

**Intraspecific comparison of *Phanerochaete*
chrysosporium strains: peroxidase production,
pollutant degradation and mycelial differentiation.**

A thesis submitted
in fulfilment of the requirements for the degree of
Doctor of Philosophy
in the Faculty of Science,
Department of Biochemistry, Microbiology and Biotechnology
Rhodes University

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Abstract

The wood-degrading basidiomycete, *Phanerochaete chrysosporium*, has been studied as a model organism in elucidating the mechanisms and pathways enabling this white-rot fungus to degrade recalcitrant lignin. These same mechanisms are implicated in the mineralisation of environmentally persistent, toxic phenolic chemicals. For this reason, *P. chrysosporium* has been exploited in a number of environmentally sound technologies, including the degradation of the indigestible lignin component in agricultural waste for the generation of digestible animal feedstocks or high sugar content raw materials for ethanol production; brightening processes in the pulp and paper industry; the detoxification and decolourisation of industrial effluents; and the bioremediation of hazardous waste sites.

The improvement of these technologies is dependant on ongoing research involving strain selection, strain development using genetic engineering approaches and process development. Strain improvement using non-recombinant methods is beneficial in that it does not limit the inherent robustness observed amongst natural variants. In this research, through a breeding programme, ten *P. chrysosporium* sibling strains were screened for variable ligninase activities and pollutant degradation capabilities in order to further describe previously identified differences between these organisms. A conventional stationary liquid culture technique was effectively miniaturised from 10 ml flask cultures to a 96-well microtitre plate format, for the assessment of multigenic traits amongst sibling strains.

Using the 96-well microtitre plate method, the relationships between *P. chrysosporium* growth kinetics, peroxidase production, pollutant sensitivity and pollutant degradation was explored. Significant correlations were primarily associated with *P. chrysosporium* growth [$P < 0.05$]. Percentage *p*-cresol removal and tannic acid tolerance were both correlated with a shorter lag phase in growth [tannic acid: $r = 0.7698$, $P < 0.05$; *p*-cresol: $r = 0.7584$, $P < 0.05$] and lower stationary phase biomass levels [tannic acid: $r = 0.8177$, $P < 0.05$; *p*-cresol: $r = 0.7803$, $P < 0.05$].

A significant correlation (linear relationship) was also detected between percentage Poly-R478 decolourisation and time of onset of MnP [$r = 0.9689$, $P < 0.001$]. No correlation was observed between dye decolourisation, *p*-cresol degradation, lignin degradation and lignin peroxidase (LiP) or manganese peroxidase (MnP) activities [$P > 0.05$]. These results imply that differences in the biosynthetic pathways for biomass accumulation in sibling strains play a significant role in the intraspecific variation observed in pollutant sensitivity, pollutant degradation, and enzyme production.

Categorical analysis of intraspecific differences was assessed according to four criteria. These included growth, extracellular peroxidase activities, tolerance to toxic pollutants and the biodegradation of model pollutants. Sibling strains showing the most variable responses in three or more of the selective criteria were recommended for further studies. These strains include *P. chrysosporium* ME446, BS 2.52, BS 13, BS 17, BS 18, and BS 24. Interestingly, BS 2.52 (a dikaryotic strain generating from the crossing of two haploid progeny) showed significantly lower degradation capabilities than the wildtype parent strain ME446.

The inherited variability observed between sibling strains is to be further explored through proteome and transcriptome analysis and genetic linkage studies aimed at describing the mechanisms or pathways conferring tolerance to or degradation of environmental pollutants. In examining fewer organisms at this next level, the number of replicates examined can be increased and thus the power of detection of experimental procedures improved, enabling the detection of multigenic traits amongst genetically related organisms.

Growth was shown to play a significant role in the intraspecific differences detected in pollutant sensitivity and degradation between sibling strains. Little is known about the mechanism of growth and differentiation, or the role of differentiation in regulating the lignolytic activity in this organism. The membrane gradostat bioreactor and a unique plug-flow membrane bioreactor were evaluated as novel tools with which to further explore the relationship between secondary metabolism, pollutant degradation and biofilm development in sibling strains. High yield MnP production at levels as high as 1478.8 U.l^{-1} was achieved using a laboratory scale membrane gradostat bioreactor. Furthermore, extensive mycelial differentiation and tissue formation are reported for *P. chrysosporium* in both the membrane gradostat bioreactor and plug-flow membrane bioreactor. Intraspecific differences in the extent of this differentiation were observed in strains ME446, BS 13, BS 17 and BS 26 cultured using the membrane gradostat bioreactor, highlighting the potential of these techniques as a platform for future strain improvement strategies.

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List of Abbreviations

%	= percentage
μ	= specific growth rate
$^{\circ}\text{C}$	= degrees Celsius
μm	= micrometer
μl	= microlitre
μM	= micro molar
2-D	= two dimensional
ABTS	= diammonium 2,2'-azinobis(3-ethyl-6-benzothiazoline sulfonate)
ANOVA	= analysis of variance
BSA	= bovine serum albumin
d	= pathlength (cm)
DMAB	= 3-(dimethylamino)benzoic acid
<i>E</i>	= extinction coefficient
EPS	= extracellular polymeric substances
GLOX	= glyoxal oxidase
G.S	= gas separation
g	= grams
H	= high
hr	= hour
hrs	= hours
i.d.	= internal diameter
IC50	= pollutant level causing 50% inhibition of growth
Kr	= colony radial growth rate
l	= litre
kPa	= kilo Pascals
LiP	= lignin peroxidase
M	= medium
mg	= milligrams
ml	= millilitres
mm	= millimetre
MBTH	= 3-methyl-2-benzothiazolinone hydrazone
MEA	= malt extract agar
M.F	= microfiltration
min	= minute
MMGB	= multiple membrane gradostat bioreactor

MnP	= magnesium chloride
n	= sample number
NH_4^+	= ammonium ion
NLM	= nitrogen limited medium
nm	= nanometre
o.d.	= outer diameter
$\text{OD}_{520 \text{ nm}}$	= optical density at 520 nm
$\text{OD}_{630 \text{ nm}}$	= optical density at 630 nm
p	= significance level
P	= probability
PAH	= polycyclic aromatic hydrocarbons
PCP	= pentachlorophenol
q	= substrate utilisation rate
r	= correlation coefficient
R^2	= R squared. Goodness of fit.
RBC	= rotating biological contactor
RFLP	= restriction fragment length polymorphism
SD	= standard deviation
SE	= standard error
SEM	= scanning electron microscopy
SFR	= single fibre bioreactor
STR	= stirred tank reactor
t	= time
TEM	= transmission electron microscopy
U	= units enzyme activity ($\mu\text{M} \cdot \text{min}^{-1}$)
UMIST	= University of Manchester Institute of Science and Technology
V	= total volume in assay mixture (ml)
v	= volume of enzyme in assay mixture (ml)
VA	= veratryl alcohol
VA^0	= veratryl aldehyde
x	= biomass concentration
$x_{60 \text{ min}}$	= biomass concentration 60 min post-inoculation
x_f	= final biomass concentration
Y	= yield coefficient

Acknowledgements

I would like to extend my sincerest gratitude to all those, who over the years, have supported, encouraged and guided me towards the completion of this degree, both in the laboratory and out.

In particular, to Winston Leukes, my supervisor, who has been a good friend. For his patience, enthusiasm and support throughout this project.

Professor Paul Broda for initiating this project by supplying the sibling strains examined in this work, and Dr Paul Sims and all those at UMIST for hosting me during an invaluable six month research exchange.

To Shirley Pinchuck and Rob Cross of the Rhodes University Electron Microscopy Unit, for their help and experience in processing SEM and TEM samples.

Professor Sarah Radloff of the Rhodes University Statistics Department and Dr. Martin Villet of the Rhodes University Entomology Department for their advice on processing the results for this thesis.

The Institute of Polymer Research, Stellenbosch, for supplying the capillary membranes from which the bioreactors are produced, and in particular Ed Jacobs and Deon Koen for their initial help in developing the plug-flow bioreactor.

To Eskom, the NRF/Royal Society, and more recently Synexa Life Sciences, for funding my research thus far. Sincerest thanks must go to my co-workers at Synexa for their continued support, and for their patience as the preparation of this thesis has dominated my time.

To my family and friends for their unconditional support, and those who have borne mild chaos in the lab with ease.

SECTION A:

STRAIN ANALYSIS USING CONVENTIONAL TECHNOLOGIES

CHAPTER 1

Literature Review

1.1. INTRODUCTION

The discovery and exploitation of microorganisms and natural products for industrial purposes has become relevant in a number of research disciplines from the production of pharmaceuticals to bioremediation and bioaugmentation of industrial wastes.

Filamentous microorganisms remain a major source of biologically active metabolites, the commercial application of which has been readily exploited. The association of natural product biosynthesis during secondary metabolism and cell differentiation or development is apparent. The differentiation and metabolism of filamentous fungi has been comprehensively studied in a number of industrially important organisms, most significantly mycotoxin-producing *Aspergillus* spp. and antibiotic-producing *Penicillium* spp. Those basidiomycetes exploited for bioremediation and bioaugmentation have not been examined as comprehensively as *Aspergillus* or *Penicillium*. While the white rot fungus *Phanerochaete chrysosporium* has been used extensively by researchers as a model organism for further unravelling the mechanism and applications of the lignolytic system in a number of biotechnology processes (Paszczyński and Crawford, 1991, Barr and Aust, 1994), a link between differentiation and lignolysis has not been established.

In its natural habitat, *P. chrysosporium* growth is saprophytic. Wood decomposition occurs primarily in dead tissue, where it is preceded by an initial swelling of the woody cell wall, possibly affected by low molecular weight reagents or radicals (Tanaka et al., 1999) allowing enzymes to penetrate the cell wall (Daniel et al., 1994, 1990). Enzymatic degradation is facilitated by specific hydrolases, including cellulases, xylanases, mannases; and redox enzymes, including peroxidases, laccase, hydrogen peroxide-producing oxidases and cellobiose dehydrogenase.

The lignin-degrading capabilities of *P. chrysosporium* has thus far largely been attributed to the action of free radicals, generated by lignin peroxidase (LiP) and manganese peroxidase (MnP) in the presence of H₂O₂-producing oxidases (Hofrichter, 2002, Paszczyński and Crawford, 1995, Barr and Aust, 1994). Of particular interest is the ability of this white rot fungus to degrade a wide variety of recalcitrant aromatic pollutants under lignolytic conditions (Cameron *et al.*, 2000, Bumpus and Aust, 1987).

