.640045	0	0.359955
	m-cresol	5	5	3.0	4.614937	0	0.385063
	m-cresol	5	5	3.5	4.589828	0	0.410172
	m-cresol	5	5	4.0	4.494416	0	0.505584
	m-cresol	5	5	4.5	4.38896	0	0.61104
	m-cresol	5	5	5.0	4.318657	0	0.681343
	m-cresol	5	5	5.5	4.313635	0	0.686365
	m-cresol	5	5	6.0	4.273461	0	0.726539
37	phenol	1.25	5	0.0	1.25	0	0
	phenol	1.25	5	0.5	1.224385	0	0.025615
	phenol	1.25	5	1.0	1.215164	0	0.034836
	phenol	1.25	5	1.5	1.19416	0	0.05584
	phenol	1.25	5	2.0	1.188012	0	0.061988
	phenol	1.25	5	2.5	1.171107	0	0.078893
	phenol	1.25	5	3.0	1.156762	0	0.093238
	phenol	1.25	5	3.5	1.138832	0	0.111168
	phenol	1.25	5	4.0	1.11168	0	0.13832
	phenol	1.25	5	4.5	1.101434	0	0.148566
	phenol	1.25	5	5.0	1.077357	0	0.172643
	phenol	1.25	5	5.5	1.069672	0	0.180328
	phenol	1.25	5	6.0	1.0625	0	0.1875

	Substrate	Concentration	Immobilisation method	Time	Substrate mM	Catechol mM	Quinone mM
38	phenol	5	5	0.0	5	0	0
	phenol	5	5	0.5	4.77602	0	0.22398
	phenol	5	5	1.0	4.732653	0	0.267347
	phenol	5	5	1.5	4.687245	0	0.312755
	phenol	5	5	2.0	4.653061	0	0.346939
	phenol	5	5	2.5	4.62602	0	0.37398
	phenol	5	5	3.0	4.576531	0	0.423469
	phenol	5	5	3.5	4.540816	0	0.459184
	phenol	5	5	4.0	4.469388	0	0.530612
	phenol	5	5	4.5	4.415816	0	0.584184
	phenol	5	5	5.0	4.355102	0	0.644898
	phenol	5	5	5.5	4.333673	0	0.666327
	phenol	5	5	6.0	4.286735	0	0.713265
39	cl-phenol	1.25	5	0.0	1.25	0	0
	cl-phenol	1.25	5	0.5	1.177015	0	0.072985
	cl-phenol	1.25	5	1.0	1.132692	0	0.117308
	cl-phenol	1.25	5	1.5	1.112993	0	0.137007
	cl-phenol	1.25	5	2.0	1.073595	0	0.176405
	cl-phenol	1.25	5	2.5	1.014498	0	0.235502
	cl-phenol	1.25	5	3.0	0.980025	0	0.269975
	cl-phenol	1.25	5	3.5	0.955401	0	0.294599
	cl-phenol	1.25	5	4.0	0.896304	0	0.353696
	cl-phenol	1.25	5	4.5	0.876605	0	0.373395
	cl-phenol	1.25	5	5.0	0.861831	0	0.388169
	cl-phenol	1.25	5	5.5	0.797809	0	0.452191
	cl-phenol	1.25	5	6.0	0.773186	0	0.476814
40	cl-phenol	5	5	0.0	5	0	0
	cl-phenol	5	5	0.5	4.853273	0	0.146727
	cl-phenol	5	5	1.0	4.804201	0	0.195799
	cl-phenol	5	5	1.5	4.735499	0	0.264501
	cl-phenol	5	5	2.0	4.710963	0	0.289037
	cl-phenol	5	5	2.5	4.676612	0	0.323388
	cl-phenol	5	5	3.0	4.617725	0	0.382275
	cl-phenol	5	5	3.5	4.583374	0	0.416626
	cl-phenol	5	5	4.0	4.544116	0	0.455884
	cl-phenol	5	5	4.5	4.475415	0	0.524585
	cl-phenol	5	5	5.0	4.450878	0	0.549122
	cl-phenol	5	5	5.5	4.401806	0	0.598194
	cl-phenol	5	5	6.0	4.200608	0	0.799392
41	p-cresol	1.25	6	0.0	1.25	0	0
	p-cresol	1.25	6	0.5	0.741489	0.00235	0.506161
	p-cresol	1.25	6	1.0	0.682634	0.006041	0.561324
	p-cresol	1.25	6	1.5	0.650523	0.010404	0.589073
	p-cresol	1.25	6	2.0	0.621437	0.014431	0.614132
	p-cresol	1.25	6	2.5	0.598354	0.014766	0.636879
	p-cresol	1.25	6	3.0	0.580591	0.018458	0.650952

	Substrate	Concentration	Immobilisation method	Time	Substrate mM	Catechol mM	Quinone mM
	p-cresol	1.25	6	3.5	0.55658	0.018458	0.674962
	p-cresol	1.25	6	4.0	0.542282	0.019129	0.68859
	p-cresol	1.25	6	4.5	0.531008	0.020136	0.698856
	p-cresol	1.25	6	5.0	0.516124	0.021142	0.712734
	p-cresol	1.25	6	5.5	0.498604	0.021478	0.729918
	p-cresol	1.25	6	6.0	0.490454	0.021478	0.738068
42	p-cresol	5	6	0.0	5	0	0
	p-cresol	5	6	0.5	3.854151	0.013088	1.13276
	p-cresol	5	6	1.0	3.771558	0.046647	1.181794
	p-cresol	5	6	1.5	3.610689	0.073494	1.315817
	p-cresol	5	6	2.0	3.503963	0.083226	1.412811
	p-cresol	5	6	2.5	3.426727	0.087589	1.485684
	p-cresol	5	6	3.0	3.37352	0.091951	1.534528
	p-cresol	5	3	3.5	3.306843	0.09665	1.596508
	p-cresol	5	6	4.0	3.258265	0.097992	1.643744
	p-cresol	5	6	4.5	3.171251	0.098999	1.729751
	p-cresol	5	6	5.0	3.153723	0.11108	1.735197
	p-cresol	5	6	5.5	3.057243	0.114771	1.827986
	p-cresol	5	6	6.0	2.998367	0.121819	1.879814
43	m-cresol	1.25	6	0.0	1.25	0	0
	m-cresol	1.25	6	0.5	0.611726	0	0.638274
	m-cresol	1.25	6	1.0	0.539305	0	0.710695
	m-cresol	1.25	6	1.5	0.516124	0	0.733876
	m-cresol	1.25	6	2.0	0.498653	0	0.751347
	m-cresol	1.25	6	2.5	0.48494	0	0.76506
	m-cresol	1.25	6	3.0	0.467176	0	0.782824
	m-cresol	1.25	6	3.5	0.450925	0	0.799075
	m-cresol	1.25	6	4.0	0.44707	0	0.80293
	m-cresol	1.25	6	4.5	0.43487	0	0.81513
	m-cresol	1.25	6	5.0	0.419351	0	0.830649
	m-cresol	1.25	6	5.5	0.409298	0	0.840702
	m-cresol	1.25	6	6.0	0.407785	0	0.842215
44	m-cresol	5	6	0.0	5	0	0
	m-cresol	5	6	0.5	3.624272	0	1.375728
	m-cresol	5	6	1.0	3.540108	0	1.459892
	m-cresol	5	6	1.5	3.453667	0	1.546333
	m-cresol	5	6	2.0	3.38919	0	1.61081
	m-cresol	5	6	2.5	3.302849	0	1.697151
	m-cresol	5	6	3.0	3.250873	0	1.749127
	m-cresol	5	6	3.5	3.214788	0	1.785212
	m-cresol	5	6	4.0	3.113822	0	1.886178
	m-cresol	5	6	4.5	3.06139	0	1.93861
	m-cresol	5	6	5.0	3.022926	0	1.977074
	m-cresol	5	6	5.5	2.978744	0	2.021256
	m-cresol	5	6	6.0	2.909155	0	2.090845

	Substrate	**Concentration**	**Immobilisation method**	**Time**	**Substrate mM**	**Catechol mM**	**Quinone mM**
45	phenol	1.25	6	0.0	1.25	0	0
	phenol	1.25	6	0.5	0.87832	0.00114	0.37054
	phenol	1.25	6	1.0	0.860867	0.00228	0.386853
	phenol	1.25	6	1.5	0.840806	0.00228	0.406914
	phenol	1.25	6	2.0	0.807505	0.003645	0.43885
	phenol	1.25	6	2.5	0.790553	0.004562	0.454884
	phenol	1.25	6	3.0	0.77962	0.005474	0.464906
	phenol	1.25	6	3.5	0.777313	0.006043	0.466644
	phenol	1.25	6	4.0	0.76638	0.006271	0.477349
	phenol	1.25	6	4.5	0.745215	0.006273	0.498511
	phenol	1.25	6	5.0	0.741203	0.006271	0.502526
	phenol	1.25	6	5.5	0.729618	0.006386	0.513996
	phenol	1.25	6	6.0	0.723449	0.007183	0.519368
46	phenol	5	6	0.0	5	0	0
	phenol	5	6	0.5	4.027311	0.005849	0.966841
	phenol	5	6	1.0	3.98773	0.007599	1.004671
	phenol	5	6	1.5	3.849198	0.011983	1.138819
	phenol	5	6	2.0	3.834356	0.01549	1.150154
	phenol	5	6	2.5	3.770038	0.018126	1.211836
	phenol	5	6	3.0	3.755195	0.021281	1.223524
	phenol	5	6	3.5	3.7453	0.02412	1.23058
	phenol	5	6	4.0	3.690877	0.024267	1.284856
	phenol	5	6	4.5	3.661191	0.026458	1.31235
	phenol	5	6	5.0	3.611716	0.027628	1.360656
	phenol	5	6	5.5	3.606768	0.028655	1.364577
	phenol	5	6	6.0	3.52266	0.030263	1.447078
47	cl-phenol	1.25	6	0.0	1.25	0	0
	cl-phenol	1.25	6	0.5	0.601237	0	0.648763
	cl-phenol	1.25	6	1.0	0.589308	0	0.660692
	cl-phenol	1.25	6	1.5	0.563063	0	0.686937
	cl-phenol	1.25	6	2.0	0.542545	0	0.707455
	cl-phenol	1.25	6	2.5	0.522026	0	0.727974
	cl-phenol	1.25	6	3.0	0.506757	0	0.743243
	cl-phenol	1.25	6	3.5	0.501508	0	0.748492
	cl-phenol	1.25	6	4.0	0.488147	0	0.761853
	cl-phenol	1.25	6	4.5	0.471446	0	0.778554
	cl-phenol	1.25	6	5.0	0.463811	0	0.786189
	cl-phenol	1.25	6	5.5	0.455699	0	0.794301
	cl-phenol	1.25	6	6.0	0.447587	0	0.802413
48	cl-phenol	5	6	0.0	5	0	0
	cl-phenol	5	6	0.5	3.566384	0	1.433616
	cl-phenol	5	6	1.0	3.460452	0	1.539548
	cl-phenol	5	6	1.5	3.450363	0	1.549637
	cl-phenol	5	6	2.0	3.444814	0	1.555186
	cl-phenol	5	6	2.5	3.410008	0	1.589992
	cl-phenol	5	6	3.0	3.358555	0	1.641445
	cl-phenol	5	6	3.5	3.352502	0	1.647498

Substrate	**Concentration**	**Immobilisation method**	**Time**	**Substrate mM**	**Catechol mM**	**Quinone mM**
cl-phenol	5	6	4.0	3.320722	0	1.679278
cl-phenol	5	6	4.5	3.304076	0	1.695924
cl-phenol	5	6	5.0	3.285412	0	1.714588
cl-phenol	5	6	5.5	3.283898	0	1.716102
cl-phenol	5	6	6.0	3.27381	0	1.72619

APPENDIX B

Data generated using the mathematical model (Section 3.3.7).

