

BIOREACTOR SYSTEMS USING THE WHITE ROT FUNGUS *TRAMETES* FOR BIOREMEDIATION OF INDUSTRIAL WASTEWATER

REPORT TO

the Water Research Commission

by

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EXECUTIVE SUMMARY

Contamination of soils, groundwater, sediments, surface water and air with hazardous and toxic chemicals is one of the major problems facing the industrialised world today. Of the various types of industry-generated effluents, those containing organic pollutants such as phenols are generally difficult to remediate. There is a need to develop new technologies that emphasize the destruction of these pollutants rather than their disposal. The bioremediation of phenolic effluents by white rot fungi, and the oxidative enzymes produced by them, has been the topic of much research and is widely reported on in literature. This study focuses on the integration of an optimised process of enzyme production/ phenol degradation by the fungi *Trametes versicolor* and *Trametes pubescens* (Figure (i)) with an Airlift Loop Reactor found suitable for the large-scale fermentation of these organisms.

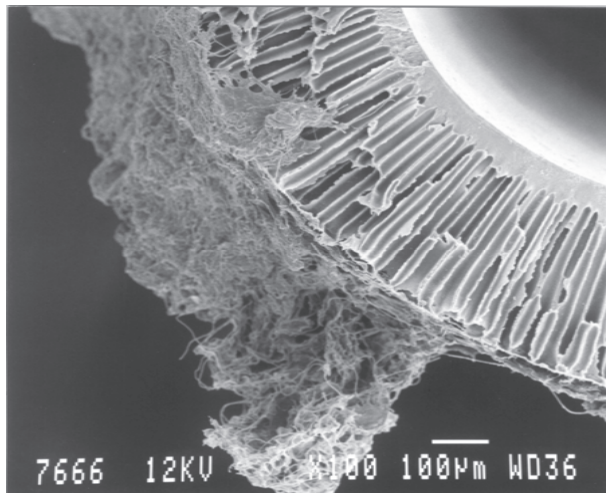
The broad aim of this project was to develop a reactor-based bioprocess utilising the white rot fungus *Trametes versicolor* for the remediation of phenol-contaminated wastewaters. To this end it was necessary to first investigate the physiology of the organism in terms of laccase production and its response to the presence of phenols in its environment. *T. versicolor* had been reported as an excellent producer of laccase but the successful application of this organism in a bioremediation process required the production of high amounts of this enzyme. The degree of success achieved in the laboratory in the mineralisation/ removal from solution of many major pollutants had so far only been realised in flask culture or in rather laboratory-specific reactor applications. A more practical (in terms of future scale-up), cost effective reactor system that provided a well-oxygenated, yet low shear environment was required.



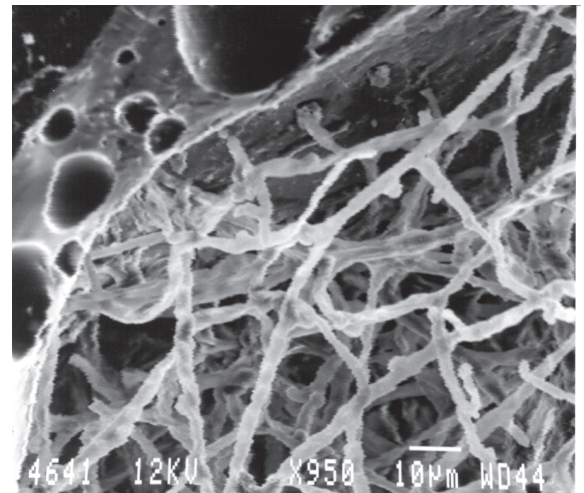
Figure (i) White rot fungal growth *in vivo* and *in vitro*

Using a comparison of the growth media recommended in literature as a basis for further experimentation, a 'nutrient sufficient' medium directed towards rapid biomass accumulation and increased lignolytic enzyme (laccase) production was developed. Enzyme activity was further increased by screening a variety of known inducers and comparing them with additions of small concentrations of stripped gas liquor from a Fischer-Tropsch plant, the target effluent in this study. In this way laccase production was increased by 700 %. The removal from solution of the principal effluent monomers (phenol, *p*-, *m*- and *o*-cresol) was studied, with up to 20 % v/v effluent being removed in flask culture. An investigation was conducted to elucidate the effect of this effluent on the morphology and physiology on the two *Trametes* species, with particular emphasis on laccase production.

Large scale, cost-effective applications of white-rot fungi to continuous treatment of liquid effluent has previously been hindered by the lack of suitable bioreactor systems. A hollow fibre membrane bioreactor and a trickle filter were investigated for suitability as supports for immobilised biofilms of *T. versicolor* and laccase production and pollutant degradation were successfully demonstrated in both reactor configurations. However, the need for a simple, cost effective, yet simple to upscale reactor system led to the investigation and development of an airlift loop reactor (ALR). These reactors have well defined flow patterns, high liquid velocities and yet provide a relatively low shear environment ideal for the growth of *T. pubescens*. The reactor configuration and aeration rate were optimised using recognised chemical engineering principles. Favourable feeding strategies, the effects of inducers/precursors and the timing of effluent addition were investigated. Increased growth (10g/L dry mass) and enzyme production (12000U/L) as well as highly efficient effluent degradation (5% v/v/day) were achieved in the ALR in fermentations over two week periods.



A



B

Figure (ii) Fungal biomass immobilised on synthetic membranes for the membrane bioreactor (A), and Glass foam support for the trickle reactor (B)

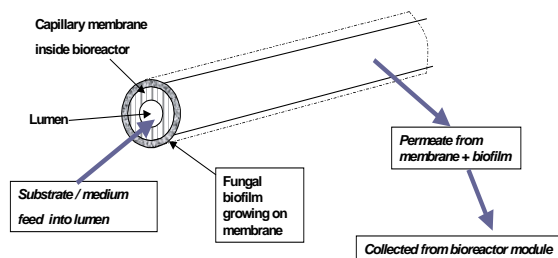


Figure (iii) How capillary membrane bioreactors operate



Figure (iv) Membrane bioreactors in the laboratory.



Figure (v) The trickle bed reactor (with the authors)

In the ALR system, a total pollutant removal of 0.1 g/g biomass/day was achieved in a 3.5L reactor, as compared with 0.5 g/g biomass/day achieved in single dose flask reactions. To our knowledge the concentrations of the phenolic monomers removed by *T. versicolor* in this system are the highest reported to date. This study demonstrated that in the ALR/*T. pubescens* system, the simplicity of design, low cost of operation and high performance that are essential for a successful biological wastewater treatment scheme, can be achieved.



Figure (vi) Airlift reactors in operation

OBJECTIVES

Overall project aim:

Development of a practicable bioremediation process for using the enzymes of *Trametes versicolor* to degrade pollutants in specific industrial wastes, namely chlorinated aromatics and phenolics produced by the pulp-and-paper and petrochemical industry.

Key objectives:

1. Characterisation of the biological conversion by *Trametes versicolor*, of the phenolics, chlorinated phenolics and polyaromatics present in these effluents. This would include characterisation of the roles and activity levels of the enzyme activities involved, and the fate of the organic compounds converted.
2. An optimised fermentation or fed-batch process in which the *Trametes versicolor* successfully degrades these pollutants.
3. A laboratory scale bioreactor involving a pneumatic fermenter linked to a membrane module, with *Trametes versicolor* growing and producing enzymes continuously at steady state with continuous operation of this system for a prolonged time to demonstrate its feasibility.
4. Development of models of the biological reactions in terms of cell growth, enzyme production, biodegradation rates and the operation of the fermenter / membrane module bioreactor for bioremediation
5. Establishment and demonstration of the process in a demonstration plant by scale-up of the laboratory scale bioreactor system, on the basis of models. (However, it was decided at the first Steering Committee meeting of the project that scale-up was outside the scope of the project.)
6. Transfer of technology to relevant industries and reporting on these results.

KEY PROJECT FINDINGS

1. The basidiomycetes *Trametes versicolor* (PPRI 3845) and *Trametes pubescens* (CBS 696.94) were shown to be outstanding producers of the oxidative enzyme laccase and are consequently resilient to the effects of aromatic compounds usually toxic to most other microorganisms.
2. The production of this enzyme was optimised in static culture by the use of a carbon and nitrogen 'sufficient' growth medium; and further increased by the addition of various inducer compounds, including a 'stripped gas liquor' effluent.
3. The complex physiological response of *T. versicolor* to the addition of the phenolic effluent was illustrated and explained in terms of enzyme production, metabolic activity and morphological differentiation. Many of the deleterious effects of effluent addition can be ameliorated by controlling the amounts added, and the time of these additions relative to culture age.
 - The pollutant compounds induce the enzymes required for their catabolism
 - increasing their concentrations resulted in increased fungal biomass
 - addition of a readily utilisable carbon source (glucose) was shown to increase and prolong oxidative enzyme production, and hence the removal of the aromatic toxins from solution

Table (i) Process parameters for *T. versicolor* growth in optimised medium

Biomass Yield ($Y_{x/s}$) (g/g)	0.023
Product Yield ($Y_{p/s}$) (U/g)	0.150
Specific Yield ($Y_{p/x}$) (U/g)	4.780
μ (per day)	0.305
GRP (U/g/day)	1.458

Growth related productivity (GRP) = $(Y_{p/x})(\mu)$ = Units of laccase produced per day per g biomass
 Units: U/g/d
 X = biomass
 P = product (laccase)
 S = substrate (glucose)

4. Immobilised biofilm reactors in the form of a Transverse Flow Membrane Bioreactor and a Trickle Bed Reactor were identified as suitable for growth, enzyme production and phenolic removal by *T. versicolor*.

5. Control of aeration rate was shown to optimise the fermentation performance for growth of *Trametes* species in an Airlift Loop Reactor and this system was found to outperform the other reactors investigated in terms of laccase production and the bioremediation of phenolic compounds.
 - ALRs are well suited to wastewater treatment and have been shown in this study to be additionally suited to the growth of *Trametes pubescens*. Control of aeration rate was shown to optimise the fermentation performance for growth of *Trametes* species in the Airlift Loop Reactor and this system was found to outperform the other reactors investigated in terms of laccase production and the bioremediation of phenolic compounds.
6. In the ALR system, a total pollutant removal of 0.1 g/g biomass/day was achieved in a 3.5L reactor, and 14 g was removed over 16 days. To our knowledge the concentrations of the phenolic monomers removed by *Trametes* in this system are the highest reported to date.

Table(ii) Phenolic removal rates for the various culture conditions and reactor configurations investigated in this study

POLLUTANT PHENOL	FLASK		TFHFBR		TFR		ALR	
	R R ^a (g/g/d) ^c	T R ^b (g)	R R (g/g/d)	T R (g)	R R (g/g/d)	T R (g)	R R (g/g/d)	T R (g)
Phenol	0.106	0.75 (7 d)			0.031	1.54 (5 d)	0.033	4.97 (15 d)
<i>p</i> -cresol	0.077	0.45 (7 d)	0.05	3.70 (7 d)	0.010	0.54 (5 d)	0.011	1.79 (15 d)
<i>m</i> -cresol	0.081	0.46 (7 d)			0.011	0.57 (5 d)	0.012	1.87 (15 d)
<i>o</i> -cresol	0.231	1.38 (7 d)			0.031	1.63 (5 d)	0.035	5.20 (15 d)

^a Maximum removal rate ^b Total removal ^c gram of pollutant/gram of biomass/day

Table (iii) Comparison of laccase activities reported in different bioremediation applications of *T. versicolor* (in flask culture)

REFERENCE	ACTIVITY (U/mL)	APPLICATION
Addleman <i>et al.</i> , (1995)	0.048	Kraft pulp degradation
Roy and Archibald (1993)	0.16	Kraft pulp degradation
Addleman and Archibald (1993)	0.58	Kraft pulp degradation
Heinfling <i>et al.</i> , (1997)	0.48	Decolourisation of dyestuffs
Rogalski <i>et al.</i> , (1991)	0.06	Optimisation of culture conditions
Swamy and Ramsay (1999)	0.41	Decolourisation of dyestuffs
This study	1.52	Degradation of cresylic effluents

7. The combination of the reactor configuration developed here and the efficiency of *T. pubescens* in the degradation of the phenolic compounds form the basis of an important, novel bioremediation process.
8. The development of this *Trametes* based treatment technology represents a departure from the traditional mixed culture biological processes commonly used to decrease the toxicity of industrial wastewater: here, a monoculture was used consistently reduce a range of compounds to low levels or, in most cases, completely remove them from solution

Complete removal of phenols in ALR was achieved over extended operation times. Repeated additions successfully removed and minimal polymer accumulation was observed.

RECOMMENDATIONS

- Since the objectives of the project were very successfully achieved, it would be valuable and meaningful to take the system to larger scale and demonstrate its effectiveness at pilot scale. This was beyond the scope of this project, however, and although much effort was put into attracting support from the relevant large-scale industrial company, their support was not forthcoming. It is strongly recommended by the research team that this piloting should be considered.
- There has been considerable international interest in collaboration on developing and extending this project, and it is a recommendation that this should be supported.

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Dr G Offringa	Water Research Commission (Chairman)
Mr G Steenveld	Water Research Commission
Professor S Burton	University of Cape Town
Professor S Harrison	University of Cape Town
Mr B Hendry	Peninsula Techikon
Dr E Jacobs	Institute for Polymer Science
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Mr G Lok	ESCOM

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PROJECT OUTPUTS

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1. Burton, S G. (2001) Development of Bioreactors for Application of Biocatalysts in Biotransformations and Bioremediation. **Pure and Applied Chemistry** **73**, 77-83.
2. Burton S G, Cowan D A and Woodley J M. (2002) The Ideal Biocatalyst: a new paradigm. (Invited review). **Nature Biotechnology**, **20**, 37-45.
3. Burton, S G. (2001) Development of Bioreactors for Application of Biocatalysts in Biotransformations and Bioremediation. (Invited paper). **Pure and Applied Chemistry** **73**, 77-83.
4. Burton S G, Cowan D A and Woodley J M. (2002) The Search for the Ideal Biocatalyst. (Invited review) **Nature Biotechnology**, **20**, 37-45.
5. Burton, S G. Oxidative biotransformations using microbial oxidases. (Invited review). **TIBTECH (Trends in Biotechnology)**, in press.
6. Burton, S G. (2003) Biotransformations with phenol oxidases and laccase: a review. **Current Organic Chemistry** **7**, 1-15

Book chapters

Biocatalysis – a complementary approach in green chemistry
Stephanie G Burton

In Inter-University Consortium “**Green Chemistry in Africa**,” Green Chemistry Series, Editor: P Tundo, Italy. IUPAC-INCA (2002).

Papers in preparation:

Fungal Bioremediation of Phenolic Wastewaters in an Airlift Loop Reactor
Daniel Ryan, Winston Leukes and Stephanie Burton (in preparation)

Conference presentations

International presentations:

1. Ryan D R, Leukes W L and Burton S G (2001) The Development of a Bioreactor System Using *Trametes versicolor* for the Bioremediation of Industrial Wastewater. **8th International Conference on Biotechnology in the Pulp and Paper Industry in Helsinki, Finland**
2. **IUPAC International Symposium on Green Chemistry:** S G Burton, Plenary Lecture title: “Biotransformations and Bioreactors”, New Delhi, India, January 2001
3. **CHEMRAWN XIV World Conference,** S G Burton, Keynote lecture: “Toward environmentally benign processes and products” Boulder, Colorado, June 2001.
4. “Biocatalysis for oxidation reactions”. S Burton, October 2001. Invited seminar, **Istituto di Biocatalisi e Riconoscimento Molecolare**, Milan,

5. Burton S, Boshoff, A, Ryan, D, Luke, T, Mhlanga C, Koteswar K, Notshe T. Biotransformation of phenolics using oxidative enzymes. **Enzyme Engineering XVI, Potsdam, 2001.**
6. "Bioremediation of phenolic pollutants using oxidases". S Burton, October 2002. Invited seminar, **Istituto di Biocatalisi e Riconoscimento Molecolare**, Milan
7. "Biotransformations using oxidative enzymes" S Burton, Keynote lecture: Biocat 2002, International conference on biocatalysis, **Hamburg, September 2002**
8. S Burton and D Ryan. An airlift bioreactor for laccase production by *Trametes*, and conversion of phenolics , **Enzyme Engineering XVII, Santa Fe, 2003**

National:

1. Ryan D R, Leukes W L and Burton S G (2001) The Development of a Bioreactor System Using *Trametes versicolor* for the Bioremediation of Industrial Wastewater **South African Institute for Chemical Engineers, Chemical Engineering R&D 2002, Cape Town**
2. Ryan D R, Leukes W L and Burton S G (2002) A Bioreactor System Using *Trametes* for the bioremediation of phenolic industrial wastewater. **Cape Biotech 2003.**
3. "**Biotransformations and bioremediation using oxidases**" S Burton, invited seminar, Department of Biotechnology, University of the Western Cape, July 2003

Theses

Honours thesis:

L van Wyk, An investigation into the bioconversion of pollutants found in industrial effluents by laccase produced by *Trametes versicolor* and *Trametes pubescens*. (Rhodes University, Dec 2001)

PhD thesis :

Ryan D R, Reactor development and process optimisation for the bioremediation of phenolic wastewaters by *Trametes* species. (April 2004).

CHAPTER 1 : BACKGROUND AND REVIEW OF LITERATURE

1.1 Introduction

This report describes the development of a fermentation process for the white rot fungus *Trametes versicolor* directed toward the optimised production of fungal biomass and oxidative enzymes, and ultimately the bioremediation of phenolic-containing wastewaters. *T. versicolor* is a common inhabitant of dead hardwoods in temperate climates where it causes a non-selective white rot decay. The lignolytic enzymes of this fungus have broad substrate specificity and have been implicated in the transformation of organopollutants with structural similarities to lignin.

Much industrial pollution can be traced to waste-management practices that involve disposal rather than treatment, or to accidental or incidental spillages that may be ignored in terms of non-existent, ineffective or uninformed environmental protection legislation. However, present legislation is increasingly designed both to ameliorate the adverse effects of past pollution, and to require immediate action to minimise the impact when industrial accidents producing new pollution do occur. A general consensus has emerged that the traditional “dilute, bury or burn” practice is no longer acceptable. To enable industrial companies to operate within increasingly stringent laws, the technology for pollution control must be made available. The challenge to biotechnology is to generate efficient, cost effective and environmentally safe bioremediation methods to replace existing technologies such as incineration, as well as to provide unique solutions for the remediation of contaminated sites.

During the past two decades certain white rot fungi (WRF) have been extensively studied in order to exploit their potential in producing enzymes that generate extracellular oxidative radicals for biotechnological applications (Schlosser, 1997). The WRF are one of the few groups of microorganisms that can degrade lignin. They produce two types of extra-cellular metalloproteins with lignolytic activities: laccases and peroxidases (Pointing, 2001). 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.

T. versicolor is one of the more extensively studied white rot fungi. It produces a group of enzymes containing various laccase (E.C. 1.10.3.2) isozymes, as well as other peroxidases e.g. lignin peroxidase (LiP, E.C. 1.11.1.14) and manganese peroxidase (MnP, E.C. 1.11.1.13). Low concentrations of the enzymes are produced constitutively in submerged culture, while increased amounts are induced by aromatic compounds such as xylinine and ferulic acid (Collins and Dobson, 1996 Galhaup *et al.*, 2002). In addition, while most other WRF's 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. This results in high total quantities of enzyme being produced. These enzymes are extracellular, and this allows high concentrations of pollutants to be detoxified without requiring the transport of substrates into cells. The enzymes are relatively non-specific with regard to aromatic pollutant structure, and thus a monoculture of the fungus can degrade a wide range of pollutants as opposed to a consortium of microorganisms that might be required in alternative processes. In a bioremediation process involving a WRF, 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.

1.2.1 The white rot fungus *Trametes versicolor*

Trametes versicolor belongs to a poroid family of the order Aphyllophorales, which is a class of the subdivision Basidiomycotina, division Eumycota. The fungus is described as a polypore, due to its polymorphic gross general morphology. Generative hyphae are of unlimited growth, possessing abundant protoplasmic contents; their wall is initially thin but may ultimately become thickened or gelatinised. Also present are specialised branches called skeletal and binding hyphae. Skeletal hyphae convey mechanical strength to the mycelial structure (Ainsworth *et. al.*, 1973). *T. versicolor* is a tetrapolar heterokaryon and each dikaryotic cell contains two non-identical nuclei derived from the fusion of two different, sexually compatible primary mycelia, each bearing a single nucleus per cell. The dikaryotic mycelia become pigmented in culture, producing brown exudates and darkening some softwoods during decay.

The mycelia of wood decay fungi operate in a dynamic environment. As a result, they exhibit considerable spatial and temporal differences in metabolic functioning related to their developmental state. Fungi are able to adopt and switch between a variety of

distinct programmed morphogenic cycles, which confer versatility and help the organism deal with local episodic pressures. It is not clear how much of this 'developmental plasticity' in fungi can be attributed to microenvironmental effects and how much to a selection of pre- and post-translational switch mechanisms. Even on relatively homogeneous laboratory medium, interconvertible morphs are frequently evident including slow-dense/fast-effuse and aerial *versus* appressed or submerged growth (White and Boddy, 1992b). Switching between modes can be correlated to different colonisation strategies. Many Basidiomycetes have two obviously different colony morphs: the first is characterised by extensive, white woolly aerial mycelia; the second is often darkly pigmented, more tolerant of adverse conditions and has greater enzyme activity. There are differences in extracellular enzyme production between different modes (Sharland, 1986). The hyphae at the margin are orientated toward rapid extension; a mode that in nature may lead to early establishment in wood i.e. primary resource capture. The septate mode is adapted to replacing other fungi and defending territory (White and Boddy, 1992b). Elevated levels of laccase in *T. versicolor* have been correlated with the slowest extension rate and low biomass production on solid culture, and there is some evidence that rate and/or extent of mycelial out-growth from wood are inversely related (Wells and Boddy, 1990).

Despite extensive investigation, the exact biological role of laccase and peroxidases has not been irrefutably determined and therefore few conclusions regarding their production can be made. Laccase has been implicated in many processes but patterns of enzyme production may simply reflect a physiological need for changes in carbon flow associated with new physiological activities. It has also been speculated that some form of metabolic coupling to phenoloxidases occurs in fungi during periods when endogenous metabolism cannot keep up with cellular demands (White and Boddy, 1992b). Rayner *et al.*, (1987) suggested that a series of superimposable switch mechanisms, rather than genetic heterogeneity, control contrasting patterns of cellular growth. This control mechanism confers on the individual fungus a developmental versatility that enables the organism to survive the changing, and often discontinuous, niches it occupies in nature.

1.2.2 The effect of nutrition and culture conditions on enzyme production in *T. versicolor*

Three extracellular fungal enzymes cause changes in lignin and lignin model compounds: laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP). *T. versicolor* produces all of these enzymes, although typically for their occurrence in *T. versicolor* there seems to be large variability between strains which may reflect the inherent heterogeneity of this species. Culture conditions are important in the production of lignolytic enzymes in this and other species. Rogalski *et al.* (1991) concluded that “the optimum culture conditions for the production of lignolytic enzymes and thus probably also for the degradation of lignin should be individually chosen for each strain of *Trametes versicolor*”. Kaal *et al.* (1995) demonstrated that higher biomass and lignolytic enzyme activities were observed in response to organic nitrogen-sufficient conditions. Collins and Dobson (1995) investigated a *T. versicolor* strain that reached maximal LiP activity when cultured in carbon-rich conditions (10 g/L glucose), a similar increase in MnP was also recorded.

Schlosser *et al.* (1997) investigated the growth of *T. versicolor* on wheat straw and beech wood compared to glucose. Growth on the lignocellulosic substrates led to a 3.5 fold increase in extracellular laccase activity in comparison with growth on glucose. However, the corresponding yields in biomass reached only about 20% of the value obtained on glucose. MnP activity appeared during growth on both wheat straw and beech, but not in cultures grown on glucose, even when the glucose in the medium was depleted. It was obvious that the efficiency (in terms of amount of enzyme produced relative to biomass) of the mycelia during growth on lignocellulosic substrates was much greater than that on glucose. During growth, the mycelial mats always developed on the surface of the cultures, whereas the solid substrates settled at the bottom of the flasks. Thus there was no contact between the mycelia and the wheat straw or beech chips. Intracellular laccase was reported in all three culture regimes (glucose, straw, beech wood) and, in assaying oxidation rates, the optimal pH values were found to be different from those of the extracellular form. However, in each case, much higher laccase activity was recovered from the culture filtrates. The origin of the intracellular laccases detected may have been due to extracellular laccase(s) before their secretion, or by ‘true’ intracellular laccase(s), or a combination of both. Obviously more information on the

regulation of laccase synthesis and activity as well as on physiological function and localisation within subcellular compartments is required

In WRF other than *T. versicolor*, enzyme production often occurs only under conditions of nutrient limitation. However, under these conditions, only low biomass yields are possible and therefore the enzymes can only be produced in low total quantities. This situation is far from ideal from a biotechnology standpoint. If these enzymes find a commercial application, it will be necessary to produce them in large quantities. Hence there is a very real advantage in the fact that *T. versicolor* has the potential to produce high levels of lignolytic enzymes under nutrient sufficient (rapid growth) conditions.

1.3.1 Laccase enzymes

Among the more than 60 laccases isolated from various plant, insect, bacterial and fungal sources, the *T. versicolor* enzyme is one of the best studied in terms of molecular properties and enzymology. The laccase molecule, in active holoenzyme form, is a dimeric or tetrameric glycoprotein usually containing four copper atoms per monomer, bound to three redox sites (Type 1,2 and 3 Cu pairs). The four Cu atoms differ from each other in their characteristic electronic paramagnetic resonance (EPR) signals. Two of them belong to the strongly coupled Type 3 site, with an EPR signal usually activated by strong anion binding. Types 1 and Type 2 Cu atoms have strong electronic absorption and well characterised EPR signals (Gianfreda *et al.*, 1999).

Laccase catalysis is believed to comprise three major steps:

1. Type 1 Cu reduction by the reducing substrate.
2. Internal electron transfer from Type 1 Cu to Type 2 and Type 3 Cu trinuclear cluster.
3. O₂ reduction (to water) at Type 2 and Type 3 Cu (Thurston, 1994; Yarapolov *et al.*, 1994).

Various physical characterisations have indicated that the Type 2 and Type 3 Cu in laccase form a trinuclear cluster, the site where the electrons from the reducing substrate (coming via the Type 1 Cu) are enzymatically transferred to O₂ to form H₂O. Experimental evidence supports a scheme in which the O₂ molecule firsts binds to the Type 2 and one of the Type 3 Cu to undergo “asymmetric” activation. The O₂ binding

pocket appears to restrict the access of oxidising agents other than O₂ (Gianfreda *et al.*, 1999).

The oxidation of a reducing substrate usually involves the formation of a free (cation) radical after the transfer of a single electron to laccase. The radical can further undergo laccase-catalysed oxidation e.g. to form a quinone product from phenol or non-enzymic reactions (e.g. hydration or polymerisation). Oxidation can be controlled by redox potential differences between the reducing substrate and the type 1 Cu in laccase (Xu, 1997). A lower oxidation potential of the substrate often results in a higher rate for substrate oxidation. The substrate specificity and affinity of laccase can vary with changes in pH. For substrates whose oxidation does not involve proton exchange (such as ferrocyanide), the laccase activity often decreases as pH increases. However, in the case of those substrates involving proton exchange (such as phenol), the pH activity profile of laccase can exhibit an optimal pH, which is dependent on the laccase rather than substrate (Bourbonnais and Paice, 1992). The difference in pH-activity profiles is attributed to the balance of two opposing effects: one generated by the redox potential difference between a reducing substrate and the Type 1 Cu of laccase (which correlates to the electron transfer rate and is favoured for a phenolic substrate by higher pH), and the other generated by the binding of an hydroxide anion to the Type 2/ Type 3 Cu of laccase (which inhibits activity at higher pH). Under turnover conditions the rate-limiting step is most likely substrate oxidation, whereas under transient or anaerobic conditions, the internal electron transfer step may become rate limiting (Gianfreda *et al.*, 1999). In general the phenol oxidase activity of laccase has a bell-shaped (biphasic) pH profile whose optimal pH varies considerably among different laccases (Xu, 1997).

Laccases can be inhibited by various reagents, particularly by small anions such as halides, azide, cyanide and hydroxide which bind to the Type 2 and Type 3 Cu, resulting in the interruption of the internal electron transfer and hence inhibition. Laccase inhibition by OH⁻ can dominate catalysis at alkaline pH. Laccases are, in fact, more stable at alkaline than at acidic pH due to the OH⁻ inhibition of autooxidation. Both the Type 1 and Type 2 Cu can be extracted reversibly in the presence of copper chelator and reducing (or sulfhydryl) reagents. Laccases can exhibit a thermal activation with apparent activation energy of 30-60 kJ/mol (Gianfreda *et al.*, 1999). Laccases are generally thermostable under physiological conditions, although this varies according to source. A

laccase that retains its full activity at a reaction temperature of 70 °C has recently been isolated (J Jordaan, 2003 pers comm).

1.3.2 Production of laccases

Several fungal strains and their diverse mutants have been investigated for their ability to produce laccases. However, production of the enzyme is dependent not only on the strain but also on the growth conditions employed. These factors include: the presence or absence of inducers, induction time, nature and composition of culture medium, type of culture conditions (static/shaking etc.), and genetic manipulation. Among these factors, the presence of an inducer, its chemical nature, the amount added, and the time of its addition influence laccase production to the greatest extent. Laccases may be constitutive or inducible enzymes and several compounds have been shown to induce and improve laccase formation. These include, mainly, phenolic compounds related to lignin or lignin derivatives. However non-ligninic compounds and extracts from different sources have also been found to be effective (Gianfreda *et al.*, 1999).

Addition of 2,5-xylydine has been found to stimulate laccase enzyme production in many basidiomycete fungi, including *T. versicolor* (Rogalski *et al.*, 1991), but no effect and even inhibition has been noted when this compound was added to other strains. Other aromatic compounds including substituted phenols, acids and anilenes have been tested as laccase inducers. *o*-Anisidine has been demonstrated to cause a 62-fold increase in laccase production in *T. versicolor* (Shuttleworth *et al.*, 1986b). Ferulic acid doubled the production of the enzyme in *T. versicolor*. Veratryl alcohol (3,4 dimethoxybenzyl alcohol) can be oxidised by laccase and seems to have important functions in the degradation of lignin, it exerts a positive effect on the production of the enzyme in several fungi; *T. versicolor* produced two to three times more laccase when supplemented with 1mM veratryl alcohol (Rogalski *et al.*, 1991)

Oxygen has been found to have an enhancing effect on many on lignin degradation by white rot fungi and many strains have to be periodically flushed with pure oxygen to ensure effective enzyme production. Laccase uses two molecules of oxygen as its primary substrate but Collins and Dobson (1997) report that purging *T. versicolor* cultures with 100% oxygen during growth had no effect on either *lcc* mRNA or laccase activity levels.

Vinasse, the wastewater produced in alcohol fermentation processes, showed good ability to enhance the production of extracellular laccase in *T. versicolor*. Other extracts from agricultural and industrial wastes e.g. cotton stalk extracts, barley and corn straw, potato waste and malt extract also increased laccase production *T. versicolor*. Surfactants have also been shown to increase laccase production (Gianfreda *et al.*, 1999). Induction effect does not always parallel inducer concentration. When xylidene was added to a *P. radiata* culture up to a concentration of 1mmol/L, laccase production was completely stopped, but it was very effective at concentrations 5 times lower (Rogalski *et al.*, 1991). In contrast, benzyl alcohol did not stimulate laccase production at a concentration of 20 µmol/L, but was very effective at a concentration 10 times higher. The effectiveness of the inducer is also dependent on the time of inducer addition.

1.3.3 Regulation of laccase production

Fungi possess numerous pathways for what can be described as 'dispensable' metabolic functions. This term is used to describe pathways that are not required for growth, or only required for growth under limiting conditions and are typically expressed to enhance survival in response to nutrient deprivation or competing organisms. Nutrient utilization pathways increase the metabolic versatility of filamentous fungi, enabling them to utilise a variety of complex compounds as alternative sources of nutrients. Catabolic pathways are essential for survival under limiting nutrient conditions. Fungi have developed complex regulatory systems to ensure that the required pathways are expressed appropriately in response to changing conditions and to limit depletion of cellular resources due to unnecessary expression (Keller and Hohn, 1997). This applies to laccases from *T. versicolor* in a number of situations that are related to the diverse physiological roles of the enzyme.

In white rot fungi, laccases are secreted as a number of isozymes encoded by different gene families. Laccase genes from a number of lignolytic fungi, including *T. versicolor*, have previously been cloned and characterised. It has been suggested that genes encoding various isozymes are differentially regulated, with some being constitutively expressed and others being inducible (Collins and Dobson, 1997). Collins and Dobson (1997) used reverse transcription PCR as a tool to determine the effects on *lcc* mRNA levels when the fungus was cultivated under a variety of physiological conditions. Copper regulates *lcc* transcription in *T. versicolor*. As the copper concentration in the

growth medium increased, increased levels of *lcc* mRNA transcript levels were observed, with concomitant increases in laccase activities. This is not surprising since laccase contains 4 copper atoms per molecule that are essential for its activity. In induced cultures, the presence of laccase protein correlated with an increase in laccase activity when copper was present in the growth medium; no activity was detected in cultures that did not contain copper. The likely explanation is that the enzyme remains inactive in these cultures because copper is not available for incorporation to produce functional laccase enzyme (Collins and Dobson, 1997).

LiP and MnP production in *P. chrysosporium* is stimulated by limiting nitrogen concentrations, and this has been shown to involve regulation at the level of gene transcription. In contrast, higher MnP and LiP titers have been observed in other white rot fungal species at high levels of nitrogen. Collins and Dobson (1997) demonstrated that nitrogen-induced laccase production in *T. versicolor* at the level of transcription. When nitrogen was provided to the fungus at increasing concentrations, corresponding increases in *lcc* mRNA and laccase activity were observed. Hence, it seems that nitrogen is an important factor in regulating expression of the three major lignolytic enzymes in the white rot fungi.

2,5-xylidine (XYL), ferulic acid and veratric acid have all been previously reported as laccase inducers. Collins and Dobson (1997) investigated their ability, along with 1-hydroxybenzotriazole (HBT), a laccase mediator, to induce *lcc* transcription. They found that only HBT and XYL induced transcription, and the mechanism by which these aromatic compounds activate *lcc* transcription is not understood. These compounds are toxic to fungi and a concentration in the growth medium above 2 mM, no fungal growth occurs. Thurston (1994) has suggested that one function of laccases is to detoxify reactive aromatic compounds by polymerising them. Therefore it is possible that *lcc* induction by XYL and HBT is indicative of a response developed by fungi to oxidize, and hence reduce the toxic effects, of such compounds. The induction of laccase production in various fungi appears to be specific for certain aromatic compounds; consequently, specific mechanisms for transcriptional activation are likely to be involved (Collins and Dobson, 1997).

1.3.4 Protein secretion in filamentous organisms

Fungal hyphae and their branches are compartmentalised by simple or complex porous septa that allow passage of nutrients, cell components and organelles. Thus, the cytoplasm within a fungal mycelium operates in a continuous system. Protein secretion in filamentous organisms is restricted to the growing hyphal tip. Even the secretion of lignolytic enzymes of *P. chrysosporium*, a process that is not growth-associated, was shown to occur only at the tips of new hyphal branches.

1.4 Substrates susceptible to degradation by *T. versicolor*

The pathways in *T. versicolor* for the oxidation of phenolic compounds and other environmental pollutants related to constituents of the lignin molecule, produce the corresponding quinones and subsequent polymerisation reactions. Subsequently, an intracellular heme-flavin adenine dinucleotide (FAD)-cofactored glycoprotein designated cellobiose dehydrogenase (CBH), donates electrons to an extremely wide range of substrates including semi-quinones, *ortho*- and *para*-quinones, azo dyes, kraft lignins, Fe(III), MnO₂ and Mn(III) complexes (Roy *et al.*, 1996). The quinone, hydroquinone and catechol reaction products can again serve as substrates for the oxidative enzymes. This ubiquitous red-ox cycle of phenolic compounds includes intra- and extra-cellular processes and is not yet fully understood. The rapid intracellular metabolism of the quinone and hydroquinone intermediates is a possible mechanism for shifting the dynamic polymerisation/depolymerisation equilibrium towards degradation and mineralisation (Schlosser *et al.*, 1997).

1.4.1 Phenols

Phenols are widely distributed in nature, where they occur naturally and as man-made aromatic compounds. Phenolic compounds enter the environment as intermediates during the biodegradation of natural polymers containing aromatic rings or during the biodegradation of xenobiotic compounds (Van Schie and Young, 1998). Phenol itself is not readily biodegradable and is toxic to most types of 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. Phenol 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 not found to be bioaccumulative in humans, individuals 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)

The biodegradability of aromatic compounds depends on the number, type and position of substituents on the aromatic ring. The relative order of biodegradability appears to correspond to *ortho*>*meta*>*para* positions of the substituents. 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)

1.4.2 Chlorophenols

Chlorophenols are components of the absorbable organic halides (AOX) present in the bleaching effluents from the pulp and paper industry. Aqueous effluents from industrial operations such as polymeric resin production, oil refining, and coking plants also contain chlorophenolic compounds. They are used as fungicides and may be formed from the hydrolysis of chlorinated phenoxyacetic acid herbicides. Pentachlorophenol (PCP) is the second most heavily used pesticide in the US, with approximately 1.25 million tons produced annually (Annachhatre and Gheewala, 1996). Soil in the vicinity of wood preserving facilities using PCP as a fungicide has been found to contain up to 1g PCP/kg (Annachhatre and Gheewala, 1996). The toxicity and bioaccumulative potential of chlorophenols increases with degree of chlorination and with lipophilicity. Bioaccumulation potential, quantified by the bioconcentration factor (BCF), is positively correlated to the octanol- water partition coefficient (K_{ow}). When PCP enters previously uncontaminated streams at concentrations of 4 ppb, fish-kill occurs. Phenol and most chlorophenolic compounds can induce mutations in certain bacteria and yeasts. Chlorophenols are considerably persistent in soils and are prone to leaching into the water table. Partial degradation of these compounds produces metabolites such as chlorocatechols or chlorosubstituted ring fission products that severely inhibit growth and development of haloaromatic utilising organisms (Annachhatre and Gheewala, 1996).

The oxidative enzymes secreted by white-rot fungi to extracellularly degrade lignin in wood have been linked to the initial degradative reactions of chlorinated aromatic compounds (Alleman *et al.*, 1995; Logan *et al.*, 1994). The use of microbial or enzyme-based treatment offers some distinct advantages over physical and chemical AOX

precipitation/absorption/filtration removal methods, in that only catalytic and not stoichiometric amounts of the reagents are required and the low organic concentrations and large volumes typical of bleachery effluents are therefore less of a problem. Also, both complete microbial systems and isolated peroxidase and laccase enzymes have been shown to reduce acute toxicity by polymerizing, and thereby rendering less soluble, many of the low-molecular weight non-chlorinated and polychlorinated phenolics (Roy-Arcand and Archibald, 1991).

Chadwick-Roper *et al.* (1995) investigated the effect of addition of co-substrates such as guaiacol and 2,6-dimethoxyphenol on the removal of chlorinated phenols by a laccase from *T. versicolor*. They found that addition of 50mM guaiacol enhanced the precipitation of 4-chlorophenol, 2,4-dichlorophenol and 2,4,5-trichlorophenol with laccase by 20, 32 and 80% respectively; while addition of 10mM 2,6-dimethoxyphenol enhanced the precipitation of 2,4,5-trichlorophenol by 98%. This co-precipitation may not require the presence of enzyme once the co-substrate is oxidised to reactive intermediates. These authors concluded that the observed phenomenon may be a useful strategy for improving the efficiency of enzymatic decontamination methods, particularly in the case of heterogeneous pollution.

Alleman *et al.*, (1995) demonstrated that toxicity is accurately quantified by the principle of chemical dose, the ratio of mass of chemical to the mass of mycelia, rather than by solution concentration. They showed that *Trametes versicolor* was the fastest growing of six commonly studied WRF able to remain viable at higher PCP doses, produced the most biomass after addition of PCP, and remained viable when grown on nutrient deficient and sufficient media.