1.2. BIOTECHNOLOGICAL SIGNIFICANCE OF LIGNIN DEGRADING WHITE ROT FUNGI

Lignin biodegradation is a key step in the recycling of carbon in terrestrial ecosystems. The unique ability of white rot basidiomycetes to oxidatively depolymerise lignin, enabling the utilisation of cellulose, has been the focus of environmentally sound biotechnology research for five decades. Several reviews on the molecular biology of lignocellulosic degradation are available (Cullen, 1997, Broda et al., 1996, 1989).

Saprophytic organisms secrete a variety of exo-enzymes enabling the biodegradation of the components of wood. Filamentous fungi are considered to be the most effective degraders of wood, with white rot fungi being the most efficient of these due to their ability to mineralise recalcitrant lignin. This same suite of enzymes facilitates the degradation of a number of polyphenolic compounds that are resistant to microbial degradation and would otherwise accumulate to toxic levels in the environment. Environmental waste generated from the utilisation of natural resources or man-made chemicals has been the focus of extensive biotechnological research involving white rot fungi.

1.2.1. Biodegradation of agricultural residues

White rot fungi have been examined in the degradation of the indigestible lignin component in agricultural waste for the generation of digestible animal feedstocks (Villas-Bôas *et al.*, 2002, Brand *et al.*, 2000, Makkar *et al.*, 1994) and high sugar content raw materials for ethanol production (Sun and Chen, 2002, Fitzgibbon *et al.*, 1995); the brightening of kraft pulp (Breen and Singleton, 1999, Buswell and Odier, 1987); and the decolourisation of spent wash from bio-ethanol distilleries (Dahiya *et al.*, 2001) and olive mill wastewaters (García *et al.*, 2000, Sayadi *et al.*, 1996).

1.2.2. Biodegradation of aromatic pollutants

Similarities between the chemical structures of many of hazardous pollutants and that of native lignin components accounts for the relatively non-specific activity of the lignolytic system and its efficient degradation of such pollutants. Early investigations into the degradation of hazardous compounds used *P. chrysosporium* BKMF-1767 almost exclusively as the experimental microorganism. More recently, investigators have focussed on the screening of white rot fungi, with the aim of isolating a more efficient organism for use in bioremediation technologies (de Jong *et al.*, 1992, Field *et al.*, 1992, Morgan *et al.*, 1991, Novotný *et al.*, 1997).

Recalcitrant toxic organic compounds, including polycyclic aromatic hydrocarbons (PAH's), chlorinated aromatics, polychlorinated biphenyl (PCB's) and dyes are present in many industrial effluents. Xenobiotics are also introduced into the environment as refrigerants, fire retardants, paints, plastics, solvents, herbicides and pesticides. Many of these chemicals end up as groundwater pollutants.

1.2.2.1. Polycyclic aromatic hydrocarbons (PAHs)

PAHs are primarily released into the environment by incomplete combustion of fossil fuels (motor vehicle emissions, forest fires and industrial processes) and oil spills. These compounds are toxic, carcinogenic and mutagenic. Degradation of PAHs is influenced by environmental parameters (Jones and Alexander, 1988), microbial type, distribution and sensitivity to the pollutant and the physicochemical factors of the pollutant itself (Volkerling *et al.*, 1995, Black *et al.*, 1983). Degradation of PAHs by microorganisms has been linked to the enzymatic mechanisms of LiP and MnP ring-fission (Hammel *et al.*, 1991), oxidation by laccase (Collins *et al.*, 1996), MnP-dependent lipid peroxidation (Bogan and Lamar, 1995) and intracellular cytochrome P450 activity (Sutherland, 1992, Sutherland *et al.*, 1991, Cerniglia *et al.*, 1985, Cerniglia and Yang, 1984, Cerniglia, 1984)

1.2.2.2. Chlorinated aromatics

Phenols and cresols are primarily found in petroleum-related industries, having widespread application in synthetic resin, explosive, petroleum, photographic, paint and agricultural industries. These substances are released into the atmosphere in auto and diesel exhaust, during coal tar refining, wood pulping and refining and metal refining and as a component of creosote, frequently appearing as contaminants of soil and groundwater as well as wastewater. Phenolic compounds are toxic.

LiP and MnP produced by *P. chrysosporium* are reported to oxidise phenolic pollutants (Aitken and Logan, 1996, Gu and Korus, 1995, Aitken *et al.*, 1994, Kennes and Lema, 1994). Degradation is favoured in the presence of biomass, suggesting further enzymatic pathways contribute to this degradation (Armenante *et al.*, 1994).

1.2.2.3. Polychlorinated biphenyls (PCBs)

The highly oxidised state of chlorinated aromatic compounds renders these chemicals very resistant to microbial degradation, becoming more recalcitrant with increasing chlorine residues. Due to their chemical stability and hydrophobicity, PCBs are used as heat transfer fluids, hydrolic fluids, solvent extenders, plastisizers, flame retardants, organic diluents and dielectric fluids (Beaudette *et al.*, 1998). Chlorinated phenolics are also used extensively as wood preservatives and have fungicidal, bactericidal, herbicidal, molluscicidal, algicidal and insecticidal activities (Crosby, 1981). These chemicals are degraded slowly and tend to accumulate in aquatic and terrestrial ecosystems. Their toxicity is a function of chemical dose and reaction products are significantly less toxic than the original compound (Aitken *et al.*, 1994).

Degradation of chlorinated aromatics is a sequential, multi-step process involving both intracellular and extracellular enzymes (Armenante *et al.*, 1994). Oxidative dechlorination and ring fission by extracellular peroxidases (Joshi and Gold, 1993) and reductive dechlorination by LiP or MnP-catalysed reduction or plasma-membrane redox system have been proposed as mechanisms by which *P. chrysosporium* is capable of degrading chlorinated aromatic compounds. Degradation is however favoured in the presence of biomass, where mycelia alone could not degrade these recalcitrant chemicals, suggesting that further pathways are involved in their degradation (Armenante *et al.*, 1994).

1.2.2.4. Dyes

A number of dyes are synthesised for use in the textile and leather industries, many of which are resistant to degradation. *P. chrysosporium* is capable of decolourising a number of these azo dyes (Fu and Viraraghavan, 2001). Poly-R478 is routinely incorporated into selective media as a means of identifying lignolytic organisms through their ability to decolourise this dye (Tekere *et al.*, 2001, de Jong *et al.*, 1992).

Although a variety of lignolytic organisms have since been screened for improved degradative capabilities; *P. chrysosporium* remains a model organism for elucidating the mechanisms controlling lignolytic activity.

1.3. *P. CHRYSOSPORIUM*

1.3.1. Taxonomy & Description

1.3.1.1. Phenotype

The wood-decaying filamentous basidiomycete, *P. chrysosporium*, conspecific to *Chrysosporium pruinosum*, *Chrysosporium lignorum*, *Sporotrichum pruinosum* and *Sporotrichum pulverulentum*, was described by Burdsall and Eslyn in 1974. This isolate was presented as the type culture for organisms of the genus *Phanerochaete* having a *chrysosporium* in an imperfect state (Burdsall and Eslyn, 1974).

P. chrysosporium shows septate, multinucleate mycelial growth and is usually propagated by asexual spores, but can also be induced into a sexual cycle resulting in the production of basidiospores. The *P. chrysosporium* basidiocarp or fruiting bodies are loosely and irregularly spreading (Burdsall and Eslyn, 1974). The basidiocarp are distinguished by their long, broad, cylindrical, smooth cystidia (Figure 1.1.1) and complete lack of clamp connections. Subicular hyphae (Figure 1.1.4) tend to be smooth near the substrate to heavily encrusted with large crystals near the subhymenium. Further, oval to spherical chlamydospore-like thick-walled cells are sometimes situated near the substrate. The subhymenium is characterised by slight wall thickening and agglutination of hyphae. The hymenium itself is comprised of cystidia (Figure 1.1.1) and basidia (Figure 1.1.2). The sexually produced basidiospores are approximately 3 x 9 µm, ovoid, thin walled and hyaline (Figure 1.1.3).

Aerial hyphae are 2-4 µm in diameter, thin walled, septate, frequently branched hyphae, lacking clamp connections (Figure 1.1.5). Aerial hyphae are occasionally encrusted with long, narrow crystals. Branching hyphae often develop as poorly defined conidiophores. Submerged hyphae are 3.5-6 µm in diameter, thick walled, septate hyphae, frequently branched at near right angles (Figure 1.1.6).

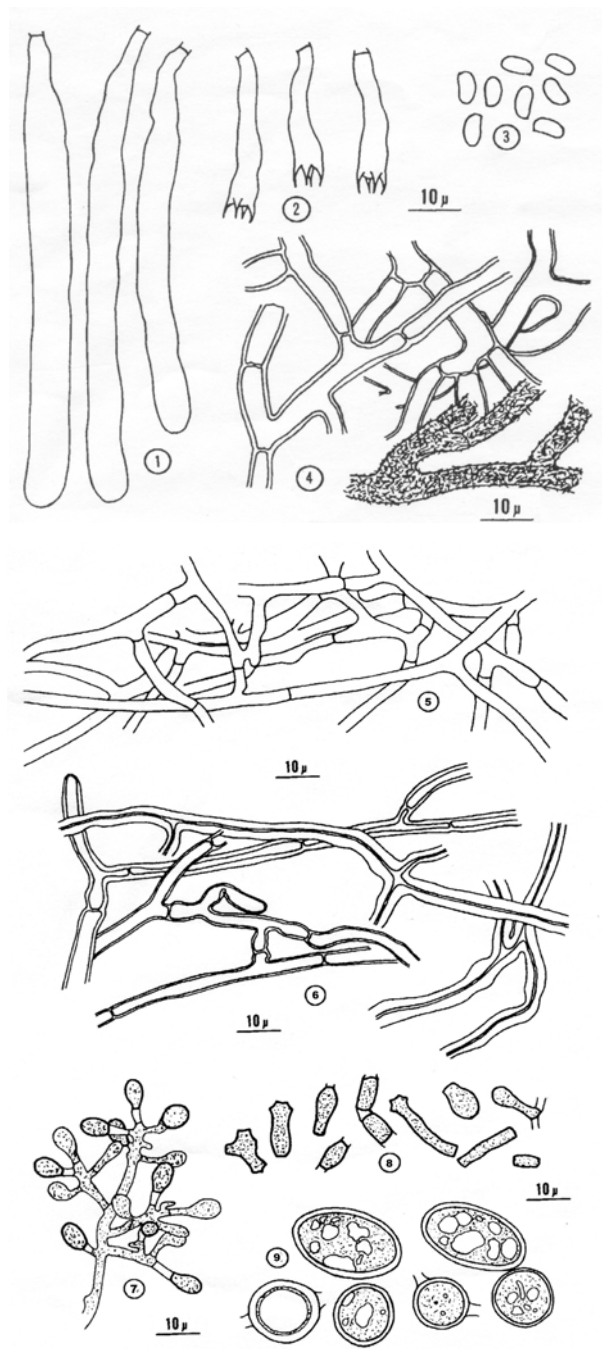


Figure 1.1: Schematic representation of microscopic observations showing *P. chrysosporium* morphology (1) cystidia (2) basidia (3) basidiospores (4) subicular hyphae (5) aerial hyphae (6) submerged hyphae (7) aleuriospores and conidiophores (8) arthrospores (9) chlamydospores (reproduced from Burdsall and Eslyn, 1974).