The mathematical model relies on both phenol and catechol data, thus the model was used for reactions where catechol was detected in solution. The percentage polyphenol oxidase inactivation (decay) was calculated for the process. Reaction rate constants k_1, k_2 and k_3 were calculated. The time at which maximum catechol was reached and the corresponding catechol concentration were also calculated.

se = sum of errors of substrate data
ce = sum of errors of catechol data
ae = average sum of errors of substrate and catechol data

%PPO decay	k_1	k_2	k_3	**se**	**ce**	**ae**	**Maximum catechol**	**Time**
run 1								
0	0.563733	0.213336	0	0.427939	0.390971	0.409455	0.691788	2.772719
5	0.562541	0.21834	0.031446	0.432901	0.39084	0.41187		
10	0.566909	0.221882	0.064597	0.433704	0.394217	0.41396		
15	0.571089	0.225159	0.099848	0.435115	0.397436	0.416275		
20	0.574537	0.228678	0.137546	0.437607	0.40027	0.418938		
25	0.580045	0.228906	0.170423	0.437828	0.403896	0.420862		
30	0.584309	0.232943	0.213482	0.440789	0.40727	0.42403		
35	0.589069	0.23739	0.260181	0.444159	0.410994	0.427576		
40	0.594407	0.242314	0.311174	0.448039	0.415108	0.431573		
45	0.600425	0.247799	0.367306	0.452565	0.419659	0.436112		
50	0.607256	0.253954	0.429689	0.457914	0.424704	0.441309		
55	0.615084	0.260926	0.499815	0.464323	0.430315	0.447319		
60	0.624173	0.268914	0.579721	0.472106	0.436587	0.454346		
65	0.634955	0.278221	0.672233	0.481671	0.443669	0.46267		
70	0.647966	0.289322	0.781505	0.493686	0.451713	0.472699		
75	0.664273	0.302862	0.91597	0.509308	0.461023	0.485166		
80	0.685401	0.319869	1.08255	0.529493	0.471892	0.500693		
85	0.714153	0.342316	1.299734	0.576906	0.503933	0.54042		
90	0.756621	0.374434	1.601686	0.600972	0.506794	0.553883		
95	0.830068	0.428298	2.080964	0.683554	0.548634	0.616094		
run2								
0	0.115854	0.522463	-1.4E-07	0.572011	0.335256	0.453634	0.721835	3.720932
5	0.116946	0.527859	0.013259	0.56721	0.338576	0.452893		
10	0.11909	0.534613	0.027117	0.560736	0.344532	0.452634	0.724185	3.71357
15	0.119365	0.540324	0.042264	0.561165	0.345874	0.453519		

% PPO decay	k_1	k_2	k_3	se	ce	ae	Maximum catechol	Time
20	0.120725	0.54779	0.058113	0.560353	0.349928	0.455141		
25	0.122181	0.555883	0.074957	0.561458	0.354209	0.457833		
30	0.123737	0.564579	0.092877	0.564819	0.358708	0.461763		
35	0.125398	0.573894	0.111965	0.570782	0.363422	0.467102		
40	0.127169	0.583852	0.132329	0.57969	0.368351	0.474021		
45	0.129066	0.594556	0.154104	0.59187	0.373511	0.482691		
50	0.1311	0.606071	0.17747	0.607668	0.378912	0.49329		
55	0.133296	0.618578	0.202762	0.627538	0.384589	0.506064		
60	0.13567	0.632097	0.230179	0.651771	0.390542	0.521157		
65	0.138256	0.646872	0.260196	0.680875	0.39682	0.538848		
70	0.141096	0.663161	0.293355	0.733061	0.413052	0.573056		
75	0.144237	0.681255	0.330331	0.775476	0.419946	0.597711		
80	0.147734	0.701514	0.371988	0.824353	0.427268	0.625811		
85	0.151661	0.724432	0.419701	0.880762	0.435067	0.657914		
90	0.156113	0.750688	0.475145	0.945788	0.443404	0.694596		
95	0.161245	0.781851	0.542982	1.023627	0.452378	0.738003		
run3								
0	0.501273	0.261651	1.02E-05	0.353064	0.111133	0.232099	0.61445	2.65047
5	0.504004	0.265232	0.029072	0.348809	0.111805	0.230307		
10	0.504702	0.268282	0.060277	0.341705	0.114509	0.228107		
15	0.46492	0.264436	0.105785	0.292091	0.154895	0.223493		
20	0.470005	0.267833	0.14332	0.286983	0.154791	0.220887		
25	0.473316	0.271868	0.185528	0.279252	0.157295	0.218274		
30	0.47677	0.276302	0.231421	0.27065	0.160375	0.215512		
35	0.480439	0.281201	0.281717	0.261047	0.164058	0.212552		
40	0.484428	0.286656	0.337335	0.250294	0.168365	0.209329		
45	0.488827	0.292772	0.399511	0.238162	0.173362	0.205762		
50	0.493175	0.299871	0.469867	0.223806	0.17965	0.201728		
55	0.498859	0.30795	0.550538	0.207989	0.186237	0.197113		
60	0.506546	0.31695	0.645075	0.190651	0.19287	0.191761		
65	0.515892	0.327499	0.758247	0.170528	0.20037	0.185449		
70	0.527502	0.340059	0.897494	0.146986	0.20885	0.177918		
75	0.538841	0.356786	1.077901	0.11522	0.221098	0.168159		
80	0.558505	0.376359	1.314372	0.08771	0.228075	0.157893	0.552942	2.474804
85	0.58819	0.401887	1.647172	0.077381	0.239309	0.158345		
90	0.635278	0.438447	2.140145	0.09896	0.250008	0.174484		
95	0.717065	0.502738	2.978759	0.224017	0.276772	0.250394		
run4								
0	0.070523	0.650388	3.3E-06	0.970334	0.779662	0.874998	0.413756	3.895082
5	0.072172	0.709422	0.017621	0.962575	0.778345	0.87046		
10	0.072963	0.718162	0.036431	0.946023	0.777053	0.861538		
15	0.073793	0.725821	0.056697	0.928195	0.775546	0.851871		
20	0.0747	0.734097	0.07862	0.898502	0.765505	0.832003		
25	0.075689	0.742825	0.102546	0.877756	0.763738	0.820747		
30	0.076785	0.752338	0.128853	0.85539	0.761794	0.808592		
35	0.078015	0.762848	0.158051	0.831141	0.759629	0.795385		
40	0.079409	0.774591	0.19091	0.804538	0.757215	0.780876		
45	0.081017	0.787785	0.228463	0.775076	0.754451	0.764764		
50	0.082914	0.80294	0.27228	0.741969	0.751233	0.746601		
55	0.085219	0.820691	0.3249	0.67697	0.738857	0.707913		

% PPO decay	k_1	k_2	k_3	se	ce	ae	Maximum catechol	Time
60	0.088148	0.842401	0.390706	0.631083	0.734175	0.682629		
65	0.092125	0.871686	0.477535	0.574821	0.728137	0.651479		
70	0.098328	0.913511	0.61009	0.482582	0.707925	0.595253		
75	0.105608	0.967448	0.754902	0.410127	0.698888	0.554508		
80	0.114086	1.029872	0.922326	0.372124	0.691766	0.531945		
85	0.122463	1.093714	1.085734	0.311276	0.682797	0.497036		
90	0.131033	1.164172	1.246745	0.297026	0.670757	0.483892	0.426584	3.275895
95	0.144247	1.278775	1.484095	0.32776	0.66465	0.496205		
run5								
0	0.464692	0.992311	-1.7E-06	0.402512	0.311221	0.356866	0.30004	1.417102
5	0.460567	1.04983	0.015578	0.408107	0.304282	0.356195	0.287995	1.399792
10	0.464461	1.069189	0.032023	0.409763	0.302951	0.356357		
15	0.467664	1.082471	0.049592	0.412877	0.30116	0.357018		
20	0.47096	1.094637	0.068434	0.429875	0.307306	0.368591		
25	0.474353	1.105649	0.088772	0.434248	0.304847	0.369548		
30	0.478234	1.119611	0.110731	0.438868	0.302354	0.370611		
35	0.482542	1.135009	0.134622	0.443995	0.29981	0.371902		
40	0.487368	1.152264	0.160782	0.444049	0.299575	0.371812		
45	0.50614	1.139454	0.182305	0.439757	0.304172	0.371964		
50	0.513605	1.171638	0.212323	0.450358	0.303007	0.376683		
55	0.522437	1.206786	0.24572	0.455016	0.300442	0.377729		
60	0.532531	1.246215	0.283429	0.453858	0.30028	0.377069		
65	0.544339	1.29181	0.326589	0.509151	0.317829	0.41349		
70	0.558425	1.345239	0.376923	0.524717	0.315173	0.419945		
75	0.575739	1.410146	0.436946	0.528338	0.313752	0.421045		
80	0.597528	1.489142	0.511033	0.560849	0.312745	0.436797		
85	0.626642	1.594399	0.606215	0.578658	0.311634	0.445146		
90	0.668535	1.741768	0.737926	0.650729	0.321595	0.486162		
95	0.739811	1.989686	0.9501	0.731445	0.32806	0.529753		
run6								
0	0.10121	1.552519	9.8E-07	0.621641	0.383792	0.502716	0.269398	1.851119
5	0.102562	1.601035	0.010054	0.600076	0.382662	0.491369		
10	0.103893	1.624402	0.020732	0.589938	0.382122	0.48603		
15	0.105312	1.650607	0.032118	0.555043	0.382637	0.46884		
20	0.106844	1.679586	0.04431	0.559631	0.3871	0.473365		
25	0.108635	1.74478	0.057406	0.517565	0.387914	0.452739		
30	0.110461	1.785609	0.071436	0.505487	0.3872	0.446343		
35	0.112449	1.827472	0.086639	0.474344	0.387522	0.430933		
40	0.114622	1.871164	0.103156	0.45262	0.396206	0.424413		
45	0.117002	1.917487	0.121121	0.482579	0.399048	0.440813		
50	0.119698	1.915438	0.143477	0.454589	0.399212	0.426901		
55	0.122381	1.970352	0.162907	0.443148	0.398831	0.42099		
60	0.125338	2.031675	0.184116	0.4208	0.398526	0.409663	0.256771	1.447561
65	0.12863	2.100683	0.207451	0.437323	0.397645	0.417484		
70	0.132339	2.179136	0.233369	0.475556	0.400099	0.437828		
75	0.136787	2.269599	0.263184	0.479229	0.407196	0.443213		
80	0.143375	2.416005	0.311196	0.557272	0.407858	0.482565		
85	0.150558	2.545857	0.359364	0.605806	0.406129	0.505968		
90	0.165984	2.885454	0.464374	0.742732	0.412096	0.577414		
95	0.196341	3.506653	0.663851	0.990293	0.413341	0.701817		

