BIOREACTOR SYSTEMS FOR THE CONVERSION OF ORGANIC COMPOUNDS IN INDUSTRIAL EFFLUENTS TO USEFUL PRODUCTS

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Report to the Water Research Commission

by

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EXECUTIVE SUMMARY

1. Background and motivation

This project aimed to develop systems in which the high activity and selectivity of biological catalysts could be used to remove pollutants from industrial residues, and at the same time, convert them into products which have some market value. The project was focussed on the application of oxidative biological reactions, catalysed by a selection of enzymes produced by a small range of organisms. These enzyme catalysts included laccases, peroxidases and polyphenol oxidases, three groups which catalyse different reactions, with varying substrate selectivities, and which produce a range of products. Thus, by utilising this group of biocatalysts, we had a broad scope for the oxidation processes which are accessible and could develop efficient systems for production of a group of related, and potentially high-value, compounds.

Specific industrial wastes were identified which contain high concentrations of the aromatic, phenolic and polyphenolic components best suited to the intended oxidative bioconversions, and these were analysed and characterised. These effluents, from petrochemical processing, pulp-and-paper processing, olive production and wine production, were used in the programme as authentic samples on which the biological activity of the selected biocatalysts could be tested.

The oxidation of aromatics by enzyme-catalysed reactions has potential for production of useful synthetic intermediates, and in particular, for activation of aromatic rings to produce more reactive intermediates. This would mean that compounds which are generally unreactive and therefore recalcitrant, could be converted to more reactive products which are more easily biodegradable or more useful for organic synthesis. The project included investigations into the biotransformation of a range of phenols and polyaromatic hydrocarbons using the various oxidative biocatalysts.

The selected enzymes are produced by particular organisms; for example, laccases are common products of certain groups of fungi, while peroxidases of different types are produced by many microorganisms and plants. We selected a narrow range of microorganisms and plants which can produce the enzymes required for the desired reactions, and investigated methodologies for optimal production of the enzymes by these organisms. The research included investigations of enzyme production and biofilm growth as well as pollutant degradation. The enzymes utilised in this study have the significant characteristic that they do not require expensive cofactors such as NAD, (in contrast with many other oxidase systems), giving us is an important advantage with respect to economic and process engineering considerations.

2. Research Objectives

The key objectives of the project were:

1. To develop biotransformation systems to facilitate the purification of effluents polluted with organic compounds
2. To convert these compounds into products which have economic value.

This required the following further objectives:

- Development of specific bioconversions, to convert the waste components and pollutants found in effluents to economically valuable products.
- Development of methods for isolation and characterisation of the chemical products of these bioremediation reactions.
- Utilisation of membrane bioreactors adapted to the specific application of such bioconversions for treatment of certain industrial effluents produced in South Africa.
- Expansion of the expertise and knowledge already developed in the group, in extending the capabilities of bioreactors supporting oxidases and biofilms.

- Contribution to the development of technologies which would make such bioconversions feasible for practical application by industries which produce organic-containing effluents.

Attention was focussed specifically on developing *oxidative* biotransformation systems, with the target groups of pollutants being phenolics, polyphenolics and related aromatic compounds.

Industrial wastes selected (see Appendix 1):

- Simple phenolics from the local petrochemical industry
- Processing effluent from pulp-and-paper production
- Plant-related phenolics from olive procession
- Polyphenolic-containing wastes from wine production

Biological systems selected:

- Fungal biofilms (*Trametes versicolor, Neurospora crassa*) producing groups of oxidising enzymes including laccase, peroxidases, polyphenol oxidase
- Bacterial whole cell biofilms (Pseudomonads)
- Bacterial oxidases (from thermophilic bacterial isolates and *Streptomyces* spp.)
- Fungal enzymes (from the above mentioned fungi and from mushroom)
- Plant peroxidases (from horseradish, sorghum)

Reactions targeted:

- Phenols to catechols
- Phenols to polyphenolics
- Phenols to carbonyl compounds
- Aromatics to aldehydes
- Polyaromatics / polyphenolics to smaller units

3. Summary of major results and conclusions

- Two fungal systems viz., *Trametes versicolor* and *Neurospora crassa*, have been shown to be highly productive in bioremediation of phenolic wastes.
- In membrane bioreactors, these fungal biofilms were found to be effective in the continuous removal of phenols such as phenol itself, and some related substituted phenols which are found in petro-chemical wastes.
- An investigation of the products of *T. versicolor* biotransformations was initiated to identify potential downstream products generated using whole cell cultures and enzyme extracts from them. Bioconversion reactions can be more readily characterised using isolated enzymes which, once they are removed from the cell, no longer form part of a complex metabolic process, and the reaction mixtures are therefore much simpler to analyse. The results indicate that the whole cell biomass utilised the organic components of real and authentic effluents as carbon sources, particularly when grown continuously in bioreactors. However, when the cells were removed from nutrient medium and exposed to solutions of substrates, products were demonstrated to be present and hence the isolation of these products can be achieved.
- We also investigated the conversion of a selection of organic substrates by enzyme extracts from *T. versicolor* and we report the preliminary results of this study. The substrates investigated were selected because they are known to be present in the effluents and their successful conversion by oxidation yielded the type of high-value compounds which are a target of the project.
- In the case of the enzyme polyphenol oxidase (PPO), common mushrooms are an inexpensive source, and we conducted a detailed study on the practical application of mushroom PPO, immobilised via a range of novel techniques, to develop an efficient process for conversion of simple phenols to catechols. Catechols are very useful as starting materials for many organic reactions, and cannot easily be synthesised by conventional methods. This study has shown that it is feasible to use immobilised PPO to synthesise catechols, and by mathematical modelling of the process, we have identified the optimal conditions for achieving this. This

modelling approach is also adaptable for optimisation of other biocatalytic reactions.

- There is a need to identify and develop new sources of enzymes, to expand the range of possible reactions and to optimise efficiency in the bioconversions. We investigated the use of two groups of microorganisms which previously have shown to have potential applications in oxidative reactions. A group of *Streptomyces* strains were selected for their thermotolerance and their ability to grow on high-lignin (polyphenolic) media, on the assumption that they would then utilise the lignin are a carbon source, thereby degrading it. The oxidative enzyme activities of the strains were characterised, and certain members of the group have been shown to produce high levels of peroxidase and PPO activity. It was a further objective of this project to improve the activities in the strains by genetic manipulation.
- Peroxidase enzymes are known to be highly effective in bioconversion of aromatic compounds, but few sources of the enzymes are economical and practical. Fungal peroxidases can be problematic to produce, whereas plant peroxidases are common and may be as effective in bioconversions. The most common plant peroxidase used generally is horseradish peroxidase (HRP). In this project, we have isolated a novel peroxidase from sorghum root, which is a cheap and readily available plant source. The biocatalytic activity of the sorghum enzyme has been demonstrated to be comparable to HRP. Our research has shown that it can be used on the type of substrates present in the effluents, and that this is a potentially useful alternative biocatalyst in the research programme.

4. Achievements

At the conclusion of this project, the most promising biocatalytic systems have been described and developed to the point where

- their potential for bioremediation has been characterised
- the production of chemical products of economic value have been demonstrated in reactors incorporating these biocatalysts and converting the components of the effluents.

Novel outcomes arising from the objectives of this work are as follows:

Specific bioconversions

Bioconversion systems have been developed for:

- Conversion of phenols and aromatics by *T. versicolor* laccase
- Conversion of phenols using PPO
- Conversion of phenolics in olive waste
- Isolation of phenol-converting thermophilic Actinomycetes
- Peroxidase conversions of phenolics.

- A specific bioconversion of the simple phenol residues produced by the petrochemical industry was developed, using polyphenol oxidase and yielding catechol products

- Specific bioconversion of ferulic acid from olive waste, using a bacterial isolate, produced the high value product vanillic acid

Methods for isolation and characterisation of the chemical products

- Efficient analytical methods were developed and are documented in the report

Utilisation of membrane bioreactors

- Membrane reactors have been demonstrated to be especially useful for immobilisation of *N. crassa* biofilms and for isolated oxidase enzyme, and alternative reactors were developed for application of *T. versicolor*

Expansion of the expertise and knowledge

- The project lead to the completion of 3 PhD degrees, 5 MSc degrees, and 3 Honours degrees. The contriubution to knowledge and expertise is demonstrated by the publication of 10 papers in international journals, presentation of 5 international papers and 6 international posters, and 16 posters at South African conferences.

Contribution to the development of technology

The project has demonstrated that bioconversions can be developed which are now available for practical application by industries producing organic-containing effluents. A substantial set of data has been generated to form the basis for further technology and process development. In particular, the technology can now be developed into processes suited to bioremediation of waters containing simple phenols or lignin-related polyphenolics. Approaches have been made to several South African business concerns where the technology may be piloted and applied.

5. Recommendations for further research and technology transfer

Extension of the technology

- There are several routes available for extension of the outcomes of this project through new collaborative initatives which are currently being developed. For instance, the Advanced Centre for Applied Microbiology (ARCAM) in the Western Cape will seek projects to develop from proof-of-concept to process and scale-up. In the present study, the systems utilising fungal whole-cell systems, and those using isolated laccase, peroxidase and polyphenol oxidase all offer such opportunities.
- In addition, the National Biotechnology Strategy which is being developed at present by DACST will offer mechanisms whereby facilities for establishment of Biotechnology business can be nurtured. This will provide a link between research and enterprise development which has been inaccessible in the past. Projects such as the present one can provide the scientific basis for building Biotechnology business, but the researchers are not usually well-equipped for, or skilled in, business development. Thus this new mechanism should be considered as a means of taking the research outputs to the market place.

Specific recommendations for further research and development

- While the membrane bioreactors have been shown to offer considerable potential in bioremediation processes, scaled-up application of this technology would require a source of less expensive membrane modules, available in quantity.
- To address the problem of a lack of suitable customised or multi-purpose bioreactors, the bioremediation systems using the bacterial strains from olive waste and the thermophilic Actinomycetes will be used as the biological agents for application in developing bioreactors in a new WRC project which now has preliminary approval.
- One modern approach to improvement of the bioconversion capacity of microorganisms is to use molecular techniques such as gene shuffling or directed evolution to increase the enzyme production and/or activity levels in the organism. Although this is a relatively new concept in modification of fungal systems, *N. crassa* is well-understood and has been genetically characterized. The genetic manipulation approach does offer an innovative opportunity for improvement of this organism for the development of a more efficient bioremediation system.
- The bacterial strains isolated from olive waste, and the thermophilic Actinomycetes isolated from compost also provide useful starting points for molecular modification and hence strain improvement. Modern molecular technology has become accepted as one of the most effective routes to efficient and cost-effective biocatalyst production. This line of research, focusing on these organisms, would lead to efficient bioremediation systems.

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"DEVELOPMENT OF BIOREACTOR SYSTEMS FOR THE CONVERSION OF ORGANIC COMPOUNDS IN INDUSTRIAL EFFLUENTS INTO USEFUL PRODUCTS"

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CHAPTER 1

1.1 INTRODUCTION

The presence of toxic organic compounds in industrial wastewaters represents an environmental hazard to the community and an expensive material waste to the producer. In South Africa, water resources are scarce and reclamation of water is an important but under-utilised means of conservation.

One choice in solving the effluent problem is bioremediation, and various aspects of this field are currently being researched in certain centres, including the Biotechnology Centre at Rhodes University. In a WRC-supported project (K5/687) entitled "Membrane-based biotechnological methods for treatment of organic pollutants, "we previously demonstrated the potential for application in bioremediation, of whole cell fungal bioreactor systems producing peroxidases, and isolated phenol oxidase enzyme bioreactors. Based on the outcome of this, the current project focussed on further applications for biotransformation systems in bioremediation, with a particular focus on enzymes which catalyse oxidative reactions. Thus, the aim of this project was to develop methods of applying the activity of oxidative enzymes in removing toxic pollutants from effluents, and converting them into useful products which can be used to offset the costs of water reclamation.

The field of Biotransformations is relatively new, and the development of bioremediation methods has been hindered by a lack of knowledge of possible reactions, their productivity and predictability, and a lack of reactor technology suited to biological agents. This project aimed to contribute knowledge to help solve these drawbacks. Success in this field would present a major advantage to industry and a breakthrough in the field of Environmental Biotechnology.

The main advantages of biotransformation methods over chemical synthetic methods are due to the mild and environmentally friendly conditions required, and the efficiency and specificity of biological catalysts. In particular, enzymes are often capable of catalysing reactions where unreactive molecules (such as benzene or xylene) can readily be converted to more reactive molecules (such as cyclohexene-diols) which also have chirality (optical activity). This selectivity (stereospecificity) is extremely important in areas such as the pharmaceutical and fine chemical industry, where different optical forms of molecules have very different biological effects, and FDA regulations require defined stereochemistry of products. Such specific conversions are not easily achieved by conventional chemical methods.

Industrial effluents present a problem for industry in South Africa, in that they must be disposed of or stored in ways which are not environmentally harmful, but many contain compounds which have the potential to be converted to valuable products. Several toxic industrial effluents which are produced locally exemplify this. (The examples given here have provided the focus of this project).

1. Coal conversion, petroleum and steel production processes produce phenolic and cresylic effluents which are highly toxic, but enzymes such as polyphenol oxidases could be used to polymerise the phenolics, producing polyhydroquinones or melanin-type polymers which have potential uses in plastics.
2. The paper-making industry generates enormous volumes of dark-coloured effluent rich in lignin-related polyphenolics as well as a high concentration of non-organic ions.
3. Various food-related industries generate aqueous streams containing high concentrations of organic, and in particular, polyphenolic, compounds eg., wine-making, olive production.

Project objectives

The primary objectives of the project were:

1. To develop biotransformation systems which would facilitate the purification of effluents polluted with organic (specifically phenolic) compounds.
2. To convert these compounds into products which have economic value.

This research required the following research activities:

1 Development of specific bioconversions to convert the waste components and pollutants found in selected effluents, to yield economically valuable products.

2 Development of methods for isolation and characterisation of the chemical products of these bioremediation reactions.

3 Utilisation of membrane bioreactors adapted for the specific application of such bioconversions for treatment of certain industrial effluents produced in South Africa.

4 Expansion of the expertise and knowledge already developed in the group, in extending the capabilities of bioreactors, particularly supporting polyphenol oxidases, laccases, peroxidases, or the biofulms producing these enzymes.

5 Contributing to the development of technologies which would make such bioconversions feasible for practical application by industries producing organic-containing effluents.

Focus of the project

The industrial wastes used in the project were selected on the basis of their being representative of typical phenolic-containing industrial residues. These were:

- Simple phenolics from petrochemical industry
- Processing effluent from pulp-and-paper production
- Plant-related phenolics from olive processing
- Polyphenolic wastes from wine production

The biological systems were selected for their capacity to produce oxidising enzymes including laccase, peroxidases, and polyphenol oxidase. Thus, the project utilised:

- fungal biofilms (*Trametes versicolor, Neurospora crassa*);
- fungal enzymes (from the above mentioned fungi and from mushroom);
- bacterial whole cell biofilms (Pseudomonads);
- bacterial peroxidases (from thermophilic *Bacillus* spp., effluent isolates and *Streptomyces* spp.); and
- plant peroxidases (horseradish, sorghum).

The biotransformations targeted were those which would produce economically valuable compounds form the pehnolics in the selected industrial residues, including:

- phenols to catechols;
- phenols to dimers;
- phenols to carbonyl compounds;
- aromatics to aldehyldes; and
- polyaromatics / polyphenolics to smaller molecular units.

The class of reactions chosen, viz., biological oxidations, arose largely from the expertise and knowledge base already established in the group. The oxidation of aromatics by enzyme-catalysed reactions has potential for production of useful synthetic intermediates, and in particular, for activation of aromatic rings to produce more reactive intermediates. This would mean that compounds which are generally unreactive and therefore recalcitrant, could be converted to more reactive products which are more readily biodegradable or useful for organic synthesis.

The enzymes utilised in this study have the significant and important characteristic that they do not require cofactors such as NAD, (in contrast with many other oxidase systems), giving us an important advantage with respect to economic and engineering considerations. We have investigated the biotransformation of a range of phenols, polyaromatic hydrocarbons and other aromatics using the various oxidative enzymes

listed above. The enzyme-catalysed reactions can be conducted in aqueous solution, organic / aqueous biphasic systems, or using enzymes immobilised in hydrogels or on synthetic membranes. Whole cell reactions can be conducted using biomass in liquid culture or biofilms immobilised on synthetic membranes in small continuously operated bioreactors.

The research programme and report structure

The research programme was conducted as a set of inter-related projects, and this report describes the outcomes of these, in the chapters which follow.

1.2 PROJECT BACKGROUND AND LITERATURE SURVEY

In the context of bioremediation, the challenge to biotechnology is to generate efficient, cost effective and environmentally safe bioremediation methods to replace existing technologies, and to provide unique solutions for the remediation of contaminated resources.

1.2.1 Phenolic pollutants

1.2.1.1 Simple phenols

Phenols are fairly ubiquitous as pollutants, appearing in wastewater streams from numerous industrial processes, including petroleum refining, petrochemical manufacture and coking and coal conversion industries, resins and plastics, dyes and other organic chemicals, textiles, timber, mining and dressing, and the pulp and paper industry (Atlow, 1984; Van Schie and Young, 1998; Klein and Lee, 1978). A South African process for conversion of coal to oil produces, in particular, two phenol-containing effluents, known as black product and cresylic effluent. These contain phenolics, mostly in the form of phenol and the cresols, to total concentrations of 20 – 25 mg/L.

The release into the environment of large quantities of phenolic wastewater has created considerable ecotoxicological problems with serious consequences for human health and for all living organisms (Alberti and Klibanov, 1981). Phenol itself is toxic to most microorganisms, even at low concentration. It can be inhibitory to the growth of even those species that have the metabolic capacity of using it as a carbon source, and it can be toxic or lethal to fish at concentrations as low as 5-25mg/L. It also contributes to off-flavours in drinking and food-processing waters. Although phenol is not found to be bioaccumulative, humans exposed to phenol at concentrations of 1300mg/L present symptoms of diarrhoea, mouth sores, dark urine and burning of the mouth (Annachhatre and Gheewala, 1996).

Numerous conventional methods for dephenolisation of industrial wastewaters exist and, although effective, suffer from serious drawbacks such as high cost, incompleteness of purification, formation of hazardous byproducts, and applicability to only a limited phenol concentration range (Atlow *et al*, 1984). It was not until recently that biological oxidation was considered for the treatment of phenolated waterwaters (Shishido and Toda, 1996). The biodegradability of aromatic compounds depends on the number, type and position of substituents on the aromatic ring. Easiest to oxidise therefore, is phenol itself, with only an -OH group in the *para* position. The relative order of biodegradability of other derivatives appears to be *ortho>meta>para*. Anomalies to this order have been found, and differences in the ability of microorganisms to degrade aromatic compounds could well depend on the environmental conditions under which they act (Annachhatre and Gheewala, 1996).

1.2.1.2 Pulp-and-paper phenolic waste

Chlorinated phenolic compounds are formed during the bleaching of pulp with chlorine. The present worldwide production of paper and board exceeds 300 million tons a year. As the pulp accounts for only 40- 45% of the original weight of the wood, these effluents have a very high organic load. Chlorophenols, which form part of the *absorbable organic*

halides (AOX), are present in bleaching effluents from concentrations from 0.1 ppm to 2.6 ppm. The chlorination process is usually followed by alkaline extraction resulting in large volumes dark, dilute pulp mill effluents containing a substantial AOX load primarily in the form of chlorophenols, chloroguaiacols, chloroaliphatics, chlorocatechols, chlorosyringols and large, polymerised chloroaromatics. Concern over the environmental effects of these compounds and increasingly stringent government emission standards require that total AOX release and overall effluent toxicity be substantially reduced.

1.2.1.3 Olive mill wastewater

Olive mill waste (OMW) is produced in large quantities during the processing of olive oil; either by means of the traditional discontinuous press or by the more recent continuous solid/liquid centrifuge system. Both processes produce two by-product streams: residual solid (husk), which contain oil to be recovered by means of solvent extraction, and olive mill wastewaters (OMW) (Boari *et al.*, 1984). This latter effluent contains large amounts of organic matter, the average concentration of volatile solids and inorganic matter being, respectively, 15% and 2% (Chakchouk *et al.*, 1994). OMW contain large concentrations of phenol compounds, up to 10g/*l* (Klibanov *et al.*, 1983), which are highly toxic, the structure of the aromatic compounds present in OMW can be assimilated to many of the compounds of lignin (Sanjust *et al.*, 1991). The maximum biological oxygen demand (BOD) and chemical oxygen demand (COD) reach 100 and 220 g/dm^3 respectively (Hamdi, 1993), leading to serious environmental problems (Borja *et al.*, 1993). In addition to its highly polluting nature, OMW usually possesses a high antibacterial and phytotoxic effect exerted by different phenolic compounds (Rodriguez *et al.*, 1988).

Phenolic wastes from wine-making processes

The wine-making process generates large amounts of pressed grape material which is then left in piles to compost, producing a run-off rich in polyphenolics of lignin-related structure. The phenolic nature of the solution makes it toxic to plants and resistant to

microbial degradation. However, it is a rich source of subtrates for enzymic reactions which can generate anti-oxidant products; these, being of food material origin, can potentially be used as dietary supplements.

1.2.2 Conventional dephenolisation procedures

Numerous physical and chemical treatment processes exist for the removal of phenolics from contaminated aqueous mixtures, including chemical precipitation, ultrafiltration/reverse osmosis, ultraviolet radiation, and treatment with titanium dioxide, ozone and hydrogen peroxide (Burton *et al*, 1998; Atlow, 1984). Several existing biological, chemical or physical treatment processes can be used to remove phenols from contaminated aqueous mixtures. Although these methods are fairly effective, they suffer serious drawbacks such as high cost, incompleteness of purification, formation of hazardous byproducts, and applicability over a limitted phenol concentration range (Atlow, 1984). However, processes such as ultrafiltration, ion exchange and lime precipitation are expensive and therefore alternate biotreatment processes have been considered (Davis, 1990). As a result increased development in the area of biological wastewater treatment has occurred and recently applications of biological oxidation for phenolated wastewater treatment have become more numerous (Shishido, 1996).

1.2.3 Biological agents for bioremediation of phenolic pollutants

The rapid development of industrial production and utilisation of enzymes and modified micro-organisms coincides with a generally growing interest in biotechnology. Because of the increasing prices of raw materials, chemicals and energy, processes, which operate with reduced raw material quality while yielding marketable product qualities, need to be developed. On the other hand, it can be expected that progress will be made in reducing chemical inputs by the use of biotechnology, which provides protection for the environment. The utilisation of enzymatic processes has become feasible by the discovery and/ or development of highly specialised micro-organisms which can be employed directly, or through the isolation of the enzymes they produce (Miletsky, 1996).

As an interesting example, most of the treatment processes used for industrial and domestic wastewaters have been tested on olive oil effluents (Boari *et al*., 1984). Several physico-chemical processes including simple evaporation, ultrafiltration and reverse osmosis have been used to detoxify this effluent. Each solution requires costly investment and maintenance which can be unprofitable for the seasonal oil extraction industry (D'Annibale *et al*., 1988; Martinez Nieto *et al*.,1993). Therefore great interest has been focused on biological treatment of OMW, as an alternative to the conventional treatment processes.

1.2.4 Whole cell systems for bioremediation

Bioremediation of toxic aromatic pollutants by whole cell culture systems can be divided into fungal and bacterial treatments. Bacteria are usually the organisms of choice as they have a much higher metabolic rate and the metabolic fates of aromatic pollutants are far better studied in bacteria than in fungi (Burton *et al*, 1998). However, bacteria can oxidise only a very narrow range of organic pollutants, and furthermore, this can only be achieved over fairly narrow concentration and pH ranges.

The use of white rot fungi has been considered as it offers a useful alternative to overcome these problems (Burton *et al*, 1998). Fungi have been important in both ancient and modern biotechnological processes. They are an important source for many important metabolites, including enzymes, organic acids, antibiotics, hormones and steroids (Gerin *et al*, 1995). Fungi are lower eukaryotes often classified into their own "Fifth Kingdom" based on their absorptive mode of nutrition (Bennet, 1998). Diverse in morphology, physiology and ecology, many are best known for their negative impact on the environment as agents of plant disease, biodeteriation or as animal pathogens. However, with the advent of recombinant DNA technology and large-scale genomic analysis a number of the microscopic species, including the yeasts and the filamentous fungi, have been harnessed for their metabolic activities, either as producers of degradative enzymes or synthesizers of useful metabolites (Bennet, 1998). With the

dawning of this mycotechnological era, researchers are looking for novel strains that can be manipulated for commercial applications.

Over the past decade, the white rot fungi have been studied for their ability or potential to produce enzymes generating extracellular oxidative radicals and hence to degrade recalcitrant organo-pollutants such as polyaromatic hydrocarbons, chlorophenols and polychlorinated biphenyls (Swamy and Ramsay, 1999; Reddy A, 1995; Ricotta *et al.*, 1996). These fungi produce two types of extra-cellular metalloproteins with lignolytic activities: laccases and peroxidases (Youn, 1995). The random nature of the structure of lignin requires lignin degradation to function in a non-specific manner; consequently, other compounds that have an aromatic structure, such as many xenobiotic compounds, are also highly susceptible to degradation by lignolytic enzymes. In particular *Phanerochaete chrysosporium* and *Trametes (coriolus) versicolor* have been implicated in the biodegradation of a number of environmental pollutants (Bumpus JA and Aust SD, 1986; Hammel KE *et al.*, 1992; Konishi K and Inoue Y, 1972). Ricotta (1996) and coworkers demonstrated the role of a laccase from *Trametes versicolor* in the degradation of pentachlorophenol. The substrate non-specificity of laccases has led to them being examined as agents for the biodegradation of xenobiotic compounds and their ability to oxidise compounds such as chlorinated phenols and polyphenols, as well as aromatic amines, has been well documented (Collins *et al*, 1996).

Fungal bleaching of pulp mill effluents is regarded as promising technique for pollutant removal. The white rot fungi appear to be most effective at the bleaching of effluents. Some species also have the ability to depolymerise the high molecular weight fractions, degrade aromatic compounds and dechlorinate and detoxify the effluent, thereby rendering these otherwise recalcitrant compounds non-toxic. These can then serve as readily available carbon sources for conventional bacterial metabolism. Thus, the *T. versicolor* system could be used to provide carbon sources for other bioremediation systems such as sulphate reducing bacteria used in the bioremediation of acid mine drainage, and the many bacteria used for the removal of excess nutrients from

wastewater, denitrifiers etc. Such a system would be of use to paper manufacturers for the direct treatment of their effluents, and also in other processes that require cheap, utilisable carbon for bioremediation regimes. WRF are fastidious in their growth requirements and have evolved to grow on a solid/air interface (wood). Hence, any reactor intended to successfully utilise these fungi has to mimic these conditions. A transverse flow capillary membrane was developed within the group for this purpose.

Neurospora crassa

Filamentous fungi are a source of many important metabolites, including enzymes, organic acids, hormones, and steroids, and in recent years, growing interest has focussed on fungi which have potential in solving environmental problems (Gerin et al, 1995). *Neurospora crassa* (*N.crassa*; the common pink bread mould) is one such filamentous fungus. N. crassa has been genetically well characterised (Kupper et al, 1990) and is favoured as a laboratory organism as it has simple nutritional requirements and straightforward biochemistry and genetics (Davis and Serres, 1970). In the past *Neurospora* was of primary value as it was a eukaryotic organism which could be handled as easily as bacteria and provided a valuable basis of comparison between prokaryotes and eukaryotes in molecular biology (Davis and Serres, 1970).

N. crassa has the capacity to produce two different oxidoreductase enzymes, a laccase and tyrosinase. These oxidative enzymes catalyse the conversion of phenolic substrates into quinoid products giving *N.crassa* the potential to be manipulated for biodegradative purposes, in particular the biodegradation of phenolics. Laccase is a multicopper oxidase capable of catalysing the biological oxidation of organic substrates (eg. mono-, di-, and polyphenols, aminophenols and diamines) with the concomittent reduction of molecular oxygen to water (Germann et al, 1988). Tyrosinase is a monooxygenase mainly involved in the biosynthesis of melanin pigments. It catalyses the ortho-hydroxylation of phenols and aromatic amines, and the oxidation of o-catechols to o-quinones and o-aminophenols to o-quinoneimines (Kupper et al, 1989). The expression of both oxidoreductase enzymes is governed by a common control mechanism and the production of both can be

co-induced in vegetatively growing cultures by the addition of a protein synthesis inhibitor, cycloheximide (Froehner and Eriksson, 1974).

Trametes versicolor

Trametes versicolor is one of the most extensively studied white rot fungi (WRF). It produces an enzyme suite containing various laccase isozymes, as well as other peroxidases e.g. lignin(LiP) and manganese peroxidases (MnP). Low concentrations are produced constitutively on wood and in submerged fungal cultures, while higher concentrations are induced by aromatic compounds such as xylidine and ferulic acid (Collins P.J. and Dobson A.D.W., 1996). In addition, while most other white rot fungi (WRF) only produce their oxidative enzymes under conditions of nutrient limitation or stress, *T. versicolor* produces high levels of these enzymes under a regime of nutrient supplementation – i.e. rapid growth conditions. These enzymes are extracellular, which allows high concentrations of pollutants to be detoxified extracellularly without requiring the transport of substrates into cells. The enzymes are relatively non-specific with regard to aromatic pollutant structure, and thus a monoculture can degrade a wide range of pollutants as opposed to a consortia of microorganisms which might be required in alternative processes. This would allow for uncomplicated re-inoculation from stock cultures after a process upset. It also allows for better prediction of the fate of the pollutants.

Actinomycetes

Actinomycetes occur in a wide range of environments, and have the ability to grow on a wide range of moist naturally occurring substrates. Some are parasites (eg *Dermatophilus*) or form symbiotic associations (eg *Frankia*). However the vast majority of Actinomycetes are saprophytes in soil, water, composts and other related substrates. It is these that are the majority of the many isolates obtained for scientific study or commercial exploitation.

Actinomycetes have considerable potential for the biotransformation and biodegradation of xenobiotics. As a result of this, a number of new recombination DNA techniques have been developed to genetically engineer microorganisms for biodegradation of environmental contaminants. Such techniques include new expression vectors, application of site-directed and random mutagenesis to increase substrate range and activity of biodegradative enzymes. Gene technology combined with a solid knowledge of catabolic pathways and microbial physiology enables the experimental evolution of new and improved catabolic activities for such pollutants (Tinnus. 1999). Such gene technology is a powerful tool for recombining existing diversity to tailor biological systems for multiple functional parameters (Ness et al. 1999).

A variety of actinomycetes are known to degrade lignin, lignocellulose and other related aromatic compounds. Actinomycetes, in particular the genus *Streptomyces*, excrete lignin-catabolizing enzymes including peroxidases and polyphenol oxidases. *Streptomyces viridosporus* T7A is the most studied strain and is known to produce several extracellular enzymes, including a type of lignin peroxidases and a group of esterases, which are all implicated in aromatic solubilization and degradation. Thermophilic actinomycetes are of particular interest as they have the added advantage of thermostability properties which is advantageous for the bioconversion process.

1.2.5 Bacteria designed for bioremediation.

The amounts of a particular enzyme produced by a particular microorganism can vary tremendously since enzyme formation is regulated in both positive and negative directions by control mechanisms such as induction, end product repression and catabolite repression. Such control mechanisms are influenced by environmental conditions such as pH, temperature, aeration etc. However, further manipulations can be used to force "overproduction of enzymes in the laboratory. Through genetic engineering, degradation pathways can be modified to widen substrate spectrum (Muller et al. 1996). The technical applicability of a microorganism for the degradation of toxic compounds is

mainly influenced by the ability to maintain high degradation rates and to mineralize the toxic substrates completely (Muller et al. 1996).

Much of the past and current focus of bioremediation has been on laboratory studies of specific microbial processes. Previous studies have ignored important properties, parameters and processes that would control the ultimate success of in situ bioremediation. A bioengineering systems approach needs to be implemented to examine the impact of some of the common bioremediation practices (Knapp and Faison, 1997). Bioremediation provides the potential cost effective, containment specific treatments to reduce concentrations of individual or mixed environmental contaminants (Jones ,1998). In situ applications involving commercially available bacterial strains have offered limited success. A study carried out by Mueller et al.(1991) indicated that it may not be possible to rely completely on indigenous microorganisms to effectively remove environmental contaminants in a reasonable time span. Many environmental pollutants can be efficiently degraded by microorganisms while others persist and constitute a serious health hazard. Persistence is a direct consequence of the inadequate catabolic potential of the available microorganisms (Tinnus, 1999) as well as the requirement of a consortia of bacteria for complete degradation (Schrijver and De Mot, 1999), constructed on the basis of synergistic and commensalistic relationships (van Ginkel, 1996). However, degradation of one surfactant by one member of the commensalistic consortium may lead to the accumulation of toxic and non-toxic metabolites (van Ginkel, 1996). Hence alternative or supplemental approaches may be necessary. The diversity of form in the Actinomycetales is well recognised due to the sustained generation of environmental isolates for industrial screening. Actinomycetes isolated from soil, compost and related substrates show primary biodegradative activity, secreting a range of extra- and intra- cellular enzymes exhibiting the capacity to degrade recalcitrant molecules. Isolation of such isolates is merely the beginning of the process. Heterologous expression for the purpose of simplifying enzyme recovery as an extracellular protein in a chosen expression host, followed by sequencing studies to determine the genes encoding these enzymes. Subsequent DNA manipulation techniques to improve the enzymes i.e

improvements in substrate specificity, thermostability etc., all contribute to the larger picture.

1.2.6 Isolated enzymes for bioconversion of phenolic pollutants

Laccase

Laccases (benzenediol:oxygen oxidoreductase EC 1.10.3.2)) are multi-copper-containing enzymes that catalyse the oxidation of various aromatic compounds, specifically phenols and anilines, while concomitantly reducing molecular oxygen to water. The ensuing oxidative coupling reaction is important in the synthesis of numerous naturally occurring complexes such as humic substances, tannins, melanins and alkaloids. The same reaction is able to transform numerous agricultural and industrial chemicals.

Polyphenol oxidase

Polyphenol oxidase is a mono-oxygenase which catalyses two sequential reactions, *viz*., the *ortho*-hydroxylation of phenols to catechols (cresolase activity) and the further oxidation of these catechols to *ortho*-quinones (catecholase activity). While the quinone products of the polyphenol oxidase reaction are highly reactive and tend to polymerise rapidly, the intermediate products, the catechols, are valuable chemical intermediates. Catechols are industrially important chemicals which are used as a raw material in the manufacture of pharmaceuticals, flavours, fragrances, insecticides and anti-oxidants (Latkar and Chakrabarti, 1994; Kakinuma *et al.*, 2000). Other uses of catechol are in photography and a polymerization inhibitor for the storage of polymeric materials. 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, using copper catalysts at high temperatures (Liu *et al.*, 1997). However these methods have failings such as low conversion rates, need for high temperatures and pressures and they result in serious environmental pollution (Liu *et al.*, 1997).

Peroxidase

Peroxidases catalyse the one-electron oxidation of a wide range of substrates, using H_2O_2 as a cosubstrate, via oxidative dehydrogenations, oxidative halogenations, H_2O_2 dismutation, or oxygen transfer reactions. Such reactions are often difficult to accomplish or control by conventional chemical syntheses, making peroxidase-catalysed biotransformations particularly useful. In addition, many of these reactions are enantioselective or regioselective. Plant peroxidases, termed Class III peroxidases, are haem peroxidases which typically oxidise aromatic amines, indoles, phenols and sulfonates to produce coupled dimeric or polymeric products via radical-based mechanisms (Smith and Veitch, 1998). Current uses of peroxidases include the well-known application of horseradish peroxidase in enzyme-linked immuno- assays as well as various oxidative biotransformations.

1.2.7 Bioreactors for bioremediation

The use of immobilised microbial cells for the treatment of polluted water and wastewater has become the main focus of many research programs (Vilchez, 1997). However, a major obstacle in the past has been the lack of suitable bioreactor systems (Ryan, 1998) and immobilisation technology (Wenhua, 1993). The application of immobilised biological systems in biotechnological processes is becoming increasingly useful, with the advantage that it can potentially prolong the retention time of microbial cells or enzymes indefinitely, without washout, even at high dilution rates (Wenhua, 1993). Membrane bioreactors designed specifically for microbial cell immobilisation are now being applied in a number of biotechnology processes, for example, to extract and biodegrade toxic organic chemicals from aqueous solutions (Livingstone, 1993). Since early reports of membranes used in combination with biological wastewater treatment, the successful treatment of numerous priority pollutants in wastewaters by membrane systems has been reported (Brindle, 1996).

CHAPTER 2

Application of a whole-cell system uing the fungus *Neurospora crassa* in the bioconversion of phenolics

2.1 INTRODUCTION

The main aim of the research reported in this section was to investigate the ability of a membrane-immobilised fungal biofilm of *Neurospora crassa* to degrade phenolic pollutants, and to compare this ability with that of a non-immobilised culture of the same fungus. The biological viability of *N.crassa* biofilm, immobilised in this way, has been previously studied using scanning electron microscopy and reported by Burton *et al.*, (1998) and Ryan *et al.* (1998). *N.crassa* has been extensively studied at a molecular level, but has not to our knowledge been studied with respect to its application involving intracellular bioconversions, which may be particularly useful in bioremediation and biotransformation.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

,4-Dihydroxyphenylalanine (DOPA), β-cyclodextrin and cycloheximide were obtained from Sigma Chemical Company. ABTS and the phenylmethylsufonylfluoride (PMSF) were obtained from Boehringer Mannheim (Germany). p-Cresol was obtained from Aldrich Chemical Company. Phenol and acetonitrile (HPLC-grade) were obtained from BDH Laboratory Supplies (England). 40% Formaldehyde (analytical grade) was obtained from Saarchem (Pty) Ltd.

2.2.2 Cultures and culture conditions

An *N.crassa* culture (strain number 2054) was obtained from the Plant Protection and Research Institute in Pretoria and maintained on agar slants according to Horowitz (1970). Mycelia were grown in chemically defined medium according to Lerch (1987) in shaken liquid cultures (120rpm) at 25°C in the dark. For the induction of polyphenol oxidase and laccase cultures were treated with 1µmol/L cycloheximide (Lerch, 1987; Froehner and Eriksson, 1974).

2.2.3 Extraction of intracellular polyphenol oxidase and laccase

Extractions were carried out on cultures of *N.crassa* grown in half strength Vogel's minimal media containing 0.5% sucrose. Cultures were induced for the production of both tyrosinase and laccase by the addition of cycloheximide (1µmol/L) after a 3 day incubation period. After a further 3 day incubation period (the time at which optimal enzymatic activity is reached) (Lerch, 1987), the mycelium was collected by filtration, rinsed to remove all growth medium and then lyophilised. Polyphenol oxidase could then be extracted from smaller aliquots of freeze-dried mycelium by homogenising in a Waring blender with liquid nitrogen, centrifuging to remove all cellular debris and finally carrying out an ammonium sulfate precipitation, using up to 60% ammonium sulfate saturation (Lerch, 1987). After dialysis, the crude extract was then freeze-dried and stored in the freezer until required.

2.2.4 Enzyme assays

Polyphenol oxidase activity was determined using dihydoxyphenylalanine (L-DOPA) as the substrate.The catecholase activity of the polyphenol oxidase was followed spectrophotometrically by following the rate of conversion of L-DOPA to DOPAchrome, a red coloured reaction product that absorbs light maximally at a wavelength of 475nm (Burton *et al*, 1993). One unit of polyphenol oxidase activity was defined as the amount of enzyme required to produce 1µmol product per minute.

Laccase activity was measured using the method of Roy-Arcand (1991) with ABTS as substrate and monitoring the increase in absorbance of the product at 420nm. Spectrophotometric measurements were made using a Shimadzu UV160 spectrophotometer.

The Bradford protein assay (Bradford, 1976) was used to determine protein concentrations.

2.2.5 Membrane-immobilisation

Capillary membrane bioreactor module

The bioreactor used incorporated capillary membrane modules manufactured at the Institute for Polymer Science, Stellenbosch University (Leukes *et al*, 1997). The bioreactor was operated in a dead-end configuration, with humidified air being passed across the shell-side of the capillary membranes, the media stream being passed through the lumen of the capillary membranes and permeate collected from the shell-side of the membranes. Polysulfone capillary membranes (o.d. 1.8 mm), each about 12 cm in length, were arranged in layers within the bioreactor module. Fungal biomass was immobilised on the shell-side (outer surface) of the capillary membranes (Ryan *et al*, 1998).

Capillary membrane bioreactor pretreatment and inoculation.

Before inoculation, the membranes in the module were pretreated with sterile deionised water to remove the glycerol wetting agent. The reactor was chemically sterilised by circulating 4% formaldehyde through the reactor system overnight and then thoroughly rinsed with sterile deionised water. At this stage the flux and flow rates were adjusted in order to achieve as low a flux as possible. With an EyeLA peristaltic pump set at a flow rate of 0.001mL/min and tubing with an internal diameter of 0.64mm, a flux as low as 3mL/h could be achieved.

A *N.crassa* conidial spore suspension (400mL), washed off the surface of a *N.crassa* culture grown on a slant in a roux flask, was used as inoculum for the reactor. The bioreactor was inoculated from the shell side of the reactor, through the permeate port in a dead-end filtration mode. The inoculum was pumped into the shell-side of the reactor using a Watson/Marlow peristaltic pump, back pressure being carefully monitored to ensure that it did not exceed the pressure limit of the system (i.e. 150kPa). The pump was switched off at regular intervals during the inoculation procedure, allowing the pressure build-up to decrease, before being switched on again. The procedure was repeated until all inoculum had been forced into the system. Half-strength Vogel's growth medium was then fed into the lumen of the capillaries, the system was closed, and left to stand overnight. This allowed time for the spores, which had been forced into to the macrovoids of the capillaries, to germinate and grow, and for the fungal biofilm to establish itself within the membrane bioreactor module.

Capillary membrane bioreactor operation.

Once the biofilm had been established, the medium vessel was attached to the reactor system and medium pumped through the lumen of each of the capillaries at a flow rate of 0.001mL/h, giving a flux of approximately 3mL/m^2/h. The humidifier was then attached to the system and moist air pumped across the shell-side of the capillaries, ensuring sufficient and continuous air supply to the developing biofilm, using an aquarium pump. Permeate was collected through a permeate collection port at the base of the reactor. Flux was also monitored in order to ascertain whether the biofilm had a major effect on the rate of diffusion of permeate through the macrovoids of the capillary membranes.

2.2.6 Localisation of intracellular oxidase enzymes

Polyphenol oxidase and laccase were located within the fungal biofilm on capillary membranes by transmission electron microscopy (TEM) using a cytochemical staining technique (Hall and Hawes, 1991). Ultrathin sections of the fungal biofilm were prepared for TEM using a method by Cross (1987) and stained with uranyl acetate and lead citrate in order to enable cell structure and organelle visualisation.

2.2.7 Phenolic degradation

Degradation by batch cultures of Neurospora crassa

Cultures of *N.crassa* were grown in liquid medium and induced for the production of both polyphenol oxidase and laccase, as described above (see Cultures and Culture conditions). After a further three days incubation, the culture medium was spiked with various phenols, the uptake of which were monitored, at regular intervals, by high performance liquid chromatography (HPLC). Two sets of flask cultures were carried out, one set spiked with *p*-cresol and the other with phenol. In both cases, the cultures were exposed to concentrations ranging from 0.5mM to 5mM (0.054 to 0.54 mg/L). To further investigate the effectiveness of free fungal biomass in catalysing the conversion of phenols to quinones, one set of flasks were incubated under static conditions, while another set of flasks were incubated with shaking.

Further batch studies were carried out in order to determine the rate of phenol bioconversion per day per mg biomass (dry mass). Conversion rates were monitored over 6 days as opposed to the initial reaction rates over the first few hours, as colour conversion was only evident after the first 2 days. Dry mass was determined by filtering biomass onto pre-weighed filter paper, drying the filtered biomass overnight at 37°C and then subtracting the weight of the biomass with the filter paper from the weight of the filter paper alone.

Degradation by capillary membrane-immobilised N. crassa cultures

Degradation of isolated phenolic pollutants (p-cresol / phenol)

Once the *N.crassa* fungal biofilm had been established on the shell-side of the capillaries in the bioreactor and the reactor was fully operational, a model pollutant (*p*-cresol or phenol) was added to the medium stream flowing through the lumen of each capillary membrane. The addition of the same model pollutants as used in the non-immobilised system, *p*-cresol and phenol, at the same concentration range (0.5mM to 5mM) allowed comparison between the non-immobilised fungal system and immobilised fungal system. Between exposures to each phenol concentration, the immobilised biofilm was allowed a

1 week recovery period, during which normal growth medium was passed through the bioreactor system, before the biofilm was exposed to the next concentration of phenol. Permeate samples were collected at regular intervals and the decrease in concentration of the model pollutant in permeate was monitored by HPLC.

Further analyses were carried out on biofilms immobilized in bioreactors in order to determine the initial rates of phenol conversion per hour per mg biomass (calculated by sacrificing bioreactor and determining total biomass present).

Degradation of phenolics in a synthetic mix of model phenolic pollutants

Similarly, membrane-immobilised biofilms were also exposed to mixtures of model phenolic compounds (i.e. phenol, *p*-, - and *o*-cresol) at initial concentrations of 1mM (0.108mg/L), then 2.5mM (0.27 mg/L) and finally 5mM (0.54 mg/L). The total phenol concentration during each exposure time being: 4mM, 10mM and 20mM (0.43, 1.08, and 2.16 mg/L), respectively. As in the previous experiment using isolated phenolic compounds, the biofilm was exposed to the mixture for a period of 6 days and then given a 1week recovery period, during which time growth medium was passed through the bioreactor system before exposing the biofilm to the next concentration of phenolic mixture.

Degradation of phenolics in authentic effluent (cresylic / phenolic)

Fully established *Neurospora* biofilms were also exposed to cresylic effluent, which had been diluted 10% with Vogel's medium N. From HPLC profiles of the effluent it was clear that the main phenolic components, and the concentrations at which they were present in the effluent before dilution, were: phenol (79.4mM; 7.5 mg/L), *p*-cresol (22mM; 2.4 mg/L), *o*-cresol (44.2mM; 4.8mg/L) and *m*-cresol (17.6mM; 1.9 mg/L). A control bioreactor, in which no biofilm was present, was run simultaneously to the experimental bioreactor. It too was exposed to the same concentration of cresylic effluent in order to determine if any change in concentration of phenolic components

could be due to adsorption of phenols to membrane or bioreactor tubing, or due to pervaporation.

2.2.8 Effluent study

The effluents used are fully described in Appendix 1. These included:

- Cresylic effluent from coal-to-oil conversion
- Black product effluent from coal-to-oil conversin
- Bleach plant effluent from pulp-and-paper production
- Digestor effluent from pulp-and-paper production
- Sorrento pond water from olive production
- La Valle pond water from olive production
- Fermentation brine from olive production

Preliminary plate study

Preliminary experiments were carried out in order to determine whether the culture could grow in the presence of the effluents. These studies were carried out on agar plates, using a defined medium. A series of plates were made up for each effluent type, with concentrations of effluent in each series ranging from 0%, 0.0788%, 0.788%, 7.88% and 31% effluent. In each case the effluent was substituted for the carbon source. Plates were inoculated using fungal spores from *N.crassa* cultures grown on agar plates and incubated at 25°C in the dark.

Effect of effluent on the growth of N.crassa in batch cultures

A series of liquid cultures were carried out using only those effluent types (and concentrations) in which cultures in preliminary studies had grown. Each series of liquid cultures was carried out in duplicate. Cultures were grown in Vogel's medium N according to Lerch (1987) and diluted to half strength using the respective effluents and milliQ water until the required effluent concentrations were obtained in each culture.

Cultures were incubated for 2 weeks at 25°C in the dark with shaking (120rpm). During the 2 week incubation period, duplicate cultures of each effluent concentration were sacrificed every two days.

These cultures were filtered from the remaining media and wet mass monitored in order to determine the effect of the effluent on growth of the fungal biomass in liquid culture. The filtrate from each culture was assayed for extracellular laccase activity to determine whether any of the phenolic components present in the effluent had an inducing effect on laccase production. In order to determine if the fungal biomass decolourises the effluent at all, an ultra violet scan was carried out using a Shimadzu-160 spectrophotometer. Finally, samples of each filtrate were analysed by HPLC in order to determine the extent (if any) of degradation of phenolics present in each effluent by the fungal biomass.

2.2.9 Catechol production by fungal biomass

Production in batch cultures

Cultures were prepared as described in section 2.2. However, before the addition of substrate, in this case *p*-cresol, the cultures were filtered and the biomass resuspended in 100mL sodium phosphate buffer (0.1 M pH6). The freshly resuspended biomass and the remaining media were then exposed to 2.5mM *p*-cresol. Samples were taken at regular intervals and analysed by HPLC in order to detect catechol production.

Production by membrane-immobilised biomass

Capillary membrane bioreactors were inoculated as described in section 2.5. Once the biofilm had been established, it was exposed to 2.5mM *p*-cresol in sodium phosphate buffer (0.1 M pH6) for a period of 24 hours. Permeate samples were collected at regular intervals and analysed by HPLC in order to monitor the appearance of any catechol peaks.

2.2.10 Analytical methods

The bioconversion of both *p*-cresol and phenol to quinone products, from both flask and bioreactor samples, was measured by reverse-phase HPLC using a Beckman System Gold HPLC unit with Beckman System Gold software and a Diode Array UV-Detector, or Anachrom HPLC Unit with Peak Windows software. Both systems were run using a reverse-phase Machery-Nagel Nucleosil 5µ column with water:acetonitrile (6:4) as the mobile phase at a flow rate of 1mL/min. To detect the phenol and *p*-cresol water : acetonitrile (6:4) was used as the mobile phase. However, when samples contained cresylic isomers as phenolic components, water:acetonitrile (8.5:1.5) together with 1.66% β-cyclodextrin were used as the mobile phase. This enabled the *p*-cresol and *m*-cresol within the sample to elute as separate peaks. The same mobile phase was used for monitoring the conversion of the phenolics present in the cresylic effluent.

Decolourisation of effluents was monitored at regular intevals spectrophotometrically by monitoring the UV absorption spectrum of each effluent-spiked culture at regular intervals for the disappearance of any absorbance peaks.

The growth and development of the fungal biofilm within the capillary membrane bioreactor was monitored by following the rate of uptake of ammonium from the growth medium.

2.3 RESULTS AND DISCUSSION

2.3.1 Growth and oxidase enzyme production

Batch cultures

Laccase is produced as both an intracellular and extracellular enzyme (Figures 2.1 and 2.2). No extracellular polyphenol oxidase could be detected in batch cultures, suggesting that this particular fungal strain produces the enzyme as an intracellular product, a common characteristic in a number of *Neurospora* strains (figure 2.3) (Gutteridge and Rob, 1975; Kupper *et al*, 1989). Both oxidase enzymes appear to be produced during the stationary growth phase of *Neurospora crassa* life cycle.

Batch studies clearly indicate that without cycloheximide-induction little or no intracellular or extracellular polyphenol oxidase or laccase activity can be detected in flask cultures (figures 2.1, 2.2 and 2.3). However, upon cycloheximide-induction (2 to 3 days after inoculation), levels of both intracellular and extracellular oxidase enzymes increase rapidly reaching optimal levels of activity 3 days after induction. Furthermore, cycloheximide-induction results in a 50% reduction in biomass yield. Although inhibition in growth (due to its toxicity) is fairly severe, cycloheximide is still one of the most effective inducers known (Froehner and Eriksson, 1974). By induction with 1μM cycloheximide it is possible to co-induce for the production of optimal oxidase enzyme activities.