1.4.3 Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are produced by chlorination of biphenyl, resulting in up to 209 different congeners. Commercial mixtures range from light oily fluids to waxes, and their physical properties make them useful as heat transfer fluids, solvent extenders, plasticisers, flame-retardants, organic diluents and dielectric fluids. The stability and hydrophobic nature of these compounds make them a persistent environmental hazard (Beaudette *et al.*, 1998). Mineralisation of PCB's was first reported by Eaton (1985), who investigated the degradation of Aroclor 1254, a recalcitrant mixture of biphenyls

chlorinated up to 54% (w/w) and containing 4-7 chlorine atoms per biphenyl. Chlorine substitution affects to some extent the oxidative attack by fungal enzyme systems, but not as much as bacterial dioxygenases, possibly due to the free radical mode of action (Novotny *et al.*, 1997). Extensive degradation of lesser-chlorinated PCBs was observed under both low-N and high-N conditions. The white-rot fungi showed the ability to degrade Aroclor 1254 mixture (PCB) after idiophasic metabolism was switched on by nitrogen limitation (Paszczynski & Crawford, 1995).

1.4.4 Polycyclic aromatic hydrocarbons (PAHs)

PAHs are compounds composed of fused benzene rings in linear, angular and cluster arrangements and are mainly formed as products of the incomplete combustion of fossil fuels (Collins *et al.*, 1996). Some of them are considered heterocyclic molecules due to the substitution in an aromatic ring carbon by nitrogen, oxygen or sulphur (Muncnerova & Augustin, 1994). PAHs are highly hazardous pollutants found widely distributed in terrestrial and aquatic environments (Anderson & Henrysson, 1996; Collins *et al.*, 1996; Muncnerova & Augustin, 1994). Paszczynski & Crawford, 1995). Some are acutely toxic, mutagenic or carcinogenic (Collins *et al.*, 1996).

Although anthracene and other members of the PAH group can easily be oxidised, they have nothing in common with typical substrate for laccase. The unexpected catalytic oxidation of anthracene *in vitro* was increased by the application of laccase with compounds forming readily available stable radicals (Johannes *et al.*, 1996). For the oxidation of anthracene to anthraquinone the presence of a mediator is essential, an ABTS mediator system achieved this with a 90 % transformation efficiency (Johannes *et al.*, 1996). These conditions were previously also shown to be necessary for the oxidation of veratryl alcohol to veratraldehyde through the laccase/ABTS system. It is believed that *in vivo* cultures of *T. versicolor* produce their own natural mediators, thereby broadening the laccase substrate range. The radicals of the mediator compound itself play no direct role in the abstraction of an electron or hydrogen atom from the anthracene and their action can only be detected by possible recombination with the anthracene intermediate radical, resulting in coupling products.

1.4.5 BTEX

BTEX (benzene, toluene, ethylbenzene and xylene), are a group of common organo-pollutants derived from gasoline and aviation fuels. Degradation of these compounds by *T. versicolor* occurs under non-lignolytic culture conditions in a nitrogen-rich medium (Alleman *et al.*, 1995; Paszczynski & Crawford, 1995). Degradation was recorded after only 5 days of incubation. Benzene was reduced by 18%, toluene by 41%, ethylbenzene by 99%, *o*-xylene by 49%, and *m*-xylene and *p*-xylene by 67% (Paszczynski & Crawford, 1995).

1.4.6 Dyes

Large amounts of structurally diverse dyestuffs are used for the textile, paper and leather industries. Dyes are classified on the basis of their chemical structure, with azo-dyes commercially the most important followed by phthalocyanines, used for the production of green and blue pigments (Hienfling *et al.*, 1997). Azo-dyes are characterised by the presence of a chromophoric azo group whose nitrogen atoms are linked to sp²-hybridised carbon atoms of the aromatic ring, which may also carry sulphonic acid groups. Sulpho- and azo- groups are not naturally occurring; thus, sulphonated azo dyes are recalcitrant to biodegradation. In the case of reactive dyes, up to 50% of the dye is lost through hydrolysis during the dying process and therefore appears in the wastewater. The recalcitrant synthetic dyestuffs are hardly removed from effluents by conventional wastewater biological treatments, such as activated sludge (Hienfling *et al.*, 1997; Paszczynski & Crawford, 1995). Textile-processing effluents also contain heavy metal ions, which may arise from materials used in the dying process or, in considerably higher amounts, from metal-containing dyes (Hienfling *et al.*, 1997).

In various studies, lignocellulolytic fungi were shown to attack not only natural and industrial lignin derivatives but also a broad range of organopollutants, including various dyes. Studies have shown the degradation of azo, anthraquinone, heterocyclic and polymeric dyes by *P. chrysosporium* (Heinfling *et al.*, 1997). Swamy and Ramsay (1999) found that *T. versicolor* displayed the greatest decolouration ability both in terms of extent and rapidity of decolourisation. In general, the overall complexity of structure alone was not an indicator of the difficulty of decolouration of a particular dye. In addition, only *T. versicolor* sustained high rates of decolouration of repeated, sequential

additions of a number of different dyes and dye mixtures. The eventual cessation of decolouration was most likely due to nutrient depletion (Swamy and Ramsay, 1999).

1.4.7 Nitro-Substituted Compounds

Most nitro-substituted compounds of interest, in terms of fungal bioremediation, belong to a larger group of explosive chemicals that also encompasses nitrate esters and nitro-amines, along with derivatives of chloric acid, perchloric acid, azides and other compounds (Paszczyński & Crawford, 1995). The toxicity and mutagenicity of nitro-aromatics and their recalcitrance to biodegradation underlie concerns about their environmental fate (Paszczyński & Crawford, 1995).

1.4.8 Xylenols

Xylenols (dimethylphenols) are major pollutants in waste-waters of industrial processes such as coal gasification and liquefaction, and more are generated by sewage disposal and petroleum exhausts. Because of their strong bacteriocidal action, unpleasant taste and odor, and toxicity to fish and other organisms, their presence is considered undesirable in water resources, even at concentrations of only 1ppb (Liu and Bollag, 1985). When Liu and Bollag (1985) incubated syringic acid, 2,6- xyleneol and laccase from *T. versicolor* together, the primary reaction appeared to be the copolymerisation of the two substrates; no oligomers of syringic acid were detected and only a small amount of a quinone dimer of 2,6-xyleneol was found. Mechanistic investigations suggested that UDP-xylose and UDP-xylosyltransferase were involved in the reaction (Kondo *et al.*, 1993). These authors showed that the xyloside formation activity of *T. versicolor* was activated by the exogenous addition of phenolic compounds in which guaiacol had the best stimulatory effect. In addition to enzyme systems, however, many abiotic factors, such as free radicals, metals and clays, contribute to the oxidative cross-coupling of xenobiotics and cross coupling of xenobiotics with humic constituents in nature.

1.4.9 Kraft pulp bleaching and delignification

The annual worldwide production of paper and board exceeds 300 million tons. As the pulp accounts for only about 40-45% of the original weight of the wood, effluent wastewaters carry an extremely heavy organic load (Annachatre and Gheewala, 1996). In response to environmental concerns and increasingly stringent emissions standards, the pulp and paper industry is seeking ways to decrease the levels of chlorinated lignin

residues in its effluents, through both production process changes and improved treatment technologies (Addleman & Archibald, 1993). Considerable interest has focused on the white-rot basidiomycetes, since they are capable of preferential degradation of native lignins and the complete degradation of wood.

The Kraft process, at present the most common commercial chemical delignification method, produces a dark pulp because of the colour of the residual modified lignin residues. These are normally bleached or removed by the use of chlorine, chlorine dioxide and caustic (NaOH) extractions (Addleman & Archibald, 1993). The ability of *T. versicolor* to bleach kraft pulps was first shown by its remarkable brightening effect on suspensions of hardwood kraft pulp. The brightness increase was accompanied by a decrease in kappa number (lignin content) of the pulp (Addleman *et al.*, 1995). Subsequently, *T. versicolor* was found to brighten and delignify softwood kraft pulps and also pulps produced by extended or oxygen delignification.

Residual lignin, the fraction remaining after the pulp has been subjected to the Kraft process, is still susceptible to biodegradation by *T. versicolor*; some of it is solubilised and a smaller fraction mineralised. One of the early events is the release of methanol from the methoxy groups on rings bearing a free phenolic hydroxyl group (Paice *et al.*, 1993). Biological bleaching of kraft pulp by *T. versicolor* is carried out by the organism's extracellular delignification system. The components of the system, enzymes and mediators, interact with the pulp structure at a molecular level. Unbleached kraft pulp is the most common substrate for biological or enzymatic bleaching experiments. The pulp pore sizes suggest easy penetration by small molecule mediators for lignolytic enzymes like ABTS or manganese oxalate. It seems likely that the oxidised ABTS must function as a diffusible electron carrier, because laccase is a large molecule ($M_w \sim 70\ 000\text{kD}$) and therefore cannot enter the secondary wall to contact the lignin substrate directly. It is postulated that ABTS can couple with phenolic groups in lignin to form hydrophilic lignin-ABTS complexes, can promote C_a-C_b cleavage of non-phenolic sites in lignin and can diffuse into regions inaccessible to enzymes. This would further increase the delignifying and depolymerising activity of the laccase-ABTS system (Addleman *et al.*, 1995)

It is widely reported that their unique enzyme suites enable white rot fungi to effectively degrade native lignin. These fungi also facilitate degradation of chlorolignins. However,

they are less suitable for treatment of other pulp mill lignins such as lignosulfonates, the EOP lignins and monomeric lignin derivatives (Bergbauer and Eggert, 1993). Since laccase is known to polymerise a wide variety of monomeric and polymeric lignin derivatives, the high laccase secretion by *T. versicolor*, together with easily polymerised substrates, may play a key role in preventing effective degradation of EOP lignins. The results obtained with chlorolignins may be due to the chlorination of the aromatic ring facilitating radical breakdown and helping to prevent the polymerisation reactions that would inhibit degradation. In addition, the ratio of laccase to peroxide activity of *T. versicolor* incubated with lignosulphonates or EOP lignins was 3 times higher than it was for a chlorine bleachery effluent (Bergbauer and Eggert, 1993).

Treatments of pulp with laccase-ABTS, followed by alkaline extraction, decreased the kappa number of softwood kraft pulp by 55%. Although this system is highly efficient, a commercial application does not seem feasible. Due to the scale of pulp bleaching, even if the ABTS was manufactured in bulk, the costs would be too high (Archibald *et al.*, 1997).

1.5 Bioreactors for the application of *Trametes* systems

The reduction of chemical residues such as phenols is an area of environmental concern. Conventional physico-chemical treatments, although effective, suffer from numerous drawbacks as discussed above. An appropriate biological treatment of contaminated waste streams with a robust biosystem that meets industrial requirements could potentially provide a less energy- and capital-intensive approach to achieving the necessary reduction of phenolic compounds. Although bioremediation is already an established technology, almost all currently employed treatments use prokaryotes and comparatively little attention has been given to the use of WRF in bioreactors (Pointing, 2001). Large scale, cost-effective application of these fungi to continuous treatment of liquid effluent has been hindered by the lack of suitable bioreactor systems.

A bioreactor is essentially a container in which a production process is carried out. The primary task of a bioreactor is mixing, the central objective of which is to provide a largely homogeneous chemical and physical environment in which all the cells are operating at their optimum with respect to the desired biochemical conversion. Mixing in the wider sense includes providing cells in aerobic culture with sufficient oxygen,

removing potentially harmful metabolites, and keeping the culture temperature within tolerable limits around its optimal value. Stirred tanks are the most common aerobic reactors used in commercial productive fermentations. Their design dates back to the early penicillin productions of the 1940's. Airlift reactors, in which all agitation is due to bubbling gas, are a relatively new invention. Other reactor designs routinely employed in fungal bioprocesses are: fixed or fluidized beds and immobilized biofilms, either in membrane systems, trickle beds or rotating biological contactors.

Over the past decade the white rot fungi have been extensively studied for their ability to degrade a broad range of xenobiotic organo-pollutants. While the bioremediation potential of these microorganisms is undoubted, nearly all the research undertaken in this regard has been done on an analytical level. Static or shake flask degradation experiments with 50-100mL volumes of culture fluid and target compound predominate. Furthermore, very little work has been done on authentic effluents as opposed to chemically pure model compounds.

1.6.1 Immobilised biofilm reactors

Solid-liquid mass transfer is known to control performance of many multiphase reactors. Mechanically agitated cultures are known to have an inhibitory effect on the production of lignolytic enzymes by white rot fungi due to the shear stress suffered by the mycelia (Nakamura *et al.*, 1999). Enzyme production is further reduced by production of proteases that degrade extracellular enzymes and oxygen limitations within pellets. In addition, immobilisation makes it possible to increase the mycelial-culture fluid interfacial contact area by preventing sedimentation and thus increasing transfer. Systems utilising immobilised biofilms enhance solid-liquid mass transfer without generating the intense shear fields that tend to damage the biofilms.

A culture of the WRF *Bjerkandera adusta* immobilized on polyurethane foam was more effective for the production of MnP and laccase than conventional static liquid culture (Nakamura *et al.*, 1999). Alleman *et al.* (1995) constructed a bioreactor that simulated the cyclical conditions of fixed film reactors trickling filters and rotating biological contactors. It was found that *T. versicolor* produced the greatest amount of biomass and PCP dehalogenation (10 g PCP/L) of the three WRF tested under these conditions. Biofilms of *T. versicolor* were also less susceptible to variations in wastewater feeds. The

use of *P. chrysosporium* immobilized on a polyethylene disc in a rotating biological contactor resulted in an efficient degradation of 4-chlorophenol. The reactor system allowed the fungus to be, alternatively, in contact with the substrate in the liquid solution and with oxygen, improving the 4-chlorophenol removal compared to agitated culture (Zouari *et al.*, 2002). A trickle bed reactor with biofilms of *P. chrysosporium* supported on ceramic carriers facilitated enzyme (LiP) extraction by ultrafiltration from the circulating growth medium. Furthermore, a relatively simple regime of pulsing the feed flow controlled the productivity per unit volume of bioreactor (Ruggeri and Sassi, 2003).

1.6.2 Fluidised bed reactors

Many bioprocesses make use of solid phase biocatalysts. The catalyst is usually suspended in a reactor–bubble column, stirred tank, fluidised bed or airlift device. Examples include suspensions of immobilised enzyme pellets and biofilms supported on suspended carriers. An effective bioreactor system directed toward the production of lignolytic enzymes should incorporate mild agitation conditions and good oxygen transfer. A fluidised bed system fulfils these requirements, but presents an additional problem in that the biomass is not suspended and is prone to the formation of preferential paths (channeling). This problem was overcome by the implementation of a pulsed flow regime that exerted periodic shear on the biomass and so removed branching hyphae (Moreira *et al.*, 1998). As a result, aggregation of agglomerates was avoided and important operational parameters such as aeration and nutrient availability were maximized. In this manner continuous MnP production from *P. chrysosporium* was achieved over a long period (40 days).

T. versicolor has also been grown in fluidised bed reactors for the production of lignolytic enzymes and the bioremediation of bleach plant effluents and pentachlorophenol (Pallerla and Chambers, 1995, 1998). A 600 mL-scale fluidised bed system containing polymer-entrapped *T. versicolor* in bead form was effective in the reduction of colour, chlorophenolics and absorbable organic halides from bleach plant effluents for an extended period (Pallerla and Chambers, 1995). *T. versicolor* was able to withstand higher concentrations in a fluidised bed reactors compared to static and agitated flask culture and a rotating biological contactor. The higher degradation levels obtained in the fluidised bed system were attributed to increased oxygen and PCP transfer, retention of higher mycelial loading and greater enzyme activities (Pallerla and Chambers, 1998).

1.6.3 Airlift loop reactors (ALR)

Airlift reactors consist of a liquid pool divided into two distinct zones, one of which is sparged by a gas. The different gas holdup in the gassed and ungassed zones results in different bulk densities of the fluid in these regions, which causes circulation in the reactor by a gas-lift action. The part of the reactor containing the gas-liquid upflow is the riser and the region containing the down-flowing fluid is called the downcomer (Fig 1.1). A widened section at the top of the airlift is known as the gas disengagement zone. In this region the fluid velocity slows (velocity = flow rate/area) and as a result, bubbles rise less rapidly and have less kinetic energy. Cell damage, which occurs when bubbles explode at the surface, is thus reduced. Bubbles disengage from the liquid and proceed to the surface instead of returning down the downcomer. Thus CO₂-rich bubbles are removed from the reactor. The widening increases turbulence in the disengagement region and it has been suggested that this region plays an important role in the overall equalization of the reactor contents

The major reason for the higher productivities of airlift bioreactors (compared to stirred tank reactors) is that the draft tube distributes shear forces throughout the reactor. As a result, cells are not exposed to large variations in shear forces and thus are able to grow in a more stable physical environment. In contrast, in stirred tank reactors, high shear conditions arise near the impeller causing cell damage or cell stress, lowering productivity. Draft tubes also prevent bubble coalescence by causing bubbles to move in one direction.

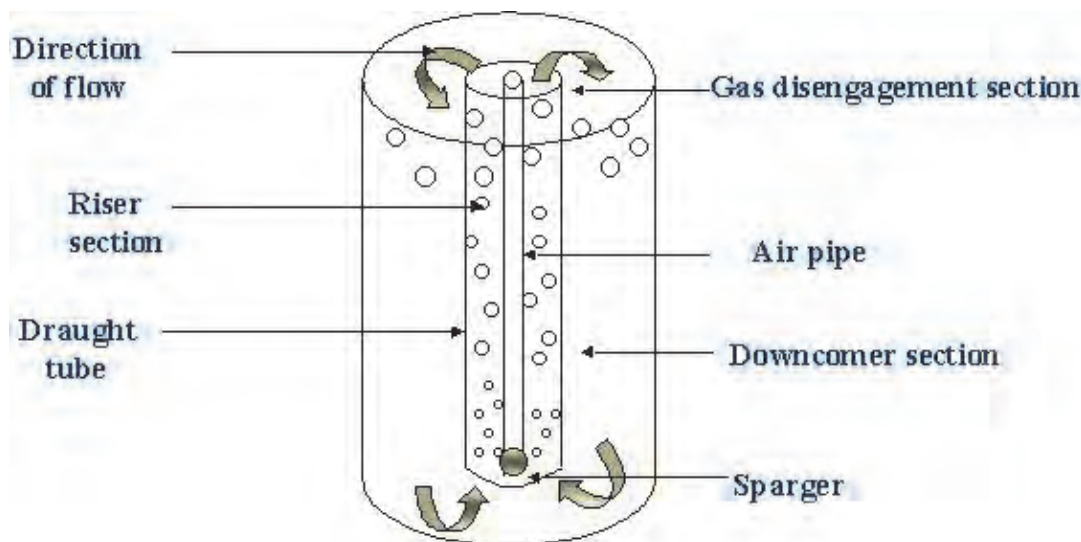


Fig 1.1 Schematic diagram of concentric draught-tube internal loop ALR

Gas-mixed reactors do not have impellers to decrease bubble diameter and increase the volumetric oxygen mass transfer coefficient ($K_L a$). To compensate for this, gas mixed reactors are designed with a larger height to diameter ratio so as to improve oxygen transfer efficiency by:

- (i) Increased hydrostatic pressure at the base of the reactor, which increases the saturation concentration of oxygen (C_o^*).
- (ii) Increasing the time the bubbles spend in the reactor and thus increases the bubble residence time and gas hold-up. This increases the air volume in the reactor available for oxygen transfer and increases the time available for each bubble to transfer oxygen into the medium.

Limits to reactor height develop because as the bubbles rise, they transfer out oxygen and receive carbon dioxide from the medium. If the reactor is too tall, bubbles at the top will be rich in carbon dioxide and poor in oxygen. The cells at the top of the reactor will therefore be starved of oxygen. Mixing time also increases with the reactor height. Thus a reactor that is too tall will suffer from mass transfer problems with cells in some areas being starved of oxygen for relatively long periods of time. A large mixing time makes process control difficult. For example, the pH may vary throughout the reactor, making it

difficult to determine when to add alkali and how much alkali to add. Construction costs also increase as the reactor height increases.

With solid phase biocatalysts, a large quantity of the catalyst must usually be maintained in the suspension so that a high volumetric productivity is achieved (Figure 1.2). The fully suspended state must be attained at lower power inputs and low levels of turbulence. The airlift system is better in this regard than its competitors because most of the energy input into them goes to creating a highly directional flow of liquid, which helps to suspend solids. Solid suspension is further improved by increasing the aspect ratio (H/D), which in turn increases the induced circulation rate. This also results in significant improvements in top to bottom fluid circulation that is often used to characterise mixing time. Compared to other low shear systems, such as bubble columns, airlift loop reactors (ALR) have significantly better solid-liquid mass transfer (Siegel *et al.*, 1986; Schugerl and Lubbert, 1994). High-density cultures typically increase productivity by orders of magnitude relative to free suspension culture (Chisti and Moo-Young, 1996). The negative aspects of high-density cultures include: a decrease in the specific productivity of the biocatalyst due to transport limitations, and problems of long term stability and the logistics of sterile manipulation.

Figure 1.2 Photograph of dense biomass in an airlift bioreactor



Bonnarme *et al.* (1993) cultivated *P. chrysosporium* in both mechanically agitated stirred tank reactor (working volume 1.7 L) and pneumatic (airlift) bioreactor (working volume 2.2 L). In the airlift loop reactor, the yields of lignin and manganese peroxidases as well

as extracellular protein were greatly increased as compared with mechanically agitated bioreactors. The authors noted two distinct growth phases for the pneumatic reactors. During phase I, more biomass was formed in the airlift reactor than in the stirred tank (STR). During phase II, biomass decreased in the airlift reactor while steadily increasing in the STR. Extracellular protein production was increased 3.8 times in the non-mechanically agitated reactor while lignolytic enzyme production was 6 to 9 times higher. Thus it appeared that the accumulation of biomass in the STR during phase II was at the expense of enzyme production. Protease activity was higher in the STR; this, in combination with the mechanical agitation, which is known to affect the secretion and stability of many enzymes, might partially explain the lower peroxidase titers obtained. The yield coefficients of the peroxidase enzymes were significantly higher in the airlift reactor than the STR demonstrating that in pneumatic reactors, the carbon and nitrogen consumption was orientated toward peroxidase synthesis at the expense of biomass.

In the design of reactors to deal with biological wastewaters, it is generally accepted that airlift loop reactors are the most economical with regard to the several important performance criteria (Siegel *et al.*, 1986; Schugerl and Lubbert, 1994):

- i) energetic oxygen transfer – almost twice as efficient as its nearest competitor (low depth clarification basin with surface aerators)
- ii) oxygen transfer rate
- iii) heat transfer – due to the strong circulatory motion the average flow velocities near the walls (cooling jackets) are higher.
- iv) application to large volume systems
- v) reduced power consumption with comparable/improved productivity relative to STR's (Schugerl and Lubbert, 1994)
- vi) decreased shear stress
- vii) extended aseptic operation (due to the elimination of stirrer shafts, seals and bearings) (Chisti and Moo-Young, 1987).

1.7.1 Project objectives

The broad aim of this project was to develop a reactor-based bioprocess utilising the white rot fungus *Trametes versicolor* for the remediation of phenol-contaminated wastewaters. To this end it was necessary to first investigate the physiology of the organism in terms of laccase production and its response to the presence of phenols in

its environment. *T. versicolor* had been reported as an excellent producer of laccase but the successful application of this organism in a bioremediation process required the production of high amounts of this enzyme. The degree of success achieved in the laboratory in the mineralisation/ removal from solution of many major pollutants had so far only been realised in flask culture or in rather laboratory-specific reactor applications. A more practical (in terms of future scale-up), cost effective reactor system that provided a well-oxygenated, yet low shear environment was required.

1.7.2 Specific objectives

To fulfill the aims outlined above, the following objectives were identified.

1. Characterisation of the biological conversion by *Trametes versicolor*, of the phenolics, chlorinated phenolics and polyaromatics present in these effluents. This would include characterisation of the roles and activity levels of the enzyme activities involved, and the fate of the organic compounds converted.
2. An optimised fermentation or fed-batch process in which the *Trametes versicolor* successfully degrades these pollutants.
3. A laboratory scale bioreactor involving a pneumatic fermenter linked to a membrane module, with *Trametes versicolor* growing and producing enzymes continuously at steady state with continuous operation of this system for a prolonged time to demonstrate its feasibility.
4. Development of models of the biological reactions in terms of cell growth, enzyme production, biodegradation rates and the operation of the fermenter / membrane module bioreactor for bioremediation
5. Establishment and demonstration of the process in a demonstration plant by scale-up of the laboratory scale bioreactor system, on the basis of models. (However, it was decided at the first Steering Committee meeting of the project that scale-up was outside the scope of the project.)
6. Transfer of technology to relevant industries and reporting on these results.

CHAPTER 2 : DEVELOPMENT OF CULTURE METHODOLOGIES FOR *TRAMETES* SPECIES (Part 1): MEDIUM DEVELOPMENT

2.1 Introduction

The first steps in generating an efficient bioremediation process are the identification of a suitable microorganism and the optimisation of the culture conditions necessary for its rapid growth and the production of the relevant enzymes. Often, aspects such as growth medium development are overlooked in favour of over-engineered, conventional or historically established formulations. The development of a simple, cost effective medium for the growth of *T. versicolor* and its production of laccase is discussed here. The genus *Trametes* is chiefly recognised as the most efficient natural producer of laccase (Jang *et al.*, 2002). Typically for the occurrence of this lignin-modifying enzyme in *T. versicolor*, there seems to be large variability between strains, which may reflect the inherent heterogeneity of this species. Neither lignin nor any pollutants degraded by these enzymes have been shown to be utilised as growth substrates, and a separate carbon source is required for metabolism (Reddy, 1995).

Culture conditions are important in the production of lignolytic enzymes in this and other species of WRF. For example, the discovery of LiP in *P. chrysosporium* in 1983 was the beginning of the extensive study of WRF and their ability to degrade xenobiotic organo-pollutants that has continued ever since. *P. chrysosporium* was regarded as a model organism in this field of research and because it only expresses lignolytic enzymes during secondary metabolism following growth, when carbon and/or nitrogen becomes limiting, the same was assumed for all other WRF. More recent work has found that this is not the case for *Trametes* species. Swamy and Ramsay (1999), in work on dye decolourisation, reported that the cessation of the process corresponded to glucose (carbon) depletion, but that replenishing the glucose restored the decolourisation. They did, however, highlight the requirement for nitrogen limitation. Jang *et al.* (2002) reported that N-rich cultures produced higher levels of laccase activity in batch experiments. Galhaup *et al.* (2002) showed that laccase production in *T. pubescens* was stimulated by high concentrations of easily utilisable carbon and nitrogen. Nutrient requirements appear therefore to be dependent on both strain and function/environment of the fungus.

Three media have been used predominantly in recent work involving growth of *T. versicolor*: those of Kirk (Tien, 1987) Yoshitake (Kondo *et al.*, 1994), and the Trametes Defined Medium (TDM) (Addleman and Archibald, 1993). The Yoshitake medium is both nitrogen (peptone 10 g/L) and glucose (30 g/l) rich. The Kirk medium is both nitrogen and carbon sufficient and TDM has both in only limiting quantities (6g/L glucose, 2.19g/L glutamine). These media were tested in terms of growth and enzyme production for the strain of *T. versicolor* used in this study and were the starting point for the development of an optimised medium.

2.2 Methods

2.2.1 Strain preservation

A slant culture of *T. versicolor* (PPRI #3845) was obtained from the Plant Protection Research Institute (South Africa). A culture of *T. pubescens* (CBS 696.94) was obtained from the Boku Institute in Austria. Both were maintained on 2% malt extract agar slants and subcultured every 60 days.

2.2.2 valuation of growth media

Detailed recipes for all growth media appear in Appendix A. The glucose and thiamine solutions for all three media were filter sterilised and added after autoclaving to prevent caramelisation and denaturing respectively. The salt solution for the *Trametes* Defined Medium was also added subsequent to autoclaving, to prevent possible precipitation.

2.2.3 Medium development

Trametes Defined Medium (TDM) was used initially as the medium for the production of biomass and enzyme, with the glutamine replaced by peptone. The medium was further simplified by the omission of the dimethyl succinate and sodium and calcium chloride. The glucose (carbon source) and peptone (nitrogen source) concentrations were varied between 20 g/L (high) and 2 g/L (low), and the 'nutrient sufficient' medium contained 10 g/L of each.

2.2.4 Inoculation

Pre-inoculum flasks were prepared by dicing two *T. versicolor* plate cultures into 5 mm × 5 mm squares and adding them aseptically to 400 mL medium in a 2L 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 d incubation, the contents of these flasks were homogenised in a sterile Sorvall bench top homogeniser. This was used as the inoculum for the production flasks.

To ensure that all the inocula were of equal biomass concentrations, a sample was spectrophotometrically scanned and an optical density (OD) peak was found at 600nm; a comparison of the three was made at this wavelength. Dilutions were made as necessary before the inocula were used.

2.2.5 Biomass determination

All flask experiments were conducted in 250mL Ehrlenmeyer flasks containing 45mL autoclaved growth medium, inoculated with 5mL of the pre-inoculum mixture. All the flasks were then incubated at 28°C in the dark. After growth, contents of the flasks were filtered through pre-dried and weighed Whatman #1 filter paper using a vacuum pump. The filter papers containing biomass were dried for 24h at 50°C and then re-weighed to give the dry mass.

2.2.6 Comparison of growth media

Media were compared in terms of biomass yield ($Y_{x/s}$), product yield ($Y_{p/s}$), specific yield ($Y_{p/x}$) and specific growth rate (μ). No appropriate term was found in literature that correlated the productivity of a micororganism with growth and to this end we defined the lump parameter 'growth related productivity (GRP) where:

$$\text{GRP} = (Y_{P/X})(\mu) \quad \boxed{\frac{\text{units of laccase (U)}}{\text{biomass (g)}} \cdot \frac{1}{\text{time (d)}}}$$

with units: U/g/d

and X = biomass
 P = product (laccase)
 S = substrate (glucose)

2.2.7 Determination of laccase enzyme activity

Laccase activities were determined by monitoring the oxidation of 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as the substrate (Wolfenden and Willson, 1982). 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). Oxidation of ABTS was measured by determining the increase in absorbance of the mixture 420 nm ($\epsilon = 36\,000\text{ M}^{-1}$). One unit (U) of enzyme activity was defined as the amount of enzyme required to oxidise 1 μmol of ABTS per min. During the testing of this assay for use in this study, 3 samples were run 10 times each to determine the error associated with this technique. A variance of less than 5 % was found and in light of the high degree of confidence associated with this assay, all subsequent samples were done in duplicate.

2.3 Results

2.3.1 Comparison of media

In initial efforts to grow *T. versicolor* in flask culture for laccase production, difficulties were encountered in culturing *T. versicolor* in the Kirk medium (Kirk and Tien, 1988), the recommended standard for culture of WRF. A survey of literature resulted in three alternative media being selected, namely those of: Kirk, Yoshitake (Kondo *et al.*, 1994) and the TDM developed by Addleman and Archibald (1993). The samples grown in TDM were the first to form a mycelial mat and reached an average total mass of 1.94 g/L after 11 days growth (Fig. 2.1). This medium was used in all subsequent experiments. Initial growth, in terms of biomass accumulation, was faster in the other two media tested but they did not progress to forming the mycelial mat at the fluid/air interface that produces high biomass yields. These mat structures are a result of the morphological nature of filamentous fungi; they are highly prone to branching and hyphal extension. In the case of glucose limitation, as would have occurred by day 9 (Fig 2.2), the formation of polysaccharides around the mycelia is enhanced, which favours the linking of pellets into conglomerates (Moreira *et al.*, 1998). Static flasks were preferred because shake flask techniques cannot be directly applied to WRF due to their sensitivity to mechanical shear and preference for high ratio of surface area to liquid volume (Alleman *et al.*, 1995).

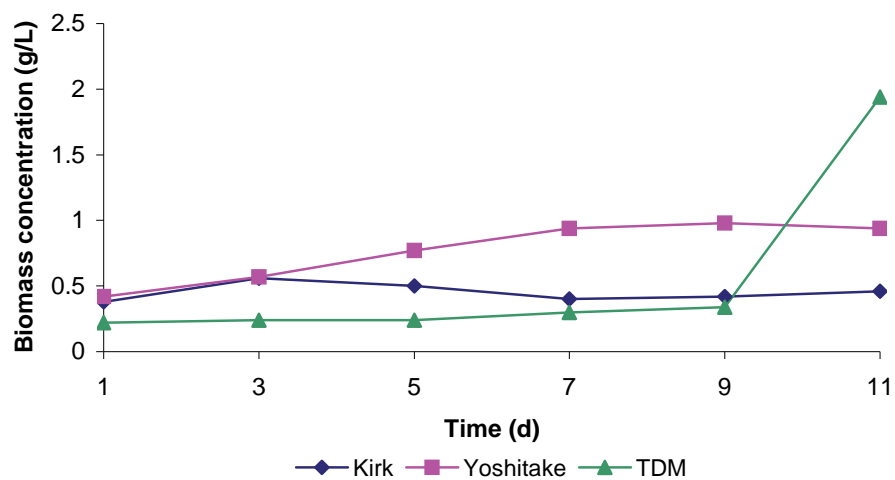


Fig 2.1 Growth of *T. versicolor*, measured as dry mass, in three different growth media

Enzyme production followed a similar trend to biomass production, with the cultures grown on TDM producing significantly more than the other two and reaching a maximum laccase yield of 1.2 U/mL (Fig. 2.2).

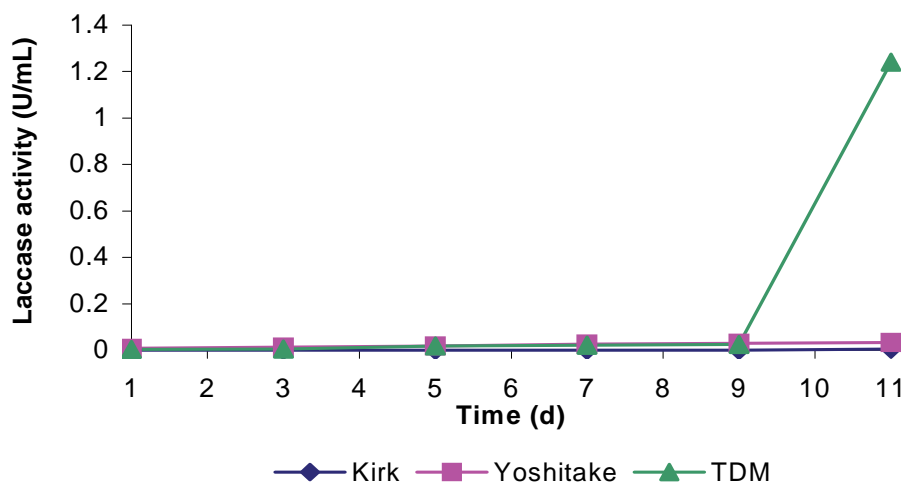


Fig 2.2 Production of the laccase enzyme by *T. versicolor* in three different growth media

To verify the above results, the experiment was repeated with TDM, maintaining growth and activity over a long period. In comparison the growth and enzyme production profiles follow very similar trends. The growth reached a maximum of 1.82 g/L and enzyme activity was found to be 1.38 U/mL (Fig 2.3). The large increase in enzyme activity was observed to occur at the same time as the formation of the continuous pellicle, or mycelial mat, which could be evidence of a relationship between growth phase and enzyme production.

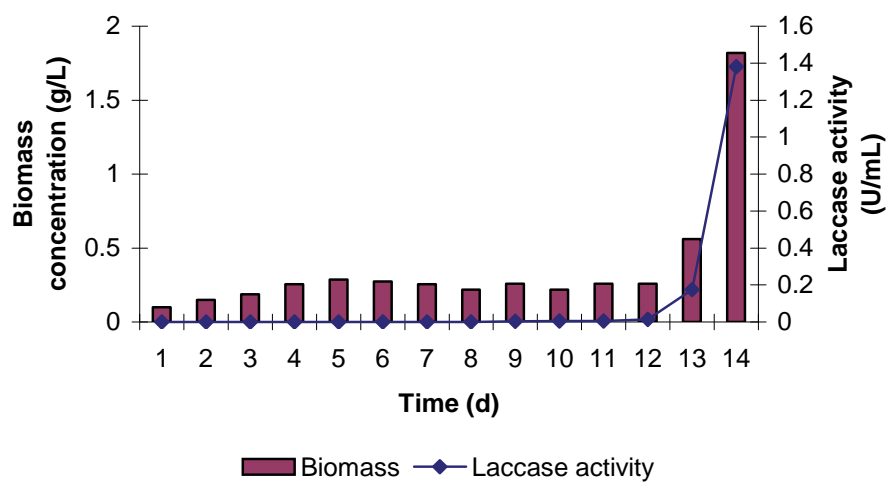


Fig 2.3 Growth and enzyme production of *T. versicolor* grown on TDM

2.3.2 Further development of TDM

On the basis of these preliminary results, TDM was used as the starting point for investigating the development of a growth medium suitable for the local strain of *T. versicolor*. The first parameters to be established were the carbon (glucose) and nitrogen (peptone) requirements of the fungal strain used here. These were investigated by varying the growth medium concentrations between 20 g/L and 2 g/L the two extreme values encountered in the literature. An intermediate third option, 10 g/L, was termed 'sufficient'. As would be expected, the cultures with the highest concentration of readily available glucose grew fastest and achieved the highest biomass accumulation. The medium containing 20 g/L of glucose produced 11.6 g/L of dry mycelial mass after 7 d (Fig 2.4). However, in terms of enzyme activity and biomass accumulation, the nitrogen- and glucose-sufficient cultures were the most productive (Table 2.1). This finding is in agreement with those of Kaal *et al.* (1995) who demonstrated that among five common

white-rot fungi that they tested (*T. versicolor* not included), higher biomass and lignolytic enzyme activities were observed in response to N (organic)-sufficient conditions. The only exception to this trend was LiP production in *P. chrysosporium*. This is notable because *P. chrysosporium* is the most commonly studied WRF and many researchers have assumed the nutrient limitation model true for all WRF; and is contrasted by the work on *T. versicolor* undertaken in this study.

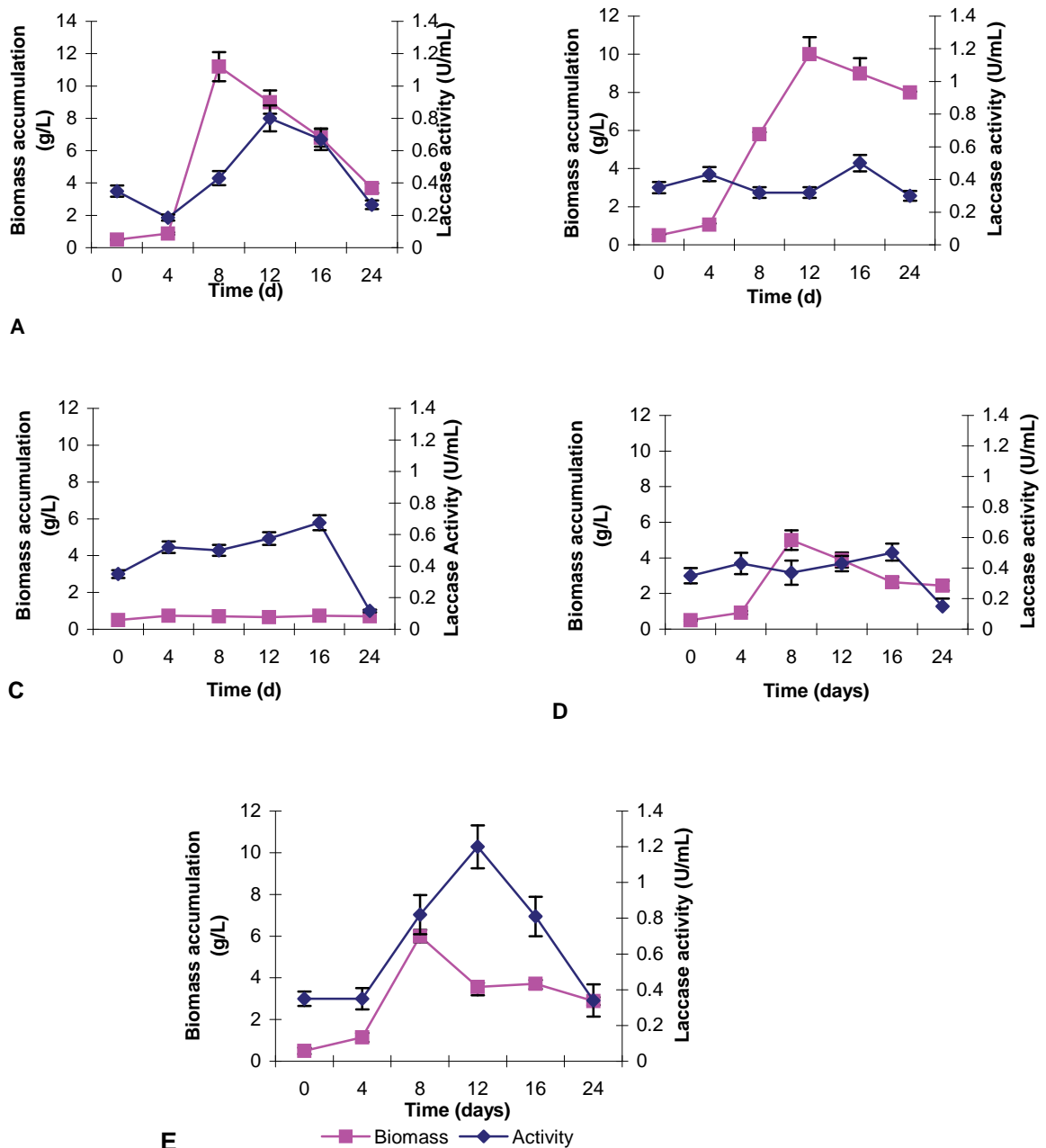


Fig 2.4 The increase in biomass and laccase activity over time for cultures of *T. versicolor* grown in media with different glucose (C) and peptone (N) concentrations: 20 g/L C: 20 g/L N (A), 20 g/L C: 2 g/L N (B), 2 g/L C: 2 g/L N (C), 2 g/L C: 20 g/L N (D) and 10 g/L C: 10 g/L N (E)

Alleman (1991) showed that fungal biomass production in flask cultures is slower in nitrogen-deficient cultures and that their long-term viability is reduced compared to nitrogen-sufficient ones. Collins and Dobson (1995) also investigated a *T. versicolor* strain, which reached maximal LiP activity when cultured under carbon-rich conditions (10 g/l glucose), and a similar increase in MnP was recorded. This increase in enzyme activity at elevated carbon levels appeared to be mediated at

transcriptional level, given that both *lip* and *mnp* mRNA transcript levels mirrored the observed enzyme activities. More recently Collins and Dobson (1997) demonstrated that nitrogen-induced laccase production in *T. versicolor* was induced at the level of transcription. When nitrogen was provided to the fungus at increasing concentrations, a corresponding increases in *lcc* mRNA was observed. Hence, it seems that nitrogen was an important factor in regulating the expression of the three major lignolytic enzymes in white rot fungi. In the flask culture investigation, the glucose concentration in all the flasks was reduced to almost zero by day 12 (Fig. 2.5). This coincided with the maximal enzyme production for media A and E, and at least 80% of maximum for the other media. The culture in medium C displayed the highest enzyme productivity in terms of initial nitrogen and carbon provided (product yield = 0.35 U/g), but because of the limited availability of these nutrients, this medium did not produce a large amount of biomass, and hence, a lower total amount of enzyme was produced (Fig 2.4). In any large-scale application, production of high yields of enzyme and biomass, for less operating expenditure, would obviously be beneficial.