% PPO decay	k_1	k_2	k_3	**se**	**ce**	**ae**	**Maximum catechol**	**Time**
run9								
0	0.105624	9.430982	-1.5E-05	0.304428	0.04905	0.176739	0.0133	0.4931
5	0.107102	9.54573	0.032239	0.315905	0.048971	0.182438		
10	0.10896	10.74586	0.065767	0.315454	0.049117	0.182286		
15	0.10996	11.6949	0.095425	0.32086	0.049319	0.18509		
20	0.111475	15.13038	0.132138	0.330297	0.050462	0.190379		
25	0.113153	20.45447	0.171084	0.336563	0.052199	0.194381		
30	0.114946	50.6468	0.211424	0.343855	0.056701	0.200278		
35	0.11696	578.3671	0.255283	0.351622	0.060297	0.205959		
40	0.119062	1062.477	0.304464	0.36034	0.060464	0.210402		
run 10								
0	0.010929	1.896978	-0.00351	0.192099	0.14439	0.168245	0.028	2.7341
5	0.011063	1.963292	0.077216	0.194071	0.144334	0.169203		
10	0.01133	2.119447	0.153321	0.199895	0.144378	0.172136		
15	0.011455	2.412869	0.234213	0.204431	0.144987	0.174709		
20	0.011617	2.932661	0.315198	0.205169	0.146461	0.175815		
25	0.011799	3.984984	0.39503	0.208687	0.149535	0.179111		
30	0.011989	6.526149	0.474346	0.212916	0.154835	0.183875		
35	0.012186	17.05487	0.554262	0.213337	0.161966	0.187651		
run 14								
0	0.057397	19.09204	1.54E-05	0.593633	0.023508	0.308571	0.01477	0.29764
5	0.058115	19.60478	0.013636	0.577647	0.023578	0.300613		
10	0.059198	20.65914	0.027872	0.561335	0.023712	0.292523		
15	0.060327	21.78998	0.042901	0.549259	0.023941	0.2866		
20	0.061085	22.55909	0.070455	0.524347	0.024174	0.274261		
25	0.062234	25.76741	0.088143	0.514364	0.025367	0.269865		
30	0.063439	29.89372	0.107016	0.495032	0.02719	0.261111		
35	0.064738	34.53702	0.127346	0.476324	0.029138	0.252731		
40	0.066214	40.76124	0.149436	0.457853	0.031392	0.244622		
45	0.067839	55.46227	0.173393	0.438183	0.035457	0.23682		
50	0.069652	86.20105	0.199602	0.419073	0.040094	0.229583		
55	0.071727	150.4931	0.229039	0.396056	0.044024	0.22004		
60	0.074137	573.6769	0.262303	0.374044	0.048291	0.211167	0.0006	0.0286
run 21								
0	0.585083	1.29403	-3.9E-06	0.14046	0.148027	0.144243	0.293505	1.136242
5	0.587848	1.303673	0.013095	0.14074	0.147282	0.144011		
10	0.590991	1.315374	0.027007	0.141301	0.146534	0.143918		
15	0.594539	1.329242	0.041823	0.13099	0.149517	0.140254		
20	0.59853	1.345421	0.057653	0.13185	0.148716	0.140283		
25	0.603	1.364096	0.074634	0.133219	0.147957	0.140588		
30	0.608011	1.385473	0.092931	0.124748	0.150974	0.137861		
35	0.613637	1.409849	0.112753	0.126677	0.150377	0.138527	0.286536	1.059204
40	0.62	1.43866	0.134354	0.144822	0.152743	0.148782		
45	0.627164	1.470392	0.15809	0.147322	0.151915	0.149619		
50	0.635316	1.506507	0.184402	0.1414	0.155475	0.148438		
55	0.644682	1.547777	0.21389	0.139924	0.159743	0.149834		
60	0.655607	1.595297	0.247376	0.163994	0.174586	0.16929		
65	0.668488	1.650729	0.286039	0.195038	0.178286	0.186662		
70	0.684094	1.716328	0.331591	0.197325	0.183418	0.190372		
75	0.703512	1.795778	0.386688	0.200041	0.185983	0.193012		

% PPO decay	k_1	k_2	k_3	se	ce	ae	Maximum catechol	Time
80	0.728604	1.895152	0.455664	0.238798	0.196807	0.217803		
85	0.762944	2.026913	0.546163	0.276917	0.214262	0.24559		
90	0.814138	2.220054	0.67422	0.318644	0.226922	0.272783		
95	0.904326	2.551678	0.885097	0.39201	0.248255	0.320132		
run 22								
0	0.122582	2.048233	1.32E-06	0.479096	0.122012	0.300554	0.250096	1.440145
5	0.124046	2.075357	0.008184	0.435843	0.122911	0.279377		
10	0.125685	2.1058	0.016914	0.423405	0.123201	0.273303		
15	0.127478	2.139265	0.026297	0.364543	0.126177	0.24536		
20	0.129431	2.175519	0.036499	0.331647	0.127651	0.229649		
25	0.131589	2.215181	0.047762	0.315073	0.128049	0.221561		
30	0.13403	2.259304	0.060483	0.25842	0.140378	0.199399		
35	0.137133	2.328069	0.076367	0.211347	0.14163	0.176489		
40	0.140226	2.37581	0.092277	0.1689	0.144117	0.156508		
45	0.143693	2.429223	0.110051	0.134393	0.145572	0.139982		
50	0.14635	2.475963	0.123605	0.119141	0.146683	0.132912		
55	0.148196	2.558575	0.132099	0.113277	0.148084	0.130681	0.243032	1.169009
60	0.149801	2.538462	0.141006	0.116794	0.148586	0.13269		
65	0.151635	2.582182	0.150193	0.118461	0.148642	0.133552		
70	0.152438	2.593076	0.154335	0.121664	0.148791	0.135227		
75	0.15134	2.55998	0.148969	0.119283	0.14889	0.134087		
80	0.169589	3.052804	0.235071	0.309297	0.168554	0.238925		
85	0.17755	3.211915	0.269271	0.432751	0.178774	0.305763		
90	0.186442	3.417621	0.307947	0.47595	0.181661	0.328805		
95	0.199192	3.727762	0.361945	0.623274	0.192086	0.40768		
run 23								
0	0.070712	1.588081	5.3E-06	0.111148	0.041258	0.076203	0.048144	2.051612
5	0.070878	1.617696	0.057826	0.107854	0.041619	0.074736		
10	0.071843	1.670122	0.115854	0.100368	0.041548	0.070958		
15	0.073035	1.748795	0.176397	0.096637	0.041055	0.068846		
20	0.074557	1.859534	0.239395	0.08923	0.041977	0.065603		
25	0.07596	2.012947	0.304702	0.087903	0.045624	0.066764		
30	0.077239	2.225158	0.372294	0.080915	0.051483	0.066199	0.03845	1.598059
35	0.078478	2.526356	0.442253	0.077407	0.059871	0.068639		
40	0.080048	2.946014	0.514988	0.074435	0.070517	0.072476		
45	0.081834	3.658861	0.590459	0.062123	0.085055	0.073589		
50	0.083577	4.868524	0.670525	0.050629	0.101725	0.076177		
55	0.086021	2.268502	0.940377	0.04808	0.04465	0.046365		
60	0.088703	2.558246	1.063051	0.037191	0.051272	0.044231		
65	0.092064	212.5238	0.9518	0.036101	0.158019	0.09706		
70	0.095742	761.1502	1.101074	0.034767	0.159058	0.096912		
run 24								
0	0.053272	3.561999	0.000101	0.506995	0.052024	0.279509	0.070158	1.193815
5	0.054016	3.622213	0.015766	0.492276	0.05238	0.272328		
10	0.054862	3.696761	0.032314	0.474346	0.052578	0.263462		
15	0.055796	3.787317	0.049855	0.469589	0.052701	0.261145		
20	0.056365	3.783879	0.069096	0.454975	0.052867	0.253921		
25	0.057178	4.027164	0.098927	0.427796	0.054351	0.241073		
30	0.058426	4.177818	0.121345	0.412442	0.055237	0.233839		
35	0.059626	4.353185	0.144795	0.390947	0.056883	0.223915		

% PPO decay	k_1	k_2	k_3	se	ce	ae	Maximum catechol	Time
40	0.060647	4.086799	0.162544	0.385997	0.054239	0.220118		
45	0.062058	4.187541	0.192083	0.363706	0.054576	0.209141		
50	0.063673	4.303656	0.225156	0.345291	0.054948	0.20012		
55	0.065542	4.439146	0.262528	0.342513	0.055158	0.198836		
60	0.067725	4.598219	0.305115	0.299135	0.056172	0.177654		
65	0.070287	4.784746	0.353881	0.29483	0.056537	0.175684		
70	0.073291	5.008008	0.409625	0.263761	0.057584	0.160673		
75	0.076813	5.269948	0.473353	0.258228	0.058479	0.158353		
80	0.081003	5.577317	0.547324	0.249428	0.059215	0.154321	0.068228	0.771829
85	0.086437	5.99073	0.640586	0.256601	0.06026	0.158431		
90	0.094725	6.595302	0.779356	0.288478	0.061854	0.175166		
95	0.111319	7.790105	1.04918	0.364606	0.063716	0.214161		
run 25								
0	0.065103	19.11179	0.001649	0.10219	0.00341	0.0528	0.004176	0.298339
5	0.065984	20.94678	0.049955	0.097765	0.003524	0.050645		
10	0.066935	31.09163	0.100192	0.093673	0.005608	0.049641	0.002656	0.199353
15	0.067969	95.60164	0.152519	0.090012	0.010169	0.05009		
20	0.069089	1608.47	0.208262	0.085106	0.012424	0.048765		
run26								
0	0.032421	20.02432	1.37E-05	0.16325	0.006718	0.084984	0.008012	0.322199
5	0.032959	21.23993	0.02491	0.158298	0.006772	0.082535		
10	0.033427	22.43337	0.04886	0.149951	0.00696	0.078456		
15	0.033931	26.4922	0.074072	0.146566	0.008441	0.077503		
20	0.034473	32.84319	0.100749	0.139604	0.011028	0.075316		
25	0.03506	46.17025	0.12903	0.135194	0.014897	0.075045	0.003776	0.1542
30	0.035698	92.67661	0.159059	0.129964	0.020291	0.075127		
35	0.036393	624.3413	0.191349	0.126624	0.025166	0.075895		
run 30								
0	0.066149	17.11702	7.43E-07	0.783366	0.038334	0.41085	0.01891	0.316937
5	0.067212	17.54905	0.011868	0.771749	0.038349	0.405049		
10	0.068295	18.13264	0.024579	0.750375	0.038383	0.394379		
15	0.069596	19.17032	0.037797	0.744241	0.038578	0.39141		
20	0.070999	20.39327	0.051808	0.727704	0.038906	0.383305		
25	0.072437	21.80718	0.066789	0.711217	0.039365	0.375291		
30	0.073996	23.84609	0.082805	0.702284	0.040239	0.371262		
35	0.075755	26.81996	0.114576	0.676806	0.041523	0.359165		
40	0.077547	31.38592	0.134127	0.652719	0.043762	0.348241		
45	0.07947	36.98108	0.155535	0.634677	0.046165	0.340421		
50	0.081631	43.25493	0.179171	0.618163	0.048356	0.33326		
55	0.084096	55.64862	0.205404	0.597367	0.051807	0.324587		
60	0.086929	66.57139	0.235207	0.576244	0.053816	0.31503		
65	0.090259	107.1227	0.268938	0.559852	0.05863	0.309241		
70	0.094196	220.3052	0.30839	0.539032	0.063197	0.301114		
75	0.099076	710.4017	0.355628	0.520066	0.066481	0.293273	0.000697	0.01277
run 31								
0	0.287369	7.298013	-5.8E-06	0.125231	0.050979	0.088105	0.043105	0.471384
5	0.29016	7.389014	0.015595	0.124965	0.050994	0.087979		
10	0.29314	7.498463	0.032037	0.136355	0.051149	0.093752		
15	0.296368	7.787443	0.049399	0.152862	0.051446	0.102154		
20	0.300144	8.165964	0.06606	0.149186	0.051617	0.100401		

