REACTOR DEVELOPMENT AND PROCESS OPTIMISATION FOR THE BIOREMEDIATION OF PHENOLIC WASTEWATERS BY *TRAMETES* SPECIES

THESIS

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ABSTRACT

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 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* with an Airlift Loop Reactor found suitable for the large-scale fermentation of these organisms.

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 of the effluent monomers being removed in flask culture. An attempt was made to characterise 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 been hindered by the lack of suitable bioreactor systems. A hollow fibre membrane bioreactor and a trickle filter were considered as supports for immobilised biofilms of *T. versicolor* and laccase production and pollutant degradation were demonstrated in both reactor configurations. 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 and favourable feeding strategies, the effects of inducers/precursors and the timing of effluent addition were ascertained. Increased final values for growth (10 g/L dry mass) and enzyme production (12000 U/L) as well as effluent degradation (5% v/v/day) were achieved in the ALR in fermentations exceeding two weeks. This study demonstrated, 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.

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---- Science is not a sacred cow. Science is a horse. Don't worship it. Feed it. ----

Aubrey Eben

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CHAPTER 1

REVIEW OF LITERATURE

1.1 Introduction

This thesis 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 xylidine 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 Physiology of *Trametes versicolor*

1.2.1 Taxonomy of the strain

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. The hyphal system can be classed into three distinct groups, namely generative, skeletal and binding hyphae. The generative hyphae ultimately give rise to the basidia, and directly or indirectly to all other structures. They are produced directly from the secondary mycelium and so frequently possess clamp connections. 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).

In *T. versicolor*, a homokaryotic mycelium is formed as a result of the germination of the basidiospore. Two compatible homokaryons then interact to form the characteristic dikaryon or secondary mycelium. Homokaryotic mycelia have been isolated from nature and may be much more common than once thought, although the dikaryotic form is the dominant phase (Alexopoulos and Mims, 1979). *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. This organism also produces "pseudo-sclerotal" plates in response to environmental stress. These structures are dense masses of darkly pigmented swollen hyphae. It has been suggested that the use of monokaryons may be preferable in the development of a biobleaching system. Monokaryons also have reduced numbers of isozymes of the essential bleaching enzymes and so would provide a genetically and biochemically less complicated delignification mechanism (Addleman and Archibald, 1993).

1.2.2 Differential enzyme production/functional modes of *T. versicolor*

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 morphogentic 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) As an example: colonies of *Phlebia sp.* have a peripheral growing front composed of sparsely branched, rapidly extending, coenocytic, appressed, non-anastomosing and non-combative hyphae, followed by a mycelial system of highly branched, submerged and aerial hyphae, with clamps if mating has occurred. 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. The mechanisms controlling the switches between mycelial modes are not fully understood. White and Boddy (1992b) hypothesized that the dysfunction of mitochondrial Krebs-cycle-associated primary metabolism represents a way of switching cells into secondary metabolism, by activating, or derepressing the acetate pathway and phenoloxidase enzyme systems. The resultant production of highly hydrophobic compounds and free radicals would then have important effects on mycelial morphology and physiology. For example, aerial mycelia must acquire a hydrophobic surface to achieve their morphology.

Iimura and Tatsumi (1997) searched for genes in *T. versicolor* that were induced by PCP by using mRNA differential display techniques. Five cDNA fragments were cloned and the DNA sequences of two of the fragments were analysed further. The sequences corresponded to novel genes that had not been previously identified in *T. versicolor*. One of the cDNA's exhibited strong sequence homology to enolase, a glycolytic protein that may have an isoform-specific role in the stress response of *T. versicolor*, and the other to a heat shock protein (HSP30). The expression of both was up-regulated in PCP-challenged *T. versicolor*. The promoter regions of both these genes are similar to those found in MnP of *Phanerochaete chrysosporium*. Li *et al.* (1995) reported that the MnP of *P. chrysosporium* was regulated at the transcriptional level by nutrients, manganous ions, and oxidative and chemical stress. It is reasonable then to presume that MnP and possibly other lignolytic enzymes of *T. versicolor* are also regulated by chemical stress.

1.2.3 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. For example, the lignolytic activity of *P. chrysosporium* only becomes apparent subsequent to the primary growth phase when carbon, nitrogen or sulfur limitation occurs. 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 The Enzymology of Laccase

1.3.1 General properties of the enzyme

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. O_2 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 O_2 to form H_2O. Experimental evidence supports a scheme in which the O_2 molecule firsts binds to the Type 2 and one of the Type 3 Cu to undergo "asymmetric" activation. The O_2 binding pocket appears to restrict the access of oxidising agents other than O_2 (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-xylidine 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 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 in *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 gene transcription

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-

hydroxybezatriole (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 at 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. An investigation of the mechanism of induction of another oxidase, mammalian cytochrome P-450c, in response to aromatic hydrocarbons revealed xenobiotic responsive elements (XRE's) in the promoter region (Collins and Dobson, 1997). The XRE receptor or binding protein is a member of a large family of regulatory proteins that activate gene transcription in response to the presence of non-polar carbon compounds. Putative XRE promoters have been found in three white rot fungi (Collins and Dobson, 1997). The *lcc* promoter in the basidiomycete PM1, a fungus closely related to *T. versicolor*, contains a sequence identical to the XRE consensus 180 bp upstream of the TATA box (Collins and Dobson, 1997). The presence of these XRE's suggests that transcription of the *lcc* genes is indeed activated by aromatic compounds.

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. Protein secretion is a highly polarised process involving the movement of the protein-containing vesicles to the hyphal tip and is believed to involve the microtubule network. This system is not unique

to fungi and is also found in some mammalian cells e.g. nerve cells. The apical localisation of protein secretion has led to the suggestion of employing morphoplogical mutants displaying an increased apical surface i.e. hyperbranching mutants as 'supersecretion' strains (Lee *et al.*, 1998). Moreover, hyperbranching strains often grow as compact pellets, resulting in low-viscosity cultures, which have additional technical advantages in the fermentation process (Conesa *et al.*, 2001). Proteins are thought to be released onto the surface of the plasma membrane at the hyphal apex, where they are taken into the wall as part of the mass flow of new wall material. As the wall thickens, the protein molecules are taken to the hyphal surface and then become part of the surface topography of the wall, or are released into the external medium. Some proteins may remain trapped in the wall as it undergoes maturation in the subapical zone. In this way, secretion is restricted to the growing hyphal tip (Conesa *et al.*, 2001).

Secretory proteins begin their journey to the extracellular medium by entering the endoplasmic reticulum (ER). In the ER proteins are folded and can undergo modifications such as glycosylation, disulfide bridge formation, phosphorylation and subunit assembly. Subsequently, proteins leave the ER in transport vesicles and undergo further modification in the Golgi compartment. Finally, again packed in secretory vesicles, proteins are directed to the plasma membrane to which they fuse and then release their contents. In some cases the proteins will not reach the extracellular space, but are targeted to intracellular compartments, such as the vacuole, either to become resident proteins or to undergo proteolytic degradation.

1.4 Substrates of interest 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), MnO_2 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 octonal- 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 substrates 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%, ethyl-benzene 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 sp^2-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.

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 (Paszczynski & Crawford, 1995). The toxicity and mutagenicity of nitro-aromatics and their recalcitrance to biodegradation underlie concerns about their environmental fate (Paszczynski & Crawford, 1995).

1.4.8 Xylenols

Xylenols (dimethylphenols) are major pollutants in waste-waters of industrial processes such as coal gasification and liquefication, 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- xylenol 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-xylenol 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 diffusable electron carrier, because laccase is a large molecule (M_w ~ 70 000kD) 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)

1.4.9.1 The Role of Laccase in Kraft Pulp Delignification

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).

1.6 BIOREACTORS FOR THE APPLICATION OF *TRAMETES* SYSTEMS

1.6.1 Introduction

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 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.

1.6.2 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 various 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).

1.6.3 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).

1.6.4 Airlift loop reactor (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 CO_2- 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.

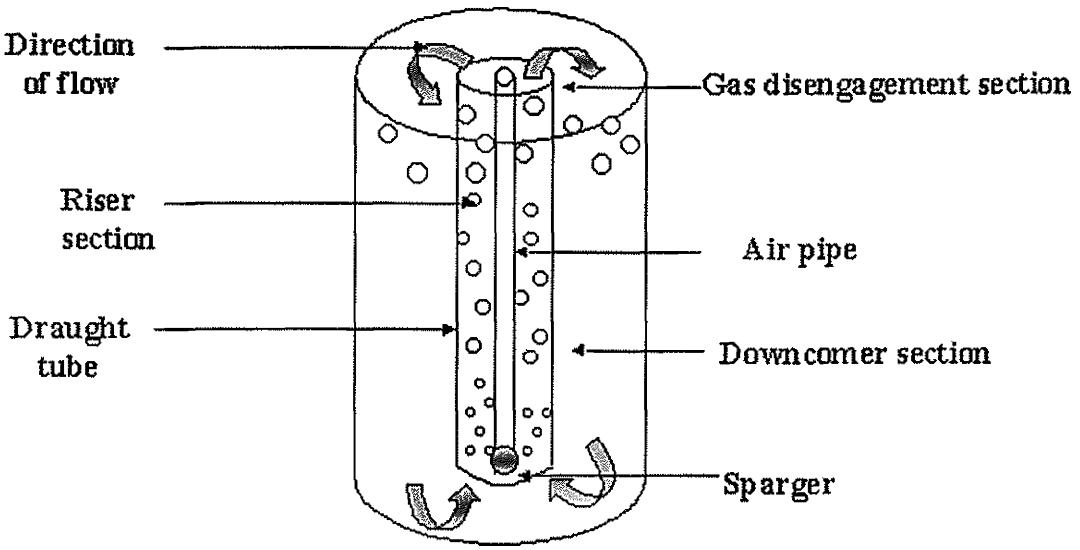


Fig 1.1 Schematic diagram of concentric draught-tube internal loop ALR

Gas-mixed reactors do not have impellers to decrease bubble diameter and increase the volumetric oxygen mass transfer coefficient (K_La). 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. 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.

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 treat biological wastewaters, it is generally accepted that airlift loop reactors are the most economical with regard to several important performance criteria:

i) energetic oxygen transfer – almost twice as efficient as its nearest competitor (low depth clarification basin with surface aerators)

ii) oxygen transfer rate

iii) application to large volume systems

iv) reduced power consumption with comparable/improved productivity relative to STR's (Schugerl and Lubbert, 1994)

v) decreased shear stress

vii) extended aseptic operation (due to the elimination of stirrer shafts, seals and bearings) (Chisti and Moo-Young, 1987).

1.7 OBJECTIVE

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 of vital importance 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.

1.7.1 Hypothesis

Increased growth and production of the laccase enzyme by *Trametes* species is a process that can be regulated by the addition of phenolic inducer compounds and further controlled by medium engineering, bioreactor design and fermentation conditions. This system can then be used for the bioremediation of phenolic-containing wastewaters.

1.7.2 Specific objectives

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

1.) Develop a suitable growth medium for *T. versicolor* and *T. pubescens* and optimise it in terms of fungal growth and enzyme production.
2.) Maximise laccase production by *T. versicolor* by the addition of inducer compounds and relate these to potential bioremediation targets.
3.) Investigate the physiological effects on *T. versicolor* of the addition of a phenolic effluent and how to relate these effects to efficient pollutant removal.

4.) Explore different bioreactor configurations in terms of fungal growth, enzyme production and bioremediation potential.

5.) Develop an effective bioremediation process at lab scale for the removal of phenolic pollutants from industrial wastewater by *T.* versicolor based on (1-4).

CHAPTER 2

THE EFFECT OF MEDIUM OPTIMISATION AND THE ADDITION OF INDUCERS ON GROWTH AND LACCASE PRODUCTION IN *TRAMETES* SPECIES

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 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 Evaluation 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 for cost reasons. Since their exclusion had no observable effects, 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 the fungal biomass from 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 media 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. 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 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 (ε = 36 000 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.

2.2.7 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 microorganism with growth and to this end we defined the lump parameter '**growth related productivity** (GRP) where:

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

with units: U/g/d

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

2.3 RESULTS AND DISCUSSION

2.3.1 Media comparison

Initial efforts to grow *T. versicolor* in flask culture for laccase production were unsuccessful in 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 2.5), 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).

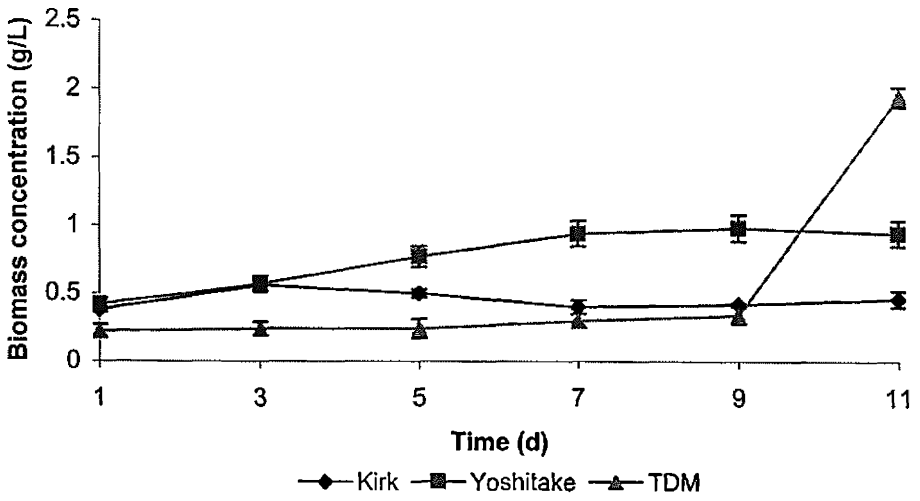


Fig 2.1 Growth of *T. versicolor*, measured as dry mass, in three different growth media

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).

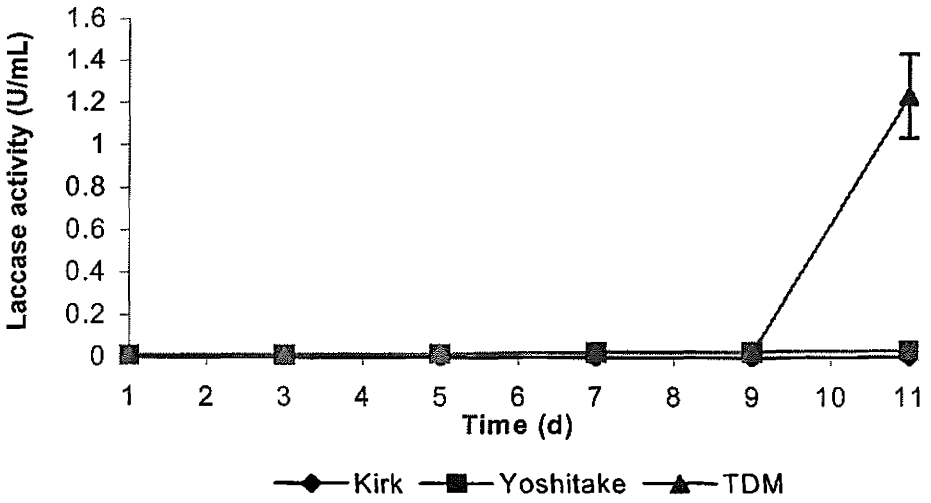


Fig 2.2 Production of the laccase enzyme by *T. versicolor* in three different growth media

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 followed similar trends, although the time period was increased, this could have been caused inoculum effects e.g. homogenised for too long. 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.

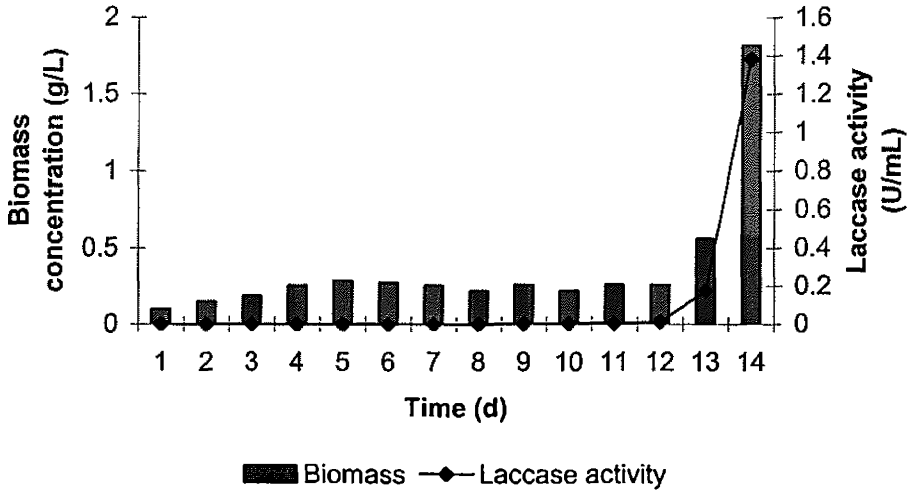


Fig 2.3 Growth and enzyme production of *T. versicolor* grown on TDM.