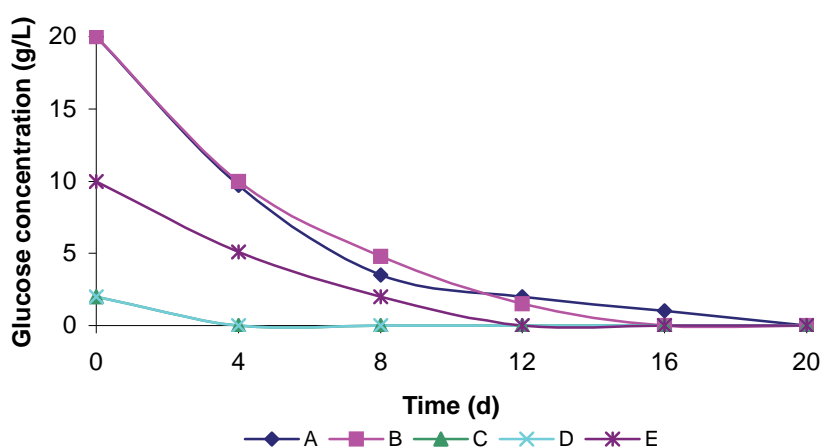


Fig 2.5 The increase in biomass and laccase activity over time for cultures of *T. versicolor* grown in media with different glucose (C) and peptone (N) concentrations: 20 g/L C: 20 g/L N (A), 20 g/L C: 2 g/L N (B), 2 g/L C: 2 g/L N (C), 2 g/L C: 20 g/L N (D) and 10 g/L C: 10 g/L N (E). Biomass yield $Y_{x/s}$ (grams of biomass produced per gram of glucose) but not overall biomass accumulation, was highest in medium D (0.125 g/g). This value may be slightly inflated as the complex nitrogen source (peptone) provided supplementation to the low initial concentration of glucose. This may not have been the case if an inorganic nitrogen source such as ammonium had been used. Product yield $Y_{p/s}$ (units of laccase produced per gram of glucose) was highest for medium C (0.35 U/g), and the overall amount of enzyme produced was far higher in E (1.2 U/mL) compared to 0.67 U/mL for C (Fig 2.4).

Specific yield $Y_{p/x}$ (units of laccase produced per gram of biomass) was highest in media C (6.96 U/g) and E (4.78 U/g). Obviously, because there was so little growth in C (0.74 g/L) the small amount of enzyme that was produced resulted in a high specific yield, but this is of limited value in process terms. Medium E, on the other hand, produced both significant growth (3.7 g/L) and a large amount of enzyme. As expected, the specific growth rate (μ) was highest in the cultures with highest initial carbon concentrations: A, B, and E. Considering the results from the growth indicators monitored (Table 2.1), nutrient regime E (10 g/L glucose and peptone) was found to be the best of the five.

Table 2.1 Table showing the process parameters for the different growth media: (20g/L C: 20g/L N (A), 20g/L C: 2g/L N (B), 2g/L C: 2g/L N (C), 2g/L C: 20g/L N (D) and 10g/L C: 10g/L N (E). The GRP term is the product of specific yield and μ .

PROCESS PARAMETER	GROWTH MEDIUM				
	A	B	C	D	E
Biomass Yield ($Y_{x/s}$) (g/g)	0.032	0.036	0.005	0.125	0.023
Product Yield ($Y_{p/s}$) (U/g)	0.034	0.029	0.350	0.215	0.150
Specific Yield ($Y_{p/x}$) (U/g)	1.000	0.020	6.960	0.412	4.780
μ (per day)	0.388	0.305	0.041	0.286	0.305
GRP (U/g/day)	0.388	0.006	0.285	0.117	1.458

The Growth Related Productivity (the product of specific yields and μ) was 3.75 times higher in medium E than the next best medium formulation, A. The term GRP was defined because it was necessary to relate laccase production with growth. Specific yield does not give an idea of growth rate or total biomass accumulation and it is then difficult to predict total enzyme produced in a fermentation. GRP provides an indication of overall or total enzyme yield, for example: medium C had a very high specific yield (6.96 U/g) but this is misleading because there was limited fungal growth and low total laccase production. On the basis of its enzyme production and cost benefits medium E was used in all further experiments.

2.3.3 Growth and enzyme activity of *T. pubescens*

An alternative strain of *Trametes*, *Trametes pubescens* was obtained from the Boku Institute in Austria, where it had been recently been identified as an excellent producer of laccase (Galhaup *et al.*, 2002). This characterisation work had utilised an extremely rich and complex growth medium containing up to 40 g/L glucose. In the present study, *T. pubescens* was grown up in flasks to test the efficiency of the medium E developed previously, for growth and enzyme production *T. versicolor*,

compared with the medium recommended by the suppliers of the *T. pubescens* culture.

The *T. pubescens* cultures grown in the recommended, glucose rich, medium produced more than double (7.8 g/L) the biomass of the optimised medium E (3.6 g/L) after 7 days growth (Fig 2.5). However, laccase production however was almost six times higher in medium E (6.35 U/mL) than in the recommended medium (1.1 U/mL) and the specific productivity was 12.6 times greater in medium E (Fig. 2.6). Thus, for a bioprocess requiring large amounts of biomass to be produced, a carbon enriched growth medium would be preferred. However, in the case of this project however, the goal of increased production of oxidative enzymes would be best served by a carbon and nitrogen sufficient medium. There is also obvious potential for a two stage process where rapid initial growth could be supported by high concentrations of readily available carbon and thereafter an 'enzyme production medium' of lower nutrient concentration would be utilised. In this manner it would be possible to ensure both high biomass and high enzyme production.

It was further suggested (Galhaup C, pers comm.) that a 5mM concentration of copper (added as CuSO_4) be used as an inducer of laccase activity and this was also tested. The addition of copper resulted in a positive effect on laccase production, increasing it in both media. The effect was greatest in the glucose rich medium where activity was increased more than nine fold up to a maximum of 10.2 U/mL, but was also evident in the optimised medium with an maximum titre of 10.5 U/mL being achieved (Fig. 2.6). Considering that most laccases contain 4 copper ions that are essential for activity, this finding is not surprising. The regulation of *lcc* expression by copper is analogous to the effect of manganese on MnP gene regulation (Brown *et al.*, 1991).

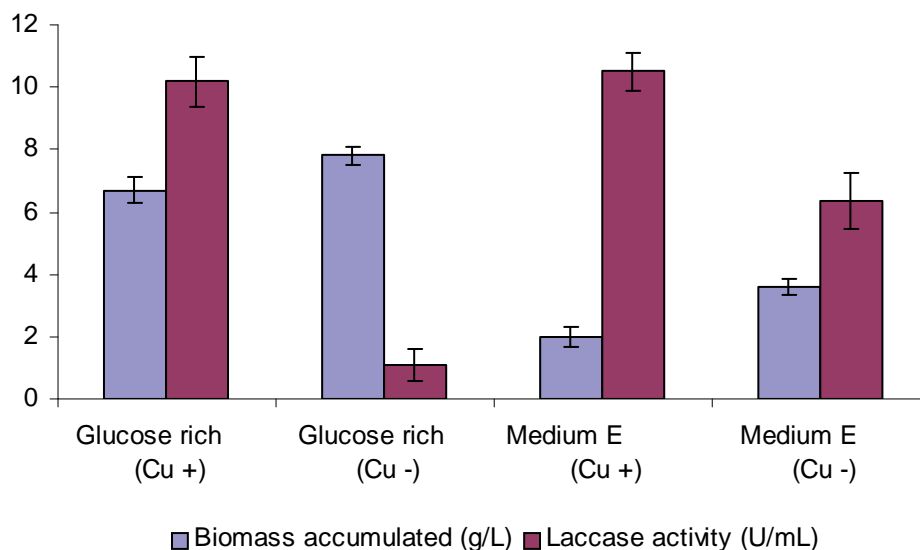


Fig 2.6 Biomass accumulation (dry mass) and laccase production in cultures of *T. pubescens* grown in a complex, glucose rich medium and medium E. (Cu +) indicates the addition and (Cu -) the omission of 5mM copper

Although copper was certainly found to be an effective inducer of laccase activity, it is an anti-microbial agent, and as such, presents a potential environmental hazard, for example, should a crude enzyme slurry be required for an *in situ* soil bioremediation process. This induction method would therefore not be applicable in a bioremediation process but could still be effective in a laccase production application where the copper could be removed by ion exchange, dialysis or some other established purification method.

2.3.4 Effect of inducers on growth and enzyme production

Laccase expression has been a topic of some research interest and laccases from a number of lignolytic fungi, including *T. versicolor*, have previously been cloned and characterised (Jonsson *et al.*, 1995). It has been suggested that genes encoding various isozymes are differentially expressed, with some being constitutive and others being inducible (Bollag and Leonowicz, 1984). In an experiment to test the efficacy of four known inducers, it was found that the presence of all the compounds did, to some extent, increase laccase enzyme activity in this strain of *T. versicolor* (Fig 2.7). Guaiacol, a lignin monomer, was the most effective, resulting in an increase of 780% in enzyme activity. This is not entirely surprising, as it most closely resembles the natural substrates of the lignolytic enzyme system and would have been the most easily recognisable to the fungus. The decrease in activity after day 3

is attributed to the removal/polymerisation of the inducer compounds and the subsequent discontinuation their affect. This could be overcome in a process situation by monitoring the laccase activity and adding more of the inducer compound as required. This was achieved in an airlift reactor study (Chapters 5 and 6).

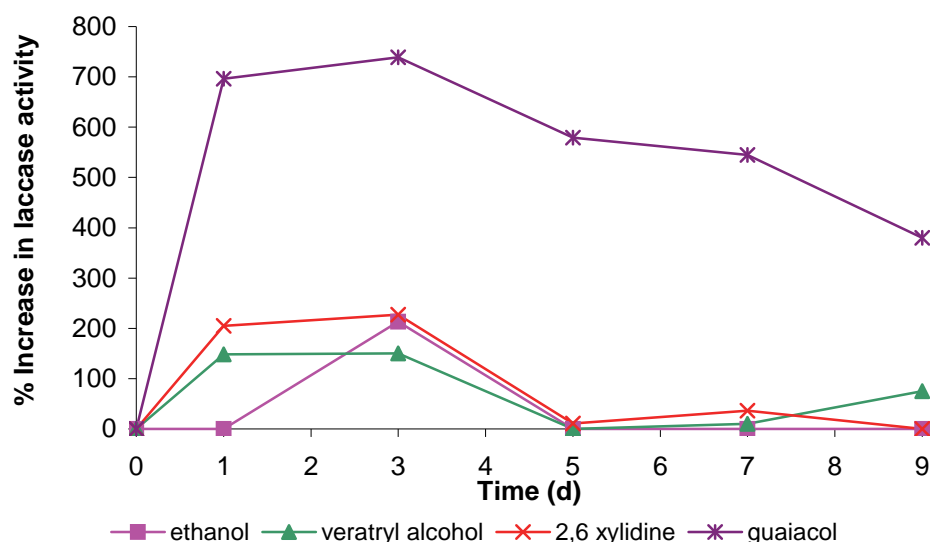


Fig 2.7 Effect of different inducers on laccase production in flask cultures of *T. versicolor*

Thurston (1994) suggested that one function of laccases is to detoxify highly reactive aromatic compounds by polymerising them. This is further verified by the fact that the increase in laccase production coincides in many instances with the formation of dark precipitates that may represent laccase-polymerised forms of the inducer. The induction effect appears to be specific for certain compounds and involve xenobiotic response elements (XRE's). This receptor or binding protein is a member of a family of regulatory proteins that activate gene transcription in the presence of nonpolar carbon compounds. The presence of putative XRE sequence 180 bp upstream from the TATA box in the lignolytic fungus PM1, a close relative of *T. versicolor*, and the fact that its laccases are also induced by xyldine (Coll, 1993) suggests that the *lcc* genes of *T. versicolor* are also activated by certain aromatic compounds. The results obtained in this study agree with the available knowledge on the induction effect of aromatic inducers on *T. versicolor*.

Aromatic inducer compounds are potentially toxic to fungi and, in the case of 2,6-xyldine, retard growth at high concentrations (> 2 mM). However, because these inducers were used at low concentrations (1 mM), they had no obvious toxic effect on

the cultures, as indicated by the similarity of the growth curves of the induced cultures to that of the control (Fig 2.8).

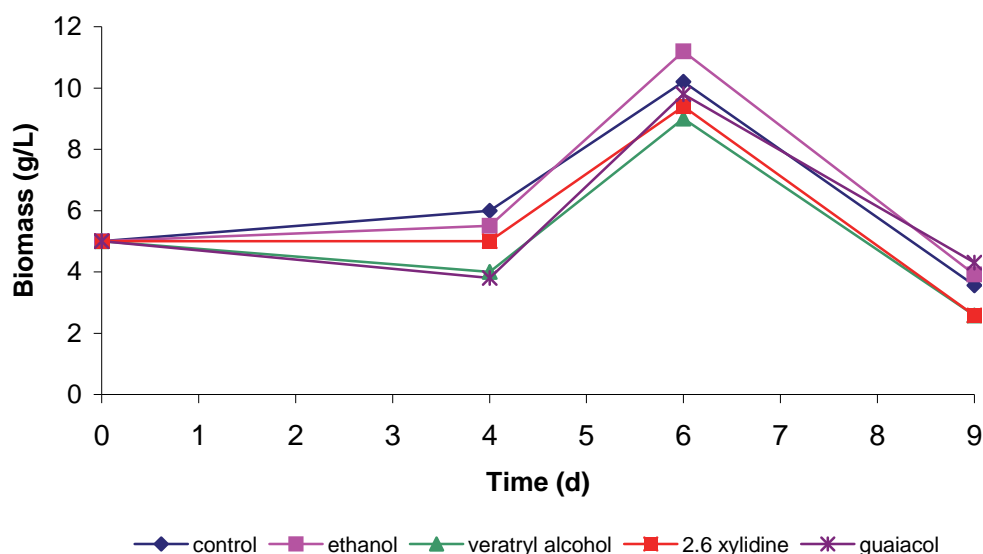


Fig 2.8 The effect of lignolytic enzyme inducers on biomass accumulation in *T. versicolor*

Clearly, using inducers would maximise enzyme production in *T. versicolor*, but in any industrial application, the cost could be prohibitive. An obvious solution would be to identify an inexpensive, readily available source of inducer. Therefore, an experiment was conducted where the induction by two industrial effluents was investigated. Guaiacol is a lignin monomer and it was reasonable to assume that an E stage kraft effluent from a pulp and paper mill would contain similar compounds. Similarly the stripped gas liquor or cresylic effluent stream from a Fischer-Tropsch (Appendix C) is known to contain phenol, cresol and other potentially useful aromatic monomers (Table 2.2). The effluents were filter sterilised and added to the flasks after 7 days growth to final concentrations of 1 and 2%. The presence of both concentrations of both effluents resulted in increased laccase production (Fig. 2.9), with 1% pulp-and-paper effluent having the greatest effect, increasing the measured laccase activity by 670%. The cresylic effluent gave a more sustained induction effect over 8 days (Fig. 2.9) and on this basis was selected for further research.

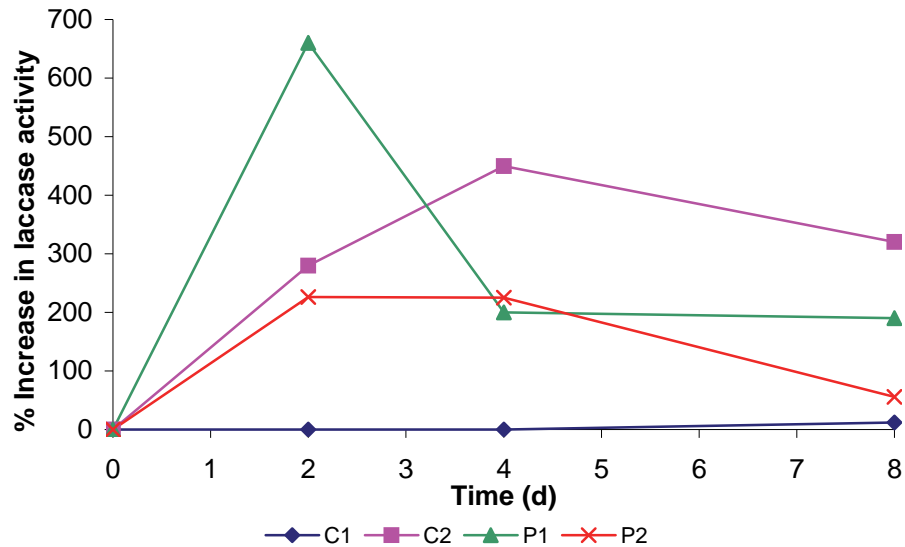


Fig 2.9 The effect of industrial effluents, as inducers, on laccase enzyme activity of *T. versicolor* (1% v/v Cresylic (S1); 2% v/v Cresylic (S2); 1% v/v Pulp mill (P1); 2% v/v Pulp mill (P2))

2.4 Conclusions

- A new, efficient and economical medium was developed for culture of the white rot fungus *T. versicolor*.
 - Of the media formulations commonly recommended in literature on white rot fungi, and specifically *T. versicolor*, the 'Trametes Defined Medium' of Addleman and Archibald (1993) proved initially to be the most successful, in terms of growth and laccase production, for the strain used in this study.
- Use of a nutrient-sufficient medium allowed superior growth-related productivity:
 - While a growth medium with an excess of glucose (20 g/l) produced the greatest biomass accumulation, the 'sufficient' glucose (10 g/L) and peptone (10 g/L) was superior in terms of overall enzyme production and 'Growth Related Productivity', and will be used in future work.
 - This is in agreement with the available literature on certain other basidiomycetes (Kaal *et al.*, 1995).

- This means that *T. versicolor* has a distinct production advantage in that it has the ability to produce high levels of laccase, under nutrient sufficient (rapid growth) conditions, producing large amounts of the enzyme.
- Furthermore, when used in flask culture for culturing of a different strain of *Trametes*, namely *T. pubescens*, the sufficient medium also gave a much higher enzyme titre (9.5 U/mL) after 7 days growth than the recommended medium (1.95 U/mL).
- The *T. versicolor* strain used here produced growth-associated (constitutive) and inducible laccases.
 - When the medium was supplemented by the addition of an aromatic-containing effluent, a large increase in the production of laccase was observed, with a maximum activity of 1.5 U/mL (cresylic effluent) achieved.
- This is one of the highest enzyme activities reported for flask cultures of *T. versicolor* (Table 2.3).
- The common monophenol, guaiacol, proved to be the most effective of the commercially available inducers, producing an increase in laccase production of nearly 800%, without noticeably affecting biomass accumulation.

The results presented in this chapter represent a sound basis from which to further investigate and optimise biomass generation and laccase production in *Trametes* species.

Table 2.2 Comparison of laccase activities reported in different bioremediation applications of *T. versicolor* (in flask culture)

REFERENCE	ACTIVITY (U/mL)	APPLICATION
Addleman <i>et al.</i> , (1995)	0.048	Kraft pulp degradation
Roy and Archibald (1993)	0.16	Kraft pulp degradation
Addleman and Archibald (1993)	0.58	Kraft pulp degradation
Heinfling <i>et al.</i> , (1997)	0.48	Decolourisation of dyestuffs
Rogalski <i>et al.</i> , (1991)	0.06	Optimisation of culture conditions
Swamy and Ramsay (1999)	0.41	Decolourisation of dyestuffs
This study	1.52	Degradation of cresylic effluents

CHAPTER 3 : DEVELOPMENT OF CULTURE METHODOLOGIES FOR *TRAMETES* SPECIES (Part 2): FACTORS INFLUENCING GROWTH AND LACCASE PRODUCTION IN FLASK CULTURE

3.1 Introduction

Although there has been much research focused on the mechanisms of enzymic degradation of lignolytic compounds and physiological control of enzyme secretion, relatively little is understood regarding the physiological adaptation of *T. versicolor* to an environmental challenge. A better understanding of this response would better enable the exploitation of the potential of *T. versicolor*'s for biodegradation. Fungi exhibit considerable developmental plasticity, being able to switch between a variety of distinct "functional modes", or programmed morphogenetic cycles, which confer versatility on the fungus, allowing it to adapt to differing environmental conditions (White and Boddy, 1992). There is some evidence of differences in extracellular enzyme production between modes. For instance, the basidiomycete *Hymenochaete corrugata* has two different colony morphs: a woolly, white form with extensive aerial mycelia, and an appressed pigmented form associated with increased laccase and tyrosinase activity, occurring in more extensively decayed wood (Sharland *et al.*, 1986). Similarly, *Rigidoporus microporus* produces one mycelial form which is tolerant of poor aeration, produces laccase, and may be responsible for development within wood, and a second form which produces mycelial cords responsible for superficial ectotrophic spread, but which does not produce laccase (White & Boddy, 1992).

A large variety of chemical products are newly synthesized and produced commercially every year. During their manufacture and use, these chemicals are often discharged into the environment. *T. versicolor* has potential for the mineralisation of one such group of compounds, phenols. The physiological response of *T. versicolor* to the presence of a phenolic effluent, generated by a fuel-from-coal plant, was investigated in this study. This effluent arises from the Fischer-Tropsch process, the greatest portion of which is known as Stripped Gas Liquour (SGL) and is produced from the plant of origin in volumes of up to 62×10^6 L/d. The SGL stream originates from gasification condensate and contains pitch, tar and naphtha as well as phenols and creosotes (Philips and du Toit, 2003). Most of these compounds are removed by physical processes, gravity separation and liquid-liquid extraction, but the resulting stream still contains xenobiotic compounds (Table 3.1).

Table 3.1: Analysis of the cresylic components of the SGL effluent

<u>COMPOUND</u>	CONCENTRATION (mM)	CONCENTRATION (g /L)	HPLC RETENTION TIME (min)
<u>phenol</u>	82.8	7.8	8.97
p- cresol	24.99	2.7	15.62
m- cresol	25.8	2.8	18.80
o- cresol	77.03	8.3	20.80

It is generally accepted, that in the degradation of aromatic compounds by WRF, the initial attack on aromatic pollutants is made by the extracellular lignolytic enzymes (Hammel, 1995). These enzymes perform a one-electron oxidation, thereby generating cation radicals of the pollutant compounds. The radicals then undergo spontaneous chemical reactions such as C-C cleavage or hydroxylation resulting in more hydrophilic products that taken up by the fungal cells and co-metabolised in the presence of the proper carbon source (cellulose) to carbon dioxide (Hammel and Moen, 1991). This is a complex process of oxidation, reduction, methylation and hydroxylation. Despite much research on the oxidative mechanism of the lignolytic enzymes, the mechanism of lignin degradation and oxidation of lignin-related compounds are not entirely understood. The reactions are complicated and involve numerous low molecular weight compounds that may serve as redox mediators.

The objective of the work discussed in this chapter was to elucidate the response of cultures of *T. versicolor* exposed to increasing concentrations of the effluent. This response was measured with respect to changes in morphology, enzyme production and metabolic activity. The morphology was assessed qualitatively by visual inspection, and then more closely by scanning electron microscopy (SEM). The ability of the different colony morphs to remove target effluent monomers from solution was determined by HPLC. Metabolic activity was determined by the measurement of rate of the hydrolysis of 3, 6- diacetylfluorescein (FDA) (Schnurer and Rosswall, 1982). FDA is hydrolysed by a number of different enzymes, such as proteases, lipases and esterases, and thus its hydrolysis is correlated with levels of general microbial activity. The product of the enzymatic conversion is flourescein, which can be quantified by spectrophotometry. This method fulfils the requirement that any technique utilised to measure total microbial activity should be non-specific and sensitive, and any incubation period necessary should be as short as possible. FDA has been used previously to determine quantities of active fungi and bacteria in soil (Schnurer & Rosswall, 1982).

The most common spectrophotometric tests performed to measure laccase activity utilise the phenolic substrates: guaiacol, 2,6-dimethoxyphenol (Prillinger and Esser, 1975) and syringaldazine. Another, more recently employed, substrate is the electron-rich 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). The oxidation of ABTS by laccase produces the readily measurable, stable, dark green cation radical ABTS^{•+} (Bourbonnaise and Paice, 1990). Furthermore, ABTS can act as a redox mediator for the indirect oxidation of non-substrate lignin compounds with high redox potentials (Xu *et al.* 2000).

In this study, it was observed during the ABTS laccase assay of samples containing high concentrations of phenols, that the measured laccase activities were consistently lower than expected. Low enzyme activities were obtained at high phenolic concentrations, even though the removal of these phenols increased with concentration. The results from the ABTS colorimetric assay was then compared to the results of a equivalent assay based on oxygen consumption, to determine whether or not the laccase activity was in fact decreasing or whether the assay was being interfered with by the residual phenols. Such an effect would result in an underestimation of actual laccase activities in the experimental samples, and hence would indicate that the culture system was less effective than was actually the case.

3.2 Methods

3.2.1 Strain preservation

A culture of *T. versicolor* (PPRI #3845) was maintained as in section 2.2.1.

3.2.2 Liquid Culture

Trametes defined medium (TDM) (Addleman and Archibald 1993) was used initially as the medium for the production of biomass and enzyme, but the glutamine was replaced by peptone, and dimethyl succinate, sodium and calcium chloride were omitted. The previously selected medium with glucose and peptone concentrations of 10 g/L was used.

3.2.3 Inoculation

Flasks were inoculated as described in section 2.2.4.

3.2.4 Biomass determination

Fungal biomass was determined as in section 2.2.5.

3.2.5 Laccase preparation for inhibition study

T. pubescens was cultured in a 4 L (3.5 L working volume) airlift reactor with an aspect ratio of 4.7:1. The modified Trametes Defined Medium (Addleman and Archibald, 1993) was used and air was supplied at 3L/min. After a 120 h fermentation the culture fluid was removed, centrifuged to remove cells and then freeze dried to give the crude enzyme extract. A portion of the crude extract was purified by acetone precipitation (1:1 vol/vol). The samples comprised of 50 μ L enzyme (20 μ g/mL) and 50 μ L dissociation buffer. They were heated in boiling water for 5 min and then cooled to room temperature. The gel was run at 120 V for 3 hours.

3.2.6 Measurement of Laccase enzyme activity

a) ABTS

Laccase activity determined by the oxidation of ABTS was carried out as described in section 2.2.7

b) Oxygen Utilisation Rate (OUR)

OUR assays were carried in a sealed 100 mL flask containing at 10x the volume of the reactants. The oxygen consumption was measured with a dissolved oxygen probe (YSI 5740) linked to a HiTech Microsystems DO/OUR meter. The meter was activated on addition of the enzyme sample; the first 10 seconds of the run were disregarded to allow for a delay in probe response time.

3.2.7 Measurement of total microbial activity

Fluorescein diacetate (FDA) (Sigma) was dissolved in analytical grade acetone and stored as a stock solution (2 mg/mL) at -20°C . The amount of FDA hydrolysed was measured as absorbance increase at 490 nm (A_{490}). Biomass samples were obtained on filter paper discs as described previously (section 2.2.5), but were not dried. These were simultaneously added with FDA (final concentration, 10 μ g/mL) to sterile 50 mL sodium phosphate buffer (60 mM, pH 7.6) and the mixture was incubated at 24°C on a rotary shaker. Experiments were run over 3h with readings taken every 30 min to determine the FDA hydrolysis curves. The buffering capacity was sufficient to maintain the pH at 7.6 for the duration of the experiments (Schnurer & Rosswell, 1982). 1.5 mL samples were drawn from each flask and centrifuged for 4 min at 5000 rpm to remove any debris. A_{490} values of the supernatant of these samples were determined and each determination was carried out in triplicate. .

3.2.8 Effect of phenolic effluent on physiology and enzyme production

Flask cultures were allowed to grow until a continuous pellicle, or mycelial mat, formed at the air-liquid interface. This occurred after 9 d growth. Varying amounts of effluent (2.5 %, 5 %, 10 % and 20 % vol/vol) were then added to the liquid medium in each flask. Samples (1 mL) were aseptically drawn from each flask every day for 4 d and then again after 12 d. Biomass (section 2.2.5) and microbial activities (section 3.2.7) were determined as before and morphology was observed in terms of colour and consistency of both the fungal mat and growth medium. A laccase activity assay (section 2.2.7) was performed on each sample.

3.2.9 Effect of culture age prior to cresylic effluent addition on growth and enzyme production

The target effluent used in this study was obtained from the cresylic stream of a local coal gasification plant. The effluent contained negligible amounts of settled solids and high concentrations of phenol and the isomers of cresol (Table 3.1). A number of flasks were inoculated as before (section 3.1.3) and left to grow for 4 d. The cresylic effluent obtained from the coal gasification plant was then added at 2.5%, 5%, 10% and 20% concentrations (v/v) to a set of flasks. Daily samples were aseptically drawn from the flasks, centrifuged and then filtered to remove any cell debris, assayed for laccase activity, and the remainder frozen for further analysis. After 4 d the flasks were removed from the incubator, the biomass filtered off, dried and weighed. At this time effluent was added to a second set of original flasks in identical concentrations and the process repeated, and similarly for a third set, after a further 4 d. This resulted in the effluent being added to cultures of different ages: 4 d (set 1), 8 d (set 2), and 12 d (set 3) and in different stages of growth.

3.2.10 HPLC analysis

Samples were aseptically drawn from the flasks, centrifuged and then filtered to remove any cell debris. The removal of the effluent components was monitored by HPLC (Merck LaChrom), utilising a Waters S5 (5 μ m), C₁₈ reverse phase column (250 x 4.6mm) with a mobile phase of water:acetonitrile 85:15 containing 1.66% w/v β -cyclodextrin, to separate the cresol isomers.

3.2.11 Electron Microscopy

The mycelial mats were removed from the stationary flask cultures and 5 mm x 5 mm sections taken from them and fixed in 4% glutaraldehyde. These were taken through a critical point drying series to remove any moisture that would interfere with the sputter coating method (Cross, 1987). The sample was placed in the sputter coater holder, clamped in position and the chamber closed. The chamber was evacuated until a vacuum of 10^{-1} Torr had been achieved. The vacuum level was then balanced at 5×10^{-1} Torr. Argon was used to flush out the residual air in this manner for 5 to 10 min. The argon leak valve was then closed and the chamber further evacuated. When a vacuum of better than 5×10^{-1} Torr had been achieved, the sputtering current was allowed to rise to 15 mA. The timer was set to 90 s and sputtering allowed to proceed. After the coating was complete, air was admitted to the chamber. The specimens were then removed from the holder and examined using a JEOL JSM-840 Scanning Electron Microscope (Cross, 1987). Photomicrographs are shown on page 53.

3.3 Results

3.3.1 Enzyme production in flasks

A single activity band, that correlated well with commercial laccase (Sigma Aldrich) was obtained for the purified enzyme samples on an SDS PAGE gel activity stained with ABTS solution (Fig. 3.1 and Table 3.2). Protein concentration was determined according to Bradford (1976).

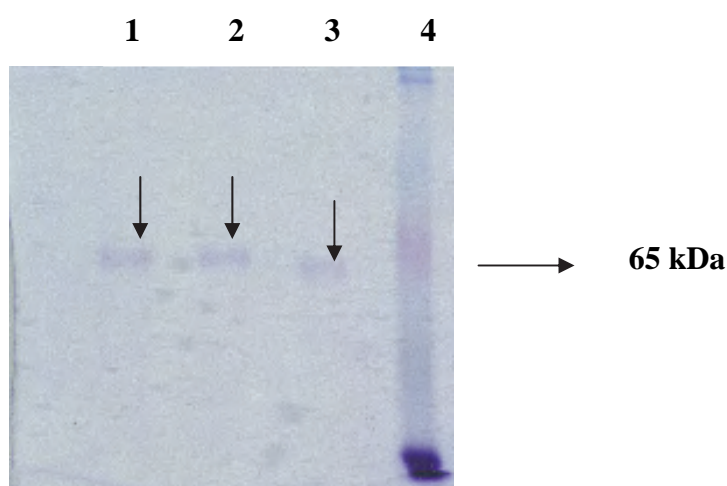


Fig 3.1 SDS PAGE gel of commercial (lane 3) and purified laccase (lanes 1 and 2) obtained from *T. versicolor* culture filtrate

Table 3.2 Specific activities of four laccase samples from different origins

SAMPLE	SPECIFIC ACTIVITY (U/ mg)
1. Purified laccase	294
2. Purified laccase	294
3. Commercial laccase	119
4. Crude Enzyme	3.21

3.3.2 Morphological effects of phenolic effluent addition on biomass of *T. versicolor*

Browning of fungal tissues often results from enzymic oxidation of phenolic substrates by phenoloxidases and peroxidases (White and Boddy, 1992). In this study, this occurred when cresylic effluent was added to flasks cultures of *T. versicolor*. As soon as the cultures showed visible mycelial mats growing on the surfaces of the growth medium (7 days), varying concentrations of cresylic effluent were added to the flasks. It was evident that high effluent concentrations had a significant physiological effect on *T. versicolor*, as demonstrated by a decrease in biomass accumulation (measured as dry mass) in the presence of the phenols (Table 3.3). This effect became more pronounced with increasing effluent concentration and was accompanied by marked changes in the morphology and appearance of the biomass and culture medium (Table 3.3).

Table 3.3 Biomass morphology and medium appearance of flask cultures of *T. versicolor* in the presence of cresylic effluent*.

EFFLUENT CONC. (mM)	BIOMASS YIELD (g/L)	BIOMASS MORPHOLOGY	MEDIUM APPEARANCE
Control = nutrient medium only	3.7	Dense, white, woolly, aerial	Fluid, clear, colourless
1.825	3.7	Dense, white, woolly, aerial	Fluid, clear, colourless
3.65	3.3	Dense, white, woolly, aerial	Fluid, clear, colourless
7.30	2.78	Less dense, white, woolly, aerial	Slightly viscous, clear, colourless
14.60	1.56	Thin, desiccated, brown, appressed	Extremely viscous, cloudy, light brown

- The undiluted cresylic effluent contained a total of 213mM (21.6g/L) mixed phenolics (Table 3.1). Dilutions were made with nutrient medium, from 1:2 to 1:12, giving molar concentrations shown

Fungal hyphae self-organise into an interconnected, branching mycelium and colonies grow by repeated branching of the hyphal tips to form a ramified mycelial network. Jones *et al.* (1996) used image analysis software to quantify the global fractal dimension, which provided an index of fungal branching morphology. The

global fractal dimension is an overall estimate of scaling complexity. It was shown that whole colonies of the WRF *Pycnoporus cinnabarinus* display a similar mode of branching initiation that can be indexed by using fractal geometry. A correlation was established between hyphal branching, and the number density of active tip production per unit area, and extracellular oxidase expression levels. Furthermore, mycelial morphology and protein expression were found to be influenced by medium composition i.e. enzymatic function was linked to colony structure (Jones and Lonergan, 1997). *P. cinnabarinus* was grown on solid growth medium (agar) supplemented with a known paramorphogen, cellobiose. A paramorphogen is thought to stimulate or induce changes in fungal cell physiology by altering either the cell wall biochemistry, by affecting the cytosol molar ratios of transport proteins that are involved with protein synthesis, or the cell-mycelium morphology by enhancing or repressing hyphal branching. The cellobiose caused profuse hyphal branching (higher fractal dimension) and a reduction in radial expansion. These changes in morphology were accompanied by a concomitant exponential increase in the expression of laccase into the extracellular medium in liquid culture. Hypersecretion was stimulated approximately 7070% over the control at 5 days. This study demonstrated a strong correlation between spatial pattern phenomena (morphology) and physiological function.

An obvious correlation exists between the effects of the paramorphogen (cellobiose) used by Jones and Lonergan (1997) the phenolic effluent used in this study. Both had adverse morphological effects on the fungi in question and both stimulated increases in laccase concentration. Morphogenic self-organisation in fungi has been shown in both cases to be an interconnected dynamic process which links spatial structure with biochemical function at the cellular and sub-cellular levels of scale.

3.3.3 Optimisation of enzyme production - Effect of time of addition of phenolic effluent on laccase production in *T. versicolor*

After several initial attempts, it became readily apparent that adding even small concentrations of effluent to flask cultures at the same time as inoculation severely inhibited growth of *T. versicolor*. It became obvious that there would be an optimum culture age at which it would be most advantageous to add effluent to the fungal cultures. The addition of varied concentrations of phenolic effluent to flask cultures at different stages of growth showed that the time of addition played a significant role in the resultant effect of that addition (Fig 3.2). At all effluent concentrations, the 12 day-old cultures (Fig 3.2 C) were better able to tolerate the pollutant addition, as indicated

by their producing more degradative enzymes, up to 3.8 U/mL compared to a maximum of 0.65 U/mL for the 4 day-old (Set 1) and 2.9 U/mL for the eight-day old (Set 2) cultures (Figs 3.2 A and 3.2 B). Furthermore, increasing the pollutant concentration in Set 3 resulted in increased enzyme production; whereas the opposite effect was observed in Set 1 and only low concentrations (1.825 mM) had a positive effect on Set 2. Laccase production by *T. versicolor* has been shown to peak after 12 days in cultures without any inducers added (section 2.3.2). This phenomenon occurred 2 to 3 days after glucose depletion and fits the model of lignolytic enzyme production as a result of nutrient limitation. The control cultures generated the highest laccase activities after pollutant addition in Set 1, but the lowest in Set 3. The flasks with the highest phenol concentration (14.6 mM) produced the lowest amounts of enzymes in Set 1, but the highest in Set 3. The control samples had a maximum laccase activity of 2.45 U/mL while a 14.6 mM addition of phenol resulted in an activity of 3.8 U/mL, both in Set 3 (Fig 3.2 C). Therefore, although nutrient limitation does increase laccase production in *T. versicolor*, it is not as an effective inducer as certain aromatic compounds.