% PPO decay	k_1	k_2	k_3	se	ce	ae	Maximum catechol	Time
25	0.304062	8.626303	0.085537	0.165396	0.052235	0.108816		
30	0.308376	9.313323	0.106329	0.180096	0.053596	0.116846		
35	0.313122	10.15439	0.128654	0.181762	0.055028	0.118395		
40	0.318336	10.93394	0.152889	0.204477	0.056659	0.130568		
45	0.324366	12.86238	0.178693	0.214918	0.060661	0.137789		
50	0.330836	14.87277	0.207423	0.226287	0.064193	0.14524		
55	0.338358	18.89176	0.238813	0.246325	0.070273	0.158299		
60	0.346969	25.59398	0.273864	0.263627	0.07683	0.170228		
65	0.356937	36.1227	0.313836	0.284232	0.082727	0.183479		
70	0.368982	118.4795	0.35834	0.308496	0.094013	0.201255		
run 32								
0	0.088807	4.666388	1.47E-05	0.190782	0.104898	0.14784	0.088118	0.866639
5	0.089906	4.735116	0.009752	0.184499	0.104858	0.144678		
10	0.091076	4.814191	0.020085	0.172633	0.105144	0.138888		
15	0.092323	4.907055	0.031002	0.147047	0.105407	0.126227	0.087173	0.826321
20	0.093646	5.014711	0.042381	0.174371	0.105534	0.139953		
25	0.095016	5.139574	0.054021	0.1648	0.106499	0.13565		
30	0.096489	5.283304	0.06637	0.159985	0.107134	0.13356		
35	0.098082	5.449568	0.079537	0.19304	0.107427	0.150234		
40	0.099819	5.63858	0.093647	0.205	0.107974	0.156487		
45	0.101724	5.838797	0.108847	0.235439	0.109638	0.172539		
50	0.103837	6.081078	0.125318	0.254472	0.110579	0.182525		
55	0.10617	6.365813	0.143298	0.26513	0.112222	0.188676		
60	0.108794	6.690126	0.163037	0.315642	0.114239	0.214941		
65	0.111757	7.072955	0.184931	0.348722	0.116233	0.232478		
70	0.115463	6.295701	0.218022	0.395849	0.110557	0.253203		
75	0.119952	6.559266	0.251713	0.477024	0.111428	0.294226		
80	0.125567	6.888028	0.293089	0.521158	0.111971	0.316565		
85	0.132913	7.319994	0.346152	0.610846	0.112942	0.361894		
90	0.1431	7.900825	0.41873	0.716835	0.11386	0.415347		
95	0.15978	8.845001	0.535303	0.856888	0.114713	0.485801		
run 42								
0	0.21755	0.958712	-2.2E-05	0.134737	0.034696	0.084717	0.183426	2.077128
5	0.220565	0.974015	0.024894	0.148968	0.033473	0.09122		
10	0.22084	0.981377	0.048671	0.155382	0.034619	0.095001		
15	0.223121	0.996235	0.07661	0.162632	0.035492	0.099062		
20	0.22562	1.013598	0.10635	0.170388	0.036618	0.103503		
25	0.228366	1.033658	0.138103	0.18517	0.047308	0.116239		
30	0.231392	1.056656	0.17214	0.19381	0.048735	0.121273		
35	0.234738	1.082872	0.208808	0.203547	0.050646	0.127096		
40	0.238456	1.112663	0.248554	0.223197	0.052716	0.137957		
45	0.242615	1.146469	0.291967	0.230828	0.055503	0.143165		
50	0.2473	1.184809	0.339834	0.253462	0.061644	0.157553		
55	0.252634	1.228419	0.393222	0.262462	0.065247	0.163854		
60	0.258855	1.278091	0.453636	0.282062	0.070814	0.176438		
65	0.26606	1.335391	0.523219	0.306803	0.076481	0.191642		
70	0.274599	1.40349	0.605222	0.320658	0.082787	0.201723		
75	0.285183	1.482907	0.704818	0.357372	0.094921	0.226146		
80	0.298654	1.578091	0.830845	0.387411	0.100993	0.244202		
85	0.316935	1.700206	0.999174	0.435601	0.107316	0.271459		

% PPO decay	k_1	k_2	k_3	se	ce	ae	Maximum catechol	Time
90	0.343698	1.869698	1.245071	0.494853	0.118201	0.306527		
95	0.391213	2.158764	1.678387	0.56346	0.128145	0.345802		
run 43								
0	0.03344	31.87123	0.000758	0.054454	0.005185	0.02982	0.001302	0.220802
5	0.033898	68.58875	0.088284	0.052587	0.005512	0.029049		
10	0.034395	649.8863	0.179865	0.050735	0.006313	0.028524	6.71E-05	0.02269
run 51								
0	0.221884	13.31092	3.51E-05	0.640016	0.038788	0.339402	0.019438	0.322653
5	0.227712	13.77888	0.018	0.6315	0.038923	0.335212		
10	0.235141	14.49558	0.036508	0.623722	0.039209	0.331466		
15	0.243124	15.73603	0.05561	0.610762	0.039652	0.325207		
20	0.250571	17.37585	0.075637	0.609128	0.040351	0.324739		
25	0.256664	19.96338	0.106824	0.595179	0.041325	0.318252		
30	0.261098	24.02088	0.130878	0.582192	0.043006	0.312599		
35	0.266029	29.12361	0.156991	0.574197	0.044854	0.309525		
40	0.271536	44.94856	0.18501	0.56454	0.048808	0.306674		
45	0.27773	81.6524	0.215836	0.552132	0.052728	0.30243		
50	0.284747	428.0923	0.249697	0.5378	0.057174	0.297487	0.000827	0.016137
run 52								
0	0.10629	4.695036	2.01E-06	1.60164	0.144494	0.873067	0.103686	0.833758
5	0.107889	4.765338	0.008328	1.563378	0.143868	0.853623		
10	0.109807	4.846095	0.01708	1.550046	0.144165	0.847105		
15	0.111779	4.933901	0.026333	1.53381	0.14536	0.839585		
20	0.113868	5.029337	0.036163	1.505399	0.145246	0.825322		
25	0.116143	5.133581	0.046646	1.464082	0.144529	0.804306		
30	0.118581	5.247165	0.057913	1.451675	0.14464	0.798157		
35	0.121241	5.370402	0.070104	1.415261	0.145478	0.780369		
40	0.124191	5.508366	0.08339	1.370181	0.144929	0.757555		
45	0.127489	5.66346	0.098017	1.35791	0.144912	0.751411		
50	0.13123	5.840918	0.114298	1.312383	0.144544	0.728463		
55	0.135531	6.045677	0.132716	1.27097	0.144783	0.707876		
60	0.140574	6.286343	0.15396	1.202737	0.144243	0.67349		
65	0.146662	6.580369	0.17903	1.173023	0.144417	0.65872		
70	0.177773	8.718142	0.2944	0.95139	0.145689	0.54854		
75	0.190329	9.332031	0.342263	0.880363	0.144512	0.512438		
80	0.205378	10.00136	0.399191	0.807089	0.142756	0.474922		
85	0.224374	10.84861	0.469393	0.753636	0.140894	0.447265		
90	0.249399	12.02024	0.559698	0.645376	0.138162	0.391769		
95	0.288329	13.72649	0.697736	0.588284	0.134673	0.361479	0.096674	0.290096
run 55								
0	0.124853	26.43042	2.53E-05	0.519307	0.00995	0.264628	0.005757	0.205905
5	0.133177	28.59234	0.033719	0.50936	0.010101	0.259731		
10	0.135142	32.74698	0.062041	0.504134	0.010373	0.257253		
15	0.137278	48.68473	0.091799	0.497149	0.011965	0.254557		
20	0.139614	123.3466	0.12318	0.49045	0.015295	0.252873		
25	0.142152	649.9302	0.156766	0.48278	0.017431	0.250106	0.000273	0.020678
run 56								
0	0.076339	16.97097	2.18E-06	1.448439	0.036506	0.742473	0.021948	0.311793
5	0.078171	17.48919	0.010378	1.426201	0.036566	0.731384		
10	0.080475	18.38748	0.021087	1.420102	0.036883	0.728493		

% PPO decay	k_1	k_2	k_3	se	ce	ae	Maximum catechol	Time
15	0.082073	19.06521	0.032482	1.3966	0.037028	0.716814		
20	0.083739	20.1785	0.045998	1.377065	0.037447	0.707256		
25	0.08775	22.05976	0.090746	1.30272	0.037825	0.670272		
30	0.089801	23.02488	0.107758	1.288545	0.038135	0.66334		
35	0.092106	25.25808	0.126301	1.260735	0.039384	0.650059		
40	0.094707	27.47379	0.146824	1.228618	0.040555	0.634587		
45	0.097686	31.10096	0.169645	1.193817	0.042649	0.618233		
50	0.101085	34.42356	0.19551	1.160184	0.044216	0.6022		
55	0.105202	38.49503	0.225337	1.115097	0.045882	0.580489		
60	0.110071	42.2954	0.260167	1.074733	0.047002	0.560867		
65	0.116037	45.87114	0.30157	1.018671	0.047631	0.533151		
70	0.123515	50.94776	0.352239	0.958759	0.04857	0.503664		
75	0.13309	55.8309	0.415066	0.897491	0.048909	0.4732		
80	0.145682	65.21808	0.494757	0.820539	0.050302	0.435421		
85	0.162492	63.46563	0.599652	0.73264	0.047065	0.389853		
90	0.186019	60.76864	0.743422	0.635798	0.042406	0.339102		
95	0.223743	63.06601	0.968556	0.528837	0.038206	0.283521	0.017386	0.087361

REFERENCES

Abel, P.U., von Woedtke, T., Schulz, B., Bergann, T., Schwock, A. 1999. Stability of immobilized enzymes as biosensors for continuous application *in vitro* and *in vivo*. J. Mol. Catal. B: Enzym. **7**: 93-100.

Aitken, M.D., Massey, I.J., Chen, T., Heck, P.E. 1994. Characterization of reaction products from the enzyme catalyzed oxidation of phenolic pollutants. Wat. Res. **28(9)**: 1879-1889.

Akyilmaz, E., Dinçkaya, E. 1999. A new enzyme electrode based on ascorbate oxidase immobilized in gelatin for specific determination of L-ascorbic acid. Talanta **50**: 87-93.

Alejandre, A., Medina, F., Fortuny, A., Salagre, P., Sueiras, J.E. 1998a. Characterization of copper catalysts and activity for the oxidation of phenol aqueous solutions. Appl. Catal. B: Environmental **16**: 53-67.

Alejandre, A., Medina, F., Salagre, P., Fabregat, A., Sueiras, J.E. 1998b. Characterization and activity of copper and nickel catalysts for the oxidation of phenol aqueous solutions. Appl. Catal. B: Environmental **18**: 307-315.

Amen-Chen, C., Pakdel, H., Roy, C. 1997. Separation of phenols from *Eucalyptus* wood tar. Biomass and Bioenergy **13(1-2)**: 25-37.

Andersen, S.O., Jacobsen, J.P., Bojesen, G., Roepstorff, P. 1992. Phenoloxidase catalyzed coupling of catechols. Identification of novel coupling products. Biochim. Biophys. Acta **1118**: 134-138.

Angelton, E.L., Flurkey, W.H. 1984. Activation and alteration of plant and fungal polyphenoloxidase isoenzymes in sodium dodecylsulfate electrophoresis. Phytochemistry. **23(12)**: 2723-2725.

Annachhatre, A.P., Gheewala,S.H. 1996. Biodegradation of chlorinated phenolic compounds. Biotechnology Advances. **14(1)**: 35-56.

Arana, G., Borge, G., Etxebarria, N., Fernández, L.A. 1999. Permeation of mixtures of four phenols through a supported liquid membrane in NaCl 1.0 mol.dm^{-3} medium. Sep. Sci. Technol. **34(4)**: 665-681.

Asimov, I., Dawson, C.R. 1950. On the reaction inactivation of tyrosinase during the aerobic oxidation of catechol. J. Am. Chem Soc. **72:** 820-828.

Atlow, S.C., Bonadonna-Aparo, L., Klibanov, A.M. 1984. Dephenolisation of industrial wastewaters catalyzed by polyphenol oxidase. Biotechnol. Bioeng. **26**: 599-603.

Bahulekar, R., Ayyangar, N.R., Ponrathnam, S. 1991. Polyethyleneimine in immobilization of biocatalysts. Enzyme Microb. Technol. **13**: 858-868.

Barnes, H.H., Ishimaru, C.A. 1999. Purification of catechol siderophores by boronate affinity chromatography: identification of chrysobactin from *Erwinia carotovora* subsp. *carotovora.* BioMetals. **12**: 83-87.

Bell, G., Halling, P.J., Moore, B.D., Partridge, J., Rees, G.D. 1995. Biocatalyst behaviour in low-water systems. TIBTECH. **13**: 468-473.

Besombes, J.-L., Cosnier, S., Labbé, P. 1996. Polyphenol oxidase-catechol: an electroenzymatic model system for characterizing the performance of matrices for biosensors. Talanta. **43**: 1615-1619.

Besombes, J.-L., Cosnier, S., Labbé, P., Reverdy, G. 1995. Determination of phenol and chlorinated phenolic compounds based on a PPO-bioelectrode and its inhibition. Anal. Lett. **28(3):** 405-424.

Blasco, R., Wittich, R.-M., Mallavarapu, M., Timmis, K.M., Pieper, D.H. 1995. From xenobiotic to antibiotic, formation of protoanemonin from 4-chlorocatechol by enzymes of the 3-oxoadipate pathway. J. Biol. Chem. **270(49)**: 29229-29235.

Bogan, B.W., Lamar, R.T. 1996. Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. Appl. Environ. Microbiol. **62(5)**: 1597-1603.