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 also tested on the basis that the medium containing 6 g/L had produced only limited, slow growth. In this study, 10 g/L was considered 'sufficient' glucose. 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 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.

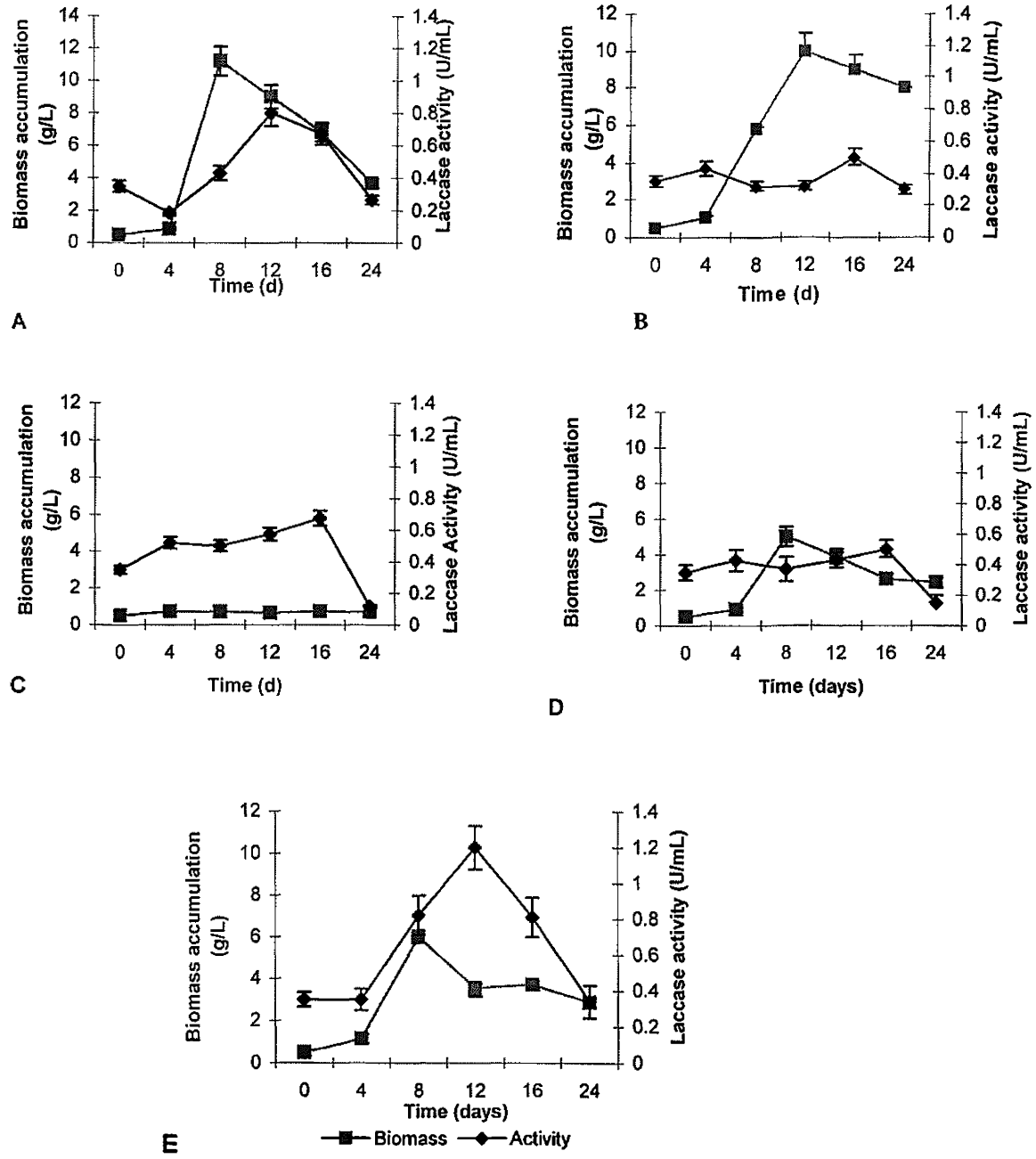


Fig 2.4 The increase in biomass and laccase activity over time for cultures of *T. versicolor* grown in TDM with different glucose (C) and peptone (N) concentrations: 20 g/L C: 20 g/L N (**A**), 20 g/L C: 2 g/L N (**B**), 2 g/L C: 2 g/L N (**C**), 2 g/L C: 20 g/L N (**D**) and 10 g/L C: 10 g/L N (**E**).

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-sufficient 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.

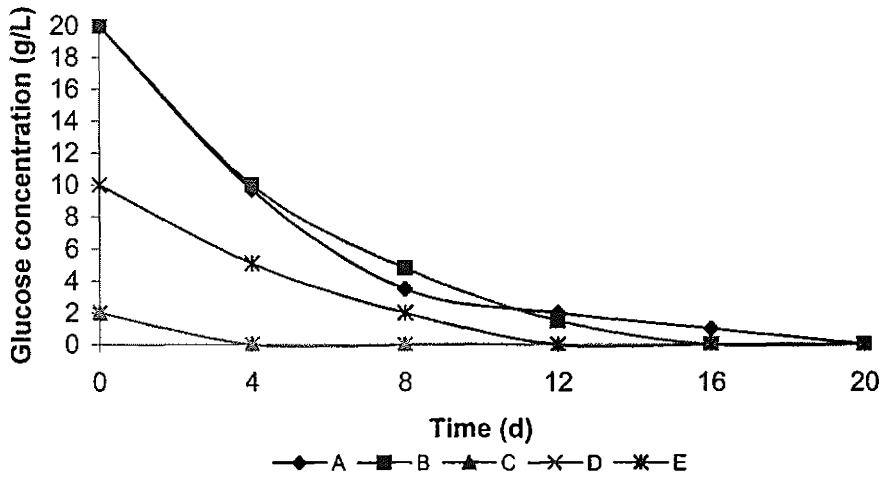


Fig 2.5 Glucose utilisation rate for different starting concentrations of glucose (20g.L^{-1} C: 20g.L^{-1} N (**A**), 20g.L^{-1} C: 2g.L^{-1} N (**B**), 2g.L^{-1} C: 2g.L^{-1} N (**C**), 2g.L^{-1} C: 20g.L^{-1}N (**D**) and 10g.L^{-1} C: 10g.L^{-1} N (**E**).

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 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 (40 g/L, Galhaup *et al.*, 2002), 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 2.6). However, laccase production 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, as this quickly produced elevated levels of laccase, without excess biomass. 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).

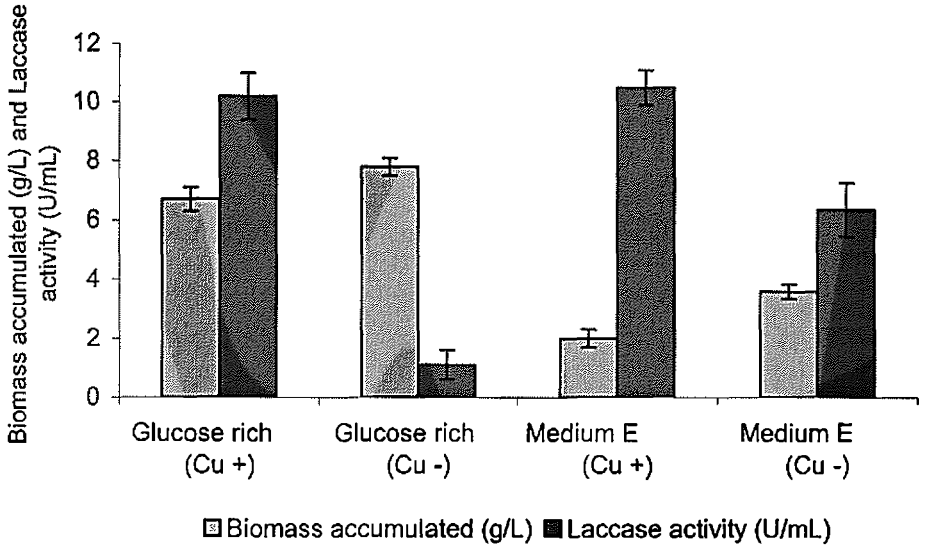


Fig 2.6 Biomass accumulation (dry mass) and laccase activity in cultures of *T. pubescens* grown in a complex, glucose rich medium and medium E. (Cu +) indicates the addition and (Cu -) the omission of 5mM copper.

Although copper was certainly found to be an effective inducer of laccase activity, at high concentrations 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 laccase genes 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 laccase 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 of 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 (section 5.3.4).

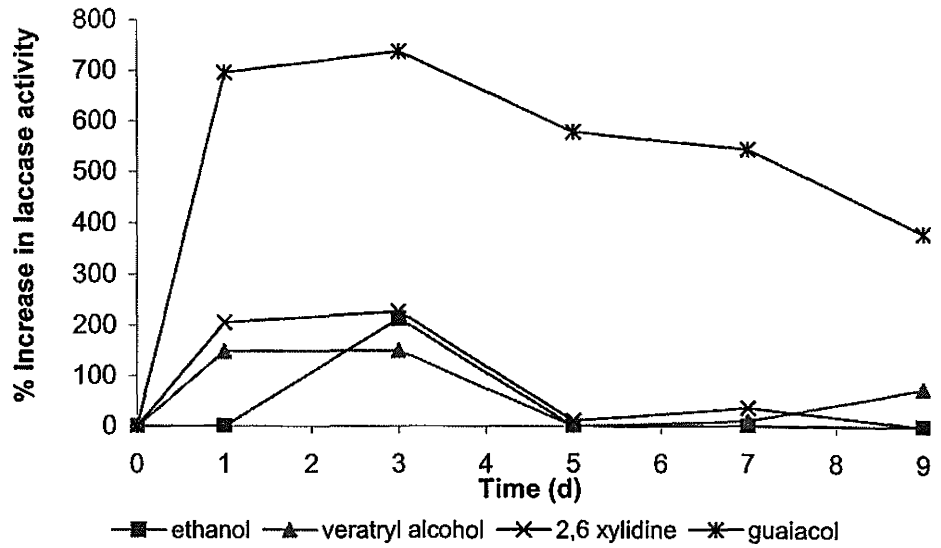


Fig 2.7 Effect of different inducers on laccase production in flask cultures of *T. versicolor*.

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 xylidine (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-xylidine, 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).

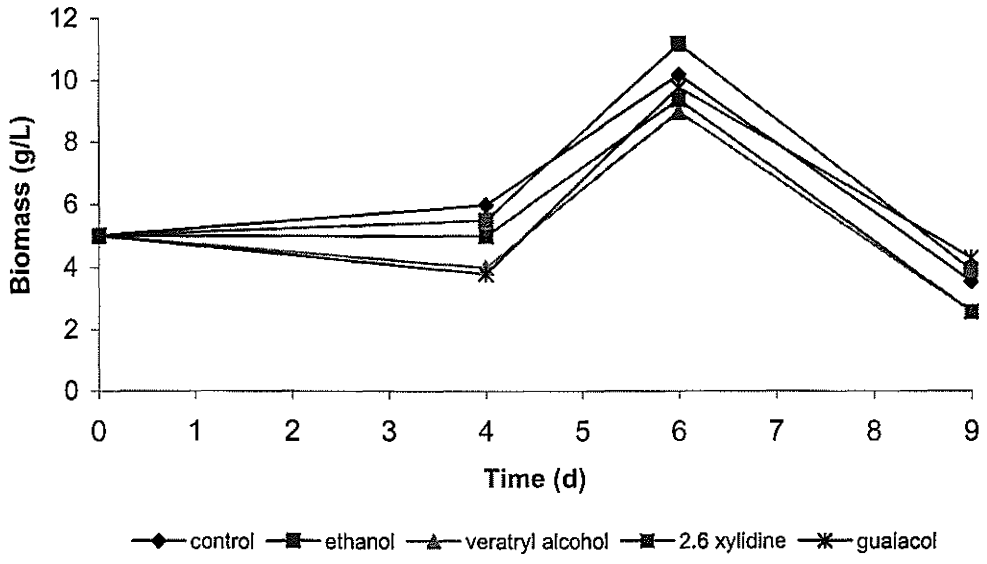


Fig 2.8 The effect of lignolytic enzyme inducers on biomass accumulation in *T. versicolor*

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 process (Appendix C) is known to contain phenol, cresol and other potentially useful aromatic monomers (Table 3.1). The effluents were filter sterilised and added to *T. versicolor* flask cultures after 7 days growth to final concentrations of 1 and 2%. The presence of both concentrations of both effluents resulted in increased laccase production over the growth-medium-only (baseline) control (Fig. 2.9), with 1% pulp-and-paper effluent having the greatest effect, increasing the measured laccase activity by 670%. However, this was a short-term effect, with the guiacol disappearing rapidly from solution along with its induction effect (see section **5.3.4/5.3.5**). The 2% cresylic effluent produced a longer lasting induction effect over 8 days (Fig. 2.9) and on this basis was selected for further research.

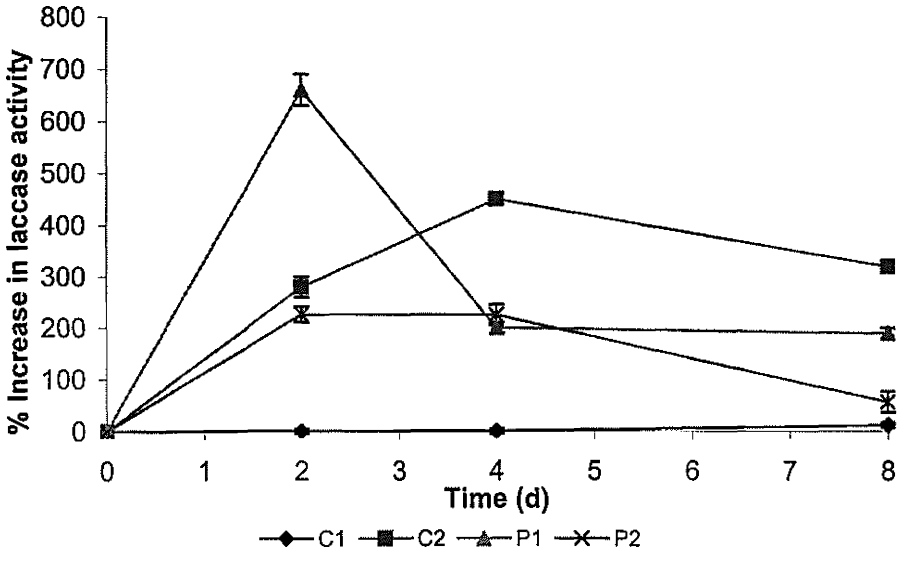


Fig 2.9 The effect of industrial effluents, as inducers, on laccase enzyme activity of *T. versicolor* (1% v/v Cresylic (C1); 2% v/v Cresylic (C2); 1% v/v Pulp mill (P1); 2% v/v Pulp mill (P2)) Percentage increase is relative to control flasks without inducers.

2.4 CONCLUSIONS

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. On further investigation it was found that 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) and 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, and so produce 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. versicolo*r 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 1.52 U/mL (cresylic effluent) achieved. This is one of the highest enzyme activities reported for flask cultures of *T. versicolor* (Table 2.2). 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 potential bioremediation applications of *T. versicolor* (in flask culture)

REFERENCE	ACTIVITY (U/mL)	APPLICATION
Addleman *et al.*, (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 *et al.*, (1997)	0.48	Decolourisation of dyestuffs
Rogalski *et al.*, (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

PROCESS DEVELOPMENT: FACTORS INFLUENCING GROWTH AND LACCASE PRODUCTION IN FLASK CULTURE

3.1 Introduction

Although there has been much research focused on the mechanisms of enzymatic 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* 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.

COMPOUND	CONCENTRATION (mM)	HPLC RETENTION TIME (min)
phenol	82.8	8.97
p- cresol	24.99	15.62
m- cresol	25.8	18.80
o- cresol	77.03	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 flouroscein diacetate (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 and 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 syringaldizine. 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 $ABTS^{+\bullet}$ (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. As their exception had no effect on growth and laccase production, 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 (as described above) 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.