The toxic effect of cresols and chlorophenols has been measured by the growth retardation, and the detoxification of these compounds by WRF by the removal of toxicity associated inhibition (Bollag *et al.* 1988). These authors found an increased lag phase in fungal growth after pollutant addition and complete inhibition of growth at concentrations approaching 2 mM. It was further reported that it was the laccase that was unequivocally responsible for detoxifying *p*-cresol, *o*-cresol and 2,6-dimethylphenol, as demonstrated by the disappearance of the parent phenol. The detoxification of these phenols allowed fungal growth to take place. In the present study, results from the dry mass determinations (Fig 3.3) indicate clearly that allowing more time for growth before adding toxic compounds reduced the negative effects of these compounds on the physiological state of the *T. versicolor* cultures. The cultures in Set 1 deteriorated markedly in terms of biomass at pollutant concentrations above 7mM, while Sets 2 and 3 actually showed increased biomass accumulation. This is possibly due to a combination of the fact that the effluent provided an additional carbon source and that the older cultures were able to detoxify their environment more quickly by removing the pollutants, and in doing so were better able continue normal growth. The fact that there was no available glucose in the culture fluid when the effluent was added to Set 3 and the highest concentrations of effluent supported the highest biomass concentrations is further evidence that *T. versicolor* must have used the phenols provided as an additional carbon source. Although this argument

contradicts previous research (Bollag *et al.*, 1988, Hammel *et al.*, 1995) it is difficult to draw another conclusion from these results.

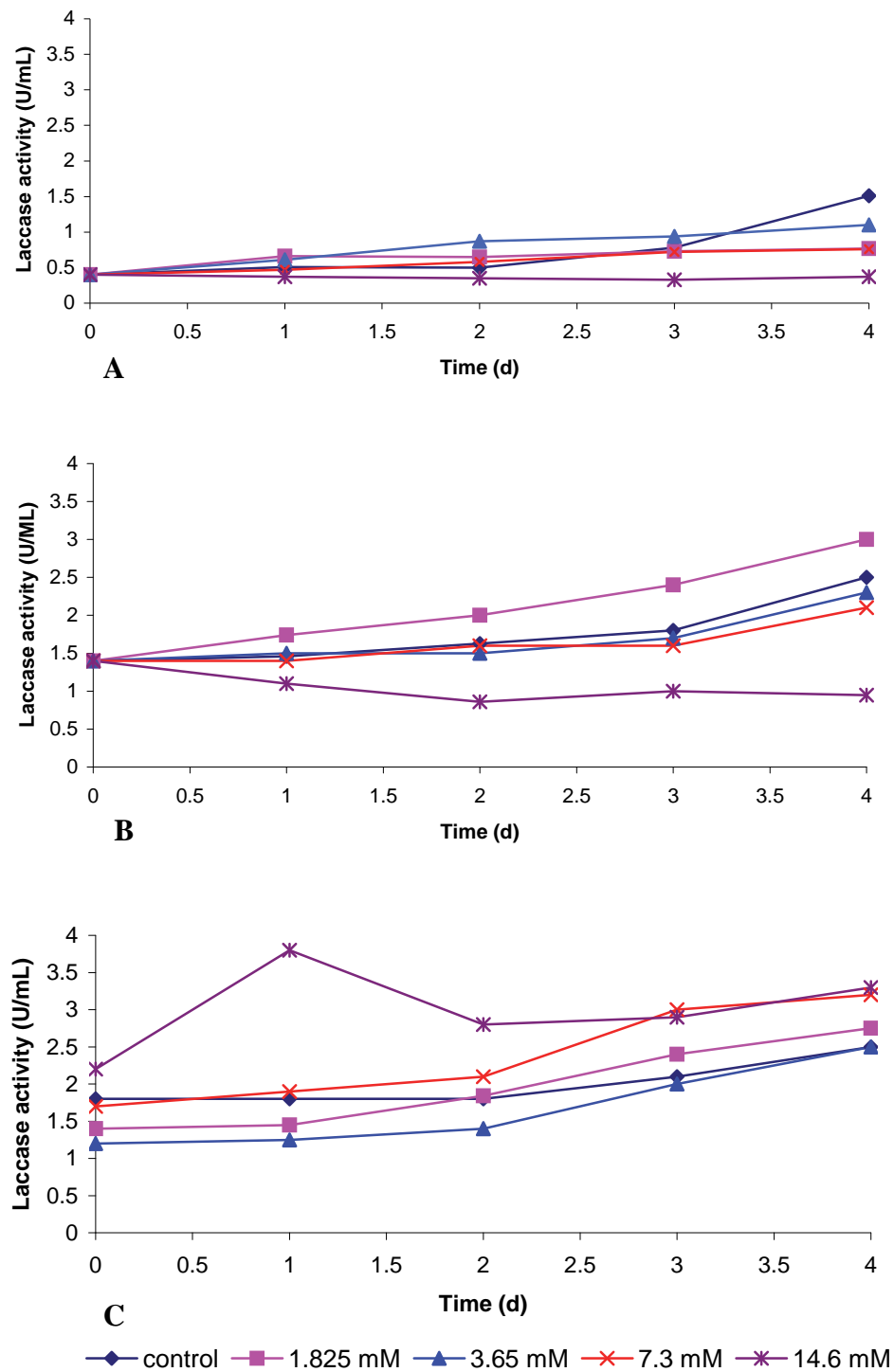


Fig 3.2 The effect of time of addition of cresylic effluent on laccase enzyme production. Flasks were allowed 4 days (A) 8 days (B) and 12 days (C) growth prior to effluent addition.

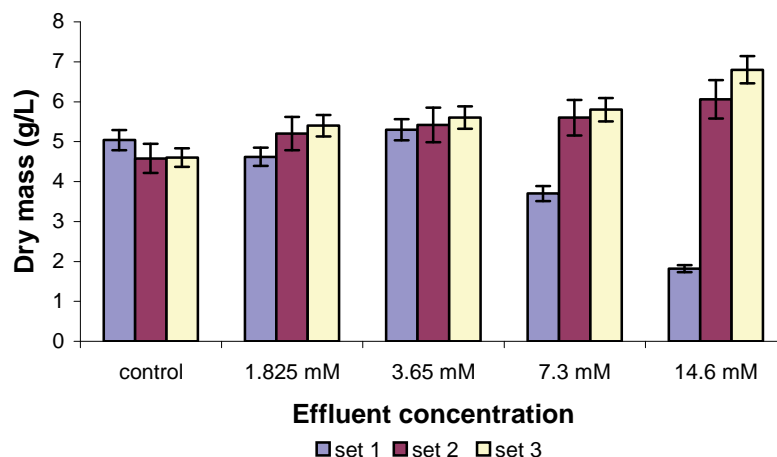


Fig 3.3 The effect of time of addition of different concentrations of cresylic effluents on biomass accumulation. Where Set 1 was allowed 4 days prior growth, Set 2, 8 days and Set 3, 12 days.

Phenol is not readily biodegradable and has been reported to be toxic or growth inhibitory to most types of microorganisms, even to those species that have the metabolic capacity of using it as a growth substrate (Annachatre and Gheewala, 1996). The ability of *T. versicolor* to grow in the presence of up to nearly 15 mM phenol is further evidence of its bioremediation potential. *T. versicolor* has been shown to be resilient to other frequently encountered environmental pollutants. Alleman *et al.* (1992) found that *T. versicolor* was able to more effectively dehalogenate PCP than the six other WRF tested by them and as a result was the also grew the fastest. Morgan *et al.* (1991) found that *T. versicolor* was best able to mineralise dichloroaniline, dieldrin and phenanthrene out of four WRF tested, including *P. chrysosporium*.

This work highlighted the importance of allowing cultures sufficient growth time to develop the systems necessary for dealing with environmental challenges before exposing them to these pollutants. This result is important in light of the fact that it has been reported previously that the addition only 2 mM *o*- and *p*- cresol to the culture medium prior to inoculation inhibited the growth of another well-studied laccase producing WRF *Rhizoctonia praticola* (Bollag *et al.*, 1988)

A closer examination (by SEM) of the morphology of *T. versicolor* indicated that fungal biomass exposed to high concentrations (14mM) of phenols had a thin, random, highly branched structure (Fig 3.4 B, D). This contrasts with the thicker walled, linearised, skeletal hyphae usually associated with generative growth that

characterized the biomass morphology in the flasks containing lower effluent concentrations (Fig 3.4 A, C). These differences in morphology are partly explained by the fact that; individual fungal mycelia are indeterminate structures and mycelial organisation is directed by interactions between the genotype of a particular organism, and the prevailing abiotic (microclimate) and biotic (other organisms) environments, mediated at the boundaries of colonies as a whole and individual hyphal tubes. Heterogeneous microclimate and resource quality have been shown previously to have a substantial influence on mycelial growth (White *et al.*, 1998). The phenolic compounds in the culture fluid had a visible effect on the biomass of *T. versicolor* (Table 3.3) and an ultrastructural one (Fig 3.4). The effect of paramorphogens (compounds that induce changes in cellular physiology) such as phenol on *T. versicolor* is discussed further in later sections. Furthermore, the reaction of laccase with phenolic compounds produces reactive quinone compounds that may be involved in oxidative polymerisations with cell surface components such as carbohydrates or proteins, resulting in a change of hydrophobicity of the hyphal walls. These reactions might serve to chemically cross-link adjacent hyphae leading to the construction of aggregated tissue (Score *et al.*, 1996). This effect can be seen in the high-density morphology of the biomass exposed to phenol (Fig 3.4 B, D).

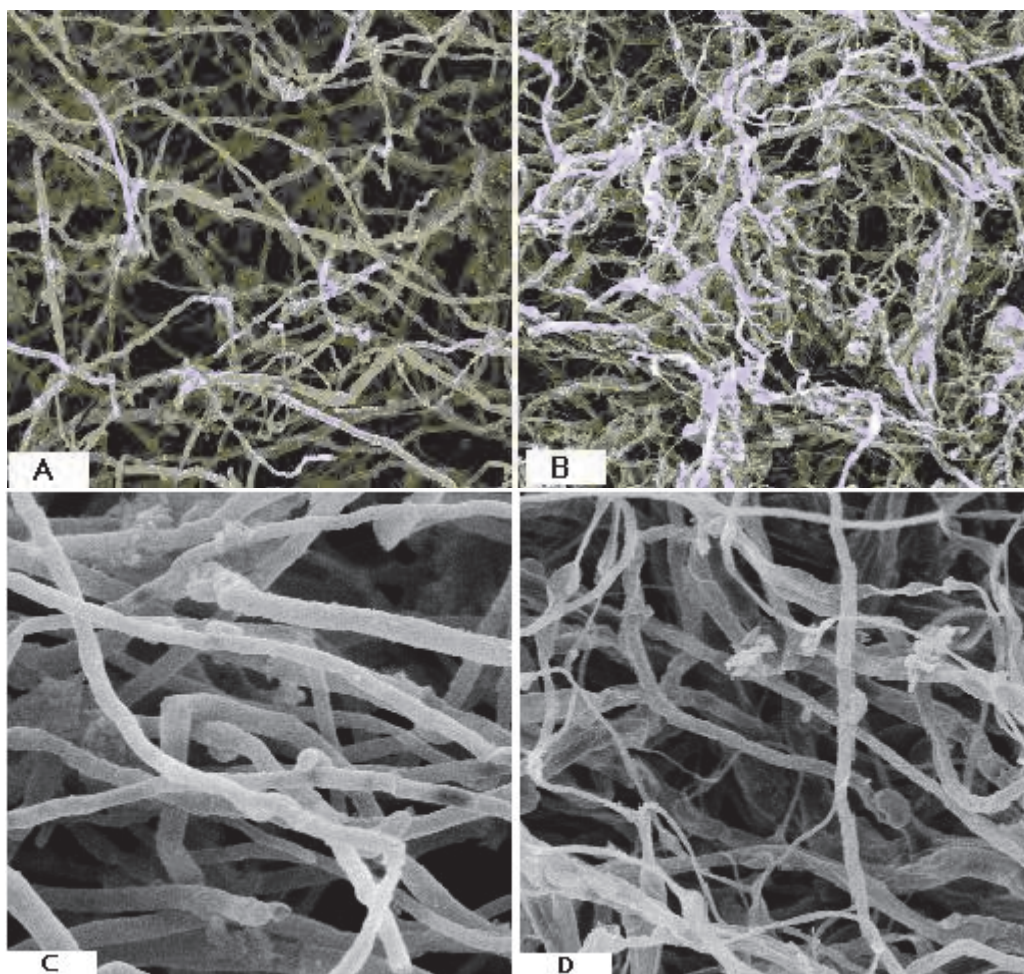


Fig 3.4 The effect of different concentrations of effluent on gross morphology of *T. versicolor*:

(A, C): Air interface of a fungal mat grown in the presence of 2.5% effluent. The hyphae are thick, turgid and loosely packed, with clamp connections visible indicating rapid, healthy growth (A 500x magnification, C 1000x magnification).

(B, D): Air interface of a fungal mat grown in the presence of 20% effluent. The hyphae are thin, tightly packed and gelatinised, often an indication of stress (B 500x magnification, D 1000x magnification).

3.3.4 Changes in microbial activity of *T. versicolor* cultures exposed to phenolic compounds

The comparative microbial activity of the cultures exposed to different effluent concentrations was investigated by the spectrophotometric determination of the hydrolysis of fluorescein diacetate (FDA) to fluorescein. FDA as a substrate for determining the overall activity of decomposer organisms is useful because it is hydrolysed by a number of different enzymes and a good correlation between FDA hydrolysis and respiration has been found (Schnurer and Roswall, 1982). Total

microbial activity in the control culture of *T. versicolor* showed a typical saturation curve, with an initial linear increase in absorbance at 490 nm up to 30 min (0.563) and thereafter the rate of absorbance decreased over time to reach 1.02 after 180 min (Fig. 3.5). The differences between the samples for the total absorbance values obtained at $t = 30$ min were found to be approximately the same as those obtained after 3 hours of incubation. For consistency therefore, when comparing the microbial activities of different samples, the initial rates were used. The decrease in hydrolysis rate over time is probably due to complete substrate utilisation limitation, as FDA has been shown to be non-toxic to microorganisms and mammalian cells at the concentrations used in this assay (Schnurer and Roswall, 1982). This concentration limitation cannot be remedied, however, as FDA solutions become cloudy at higher concentrations, reducing reproducibility.

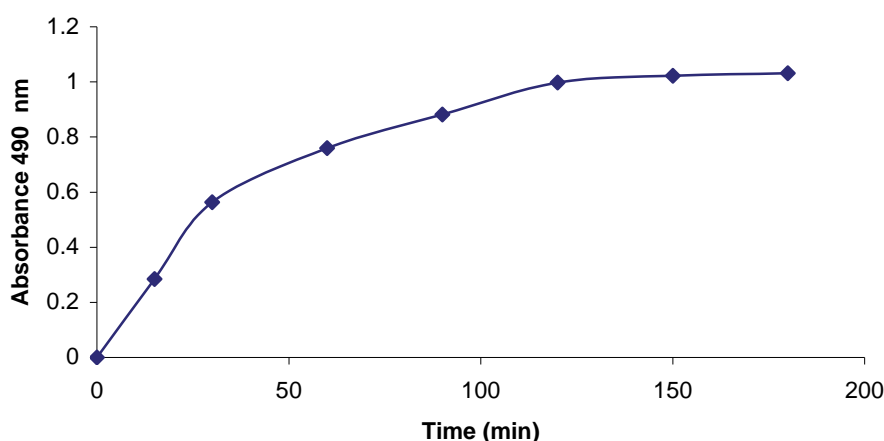


Fig 3.5 Measurement of FDA hydrolysis over time as a measure of total metabolic activity of *T. versicolor*

The total microbial activity of *T. versicolor* cultures in the absence of a pollutant challenge was correlated with the amount of biomass accumulated during the exponential growth phase of the fungus and a linear relationship ($R^2 = 0.99$) was found. This is not entirely surprising, as one would expect almost the entire culture to be uniformly active during this period. Schnurer and Roswall (1984) also found a linear relationship between FDA hydrolysis and different amounts of microbial biomass in a suspended soil sample but warned against using this assay as a biomass determination, and recommend it as a measure of microbial activity only. In this study it has been used as such, but the linearity of the relationship between biomass and FDA hydrolysis demonstrated (Fig. 3.6) indicates the potential to extend the use of this assay to estimations of biomass within certain constraints e.g. for a synchronously active culture.

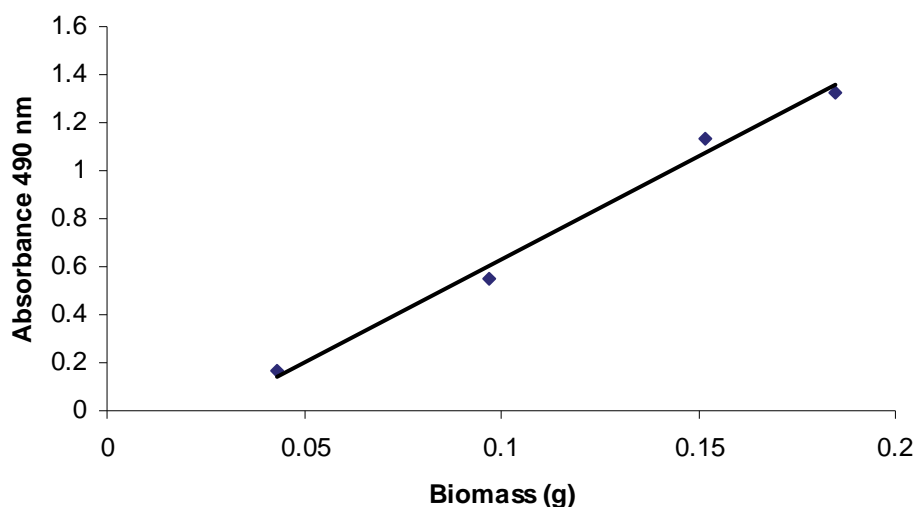


Fig 3.6 The linear relationship ($R^2 = 0.99$) between biomass and metabolic activity, as measured by FDA hydrolysis, during the growth phase of *T. versicolor* in flask culture

3.3.5 Effects of effluent addition on metabolic activity of *T. versicolor*

It has been already been shown that the addition of a phenolic effluent had profound morphological effects on *T. versicolor*. One would also expect changes to occur in the physiology of the fungus as its metabolism shifts to overcome an environmental challenge by producing oxidative enzymes. The proportion of metabolically active biomass measured as a ratio of activity (FDA hydrolysis) to biomass was measured for cultures of different ages, as done before for laccase activity. This ratio was highest in Set 1 (4 day-old cultures) at low phenol concentrations (3.1 at 1.87 mM) but decreased to less than half at phenol concentrations above 7 mM (Fig 3.7). Since increasing the concentrations of phenol in the medium also decreased the biomass of the exposed Set 1 cultures, the decrease in activity is even more marked. Clearly cultures at this early stage of development are not able to survive the adverse conditions resulting from high phenolic concentrations. This is not simply a function of less biomass in the younger cultures resulting in an increased chemical dose effect because the activity was measured as a function of total biomass.

The 8 day-old cultures of Set 2 maintained the level of activity of their zero effluent control and had the highest ratio of metabolic activity to biomass across the concentration range tested (Fig 3.7). This could be attributed to the fact that, even though they were approaching stationary phase, they would still have been producing some of the enzymes required for trophophasic growth and that they were able to

produce enough laccase to remove the phenols added to their environment and thus avert any negative effects they might have. The Set 3 cultures also showed a slight increase in activity at low concentrations (up to 3.65 mM) but activity decreased when the biomass was exposed to increased amounts of phenolic compounds (Fig 3.7). After 12 days of growth before the effluent addition, these cultures had entered idiophasic growth and one would expect their general metabolism to have decreased. Laccase production was highest in these cultures (Fig 3.2), a consequence of both the glucose limitation and the induction effect of the added phenols.

The detoxifying effect of this increased laccase activity enabled these cultures to better tolerate the addition of the effluent; illustrated by the fact that they were able to begin another period of growth, sustained possibly by the phenolic carbon being made bio-available. This speculation is supported by the greatest increase in biomass observed for the cultures that had the most effluent added to them. Again it has been made clear that time of addition was critical in the bioremediation of a phenolic effluent, (and possibly therefore any potentially toxic substance). At least 8 days growth (flask culture) is required before addition of phenols to cultures of *T. versicolor* in order to best utilise the potential of this organism.

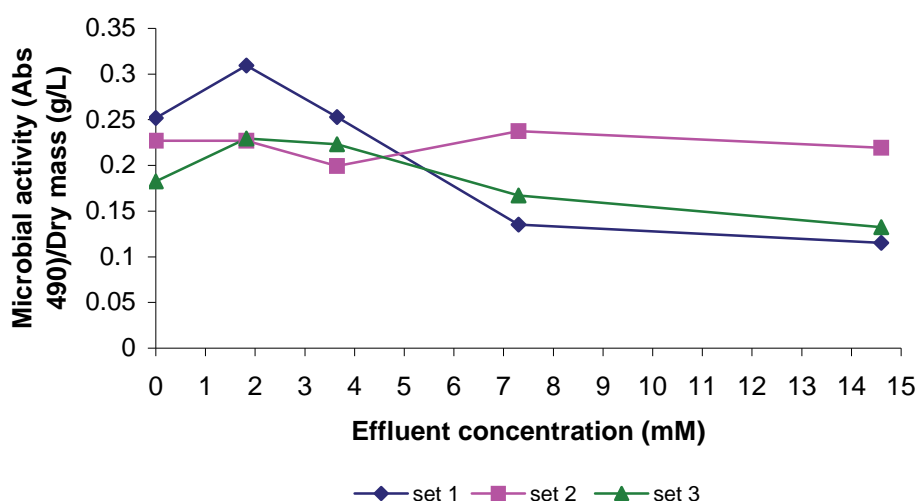


Fig 3.7 The effect of different effluent concentrations on the total metabolic activity per gram of biomass as a function of culture age. Where Set 1 was allowed 4 days prior growth, Set 2, 8 days and Set 3, 12 days.

3.3.6 Inhibition of the ABTS laccase assay

The activity of laccases *in vivo* or as purified iso-enzymes is often determined by spectrophotometric tests using phenolic substrates and by monitoring the coloured oxidation products. The most widely used laccase assay in recent literature utilises the electron- rich, non- phenolic substrate 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), whose oxidation proceeds in one step resulting in a coloured radical cation ABTS^{•+}. This assay is not without its complications however. The ABTS^{•+} complex can react with some of the components in a given sample and its absorption in solution depends on the amount of remaining, unreacted ABTS, which can result in an underestimation of the enzyme activity (Johannes and Majcherczyk, 2000).

During this investigation of the removal of phenolic compounds by *T. versicolor* it was noticed that as concentrations of effluent phenols were increased, although phenol removal rates (as measured by HPLC) increased, the ABTS assay indicated lower laccase activities. This was unexpected, as the laccase was considered to be primarily responsible for the bioconversion of the phenols. Laccase was the only oxidative enzyme measured in significant quantity in the whole cell removal experiments with *T. versicolor* and is well known to remove phenols from solution (Bollag *et al.*, 1988, Davis and Burns, 1990, Morgan *et al.*, 1991, Reddy 1995). To better quantify this effect and to remove any possible interference from artefacts in whole cell culture, partially purified laccase was reacted with ABTS in the presence of various concentrations of the effluent phenols. In this simplified system the laccase activity again decreased in the presence of the phenols and this decrease was found to correlate with increasing phenol concentrations (Fig 3.8).

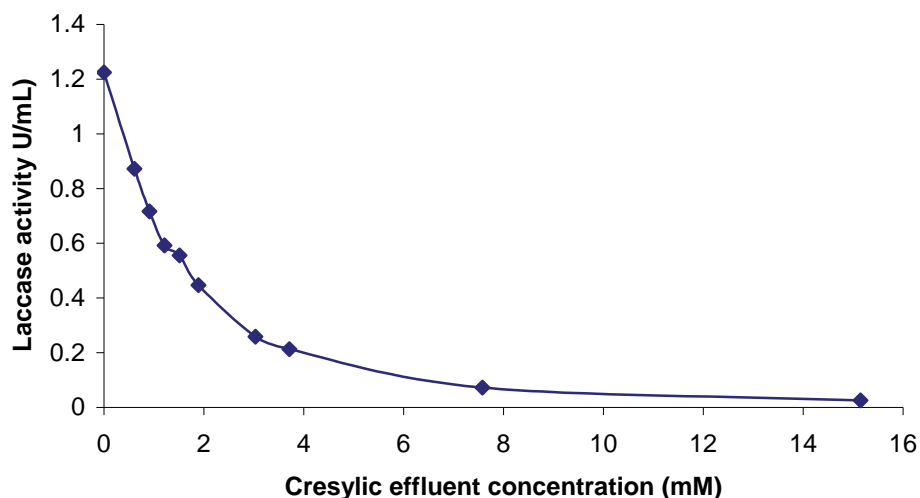


Fig 3.8 Effect of increasing concentrations of cresylic effluent on laccase activity (as measured by the ABTS colourimetric assay). Errors generated in measuring laccase activity are <5 %

When this data was plotted in a Lineweaver-Burk format, the results showed a classic plot for competitive inhibition (Fig 3.9). In our case this meant that the phenolic effluent acted as an inhibitor (I) that bound reversibly to the laccase active site preventing the intended substrate, ABTS, (S) from binding. The data was obtained by plotting the reaction rates (v) for several concentrations of ABTS [S] at different concentrations of phenolic inhibitor [I], in the form:

$$1/v = 1/V_{\max} + K_M/V_{\max}[S] \quad (\text{Ferscht, 1997})$$

Calculation gave a K_m of 3.42 (+/- 0.15) mM and a V_{\max} of 2.55 (+/- 0.11) mM/mL/min for the uninhibited ABTS reaction and a K_i of 1.913 mM for the phenolic inhibitor, calculated according to Ferscht (1997). The V_{\max} remained unchanged for all concentrations of inhibitor (i.e. the double reciprocal plots for all [I] intersect at $1/V_{\max}$), which is diagnostic of competitive inhibition. Furthermore, high concentrations of substrate (ABTS) gave reaction rates similar to that of the control ([I] = 0), also indicative of competitive inhibition. Ideally, the plots for different [I] should have intersected at the Y axis of the graph and the discrepancy is 0.72 mM, but, the regressions for the data series plotted had correlation coefficients of at least $R^2 = 0.97$, and the reaction rates were calculated in duplicate with a variation of less than 5%. ABTS has recently been reported to be inhibitory at high concentrations in the laccase assay; Maremonti *et al.* (in preparation) estimated a K_i value of 20 mM for the inhibition of laccase by ABTS. However, this value is sufficiently high to

assume that substrate inhibition is negligible at the conditions adopted for the laccase assays conducted in this study (0.5 mM ABTS). The concentration of oxygen, the other substrate participating in this reaction may be considered in excess for the duration of the reaction (Gianfreda *et al.* 1998)

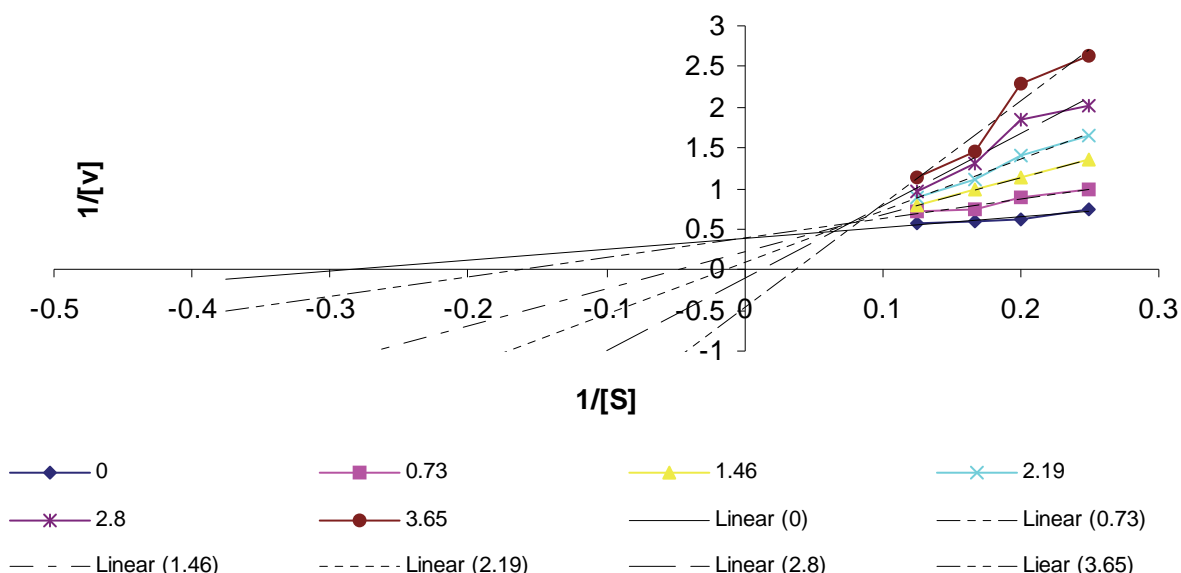


Fig 3.9 Lineweaver-Burke double reciprocal plot for the inhibition of the laccase colour reaction with ABTS by a phenolic effluent

3.3.7 The Oxygen Utilisation Rate (OUR) Assay for Laccase

Johannes and Majcherzyk (2000) suggested that while the ABTS assay was one of the better tests determining the activity of oxidative enzymes, in the case of laccase the best (but also more laborious) alternative was to perform a direct measurement of the oxygen consumption. In this study the accuracy of using oxygen consumption as a measure of laccase activity was investigated. A near linear relationship ($R^2 = 0.999$) between enzyme concentration and activity as measured by ABTS and oxygen utilisation rate was shown (Fig 3.10). This demonstrated that the colourimetric ABTS assay could be substituted safely with the oxygen consumption assay and it now seemed reasonable to relate laccase activity to oxygen consumption. The reactions with laccase and the cresylic effluent in presence and absence of ABTS were then repeated, using oxygen consumption as a measure of the rate of reaction. There was only a 7 % increase in OUR between reactions using 5 mM ABTS (the assay concentration used in the colourimetric assay) and 25 mM ABTS, indicating no that substrate limitation had occurred previously. It was decided

that 5mM ABTS could reasonably be used for all further experiments requiring the use of the colourimetric assay.

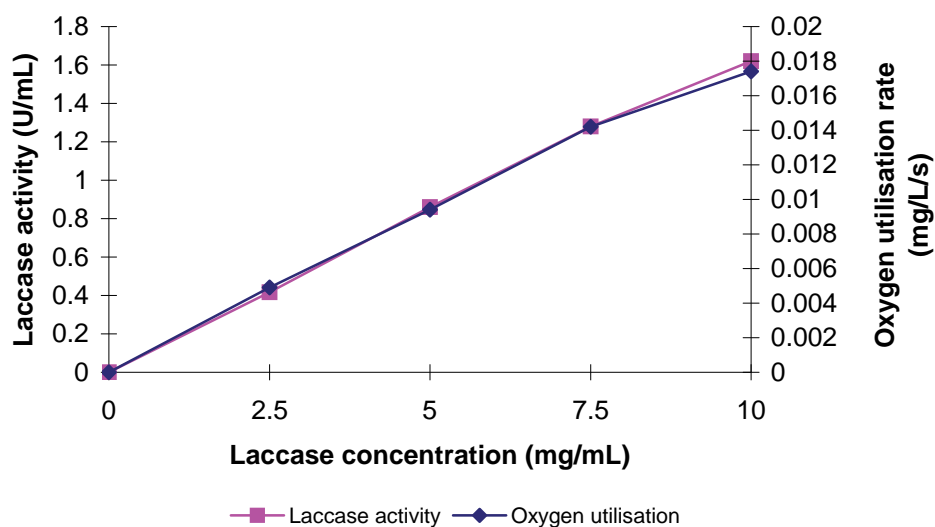


Fig 3.10 Correlation between laccase activity, as measured by ABTS and OUR at different concentrations of laccase

The OUR increased with increasing concentration of cresylic effluent (Fig 3.11), a result that supports the hypothesis that the laccase was responsible for the removal of the phenols in the effluent. However, when ABTS was added to the reaction, the total OUR should have been cumulative due to additional reaction with the ABTS i.e. the reaction of laccase and the cresylic effluent in the presence of ABTS should have produced an OUR approximately equal to the sum of the reaction without ABTS and the ABTS control. The fact that it was not indicates that it was competing with the effluent components for binding sites and confirms the suggestion that it was an inhibitor.

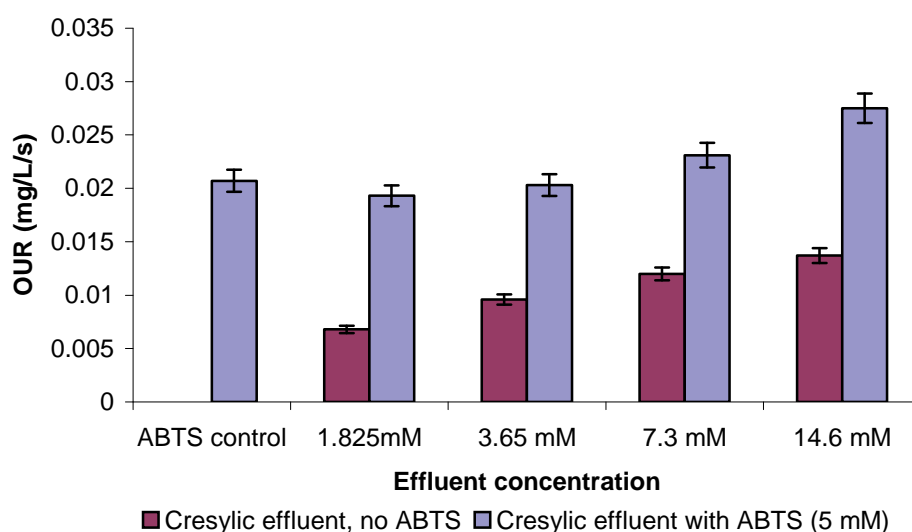


Fig 3.11 Increasing oxygen utilisation rate (OUR) of the laccase reaction with increasing concentrations of cresylic effluent, in the presence and absence of 5 mM ABTS

Kadhim *et al.* (1999) investigated the removal of phenolic compounds from water by *T. versicolor* and found that both laccase and Mn-dependent peroxidase were inactivated to some degree during the removal reaction. This effect was ascribed to the formation of reaction intermediates and was also observed with the laccase from the soil fungus *Rhizoctonia praticola* used for the removal of substituted phenols. The effect in both cases was most pronounced with 2,4,6- trichlorophenol that inactivated the laccase by 96%.

To test whether the laccase was being inactivated by the aromatic compounds being investigated, 2mM slugs of *p*-cresol were added to a fixed quantity of enzyme and the resultant activity measured by monitoring the OUR. As is evident from Fig 3.12, increasing the amount of *p*-cresol in the reaction mixture increased the laccase activity, as measured by oxygen consumption. Gianfreda *et al.* (1998) speculated that the oxidative transformation of certain phenolic compounds resulted in the formation polymers in which enzyme molecules were entrapped and progressively removed from solution. This situation seemed unlikely in the scenario investigated here, no visible precipitates were observed to form, and laccase activity only increased with increased substrate concentration. It should be noted that Gianfreda *et al.* (1998) measured laccase activity by the ABTS assay alone, interference of their test substrate, 2,4-dichlorophenol, with the assay may have been the cause of the decrease in laccase activity observed by them.

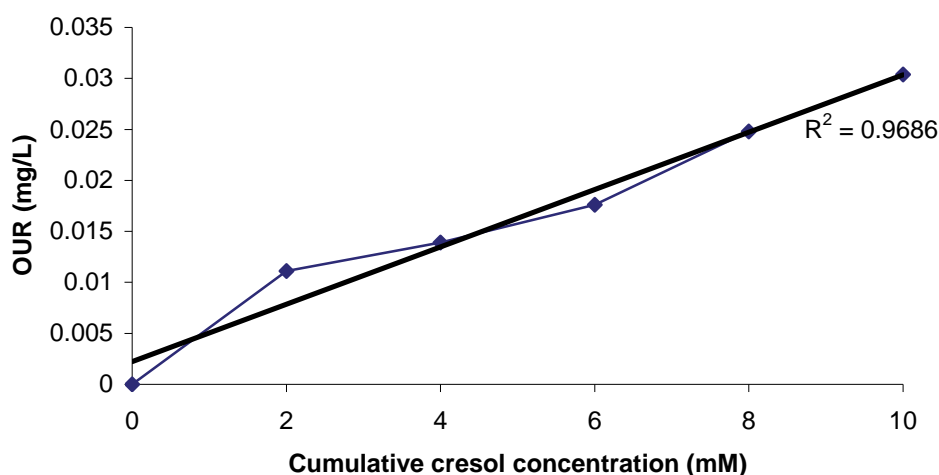


Fig 3.12 The linear increase in laccase activity (as measured by oxygen consumption) with *p*-cresol concentration

Although the ABTS assay for laccase activity is widely used, quick to perform and reproducible, it is apparent that a flaw exists in the assay when there are other laccase substrates present in the reaction mixture. Tests based on a complex, partially non-enzymic oxidation of phenolic substrates present a series of other problems and do not seem to be viable alternatives (Johannes and Majcherzyk, 2000). For accurate quantification of laccase in a solution containing potential laccase substrates, these would either have to be removed prior to performing the ABTS assay, an assay based on oxygen consumption appears to be the most accurate.

3.3.8 Utilisation of a new strain of *Trametes* – *Trametes pubescens*

While attending the 'Biotechnology in the Pulp and Paper' conference in Finland in June 2001, D Ryan was made aware of a new, potentially valuable strain of *Trametes*, *Trametes pubescens*. This strain was reported to exhibit laccase production far higher than that of the *T. versicolor* strain currently used by us in this project. After receiving a culture from the 'Universitat fur Bodenkultur' in Austria we set about determining its usefulness in the context of this project. We grew it in the culture medium recommended to us and compared the growth and productivity with a culture inoculated into the medium developed by us for optimum results using *T. versicolor* (medium E, see above). Copper (added as copper sulphate), at a concentration of 2mM, was added as an inducer to both growth media, as suggested (Galhaup, C pers. comm., 2001).

Cultures of *T. pubescens* grown in the nutrient rich medium that came suggested for use with this strain showed a lag phase of six days before large amounts of laccase was secreted into the medium. Medium E induced laccase production almost immediately and showed a steady increase in production of up to 10 U; both media produced laccase to an enzyme activity to a similar maximum (10 U, Figure 3.13). A further advantage in using medium E, apart from cost, is that more than 90% of the maximum enzyme production can be achieved without copper induction, while an absence of copper resulted in an 80% decrease in laccase production in the 'Austrian' medium (Figure 3.13). Copper is toxic in high concentrations and would need to be removed from any product/waste stream arising from a process using this fungus.

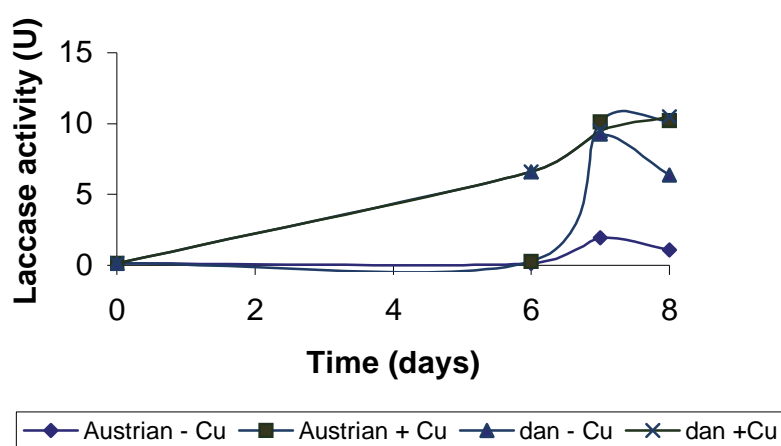


Fig 3.13 Laccase enzyme production by *T. pubescens* in shake flask culture in two different growth media, in the presence and absence of a copper as an inducer

3.4 Conclusions

- This study has shown that the response of *T. versicolor* to an environmental challenge is a complex process involving enzyme induction and morphological differentiation.
 - The hydrolysis of FDA was shown to provide an appropriate means of measurement of total metabolic activity in cultures of this fungus, and this procedure indicated a linear relationship between biomass accumulation and microbial (metabolic) activity during primary growth phase.
 - By comparison of the biomass/ microbial activity ratio for cultures subjected to increasing cresol concentrations, *T. versicolor* was shown

to adapt its morphogenetic cycles to local episodic environmental pressures, as reported for other WRF by White and Boddy (1992).