Asexually produced conidia are found only on aerial hyphae; aleuriospores, borne in intercalary position at the tips of conidiophores, are approximately $3 \times 10 \mu\text{m}$ ovate to elliptical spores (Figure 1.1.7). Arthrospores, sometimes outnumbering aleuriospores, are arthritic, retrogressive, variably sized conidia, formed by fragmented hyphae (Figure 1.1.8). Under adverse conditions, vegetative hyphae form chlamydospores, large (up to $60 \mu\text{m}$) terminal or intercalary, spherical to pear-shaped resting spores with thick walls and densely granular contents (Figure 1.1.9).

1.3.1.2. Genotype

The last decade has shown an increased tendency towards examining the molecular biology or genomics of *P. chrysosporium*'s degradative system. *P. chrysosporium* exists in both dikaryotic and monokaryotic forms, initially complicating molecular biology research in this organism. RFLP analysis showed the *P. chrysosporium* genome to contain seven to nine chromosomes (Gaskell *et al.*, 1991, Raeder *et al.*, 1989a).

In 2004, Martinez *et al.* reported the first full-length genome sequence of the haploid *P. chrysosporium* strain RP78, the first fully sequenced basidiomycete genome. *P. chrysosporium*'s ± 30 Mbp genome was shown to encode a predicted 11 777 genes (Martinez *et al.*, 2004). General features describing the *P. chrysosporium* genome are summarised in Table 1.1.

Table 1.1: General features of the *P. chrysosporium* genome (Martinez *et al.*, 2004).

Assembly size	29.9 Mb
GC content overall	57%
GC content (coding)	59%
Protein coding genes	11 777
tRNAs	200
% coding	45%
Average intergenic distance	1 339 bp
Intron size (average)	117 bp
Intron size (mode)	54 bp
Exon size (average)	232 bp
Exon size (mode)	89 bp
Exon size (mode)	89 bp

With the full genome sequence published, the next milestone remains in identifying and understanding the role of the predicted genes in *P. chrysosporium*'s biological development and the functioning of the lignolytic system.

1.3.2. Secondary metabolism

Primary metabolism, occurring during trophophase, provides a number of small molecules that serve as starting material for all important secondary metabolite pathways, initiated during idiophasic growth, under nutrient-limiting conditions. The lignolytic system is a complex interplay of metabolic pathways feeding into and activating enzymatic suites. Two-dimensional (2-D) gels reveal many changes in the extracellular enzymes produced during lignolytic growth (Wallace *et al.*, 1983). Key enzymes and compounds produced as secondary metabolites and found to influence lignolytic activity are introduced below.

1.3.2.1. Extracellular polymeric substances (EPS)

P. chrysosporium cell walls include β -1,3-1,6-linked glucans, which, under nitrogen-limited (Leisola *et al.*, 1982) or carbon-limited (Rothschild *et al.*, 1995) conditions are produced in excess. Glucan-type EPS are deposited as an extracellular sheath (Sietsma and Wessels, 1981) or secreted into the culture medium (Bes *et al.*, 1987). The production and degree of branching of β -1,3-1,6-D-glucans produced by basidiomycetes varies with culture conditions and age (Bartnicki-Garcia, 1973, 1969).

EPS is produced as a secondary metabolite, the appearance of which frequently occurs simultaneously with the appearance of lignolytic activity. Electron microscopy of degradative mycelia have shown the glucan sheath surrounding hyphae to play a role in the support and transport of depolymerising enzymes in wood decay (Palmer *et al.*, 1983a, 1983b). Cellulase, hemicellulase (Ruel *et al.*, 1989), xylanase (Blanchette *et al.*, 1989) and ligninase (Daniel *et al.*, 1989, 1990) form associations with the glucan network of the sheath in *P. chrysosporium*. This mucilage is seen to associate with wood cell walls at the site of degradation (Ruel and Joseleau, 1991). Retention of water by glucan may help maintain an environment suited for enzymatic catalysis (Ruel and Joseleau, 1991). Diffusion of enzymes is restricted to the distance and extent of the glucan network, while hydrolysis of glucan to glucose releases H_2O_2 required for peroxidase activity.

However, the association of EPS and ligninases is conflicting as free and attached EPS may also offer resistance to oxygen and nutrient diffusion (Dosoretz *et al.*, 1990a); hence influencing oxygen availability (Rothschild *et al.*, 1995). While EPS secretion is more pronounced when grown under air than oxygen (Leisola *et al.*, 1982), oxygen is the rate-limiting factor in ligninase production. EPS is also produced in response to oxygen toxicity (Zacchi *et al.*, 2000, Miura *et al.*, 2004), supporting its role in limiting oxygen diffusion.

1.3.2.2. Veratryl alcohol (VA)

VA biosynthetic pathways in *P. chrysosporium* have been explored (Harper *et al.*, 1990, Mester *et al.*, 1995, Jensen *et al.*, 1994, Harper *et al.*, 1990, Shimada *et al.*, 1981) and linked to LiP production, activity and stability. VA was first thought to induce elements of the lignolytic system (Faison *et al.*, 1986, Faison and Kirk, 1985). However, this has been shown not to be the case (Cancel *et al.*, 1993). Rather, VA has a protective effect on LiP activity (Cancel *et al.*, 1993, Tonon and Odier, 1988), but does not protect against Mn^{2+} inhibition (Rothschild *et al.*, 1999).

VA stabilises LiP and protects it against H_2O_2 -mediated inactivation (Wariishi and Gold, 1989, Tonon and Odier, 1988, Haemmerli *et al.*, 1986) and enhances oxidation of lignin by LiP *in vitro* (Haemmerli *et al.*, 1986). It has been suggested that VA may act as an electron redox mediator or shuttle (Harvey *et al.*, 1986), promoting the oxidation of recalcitrant or inaccessible substrates (Akamatsu *et al.*, 1990, Harvey *et al.*, 1986) or as a co-substrate completing enzyme turnover (Paszczynski and Crawford, 1991, Tien *et al.*, 1986). Intracellular levels of VA were found to increase dramatically 1 hr following an oxygen purge, supporting its role as a redox scavenger (Miura *et al.*, 2004)

The addition of VA to culture medium has also been shown to decrease glyoxal oxidase (GLOX) activity and acid protease production during early secondary metabolism (Cancel *et al.*, 1993).

1.3.2.3. Organic acids

Organic acid biosynthesis in *P. chrysosporium* occurs under both nitrogen sufficient and nitrogen-limited culture conditions (Dosoretz *et al.*, 1993). The secretion of organic acids facilitates pH control, maximising extracellular enzyme activity. Physiological concentrations of oxalate are seen to stimulate MnP activity (Kuan and Tien, 1993). Organic acid production in liquid culture is evidenced by a pH drop coinciding with MnP and LiP production (Kuan and Tien, 1993). Oxygen is required for oxalate-supported oxidative activity (Kuan and Tien, 1993).

Organic acids also function as metal chelators that enhance Mn^{2+} -dependent MnP activity. Further, oxalate is also able to reduce iron-producing hydroxyl radicals and sequester calcium; features proposed to promote accessibility of wood matrix to degradative extracellular lignolytic enzymes (Kuan and Tien, 1993, Linko, 1992).

1.3.2.4. *Proteases*

An initial increase in acid protease is associated with change from primary to secondary metabolism, decreasing during early stages of secondary metabolism (Cancel *et al.*, 1993). This correlates with a rapid decay of LiP activity in extracellular culture fluid (Bonnarme *et al.*, 1993, Datta, 1992, Lackner *et al.*, 1991, Dosoretz *et al.*, 1990b). Proteases secreted during the primary growth phase were shown to be more active against LiP than MnP (Bonnarme *et al.*, 1993, Feijoo *et al.*, 1995). Proteases secreted during the secondary growth phase have little effect on ligninase (Bonnarme *et al.*, 1993, Datta, 1992).

Synthesis of proteases correlated to a decay of LiP activity could be the mechanism of autolysis reported in filamentous fungi (Dosoretz *et al.*, 1990b).

1.3.2.5. *Oxidases*

Extracellular oxidases such as GLOX are involved in the generation of H₂O₂ (Feijoo *et al.*, 1995, Kersten and Kirk, 1987). GLOX synthesis is subject to nitrogen catabolite repression (Dosoretz *et al.*, 1993) and is correlated with LiP production (Rothschild *et al.*, 1995, Kersten and Kirk, 1987). In addition to GLOX several further oxidase-like gene sequences, which may play a role in the lignolytic system, have recently been identified within the *P. chrysosporium* RP78 haploid genome sequence (Martinez *et al.*, 2004).

1.3.2.6. *Cyclic AMP (cAMP)*

cAMP plays a key role in regulating cell metabolism in a variety of biological systems, including a wide range of morphogenetic and developmental processes in fungi (Calvo *et al.*, 2002). Regulation occurs primarily at the level of transcription (MacDonald *et al.*, 1985).

cAMP biosynthesis is activated at a lower pH (section 1.3.3.2) as a result of activation of adenylate cyclase and hampered at higher pH as a result of activation of phosphodiesterase (Thevelein *et al.*, 1987a, Busa and Nuccitelli, 1984).

While cAMP levels increase as a function of growth during primary metabolism (MacDonald *et al.*, 1985), a transition from primary to secondary metabolism in filamentous fungi is correlated with a sharp rise in intracellular cAMP levels (Calvo *et al.*, 2002). cAMP levels are elevated in nitrogen-limited lignolytic cultures and are presumably connected with the control of biosynthesis of catabolically repressed ligninases (Broda *et al.*, 1989). Intracellular concentration of cAMP in LiP active cultures increases 10-fold at the onset of LiP activity (MacDonald *et al.*, 1984). In comparison

to LiP, lower levels of cAMP are required to stimulate MnP activity. This occurs at the level of transcription where LiP and MnP appear to be differentially regulated by cAMP (Boominathan and Reddy, 1992). The inhibition of LiP activity by Mn^{2+} may be the result of reduced cAMP levels, due to the stimulation of phosphodiesterase activity by Mn^{2+} . Further, cAMP induction may differentially regulate the different LiP isoenzymes (Boominathan and Reddy, 1992).