Boshoff, A., Burton, M.H., Burton, S.G. 2001. Optimisation of catechol production by membrane-immobilised polyphenol oxidase - a modelling approach. (Submitted to Biotechnol. Bioeng., December 2000).

Bouchilloux, S., McMahill, P., Mason, H.S. 1963. The multiple forms of mushroom tyrosinase. Purification and molecular properties of the enzymes. J. Biol. Chem. **238(5)**: 1699-1707.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

Brindle, K., Stephenson, T. 1996. The application of membrane biological reactors for the treatment of wastewaters. Biotechnol. Bioeng. **49(6)**: 601-610.

Brookes, P.R. Livingston, A.G. 1994. Biotreatment of a point-source industrial wastewater arising in 3,4-dichloroaniline manufacture using an extractive membrane bioreactor. Biotechnol. Prog. **10**: 65-75.

Bryjak, J., Bryjak, M., Noworyta, A. 1996. Membrane reactor with soluble forms of penicillin acylase. Enzyme Microb. Technol. **19**: 196-201.

Buchanan, I.D., Nicell, J.A. 1997. Model development for horseradish peroxidase catalyzed removal of aqueous phenol. Biotechnol. Bioeng. **54(3)**: 251-261.

Buchanan, I.D., Nicell, J.A. 1998. Kinetics of peroxidase interactions in the presence of a protective additive. J. Chem. Technol. Biotechnol. **72**: 23-33.

Bull, A.T., Bunch, A.W., Robinson, G.K. 1999. Biocatalysts for clean industrial products and processes. Curr. Opin. Microbiol. **2**: 246-251.

Burton, S.G. 1993. Biocatalytic and biomimetic studies on polyphenol oxidase. Ph.D. thesis, Rhodes University.

Burton, S.G. 1994. Biocatalysis with polyphenol oxidase: a review. Catalysis Today. **22**: 459-487.

Burton, S.G., Boshoff, A., Edwards, Jacobs, E.P., Leukes, W.D., Rose, P.D., Russell, A.K., Russell, I.M., Ryan, D. 1998. Membrane-based biotechnological systems for treatment of organic pollutants. Water Research Commission, Report No. 687/1/98, South Africa.

Burton, S.G., Duncan, J.R. 1995a. Analysis of enzyme kinetic measurements for an organic-medium biocatalyst. Biotechnol. Tech. **9(1)**: 7-12.

Burton, S.G., Duncan, J.R. 1995b. Activation of mushroom polyphenol oxidase in organic medium by the detergent SDS. Biotechnol. Lett. **17(6)**: 627-630.

Burton, S.G., Duncan, J.R., Kaye, P.T., Rose, P.D. 1993. Activity of mushroom polyphenol oxidase in organic medium. Biotechnol. Bioeng. **42(8)**: 938-944.

Byrne, J.A., Eggins, B.R., Brown, N.M.D., McKinney, B., Rouse, M. 1998. Immobilisation of TiO_2 powder for the treatment of polluted water. Appl. Catal. B: Environmental. **17**: 25-36.

Cabanes, J., Sánchez-Ferrer, A., Bru, R., Garcíá-Carmona, F. 1988. Chemical and enzymic oxidation by tyrosinase of 3,4-dihydroxymandelate. Biochem. J. **256**: 681-684.

Campanella, L., Beone, T., Sammartino, M.P., Tomassetti, M. 1993. Determination of phenol in wastes and water using an enzyme sensor. Analyst. **118**: 979-986.

Campanella, L., Favero, G., Pastorino, M., Tomassetti, M. 1999. Monitoring the rancidification process in olive oils using a biosensor operating in organic solvents. Biosens. Bioelectron. **14**: 179-186.

Casella, L., Gullotti, M., Radaelli, R., Di Gennaro, P. 1991. A tyrosinase model system. Phenol *ortho*-hydroxylation by a binuclear three-coordinate copper(I) complex and dioxygen. J. Chem. Soc. Chem. Commun. 1611-1612.

Chae, H.J., Yoo, Y.J. 1997. Mathematical modelling and simulation of catechol production from benzoate using resting cells of *Pseudomonas putida.* Process Biochem. **32(5)**: 423-432.

Cheetham, P.S.J. 1998. What makes a good biocatalyst? J. Biotechnol. **66**: 3-10.

Chen, H-Y., Yu, A-M., Xu, D-K. 1997. Development of an amperometric detector for the determination of phenolic compounds. J. Anal. Chem. **359**: 542-545.

Cheynier, V., Moutounet, M. 1992. Oxidative reactions of caffeic acid in model systems containing polyphenol oxidase. J. Agric. Food Chem. **40**: 2038-2044.

Chioccara, F., Di Gennaro, P., La Monica, G., Sebastiano, R., Rindone, B. 1991. Selective ortho-hydroxylation of phenols in copper(I) complexes. Tetrahedron **47(25)**: 4429-4434.

Clark, D.S. 1994. Can immobilization be exploited to modify enzyme activity? TIBTECH. **12**: 439-443.

Claus, H., Filip, Z. 1990. Enzymatic oxidation of some substituted phenols and aromatic amines and the behaviour of some phenoloxidases in the presence of soil related adsorbents, Wat. Sci. Tech. **22(6)**: 69-77.

Coche-Guérente, L., Desprez, V., Diard, J.-P., Labbé, P. 1999a. Amplification of amperometric biosensor responses by electrochemical substrate recycling Part I. Theoretical treatment of the catechol-polyphenol oxidase system. J. Electroanal. Chem. **470**: 53-60.

Coche-Guérente, L., Desprez, V., Labbé, P., Therias, S. 1999b. Amplification of amperometric biosensor responses by electrochemical substrate recycling Part II. Experimental study of the catechol-polyphenol oxidase system immobilized in a laponite clay matrix. J. Electroanal. Chem. **470**: 61-69.

Collins, L.D., Daugulis, A.J., 1997. Biodegradation of phenol at high initial concentrations in two-phase partitioning batch and fed-batch bioreactors. Biotechnol. Bioeng. **55(1)**: 155-162.

Conrad, J.S., Dawso, S.R., Hubbard, E.R., Meyers, T.E., Strothkamp, K.G. 1994. Inhibitor binding to the binuclear active site of tyrosinase: temperature, pH, and solvent deuterium isotope effects. Biochemistry. **33(19)**: 5739-5744.

Constantinides, A., Moustoufi, N. 1999. Numerical methods for chemical engineers with MATLAB applications. p. 502. Prentice Hall, New Jersey.

Cooper, V.A., Nicell, J.A. 1996. Removal of phenols from a foundry wastewater using horseradish peroxidase. Wat. Res. **30(4)**: 954-964.

Cosnier, S., Lepellec, A., Guidetti, B., Rico-Lattes, I. 1998. Enhancement of biosensor sensitivity in aqueous and organic solvents using a combination of poly(pyrrole-ammonium) and poly(pyrrole-lactobionamide) films as host matrices. J. Electroanal. Chem. **449**: 165-171.

Crecchio, C., Rugierro, R, and Pizzigallo, M.D.R. 1995. Polyphenoloxidases immobilized in organic gels: properties and applications in detoxification of aromatic compounds. Biotechnol. Bioeng. **48**: 585-591.

Crestini, C., Kovac, B., Giovannozzi-Sermanni, G. 1996. Production and isolation of chitosan by submerged and solid-state fermentation from *Lentinus edodes*. Biotechnol. Bioeng. **50**: 207-210.

Czapski, J. 1994. Substrate specificity and inhibitors of polyphenol oxidase in aspect of darkening of fresh and frozen mushrooms (*Agaricus bisporus* (Lange) Sing.). Acta Agrobot. **47(1)**: 103-110.

Daley, R.F., Daley, S.J. 1996. Organic chemistry: a preliminary first edition. p. 1142. In: J. Berns (ed.), Organic chemistry: a preliminary first edition. Times Mirror Higher Education Group, Inc., Dubuque.

Dai, P.-S.E., Petty, R.H., Ingram, C.W., Szostak, R. 1996. Metal substituted aluminophosphate molecular sieves as phenol hydroxylation catalysts. Appl. Catal. A **143**: 101-110.

Dantoni, P., Serrano, S.H.P., Brett, A.M.O., Gutz, I.G.R. 1998. Flow-injection determination of catechol with a new tyrosinase/DNA biosensor. Anal. Chim. Acta. **366**: 137-145.

Davis, S., Burns, R.G. 1990. Decolorization of phenolic effluents by soluble and immobilized phenol oxidases. Appl. Microbiol. Technol. **32:** 721-726.

Dawson, C.R., Tarpley, W.B. 1990. On the pathway of catechol-tyrosinase reaction. p. 937-949. In: Annals of the New York Academy of Sciences.

Desprez, V., Labbé, P. 1996. A kinetic model for the electroenzymatic processes involved in polyphenol-oxidase-based amperometric catechol sensors. J. Electroanal. Chem. **415**: 191-195.

Dhawale, S.W., Dhawale, S.S., Dean-Ross, D. 1992. Degradation of phenanthrene by *Phanerochaete chrysosporium* occurs under ligninolytic as well as non-ligninolytic conditions. Appl. Environ. Microbiol. **58(9)**: 3000-3006.

Díaz-García, M.E., Valencia-González, M.J. 1995. Enzyme catalysis in organic solvents: a promising field for optical biosensing. Talanta. **42**: 1763-1773.

Doddema, H.J. 1988. Site-specific hydroxylation of aromatic comounds by polyphenol oxidase in organic solvents and in water. Biotechnol. Bioeng. **32**: 716-718.

Duckworth, H.W., Coleman, J.E. 1970. Physicochemical and kinetic properties of mushroom tyrosinase. J. Biol. Chem. **245(7)**: 1613-1625.

Edwards, W., Bownes, R., Leukes, W.D., Jacobs, E.P., Sanderson, R., Rose, P.D., Burton, S.G. 1999a. A capillary membrane bioreactor using immobilized polyphenol oxidase for the removal of phenols from industrial effluents. Enzyme Microb. Technol. **24**: 209-217.

Edwards, W., Leukes, W.D., R., Rose, P.D., Burton, S.G. 1999b. Immobilization of polyphenol oxidase on chitosan-coated polysulphone capillary membranes for improved phenolic effluent bioremediation. Enzyme Microb. Technol. **25**: 769-773.

Emnéus, J., Gorton, L. 1993. Comparison between different inorganic supports for the immobilization of amyloglucosidases and α-amylase to be used in enzyme reactors in flow-injection systems. Anal. Chim. Acta. **276**: 303-318.

Espín, J.C., Jolivet, S., Overeem, A., Wichers, H.J. 1999. Agaritine from *Agaricus bisporus* is capable of preventing melanin formation. Phytochemistry. **50**: 555-563.

Espín, J.C., Morales, M., García-Ruiz, P.A., Tudela, J., García-Cánovas, F. 1997. Improvement of a continuous spectrophotometric method for determining the monophenolase and diphenolase activities of mushroom polyphenol oxidase. J. Agric. Food Chem. **45**: 1084-1090.

Estrada, P., Baroto, W., Castillon, M.P., Acebal, C., Arche, R. 1993. Temperature effects on polyphenol oxidase activity in organic solvents with low water content. J. Chem. Tech. Biotechnol. **56**: 59-65.

Fadnavis, N.W., Koteshwar, K. 1999. An unusual reversible sol-gel transition phenomenon in organogels and its application for enzyme immobilization in gelatin membranes. Biotechnol. Prog. **15**: 98-104.

Fava, F., Di Giola, D., Marchetti, L. 1993. Characterization of a pigment produced by *Pseudomonas fluorescens* during 3-chlorobenzoate co-metabolism. Chemosphere **27(5)**: 825-835.

Fayad, N., Marchal, L., Billaud, C., Nicolas, J. 1997. Comparison of β-cyclodextrin effect on polyphenol oxidation catalyzed by purified polyphenol oxidase from various sources. J. Agric. Food Chem. **45**: 2442-2446.

Fernández-Lafuente, R., Rodríguez, V., Mateo, C., Fernández-Lorente, G., Arminsen, P., Sabuquillo, P., Guisán, J.M. 1999. Stabilization of multimeric enzymes via immobilization and post-immobilization techniques. J. Mol. Catal. B: Enzym. **7**: 181-189.