3.2.6 Verification of laccase authenticity by gel electrophoresis

A portion of the crude extract obtained above was partially purified by acetone precipitation (1:1 vol/vol). Thereafter samples where prepared for electrophoresis on an SDS-PAGE activity gel, these samples comprised of 50 µL enzyme (20 µg/mL) and 50 µL dissociation buffer. They were heated in boiling water for 5 min and then cooled to room temperature. 10 µL of each sample was then separated on a PAGE (4 %) gel electrophoresed in a 1 x SDS running buffer. The gel was run at 120 V for 180 min and activity stained with 0.5 mM ABTS solution.

3.2.7 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 10 x the volume of the reactants as used in the ABTS laccase assay (section **2.2.7**). The oxygen consumption was measured with a dissolved oxygen probe (YSI 5740) linked to a HiTech Microsystems DO/OUR meter. The system was activated on addition of the enzyme sample (isolated in **3.2.5** and **3.2.6**) 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 µg/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 and 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 7 d growth. Varying amounts of filter sterilised cresylic SGL 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 **3.2.3**) 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. Each set was run in triplicate.

3.2.10 HPLC analysis

Daily 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.

3.2.11 Electron Microscopy

The mycelial mats were removed from the stationary flask cultures after 9 days incubation 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 $x10^{-1}$ 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 $5x10^{-1}$ 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).

3.3 RESULTS AND DISCUSSION

3.3.1 Protein (laccase) extraction from culture fluid.

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). Protein concentration was determined according to Bradford (1976) and specific activities were calculated. The purified laccase samples from the crude filtrate obtained showed more than double the activity of the commercial product (Table 3.1).

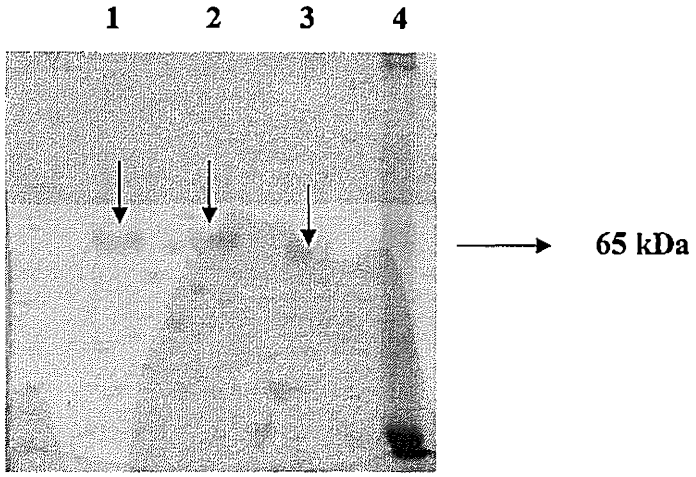


Fig 3.2 SDS PAGE gel of commercial (lane 3), and purified laccase (lanes 1 and 2) obtained from *T. versicolor* culture filtrate. Lane 4 carried molecular weight markers.

Table 3.2 Specific activities of four laccase samples from different origins

SAMPLE	SPECIFIC ACTIVITY (U/ mg protein)
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, usually after 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* after 12 d in the presence of cresylic effluent.

EFFLUENT CONC. (mM)	BIOMASS (g/L)	BIOMASS MORPHOLOGY	MEDIUM APPEARANCE
Control	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

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. 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 also observed in liquid culture and were accompanied by a concomitant exponential increase in the expression of laccase into the extracellular medium. Hypersecretion was stimulated approximately 7070% over the control at 5 days. Jones and Lonergan (1997) demonstrated a strong correlation between spatial pattern phenomena (morphology) and physiological function.

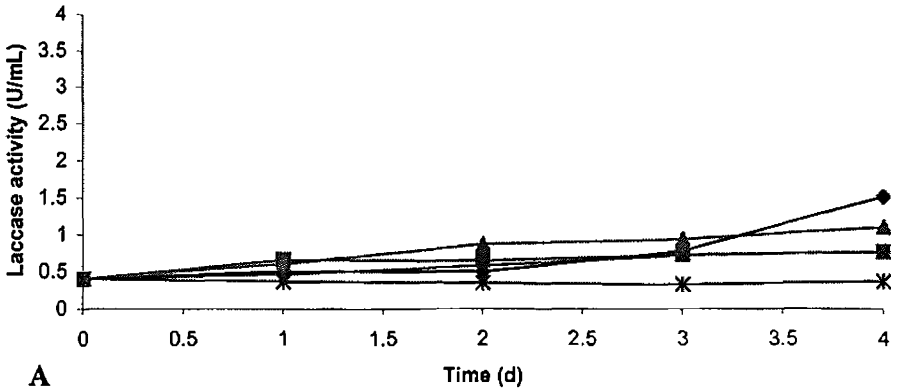
An obvious correlation exists between the effects of the paramorphogen (cellobiose) used by Jones and Lonergan (1997) and the phenolic effluent used in this study. Both had adverse (limiting) morphological effects on the fungi in question and both stimulated increases in laccase concentration. Morphogenic differentiation in fungi has thus 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 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 effluent monomers 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 the 12 day-old cultures (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, for 10 g/L starting concentration (Fig 2.5) and fits the model of lignolytic enzyme production as a result of nutrient limitation. Later experiments that included a daily feeding regime of low (non-repressing) concentrations of glucose resulted in this effect being kept up for several days (section **5.3.5**) The control (zero effluent) 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.



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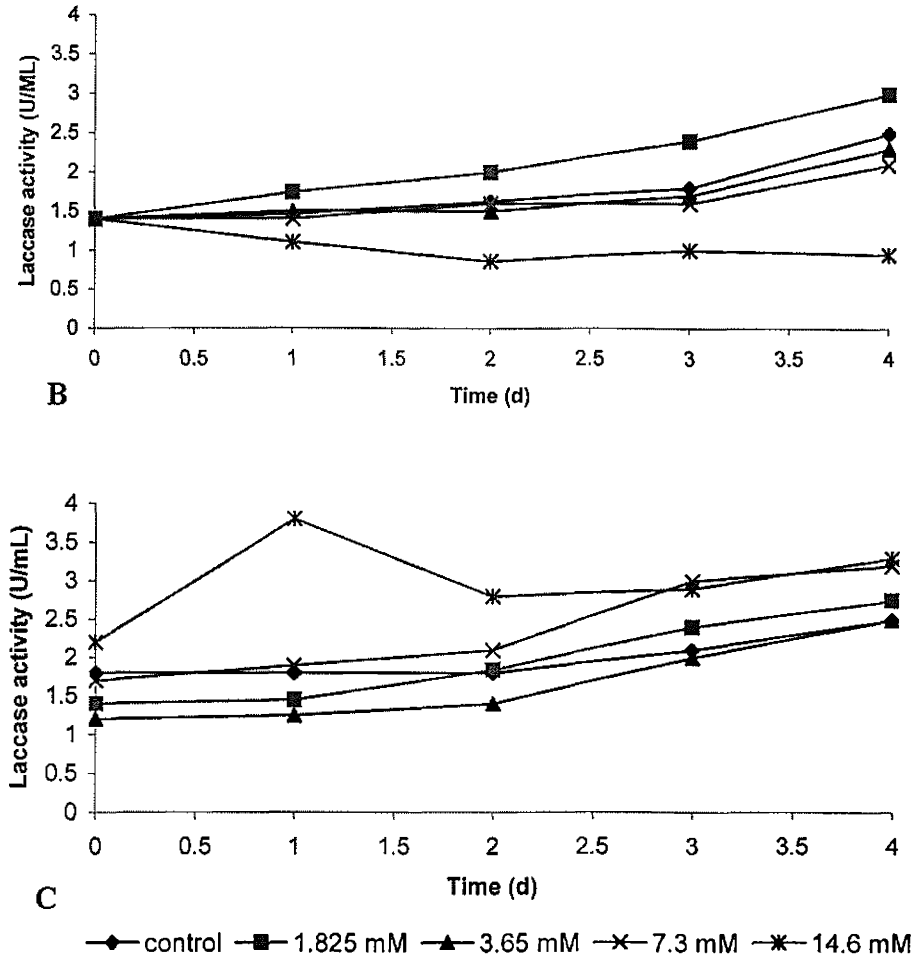


Fig 3.2 The effect of time of addition of cresylic effluent on laccase enzyme production. Flasks were allowed 4 days (A) 8 days (B) and 12 days (C) growth prior to effluent addition. The control contained zero effluent. All data points are means of triplicate samples; errors encountered are less than 5%, and are thus not shown.

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 to 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.

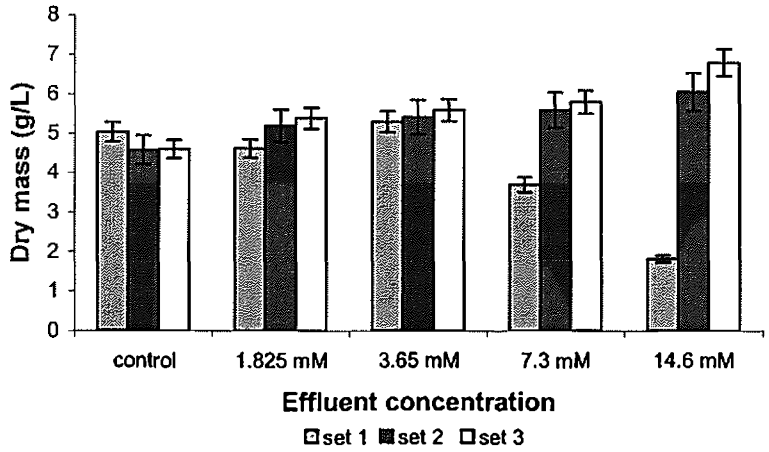


Fig 3.3 The effect of time of addition of different concentrations of cresylic effluents on biomass accumulation. Where Set 1 was allowed 4 days prior growth, Set 2, 8 days and Set 3, 12 days.

Phenol is not readily biodegradable and has been reported to be toxic or growth inhibitory to most types of micoroorganisms, 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, 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 of 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, 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 3.2) 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 section **5.3.3**. 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 higher concentrations of phenol (Fig 3.4 B, D).

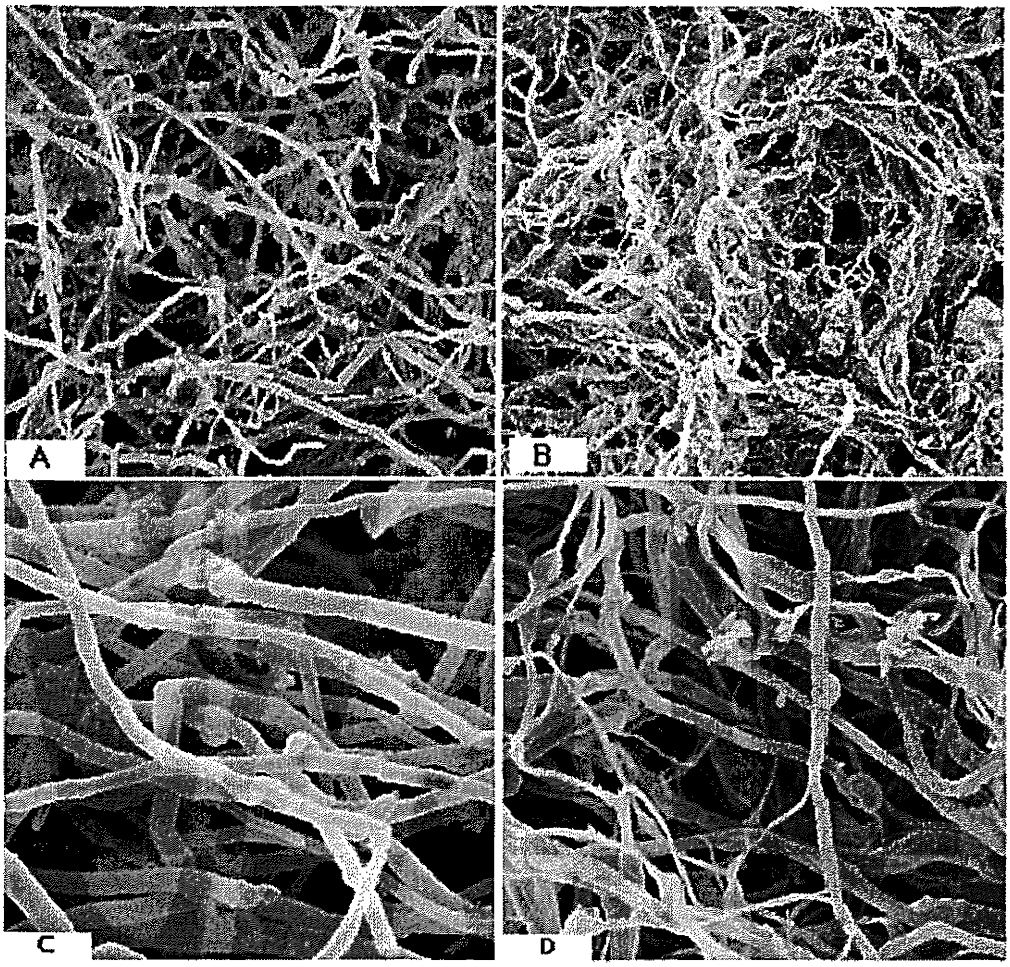


Fig 3.4 The effect of different concentrations of effluent on gross morphology of *T. versicolor*:
A, C) Air interface of a fungal mat grown in the presence of 1.825 mM effluent. The hyphae are thick, turgid and loosely packed, with clamp connections visible indicating rapid, healthy growth (A 500x magnification, C 1000x magnification).
B, D) Air interface of a fungal mat grown in the presence of 14.6 mM effluent. The hyphae are thin, tightly packed, often an indication of stress (B 500x magnification, D 1000x magnification).

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

The comparative microbial activity of the cultures exposed to different effluent concentrations was investigated by the spectrophotometric determination of the hydrolysis of flourescein diacetate (FDA) to flourescein. 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 increase in 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.

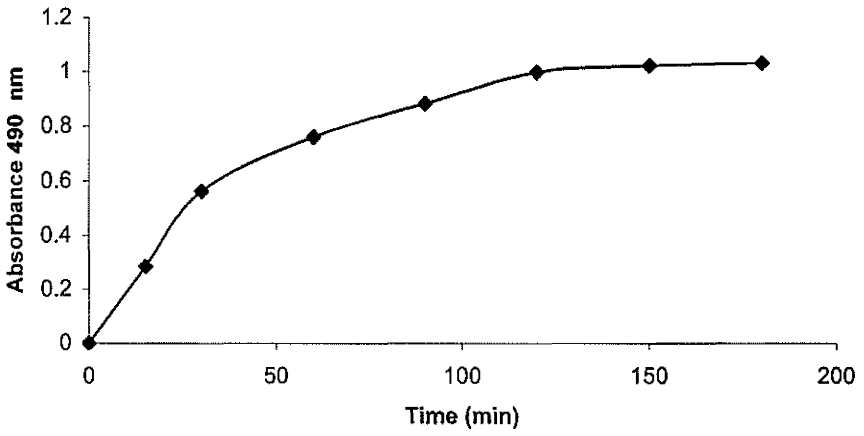


Fig 3.5 Measurement of FDA hydrolysis over time as a measure of total metabolic activity of *T. versicolor*.

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

(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.

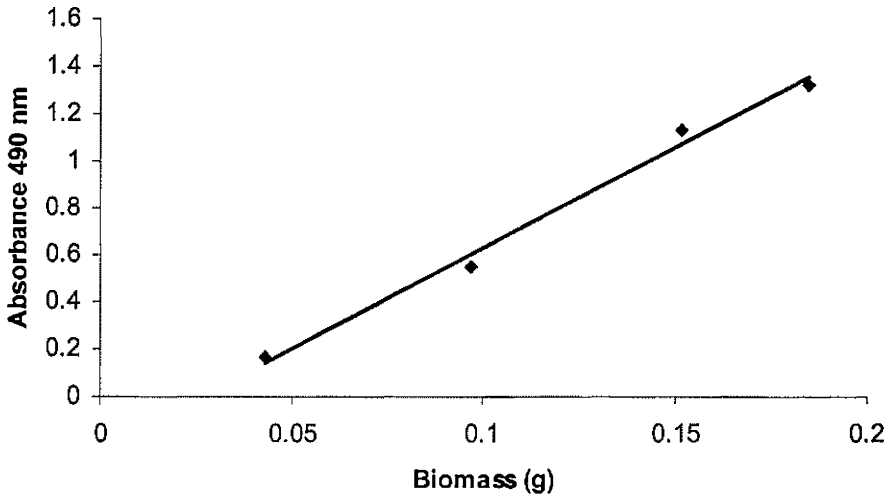


Fig 3.5 The linear relationship (R^2 = 0.99) between biomass and metabolic activity, as measured by FDA hydrolysis, during the growth phase of *T. versicolor* in flask culture

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

It has been already been shown (section **3.3.2**) 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 for laccase activity (section **3.3.2**). 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. Because the activity was measured as a function of total biomass, this effect is not simply a function of less biomass in the younger cultures resulting in an increased chemical dose. The 8 day-old cultures of Set 2

maintained the level of activity similar to their zero effluent control and had the highest ratio of metabolic activity to biomass across the concentration range tested (Fig 3.6). 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 3.6). 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 (Fig 3.2), 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 (Fig 3.3).