- This conclusion was validated by visual inspection and further by electron microscopy, and confirmed by the ability of the fungus to tolerate and remove the model pollutant from solution, even at high concentrations.
- The physiological response to high concentrations of aromatic compounds and their subsequent removal by whole-cell cultures of *T. versicolor* have not been previously reported. Phenol is not readily biodegradable and has been reported to be toxic or growth inhibitory to many microorganisms.
- The ability of *T. versicolor* to grow in the presence of up to nearly 15 mM phenol is strong evidence of its bioremediation potential.
- This work has highlighted the importance of allowing cultures sufficient growth time to develop the systems necessary for dealing with environmental challenges before exposing them to these pollutants.
 - Culture age prior to the addition of a pollutant is an important factor in the survival of the fungus its production of the enzymes necessary to degrade the pollutant. The importance of this parameter has been previously speculated on, but never investigated nor quantified.
 - Moderate cresol concentrations (up to 15 mM) were found to have a stimulatory effect on biomass accumulation and laccase enzyme production.
 - The physical adaptation to a pollutant challenge demonstrated here, indicates the need for consideration of metabolic differentiation in the design of a bioreactor for the fungal bioremediation of cresylic pollutants.
- The detoxifying effect of increased laccase activity enabled the cultures to better tolerate the addition of effluent. This is suggested to be due their ability to begin a further period of growth, sustained by the phenolic carbon being made bio-available. This is supported by the greatest

increase in biomass being observed for the cultures where the most effluent was added.

- While the ABTS colorimetric assay is one of the most popular ways to measure the activity of oxidative enzymes, specifically laccase, this study has shown that for the assaying of samples containing phenolic compounds, this method may not be suitable. Similar problems could well be encountered when using other common laccase assay substrates eg syringaldazine. To obtain a more accurate measurement of laccase activity, the competing phenols would need to be removed, or the assay should be based on oxygen consumption.

CHAPTER 4 : DEVELOPMENT OF BIOREACTOR SYSTEMS (PART 1): IMMOBILISED BIOFILM REACTORS FOR THE CULTIVATION OF *T. VERSICOLOR*

4.1 Introduction

The immobilisation of microbial cells is increasingly being applied in biotechnological processes (Vilchez, 1997) and has been found to prolong the retention time of microbial cells or enzymes indefinitely without washout, even at high dilution rate. The application of enzyme immobilisation technology to treatment of wastewater and drinking water was first considered in the early 1980s (Wenhua, 1993). Ideally a bioreactor must maintain a sterile culture of cells in medium conditions that maximize cell growth and productivity. A number of complex interactions (physical, chemical and biochemical) exist in a bioreactor. These include:

- i) Mixing and aeration.
- ii) Oxygen demand and supply.
- iii) Cell growth and product formation kinetics in relation to the nutritional requirements of the cells.
- iv) Waste and toxic factor accumulation.
- v) Detrimental effects to the culture due to mixing or aeration devices

This chapter reports the development and use of a membrane bioreactor and a trickle bed bioreactor as supports for immobilised cultures of *T. versicolor* to facilitate the growth of the fungus, laccase production and effluent bioremediation.

4.1.1 Transverse flow hollow fibre bioreactor (TFHFBR)

Previous studies by Burton *et al.*, (1996) and Leukes *et al.* (1995) have shown that the white-rot fungus *Phanerochaete chrysosporium* immobilised on capillary membranes achieves a steady state of growth after a certain period of time. This concept has been termed the membrane-gradostat concept and is illustrated in Fig 4.1. Fungi immobilised in this manner reach a steady state and may be manipulated, either for the continuous production of secondary metabolites, or possibly for bioremediation purposes. The rationale behind the gradostat concept is that depletion of nutrients from the growth medium as it perfuses across the membrane into the biofilm creates a radial nutrient gradient. This means that the biomass closest to the lumen of the hollow fibre membrane is in contact with a nutrient rich environment, while biomass that is radially distant from the lumen is nutrient limited;

there is a concurrent oxygen gradient decreasing towards the lumen. This induces a switch to stationary growth and secondary metabolism with the accompanying increase in enzyme production. This biomass eventually dies and is sloughed off the outside of the biofilm by an air stream. The continuous formation of new biomass at the edge of the lumen and the continuous enzyme production and sloughing off on the outside, lead to a stable, steady state equilibrium. Essentially this concept converts the normally temporal events of a typical growth curve in batch culture into spatial events resulting in continuous production of enzyme over time. This concept was applied, in this study, to determine the suitability of a gradostat reactor for *T. versicolor*-mediated bioremediation.

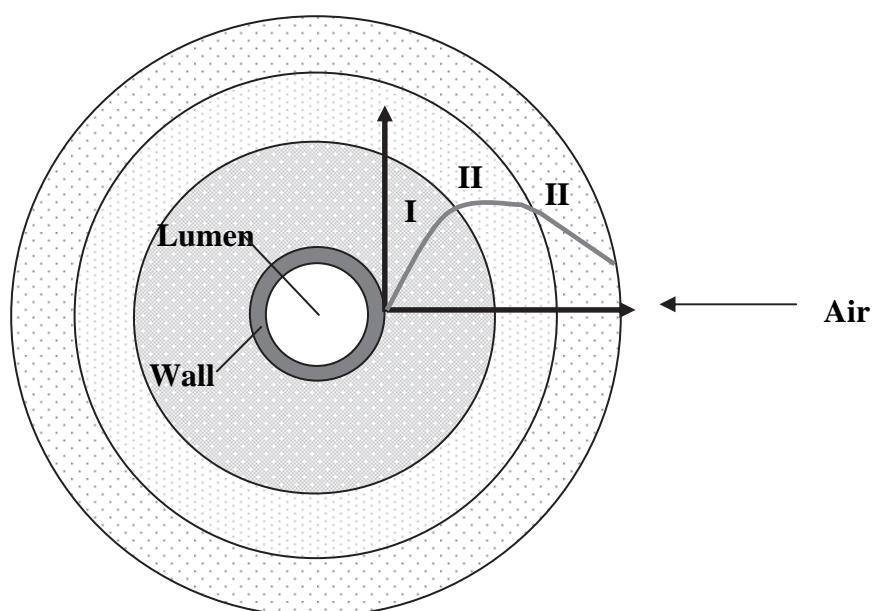


Fig 4.1 The gradostat concept developed for continuous production of secondary metabolites from white rot fungi. Nutrient gradients are established over space instead of time as in the case in batch culture. Exponential growth (I), stationary (II) and decline (III) phases are the same as for batch cultures except that these phases occur over space instead of time so that part of the culture is continuously in secondary metabolic phase. There is no lag phase.

4.1.2 Trickle Bed Bioreactor (TBR)

TBRs consist of a porous bed of an organic or inert synthetic material that supports a biofilm. A liquid nutrient scheme is introduced into the bed via a nozzle at the top of the reactor and flows downward over the biocatalyst (immobilised fungus) surface as a film within a gas continuum. In the configuration used in this study, humidified air was pumped from the bottom of the reactor, counter current to liquid whereas traditionally it is introduced co-currently. This system is advantageous in that it can be packed into tall towers, which gives it plug-flow characteristics unless, of course, a

recycle is introduced. TBR's are inherently continuous in operation and are used mainly in the petrochemical industry for hydrogenation-based reactions and in bioremediation for gas stripping. Although these reactors generally have low operating costs, they can become gas-liquid mass transfer-limited at high flow rates (Stitt, 2002).

There has been a previous attempt to produce lignolytic enzymes in a TBR. Ruggeri and Sassi (2003) reported on the growth of and LiP production by *P. chrysosporium* in a 500cm³ TBR with an aspect (H/D) ratio of 17. These authors found that the superficial velocity of the feed gas controlled the oxygen mass transfer to biomass, but also that the liquid flow regime played an important part in the oxygen supply pathways. A more direct contact between gas and biotic phase increased reactor productivity. The use of an alternating continuous/pulsed flow regime was used to achieve this to an extent. Pulsed flow increased the variability of the liquid film thickness and hence the portion of biomass directly exposed to the gaseous phase. Ruggeri and Sassi (2003) reported further on the advantages of the system in terms of ease of product separation. In order to evaluate support matrices for potential use in an Airlift Loop Reactor (ALR), and also as an alternative reactor system, a trickle filter reactor was developed for the growth of *T. versicolor*. The supports tested in this study included Oasis® florists' material, silicate pebbles used in hydroponic growth systems and woven glass beads (Dennert Poravar GmbH).

4.2 Methods

4.2.1 Transverse flow hollow fibre bioreactor (TFHFBR) set- up

Membrane modules were received from the Institute for Polymer Science of the University of Stellenbosch. The reactors were built on a frame of stainless steel rods and made watertight by the addition of greased gaskets between the module and the manifolds.

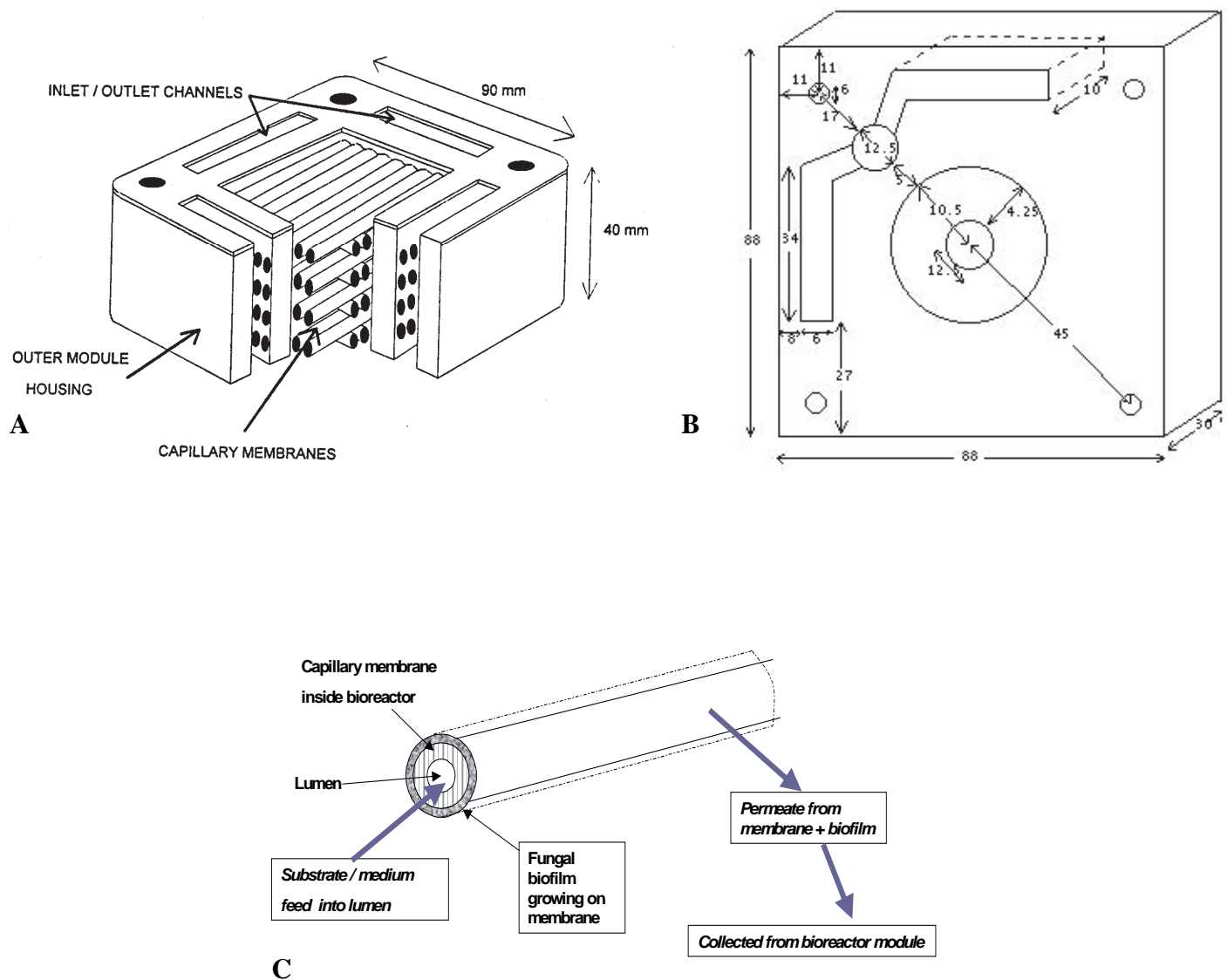


Fig 4.2 Modular components of the TFHFBR: (A) Transverse flow membrane module (B) Polyurethane manifold with central mass dissipater (C) Single hollow fibre membrane (Leukes, 1996, Burton *et al*, 1998, Edwards 1999).

4.2.2 TFHFBR system configuration

The TFHFBR was operated in a dead end filtration mode in the configuration shown in Fig 4.3. The air was humidified by passing it through distilled water and pumped through the system by an aquarium pump. A peristaltic pump circulated the medium. The membrane module was initially pre-treated with Milli-Q water to remove the glycerol wetting agent. Feed flow rates were determined and adjusted to 1mL/h.

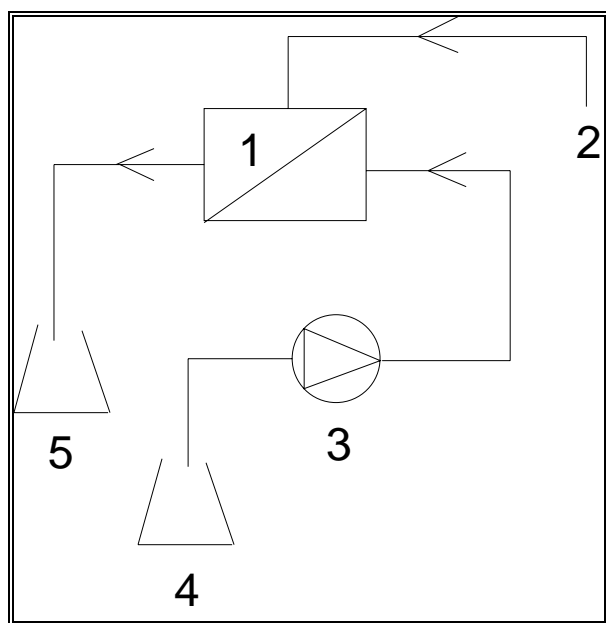


Fig. 4.3 Schematic TFHFBR set-up indicating dead-end ultrafiltration configuration (1= Transverse flow module (TFR), 2= air line, 3= peristaltic pump, 4= medium reservoir, 5= permeate reservoir)

4.2.3 TFHFBR operation

The TFHFBR was chemically sterilised by running 4% (v/v) formaldehyde through the system overnight. Formaldehyde solution was rinsed from the HFBR with autoclaved Milli-Q water. The 500mM inoculum was prepared as for flask culture and decanted into a modified 1 L Ehrlenmeyer flask with an inoculation port at the base. The TFHFBR was inoculated from the shell-side using the airline in dead-end filtration mode with a peristaltic pump. The inoculum was forced into the module until a backpressure of 150 kPa was reached (the maximum safe pressure of the system). The pump was then switched off until the pressure subsided to approximately 130 kPa at which point the cycle was repeated. The reason for doing this was to force the homogenised mycelia onto the hollow fibres and so initiate attachment. Growth medium was run into the lumen and then left overnight. The fungal mycelium grows towards the growth medium in the lumen and in so doing the biofilm attaches to the hollow fibres. Thereafter feed flow rates were determined and adjusted to achieve a flux of 0.177 L/m²/h. The TFHFBR was operated for 20 days in a dead-end filtration mode with the substrate perfusing through the lumen and the immobilised biomass situated on the shell side of the hollow fibre membranes. On day 20, *p*-cresol (0.1 mM) was added to the reactor feed. The reactor permeate was assayed daily for changes in pH, oxidation-reduction potential and laccase activity.

4.2.4 TBR set-up

The TBR vessel used had a height of 400mm and an internal diameter of 70mm. The air was introduced through a sparger at 40mm and a sampling/drain port inserted at 20 mm. Four lengths of glass tubing (I.D. 8mm) were inserted into and through a rubber stopper, these acted as droppers through which the growth medium and pollutant was introduced (Fig 4.4).

4.2.5 TBR operation

The reactor vessel was filled with support matrix until a headspace of 75 mm was achieved. The stopper was then fixed firmly in place and the entire reactor, including all feed and airlines, was sterilised as a unit by autoclaving. A 1 L inoculum was prepared as for flask culture and poured aseptically into the reactor. This was left in the vessel for 24 hours to allow fungal attachment. After this time, the drain valve was opened and the residual inoculum wasted.

The feed line attached to the droppers was then attached to a medium reservoir and growth medium was pumped into the reactor at a flow rate of 10 mL/h. The stopper was periodically rotated in an attempt to avoid channelling of the medium down the length of the reactor. After 7 d of operation in this fashion, the growth medium was replaced with one made up in 20 % cresylic effluent. This medium was recycled after passage through the reactor. Samples of reactor permeate were taken daily at the sampling port and assayed for laccase activity and residual cresols as required.

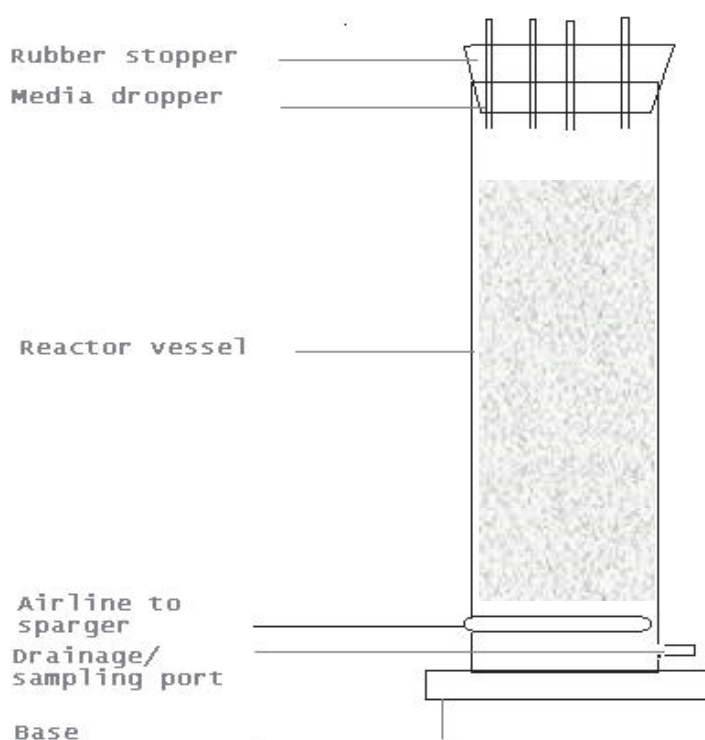


Fig 4.4 Schematic diagram of the TBR used in this study

4.2.6 SEM preparation

Sections of hollow fibre and glass bead with attached biomass were incubated in 2.5% glutaraldehyde at 4 °C for 90 min to cross-link the biofilm and prevent detachment from the support matrix. Thereafter preparation as in previous chapter.

4.3 Results

4.3.1 Growth in the TFHFBR

Membrane bioreactors designed specifically for microbial cell immobilisation are now used in a number of biotechnological processes, often in extraction applications. The first report of membrane use in biological wastewater treatment was made in 1969 and since then the successful treatment of numerous priority pollutants has been reported, for example, Luke and Burton (2001). The hollow fibres used in this system present a compromise between solid and liquid growth medium combining the best of both environments by providing a secure point of attachment and access to readily utilisable nutrients in the form of liquid growth medium. The reactors were inoculated and run initially with growth medium only, Fig. 4.5 shows the changes in pH and

oxidation-reduction potential in the reactor permeate over time. Both these parameters are indications of fungal activity; one of the survival strategies of WRF is to lower the pH of their environment. Significant alterations in these values were observed after the airflow was increased from 1 L/min to 2 L/min on day 9: pH decreased and redox potential increased. Addition of 0.1 mM *p*-cresol on day 20 had the effect of reversing these changes.

It is likely that increasing the airflow increased the growth rate of *T. versicolor* in the reactor, as increased oxygen tension is known to stimulate the growth of WRF. The addition of *p*-cresol retarded fungal growth, as measured by the pH and redox activity indicators. Laccase activity was measured in the permeate samples of days three to nine, showing a maximum of 0.7 U/mL. There was no measurable laccase activity in the permeate after the *p*-cresol was added to the medium reservoir. This may have been coincidental with the increased airflow in the reactor as White and Boddy (1992b) showed that maximum enzyme activity produced by *T. versicolor* in solid state was achieved at an environmental oxygen concentration of only 5 %. These authors further correlated decreased fungal growth activity with insufficient airflow, and this may be another switch that induces secondary metabolism and enzyme production. HPLC analysis of the permeate after day 20 showed no remaining cresol in the permeate but an increase in the concentration of cresol reaction products.

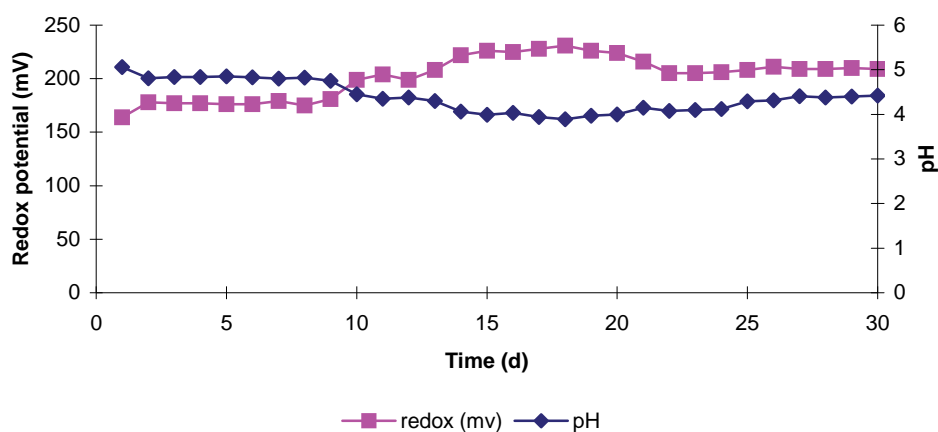


Fig 4.5 TFHFBR history showing changes in pH and oxidation/reduction potential

Two further reactors were configured, inoculated and operated with growth medium only for 7 days. After this time, different (1mM, 2.5 mM, 5mM and 7.5 mM) concentrations of *p*-cresol were added to the medium reservoir of both. The reactor permeate was assayed daily for residual cresol. Cresol was continuously and completely removed up to a concentration of 5mM, above which there appeared to be a breakthrough point (Fig 4.6). The 7.5 mM *p*-cresol feed was initially all removed, representing a removal rate of 0.0486 g *p*-cresol/g biomass/d but over time increasing amounts were observed in the reactor permeate. Continuous dosing at this concentration was apparently above the threshold of the fungus in this reactor configuration, possibly due to a build-up of toxic intermediates.

While investigating the effects of biocarriers on the production of lignin-degrading peroxidase activity in *P. chrysosporium*, Shim and Kawamoto (2002) found that a static culture, immobilised on a 'Biostage' support had distinct advantages over free cultures in terms of (1) better growth of fungi (2) higher enzyme activity; and (3) longer lasting enzyme activity. These authors ascribe these effects to a lack of oxygen supply to the free fungal cells in submerged culture, a result being that the cells were prone to sedimentation. This immobilised method of incubation was found to increase the contact area between cells and the sparged oxygen without increasing shear. Shuttleworth and Bollag (1986) found that the most promising alternative to direct enzyme treatment to transform phenols was to immobilise the mycelia in such a way as to provide continuous production of oxidative enzymes, as has been achieved in this study.

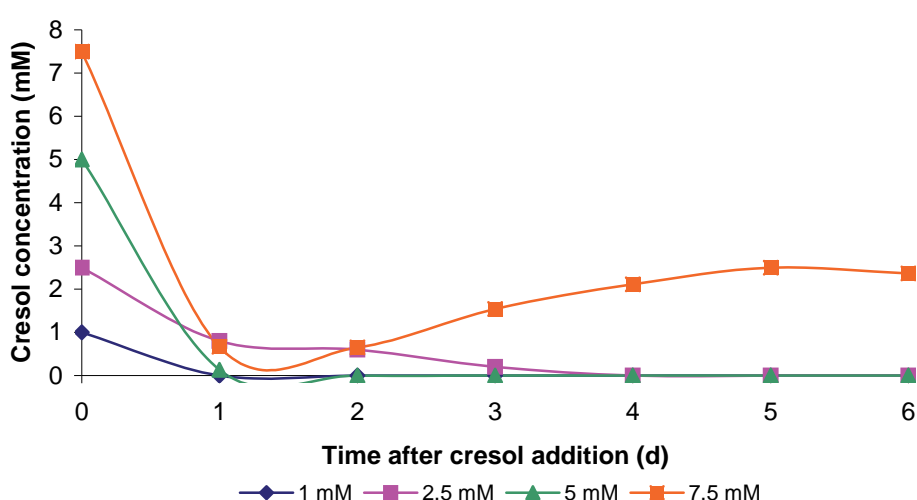
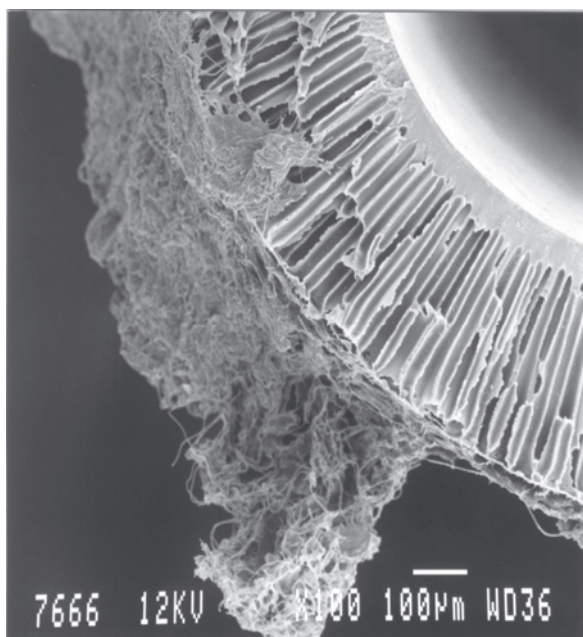
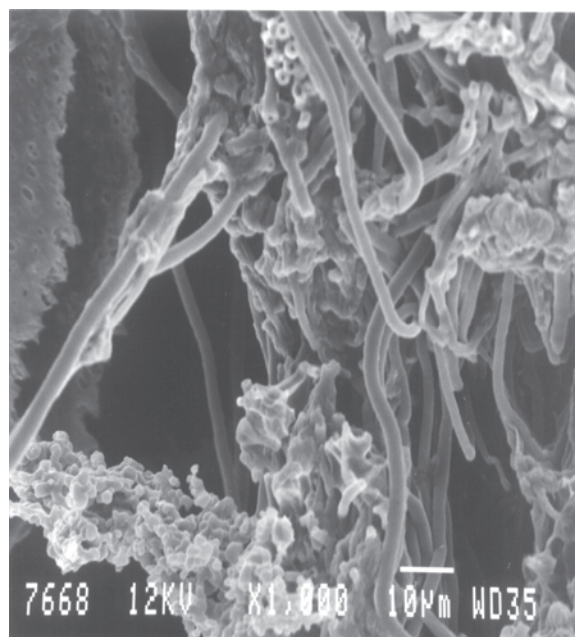


Fig 4.6 Removal of different concentrations of cresol by *T. versicolor* immobilized in a TFHFBR

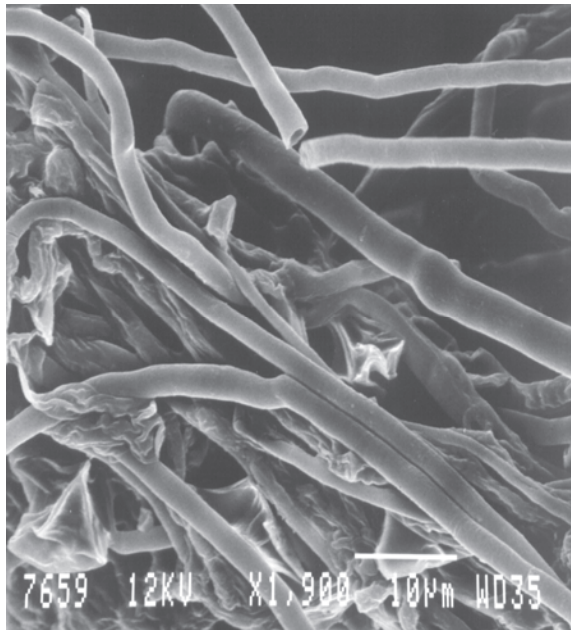
The TFHFBR represents an attempt to mimic conditions encountered by fungi in nature and promoted fungal growth at the solid/air interface that these organisms have evolved to colonise. To ascertain whether this environment was achieved in the reactor it was necessary to visually inspect the growth morphology of *T. versicolor*. The TFHFBR was sacrificed and sections of the hollow fibre membranes examined using scanning electron microscopy (SEM). The SEM photographs show growth on the membrane (Fig 4.7A) and that the differentiation within the biofilm of *T. versicolor* grown on hollow fibre membranes also exhibited the developmental variation reported for growth on wood. The biomass closest to the lumen displayed the characteristic fast-effuse growth associated primary growth phase under optimum nutrient conditions with binding and skeletal hyphae the dominant morphs (Fig. 4.7B, C). The next region in the cross section is dominated by generative growth, where the biomass is highly branched and has become gelatinised an indication of slow-dense growth (Fig 4.7D). The outside edge of the biofilm is characterised by less dense, desiccated hyphae in the process of being sloughed off (Fig 4.7E).



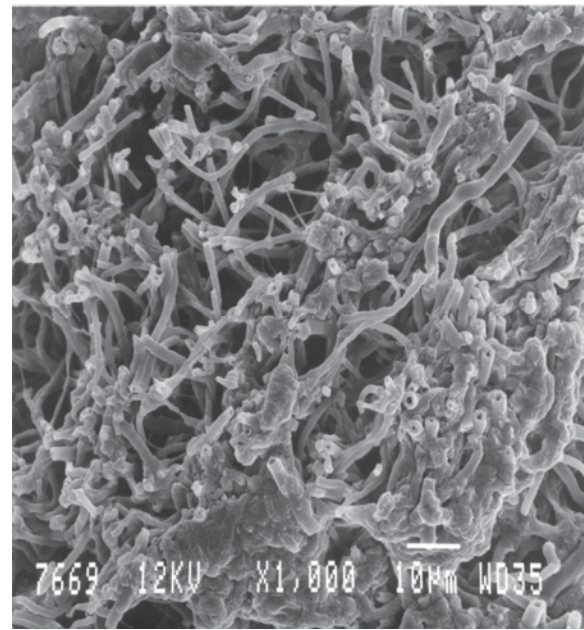
A



B



C



D

Fig 4.7 Cross section of a *T. versicolor* biofilm attached to a tubular membrane

A) Hollow fiber membrane with attached biofilm of *T. versicolor*

B) Biomass near the membrane wall, loose growth with a predominance of skeletal fibers

C) Linear, thick, skeletal hyphae near the membrane wall indicating rapid primary growth.

D) Generative hyphae close to the outside of the biofilm, showing some gelatinisation

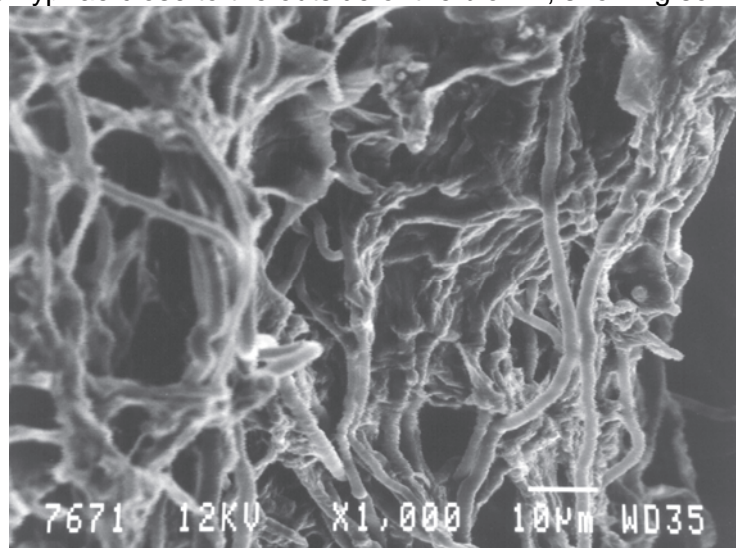


Fig 4.7 E) Outer edge of membrane-bound biofilm: biomass becomes more diffuse and desiccated and eventually sloughs off

These results support the gradostat concept and show clearly that the capillary membrane did provide the nutrient gradient it was expected to. In any long- term operation of a fungal bioreactor, a number of operational problems such as: increase of broth viscosity, formation of mycelial aggregates, electrode fouling and clogging

often occur. These are caused by the uncontrolled morphology and growth of mycelia that limits biomass transfer and hampers performance (Moreira *et al.*, 1998). The configuration of the TFHFBR and the growth morphology that it promotes ameliorate these problems to some extent. The thickness of the biofilm is limited by the perfusion of the growth medium from the membrane lumen; the dead biomass is continuously sloughed off and washed out of the reactor. Therefore the reactor contains only living, productive biomass and successfully maintains stable conditions for secondary metabolism and controls fungal growth. This enables constant enzyme production and phenolic degradation.

4.3.2 Growth of *T. versicolor* in a TBR

The growth on the glass beads was dense, white and covered all the available surfaces. When the beads in the trickle filter were fully covered, new growth medium was added to the reservoir and samples of the permeate were assayed for laccase enzyme activity. After a growth period of 7 days, the growth medium in the reservoir was replaced with 20% vol/vol cresylic effluent (made up in growth medium). The addition of the effluent resulted in a substantial increase in laccase activity (Fig 4.8). The fungus became slightly discoloured, with brown residue forming on the beads. A similarly coloured pellet was formed when samples taken from the reactor were centrifuged. The components of the pellet were assumed to comprise of polymerized phenolic monomers.

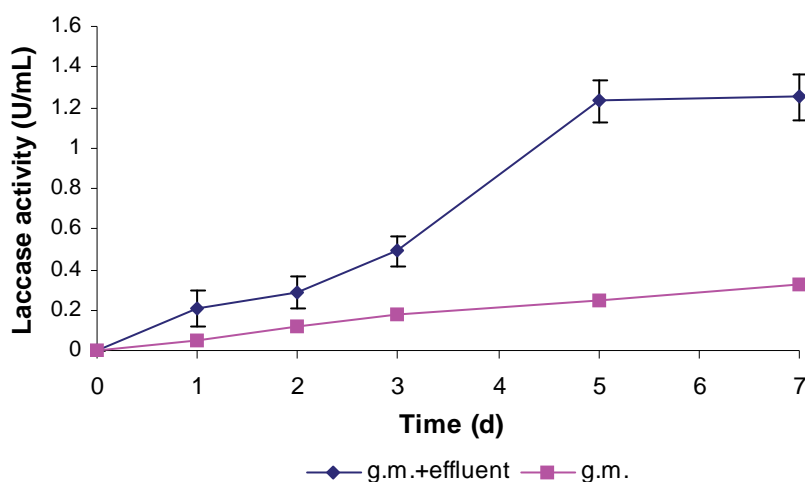


Fig 4.8 The induction effect of cresylic effluent (20%) added to *T. versicolor* immobilised in a TBR. The growth medium containing the effluent was employed after the normal medium

It is interesting to note that a high effluent concentration (20%) resulted in an increase of secreted laccase enzyme (Fig 4.8) whereas in unimmobilised cultures, this concentration retarded both growth and enzyme production. These results are in agreement with those of Nakamura *et al.* (1999) who compared free versus immobilized culture conditions for another widely utilized WRF, *Bjerkandera adusta*. No significant differences in cell growth and glucose utilization were observed, but a significantly higher enzyme activity, coupled to more extensive delignification activity, was reported for the immobilised cultures. This result confirmed the suitability of the glass support for growth of *T. versicolor* and the advantages of biomass immobilization in terms of resistance to toxic compounds. The medium containing the effluent was recycled for the duration of the experiment and all the phenolic monomers had been removed from solution by the end of the operational period (Table 4.1).

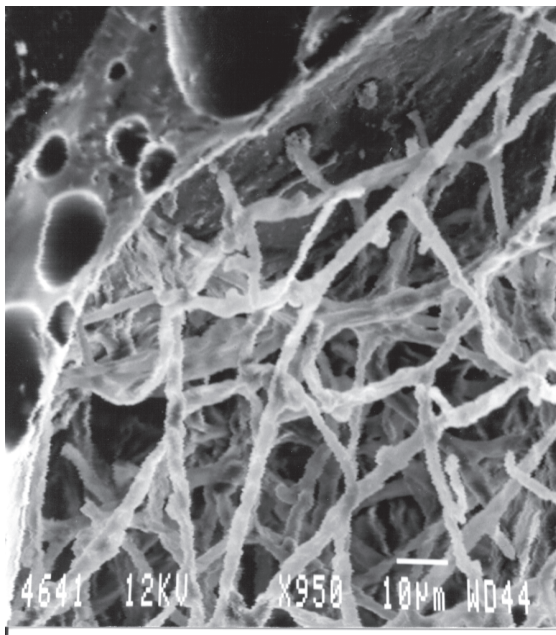
Table 4.1 Rate of removal of phenolic effluent monomers in a TBR

EFFLUENT MONOMER	REMOVAL RATE (g/L/d)	BIOMASS ASSOCIATED REMOVAL g/g biomass/d
Phenol	0.524	0.031
<i>p</i> -cresol	0.180	0.010
<i>m</i> -cresol	0.196	0.011
<i>o</i> -cresol	0.546	0.031

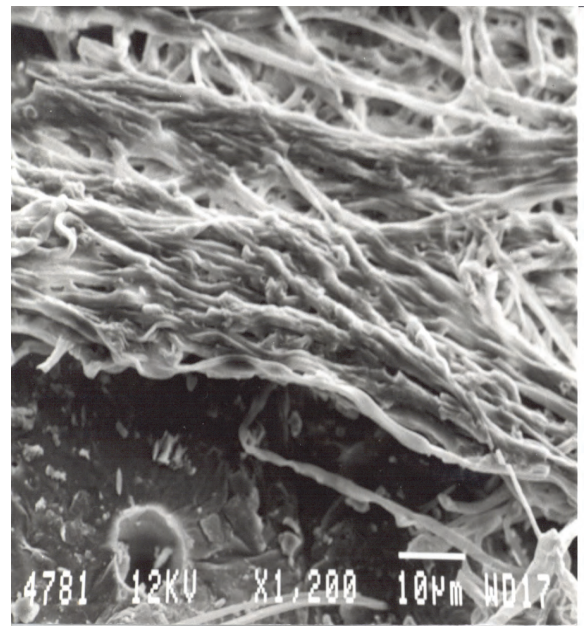
Closer inspection of sections of the glass beads by scanning electron microscopy (SEM) showed thick, linear hyphae covering individual beads with a continuous fungal film of fungi (Figs 4.9B, 4.9C). The hyphae had penetrated and attached to the glass beads (Fig 4.9A) and had formed septate clamp connections which only occur during generative (healthy) growth. The linear hyphae indicated rapid growth and were not covered in polysaccharide, as would be expected under conditions of stress (Fig 4.9B). These factors indicate that the culture was growing well, even after being exposed to 20% cresylic effluent for 7 days. The glass beads used in the support were uniformly covered with a mat of fungus. In Fig 4.9 C the mat had pulled away slightly from the support, probably due to the critical point drying process in the EM preparation, but nevertheless can be seen to be continuous.

Due to the hydrophobic nature of the glass that makes up the beads and their high porosity, the fungus formed a secure attachment to the porous glass support. The anchoring effect of the glass beads and the ability of the fungus to withstand up to

20% concentration of the cresylic effluent while attached to them indicate that these beads are a suitable support for the growth of *T. versicolor*. Pallerla and Chambers (1997) observed similar results in a study on the bioremediation of foul condensates from a pulp and paper plant, where immobilised *T. versicolor* was found to withstand the inhibiting nature of the effluent more than was observed in batch culture. The authors showed that immobilised cells were able to tolerate higher concentrations of toxic compounds and degrade higher concentrations of reactants as compared to free cells.