1.3.2.7. Ligninases

Until recently it was believed that *P. chrysosporium* did not produce the enzyme laccase. This belief is supported by the absence of laccase-like gene sequences in the *P. chrysosporium* genome (Martinez *et al.*, 2004). Laccase activity described in *P. chrysosporium* strain BKMF-1767 under both liquid (Srinivasan *et al.*, 1995) and semi-solid (Rodríguez Couto *et al.*, 1997) culture conditions is most likely due to distantly related, previously unidentified, multicopper oxidases (MCO) described by Martinez *et al.* (2004).

Srinivasan *et al.* (1995) attributed the detection of low levels of “laccase activity” to the use of a complex carbon source (cellulose powder) instead of the more frequently used glucose. However, Rodríguez Couto *et al.* (1997) showed that “laccase activity” is not only determined by the nature of the carbon source, but is also influenced by immobilisation and the addition of MnO_2 to the culture medium. Furthermore, laccase activity is readily influenced by the chemical composition of the immobilisation matrix and not the physical characteristics of the carrier.

Although laccase has been implicated directly in pollutant degradation by white rot fungi such as *Trametes versicolor*, this enzyme has not been shown to play a major role in biodegradation pathways of *P. chrysosporium*. Most biodegradation assays have been undertaken using liquid culture systems containing nitrogen-limiting medium with glucose as the carbon source. Such conditions favour the production of LiP and MnP, but not laccase-like enzymes.

Two classes of extracellular peroxidases are detected in *P. chrysosporium* these are LiP and MnP. These two classes differ in their reaction mechanisms, where LiP directly catalyses the oxidation of nonphenolic aromatic substrates and MnP catalyses the oxidation of Mn^{2+} to Mn^{3+} . Mn^{3+} complexed with an organic acid chelator such as oxalate or malonate, further mediates the oxidation of phenolic substrates (van Deurzen *et al.*, 1997). The appearance of each of these enzymes has been correlated to the disappearance of lignin, lignin model compounds and a variety of aromatic pollutants.

Both LiP and MnP are produced by *P. chrysosporium* during idiophasic growth. Enzyme activity studies have shown *P. chrysosporium* to produce large amounts of LiP or MnP depending on the culture medium composition and culture system used. Initial experiments showed the activity of these enzymes to be affected by nutrient limitation; namely carbon, nitrogen and sulphur limitation, oxygen availability, and agitation. The regulation of both enzymes has been shown to be uncoupled and occurs at the level of gene transcription (Reddy and D'Souza, 1994).

Both LiP and MnP are comprised of a family of isoenzymes. In *P. chrysosporium*, six different LiP isoenzymes and four different MnP isoenzymes have been identified. Within each peroxidase family, the isoenzymes are structurally and functionally related; showing similar catalytic properties (Tien and Kirk, 1988). The relatedness of these isoenzymes and their regulation will now be reviewed.

Lignin peroxidase (LiP)

Oxidative depolymerisation of lignin by white rot fungi is a random process mediated by cation radicals. LiP and MnP produced by *P. chrysosporium* under nitrogen-limiting culture conditions, react with H_2O_2 to catalyse the single-electron oxidation of organic compounds and Mn^{2+} , respectively. The resultant cationic radicals formed by LiP, and the Mn^{3+} formed by MnP, diffuse from the active sites of these enzymes to catalyse the non-specific cleavage of the lignin polymer. The low molecular weight products are then metabolised to CO_2 . This non-specific free radical mechanism has been shown to mediate the catalytic oxidative transformation of many aromatic compounds (van Deurzen *et al.*, 1997).

Initial studies involving the characterisation of peroxidase isoenzymes were undertaken using gel electrophoresis and isoelectric focusing techniques. Further separation of distinct isoenzymes was accomplished with fast protein liquid chromatography (FPLC) and high performance liquid chromatography (HPLC). Present isoenzyme classification is based on chromatograms generated by Kirk *et al.* (1986a), where six different VA oxidising enzymes (LiP) and four Mn^{2+} -dependent isoenzymes (MnP) were identified. These isoenzymes are referred to as peroxidases H1 to H10.

LiP isoenzymes include H1, H2, H6, H7, H8, and H10. LiP isoenzymes have been shown to differ from one another in terms of their isoelectric points, molecular mass (ranging from 38-43 kDa), sugar content, spectral characteristics, substrate specificity and stability. Some of these differences are listed in Table 1.2. Certain differences, for example sugar content, are a result of differences in post-translational modification. However, differences in the N-terminal sequence of each isoenzyme suggest different gene products. Each isoenzyme type, as identified by HPLC and V8 proteolysis, can include the products of several genes (Gold and Alic, 1993).

Table 1.2: Characteristics of LiP isoenzymes.

Isoenzyme	Size ^a	PI ^b	Km (μM) ^b	
			H ₂ O ₂	VA
H1	38,000	4.7	13	86
H2	38,000	4.4	42	250
H6	43,000	3.7	34	122
H7	42,000	3.6	77	483
H8	42,000	3.5	17	89
H10	46,000	3.3	24	190

^a From Tien and Kirk (1988). ^b From Farrell *et al.* (1989).

The organisation and number of genes encoding LiP isoenzymes is uncertain. The situation has been complicated by the existence of a number of allelic variants, inconsistent nomenclature and the use of different *P. chrysosporium* strains; namely BKM-F1767, OCG101, ME446 and now RP78. Classification of sequenced genes, shown in Table 1.3, is best summarised by Gaskell *et al.* (1992). The full genome sequence of *P. chrysosporium* RP78 shows 10 distinct *lip* genes (Martinez *et al.*, 2004).

Table 1.3: Gene sequences reported to encode lignin peroxidase isoenzymes (modified from Gaskell *et al.*, 1994).

Designation	Isoenzyme	Source	Synonyms	Reference
<i>lipA1</i>	H8	BKM-F-1767	ML1(c) (g) H8(g) LPOA(g)	Tien and Tu, 1987, Holzbauer and Tien, 1988, Smith <i>et al.</i> , 1988 Schalch <i>et al.</i> , 1989 Huoponen <i>et al.</i> , 1990, Gaskell <i>et al.</i> , 1991
<i>lipA2</i>		BKM-F-1767	LiPA(g) ML4 (c)	Andrawis <i>et al.</i> , 1989, Gaskell <i>et al.</i> , 1992, Tien and Tu, 1987
<i>lipA3</i>		ME446	LIG2(g)	Broda <i>et al.</i> , 1989
<i>lipA4</i>		ME446	LG1(g)	Asada <i>et al.</i> , 1988
<i>lipB1</i>		BKM-F-1767	none	Gaskell <i>et al.</i> , 1991
<i>lipB2</i>		BKM-F-1767	LPOB(g)	Huoponen <i>et al.</i> , 1990
<i>lipB3</i>		ME446	LIG3(g)	Broda <i>et al.</i> , 1989
<i>lipC1</i>	H10	BKM-F-1767	CLG5(c) GLG5(g)	de Boer <i>et al.</i> , 1988, Gaskell <i>et al.</i> , 1991
<i>lipC2</i>	H10	BKM-F-1767	LiP6(g) GLG2	Zhang <i>et al.</i> , 1991
<i>lipD1</i>	H2	BKM-F-1767	GLG4 (g) CLG4(c)	de Boer <i>et al.</i> , 1987 and 1988
<i>lipD2</i>		BKM-F-1767	LiP2(g)	
<i>lipD3</i>	H2	ME446	LIG5	James <i>et al.</i> , 1992
?	H2	BKM -F1767	LG4 (gt-11) (c)	de Boer <i>et al.</i> , 1987 (Cai and Tien, 1991)
<i>lipE1</i>	H7	BKM-F-1767	LPO811(c)	Reiser <i>et al.</i> , 1993
<i>lipE3</i>		OGC101	L189(c) LG2(g)	Ritch <i>et al.</i> , 1991 Ritch and Gold, 1992
<i>lipF1</i>		BKM-F-1767	GLG6(c)(g)	Padmavathy <i>et al.</i> , 1991
<i>lipG1</i>		BKM-F-1767	ML-5(c)	Andrawis <i>et al.</i> , 1989
<i>lipH3</i>		ME446	LIG1(g) L13 (g)	Brown <i>et al.</i> , 1988
<i>lipI1</i>		BKM-F-1767	O282(g)	Schalch <i>et al.</i> , 1989
<i>lipI2</i>	Pce1 heritable insert. Inactive	BKM-F-1767		Gaskell <i>et al.</i> , 1994
<i>lipJ1</i>		BKM-F-1767	V4(g)	Schalch <i>et al.</i> , 1989
<i>lipJ3</i>		ME446	LIG4(g) L13 (c)	Broda <i>et al.</i> , 1989

[No distinct gene has been found for H1 - which appears to be a breakdown product of H2 (Holzbauer and Tien, 1988)]

lip genes are found in unlinked clusters and are thought to be differentially regulated. Limited information is available on the mechanism of regulation of *lip* genes. The temporal expression of isoenzymes is seen to differ depending on culture conditions. LiP production is induced by nutrient depletion at the level of gene transcription (Tien and Tu, 1987). Different isoenzymes appear to be regulated by different nutrient sources. Under carbon-limiting conditions isoenzyme H2 predominates; whereas under nitrogen-limiting conditions, the isoenzyme H8 predominates (Holzbauer and Tien, 1988). Carbon and nitrogen regulation of LiP may not be separated in *P. chrysosporium* (Tonon *et al.*, 1990). The differential regulation of *lip* genes is strain dependent, further suggesting the existence of multiple copy genes or alleles for a given isoenzyme. Although the open reading frames of multiple genes are similar, differences are detected in their promoters (Broda *et al.*, 1996). Until recently, experiments have focused on the isolation of genes encoding LiP isoenzymes. However, the differential regulation of these genes has currently become of interest.

Manganese peroxidase (MnP)

The four distinct MnP isoenzymes, as defined by Kirk *et al.* (1984), are isoenzymes H3, H4, H5 and H9. Subsequent studies have separated a further two isoenzymes on the basis of their pIs (Datta *et al.*, 1991). The presence of these isoenzymes have, however, not been confirmed.