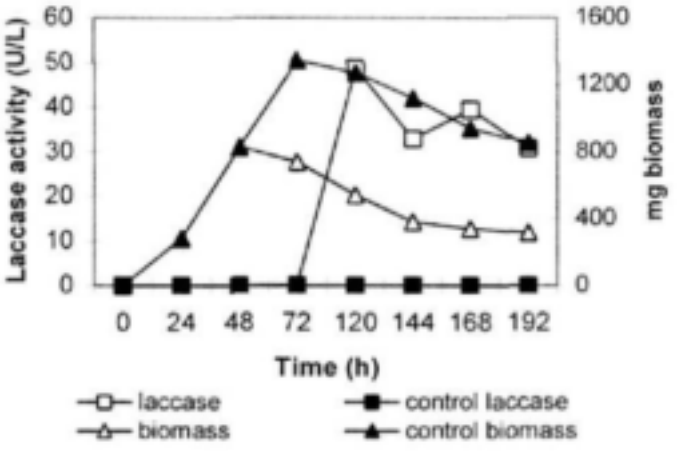


Figure 2.1. **Biomass yield and extracellular laccase production by non-induced and cycloheximide-induced (1μM) cultures of *N.crassa***

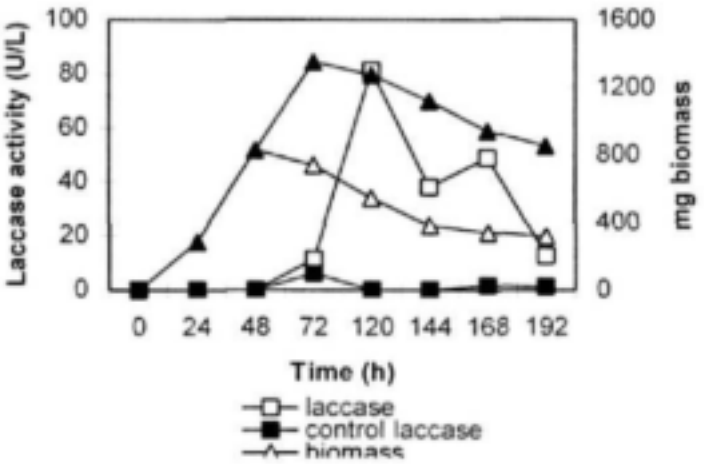


Figure 2.2. **Biomass yield and intracellular laccase activity in non-induced and cycloheximide-induced (1μM) cultures of *N.crassa***

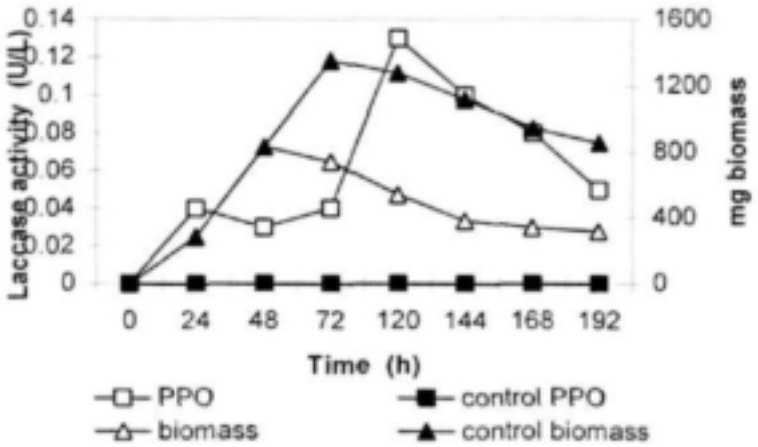


Figure 2.3. Biomass yield and intracellular polyphenol oxidase activity in non-induced and cycloheximide-induced (1μM) cultures of *N.crassa*

Membrane-immobilised cultures

In order to determine the viability of capillary membrane-immobilised biofilms, without having to sacrifice the immobilised biomass, the uptake of ammonium from the growth medium was monitored as it permeated through the fungal biofilm. Levels of ammonium in permeate collected from a control bioreactor, where no biomass had been immobilised, rapidly increased and then leveled off at the expected ammonium concentration. The opposite effect was seen in the ammonium concentrations from experimental bioreactor permeate, which had been inoculated with fungal spores. The ammonium concentration rapidly declines at first and then slowly levels off and increases slightly again (Figure2.4). This is a common characteristic in viable biofilms, which grow very rapidly at first utilising the nitrogen source very quickly and then as they reach stationary growth phase so there is a decline in growth and less utilisation of the nitrogen source. Toward the end the fungal biofilm begins to decline, which would explain the slight increase in ammonium concentration toward the end.

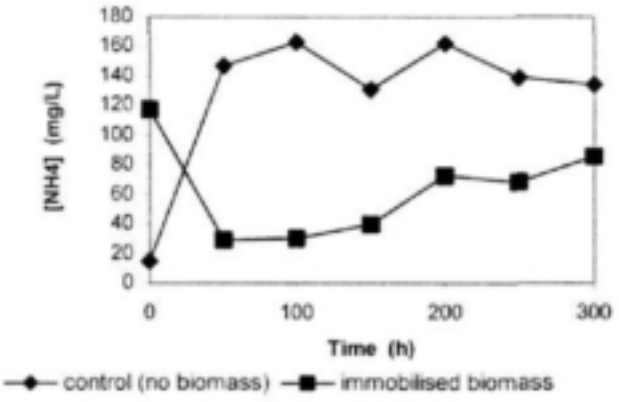


Figure 2.4. Uptake of ammonium from bioreactor permeate of the control and experimental capillary membrane bioreactors

The flux was monitored throughout the life of the bioreactors in order to ensure that it remained fairly constant whether the biofilm was present or not. In a control bioreactor, where no biomass had been immobilized, the flux was around 3mL/ m^2/hr; while in an experimental bioreactor, where a biofilm had been well established, the flux fluctuated around 3mL/m^2/hr (Figure 2.5).

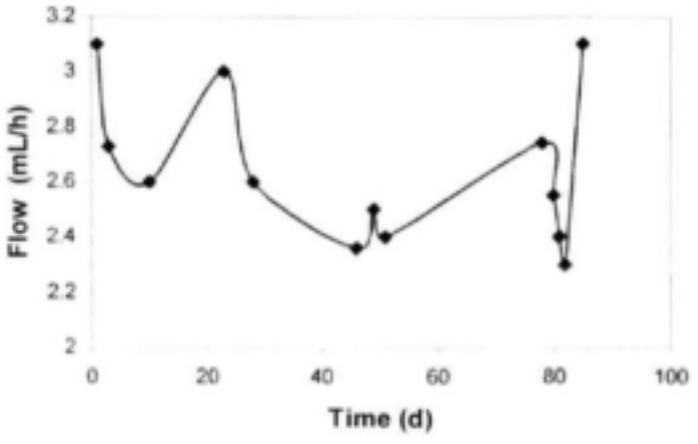


Figure 2.5. Flow measurements across a well established membrane-immobilised biofilm

Bioreactors were cycloheximide-induced for oxidase enzyme activity 6 days after inoculation, at which time an increase in extracellular laccase, detected in the bioreactor permeate, was evident. An increase in laccase activity was observed in bioreactor permeate after 33 days, but no polyphenol oxidase activity could be detected (figure 2.6). This supports the findings by Germann *et al* (1988), that polyphenol oxidase is produced solely as an intracellular product, as opposed to laccase, which is produced predominantly as an extracellular product. While quantitative measurements for intracellular oxidative enzyme production were not possible without the destruction of the biofilm, transmission electron microscopy of thin cytochemically stained sections of capillary membrane-immobilised biofilm have previously indicated the presence of oxidase enzyme activity within the immobilised fungal biofilm (Burton *et al*., 1998).

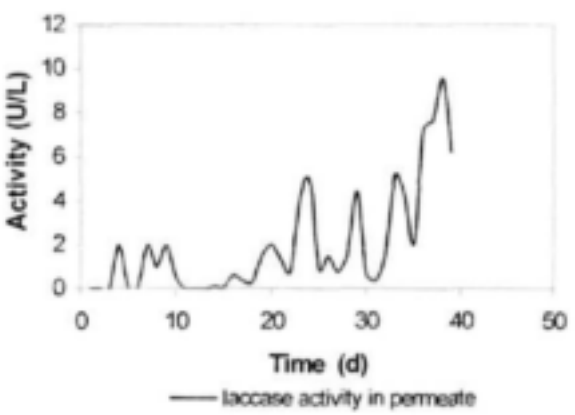


Figure 2.6. Extracellular laccase activity in bioreactor permeate from cycloheximide-induced (1μM) biofilm of *N.crassa*

Comparison of Neurospora oxidase enzyme activities with those of other Neurospora strains and other fungal strains

The activity of both laccase and polyphenol oxidase, from the *Neurospora crassa* strain under study, compare favourably with those of other *Neurospora crassa* strains, as well as other fungal strains (Table 2.1 – 2.3 and Figure 2.7). The favourable yields of both oxidase enzymes produced, give *Neurospora crassa* (strain number 2054) a capacity to be utilised in the degradation of phenolic pollutants. According to literature a good strain of *Neurospora* will produce 200U of activity per gram wet weight mycelium (Horowitz *et al*, 1970).

Table 2.1. *Neurospora crassa* (strain number 2054) polyphenol oxidase activity

Enzyme	Biomass (g)	Crude extract/ (mg)	Specific activity/ ($\mu mol.min^{-1}.mg^{-1}$)	Unit activity (g wet biomass)
Polyphenol oxidase	11.86	16.30	0.494	0.697

Table 2.2. *Neurospora crassa* (strain number 2054) laccase (extracellular)

Activity (U/mL)	Strain number
45	2054
25	69-1113a (Horowitz *et al*, 1970)

Table 2.3. Comparison of *Neurospora crassa* (strain number 2054) with other fungal laccases

Activity / (U/mL)	Source
45	*N.crassa*
14	T.versicolor (Bollag and Leonowicz, 1984)
8	*P.ostreatus* (Palmieri *et al*, 1997)

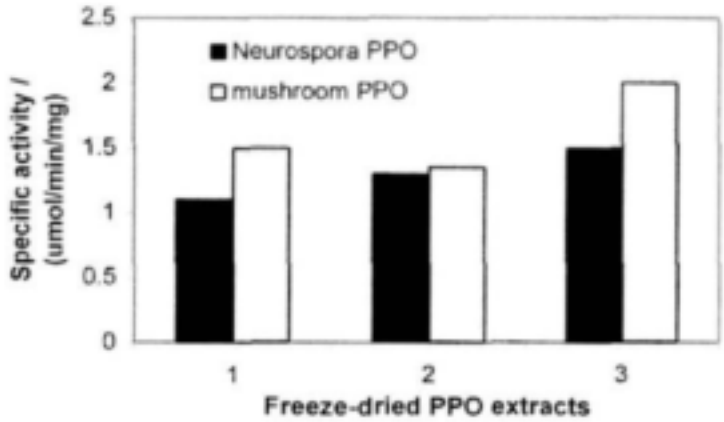


Figure 2.7. Specific activities of freeze-dried polyphenol oxidase extracts from *A.bisporus* and *N.crassa*

2.3.2 Capacity of *Neurospora* to degrade phenolic compounds

Batch cultures (shaken and static):

Degradation of isolated phenolic pollutants (p-cresol / phenol)

Batch cultures exposed to concentrations of *p*-cresol or phenol ranging from 0.5mM to 5mM (approx. 0.05 – 0.5 mg/L) were effective in catalysing the conversion of the isolated phenolic compounds to quinones. This was evident from HPLC analysis of samples taken from the batch cultures grown under shaken or static conditions (Table 2.5) and confirmed by the orange-brown appearance of culture media upon exposure to each phenolic compound. Conversion of *p*-cresol appears to be slightly more effective in static cultures than in shaken cultures. However, this could not be said of the phenol degradation by batch cultures where no real trend could be seen. This could be due to the higher toxicity of phenol.

Conversion rates were determined for the batch cultures and were found to vary from between 20 – 55mg of pollutant converted per g dry biomass per day (Table 2.4).

Table 2.4 Conversion of substrate by non-immobilised *Neurospora* cultures

Model Pollutant	Initial substrate concentration (mM)	Initial substrate concentration (mg/L)	Conversion of substrate (mg) per g dry biomass per day
p-cresol	2.5	0.27	20.0
	5	0.54	31.6
Phenol	2.5	0.23	24.78
	5	0.46	54.5

Dry mass determinations were done in triplicate on cultures that had been exposed to the pollutant for a period of 6 days

Table 2.5. *p*-Cresol and phenol degradation by non-immobilised *N.crassa* cultures

Model pollutant	Initial substrate concentration (mM)	Initial amount of substrate present[1] (mg)	Shaker cultures		Static cultures	
			Total substrate removed after 6 days (mg)	% substrate removed	Total substrate removed after 6 days (mg)	% substrate removed
p-cresol	0.5	2.70	1.9	**70**	2.48	**92**
	1.5	8.11	6.41	**79**	7.46	**92**
	2.5	13.52	8.65	**64**	12.03	**89**
	5	27.04	16.49	**61**	18.17	**67**
Phenol	0.5	2.35	2.35	**100**	1.13	**48**
	1.5	7.06	2.97	**42**	3.25	**46**
	2.5	11.76	7.88	**67**	11.64	**99**
	5	23.53	23.53	**100**	23.29	**99**

(Values shown are a mean of triplicate data) [1] Amount present in 50mL

Degradation of phenolics in authentic effluents (cresylic/phenolic)

Neurospora crassa cultures were inoculated onto a series of plates containing known amounts of various effluents (as explained above) in order to determine its capacity to grow in the presence of each. Only if *Neurospora* could grow on plates containing the pollutants could we consider its potential to degrade pollutants within that particular effluent. Cultures were found to grow in up to 31% concentrations of certain effluents used. *Neurospora* was able to grow fairly effectively on 31% concentrations of effluents 1, 5 and 7, 7.88% of effluents 6 and 7, as well as 0.788% of effluent 6 (Table 2. 6). Unexpectedly, the growth was not as effective on the cresylic (2) and the black product (4) effluent, which were the effluents containing highest amounts of the simple phenols.

Table 2.6. Preliminary plate study: Score of fungal growth on each effluent-spiked agar plate (after 5 days incubation)

Effluent type	0.0788%	0.788%	7.88%	31%
1. Sorrento (olive oil)	+	+	++	+++
2. Cresylic	+	+	-	-
3. La Valle (olive oil)	+	++	++	++
4. Black product	++	++	+	-
5. Bleach plant	+	+	++	+++
6. Digestor	++	+++	++++	-
7. Fermentation brine	+	++	+++	++++

- no growth
+ some growth
++ moderate growth
+++ growth / sporulation
++++ a lot of growth / sporulation

Batch studies were carried out using effluents in which plate cultures had exhibited good growth, in order to determine the effect of each effluent on the biomass yield and extracellular laccase activity. The capacity of the fungal biomass to degrade pollutants in each effluent and the extent to which each could be decolorised by the fungal biomass was also monitored. Biomass yields in batch cultures were significantly lower than in the control cultures (figure 2.8). Growth in 31% fermentation brine was fairly effective reaching yields as high as in control cultures. The fermentation brine did not appear to contain as toxic pollutants as the other effluents. Oxidative enzyme production was not as high as normal in effluent exposed cultures. Little decolorisation of the effluents by the fungal biomass was evident and HPLC chromatograms taken at regular intervals were not effective in monitoring the conversion of substrate peaks to product peaks as the effluents contained far too complex a mixture of pollutants to be effectively analysed.

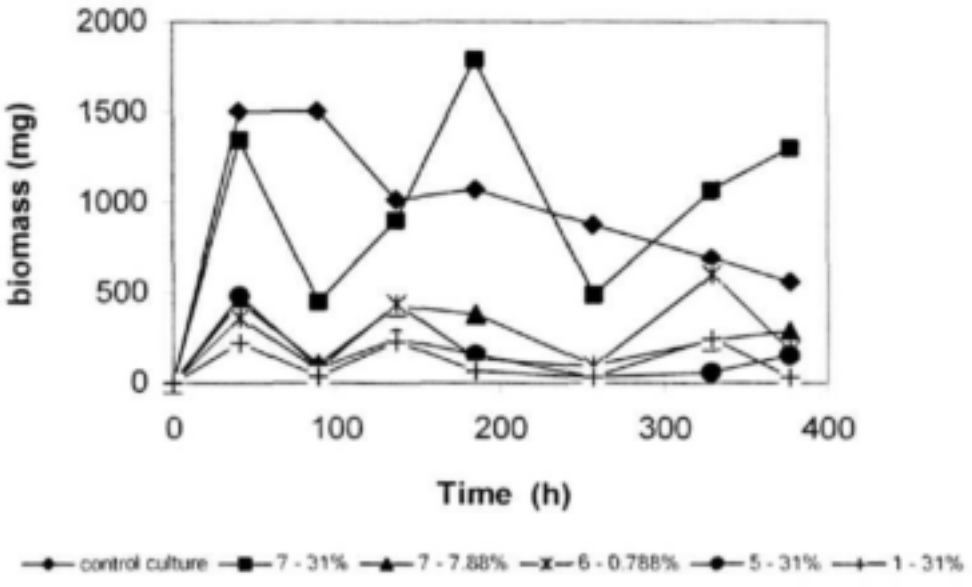


Figure 2.8. Biomass yields in batch cultures of *N. crassa* exposed to different effluents

Capillary membrane-immobilised biomass

Degradation of isolated phenolic pollutants (p-cresol / phenol)

Membrane-immobilised fungal biomass exposed to increasing concentrations of *p*-cresol showed high tolerance and a high capacity for the bioconversion of *p*-cresol. The immobilised biofilm, exposed to concentrations of 1mM, 2.5mM and 5mM (0.11, 0.27 and 0.54 mg/L) p-cresol for 6-10 day periods showed a capacity to convert 100%, 100% and 92% of the *p*-cresol present, respectively (Table 2.7).

The bioreactor used for this study was operated continuously for a period of 4 months. The immobilised biofilm was exposed to a range of different *p*-cresol concentrations followed by the same range of phenol concentrations. The biofilm was allowed a 10-day recovery period, between exposures to successive phenol concentrations, during which normal growth medium was passed through the bioreactor system, before being exposed to the next concentration of phenol. Permeate samples were collected at regular intervals and the decrease in concentration of the model pollutant in the permeate was monitored by HPLC. Conversion rates for each pollutant were determined for immobilised biomass (Table 2.8). Conversion rates compared favourably with those obtained from the batch study, especially as the conversion rates from immobilised cultures were determined using wet biomass as opposed to the dry biomass used for determining conversion rates in batch studies.

The total time for which the immobilised biomass was exposed to varying concentrations of *p*-cresol, was 31days, over an 8 week period. During this time the total mass of *p*-cresol converted by the biofilm within the membrane bioreactor was 492mg. The effective membrane surface area within the capillary membrane bioreactor is 0.0113m^2 and therefore, the conversion achieved was 43.53 g/m^2. This is equivalent to a conversion of 1.4 g/m^2/day. Immobilised cultures were capable of catalysing a maximum conversion of 497 mg/L (0.083g/L/.day) *p*-cresol over a 6 day period, whereas flask cultures were capable of converting a maximum of 380 mg/L (0.063 g/L/day) *p*-cresol over a 6-day period. The immobilised system therefore has a higher capacity for *p*-cresol degradation and thus, together with the facility of continuous operation, greater potential for use in the biotransformation of *p*-cresol and possibly other aromatic compounds (Figure 2.9).

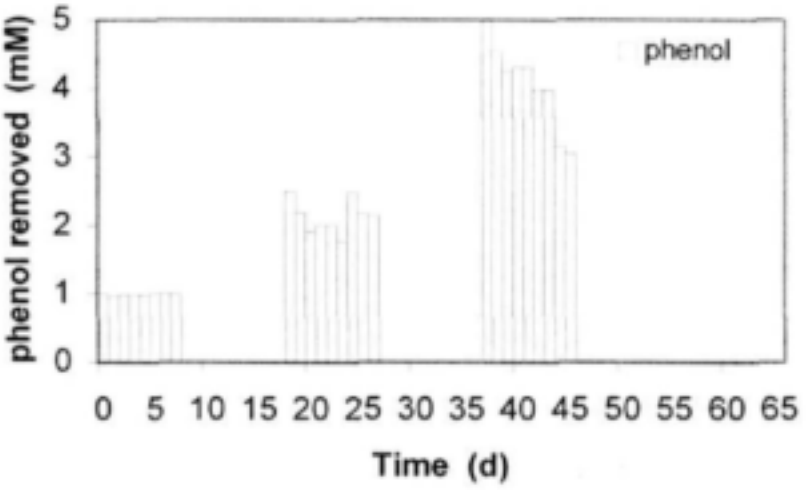


Figure 2.9. **Phenol degradation by capillary membrane-immobilised *N.crassa* biomass exposed to 1mM, 2.5mM and 5mM concentrations of phenol**

Table 2.7. ***p*-Cresol and phenol degradation by capillary membrane-immobilised *N.crassa* biomass**

Model pollutant	Initial substrate Concentration (mM)	Substrate (mg) / total volume permeate	Substrate concentration in Permeate after 6 days (mM)	Total substrate removed After 6 days (mg)	% substrate removed
p-cresol	1	46.72	0	46.72	**100**
	2.5	116.79	0	116.79	**100**
	5	233.58	0.4	214.89	**92**
Phenol	1	40.66	0.08	37.41	**92**
	2.5	101.64	0.343	87.70	**86**
	5	203.28	1.025	161.61	**80**

(Total permeate volume after 6 day period was 432mL)

Table 2.8 Conversion rates of phenolic pollutants by membrane-immobilised biomass

Model Pollutant	Initial substrate concentration (mM)	Conversion rates	
		Conversion of substrate (mg) per g wet biomass per hour	Conversion of substrate (mg) per g wet biomass per day
p-cresol	1	0.41	9.84
	2.5	1.01	24.2
	5	1.88	45.1
Phenol	1	0.356	8.54
	2.5	0.969	23.26
	5	1.49	35.76

Biomass present in bioreactor was calculated to be approximately 20g / bioreactor
Biofilms were exposed to phenolic pollutants for 6 days

Immobilised *Neurospora* biofilm systems were found to have an apparently lower capacity for phenol degradation than free fungal systems. Immobilised cultures catalysed a maximum conversion of 374mg.L^{-1} phenol over a 6 day period, whereas flask cultures were capable of converting a maximum of 466mg.L^{-1} phenol. However, the total removal of both *p*-cresol and phenol were achieved by immobilised *Neurospora* biofilms, whereas the removal capacities of free *Neurospora* systems were observed to be erratic, giving between 42% to 100% removal of *p*-cresol or phenol.

Degradation of phenols in a synthetic phenol mix (phenol, p-, m- and o-cresol)

As the biofilm had successfully degraded phenol and *p*-cresol when exposed to each in isolation, the next step was to see how the biofilm reacted when exposed to phenols within a mixture. A solution containing four model phenolic compounds was used. These components were chosen as they form the main components of most cresylic / phenolic effluents.

The immobilised biofilm showed a high capacity to degrade phenolics within a 1mM, 2.5mM and 5mM solutions of the phenols mix (Table 2.9, 2.10 and 2.11). The most effective conversion of the phenolics appeared to be at 2.5mM concentrations and the least effective at 5mM concentrations. The phenol in each case is not as readily degraded as the cresylic compounds,

especially when present at concentrations of 5mM. In total, the biofilm during each exposure time, was exposed to a total phenol concentration of 4mM, 10mM and 20mM (approx. 0.4mg/L, 0.3mg/L and 2.0 mg/L). In each case between 75 and 100% of the phenols present were successfully converted, except for the phenol present in the 20mM solution, which only gave a 38% conversion.

Degradation of phenolics in authentic (cresylic/phenolic) effluents

As the biofilm had successfully catalysed the conversion of phenolics within a synthetic mixture of model phenolic pollutants, the biofilm was then exposed to a cresylic effluent containing the same four phenolic components as the synthetic mix. The initial concentrations of each phenolic component can be seen in Table 2.12. A 10% dilution of effluent in growth medium was used for experimental purposes. The conversion of the phenolics present in the effluent were slightly lower than those obtained from the synthetic mix (Table 2.13), which is to be expected as there are other components present in the effluent which may exert effects on conversion as well. Also, the biomass was exposed to a total of 16.32mM pollutant, which is much higher than previous exposures.

Table 2. 9. Exposure of biomass to a synthetic mixture of phenolics (4mM total concentration)

Model pollutant	Initial substrate concentration (mM)	Mass substrate (mg) per total volume permeate collected	Substrate concentration in permeate after 6 days (mM)	Total substrate removed after 6 days (mg)	Percentage substrate removed
Phenol	1	40.7	0.25	30.5	75
p-cresol	1	46.7	0.24	35.5	76
m-cresol	1	46.7	0.15	39.7	85
o-cresol	1	46.7	0.12	41.1	88

Permeate from bioreactor ± 72mL / day

Table 2.10. Exposure of biomass to a synthetic mixture of phenolics (10mM)

Model pollutant	Initial substrate concentration (mM)	Mass substrate (mg) per total volume permeate collected	Substrate concentration in permeate after 6 days (mM)	Total substrate removed after 6 days (mg)	Percentage substrate removed
Phenol	2.5	101.6	0.05	99.57	**98**
p-cresol	2.5	116.8	0.00	116.8	**100**
m-cresol	2.5	116.8	0.01	115.6	**99**
o-cresol	2.5	116.8	0.01	115.6	**99**

Permeate from bioreactor ±72mL / day

Table 2.11. Exposure of biomass to a synthetic mixture of phenolics (20mM)

Model pollutant	Initial substrate concentration (mM)	Mass substrate (mg) per total volume permeate collected	Substrate concentration in permeate after 6 days (mM)	Total substrate removed after 6 days (mg)	Percentage substrate removed
Phenol	5	203.3	3.1	77.3	**38**
p-cresol	5	233.6	1.02	186.0	**79.6**
m-cresol	5	233.6	1.34	170.9	**73.2**
o-cresol	5	233.6	1.11	181.7	**77.8**

Permeate flow from bioreactor ±72mL / day

Table 2.12. Concentration of components in cresylic effluent (undiluted effluent)

Phenolic pollutant	Concentration / (mM)	Concentration (g/L)
Phenol	82.80	7.8
p-cresol	24.99	2.7
m-cresol	25.80	2.8
o-cresol	77.03	8.3

Table 2.13. Conversion of phenolics in 10% cresylic effluent in bioreactor

Model pollutant	Initial substrate concentration (mM)	Mass substrate (mg) per total volume permeate collected	Substrate concentration in permeate after 2 days (mM)	Total substrate removed after 2 days (mg)	Percentage substrate removed
Phenol	7.94	322.8	6	77.5	**24**
p-cresol	2.2	102.8	1.48	33.9	**33**
m-cresol	1.76	82.2	1.22	25.5	**31**
o-cresol	4.42	206.4	2.52	88.8	**43**

Permeate from bioreactor ± 72mL / day

2.3.3 Catechol Production by *Neurospora* biomass

In batch culture experiments, attempts to detect catechol production by HPLC were largely unsuccessful. Catechols may well be produced from the conversion of the *p*-cresol and may not have been detected as the biomass may have utilized the catechols as a carbon source once the *p*-cresol had become depleted. However, the biofilms immobilized in capillary membrane reactors showed somewhat different results to the batch studies. Here the biomass was continually exposed to 2.5mM *p*-cresol and therefore the carbon source never became depleted. Catechols that were produced could readily be detected by HPLC. The growth of the biofilm was also effectively stopped or slowed down by having the substrate in a buffer solution rather than in medium, thereby preventing the catechols from being utilized by the fungal biomass. Analysis of the permeate samples by HPLC showed three different peaks eluting from the column, representing, in order of elution, quinone product peaks, a catechol product peak and the substrate peak. The catechol peak eluted with exactly the same retention time as standard methylcatechol, and the production of this product is shown in Figure 2.10. Thus, *Neurospora* biofilms can be used in the production of methylcatechol and other types of catechols. Once a catechol has been produced it may undergo spontaneous or enzymic reaction to form quinone, which would explain the low concentrations of catechol shown. Control of reactor conditions eg., flux rates and substrate concentrations could be manipulated to decrease quinone production and to enhance catechol production.

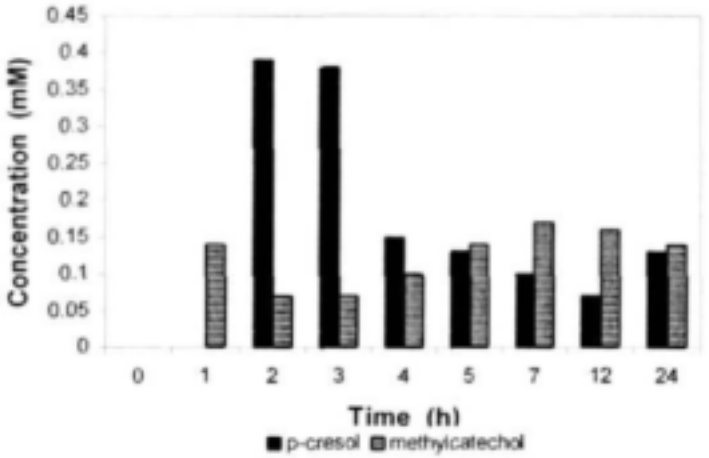


Figure 2.10. Methylcatechol production by membrane-immobilised *Neurospora* biomass exposed to 2.5mM *p*-cresol.

2.4 CONCLUSIONS

- This is the first report on the production of oxidase enzymes by capillary membrane-immobilised *Neurospora* biomass (Luke and Burton, 2000).
- Immobilised cultures of *Neurospora crassa* are capable of sustaining oxidase enzyme production continuously, whereas in the non-immobilised cultures, the activity levels are maximal after 6 days after which they decline.
- The use of *Neurospora crassa* for the bioremediation of phenols is novel.
- Membrane-immobilised cultures are capable of catalysing the conversion of 497mg/L *p*-cresol and 374 mg/L phenol, with rates of 160 mg/L/d/ g biomass.
- The biodegradative capacity of immobilised biomass could be sustained for a much longer period of time than in batch systems.
- Biodegradation could be sustained in immobilised cultures for a period of 30 days, whereas batch cultures lasted only 6 days after which they were no longer viable.
- Membrane-immobilised biofilms were effective in catalyzing the conversion of phenolics within synthetic mixtures of model phenolic compounds as well as in a cresylic effluent.
- Phenol bioconversion, by membrane-immobilised biofilms, was more consistent than reactions batch systems.
- Catechol production was achieved by membrane-immobilised cultures, but was not detected in batch cultures.

CHAPTER 3

Application of the fungus T*rametes versicolor* and its enzymes in the bioconversion of phenolics

3.1 INTRODUCTION

The objective of this section of the research was to investigate the capacity of the oxidising enzymes produced by *T. versicolor* to convert phenolics present in industrial effluents. Since the enzymes are produced extracellularly, the bioconversion reactions could be extended to the use of isolated enzyme extracts as well as whole-cell systems. Thus, both approaches were investigated. The work reported here was based on previous research (Burton et al., 1997) which is discussed in the Results and Discussion section. In summary, the previous research had indicated that the laccase of *T. versicolor* was readily produced in flask culture, and under optimal conditions high oxidase activities could be measured in the extracellular culture medium.

These results were used to develop two bioreactor systems for investigation of the capacity of the whole-cell fungal biomass for laccase production and bioconversion of some examples of industrial effluents, selected on the basis of plate culture experiments. In the next set of experiments *Trametes versicolor* cultures were challenged with four different aromatic pollutants (*viz.*, *p*-cresol, 4-chlorophenol, naphthalene and toluene), and the response of the biomass to these pollutants was monitored and analysed.

The laccase enzyme of *T. versicolor* has been found to have a broad substrate specificity, and because production of the enzyme is extracellular, extraction of the enzyme for use in biocatalytic reactions can readily be achieved. Thus, further investigation was conducted to isolate the extracellular laccase enzyme, and to observe the conversion of aromatic pollutants by the isolated crude enzyme extracts, and compare it to the conversion of the same pollutants by commercial enzyme extracts, and the whole-cell systems. This section describes the use of a crude extracellular extract from *Trametes versicolor* culture medium, containing laccase activity, for the bioconversion of some model pollutants. These compounds were selected because they

are known to be present in the effluents investigated in this research programme. However, the analysis and characterisation of organic products present in authentic industrial residues is complicated by the complexity of the matrix, and thus, model compounds were used in these initial studies.

3.2 METHODS AND MATERIALS

3.2.1 Strain preservation

A slant culture of *T. versicolor* (PPRI #3845) was obtained from the Plant Protection Research Institute in Pretoria and was maintained on 2% malt extract agar slants. These were subcultured every 60 days.

3.2.2 Liquid Culture

Trametes defined medium (TDM) (Addleman and Archibald, 1993) was found to be optimal for biomass and enzyme production. TDM contained (per litre):83 mM glucose; 5 mM glutamine; 5 mM NaCl; 5mM KH_2PO_4; 1mM $MgSO_4$; 0.1mM $CaCl_2$; 10mM 2,2- dimethyl succinate; 2.4µM thiamine. The trace metal solution contained 20µM $FeSO_4.7H_2O$; 2µM $CuSO_4.5H_2O$; 5µM $ZnCl_2$; 20µM $MnSO_4.H_2O$, 6µM $CoCl_2.6H_20$, 0.1µM $NiCl_2.6H_20$ and 0.5 µM $(NH_4)_6Mo_7O_{24}.2H_2O$.

3.2.3 Inoculation

To acclimatise the cultures to growth in liquid media, a pre-inoculum flask was prepared. In each, two *Trametes* plate cultures were diced and added aseptically to 200 mL medium in a 1 L flask. These flasks were incubated at 29 °C in an orbital shaker at 200 rpm, with a glass bead in each to prevent a mycelial mat forming. After 4 days of incubation, the contents of these flasks were homogenised in a sterile Sorvall benchtop homogeniser.

3.2.4 Measurement of Laccase enzyme activity

The filtrate from the biomass determination was retained and assayed for the presence of the laccase enzyme, by monitoring the oxidation of 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The reaction mixture contained 2.5 mL 0.1 M sodium acetate buffer, 0.33 ml 5 mM ABTS and 0.17 mL sample (Roy-Arcand *et al.*, 1991). The oxidation of ABTS was monitored by measuring the increase in absorbance at 420 nm (A_{420}) over 10 minutes. Enzyme activity was expressed in units (U) (1U = 1µmole product formed /ml /minute).

3.2.5 Authentic effluent studies:

Effluent preparation

500mL of each of the effluents was diluted by 50% using distilled water. These effluents were autoclaved and allowed to cool to room temperature before use. These solutions were then used as stock solutions for all further analysis.

Preliminary plate study

Plate experiments were conducted to determine the feasibility of growing the fungus using the various effluents as a sole carbon source. These studies were conducted using a chemically defined medium. A series of plates were made with final effluent concentrations of: 0%, 0.0788%, 0.788%, 7.88%, and 31%. The plates were innoculated with *T. versicolor* from a nutrient agar plate. The plates were incubated for 14 days at 25°C in a constant environment hood.

Preparation of liquid cultures

Only those concentrations of the various effluents that allowed growth in the plate study were utilised in this study (Table 3.2). The flask cultures were made as before, with a 10 mL inoculum, 15 mL of TDM and 25 mL of an effluent/water mix to obtain the desired concentrations. These were then incubated at 28°C with shaking for two weeks. All cultures were done in duplicate. Every 2 days destructive sampling was carried out for each effluent. Parameters measured included: dry mass, laccase activity and UV spectra to monitor the (dis)appearance of aromatic compounds (these results were later verified by HPLC analysis).

3.2.6 Membrane Bioreactor

The membrane bioreactor (Leukes et al., 1997) was operated in a dead end filtration mode in the configuration shown in Figure 3.1. The air was humidified by passing it through distilled water and pumped through the system by an aquarium pump. The membrane module was initially pre-treated with Milli-Q water to remove the glycerol wetting agent. The reactor was chemically sterilised by running 4% formaldehyde through the system overnight.

The inoculum was prepared by dicing 4 petri plate cultures of *T. versicolor* into 400 mL of growth medium and incubated for 3 days on a rotary shaker at 200rpm. It was then decanted into a modified 1L Erlenmeyer flask with an inoculation port at the base. The reactor was inoculated from the shell side using the humidified dead-end filtration mode with a peristaltic pump. The inoculum was forced into the module until a back-pressure was achieved. The pump was then switched off until the pressure subsided and the cycle was repeated. The reason for doing this was to force the homogenised mycelia onto the hollow fibers and so initiate attachment. After inoculation, growth medium was circulated into the lumen of the capillaries and the fungus left to attach for 48 hours. Thereafter feed flow rates were determined and adjusted to achieve a flux of 0.177 $L/m^2/h$.

Once a viable biofilm had been established, the ability of the bioreactor to treat an authentic waste was investigated. An industrial cresylic effluent from a local coal gasification plant was chosen as it contained various organic compounds of interest (Table 2.12). The effluent was diluted 50% with water and supplemented with additional carbon, nitrogen, minerals and trace elements.

The removal/conversion of the monomers was monitored by HPLC (Beckman System Gold), utilising a Waters S5, ODSI column with a mobile phase of 60% water and 40% acetonitrile with a flow rate of 1ml/min. The removal of the effluent monomers was analysed using a mobile phase of 85%water, 15% acetonitrile and 1.66% β-cyclodextrin, to separate the cresol isomers.

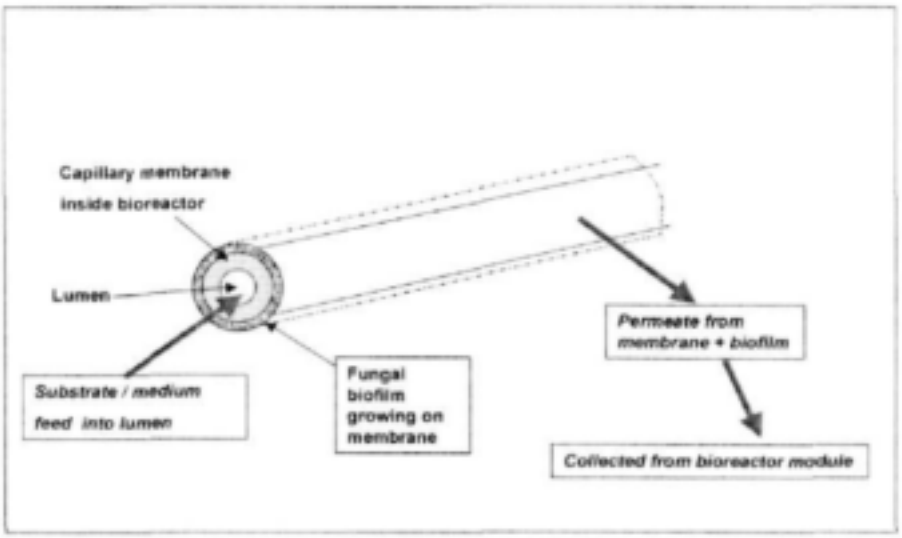


Figure 3.1. Cpillary membrane reactor set-up, indicating dead-end ultrafiltration configuration

3.2.7 Airlift reactor

A 3.5L Bioflow upflow reactor was used. The reactor was chemically sterilised by first rinsing with Biocide, and then by adding 300mL of 4% formaldehyde solution l and leaving this to evaporate in the vessel for 2 days. The vessel was then thoroughly rinsed with autoclaved distilled water. The inoculum was prepared by adding a stationary phase flask culture (50 mL) to 250 mL growth medium and then shaking at 200rpm at 30°C until a suitable density of mycelial pellets had formed. This was then added to 2.7 L of sterilised growth medium in the vessel (10% inoculum), leaving 500 mL headspace. The air was turned on and flow increased until just before foaming occurred in the reactor. Compressed air was sparged into the section of the vessel called the riser. Gas hold-up and decreased fluid density cause liquid in the riser to move upwards. Gas disengages at the top of the vessel leaving heavier bubble-free liquid to recirculate through the downcomer (Figure 3.2).

The fermentation was allowed to proceed and changes in pH, redox potential and laccase activity were monitored. Once an apparent steady state was reached it was decided to add bleach plant effluent to the reactor. The effluent was diluted to 50% with distilled water and nutrients added:

glucose 0.8%, $(NH_4)_2SO_4$ 0.16%, K_2HPO_4 0.3% and KH_2PO_4 0.2% with mineral salts and thiamine as before. The pH was adjusted to 5.0 with HCl. Once again pH and redox potential were monitored and samples taken for HPLC analysis.

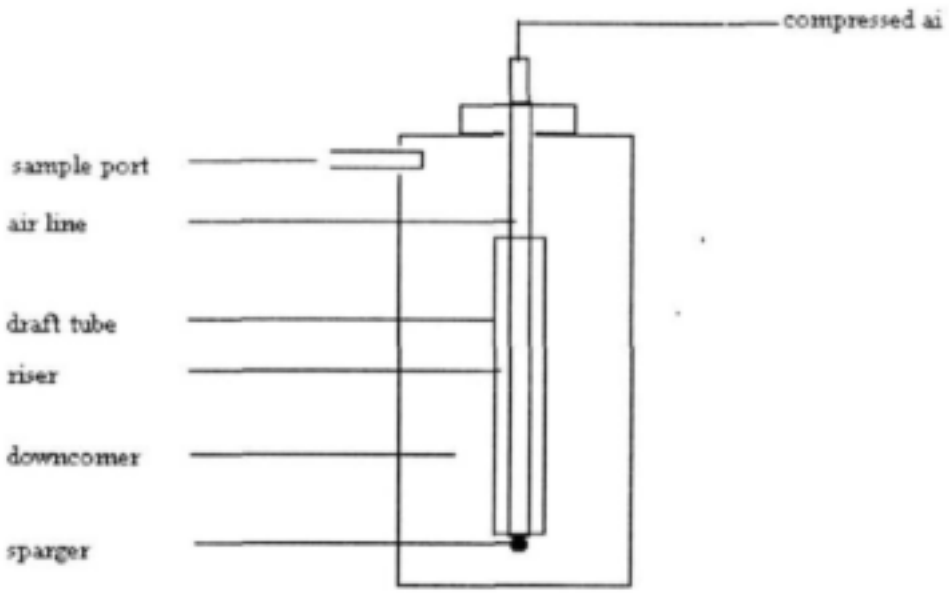


Figure 3.2. Schematic of the uplift reactor configuration

3.2.8 Biotransformations of model pollutants

Preparation of whole cell extract

Four 2 L static cultures of *T. versicolor* were grown to stationary phase in flasks. Harvesting of the cells was carried out by centrifugation (Beckman, J2-J21 centrifuge), in pre-weighed centrifuge tubes (7000 rpm, 10 minutes). The supernatant was decanted and the cells washed twice in half volumes of 0.1 M sodium acetate buffer pH 5. The drained tubes were then re-weighed, and the wet cell mass calculated:

Wet cell mass (mg) = [Final mass (mg) – Initial mass (mg)]

The pellet was then resuspended in the required volume of 0.1 M sodium acetate buffer, pH 5, to yield a 40 mg/mL suspension.

Volume of buffer (mL) = wet cell mass (mg)/40.

This cell suspension was used immediately for the resting cell reaction.

Resting cell reaction

Resting cell reactions were carried out using a 40mg/mL cell suspension, 0.1 M sodium acetate buffer (pH 5) and four different pollutants, namely; *p*-cresol, 4-chlorophenol, naphthalene and toluene. 7.5 mM stock solutions of *p*-cresol and 4-chlorophenol were made up in 0.1 M sodium acetate buffer, pH 5, while the naphthalene and toluene stock solutions were made up in ethanol.

Whole cells, pollutant and buffer were added together to give a final volume of 2 mL, the final concentrations of the pollutants being 1.25 mM, 2.5 mM and 3.75 mM. The reaction was allowed to proceed for 4 h at 28 ^{0}C with gentle rocking. After this time the reaction was centrifuged to remove cell debris. 1 mL of the supernatant was then extracted with 1 mL of chloroform. The chloroform was then evaporated almost to dryness and the concentrate used for thin layer chromatography (TLC).

Analysis by thin layer chromatography (TLC)

Thin layer chromatography was carried out using silica gel 60 F_{254} plates (Merck). The mobile phase used was ethanol-chloroform (1:1).

Growth in flask culture

One hundred 250 mL Erlenmeyer flasks were inoculated and incubated as static cultures in a constant environment (CE) room at a temperature of 28 ^{0}C. Care was taken to disturb the flasks as little as possible after inoculation, so as not to disturb mycelial pellet formation. On day 12 (stationary phase), the flasks were spiked with three different concentrations of four aromatic pollutants (p-cresol, 4-chlorophenol, naphthalene and toluene). Stock solutions of 90 mM were made up for all four aromatic pollutants and added to the 60 mL growth media with established mycelial mat to yield final concentrations of 2.5 mM, 5 mM and 7.5 mM. Directly after spiking, 0.5 mL samples were taken, in triplicate, from flasks of each of concentration and the controls: biomass but no pollutant, pollutant but no biomass and dead biomass with pollutant. 0.5 mL samples were taken at time intervals of 0 h, 2 h, 4 h, 6 h, 12 h, 1 d, 2 d, 3 d, 6 d and 9 d, and thus the whole experiment was run for a total of 21 d. The samples were frozen and kept for HPLC analysis.

Dry mass determination

Destructive sampling (in triplicate) of flasks with each pollutant and at each concentration, was carried out on day 0, 3 and 9 after spiking with pollutants. The contents of the flasks were filtered through pre-dried and weighed Whatman #1 filter paper under pressure from a vacuum pump. The filter papers containing biomass were then incubated for 48 h at 37 ^{0}C to dryness and then re-weighed. The dry mass of the mycelium was calculated by subtracting the initial mass of the paper from the final.

Physiology assessment

Visual assessment of the physiology and morphology of the cultures were monitored throughout the experiments to determine the effects the pollutants have on physical appearance of the fungus.

Analysis by high performance liquid chromatography (HPLC)

HPLC analysis was carried out on the flask cell cultures with naphthalene and toluene, to monitor a decrease in the pollutant over a period of time. The HPLC system used was Beckman, System Gold using a reverse phase C_{18} column. The mobile phase for naphthalene and toluene was 2:8 water-acetonitrile.

3.2.9 Biotransformations using enzyme extract

Preparation of freeze-dried laccase extract

Permeate, obtained from an experiment in which peptone was used as the nitrogen source, was used to prepare the freeze-dried laccase extract. Destructive sampling of 10 500 mL flask cultures was carried out. The contents of the flasks were spun at 7000rpm for 10 min in a Beckman J2-J21 centrifuge. The supernatant (approx. 1L) was freeze-dried in liquid nitrogen, the freeze-dried powder was then stored at 4 °C until needed. Laccase activity was measured before and after freeze drying (see Appendix B).

Enzyme activity of crude extract and commercial enzymes

Enzyme activity (U/mL) and specific activity (U/µg) were determined for commercial horseradish peroxidase (HRP) (Serevac), commercial laccase (Sigma) and the freeze-dried

laccase extract from the local *T. versicolor* strain (see Appendix B). Protein determination was carried out using the method of Bradford (Smith, 1987).

Biocatalytic reaction using only crude laccase extract

Biocatalytic reactions were carried out using freeze-dried laccase powder, buffer and four different pollutants, namely; *p*-cresol, 4-chlorophenol, naphthalene and toluene. 80 mM stock solutions of *p*-cresol and 4-chlorophenol were made up in 0.1 M sodium acetate buffer, pH 5, while the naphthalene and toluene stocks were made up in ethanol. A 1mg/mL solution of freeze-dried enzyme powder was made using 0.1 M sodium acetate buffer, pH 5.

Stock solutions of enzyme (1 mg/mL) and pollutant (80 mM) were added together to give a final volume of 2 mL. Final enzyme concentration was 0.5 mg/mL and final concentrations of the pollutants, 20 mM and 40 mM. The reaction was allowed to proceed for four hours at 28 °C with gentle rocking. After this time the reaction was centrifuged to remove any enzyme debris. 1 mL of the supernatant was then extracted in 1 mL of chloroform, and the chloroform extract was then evaporated almost to dryness, and the concentrate used for thin layer chromatography (TLC).

Analysis by thin layer chromatography (TLC)

Thin layer chromatography was carried out using silica gel 60 F_{254} plates (Merck). The mobile phase used was 50/50 ethanol:chloroform.

3.2.10 Biocatalytic reaction with crude laccase extract and the commercial enzymes, horseradish peroxidase and laccase

Biocatalytic reactions were carried out using three enzymes; crude laccase extract, commercial HRP (Serevac) and commercial laccase (Sigma), and four aromatic pollutants. The reactions were allowed to proceed for four hours with sampling after 2 and 4 hours. Final concentrations of pollutants were as follows: 5 mM *p*-cresol, 1 mM 4-chlorophenol, 1 mM naphthalene, 1 mM toluene. Enzyme units used were, as follows: HRP 0.4046 U, commercial laccase 0.0934 U and *T. versicolor*, laccase 0.03584 U. Solutions were made up in their respective buffers, with a final reaction volume of 10 mL. For HRP reactions 0.003% H_2O_2 was added at the beginning of the

reaction and again after two hours. The second addition of H_2O_2 is because H_2O_2 is toxic to the enzyme in large amounts and two additions eliminate this problem. The experiments using laccase enzyme had an extra variable; the reaction was carried out in the presence of ABTS (in a ratio of 1:100 ABTS:pollutant) and absence of ABTS.

Analysis by high performance liquid chromatography

HPLC analysis was carried out on the biocatalytic reactions, at he time of sampling, to monitor a decrease in the pollutant over the period of 4 h. The HPLC system used was Beckman, System Gold using a reverse phase C_{18} column. The mobile phase for p-cresol and 4-chlorophenol was 6:4 water-acetonitrile, while the system used for naphthalene and toluene was 2:8 water-acetonitrile.

Analysis by ultra violet spectrophotometry

UV scans were carried out on biocatalytic reaction mixtures after 4 h to determine the wavelength of potential products and if products were produced. The scan was generated between wavelengths 200 to 800 nm on a UV-160A Shimadzu spectrophotometer.

3.3 RESULTS

3.3.1 Previous research

Previous research results (Burton et al., 1997) can be summarised as follows:

- The fungus could be grown optimally on *Trametes* defined medium (TDM) (Addleman and Archibald, 1992) and was found to reach stationary phase after 7 d and maximum enzyme production of 1.14 U, when induced.
- Addition of an aromatic compound, *p*-cresol, to stationary cultures resulted in physiological changes in the fungus, manifesting in marked changes in morphology and appearance of the biomass and culture medium.
- Total microbial activity, as measured by the hydrolysis of fluorescein diacetate to fluorescein, maintained a linear relationship with accumulated biomass. A ratio of microbial activity to biomass was found to actually increase with increasing concentrations of the pollutant (*p*-cresol).

- Addition of *p*-cresol was found to either induce production, or increase the activity of, the oxidative enzymes (laccase) produced by *T. versicolor*.
- Flask cultures of *T. versicolor* was found to effectively remove varying concentrations of *p*-cresol from solution (Table 3.1).
- *p*-Cresol was also effectively and continuously removed from solution by *T. versicolor* immobilised in a transverse flow capillary membrane bioreactor.

Table 3.1. Removal of *p*-cresol from solutions at different initial concentrations, by *T. versicolor* in flask culture

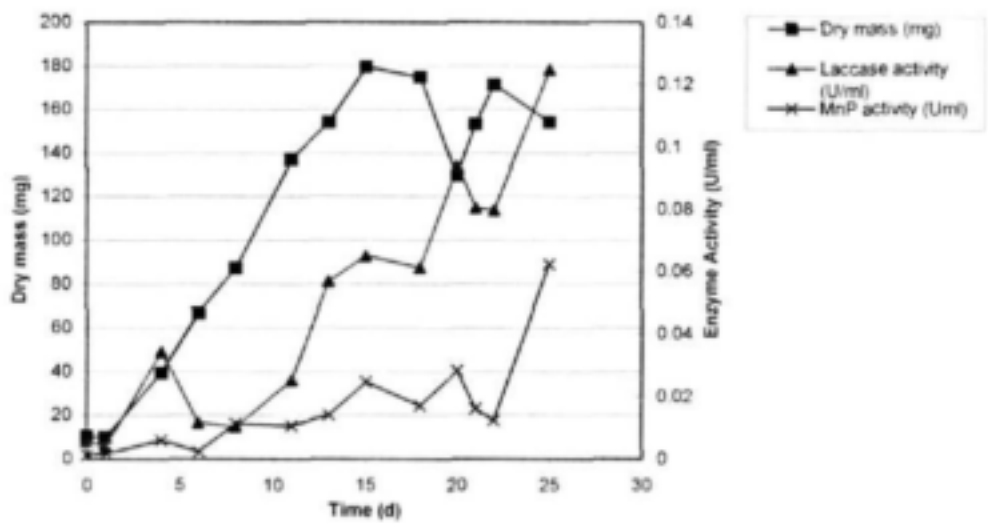
Initial [cresol] (mg/mL)	Cresol removal (%)		Total cresol removed (mg/mL)	
	Day 3	Day 12	Day 3	Day 12
0.1	80	100	0.08	0.1
0.25	95	100	0.24	0.25
0.50	95	100	0.48	0.5
0.80	67	92	0.536	0.74
1.0	65	89	0.65	0.89

3.3.2 Growth of *T. versicolor* and enzyme production

The growth of the *T. versicolor* cultures was correlated with production of laccase and peroxidase (Figure 3.3). Growth curves of the local *Trametes* strain were carried out over a period of 21 to 25 d. At certain time intervals, dry mass and enzyme activities for laccase and manganese peroxidase were determined according to the methods listed above.