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). Between 4 and 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.

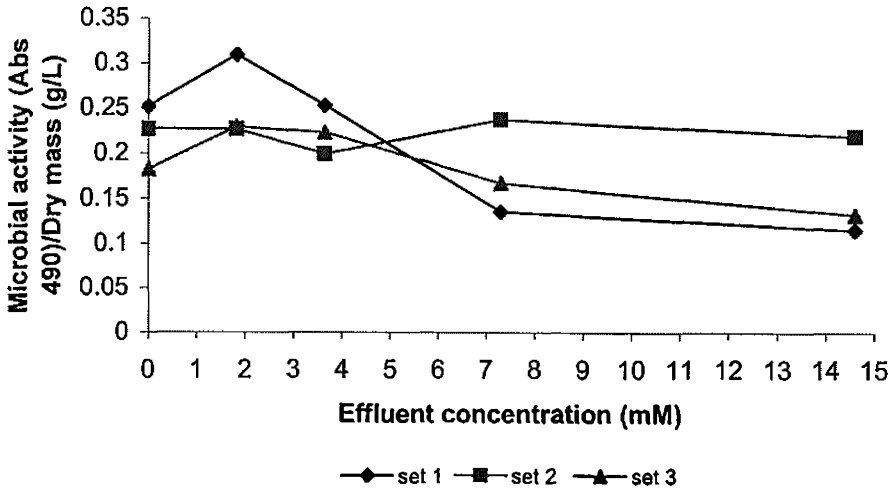


Fig 3.6 The effect of different effluent concentrations on the total metabolic activity per gram of biomass as a function of culture age. Set 1 was allowed 4 days prior growth, Set 2, 8 days and Set 3, 12 days.

3.3.6 Removal of cresylic effluent components from culture medium

Many phenols are toxic and some are known to be human carcinogens. Therefore their removal from industrial aqueous effluents is of great practical significance (Klibanov *et al.*, 1980). 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). 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 (section **2.3.5**).

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 3.8). 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 (section **3.3.2**). 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 substrate transformed).

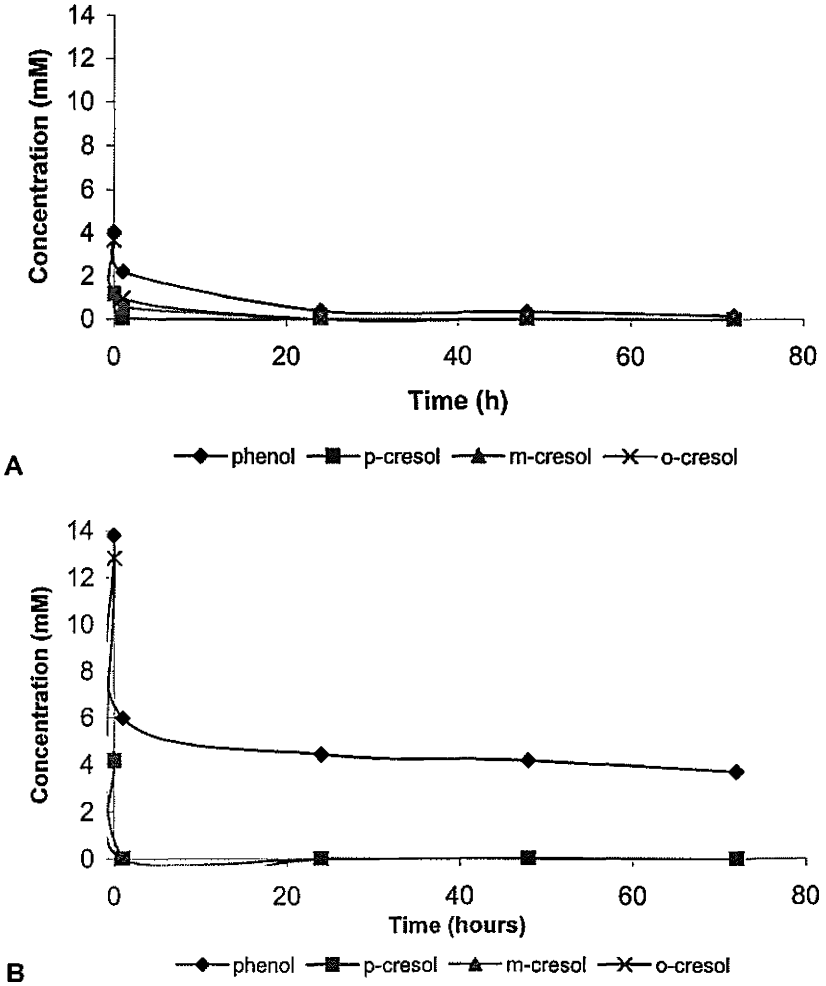


Fig 3.7 The removal from solution of the different monomers found in the phenolic effluent by flask cultures of *T. versicolor* from 5 % vol/vol (A) and 20 % vol/vol (B) final concentrations.

To our knowledge the concentrations of the phenolic monomers removed by *T. versicolor* in this system are the highest reported to date (Table 3.3). However, the removal of single doses of the target effluent monomers from flasks does not constitute an efficient bioremediation process. The preliminary success shown here needed to be further developed into removal of multiple doses and eventually a (semi) continuous system.

Table 3.3 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

* removal had been was completed by day 2

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.

3.3.7 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-ethylbenzothiozoline-6-suphonic acid) (ABTS), whose oxidation proceeds in one step resulting in a coloured radical cation $ABTS^{+\bullet}$. This assay is not without its complications however. The $ABTS^{+\bullet}$ 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 Majchercyzk, 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 (as prepared in section **3.1**) 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).

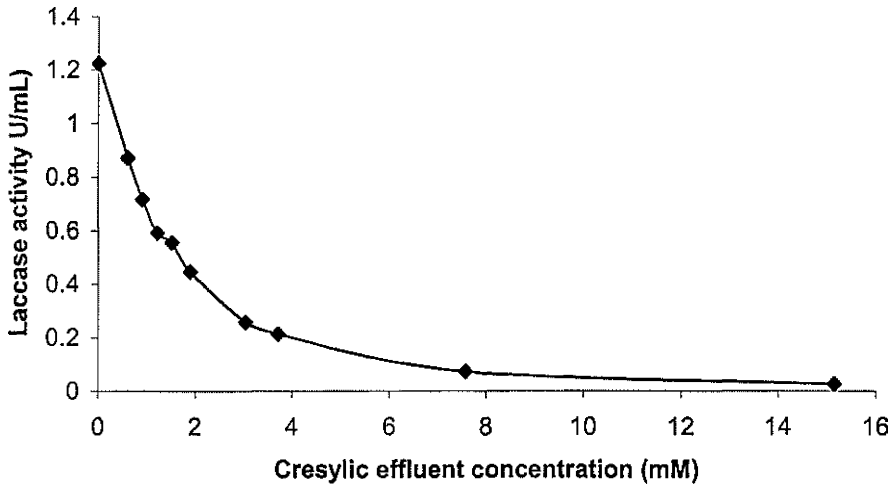


Fig 3.8 Effect of increasing concentrations of cresylic effluent on laccase activity (as measured by the ABTS colourimetric assay). Errors generated in measuring laccase activity are <5 %.

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:

$1/v = 1/V_{max} + K_M/V_{max}[S]$ (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)

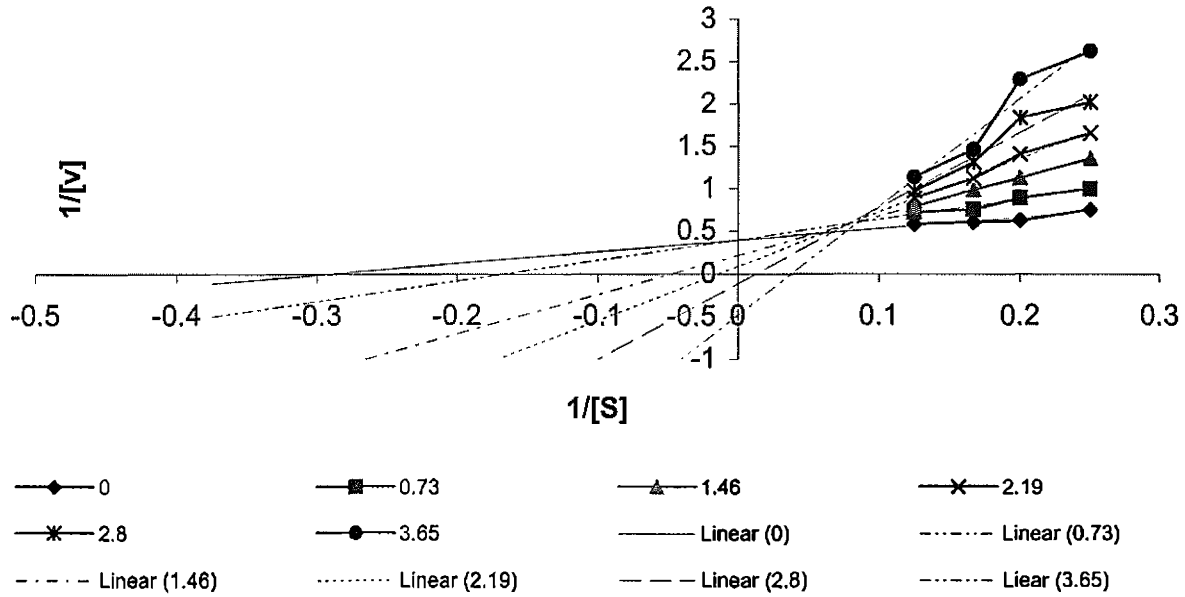


Fig 3.9 Lineweaver-Burk double reciprocal plot for the inhibition of the laccase colour reaction with ABTS by a phenolic effluent

3.3.7.1 The Oxygen Utilisation Rate (OUR) Assay for isolated 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.

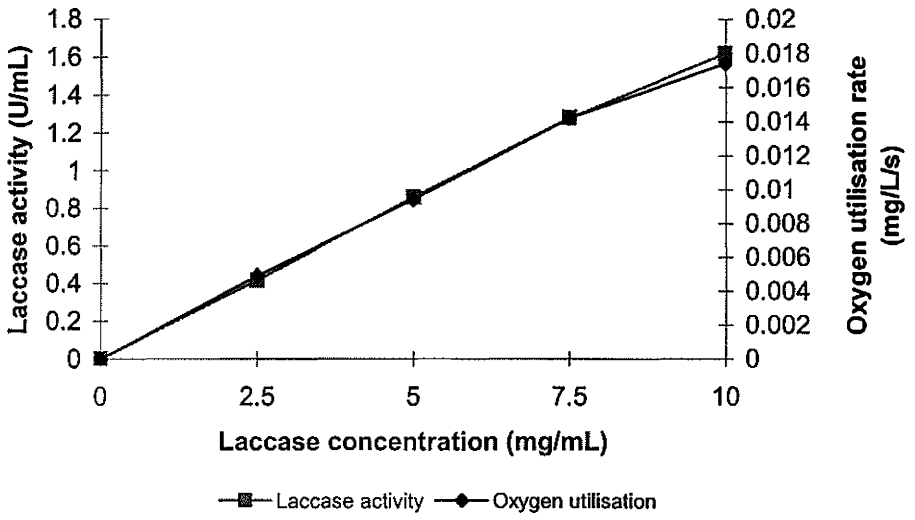


Fig 3.10 **Correlation between laccase activity, as measured by ABTS and OUR at different concentrations of laccase. The laccase used here was isolated as indicated in section 3.2.6**

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 (Fig 3.10) 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.

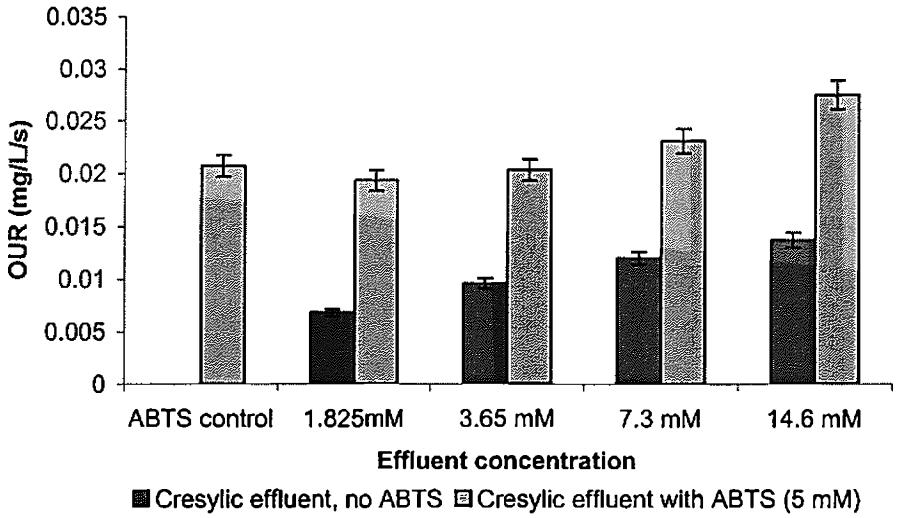


Fig 3.11 **Increasing oxygen utilisation rate (OUR) in the laccase reaction with increasing concentrations of cresylic effluent, in the presence and absence of 5 mM ABTS**

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, aliquots of *p*-cresol were added to a fixed quantity of enzyme and the resultant activity measured by monitoring the OUR (section **3.2.6b**). 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.

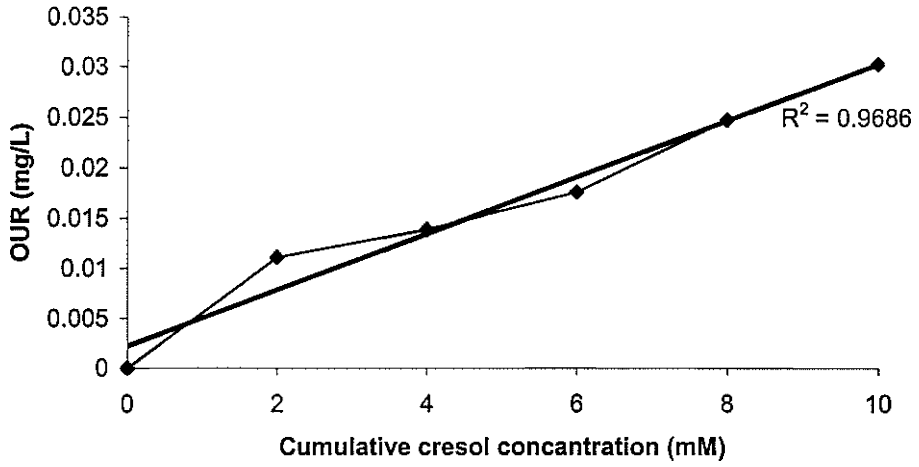


Fig 3.12 The linear increase in laccase activity (as measured by oxygen consumption) with *p*-cresol concentration

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 isolated 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.

3.4 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. Both the physiological response to high concentration of aromatic compounds and their subsequent removal by whole-cell cultures of *T. versicolor* has not been previously reported. A period of growth prior to the addition of a pollutant is also 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 confirmatory/quantifiable data has not been produced. Moderate cresol concentrations (up to 15 mM) were found to have a stimulatory effect on biomass accumulation and laccase enzyme production. The demonstrated physical adaptation to a pollutant challenge indicates the need for consideration of metabolic differentiation in the design of a bioreactor for the fungal bioremediation of cresylic pollutants.