From Table 1.4 it can be seen that all identified MnP isoenzymes are of a similar molecular size. However, the total amino acid composition of each isoenzyme differs (reflected as differences in pI), as does the N-terminal amino acid sequence (Pease and Tien, 1992). These findings suggested that separate, closely related *mnp* genes encoded each isoenzyme. Three distinct *mnp* genes have been isolated and sequenced as follows: *mnp-1* encoding H4; *mnp-2* encoding an unidentified protein product and *mnp-3* encoding H3 (Bogan *et al.*, 1996). It appears that there is a multiplicity of genes encoding each isoenzyme, as shown for LiP (Pribnow *et al.*, 1989). Genes encoding isoenzymes H4 and H9 have thus far not been identified. However, the recently sequenced genome of *P. chrysosporium* RP78 identified a total of five *mnp* genes, including two new sequences (Martinez *et al.*, 2004).

Table 1.4: Characteristics of Mn²⁺-dependent peroxidase isoenzymes.

Gene	Source	Isoenzyme ^a	Size ^b	PI ^c	Km (μM)		Reference
					Mn ²⁺	H ₂ O ₂	
mnp-3 [MP-2]		H3	≈ 46 K (37,490 K)	4.9			Orth <i>et al.</i> , 1994
[MnP-1]	OGC101	H3?	(37,439 K)	4.9	16	34	Pribnow <i>et al.</i> , 1989
mnp-2 [mnp1]	ME446	?					Godfrey <i>et al.</i> , 1990
mnp-1 [MP-1]	BKMF-1767	H4	≈ 46 K (37,711 K)	4.55			Pease <i>et al.</i> , 1989
		H5	≈ 46 K	4.2	9	32	
		H9	≈ 46 K	4.3	4	21	

^a As defined by Kirk *et al.* (1984). ^b Gold and Glenn (1988). Predicted molecular size of MnP proteins are in brackets. ^c Kirk and Farrell (1987).

MnP production is regulated at the level of transcription by a hierarchy of controls. The first prerequisite appears to be nutrient limitation, in particular the level of carbon and nitrogen nutrient sources (Pribnow *et al.*, 1989). Under nutrient-limiting conditions, induction of transcription is effected by Mn²⁺ and heat shock, the former being a more efficient inducer of *mnp* expression (Brown *et al.*, 1993, 1991). Putative metal response elements (MREs) and heat shock elements (HSEs) have been detected within the promotor regions of *mnp* genes (Godfrey *et al.*, 1990). Mn²⁺ induction can be further enhanced by combining Mn²⁺ with heat shock, H₂O₂, chemical stress or molecular oxygen (Li *et al.*, 1995, Brown *et al.*, 1993). Each of these inducers appears to work at a different level. However, *mnp* gene expression appears to be dependent on the presence of Mn²⁺ and H₂O₂, as does MnP activity.

mRNA production and turnover, as a reflection of transcription, has been monitored using both northern blot analysis (Li *et al.*, 1995) and RT-PCR (Gettemy *et al.*, 1998). The latter technique was found to be 10³ to 10⁴ times more sensitive. Quantification of mRNA templates was performed by competitive reverse transcription polymerase chain reaction (RT-PCR), where a series of internal standards were used to calculate initial template concentrations. Competitive RT-PCR has been used to monitor the temporal expression of *mnp* genes in response to various culture conditions (Gettemy *et al.*, 1998) and during pollutant degradation (Bogan *et al.*, 1996).

Differences in the affinity of different MnP isoenzymes for Mn^{2+} and H_2O_2 (Table 1.4), in addition to differences in the catalytic efficiency of certain substrates, suggest these isoenzymes have different functions and are produced in response to different environmental conditions. This has led to the proposal that the differential regulation of *mnp* genes may be linked to differences in induction pathways of these isoenzymes (Pease and Tien, 1992).

1.3.3. Effect of culture conditions on ligninase production

The manipulation of growth medium and culture conditions usually differs for optimal growth and optimal product formation. Primary metabolites synthesised during the growth phase or trophophase provide the basis for subsequent biosynthetic pathways, resulting in the production of secondary metabolites. The production of secondary metabolites routinely occurs during idiophasic growth under nutrient-limiting conditions. Ligninase production by *P. chrysosporium* is no exception. Nutrients, trace metals, pH, stimulants and oxygen influence LiP and MnP levels. Similarly, the profile and activity of specific isoenzymes is linked to culture age and growth conditions.

1.3.3.1. Temperature

Several studies have shown optimal temperatures for maximum LiP production in liquid culture by *P. chrysosporium* BKMF-1767 to vary between 37 °C and 40 °C (Kirk *et al.*, 1986a, Leisola and Fiechter, 1985). However, Asther *et al.* (1988a) ascribed two temperature optima connected with LiP synthesis by INA-12, where 37 °C was optimal for the mycelial growth phase, and 30 °C was optimal for the LiP-producing phase. Similarly, Hatakka and Uusi-Rauva (1983) observed the ability of *P. chrysosporium* to degrade poplar wood lignin was higher at 28 °C than 39 °C. LiP activity coincided with temporary cessation of biomass increase and a reduction in relative active biomass concentration.

1.3.3.2. pH

Bonnarme *et al.* (1993) measured an increase in the growth medium pH and in dissolved oxygen levels; coinciding with the onset of LiP activity. Similarly, during LiP activity a decrease in intracellular pH and an increase in growth medium pH were determined in cultures supplemented with Tween 80® (Leštan *et al.*, 1994).

An increase in intracellular pH of only 0.12 pH units has been shown to increase the glycolysis rate by 43 % (Leštan *et al.*, 1994) and influences cAMP biosynthesis (Thevelein *et al.*, 1987a, 1987b, Busa and Nuccitelli, 1984). cAMP is correlated with the onset of LiP activity (MacDonald *et al.*, 1984) and is proposed to control the production of catabolically repressed LiP (Broda *et al.*, 1989).

1.3.3.3. Nutrient composition

Peroxidase production is regulated by carbon and nitrogen at the level of transcription (Gold and Alic, 1993, Pérez and Jeffries, 1992, Brown *et al.*, 1991, Bonnarme and Jeffries, 1990, Tonon *et al.*, 1990, Tien and Tu, 1987, Fenn and Kirk, 1981, Jeffries *et al.*, 1981, Keyser *et al.*, 1978). The composition of isoenzymes is effected by levels of nitrogen. H1 and H2 are predominant LiP isoenzymes in high nitrogen content medium, whereas H2 (low) H6 and H8 are predominant in nitrogen-limited medium such as the basal III medium described by Tien and Kirk (1988) (Rothschild *et al.*, 1999, Dosoretz *et al.*, 1993, Gold and Alic, 1993, Holzbauer and Tien, 1988).

Supplementation of the growth medium with peptone (Kaal *et al.*, 1995), VA and trace elements (Kirk *et al.*, 1986a), particularly Cu^{2+} and Mn^{2+} (Boominathan and Reddy, 1992, Buswell and Odier, 1987), stimulate peroxidase production. The relative amount of each isoenzyme varies depending on culture additives such as VA and the trace elements solution (Rothschild *et al.*, 1999, Cancel *et al.*, 1993, Kirk *et al.*, 1985).

While *P. chrysosporium* produces VA at low levels (Lundquist and Kirk, 1978), its addition to culture medium was found to increase LiP activity (Tonon and Odier, 1988, Faison *et al.*, 1986, Kirk *et al.*, 1986a, Faison and Kirk, 1985, Leisola and Fiechter, 1985, Leisola *et al.*, 1985), but not MnP (Cancel *et al.*, 1993). Increased activity of isoenzymes H1 and H2 were most noticeable, attributed to the protection of these isoenzymes from inactivation (Cancel *et al.*, 1993).

Mn^{2+} is known to regulate a number of cell functions (Korc, 1993), where Mn^{2+} deficiency results in lower biomass and lower glucose consumption rates in nutrient sufficient cultures (Rothschild *et al.*, 1999). Further, Mn^{2+} enhances Mn superoxide dismutase activity, which acts as an antioxidant through the conversion of superoxide radicals to H_2O_2 (Rothschild *et al.*, 1999). The addition of Mn^{2+} to *P. chrysosporium* cultures regulates *mnp* transcription and has an inhibitory effect on LiP, where inactivation of LiP is in response to elevated H_2O_2 levels and is not affected at the level of transcription (Mester *et al.*, 1995, Li *et al.*, 1994, Gold and Alic, 1993, Pérez and Jeffries, 1992, Bonnarme and Jeffries, 1990). The effects of Mn^{2+} on LiP are partially counteracted by Ca^{2+} and Mg^{2+}

(Michel *et al.*, 1991) and alleviated only by increased oxygen availability (Rothschild *et al.*, 1999). Mn^{2+} -deficient cultures are more susceptible to oxidative stress as Mn^{2+} acts as a free radical scavenger (Rothschild *et al.*, 1999). Profiles and relative ratio of isoenzymes vary with Mn^{2+} supplementation. Isoenzyme ratio H1:H2 is affected by the addition of Mn^{2+} to culture medium, as is the production of H4, which would otherwise be absent under low nitrogen culture conditions. The altered ratio of H1:H2 under these conditions is attributed to the dephosphorylation of H2 to H1 by an extracellular phosphatase, the activity of which is enhanced by Mn^{2+} (Rothschild *et al.*, 1999).

1.3.3.4. Stimulants

A number of stimulators have been used in attempts to enhance ligninase production in *P. chrysosporium* fermentations. Tween 80[®], Tween 20[®], the alcoholic moiety of both Tween 80[®] hydrolysate and polyoxyethylene oleate and CHAPS enhance LiP production (Leštan *et al.*, 1993, Asther *et al.*, 1987, Jäger *et al.*, 1985). These surfactants were initially thought to act at the cell membrane, affecting the release of enzymes (Leštan *et al.*, 1993, Reese and Maguire, 1969). However, Leštan *et al.* (1993) showed this not to be the case. These authors correlated high extracellular LiP activity with high intracellular LiP activity (Leštan *et al.*, 1993).

Reports on the mechanism of stimulants are conflicting. Tween 80[®] has been shown to increase LiP levels by addition after primary growth (Venkatadri and Irvine, 1990), contradicting Jäger *et al.* (1985) who demonstrated Tween 80[®] to be effective only if added at the beginning of primary growth. Leštan *et al.* (1993) report the disappearance of Tween 80[®] within the first three days, also suggesting a role in primary metabolism.