It was apparent that enzyme production followed that of the rate of growth of the fungus itself up to stationary phase. When growth reached stationary phase, laccase, as well as manganese peroxidase (MnP) enzyme activities attained their peak. Growth was followed for 25 d, with the enzymes reaching a peak at day 20, followed by a decline and a second peak at day 25. The enzyme maximum was reached somewhat later than had been obtained in previous work carried out by Ryan, (1996). In the experiments carried out by Ryan (1996), maximum laccase enzyme production occurred at day 11. The fact that the formation of the surface mat and enzyme production took longer to form (20 days) can be ascribed to the fact that the original inoculum plates were older and hence in a less active growth state.

Figure 3.3. Growth of *Trametes* in TDM, also showing laccase and MnP activity



The use of a cheaper nitrogen source was investigated, by using TDM with ammonium sulphate. Using ammonium sulphate as a nitrogen source appeared to slow down the growth but did however, increase laccase production (Figure 3.4) Laccase production reached a maximum at day 13, while manganese peroxidase (MnP) appeared to stay more or less constant between days 10 and 13. High laccase production was probably due to the nitrogen source, (ammonium sulphate) being less available to the biomass for utilisation. Laccase activity for TDM reached a maximum value of 0.125 U/mL while for TDM(ammonium sulphate), the maximum activity was recorded as 0.185 U/mL.

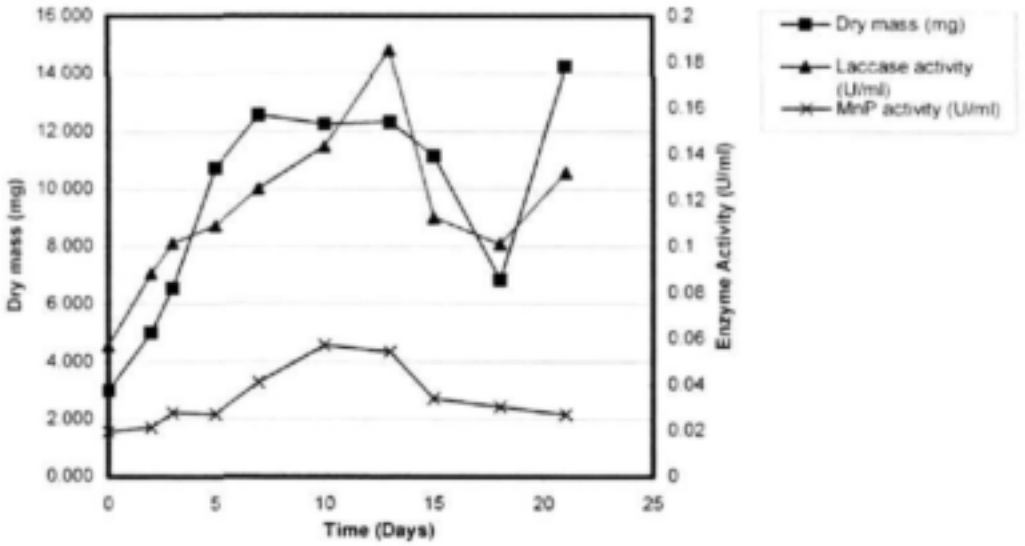


Figure 3.4. Growth of *T. versicolor* in TDM(ammonium sulphate), also showing laccase and MnP activity.

3.3.3 Authentic effluent studies

In a survey to investigate growth in various effluents, *T. versicolor* was only grown on plates supplemented with the effluents (Table 3.2). The fungus was grown successfully on the SAPPI effluents. This is not an unexpected result given the nature of the constitutive enzyme suite of this fungus and its role in nature. A possible shortcoming in this experiment is that the fungus was inoculated directly into the effluents; it may have been advisable to first grow it up in a more favourable growth medium, and then expose it to the undoubtedly stressful conditions imposed by the effluents. This refers particularly to the olive mill wastes and the cresylic effluents. The growth was then measured in flask culture (Table 3.3, Figure 3.4)

Very little can be concluded from the absolute values of the data presented. However, reduction in absorbance maxima, increases in dry mass and general increases in enzyme activities with time indicate that the effluent components are being altered in some way (Figure 3.5).

Table 3.2. Growth of *T. versicolor* on effluent supplemented plates

	Effluent concentration (%)				
Effluent type	0	0.0788	0.788	7.88	31
1	++++	-	-	-	-
2	++++	+	-	-	-
3	++++	-		-	-
4	++++	-	-	-	-
5	++++	+	+	+++	++++
6	++++	+	++++	+++	-
7	++++	+	+	-	-

Effluents used (see Appendix 1):

1) Sorrento (olive)
2) Cresylic
3) La Valle (olive)
4) Black product
5) Bleach Plant
6) Digester
7) Fermentation brine

Key: ++++ excellent growth
+++ good growth
++ moderate growth
+ poor growth
- no growth

Table 3.3. Growth characteristics of *T. versicolor* in flask culture uitlising the most successful effluents from preliminary plate studies; percentage values indicate percent of the solution made up by the effluents.

	Bleach Plant (10%) {tc "**Bleach Plant (10%)** " \l 4}			**Bleach Plant (50%)**			**Digester (1%)**			**Digester (10%)**		
Time	**Abs**	**Enz**	**DM**	**Abs**	**Enz**	**DM**	**Abs**	**Enz**	**DM**	**Abs**	**Enz**	**DM**
D_0	1.76	-	-	2.03	-		2.09	-		2.50	-	-
D_2	1.34	0.05	0.08	2.49	.005	0.03	0.57	.004	0.01	2.44	-	0.09
D_5	1.5	0.12	0.09	1.9	.009	0.03	2.09	0.08	0.05	2.13	-	0.04
D_9*	2.49	0.03	0.07	1.99	0.03	0.04	2.14	0.18	0.09	2.49	0.15	0.2
D_{14}	1.4	0.11	0.1	1.56	0.04	0.05	1.82	0.24	0.09	2.49	0.09	0.22

Key: Abs. – absorbance maximum in the UV range
Enz – laccase enzyme activity (U)
DM – dry mass (g) * definite pellet formation in the wet mass obtained from the digester effluent

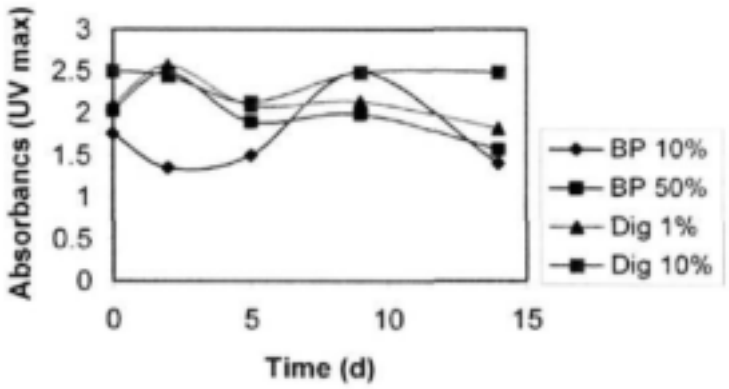


Figure 3.5a. Changes in absorbance of maximum peaks in the ultraviolet range of the 4 test effluents, where: BP: Black Product and Dig: Digester effluent. Percentage values indicate percent of the solution made up by the effluents.

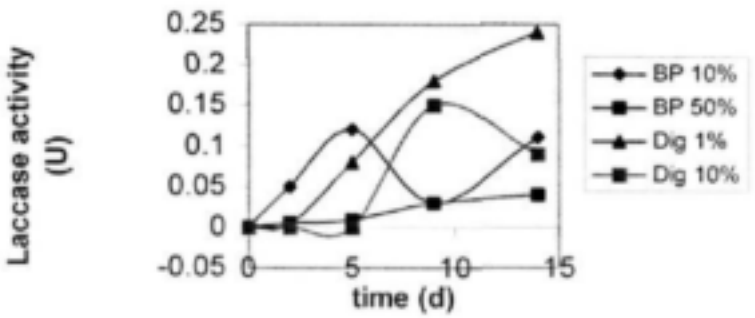


Figure 3.5b. Increase in laccase activity over time, where: BP: Black Product and Dig: Digester effluent. Percentage values indicate percent of the solution made up by the effluents.

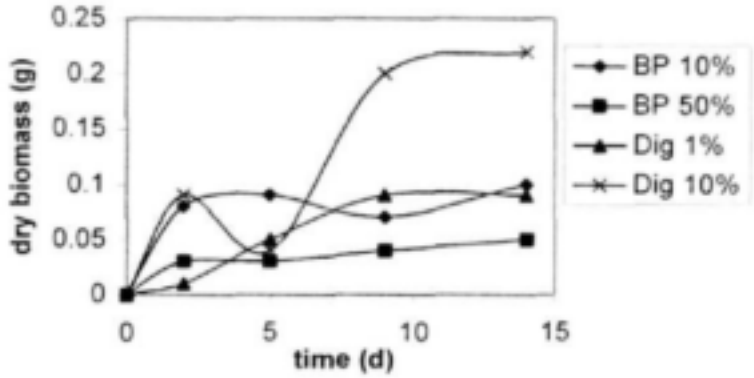


Figure 3.5c. Growth of *T. versicolor* as indicated by increases in dry mass over time, where: BP: Black Product and Dig: Digester effluent. Percentage values indicate percent of the solution made up by the effluents.

HPLC analysis was carried out on the two most concentrated samples, namely 50% bleach plant effluent and 10% digester effluent. Because of the complexity of the effluents, only changes in the total peak areas are reported to indicate changes occurring in the effluents due to the action of *T. versicolor* (Table 3.4). There was an initial decrease in the sum of the peaks and this was followed by a slight increase, possibly due to repolymerisation of the monomers over time.

Table 3.4. Changes in total peak area for the concentrated effluents.

	Bleach Plant (50%)	**Digester (10%)**
Control	174	218
Day 2	135	123
Day 14	190	167

3.3.4 Use of airlift reactor to grow T. versicolor

Attempts were made to establish a fermentation of *T. versicolor* in a 3.5L pneumatic uplift fermenter. On the basis of results from the flask culture experiments, the pulp-and-paper bleach plant effluent was chosen as a model pollutant to treat. As the fungus grows it lowers the pH and raises the redox potential in its environment. These parameters were measured over time as growth indicators (Figure 3.6). The cultures establishes itself (days 0-2), then reaches steady

state (days 2-6) . At day 7 the nutrients begin to run out and growth slows. The effluent was added on day 8 and the culture resumes exponential growth (days 8-12) until a steady state is again achieved (days 12-15). This experiment indicates that *T. versicolor* can be grown in a fermentation system.

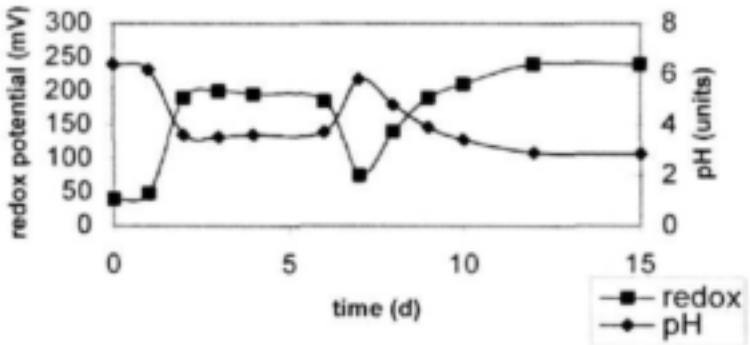


Figure 3.6. Growth progression of *T. versicolor* in an uplift reactor as measured by changes in pH and relative redox activity

The enzyme activity in the reactor was also measured; as was observed in previous studies on the capillary membrane reactors (Burton et al., 1998), there is little or no free enzyme activity measured in the reactor. The reason for this is not known at this time. The performance of airlift reactors is influenced significantly by the design of the vessel. For example, the distance between the lower edge of the draft tube and the base of the reactor alters the pressure drop in this region and affects liquid velocity and gas hold-up. The depth of the draft tube submersion from the top of the liquid also influences mixing and mass transfer characteristics. These and other parameters will be varied in a factorial analysis to optimise performance in our system.

Other parameters measured in the uplift reactor were the changes in turbidity and the decrease in colour of the bleach plant effluent with time. The results showed a 75% decrease in turbidity and a 27% decrease in colour in the 15 d assay period. The HPLC analysis of the changes occurring in the reactor follow a similar trend to those observed in flask culture: an initial decrease in total peak area, followed by a gradual increase. The peak area in the 7-17 minute range was decreased significantly (this being the time interval during which the phenols elute).

Table 3.5 Removal of colour and turbidity from bleach plant effluent with time.

Time (d)	Colour (Abs_{465nm})	Turbidity (Abs_{665nm})
1	1.025	0.190
3	1.153	0.105
5	0.889	0.059
7	0.758	0.053
9	0.768	0.052
11	0.699	0.050

3.3.5 Membrane bioreactor

Considering the success of the capillary membrane bioreactor in removing *p*-cresol from solution, it was decided to investigate treatment of an authentic industrial effluent. The coal-to-oil process cresylic effluent proved to be an ideal test case and most of the aromatics of interest were continuously removed from solution (Figure 3.7) over a period 10 d. The exception was *o*-cresol which was only removed in the initial stages, up to day 5. The slight decrease in removal efficiency for the rest of the effluents also around day 5 is unexpected and may be a saturation effect. The HPLC results are an average of duplicate samples. These results have formed the basis for a separate WRC project (K5/1129), initiated in 2000, and focused specifically on development of bioreactors for application of T. *versicolor in* bioremediation of aromatic-polluted waters.

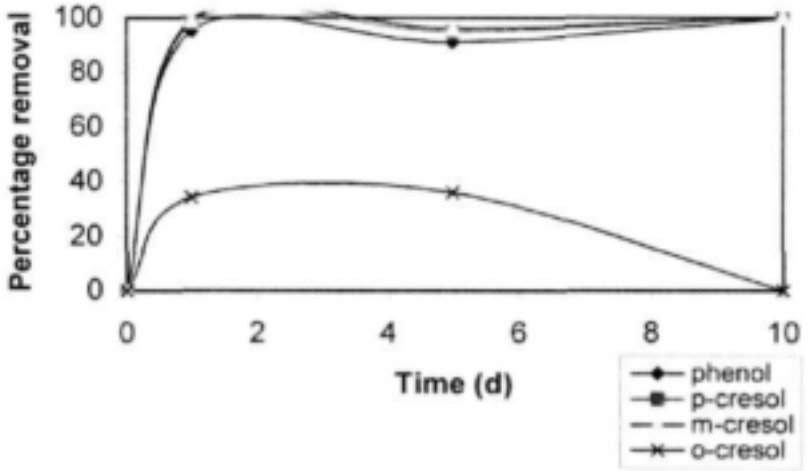


Figure 3.7 The removal of mixed aromatic pollutants from a cresylic effluent.

3.3.6 Application of *T. versicolor* in bioconversion of model aromatic pollutants

Resting cell reactions

Resting cell reactions, where biomass was added to solutions of model pollutants in buffer, were used to determine whether the whole system could convert the pollutants. A range of concentrations of the various pollutants was tested, and TLC analysis was used for initial analysis. *p*-Cresol was demonstrated to be converted by the whole cells when the pollutant was present at a concentration of 7.5 mM (0.8 mg/L).

Growth in flask culture

Flask culture experiments were carried out to determine the effect of the pollutants on the fungus morphology, and to determine whether the fungus was utilising the pollutants. This system should behave differently to that of the resting cell reaction as the cells were in the presence of growth media and not only buffer.

Dry mass determination

Dry mass determination is an indication as to whether or not the fungus was still growing (i.e. an increase in biomass) in the presence of the pollutant. Thus, it can determined whether the

pollutant was having an effect on the growth of the fungus. Dry mass was measured in control samples as well as experimental samples on days 0, 3 and 9.

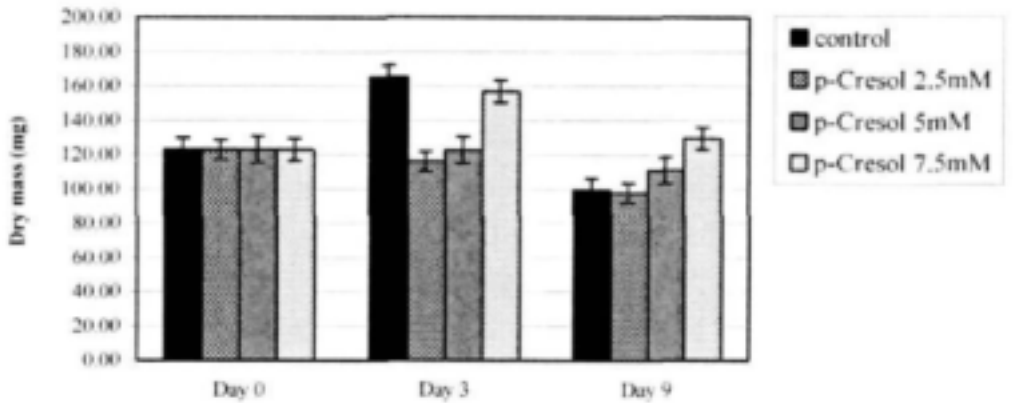


Figure 3.8. Dry fungal biomass produced in flasks containing *p*-cresol and control flasks containing biomass but no pollutant

Dry mass in the control increased in the first three days as did the experiments containing 7.5 mM (0.8 mg/L) *p*-cresol. The two other experimental concentrations decreased slightly or remained the same. It had been expected that the lower concentration of *p*-cresol would have no effect on growth and that the 2.5 mM (0.27 mg/L) experiments would increase in growth for the first three days, using the cresol as an additional carbon source. After 9 d the biomass of the control experiments decreased as did the experimental cultures containing the *p*-cresol. At this stage, the fungi in the control experiment were probably running out of nutrients and the *p*-cresol experiments were adapting to be able to use the *p*-cresol as a carbon source. Overall, the percentage decrease in biomass for the experimental flasks was less than that of the control experiments, with an increase in biomass in the experiments that contained 7.5 mM *p*-cresol. Thus, fungal biomass was able to convert *p*-cresol by using it as a carbon source.

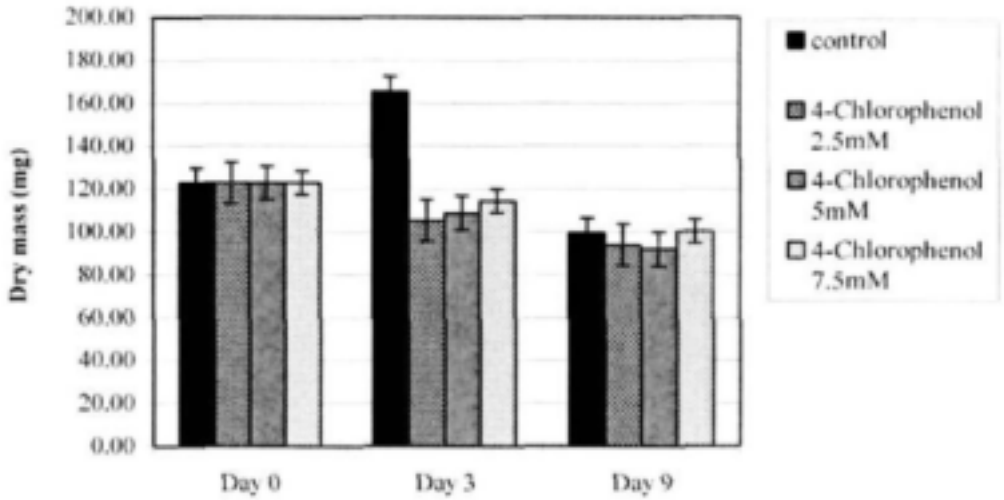


Figure 3.9 Dry fungal biomass produced in flasks containing 4-chlorophenol and control flasks containing biomass but no pollutant

When 4-chlorophenol was used as substrate (Figure 3.10), it seemed to affect the biomass within the first three days of spiking. While the control biomass increased over the 3 d period, the treated cultures decreased. After 9 d, the control biomass decreased, probably due to lack of nutrients, while the biomass in experimental cultures declined more, due to 4-chlorophenol presence as well as decrease in nutrients. Thus, 4-chlorophenol was apparently toxic to the fungal biomass at these concentrations.

When naphthalene was used as substrate, the dry mass for in the experimental flasks decreased dramatically during the first 3 d. The biomass in the naphthalene experimental flasks declined further than the control flasks, due to lack of nutrients and toxic effects of the naphthalene.

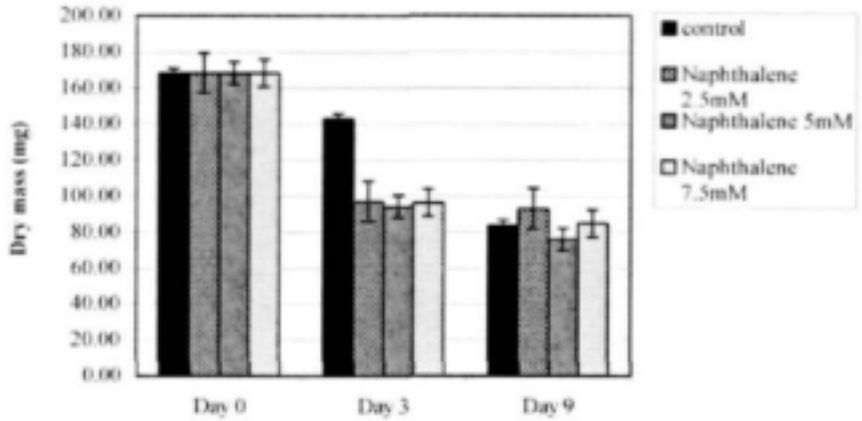


Figure 3.10 Dry fungal biomass produced in flasks containing naphthalene and control flasks containing biomass but no pollutant

Toluene did not appear to affect growth of the fungus as both the control and experimental cultures showed an increase in dry mass after the first 3 d (Figure 3.11). Thereafter, both showed a decline, probably due to lack of nutrients and not necessarily because of the presence of the toluene.

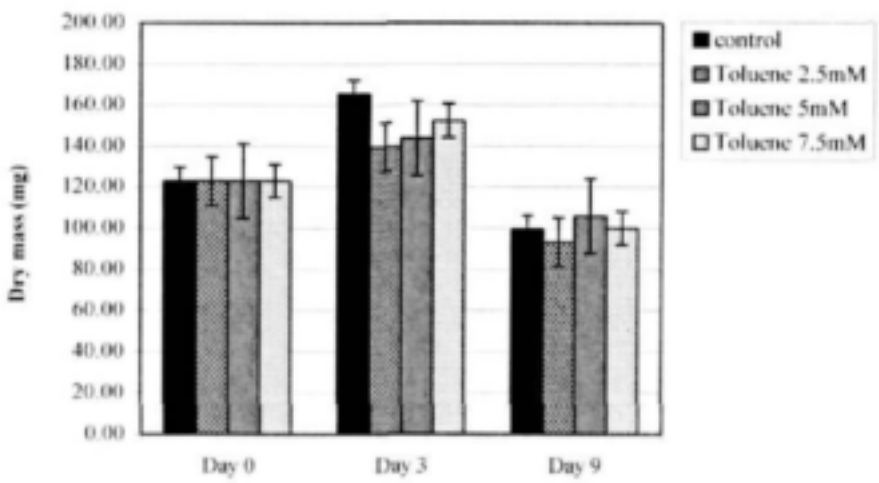


Figure 3.11 Dry fungal biomass produced in flasks containing toluene and control flasks containing biomass but no pollutant.

Physiological assessment

The physical appearance of the fungal cultures was observed over the period of the experiments, and below is a summary of characteristics noted for the three different concentrations of pollutants (i.e. 2.5 mM, 5 mM and 7.5 mM). Directly after spiking of the flasks with the various pollutants, no change in the physical appearance of the cultures was observed. This was used as the reference point for subsequent physiological assessments. After a period of 3 d, the appearance had changed significantly. In general the biomass started to degrade, therefore making the medium appear opaque. After 9 d of incubation of the fungus with pollutant, it was clear to see that the biomass had been challenged. The control experiments contained healthy biomass that only deteriorated after 9 d due to nutrient stress.

Table 3.6. Effects of pollutants on biomass morphology and medium appearance

Day 0

Pollutant	Biomass morphology	Medium appearance
p-Cresol	Dense, white and wooly	Fluid, clear and colourless
4-Chlorophenol	Dense, white and wooly	Fluid, clear and colourless
Naphthalene	Dense, white and wooly	Fluid, clear and colourless
Toluene	Dense, white and wooly	Fluid, clear and colourless

Table 3.7 Effects of pollutants on biomass morphology and medium appearance

Day 3

Pollutant	Biomass morphology	Medium appearance
p-Cresol	Retracting biomass, becoming thin and yellowish colour	Murky and yellowish in colour, becoming viscous
4-Chlorophenol	Retracting biomass, becoming thin and brown in colour	Quite clear, but orange in colour, viscous
Naphthalene	Retracting biomass, very thin, becoming transparent	Milky colour and very viscous
Toluene	Dense, white and wooly	Murky and slightly yellowish in colour, fluid

Table 3.8 Effects of pollutants on biomass morphology and medium appearance

Day 9

Pollutant	Biomass morphology	Medium appearance
p-Cresol	Retracted biomass, very thin and yellowish in colour	Cloudy, viscous and yellow in colour
4-Chlorophenol	Retracted biomass, very thin and orange brown in colour	Orange brown in colour and viscous
Naphthalene	Retracted biomass, very thin and transparent	Milky colour, very viscous
Toluene	Slightly retracted and white in colour	Murky and slightly yellowish in colour, becoming viscous

These results can be summarized as follows:

1) Static flask culture experiments using *p*-cresol showed that growth was sustained in the medium containing *p*-cresol for a few days, and thereafter the system became stressed and the cell-mat began to break up.
2) Static flask cultures containing 4-chlorophenol showed that 4-chlorophenol hinders growth of the biomass and eventually stresses the system such that the biomass breaks up and the medium becomes more viscous.
3) Static flask cultures with naphthalene showed that growth of the fungus is inhibited by the presence of naphthalene and the biomass becomes very thin and transparent.
4) Static flask cultures with toluene suggested that growth of the fungus was not affected initially by the toluene, but subsequently the growth was inhibited.

Use of laccase extracted from T. versicolor growth medium

Activity of freeze-dried laccase extract

Laccase was obtained from the growth medium which was freeze-dried, and then assayed as a crude enzyme extract. The enzyme extract was still very active even after freeze-drying (Table 3.9).

Table 3.9 **Laccase activity of permeate from *Trametes versicolor*, before and after freeze drying.**

Sample	ΔA/min	Activity U/mg
Before freeze drying	1.812	0.888
After freeze drying	1.462	0.717

3.3.7 Enzyme activity of commercial enzymes and crude *Trametes* extract

Biotransformations of model pollutants were carried out using this extract, and comparing substrate conversion with that effected by other oxidative enzymes. In a comparison of the crude extract with other commercial enzymes (Table 3.10), commercial HRP was found to be extremely active and had to be diluted 1000 times to calculate enzyme activity. The commercial laccase had lower activity than was expected and considerably high protein content, especially when compared to the crude laccase extract. For convenience in the biocatalytic reactions, the following quantities were chosen:

200 µL of 1000x diluted commercial HRP (0.4046 U),

200 µL of undiluted commercial laccase (0.0934 U) and,

50 µL of undiluted crude laccase (0.03584 U).

Table 3.10 **Enzyme activity and specific activity of commercial HRP and laccase, and for crude laccase from *Trametes versicolor*.**

Enzyme	Activity U/ml	Protein µg/ml	Specific activity U/µg
Commercial HRP (Serevac)	1657	34.220	48.42
Commercial laccase (Sigma)	0.4672	9.812	0.0476
Crude extract (*T. versicolor*)	0.7168	5.559	0.1289

Conversion of model pollutants by laccase and HRP

Bioconversion reactions were carried out to measure conversion of the model pollutants by laccase and HRP. The changes in pollutant concentrations were monitored for 4 h. HPLC analysis was carried out at time 0, 2 and 4 h, to monitor decreases in pollutant concentration, and to detect product peaks. Each pollutant was allowed to react with the three different enzymes, with the laccase enzyme reactions being carried out in the presence and absence of ABTS as cosubstrate. The reaction mixtures were also analysed by UV spectrophotometry, where changes in the spectra in the UV and visible region would also indicate products with different colour or absorbance being formed.

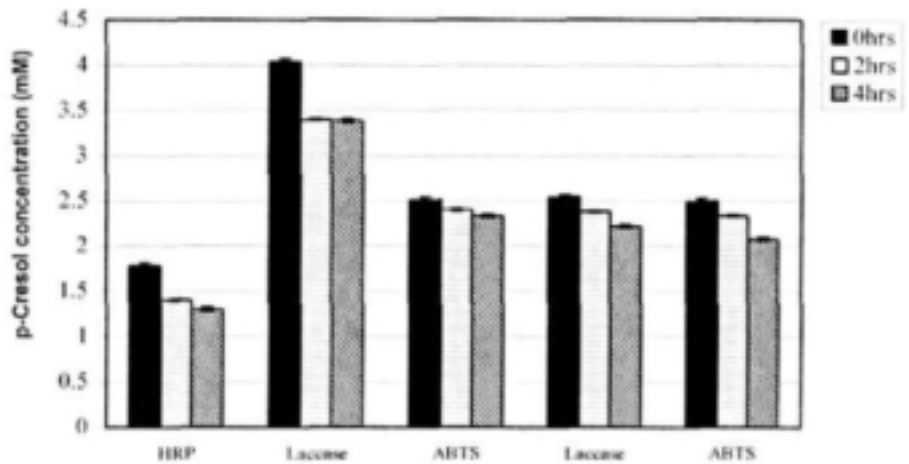


Figure 3.12 Changes in *p*-cresol concentration (mM) over 4 h reaction with *T. versicolor* laccase.

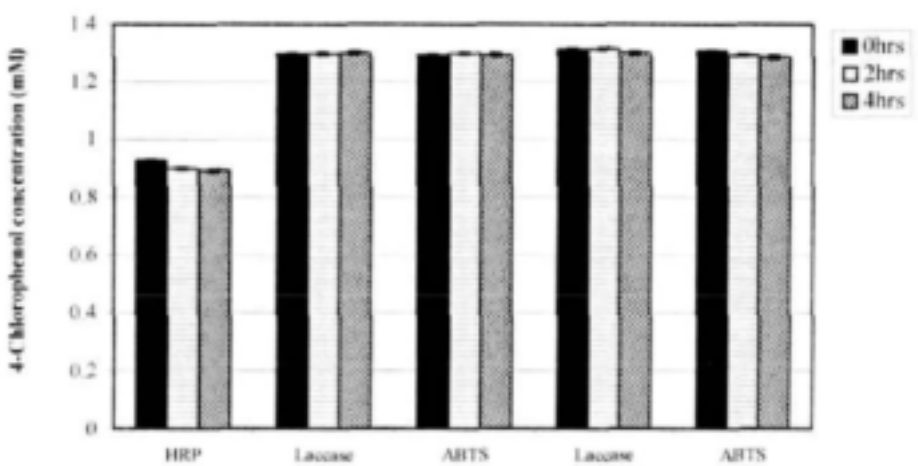


Figure 3.13 Changes in 4-chlorophenol concentration (mM) over 4 h reaction with *T. versicolor* laccase.

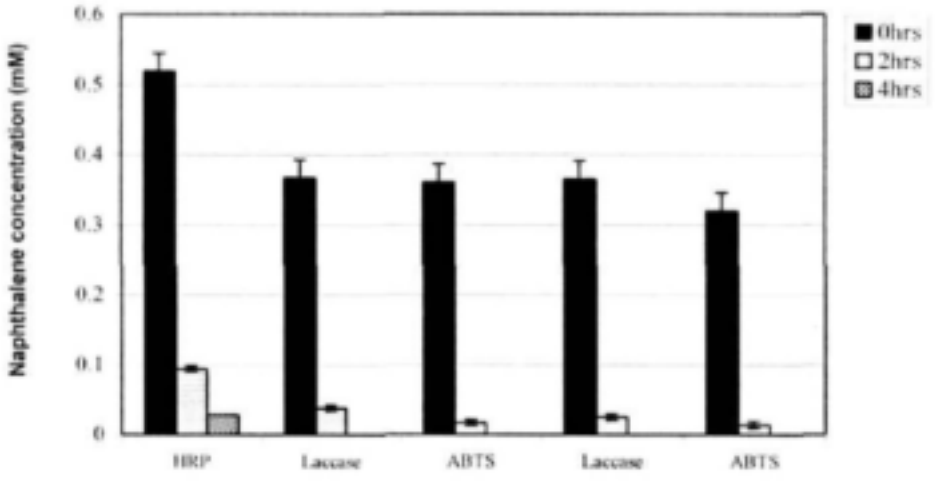


Figure 3.14 **Changes in naphthalene concentrations (mM) over 4 h reaction with *T. versicolor* laccase.**

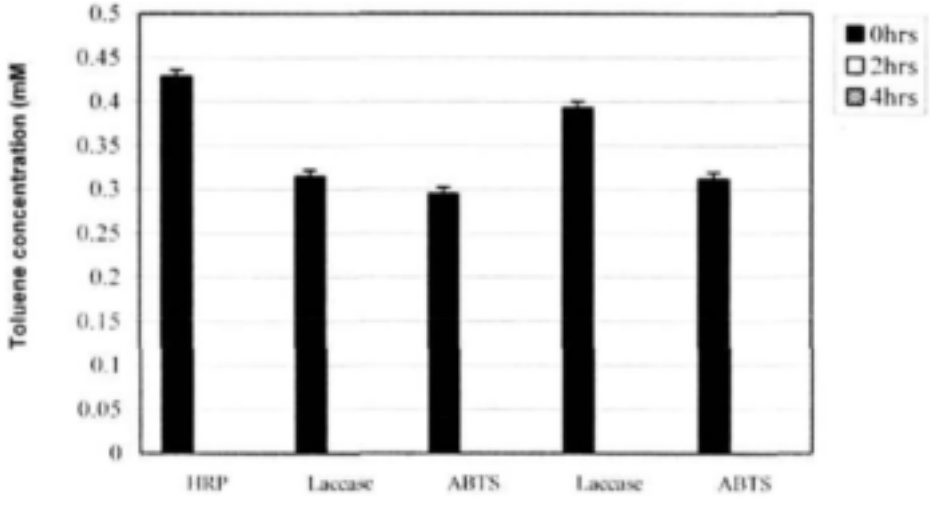


Figure 3.15 **Changes in concentration (mM) of toluene over 2 h reaction with *T. versicolor* laccase.**

Table 3.11 Percentage decrease of pollutants by the different enzymes after 4 h.

Pollutants	HRP	Laccase	Laccase + ABTS	Crude laccase	Crude laccase + ABTS
p-Cresol	18.9	11.8	2.76	8.3	12.4
4-Chlorophenol	2.7	0	0	1.1	1.6
Naphthalene	0	2.1	2.1	2.1	2.1
Toluene	0	0.2	0.2	0.2	0.2

p-Cresol conversion

A decrease in *p*-cresol concentration occurred within the 4 hour period, with HRP being the most reactive, followed by the crude extract of laccase in the presence of ABTS. ABTS acts as a single electron donor and activator of the enzyme (termed a mediator), and is therefore itself, oxidised by the enzyme (Johannes *et al.*, 1996). Once the ABTS has been oxidised, it then oxidises the substrate. During the reaction, the laccase solutions containing ABTS changed to a green colour within the first few minutes of addition of enzyme; this is due to the oxidation of the ABTS. The reaction carried out in the absence of ABTS still occurred, but took longer than when the ABTS was present. After a period of about 30 minutes (1h when no ABTS was present) the solution become a pink-purple colour, indicating the formation of product.

4-Chlorophenol conversion

There was a small percentage of conversion of the 4-chlorophenol by HRP and the crude laccase enzymes. No decrease in concentration was observed by commercial laccase. After addition of laccase enzyme to the reactions containing ABTS, the solution once again turned a light green colour due to the oxidation of the ABTS. After about 30 minutes, the enzyme reaction using crude laccase extract in the presence of ABTS changed from pale green to pale lilac, again indicating the possibility of product formation.

Naphthalene conversion

Naphthalene that was added to the control experiments (i.e. no enzyme present) showed almost 100 % decrease in concentration in all cases. This indicates that the naphthalene may have been sticking to the glassware, despite continuous gentle shaking.

Toluene conversion

Although there was a decrease in concentration of 100 %, this decrease was thought to be due to evaporation of the toluene within the first two hours and not due to enzyme action.

UV spectral analysis of laccase bioconversions

UV spectral analysis indicated product formation in the reaction of laccase with *p*-cresol, but less so with HRP. *p*-Cresol absorbs at a wavelength of 270 nm while, the peak at 320 nm indicates product formation from the reaction with HRP. The *p*-cresol absorbance peak could be seen at 270 nm (Figure 3.16a), and it decreased slightly (Figure 3.16 b) indicating the degradation of the *p*-cresol. The peak at 340 nm corresponds to ABTS. This could be seen to decrease (Figure 3.17) due to it being oxidised by the enzyme and the oxidised form is detected at 410 nm. The product of the *p*-cresol – laccase reaction appears to absorb at 550 nm and is considerably greater in area for the *p*-cresol – crude laccase sample (Figure 3.17c). In the case of 4-chlorophenol, the substrate peak appeared at a wavelength of 280 nm (Figure 3.18). There was no change in the peak when HRP was added to the reaction (Figure 3.18b) therefore indicating no product formation. The 4-chlorophenol peak at 280 nm was seen to decrease slightly after treatment with laccase, indicating a small amount of degradation of 4-chlorophenol (Figure 3.19). The ABTS peak at 340 nm, decreased (Figure 3.19b and c) due to it oxidation by the enzyme. The oxidised form of ABTS was observed as a peak at 410 nm. A broad peak at 570 nm indicated the start of product formation from the reaction with laccase and crude laccase.

In the toluene reaction, a small peak at 270 nm, due to toluene, was shown to decrease (Figure 3.20 b and c), as did the ABTS peak (340 nm). The decrease in ABTS showed that it was being oxidised by the enzyme. Peaks at 410 nm indicate the oxidised ABTS. A small peak could be observed at 720 nm indicating a small amount of product that may have been formed from the enzyme reaction.

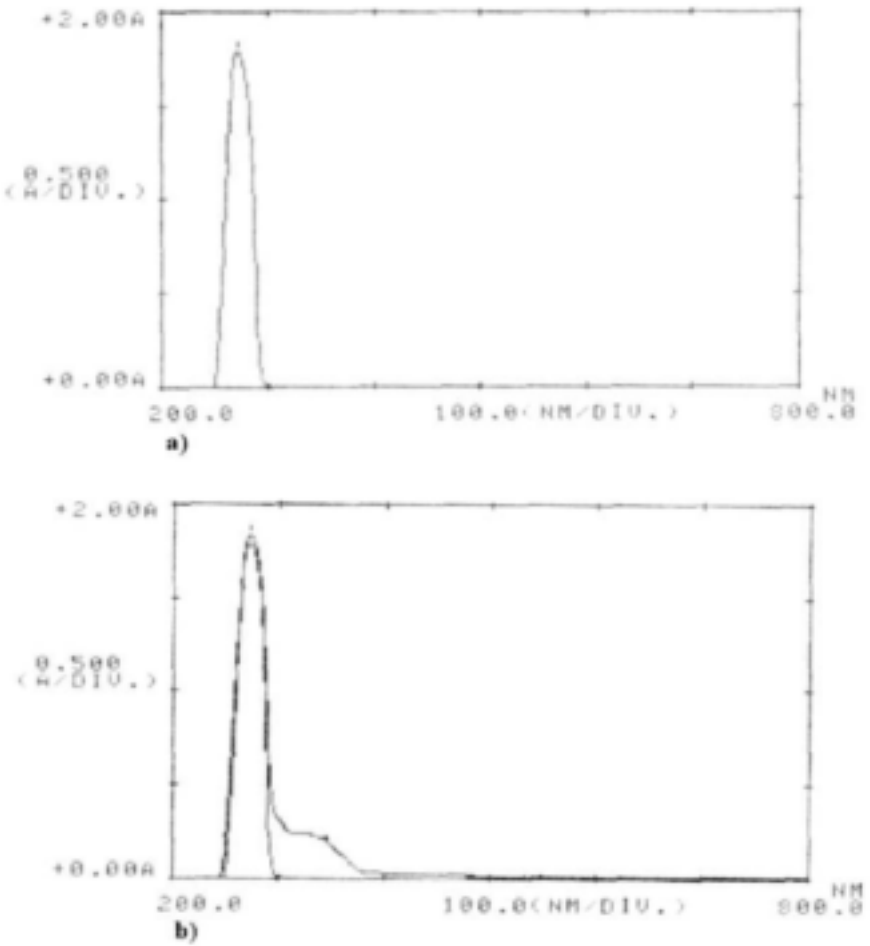


Figure 3.16 UV spectra of a) p-Cresol control and b) p-Cresol + HRP reaction.

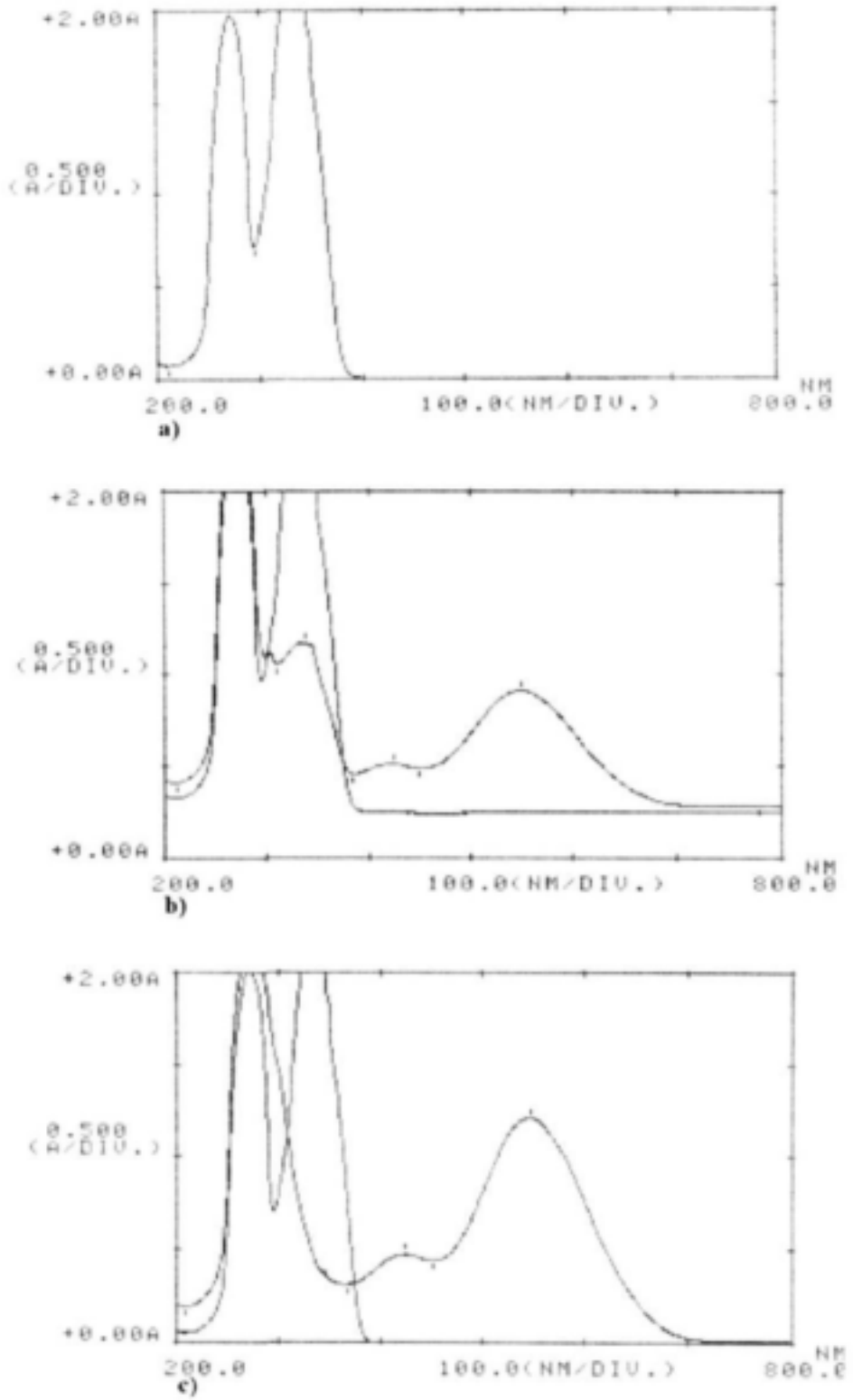


Figure 3.17 **UV scans of a) p-Cresol control and ABTS, b) p-Cresol + laccase and ABTS, and c) p-Cresol + crude laccase and ABTS.**

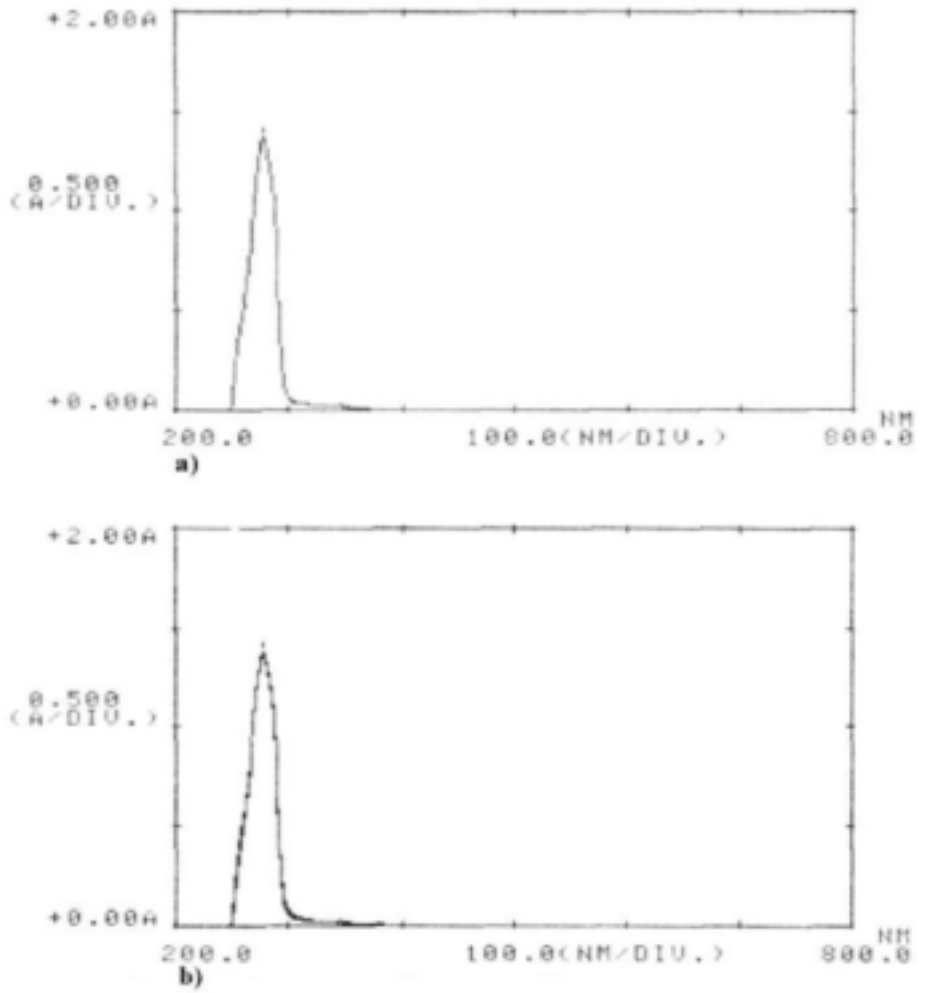


Figure 3.18 **UV spectra of a) 4-Chlorophenol control and b) 4-Chlorophenol + HRP reaction**

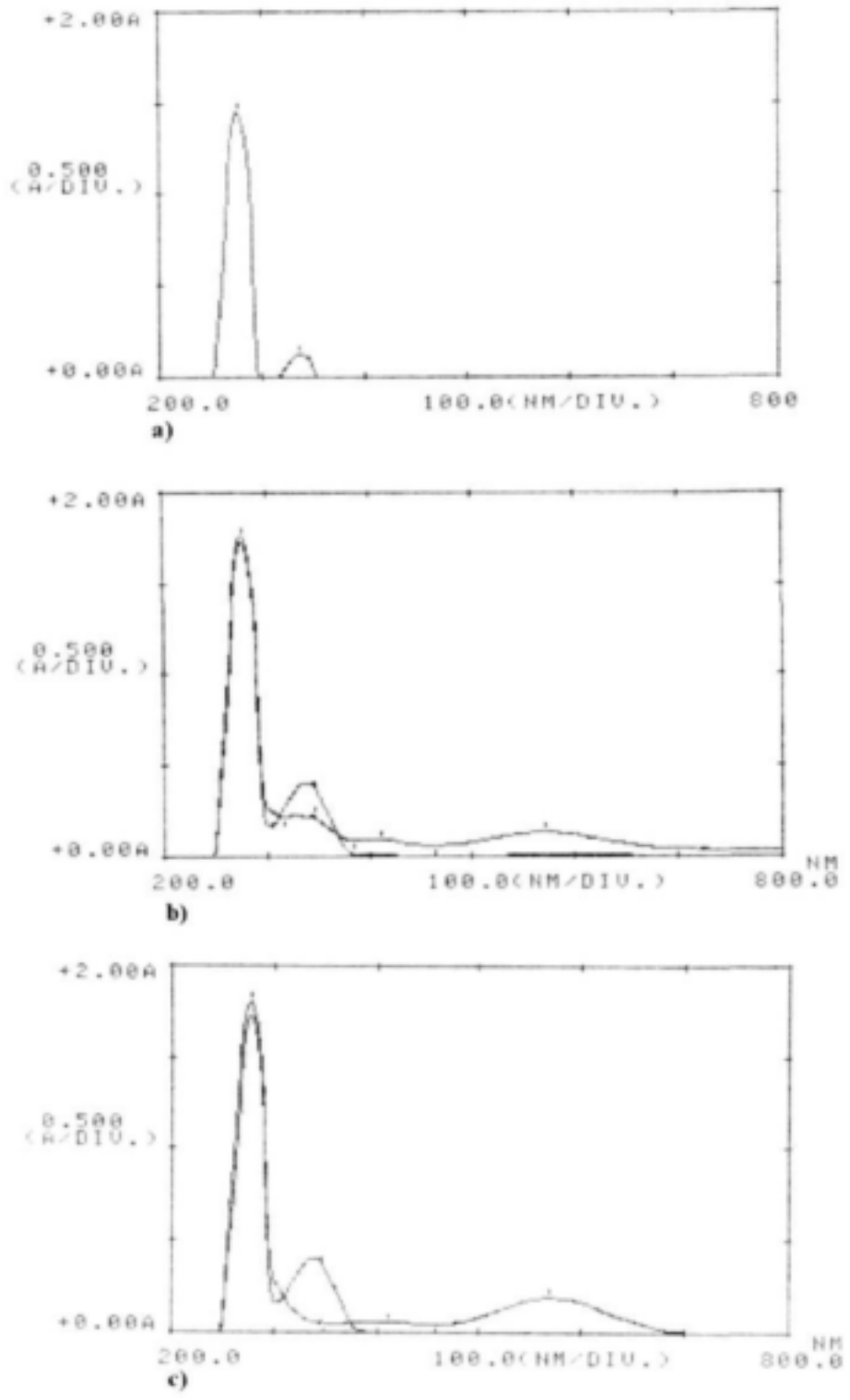


Figure 3.19 UV spectra of a) 4-chlorophenol control, b) 4-chlorlphenol +ABTS , and c) 4-Chlorophenol + crude laccase and ABTS reaction.