The ABTS colourimetric assay is one of the most popular ways to measure the activity of oxidative enzymes in solution, specifically laccase. It is accurate, reliable, and easy to use, but this study has shown that for the assaying of samples containing phenolic compounds, this method may not be suitable. It has been shown here that the assay is competitively inhibited (K_I = 1.263 mM) by the phenols acting as secondary substrates. Similar problems could well be encountered when using other common laccase assay substrates eg syringaldizine. 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
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 would maintain an axenic culture of cells in medium conditions that maximize cell growth and productivity. When dealing with wastewater treatment it is usually more realistic to ensure that a specific culture dominates the reactor. 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 study reports on 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 Leukes *et al.* (1995) have shown that the white-rot fungus *Phanorochaete 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. This phenomenon was confirmed by enzyme specific staining that confirmed the presence of enzymes characteristic of exponential and stationary phase in different areas of the membrane bound biofilm (Leukes, W D pers. comm., 2004). 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.

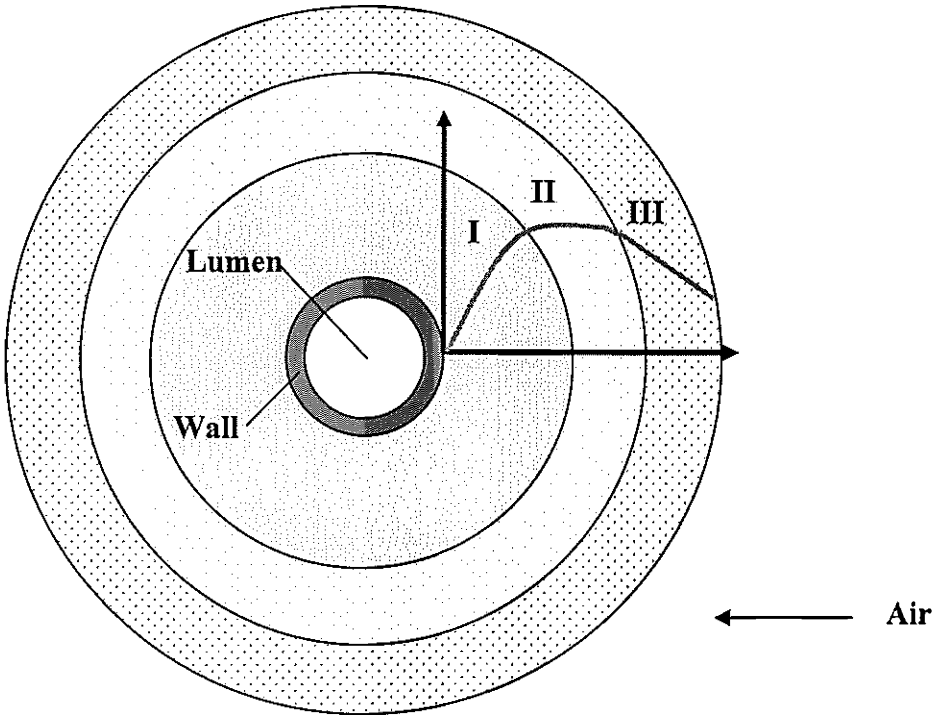


Fig 4.1 The gradostat concept developed for continuous production of secondary metabolites from white rot fungi. Nutrient gradients are established over space instead of time as in the case in batch culture. Rapid growth (I), stationary (II) and decline (III) phases are the same as for batch cultures 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 500cm^3 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 initially considered in this study included Oasis® florists' material, silicate pebbles used in hydroponic growth systems and porous woven glass beads (Dennert Poravar GmbH). Preliminary evaluations carried out on the different support matrices indicated operational difficulties with the Oasis material (contamination) and the silicate pebbles (attachment). All further work in the TBR was therefore conducted using the glass beads

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.

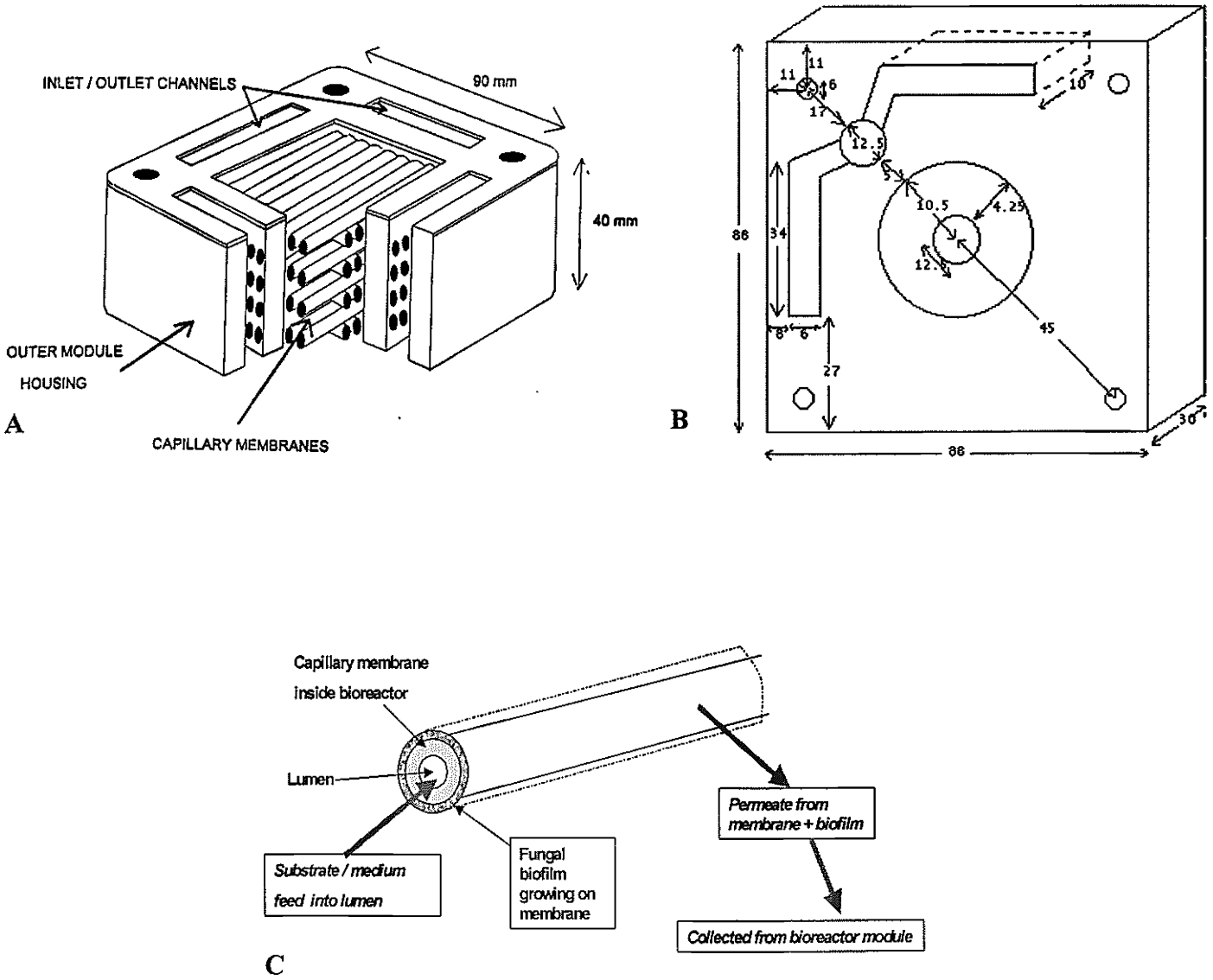


Fig 4.2 Modular components of the TFHFBR: (A) Transverse flow membrane module (B) Polyurethane manifold with centralised mass dissipater (C) Single hollow fibre membrane (Leukes *et al*, 1996, Burton *et al*, 1998, Edwards 1999).

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

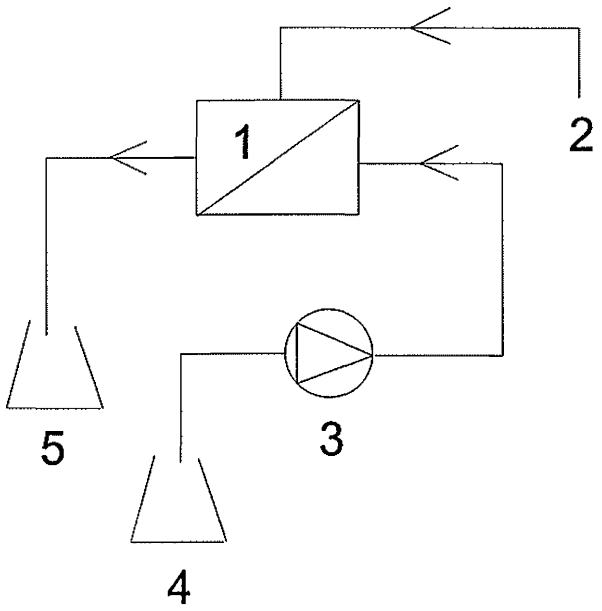


Fig. 4.3 Schematic TFHFBR set-up indicating dead-end ultrafiltration configuration (1= Transverse flow module (TFR), 2= air line, 3= peristaltic pump, 4= medium reservoir, 5= permeate reservoir). The air exits with the permeate

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 500 mL 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 immobilisation. 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 $L/m^2/h$. 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 400 mm and an internal diameter of 70 mm. 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. 8 mm) 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

All the packing materials used in the TBR had similar dimensions (d = 0.5-0.7mm) and where roughly spheroid shaped. The reactor vessel was filled with the desired 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 sent to waste.

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 filter sterilised 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.

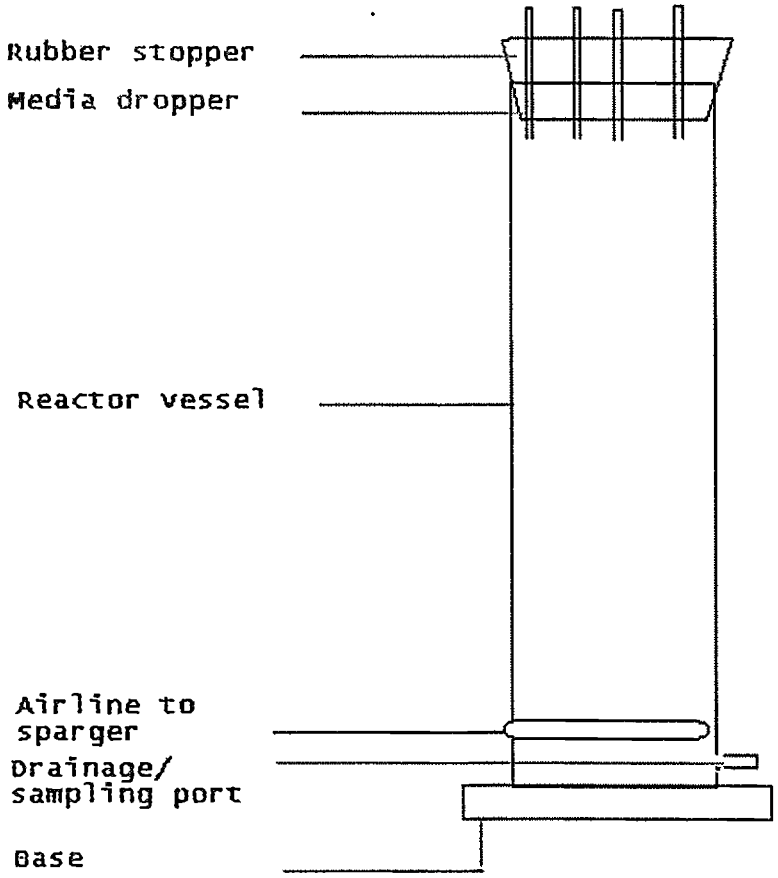


Fig 4.4 Schematic diagram of the TBR used in this study

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 section **3.1.11**

4.3 RESULTS AND DISCUSSION

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. Noteworthy 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 partially 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. Following the addition of the effluent to the medium, HPLC analysis of

the permeate showed no remaining cresol in the permeate but an increase in the concentration of cresol reaction products (results not shown).

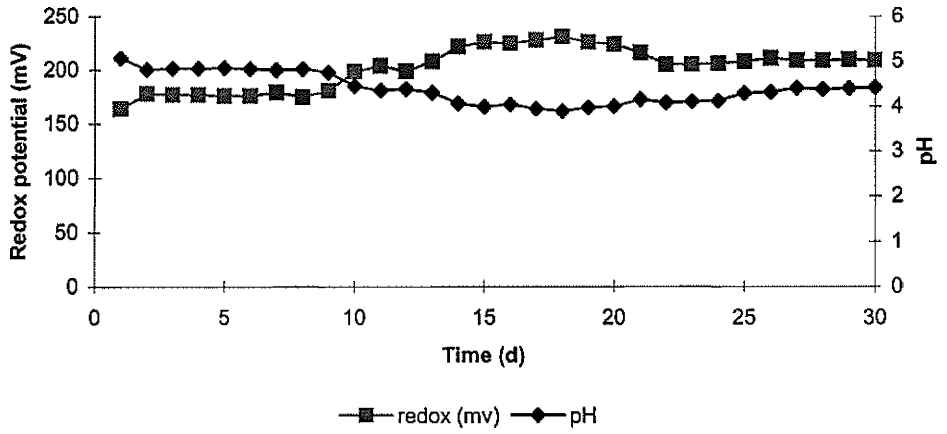


Fig 4.5 TFHFBR history showing changes in pH and oxidation/reduction potential

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 reactors were run for 7 d at each concentration of cresol and the 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. Although bioaccumulation cannot be ruled, it is unlikely due to the fact that *p*-cresol was removed continuously for 23 days prior to breakthrough.

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.

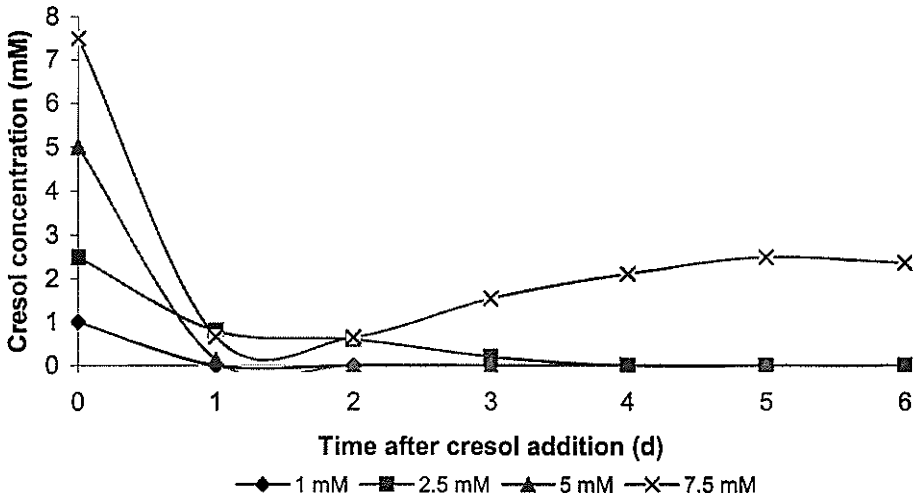
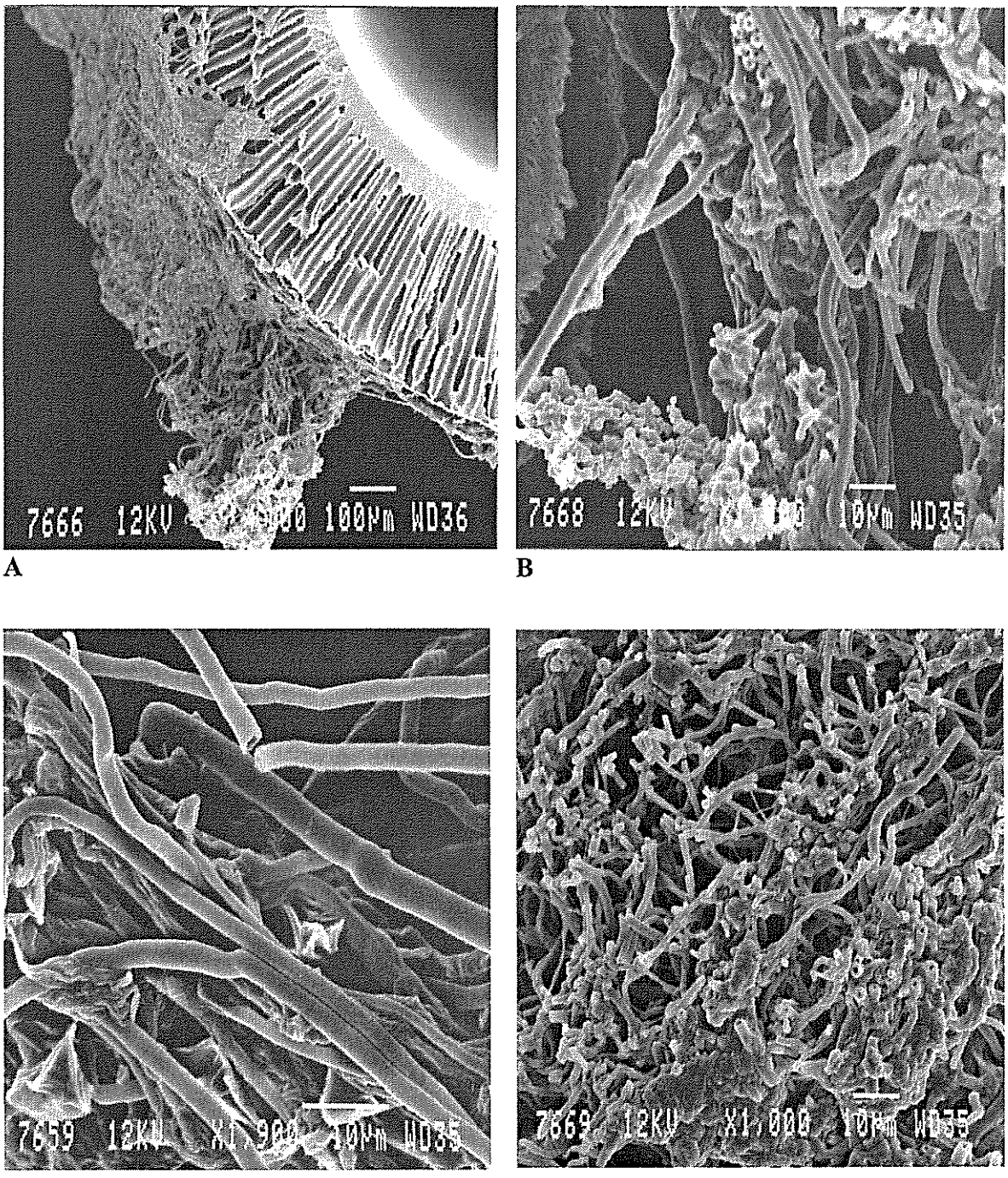


Fig 4.6 Removal of different concentrations of cresol by *T. versicolor* immobilized in a TFHFBR.