Fernández-Romero, J.M., Luque de Castro, M.D., Valcárcel, M. 1993. Approaches to the development of spectrophotometric reaction-rate methods by use of immobilized enzymes in continuous-flow systems. Anal. Chim. Acta. **274**: 99-107.

Ferrari, R.P., Laurenti, E., Ghibaudi, E.M., Casella, L. 1997. Tyrosinase-catecholic substrates *in vitro* model: kinetic studies on the *o*-quinone/*o*-semiquinone radical formation. J. Inorg. Biochem. **68**: 61-69.

Forsyth, W.C.G., Quesnel, V.C. 1957. Intermediates in the enzymic oxidation of catechol. Biochim. Biophys. Acta. **25**: 155-160.

Forsyth, W.C.G., Quesnel, V.C., Roberts, J.B. 1960. Diphenylenedioxide-2,3-quinone: an intermediate in the enzymic oxidation of catechol. Biochim. Biophys. Acta. **37**: 322-326.

Frieden, E., Ottesen, M. 1959. A simplified method for the purification of mushroom polyphenol oxidase. Biochim. Biophys. Acta. **34**: 248-251.

Games, D.E., Foster, M.G., Meresz, O. 1984. Application of LC-MS to the analysis of water. Anal. Proc. **21**(**5**): 174-177.

García, I.G., Peña, P.R.J., Venceslada, J.L.B., Martín, A.M., Santos, M.A.M., Gómez, E.R. 2000. Removal of phenol compounds from olive mill wastewater using *Phanerochaete chrysosporium*, *Aspergillus niger*, *Aspergillus terreus* and *Geotrichum candidum*. Process Biochem. **35**: 751-758.

Gardner, A.R., Cadman, T.W. 1990. Product deactivation in recombinant *Streptomyces*. Biotechnol. Bioeng. **36**: 243-251.

Gauillard, F., Richard-Forget, F., Nicolas, J. 1993. New spectrophotometric assay for polyphenol oxidase activity. Anal. Biochem. **215:** 59-65.

Geißler, A., Schöler, H.F. 1994. Gas chromatographic determination of phenol, methylphenols, chlorophenols, nitrophenols and nitroquinones in water at 0.1 $\mu g.l^{-1}$. Wat. Res. **28**(**10**): 2047-2053.

Gekas, V.C. 1986. Artificial membranes as carriers for the immobilization of biocatalysts. Enzyme Microb. Technol. **8**: 450-460.

Ghioureliotis, M., Nicell, J.A. 1999. Assessment of soluble products of peroxidase-catalyzed polymerization of aqueous phenol. Enzyme Microb. Technol. **25**: 185-193.

Golan-Goldhirsh, A., Whitaker, J.R. 1984. Effect of ascorbic acid, sodium bisulfite and thiol compounds on mushroom polyphenol oxidase. J. Agric. Food Chem. **32**: 1003-1009.

Golan-Goldhirsh, A., Whitaker, J.R. 1985. K_{cat} inactivation of mushroom polyphenol oxidase. J. Mol. Catal. **32**: 141-147.

Greco, G., Toscanoa, G., Cioffi, M., Gianfreda, L., Sannino, F. 1999. Dephenolisation of olive mill waste-waters by olive husk. Wat. Res. **33**(**13**): 3046-3050.

Halder, J., Tamuli, P., Bhaduri, A.N. 1998. Isolation and characterization of polyphenol oxidase from Indian tea leaf (*Camellia sinensis*). J. Nutr. Biochem. **9**: 75-80.

Hasebe, Y., Yokobori, K., Fukasawa, K., Kogure, T., Uchiyama, S. 1997. Highly sensitive electrochemical determination of *Escherichia coli* density using tyrosinase-based chemically amplified biosensor. Anal. Chim. Acta. **357**: 51-54.

Hayashi, T., Ikada, Y. 1990. Protease immobilization onto polyacrolein microspheres. Biotechnol. Bioeng. **35**: 518-524.

Healey, D.F. Strothkamp, K.G. 1981. Inhibition of the catecholase and cresolase activity of mushroom tyrosinase by azide. Arch. Biochem. Biophys. **211**(**1**): 86-91.

Heath, C.A., Belfort, G. 1992. Synthetic membranes in biotechnology: realities and possibilities. p. 45-88. In: Advances in Biochemical Engineering Biotechnlogy, Vol. 47. Springer-Verlag, Berlin.

Held, M., Schmid, A., Kohler, H-P.E., Suske, W., Witholt, B., Wubbolts, M.G. 1999. An integrated process for the production of toxic catechols from toxic phenols based on a designer biocatalyst. Biotechnol. Bioeng. **62(6)**: 641-648.

Held, M., Suske, W., Schmid, A., Engesser, K.-H., Kohler, H.-P.E., Witholt, B., Wubbolts, M.G. 1998. Preparative scale production of 3-substituted catechols using a novel monooxygenase from *Pseudomonas azelaica* HBP 1. J. Mol. Catal. B: Enzym. **5**: 87-93.

Holland, H.L. 1998. Microbial transformations. Curr. Opin. Chem. Biol. **2**: 77-84.

Huh, J.-K., Song, D.-I., Jeon, Y.-W. 2000. Sorption of phenol and alkylphenols from aqueous solution onto organically modified montmorillonite and applications of dual-mode sorption model. Sep. Sci. Technol. **35(2)**: 243-259.

Ikehata,K., Nicell, J.A. 2000. Characterization of tyrosinase for the treatment of aqueous phenols. Biores. Technol. **74**: 191-199.

Jacobsohn, G.M., Iskandar, R., Jacobsohn, M.K. 1993. Activity of mushroom tyrosinase on catechol and on a catechol estrogen in an organic solvent. Biochim. Biophys. Acta. **1202**: 317-324.

Jiménez, M., Escribano-Cebrián, J., García-Carmona, F. 1998. Oxidation of the flavonol fisetin by polyphenol oxidase. Biochim. Biophys. Acta. **1425**: 534-542.

Jiménez, M., García-Carmona, F. 1993. *In vitro* activation of grape polyphenol oxidase by polyglucan type elicitors. Plant Physiol. Biochem. **31(4)**: 541-546.

Jiménez, M., García-Carmona, F. 1999a. Myricetin, an antioxidant flavonol, is a substrate of polyphenol oxidase. J. Sci. Food Agric. **79**: 1993-2000.

Jiménez, M., García-Carmona, F. 1999b. Oxidation of the flavonol quercetin by polyphenol oxidase. J. Agric. Food Chem. **47**: 56-60.

Jiménez-Atiénzar, M., Pedreño, M.A., García-Carmona, F. 1991. Activation of polyphenol oxidase by polyamines. Biochemistry International. **25(5)**: 861-868.

Jolivet, S., Arpin, N., Wichers, A., Pellon, G. 1998. *Agaricus bisporus* browning: a review. Mycol. Res. **102(12)**: 1459-1483.

Juang, R.-S., Shiau, J.-Y., Shao, H.-J. 1999. Effect of temperature on equilibrium adsorption of phenols onto nonionic polymeric resins. Sep. Sci. Technol. **34(9)**: 1819-1831.

Kahn, V. 1985. Effect of proteins, protein hydrolysates and amino acids on *o*-dihydroxyphenolase activity of polyphenol oxidase of mushroom, avocado and banana. J. Food Sci. **50**: 111-115.

Kakinuma, K., Nango, E., Kudo, F., Matsushima, Y., Eguchi, T. 2000. An expeditious chemo-enzymatic route from glucose to catechol by the use of 2-deoxy-*scyllo*-inisose synthase. Tetrahedron Lett. **41**: 1935-1938.

Kazandjian, R.Z., Klibanov, A.M. 1985. Regioselective oxidation of phenols catalyzed by polyphenol oxidase in chloroform. J. Am. Chem. Soc. **107**: 5448-5450.

Kermasha, S., Goetghebeur, M., Monfette, A., Metche, M., Rovel, B. 1993a. Inhibitory effects of cysteine and aromatic acids on tyrosinase activity. Phytochemistry. **34(2)**: 349-353.

Kermasha, S., Goetghebeur, M., Monfette, A., Metche, M., Rovel, B. 1993b. Studies on inhibition of mushroom polyphenol oxidase using chlorogenic acid as substrate. J. Agric. Food Chem. **41**: 526-531.

Khmelnitsky, Y.L., Rich, J.O. 1999. Biocatalysis in nonaqueous solvents. Curr. Opin. Chem. Biol. **3**: 47-53.

Klibanov, A.M. 1983. Immobilized enzymes and cells as practical catalysts. Science. **219:** 722-727.

Klibanov, A.M., Alberti, B.N., Morris, E.D., Felshin, L.M. 1980. Enzymatic removal of toxic phenols and anilines from waste waters. J. Appl. Biochem. **2**: 414-421.

Klibanov, A.M., Tu, T-M., Scott, K.P. 1983. Peroxidase-catalyzed removal of phenols from coal-conversion waste waters. Science. **221**: 259-260.

Kragl, U., Kruse, W., Hummel, W., Wandrey, C. 1996. Enzyme engineering aspects of biocatalysis: cofactor regeneration as example. Biotechnol. Bioeng. **52(2)**: 309-319.

Krol, E.S., Bolton, K.L. 1997. Oxidation of 4-alkylphenols and catechols by tyrosinase: *ortho*-substituents alter the mechanism of quinoid formation. Chemico-Biol. Interact. **104**: 11-27.

Kumar, G., Bristow, J.F., Smith, P.J., Payne, G.F. 2000. Enzymatic gelation of the natural polymer chitosan. Polymer. **41**: 2157-2168.

Lacki, K., Duvnjak, Z. 1997. Enzymatic transformation of sinapine using polyphenol oxidase from *Trametes versicolor*: Effect of pH and temperature and model development. The Chem. Eng. J. **65**: 27-36.

Lahiere, R.J., Goodboy, K.P. 1993. Ceramic membrane treatment of petrochemical wastewater. Environ. Progress. **12(2)**: 86-96.

Latkar, M., Chakrabarti, T. 1994. Resorcinol, catechol and hydroquinone biodegradation in mono and binary substrate matrices in upflow anaerobic fixed-film fixed-bed reactors. Wat. Res. **28**(**3**): 599-607.

Lee, S.-G., Hong, S.-P., Sung, M.-H. 1996. Removal and bioconversion of phenol in wastewater by a thermostable ß-tyrosinase. Enzyme Microb. Technol. **19**: 374-377.

LeJeune, K.E., Swers, J.S., Hetro, A.D., Donahey, G.P., Russell, A.J. 1999. Increasing the tolerance of organophosphorus hydrolase to bleach. Biotechnol. Bioeng. **64**(**2**): 250-254.

LeJeune, K.E., Russell, A.J., 1996. Covalent binding of nerve agent hydrolyzing enzyme within polyurethane foams. Biotechnol. Bioeng. **51**: 450-547.

Lenhart, J.L., Chaubal, M.V., Payne, G.F., Barbari, T.A. 1998. Enzymatic modification of chitosan by tyrosinase. A.C.S. Symp. Enzymes in polymer synthesis. **684**: 188-198.

Leukes, W.D., Burton, S.G., Rose, P.D., Jacobs, E., Sanderson, R.D. 1995. Method for continuous production of secondary metabolites using hollow fibre membranes. WRC patent. Provisional, No. 95/7366.

Li, J., Chia, L.S., Goh, N.K., Tan, S.N. 1998. Silica sol-gel immobilised amperometric biosensor for the determination of phenolic compounds. Anal. Chim. Acta. **362**: 203-211.

Lisdat, F., Wollenberger, U. 1998. Trienzyme amplification system for the detection of catechol and catecholamines using internal co-substrate regeneration. Anal. Lett. **31**(**8**): 1275-1285.

Liu, C., Ye, X., Wu, Y. 1997. A novel catalyst for clean production of diphenols - ferric trisacetylacetonate/MCM-41. J. Chem. Tech. Biotechnol. **70**: 384-390.

Livingston, A.G. 1993a. A novel membrane bioreactor for detoxifying industrial wastewater: I. Biodegradation of phenol in a synthetically concocted wastewater. Biotechnol. Bioeng. **41**(**10**): 915-926.