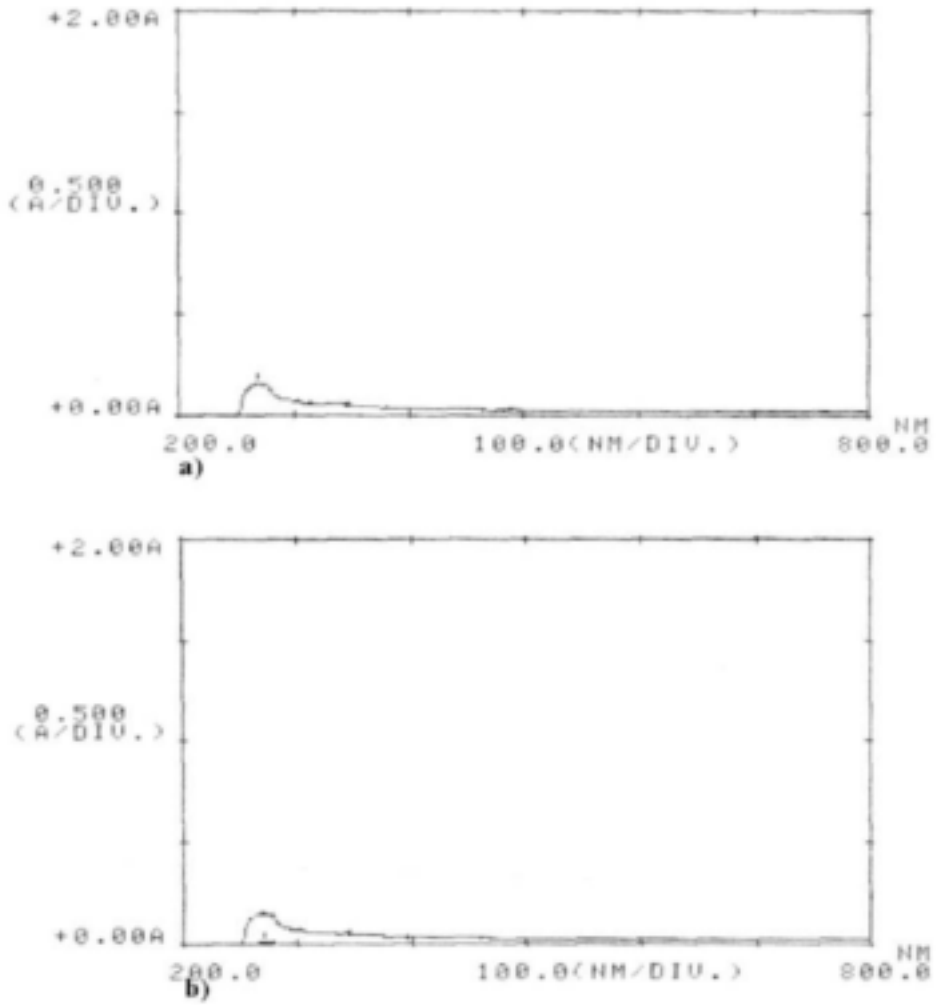


Figure 3.20 UV spectra of a) Toluene control and b) Toluene control + HRP reaction.

3.3.8 Biotransformations of model pollutants using purified and immobilized *T. versicolor* laccase

Bioconversion reactions of compounds found in the effluents by purified laccase were used to demonstrate and characterise the reaction products, and to ascertain the value of purifying the laccase. Laccase is known to react with a wide range of compounds via free radical mechanisms, and not only are the products potentially valuable, but the enzyme itself can have commercial value. Laccase mediated reactions were carried out with and without ABTS as mediator, in buffer solution. The interesting observation was found that with different substrates in presence of ABTS produced different color.

Table 3.12 UV spectral analysis of reaction of laccase with a range of substrates

Substrate	Wavelength of max absorbance in aqueous solution (nm)	Colour of solution
ABTS	343,420	dark green
Toluene	342	light green
2-nitrotoluene	341	dark green
p-cresol	342, 555	purple (acetonitrile soluble)
m-cresol	342	light yellow
veratryl alcohol		light reddish brown
4-methylcatechol	342, 260	Yellow
4-chlorophenol	361, 342, 357,217	light green

Biotransformation reactions

The purpose of this section was to establish that products of the laccase reaction could be detected and analysed, and to give a preliminary indication of which might later be developed further. (The following summarises the results of the biotransformation experiments, but not all data is shown).

Toluene

HPLC analysis of the reaction mixture showed formation of benzaldehyde.

4-methoxy phenol

HPLC analysis of this reaction mixture indicated peaks at 4.8min (parent compound) and two product peaks at 7.5 and 13.6 min.

p-Cresol

Reaction with laccase and ABTS resulted in conversion to 4-methylcatechol. The reaction then lead to ring cleavage products. This was confirmed by the NMR and moreover, when 4-methyl catechol was used as substrate, the reaction gave the same type of product. The aqueous phase was turned a deep purple color; the water was evaporated under vaccum the product was dissolved in acetonitrile for UV-visible spectrophotometric analysis.

4-Chlorophenol.

The HPLC analysis showed formation of more polar compounds than the 4-chlorophenol, which may be di-hydroxy compounds and quinones.

3.4 CONCLUSIONS

- *T. versicolor* was shown to grow successfully in flasks and to produce high levels of laccase and manganese peroxidase activity under the culture conditions used. The laccase specific activity was higher than that of the commercially available laccase. The peroxidase activity was found to be mainly manganese peroxidase, rather than lignin peroxidase.
- The culture medium for *T. versicolor* was investigated for optimal biomass accumulation and enzyme production. Of the two media tested (TDM and the variation using ammonium sulphate as a nitrogen source), it was found that TDM was better, based on biomass accumulation in the medium. A more established biomass facilitated the fungus tolerating a pollutant challenge.

Conversion of pollutants with laccase extracts

- Conversion of cresol, chlorophenol, toluene, and naphthalene by the *T. versicolor* extracts was found to be comparable with that of commercially available enzymes in terms of conversion yield and the nature of the products. The presence of the pollutants was found to have little effect on the growth of the fungus in flask culture.
- It was found that the common pollutant *p*-cresol was converted by *T.versicolor* laccase. This reaction can be optimized for higher yield by using a higher enzyme concentration or longer reaction time.
- 4-Chlorophenol, naphthalene and toluene were less readily converted in preliminary testing and these reactions require further investigation.Crude laccase extract from the local strain of *T.versicolor* showed greater promise for aromatic pollutant conversion than did the commercial strain of laccase.

Conversion of pollutants using T. versicolor biomass

- Whole cell systems were considerably more difficult to investigate as they are more complex due to the presence of growth medium, longer experimentation times and contamination risks. Increasing pollutant concentrations had visibly increasing effects on the morphology of the fungus. The surface mat cultures were reduced in size, took on a desiccated appearance and darkened in colour, the medium became more viscous and also darkened in colour. Growth of the fungus appeared to be decreased in the presence of 4-chlorophenol and naphthalene.
- Product formation was only convincingly evident in the reactions carried out using *p*-cresol. The reaction products, when using laccase in the presence of ABTS, were pink-purple in colour and had an absorbance in the region of 555 nm. A peak in the HPLC chromatogram was observed at a retention time of 2.2-2.3 minutes, while *p*-cresol had a retention time of between 4.94 and 5.05 min. This product would need to be produced in larger quantities for isolation and identification.

Bioreactors for T. versicolor application

- The membrane bioreactor system was successfully used to treat an industrial effluent containing a variety of aromatic pollutants, with all the measured compounds being removed from solution continuously after 1 day and thereafter for a further 10 days. An uplift reactor was evaluated and found to have potential for the growth of *T. versicolor* and treatment of certain effluents and represents the most likely option for a large scale bioremediation application.

Few other studies on laccase have focused on the characteristics of reaction products relevant to waste treatment objectives, and these must be understood if enzyme technology is to be developed as a viable treatment process. One of the most promising prospects for *Trametes versicolor* is to use the extracellularly produced laccase enzyme to convert aromatic pollutants to useful products.

CHAPTER 4

Application of polyphenol oxidase from the mushroom *Agaricus bisporus* in bioconversion of simple phenolic pollutants to catechol products

4.1 INTRODUCTION

Polyphenol oxidase is a highly active enzyme with a broad substrate range, which makes it a good candidate for the biotransformation of phenols (Burton, 1994). One advantage in the application of mushroom polyphenol oxidase is that relatively crude extracts prepared from mushrooms are as effective as purified enzyme (Wada *et al.*, 1993; Burton *et al.*, 1993). Much research has focussed on phenol removal efficiencies with the enzyme in solution or immobilised on various supports, and on the subsequent removal of *ortho*-quinones by precipitation or adsorption onto chitosan (Wada *et al.*, 1993; Payne, 1992; Wada *et al.*, 1995; Edwards *et al.*, 1999). Less attention has been paid to the potential of the polyphenol oxidase reaction for the production of catechol, or to characterisation of the reaction products from the polyphenol oxidase-catalysed reaction with phenols.

In this study, analysis of the reaction mixtures by HPLC and LC-MS were used to characterise the products of the reaction. The HPLC retention times of commercially available catechols were compared to catechols produced enzymatically and LC-MS was used to confirm the identity of the individual catechol product peaks.

The effect of additives, viz., chitosan, gelatin and polyethylene glycol (PEG), on the activity of polyphenol oxidase was also investigated. The aim of these experiments was to use additives to increase the production of catechol and suppress the production of quinones. According to Wada *et al.* 1993, the quinones may react with a free amino group in the active site of polyphenol oxidase resulting in inactivation. Chitosan is a natural polymer produced by shellfish industries, and polyphenol oxidase-generated quinone can be chemisorbed onto chitosan removing it from solution (Sun *et al.*, 1992). In previous studies by Nakamoto (1992), using horseradish peroxidase to remove phenols from solution, it was found that the apparent inactivation of

peroxidase during the phenol polymerizing reaction was caused by adsorption of the enzyme by polymerized phenol. With the addition of additives, such as proteins (gelatin) or hydrophilic synthetic polymers (PEG), the enzyme adsorption was suppressed and the enzyme inactivation was lowered to significantly reduce the amount of enzyme required.

The reaction of polyphenol oxidase was also carried out in the presence of different buffer solutions to determine their effects on catechol production. Extraction and isolation methods were also sought for the extraction of catechols from the reaction mixture. Numerous organic solvents are capable of extracting phenols and catechols, but isolating catechol from residual phenol can be problematic.

The effect of different immobilisation matrices on the activity of polyphenol oxidase and on the enzyme-catalysed formation of catechol was investigated. Immobilisation allows for enzyme re-use and increases enzyme stability, and has the advantage that it results in partitioning between the immobilized enzyme phase and the aqueous phase, allowing separation of the enzyme from substrates and products (Klibanov, 1983). However, it also leads to alteration in the micro-environment of the enzyme, which can change the behaviour of the biocatalyst. Thus, polyphenol oxidase was immobilised on a wide range of supports using various immobilisation techniques, such as covalent coupling, cross-linking, adsorption or a combination of these techniques. Initial experiments focussed on use of nylon membranes to optimise the immobilisation procedure. When the immobilisation procedure had been optimised it was used to determine the effect of different immobilisation matrices on the activity of polyphenol oxidase and on the enzyme-catalysed formation of catechols.

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 (in collaboration with Dr. M.H. Burton from the Department of Mathematics) was used to determine reaction rate constants and to elucidate the effects of the nature of the immobilisation matrix. The extent of product inactivation was also investigated by comparing models of the reaction systems which included or excluded the effects of enzyme inhibition by the quinone. This provided a useful indicator of the extent to which product inactivation affects the polyphenol oxidase reaction under the experimental conditions

applied here. An investigation into the reuse and stability of polyphenol oxidase immobilised on nylon membranes was also carried out.

In an investigation into alternative immobilisation methods, polyphenol oxidase was immobilised, by entrapment, in polyurethane foams (in collaboration with Professor A Russell and Dr. A LeJeune, University of Pittsburgh, USA). The effect of various surfactants on the formation of the foams was also investigated. Polyphenol oxidase was also immobilised by cross-linking the enzyme to gelatin using a chemical cross-linking agent, glutaraldehyde.

The activity of polyphenol oxidase on authentic industrial effluents was then investigated. Previous studies had involved the action of polyphenol oxidase on synthetic effluents.

4.2 MATERIALS AND METHODS

4.2.1 Polyphenol oxidase preparation

An extract of polyphenol oxidase was prepared from the mushroom *Agaricus bisporus*, and partially purified by salt fractionation, according to the method of Burton *et al.*, (1993).

4.2.2 Measurement of polyphenol oxidase activity

The activity of polyphenol oxidase was determined by monitoring the production of dopachrome at 475nm in 3 ml 10mM L-DOPA in potassium phosphate buffer (50mM, pH 6) (Burton *et al.*, 1993), using a Shimadzu UV-160A UV/VIS spectrophotometer. 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 1μmol./.min where the extinction coefficient (ϵ) is 3600/M/min.

4.2.3 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 (250 x 4.6 mm) column and mobile-phase of water (0.1 % acetic acid):acetonitrile (60:40) at a flow rate of 1ml.min^{-1}. 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 (Aldrich Chemical Company, Milwaukee, U.S.A.), 4-methylcatechol (Sigma Chemicals, St. Louis, MO) and catechol (Sigma Chemicals, St. Louis, MO), 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.

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

The reaction system consisted of 20ml of 2 mM 4-methoxyphenol, 0.6U polyphenol oxidase and addition of varying amounts of chitosan, gelatin and PEG 6000 respectively. 0.3g, 0.5g, 0.7g and 1g of chitosan; 0.5g, 0.75g and 1g gelatin; 0.5g, 1g, 1.5g and 1.75g PEG 6000 were added to the substrate and enzyme solution respectively. Experimental controls were also used, the first control consisted of substrate and enzyme and further controls consisted of substrate and chitosan, gelatin or PEG with no enzyme present in the solution. The solutions were allowed to stir continuously on an orbital shaker. Samples were analysed by HPLC.

4.2.5 The effects of buffers on polyphenol oxidase activity

20 ml 2.5 - 3mM solutions of phenol, *p*-cresol, *m*-cresol and 4-chlorophenol were prepared in 3 different buffers, namely, 75mM borate buffer (pH 7.8), 50mM potassium phosphate buffer (pH 6.8) and 50mM sodium acetate buffer (pH 6.8). 1.4 - 1.9 U polyphenol oxidase was added to the solutions. The solutions were allowed to stir continuously on an orbital shaker. Samples were analysed using the HPLC.

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

20 ml 1 - 3.5mM solutions of phenol, *p*-cresol, *m*-cresol and 4-chlorophenol were prepared in borate buffers of varying concentrations, ranging from 25mM - 100mM borate. Different concentrations of ascorbate (6mM, 12,5mM, 15mM and 18mM) were added to the substrate solutions prepared in borate buffers. 0.9 to 2.4 U polyphenol oxidase was added to the reaction systems and allowed to stir continuously on an orbital shaker. Samples were withdrawn every 30 min for 2 to 3 h and analysed by HPLC. Acetic acid was included in the mobile phase as it prevents the ionisation of catechol and ascorbate resulting in sharper HPLC peaks .

4.2.7 The effect of pH on catechol extraction using organic solvents

Ethyl acetate and diethyl ether were used to extract catechol from the reaction medium containing 75 mM borate buffer (pH 6.8) and 12.5 mM ascorbate. Catechol was extracted twice using 50 mL organic solvent and 50 mL solution of catechol in a separating funnel. The extractions were carried out using different pHs to determine its effect on the efficiency of the catechol extraction. Catechol dissolved in water was used as an experimental control to determine the effect of borate on the extraction.

4.2.8 Product purification by recrystallization from hexane

For recrystallization of 3-phenylcatechol, 100 mL hexane was used for each gram of catechol (Held *et al.*, 1999). During the initial experiments 50 mM catechol and 25 mM phenol were prepared in100 mL 75 mM borate buffer which was acidified to pH 2 using 0.5 M H_2SO_4. The solution was extracted into 200 mL diethyl ether. The organic layer was dried using Na_2SO_4 and then filtered through Whatman paper. A rotary evaporator was used to remove the organic solvent. The residue was re-dissolved in 100mL hexane and refluxed for 2 h 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, except 0.1% acetic acid was present in the water of the mobile phase.

The experiment was repeated using a significantly lower phenol concentration and polyphenol oxidase was used to produce catechols using phenol as the substrate. 250 mL of 2.7 mM phenol was prepared in 75 mM borate buffer (pH 6.8) containing 12.5mM ascorbate, 19 U polyphenol oxidase was added to the phenol solution. The solution was placed onto a Labcon shaker at 180 rpm, samples were removed every 30 min for 2 h and analysed by HPLC. After 2 h 1.98 mM catechol and 0.72 mM phenol were present in the solution which was acidified to pH 2 using 0.5 M H_2SO_4. The solution was extracted into 500 mL ethyl acetate. The organic layer was dried using Na_2SO_4 and then filtered through Whatman paper. A rotary evaporator was used to remove the organic solvent. The residue was re-dissolved in 20mL hexane and refluxed for 2 h 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.

4.2.9 Immobilisation of polyphenol oxidase on nylon membranes

Nylon (MAGNA, 47mm, 0.45μm), obtained from Micron Separation Inc. (Westborough, MA, U.S.A.), 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 50 mL of 3M HCl for 10 minutes. The nylon membranes were then washed in 150 ml of HPLC grade water and incubated for 24 hours in 30 mL methylene chloride solution containing *N,N'*- dicyclohexylcarbodiimide (1% w/v) and 3,3' ,5,5' - tetramethylbenzidine (1% w/v). 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% gluaraldehyde for 2.5 hours at 4°C with gentle stirring. The membranes were then washed with 50 mL 0.1M sodium phosphate buffer (pH 7), then placed in a 3 ml solution of 0.1M phosphate buffer (pH 7) containing 20.4 U polyphenol oxidase. The membranes were incubated for 24 h at room temperature and at 4°C to allow for polyphenol oxidase attachment. A control was set up which contained the same number of units of polyphenol oxidase but no membrane was added. The membranes were then washed with 0.1 M 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.

4.2.10 Optimised polyphenol oxidase immobilisation procedure on hydrophilic membranes

The nylon, glass-fibre and nitrocellulose membranes were soaked overnight in distilled water. Polyphenol oxidase was dissolved in 50mM phosphate buffer pH 6.8. Each membrane was then placed into a solution of the enzyme containing a sufficient number of enzyme units to ensure that 0.9 U enzyme was immobilised onto each membrane. This was determined previously by placing the membranes into solutions containing varying concentrations of polyphenol oxidase and determining the percentage of enzyme immobilised. The membranes were left in the enzyme solution for 1 h at 4°C. Glutaraldehyde (25%) was then added to give a final concentration of 1% glutaraldehyde, and a further 30 min was allowed for cross-linking. Membranes were removed, washed in 50 mM phosphate buffer pH 6.8 and then used for biocatalytic reactions.

4.2.11 Immobilization of polyphenol oxidase on hydrophobic membranes

PTFE membranes were pre-wetted using ethanol. Polysulfone membranes were rinsed in water to remove glycerine. Each membrane was placed in a stirred cell (Spectrum, Houston, Texas) and a solution containing a sufficient number of enzyme units to ensure that 0.3 U enzyme was immobilised onto each membrane was then placed in the reservoir, and a positive pressure, using nitrogen gas, was applied to the enzyme solution. This was followed by treatment with 1% glutaraldehyde solution in water.

4.2.12 Immobilization of polyphenol oxidase in polyurethane foams

The effect of different surfactants on foam formation and substrate and product adsorption was first investigated. Four different surfactants were used:- Pluronic L-62, Pluronic F-68, Pluronic P-65 and Emolgrade. 5mL of 1% (w/v) in 50 mM phosphate buffer pH 6 of these surfactants was added to 5g of pre-polymer. The solution was aerated and the foam was formed. The foams were allowed to dry overnight in a fumehood. Adsorption of the substrates to the foams was determined by placing 200 mg of each foam into 20mL of 5mM phenol and 5mM *p*-cresol. Samples were withdrawn and analysed using HPLC. The mobile phase used was 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. Polyphenol oxidase was then immobilised into the foams. 1% (w/v) surfactant in 5 mL 50 mM phosphate buffer (pH 6) and 70 mg of polyphenol oxidase were mixed with 5 g pre-polymer. The solution was then aerated and the foam was formed, the foam was then allowed to dry overnight.

4.2.13 Immobilization of polyphenol oxidase in a gelatin gel

5g of gelatin was dissolved in 8.5 mL water. 50 mL of 0.3M AOT (sodium bis-diethylhexyl sulfosuccinate) dissolved in heptane was added to the gelatin solution at 50°C. Solution was agitated until viscous. Solution was then cooled to 5°C, a 25 mL portion was removed and 1ml of polyphenol oxidase, containing 24.95 U, was added. Solution was allowed to stir for 5 - 10 minutes. The enzyme was cross-linked by adding 1 mL glutaraldehyde (25%) to the solution and allowing it to stir for 10 - 15 min. The gel was poured into 3 petri dishes and dried overnight, rinsed in hexane and washed extensively in water to remove traces of glutaraldehyde. The dried gel was then ground in liquid nitrogen using a mortar and pestle, 0.35 g of freeze-dried gel was

placed into 10 mL of 1.25 mM and 5 mM *p*-cresol, and the mixtures were allowed to swirl continuously on an orbital shaker. Samples were filtered before being analysed on the HPLC.

4.2.14 Effectiveness of immobilization

The number of active enzyme units was determined after the enzyme was immobilised on hydrophilic membranes, hydrophobic membranes, incorporated into polyurethane foams and gelatin gel. The amount of enzyme immobilised on the hydrophilic membranes was determined by measuring the polyphenol oxidase activity in the enzyme solution before and after incubation with the membrane. In the case of the hydrophobic membranes, a solution of polyphenol oxidase was placed into the reservoir of the stirred cell, and the permeate from the stirred cell was assayed for enzyme activity to determine whether the enzyme had passed through the pores of the membrane. Each type of membrane was also placed in 30 mL 10 mM L-DOPA in phosphate buffer (50 mM, pH 6). The membranes were allowed to stir continuously while samples were withdrawn every 2 min for 10 min, and the absorbance was read at 475nm. After 10 min the membranes were removed and further absorbance readings were taken to detect any residual enzyme activity in the L-DOPA solution.

In the case of the polyurethane foam, 200 mg of each foam was placed into 20 mL 10mM L-DOPA and allowed to stir. Samples were taken every 2 min and the absorbance was read at 475nm. For gelatin gels, 200 mg of the gelatin gel was placed into 3 mL 10mM L-DOPA, samples were removed every 10 min. These assay methods were used regularly to monitor the activity of the immobilised biocatalyst.

4.2.15 Reaction of the substrates with soluble and 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 20 ml substrate solution at two different concentrations (1.25mM and 5mM). After the addition of soluble or immobilised enzyme the reaction mixture was allowed to stir continuously on an orbital shaker.

4.2.16 Chromatographic analysis of membrane-immobilised 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, U.S.A.) using reverse-phase 5μm C_{18} Spherisorb (250mm x 4.6 mm) column with a mobile-phase of water/acetonitrile (60:40) at a flow rate of 1ml.min^{-1}. Peaks were detected at 270nm and analysed with Beckman System Gold Chromatography Software, using standard compounds for confirmation, where available. Products peaks (which are more polar) were observed to elute before the phenolic substrates.

4.2.17 Industrial effluent analysis and reaction with non-immobilised polyphenol oxidase

Two different industrial effluents were used, *viz*., cresylic and phenolic (also known as Black Product) effluents. Two samples of each industrial effluent were used. (The two samples were collected from the same effluent at different locations in the industrial process). The effluents were analysed by HPLC and found to contain phenol, *o* -, *m* - and *p*-cresol. The concentrations of the phenolic pollutants were determined using HPLC (Table 4.1).

Table 4.1. Phenolic concentrations present in each of the industrial effluents. (See also Appendix 1)

	Phenolic pollutant							
	Phenol		*p*-Cresol		*m*-Cresol		*o*-Cresol	
	(mM)	(g/L)	(mM)	(g/L)	(mM)	(g/L)	(mM)	(g/L)
Cresylic 1	87.1	8.19	25.2	2.72	26.9	2.91	81.1	8.76
Cresylic 2	27.6	2.59	35.5	3.83	36.8	3.97	70.8	7.65
Black product 1	11.7	1.10	0.8	0.86	0.5	0.54	0.3	0.32
Black product 2	16.3	1.53	1.0	0.11	1.1	1.18	1.1	1.18

The phenolic content of these effluents was high, and therefore dilution of the effluents was necessary. The effluents were filtered using a 0.45 micron disposable filter (Micron Separation Inc.), diluted in water and the pH was adjusted to neutrality using 0.1M NaOH. Cresylic effluents 1and 2 were diluted 25 and 20 times respectively and the Black Product effluents were diluted 10 times. 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 removed and analysed by HPLC.

4.2.18 Chromatographic analysis of the reaction of polyphenol oxidase with the diluted effluents

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 4.6 mm) 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.

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

Different dilutions of the effluent were prepared. Cresylic effluent 1 was diluted 30 and 50 times respectively using 75mM borate buffer (pH 7.8) containing 12.5mM ascorbate. 19U polyphenol oxidase was added to 50ml diluted effluent. The experiments were carried out in duplicate with a control experiment which contained no enzyme. The samples were allowed to stir continuously on an orbital shaker and samples were removed and analysed by HPLC.

4.2.20 Chromatographic analysis of the reaction of polyphenol oxidase with the effluents prepared in borate buffer

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 4.6 mm) column with a mobile-phase of water/acetonitrile (90:10), containing 10g/L ß-cyclodextrin, at a flow rate of 1ml.min^{-1}. Peaks were detected at 270nm and analysed using Peak Simple Software (version 1.59).

4.3 RESULTS AND DISCUSSION

4.3.1 Identification of catechols

The identity of the catechol products was confirmed by LC-MS. Three of the four catechols are available commercially, and the molecular masses of these were compared to those obtained from the enzymatically produced catechols. In cases where catechols were not available commercially, the molecular masses of the enzymatically produced catechols were taken as confirmation that the product was the catechol (Table 4.2). The results indicate that LC-MS can be used to accurately determine the molecular masses of catechols generated by polyphenol oxidase.

Table 4.2. 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 with Substrate, from LC-MS
m-cresol	3-methylcatechol	123.14	123.90	123.81
p-cresol	4-methylcatechol	123.14	123.87	123.83
Phenol	catechol	110.12	109.70	109.70
4-chlorophenol	4-chlorocatechol	145.57	Not available	145.50

4.3.2 The influence of chitosan, gelatin and PEG on the 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. Substrate utilization was measured and compared to the control experiments as increased utilization would signify decreased enzyme inactivation. The ability of chitosan, gelatin and PEG to remove *o*-quinones from solution was measured in terms of *o*-quinone HPLC peak areas. Changes in catechol concentrations were monitored and compared to the controls.

After the reaction of polyphenol oxidase with 4-methoxyphenol, in the presence of chitosan, there was an approximately 30-fold decrease in the *o*-quinone levels compared to the control (no chitosan). There was also a significant increase in *o*-quinone removal when more than 0.3g of chitosan was used, with little difference in *o*-quinone removal with chitosan varying from 0.5g to 1g. In the presence of gelatin, there was an approximately 2-fold decrease in the levels of *o*-quinone with little variation in *o*-quinone levels with the amount of gelatin added to the reaction (Figure 4.2). The presence of 1.75g PEG 6000 resulted in half the amount of *o*-quinone in solution (Figure 4.3).

There was a 6-fold decrease in the amount of 4-methoxycatechol in solution after 30 minutes, in the presence of chitosan, and the concentration of 4-methoxycatechol decreased further with time (Figure 4.4). In the presence of gelatin, there was also a decrease in the amount of 4-methoxycatechol in solution (Figure 4.5). However, when PEG was present in the reaction mixture, there was a slight increase in the levels of 4-methoxycatechol in solution (Figure 4.6). The presence of these compounds in solution, with no polyphenol oxidase, did not result in the removal of 4-methoxyphenol by adsorption. There was increased substrate removal by polyphenol oxidase in the absence of chitosan and gelatin while the presence of PEG had no effect on substrate removal by the enzyme.

In the presence of chitosan and gelatin, the amount of *o*-quinone in solution decreased significantly. Amine groups in the chitosan polymer are able to react with *o*-quinones while gelatin also contains functional groups capable of reacting with *o*-quinones. However, the removal of *o*-quinones from solution did not decrease polyphenol oxidase inactivation as substrate removal by the enzyme was not increased in the presence of chitosan or gelatin. An unexpected result was the considerable reduction in the amount of 4-methoxycatechol in the presence of chitosan and gelatin as catechol uptake by chitosan has not been indicated in the literature. The inclusion of the hydrophilic polymer PEG 6000, during the reaction of polyphenol oxidase with 4-methoxyphenol, resulted in slightly increased levels of catechol. The result indicates that an increased hydrophilic environment, during the reaction of polyphenol oxidase with phenols, is best suited for catechol production and warrants further investigation using hydrophilic solvents or compounds that can increase the hydrophilic environment of the reaction medium.

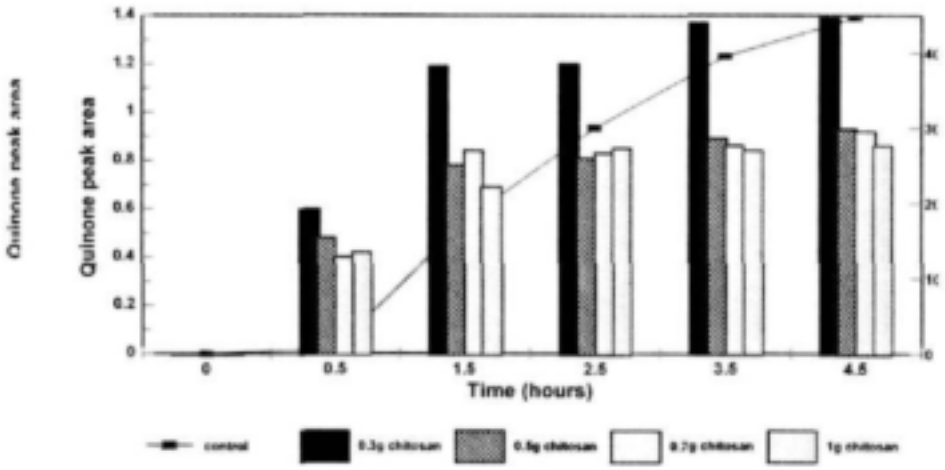


Figure 4.1 Changes in *o*-quinone production during the reaction of polyphenol oxidase with 4-methoxyphenol in the presence of varying amounts of chitosan.

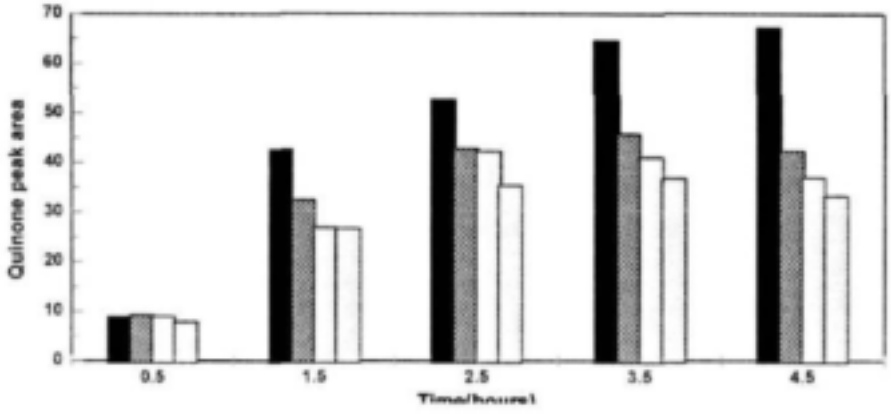


Figure 4.2 Changes in *o*-quinone production during the reaction of polyphenol oxidase with 4-methoxyphenol in the presence of varying amounts of gelatin.

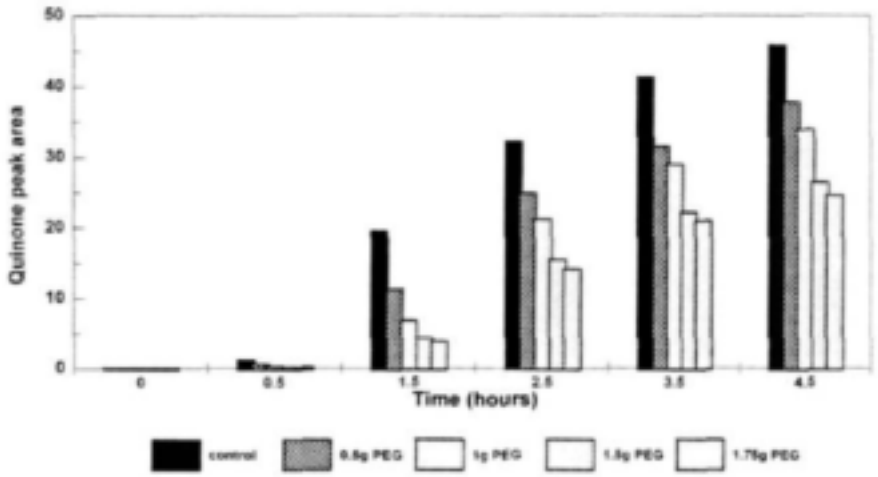


Figure 4.3 **Changes in *o*-quinone production during the reaction of polyphenol oxidase with 4-methoxyphenol in the presence of varying amounts of PEG 6000.**

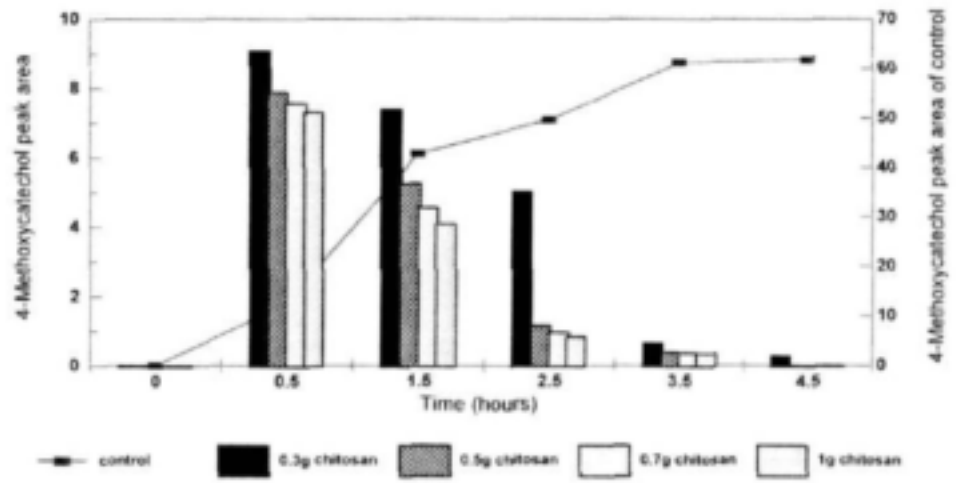


Figure 4.4 **Changes in 4-methoxycatechol production during the reaction of polyphenol oxidase with 4-methoxyphenol in the presence of varying amounts of chitosan.**

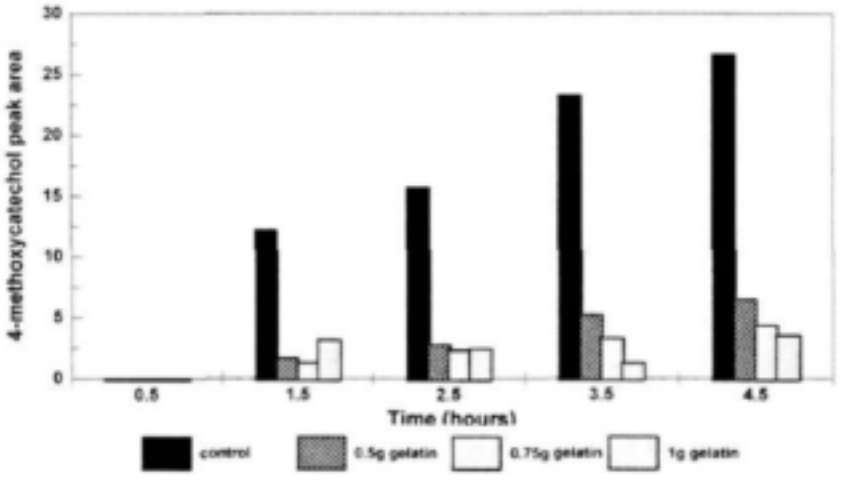


Figure 4.5 **Changes in 4-methoxycatechol production during the reaction of polyphenol oxidase with 4-methoxyphenol in the presence of varying amounts of gelatin.**

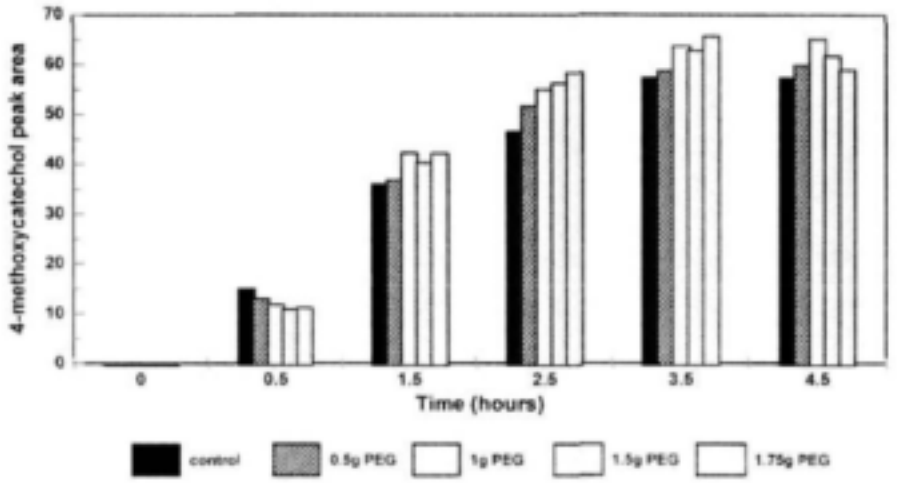


Figure 4.6 **Changes in 4-methoxycatechol production during the reaction of polyphenol oxidase with 4-methoxyphenol in the presence of varying amounts of PEG 6000.**

4.3.3 Effect of buffers on phenol removal and catechol production

Phenols were dissolved in various buffers at pH 6.8 which is the pH suited for optimum polyphenol oxidase activity. Phenol utilization and catechol production were monitored using HPLC in the presence of various buffers at pH 6.8.

During the biocatalytic activity of polyphenol oxidase, with phenol (Figure 4.7), *p*-cresol (Figure 4.8) and *m*-cresol (Figure 4.9), the highest substrate removal occurred in water, while the greatest 4-chlorophenol removal occurred in sodium acetate buffer (Figure 4.10). The accumulation of each catechol was greater in water than in buffer. The borate buffer had an inhibitory effect on the cresolase activity of polyphenol oxidase. In potassium phosphate and sodium acetate buffers catechols were rapidly oxidized to *o*-quinones as a result of the higher catecholase activity of polyphenol oxidase in these buffers. These results show that water is the best reaction medium for catechol production.

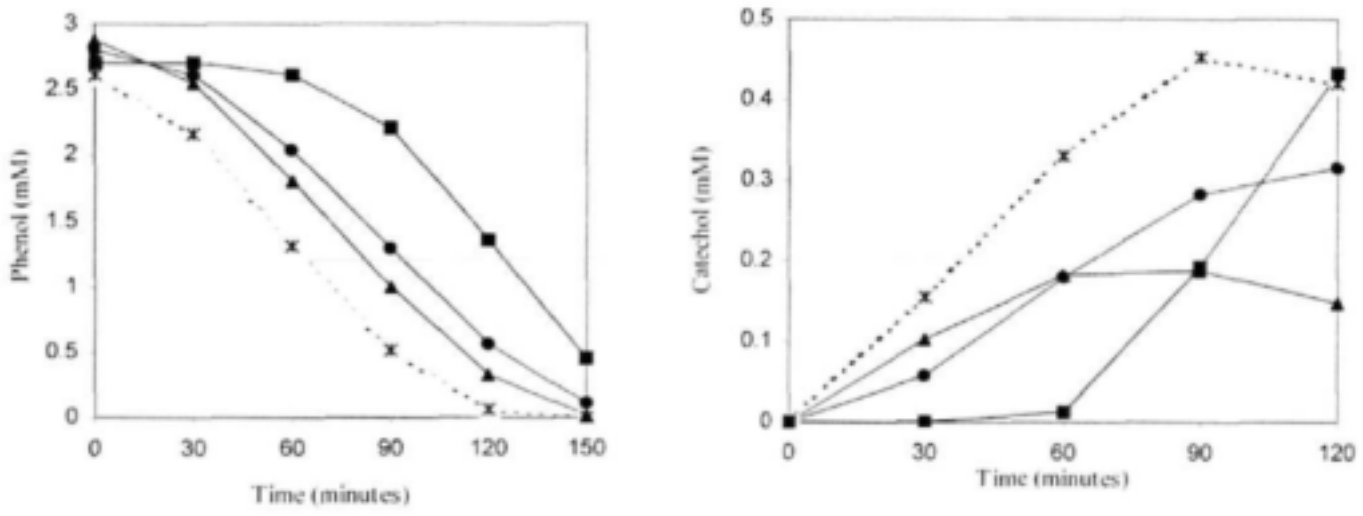


Figure 4.7 Reaction of 1.87U polyphenol oxidase with 2.5mM phenol demonstrating phenol conversion and catechol production in water (x); 75mM borate buffer (■); 50mM potassium phosphate buffer (▲) and 50mM sodium acetate buffer(●).

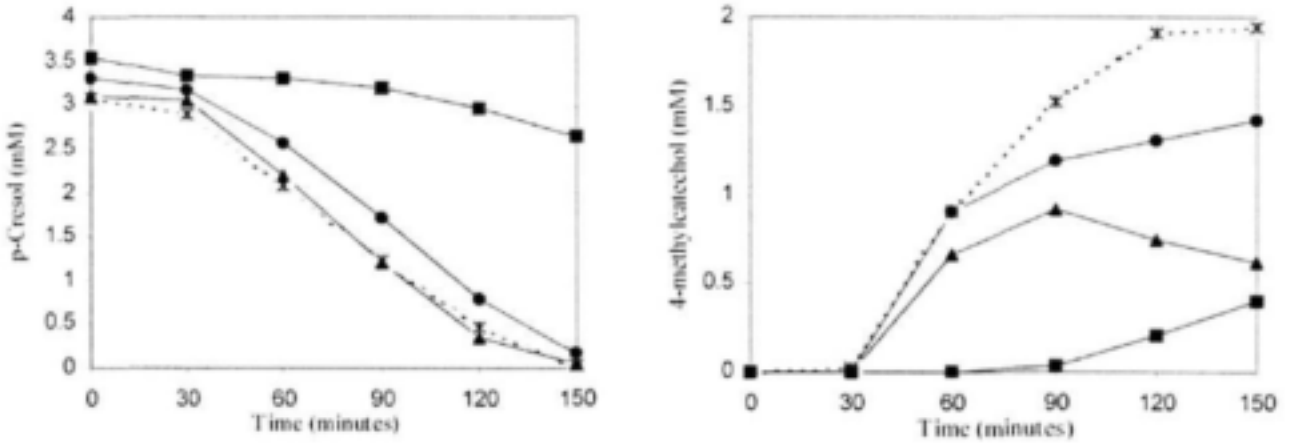


Figure 4.8 **Reaction of 1.35U polyphenol oxidase with 3 mM *p*-cresol demonstrating *p*-cresol conversion and 4-methylcatechol production in water (x); 75mM borate buffer (■); 50mM potassium phosphate buffer (▲) and 50mM sodium acetate buffer(●).**

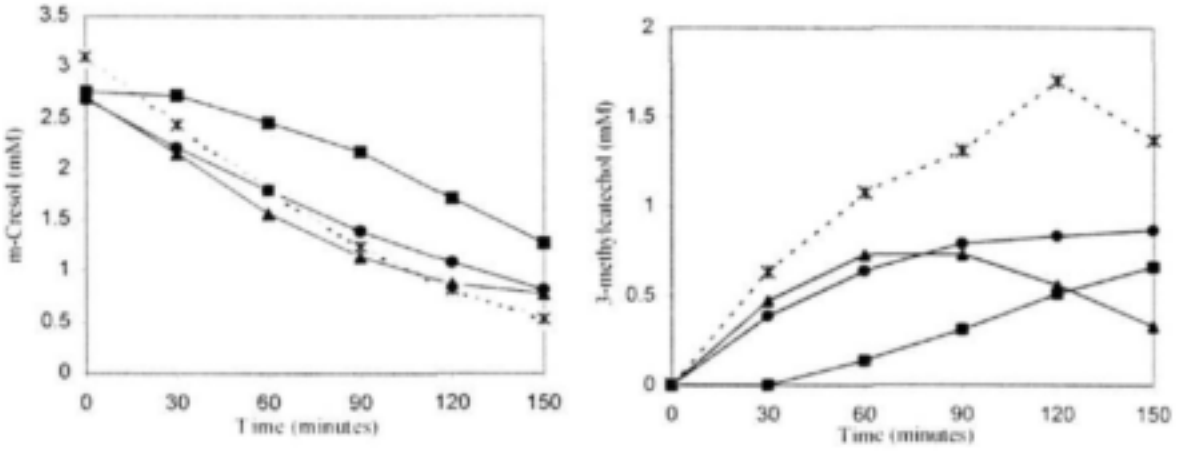


Figure 4.9 **Reaction of 1.41U polyphenol oxidase with 2.5 mM *m*-cresol demonstrating *m*- cresol removal and 3-methylcatechol production in water (x); 75mM borate buffer (■); 50mM potassium phosphate buffer (▲) and 50mM sodium acetate buffer(●).**

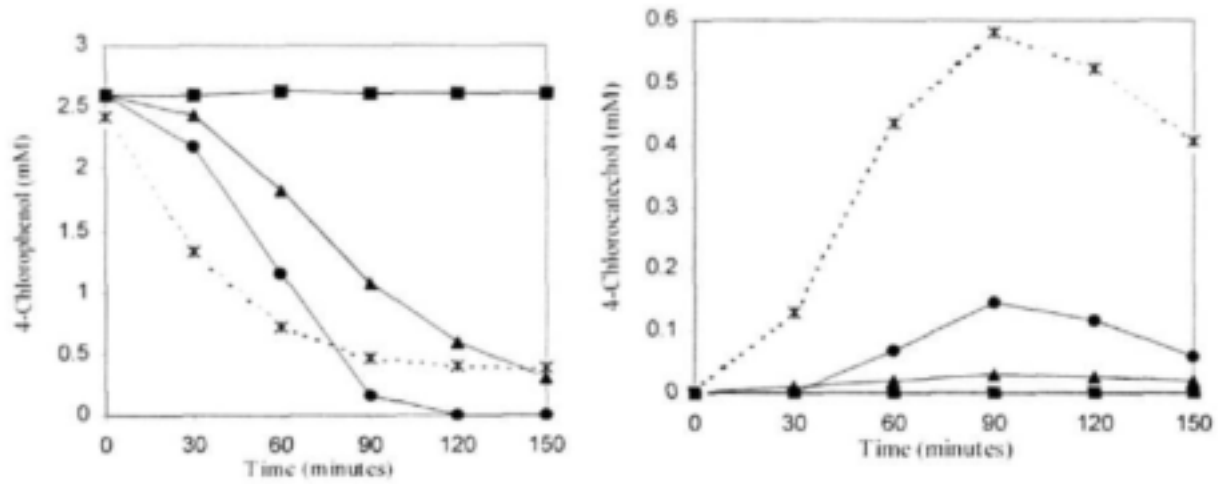


Figure 4.10 Reaction of 1.8U polyphenol oxidase with 2.5 mM 4-chlorophenol demonstrating 4-chlorophenol removal and 4-chlorocatechol production in water (x); 75mM borate buffer (■); 50mM potassium phosphate buffer (▲) and 50mM sodium acetate buffer(●).

4.3.4 Optimization of ascorbate and borate concentrations for catechol production

After incubating polyphenol oxidase for 2 h in different concentrations of ascorbate, dissolved in 75mM borate buffer, ascorbate oxidase activity was not detected. Thus, the decrease in ascorbate levels in solution can be attributed to its activity as a reducing agent for the reduction of *o*-quinones to catechols. The formation of the borate-ascorbate complex was exploited for the production of catechols while minimising polyphenol oxidase inactivation by ascorbate. The effects of ascorbate and borate, on the cresolase activity of polyphenol oxidase, were monitored. The cresolase activity of polyphenol oxidase, in the presence of borate or ascorbate (used independently or together), during the reaction with *p*-cresol (Figure 4.11) and 4-chlorophenol (Figure 4.12), was monitored using HPLC. The presence of 75 mM borate buffer inhibited the cresolase activity of polyphenol oxidase. Complete inhibition was observed using 4-chlorophenol as the substrate. The addition of 12.5 mM ascorbate resulted in enzyme inactivation after 60 min using *p*-cresol and after 30 min using 4-chlorophenol as the substrates. 5 mM ascorbate caused 50% polyphenol oxidase inactivation after 130 minutes (Golan-Goldhirsh and Whitaker, 1984). The combination of borate and ascorbate in the reaction system lead to the substrates being utilised faster than in the presence of water.

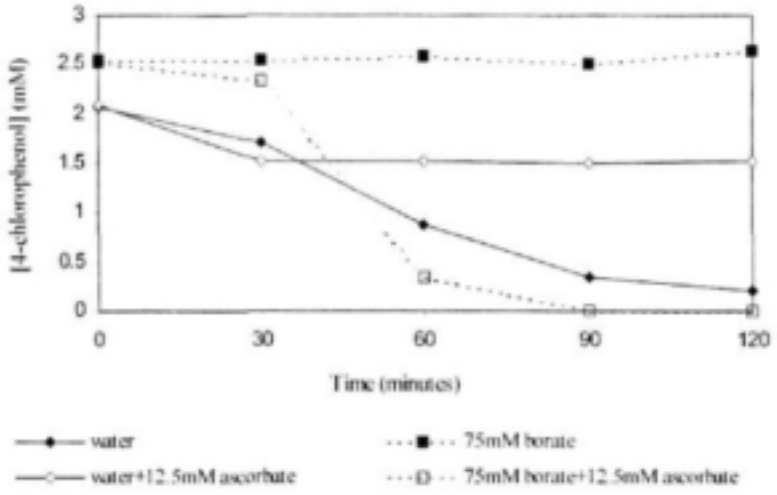


Figure 4.11 ***p*-Cresol utilisation by polyphenol oxidase in the presence of ascorbate and borate.**

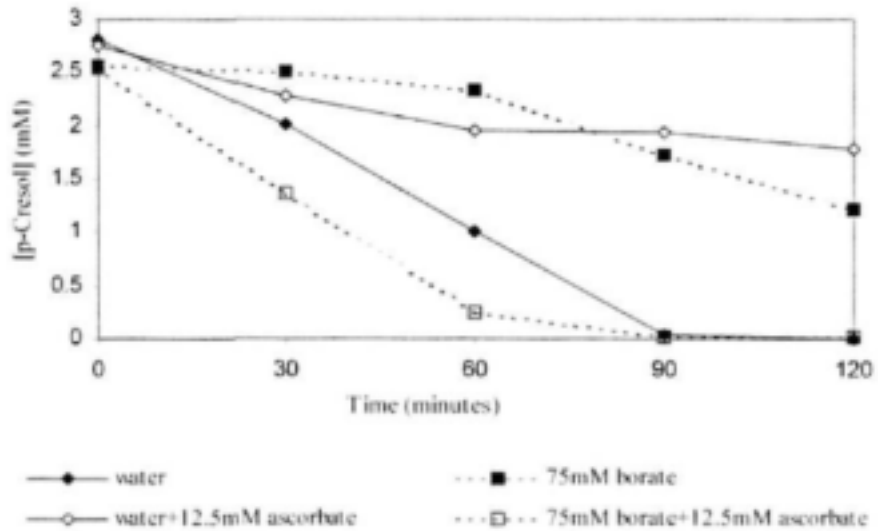


Figure 4.12 4-Chlorophenol utilisation by polyphenol oxidase in the presence of ascorbate and borate.