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

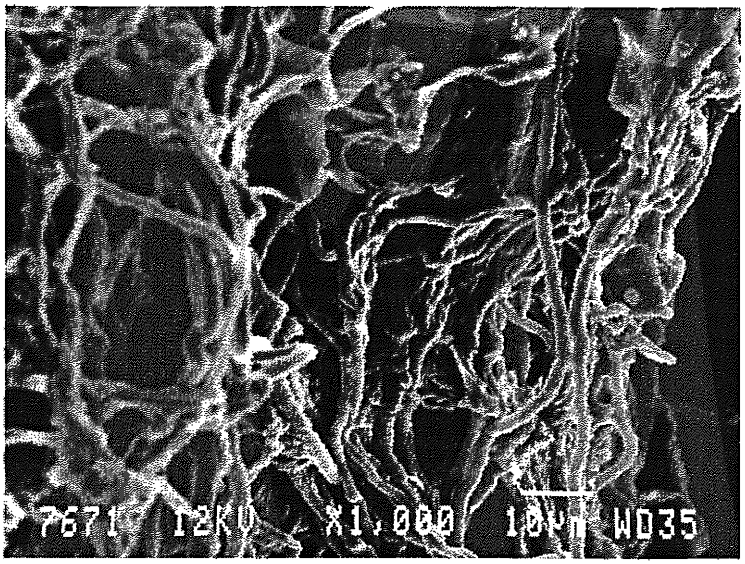


Fig 4.7 E) Outer edge of membrane-bound biofilm: biomass becomes more diffuse and desiccated and eventually sloughs off.

These results support the gradostat concept and indicate 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 mainly 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. It is very possible that these pellets were composed of polymerized phenolic monomers, as this had been observed in other experiments conducted in the research group.

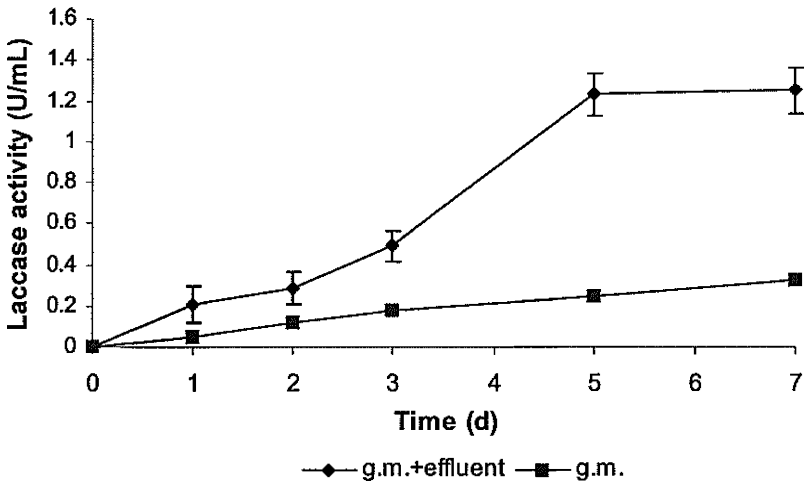


Fig 4.8 The induction effect of cresylic effluent (20%) added on day 0 to *T. versicolor* immobilised in a TBR. The growth medium containing the effluent was employed after the normal medium, day 0 on this chart.

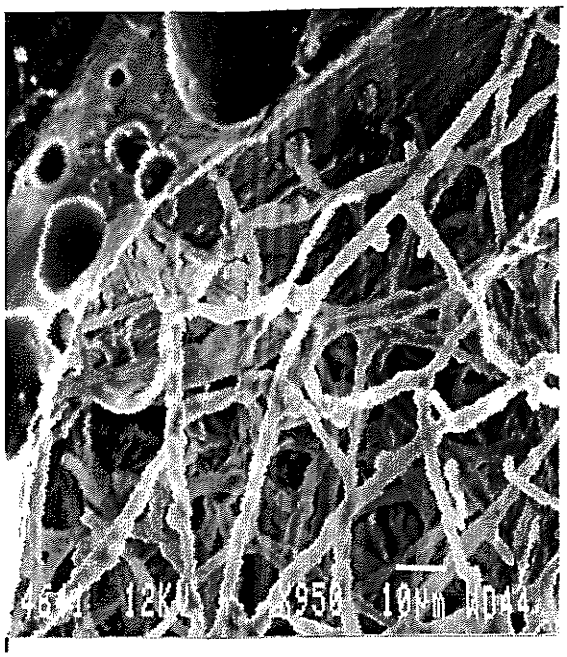
In contrast with the TFHFBR, it is interesting to note that a high effluent concentration (20%) resulted in an increase of secreted laccase enzyme (Fig 4.8) whereas in unimmobilised cultures, this concentration retarded both growth and enzyme production. These results are in agreement with those of Nakamura *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 of in terms of resistance toxic compounds. The medium containing the effluent was recycled for 7 days 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

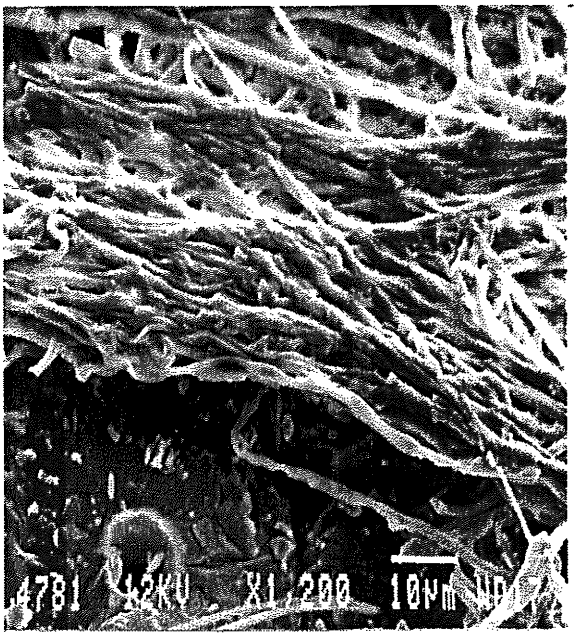
EFFLUENT MONOMER	REMOVAL RATE (g/L/d)	BIOMASS ASSOCIATED REMOVAL g/g biomass/d	RESIDUAL EFFLUENT (g/L)
Phenol	0.524	0.031	0.0
p-cresol	0.180	0.010	0.0
m-cresol	0.196	0.011	0.0
o-cresol	0.546	0.031	0.0

Closer inspection of sections of the glass beads by scanning electron microscopy (SEM) showed thick, linear hyphae covering individual beads with a continuous mat of fungi (Figs 4.9B, 4.9C). The hyphae had penetrated and attached to the glass beads (detail shown in 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 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 glass 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. versiciolor* 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



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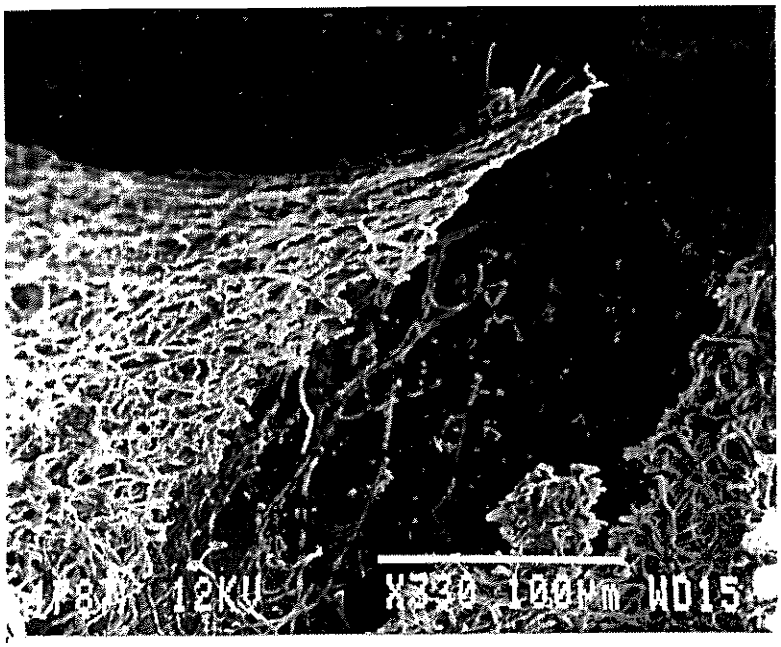


C

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 CONCLUSION

The TFHFBR promoted good fungal growth and differentiation according to the gradostat concept for the production of secondary metabolites. Although there was very little measurable free laccase in the reactor permeate, the system facilitated the removal of cresol up to a concentration of 5.0 mM (0.0486 g/g/d). However, the low flow rates associated with this reactor configuration and the difficulty of scale-up would impede its application in a wastewater treatment role. Instead, the true 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. Immobilising the fungus enabled it to tolerate and remove high (20%) concentrations of cresylic effluent in the reactor configuration and produce over 1 U/mL/d of laccase. An obvious advantage to the TBR is the almost complete lack of shear forces and the potential for excellent oxygen mass transfer; two factors that are widely acknowledged to favour growth of white rot fungi. 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 lower. It was decided to investigate a bioreactor offering low shear agitation coupled with good oxygen transfer, to this end an airlift loop reactor was considered.

CHAPTER 5

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). The mechanics of the reactor were more thoroughly described or in section 1.6.

5.1.2 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).

5.1.3 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).

5.1.4 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 concluded 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 in 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).

5.1.5 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 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, 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 investigated. 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 be 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 by investigating range of increasing airflows. The reactor was filled with 3.15 L of growth medium (TDM) 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. Initial reactor runs where carried out using sterilised effluent. In an effort to simulate real-life scenarios, later experiments utilized unsterilised effluent, however, sterile technique was still employed in the handling of the effluent and the running of the experiment

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 (dinitrosalicyclic acid) method according to Ghose (1987).

5.2.6 Measurement of oxygen concentration

The oxygen consumption was measured with a dissolved oxygen probe (YSI 5740) linked to a HiTech Microsystems DO/OUR meter. The probe was immersed in the reactor and the meter took a reading every second.

5.3 RESULTS AND DISCUSSION

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). However, at high cell densities, pelletted 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 where 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 5.1). For aeration rates above 3 L/min there was little difference in the maximum dissolved oxygen achieved in the reactor.

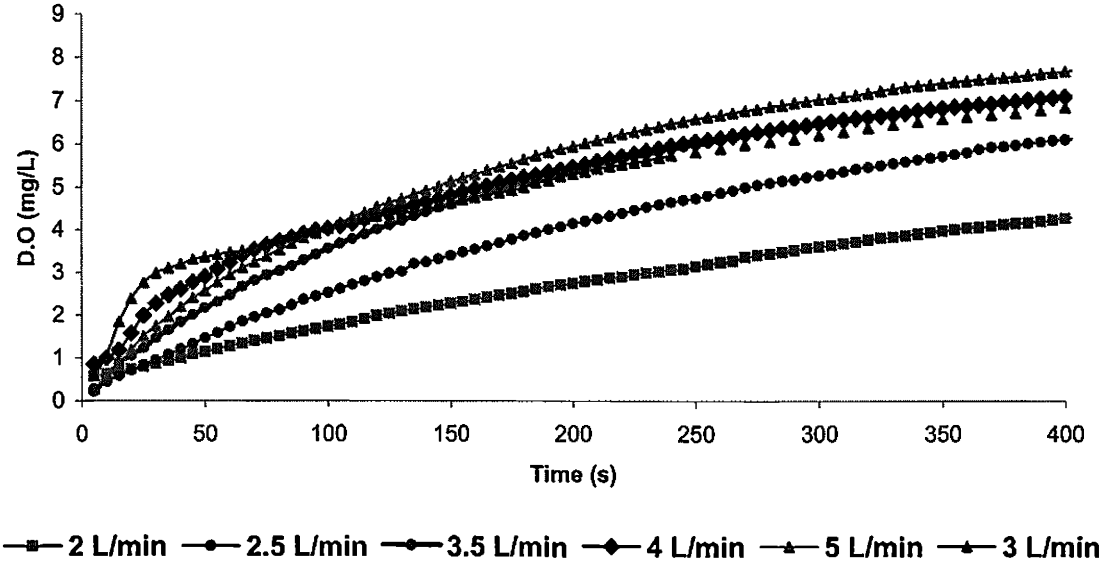


Fig 5.1A The effect of increasing airflow rate supplied to an ALR on dissolved oxygen concentrations in the reactor fluid.

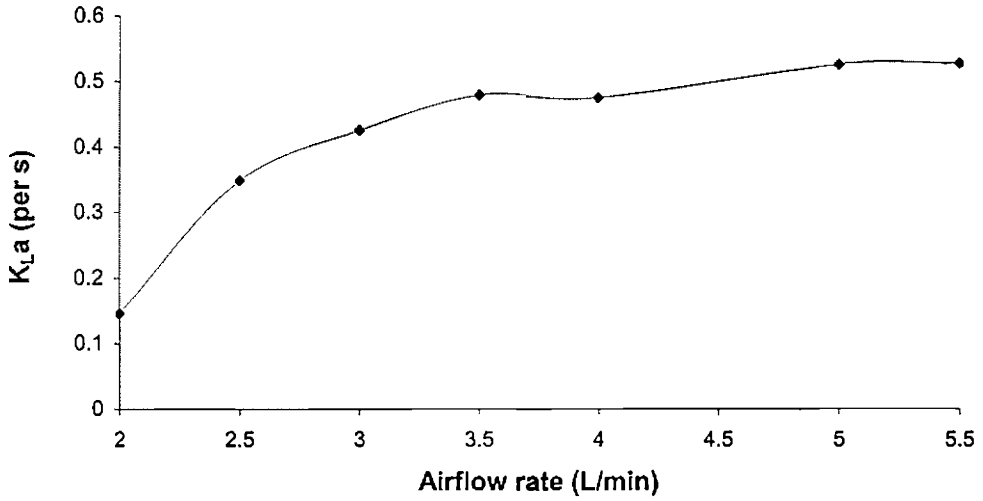


Fig 5.1B The effect of increasing airflow on the oxygen mass transfer coefficient (K_La) in an ALR.

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 (Fig. 5.1B). However, a flow rate of 3.5 L/min delivered a K_La of 0.45 /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 be 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 was calculated from a plot of dC_{O2L}/dt and 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 = $dC/dt = K_La_L*(C_{sat}-C_L)$.