A



B

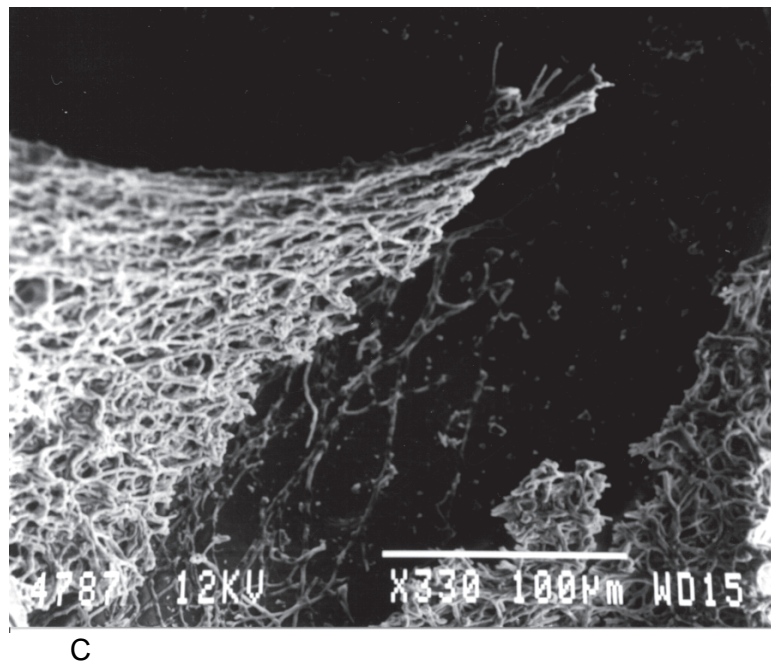


Fig 4.9 Fungal morphology and attachment in a TBR. A) SEM image of *T. versicolor* hyphae attached to, and growing on, woven glass beads in a TBR; B) SEM image showing thick bunches of linear hyphae colonizing the surface of a glass bead; C) SEM image showing a continuous mat of *T. versicolor* covering the glass support.

4.4 Conclusions

- The TFHFBR promoted good fungal growth and differentiation according to the gradostat concept for the production of secondary metabolites.
 - The system facilitated the removal of cresol up to a concentration of 7.5 mM (0.05 g/g biomass/d) although there was very little measurable free laccase in the reactor permeate.
 - The low flow rates associated with this reactor configuration and the difficulty of scale-up would impede its application in a wastewater treatment role
 - A good application of this system could be in low volume, high value biotransformations of phenolic substrates by fungal oxidases (e.g. antioxidants) or in the production of valuable secondary metabolites from other filamentous organisms.
- The TBR facilitated excellent growth when operated with a culture of *T. versicolor* immobilised on woven glass beads.

- The immobilised fungus tolerated and removed high (20%) concentrations of cresylic effluent in the reactor configuration and produce over 1 U/mL/d of laccase.
- An obvious advantage of the TBR is the almost complete lack of shear forces and the potential for excellent oxygen mass transfer; two
- This system represents a low maintenance alternative to other bioreactor configurations. Its resistance to high concentrations of effluent presents a possible application for the TBR as pre-treatment for another system to buffer possible variations in effluent strength (shock loading).
- A possible improvement to the TBR design would be to introduce baffles at intervals along the length of the reactor vessel to break up the paths and prevent the channelling of liquid that is often inevitable in these systems.
- Although this bioreactor system performed at least as well as anticipated, the growth of *T. versicolor* was slow compared to agitated free culture and enzyme production was lower.

CHAPTER 5 : DEVELOPMENT OF BIOREACTORS (PART 2) - AIRLIFT LOOP REACTOR (ALR) FOR LACCASE PRODUCTION AND BIOREMEDIATION BY *TRAMETES* SPECIES

5.1 Introduction

Although reasonably effective as bioremediation tools, both the membrane gradostat reactor and the trickle filter had shortcomings in their potential for large volume wastewater treatment due to difficulty in up-scaling them. Airlift reactors have been found suitable for the fermentation of filamentous microorganisms due to the low shear, highly oxygenated environment they provide. Furthermore, the upscale of these reactors is an established field of study. Therefore an investigation of an ALR system seemed to be the next logical step, as these reactors are understood to have potential in fungal bioprocesses. They provide a well-mixed, highly oxygenated environment, with well-defined flow pattern and high liquid velocities that occur despite an absence of a mechanical agitator and can create an optimal environment for many productive microorganisms (Klein *et al.*, 2002).

5.1.1 Bubble recirculation and gas holdup in ALRS

Siegel *et al.* (1986) described the existence of different flow regimes in internal-loop airlift reactors, which depended in the design of the gas-liquid separator and the liquid velocity in the downcomer. More recently Heijnen *et al.* (1997) described the existence of three distinct regimes. At very low air input rates, no air bubbles are present in the downcomer as the liquid velocity in the downcomer is lower than the average slip velocity of the air bubbles in the liquid, this is referred to as *heterogeneous flow*. The liquid velocity increases rapidly when the superficial gas velocity is increased until the liquid velocity in the downcomer is equal to the slip velocity of the air bubbles (bubble slip velocity is the difference between the linear velocity of the gas and the liquid in the column). This results in stationary air bubbles in the downcomer i.e. the velocity of the bubbles relative to the reactor is zero. The downcomer can be either partially or completely filled with bubbles, called *transition flow*. As the superficial gas velocity is further increased, the liquid velocity in the downcomer becomes higher than the slip velocity of the air bubbles and the bubbles are recirculated with the liquid from the downcomer into the riser, called *uniform bubbly flow*. Gas holdup, the volume fraction of gas in the dispersion, in the riser can be as high as 20%. Knowing the relationships between gas holdup and superficial gas velocity is important because it determines liquid circulation velocity. On this basis, internal loop ALRs can be designed and the operational parameter, superficial gas velocity can be coupled with mixing time and gas liquid transfer rate (van

Benthum *et al.*, 1999). The head region of the airlift reactor can be designed to ensure near complete gas liquid separation, so that the downcomer gas holdup is zero, ensuring optimal liquid circulation. This design (for an internal loop reactor) is based on the principle of reducing the downward liquid velocity in the entrance of the downcomer to less than the rise velocity, if the bubbles are to be kept out of the downcomer. This can be achieved by expanding the cross-sectional area of the downcomer in the head region of the reactor. Care must be taken to avoid boundary layer separation and the formation of stagnant regions (Chisti and Moo-Young, 1993).

5.1.2 Effect of sparger aperture size (porosity)

In bubbly flow, bubbles rise in the riser and descend in the downcomer independently, with fairly uniform spacing between them, and therefore, holdup rises almost linearly as gas velocity increases. In transition flow the uniform bubble swarms begin to meander, revealing the formation of small eddies in the liquid. The change from uniform to transition flow is gradual, and has been attributed to various factors that affect the size of the bubbles by altering the degree of coalescence and therefore their rise velocity (Contreras *et al.*, 1999). Because spargers with larger pore sizes produce bubbles with greater diameter coalescence begins at lowest gas velocity with these spargers. The differences in circulation time in the uniform bubbly and transition flow regimes are directly related to downcomer holdup. In these two flow regimes the recirculation of gas to the downcomer is highest for spargers with the lowest pore diameter, producing the smallest bubbles. This implies lower rise velocities and thus greater gas recirculation. When pore size increases, both the mean size of the bubbles and their rise velocity increase, the gas recirculates less so the difference between riser and downcomer densities increases, and therefore, liquid circulation time is decreased. In heterogeneous flow, turbulence in the bulk fluid controls the hydrodynamic behaviour. This means that bubble size is ultimately not determined by the porosity of the sparger but the degree of coalescence and therefore holdup and liquid circulation time are similar for all spargers used (Contreras *et al.*, 1999).

Reactor performance in ALRs is strongly affected by the coalescence and viscosity of the liquid, and these factors often change considerably during batch culture (Poulsen and Iverson, 1999). Since both gas-liquid mass transfer (optimised using small bubbles) and mixing (when larger bubbles are required) are driven by the gas stream, the bubble size is an important variable. Bubble size is determined by

sparger design, liquid properties, and reactor geometry. In coalescent liquids the equilibrium bubble size (4-6mm in water) is obtained very rapidly, and the formation of smaller bubbles at the sparger has no influence on the actual bubble size. In noncoalescent liquid the actual bubble size is very close to the size at the sparger (Poulsen and Iverson, 1999).

Poulsen and Iverson (1999) developed a rubber membrane sparger, which produced small bubbles, giving high mass transfer. They then designed a combined membrane - ring sparger with unique features with respect to efficiency of utilisation of energy and substrate gases. Their experiments showed that the small bubbles from the membrane sparger did not coalesce with the large bubbles from the ring sparger. In the dual sparging mode the energy input for mass transfer and mixing is divided. Therefore, the energy consumption can be minimised if the flow distribution through the membrane and ring sparger is controlled by the oxygen demand and the inhomogeneity of the culture medium respectively. In addition it would be possible to deliver a hazardous or expensive gas quantitatively to the liquid through the membrane sparger (Poulsen and Iverson, 1999).

5.1.3 Shear forces in ALRs

Gas-liquid mass transfer rate depends strongly on the hydrodynamics near the gas-liquid interface of a system; therefore, interfacial shear rate affects mass transfer (Contreras *et al.*, 1999). In most chemical processes, the shear rate is not important in itself, except as a means of increasing heat and mass transfer. This is not the case in less robust biological systems such as microbial plant and animal cell culture. Excessive shear is known to damage suspended cells, leading to loss of viability and even disruption. However, a certain degree of shearing is required to attain sufficient mass transfer rates and to achieve a homogenous distribution of transferred components into the bulk fluid and to prevent anoxic dead zones in the reactor. Consequently, bioreactors that provide a gentle culture environment are in increasing demand. One of the principal reasons for the success of ALRs is the mildness of the shear effects on the particles suspended in the liquid. This mildness is due to homogeneity of the shear field and the absence of zones of excessive turbulence (high shear regions) in this type of reactor, in contrast to STRs where the energy input is highly localised (Merchuk and Berzin, 1995). The more ordered, less turbulent flow seems to have a positive effect on the yields of shear sensitive cultures.

The shear stress in the liquid of each region of an ALR can be defined as equal to the energy dissipated divided by the mean path of circulation in the region and by the sum of the area of all the bubbles (Merchuk and Berzin, 1995). It is well known that energy is dissipated in ALRs by two main mechanisms: wall friction and bubble associated dissipation. Merchuk and Berzin (1995) confirmed that the energy lost at the walls of the reactor is a minor contribution. Therefore, the energy dissipation in the bulk of the liquid is unequivocally related to the bubbles present in the system and gives a global shear rate related to the interfacial area of the dispersed gas, which is in turn related to the mass transfer of the system. They conclude that this global shear rate is a function of both fluid dynamics and rheology and can be calculated for each region in the ALR. It increases sharply at low gas flow rate and then remains almost constant, with the riser playing a predominant role as long as the bottom clearance is sufficient.

The shear rate behaves differently in different flow regimes. In bubbly flow, the shear rate increases slightly with increasing gas flow rate and tends to flatten out in the heterogeneous flow regime. At the low gas velocities, the spargers with the lowest pore sizes produce the lowest shear rates, at very high velocities shear rate become independent of pore size (Contreras *et al.*, 1999). The highest shear rates occur in the bottom zone, followed by the gas-liquid separator and the riser, showing the importance of the bottom zone and separator in overall behaviour and scale up of this type of system.

The lowest shear rates are found in the downcomer where turbulence is minimised. Shear rate is also strongly related to the characteristic length or internal scale of the micro-eddies found in the bulk fluid. Shear rate declines as the eddy length increases. For a fixed characteristic length the shear rate decreases when pore size i.e. bubble size, diminishes. This indicates that the smaller the eddy sizes the greater the shear rate and thus hydrodynamic stress and the possibility of cell damage (Contreras *et al.*, 1999).

5.1.4 Mass transfer in ALRs

Gas-liquid transfer is often a limiting factor in many aerobic fermentations; therefore the determination of the volumetric mass transfer coefficient, K_La , is an important factor in the design and operation of ALRs. Despite many previous attempts to model the rate of mass transfer at the gas-liquid interface, it is not yet possible to give a clear-cut theoretical explanation of experimental data relating to K_La (Ayazi Shamlou

et al., 1995). This is due to the abundance of parameters that are known to influence the volumetric mass transfer coefficient and the complex interactions that exist among them. Experimental measurements show that the rate of mass transfer in an ALR is influenced by the physicochemical properties of the two-phase mixture, the superficial gas velocity, the gas hold-up and the liquid circulation velocity (Ayazi Shamlou *et al.*, 1995). In general, for draught tube, internal loop ALRs there is no influence of either the presence of a draught tube or of its relative area with respect to the annulus on the overall gas holdup i.e. they can be related to simple bubble columns. This does not mean, however, that K_La is the same. The enhanced K_La observed in ALRs is explained by of the different bubble size distributions that occur in bubble columns and ALRs under otherwise identical conditions (Chisti and Moo-Young, 1987).

When a population of bubbles reaches the disengagement zone at the top of the riser, the larger bubbles separate relatively easily from the liquid and therefore the bubbles recirculating in the downcomer at the top are likely to have a lower diameter than those in the riser. These bubbles can subsequently grow through coalescence further downstream in the downcomer. The gas holdup and liquid circulation velocity in the downcomer are in fact lower than that of the riser, but the K_La in the upper portion of the downcomer exceeds that of the riser. In the lower part of the downcomer the K_La values are comparable to those in the riser. It appears, therefore, that the effect of bubble diameter on the volumetric mass transfer coefficient dominates over the combined effect of gas holdup and liquid circulation velocity (Ayazi Shamlou *et al.*, 1995). For the lower gas velocities spargers with small pore sizes produce the highest K_La with the lowest global shear rates. The smallest pore sizes are more efficient for mass transfer because the small size of bubbles is conserved in the reactor is conserved in the reactor, rendering a high interfacial area (Contreras *et al.*, 1999).

In this chapter the development of an ALR reactor system employing a whole cell culture of *T. pubescens* to remove phenolic compounds from a coal gasification plant is described. Although these reactors were tested initially with *T. versicolor*, when the *T. pubescens* strain became available, the elevated laccase production and superior growth rates of this strain indicated that it would provide a better agent for a bioremediation bioprocess.

5.2 Methods

5.2.1 Culture and growth conditions

Trametes pubescens (CBS 696.94) was maintained on 2% malt agar plates. The culture medium used contained 10g/L glucose and peptone and 2g/L KH_2PO_4 .

5.2.2 Inoculum preparation

T. pubescens from plate culture was homogenized in growth medium and used to inoculate 300mL-sterilized medium in 2L Erlenmeyer flasks. These flasks were incubated at 28°C and 175 rpm. The gyratory motion of the shaker promoted uniform pellet formation and this speed was found to produce small, dense pellets. Four-day-old pellets from these cultures were used as a reactor inoculum.

5.2.3 ALR operation

The concentric draught-tube internal loop airlift reactor used in this work was manufactured in the workshop of the Chemistry Dept., Rhodes University. The vessel had a height of 500mm, an aspect ratio (H/D) of 4.5 and a working volume of 3.5L. The draught tube had a diameter of 55mm. Initially air was supplied by an aquarium pump at a fixed rate of 2.2L/min, but this was later optimized. The reactor was filled with 3.15 L of growth medium and autoclaved. After cooling a 350mL (10%) inoculum was aseptically introduced and the air supply attached, initiating the fermentation. In the pollutant degradation experiments, a three-day initial growth period was allowed before any stripped gas liquor (SGL) effluent was added. Thereafter 2% v/v effluent was added daily to one reactor, 3.5% to a second and 5 % to a third.

5.2.4 ALR Sampling

5mL samples were aseptically drawn from the ALR and replaced with glucose-free growth medium. Samples were centrifuged to remove suspended biomass and glucose and laccase activity assays were performed on the supernatant. In the experiments where pollutants were added to the reactor, the air supply was terminated for 10 minutes to allow the biomass to settle and 100mL of cell-free culture medium withdrawn from the reactor. This volume was replaced with effluent appropriately diluted with growth medium.

5.2.5 Glucose determination

Glucose concentrations in culture media were determined spectrophotometrically by the DNS (dinitrosalicylic acid) method according to Ghose (1987).

5.3 Results

5.3.1 Optimisation of air supply rate and oxygen mass transfer

A disadvantage of high-density fermentations of filamentous biomass is the reduced efficiency of oxygen transfer in such cultures. Since oxygen is an essential nutrient for aerobic biochemical processes, optimisation of oxygen transfer in the bioreactor is essential for the successful application of these fungi. Initial experiments were conducted with a fixed airflow rate (2.2 L/min for the 3L reactor volume). However, at high cell densities, pelleted biomass was observed to accumulate in parts of the bioreactor, particularly at the base of the vessel. Measurement with a submerged dissolved oxygen (DO) probe showed that these areas were anoxic, indicating that the airflow rate being used was insufficient and needed to be optimised. All the main performance indicators of pneumatic reactors: gas hold-up, mixing time, axial dispersion coefficients for gas and liquid phases and the overall mass transfer coefficient have been correlated with gas throughput (Chisti and Moo Young, 1987). Accordingly, several flow rates were investigated. The increase in dissolved oxygen (DO) in the reactor fluid was measured for airflow rates between 2 and 5.5 L/min to determine the airflow required for optimum mass transfer (Fig 5.1A). For aeration rates above 3 L/min there was little difference in the maximum dissolved oxygen achieved in the reactor.

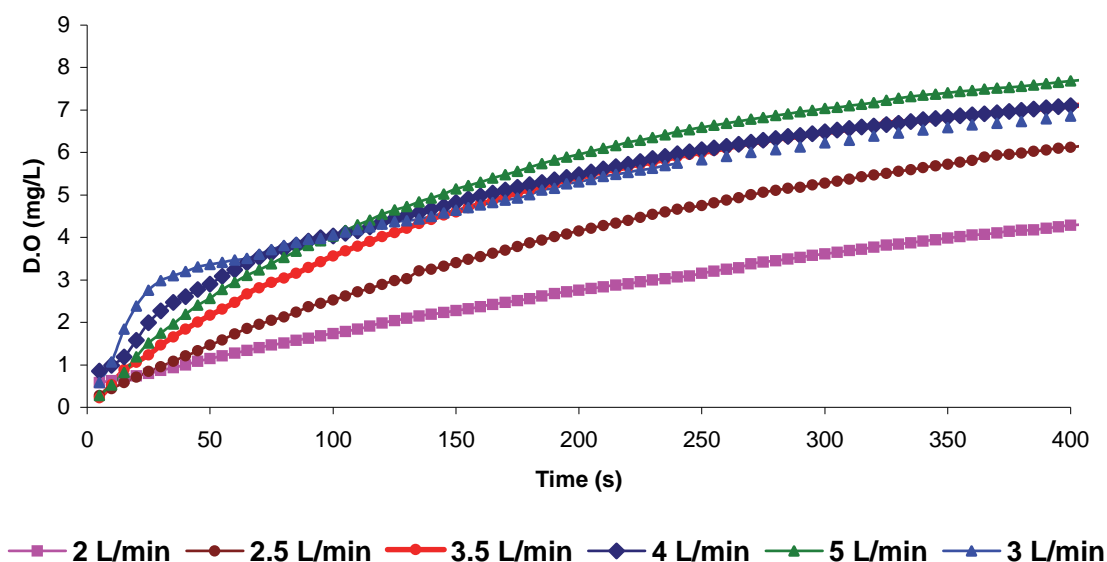


Fig 5.1A The effect of increasing airflow rate supplied to an ALR on dissolved oxygen concentrations in the reactor fluid

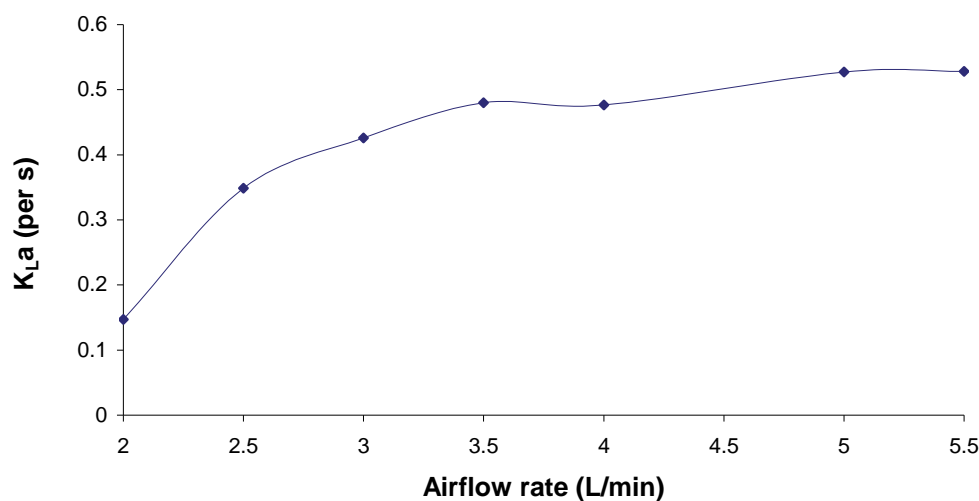


Fig 5.1B The effect of increasing airflow on the oxygen mass transfer coefficient (K_La) in an ALR

Oxygen mass transfer increased non-linearly with increasing airflow into the reactor to a maximum of $K_La = 0.52$ /s achieved at a flow of 5.5 L/min. However, a flow rate of 3.5 L/min delivered a K_La of 0.48 /s. Thus, 87% of the maximum oxygen mass transfer was achieved from only 64% of the corresponding airflow rate. From a power usage and cost benefit position, the lower flow would be preferable. These measurements were all obtained with only growth medium in the ALR. Having respiring fungi in the reactor would obviously have an effect on oxygen transfer. Su and He (1997) found that the effect of aeration rate becomes more pronounced at higher biomass loading. Testing at the maximum biomass concentration achieved in this study indicated that a gas (air) injection of 3.5 L/min was sufficient to prevent any anoxic zones forming in the reactor and all further experiments were conducted using this flow rate

The OTR in the ALR was measured at the top and bottom of the draught tube and was found to be different at different positions in the reactor. This may have been due to the different local dissolved oxygen concentrations in the liquid and oxygen partial pressures in the bubbles. Both determine the driving force of oxygen into solution. Also, different transport cross-sections are to be expected to be different, in different parts of the internal loop reactor this is due to different bubble sizes and number densities. The highest dissolved oxygen concentrations in the reactor used in this study were found at the top of the downcomer. Initially, this was surprising, as one would expect the highest concentrations close to where the air was introduced i.e.

the bottom of the riser. However this phenomenon can be explained by the fact that at the top of the riser there was an accumulation of small bubbles. There was a balance achieved in this area for a certain bubble size where their rise velocity is almost balanced by the effect of the fluid flow in the downcomer. The increased concentration of bubbles resulted in increased dissolved oxygen. The OTR was obviously also affected by airflow rate. The rate calculated from a plot of dC_{O_2L}/dt correlated very well with that obtained from a fitted model, based on K_La values, (Table 5.1) where calculated K_La values were substituted into the equation:

$$OTR = dC/dt = K_La*(C_{sat}-C_L).$$

Table 5.1 Relationship between airflow rate and maximum OTR (both calculated and experimentally obtained)

AIRFLOW RATE (L/min)	OTR (experimental) (mg O ₂ /L/s)	OTR (calculated) (mg O ₂ /L/s)
2	0.019 ± 0.005	0.0187 ± 0.002
2.5	0.036 ± 0.016	0.0352 ± 0.003
3	0.038 ± 0.004	0.0375 ± 0.004
3.5	0.07 ± 0.002	0.068 ± 0.004
4	0.083 ± 0.003	0.0801 ± 0.003
5	0.072 ± 0.004	0.0702 ± 0.003

An alternative to increasing airflow would be to increase the oxygen content of the air provided. Increasing the oxygen concentration (Y_{O_2}) of the influent air would result in a higher value for saturation concentration (C_{sat}) and hence an increase in OTR. Increasing the oxygen content to 30% (from 21% in air) resulted in an increase in specific growth rate (μ) of 40% from 0.251 to 0.422 per day. The biomass formed smaller, pellets and achieved an overall concentration of 16.93 g/L (dry mass).

5.3.2 Growth in an ALR - effects on fungal morphology

When grown in submerged culture, filamentous fungi exhibit different morphological forms, ranging from free mycelial trees to densely interwoven mycelial masses referred to as pellets. The particular form exhibited is determined not only by the genotype of the strain, but also by the nature of the inoculum as well as the physical, chemical and culture conditions (Papagianni and Moo-Young, 2002). The mechanical forces in a fermenter, which, in the case of an ALR would primarily be aeration rate, have a critical role in determining the morphology type. When discussing pellet morphology, the quantitative measurement is pellet size, which does not include the hyphae on the outer zone of the pellets that are called the 'hair'. It is known that

agitation can influence the size of the pellets and the structure of the pellets surface (smooth or fluffy). Since higher agitation will result in higher oxygen tension and more branching of hyphae, the pellets formed may be denser and stronger. Cui *et al.* (1997) proposed a shaving mechanism caused by the effects of agitation on pellet morphology. The hairs on the outer zone of the pellets are chipped off by the mechanical forces induced by turbulent flow in the fluid. The severity of the cutting is a function of hyphal strength and the magnitude of these forces. The chipped hyphae form filamentous mycelia and reseed the formation of new pellets. This shaving motion also restricts pellet size. Pellet size is thus related to energy dissipation rate, but this applies mainly to stirred tank reactors, in an ALR system the same could be said of aeration rate, as it is this parameter that provides the agitation. Therefore, in the growth phase of the fermentation in an ALR, a higher rate of aeration is required, to stimulate new pellet formation, thereafter it could be decreased to a level where it just ensures sufficient oxygenation of the biomass. Controlling aeration, therefore, is another potential method for controlling biomass formation, and may find particular use preventing overproduction of biomass.

Morphological growth forms can have a significant effect on the rheology of the fermentation broth and thus on bioreactor performance. Pelleted morphologies result in Newtonian (constant viscosity) broths and better mass transfer rates as compared to viscous and often pseudoplastic (where apparent viscosity decreases with increasing shear) filamentous broths. However, autolysis due to oxygen limitation, in pellets that become too large results in a large portion of the fungal mass being metabolically inactive. The extensive morphological differentiation exhibited by filamentous organisms is often associated with their involvement in chemical differentiation. A close relationship between a particular morphological form and increased process productivities is characteristic for industrially important fermentations e.g. citric acid and penicillin. Furthermore, pelleted morphologies are related to lower levels of formation and release of harmful proteases (Su and He, 1997; Jin *et al.*, 2002; Papagianni and Moo-Young, 2002). It is apparent therefore that mycelial aggregates should be viewed as complex differentiated tissues and not as mechanical conglomerates (Papagianni and Moo-Young, 2002)

Inoculum quality (size and age) is also of great importance in determining the outcome of filamentous fermentations (Papagianni and Moo-Young, 2002). By controlling and manipulating this factor, together with feed and aeration rates, it

should be possible to keep the pellets of *T. pubescens* from becoming oversized and from sedimenting out and so creating mass transfer and mixing problems.

5.3.3 Laccase production during fermentation of *T. pubescens*

To establish the details of growth of *T. pubescens* in an ALR, a fermentation was initiated by a 10 % inoculum and a starting concentration of 10 g/L glucose, aeration was fixed at 3.5 L/min. Figure 5.4 illustrates the relationship between glucose utilisation, biomass accumulation and product (laccase) formation for a fermentation of *T. pubescens* in an ALR. The shapes of the curves are in agreement with those of classic concentration-time profiles for batch operation. The culture had a specific OUR of 0.02 mgO₂/L/s/g biomass and plotting $\ln X/X_0$ gave a specific growth rate (μ) of 0.25 per day. The fermentation reached a maximum biomass concentration of 8.7 g/L dry mass. This result compares favourably with a report on an optimised stirred tank fermentation of *T. pubescens* where a growth (biomass) maximum of 10.6 g/L was reported after 12.5 days of cultivation, from a starting concentration of 40 g/L glucose (Galhaup *et al.*, 2002). This is a significantly lower biomass yield (0.265 g biomass/g glucose) than that achieved in the ALR fermentation in this study (0.87 g/g). Too much biomass would not always be beneficial to a process as this can lead to blockages and sludge problems. Bioreactor cultures can become too crowded and at increased cell densities some pellets can be forced into settling (Su and He, 1997).

Although Rodriguez Couto *et al.* (2002) reported that attempts to produce laccase at a bioreactor scale have been unsuccessful, Rancano *et al.* (2003) cultured *T. versicolor* in an airlift reactor with a working volume of 2 L, reporting a glucose consumption rate of 0.42 g/L/day from a starting concentration of 20 g/L and a maximum (2,5-xylydine induced) laccase activity of 1.67 U/mL achieved after 13 days of cultivation; cellular growth was not recorded. Laccase activity in the ALR fermentation in this study reached a maximum of 2.54 U/mL after 7 days without induction. It is difficult to compare data from different size reactors with different configurations and operating conditions, but the increased glucose consumption shown in Fig 5.4 (1.58 g/L/d) is certainly an indicator of an increased growth rate and the laccase activity in the ALR was 1.5 times that of Rancano *et al.* (2003), and was achieved in half the time (Fig 5.2).

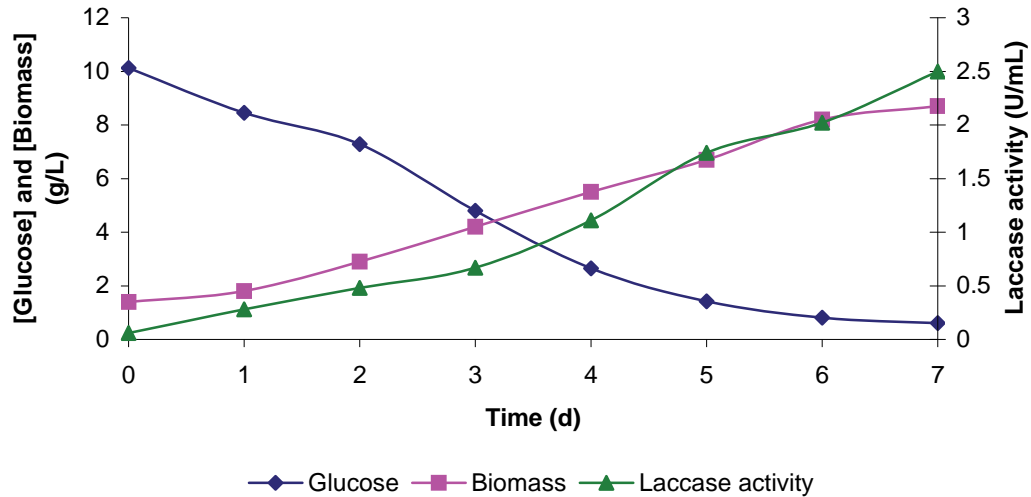


Fig 5.2 Time courses of glucose depletion, biomass accumulation and laccase production during a fermentation of *T. pubescens* in an ALR. The initial glucose concentration was 10 g/L and aeration was fixed at 3.5 L/min

An additional measure of cell growth or activity is respiration, measured as oxygen utilisation rate (OUR). OUR is dependent on both the amount of biomass and phase of growth. As $OUR = -dC_{O_2}/dt$, it was possible to calculate it from the rate (slopes) of oxygen depletion measured for samples of biomass drawn from the ALR over the time course of the fermentation (Fig. 5.3). As would be expected, the OUR (as calculated by the decrease in DO) increased rapidly during the growth phase of the fermentation, until day 4, and then gradually decreased as the growth slowed and entered stationary phase. When related to the amount of biomass in the reactor, giving specific OUR (Fig 5.4), there was an increase during the short lag phase followed by a period where it remains fairly constant, showing that the increase in OUR was correlated with an increase in biomass, as the culture enters stationary phase, the specific OUR decreased rapidly, indicating that the total amount of biomass, although it increased, became less metabolically active.

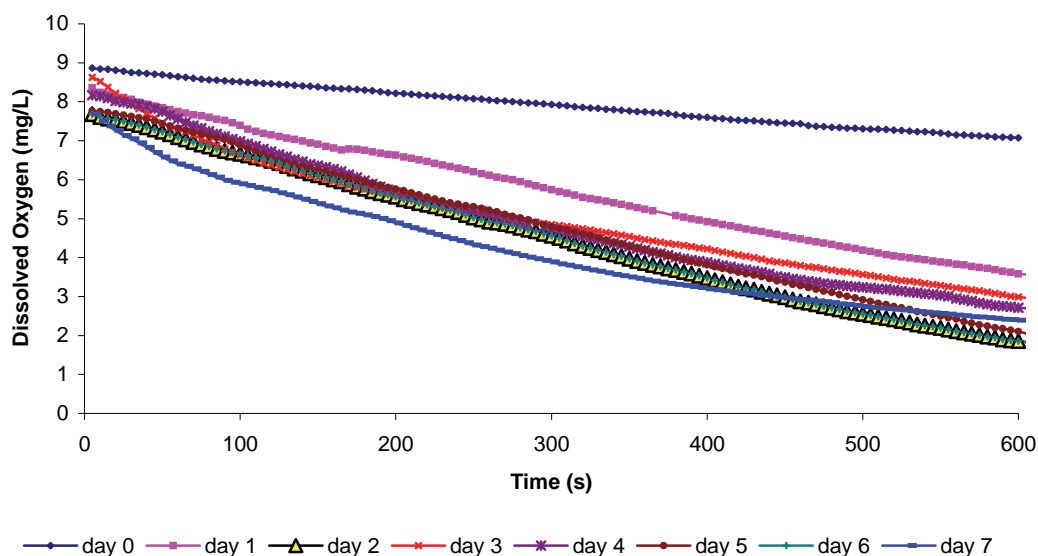


Fig 5.3 Decrease in oxygen concentration over time for *T. pubescens* grown in an ALR. The initial glucose concentration was 10 g/L and aeration was fixed at 3.5 L/min.

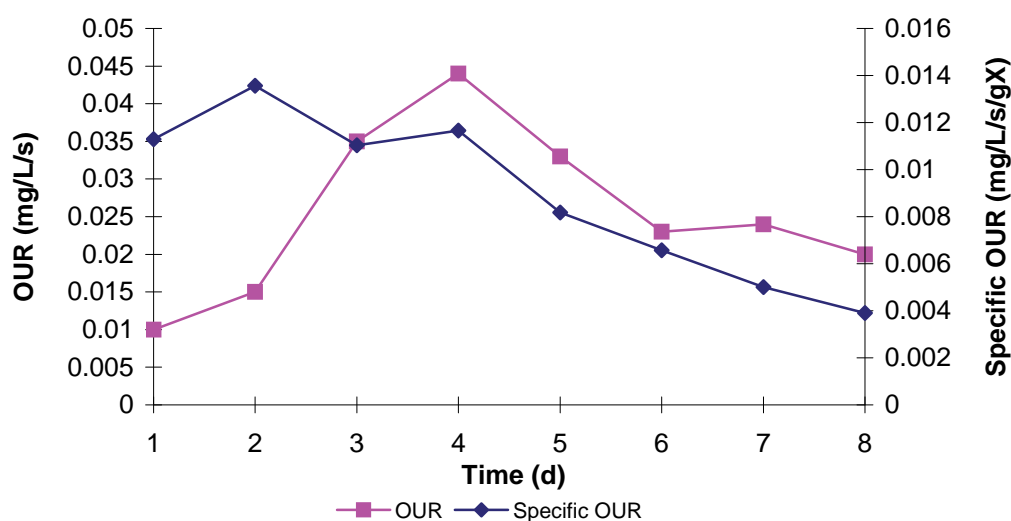


Fig 5.4 Comparison of OUR and Specific OUR of *T. pubescens* growing in an ALR. The initial glucose concentration was 10 g/L and aeration was fixed at 3.5 L/min

5.3.4 Effect of effluent addition on biomass morphology and laccase production

Airlift reactors containing TDM were inoculated (10%) with *T. pubescens* and the fermentation allowed to progress with the airflow fixed at 3.5 L/min. At the end of the initial growth period of 7 days, increasing concentrations of the phenolic effluent used previously was added to the ALR. These additions of SGL effluent had visible effects on biomass morphology and the appearance and consistency of the reactor fluid.

The size and gross morphology of the control and 2 % (v/v) reactors was also more consistent, with the pellets consisting of a dense core surrounded by fine hairs (Fig 5.5 A, B). The pellets in the reactors inoculated with 3.5 and 5 % (v/v) effluent settled faster and were darker in colour than those of the control and 2 % reactors. Closer inspection by light microscopy revealed that the pellets in the heavily dosed reactors (3.5 and 5 %) were irregularly shaped, smaller overall, less hairy and appeared to be simply a loose conglomeration of mycelia, with loose filaments extruding from mycelial masses (Fig 5.5 C, D). Su and He (1997) reported that over-stressed cultures of *Neurospora crassa* developed long mycelia on the pellets and that this was an indication of loss of viability. Control of pellet morphology has been achieved previously by the application of pulsing flow to fluidised bed reactor (Moreira *et al.*, 1996). It was reported here that decreasing pellet size and increased hairiness increased the active surface area of mycelia and resulted in better oxygen availability and higher productivity.

Changes to mycelial mats observed in the present study in flask cultures of *T. pubescens* after effluent addition included browning, shrinkage and fewer aerial mycelia. Electron microscopy revealed thinner more highly branched mycelia (section 3.3.2). These physiological adaptations have been correlated with higher laccase activity and increased removal of phenols (sections 3.3.4 and 3.3.5). Browning of fungal tissues often results from enzymic oxidation of phenolic substrates by phenoloxidases and peroxidases (White and Boddy, 1992), as would be expected in this case, after addition of a phenolic pollutant. Careful control of pollutant addition could actually become a tool in manipulating the size and activity of the pelleted culture in the ALR system. Should the biomass become too dense or the mycelial pellets too large, effluent could be added to slow the growth and reduce pellet size.

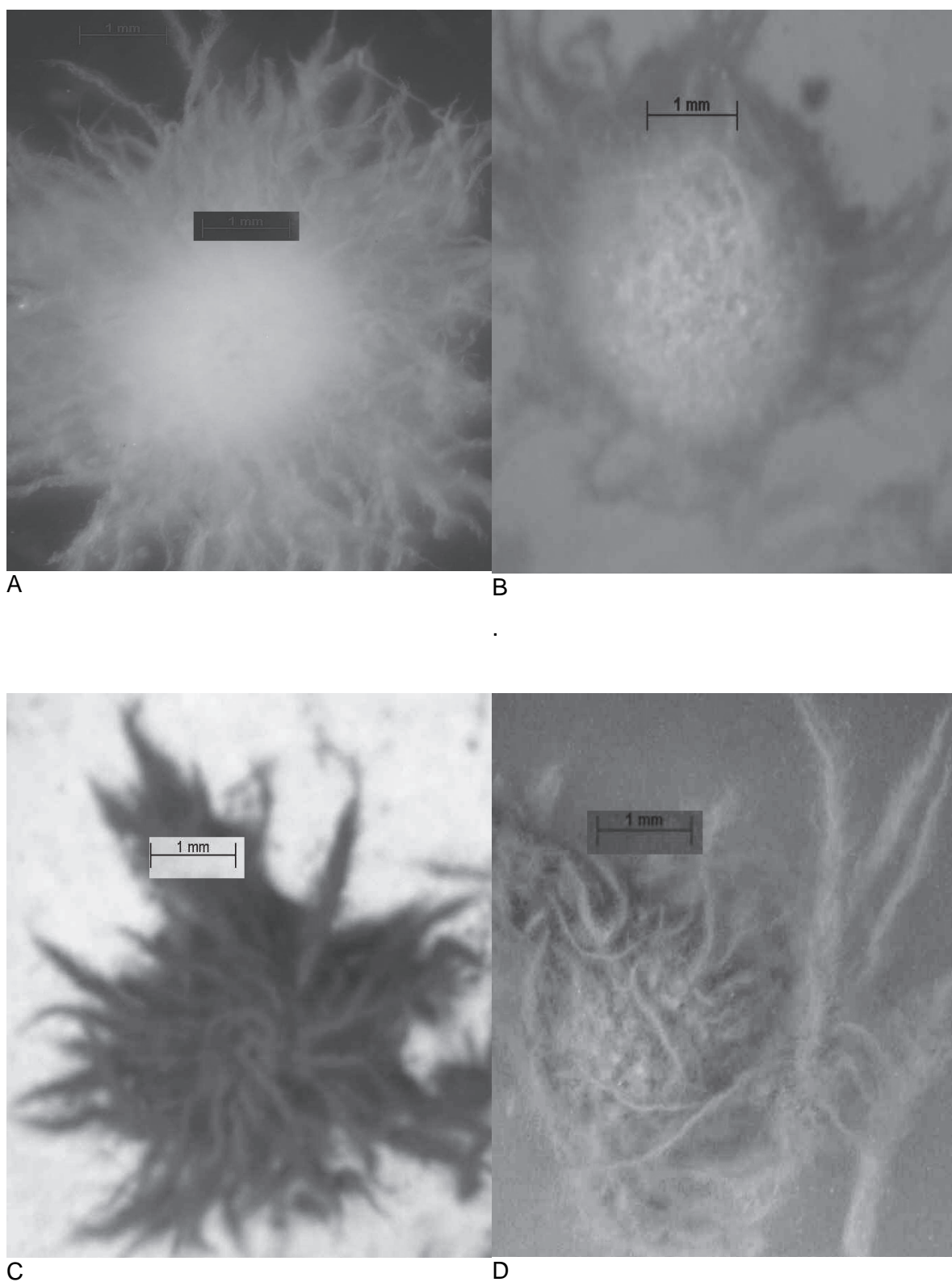


Fig 5.5 Light microscope images of pellets of *T. pubescens* grown in an ALR at increasing concentrations of a phenolic effluent: A (control, zero effluent), B (2.5 % v/v), C (3.5 % v/v) and D (5 % v/v)

Although laccase formation in *T. pubescens* has previously been reported as not being growth associated (Galhaup *et. al.*, 2002) the parallel increase of laccase activity with biomass accumulation in ALR cultivation is clear (Fig. 5.2). This consistent production of laccase, even in uninduced fermentations, contrasts with other reports of unreliable enzyme production by white rot fungi in the 'unfamiliar' environment of liquid fermentation (Robinson *et. al.*, 2001). Since enzyme production and bioremediation or even biotransformations in liquid fermentation have obvious advantages over solid substrate in terms of mixing, control and product recovery, this result is an important finding in terms of use of *T. pubescens* and other white rot fungi.