The effects of ascorbate and borate concentrations on the catecholase activity of polyphenol oxidase using *p*-cresol (Table 4.3), phenol (Table 4.4), *m*-cresol (Table 4.5) and 4-chlorophenol (Table 4.6) as substrates were also monitored using HPLC. Using phenol and 4-chlorophenol resulted in 9.6% and 16.8% yields of catechol and 4-chlorocatechol, respectively, when the reactions were carried out in water. While the use of *p*-cresol and *m*-cresol as substrates resulted in higher catechol yields of 41.4% and 61.3% yields of 4- and 3-methylcatechol respectively in water.

During the initial experiments *p*-cresol was used as the substrate in the presence of 25mM borate, with the addition of 6mM and 12.5mM ascorbate (Table 4.3). 6mM ascorbate was insufficient to prevent *o*-quinone formation and after 30 minutes *o*-quinones were detected in solution and the catechol concentration decreased. 12.5mM ascorbate resulted in enzyme inactivation after 60 minutes because the borate concentration was too low to complex sufficient ascorbate to minimise polyphenol oxidase inactivation. The concentration of the borate was increased to 50mM. Experiments were carried out using phenol, in the presence of 50mM borate with the addition of 12.5mM, 15mM and 18mM ascorbate (Table 4.4). The highest catechol yield of 77.7% was obtained using 12.5mM ascorbate as the borate concentration was too low to complex the ascorbate at the higher concentrations to prevent enzyme inactivation. However, in the presence of 50mM borate and 12.5mM ascorbate the catechol concentration started to decrease after 60 minutes. The borate concentration was further increased to 75mM borate. 100% yield of catechol, 4-methylcatechol and 4-chlorocatechol was obtained using 75mM borate and 12.5mM ascorbate. 56 to 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).

Table 4.3 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)	Initial [*p*-cresol] (mg/L)	Borate buffer (mM)	[Ascorbate] (mM)	Units PPO (U)	Reaction time (minutes)	Final [4-methylcatechol] (mM)	% yield
1.16	125	0	0	1.20	120	0.66	41.4
2.75	297	0	12.5	1.80	120	1.06	38.6
1.69	183	25	6	0.90	120	1.05	62.1
1.69	183	25	12.5	0.90	120	1.22	72.0
1.31	141	50	12.5	1.20	120	1.09	80.0
2.56	2767	75	0	1.80	120	0.67	26.2
2.61	282	75	12.5	1.50	180	2.36	90.87
3.06	330	75	12.5	1.20	180	3.04	99.4
2.6	281	75	12.5	1.50	165	2.6	100.00
3.06	330	75	15	1.20	180	3.03	99.0
2.66	287	100	12.5	1.50	180	2.57	96.6

Table 4.4 Catechol yields obtained after the polyphenol oxidase-catalysed reaction using phenol in the presence of different borate and ascorbate concentrations.

Initial [phenol] (mM)	Initial [*p*-cresol] (mg/L)	Borate buffer (mM)	[Ascorbate] (mM)	Units PPO (U)	Reaction time (minutes)	Final [catechol] (mM)	% yield
2.92	315	0	0	1.64	150	0.28	9.6
2.13	230	0	12.5	1.95	120	0.57	26.8
2.53	273	50	12.5	1.64	150	1.97	77.7
2.54	274	50	15	1.64	150	1.82	71.6
2.53	273	50	18	1.64	150	1.49	58.8
2.30	248	75	0	1.95	120	0.13	5.7
2.60	281	75	12.5	1.64	150	2.34	89.9
3.02	326	75	12.5	2.40	120	3.02	100.0
2.62	282	75	15	1.64	150	2.33	88.9
2.62	282	75	18	1.64	150	2.2	84.0

Table 4.5 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)	Initial [*p*-cresol] (mg/L)	Borate buffer (mM)	[Ascorbate] (mM)	Units PPO (U)	Reaction time (minutes)	Final [3-methylcatechol] (mM)	% yield
2.43	262	0	0	1.95	120	1.49	61.3
2.42	261	0	12.5	1.95	120	0.25	10.3
2.00	216	75	0	1.95	120	1.02	51.0
2.78	300	75	12.5	1.62	150	0.9	32.4
1.99	215	75	12.5	1.80	120	0.67	34.0
2.17	234	100	12.5	1.80	180	0.48	22.1

Table 4.6 Reaction using 4-chlorophenol in the presence of different borate and ascorbate concentrations.

Initial [4-chlorophenol] (mM)	Initial [*p*-cresol] (mg/L)	Borate buffer (mM)	[Ascorbate] (mM)	Units PPO (U)	Reaction time (minutes)	Final [4-chlorocatechol] (mM)	% yield
2.56	276	0	0	1.46	120	0.43	16.8
2.08	225	0	0	1.95	120	0.73	35.1
2.09	226	0	12.5	1.95	120	0.52	24.8
2.54	274	75	0	1.95	120	0.00	0.0
2.35	254	75	12.5	1.80	180	1.55	65.9
2.52	272	75	12.5	1.95	120	1.96	77.7
3.17	342	75	12.5	2.40	180	3.17	100
2.4	259	100	12.5	1.80	180	1.62	67.5

4.3.5 Effect of pH on catechol extraction using organic solvents

Ethyl acetate and diethyl ether were used to extract catechol dissolved in 75mM borate buffer and 12.5mM ascorbate. The effect of the pH of the catechol solution before extraction was determined in the presence of borate (Table 4.7). Catechol is known to form a complex with borate under weakly basic conditions and under these conditions 20% catechol was extracted into the organic solvent. Acidification of the catechol solution, prepared in borate buffer, resulted in nearly 100% extraction of catechol.

Table 4.7 Extraction of catechol from solutions of different composition and pH

	water (no pH adjustment)	75mM borate buffer (pH 6.8)	75mM borate buffer (acidified to pH 5)
[Initial catechol] (mM)	2.85	2.52	2.76
(mg/L)	314	277	303
[Catechol] after first solvent extraction (mM)	0.41	2.31	0.46
(mg/L)	45	254	51
[Catechol] (mM) after second extraction (mM)	0.039	2.00	0.037
(mg/L)	4.3	220	4

4.3.6 Product purification by recrystallization from hexane

Using high concentrations of catechol and phenol resulted in 98.5% catechol recovery with 0.07% phenol present as a contaminant. However, when significantly lower catechol concentrations were used, between 40 and 60% catechol was recovered. This yield is similar to that obtained using a whole cell biocatalyst for the production of 3-substituted catechols where 59% overall yield was obtained (Held *et al.*, 1999).

4.3.7 Immobilisation of polyphenol oxidase on nylon membranes

The immobilisation procedure of Pialis *et al.* (1996) involved the covalent linkage of polyphenol oxidase to nylon membranes, which had subsequently 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. Measurements of polyphenol oxidase activity before and after the immobilisation procedure revealed that the immobilisation procedure was unsuccessful as the activity in solution was almost identical after immobilisation. The immobilisation procedure was tedious and involved many chemicals, and 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 on several types of immobilisation support.

4.3.8 Effectiveness of polyphenol oxidase immobilisation on different membrane supports

A simple immobilisation method was adopted which could be used with many different immobilisation supports, and which minimised chemical modification of the enzyme. Polyphenol oxidase was adsorbed on to the membranes and then cross-linked using glutaraldehyde. The effects of glutaraldehyde concentration upon polyphenol oxidase uptake and length of time needed for immobilisation were investigated. 1% glutaraldehyde was sufficient for immobilisation and the optimum immobilisation time was 1.5 h.

Different immobilisation procedures were necessary to immobilise polyphenol oxidase on hydrophilic and hydrophobic membranes. Polyphenol oxidase would not immobilise on hydrophobic membranes using static immobilisation (the method used for hydrophilic membranes). Using a stirred cell and applying a positive pressure ensured that polyphenol oxidase was immobilised on hydrophobic membranes with no polyphenol oxidase leakage from the support.

The amount of polyphenol oxidase immobilised on the membranes was determined by placing the membranes in the substrate L-DOPA, and using linear regression to find the activity. After polyphenol oxidase was immobilised onto flat-sheet membranes, the number of units of active enzyme was determined for each type of membrane before it was reacted with substrate, to facilitate comparison of different immobilisation supports (Table 4.8). In order to determine the effect of immobilisation on polyphenol oxidase activity, the same number of units of polyphenol oxidase were used in a non-immobilised form.

Table 4.8.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)	
Non-immobilised polyphenol oxidase	0.32±0.03
Immobilised polyphenol oxidase	
nylon	0.31±0.01
nitrocellulose	0.29±0.01
glass-fibre	0.30±0.03
PTFE	0.28±0.04
polysulfone	0.31±0.07

[a] : mean±SD

4.3.9 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 four phenol substrates, over a period of 6 hours, using two different starting concentrations of substrate. The concentrations of phenolic substrate and catechol intermediates were monitored by HPLC. The resulting data set, which was standardized to account for small differences in polyphenol oxidase immobilised on the membranes, was used as the basis for formulating a mathematical model of the process as described below.

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

A mathematical model was developed as follows, in order to characterise the reaction and compare catechol concentrations which could be produced in the reaction.

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

$$E+S \xrightarrow{k_1} E+C$$
$$E+C \xrightarrow{k_2} E+Q$$
$$E+Q \xrightarrow{k_3} P$$

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.

$$\frac{ds}{dt}=-k_1 se$$
$$\frac{dc}{dt}=k_1 se-k_2 ce \qquad (1)$$
$$\frac{de}{dt}=k_3 qe$$

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

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

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

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

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. 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 1.25 mM *p*-cresol with polyphenol oxidase immobilised on a nylon membrane is shown in Figure 4.13.

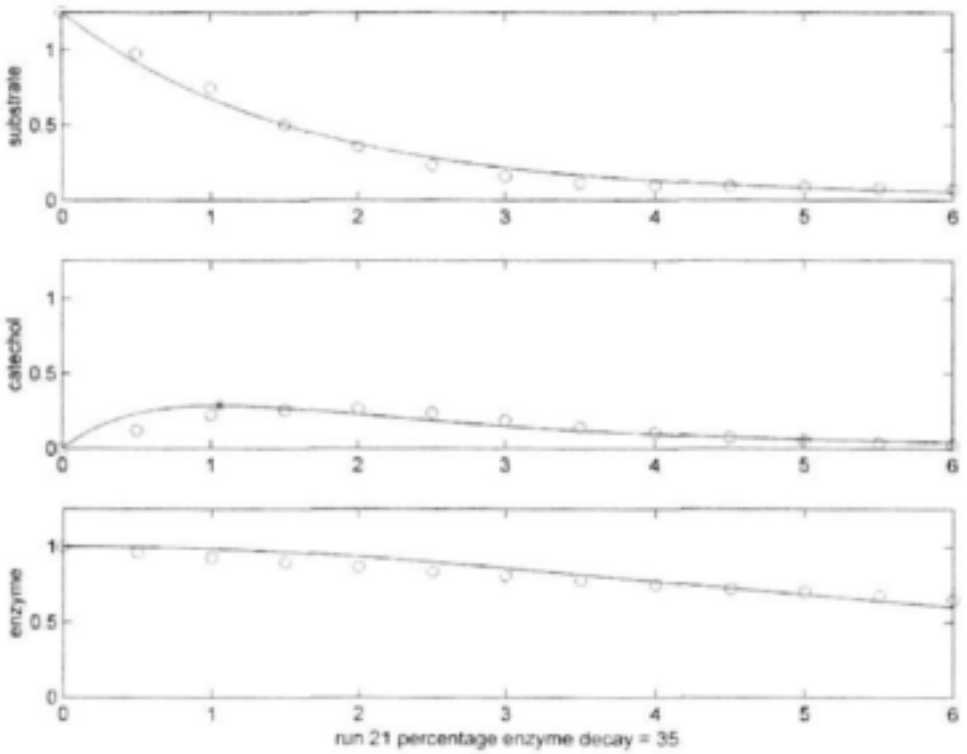


Figure 4.13 Polyphenol oxidase immobilised on nylon and placed into 1.25mM *p*-c resol.

Program 2:

The second program ran the first program for d varying from 0 to 95 and identified that value of d which produced the best fit in terms of a minimum value of r.

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.

4.3.11 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 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 demonstrated that the product inhibition was not present to a degree that would alter the experimental results *i.e.*, it could be assumed to be negligible. 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 where 1.25mM substrate concentrations were used, the rate constants k_1 and k_2, for each substrate are shown in Table 4.9.

Table 4.9 A comparison of the reaction rate constants k_1 and k_2, using 1.25mM substrate

	Reaction rate constant[a]	Reaction rate constant[b]	Reaction rate constant (k_1) using Program 2 [c]		Reaction rate constant (k_2) using	Reaction rate constant (k_2) using	k_1/k_2 using Program 1	k_1/k_2 using Program 2
1.25mM substrate	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	0.60	-	-	-	-	-	-	-
Nylon	0.60	0.59	0.61	35	1.29	1.41	0.46	0.43
glass-fibre	0.29	0.29	0.29	0	7.30	7.30	0.04	0.04
PTFE	0.22	-	-	-	-	-	-	-
polysulphone	0.24	0.22	0.29	50	13.31	428.10	0.02	0.0007
***m*-cresol**								
non-immobilised	0.43	0.50	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	0.00005
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 4.9). 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 4.9) 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 $k_2 = 26.43\ s^{-1}$ (using Program 1) and $k_2 = 649.93\ s^{-1}$ (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 4.10.

Table 4.10 A comparison of the reaction rate constants k_1 and k_2, using 5mM substrate

	Reaction rate constant	Reaction rate constant (k_1) using	Reaction rate constant (k_1) using Program 2 [a]		Reaction rate constant (k_2) using	Reaction rate constant (k_2) using	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^{-1})	% 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	0.001
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 4.10). 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 0.22 s^{-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 = 0.12 s^{-1} using non-immobilised polyphenol oxidase, k_1 = 0.13 s^{-1} for polyphenol oxidase immobilised on nitrocellulose and k_1 = 0.12 s^{-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 4.11 and 4.12). The ratio of the catechol concentration to the quinone concentration ([catechol] / [quinone]) was calculated as a

high ratio would imply that 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 in biotransformation processes using an immobilised enzyme rather than a soluble one, and hence consideration was given to the membrane providing the best immobilisation conditions for catechol production.

Table 4.11 Maximum catechol and corresponding quinone concentrations determined using 1.25mM substrate

1.25mM substrate		maximum [catechol] (mM) * no inactivation	corresponding [quinone] (mM) no inactivation	maximum [catechol] (mM) * 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	0.00007	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	-

*: catechol was not detected in all cases

Table 4.12 Maximum catechol and corresponding quinone concentrations determined using 5mM substrate

5mM substrate		maximum [catechol] (mM) * no inactivation	corresponding [quinone] (mM) no inactivation	maximum [catechol] (mM) * 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

*: catechol was not detected in all cases

Based on the high [catechol]/[quinone] ratios (Tables 4.11 and 4.12), *p*-cresol and *m*-cresol are the substrates best suited for catechol production by 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 1.25 mM and 5 mM 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. Use of starting substrate concentrations of 1.25mM *p*-cresol and *m*-cresol, resulted in a higher accumulation of 4-methylcatechol and 3-methylcatechol, respectively, than when the starting concentration was 5mM *p*-cresol and *m*-cresol (Table 4.13). However when polyphenol oxidase was immobilised

negligible accumulation of 3-methylcatechol occurred. Using 4-chlorophenol and phenol as substrates resulted in very low or negligible concentrations of 4-chlorocatechol and catechol, respectively, being accumulated in solution due to the rapid conversion of these catechols to their respective quinones (Table 4.13). Correlation of these results indicate that polyphenol oxidase, immobilised on hydrophilic supports or non-immobilised can feasibly be applied in the production of catechols, particularly 3- and 4-methylcatechol.

Table 4.13 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 (%)*	5mM *p*-cresol (%) *	1.25mM *m*-cresol (%) *	5mM *m*-cresol (%) *	1.25mM phenol (%) *	5mM phenol (%) *	1.25mM 4-chlorophenol (%) *	1.25mM 4-chlorophenol (%) *
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	0.003	0.002	-	-
Nitrocellulose	25.91	6.04	0.003	0.002	-	-	-	-
glass-fibre	4.33	1.39	-	-	-	-	-	-
Polysulphone	0.01	0.004	0.002	-	0.004	0.003	-	-
PTFE	15.05	2.94	-	-	-	-	-	-

* calculated using maximum catechol concentration produced / substrate concentration

4.3.12 Operational stability of polyphenol oxidase immobilised on nylon membranes

Characterisation of membrane supports, derived from the mathematical model, revealed that nylon membranes were the choice support for maximum catechol production and these membranes were therefore used for a stability study. 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 and is shown in Figure 4.14.

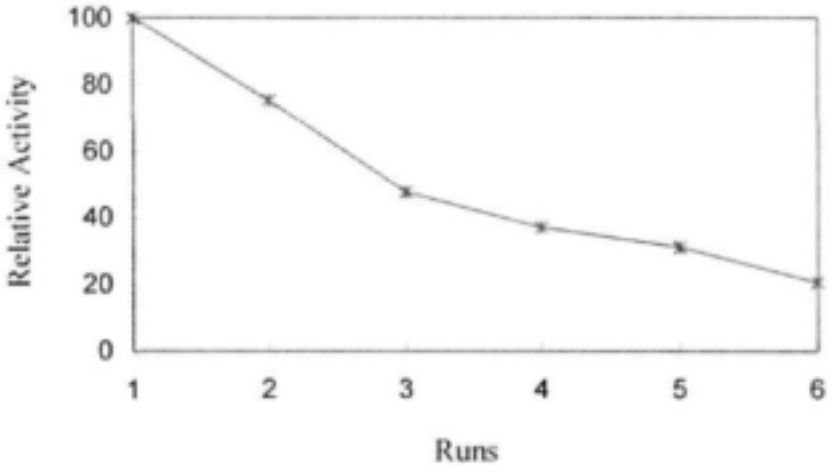


Figure 4.14 Activity of nylon-immobilised polyphenol oxidase after 6 batch experiments using L-DOPA as the substrate.

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 4.15. 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.

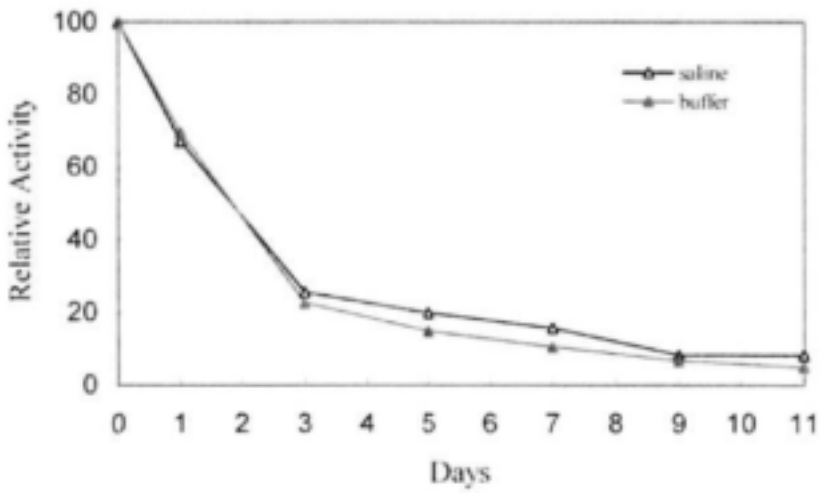


Figure 4.15 Activity of immobilised polyphenol oxidase, monitored over 11 days, whilst stored in saline and potassium phosphate buffer, using L-DOPA as the substrate.

4.3.13 Effect of incorporating different surfactants on polyurethane foam properties

According to the literature polyphenol oxidase has not been immobilised within polyurethane foams. A preliminary investigation was carried out to determine the activity of polyphenol oxidase immobilised 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 4.14). 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.

Table 4.14 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

4.3.14 Substrate and product partitioning 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 4.15). The highest adsorption of 17.7% *p*-cresol occurred using polyurethane foam containing Pluronic F-68. Low levels of 4-methylcatechol and catechol were adsorbed onto each of the foams.

Table 4.15 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	17.70
Product	catechol	7.61	10.65	7.61
	4-methylcatechol	3.92	6.28	2.75

4.3.15 Immobilisation of polyphenol oxidase within polyurethane foam

70mg of crude polyphenol oxidase extract was used to synthesise each foam. The amount of active polyphenol oxidase in the polyurethane foams was determined by placing 200mg of the foams into 20ml 10mM L-DOPA and monitoring the appearance of dopachrome at 475nm (Figure 4.16).

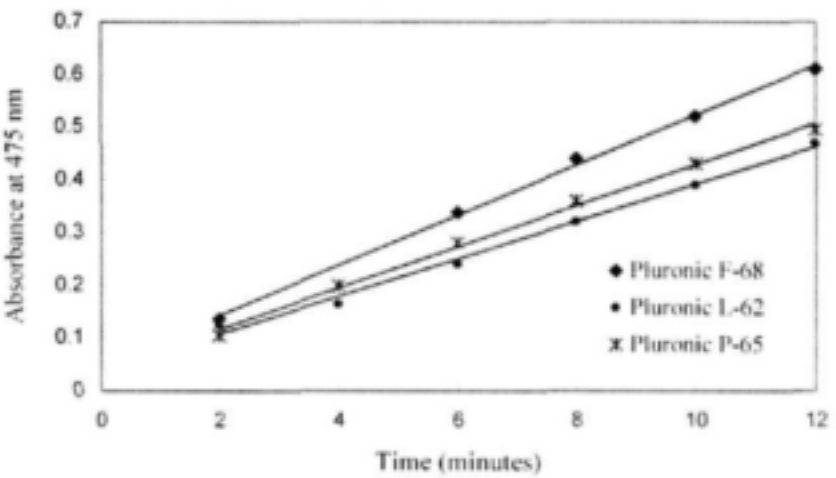


Figure 4.16 Monitoring formation of dopachrome from L-DOPA at 475nm catalysed by polyphenol oxidase immobilised in polyurethane foams.

The percentage polyphenol oxidase activity in the foams, synthesised using different surfactants, was calculated using the change in absorbance at 475nm determined from Figure 4.16 (Table 4.16). The foam containing Pluronic F-68 retained the most polyphenol oxidase activity of

33.21%. Due to the high enzymatic loading required for the synthesis of the foam, commercial polyphenol oxidase, with a specific activity 17 times higher than the polyphenol oxidase extract, was used to synthesise polyphenol oxidase-polyurethane foams. Commercial polyphenol oxidase retained 36 % activity when immobilised in polyurethane foam synthesised using Pluronic F-68.

Table 4.16 Percentage of active polyphenol oxidase present in polyurethane foams synthesised in the presence of Pluronic F-68, L-62 and P-65 surfactants.

Surfactant	ΔA/min	Units polyphenol oxidase (U) /g foam	% polyphenol oxidase activity
Pluronic F-68	0.0474	1.317	33.21
Pluronic L-62	0.0355	0.986	25.12
Pluronic P-65	0.0389	1.081	26.82

4.3.16 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. The relative activity of polyphenol oxidase was calculated after each run and is shown in Figure 4.17. After 6 runs polyurethane-immobilised polyphenol oxidase had retained 36%.

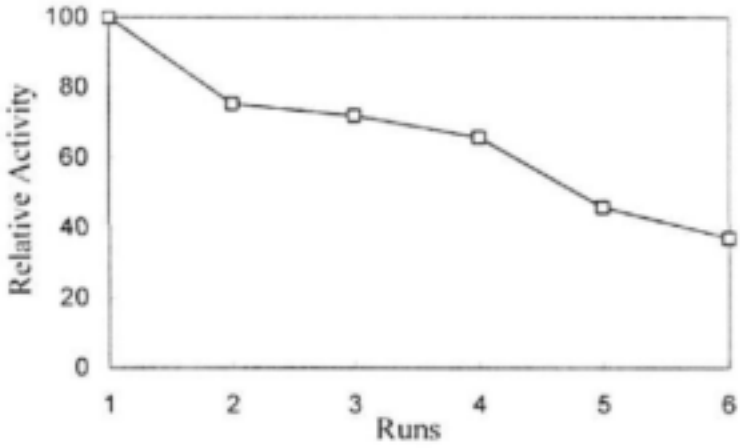


Figure 4.17 Activity of polyurethane-immobilised polyphenol oxidase after 6 consecutive batch reactions using 10mM L-DOPA as substrate.

The stability of polyphenol oxidase, immobilised in polyurethane foams and stored at room temperature, was monitored over 15 days by determining the relative activity of the enzyme using L-DOPA as the substrate (Figure 4.18). After 15 days 65% polyphenol oxidase activity was present in the polyurethane foam.

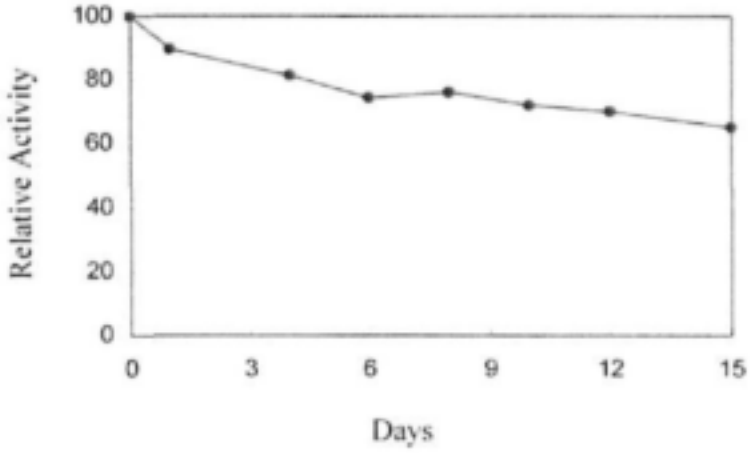


Figure 4.18 Relative activity of polyurethane-immobilised polyphenol oxidase over 15 days at room temperature using 10mM L-DOPA as substrate.

The effect of storing the immobilised biocatalyst in saline and potassium phosphate buffer at 4 °C was investigated by periodically monitoring the enzyme activity using L-DOPA as the substrate.

The relative activity of polyphenol oxidase was calculated over 11 days and is shown in Figure 4.19. The activity of the immobilised biocatalyst was slightly higher when stored in saline than buffer; there was approximately 15% polyphenol oxidase activity after 11 under these storage conditions.

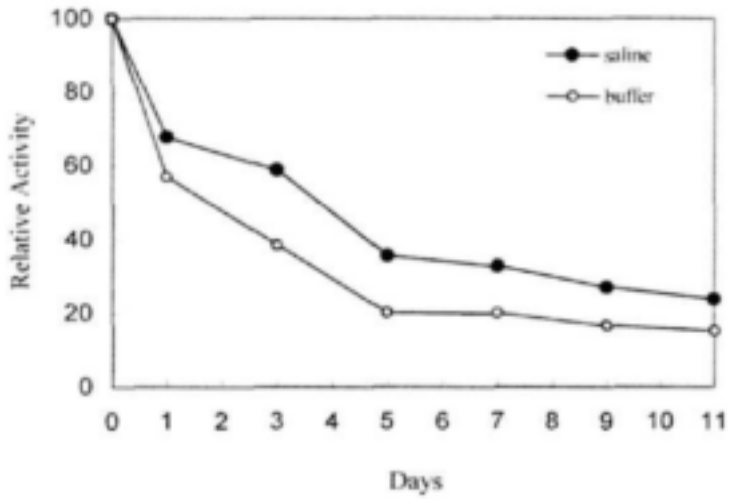


Figure 4.19 The activity of polyphenol oxidase immobilised in polyurethane foam and stored in saline and potassium phosphate buffers, using L-DOPA as the substrate.

4.3.17 Immobilisation of polyphenol oxidase in gelatin gel

Immobilisation of polyphenol oxidase in gelatin gel 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 rate of the reaction of polyphenol oxidase in the gel was very slow with low levels of activity being detected after 60 minutes (Figure 4.20). The retained activity in the gel was found to be very low, 2.6 % of the original number of units of polyphenol oxidase were active. However, when the gel was placed into 1.45mM and 5.2mM *p*-cresol solutions (Figures 4.21 and 4.22) and allowed to react for 3 hours, significant substrate removal was observed. 80 % removal of *p*-cresol was observed after 3 hours using polyphenol oxidase

immobilised in gelatin gel. 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 in the gel. In terms of product formation, very low levels of 4-methylcatechol were detected in solution.

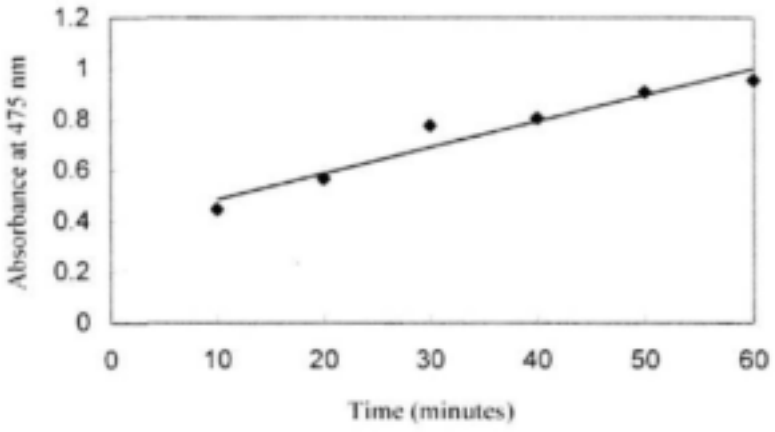


Figure 4.20 **Increasing absorbance at 475nm showing formation of dopachrome from L-DOPA catalysed by polyphenol oxidase immobilised in gelatin gel.**

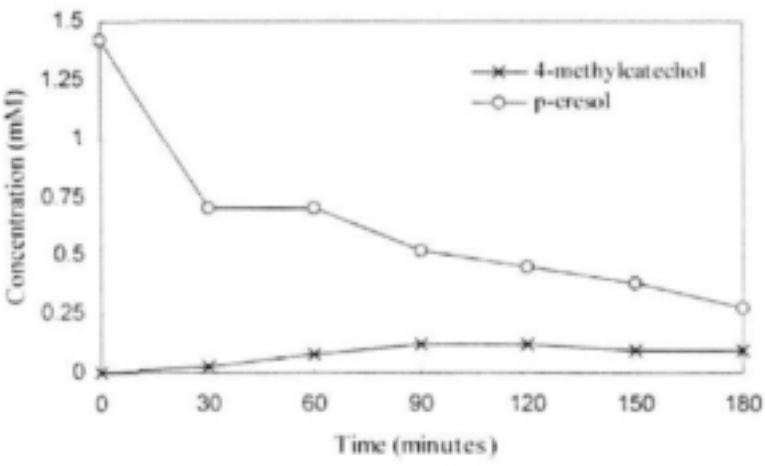


Figure 4.21 **The utilisation of 1.45mM *p*-cresol and the formation of 4-methylcatechol over 3 hours using polyphenol oxidase immobilised in gelatin gel.**

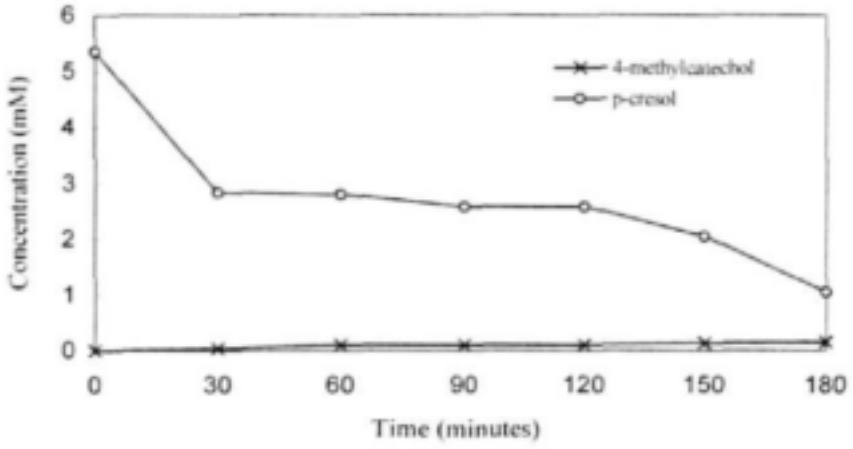


Figure 4.22 The utilisation of 1.45mM *p*-cresol and the formation of 4-methylcatechol over 3 hours using polyphenol oxidase immobilised in gelatin gel.

4.3.18 Application of non-immobilised polyphenol oxidase in the treatment of phenolic effluents

The inclusion of ß-cyclodextrin in the mobile phase facilitated the complete separation of the structural isomers of cresol. The addition of polyphenol oxidase to the effluents resulted in decreases in the concentrations of the phenolic pollutants. The utilisation of phenolic pollutants by the addition of polyphenol oxidase in Cresylic effluent 1 is shown in Figure 4.23. There was an approximately 25% reduction in the concentration of *o*-cresol in the control, while there was very slight reduction in the concentrations of the phenol, *m* - and *p*-cresol controls.

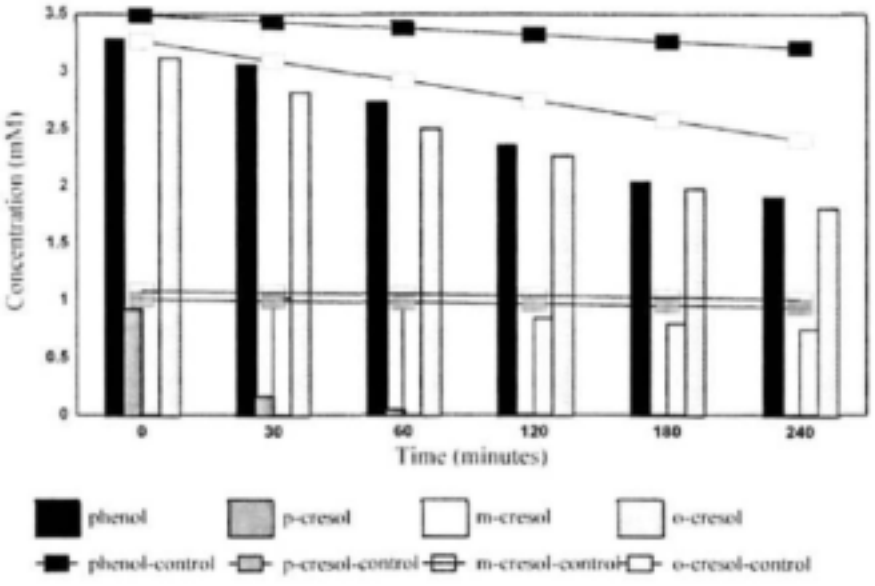


Figure 4.23 Utilisation of phenolic pollutants present in Cresylic effluent 1 by the addition of 6U polyphenol oxidase.

The percentage utilisation of phenol and the cresol isomers, present in each effluent, by polyphenol oxidase is shown in Table 4.17. There was 100% removal of *p*-cresol in all the effluents indicating a preference for *p*-cresol by polyphenol oxidase. Significant *m* - and *o*-cresol removal occurred during the reaction with polyphenol oxidase. Greater phenol utilisation occurred in the Black Product effluents as the initial concentrations of phenol were lower than in the Cresylic effluents. High concentrations of phenol inhibit polyphenol oxidase activity which could be attributed to the inhibitory nature of its reaction products. These results indicate that polyphenol oxidase is capable of reacting with phenolic pollutants in industrial effluents.

Table 4.17 Utilisation of phenolic pollutants present in Cresylic and Black Product effluents, by 6U polyphenol oxidase.

	Phenolic pollutants			
	phenol	*p*-cresol	*m*-cresol	*o*-cresol
Cresylic 1	42.3%	100%	30.4%	42.1%
Cresylic 2	44.3%	100%	31.1%	36.9%
Black Product 1	92.5%	100%	74.3%	95.9%
Black Product 2	78.6%	100%	59.8%	63.4%

4-Methylcatechol was detected during the reaction of polyphenol oxidase with the Cresylic effluents using HPLC (Figure 4.24. The concentration of 4-methylcatechol increased 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. A higher concentration of 4-methylcatechol was detected in the Cresylic 2 than Cresylic 1 effluent as there was a higher starting concentration of *p*-cresol in the Cresylic 2 effluent. Catechol and 3-methylcatechol were not detected by HPLC due to their rapid oxidation to *o*-quinones.

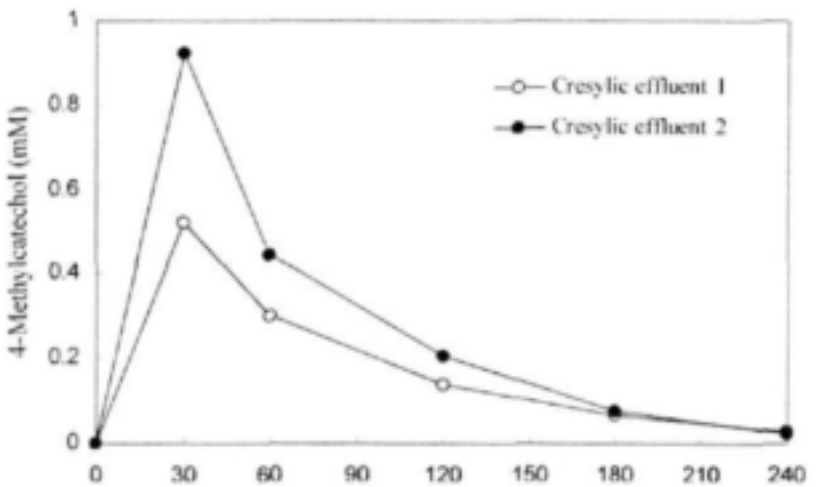


Figure 4.24 Production of 4-methylcatechol during the reaction of polyphenol oxidase with Cresylic effluents.

4.3.19 Non-immobilised polyphenol oxidase in the treatment of phenolic effluents using borate buffer

The effluent was prepared in 75mM borate buffer, with the addition of ascorbate in an attempt to increase the yield of catechol products. The percentage utilisation of phenol and the cresol isomers, present in each effluent, by polyphenol oxidase is shown in Table4.18. There was 100% removal of *p*-cresol in the more dilute effluent.

Table 4.18 Utilisation of phenolic pollutants present in two dilutions of Cresylic effluent 1

	30x dilution		50x dilution	
Phenolic pollutants	**Initial concentrations (mM)**	**% Utilisation by polyphenol oxidase**	**Initial concentrations (mM)**	**% Utilisation by polyphenol oxidase**
phenol	3.0	35.4	1.6	65.1
p-cresol	0.9	96.3	0.5	100.0
m-cresol	0.8	14.0	0.5	26.7
o-cresol	2.6	18.3	1.4	22.7

4-Methylcatechol and catechol were detected during the reaction of polyphenol oxidase with the Cresylic effluent 1 (diluted 30 times) (Figure 4.25). The concentration of 4-methylcatechol increased during the first 270 minutes and then started to decrease rapidly as the ascorbate was completely utilised, which resulted in the further oxidation of 4-methylcatechol to *o*-quinone. The catechol concentration, which reached 0.8mM, did not decrease during the reaction which indicates a preference of polyphenol oxidase for 4-methylcatechol. 4-Methylcatechol and catechol were also detected during the reaction of polyphenol oxidase with the Cresylic effluent 1 (diluted 50 times) (Figure 4.26). The concentration of 4-methylcatechol and catechol increased during the first 180 minutes and then started to decrease rapidly as the ascorbate was completely utilised which resulted in the further oxidation of 4-methylcatechol and catechol to *o*-quinon.e

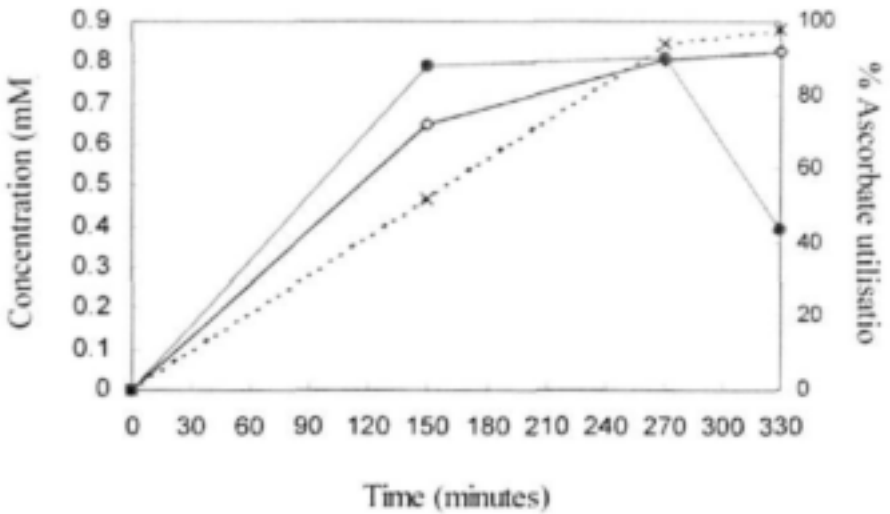


Figure 4.25 **Production of catechol (◊), 4-methylcatechol (●) and utilisation of ascorbate (- -x- -), during the reaction of polyphenol oxidase with Cresylic effluent 1, diluted 30 times in 75mM borate buffer (pH 7.8) containing 12.5mM ascorbate.**

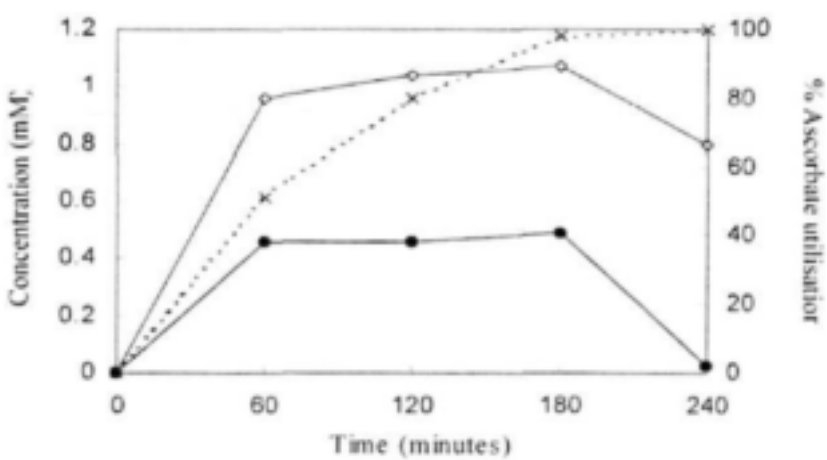


Figure 4.26 **Production of catechol (◊), 4-methylcatechol (●) and utilisation of ascorbate (- -x- -), during the reaction of polyphenol oxidase with Cresylic effluent 1, diluted 50 times in 75mM borate buffer (pH 7.8) containing 12.5mM ascorbate.**

4.4 CONCLUSIONS

- This study has demonstrated that catechols are true intermediate products formed during the reaction of phenols with polyphenol oxidase. The enzymatic products were successfully separated by HPLC and the catechol products identified using LC-MS.
- Conditions resulting in maximum catechol production were determined for the reaction of polyphenol oxidase with phenols.

  The presence of PEG resulted in higher catechol concentrations implying that a more hydrophilic environment, during the reaction of polyphenol oxidase with phenols, is best suited for catechol production.

  The stable complex formed between ascorbate and borate was exploited for the production of catechols. By manipulating the borate and ascorbate concentrations, 100% yield of catechol was possible without *o*-quinones present as contaminants.

- Polyphenol oxidase was efficiently immobilised on a variety of synthetic membranes and used to convert phenolic substrates.
- The reaction conditions and properties of the immobilisation support were shown to influence catechol formation.
- The influences of the hydrophobic or hydrophilic properties of the synthetic immobilisation supports were studied for the synthesis of catechols.

- The application of a novel mathematical model was demonstrated as a useful aid in the interpretation of reaction kinetic data which could then be used to determine which substrates and support materials allow 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. This mathematical modelling approach could be extended to other immobilised biocatalyst systems for the interpretation of kinetic data.

- In the application of the system for catechol production 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 where the catechols were rapidly oxidised to the corresponding *o*-quinones. Thus, the nature of the immobilisation support influenced catechol production. Although non-immobilised polyphenol oxidase yielded higher catechol concentrations, nitrocellulose- and nylon-immobilised polyphenol oxidase were shown to be effective in the production of 4-methylcatechol and 3-methylcatechol.

- Immobilisation of polyphenol oxidase on nylon membranes allowed for reuse of the enzyme over 11 days. However, immobilisation was not shown to greatly increase the catalytic lifetime of the enzyme. Polyphenol oxidase was successfully immobilised into polyurethane foams. Different surfactants resulted in differences in the structure of the foam, polyphenol oxidase activity and adsorption of substrates and products. Significant substrate utilisation occurred with polyphenol oxidase immobilised in gelatin gels, although minimal catechol accumulation occurred.

- The results of the 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.

- Polyphenol oxidase was shown to be effective in converting phenols in phenolic industrial effluents and in producing catechols. Although the catechol yields were increased in the presence of the borate buffer containing ascorbate, it is clear that these additives increase the practical cost of the system, and future research should be directed to development of a more economically efficient process. The process would lead to value addition to phenolic wastes in real effluents.

SECTION B

CHAPTER 5

Application of bacterial strains in bioconversion of olive processing waste components

5.1 INTRODUCTION

While olive processing waste is clearly problematic in terms of environmental pollution and disposal, from another point of view it has much potential for beneficiation. It contains a range of potentially valuable compounds which could be recovered and/or used as substrates in biotechnological processes, for the production of other valuable products. Many of the aromatic compounds present in olive waste are structurally related to many of the monomers of lignin (Sanjust *et al.*, 1991). Various phenolic compounds have been detected, including catechol, 4-methylcatechol, tyrosol and hydroxytyrosol (Capasso *et al.*, 1992). High performance liquid chromatography has been used to evaluate simple and hydrolizable phenolic compounds from virgin olive oil, including gallic acid, 3,4-(dihydroxyphenyl)-ethanol, protocatechuic acid, (*p*-hydroxyphenyl)-ethanol, *p*-hydroxybenzoic acid, vanillic acid, ferulic acid, caffeic acid, syringic acid, *p*-coumaric acid, *o*-coumaric acid, oleuropeine glycoside and cinnamic acid (Montedoro *et al.*, 1992).

The aim of this study was to identify such components in olivewaste, and to apply bacterial enzyme systems which can tolerate the conditions of the waste, *via* oxidative biotransformations, to convert the organic components in economically valuable products.

5.2 METHODS AND MATERIALS

5.2.1 Characterisation of olive processing effluents

Three types of olive effluents were obtained from an olive processing plant in the Western Cape and coded as follows (see Appendix 1):

FB - collected from the fermentation tank.

LV - collected from the surface of the digester tank.

SO – collected from the evaporation pond

5.2.2 Extraction of organic compounds present in oliveprocessing effluents

Olive effluent (200 mL) and olive effluent adjusted to pH 1 with H_2SO_4 (200 mL) were each extracted with ethyl acetate (200 mL). The organic extract was dried over anhydrous Na_2SO_4 and then filtered and evaporated. The residue was re-dissolved in 5 mL of water-acetonitrile (60:40) or methanol and analysed by HPLC (see below) and UV analysis was carried out to determine the wavelength at which maximum absorbance for the extracted effluents occurs in the range 200 - 800 nm using UV-visible spectrophotometry.

5.2.3 Analyses of phenols

Olive effluents and olive effluents acidified to pH 2 with HCl were extracted with ethyl acetate at room temperature. The organic fraction was dried with anhydrous ammonium sulphate for 30 to 40 minutes. The extract was concentrated to dryness in a rotary evaporator and re-dissolved with a mixture methanol-water (60: 40). Total phenolic content was determined by the Folin – Ciocalteau assay.

5.2.4 Thin layer chromatography

The standard compounds and effluent extracts were dissolved in chloroform-methanol (1:1). Analysis was carried out on silica plates with mobile phase 10% acetic acid in chloroform. The constituents were visualised by exposure to UV light.

5.2.5 High performance liquid chromatography(HPLC)

HPLC conditions: UV detection at 280 nm, flow rate 1 mL/min, mobile phase water-acetonitrile (60 : 40), 2% acetic acid in water, and water-methanol-acetic acid (68 : 30 : 2), run times 40 minutes. Samples of standard compounds were dissolved in mobile phase (1:10) and extracted

effluents were dissolve in mobile phase. The injection volume was 20 µL . The retention time for each sample was recorded. To confirm the identity of the peaks in the olive effluent extract (FB), after comparison of retention times of the standard compounds, the extract was spiked with the standard compounds one at a time and analysed by HPLC.

5.2.6 Standard compounds

Caffeic acid, ferulic acid, *p*-coumaric acid, *m*-coumaric acid, protocatechiuc acid, *p*-hydroxyphenylacetic acid, *p*-hydroxybenzaldehyde, syringic acid, vanillic acid, 4-chlorophenol, 2,4-dichlorophenol, *p*-cresol, 4-methoxyphenol, *m*-cresol and phenol were analytical grade products obtained from Sigma Aldrich. 10mM of these standard compounds were prepared in methanol and 1:10 dilution of each standard compound in methanol: mobile phase was prepared and analysed by HPLC. The retention times of standard compounds were compared with those of extracted organic compounds of olive effluents.

5.2.7 Isolation and characterisation of microorganisms present in olive processing effluents

Isolation of microorganisms

100 µL of each type of effluent was spread on nutrient agar (NA) plates and incubated at 28 °C. The different colonies obtained were streaked on NA plates until a pure culture of each colony was found. The pure cultures were streaked on olive effluent based agar plates (10 - 100%) and incubated at 28°C. The strains that grew on 10% up to 100% of olive effluent were kept on NA at 4 °C and transferred every four weeks.

Characterisation of bacterial strains isolated from olive effluents

The isolated strains were characterised physiologically by their growth temperature, growth pH and their ability to grow in the presence of NaCl. The strains were Gram stained and evaluated for ability to degrade phenolic compounds found in olive effluents.

5.2.8 Degradation of model compounds by bacterial strains isolated from olive effluents

Growth and substrate utilization by five bacterial strains isolated from the effluents was measured in 250 mL Erlenmeyer flasks containing 100 mL of nutrient broth and 1 mM of the

model compounds. The flasks were inoculated with each of the bacteria and incubated at 28 °C with shaking at 200 rpm. Everyday, from day 0 (which was the day of inoculation) to day 6, a 1mL of the culture medium was removed. Growth was measured by turbidity readings at $OD_{600\ nm}$, the culture medium was then centrifuged at 13 000 rpm for 5 minutes. The supernatants were kept at – 20 °C until they were analysed by HPLC.

5.2.9 HPLC quantitative analysis of model compounds

The supernatants were analysed on a LaChrom HPLC system equipped with a UV detector and a autosampler, with a Wakosil II C 18 Reverse phase column and detector wavelength 280 nm. The mobile phase was water : methanol : acetic acid (68:30:2), with a flow rate of 1 mL per minute.

5.2.10 Screening of microorganisms for growth in olive effluents

FB and LV olive effluents were used for this study. Each effluent was diluted to 50% using distilled water. The effluents were centrifuged and filtered to remove suspended solids, then autoclaved and allowed to cool to room temperature before use. The 50% solutions were used as the stock solutions for all further analysis.

Bacterial strains used: RU – LV1, RU – FB1, RU – FB2, RU – SO1, RU – SO2,
Fungal strains used: *Trametes versicolor*, *Neurospora crassa*

Plate experiments were conducted to determine the feasibility of microorganisms to grown on olive effluents. The solid media for each microorganism was supplemented with the respective olive effluent. A series of plates were made with the final effluents concentration of : 10, 20, 30, 40 and 50%. The plates were inoculated with the different microorganisms and incubated at28°C. All experiments were done in triplicate.

5.2.11 Flask culture study

Bacterial strains

A series of 250 mL flasks were prepared containing 100 mL of culture medium based on the results of the plate experiments. Media were prepared in the same way as for the plate study except that the agar was omitted and replaced with broth and only 20% effluent final

concentration was used. The flasks were inoculated with the selected bacterial strain and incubated with shaking (200 rpm) at a temperature suitable for that microorganism. All experiments were done in duplicate.

Fungal strain

T. versicolor from a malt extract agar was homogenized under sterile conditions and 10 mL of the homogenate was transferred to 250 mL flasks each containing 50 mL of Trametes defined media (TDM) supplemented with 20% or 10% of LV and 10 or 5% FB effluent final concentration. These were then incubated at 30° C with or without shaking (200 rpm) for seven days.