Bonnarme and Jeffries (1990), Capdevila *et al.* (1990, 1989) and Asther *et al.* (1988b) report LiP synthesis is stimulated by exogenous phospholipids (oleic acid, soybean phospholipids or vegetable oils), which also enhanced energy metabolism and modify fungal physiology. In a later publication, Leštan *et al.* (1994) suggest that Tween 80[®] confers higher oxygen permeability of cell membranes due to altered fatty acid composition of polar lipids and thus increased oxygen supply to cells.

1.3.3.5. Aeration

Oxygen is routinely shown to be the rate-limiting nutrient in aerobic fermentations (Dosoretz *et al.*, 1990a). While high oxygen levels do not affect growth in *P. chrysosporium* cultures (Reid and Siefert, 1980, 1982), high oxygen tension increases LiP (Dosoretz *et al.*, 1990a, Faison and Kirk, 1985) and MnP levels (Rothschild *et al.*, 1999, Li *et al.*, 1995).

Most research into the degradation capabilities of LiP and MnP have been undertaken in liquid culture systems. Oxygen transfer in stationary cultures is restricted (Leisola *et al.*, 1983), and consequently a high partial pressure of oxygen in the culture headspace is needed to make sufficient oxygen available to the submerged hyphae (Michel *et al.*, 1992, Leisola *et al.*, 1983). In agitated cultures, surfactants (section 1.3.3.4) are used to reduce the concentration boundary layer between media and hyphae thus enhancing LiP production (Leštan *et al.*, 1994). Oxygen concentration is less rate limiting in solid-state cultures (Highley *et al.*, 1982), partially due to the ability of fungal hyphae to take up oxygen directly from air (Raghavarao *et al.*, 2003).

1.3.3.6. Liquid culture systems

The reproducibility of LiP and MnP production, activity and isoenzyme profiles in liquid culture is poor (Linko, 1992, Leisola *et al.*, 1987). Research into culture parameters, initially undertaken in shallow stationary flask cultures (Tien and Kirk, 1988), has proven difficult to scale up via proportional increase in culture volume/flask size, resulting in lower activity (Tien and Kirk, 1988). The shape of the flask, volume of culture and oxygen partial pressure significantly affects enzyme production.

Ligninase production in agitated cultures has proven problematic (Linko, 1992). Factors including the nature and concentration of nutrients, the rate and type of shaking, aeration, culture volume and inoculum type all influence product amount, isoenzyme composition and stability. The inhibitory effects of agitation (Venkatadri and Irvine, 1990) have been overcome only by the addition of surfactants to culture medium (section 1.3.3.4).

While submerged agitated cultures have proven easier to scale up from flask size to the level of stirred tank reactors (Linko, 1992), large scale fermentations introduce the problem of sterilizing large volumes, sufficient aeration and temperature control, impeller damage of cells, foaming and high downstream processing costs.

1.3.3.7. Immobilisation

Mycelial differentiation is observed to some extent in ageing batch cultures and as pellet formation in agitated cultures. In its natural growth habitat the *P. chrysosporium* thallus is resupinate, growing as a thin crust on woody surfaces. Thus, biofilm formation by this fungus is considered to be more stable than growth in liquid suspension. Immobilisation of *P. chrysosporium* has been undertaken both actively through entrapment within agar, agarose and k-carrageenan gel beads (Venkatadri and Irvine, 1990, Linko, 1992); and passively through biofilm formation on a solid substratum (Table 1.5).

Table 1.5: Immobilisation substrates used for ligninase production in *P. chrysosporium*.

Immobilisation Matrix	References
Entrapment: agar, agarose, K-carrageenan gel beads	Venkatadri and Irvine, 1990
Celite	Endo <i>et al.</i> , 1988
Polyurethane	Bonnarme <i>et al.</i> , 1991, Bonnarme and Jeffries, 1990, Ma <i>et al.</i> , 1990, Capdevila <i>et al.</i> , 1989, Kirkpatrick and Palmer, 1987
Nylon Web	Polvinen <i>et al.</i> , 1991, Glumoff <i>et al.</i> , 1989, Linko, 1988
Silicon tubing	Venkatadri and Irvine, 1993, References in Linko, 1992
Sintered glass	Jäger <i>et al.</i> , 1991
Stainless steel	Asther <i>et al.</i> , 1990
Porous ceramics	Cornwell <i>et al.</i> , 1990
Polypropylene	Asther <i>et al.</i> , 1990
Polysulphone	Leukes, 1999

The main advantages of immobilised viable cells include:

1. Ease of separation and reuse of cells;
2. High cell concentrations;
3. Flexibility in reactor design and operation; and
4. Stabilisation of cell functions.

Immobilisation of *P. chrysosporium* stabilises filamentous growth and improves peroxidase yield. A variety of bioreactor designs and immobilisation matrixes have been examined for this purpose. The production of peroxidase achieved in some of these reactors is outlined in Table 1.6.

Table 1.6: Partial listing of *P. chrysosporium* immobilised bioreactors and their yield (modified from Linko, 1992).

Bioreactor System	Immobilised	Strain	Growth conditions	Working vol. (l)	Max. LiP activity (U.l ⁻¹)	Max. MnP activity (U.l ⁻¹)	Reference
Stationary Batch							
Stationary batch	Polyurethane cubes	INA-12	2.5 mM VA, glycerol, soybean phospholipids	0.03	2430		Capdevila <i>et al.</i> , 1989
Agitated							
Agitated batch	Polyurethane cubes	BKMF-1767 INA-12		0.1	1296	252	Bonnarme <i>et al.</i> , 1991
Stirred tank reactor	Spiral silicon tubing			0.1	2580	630	References in Linko, 1992
Agitated submerged	Polyurethane		175 rpm, 2 mM VA, 0.05 % Tween 80 [®] , 37 °C	1	200		Chen <i>et al.</i> , 1991
Agitated submerged, repeated batch	Polyurethane		125/60 rpm, 1 mM VA, 37 °C	0.09	340		Kirkpatrick and Palmer, 1987
Agitated submerged	Nylon		60 rpm, 2.5 mM VA, 37°C, carbon-limited	0.2	100		Linko, 1988
Repeated batch				0.4	730		
Static-bed	Polyurethane sponges	BKMF-1767		1	194	154	Rodríguez Couto <i>et al.</i> , 2001a
Immersion	Silicon tubing		2 mM VA and 0.05 % Tween 80 [®]	1	277	1477	Polvinen <i>et al.</i> , 1991
STR, semi-continuous			2.5 % glucose, 2.5 % yeast extract, 30°C	800	336		
STR	Nylon sheet	TKK 20512	250/220 rpm, 2.5 mM VA carbon-limited, 37/30 °C	8	1035		Linko, 1992
STR	Nylon cubes		2.5 mM VA 37 °C, carbon-limited	8	730		Linko, 1988
STR	Silicon tubing		250 rpm, glycerol, 40 °C	1	232		Willerhausen <i>et al.</i> , 1987
Air Lift Reactor							
Air lift reactor	Nylon sheet	BKMF-1767 /ME446		8	1035		References in Linko, 1992
Air lift reactor	Sintered glass	BKMF-1767 /ME446		40	2200		Linko, 1992
Transverse flow membrane gradostat bioreactor	Capillary membranes	ME446	Continuous		1961		Leukes, 1999

Table 1.6 continued.

Bioreactor System	Immobilised	Strain	Growth conditions	Working vol. (l)	Max. LiP activity (U.l ⁻¹)	Max. MnP activity (U.l ⁻¹)	Reference
<u>Rotating Biological Contactor</u>							
Rotating biological reactor		BKMF-1767 and SC26		2.8	80		References in Linko, 1992
Rotating disk reactor	PMM disks	SC 26		2.5	130		Kirk <i>et al.</i> , 1986a
<u>Fixed Bed</u>							
	Nylon web	Various mutant strains of WRF		10	100-750		Linko, 1988
Fixed bed-loop	Sintered glass		0.1 % yeast extract, glycerol, 39/30 °C	0.3 40	2200		Jäger <i>et al.</i> , 1991
Pulsed-Bed Bioreactor - Fluidised bed reactor	Polyurethane foam	BKMF-1767	Continuous	0.167	25-50	250	Moreira <i>et al.</i> , 1997
		BKMF-1767 and ME446		8	260-280		Shim and Kawamoto, 2002
<u>Membrane Bioreactors</u>							
Hollow fibre reactor		BKMF-1767		0.2	190		Venkatadri and Irvine, 1993
Silicon membrane reactor		BKMF-1767		1.5	230		Venkatadri and Irvine, 1993
Transverse flow membrane gradostat bioreactor		ME446	continuous			1916	Leukes, 1999

1.3.3.8. Membrane bioreactors

The successful application of membrane bioreactors as whole cell culture systems was first reported in 1972 (Knazec *et al.*, 1972). These reactors have primarily been operated as submerged immobilisation systems for mammalian cell culture (Sardonini and DiBiasio, 1992, Belfort, 1989). The use of membrane immobilised *P. chrysosporium* BKMF-1767 in the production of extracellular peroxidase was first reported by Venkatadri and Irvine (1993). While the peroxidase yields achieved by these authors were lower than obtained for alternative immobilisation systems, membrane bioreactors offer continuous production (Table 1.6).

The membrane bioreactors are advantageous in that they provide:

1. A hydrophobic substratum encouraging biofilm attachment;
2. High surface area to volume ratios;
3. Cell separation and high cell density;
4. High volume productivity;
5. Good oxygen transfer with low shear;
6. Continuous nutrient supply and product removal. (Heath and Belfort, 1992)

A novel approach to membrane bioreactors, termed the membrane gradostat and its application to secondary metabolite production, was first conceptualised by Leukes (1999). The membrane gradostat bioreactor harnesses the problem of nutrient gradients previously reported for membrane bioreactors (Hammer *et al.*, 1990, Piret and Cooney, 1991) and uses these gradients to establish steady state nutrient and oxygen gradients across a membrane-immobilised biofilm (Leukes, 1999).

This system was initially developed with the objective of reflecting the natural growth characteristics of *P. chrysosporium*, where the immobilisation of this filamentous fungus on the outer surface of a capillary membrane allows for a unique liquid, solid, air interface i.e. the biofilm attached to the surface of a capillary membrane is supplied with nutrients from below (diffusion from the membrane lumen) and oxygen from above. A specialised externally unskinned polysulphone ultrafiltration capillary membrane with uniquely porous substructure was developed for this purpose (Jacobs and Leukes, 1996). The gradostat theory has been patented (Leukes *et al.*, 1996) and since applied to a number of organisms including *P. chrysosporium* (Garcin, 2002, Leukes, 1999), *Trametes versicolor* (Ryan *et al.*, 1998), *Neurospora crassa* (Luke and Burton, 2001), *Penicillium* sp. (Purohit, 2001), *Microcystis aeruginosa* (Strong, 2002) and *Streptomyces* sp. (Purohit, 2001).