Livingston, A.G. 1993b. A novel membrane bioreactor for detoxifying industrial wastewater: 11. Biodegradation of 3-chloronitrobenzene in an industrially produced wastewater. Biotechnol. Bioeng. **41**(**10**): 927-936.

Long, T.J., Alben, J.O. 1968. Preliminary studies of mushroom tyrosinase (polyphenol oxidase). Mushroom Sci. **7**: 69-79.

Longa, S.D., Ascone, I., Bianconi, A., Bonfigli, A., Castellano, A.C., Zarivi, O., Miranda, M. 1996. The dinuclear copper site structure of *Agricus bisporus* tyrosinase in solution probed by X-ray absorption spectroscopy. J. Biol. Chem. **271**(**35**): 21025-21030.

Lye, G.J., Woodley, J.M. 1999. Application of *in situ* product-removal techniques to biocatalytic processes. TIBTECH. **17**: 395-402.

Mantzavinos, D., Hellenbrand, R., Metcalfe, I.S., Livingston, A.G. 1996. Partial wet oxidation of *p*-coumaric acid: oxidation intermediates, reaction pathways and applications for wastewater treatment. Wat. Res. **30(12)**: 2969-2976.

Martinez, M.V., Whitaker, J.R. 1995. The biochemistry and control of enzymatic browning. Trends in Food Science and Technol. **6**: 195-200.

Martirani, L., Giardina, P., Marzullo, L., Sannia, G. 1996. Reduction of phenol content and toxicity in olive mill wastes with the ligninolytic fungus *Pleurotus ostreatus*. Wat. Res. **30(8)**: 1914-1918.

Massey, I.J., Aitken, M.D., Ball, L.M., Heck, P.E. 1994. Mutagenicity screening of reaction products from the enzyme-catalyzed oxidation of phenolic pollutants. Environ. Toxicol. Chem. **13(11)**: 1743-1752.

Maumy, M., Capdevielle, P. 1996. Copper-catalyzed *ortho*-oxidation of phenols by dioxygen (tyrosinase mimics) do yield catechols as primary products. J. Mol. Catal. A: Chem. **113**: 159-166.

Messing, R.A. 1985. Immobilization techniques - enzymes. p. 191-201. In: Moo-Young, M. (ed.), Comprehensive Biotechnology - The principles, applications and regulations of biotechnology in industry, agriculture and medicine, vol. 2. Pergamon Press Ltd, Great Britain.

Moore, B.M., Flurkey, W.H. 1990. Sodium dodecyl sulfate activation of a plant polyphenoloxidase. J. Biol. Chem. **265(9)**: 4982-4988.

Morrison, M.E., Cohen, G. 1983. Novel substrates for tyrosinase serve as precursors of 6-hydroxydopamine and 6-hydroxy-dopa. Biochemistry. **22(24):** 5465-5467.

Mulder, M.H.V. 1996. Basic principles of membrane technology. 2nd edition. Kluwer Academic Publishers, Nordrecht.

Naish-Byfield, S., Cooksey, C.J., Riley, P.A. 1994. Oxidation of monohydric phenol substrates by tyrosinase: effect of dithiothreitol on kinetics. Biochem. J. **304**: 155-162.

Naish-Byfield, S., Riley, P.A. 1992. Oxidation of monohydric phenol substrates by tyrosinase. An oximetric study. Biochem. J. **288**: 63-67.

Nakamoto, S., Machida, N. 1992. Phenol removal from aqueous solutions by peroxidase-catalyzed reaction using additives. Wat. Res. **26(1)**: 49-54.

Negishi, O., Ozawa, T. 1997. Effect of polyphenol oxidase on deodorization. Biosci. Biotech. Biochem. **61(12)**: 2080-2084.

Nelson, R.M., Mason, H.S. 1970. Tyrosinase (mushroom). p. 626-633. In: Tabor, H., Tahoor, C.W., Methods in Enzymology, vol.17. Academic Press, New York

Neves, A., Rossi, L.M., Horn, A., Vencato, I., Bortoluzzi, A.J., Zucco, C., Mangrich, A.S. 1999. Synthesis, structure and properties of the first dinuclear copper(II) complex as a structural model for the phenolic intermediate in tyrosinase-cresolase activity. Inorg. Chem. Commun. **2**: 334-337.

Nicell, J.A., Bewtra, J.K., Biswas, N., Taylor, E. 1993. Reactor development for peroxidase catalysed polymerization and precipitation of phenols from wastewater. Wat. Res. **27(11)**: 1629-1639.

Nicell, J.A., Bewtra, J.K., Taylor, K.E., Biswas, N., St. Pierre, C. 1992. Enzyme catalyzed polymerization and precipitation of aromatic compounds from wastewaters. Wat. Sci. Tech. **25(3)**: 157-164.

Nistor, C., Emnéus, J. 1999. Bioanalytical tools for monitoring polar pollutants. Waste Management. **19**: 147-170.

Nistor, C., Emnéus, J., Gorton, L., Ciuca, A. 1999. Improved stability and altered selectivity of tyrosinase based graphite electrodes for detection of phenolic compounds. Anal. Chim. Acta. **387**: 309-326.

Osaki,S., 1963. The mechanism of tyrosinase oxidation by mushroom tyrosinase. Arch. Biochem. Biophys. **100**: 374-384.

Pal, S., Bollag, J-M., Huang, P.M. 1994. Role of abiotic and biotic catalysts in the transformation of phenolic compounds through oxidative coupling reactions. Soil Biol. Biochem. **26(7)**: 813-820.

Park, J-W., Dec, J., Kim, J-E., Bollag, J-M. 1999. Effect of humic constituents on the transformation of chlorinated phenols and anilines in the presence of oxidoreductive enzymes or birnessite. Environ. Sci. Technol. **33(12)**: 2028-2034.

Parker, W.J., Bell. J.P., Melcer, H. 1994. Modelling the fate of chlorinated phenols in wastewater treatment plants. Environ. Progress. **13(2)**: 98-104.

Passi, S., Nazzaro-Porro, M. 1981. Molecular basis of substrate and inhibitory specificity of tyrosinase: phenolic compounds. Brit. J. Dermatol. **104**: 659-665.

Patel, A.R., Sun, W-Q., Payne, G.F. 1994. Tyrosinase reaction/chitosan adsorption: potential for removing polymerization storage inhibitors. Ind. Eng. Chem. Res. **33**: 2168-2173.

Payne, G.F., Elabd, Y.A., Lenhart, J.L., Ramirez, D.M. 1996. Enzymatic modification of chitosan by tyrosinase. Polym. Mater. Sci. Eng. **74(36)**: 36.

Payne, G.F., Sun, W-Q., Sohrabi, A. 1992. Tyrosinase reaction/chitosan adsorption for selectively removing phenols from aqueous mixtures. Biotechnol. Bioeng. **40(9)**: 1011-1018.

Payne, G.F., Sun, W-Q. 1994. Tyrosinase reaction and subsequent chitosan adsorption for selective removal of a contaminant from a fermentation recycle stream. Appl. Environ. Microbiol. **60(2)**: 397-401.

Pérez, J., De la Rubia, T., Moreno, J., Martínez, J. 1992. Phenolic content and antibacterial activity of olive oil waste waters. Environ. Tox. Chem. **11**: 489-495.

Pialis, P., Hamann, M.C.J., Saville, B.A. 1996. L-Dopa production from tyrosinase immobilized on nylon 6,6. Biotechnol. Bioeng. **51**: 141-147.

Pialis, P., Saville, B.A. 1998. Production of L-Dopa from tyrosinase immobilized on nylon 6,6: enzyme stability and scaleup. Enzyme Microb. Technol. **22**: 261-268.

Pifferi, P.G., Baldassari, L. 1973. A spectrophotometric method for the determination of the catecholase activity of tyrosinase by Besthorn's hydrazone. Anal. Biochem. **52**: 325-335.

Pras, N., Hesselink, P.G.M., Tusscher, J., Malingré, T.M. 1989. Kinetic aspects of the bioconversion of L-tyrosine into L-DOPA by cells of *Mucana pruriens* L. entrapped in different matrices. Biotechnol. Bioeng. **34**: 214-222.

Prazeres, D.M.F., Cabral, J.M.S. 1994. Enzymatic membrane reactors and their applications. Enzyme. Microb. Technol. **16**: 738-748.

Prenosil, J.E., Hediger, T. 1988. Performance of membrane fixed bioreactors. 1: Membrane reactor systems and modelling. Biotechnol. Bioeng. **31**: 913-921.

Rebhun, M., Galil, N. 1994. Technological strategies for protecting and improving the biological treatment of wastewater from a petrochemical complex. Wat. Sci. Tech. **29(9)**: 133-141.

Rescigno, A., Sanjust, E., Montanari, L., Sollai, F., Soddu, G., Rinaldi, A.C., Oliva, S., Rinaldi, A. 1997. Detection of laccase, peroxidase, and polyphenol oxidase on a single polyacrylamide gel electrophoresis. Anal. Lett. **30(12)**: 2211-2220.

Ricquebourg, S.L., Robert-Da Silva, C.M.F., Rouch, C.C., Cadet, F.R. 1996. Theoretical support for a conformational change of polyphenol oxidase induced by metabisulfite. J. Agric. Food Chem. **44**: 3457-3460.

Robb, D.A. 1984. Tyrosinase. p. 208-240. In: Lontie, R. (ed.), Copper proteins and copper enzymes, vol II. CRC Press, Boca Raton.

Robb, D.A. 1995. Exploiting tyrosinase activity in aqueous and non-aqueous media. ACS symposium (preprint).

Robinson, A.V. 1979. A rapid column chromatographic method for the isolation of catechol-type siderophores. Anal. Biochem. **95**: 364-370.

Rodríguez-López, J.N., Escribano, J., García-Cánovas, F. 1994. A continuous spectrophotometric method for the determination of monophenolase activity of tyrosinase using 3-methyl-2-benzothiazolinone hydrazone. Anal. Biochem. **216**: 205-212.

Ros, J.R., Rodríguez-López, J.N., Espín, J.C., Varón, R., García-Cánovas, F. 1996. Oxymetric and spectrophotometric study of the ascorbate oxidase activity shown by frog epidermis tyrosinase. Int. J. Biochem. Cell Biol. **28(8)**: 917-923.

Ros, J.R., Rodríguez-López, J.N., García-Cánovas, F. 1993. Effect of ferrous ions on the monophenolase activity of tyrosinase. Biochim. Biophys. Acta. **1163**: 303-308.

Ros, J.R., Rodríguez-López, J.N., García-Cánovas, F. 1994. Tyrosinase: kinetic analysis of the transient phase and the steady state. Biochim. Biophys. Acta. **1204**: 33-42.

Ros, J.R., Rodríguez-López, J.N., Varon-Castellanos, R., García-Cánovas, F. 1995. Mushroom tyrosinase has an ascorbate oxidase activity. Biochem. Mol. Biol. Int. **36(2)**: 301-309.

Rothschild, N., Hadar, Y., Dosoretz, C. 1995. Lignolytic system formation by *Phanerochaete chrysosporium* in air. Appl. Environ. Microbiol. **61(5)**: 1833-1838.

Russell, I.M. 1998. The development of an immobilised-enzyme bioprobe for the detection of phenolic pollutants in water. M. Sc. thesis, Rhodes University.

Russell, I.M., Burton, S.G. 1999. Development and demonstration of an immobilised-polyphenol oxidase bioprobe for the detection of phenolic pollutants in water. Anal. Chim. Acta. **389**: 161-170.

Ruzgas, T., Emnéus, J., Gorton, L., Marko-Varga, G. 1995. The development of a peroxidase biosensor for monitoring phenol and related aromatic compounds. Anal. Chim. Acta. **311**: 245-253.

Sánchez-Ferrer, A., Rodríguez-López, J.N., García-Cánovas, F. 1995. Tyrosinase: a comprehensive review of its mechanism. Biochim. Biophys. Acta. **1247**: 1-11.

Sariaslani, F.S., Rosazza, J.P.N. 1984. Biocatalysis in natural products chemistry. Enzyme Microb. Technol. **6**: 242-253.