T.versicolor was also grown statically in TDM or TDM supplemented with olive effluent. After seven days, the fungi was transferred to fresh media with or without the olive effluent and incubated at 30° C as static cultures for seven days.

All experiments were done in duplicate.

Analyses of phenols

Samples were acidified with HCl to pH 2 and extracted with ethyl acetate (v/v) at room temperature. The organic fraction was dried with anhydrous ammonium sulphate for 30 to 40 minutes. The extract was concentrated to dryness in a rotary evaporator and redissolved in a mixture methanol-water (60 : 40). The total phenolic content was determined by the Folin – Ciocaltaeu assay.

5.2.12 Bionconversion of vanillic acid and ferulic acid by bacterial strain RU – LV1

Growth conditions

RU – LV1 was grown in minimal medium with glucose as a carbon source, or minimal media supplemented with 0.01% vanillic acid as a carbon source instead of glucose, or minimal media with glucose and 0.01% vanillic acid added after 12 hours of growth of the microbe. The culture medium was incubated at 28 °C, with shaking, for 24 hours.

Resting cells

Cells cultivated in 250 mL shake flasks containing 100 mL medium were harvested when they reached 1.00 to 1.50 OD_{600} density. The bacteria were collected by centrifugation at 10000 rpm for 10 minutes, washed in sterile 0.1 M sodium phosphate buffer (pH 7) and then resuspended in the same buffer to a concentration of 100mg of cells per mL of the buffer. The cell suspension was used immediately.

French pressed extract , cell free extract and cell debri

The suspension which contained 100 mg of cells per mL of buffer was passed through a French press. The French pressed extracts were used as they were or were centrifuged at 10000 rpm for 10 minutes to remove cell debris. The cell free extracts were used immediately. The cell debris was kept at – 20 °C freezer until further use, when it was suspended in sterile 0.1 M phosphate buffer (pH7).

Biotransformation of vanillic acid by resting cells, French pressed extract, cell free extracts or cell debris

One mL of vanillic acid in phosphate buffer (0.1 M, pH 7) was mixed with 1 mL of resting cells, French pressed extract, cell free extract or cell debris in buffer. The mixture was incubated with shaking at 200 rpm at 28 ^{0}C. Samples were taken over time. The samples were centrifuged at 13000 rpm to remove cell debris and 500 uL of 12% tricarboxylic acetic acid was added to stop the reaction. The supernatants were analysed by HPLC as described above.

5.3 RESULTS AND DISCUSSION

5.3.1 Characterisation of olive processing effluents

Total phenolic content of olive effluents

The total phenolic content of the olive effluents FB, LV and SO, and that of the same olive effluents adjusted to pH 2, were measured by solvent extraction and assay (Figure 5.1). After evaporating the organic fraction to dryness an oily residue was obtained. FB was found to contain a higher phenolic content than the other two effluents. A possible explanation for the lower phenolic content in LV and SO as compared to FB might be the partial natural biodegradation of the phenolic compounds prior to sampling. FB is collected from the

fermentation tank, which is the first effluent resulting from the olive processing. From the fermentation tank the effluent goes to the digester and then to the evaporation pond. The storage of olive mill wastes in evaporation ponds partially reduces contamination of the effluents with phenolic compounds (Moreno *et al.*, 1987). This was observed in this study, since SO collected from the evaporation pond had the lowest total phenolic content as compared to the other olive processing effluents. The phenolic content of FB increases when adjusted to pH2, whereas the phenolic content of the other two effluents decreases. The pH of the olive effluents is shown in Table 5.1.

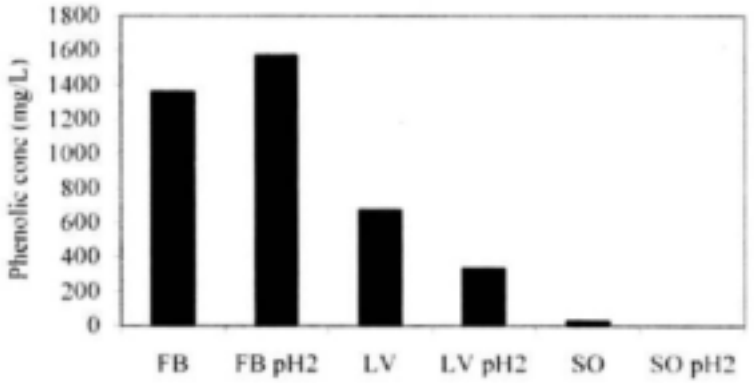


Figure 5.1 The total phenolic content of olive effluents and olive effluents adjusted to pH 2.

Table 5. 1 The pH of the olive effluents

Effluent	pH
FB	**3.51**
LV	**7.86**
SO	**7.70**

UV analysis

The wavelengths of maximum absorbance for each effluent extract are shown in Table 5.2. In methanol and water:acetonitrile, the organic compounds in the effluents absorb at almost the same wavelength.

Table 5. 2 Wavelengths of maximum absorbance of extracted effluents

Effluent type	Solvent		
	Methanol	Ethanol	Water:Acetonitrile(60:40)
LV	275 nm	280 nm	299 nm
FB	278 nm	442 nm 293 nm	301 nm
LV (pH1)	277 nm	288 nm	302 nm
FB (pH1)		309 nm 435 nm	301 nm

Thin layer chromatography

In a preliminary investigation using TLC analysis of extracts, FB separated into four components, LV into two components, FB (pH1) into three components and LV (pH1) into two components.

High performance liquid chromatography

The retention times of the standard compounds were comparable to those of the extracted organic compounds in the effluents. However, some of the standard compounds were eluted with almost identical retention time using acetonitrile-water (60 : 40) as a mobile phase and this mobile phase did not give a good separation for the peaks in olive effluent extracts. The mobile phase that gave better separation of peaks in olive effluents was water-methanol-acetic acid (68 : 30 : 2). Therefore water-methanol-acetic acid was used in all the other HPLC analyses. The standard compounds, protocatechuic, vanillic and ferulic acids were identified in FB using the spiking experiments.

5.3.2 Isolation and characterization of microorganisms present in olive processing effluents

Strains RU-LV1, RU – FB1 and RU – FB2, and RU-SO1 and RU-SO2, isolated from the effluents LV, FB and SO respectively, were found to grow in 10% to 100% of the respective olive effluent- based agar plates. This is not surprising since the strains were isolated from these effluents.

All these isolates are Gram negative and rod shaped. They were found to tolerate a wide pH and temperature range (Table 5.3), which could be important for treating wastewaters where pH and temperature fluctuations are common. The optimal growth temperature for all these bacterial strains was determined to be 28°C, and therefore in all the other experiments these bacterial strains were grown at 28 °C. Although these olive effluents do not contain high salt concentrations, the isolated bacterial strains tolerated high salt concentrations (Table 5.3). These strains have not been identified and very little is known about the properties of strains cultivated in olive oil wastewater (Moreno et al., 1987). Further studies are needed for their genetic identification.

Table 5.3 Physiological characteristics of bacterial strains isolated from olive effluents.

Physiological characteristic	RU – LV1	RU – FB1	RU – FB2	RU – SO1	RU – SO2
Growth temp	20 – 40 °C	15 – 45 °C	15 – 45 °C	15 – 45 °C	15 – 40 °C
Growth pH	6 - 10	5 – 9	5 - 9	5 - 9	5 – 10
Growth in NaCl solution	0.015 – 9.00%	0.015 – 6.00%	0.015 – 6.00%	0.015 – 4.00%	0.015 – 5.00%

5.3.3 Growth characteristics of isolated strains and degradation of model pollutants

All the bacterial strains were found to grow in nutrient broth supplemented with caffeic, *p*–coumaric, protocatechuic, syringic and vanillic acids Figure 5.2 – 5.5. No lag phase was observed during the growth of these strains in all the phenolic compounds at 1 mM. The removal of the model compounds by the bacterial isolates is shown in Table 4.

Table 5.4 Removal of the model pollutants by the bacterial isolates.

Strain	Model compounds			
	Caffeic acid	Protocatechuic acid	p–coumaric acid	Vanillic acid
RU – LV1	96%	100%	73%	100%
RU – FB1	96%	50%	0%	76%
RU – FB2	96%	59%	0%	0%
RU – SO1	96%	97%	68%	0%
RU – SO2	97%	97%	0%	0%

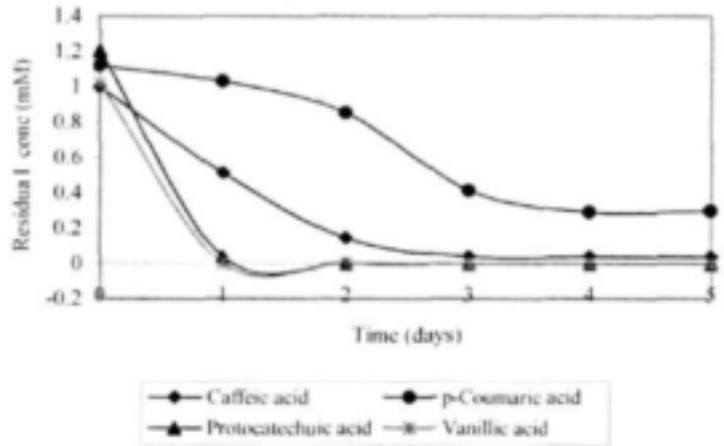


Figure 5.2 Degradation of model compounds (1mM) by RU – LV1

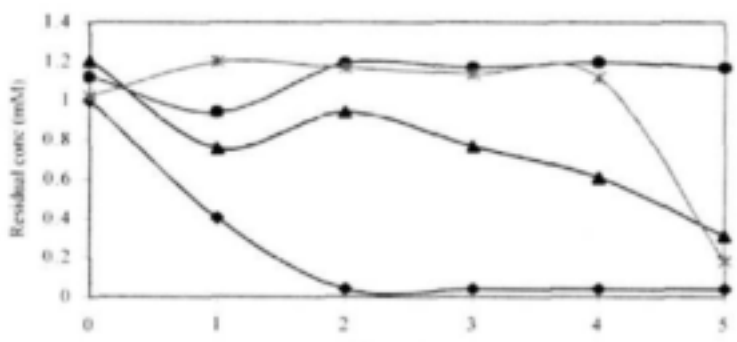


Figure 5.3 Degradation of model compounds (1 mM) by RU – FB1

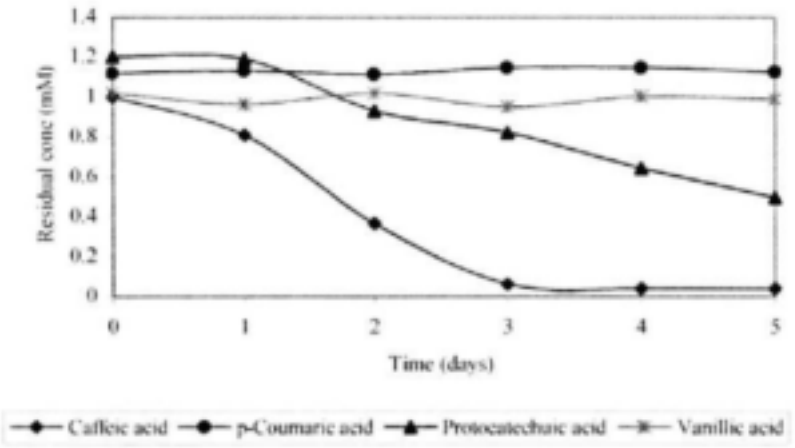


Figure 5.4 Degradation of model compounds by RU – FB2

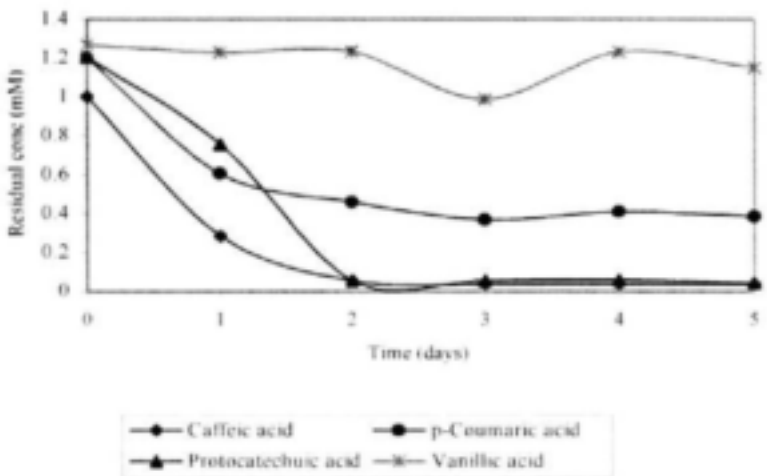


Figure 5.5 Degradation of model compounds (1mM) by RU – SO1

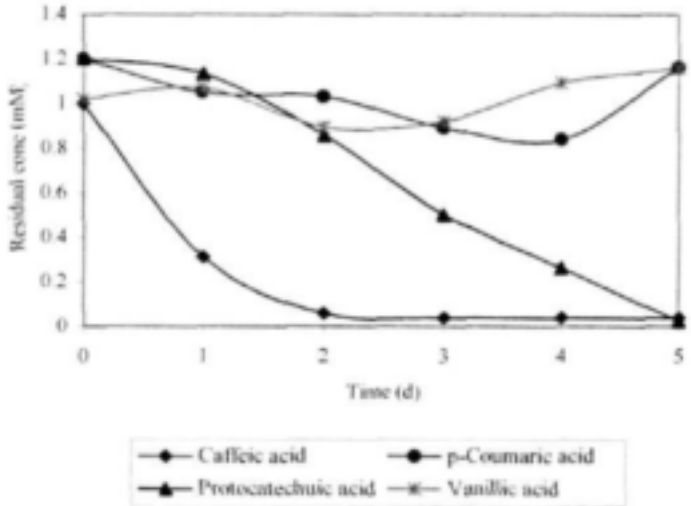


Figure 5.6 Degradation of model compounds (1mM) by RU – SO2

During the experiments on the growth of all the isolates in media containinng caffeic and protocatechuic acid, the media became black and dark-brown respectively. The colour change is likely to be due to quinoid products. None of the bacterial strains degraded syringic acid. All the isolated bacterial strains degraded caffeic and protocatechuic acids at 1 mM. These results could suggest tolerance due to the content of these acids in olive wastes and induction of appropriate enzymes in the bacterial strains. RU – LV1 showed higher efficiency in degrading the model compounds used in this study than all the other isolates. Thus, useful strains potentially capable of degrading phenolics in wastewater from olive mills were isolated in this study.

5.3.4 Screening of a range of microorganisms for growth in authentic olive effluents

Plate cultures

The results of a survey growth of microorganisms grown on agar plates supplemented with olive effluents is shown in Table 5.5 and 5.6. Growth inhibition of the isolated bacterial strains occurred in FB. LV is more acidic (pH 3.5) than LV (pH 7.9) and in general bacteria prefer a neutral pH to an acidic one, which may explain why these bacteria grew only on LV. Both *N. crassa* and *T. versicolor* grew on 10 to 50% of each of the olive effluents used. There was less

growth of *T. versicolor* at higher concentrations (40 to 50%) of FB possibly due to a decrease in available nutrients.

Table 5.5 Growth of bacterial strains isolated from olive effluents on agar plates supplemented with the olive effluent.

Strain	10%	20%	30%	40%	50%
RU – LV1	+ + +	+ + +	+ + +	+ + +	+ + +
RU – FB1	+ +	+	+ +	+ +	+ +
RU – FB2	+	+	+ +	+ +	+
RU – SO1	+ +	+ +	+ +	+	+
RU – SO2	+ +	+	+ +	+	+

+ + + = Significant growth, + + = Growth, + = Poor growth

Table 5.6 Growth of fungal strains on agar plates supplemented with olive effluents.

Effluent	Strain	10%	20%	30%	40%	50%
LV	*T. versicolor*	+ + +	+ + +	+ +	+ +	+
	N. crassa	+ + +	+ + +	+ + +	+ + +	+ +
FB	**T. versicolor**	+ +	+ +	+ +	+	+
	N. crassa	+ + +	+ + +	+ +	+ +	+

+ + + = Significant growth, + + = Growth, + = Poor growth

Flask cultures

All the isolated bacterial strains were found to grow in liquid media supplemented with 20% LV. Degradation of phenolics in LV by these microorganisms occurred after three days, and did not increase further, and therefore the phenolic content determined after three days of growth is reported. RU – SO1 showed the highest phenolic removal than the other isolates (Table 5.7 and Figure 5.7).

Table 5.7 Removal of the phenolic content from 20% LV by the bacterial isolates.

Strain	% Removal
RU – SO1	47%
RU – LV1	40%
RU – FB2	30%
RU – FB1	26%
RU – SO2	20%

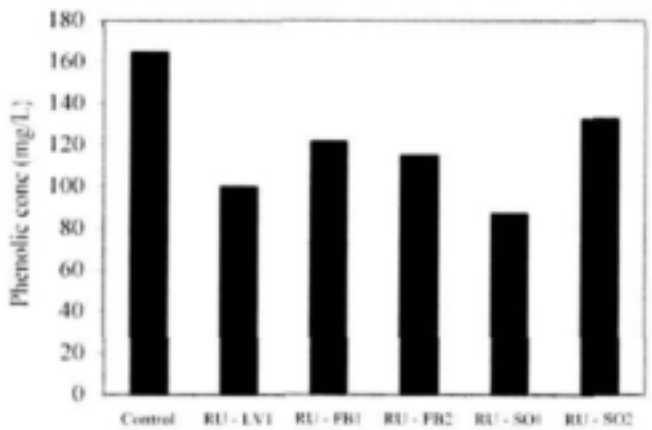


Figure 5.7 The total phenolic content in 20% LV after three days of treatment with bacteria isolated from olive processing effluents.

Since *T. versicolor* normally takes seven days to grow, the reduction in total phenolic content of olive effluents by the fungus was determined after seven days. The fungus was not able to grow in media supplemented with 20% LV, therefore 10% of LV and 10% FB and 5% FB effluent were used. Lower concentrations of FB were used because this effluent contains a higher phenolic content than LV. The removal of the phenolic content of FB and LV by *T. versicolor* is shown in Table 5.8. For both FB and LV shaking the culture lead to a higher reduction in the phenolic content. Growing the fungus first and subsequently adding effluent to the medium to reduce the total phenolic content lead to a higher phenolic removal from FB. Under static conditions growing *T. versicolor* in TDM lead to higher phenolics conversion than growing the fungus in TDM supplemented with the olive effluents (Figure 5.8, 5.9).

Table 5.8 Removal of the phenolic content of FB and LV by *Trametes versicolor*

Effluent	With shaking	Without shaking
10% LV	78%	71%
5% FB	51%	30%
10% FB	87%	50%

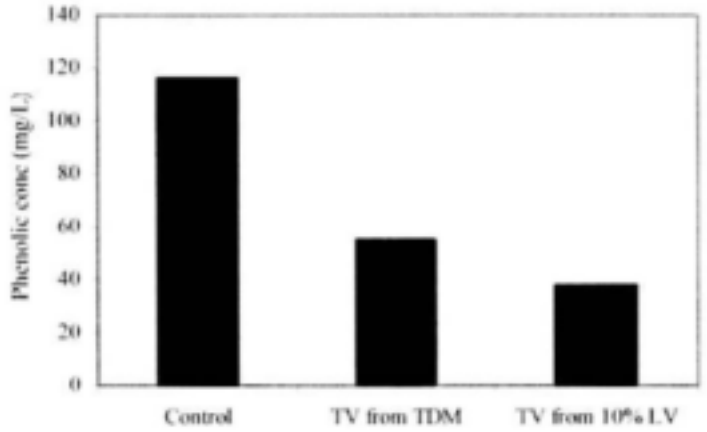


Figure 5.8 Degradation of the total phenolic content of 10% LV by *Trametes versicolor* (TV) grown in Trametes defined media (TDM) or in 10% LV.

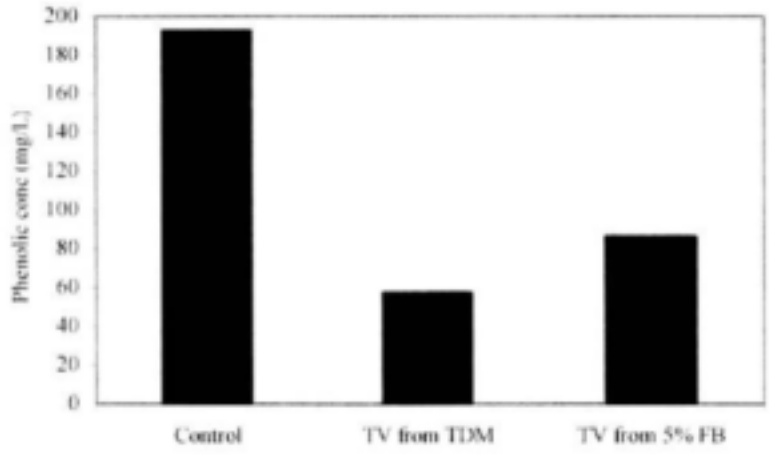


Figure 5.9 The residual total phenolic content of 5% FB treated with *Trametes versicolor* grown in TDM or in 5% FB.

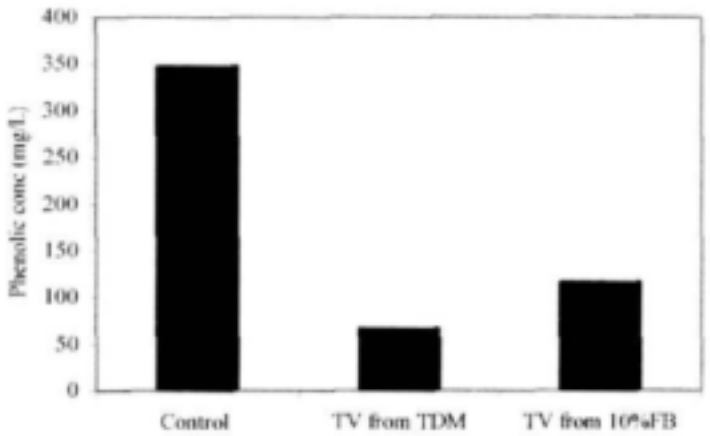


Figure 5.10 The residual total phenolic content of 10% FB treated with *Trametes versicolor* grown in TDM or in 10% FB.

The results indicated white rot fungushad more capacity to reducithe total phenolic content of olive effluents than the bacterial strains. White rot fungi have been studied in degrading lignin related compounds (Aust, 1990; Field *et al.*, 1993). Aromatic compounds known to be found in olive mill waste are known to be lignin related (Sanjust *et al.*, 1991). Fungi have also been studied for their ability to degrade olive wastes, which sometimes resulted in the removal of the dark colour of these wastes (Borja *et al.*, 1993; Hamdi & Garcia, 1993; Flouri *et al.*, 1996; D'Annibale *et al.*, 1998; Garcia Garcia *et al.*, 2000). A possible explanation for the lower phenolic content of the effluents after treatment with *T. versicolor* might be the capacity to mineralize the phenolic compounds in the effluents to carbon dioxide and water, since the fungi are known to mineralize lignin related compounds to carbon dioxide and water (Aust, 1990).

5.3.5 Bioconversion of vanillic acid by RU – LV1

RU-LV1 was selected as the bacterial strain exhibiting the greatest bioremediation potential, and further investigation of its bioconversion of phenolics was carried out.

Cells of RU – LV1 were grown in 0.01% vanillic acid as a carbon source, and complete conversion of 0.5 mM vanillic acid was observed after two hours, as compared to the same microbe grown in glucose as a carbon source, which degraded 56% of vanillic acid after 24 hours (Figure 5.11). However no metabolic products were detected in the supernatant of the reaction mixture. High concentrations (1 mM) of vanillic acid was not degraded by RU – LV1.

Cell free extracts, of induced and non induced cells, obtained by French pressing the cells and centrifugation, completely degraded 0.5 mM vanillic acid after 11 and 14 hours respectively (Figure 5.12). Cell debris obtained after centrifugation of the French pressed cells completely degraded 0.5 mM vanillic acid after 18 and 24 hours respectively (Figure 5.13).

Increasing the concentration of vanillic acid from 0.5 mM to 1 mM was found to retard the degradation of vanillic acid by the cell free extracts and the cell debris obtained from RU – LV1. The results of 1 mM vanillic acid are comparable to those of 0.5 mM whereby cell free extracts and cell debris from induced RU – LV1 degraded 1 mM vanillic acid faster than those from non-induced cells (Figure 5.15 and 5.16). The results of treating 1 mM vanillic acid by the French pressed extract of induced cells and those obtained using the cell debris indicate that the enzymes used for the degradation of vanillic acid are still in the cell debris since cell free extracts took a longer time to degrade vanillic acid.

RU – LV1 was also grown in a media with glucose for 12 h, after which 0.01% vanillic acid was added and the culture grown for another 24 hours. Cells, French pressed extract, cell free extracts and cell debris of this culture were used in degrading 1 mM vanillic acid. The French pressed extract and the cell debris completely degraded vanillic acid after 24 hours. Resting cells and cell free extracts degraded 76 and 60 % of 1 mM vanillic acid after 24 hours (Figure 5.17). The results of the degradation of vanillic acid by resting cells, French pressed extract, cell free extracts and cell debris indicate the induction of an enzyme for the degradation of vanillic acid since suspensions obtained from cells grown in 0.01% vanillic acid (used an inducer) always degraded vanillic acid faster than the cells grown in a medium with glucose as a carbon source.

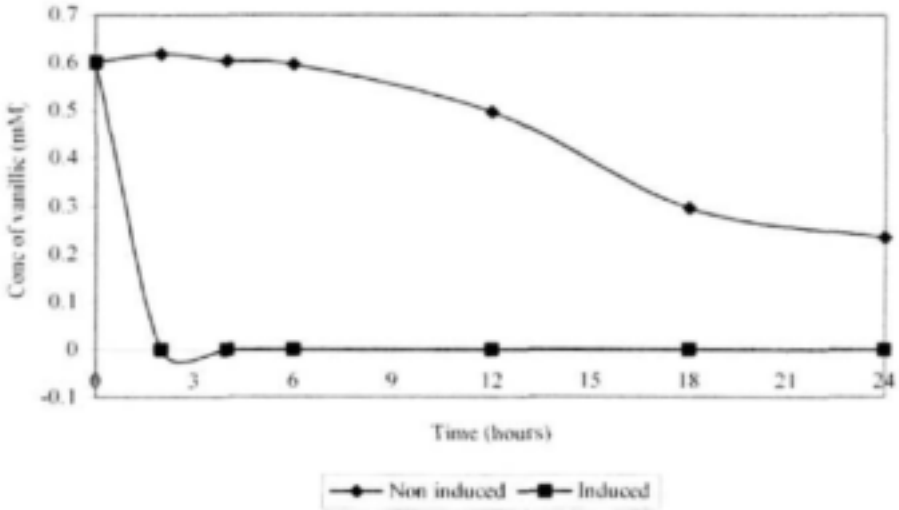


Figure 5.11 Degradation of 0.5 mM vanillic acid by resting cells of RU – LV1.

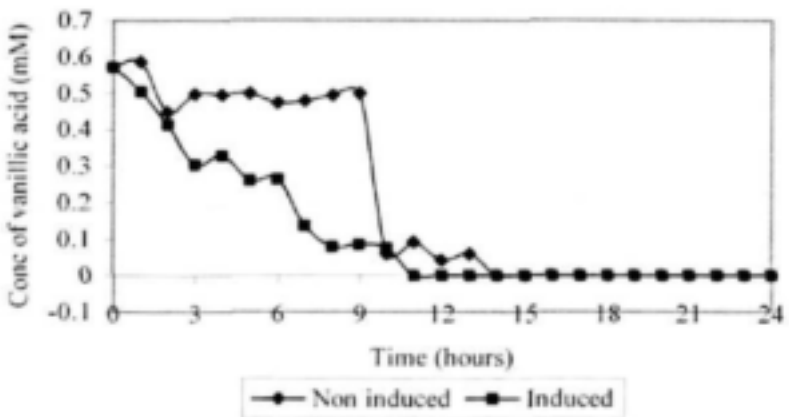


Figure 5.12 Degradation of 0.5 mM vanillic acid with the cell free extracts of RU – LV1.

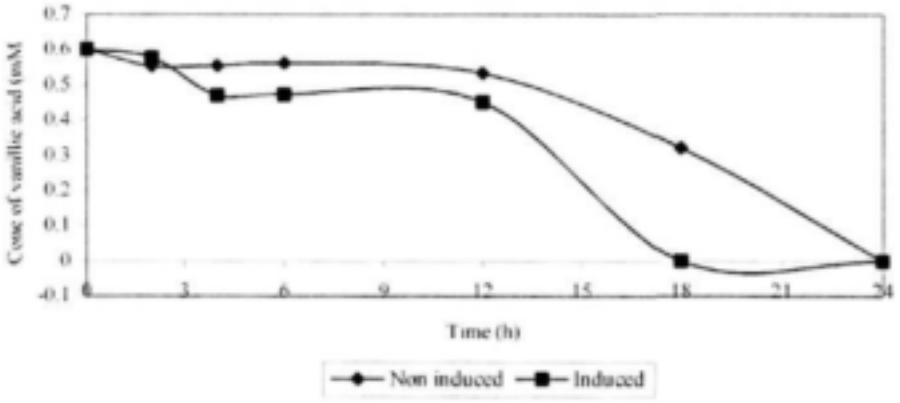


Figure 5.13 Degradation of 0.5 mM vanillic acid with the cell debris of RU – LV1.

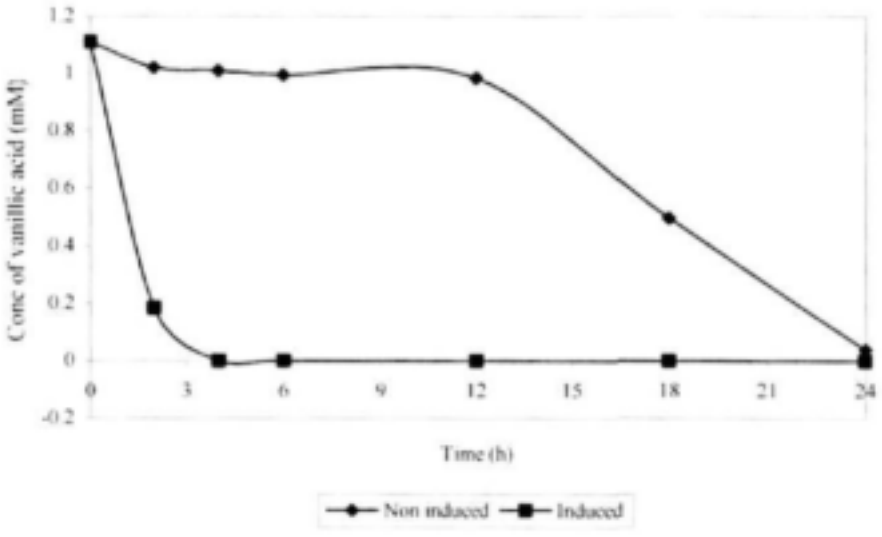


Figure 5.14 Degradation of 1 mM vanillic acid by French pressed extracts of RU – LV1.

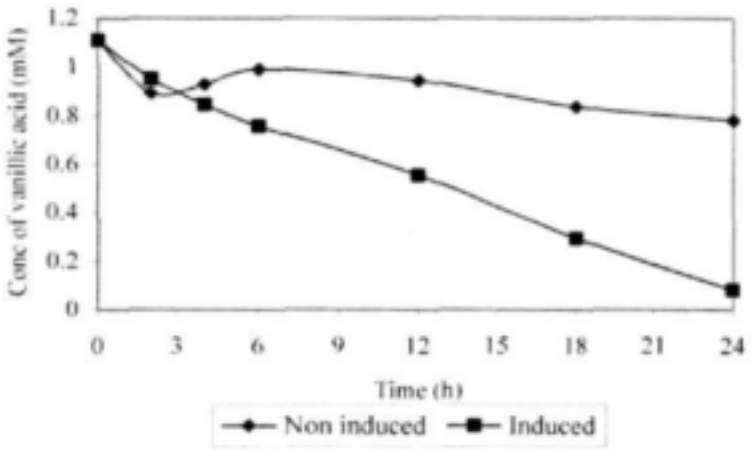


Figure 5.15 Degradation of 1 mM vanillic acid with cell free extracts of RU – LV1.

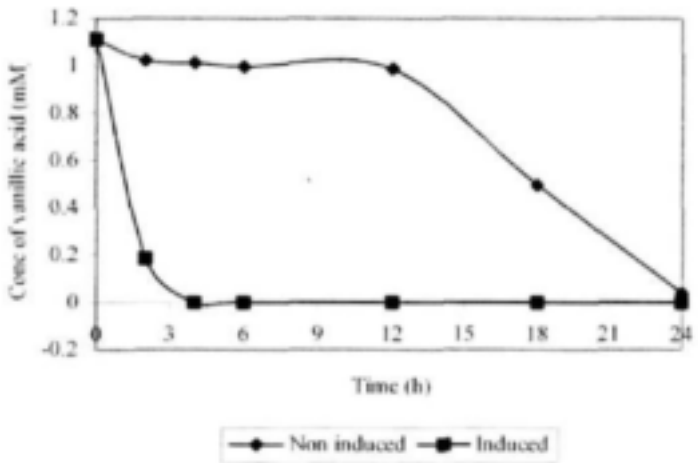


Figure 5.16 Degradation of 1 mM vanillic acid by the cell debris of RU – LV1.

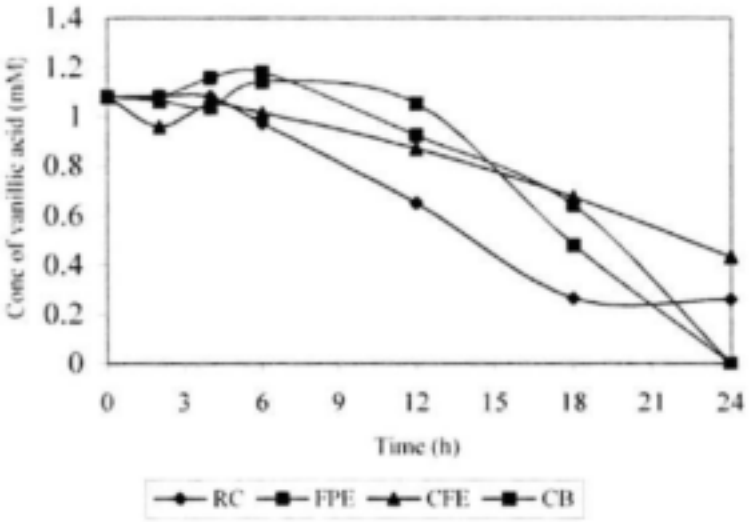


Figure 5.17 Degradation of 1 mM vanillic acid with resting cells (RC), French pressed extract (FPE), cell free extract (CFE) and cell debris (CB) of RU – LV1 grown in glucose and 0.01% vanillic acid added after 12 hours of growth in a medium with glucose.

5.3.6 Bioconversion of ferulic acid by strain RU-LV1

It has been reported that some bacterial strains can convert ferulic acid to vanillic acid or vanillin by a reductive process. This possibility was investigated with RU-LV1. Resting cells of induced and non induced RU – LV1 did not degrade 0.4 mM ferulic acid even after 24 hours of incubation. However, French pressed extract of both induced and non induced RU – LV1 completely removed 0.4 mM of ferulic acid from the solution after 18 hours. Vanillic acid was observed as a product of ferulic acid degradation, with vanillic acid accumulating over the 10 to 16 h reaction period, and thereafter the product was degraded or converted further.

The highest yield of vanillic acid from 0.4 mM ferulic acid was found to be 0.13 mM after 16 hours using the French extract of both induced and non induced cells. When RU – LV1 was grown in media with glucose for 12 hours and then 0.01% vanillic acid added as an inducer, the French pressed extract degraded 100% of 0.4 mM ferulic acid after 20 hours. Vanillic acid yield was 0.1 mM after 16 hours and then the concentration of vanillic acid gradually decreased until it was completely removed from the reaction mixture.

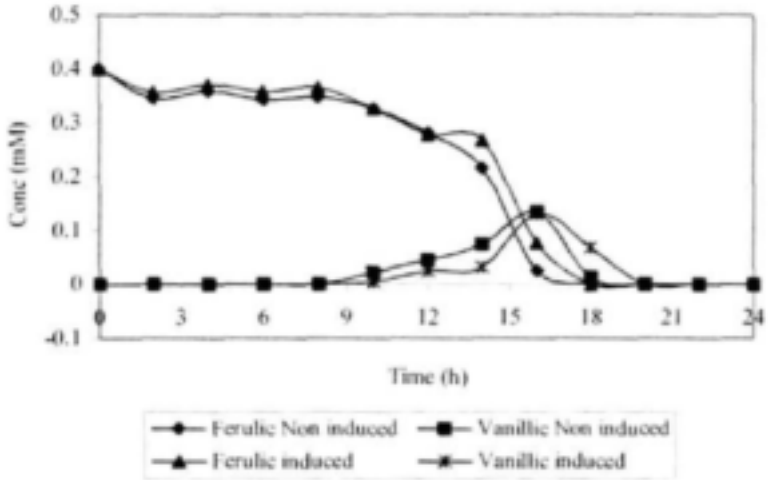


Figure 5.18 Degradation of ferulic acid into vanillic acid by French pressed extract of RU – LV1.

Cell free extracts obtained by centrifuging the French pressed extract, and using the supernatant, were used for the biotransformation of ferulic acid. These cell free extracts of non induced cells degraded 82 % of ferulic acid (0.4 mM) and the vanillic acid accumulated from 16 hours with a gradual increase from 0.014 mM to 0.076 mM after 24 hours. The cell free extracts from induced cells degraded 80 % of 0.4 mM ferulic acid after 16 hours and only 0.036 mM vanillic acid was observed in the reaction after 16 hours. The vanillic acid gradually disappeared from the reaction mixture. Cell free extracts from cells grown in glucose and 0.01% vanillic acid degraded 88% of ferulic acid at 0.4 mM after 24 hours. Vanillic acid was observed from 14 hours and it reached its maximum yield of 0.14 mM after 22 hours.

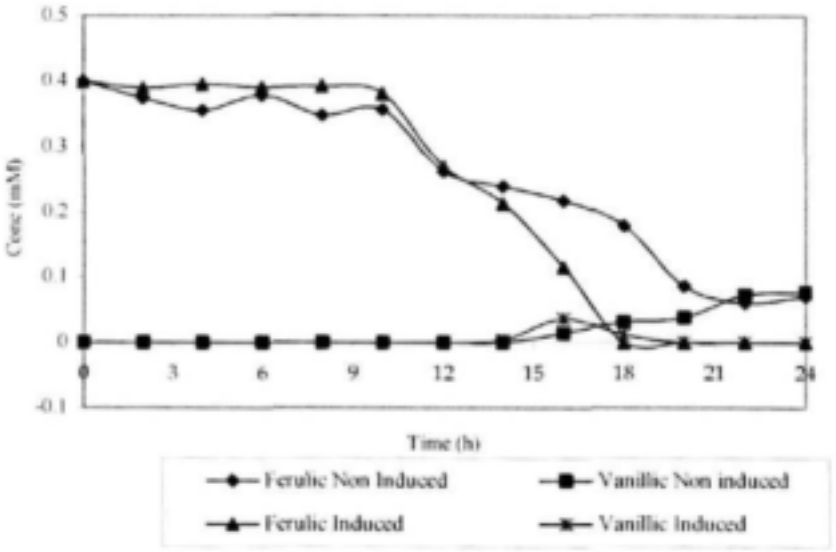


Figure 5.19 Degradation of ferulic acid into vanillic acid by cell free extracts of RU – LV1.

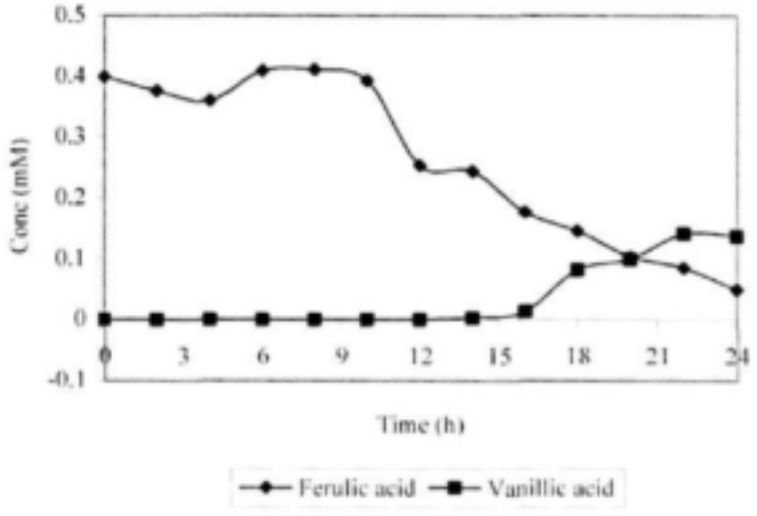


Figure 5.20 Degradation of ferulic acid into vanillic acid by cell free extracts of RU – LV1 grown in glucose and 0.01% vanillic acid.

With the application of cell free extract, the maximum yield of vanillic acid was found using an extract from cells grown in glucose and vanillic acid. Trace amounts of vanillin were also observed with cell free extracts from non induced cells and cells grown in glucose and vanillic acid. The vanillin appeared from 20 hours, and it accumulated until 24 hours.

In attempts to improve enzyme concentration in the cell-free extracts, the French pressed extract was sonicated and then centrifuged, and the supernatant was used as a cell free extract for ferulic acid bioconversion. The sonicated French pressed cell free extract of non induced cells degraded 75% of 0.4 mM ferulic acid after 24 hours. The vanillic acid appeared from 12 hours and vanillic acid concentration increased from 0.025 mM (after 12 hours) to 0.142 mM after 24 hours. Trace amounts of vanillin were observed after 24 hours of the reaction. Ferulic acid was completely removed from the reaction mixture after 14 hours when sonicated French pressed cell free extracts of induced cells were used. Vanillic acid appeared after 12 hours at 0.057 mM. However, no vanillic acid was observed after 14 hours. No trace amounts of vanillin were observed.

Cell free extracts which were French pressed and sonicated obtained from cells grown in glucose and vanillic acid degraded 74 % of ferulic acid after 24 hours. The yield of vanillic acid reached 0.23 mM after 24 hours and this was the maximum yield of vanillic acid with 0.4 mM ferulic acid.

These results illustrate that the enzymes for the biotransformation of ferulic acid to vanillic acid have been released into the cell free extract by French pressing and sonication. The high commercial value of vanillic acid indicates that further development of this reaction would be useful.

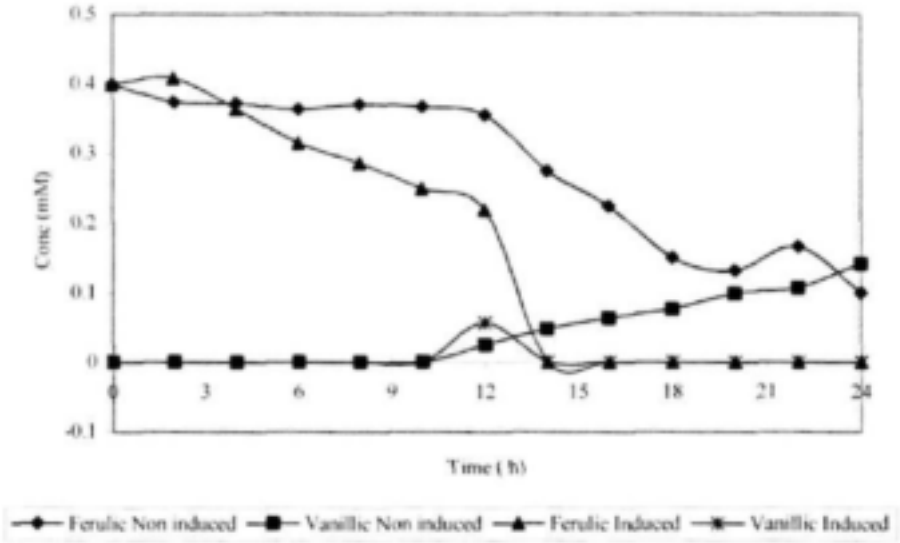


Figure 5.21 **Degradation of ferulic acid into vanillic acid by cell free extracts of RU – LV1 obtained by sonication after French pressing the cells.**

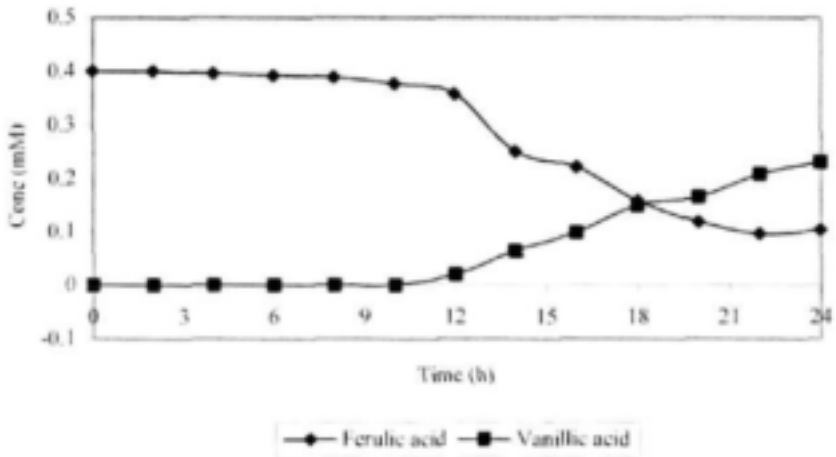


Figure 5.22 **Degradation of ferulic acid into vanillic acid by cell free extracts of RU – LV1 obtained by sonication after French pressing. The cells were grown in glucose and 0.01% vanillic acid.**

5.4 CONCLUSIONS

- The effluent coded FB has a high phenolic content, and lower but significant concentrations were measured in effluents coded LV and SO.
- Five bacterial strains isolated from olive effluents were characterised and found to tolerate a broad temperature and pH range, and also high salt concentration, with RU – LV1 tolerating up to 9 % NaCl. The five isolates degraded caffeic and protocatechuic acids at 1 mM. RU – LV1 and RU – FB1 degraded 1 mM vanillic acid and RU – LV1 and RU – SO1 degraded 1 mM *p*– coumaric acid.
- The white rot fungus *T. versicolor* exhibited high efficiency in reducing the total phenolic content of olive effluents as compared with the isolated bacterial strains.
- The resting cells, French pressed extract, cell free extract and cell debris of RU – LV1 degraded 1 mM vanillic acid.
- Sonicated and French-pressed cell-free extracts of cells, induced with vanillic acid, were shown to be capable of catalysing the economically important reaction converting ferulic acid to vanillic acid, with high efficiency.

CHAPTER 6

Application of lignin degrading enzymes from thermophilic actinomycetes in bioconversion of phenolics

6.1 INTRODUCTION

The basis of this study was to isolate thermophillic actinomycetes which have the ability to produce and secrete lignin degrading enzymes which play a role in the degradation of phenolic effluents. The aims of this study were to 1) assess these thermophiles ability to degrade phenols and 2) express these enzymes as recombinant proteins in an expression host, 3) identify the genes responsible for these enzyme production and finally 4) identify these thermophiles.

To address these goals actinomycetes were isolated from compost and assayed for polyphenol oxidase, peroxidase and laccase production as well as their ability to degrade small model lignin-related compounds. Three thermophiles (RU-A01, RU-A03 and RU-A06) were isolated, all producing polyphenol oxidase and peroxidases. These were then exposed to varying concentrations of a cheap phenolic source in the form of grape waste and grown in liquid media batches. UV scans, phenol determinations and enzyme assays were carried out to determine phenol breakdown and possible bioconversion products. DNA was extracted from these thermophiles and, using restriction enzymes, were restricted to fragment sizes of 1-2Kb and ligated into an *E.coli* expression host and transformed into *E.coli*, creating a gene library. Clones were assayed for enzyme activity. One clone was selected due to its consistent high producing activity with an insert size of 1.1Kb and sent for sequencing. 16s rDNA analysis was carried out on the thermophiles using two sets of primers to identify the actinomycetes.

6.2 MATERIAL AND METHODS

6.2.1 Isolation of Microorganisms.

Thermophillic actinomycetes were isolated from soil samples obtained from a compost heap. 1 g of soil was placed into 20 ml distilled water in 100 mL flasks and left shaking at 150 rev/min for 1 h before serially diluting in distilled water and plating out 10μl onto M65 agar plates and

incubated at 50°C overnight. Single actinomycete colonies were then selected and streaked out onto to fresh M65 agar plates and incubated overnight at 50°C.

6.2.2 Batch experiments

Medium and culture conditions

The thermophiles were grown in M65 broth and agar containing (g/l): 10 g Malt extract, 4 g yeast extract, 4 g glucose and 2 g calcium carbonate buffered to pH 8.0 in agitated (200 rev/min) 1 L flasks containing 200 mL of growth medium. Flasks were inoculated with spores from 2- to 3- week old agar slants and cultures were incubated at 50°C.

Phenol breakdown assays

Red grape waste was added to M65 broth and agar at different concentrations ranging from 0.1 % through to 50 %. Enzyme activities and phenol degradation were determined as described below. UV scans were taken before and after inoculation, as well as phenol determination assay using the Folin- Ciocalteau total phenol determination assay.

6.2.3 Determination of enzyme activities.

Peroxidase activity.

Peroxidase activity was assayed with 2,4-dichlorophenol (2,4-DCP) (Sigma) as a substrate. The reaction mixture (total volume 1.0ml) contained 200μl of 100 mM potassium phosphate buffer (pH 7.0); 200 μl of 16 mM 4-aminoantipyrine; 200 μl of 25 mM 2.4-DCP; 200 μl of culture supernatant or sonicated cells. The reaction was initiated with the addition of hydrogen peroxide (200 μl of 50 mM) and the reaction was monitored at 50°C for 1 min at a wavelength of 510nm (Ramachandra et al, 1988).

LiP activity

LiP activity was determined according to Tien and Kirk using a final veratryl alcohol concentration of 10 mM. 0.4 mL distilled water was added to 0.3 mL sodium tartrate buffer (pH 2.5, 0.25 mM), 0.1 mL veratryl alcohol and 0.1 mL crude enzyme extract. The reaction was initiated by the addition of 6μl of hydrogen peroxide (10 mM). Reaction was determined at absorbance 310 nm for 5 min.

Polyphenol oxidase activity

Polyphenol oxidase activity was measured using the Dopachrome assay. A fresh solution of L-DOPA (10mM) in 50mM of phosphate buffer, pH 6 was made up and 0.1 mL enzyme solution was added to 3 mLof the DOPA solution in a cuvette. The change in absorbance over 180 s was measured spectrophotometrically at 475 nm.

Protein estimation

Intra- and extracellular protein concentrations were determined by the method of Bradford (1976). Culture pellets were washed twice in phosphate buffer and then resuspended in phosphate buffer, sonicated on ice at maximum amplitude for 2 cycles of 10 seconds each. The mass was then spun down for 3 mins at 5000g and supernatant was then aliquoted in to 1.5 ml Eppendorfs. Extracellular protein were collected by pouring 1 ml pf phosphate buffer pH 7.0 onto dense lawns of growth on agar plates and gently removed using a loop. Total concentrations of supernatant and biomass protein were estimated from a calibration curve constructed using bovine serum albumin. Growth was measured as mg of intracellular protein per ml of culture supernatant. In addition, the specific activity is defined as the unit of enzyme activity per mg of protein.

Gel Electrophoresis

Native PAGE and SDS PAGE gels were performed using "Tall Mighty Small" electrophoresis apparatus. Gels were prepared with 4% stacking nd 10% resolving gel concentrations and were run for approximately 3 hours at 100V. Peroxidase bands were detected by incubating the gels in 10mM o-dianisidine in the presence of 1.6 mM hydrogen peroxide for 30 min and for polyphenol oxidase, gels were incubated for 30 min in a 10 mM solution of L-DOPA.

6.2.4 Metabolism of low molecular weight lignin-related compounds

Degradation of lignin model compounds

Although degradation of lignin model compounds by microorganisms may not reflect true lignolytic capacity, this approach has proved successful in studies with filamentous fungi. 10mM concentrations of caffeic, p-coumaric, syringic acid, ferulic acid, vanillic acid and protocatechuic acid were used as sole carbon sources in minimal liquid media. Samples were taken at regular intervals and run on HPLC to trace degradation of the compounds and possible bioconversion

products. Standard curve graphs were prepared from each model compound to convert peak areas into concentrations.