P. chrysosporium ME446, cultured using a laboratory scale transverse flow membrane bioreactor, showed ligninolytic enzyme production of 1916 U.l⁻¹ for manganese peroxidase (Leukes, 1999). This compared favourably with the best-reported systems (Table 1.6). The transverse flow module was however disadvantageous as it proved difficult to scale up and could not be operated for extended periods of time, primarily due to extensive biomass accumulation preventing adequate oxygenation and product removal.

1.4. STRAIN IMPROVEMENT

Strategies for genetic improvement, which are favoured in process optimisation, are a difficult undertaking due to the complexity of the lignolytic system.

The need for strain improvement arises from the existence of rate-limiting steps within all metabolic pathways. These mechanisms are not always measurable during the course of an industrial fermentation. The impact of metabolic flux on secondary metabolite biosynthesis is facilitated through an integrated network of biological reactions including enzyme specific biosynthesis; primary metabolism for the supply of precursors to growth and secondary metabolites; and regulatory circuits involved in cell growth and differentiation (Vinci and Byng, 1999).

Improvement of peroxidase production has been achieved through traditional methods of screening white rot fungi, natural variants and mutants for improved yields.

Initial experiments into ligninase production by *P. chrysosporium* used strain BKMF-1767 almost exclusively. BKMF-1767 produces significantly higher levels of ligninase activity than *P. chrysosporium* ME446 when grown in shallow stationary culture (Kirk *et al.*, 1986b, Faison and Kirk, 1985). Wildtype isolates (Table 1.7) have similar growth rates, differ in EPS production, onset of LiP and maximum enzyme activities detected, yet show similar isoenzyme profiles (Kirk *et al.*, 1986a, 1986b).

Table 1.7: Wildtype and mutant *P. chrysosporium* strains reported to produce high levels of ligninase activity.

<i>P. chrysosporium</i> strain	Mutant (Parent)	Characteristic	Reference
BKMF-1767	WT (ATCC 24725)		Kirk <i>et al.</i> , 1986a, 1986b, Burdsall <i>et al.</i> , 1974
ME446 (dikaryon)	WT (ATCC 34541)		Burdsall <i>et al.</i> , 1974
K-3 (heterokaryon)	WT (ATCC 32629)		Johnsrudd and Eriksson, 1985 Burdsall <i>et al.</i> , 1974
SC 26	UV (ATCC 24725)		Kirk <i>et al.</i> , 1986a, 1986b
TKK 20512		High ligninase under carbon-limiting conditions	References in Linko, 1992
INA-12		Nitrogen deregulated	Buswell <i>et al.</i> , 1984
MA 4	UV (ATCC 34541)	Nitrogen deregulated	References in Linko, 1992
FL 21		Nitrogen deregulated	References in Linko, 1992
PSBL-1	Mutant	Lysine auxotroph LiP production during primary metabolism	Tien and Myer, 1990 Orth <i>et al.</i> , 1991
		Nitrogen deregulated	Boominathan <i>et al.</i> , 1990

Subsequent mutation-selection using *P. chrysosporium* has resulted in a number of mutants and progeny with altered properties (Table 1.7). These include nitrogen deregulated mutants showing hypersecretion of peroxidase enzymes under non-lignolytic conditions (Boominathan *et al.*, 1990); auxotrophic mutants also producing high ligninase under non-lignolytic conditions (Orth *et al.*, 1991, Tien and Myer, 1990); pleiotropic mutants deficient in phenol oxidase activity and thus intracellular cAMP levels, VA, EPS and sporulation (Ander and Eriksson, 1976, Gold *et al.*, 1982); cellulase negative mutants with high lignolytic activity (Johnsrudd and Eriksson, 1985); and the mutant SC26 showing an altered phenotype found to be better suited to immobilisation (Kirk *et al.*, 1986b). Of these mutants, INA-12 has been the most productive and frequently reported (Buswell *et al.*, 1984, Capdevila *et al.*, 1989, Bonnarne and Jeffries, 1990, Asther *et al.*, 1988a, 1988b).

The number of functions affected by mutagenesis suggests that the lignolytic system is closely integrated with other functions such as carbohydrate metabolism (Broda *et al.*, 1989) and that multiple alleles contribute to the complexity of this system. Heritable variation in filamentous fungi has been linked to mating type, fruiting and growth rate, for example (Gay *et al.*, 1993, Simchen, 1966a, 1966b, Croft and Simchen, 1965).

A rational approach to strain improvement in *P. chrysosporium* was first proposed by Wyatt and Broda (1995). These authors suggest that through a breeding programme, improved lignin degradation could be achieved while maintaining the inherent robustness of wildtype strains. In the event of genetic crossover during sexual reproduction in a dikaryon (ME446), the haploid strains (sibling strains) produced may differ in their genetic organisation, affecting peroxidase production either directly or indirectly. Wyatt and Broda (1995) showed that strain performance was improved in several ME446 progeny and in certain heterozygous crosses of those haploid progeny.

The rationale behind the use of sibling strains in strain improvement extends towards the identification and linkage of performance with heritable traits. The linking of performance to multiple genes or alleles at a particular locus would enable further strain improvement through genetic engineering approaches, where multiple genes under the control of a single promotor could be simultaneously introduced into a suitable expression system (Wyatt and Broda, 1995).

Initial experiments by Raeder and Broda (1986) involved the propagation of haploid genome equivalents of *P. chrysosporium* ME446, a dikaryon, by stimulating fruiting in ME446. Of the 53 monokaryons generated, 26 strains were then used for RFLP analysis and genetic linkage studies (Raeder *et al.*, 1989a and 1989b). Wyatt and Broda (1995) found no correlation between biological activity (lignolytic enzyme activities) and performance (lignin degradation). Synthetic lignin (DHP) degradation could not be attributed to any specific allele or loci (Wyatt and Broda, 1995). Similarly, earlier intraspecific comparison of wildtype isolates by Kirk *et al.* (1986a, 1986b) found no correlation between LiP activity and ¹⁴C-labelled synthetic lignin (DHP) degradation.

This thesis continues with the research of Wyatt and Broda (1995), using *P. chrysosporium*. In this work, the wildtype strain *P. chrysosporium* ME446; eight monokaryons (strains BS 2, BS 3, BS 13, BS 17, BS 18, BS 24, BS 26 and BS 32) and a single genetic crossover (dikaryotic strain BS 2.52) were selected from the UMIST culture collection (further referred to as sibling strains). Each of these strains showed different degrees of LiP activity levels (Raeder *et al.*, 1989b) and DHP-mineralisation (Wyatt and Broda, 1995).

1.5. RESEARCH HYPOTHESES

The exact role of peroxidase isoenzymes in lignin or pollutant degradation is still unknown. The amount, ratio and nature of isoenzymes transcribed by *P. chrysosporium* are influenced by both strain and the growth conditions. Similarly, the degradation of lignin, lignin models and aromatic pollutants also differs according to the strain and growth conditions examined.

Differences in enzyme production or activity and the pollutant or lignin degradation capabilities of different *P. chrysosporium* strains may not only be related to differences in growth rates, but also to the ability of individual strains to adapt to their environment. Growth kinetics and morphological characteristics of *P. chrysosporium* are seen to differ, depending on whether the fungus is grown as stationary or agitated cultures, solid surface fermentations, or immobilised on an artificial surface.

Therefore, using the rationale behind Wyatt and Broda (1995) strain improvement approach we hypothesised that:

HYPOTHESIS 1: Under controlled growth conditions the amount, ratio or nature of peroxidases produced may be correlated with the biodegradation capabilities of sibling strains.

HYPOTHESIS 2: Intraspecific differences in peroxidase production and biodegradation capabilities of sibling strains may be linked to differences in growth rate and differentiation in these organisms.

In order to test each of the above hypotheses a stable experimental system was required for the discrimination of phenotypic differences between the sibling strains examined.

1.6. OBJECTIVES

The research objectives of this thesis were therefore to:

1. Evaluate the reproducibility of traditional stationary flask culture techniques in the comparison of inherent differences between sibling strains;
2. Evaluate the feasibility of scaling down stationary liquid culture methods to 96-well microtitre plates, which allows for the analysis of larger sample sizes and thus increased power of detection;
3. Further examine the *in vitro* pollutant sensitivities and degradation capabilities of sibling strains;
4. Evaluate the potential of the gradostat membrane bioreactor as a tool for detecting morphological differences in sibling strains;
5. Correlate the observed differences between sibling strains with their degradation capabilities; and
6. Critically discriminate between sibling strains, selecting individuals showing significantly different characterisation for further physiological and genetic linkage analysis.

CHAPTER 2

Evaluation of stationary liquid culture techniques for the assessment of physiological differences between *P. chrysosporium* strains

2.1. INTRODUCTION

P. chrysosporium growth has primarily been evaluated in liquid culture systems despite this being an unnatural growth environment for a saprophytic fungus. Basidiomycetes preferentially form biofilms on porous, solid substrates such as woody tissue and soil. Culturing white rot fungi, including *P. chrysosporium*, in liquid culture systems is notoriously inconsistent. This is attributed to sensitivity to small differences in inoculum size, type and age, sensitivity of mycelial growth to agitation, and differences in oxygen and nutrient mass transfer rates (Baldrian and Gabriel, 2002, Vinci and Byng, 1999, Linko, 1992, Leisola *et al.*, 1987). The intrinsic variation observed in the behaviour of *P. chrysosporium*, using traditional methods to discriminate between cultures, renders the power of detection of small differences unacceptable due to the high standard deviations of replicate samples (Raeder *et al.*, 1989b). Standardisation of methods is an important process in decreasing variability due to experimental error. Increasing the sample size within a given experiment usually accommodates this inconsistency. However, scaling up an experiment can become logistically difficult when resources and time constraints become a limiting factor in the comparison of a large number of organisms.

This chapter serves two functions: firstly in assessing the growth kinetics of ten *P. chrysosporium* strains and secondly in evaluating the potential of the 96-well microtitre plate method as a means of realistically accommodating the comparison of a large number of organisms simultaneously under the same conditions.