Laccase production was stimulated by the addition of cresylic effluent to the reactor. *T. pubescens* has various physiological responses to local environmental challenges, in this case the addition of the pollutant. One of them is to produce more lignolytic enzymes. It had already established from flask culture that the fungus was able to withstand up to 10% v/v of the effluent without obvious harmful effects. Biomass was largely unaffected and enzyme production was increased relative to the control. However, these concentrations had been 'one-off' additions. Sequential additions of effluent would conceivably have a cumulative effect and this was investigated in an ALR. The addition of 2.5 % (v/v) effluent increased laccase activity by almost 3 times; the subsequent addition of 5 % and 10 % appeared to have negative effects on the culture. The biomass decreased, became more prone to settling, and enzyme activity decreased. HPLC analysis showed that there were no residual phenol or cresols present in the reactor prior to the further additions of effluent, and therefore there was no cumulative build up of these compounds. This indicates that even though there were some negative effects associated with effluent addition, the fungus removed all the aromatic toxins from solution.

Free enzyme activity increased after falling from 2.7 U/mL, to 0.8 U/mL after the 5 % (v/v) addition (day 13) only to decrease from 1.95 U/mL, to almost zero after the 10 % (v/v) addition (day 19) after which time the culture died (Figure 5.6). It is possible however that these final effect effects were related to lack of glucose, rather than a phenolic concentration above the physiological limit of the fungus. This argument was at least partially validated in a further experiment in which the addition of glucose along with the aromatic pollutants allowed the fungus to contend with multiple doses of

effluent. The brown colour of the biomass and the culture fluid was also observed in flask culture (chapter 30. Klonowska *et al.* (2001) reported a similar colouration effect produced by the aromatic acid, and known fungal toxin, *p*- hydroxybenzoic acid. These authors suggested that the brown colour was evidence of the fungus getting rid of toxin by polymerisation.

Basidiomycetes are known to exhibit widely varying responses in terms of effects elicited by aromatic compounds, particularly in laccase production. An individual aromatic compound may increase laccase titres without altering the isozyme pattern (Eggert *et al.*, 1996), induce new isoforms (Bollag and Leonowicz, 1984) or have no induction effect (Coll *et al.*, 1993). Induction is considered to be a proactive response to toxic compounds produced either the degradation of lignin component of lignocellulosic residues (which serve as natural growth inhibitors to fungi) or to antimicrobial agents secreted by microbial competitors (Collins and Dobson, 1997, Chen *et al.*, 2003). Imura and Tatsumi (1997) isolated cDNAs of PCP-stressed *T. versicolor* by differential display and found bands that were common and control/PCP-treated sample specific. RNA analysis found a 9.7-fold increase in the stress response protein HSP70, which is involved in adaptive response mechanisms, after PCP treatment. It is apparent therefore that induction using potentially toxic aromatic compounds is beneficial in terms of laccase production and bioremediation efficacy, as long as the toxic loading limit is not exceeded.

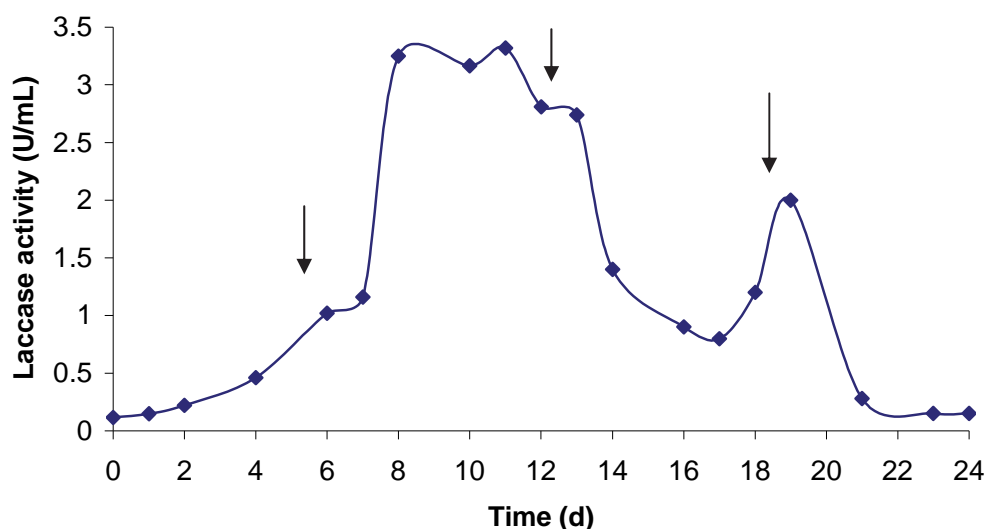


Fig 5.6 Time course of laccase production over an ALR fermentation of *T. pubescens*; 2.5% (v/v) phenolic effluent added on day 6, 5 % (v/v) on day 13 and 10 % (v/v) on day 19.

5.3.5 Optimisation of laccase production in an ALR

A fed batch experiment was set up to evaluate the effect of sequential effluent additions of effluent fermentation cultures of *T. pubescens*. Multiple ALRs were run in parallel with different concentrations (2 %, 3.5 % and 5 % v/v) of phenolic effluent were then added daily to each. The effluent was added with a glucose stock to a final concentration of 0.5 g/L. The addition of the co-metabolite had the effect of both increasing and maintaining laccase production compared with sequential additions attempted before. This effect is in agreement with other recent studies that employed a 'laccase production medium' containing low, non-repressing concentrations of glucose to stimulate laccase production after primary cell growth was completed (Jang *et al.*, 2002; Galhaup *et al.*, 2002). The glucose consumption rate during secondary metabolism has been shown to be ten times lower in an agitated culture of WRF than that observed under static conditions (Ulmer *et al.*, 1983). This indicates that the maintenance coefficient, and hence glucose feeding required, depends on operating conditions. Lignolytic activity of WRF is associated with polysaccharide formation, which hydrolyses in the acidic culture conditions of *T. pubescens*, creating another potential carbon source, a further indication that some other easily utilisable carbon source is required in small quantities for successful production of lignolytic enzymes.

All the effluent concentrations used resulted in an initial increase in laccase production relative to the control. The most pronounced effect was observed for 2% v/v effluent, with a maximum activity of 11.8 U/mL being reached after 9 days (Fig 5.7).

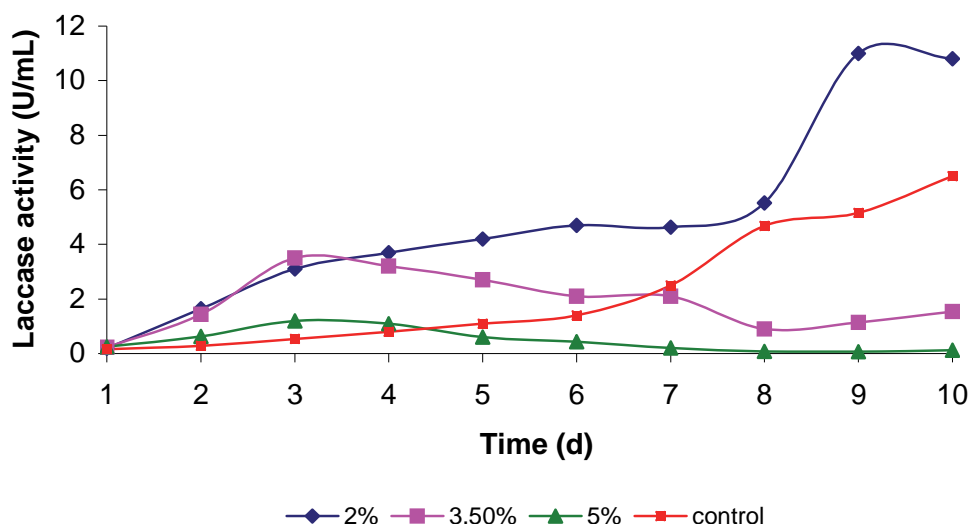


Fig 5.7 Induction effect of daily addition of different concentrations of phenolic effluent to a fermentation of *T. pubescens* in an ALR

Two interesting physiological phenomena were observed on addition of effluent to the ALR cultures of *T. pubescens*. Immediately (within 5 min) after addition of an aliquot of effluent (2 % v/v), there was an increase in laccase activity in the culture medium and the effect became more pronounced over successive additions (Fig 5.8). It was not sustained, however, and the peak disappeared in 2-3 hours. In one instance, the laccase activity spiked up to 70 U/mL immediately following the 4th addition of a 2% slug of effluent, and up to 50 U/mL in the 5% reactor. There was no sudden large increase in protein in the culture fluid, as measured by the Bradford's dye binding assay. This is not entirely surprising as increases in laccase activity are almost undetectable in terms of increase in total protein, but it does indicate that it is unlikely that the increase in laccase activity is due to cell lysis, in which case a large, measurable release of cellular protein would occur. This suggests some sort of adaptation by the culture where there is storage of laccase taking place in vesicles or vacuoles, which is then released as required.

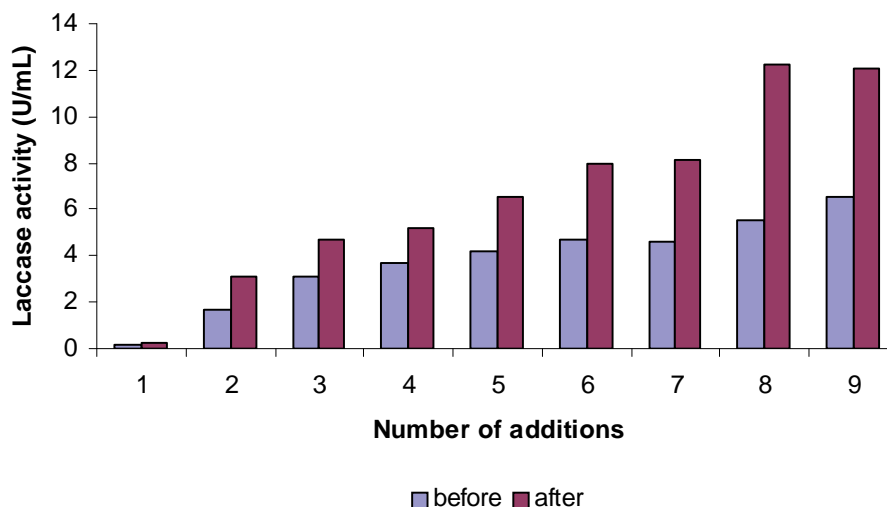


Fig 5.8 The immediate increase in laccase activity after the addition of phenolic effluent (2% v/v/day) in ALR fermentations of *T. pubescens*. Readings were taken on the same day immediately before, and 5 min after effluent addition

At the conclusion of this set of ALR fermentations a decreased pellet density was observed in the culture fluid from the reactors to which higher concentrations of effluent had been added. Inspection of the respective fermenters showed large numbers of pellets attached to the walls of the vessel and to the draught tube, and the relative amounts were quantified as 'suspended' and 'attached' biomass (Fig 5.9). A proportionate decrease of suspended biomass with increasing effluent concentration ($R^2 = 0.96$) was observed. There was hardly any difference in total biomass, however, with the lowest biomass concentration, 4.55 g/L for the 5 % v/v ALR, constituting 85 % of that of the highest (5.4 g/L) recorded for the control. Immobilisation affects the physiology of microorganisms and has in many cases been linked to increased enzyme production in fungi.

Immobilised organisms are known to be more resilient to environmental stress compared to free cells. These factors seem to indicate that in an optimised bioremediation process intended for high pollutant concentrations, better results might be obtained by providing some sort of support matrix inside the reactor. This will, however limit the chief advantage of fungal growth in an ALR, namely effective oxygen transfer.

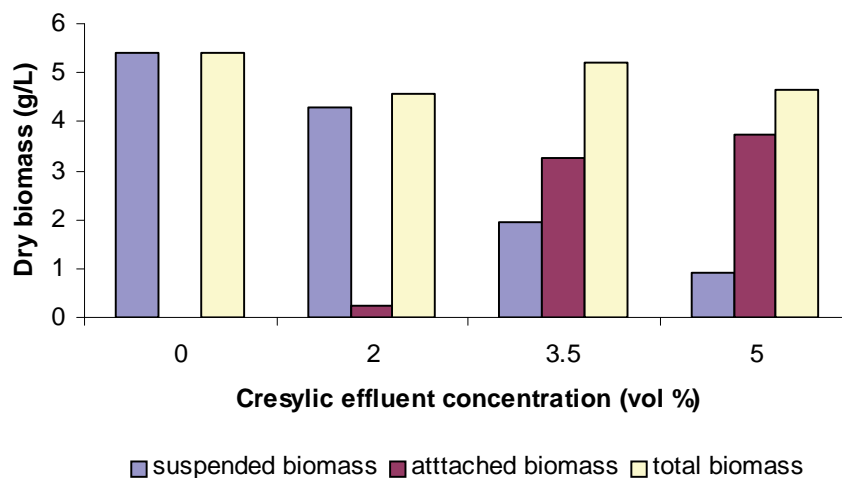


Fig 5.9 The relationship between freely suspended and attached biomass in ALR's containing different amounts of phenolic effluent. The masses are an average from two reactors. The biomass readings are an average from 2 reactors

5.4 Conclusions

- The ALR system proposed in this study has been shown to provide a suitable environment for the successful growth (10 g/L) of the laccase hyper-secreting fungus (12 U/mL), *T. pubescens*.
- The application of WRF in large-scale waste treatment has been impeded by lack of bioreactor systems yielding consistent production of lignolytic enzymes and controlled growth of fungi (Zhang *et al.*, 1999).
- The aeration supplied to an ALR, which provides control of both the agitation and OTR in the reactor, was optimised to achieve sufficient oxygenation and mixing while avoiding surplus supply, thus maintaining a suitable cost-benefit relationship.
- Laccase activity levels were found to outperform those obtained in flask culture for both uninduced fermentations and those induced with phenolic effluent.
- The management of aeration rate and the addition of inhibitory compounds (phenolic effluent) both have potential in controlling fungal growth.

CHAPTER 6 : REACTION CHARACTERISATION AND OPTIMISATION OF THE BIOREMEDIATION PROCESS

6.1 Introduction

Because of their biocidal nature most phenols cannot be satisfactorily treated by conventional biological wastewater treatment. The presence of phenols in high concentrations has the potential to irreversibly shock and damage live microbial systems (Kauffman *et al.*, 1999; Klibanov *et al.*, 1980). In order for the fungal system developed in this study to be applied in bioremediation, it would need to both survive and affect the removal the pollutant monomers from the effluent at concentrations much higher than those already shown to be effective inducers of laccase activity. This was investigated in this project, using flask and bioreactor culture systems.

It is important that degradation studies focus on realistic bioreactor operating conditions, and information on biodegradation mechanisms and kinetic data are required to allow an engineered reactor design (Pallerla and Chambers 1998). The ALR developed here was shown to be capable of biomass and laccase production by *T. pubescens*, but these investigators had not demonstrated degradation of pollutants. Royer *et al.*, (1991) could not demonstrate a correlation between laccase activity and decolourisation of bleach plant effluents and concluded that laccase production did not reflect the detoxifying activity of fungal pellets of the *Trametes* species used. This chapter reports the results of our investigation into the bioremediation using the *Trametes* ALR system.

The oxidation of phenolic compounds and other environmental pollutants by *Trametes* produce quinones and further polymerised products, and some of the pollutants are converted to carbon dioxide. An intracellular heme-flavin adenine dinucleotide (FAD)-cofactored glycoprotein designated cellobiose dehydrogenase (CBH), donates electrons to an extremely wide range of substrates including semi-quinones, *ortho*- and *para*-quinones, azo dyes, kraft lignins, Fe(III), MnO₂ and Mn(III) complexes (Roy *et al.*, 1996). The quinone, hydroquinone and catechol reaction products can again serve as substrates for the oxidative enzymes. This ubiquitous redox cycle of phenolic compounds includes intra- and extra-cellular processes and is not yet fully understood. The rapid intracellular metabolism of the quinone and hydroquinone intermediates is a possible mechanism for shifting the dynamic polymerisation/depolymerisation equilibrium towards degradation and mineralisation (Schlosser *et al.*, 1997). This

chapter also reports a preliminary investigation conducted to confirm that the system could be applied to other authentic phenolic effluent streams.

6.2 Methods

6.2.1 Culture and growth conditions

Trametes pubescens (CBS 696.94) was maintained on 2% malt agar plates. The culture medium used contained 10g/L glucose and peptone and 2g/L KH_2PO_4 .

6.2.2 Inoculum preparation

T. pubescens from plate culture was homogenized in growth medium and used to inoculate 300mL-sterilized medium in 2L Erlenmeyer flasks. These flasks were incubated at 28°C and 175 rpm. The gyratory motion of the shaker promoted uniform pellet formation and this speed was found to produce small, dense pellets. Four-day-old pellets from these cultures were used as a reactor inoculum.

6.2.3 Determination of laccase enzyme activity

Laccase activities were determined by monitoring the oxidation of 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as the substrate (Wolfenden and Willson, 1982). 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). Oxidation of ABTS was measured by determining the increase in absorbance of the mixture 420 nm ($\epsilon = 36\,000\text{ M}^{-1}$). One unit (U) of enzyme activity was defined as the amount of enzyme required to oxidise 1 μmol of ABTS per min.

6.2.4 HPLC analysis of phenolics

Samples were aseptically drawn from the flasks, centrifuged and then filtered to remove any cell debris. The removal of the effluent components was monitored by HPLC (Merck LaChrom), utilising a Waters S5 (5 μm), C_{18} reverse phase column (250 x 4.6mm) with a mobile phase of water:acetonitrile 85:15 containing 1.66% w/v β -cyclodextrin, to separate the cresol isomers.

6.2.5 ALR operation

The concentric draught-tube internal loop airlift reactor used in this work was described in section 5.2.3. The reactor was filled with 3.15 L of growth medium and autoclaved. After cooling a 350mL (10%) inoculum was aseptically introduced and the air supply attached, initiating the fermentation. In the pollutant degradation experiments, a three-day initial growth period was allowed before any stripped gas liquor (SGL) effluent was added. Thereafter 2% v/v effluent was added daily to one reactor, 3.5% to a second and 5 % to a third.

6.2.6 ALR Sampling

5mL samples were aseptically drawn from the ALR and replaced with glucose-free growth medium. Samples were centrifuged to remove suspended biomass and glucose and laccase activity assays were performed on the supernatant. In the experiments where pollutants were added to the reactor, the air supply was terminated for 10 minutes to allow the biomass to settle and 100mL of cell-free culture medium withdrawn from the reactor. This volume was replaced with effluent appropriately diluted with growth medium.

6.3 Results

6.3.1 Removal of phenols from effluents in flask reactions

To quantify the removal of effluent phenolic monomers from solution, different concentrations of the effluent were added to flask cultures of *T. versicolor* and samples were taken over time for HPLC analysis. The removal of the effluent monomers from solution in flask cultures with up to 20% v/v (15mM) concentration of effluent was successfully completed after 12h (Fig 6.1). The phenol proved to be more recalcitrant than the cresol (*o*, *m* and *p*) isomers. This was not an unexpected result because the efficacy of laccase reactions in removing various phenolic substrates has been shown previously to depend on both substituent group and position of substitution. Shuttleworth and Bollag (1986) showed that at a concentration of 0.1 mg/mL (0.925 mM) nearly 100 % of all the cresols were removed, whereas only 10 % or less of the chlorophenols were transformed. The ability of the laccase to remove cresol was dependent on the position of the methyl group with *meta*- substituted cresol being the least oxidised and *para*-

cresol the most. Interestingly, the authors found also that 2,6-dimethylphenol was a better laccase substrate than any of the cresols (based on total amount of transformed).

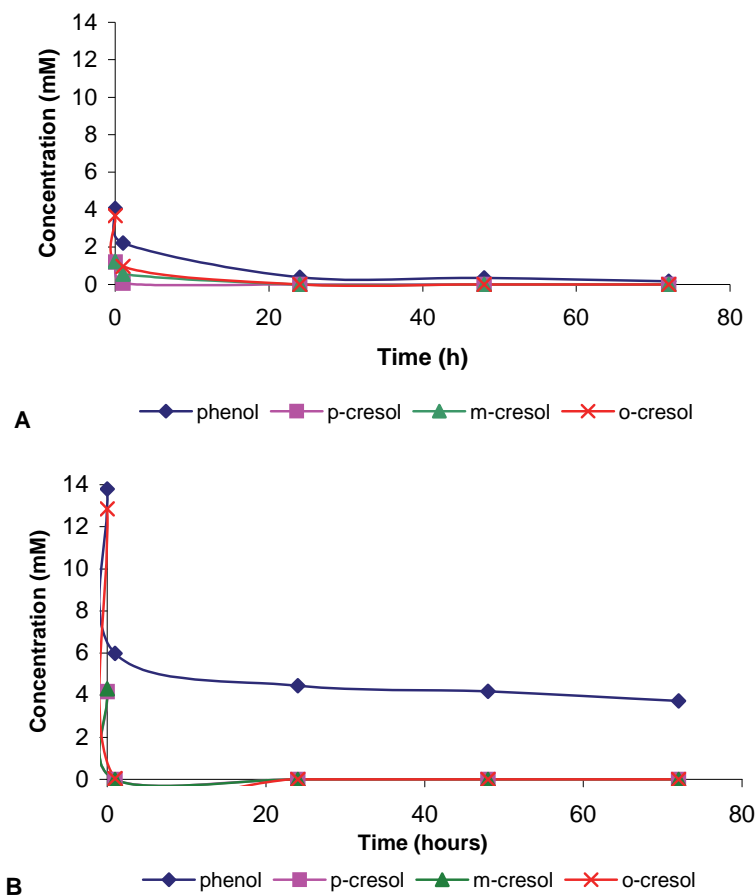


Fig 6.1 The removal from solution of the different monomers found in the phenolic effluent by flask cultures of *T. versicolor* from 5 % vol/vol (A) and 20 % vol/vol (B) final concentrations

To our knowledge the concentrations of the phenolic monomers removed by *T. versicolor* in this system are the highest reported to date (Table 6.1). This is attributed to the optimised conditions developed in this study.

However, the removal of single doses of the target effluent monomers from flasks does not constitute an efficient bioremediation process, and the preliminary success shown here needed to be further developed into removal of multiple doses and eventually a (semi) continuous system, and this was achieved using the ALR bioreactors described earlier in this report.

Table 6.1 Removal of effluent (20 % v/v) monomers from solution by flask cultures of *T. versicolor*

EFFLUENT MONOMER	INITIAL CONCENTRATION (mM)	RESIDUAL AFTER 1 DAY (mM)	REMOVAL RATE (g/g biomass/day)
Phenol	13.8	5.99	0.125
p- cresol	4.17	0.0	0.075
m- cresol	4.3	0.0	0.077
o- cresol	12.84	0.04	0.231
TOTAL			0.498

It is difficult to compare the removal rates reported here with other studies. While there are numerous studies on the removal of organic pollutants using the enzymes of *T. versicolor* there are very few that deal with whole cell degradation of phenolic effluents. Novotny *et al.*, (1997) reported a 50% removal of a 0.9 ppm concentration of a commercially available mixture of PCB's, Delor 106, after 3 weeks incubation in static flasks of *T. versicolor*. This was the best removal out of the four WRF tested. Logan *et al.* (1994) found a removal of 6 mg PCP/L over a 12 day incubation period. The authors reported further that PCP removal in 250 mL flasks was greater in shallow (10 mL) cultures than in deep (50 mL) culture. This indicates that the ratio of surface area to volume of liquid media is an important factor in the extent of PCP (and possibly other phenolic compounds) removal by WRF. Interestingly, they also acknowledged that culture age prior to addition of PCP had an effect on its removal, but this was never quantified. Collins and Dobson (1996) found a 50 % removal of 20 mg/L phenanthrene in 3 days in whole culture and Morgan *et al.* (1991) reported a mineralisation rate of 2.8 µg/g dry weight/day for Dieldrin in flask culture. Based on comparisons with these results available from literature it appears that *T. versicolor* is highly suitable to the removal of phenolic compounds from solution. Further, the system developed here is apparently superior to those reported previously.

6.3.2 Removal of phenolic monomers from solution in the ALR

Based on prior results in this study, the bioremediation capacity of *T. pubescens* in flask culture was shown to be successful, but these results needed to be extrapolated to a bioreactor system. After 4 days growth period 2.5 % (v/v) effluent was introduced into

the ALR, after 3 more days, another 5 % and 3 days later another 10 %. Samples were taken daily and assayed for residual phenols. The target monomers (phenol, *p*-cresol, *m*-cresol and *o*-cresol) were completely removed from sequential additions of effluent at increasing concentrations over a period of 8 d (Fig 6.2).

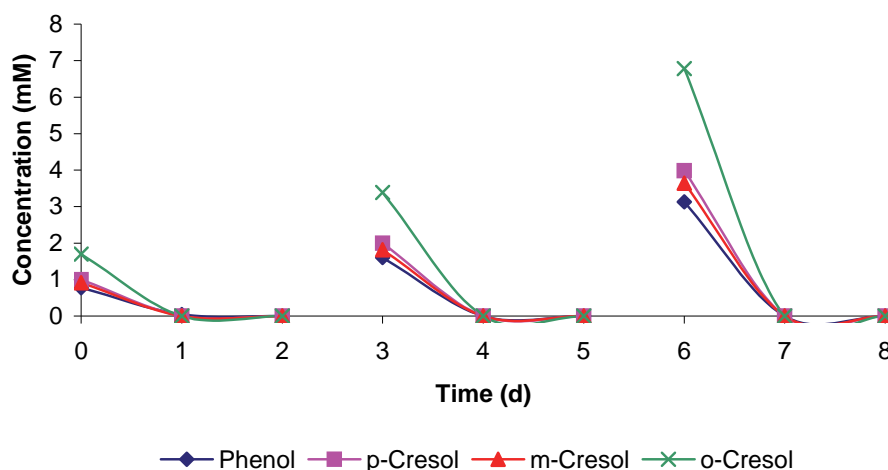


Fig 6.2 Removal of target monomers from successive increasing doses of phenolic effluent (2.5%, 5% and 10% v/v) by *T. pubescens* in an ALR

Although dephenolisation of the effluent is considered to be primarily due to the action of extracellular enzymes, there are reports on the involvement of intracellular enzymes in the detoxification/decolourisation process. Watanabe *et al.* (1982) showed that in the presence of a simple sugar and oxygen, sorbose oxidase, an intracellular enzyme in *Coriolus (Trametes) versicolor* contributed to the decolourisation of a distillery effluent. Royer *et al.* (1991) report the loss of activity of pellets of *T. versicolor* in a packed bed upflow type reactor in repeat batch additions of a kraft effluent. No such loss of activity was evident in this study, where all the phenols being removed within one day of addition for all three additions (Fig 6.2). Swamy and Ramsay (1999) presented further evidence of the requirement of *Trametes* cultures for a carbon co-substrate in long-term lignolytic/bioremediation activity. It was demonstrated by them that sequential dye decolourisation required adequate supplies of glucose, but that above a certain critical concentration, this rate was unaffected by increases in glucose levels. The requirement for glucose appeared to be associated with a rate-limiting step in the decolouration mechanism, rather than heightened metabolic activity.

To further establish the potential of a *T. pubescens* bioremediation process, the ALR system was operated in a fed batch mode where effluent and glucose (0.5 g/L) were added daily to the reactor. A 5 % (v/v) effluent concentration was the highest tested and all target monomers were removed before the next addition (Fig 6.3). In the only other report of *Trametes* sp. grown in an ALR, the authors (Rancano *et al.*, 2003) measured the performance of the fermentation in terms of laccase production. The decolourisation of the sulphonephthalein dye Phenol Red was investigated, but only in the extracellular fluid obtained from the reactor, not *in situ*. This does present an alternative treatment methodology, but removes the potential for the whole cell mineralisation of the target compounds and overlooks the role of the mediator systems produced by WRF that facilitate lignolysis and the degradation of xenobiotic compounds. This could explain the relatively poor results achieved by them, with a decolourisation of 34 % reached in 27 h.

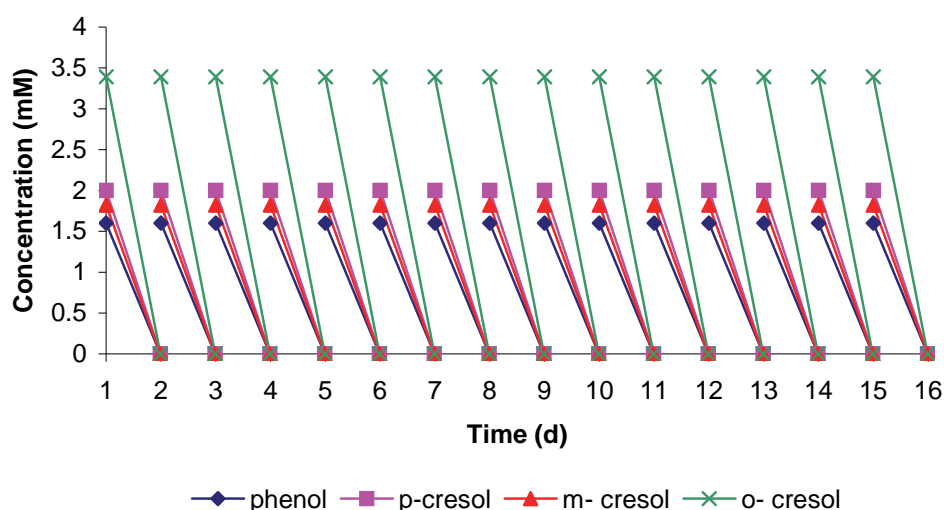


Fig 6.3 Removal of target monomers from phenolic effluent (5% v/v) added daily to *T. pubescens* immobilised in an ALR

The initial attack on aromatic compounds by WRF is made by the extracellular lignolytic enzymes performing a one-electron abstraction, thereby generating cation radicals of the contaminants. These radicals undergo spontaneous chemical reactions such as C-C cleavage or hydroxylation resulting in more hydrophilic products (Hammel, 1995). Those products are taken up by the fungal cells and co-metabolised in the presence of a proper (easily utilisable) carbon source to carbon dioxide (Hammel *et al.*, 1991), still a complex process of oxidation, reduction, methylation and hydroxylation. This presents further motivation to provide a constant supply of a readily utilisable carbon source to *T.*

pubescens functioning in a bioremediation capacity. The removal rates of the individual phenols are shown in Table 6.2.

Table 6.2 Removal of cresylic effluent phenols over 16 days in the ALR

Effluent monomer	Amount of phenol added daily (mM)	Residual phenol (mM)	Removal rate (g phenol/ g biomass/d)	Total removal of phenol over 16 d (g)
Phenol	3.45	0	0.033	4.97
p- cresol	1.04	0	0.011	1.79
m- cresol	1.08	0	0.012	1.87
o- cresol	3.21	0	0.035	5.20
TOTAL			0.10	13.83

These results (Table 6.2) represent the highest total removal as well as rate of removal of toxic aromatic compounds from water by WRF yet reported.

Other researchers have used bioreactors based on WRF to treat toxic aromatic compounds: Shim and Kawamoto (2002) utilised *P. chrysosporium* in a packed bed system to remove PCP from solution at a rate of 0.06g/d for 20 days, an overall removal of 1.2 g achieved in an 8 L (solid substrate) reactor, i.e. double the size of the one used in this study. Pallerla and Chambers (1997) developed a 0.6 L fluidised bed bioreactor utilising *T. versicolor* immobilised in alginate beads to treat bleach plant effluents (2 % v/v) that achieved a colour reduction of 75 % and AOX reduction of 70 %. The same reactor was used to remove 0.01 gPCP/g biomass/day (Pallerla and Chambers, 1998). Mielgo *et al.* (2001) developed a *P. chrysosporium* based packed bed reactor that removed 0.2g/L/d of the azo-dye Orange II reliant on the production of MnP.

6.3.3 Characterisation of reaction products from bioremediation

An investigation was conducted to characterise the changes in the pollutant substrates when treated with laccase from *Trametes versicolor* and *Trametes pubescens*. The results showed that crude laccase extracts from *Trametes versicolor* and *Trametes pubescens*, and partially purified laccase from *Trametes versicolor* would totally or partially convert a number of model pollutants in a synthetic effluent solution and in authentic industrial effluents. The pollutants present in three local industrial effluents i.e. phenolic Black Product and cresylic (SGL) petrochemical effluent, and Pulp and

Paper effluent from a paper mill were used. The crude laccase extract, from *Trametes versicolor*, was used for the bioconversion of phenol, *m*-cresol, *p*-cresol and *o*-cresol. 69.2% and 43.6% bioconversion of *o*-cresol and phenol occurred respectively, at 5mM concentrations.

Table 6.3 Initial concentration of phenols present in the authentic effluents

Effluent	Pollutant	Concentration (mM)
Phenolic Black Product	Phenol	4.28
Cresylic Effluent	Phenol	31
	<i>p</i> -cresol	6.25
	<i>m</i> -cresol	6.7
	<i>o</i> -cresol	87.65
Pulp and Paper Effluent	Guaiacol	32.47
	4-chlorophenol	5.01

The percentage conversion of each pollutant in the pulp-and-paper waste was not dependent on the starting concentration, but 24.5% 5mM 2,4-dichlorophenol was converted after treatment with crude laccase extract. The laccase enzyme showed selectivity for 2,4,6-trichlorophenol and 4-chlorophenol at 5mM concentrations. The lower levels of conversion achieved in this study in comparison to the phenolic compounds in the petrochemical effluents, might be attributable to the methanol used to dissolve the insoluble chlorophenolic compounds, which may have had a slight inhibitory effect on laccase enzyme. The highest conversion of the pulp and paper pollutants appeared to be for 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol, at 5mM concentrations. The use of whole cell biomass was also investigated and compared to the results obtained for the free laccase enzyme. *Trametes versicolor* biomass, grown in TDM, were used to treat model pollutants found in the petrochemical and pulp and paper effluents. The whole cell system appeared to be an efficient method for the bioconversion of pollutants, catalysing 100% and 26.36 % conversion of *o*-cresol and *m*-cresol respectively at a 5mM concentration. The *Trametes versicolor* whole cell system did not necessarily offer a better alternative for the treatment of the pollutants, with respect to percentage conversion, than laccase enzyme, but whole cell systems do represent a more cost effective method of treating wastes. The selectivity in the treatment of pollutants present in the three industrial effluents was also considered. After treatment with crude laccase extract from *Trametes pubescens*, *p*-cresol and *o*-cresol

present in the cresylic SGL effluent had undergone 100% conversion and 98.5% conversion respectively. In the pulp-and-paper effluent, after treatment with laccase from *Trametes pubescens*, the guaiacol decreased by 65.9% and the 4-chlorophenol by 14.3%. The laccase enzyme was thus effectively able to reduce the toxicity of the selected pollutants in both the effluents. This clearly demonstrates the general applicability of the system.

6.4. Further development of the ALR bioremediation system

6.4.1 Investigation of physical design parameters (Pentech contribution)

Initial experiments concentrated on mixing effects in a small reactor (63 mm diameter). Later work used a reactor of similar dimensions as that used at UCT. A 63 mm diameter 500 mm deep vessel with a 32mm draft tube was set up. An air flowrate of between 40 ml/min and 200 ml/min had been tried in this unit and the apparent mixing time recorded. It did not vary significantly with air flowrate as would be expected. Values between 270 and 390 seconds were observed but with no repeatable relation to the air flowrates used.

Table 6.4 Air flow rate vs. mixing time in small airlift reactor

Air flowrate (ml/min)	Seconds to reach equal concentration top and bottom	
	Early estimate	Last estimate
41	150	270
96	150	300
153	210	390
166	240	270
231	120	330

It was concluded that in these experiments the delay in salt tracer reaching the active elements of the (occluded or “shrouded”) conductivity cells used probably accounted for most of the time delays observed and that the real mixing times were far shorter than the apparent values recorded. Thus the results were discounted as being of little conclusive value. These early experiments concentrated on the mixing effects but did not attempt to measure the oxygen transfer rates. There was also no attempt to simulate the viscosity effects that would be present in an operating fungal bioreactor. It was expected that an optimum bubble size would be demonstrated. Literature had

suggested that 2mm bubbles seemed to give the best mass transfer rates. Bubbles this size were difficult to attain in the small reactor. The type of home-made sparger (perforated plastic tube) employed could not deliver regular bubble streams from the relatively large orifices used. The aquarium pump employed could also not allow higher pressures and smaller sparger orifices to be employed.

A larger unit was desirable that would allow a larger air flow rate to be used and thus constructed in 2001. A range of interchangeable single-orifice nozzles was selected so that the effect of pressure on the sparger discharge and its air dispersion characteristics could be studied and varied. A 160 mm diameter vessel was then constructed using funds allocated to Pentech from the project budget. This unit could be varied in height from 500 mm to 2000 mm and greater, if desired, by bolting together flanged clear pvc sections each of 500 mm depth. These segments were originally purchased for an ion exchange column in another project funded by NRF and re-allocated to the airlift reactor project.

The draft tubes were of clear Perspex or pvc of five different (32; 50; 63; 75 and 90 mm) diameters. Thus the draft tube could be varied in both diameter and height and its position above the base of the vessel and/or below the top liquid level could be adjusted by means of a plastic "spider" with rubber friction pads holding it in place.. The point of injection of air was also variable relative to the base of the draft tube by moving a central air inlet tube that carried the nozzle and that was inserted through a gland at the base of the vessel.

The air nozzles could be changed starting with single orifice nozzles or various types of frit. The nozzles included a simple orifice plate (170microns hole); a slit and a circular orifice in two solid body jet type spray nozzles (used for water spray purposes) and a nozzle with a very fine "long" orifice (approximately 2mm deep and 50 microns diameter) in the form of a readily available and inexpensive (R10-00) "Cadac" cooker top gas nozzle, obtained from a local gas appliance store.

Domed ends of light gauge material were fitted at top and bottom for operation at atmospheric pressure only. Heavy gauge clear Perspex spherical domed ends were later obtained from a fabricator that would allow the vessel to be operated under

pressures above atmospheric in order to test the effects on bubble size and oxygen mass transfer coefficient.

6.4.2 Measurements on 160 mm diameter reactor

Mixing effects could be monitored from a residence time between two conductivity probes and a salt injection “lance” that could be placed at different points to experiment with the dispersion of a salt tracer ahead of the conductivity detectors. Variable height conductivity cells were made up that could, similarly to the salt injection lance, be placed at different heights relative to the draft tube – diametrically and vertically.

The air flow rate in this unit could be altered by adjusting a pressure regulator. And measured by reading a rotameter situated on the outlet. Typical air rates obtainable were up to 600 ml/min at 1,5 bar inlet pressure (or higher if desired).

The mass transfer coefficient measurements were to be obtained by using a sodium sulphite solution catalyzed with cobalt to simulate a reactive organism “medium”. The chemical reaction (reduction of oxygen by sulphite) would maintain a nearly zero concentration of oxygen in the liquid phase at all points in the reactor at all times during an experiment. This would obviate the need to arrange a measurement of oxygen in continuous inlet and outlet liquid streams. It would also avoid having to attempt live culture work that would have been expensive in terms of medium, disposal and probably impractical for us to perform at this stage. The solution of sulphite would be introduced as a batch and as long as there remained unreacted sulphite it was expected that oxygen concentration in the solution phase could be assumed to be close to zero at all points.

The mass transfer coefficient effect was to be derived from changes in the oxygen concentration in the air leaving the top of the unit. Oxygen concentrations were measured by placing a DO probe in a small Buchner flask connected into the outlet gas stream. The probe was calibrated in fresh air and the oxygen consumed was then deduced by a mass balance based on the air outlet volumetric flow rate measured on a rotameter. The rotameter was calibrated using the standard displacement technique by means of an inverted 2 litre measuring cylinder.