Grape waste preparation.

Red grape skins and pips were thawed and shredded using a Sorvall blender with distilled water. The resultant mixture was then passed through cheesecloth to collect the watersoluble phenols which was labelled grape waste 1. Acetone was then added to the remaining mass and left to stand for one hour before filtering off the liquid. An equal volume of distilled water was then added and then solvent extracted leaving the phenols in solution. This second batch was labelled grape waste 2.

6.2.5 Genomic DNA extraction

DNA used in this report was extracted using the Qiagen Dneasy Tissue extraction kit. The protocol used was provided in the handbook.

6.2.6 Restriction endonuclease digestion of genomic DNA

1 μl of DNA (concentration of 5 micrograms) was incubated in a final volume of 11μl of water, containing 2μl of the recommended digestion buffer (see table 2.1) and 1 μl of the restriction enzyme (5-10units/μl). DNA digestion was carried out at 37°C for 3 hours or overnight, followed by heating at 65° for 10 minutes to inactivate the enzyme.

6.2.7 Preparation of digested genomic DNA for electrophoresis

To test whether the digestion was complete, 8 μl of the digestion mix was added to 4 μl loading buffer and 1μl of sybr green (Material) and 10 μl was loaded onto 1% agarose (1X TBE, pH 8.3) (Materials). The DNA was electrophoretically fractionated at 80V. The DNA was visualised under an ultraviolet light. An even smear indicated complete digestion.

6.2.8 Gel extraction and purification

The desired bands were excised and purified using Separations and Qiagen gel purification kits according to the manufacturer's instructions.

6.2.9 Plasmid preparations

Culture of E.coli

E.coli cells containing plasmids pSE280 and pSE380 (Fig 6.10) were placed onto LB Amp (50μg/ml) plates, and incubated at 37°C overnight. Plasmid pSE280 and pSE380 were first transferred into competent E.coli cells by electroporation before being placed onto LB Amp plates. A single colony was picked from respective plates with a disposable loop and inoculated into 5mls of LB containing 50 μg/ml ampicillin, and incubated at 37°C by shaking for not longer than 16 hours.

6.2.10 Mini preps

Plasmid DNA extraction (CTAB method)

Pellet 1.5ml bacterial cells by centrifugation for 5 min at 13000g. Discard supernatant and resuspend cells in 200μl STET. Add 5 μl lysozyme and incubate at room temperature for 5 min. Boil the eppendorfs for 45 s. Centrifuge at 13000g for 15 min. Pour supernatant in a sterile eppendorf and discard pellet. Add 8 μl CTAB and centrifuge at 13000g for 5 min. Resuspend pellet in 300 μl NaCl and add 750 μl absolute ethanol and spin mixture at 13000g for 10 min. Discard supernatant and wash pellet with 800 μl 70% ethanol. Dry under vacuum for 30 min to 1 h. When almost dry, resuspend in 20 μl TE buffer.

The plasmid was screened as follows:

5 μl sample of plasmid DNA and 1μl of sybr green mixed togetehr and allowed 15 min to react. 3.5 μl of loading buffer was then added and the mixture was then loaded into a 1% agarose gel and electrophoresed at 80 V . The gel was then visualised under UV light.

Restriction endonuclease digestion of plasmid DNA

Plasmid DNA derived from mini-plasmid preparations (Fig 6.10). Two microliters of plamid DNA was incubated in a final volume of 11μL of water with 2μl of the recommended digestion buffer, containing approximately 1μL(5-10 units/μl) of the respective restriction endonuclease. Digestion was allowed to proceed at 37°C for 1-3 hours, followed by heating at 65°C for 10 minutes to inactivate the enzyme.

Preparation of digested plamid DNA for electrophoresis

Ten μL of the digestion mixture was aliquoted, with 1μL syber green and 4 μL of loading buffer from which 15 μL was loaded onto 1% agarose gel and electrophoretically frationated as described above.

6.2.11 Ligation of DNA framents into vectors

Linearisation of vector

The vector DNA, pSE280 and pSE380 was linerised by BamH1, Bgl11 and Sau3A restriction endonuclease at the multiple cloning site.

Ligation of foreign DNA into linerised vectors

Ligations were carried out using the following protocol:

	Std Rxn	**Positive control**	**Background control**
2 x Rapid ligation buffer	2.5μl	2.5μl	2.5μl
pSE vector	0.5μl	0.5μl	0.5μl
DNA	1.5μl	-	-
Control insert DNA	-	1.0μl	-
T4 DNA ligase	0.5μl	0.5μl	0.5μl
dH2O to 5μl final vol	-	0.5μl	1.5μl
Total volume	5.0μl	5.0μl	5.0μl

Reactions were mixed by pipetting several times and the 0.5μl eppendorf tubes with reactions are incubated at 4°C overnight to maximise the number of transformants obtained.

6.2.12 Preparation of *E.coli* competent cells

5mL of LB broth was added in a sterile test tube. A single colony was inoculated into a test tube and incubated at 37°C overnight. Into 500 mL flasks 100mL LB broth was added .

Over night cultures were inoculated as follows:

Flask 1 1.5 mL ON culture

Flask 2 1.0 mL ON culture

Flask 3 0.7 mL

Flask 4 0.3mL

Flasks were incubated on a shaker at 37°C for 2 hours. OD_{600} absorbance readings were taken of Flask 2 after 2 hours. OD readings were taken until OD600 readings were within the range of 0.8 (normally obtained after 2.5-2.75 hours). The flasks were placed on ice for 10 min and then contents were transferred into pre-autoclaved centrifuge bottles. The cells were centrifuges at 5000 rpm for 10 min at 4°C. The supernatant was decanted and the pellet resuspended in 50 mLs RF1 solution and incubated on ice for 20 min. The suspension was centrifuged using the same conditions in previous steps. The supernatant was decanted and each pellet was resuspended in 4 mLs RF2.The flask contents were all pooled together and 50 μL were aliquoted into sterile and pre-cooled eppendorfs and .stored at -80°C.

6.2.13 Transformation Procedure

The vial(s) containing the ligation reaction(s) were centrifuged briefly and placed on ice.

One 50μLvial of competent E.coli TOP10F' cells was thawed on ice for each ligation/transformation.

To transform, one of the following was used:

1 to 5μl of a ligation reaction

10pg supercoiled plasmid (i.e pUC18 as the transformation control)

DNA was directly added to the competent cells and mixed by tapping gently. Do not mix cells by pipetting. The remaining ligation reactions could be stored at -20°C.

The vials were incubated on ice for 30 min. Then the vial(s)were incubated for exactly 30 s in the 42°C water bath without mixing or shaking. The vials were removed from the 42°C bath and quickly placed them on ice. 250 μL of pre-warmed SOC medium was added to each vial and placed in a microfuge rack on its side and secured with tape to avoid loss of the vial(s). The vials were shaken 37°C for 1 h at 225 rpm in a rotary shaker-incubator. 10 to 50μL from each transformation vialwas spread on separate, labelled LB agar plates. It was necessary to plate two different volumes to ensure at least one plate has well space colonies. For plating small volumes, add 20μlL of SOC to allow even spreading. Note: Plate 50μl for the transformation control. Plates were inverted and incubated at 37°C overnight. Colonies were selected for further analysis by plasmid isolation.

6.2.14 Pilot expression

For each strain 2mL of SOB or LB containing 50 μg/mL ampicillin with a single recombinant *E.coli* colony was inoculated and grown overnight at 37°C, shaking at 225-250 rpm. The next day 10 mL of SOB or LB containing 50μg/mL ampicillin was inoculated with 0.2 mL of over night culture, grown at 37°C with vigourons shaking to an OD600= 0.6 (mid log phase). A 1 mL aliquot of cells was removed, centrifuged at maximum speed for 30 s and the supernatant aspirated. The pellet was frozen at -20°C. This was the zero time point sample. IPTG was added to the final concentration of 1mM (10 μlLof a 1M IPTG stock to 10 mL and grown at 37°C shaking. 1mL samples were taken every hour for 5 hours (or more) and treated by centrifugation at maximum speed for 30 seconds and aspiration of the supernatant. The tube were labelled to correspond to the number of hours post-induction.

6.2.15 cale up expression

2 mL of SOB or LB containing 50μg/mL ampicillin was inoculated with a single recombinant *E.coli* colony and grown overnight at 37°C shaking at 225-250 rpm.

The next day 10 ml of SOB or LB containing 50μg/ml ampicillin was inoculted with 0.2 ml of over night culture and grown at 37°C with vigourous shaking to an OD600= 0.6 (mid log phase). IPTG was added to the final concentration of 1mM (50 μL of a 1M IPTG stock to 50 mL and grow at 37°C shaking until the optimal time point was reached. Cells were harvested by centrifugation at3000 x g for 10 mins at 4°C.

6.3 RESULTS AND DISCUSSION

6.3.1 Isolation of strains

Four thermophillic actinomycetes were isolated from soil samples. Each strain was characterized by phenotype.

6.3.2 Whole cell treatment of grape waste

The strains were successfully inoculated on agar plates and into liquid medium containing various concentrations of red grape waste (10 to 50%). UV spectrophotometric scans showed shifts in absorbance peaks which indicated phenolic breakdown. Colour change in the growth medium was observed, from a brick-red colour to a pale orange colour for RU-A03 and a deep

purple colour for RU-A01. The latter may well be the result of polyphenol oxidase activity. The phenol content of the extracts was measured before and after the growth of the strains (Table 6.1).

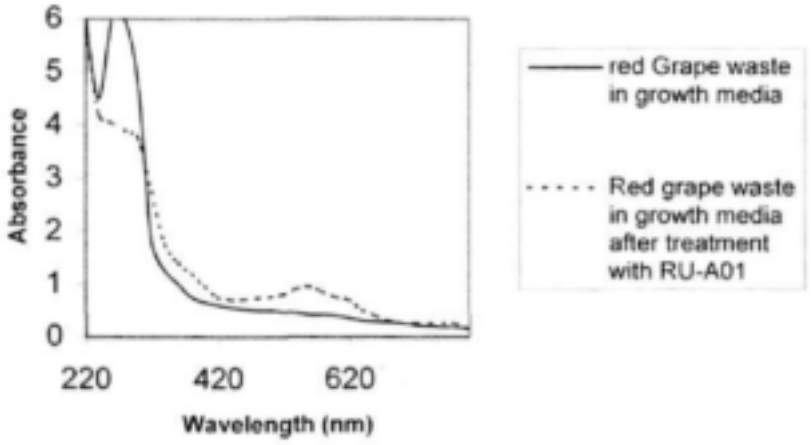


Figure 6.1 UV spectrum of red grape waste before and after treatment with strain RU-A01.

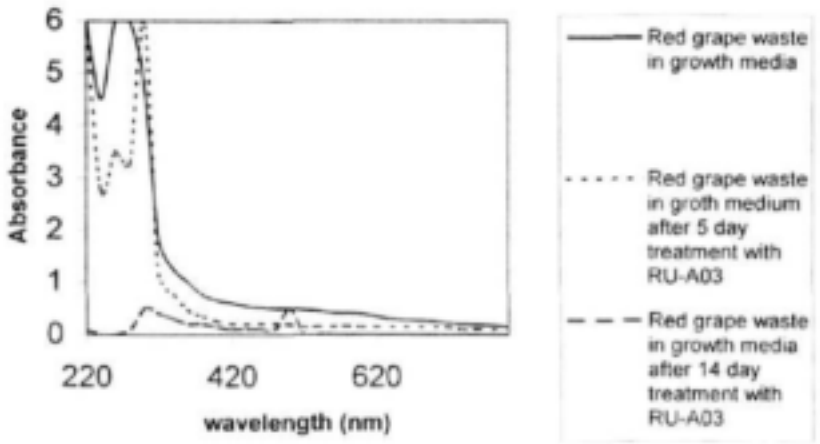


Figure 6.2 UV spectrum of 30% red grape waste before and after treatment with RU-A03 over a period of 7days and 14 days.

Table 6.1 Phenolic concentrations in grape waste before and after growth of soil isolates

Sample	Phenol Concentration (mg/L)
Red grape waste	1099
30% red grape waste in growth medium	279.2
RU-A01	31.7
RU-A03	59.2
RU-A06	108.3

6.3.3 Metabolism of low molecular mass lignin-related compounds

The strains were inoculated into minimal medium containing 10mM concentrations of a selection of model phenolic pollutants as sole carbon sources. These compounds were: ferulic acid, vanillic acid, syringic acid, protocatchuici acid and *p*-coumaric acid. The thermophiles were found to degrade these standard lignin–related compounds with a 95% decrease in the compounds within 24 hours (Figures 6.3 to 6.6).

Lignin-related aromatic acids, which contain phenylpropane (C_6 –C_3) type structures (such as ferulic acid and related compounds), are abundant molecules that play important functions in plant cells. The catabolism of these compounds is an important aspect for the mineralization of plant wastes as they are released during the breakdown of lignin. Caffeic, vanillic, ferulic, protocatechuic, syringic and p-coumaric acids are all low molecular mass lignin-model like compounds, which are also found in the lignin degradation pathway. Although degradation of lignin model compounds by microorganisms may not reflect true lignolytic capacity, this approach has proved successful in studies with filamentous fungi.

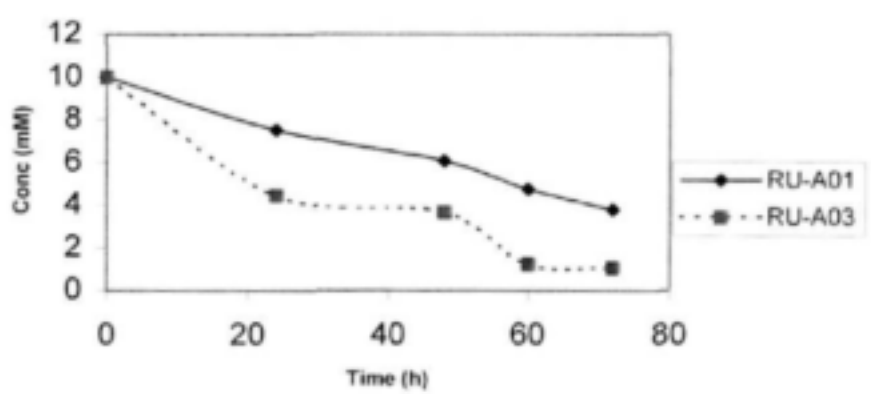


Figure 6.3 Ferulic acid degradation by RU-A01 and RU-A03

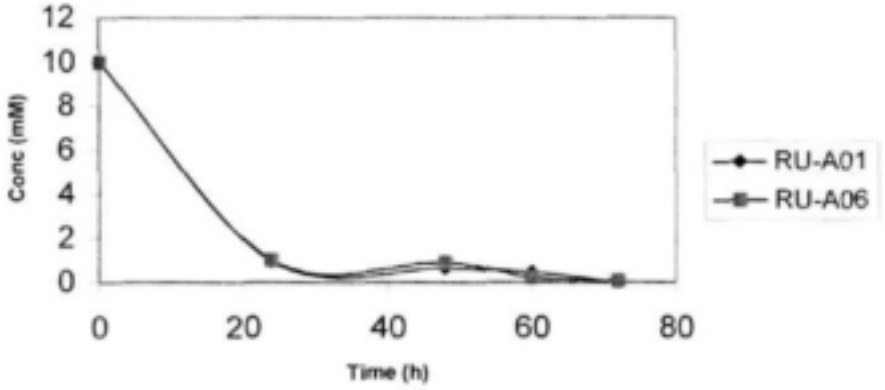


Figure 6.4 Vanillic acid degradation by RU-A01 and RU-A06

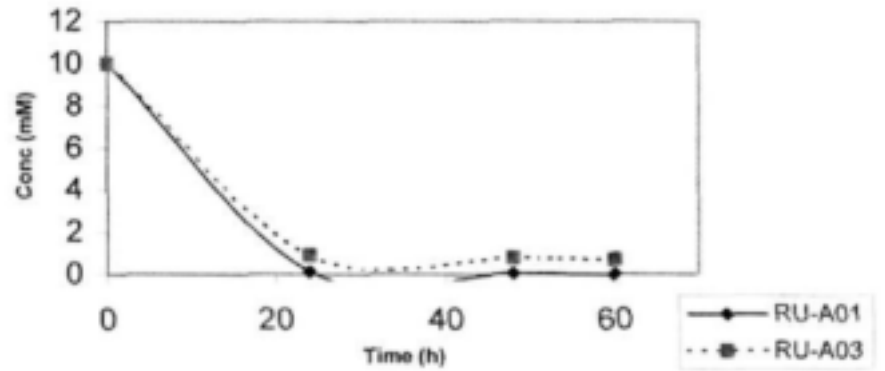


Figure 6.5 Protocatechuic acid degradation by RU-A01 and RU-A03

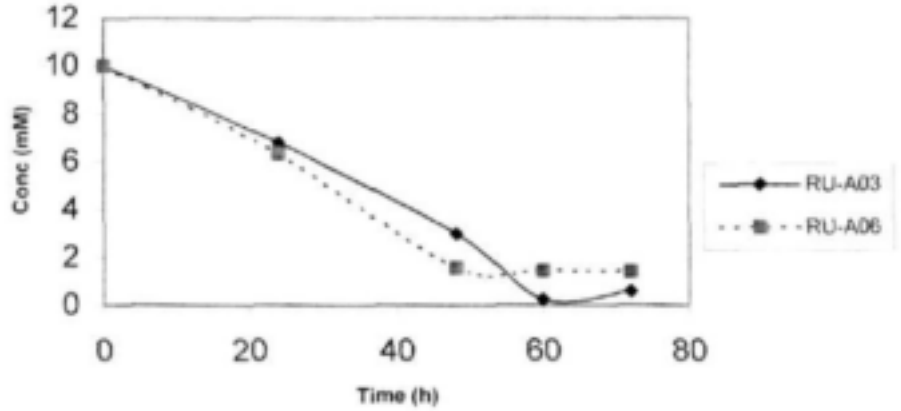


Figure 6.6 p-Coumaric acid degradation by RU-A03 and RU-A06

6.3.4 Oxidative enzyme activities in actinomycete isolates

Assays were conducted to detect and measure oxidase enzyme activity in the extracellular growth medium and in intracellular (cell) extracts; peroxidase, laccase and polyphenol oxidase activities were measured (Figures 6.7 to 6.9).

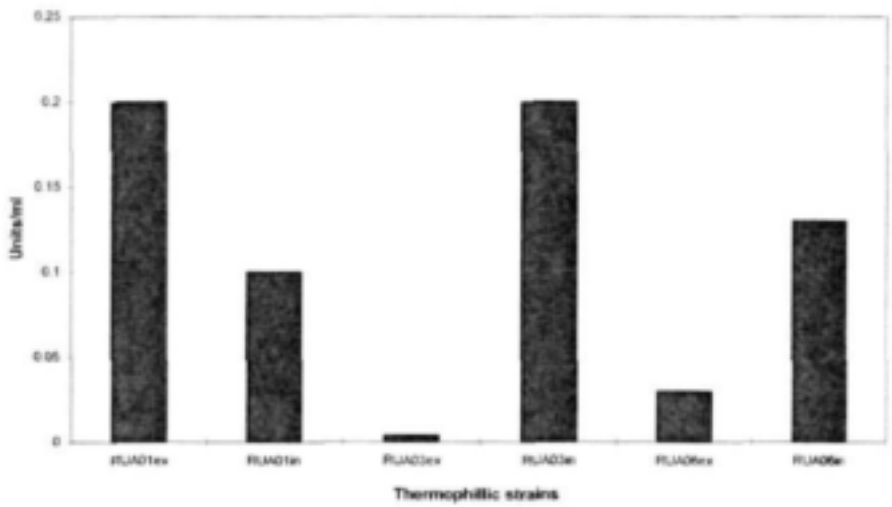


Figure 6.7 Intra- and extracellular lignin peroxidase activity using veratryl alcohol as substrate.

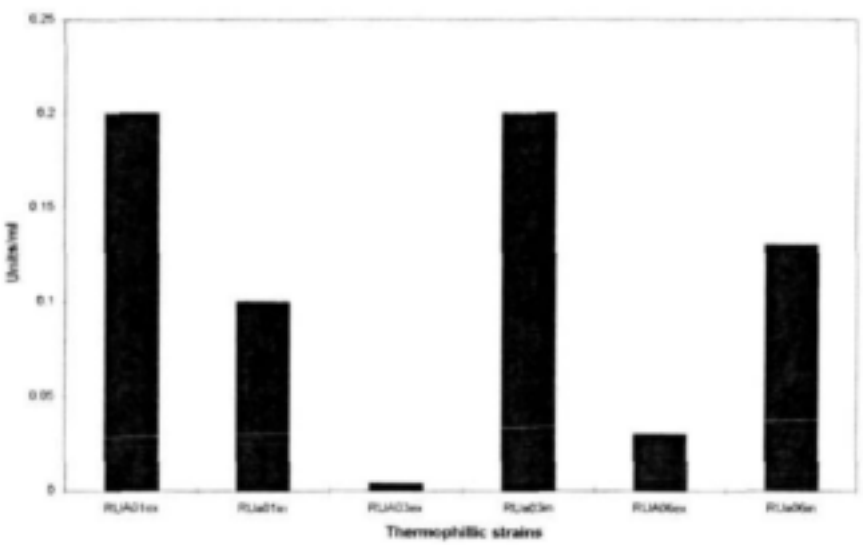


Figure 6.8 Intra- and extracellullar peroxiase activity of the 3 thermophiles

6.3.5 Heterologous expression and secretion of thermostable peroxidases and polyphenol oxidases from thermophillic actinomycetes in *E.coli.*

Heterologous genes need to be expressed at a very high level for basic research and for production of useful biologicals. These proteins then need to be harvested and purified for either purpose. Thus the most important parameter is the fractional abundance of the protein at the time the culture is harvested. *E.coli* plasmids are widely used in recombinant DNA-based technologies as vectors for the over production of proteins with theoretical and practical values. Standard cloning procedures have become routine and a larger variety of host/vector systems for the expression of genes are available, difficulties may arise when the theoretical strategies for over-production of a protein are put into practice.

In plasmid-based systems, the minimal elements that an expression plasmid vector should supply are a well-characterized origin of replication and a selection marker for plasmid propagation and maintenance, a strong promoter. Based on this criteria the plasmid vectors used in this study were selected, pSE 280 and pSE 380 superlinker vectors supplied by Invitrogen. These cloning vectors are extended polylinker regions containing the recognition sequences of 49 uninterrupted hexameric restriction sites in addition to 15 palindromic hexamers from which enzymes have not yet been discovered.. pSE280, a pKK233-2 derivative and pSE380 a pTrc99A derivative can both be used for inducible expression of genes from the strong *trp-lac* promoter. pSE280 and pSE380 each have a *Nco1* cloning site at the beginning of the superlinker which contains a translational ATG start codon and a strong rrnB ribosomal RNA transcription terminator at the end of the superlinker to ensure correct termination. The two plasmids differ from one another in that pSE380 contains the Laq Iq repressor gene which allows induction in strains which do not contain *Laq Ip.*

The *E.coli* strain used was TOP10F' supplied by Invitrogen, a recombination negative strain designed for stable replication of high copy number plasmids, with a transformation efficiency of 1 x 10^9 cfu/µg. Other features are the *recA1* for increased stability of inserts, *endA1* for improved quality of the minipreps and *hsdRMS* which eliminates cleavage by endogenous restriction enzymes. DNA was extracted using the Qiagen DNeasy Tissue extraction kit. Extraction yield averages of x µg/ml in the first elution and x µg/ml in the second elution were obtained. DNA

obtained was of molecular weight of approxmiately 20 000 base pairs and Molecular weight marker used was Molecular weight marker 111. Cloning vectors pSE280 and pSE380 were first transformed into *E.coliTOP10F`* host and then extracted for further use using the CTAB plasmid DNA extraction method (Nucleic Acid Res, 1988).

Ligation and transformation efficiencies

Transformations and ligations were carried out according to standard protocols. Two volumes of ligations reactions transformed into *E.coli* were used (10 μl and 50μl) to determine which volume gave well spaced colonies and for the calculation of transformation efficiencies. Colonies were randomly selected for plasmid DNA extraction using the CTAB extraction method, and then run on a 1% agarose gel to determine the presence of DNAinserts in the clones (Fig 6.9). Enzyme restriction using an enzyme that cut outside the vector was used to determine DNA insert size frequency.

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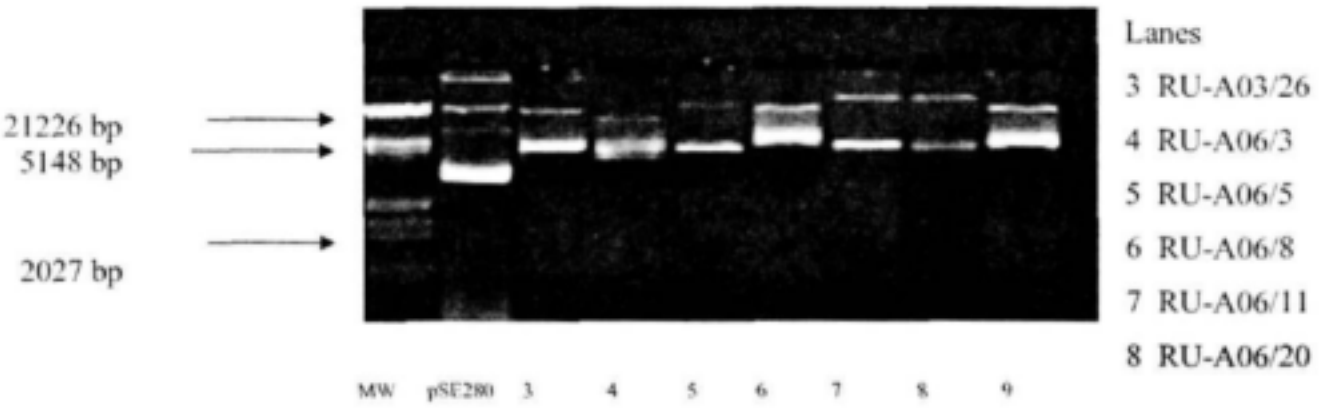

Figure 6.9 Plasmid extraction of clones to determine the presence of DNA inserts.

6.3.6 Comparative enzyme assays between selected clones and thermophiles.

Peroxidase, poly phenol oxidase and lignin peroxidase activity assays were carried out on the clones. Three of the highest producing clones were selected 9 RU-A03/5, RU-A06/8 and RU-A06/27) and further assayed for consistent activity and to ensure that the DNA inserts were stable. Enzyme activity of the clones were compared with enzyme activity of the thermophiles RU-A01, RU-A03 and RU-A06 and showed a significant increase in activity in the clones (Figures 6.15, 6.16). Peak activity was also determined after induction with IPTG, peaking at time 6 hours after induction with IPTG (Figure 6.10). Activity gels were run and stained for polyphenol oxidase activity and peroxidase activity (Figure 6.11). Thermophiles showed numerous bands however the selected clones showed 2 distinct bands of different molecular weight to those of the thermophiles. A control was run which was the vector alone without a DNA insert and this produced one activity band for polyphenol oxidase, possibly indicating and not previously reported before that the *E.coli* strain used produces its own polyphenol oxidase. The protein gels clearly show that after induction there was a general increase in protein production in the clones. For the Control (the vector alone with no DNA insert) protein concentration appeared to remain the same before (basal expression) and after induction with IPTG. One band was found in the clones and not in the control, which could possibly represent the polyphenol oxidase band found in the activity gel. No conclusions can be drawn from this as this must be confirmed by Western blotting, using antibodies specific to polyphenol oxidase.

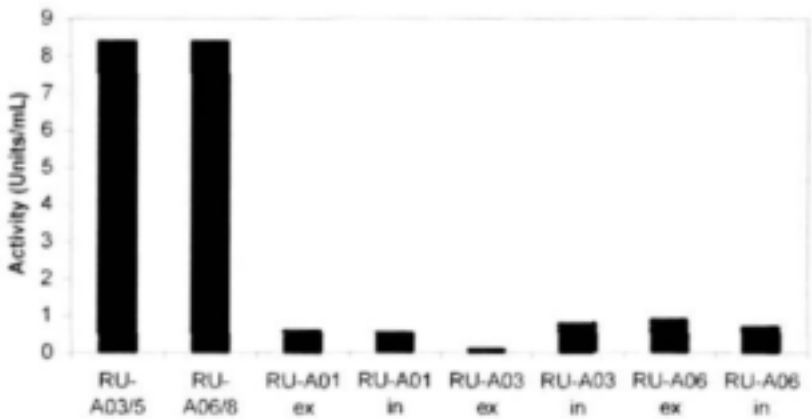

Figure 6.10 Comparison of polyphenol oxidase activity between clones and thermophiles

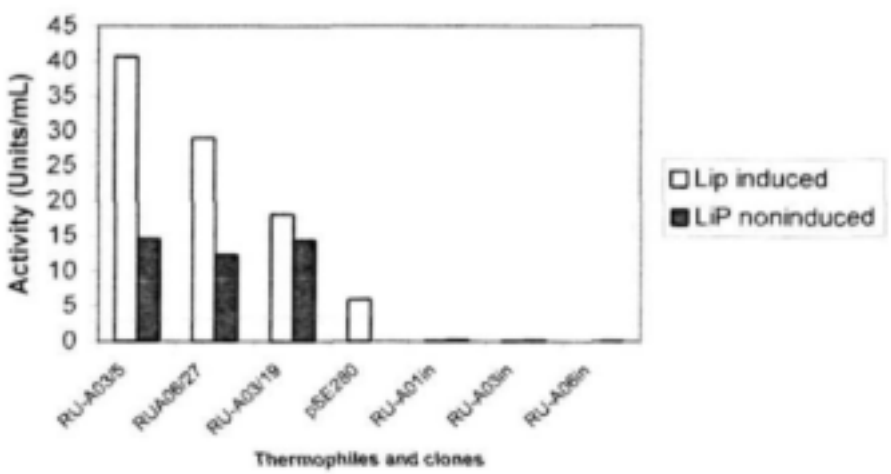

Figure 6.11 Lignin peroxidase activity using veratryl alcohol as substrate

6.4 CONCLUSIONS

- Thermophilic strains RU-A01, RU-A03 and RU-A06 were isolated form compost.
- The strains displayed the capacity to degrade phenols to some extent.
- The peroxidase and polyphenol oxidase activity of these strains is within activity range of the common producing, widely studied streptomycete *Streptomyces viridosporus* T7A.
- The use of a prokaryotic vector system in this study was used with the aim of increasing enzyme production as well as identifying the genes responsible for the production of these enzymes. This process, if successful, could identify these genes which have not been previously sequenced before in Actinomycetes and which in turn enable gene manipulation techniques to improve and increase enzyme activity.

CHAPTER 7

Sorghum root peroxidase, a novel enzyme with the potential to oxidize aromatic pollutants in water

7.1 INTRODUCTION

Much is known about how microorganisms enzymatically purify aquatic and terrestrial ecosystems from organic pollutants (Chrost, 1991; Siqueira ***et al.***, 1991; Rheinheimer, 1992). For example, microbial peroxidases and polyphenol oxidases polymerize phenolic acids thereby detoxifying phenolics into stable humic substances (Siqueria *et al.*, 1991). Studies on the role of plant roots in the removal of organic pollutants, however, has been limited to uptake and internal metabolism (Bellin and O'Conner, 1990), rather than enzymatic detoxification at the soil-root interface. In this study, sorghum peroxidase from roots has been studied and shown to have a wide substrate range. It is thus a potentially cheap peroxidase that can be used for the conversion of aromatic compounds in water to less toxic or indeed useful compounds.

Peroxidases have been identified in many plant species (Mueller and Beckman, 1978; Smith and O'Brien, 1979; Zaar, 1979; Albert *et al.*, 1986) and have been shown to polymerize phenolic compounds *in vitro* (Klibanov *et al.*, 1980; Alberti and Klibanov, 1981; Klibanov *et al.*, 1983; Nakamoto and Machida, 1992). Adler *et al.* (1994) have suggested that plants may be utilized as a source of peroxidases for the removal of phenolic compounds and that root-surface peroxidases may minimize the absorption of phenolic compounds into plants by precipitating them at the root surface. They have identified a new use for root-associated proteins by ecologically engineering plant systems for bioremediation of phenolic compounds in the soil and water environment.

Peroxidases have the potential to oxidise a wide range of substrates, and have the advantage that only hydrogen peroxide is required as a cofactor. The reactivity and characteristics of peroxidases are described in the previous section. The nature of the products produced by the reactions of known plant peroxidases is relatively well documented, and one aim of this study was to compare the activity of sorghum root peroxidase (SRP) with that of the well-known

horseradish peroxidase (HRP) which is often used in analytical reactions. The reaction typically involves oxidation of an electron donating substrate by H_2O_2 and involves the overall transfer of two electrons, although most reactions catalyzed by HRP and other peroxidases occur in sequential one electron steps.

While a number of peroxidase enzyme obtained plant, animal and microbial sources have been investigated for their ability to catalyze the removal of aromatic compounds from waste waters, the majority of studies have focused on using commercially available pure HRP. However, it is expensive and susceptible to inactivation by various side products from reactions occurring in the treatment process.

The major limitation for wide spread application of peroxidases other than HRP is their high cost and low commercial availability. Thus, there is value to investigate alternative sources of peroxidase activity. In this study we have investigated a novel peroxidase enzyme which was extracted from sorghum roots. Sorghum is a common crop which is readily available, and the root material has almost zero cost since it is normally disposed of. Biotransformations of a range of model phenolics, which are present in different industrial effluents, were carried out and and characterisation of products was achieved using analytical techniques including UV-Visible absorption spectroscopy, NMR, HPLC, and HPLC-Mass spectroscopy.

7.2 METHODS AND MATERIALS

Sorghum bicolor seeds, a gift from Mr. C. Muzariri, University of Zimbabwe and Dr. Kamugira of the Kawanda Agricultural Research Institure, Uganda, were planted in the Grounds and Gardens Department of Rhodes University and the fruit was used for all experiments described. Root tissue was harvested at the stage where fruit was ripe, and was stored at -20^0C until used. Before extraction, the root tissue was powdered by liquidising in liquid nitrogen.

7.2.1 Assay Methods

All assays were conducted in duplicate. Peroxidase assays were conducted by the following methods: (1) Substrate solution, *o*-dianisidine,(50 µL, 50 mM) was added to 6mL sodium phosphate buffer, pH 6, containing 0.01 M H_2O_2. The peroxidatic reaction was initiated by

adding 100 µL of enzyme solution to 2.9 mL of the substrate mixture in a plastic cuvette and monitored at 460 nm (Vágújfalvi et al., 1982).

(2).Using 0.02 M 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as a substrate, with the following reaction mixture: 500 µL enzyme extract, 500 µL 0.1 M sodium phosphate buffer, pH6, 100 µL ABTS and 100 µL 0.01 M H_2O_2 which was added to initiate the reaction. The change in absorbance was monitored at 465 nm. (Pütter (1975).

(3). The reaction mixture, with 10 mM guaiacol as substrate, consisted of 100 µL of enzyme extract, 700 µL of guaiacol and 200 µL of 0.01 M H_2O_2 to initiate the reaction. The change in absorbance was monitored at 465 nm.

(4). The reaction mixture, with 2,4-dichlorophenol (2,4-DCP), consisted of 100 µL enzyme extract, 200 µL of sodium phosphate buffer, pH 6, 200 µL 1 mM 4-aminoantipyrine, 300µL 5 mM 2,4-DCP and 200 µL of 0.01 M H_2O_2 to initiate the reaction. The reaction was monitored at 510 nm. Assays were performed in duplicate.

Peroxidase activity was expressed as µmol of product formed per minute per mL enzyme.

7.2.2 Purification of Sorghum Root Peroxidase

Plant tissue was ground to a powder using a Waring blender. The powder (100 g) was homogenized by stirring in 250 mL 50 mM MES buffer, pH 5.5 containing 1 M NaCl, 30 mM ascorbic acid, and 1 mM EDTA. The homogenate was filtered through two layers of cheese cloth and centrifuged at 9000 rpm for 30 minutes. The supernatant (crude extract) was then assayed for peroxidase activity (section 2.2.2.3 - method 1) and protein content (section 2.2.2.4). The first purification step involved blending 10 % polyvinylpolyprrolidone (PVPP) in the crude extract using a Waring blender operated at maximum speed for 1 minute. This mixture was centrifuged at 9000 rpm for 30 minutes. The supernatant was dialysed against a 25 mM malonate solution, pH 5.5, containing 10 mM of ascorbic acid and then centrifuged to remove the solids. The supernatant was analysed using polyacrylamide gel electrophoresis (PAGE) (section 3.2.4) and then examined for peroxidase activity and protein content. It was then subjected to ultrafiltration through a cellulose Molecular/Por filtration membrane (MWCO 0

10,000) at a pressure of 3000 tm in a Molecular/Por Stirred Cell. A portion of the filtrate was used for to PAGE analysis (section 3.2.4). Column chromatography was employed as the final purification step, using phenyl sepharose Cl-4B equilibrated in buffer, and applying the filtrate to the column and eluting it with distilled water. The eluate was assayed for peroxidase activity (section 2.2.2.3 - method 1) and a sample was subjected to gel electrophoresis (section 3.2.4).

7.2.3 Polyacrylamide Gel Electrophoresis Analysis

Denaturing PAGE was carried out according to the method described by Laemmli (1970) using 10% resolving gel and 4% stacking part of the gel containing 10% SDS. For non-denaturing gels SDS was omitted. 30 µl of enzyme samples or 5 µL of protein marker solutionwere applied to the gel. After electrophoresis, the gels were soaked in Coomassie Brilliant Blue (CBB) staining solution or, for activity gels, the gels were soaked in a dilute substrate solution (100 µL of 50 mM *o*-dianisidine in 30 mL H_2O containing 50 µL H_2O_2). Gels were also stained for protein detection using the silver stain method as described in the silver stain kit booklet supplied by Sigma Chemicals Co. (USA).

7.2.4 Characterization Experiments

Temperature dependence of sorghum root peroxidase

Experiments to determine the optimal temperature for sorghum root peroxidase activity involved measuring the activity at varying temperatures (in duplicate). The assay mixture containing *o*-dianisidine as substrate, with H_2O_2, in sodium phosphate buffer (section 2.2.2.3 - method 1), was adjusted to the relevant temperature, and the enzyme solution was added to initiate the reaction. Temperatures ranged from 20 to 60°C and at pH 6.

Thermostability of sorghum root peroxidase

Enzyme samples were incubated at the relevant temperature for a specific period in a water bath. The temperatures ranged from 40 to 100°C and peroxidase activity was monitored hourly for a total of 6 h. At 100°C, peroxidase activity was monitored every 2 min for a total of 8 min. The extract was removed from the water bath and allowed to equilibrate to room temperature for 15 min before assaying for activity.

Effect of hydrogen peroxide concentration on sorghum root peroxidase

To study the effect of hydrogen peroxide concentration on peroxidase activity, 0.1 M sodium phosphate buffer, pH 6, was prepared. A series of buffer solutions were prepared containing varying concentrations of hydrogen peroxide ranging from 0.005 to 0.9 M. Peroxidase were then performed using the relevant buffer at room temperature.

Optimal substrate concentration for sorghum root peroxidase

This experiment involved assaying for peroxidase activity using different substrate (*o*-dianisidine) concentrations, at room temperature and pH 6. The substrate concentration ranged from 10 to 150 mM.

Substrate range for assay of sorghum root peroxidase

Assays were carried out using other substrates to determine the substrate range of sorghum peroxidase. The other substrates used to measure the activity of sorghum peroxidase were guaiacol, 2,4-dichlorophenol and 2.2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS).

Effect of $CaCl_2$ on sorghum root peroxidase

This experiment involved adding 10 µL of the appropriate $CaCl_2$ concentration to 100 µL samples of enzyme extracts and proceeding with the assay procedure. The $CaCl_2$ concentration ranged from 0.001 to 10 M.

7.2.5 Optimal pH of sorghum root peroxidase

A series of 0.1 M sodium phosphate buffers with pH values ranging from 3 to 8 were prepared. The assay mixture was prepared using sodium phosphate buffer and *o*-dianisidine as substrate, at room temperature, and enzyme extract was added to initiate the reaction.

7.2.6 Bioconversions of model pollutants using sorghum peroxidase

SRP was used as the biocatalyst in the biotransformation reactions. The reaction mixture consisted of equal volumes of buffer and ethyl acetate, with 1mM substrate and crude enzyme extract added. Then the reaction was initiated by addition of the 1mM H_2O_2 and this was repeated periodically. After 24 hours the reaction mixture was allowed to separate into two layers and the organic layer was separated, washed with brine, dried, and evaporated. The

formation of the reaction products from a given phenolic substrate was determined by C18 reverse phase HPLC with ultraviolet detection. Any product eluting before the parent compound was assumed to be more polar than parent compound.

7.3 RESULTS AND DISCUSSION

7.3.1 Extraction and purification of sorghum root peroxidase

Sorghum peroxidase from roots was extracted in MES buffer and partially purified as demonstrated in Table 7.1.

Table 7.1 Purification table for isolation of sorghum peroxidase

Fraction	Crude	Centrifuged	PVPP*	Ultrafiltration	Chromatography
Vol (mL)	210	200	100	14	5
Protein (mg/mL)	0.053	0.0298	0.0258	0.0799	0.0336
Activity (µmols/min/ mL)	1.24	1.19	1.4	5.4	4.11
Specific activity (µmols/min/ mg)	23.3	39.9	54.3	69.3	122.32
Total activity (µmols/min)	260.4	238	140	75.6	20.55
Total protein (mg)	11.13	5.96	2.58	5.2	0.168
Yield (%)	100	91.4	57.3	29	7.9
Relative Purification	1	1.7	2.3	2.9	5.2

PVPP* - Polyvinylpyrrolidone for the removal of phenols

7.3.2 Assay Optimization

Optimization experiments showed that the highest activity took place at a temperature of 60^{0}C (Fig. 7.1). The optimal pH for sorghum root peroxidase (SRP) activity was found to be 6 (Fig. 7. 2) while the optimal substrate (*o*-dianisidine) concentration was 50mM (Fig. 7.3). Low concentrations of hydrogen peroxide elicited an oxidative reaction with the optimal concentration being 0.01M (Fig . 7.4). A relatively high concentration of calcium chloride increased the enzyme activity slightly as is seen in figure 7.5.

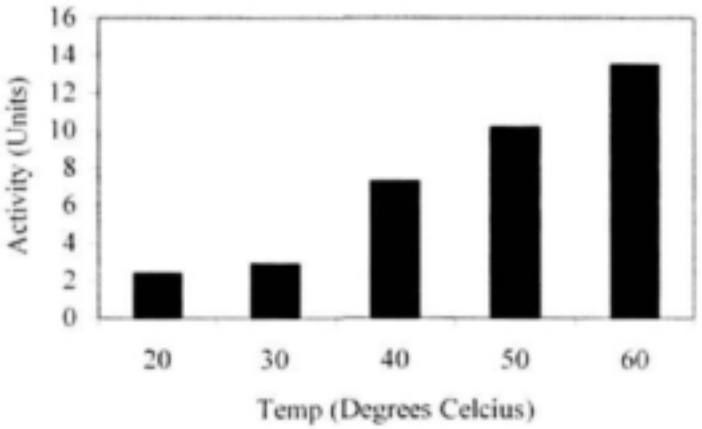

Figure 7.1 Effect of temperature on SRP activity

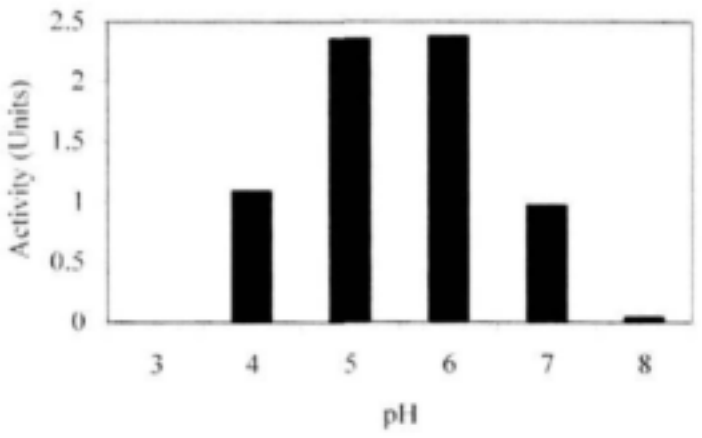

Figure 7.2 Effect of pH on SRP activity

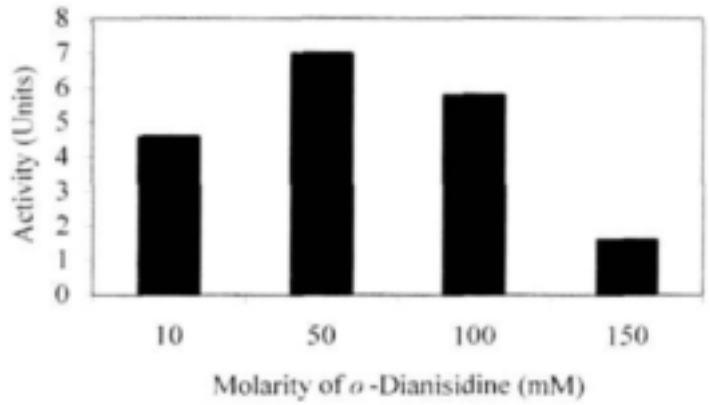

Figure 7.3 Effect of substrate (o-dianisidine) concentration on SRP activity was found to be 50mM

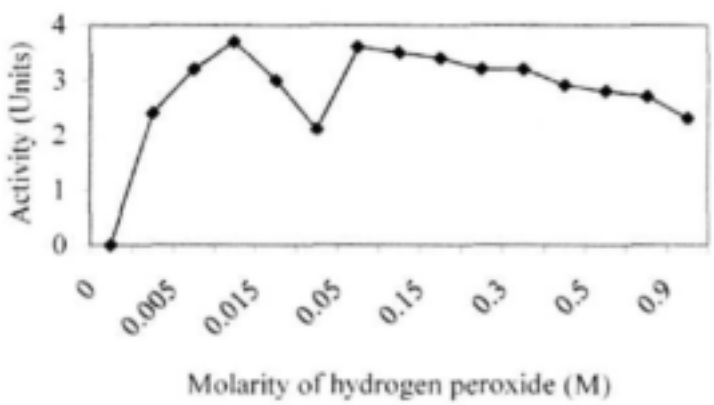

Figure 7.4 Effect of hydrogen peroxide molarity for SRP activity

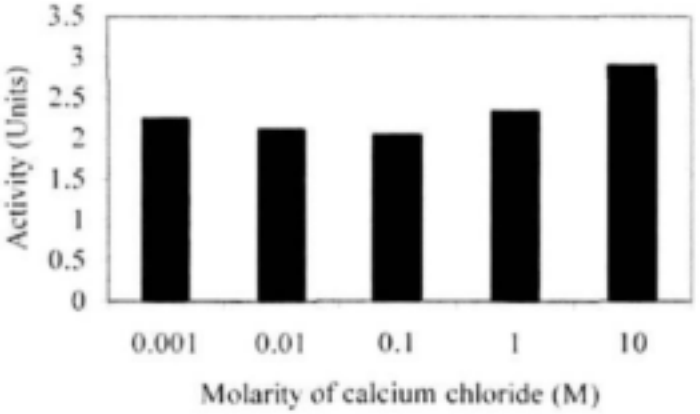

Figure 7.5 Effect of calcium chloride on SRP activity slightly

7.3.3 Substrate Range

Apart from *o*-dianisidine (Fig. 7.3), three other substrates were oxidized by SRP. Figures 7.6 and 7.7 show the activity of the enzyme with different molarities of guaicol and 2,4-dichlorophenol respectively. 2.2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was also oxidized by the enzyme as indicated by the a colourless ABTS solution turning dark green and eventually purple.

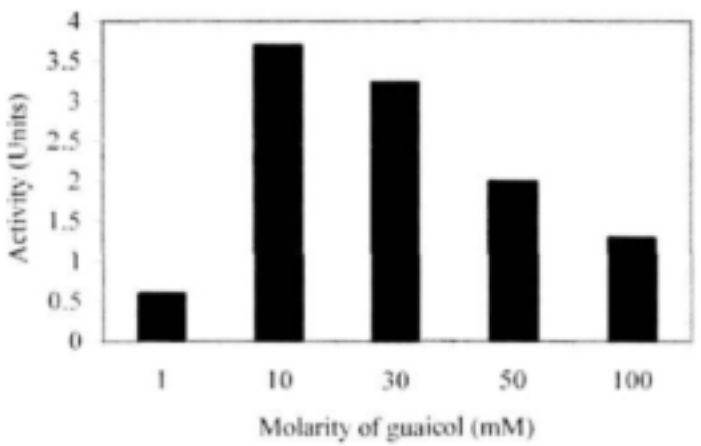

Figure 7.6 Substrate range of SRP - oxidation of guaiacol

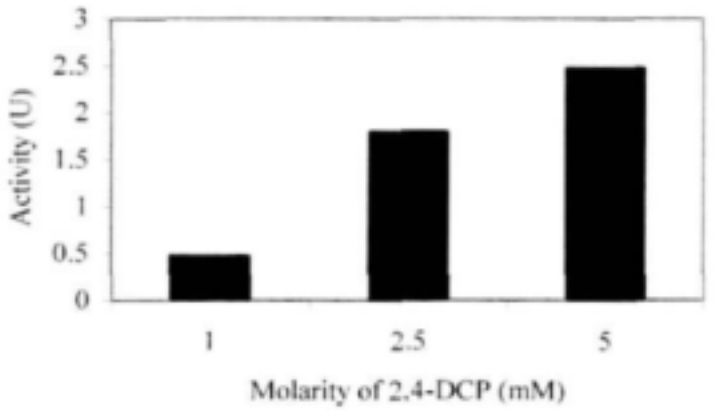

Figure 7.7 Substrate range of SRP - oxidation of 2,4-DCP by SRP

7.3.4 Thermostability experiments

Plant peroxidases are known for a high thermostability (Robinson, 1991). Sorghum rppt peroxidase was also shown to have a high thermostability at 50□C but less at higher tempreatures, as shown in figures 7.8 and 7.9.

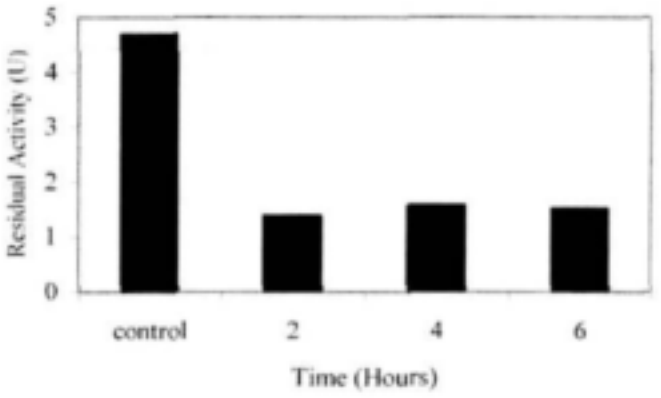

Figure 7. 8 Thermostability of SRP at 50^0C

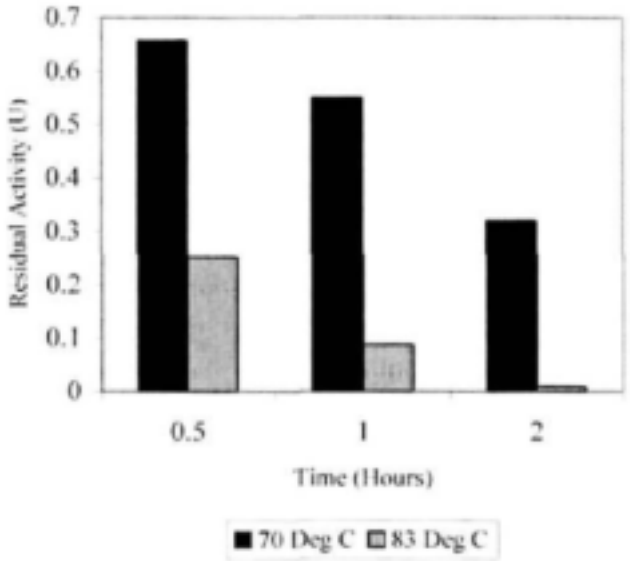

Figure 7. 9 Residual actvity of SRP at 70 and 83^{0}C

7.3.5 Molecular mass of sorghum root peroxidase

The molecular mass of sorghum root peroxidase (SRP) was determined by SDS-PAGE. Denatured protein was run on the gel with known molecular mass standards. Denatured HRP and lignin peroxidase (LiP) were also run in the gel as a comparative measure. SRP had three isoenzymes with molecular mass of 36000, 25000 and 20000 Da. HRP had isoenzymes of molecular mass of 20000, 32000 (the most visible band) and 63000 Da while LiP had isoperoxidases of molecular mass of 50 118 and 32 000 Da. In literature, the molecular mass of HRP ranges from 32000 – 33000 Da (Welinder *et al.*, 1972). The molecular masses of isoperoxidases generally occur in the range 40000 to 50000 Da (Robinson, 1991). HRP and LP have an isoenzyme of the same molecular mass (32000 Da) while sorghum has an isoenzyme of similar molecular mass (36000 Da) to those of HRP and LP. It can thus be assumed that SRP has similar properties to HRP, a thoroughly studied enzyme.