(see appendix C for details of the calculation)

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_{O2}) 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 fermentation, 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 $mgO_2/L/s/g$ 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-xylidine 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 5.2 (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 5.4).

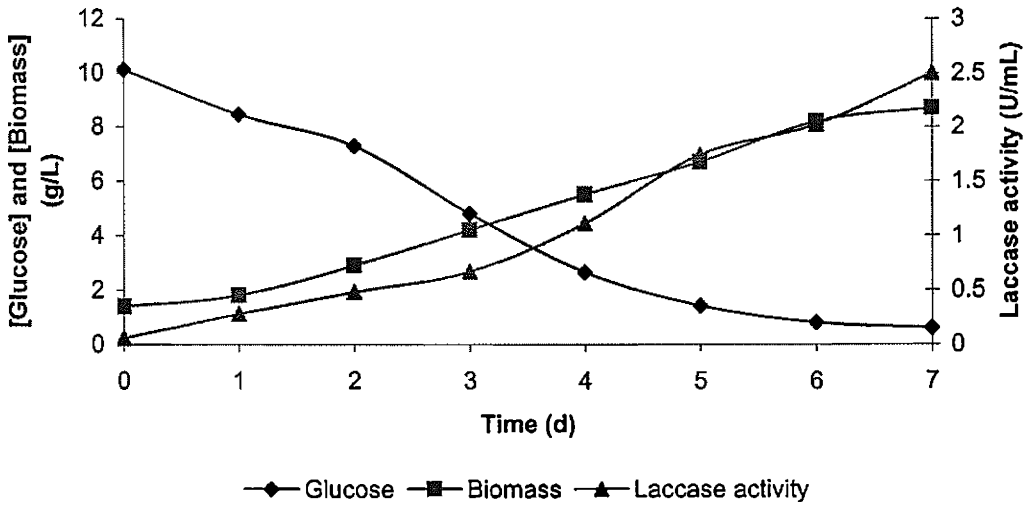


Fig 5.2 Time courses of glucose depletion, biomass accumulation and laccase production during a fermentation of *T. pubescens* in an ALR. The initial glucose concentration was 10 g/L and aeration was fixed at 3.5 L/min.

An additional measure of cell growth or activity is respiration, which ca be measured as oxygen utilisation rate (OUR) by the biomass in the reactor. OUR is dependent on both the amount of biomass and phase of growth. As OUR = $-dC_{02}/dt$, 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. 5.1B). 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. It was observed previously that cultures grown under these conditions reach stationary phase after 7 days, at this time the specific OUR decreased rapidly, indicating that the total amount of biomass, although it increased, became less metabolically active.

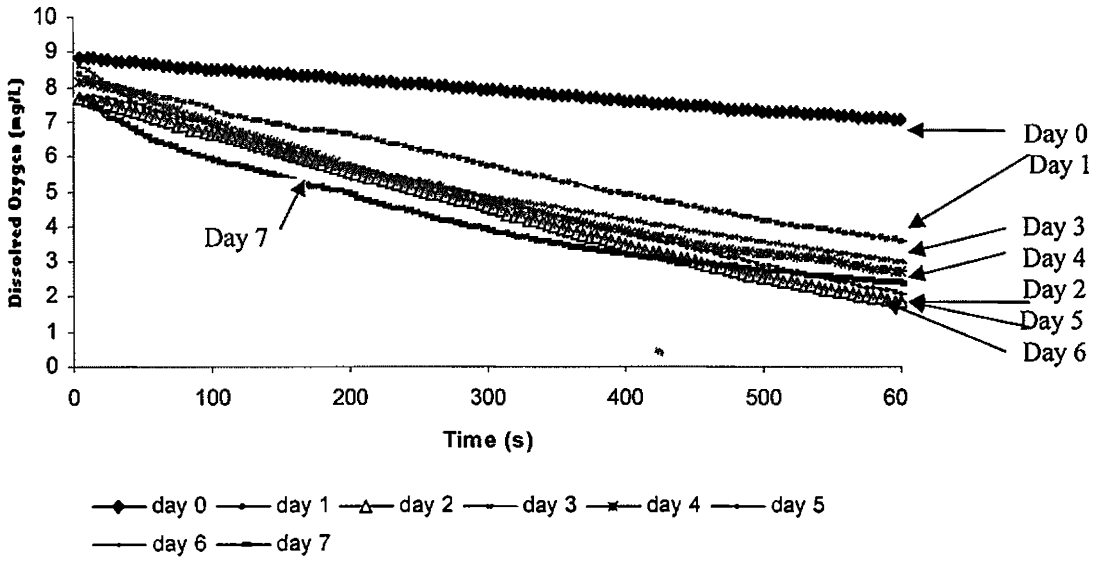


Fig 5.3 Decrease in oxygen concentration over time for *T. pubescens* grown in an ALR. The initial glucose concentration was 10 g/L and aeration was fixed at 3.5 L/min.

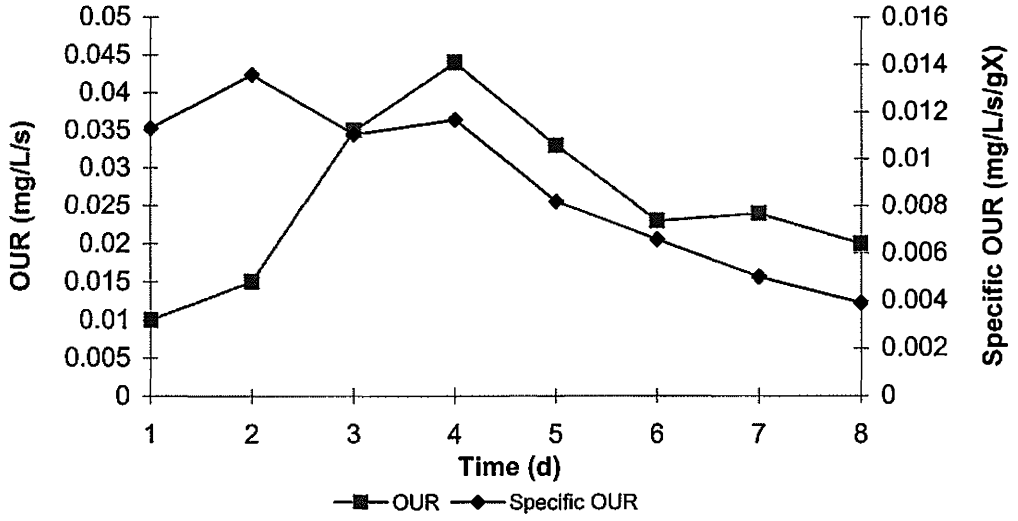


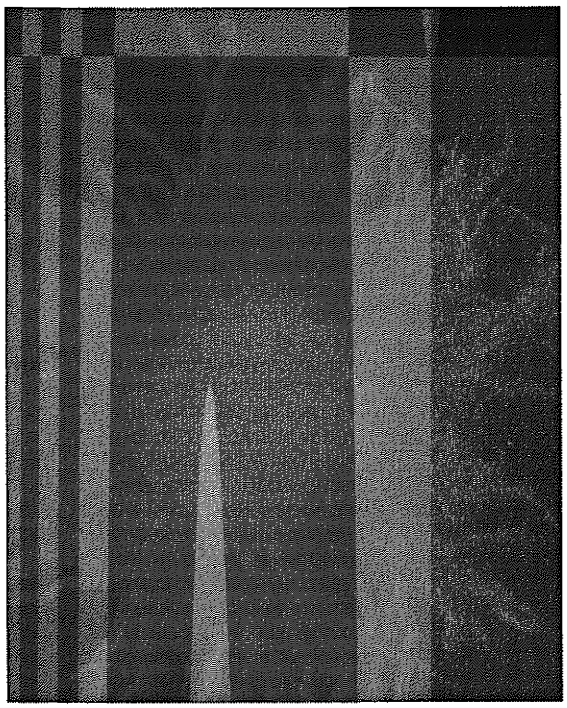
Fig 5.4 Comparison of OUR and Specific OUR of *T. pubescens* growing in an ALR. The initial glucose concentration was 10 g/L and aeration was fixed at 3.5 L/min.

5.3.4 Effect of effluent addition on biomass morphology and laccase production

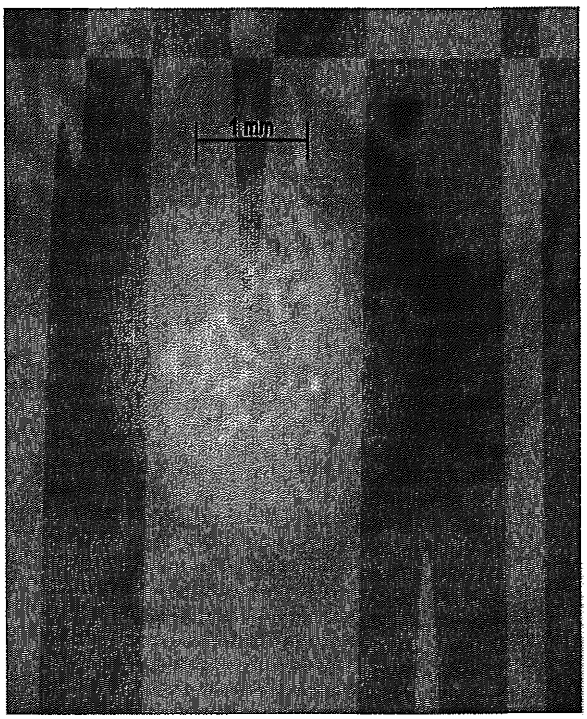
To test the effect of effluent on reactor biomass, airlift reactors containing TDM were inoculated (10%) with *T. pubescens* and the fermentation allowed to progress with the airflow fixed at 3.5 L/min. At the end of the initial growth period of 7 days, increasing concentrations of the phenolic effluent 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 pellets between 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.

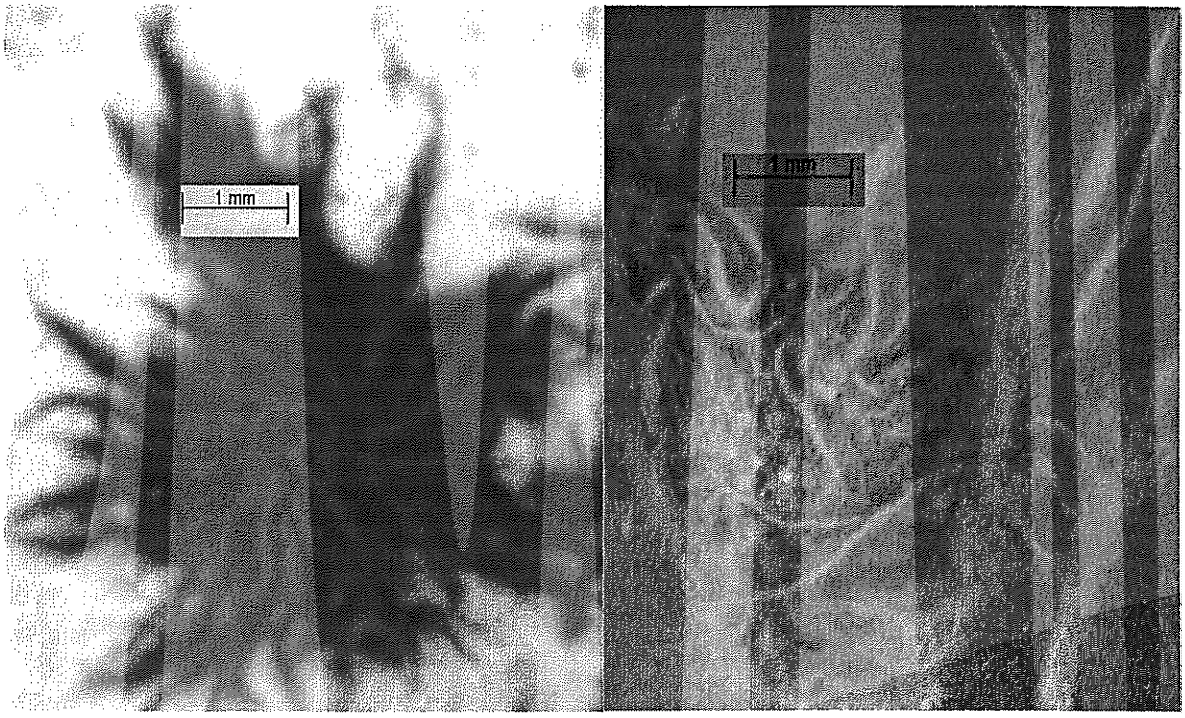
Changes to mycelial mats observed in the present study in flask cultures of *T. pubescens* after effluent addition included browning, shrinkage and fewer aerial mycelia. Electron microscopy revealed thinner more highly branched mycelia (section 3.3.2). These physiological adaptations have been correlated 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 pelletted 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.



A



B



C D

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

(section **3.3**). However, these were '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 free laccase activity by almost 3 times; the subsequent addition of 5 % and 10 % appeared to have negative effects on the culture (Fig 5.6). 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.

There is a case for non-laccase mediated removal of effluent, but correlation of the amount free enzyme with the concentration of phenolic monomers has been shown previously (section **3.3.7**) to result in an underestimation of true activity. When the added phenols had been eliminated the inhibition/masking effects were removed and enzyme levels were observed to increase again (Fig 5.6), supporting the theory that phenolic removal is laccase associated. Free enzyme activity increased after falling from 2.7 U/mL, to 0.8 U/mL after the 5 % (v/v) addition (day 13). Thereafter it increased again up to 1.95 U/mL, but dropped to almost zero after the 10 % (v/v) addition (day 19) after which time the culture died. It is possible however that these final 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 slugs of effluent (section **5.3.5**). The brown colour of the biomass and the culture fluid was also observed in flask culture (section **3.3**). 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 by 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). Iimura 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.

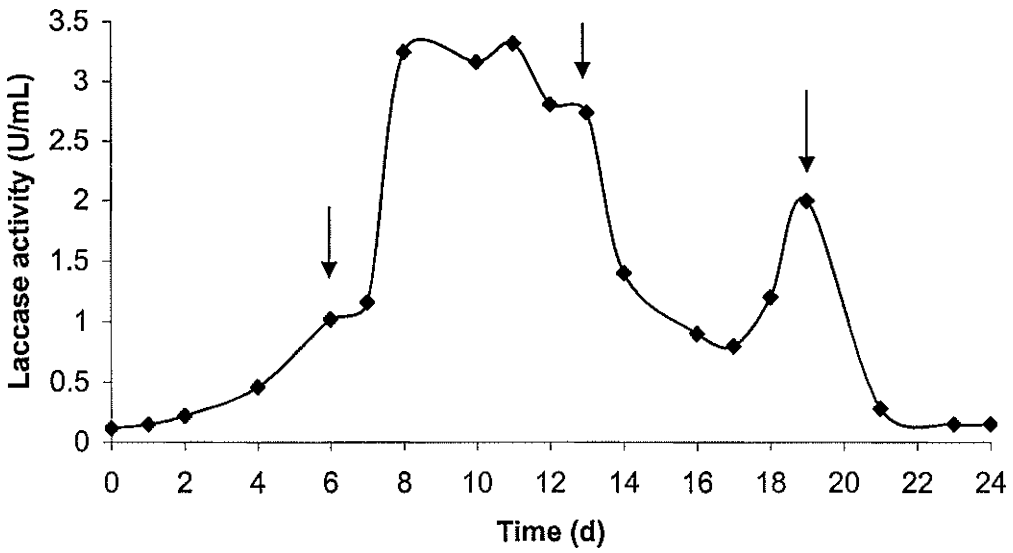


Fig 5.6 Time course of laccase production over an ALR fermentation of *T.pubescens;* 2.5% (v/v) phenolic effluent added on day 6, 5 % (v/v) on day 13 and 10 % (v/v) on day 19.

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 on *T. pubescens*. Multiple ALRs were run in parallel with different concentrations (2 %, 3.5 % and 5 % v/v) of phenolic effluent 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 (section 5.3.6). 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.

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).