6.5 Scale up of the ALR

In the final stages of this project, a larger scale ALR has been designed (Figure 6.4) and built, and although it was not an objective of this project to run the system at pilot scale, the larger scale bioreactor has been commissioned and will be used in further development of the bioremediation system subsequent to completion of this project.

6.6 Inclusion of a membrane separation unit

The original plan for the reactor developed in the project included a membrane unit to separate or retain biomass within the reactor. This was found to be unnecessary in the ALR, since the biomass is retained automatically within the reactor, and medium / reaction solution is removed by overflow without loss of biomass incurring. This is an advantage, in fact, since it means that the considerable expense that would be incurred in including a membrane unit can be avoided.

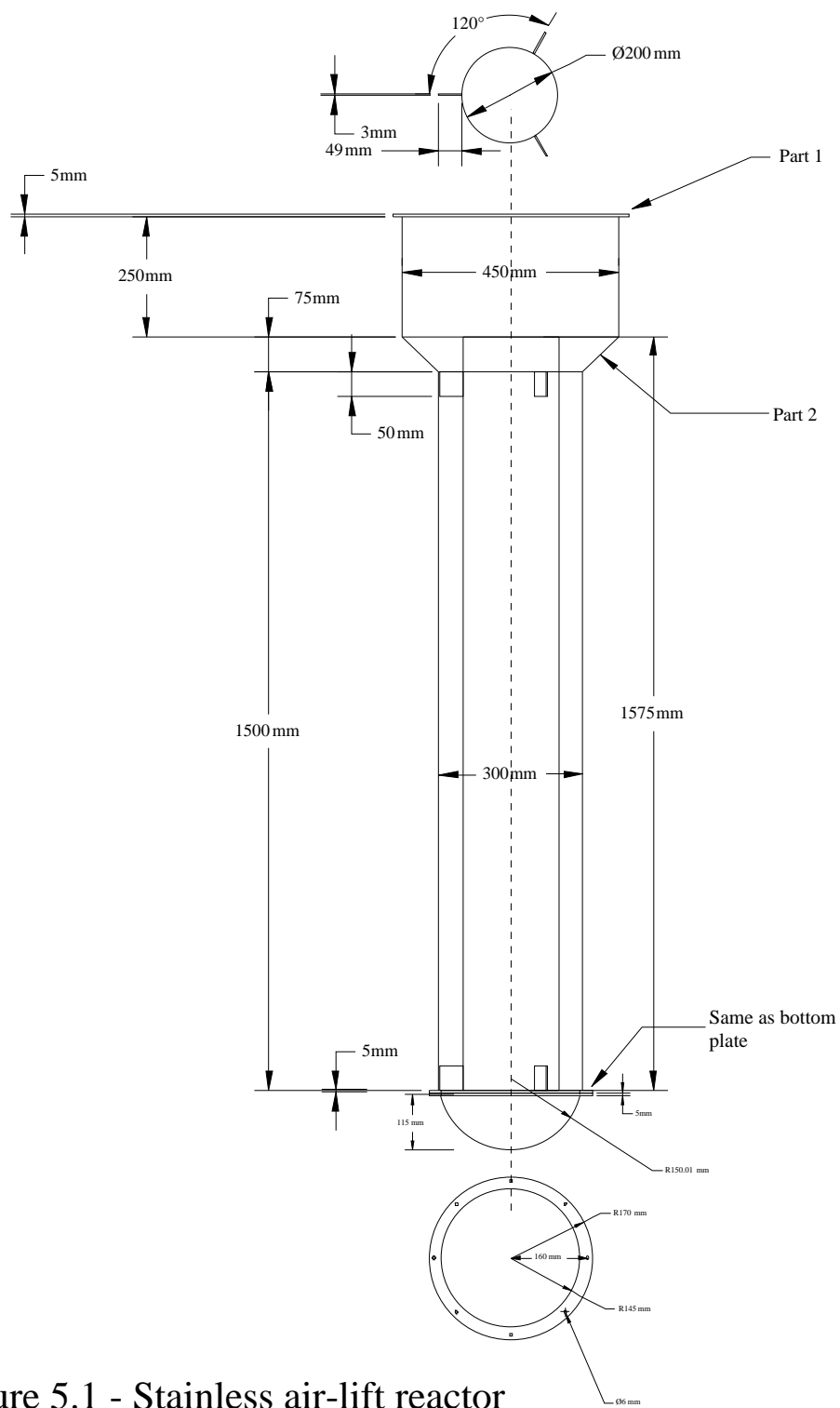


Figure 5.1 - Stainless air-lift reactor

Figure 6.4 Scale-up Airlift reactor design

6.7 Conclusions

- The combination of *Trametes pubescens* and the ALR reactor system has been shown to be highly suited for the removal of phenols from solution.
- The results achieved here represent the highest total removal of toxic phenols yet reported
- These results also demonstrate the highest rates of removal of toxic aromatic compounds from water by WRF yet reported.
- This study has elucidated the biological aspects specific to *T. pubescens* and has shown the potential to provide a practical, cost effective bioremediation process for the treatment of phenolic wastewaters.
- A direct comparison of the bioreactors tested in the course of this study (Table 6.5) shows the superiority of the ALR as compared with the TFHFBR which reached pollutant concentrations where breakthrough occurred and the system was no longer effective.

Table 6.5 Phenolic removal rates for the various culture conditions and reactor configurations investigated in this study

POLLUTANT PHENOL	FLASK		TFHFBR		TFR		ALR	
	R R ^a (g/g/d) ^c	T R ^b (g)	R R (g/g/d)	T R (g)	R R (g/g/d)	T R (g)	R R (g/g/d)	T R (g)
Phenol	0.106	0.75 (7 d)			0.031	1.54 (5 d)	0.033	4.97 (15 d)
<i>p</i> -cresol	0.077	0.45 (7 d)	0.05	3.70 (7 d)	0.010	0.54 (5 d)	0.011	1.79 (15 d)
<i>m</i> -cresol	0.081	0.46 (7 d)			0.011	0.57 (5 d)	0.012	1.87 (15 d)
<i>o</i> -cresol	0.231	1.38 (7 d)			0.031	1.63 (5 d)	0.035	5.20 (15 d)

^a Maximum removal rate

^b Total removal

^c gram of pollutant/gram of biomass/day

CHAPTER 7 : GENERAL CONCLUSIONS

The work described in this report represents a broad-based, interdisciplinary approach to the development of a fungal bioremediation process for the production of the lignolytic enzyme laccase, and the subsequent treatment of phenolic wastewaters of industrial origin.

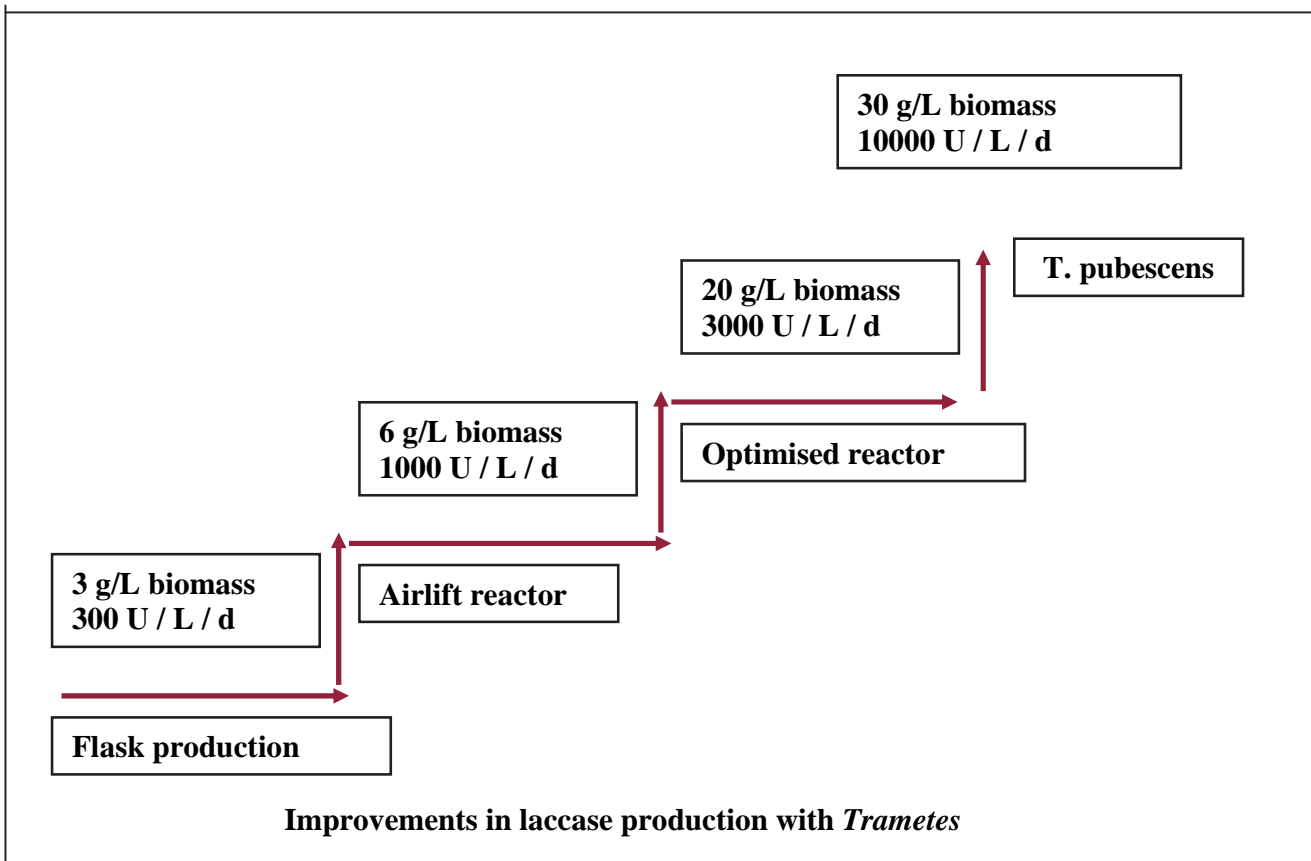
7.1 Key findings

The major findings of this work can be summarised as follows:

7.1.1 Physiology of *Trametes* strains with respect to bioremediation potential

- The basidiomycetes *Trametes versicolor* (PPRI 3845) and *Trametes pubescens* (CBS 696.94) have been shown to be outstanding producers of the oxidative enzyme laccase and are consequently resilient to the effects of aromatic compounds usually toxic to most other microorganisms.
- The production of this enzyme was optimised in static culture by the use of a carbon and nitrogen 'sufficient' growth medium; and further increased by the addition of various inducer compounds, including a 'stripped gas liquor' effluent.
- The complex physiological response of *T. versicolor* to the addition of the phenolic effluent was illustrated and explained in terms of enzyme production, metabolic activity and morphological differentiation. Many of the deleterious effects of effluent addition can be ameliorated by controlling the amounts added, and the time of these additions relative to culture age.
- The pollutant compounds induce the enzymes required for their catabolism
 - increasing their concentrations resulted in increased fungal biomass and
 - addition of a readily utilisable carbon source (glucose) was shown to increase and prolong oxidative enzyme production, and hence the removal of the aromatic toxins from solution
- Immobilised biofilm reactors in the form of a Transverse Flow Membrane Bioreactor and a Trickle Bed Reactor were identified as suitable for growth, enzyme production and phenolic removal by *T. versicolor*.
- Control of aeration rate was shown to optimise the fermentation performance for growth of *Trametes* species in an Airlift Loop Reactor and this system was found to outperform the other reactors investigated in terms of laccase production and the bioremediation of phenolic compounds.

- The Growth Related Productivity of 1.46 units of laccase produced per day per g biomass in the airlift bioreactor, under optimised conditions, represents a 33-fold improvement in laccase production over the initial levels obtained in flasks



7.1.2 Bioreactors for application of *Trametes* strains in bioremediation

- Control of aeration rate was shown to optimise the fermentation performance for growth of *Trametes* species in the Airlift Loop Reactor and this system was found to outperform the other reactors investigated in terms of laccase production and the bioremediation of phenolic compounds. The ALR design has been scaled up to 100L
- ALRs are well suited to wastewater treatment and have been shown in this study to be additionally suited to the growth of *Trametes pubescens*.

- The combination of the reactor configuration developed here and the efficiency of *T. pubescens* in the degradation of the phenolic compounds form the basis of an important, novel bioremediation process.
- The study has shown the potential for providing a practical, cost-effective bioremediation process.
- Complete removal of phenols in the ALR was achieved over extended operation times.
- A total of 14 g mixed phenolics was removed over 16 days in a 3.5L airlift reactor.
- These results represent the highest total removal and rate of removal of toxic phenols by WRF yet reported.

7.1.3 Usefulness of this bioremediation system

- The combination of the reactor configuration developed here and the proven efficacy of *T. pubescens* in the degradation of certain phenolic compounds form the basis of an important, novel bioremediation process.
- The evaluation of waste treatment procedures is no longer based solely on the reduction of gross indicators (e.g. BOD and COD); there is an increased emphasis on the removal of specific pollutants from waste mixtures. The development of this *Trametes* based treatment technology represents a departure from the traditional mixed culture biological processes commonly used to decrease the toxicity of industrial wastewater: here, a monoculture was used consistently reduce a range of compounds to low levels or, in most cases, completely remove them from solution.

7.2 Implications of the research carried out in this study

To achieve success using a biocatalyst it is necessary to clearly define both the market need, in a properly focused and quantified form, and the performance criteria required of the biocatalyst. Unlike other applications (e.g. the pharmaceutical industry), bioremediation does not result in the production of high value-added products. Thus, venture capital has been slow to invest in the technology and as a consequence, commercial activity in research and development has lagged far behind other industrial sectors involving bioprocesses. However, heightened environmental awareness has prompted regulatory organisations to assign economic value to environmental factors,

making by-product recovery and effective waste disposal part of standard industrial practice. With the worldwide market now estimated at \$50 billion, there are clearly tremendous opportunities available for bioremediation technologies and practitioners. The bioprocess developed in this study fulfils, at least in part, the wastewater treatment needs of various petrochemical industries.

For a bioremediation process to be successful, the bioremediation methods depend on having the right microbes in the right place with the right environmental factors for degradation to occur. Both of the *Trametes* species tested in this work were demonstrated to have the physiological and metabolic capabilities to grow successfully in the presence of, and to degrade, the target pollutants. Many researchers have argued against these fungi utilising the aromatic pollutant compounds investigated in primary metabolism. This study has demonstrated that these compounds induce the enzymes required for their catabolism, and increasing their concentrations resulted in increased fungal biomass; thus, at least partially, fulfilling the definition of primary metabolism. It must be noted however that the addition of a readily utilisable carbon source (glucose) was shown to increase and prolong oxidative enzyme production, and hence the removal of the aromatic toxins from solution, indicating a co-metabolic role.

Bioreactors are used extensively in bioremediation and their roles in the aerobic and anaerobic treatments of solid, slurry and liquid wastes are already well established. Due to their low capital and maintenance costs, ALRs are well suited to wastewater treatment and have been shown in this study to be additionally suited to the growth of *Trametes pubescens*.

The evaluation of waste treatment procedures is no longer based solely on the reduction of gross indicators (e.g. BOD and COD); there is an increased emphasis on the removal of specific pollutants from waste mixtures. Selective removal of toxic compounds may be required to meet regulatory requirements or to facilitate subsequent treatment. The development of this *Trametes* based treatment technology represents a departure from the traditional mixed culture biological processes commonly used to decrease the toxicity of industrial wastewater: in this instance a monoculture was able to consistently reduce a range of compounds to low levels or, in most cases, completely remove them from solution.

7.3 Further development

While this report shows that the objectives of the project as proposed were achieved, it would be valuable and meaningful to take the system to larger scale and demonstrate its effectiveness at pilot scale. Unfortunately this was beyond the scope of this project, and although much effort was put into attracting support from the relevant large-scale industrial company (who were kept informed about the research as it progressed), the support was not forthcoming.

7.4 Recommendations

- It is a strong recommendation of the research team that piloting of the airlift bioreactor system should be carried out.
- A link should be made with the research currently being conducted in project K5/1361 where bioremediation of agricultural wastes is leading to beneficiation of the phenolics in the waste. The bioreactor system developed here can be extended to enhance the economic value of the beneficiation process.
- There is a significant opportunity for international development of this research. Collaboration with researchers at the Department of Chemical Engineering, University of Sydney is currently being investigated as a route to achieve this. This has enormous potential in terms of international extension of the research already achieved, since researchers in this institution have expertise and interest in both bioremediation and process modelling. It is recommended that the development of this collaboration should be supported.

REFERENCES

- Addleman K and Archibald F (1993) Kraft pulp bleaching and delignification by dikaryons and monokaryons of *Trametes versicolor*. Appl. Environ Microbiol. 59 266-273.
- Addleman K, Dumonceaux T, Paice M. G, Bourbonnais R and Archibald F S (1995) Production and Characterisation of *Trametes versicolor* Mutants Unable to Bleach Kraft Pulp Appl. Environ. Microbiol. 61 3687- 3694.
- Ainsworth C G, Sparrow F K and Sussman A S (1973) The Fungi vol IVB Academic Press, London
- Alberti B and Klibanov A (1981) Enzymatic removal of dissolved aromatics from industrial aqueous effluents. Biotechnol Bioeng Symp 2: 373-379
- Alleman B C (1991) Degradation of pentachlorophenol by selected species of WRF PhD dissertation, University of Arizona, Tucson
- Alleman B C, Logan B E and Gilbertson R L (1995) Degradation of pentachlorophenol by fixed films of white rot fungi in rotating tube bioreactors. Wat. Res. 29 61-67.
- Anderson B E and Henrysson T (1996) Accumulation and degradation of dead-end metabolites during treatment of soil contaminated with polycyclic aromatic hydrocarbons with five strains of white-rot fungi. Appl. Microbiol. Biotechnol 46 647-652.
- Annachhatre A P and Gheewala S H (1996) Biodegradation of Chlorinated Phenolic Compounds Biotechnol Adv 14 35-56
- Archibald F S, Bourbonnais R, Jurasek L, Paice M G and Reid I. D (1997) Kraft pulp bleaching and delignification by *Trametes versicolor*. J. Biotechnol. 53 215-236.
- Ayazi-Shamlou P, Pollard D and Ison A Volumetric mass transfer coefficient in concentric-tube airlift bioreactors Chem. Eng. Sci 50 1579-1590

Beaudette L E, Davies S, Fedorak P M, Ward O P and Pickard M. A (1998) Comparison of gas chromatography and mineralisation experiments for measuring loss of selected polychlorinated biphenyl congeners in cultures of white rot fungi. Appl. Environ. Microbiol. 64 2020-2025.

Bergbauer M and Eggert C (1993) Degradability of chlorine-free bleachery effluent lignins by two fungi: effects on lignin subunit type and on polymer molecular weight

Bollag J-M and Leonowicz A (1984) Comparative study of extracellular fungal laccases Appl Environ Microbiol 48 849-854

Bollag J-M, Shuttleworth K L & Anderson D H (1988) Laccase-mediated detoxification of phenolic compounds. Appl. and Environ. Microbiol. 54 3086-3091.

Bonnarme P, Delattre M, Drouet H., Corrieu G and Asther M (1993) Towards a control of Lignin and Manganese Peroxidases Hypersecretion by *Phanerochaete chrysosporium* in agitated vessels: Evidence of the Superiority of Pneumatic Bioreactors on Mechanically Agitated Bioreactors Biotech Bioeng 41 440-450

Bourbonnais R. and Paice M (1990) Oxidation of non-phenolic substrates, an expanded role for laccase in lignin biodegradation FEBS Letters 267 99-102

Bradford M M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding Anal. Biochem. 72, 248

Brown J A, Alic M and Gold M H (1991) Manganese peroxidase gene transcription in *Phanerochaete chrysosporium*: activation by manganese J Bacteriol 173 4101-4106

Burton S G, Boshoff A, Edwards W, Jacobs E P, Leukes W D, Rose P D, Russel A K, Russel I M and Ryan D R (1998) Membrane-based biotechnological systems for the treatment of organic pollutants: Report to the Water Research Commission by the Department of Biochemistry and Microbiology, Rhodes University. Pp1-7. 41-54.

Chadwick Roper J, Sarkar J M, Dec J and Bollag J-M (1995) Enhanced enzymatic removal of chlorophenols in the presence of co-substrates. Wat. Res 12 2720-2724.

Chen S, Ma, D, Ge W and Buswell J (2003) Induction of laccase activity in the edible straw mushroom *Volvariella volvacea* FEMS Mic Lett 218 (143-148)

Chisti Y and Moo-Young M (1987) Airlift Reactors: Characteristics, Applications and Design Considerations Chem Eng Comm 60 195-242

Chisti Y and Moo-Young M (1996) Bioprocess Intensification through Bioreactor Engineering Trans IchemE 74 575-583

Coll P M, Fernandez-Abalos J M, Villanueva J R, Santamaria R and Perez, P (1993) Purification and characterization of a phenoloxidase (laccase) from the lignin degrading basidiomycete PM1 (CECT 2971) Appl Env Micro 59 2607-2613

Collins P J and Dobson D W (1995) Extracellular lignin and manganese peroxidase production by the white rot fungus *Coriolus versicolor* 290. Biotechnol. Letters 17 989-992

Collins P J and 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 P J, Kotterman M J, Field J A, Dobson D. W (1996) Oxidation of anthracene and benzo[a]pyrene by laccase from *Trametes versicolor*. Appl. Environ. Microbio 62 4563-4567.

Collins P J and Dobson D W (1997) Regulation of laccase gene transcription in *Trametes vesicolor*. Appl. Environ. Microbiol. 63 3444-3450.

Contreras A, Garcia F, Molinea E and Merchuk J (1999) Influence of sparger on energy dissipation, shear rate, and mass transfer to sea water in a concentric-tube airlift bioreactor Enz. Micro. Technol. 25 820-830

Cross, R H M (1987) A Handbook on the Preparation of Biological Material for Electron Microscopy, Rhodes University, Grahamstown

Cui Y Q, van der Lans R G J M and Luyben K C A M (1997) Effect of agitation intensities on fungal morphology of submerged fermentation Biotech Bioeng 55 715-726

Davis S and Burns R G (1990) Decolorisation of phenolic effluents by soluble and immobilised phenol oxidases. Appl. Microbiol. Biotechnol. 32 721-726.

Eggert C, Temp U, Dean J and Eriksson K L (1996) A Fungal Metabolite Mediates Degradation of Non-Phenolic Lignin structures and Synthetic Lignin by Laccase FEBS Lett 391 144-148

Edwards, W (1999) Capillary membrane-immobilised polyphenol oxidase and the bioremediation of industrial effluent. PhD thesis, Rhodes University

Ferscht A (1997) Enzyme structure and mechanism, 2nd Edition, W H Freeman and Company, New York

Galhaup C, Wagner H, Hinterstoisser B and Haltrich D (2002) Increased production of laccase by a wood degrading basidiomycete *Trametes pubescens* Enz Micro Tech 30 529-536

Ghose T K (1987) Measurement of cellulase activities, Pure Appl. Chem. 59 257-268

Gianfreda L, Sannino F, Filazzola M and Leonowicz A (1998) Catalytic behavior and detoxifying ability of a laccase from the strain *Cerrena unicolor* J. Molec. Cat. B 4 13-23

Gianfreda L, Xu F and Bollag J-M (1999) Laccases: A useful group of oxidative enzymes J. Bioremediation 3 1-25

Hammel, K E and Moen M A (1991) Depolymerisation of a synthetic lignin in vitro by peroxidase Enz. Microb. Technol. 13 15-18

Hammel, K E (1995) Mechanism for polycyclic aromatic hydrocarbon degradation by lignolytic fungi Environ Health Perspect 103 41-43

Heijnen J, Hols R, van der Lans R, van Leeuwen H, Mulder A and Weltevrede R (1997) A simple hydrodynamic model for the liquid circulation velocity in a full-scale two- and three-phase internal airlift reactor operating in the gas recirculation regime Chem Eng. Sci. 52 2527-2540

Heinfling A, Bergbauer M and Szewzyk U (1997) Biodegradation of azo and phthalocyanine dyes by *Trametes versicolor* and *Bjerkandera adusta*. Appl. Microbiol. Biotechnol. 48 261-266.

Jang M. Y, Ryu W R, and Cho M H (2002) Laccase production from repeated batch cultures using free mycelia of *Trametes* sp. Enz Micr Tech 30 741-746.

Jin B Yan X Q, Yu Q and van Leeuwen J H (2002) A comprehensive pilot plant system for fungal biomass protein production and wastewater reclamation Adv Environ Res 6 179-189

Johannes C and Majcherczyk A (2000) Laccase activity tests and laccase inhibitors J Biotech 78 193-199

Johannes C, Majcherczyk A and Huttermann A (1996) Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds. Appl. Microbiol. Biotechnol. 46 313-317.

Jones C, Mainwaring D, and Lonergan G. "Prediction of fungal oxidase exo-enzyme expression using the fractal dimension". In *10th. International Biotechnology Symposium*, Sydney - Australia, August 25-30 1996

Jones C and Lonergan G (1997) Prediction of phenol- oxidase expression in a fungus using fractal dimension Biotechnol Lett 19: 65-69.

- Jonsson L, Sjöström K, Haggström I and Nyman P (1995) Characterisation of a laccase gene from the white rot fungus *Trametes versicolor* Biochim. Biophys. Acta 1251 210-215
- Kaal E E, Field J A and Joyce T W (1995) Increasing lignolytic enzyme activities in several white-rot basidiomycetes by nitrogen-sufficient media. Biores. Technol. 53 133-139.
- Kadhim H, Graham C, Barratt P, Evans C and Rastall R (1999) Removal of phenolic compounds in water using *Coriolus versicolor* grown on wheat bran. Enz. Microbial Technol. 24 303-307.
- Kauffman C, Petersen B R, and Bjerrum M J (1999) Enzymatic removal of phenols from aqueous solutions by *Coprinus cinereus* peroxidase and hydrogen peroxide J Biotech 73 71-74
- Keller N and Hohn P (1997) Metabolic pathway gene clusters in filamentous fungi Fungal Gen Biol 21 17-29
- Klein J, Rosenberg M, Markos J, Dolgos O, Kroslak M and Kristofikova L (2002) Biotransformation of glucose to gluconic acid by *Aspergillus niger* – study of mass transfer in an airlift bioreactor. J Biochem Eng 10 197-205
- Klibanov, A M, Alberti B N, Morris, E D and Felshin L M (1980) Enzymatic removal of toxic phenols and anilines from waste water. J Appl. Biochem. 2 414-421
- Klonowska A, Petit J and Tron T (2001) Enhancement of minor laccase production in the basidiomycete *Marasmius quercophilus* C30 FEMS Lett 200 25-30
- Kondo R, Yamagami H. and Sakai K (1993) Xylosylation of Phenolic Hydroxyl Groups of the Monomeric Lignin Model Compounds 4-Methylguaiacol and Vanillyl Alcohol by *Coriolus versicolor* Appl. Environ. Microbiol. 59 438- 441

Kondo R, Kurashiki K and Saka K (1994) In Vitro Bleaching of Hardwood Kraft Pulp by Extracellular Enzymes Excreted from White Rot Fungi in a Cultivation System Using a Membrane Filter Appl. Environ. Microbiol. 60 921- 926

Kowalska M, Bodzek M and Bohdziewicz J (1998) Biodegradation of phenols and cyanides using membranes with immobilised microorganisms. Process Biochem. 33 189-197.

Lacki K and Duvnjak Z (1999) A Method for the Decrease of Phenolic Content in Commercial Canola Meal Using an Enzyme Preparation Secreted by the White-Rot Fungus *Trametes versicolor* Biotech. Bioeng. 62 422-433

Lee J, Lee S Y, Park S and Middelberg A P (1999) Control of Fed- Batch Fermentation Biotechnol Adv 17 29-48

Lee I H, Walline R G and Plamman M (1998) Apolar growth of *Neurospora crassa* leads to increased secretion of extracellular proteins Mol Microbiol 29 209-218

Leukes W D Jakobs E, Rose P, Sanderson R and Burton S (1995) Method for Producing Secondary Metabolites, Patent #7366 (RSA)

Liu S Y and Bollag J M (1985) Enzymatic Binding of the Pollutant 2,6- Xylenol to a Humus Constituent Water, Air Soil Pollut 25 97-106

Logan B E, Alleman B C, Amy G L and Gilbertson R L (1994) Adsorption and removal of pentachlorophenol by white rot fungi in batch culture. Wat. Res. 28 1533-1538.

Luke A and Burton S (2001) A novel application for *Neurospora crassa*: Progress from batch culture to a membrane bioreactor for the bioremediation of phenols Enz. Microb. Technol. 7 348-356

Majcherczyk A, Johannes C and Huttermann A (1998) Oxidation of polycyclic aromatic hydrocarbons (PAH) by laccase of *Trametes versicolor*. Enz. Microb. Technol. 22 335-341.

Maremonti M, Sannino F, Gianfreda L and Greco G Jnr in preparation

Merchuk J and Berzin I Distribution of energy dissipation in airlift reactors Chem. Eng. Sci. 50 2225-2233

Merchuck J C, Contreras A, Garcia F and Molina E (1998) Studies of mixing in a concentric tube airlift bioreactor with different spargers. Chem Eng Sci 53 (4) 709-719

Mielgo I, Moreira M, Feijoo G, Lema J (2001) A packed-bed fungal bioreactor for the continuous decolourisation of azo-dyes (Orange II) J Biotechnol 89 99-106

Moreira M T, Sanroman A, Feijoo G and Lema J M (1996) Control of pellet morphology of filamentous fungi in fluidized bed bioreactors by means of a pulsing flow. Application to *Aspergillus niger* and *Phanerochaete chrysosporium* Enzyme Micob. Technol. 19 261- 266

Morgan P, Lewis S and Watkinson J (1991) Comparison of abilities of white-rot fungi to mineralize selected xenobiotic compounds Appl. Microbiol Biotechnol 34 693-696

Morgan P, Lee SA, Lewis ST, Sheppard A N and Watkinson R J (1993) Growth and Biodegradation by White-Rot Fungi Inoculated into Soil Soil Biol Biochem 2 279-287

Muncnerova D and Augustin J (1994) Fungal metabolism and detoxification by polycyclic aromatic hydrocarbons: A review. Bioresource Technol. 48 97-106.

Nakamura Y, Sungusia M, Sawada T and Kuwahara M (1999) Lignin- degrading enzyme production by *Bjerkandera adusta* immobilized on polyurethane foam J Biosci Bioeng 88 41-47

Novotny C, Vyas B, Erbanova P, Kubatova A and Sasek V (1997) Removal of PCBs by various white rot fungi in liquid cultures. Folia Microbiol. 42 136-140.

Paice, M G, Reid, I D, Bourbonnais, R, Archibald, F.S. and Jurasek, L. (1993) Appl. Environ. Micro. 59 260- 265

Pallerla S and Chambers R (1995) Continuous decolorisation and AOX reduction of bleach plant effluents by free and immobilised *Trametes versicolor*. J. Environ. Sci. Health A30 423-437.

Pallerla S and Chambers R P (1997a) characterization of a Ca-alginate-immobilised *Trametes versicolor* bioreactor for decolorization and AOX reduction of paper mill effluents Bioresource Technol 60 1-8

Pallerla S and Chambers R P (1997b) Biotreatability of Foul Condensates in Combination with Caustic Bleach Filtrate from the Paper Industry Environmental Engineering Science 14 105-111

Pallerla S and Chambers R P (1998) Reactor development for the biodegradation of pentachlorophenol Catalysis Today 40 103-111

Papagianni, M and Moo-Young M (2002) Protease secretion in glucoamylase producer *Aspergillus niger* cultures: fungal morphology and inoculum effects Process Biochem 37 1271-1278

Paszczyński A & Crawford R. L (1995) Potential for bioremediation of xenobiotic compounds by the white-rot fungus *Phanerochaete chrysosporium* . Biotechnol. Prog. 11 368-379.

Philips T and du Toit K (2003) Water Reuse and Recycling at SASOL, submitted for publication

Pointing S B (2001) Feasibility of bioremediation by white rot fungi Appl Microbial Biotechnol 57 20-33

Poulsen B R and Iverson J J L (1999) Membrane Sparger in Bubble Column, Airlift, and Combined Membrane-Ring Sparger Bioreactors Biotech Bioeng 64 452-458

Prillinger H and Esser K (1975) The phenoloxidases of the ascomycete *Podospora anserina* XIII. Action and interaction of genes controlling the formation laccase Molec Gen Genet 156 333-345

Rancano G, Lorenzo M, Molares N, Rodriguez Couto S and Sanroman M (2003) Production of laccase by *Trametes versicolor* in an airlift fermentor Proc Biochem in press

Rayner A, Boddy L and Dowson C (1987) Genetic interactions and developmental versatility during establishment of decomposer basidiomycetes in wood and tree litter. In Ecology of Microbial Communities Cambridge University Press

Reddy A (1995) The potential for white rot fungi in the treatment of pollutants. Curr. Opin. Biotechnol 6 320-328

Robinson T, McMullan G, Marchant R and Nigam P (2001) Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative Bioresource Tech 77 247-255

Rodriguez Couto S, Moldes D, Liebanas A and Sanroman A (2003) Investigation of several bioreactor configurations for laccase production by *Trametes versicolor* operating in solid-state conditions J. Biochem. Eng. 15 21-26

Rogalski J, Lundell T, Leonowicz A and Hatakka, A. (1991) Production of Laccase, Lignin Peroxidase and Manganese Dependant Peroxidase by Various Strains of *Trametes versicolor* Depending on Culture Conditions Acta Microbiol Pol 40, 221- 234

Roy B P and Archibald F (1993) Effects of Kraft Pulp and Lignin on *Trametes versicolor* Carbon Metabolism Appl. Environ. Microbiol. 59 1855-1863

Roy B P, Dumonceaux T, Koukoulas A A and Archibald F S (1996) Purification of Cellobiose Dehydrogenases from the White Rot Fungus *Trametes versicolor* Applied and Environmental Microbiology 62 4417-4427

Roy-Arcand L and Archibald F S (1991) Direct dechlorination of chlorophenolic compounds by laccases from *Trametes (Coriolus) versicolor* Enzyme Microb. Technol. 13 194- 203

Royer G, Yerushalmi L, Rouleau D and Desrochers M (1991) Decolourisation of bleached kraft effluents by *Coriolus versicolor* in the form of pellets J Ind Microbiol 7 269-278

Ruggeri B and Sassi G (2003) Experimental sensitivity analysis for lignin peroxidase production by *P. chrysosporium* Process Biochem in press

Ryan D (1996) A preliminary investigation into the potential of a local strain of the fungus *Trametes (Coriolus) versicolor* for the bioremediation of aromatic pollutants. Honours Thesis, Rhodes University, Grahamstown, South Africa

Schlosser D, Grey R & Fritsche W (1997) Patterns of lignolytic enzymes in *Trametes versicolor*. Distribution of extra- and intracellular enzyme activities during cultivation on glucose, wheat straw and beech wood. Appl. Microbiol. Biotechnol. 47 412-418.

Schnurer J and Rosswall R (1982) FDA Hydrolysis as a Measure of Total Microbial Activity in Soil and Litter Appl. Environ. Microbiol. 43 1256- 1261

Schugerl K and Lubbert A (1994) Design of pneumatically agitated bioreactors, Chap 7, pp257-303 in: Bioreactor system design, Asenjo J A and Merchuk J C eds, Marcel Dekker, New York

Score A J, Palfreyman J W and White N A (1997) Extracellular phenoloxidase and peroxidase enzyme production during interspecific fungal interactions Internat Biodeter & Biodegradat 39 225-233

Sharland P R, Burton J L and Rayner A D M (1986) Mycelial dimorphism, interactions and pseudosclerotial plate formation in *Hymenochaete corrugata* Trans Brit Myc Soc 86 158-163

Shim S and Kawamoto K (2002) Enzyme production activity of *Phanerochaete chrysosporium* and degradation of pentachlorophenol in a bioreactor Wat Res 36 4445-4454

Shuttleworth K L and Bollag J-M (1986) Soluble and immobilised laccases as catalysts for the transformation of substituted phenols Enzyme Microb. Technol. 8 171- 176

Shuttleworth K L, Postie L, and Bollag J-M (1986b) Production of induced laccase by the fungus *Rhizoctonia praticola* Can. J. Microbiol 32 867-870

Siegel M H, Merchuk J C and Schurgel K (1986) Airlift reactor analysis: Interrelationships between riser, downcomer and gas-liquid separator behaviour including gas recirculation effects AIChE J. 32 1585-1596

Su W and He B (1997) Secreted enzyme production by fungal pellets in a perfusion bioreactor J Biotechnol 54 43-52

Swamy J and Ramsay J A (1999) The evaluation of white rot fungi in the decoloration of textile dyes. Enz. Microbial Technol. 24 130-137.

Stitt E H (2002) Alternative multi phase reactors for fine chemicals. A world beyond stirred tanks Chem Eng J 90 47-60

Tariq V-N and Irvine W T (1995) Lignolytic Enzyme Activity: A comparison of the Veratryl Alcohol and Azure B Assay Systems Biotech Tech 9 197-202

Tien M (1987) Properties of ligninase from *Phanerochaete chrysosporium* and their possible applications Crit. Rev. Microbiol 15 141-167

Taspinar A and Kolankaya N (1998) Optimisation of Enzymatic Chlorine Removal from Kraft Pulp. Bull. Environ. Contam. Toxicol 61 15-21

Thurston C F (1994) The structure and function of fungal laccases. Microbiology. 140 19-26.

Ulmer D, Leisola M, Puhakka J and Fiechter A (1983) *Phanerochaete chrysosporium*: growth patterns and lignin degradation Eur. J. Appl. Microbiol. Biotechnol 18 153-157

- van Benthum W, van der Lans R, van Loosdrecht M and Heijnen J (1999) Bubble recirculation regimes in an internal loop airlift reactor Chem. Eng. Sci. 54 3995-4006
- van Schie D and Young P (1998) Isolation and characterisation of phenol-degrading denitrifying bacteria. Appl Environ. Microbiol 64 24432-2438
- Vilchez M J, Vigara J, Garbayo I. and Vilchez C (1997) Electron microscopic studies on immobilised growing *Chlamydomonas reinhardtii* cells Enzyme Microb. Technol. 21 45-47
- Watanabe Y, Sugi R, Tanaka Y and Hayashida S (1982) Enzymatic decolorization of melanoidin by *Coriolus* sp. Agric. Biol. Chem. 46 1623-1628
- Wang S-J. and Loh K-C. (1999) Modelling the Role of Metabolic Intermediates in Kinetics of Phenol Biodegradation Enz Micro Techn 25 177-184
- Wells J and Boddy L (1990) Wood decay and phosphorus and fungal biomass allocation in mycelial cord systems New Phytol 116 285-295
- Wenhua H and Ding Z (1993) Treatment of phenol-containing wastewater by immobilised microorganisms, Water Treatment 8 119-126
- White N A and Boddy L (1992) Extracellular Enzyme Localisation during Interspecific Fungal Interactions FEMS Micro Let 98 75-80
- White N A and Boddy L (1992b) Differential Enzyme Production in Colonies of *Coriolus versicolor*, *Phlebia radiata* and *Phlebia rufa*: Effect of a Gaseous regime J. Gen Microbiol 138 2589- 2598
- White N A, Palfreyman J W and Crawford J (1998) Interspecific fungal interactions on spatially heterogeneous systems FEMS Microbiolo Eco 27 21-32
- Wolfenden B S and Willson R L (1982) Radical-cations as reference chromogens in kinetic studies of one-electron transfer reactions J Chem Soc Perkin Trans II 1982 805-812
- Xu F (1997) Effects of Redox Potential and Hydroxide Inhibition on the pH Activity Profile of Fungal Laccases J Biological Chemistry 272 924-928

Xu F, Kulys J, Duke K, Kaichang L, Krikstopaitis K, Deussen H, Abbate E, Galinyte V, and Schneider P (2000) Redox chemistry on laccase catalysed oxidation of N-hydroxyl compounds App Environ Micro 66 2052- 2056

Yarapolov A I., Skorobgatko O V, Vartanov S S, and Varfolomeyev S D (1994) Laccase properties, catalytic mechanism and applicability Applied Biochemistry and Biotechnology 49 257-280

Zhang F-M, Knapp J S and Tapley K N (1999) Development of bioreactor systems for decolorization of Orange II using white-rot fungus. Enz Microb. Technol. 24 48-53.

Zouari H, Labat M and Sayadi S (2002) Degradation of 4- chlorophenol by the white rot fungus *Phanerochaete chrysosporium* in free and immobilized cultures Biores Technol 84 145-150.