Undenatured protein (SRP and HRP) was subjected to gel electrophoresis and the gel was soaked in a dilute solution of substrate (*o*-dianisidine). Denatured protein was run on the same gel and this section of the gel was stained in Coomassie Brilliant Blue (CBB gel). The SP bands in the activity gel were formed within 10 minutes of soaking. The corresponding SP bands in the CBB

gel were barely visible due to low concentration of protein. Thus, even at very low concentrations sorghum peroxidase has high activity in the oxidation of *o*-dianisidine.

7.3.6 Bioconversion of model pollutants

The following reactions were used to demonstrate that sorghum peroxidase is capable of converting a range of compounds commonly found in industrial effluents.

p-Dianisidine

This compound would be found in effluents from processes involving dyestuffs. The *p*-amino groups are very prone to oxidation and form a diazo linkage between the molecules, which leads to reddish brown colour and finally the solid separating out. The yield was 161 mg (70%) and HPLC analysis of this compound showed a single peak, detected at 300 nm, with retential time 3.6 min.

Phenol

Generally when phenol reacts with a peroxidase in the presence of H_2O_2, it gives polymeric products which are insoluble in aqueous system and which deactivate the enzyme. When a biphasic system was used, however, the polymeric materials remained in the organic phase, and no enzyme inactivation was observed. HPLC analysis showed the starting material peak was observed at 6.3 min, and other products at 8.2 and 10.0 min which may be polymeric products. After reaction 70 mg product was recovered.

p-Cresol

This reaction has been thoroughly investigated for HRP, and *p*-cresol will give Pummerer's ketone with other polymeric compounds, such as the dimer and trimer, in presence of the H_2O_2 / preoxidase system. In the presence of sorghum peroxidase and H_2O_2, we have observed the formation of the same compounds. This was confirmed by using the HPLC analysis and HP-Mass spectral analysis. The retention times were observed at 4.3min (p-cresol), 6.46 min (Pummerer's ketone) >5%, 8.3 min (dimer) <5% and 13 min (trimer) (very little). These exactly match with information in the literature. From HP-mass spec analysis, the molecular ion peak 214m/z. was observed.

Guaiacol

Guaiacol is a common component of lignin-related wastes such as pulp-and-paper wastestreams. During the guaiacol reaction with SP in presence of H_2O_2, the solution which was intially colourless, turns yellow and later becomes pink. This indicates that it first forms *o*-quinone, which undergoes rapidly polymerization and leads to formation of tetraguaiacol.

4-Methoxyphenol

This compound is a common component of lignin-related wastes such as pulp-and-paper effluents. It was observed from HPLC analysis that the 4-methoxy phenol reacts with peroxidase and H_2O_2 gave two different peaks apart from the starting material, where one is more major than other.The retention times were 5.54min (4-methoxy phenol), 11.89 min and 20.74min.These peaks may indicate the dimer and trimer.

Veratryl alcohol

3, 5- dimethoxybenzyl alcohol (veratryl alcohol) is a common component of lignin-related wastes such as pulp-and-paper wastestreams. With peroxidase and H_2O_2 , it reacted to give 3,5-dimethoxybenzaldehyde, which has maximum absorbance at wavelength 310 nm, while the alcohol absorbs at 280 nm. HPLC analysis indicates that the oxidized product aldehyde is less polar than parent compound, since it eluted later. The retention times are 5.391 min (aldehyde) and 3.6 min (alcohol).

2-Napthol

Naphtol is a representative of the polyaromatic hydrocarbon group of pollutants. During this reaction the colour changed to yellowish and the absorbances were observed at 304 nm for parent compound and 304 nm, 346 nm, 343 nm and 257 nm for the reaction products. It is expected that bi-napthol was formed of bi-napthol, as is reported in the literature for the reaction of HRP and H_2O_2 with napthol.

Protocatechuic acid

3,4-dihydroxybenzoic acid (protocatechuic acid) is a product of lignin breakdown. In the presence of H_2O_2 and peroxidase this compound gave a colourless solution which turned yellow, possibly due to formation of the *o*-quinone, in organic phase, and reddish brown in aqeuous

phase. As the reaction progressed, the yellowish organic phase changed to brown. TLC showed that the product was very in comparison with the parent compound. UV spectrophotometric analysis showed a bathochromic shift in wavelength of 20nm.

4-Hydrox phenyl acetic acid

This compound is also a product of lignin breakdown. change in wavelength of absorption was observed. The aqeuous solution, was acidified and extracted with ethyl acetate. From the NMR analysis of the compound, no acid remained after the reaction. TLC analysis showed that the product was more polar than the parent compound.

F*erulic acid*

Ferulic acid is a major component of lignin-realted wastes. During the reaction, the solution changed from light brown to yellow. HPLC analysis showed peakes with retention times 4.9min (Ferulic acid) and a product at 18.2min. HPLC-Mass spec analysis showed major ion peaks at 298, 358, 360, and 390 as major ones, corresponding to the structures shwon below. The formation of the above type of compounds with ferulic acid and HRP are known in the literature.

In order to avoid the formation of a large number of product compounds, the ferulic acid ester was prepared and reacted with the enzyme system. After the reaction, the ester and unreacted acid were separated by silica gel coloumn chromatography. The pure ester was obtained in 70% yield. The ester was reacted with H_2O_2 and peroxidase in aqueous acetone and the reaction was monitered by TLC. Three different products were observed and and these were seperated by coloumn chromatography. NMR analysis showed the three different products in yields: I, 108 mg (ester); II,30 mg; III, 40 mg; and IV,60 mg.

7.4 CONCLUSIONS

Sorghum peroxidase was isolated and partially purified from the roots of *Sorghum bicolor*.

- Sorghum peroxidase was characterized as follows:
  - optimal pH:- 6
  - optimal substrate molarity (*o*-dianisidine) : 50mM
  - optimal hydrogen peroxide molarity : 0.01M
- Like most plant peroxidases studied, sorghum peroxidase has a wide substrate range and is relatively stable at high temperatures.
- An isoenzyme of sorghum peroxidase was found to have a similar molecular weight (36000 Da) to an HRP isoenzyme (32000 Da).
- The work reported here represents a preliminary study, and much of the analysis of the products must still be confirmed. However, it is clear that sorghum peroxidase is a highly active enzyme and that its reactions are comparable with those of HRP. This means that SP could feasibly be used in bioremediation and biotransformation reactions.
- The substrates selected for trial biotransformation reactions are representative of the aromatic compounds found in common industrial residues, and in particular, olive waste. The fact that they were transformed by the SP indicates that the beneficiation of industrial residues using peroxidases in general, and with SP inparticular, is feasible.

CHAPTER 8

CONCLUSIONS AND FUTURE OBJECTIVES

8.1 GENERAL SUMMARY

This project aimed to develop systems in which the high activity and selectivity of biological catalysts could be used to remove pollutants from industrial residues, and at the same time, convert them into products which have some market value. The project was focussed on the application of oxidative biological reactions, catalysed by a selection of enzymes produced by a small range of organisms.

The enzyme catalysts included laccases, peroxidases and polyphenol oxidases, three groups which catalyse different reactions, with varying substrate selectivities, and which produce a range of products. Thus, by utilising this group of biocatalysts, we had a broad scope for the oxidation processes which are accessible and could potentially develop efficient systems for production of a group of related, and potentially high-value, compounds.

Specific industrial wastes were identified which contain high concentrations of the aromatic, phenolic and/or polyphenolic components best suited to the intended oxidative bioconversions, and these were analysed and characterised. These effluents, from petrochemical processing, pulp-and-paper processing, olive production and wine production, were used in the programme as authentic samples on which the biological activity of the selected biocatalysts could be tested.

The oxidation of aromatics by enzyme-catalysed reactions has potential for production of useful synthetic intermediates, and in particular, for activation of aromatic rings to produce more reactive intermediates. This would mean that compounds which are generally unreactive and therefore recalcitrant, could be converted to more reactive products which are more easily susceptible to further biodegradation, or more useful for organic synthesis. The project included investigations into the biotransformation of a range of phenols and polyaromatic hydrocarbons using the various oxidative biocatalysts.

The selected enzymes are produced by particular organisms; for example, laccases are common products of certain groups of fungi, while peroxidases of different types are produced by many microorganisms and plants. We selected a narrow range of microorganisms and plants which can produce the enzymes required for the desired reactions, and investigated methodologies for optimal production of the enzymes by these organisms.

The research included investigations of enzyme production and biofilm growth as well as pollutant degradation. The enzymes utilised in this study have the significant characteristic that they do not require expensive cofactors such as NAD, (in contrast with many other oxidase systems), giving us is an important advantage with respect to economic and process engineering considerations.

8.2 RESEARCH CONCLUSIONS

8.2.1 Application of a whole-cell system using the fungus *Neurospora crassa* in the bioconversion of phenolics

The capacity of a *Neurospora crassa* fungal system to produce oxidative enzymes, and their application in the biodegradation of phenolic compounds, was demonstrated in non-immobilised batch (flask) cultures, and by capillary membrane-immobilised biofilms.

Extracellular laccase activity was produced at 10 to12 U/mL (800 U/g wet mass) in static flask cultures, over 8 – 15 days. Polyphenol oxidase was shown to be produced as an intracellular enzyme, at levels of 374 U/g wet mass. The production of laccase in a capillary membrane bioreactor was sustained at a level of 10 U/mL of permeate (1080 U/g wet biomass), typically over periods of approximately 30 days.

Two phenolic substrates, phenol and *p*-cresol, both components of petrochemical industrial effluents, were used as model pollutants for bioremediation studies using the *N.crassa* enzyme system. In flask cultures, 18 mg *p*-cresol and 23 mg phenol respectively were removed from 0.5 mg/L solutions per g wet biomass, over a 6 day period. Over the same

time period, immobilised cultures were found to convert 10 mg *p*-cresol or 8 mg phenol per g biomass.

The immobilised biomass in a continuous reactor was found to have the capacity to sustain this removal efficiency continuously for a 4-month period, whereas the batch liquid culture systems remained active for approximately 8 to 15 days, after which cultures were no longer viable. The effective membrane surface area within the capillary membrane bioreactor is $0.0113m^2$ and therefore, the conversion achieved was 43.5 $g.m^{-2}$, which is equivalent to a conversion of cresol, of $1.4g.m^{-2}.day^{-1}$ under the conditions used in this study. This is the first demonstration of the use of immobilised *N.crassa* biofilms and their continuous application for bioremediation of phenols.

Furthermore, the immobilisation of a *N.crassa* biofilm allows the continuous production of both intracellular and extracellular enzymes which have been shown to be extremely effective in catalysing the conversion of toxic substances, such as *p*-cresol and phenol, into quinone products which later polymerise and can be separated out. In view of these findings it would be beneficial to further characterise and quantify the products produced from the enzyme-catalysed conversion of phenolic substrates to catecholic or quinoid products. The next step would therefore be to establish which enzyme is responsible for the production of which product and to determine the identity and quantify of each product produced. This would allow effective application of *N.crassa* for bioremediation purposes and for biotransformation purposes.

8.2.2 Application of the fungus *Trametes versicolor* and its enzymes in the bioconversion of phenolics

T. versicolor was grown successfully in flasks and used to produce high levels of laccase and manganese peroxidase activity. The laccase specific activity achieved was higher than that of the commercially available laccase. The peroxidase activity was found to be mainly manganese peroxidase, rather than lignin peroxidase. The culture medium for *T. versicolor* was investigated for optimal biomass accumulation and enzyme production.

In the conversion of pollutants with *T. versicolor* laccase extracts, cresol, chlorophenol, toluene, and naphthalene, eactions were demonstrated to be comparable with those of commercially

available enzymes. The presence of the pollutants did not adversely affect the enzyme production, but detection of reaction products was problematic. In the conversion of pollutants using *T. versicolor* biomass as whole cell systems, high pollutant concentrations had visibly increasing effects on the morphology of the fungus. Product formation was only convincingly evident in the reactions carried out using *p*-cresol.

Few other studies with the laccase enzyme have focused on the characteristics of reaction products relevant to waste treatment objectives, and these must be understood if enzyme technology is to be developed as a viable treatment process. Therefore, one of the most promising prospects for *Trametes versicolor* is to use the extracellularly produced laccase enzyme to convert aromatic pollutants to useful products.

The vigorous growth and high enzyme production capacity of *T. versicolor* make it a particularly useful microorganism for bioremediation applications. Initial studies demonstrated that the use of a small-scale membrane bioreactor was not suitable for *T. versicolor* because of its rapid growth and high biomass productivity. Thus, alternative bioreactor systems were explored, and this section of the project became the subject of a separate WRC-supported project focusing specifically on developing bioreactors for this system.

8.2.3 Application of polyphenol oxidase from the mushroom *Agaricus bisporus* in bioconversion of simple phenolic pollutants to catechol products

Non-immobilised and immobilised polyphenol oxidase were shown to be capable of producing catechol products from phenolic substrates. The reactions catalysed by the enzyme were characterised and the catechol products were successfully identified and quantified. In the presence of borate and ascorbate it was possible to obtain 100% conversion of the phenolic substrate to catechol. There are various methods available to extract the catechols from the reaction system and the choice would depend on the scale of the reaction.

Polyphenol oxidase was successfully immobilised on a range of immobilisation supports and the results demonstrated that a hydrophilic support was the most suitable for catechol production. Immobilisation would allow for reuse of the enzyme. The development of a continuous process for catechol production using immobilised polyphenol oxidase will need to be developed.

A mathematical model was developed and used to determine reaction rate constants of the cresolase and catecholase activities of the enzyme. The results indicate that the biotransformation of the phenols to their corresponding catechols was strongly influenced by the immobilisation support resulting in differing yields of catechols. Use of nylon as the biocatalyst support resulted in the highest catechol yield, with 0.006 mmoles 4-methylcatechol produced from 0.025 mmoles *p*-cresol after 2 hours, using 0.3U of immobilised polyphenol oxidase, representing a 25% yield, using a very small amount of enzyme. The reaction can be optimised by application of larger amounts of biocatalyst.

The extent of polyphenol oxidase inactivation during the reactions using both non-immobilised and immobilised polyphenol oxidase was also investigated using the mathematical model. The analysis indicated that under the immobilisation conditions used, and at low substrate concentrations, the product inactivation was not significant.

Thus the polyphenol oxidase system offers a useful route for conversion of phenols in the waste streams. The low concentration of the substrates in these wastes does not preclude efficient conversion by the enzyme, but an effective method for extraction of the products would be required.

8.2.4 Bacterial strains present in olive processing waste components and their application

Olive processing yields an effluent rich in polyphenolics and lignin-related monomers, which serve as a nutrient source for the group of microorganisms capable of converting these phenolics. These microorganisms are also tolerant of the harsh conditions pertaining in the wastes and thus are potentially useful in industrial applications. Five bacterial strains were isolated from olive effluents and characterized. They were shown to tolerate broad temperature and pH ranges, and high salt concentrations (RU – LV1 tolerated up to 9 % NaCl). The five isolates were shown to be capable of phenolic conversion, in reactions with substrates typically found in plant-related wastes (caffeic acid, protocatechuic acid, vanillic acid and *p*– coumaric acid).

The strain RU-LV1 was selected for further investigation, because of its capacity to convert ferulic acid to vanillic acid, which is a high-value product. These studies were continued and

completed *via* a WRC consultancy during 2001. The outcome of the research has shown that RU-LV1 has significant potential for this set of reactions, and this strain will be further researched in a new WRC project beginning in 2002.

8.2.5 Lignin degrading enzymes from thermophilic Actinomycetes in bioconversion of phenolics

Three novel thermophillic bacteria were isolated, each of which showed the ability to degrade phenolic plant residues in wine waste. The presence of oxidase enzyme activity was demonstrated in these isolates. Genetic techniques in the form of Southern blotting to identify the genes responsible for enzyme production is ongoing and could be developed further by identifying clones with this gene and used for genetic manipulations such as mutagenesis and gene shuffling.

In addition, studies have been ongoing towards the search for the genes responsible for the production of peroxidases and polyphenol oxidases, which will allow for genetic manipulation to produce ideal enzymes. The final stage of molecular investigation into the identification of the genes responsible for the production of peroxidases and polyphenol oxidases through sequencing will enable the use of gene manipulation techniques to improve the quality and quantity of these enzymes. With such data, it would be possible to employ techniques such as random gene shuffling or more specific gene manipulations such as point mutations. Thus, not only would it be possible to externally optimise enzyme production (eg., using nutrients, inducers etc), but manipulation of the genes can further optimise productivity.

These thermophilic isolates have considerable potential value, since they can be readily cultured, are thermotolerant and novel. They will be further investigated and bioremediation systems will be developed for their application, in a new WRC project beginning in 2002.

8.2.6 Sorghum root peroxidase

A novel peroxidase was isolated from the roots of sorghum plants, and characterised. The enzyme was shown to have useful stability properties. It has significant potential for application in bioremediation, in the conversion of phenolic compounds, and also for biocatalysis applications. The range of compounds it was shown to oxidize was broad, and several potantial

products have industrial of synthetic value. Further development of the process for the production of this enzyme would yield a potentially commercialisable product.

8.3 SUMMARY OF OUTPUTS

The following objectives of the project have been achieved:

8.3.1 Development of specific bioconversions to convert the waste components and pollutants found in selected effluents, to yield economically valuable products.

Bioconversion systems have been developed for:

- Conversion of phenols and aromatics by *T. versicolor* laccase
- Conversion of phenols using PPO
- Conversion of phenolics in olive waste
- Isolation of phenol-converting thermophilic Actinomycetes
- Peroxidase conversions of phenolics.

8.3.2 Development of methods for isolation and characterisation of the chemical products of these bioremediation reactions.

- Methods are now available for TLC, HPLC and HPLC-MS analysis of aromatic and phenolic components and oxidised products of the selected industrial wastes, including petrochemical black product and cresylic waste, olive production wastewater, wine production waste and pulp and paper waste.

8.3.3 Utilisation of membrane bioreactors adapted for the specific application of such bioconversions for treatment of certain industrial effluents produced in South Africa.

- Membrane bioreactors were developed and used for *N. crassa* bioremediation and *T.versicolor* application.

8.3.4 Expansion of the expertise and knowledge already developed in the group, in extending the utilisation of bioreactors, particularly supporting polyphenol oxidases, laccases, peroxidases, or the biofilms producing these enzymes.

- List of publications and presentations included at the end of this chapter.

8.4 TECHNOLOGY TRANSFER ACTIVITIES

- We have had discussions with two South African businesses in the chemical industries sector about the possibilities of commercialising the catechol production process.

- We have submitted an application to a major petrochemical company for the establishment of a pilot scale plant to test the T. versicolor laccase bioremediation system.

- We have submitted a proposal to two large industrial corporates to consider catechol production from Black product wastewater.

- We have recently set up a Materials Transfer Agreement with Albany Chemicals (Enzymed) (USA) for their use of our oxidase enzymes in combinatorial screening for biotransformations applications.

- Our collaboration with the technikons, particularly Pentech, have resulted in fruitful interactions whereby technology and understanding developed in our group has been transferred and have become the basis for successful research activities in the Technikon groups.

8.5 RECOMMENDATIONS

Extension of the technology

- There are several routes available for extension of the outcomes of this project through new collaborative initatives which are currently being developed. For instance, the Advanced Centre for Applied Microbiology (ARCAM) in the Western Cape will seek projects to develop from proof-of-concept to process and scale-up. In the present study, the systems utilising fungal whole-cell systems, and those using isolated laccase, peroxidase and polyphenol oxidase all offer such opportunities.
- In addition, the National Biotechnology Strategy which is being developed at present by DACST will offer mechanisms whereby facilities for establishment of Biotechnology business can be nurtured. This will provide a link between research and enterprise development which has been inaccessible in the past. Projects such as the present one can provide the scientific basis for building Biotechnology business, but the researchers are not usually well-equipped for, or skilled in, business development. Thus this new mechanism should be considered as a means of taking the research outputs to the market place.

Specific recommendations for further research and development

- While the membrane bioreactors have been shown to offer considerable potential in bioremediation processes, scaled-up application of this technology would require a source of less expensive membrane modules, available in quantity.
- To address the problem of a lack of suitable customised or multi-purpose bioreactors, the bioremediation systems using the bacterial strains from olive waste and the thermophilic Actinomycetes will be used as the biological agents for application in developing bioreactors in a new WRC project which now has preliminary approval.
- One modern approach to improvement of the bioconversion capacity of microorganisms is to use molecular techniques such as gene shuffling or directed evolution to increase the enzyme production and/or activity levels in the organism. Although this is a relatively new concept in modification of fungal systems, *N. crassa* is well-understood and has been genetically characterized. The genetic manipulation approach does offer an

innovative opportunity for improvement of this organism for the development of a more efficient bioremediation system.

- The bacterial strains isolated from olive waste, and the thermophilic Actinomycetes isolated from compost also provide useful starting points for molecular modification and hence strain improvement. Modern molecular technology has become accepted as one of the most effective routes to efficient and cost-effective biocatalyst production. This line of research, focusing on these organisms, would lead to efficient bioremediation systems.

OUTPUTS OF THIS PROJECT

PUBLICATIONS IN INTERNATIONAL PEER-REVIEWED JOURNALS

1. Burton S G, Boshoff A, Edwards W and Rose P D. (1998). Biotransformation of phenols using immobilised Polyphenol oxidase. **J. Molec. Catal. (Enzymic, 224)**, 411 – 416.

2. Ryan D R, Russell,A K, Leukes W D, Rose P D and Burton, S G. (1998) Suitability of a modified capillary membrane for growth of fungal biofilms. **Desalination: Special Issue on Membrane Technology 115(3),** 303 – 306.

3. Boshoff A, Edwards W, Leukes W D, Rose P D and Burton S G. (1998). Immobilisation of polyphenol oxidase on nylon and polyethersulphone membranes: effect on product formation. **Desalination: Special Issue on Membrane Technology 115(3),** 307 – 312.

4. Edwards W, Bownes R, Leukes W D, Jacobs E, Sanderson R, Burton S G, and Rose P D. (1999) A capillary membrane bioreactor using immobilised polyphenol oxidase for the removal of phenols from industrial effluent. **Enzyme and Microbial Technology, 24 (3-4),** 209 - 217.

5. Russell I and Burton S G (1999). An immobilised-enzyme bioprobe using polyphenol oxidase to detect low concentrations of phenols. **Analytica Chimica Acta (389, 1-3),** 161 - 170.

6. Edwards W, Leukes W D, Rose P D, Burton S G. (1999) Immobilisation of tyrosinase on chitosan coated polysulphone capillary membranes for phenolic effluent bioremediation. **Enzyme and Microbial Technology, 25,** 769 - 773.

7. Burton S G, Cowan D A and Woodley J M. The Ideal Biocatalyst: a new paradigm. (**Invited Review** for **Nature Biotechnology,** submitted September 2000; in press).

8. Luke, A K and Burton, S G. A novel application for *Neurospora crassa*: Bioremediation of phenols in a membrane bioreactor. **Enzyme and Microbial Technology** (in press)

9. Boshoff, A, Burton M H, Burton S G. Optimisation of catechol production using membrane-immobilised polyphenol oxidase – a modeling approach. (**Biotech and Bioeng.** Accepted, under revision).

10. Burton, S G. Development of Bioreactors for Application of Biocatalysts in Biotransformations and Bioremediation. (2000) **Pure and Applied Chemistry 73,** 77-83.

PLENARY LECTURES / INVITATIONS

1. "Biotransformations research at Rhodes University". **Biotech SA '97** Grahamstown

2. **A workshop on "Biotransformations into Practice"**. Hosted by Professor H Griengl, Department of Organic Chemistry, Technical University of Graz. Graz, Austria (May 2000).

3. **Royal Society Lecture**: "Biotranformations: Enzymes that work" Grahamstown, September 2000.

4. **IUPAC International Symposium on Green Chemistry:** Lecture title: "Biotransformations and Bioreactors", New Delhi, India, January 2001

5. **CHEMRAWN XIV World Conference** "Toward environmentally benign processes and products"" Boulder, Colorado, June 2001.

INTERNATIONAL CONFERENCE POSTERS

1. S G Burton, A Boshoff, W Edwards, W Leukes, D Ryan, T Russell, I Russell. Bioremediation and Biotransformation of organic pollutants using oxidative enzymes. **20th Symposium on Biotechnology for Fuels and Chemicals,** Gatlinburg, Tenn., USA, 1998.

2. S G Burton, K Buchanan, G Matcher and Z Skepu. A Novel *Pseudomonas* strain with high levels of hydantoinase activity. **Biotrans '99,** Sicily, 1999.

3. S G Burton, A Boshoff, W Edwards, K Koteshwar, P Nganwa, D Ryan and T Luke. Biotransformation of aromatics using oxidative enzymes. **Enzyme Engineering, XIV**, USA, 1999.

4. S G Burton, M H Burton and A Boshoff. Optimisation of catechol production using immobilised polyphenol oxidase. **Enzyme Engineering, XIV**, USA, 1999.

5. S G Burton. Biotransformations into Practice. Workshop hosted by Professor H Griengl, Department of Organic Chemistry, Technical University of Graz, Austria 2000.

6. Burton S, Boshoff, A, Ryan, D, Luke, T, Mhlanga C, Koteshwar K, Notshe T. Biotransformation of phenolics using oxidative enzymes. **Enzyme Engineering XVI**, Potsdam, 2001.

SOUTH AFRICAN CONFERENCE PRESENTATIONS

1. W Edwards, W Leukes, E Jacobs, R Sanderson, P Rose and S Burton. Upscale of a transverse-flow capillary membrane module to a demonstration size unit for the treatment of phenol-containing industrial effluent. **Water Institute of South Africa**, Biennial conference, Cape Town, May 1998.

2. I M Russell and S G Burton. The development of an immobilised-enzyme bioprobe for detection of phenolic pollutants in water. **Water Institute of South Africa**, Biennial conference, Cape Town, May 1998.

3. A Boshoff and S G Burton. Application of membrane-immobilised polyphenol oxidase for the biotransformation of phenolic pollutants. **Water Institute of South Africa**, Biennial conference, Cape Town, May 1998.

4. A K Russell, W Leukes, and S G Burton. The biocatalytic potential of fungal biomass and membrane-immobilised polyphenol oxidase from *Neurospora crassa* in the biotransformation of phenols. **Water Institute of South Africa**, Biennial conference, Cape Town, May 1998.

5. D R Ryan, W D Leukes, E P Jacobs, R D Sanderson and S G Burton. Bioremediation of waste waters polluted with aromatic compounds by the white-rot fungus *Trametes versicolor*. **Water Institute of South Africa**, Biennial conference, Cape Town, May 1998.

6. A Boshoff and S G Burton. The effect of polyphenol oxidase immobilisation on the biotransformation of phenolic pollutants. **15th symposium, South African Society for Biochemistry and Molecular Biology,** Potchefstroom, September 1998.

7. P Nganwa and S G Burton. Detection and characterisation of enzymes in sorghum plants for the enhancement of sorghum utilisation. **15th symposium, SASBMB,** Potchefstroom, September 1998.

8. S G Burton. The future of Biotechnology in South Africa. **FRD Biotechnology Workshop**, August, 1998.

9. S G Burton. The way forward: Biochemical Processes. Report back to **FRD Biotechnology Task team meeting**, September 1998.

10. A Boshoff, M H Burton, S Burton. The effects of membrane immobilisation on the activity of polyphenol oxidase. **3rd WISA-MTD workshop**, Drakensberg, 1999

11. A Luke and S Burton. Application of *Neurospora crassa* biofilms in capillary membrane bioreactors for phenol bioremediation. **3rd WISA-MTD workshop**, Drakensberg, 1999

12. R D Ryan, W D Leukes, W Edwards, E P Jacobs, R Sanderson and S G Burton. Bioremediation of aromatic pollutants by the white-rot fungus *Trametes versicolor* immobilised in a transverse flow capillary membrane bioreactor. **3rd WISA-MTD workshop**, Drakensberg, 1999

13. A. Boshoff, M H Burton and S.G. Burton. The effects of membrane immobilization on the activity of polyphenol oxidase. **BIOY2K, Grahamstown, 2000**.

14. K.Koteshwar and S.G Burton . Transformation of organic compounds in different industrial wastes by using a novel peroxidase. **BIOY2K, Grahamstown, 2000**

15. S G Burton and T. L. Notshe Investigation into the bioconversion of constituents in olive waste effluents to produce valuable chemical compounds. **BIOY2K, Grahamstown, 2000**

16. **AK Luke and SG Burton. Application of *N.crassa* biofilms in capillary membrane bioreactors for phenol bioremediation.** BIOY2K, Grahamstown, 2000

STUDENTS SUPERVISED AND DEGREES OBTAINED 1998 - 2000

Degrees completed

PhD:
Mr W Leukes - Completed PhD, May 1999.
Mr W Edwards - Completed PhD, May 1999.
Ms A Boshoff - Completed November 2001.

MSc:
Ms I Russell - Degree awarded with Distinction, 1999
Ms P Nganwa - MSc awarded April 2001
Ms M Joseph – MSc awarded April 2001

Ms T Notshe – Thesis submitted Nov 2001
Ms C Mhlanga - Thesis submitted Nov 2001

Hons:
Ms L McIntosh - Hons, Biotechnology 1999.
Ms T Clifford - Hons, Biotechnology, 1999.
Ms S Schnablegger - Hons, Biotechnology, 2000.
Ms A Edkins - Hons, Biotechnology, 2000.

Post-Doc:
Dr K Koteshwar - Jan 2000 – Nov 2000.

REFERENCES

Aitken M D and R .L .Irvine, (1987) Proc. of water pollution control federation post conference workshop on hazardous waste treatment processes Design, PhilAdelphia, PA.

Aitken, M.D.; Irvine, R . L.; and Venkatadri, R. (1989) Oxidation of phenolic pollutants by lignin degrading enzyme from white rot fungus phanerochate Chrysosporium *Wat . Res.* 23, 443-450.

Alberti M and Klibanov A (1981) Enzymatic removal of aromatics form industrial aqueous effluents, *Biotechnol.Bioeng.Symp.* 11: 373-379

Allsop, P.J.; Moo-Young M. and Sullivan ,G. R. (1990) The dyanamics and control of substrate inhibition in activated sludge , Crit .Rev. Envir. Control 20, 115-167.

Annachhatre AP and Gheewala SH (1996) Biodegradation of chlorinated phenolic compounds. *Biotechnol.Adv.* 14: 35-56

Atlow SC, Bonadonna-Aparo L and Klibanov AM (1984) Dephenolisation of industrial wastewaters catalysed by polyphenol oxidase, *Biotechnol.Bioeng.* 26: 599-603

Aust S D (1990) Degradation of environmental pollutants by *Phanerochaete chrysosporium. Microb Ecol* 20: 197 – 209.

Benedick M J, P.R. Gibbs , R. R. Riddlea and C. Willson. (1998) Microbial denitrogenation of fossil fuels. *TIBTECH* 16, 390-395.

Bennet JW (1998) Mycotechnology: role of fungi in biotechnology. *J.Biotechnol.* 66: 101-107

Boari G, Brunetti A, Passino R, Rozzi A (1984) Anaerobic digestion of olive oil mill wastewaters. *Agricultural W astes* 10: 161 – 175.

Bollag J-E and Leonowicz A (1984) Comparative studies Of extracellular fungal laccases, *Appl.Environ.Microbiol.* 48: 849-854

Borja R, Martin A, Garrido A (1993) Anaerobic digestion of black-olive wastewater. *Bioresource Technology* 45: 27 – 32.

Borja R, Martin A, Gomez L F, Ramos-Cormenzana A (1993) Anaerobic digestion of olive mill wastewater pretreated with *Azotobacter chroococcum. Resources, Conservation and Recycling* 9: 201 – 211.

Bower E J and Zehnder A J (1993). Bioremediation of organic compounds-putting microbial metabolism to work. *Trends in Biotechnol* 11(8):360-367.

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

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

Buchmann, I.D.; Nicell, J. A (1997) Model development for HRP catalyzed removal of acquoes phenol. *Biotech and Bioeng.* 54(3), 257-261.

Bumpus JA and Aust SD (1986) Biodegradation of environmental pollutants by the white rot fungus *Phanerochaete chrysosporium*: Involvement of the lignin degrading system. *Bioessays* 6:166-170

Burnette F. (1977) Peroxidase and its relation to food flavour and quality: A review : *J.Food.Sci.* 42, 1-6.

Burton SG, Boshoff A, Edwards W, Jacobs E, Leukes WD, Rose PD, Russell AK, Russell IM and Ryan D (1998) Water Research Commission report number 687/1/98: Membrane-based biotechnological systems for the treatment of organic pollutants. Department of Biochemistry and Microbiology, Rhodes University.

Burton SG, Duncan JR, Kaye PT and Rose PD (1993) Activity of mushroom polyphenol oxidase in organic medium, *Biotechnol.Bioeng.* 42: 938-944

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

Capasso R, Evidente A, Scognamiglio F (1992) A simple thin layer chromatographic method to detect the main polyphenols occuring in olive oil vegetation waters. *Phytochemical Analysis* 3: 270 – 275.

Chakchouk M, Hamdi M, Debellefontaine H (1994) Complete treatment of olive mill wastewaters by a wet air oxidation process coupled with a biological step. *Environmental Technology* 15: 323 – 332.

Collins P. J & Dobson D. W (1996) Oxidation of fluorene and phenanthrene by Mn(II) dependent peroxidase activity in whole cultures of *Trametes (Coriolus) versicolor*. Biotechnol. Letters **18** 801-804.

Collins PJ, Kotterman MJJ, Field JA and Dobsen ADW (1996) Oxidation of anthracene and benza[a]pyrene by laccases from *Trametes versicolor*. *Appl.Environ.Microbiol.* 62: 4563-4567

Cooper, V.A.; Nicell, J .A. (1994) removal of phenols from a foundry waste water using HRP Water. Res. 30(4), 954-964.

Cross RHM (1987) The preparation of biological material for electron microscopy: A practical guide, Rhodes University

D'Annibale A, Crestini C, Vinciguerra V, Sermanni G G (1988) The biodegradation of recalcitrant effluents from an olive mill by a white rot fungus. *Journal of Biotechnology* 61: 209 – 218.

D'Annibale, Claudia Crestini, Vittorio Vimiciguerra , Giovanni Giovannozzi Sermani
Davis RH and de Serres FJ (1970) Genetic and microbiological research techniques for *N.crassa*. *Methods Enzymol.* 16: 80- 140

Davis S and Burns RG (1990) Decolourisation of phenolic effluents by soluble and immobilized phenol oxidases. *Appl.Microbiol.Biotechnol.* 32: 721-726

De Schrijver A and De Mot R (1999). Degradation of pesticides by actinomycetes. *Crit Rev Microbiol.* 25(2):85-119.

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

Dunford, H . B; HRP: structure and kenetic properties. (1994) In Peroxidases in Chemistry and Biology [(edited by Everse J.; Everse, K. E and Grishman , M. B.) vol .II pp 1-24, CRC press, Boca Raton .Fla.]

Field J A, De Jong E, Feijoo-Costa G, de Bont J A M (1993) Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. *Tibtech* 11: 44 – 49.

Flouri F, Sotirchos D, Ioannidou S, Balis C (1996) Decolorization of olive oil mill liquid wastes by chemical and biological means. *International Biodeterioration and Biodegradation* : 189 – 192.

Froehner SC and Eriksson K-E (1974) Induction of *Neurospora crassa* laccase with protein synthesis inhibitors. *J.Biol.Chem.* 120: 450-457

Froehner SC and Eriksson K-E (1974) Purification and properties of *Neurospora crassa* laccase. *J.Biol.Chem.* 120: 458-465

Garcia I G, Pena P R J, Venceslada J L B, Martin A M, Santos M A M, Gomez E R (2000) Removal of phenol compounds from olive mill wastewater using *Phanerochaete chrysosporium, Aspergillus niger, Aspergillus terreus* and *Geotrichum candidum*. *Process Biochemistry* 35: 751 – 758.

Gerin P, Bellon-Fontaine MN, Asther M and Rouxhet PG (1995) Immobilisation of fungal spores by adhesion. *Biotechnol.Bioeng.* 47: 677-687

Germann UA, Muller G, Hunziker PE and Lerch K (1988) Characterisation of two allelic forms of *Neurospora crassa* laccase, *J.Biol.Chem.* 263: 885-896

Grady, C.P. L (1990). Bio-degradation of toxic compounds : status and potential. *J. Envir. Engng.* 116, 805-828.

Gutteridge S and Robb D (1975) The catecholase activity of *Neurospora* tyrosinase, *Eur.J.Biochem.* 54: 107-116

Hall JL and Hawes C (1991) Electon microscopy of plant cells. Academic Press, London, 105-180

Hamdi M (1991) Effects of agitation and pretreatment on the batch anaerobic digestion of olive mill wastewater. *Bioresource Technology* 36: 173 – 178.

Hamdi M (1993) Future prospects and constraints of olive mill wastewaters use and treatment : A review. *Bioprocess Engineering* 8: 209 – 214.

Hamdi M, Ellouz R (1992) Bubble column fermentation of olive mill wastewaters by *Aspergillus niger*. *J. Chem. Tech. Biotechnol.* 54: 331 – 335.

Hamdi M, Garcia J L (1993) Anaerobic digestion of olive mill wastewaters after detoxification by prior culture of *Aspergillus niger*. *Process Biochemistry* 28: 155 – 159.

Hammel KE, Gai WZ, Green B and Moen MA (1992) Oxidative degradation of phenanthrene by the lignolytic fungus *Phanerochaete chrysosporium*. *Appl.Environ.Microbiol.* 58: 1832-1838

Harayama S (1998). Artificial evolution by DNA shuffling. *Trends in Biotechnol* 16(2): 76-82.

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. *J. Mol. Catal. B: Enzym.* 5: 87-93.

Horowitz NH, Feldman HM and Pall ML (1970) Derepression of tyrosinase synthesis in *Neurospora* by cycloheximide, actinomycin D and puromycin, *J.Biol.Chem.* 245: 2784-2788

Jones W R (1998). Practical applications of marine bioremediation. *Curr Opin Biotechnol* 9(3):300-304.

Jucovic M and Poteete A R (1998). Delineation of an evolutionary salvage pathway by compensatory mutations of a defective lysozyme. *Protein Sci* 7(10):2200-2209.

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.

Karapinar M, Worgan J T (1983) Bioprotein production from waste products of olive oil extraaction. *J. Chem. Tech. Biotechnol.* 33B: 185 – 188.

Klibanov A M, Tu T M, Scott K P (1983) Peroxidase catalysed removal of phenols from coal conversion wastewaters. *Science* 221: 259 – 261.

Klibanov A, B . N . Alberti, E.D. Morris and L .M . Felshin *J.Applied Biochemistry*, 1980, 2, 414-421.

Klibanov A, B . N . Alberti, Scott, K. P.; Peroxidase catalyzed removal of phenols from coal conversion waste waters *Science* 1983,221, 259-261.

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

Knapp R B and Faison B D (1997). A bioengineering system for *in situ* bioremediation of contaminated groundwater. (Abstract). *J Ind Microbial Biotechnol* 18(2-3):189-197.

Konishi K and Inoue Y (1972) Detoxification mechanism of pentachlorophenol by the laccase of *Coriolus versicolor*. *Mokuzai Gakkaishi*. 18: 463-469

Kupper U, Linden M, Cao K and Lerch K (1990) Expression of tyrospinase in vegetative cultures of *Neurospora crassa* transformed with a metallothionein promotor/protyrosinase fusion gene. *Curr.Genet.* 18: 331-335

Kupper U, Niedermann DM, Travaglini G and Lerch K (1989) Isolation and characterisation of the tyrosinase gene from *Neurospora crassa*, *J.Biol.Chem.* 264: 17250-17258

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.

Lerch K (1987) Monophenol monooxygenase from *Neurospora crassa*, *Meth.Enzymol.* 142: 165-169

Leukes WD, Burton SG, Rose PD, Jacobs E and Sanderson RD Method for continuous production of secondary metabolites using hollow fibre membranes. WRC patent. (95/7366, International patent)

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.

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

Martin A, Borja R, Garcia I, Fiestas J A (1991) Kinetics of methane production from olive mill wastewater. *Process Biochemistry* 26: 101 – 107.

Martinez Nieto L M, Hoyos S E G, Rubio F C, Parcja M P G, Cormenzana A R (1993) The biological purification of waste products from olive oil extraction. *Bioresource Technology* 43: 215 – 219.

Martirani L, Giardina P, Marzullo L, Sannia G (1996) Reduction of phenolic content and toxicity in olive oil mill waste waters with the ligninolytic fungus *Pleurotus ostreatus*. *Water Research* 30 (8): 1914 – 1918.

Michel D. Atkin (1993) Waste treatment applications of enzymes : Opportunities and obstacles. *The Chemical Engineering Journal*, 52, B 49-B58.

Minshull J (1999). DNA shuffling of subgenomic sequences of subtilisin. *Nat Biotechnol* 17(9):893-896.

Mliki A and Zimmermann W (1992). Purification and characterization of an intracellular peroxidase from *Streptomyces cyaneus*. *Appl Microbiol Enivron* 58(3):916-919.

Mliki A and Zimmermann W (1992). Purification and characterization of an intracellular peroxidase from *Streptomyces cyaneus*. Appl Microbiol Enivron 58(3):916-919.

Montedoro G, Servili M, Baldioli M, Miniati E (1992) Simple and hydrolyzable phenolic compounds in virgin olive. 1. Their extraction, separation and quantitative and semiquantitative evaluation by HPLC. *J. Agric. Food Chem.* 40: 1571 – 1576.

Moreno E, Perez J, Ramos-Comenzana A, Martinez J (1987) Antimicrobial effect of water from olive oil extraction plants selecting soil bacteria after incubation with diluted waste. *Microbios* 51: 169 – 174.

Moreno E, Perez J, Ramos-Comenzana A, Martinez J (1987) Antimicrobial effect of water from olive oil extraction plants selecting soil bacteria after incubation with diluted waste. *Microbios* 51: 169 – 174.

Muller R, Deckwer W-D and Hecht V (1996). Degradation of chloro- and methyl substituted benzoic acids by a genetically modified microorganism . *Biotechnol Bioeng* (51): 528-537.

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

Nakamoto, S.; Machida, N.; Phenol removal from aqueous solutions by peroxidase -catalysed reaction using additives. *Wat .Res.*1991, 26, 49-54.

Ness J E, Welch M, Giver L, Bueno M, Cherry J R, Borchert T V, Stemmer W P and Minshull J (1999). DNA shuffling of subgenomic sequences of subtilisin. *Nat Biotechnol* 17(9):893-896.

Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A and Sannia G (1997) A Novel white laccase from *Pleurotus ostreatus*, *J.Biol.Chem.* 272: 31301-31307

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.

Pekka Pietikaien and Patrik Adlercreutz (1990) Influence of reaction medium on the product disribution of peroxidase -catalyzed oxidation of p-cresol *Appl.Microbiol.Biotechnol.* 33, 455-458.

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.

Pussemier, L.; Szabo G.; and Bulman, R.A.; Prediction of solid sorption coefficient for aromatic pollutants. *Chemosphere* 1990, 21, 1199-121.

Ramachandra M, Crawford D L and Hertel G (1988). Characterization of an extracellular lignin peroxidase of the lignocelluloytic Actinomycete *Streptomyces viridosporus. Appl. Environ. Microbial.* 54: 3057-3063.

Ramachandra M, Crawford D L and Pometto A (1987). Extracellular enzyme activities during lignocellulose degradation by *Streptomyces* spp: A comparative study of wild type and genetically manipulated strains. *Appl Microbial Enivron* 53(12):2754-2760.

Ramos-Cormenzana A, Monteoliva-Sanchez M, Lopez M J (1995) Bioremediation of alpechin. *International Biodeterioration and Biodegradation* 35 (1-3): 249 – 268.

Reddy A (1995) The potential for white rot fungi in the treatment of pollutants. *Curr.Opin.Biotechnol.* 6: 320-328

Ricotta A, Unz RF and Bollag J-M (1996) Role of a laccase in the degradation of pentachlorophenol. *Bull.Environ.Contam.Toxicol.* 57: 560-567

Rodriguez M, Perez J, Ramos-Comenzana A, Martinez J (1988) Effect of extracts from olive oil mill waste waters on *Bacillus megaterium* ATCC 33085. *Journal of Applied Bacteriology* 64 : 219 – 226.

Rosazza,J.P.N. Huang, Z.; Dostal, L.; Volm, T.; and Rousseau, B. (1995) Review: Biocatalytic transformations of ferulic acid:an abundant aromatic natural product. *Journal of Industrial Microbiology*, 15, 457-471.

Roy-Arcand L and Archibald FS (1991) Direct dechlorination of chlorophenolic compounds by laccases from *Trametes (Coriolus) versicolor, Enzym.Microb.Technol.* 13: 194-203

Rozgaj R (1994). [Microbial degradation of xenobiotics in the environment]. Abstract. Arh hig Rada Toksikol 45(2):189-198.

Rüttimann C, Seelenfreund D and Vicuña R (1987). Metabolism of low molecular weight lignin-related compounds by *Streptomyces viridosporus* T7A. Enzyme Microb Technol (9):526-530.

Ryan D, Russell AK, Leukes WD, Rose PD and Burton SG (1998) Suitability of a modified capillary membrane for growth of fungal biofilms, *Desal.* 115: 303-306

Sanjust E, Pampei R, Rescigno A, Rinal A, Ballero M (1991) Olive milling wastewater as a medium for growth of four *Pleurotus* species. *Applied Biochemistry and Biotechnology* 31: 223 – 235.

Sepic E, M . Bricelji and H. Lescovisek Degradation of flouranthene by Pastarulla species IFA and Mycobacterium species PYR-1: isolation and identification of metabolites. *J. Appl .Microbiology* 1998, 85, 746-754.and ref there in.

Shishido M and Toda M (1996) Apparent zero-order kinetics of phenol biodegradation by substrate-inhibited microbes at low substrate concentrations, *Biotechnol.Bioeng.* 50: 709-717

Shishido M and Toda M (1996) Apparent zero-order kinetics of phenol biodegradation by substrate-inhibited microbes at low substrate concentrations. *Biotechnol.Bioeng.* 50: 709-717

Sin, Kwang-Soo and Kim, Chang-Jin (1998) Decolourization of artificial dyes by peroxidase from the white rot fungus, Pleurotus Ostreatus. *Biotechnology Letters* 20(6), 569-572.

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: 179-186.

Swamy J and Ramsay JA (1999) The evaluation of white-rot fungi in the decolorisation of textile dyes. *Enzyme Microb.Technol.* 24: 130-137

Thomas L and Crawford D L (1998). Cloning of clustered *Streptomyces viridosporus* T7A lignocellulose catabolism genes encoding peroxidase and endoglucanase and their extracellular expression in *Pichia pastoris*. *Can J Microbial* (44):364-372.

Tinnus K N and Pieper D H (1999). Bacteria designed for bioremediation. *Trends Biotechnol* 17(5):200-204.

Vagujgfalvi Enzymatic transformations of morphinane alkaliods *Phytochemistry* 1982, 21(7), 1533-1536.

Van Schie PM and Young LY (1998) Isolation and characterization of phenol-degrading denitrifying bacteria. *Appl.Environ.Microbiol.* 64: 2432-2438

Vilchez MJ, Vigara J, Garbaya I and Vilchez C (1997) Electron microscopic studies on immobilised growing *Chlamydomonas reinhardtii* cells, *Enzyme Microb.Technol.* 21: 45-47

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.

Wada, S., Ichikawa, H., Tatsumi, K. 1993. Removal of phenols from wastewater by soluble and immobilized tyrosinase. *Biotechnol. Bioeng.* 42(7): 854-858.

Wenhua H and Ding Z (1993) Treatment of phenol-containing wastewater by immobilised microorganisms, *Water treatment*, 8: 119-12

Yamada, Y.; Kobayashi, S.;Watanabe, K.; and Hayashi, U. (1987) Production of Horse raddish peroxidase by plant cell cultures. *J.Chem. Tech. Biotechnology* 38, 31-39.

Yesilada O, Fiskin K, Yesilada E (1995) The use of the white rot fungus *Funalia trogii* (Malatya) for the decolorization and phenol removal from olive mill wastewater. *Environmental Technology* 16: 95 – 100.

APPENDIX 1

Industrial effluents used in this study

The industrial wastes used in the project were selected on the basis of their being representative of typical phenolic-containing industrial residues. These were:

- Simple phenolics from petrochemical industry
- Processing effluent from pulp-and-paper production
- Plant-related phenolics from olive processing
- Polyphenolic wastes from wine production.

The organic content of each set is described in the sections and tables below.

A1. Simple phenol-containing effluent from petrochemcial industry

South Africa has a unique and very large industry converting coal to oil for supply to the petroleum industry. This process generates large (ML per day) volumes of wastewaters which contain high concentrations of simple phenols. Table A1 show concentrations in the samples donated to this group byt the company involved. Two sets of data are shown, indicating small variations between samples.

Table A1: Phenolic concentrations present in petrochemical industry effluents

	Phenolic pollutant							
	Phenol		***p*-Cresol**		***m*-Cresol**		***o*-Cresol**	
	(mM)	**(g/L)**	**(mM)**	**(g/L)**	**(mM)**	**(g/L)**	**(mM)**	**(g/L)**
Cresylic 1	87.1	8.19	25.2	2.72	26.9	2.91	81.1	8.76
Cresylic 2	27.6	2.59	35.5	3.83	36.8	3.97	70.8	7.65
Black product 1	11.7	1.10	0.8	0.86	0.5	0.54	0.3	0.32
Black product 2	16.3	1.53	1.0	0.11	1.1	1.18	1.1	1.18

A2. Polyphenolic wastewaters from olive processing

The olive processing industry generates high saline wastewaters containg high concentrations of phenolics derived form the lignin-related polyphenols in the olive tissue. These are leached out during the curing process. Data supplied by the relevant olive production company is shown in Table A2.

Table A2. Organic content in olive processing wastewaters

	Fermentation effluent FB	**Digestor tank water LV**	**Evaporation pond water SO**
Chemical oxygen demand (mg/mL)	32758	2238	562
Salinity (mg/L chloride)	37071	4890	3624
Total phenols (mg/L)	1573	336	1.7

A3. Wine waste

The wine industry generates large quantities of grape waste (pomasse) which is left to compost, during which time lignin-related polyphenolics leach into ground waters. This project utilized this waste as a source of the polyphenols, by grinding frozen grape waste and using aqueous and organic-aqueous extracts as represetative of the leachate.

Table A3. Phenolic content of grape waste waters

	Green grape waste aqueous extract	**Red grape waste aqueous extract**	
Total phenols (mg/g waste)	1.7	1.5	

APPENDIX 2

Potential economic value of products

One objective of the project was to demonstrate the generation of high-value products from bioconversion of industrial residues. The table below indicates the approximate current value of selected examples of products which were generated by the biological systems investigated in this study.

Table A4: Current prices for potential products from commercial chemical companies

Product	**Current price for pure compound ($ per 100 g)**
4-Methylcatechol	50
4-Chlorocatechol	Not available commercially
Catechol	30
Benzaldehyde	30
Vanillin	160
Vanillic acid	150

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