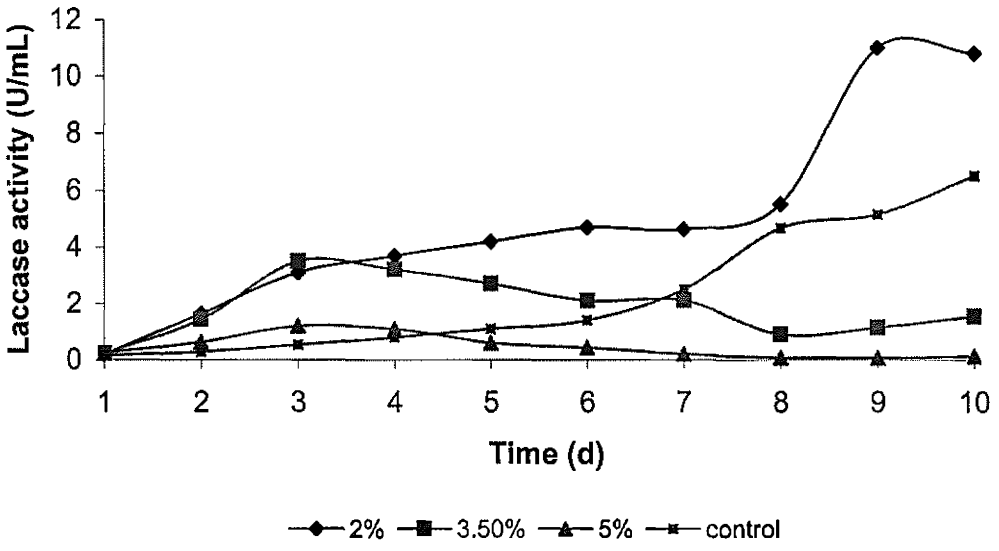


Fig 5.7 Induction effect of daily addition of different concentrations of phenolic effluent to a fermentation of *T. pubescens* in an ALR

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.

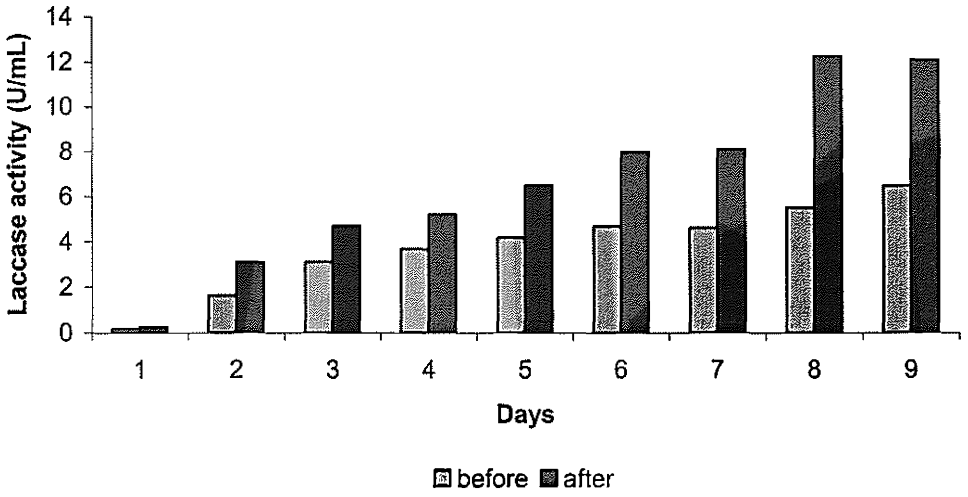


Fig 5.8 The immediate increase in laccase activity after the addition of phenolic effluent (2% v/v/day) in ALR fermentations of *T. pubescens*. Readings were taken daily immediately before, and 5 min after effluent addition.

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 fermentors 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 (Venkatadri and Irvine, 1990, Linko, 1992)

Immobilised organisms are known to be more resilient to environmental stress compared to free cells (Venkatadri and Irvine, 1990). 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.

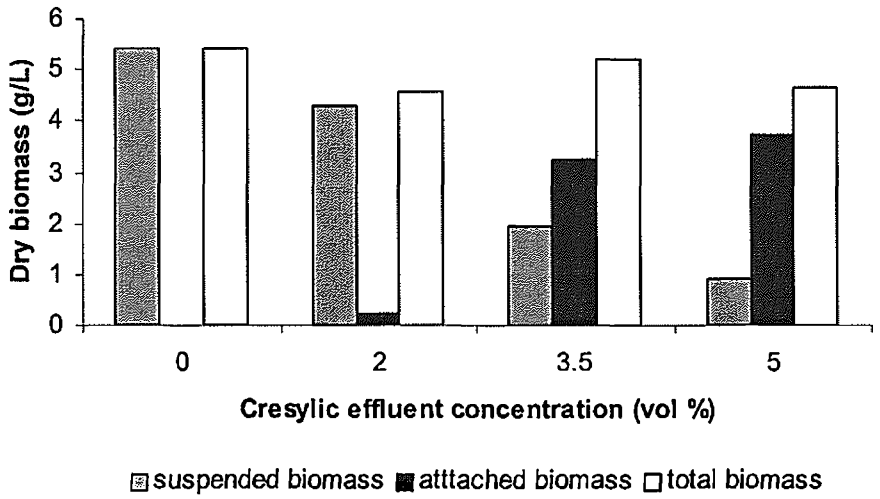


Fig 5.9 **The relationship between freely suspended and attached biomass in ALR's containing different amounts of phenolic effluent. The biomass readings are an average from 2 reactors.**

5.3.6 Removal of phenolic monomers from solution

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. Based on prior results in this study, the bioremediation capacity of *T. pubescens* in flask culture was indicated, 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 5.10).

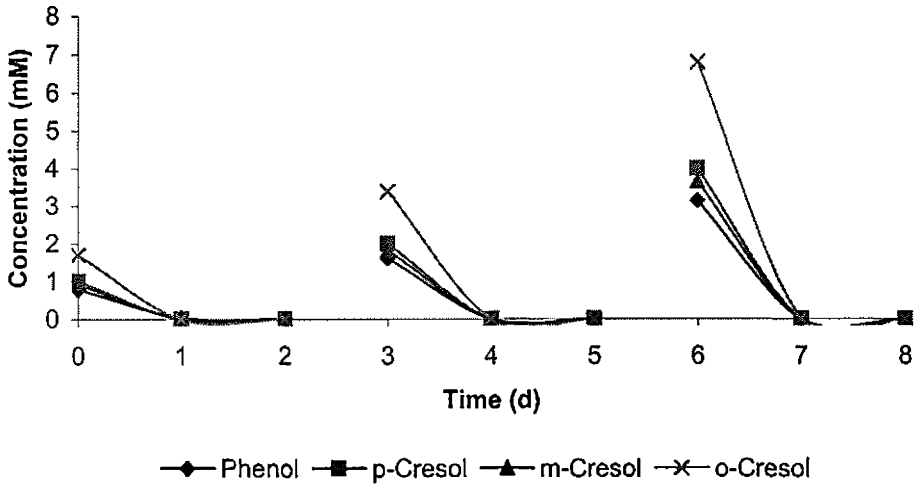


Fig 5.10 **Removal of target monomers from successive increasing doses of phenolic effluent (2.5%, 5% and 10% v/v) by *T. pubescens* in an ALR**

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) reported 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 5.10). 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 5.11). 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 (discussed in section **5.3.3**). 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.

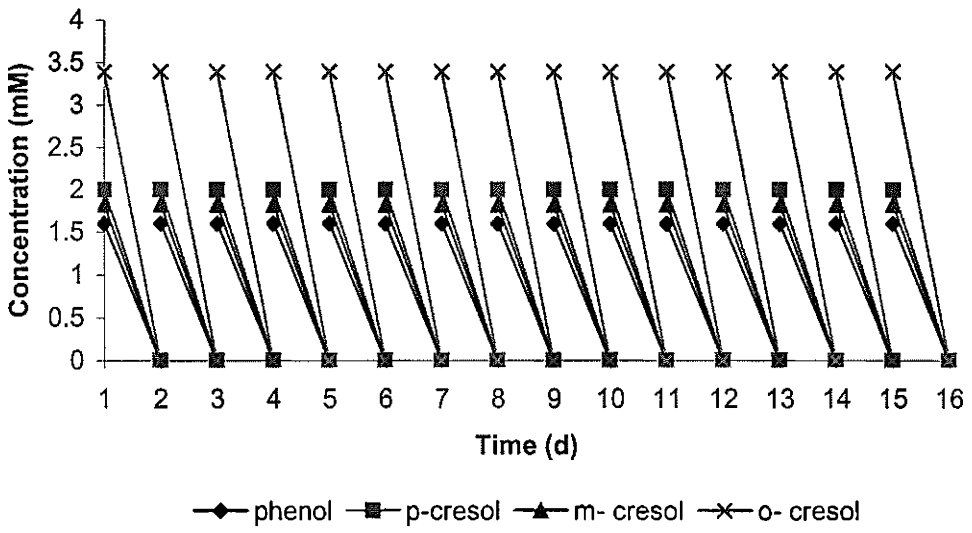


Fig 5.11 Removal of target monomers from phenolic effluent (5% v/v) added daily to *T pubescens* immobilised in an ALR

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 5.2.

Table 5.2 Removal rates in the ALR for the individual phenols in the cresylic effluent

Effluent monomer	Amount of phenol added daily (mM)	Residual phenol (mM)	Removal rate (g phenol/ g biomass/d)	Total removal of phenol (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

These results (table 5.2) 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.

5.4 CONCLUSIONS

Various reactor systems used for the production of lignolytic enzymes by white rot fungi (especially *P. chrysosporium*) have been investigated by other researchers. These have included stirred tanks, packed beds and rotating disc reactors. Far fewer studies have reported the use of reactor systems employing these fungi for waste treatment. 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 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 combination of this organism and reactor system has been shown to be highly suited for the removal of phenols from solution (Table 5.3). It should be noted that only the TFHFBR was operated with increasing pollutant concentrations until the breakthrough occurred and the system was no longer effective.

Table 5.3 Phenolic removal rates for the various culture conditions and reactor configurations investigated in this study

MONOMER	FLASK		TFHFBR		TFR		ALR	
	RR[a] (g/g/d)[c]	TR[b] (g)	RR (g/g/d)	TR (g)	RR (g/g/d)	TR (g)	RR (g/g/d)	TR (g)
Phenol	0.106	0.75 (7 d)			0.031	1.54 (5 d)	**0.033**	**4.97** (15 d)
p-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)
m-cresol	0.081	0.46 (7 d)			0.011	0.57 (5 d)	**0.012**	**1.87** (15 d)
o-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

The aeration supplied to an ALR provides control of both the agitation and OTR in the reactor. This operating parameter was optimised for a 4 L reactor so as 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. Fungi can lose their ability to degrade lignin and chlorinated aromatic compounds due to the mechanical forces acting on them that result in enzyme inactivation, production of proteases and oxygen limitations within pellets (Alleman *et al.*, 1995). In this study all the effluent added to the reactor was removed effectively, even during operation in a fed-batch mode with daily additions of effluent over 16 days. It was necessary to supplement the fermentation with glucose to maintain the viability of the fungal culture as well as to sustain its phenolic degradation capability.

The technical and economic feasibility of using airlift devices has been conclusively established for a number of processes, including aerobic fermentations and wastewater treatment. 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.

CHAPTER 6

GENERAL CONCLUSIONS

The work described in this thesis 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.

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

i) 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.

ii) 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.

iii) The complex physiological response of *T. versicolor* to the addition of the phenolic effluent was illustrated and partly 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.

iv) 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* (at lab scale).

v) 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.

6.1 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 practioners. 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. At practical scale there would invariably be sterility issues involved in using a monoculture. Medium preparation and reactor inoculation would have to kept sterile, but thereafter operation would be carried out under hygienic conditions. Basidiomycetes are slower growing than many potential contaminants, but one established are aggressively territorial. Together with the harsh environment caused by the addition of phenolic effluent to the system, these factors should prove sufficient to ensure that *Trametes* would at least remain that dominant culture in reactor.

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.

6.2 Potential for scale-up

With the long-term goal of this project being the development of a wastewater treatment system, the next phase of this work would obviously be scale-up of the ALR developed. Successful use has been made of very deep airlifts both for municipal and industrial water treatment. Using the knowledge acquired in the design and operation of the ALR developed in Chapter 5 and 'rules of thumb' obtained from literature, specifically Chisti and Moo-Young (1987) a 100 L ALR was designed (Fig 6.1). These design points included:

1.) Placing the sparger just inside the riser.
2.) Reducing the size of the 'dead zone' at the base of the reactor by making it a concave dome.
3.) The ratio of draught tube/reactor diameter was increased to 0.66, as this had been shown to increase gas holdup in solutions more viscous than water, as would be the case in an operational reactor.
4.) The reactor headspace volume (the area of best mixing) was increased by adding a gas disengagement zone.
5.) This zone (4) was designed to 250mm as the positive effects on mixing of clear liquid height above the top of the draught tube tail off after this height.
6.) The reactor was constructed out of polished stainless steel, with standard dairy fittings.

Testing and operation of this reactor will be the future work undertaken in this project.

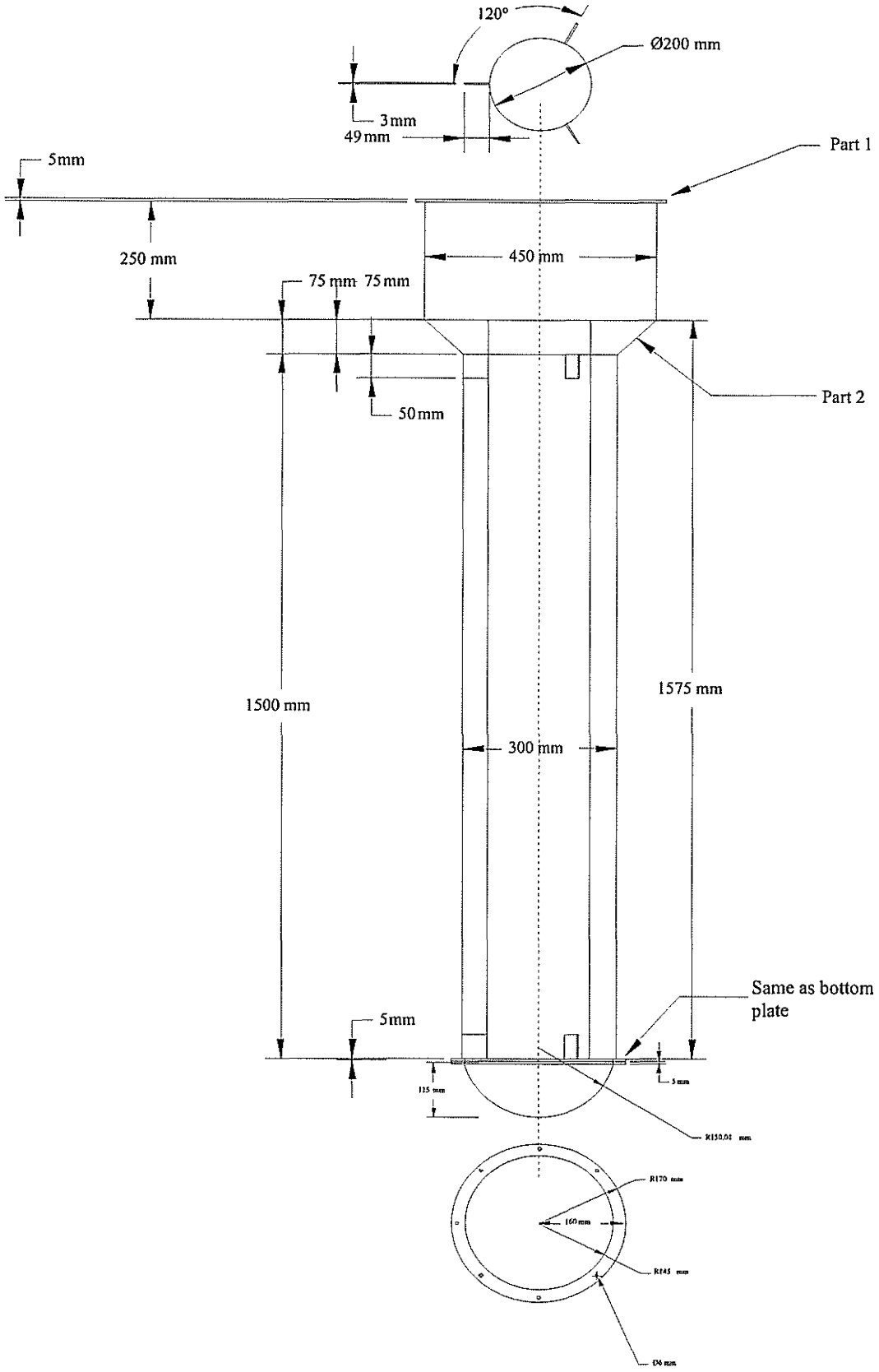


Fig 6.1 Design for scaled up (100 L) ALR for the bioremediation of phenolic wastewater by *T. versicolor*

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