Sayre, L.M., Nadkarni, D.V. 1994. Direct conversion of phenols to *o*-quinones by copper(I) dioxygen. Questions regarding the monophenolase activity of tyrosinase mimics. J. Am. Chem. Soc. **116**: 3157-3158.

Scheper, T.H., Hilmer, J.M., Lammers, F., Müller, C., Reinecke, M. 1996. Biosensors in bioprocess monitoring. J. Chromatogr. A. **725**: 3-12.

Schmid, A., Kohler, H.-P.E., Engesser, K.-H. 1998. *E. coli* JM109 pHBP461, a recombinant biocatalyst for the regioselective monohydroxylation of *ortho*-substituted phenols to their corresponding 3-substituted catechols. J. Mol. Catal. B: Enzym. **5**: 311-316.

Schulze, B., Wubbolts, M.G. 1999. Biocatalysis for industrial production of fine chemicals. Curr. Opin. Biotechnol. **10**: 609-615.

Schved, F., Kahn, V. 1992. Synergism exerted by 4-methyl catechol, catechol and their respective quinones on the rate of *DL*-DOPA oxidation by mushroom tyrosinase. Pigment Cell Res. **5:** 41-48.

Scouten, W.H., Luong, J.H.T., Brown, R.S. 1995. Enzyme or protein immobilization techniques for applications in biosensor design. TIBTECH. **13**: 178-184.

Semple, K.T., Cain, R.B. 1995. Metabolism of phenols by *Ochromonas danica*. FEMS. **133**: 253-257.

Shao, L., Kumar, G., Lenhart, J.L., Smith, P.J., Payne, G.F. 1999. Enzymatic modification of the synthetic polymer polyhydroxystyrene. Enzyme Microb. Technol. **25**: 660-668.

Shuttleworth, K.L., Bollag, J.-M. 1986. Soluble and immobilized laccase as catalysts for the transformation of substituted phenols. Enzyme Microb. Technol. **8**: 171-177.

Siddique, M.H., St. Pierre, C.C., Biswas, N., Bewtra, J.K., Taylor, K.E. 1993. Immobilized enzyme catalyzed removal of 4-chlorophenol from aqueous solution. Wat. Res. **27(5)**: 883-890.

Sikkema, J., de Bont, J.A.M. 1991. Biocatalytic production of hydroxylated aromatic and alicyclic compounds: products derived from tetralin. Recl. Trav. Chim. Pays-Bas. **110**: 189-194.

Siuzdak, G. 1996. Mass spectrometry for biotechnology. p. 1-3. In: Siuzdak, G. (ed.), Mass spectrometry for biotechnology, Academic Press Inc., California.

Siuzdak, G., Lewis, J.K. 1998. Applications of mass spectrometry in combinatorial chemistry. Biotechnol. Bioeng. **61(2)**: 127-134.

Sizer, I.W. 1953. Oxidation of proteins by tyrosinase and peroxidase. Adv. Enzymol. **14**: 129-161.

Smit, M.H., Rechnitz, G.A. 1993. Toxin detection using a tyrosinase-coupled oxygen electrode. Anal. Chem. **65**: 380-385.

Spain, J.C., Zylstra, G.J., Blake, C.K., Gibson, D.T. 1989. Monohydroxylation of phenol and 2,5-dichlorophenol by toluene dioxygenase in *Pseudomonas putida* F1. Appl. Environ. Microbiol. **55(10)**: 2648-2652.

Still, W.C., Kahn, M., Mitra, A. 1978. Rapid chromatographic technique for preparative separations with moderate resolution. J. Org. Chem. **43(14)**: 2923-2925.

Streffer, K., Kaatz, H., Bauer, C.J., Makower, A., Schulmeister, T., Scheller, F.W., Peter, M.G., Wollenberger, U. 1998. Application of a sensitive catechol detector for determination of tyrosinase inhibitors. Anal. Chim. Acta **362:(1)**: 81-90.

Suguraman, M., Dali, H., Semensi, V. 1990. Formation of a stable quinone methide during tyrosinase-catalyzed oxidation of α-methyl DOPA methyl ester and its implication in melanin biosynthesis. Bioorg. Chem. **18**: 144-153.

Suguraman, M., Lipke, H. 1982. A procedure for the isolation and concentration of catechols by chromatography on dihydroxyboryl cellulose. Anal. Biochem. **121**: 251-256.

Suguraman, M., Nelson, E. 1998. Model sclerotization studies. 4. Generation of N-acetylmethionyl catechol adducts during tyrosinase-catalyzed oxidation of catechols in the presence of N-acetylmethionine. Archives Insect Biochem. Physiol. **38**: 44-52.

Sun, W-Q., Payne, G.F. 1996. Tyrosinase-containing chitosan gels: a combined catalyst and sorbent for selective phenol removal. Biotechnol. Bioeng. **51**: 79-86.

Sun, W-Q., Payne, G.F., Moas, M.S.G.L., Chu, J.H., Wallace, K.K. 1992. Tyrosinase reaction/chitosan adsorption for removing phenols from wastewater. Biotechnol. Prog. **8(3)**: 179-186.

Takeuchi, W., Takahashi, H., Kojima, M. 1996. Production of diphenols by polyphenol oxidase from mushroom and mung bean (*Vigna mungo*). Nippon Shokuhin Kagaku Kogaku Kaishi. **43(10)**: 875-879.

Tatsuma, T., Oyama, N. 1996. H_2O_2-generating peroxidase electrodes as reagentless cyanide sensors. Anal. Chem. **68(9)**: 1612-1615.

Toussaint, O., Lerch, K. 1987. Catalytic oxidation of 2-aminophenols and ortho-hydroxylation of aromatic amines by tyrosinase. Biochemistry. **26**: 8567-8571.

Tse, M. T., Kermasha, S., Ismail, A. 1997. Biocatalysis by tyrosinase in organic solvent media: a model system using catechin and vanillin as substrates. J. Mol. Catal. B: Enzym. **2**: 199-213.

Ulbricht, M., Papra, A. 1997. Polyacrylonitrile enzyme ultrafiltration membranes prepared by adsorption, cross-linking and covalent binding. Enzyme Microb. Technol. **20**: 61-68.

Van Gelder, C.W.G., Flurkey, W.H., Wichers, H.J. 1997. Sequence and structural features of plant and fungal tyrosinases. Phytochem. **45(7)**: 1309-1323.

Vazquez-Duhalt, R., Fedorak, P.M., Westlake, D.W.S. 1992. Role of enzyme hydrophobicity in biocatalysis in organic solvents. Enzyme Microb. Technol. **14**: 837-841.

Venkatadri, R., Tsai, S.P., Vukanic, N and Hein, L.B. 1992. Use of a biofilm membrane reactor for the production of lignin peroxidase and treatment of pentachlorophenol by *Phanerochaete chrysosporium*. Hazardous Waste and Hazardous Materials. **9(3):** 231-243

Wada, S., Ichikawa, H., Tatsumi, K. 1992. Removal of phenols with tyrosinase immobilized on magnetite. Wat. Sci. Tech. **26(9-11)**: 2057-2059.

Wada, S., Ichikawa, H., Tatsumi, K. 1993. Removal of phenols from wastewater by soluble and immobilized tyrosinase. Biotechnol. Bioeng. **42(7)**: 854-858.

Wada, S., Ichikawa, H., Tatsumi, K. 1995. Removal of phenols and aromatic amines from wastewater by a combination treatment with tyrosinase and a coagulant. Biotechnol. Bioeng. **45(4)**: 304-309.

Waite, J.H. 1976. Calculating extinction coefficients for enzymatically produced *o*-quinones. Anal. Biochem. **5**: 211-218.

Wang, J., Lin, Y. 1993. On-line organic-phase enzyme detector. Anal. Chim. Acta. **271**: 53-58.

Wang, J., Lin, Y., Chen, Q. 1993. Organic-phase biosensors based on the entrapment of enzymes within poly (ester-sulfonic acid) coatings. Electroanalysis. **5**: 23-28.

Wang, S-J., Loh, K-C. 1999. Modeling the role of metabolic intermediates in kinetics of phenol degradation. Enzyme Microb. Technol. **25**: 177-184.

Warhurst, A.M., Clarke, K.F., Hill, R.A., Holt, R.A., Fewson, C.A. 1994. Production of catechols and muconic acids from various aromatics by the styrene-degrader *Rhodococcus Rhodochrous* NCIMB 13259. Biotechnol. Lett. **16(5)**: 513-516.

Warrington, J.C., Saville, B.A. 1999. Tyrosinase inactivation in organic solvents. Biotechnol. Bioeng. **65(3)**: 325-333.

Weemaes, C.A., Ludikhuyze, L.R., Van den Broeck, I., Hendrickx, M.E. 1999. Influence of pH, benzoic acid, glutathione, EDTA, 4-hexylresorcinol, and sodium chloride on the pressure inactivation kinetics of mushroom polyphenol oxidase. J. Agric. Food Chem. **47**: 3526-3530.

Weith, H.L., Wiebers, J.L., Gilham, P.T. 1970. Synthesis of cellulose derivatives containing the dihydroxyboryl group and a study of their capacity to form specific complexes with sugars and nucleic acid components. Biochemistry. **9(22)**: 4396-4401.

Won, K.W., Prausnitz, J.M. 1975. Distribution of phenolic solutes between water and polar organic solvents. J. Chem. Thermodynamics. **7**: 661-670.

Wu, J., Taylor, K.E., Bewtra, J.K., Biswas, N. 1993. Optimization of the reaction conditions for enzymatic removal of phenol from wastewater in the presence of polyethylene glycol. Wat. Res. **27(12)**: 1701-1706.

Wu, Y., Taylor, K.E., Biswas, N., Bewtra, J.K. 1998. A model for the protective effect of additives on the activity of horseradish peroxidase in the removal of phenol. Enzyme Microb. Technol. **22**: 315-322.

Yamamoto, H., Kuno, S., Nagai, A., Nishida, A., Yamauchi, S., Ikeda, K. 1990. Insolubilizing and adhesive studies of water-soluble synthetic model proteins. Int. J. Biol. Macromol. **12**: 305-310.

Yang, F., Russell, A.J. 1995. A comparison of lipase-catalyzed ester hydrolysis in reverse micelles, organic solvents and biphasic systems. Biotechnol. Bioeng. **47(1)**: 60-70.

Yang, Z., Robb, D.A. 1993(a). Comparison of tyrosinase activity and stability in aqueous and nearly nonaqueous environments. Enzyme Microb. Technol. **15**: 1030-1036.

Yang, Z., Robb, D.A. 1993(b). Use of salt hydrates for controlling activity of tyrosinase in organic solvents. Biotechnol. Techniques. **7(1)**: 37-42.

Yang, Z., Robb, D.A. 1994. Partition coefficients of substrates and products and solvent selection for biocatalysis under nearly anhydrous conditions. Biotechnol. Bioeng. **43(5)**: 365-370.

Yasunobu, K.T., Norris, E.R. 1957. Mechanism of borate inhibition of diphenol oxidation by tyrosinase. J. Biol. Chem. **227:** 473-482.

Yoshikawa,M., Taguchi, Y., Arashidani, K., Kodama, Y. 1986. Determination of cresols in urine by high-performance liquid chromatography. J. Chromatogr. **362**: 425-429.

Yun, C.H., Prasad, R., Sirkar, K.E. 1992. Membrane solvent extraction removal of priority organic pollutants from aqueous waste streams. Ind. Eng. Chem. Res. **31**: 1709-1717.

Zaks, A., Russell, A.J. 1988. Enzymes in organic solvents: properties and applications. J. Biotechnol. **8**: 259-270.

Zamorani, A., Spettoli, P., Lante, A., Crapisi, A., Pasini, G. 1993. Immobilized laccase and tyrosinase: an approach for wine stabilization. Ital. J. Food Sci. **4**: 409-414.

Zawistowski, J., Biliaderis, C.G., Eskin, N.A.M. 1991. Polyphenol oxidase. p. 217-255. In: Robinson, D.S., Eskin, N.A.M. (ed.), Oxidative enzymes in Foods. Elsevier Science Publishers, England.

Zelder, O., Hauer, B. 2000. Environmentally directed mutations and their impact on industrial biotransformation and fermentation processes. Curr. Opin. Microbiol. **3**: 248-251.