2.1.1. Growth kinetics and liquid culture

Growth in multicellular organisms such as filamentous fungi cannot be assumed to be exponential as in unicellular organisms growing by division, as growth occurs only at hyphal tips and where branching and fragmentation of hyphae create new tips. Furthermore, in biofilms or pellet growth no constant specific growth rate can be assumed due to differential substrate and oxygen diffusion constraints (Lejeune and Baron, 1998). Exponential growth does however occur under a number of experimental conditions and can be assumed if unrestricted growth occurs in the absence of

fragmentation and mass transfer limitation, as with unrestricted growth of filamentous fungi on agar surface cultures (Steele and Trinci, 1975) or pellicle formation on the surface of shallow stationary cultures, where a single hyphal growth unit can be defined (Prosser, 1995).

Growth in batch culture is limited by the amount and availability of nutrients and continues until the exhaustion of nutrients or the development of unfavourable conditions restricts growth. This growth is typically divided into three growth phases (Prosser, 1995):

1. Lag phase: a period allowing for spore germination and physiological adaptation of the organism to its environment
2. Acceleration phase: which in filamentous organisms results through the exponential production of hyphal branches, extending at a linear rate. The increase in biomass concentration (x) over time (t) is described as the specific growth rate of an organism (μ) for those conditions under which it was examined (equation 1).

$$\frac{dx}{dt} = \mu x \quad \dots\dots\dots (1)$$

Decrease of the specific growth rate (μ) occurs as the conditions become unfavourable due to nutrient or oxygen limitation, altered medium pH or the accumulation of inhibitory compounds in the growth medium.

3. Stationary phase: occurs as biomass is maintained at relative constancy where mycelial growth and autolysis are in equilibrium. The amount of biomass formed at the onset of stationary phase (x_f), a function of the substrate utilisation rate (q), is described by the yield coefficient (Y) and is assumed to be constant for a given medium. Y can be computed from equation 2.

$$Y = \frac{\mu}{q} \quad \dots\dots\dots (2)$$

2.1.2. Growth kinetics of *P. chrysosporium*

Initial laboratory-scale characterisation of *P. chrysosporium* ME446 growth in liquid culture was undertaken as early as 1978 (Keyser *et al.*, 1978). These authors describe a reproducible sequence of metabolic events following inoculation of stationary batch cultures with a spore suspension. From 0-24 hrs, spore germination occurred followed by linear growth and nutrient nitrogen depletion; from 24-48 hrs, linear growth ceased (after 30 hrs) and ammonium permease activity was de-repressed, demonstrating nitrogen starvation; lignolytic activity was only observed 72-96 hrs post-inoculation (Keyser *et al.*, 1978). Similarly Tien and Kirk (1988) report that mycelial growth stops by day 2 (48 hrs) and lignolytic activity starts on day 4 (96 hrs); maximum activities recorded on days 5 (120 hrs) and 6 (144 hrs).

P. chrysosporium produces both MnP and LiP under stationary batch culture conditions. MnP activity precedes LiP activity by approximately two days (Tien and Kirk, 1988, Keyser *et al.*, 1978). The onset of enzyme activity is regulated by a complex interplay of nitrogen-, carbon- or sulphur-limitation in addition to medium composition including the nature of carbon and nitrogen sources, trace element concentrations, the presence of additives, physiologically produced secondary metabolites and oxygenation conditions (section 1.3.3). The time profiles and concentrations of peroxidase enzymes also differ according to the *P. chrysosporium* strain examined and the type and age of the inoculums used.

2.1.3. Intraspecific differences in *P. chrysosporium* growth

Intraspecific comparison of *P. chrysosporium* was initially reserved for wildtype strains including BKMF-1767 and ME446. Under nitrogen-limiting conditions, these organisms were shown to have similar growth rates, while differing in the extent of EPS production and ligninase activities (Kirk *et al.*, 1986a). *P. chrysosporium* BKMF-1767 and ME446 are reported to differ from one another in both the time of onset and maximum levels of lignolytic activity recorded under shallow stationary flask conditions. Although BKMF-1767 and ME446 show similarities in peroxidase isoenzyme profiles generated, BKMF-1767 lignolytic activity was shown to peak one and a half days before ME446 at approximately nine times higher than was recorded for ME446 when grown as shallow stationary cultures (Kirk *et al.*, 1986a, Faison and Kirk, 1985).

Strain improvement approaches utilising mutants or progeny of wildtype strains, have also generated intraspecific comparisons in the lignolytic activities of *P. chrysosporium* strains (Section 1.4). In the screening of 53 haploid progeny of *P. chrysosporium* ME446 for genetic segregation analysis, intraspecific differences were detected in the LiP activity (Raeder *et al.*, 1989b), MnP activity (Wyatt and Broda, 1995) and lignin biodegradation capabilities (Wyatt and Broda, 1995) of these organisms. Using stationary flask culture, these authors identify an intrinsic variability between replicates as high as 65 % for DHP-degradation, their assessment of intraspecific variability, based on sample means, was therefore qualitative and not quantitative (Wyatt and Broda, 1995).

A selection of ten different isolates with different levels of LiP activities (Raeder *et al.*, 1989b) and lignin degradation capabilities (Wyatt and Broda, 1995) were selected for subsequent analysis (Table 2.1). This chapter describes the evaluation of liquid culture methods with the view of identifying a more robust system with which quantitative assessment of intraspecific variability is made possible.

Table 2.1: *P. chrysosporium* strains evaluated in this project. Phenotype reported as identified by Raeder *et al.* (1989b) and Wyatt and Broda (1995).

Strain	Phenotype: LiP activity Raeder <i>et al.</i> (1989b)		DHP-degradation Wyatt and Broda (1995)	
	Rank Order	*Activity	Rank Order	*Activity
ME446			6	H
BS 2.52				
BS 2	3	H	11	M
BS 3	47	L	25	L
BS 13	1	H	12	M
BS 17	52	L	9	M
BS 18	29	M	1	H
BS 24	42	L	2	H
BS 26	2	H	3	H
BS 32	6	H	27	L

*Categorically described according to high (H), medium (M) or low (L) characteristics

2.1.4. Aims

P. chrysosporium strains grown under control, nitrogen-limiting stationary liquid culture conditions, were classified according to differences observed in their growth kinetics and extracellular LiP and MnP activities. A 96-well microtitre plate method was evaluated as an alternative to the conventional stationary flask culture method for the detection of significant differences between *P. chrysosporium* strains.

2.2. MATERIALS & METHODS

2.2.1. Organisms

For all experiments, ten genetically related *P. chrysosporium* strains were used (Table 2.1). Stock cultures of each strain were maintained on spore and maintenance agar slants at 4 °C (Appendix I:A-1). Spore inoculums were prepared as described in Appendix I:B. Spore suspensions were stored at 4 °C and a standard concentration of approximately 1×10^5 spores was routinely used per ml culture fluid.

2.2.2. Culture conditions

2.2.2.1. Stationary culture

Experiments involving growth of *P. chrysosporium* strains in liquid culture, under shallow stationary conditions, were undertaken in rubber stoppered 125 ml Erlenmeyer flasks containing a final volume of 10 ml basal III medium [Appendix I:A-2]. The air space above the shallow cultures was flushed with pure oxygen at a flow rate of 1 l.hr^{-1} at the time of inoculation, on day 3 and every second day thereafter, facilitated by injection ports situated in the rubber stoppers. Stationary cultures were inoculated with 1×10^6 spores [Appendix I:B] and incubated at 37°C for a period of ten days. In a time course study, inoculated flasks were removed at 24 hr intervals, filtered through Whatman GF/C filter paper, the supernatants combined and analysed in triplicate ($n = 3$).

2.2.2.2. Microtitre plate cultures

Growth curves using 96-well, sterile microtitre plates (Greiner) were introduced as a means of logistically enabling the comparison of multiple strains. 96-well microtitre cultures were undertaken using a final volume of 300 µl basal III medium [Appendix I:A-2] per well. Each well was inoculated with 2.5×10^4 spores [Appendix I:B] giving a final concentration of 1×10^5 spores per ml. Plates were incubated at 37 °C for a period of six days (longer time course was hampered by evaporation even with plates sealed by parafilm). 96-well microtitre plates have a 12 x 8 well configuration, where the twelve columns comprised eight wells each. A single, eight-well containing column was sampled for subsequent analysis, from each 96-well microtitre plate, at 12 hr intervals. Within a given column, two wells served as uninoculated controls (absorbance blanks) and six inoculated wells ($n = 6$) from which growth kinetics and enzyme activities were quantified.

2.2.3. Growth measurements

2.2.3.1. Growth rate

Dry weight

Growth curves for flask cultures were obtained by measuring the accumulation of dry mycelial weight. Culture fluid from flasks was filtered through pre-weighed glass filter paper disks using a vacuum pump. Filtrate was retained for further analysis. Biomass was rinsed with distilled water and dried at 100 °C overnight. Disks were re-weighed and biomass dry weight recorded. For each *P. chrysosporium* strain, a time course profile of biomass accumulation was generated from triplicate samples and μ (mg dry weight per day) was calculated from the linear slope of each graph [$R^2 > 0.95$].

Absorbance at 630 nm

Microtitre plate growth curves were generated by measuring the absorbance of biomass at 630 nm using a microplate reader (BioTek Powerwave) as described by Langvad (1999). Absorbance measurements were taken at 12 hr intervals. For each strain, a time course profile reflecting the mean OD_{630 nm} of unsampled wells was generated. Assuming a standard of 4.2 mg.ml⁻¹ dry weight per absorbance unit for filamentous fungi (Langvad, 1999) the slope of each curve [$R^2 > 0.95$] was used to calculate μ (in mg dry weight per day). Maximum biomass (x_t) formed at stationary phase growth was also calculated from absorbance readings.

2.2.3.2. Substrate utilisation

Substrate concentration of extracellular medium was assayed at 24 hr or 12 hr intervals for stationary flask or 96-well microtitre cultures, respectively. NH₄⁺ concentration was determined using a modified version of the Nessler method (Spectroquant, Merck) [Appendix I:C-1] while glucose concentration was determined using the Nelson-Somogyi method [Appendix I:C-2]. The linear slope of glucose uptake was used to calculate q [mg.day⁻¹]. From equation 2 (section 2.1.1), q and μ were subsequently used to calculate the biomass yield per substrate converted (Y) in mg.mg⁻¹, for each *P. chrysosporium* strain.

2.2.3.3. Enzyme activities

LiP and MnP activities in extracellular culture fluid were quantified using the VA oxidation method (Appendix I:D-1) and ABTS (Appendix I:D-2.1) or MBTH/DMAB (Appendix I:D-2.2) methods, respectively. Enzyme activities are expressed as U.l⁻¹ (μ M.min⁻¹.l⁻¹).

Initial stationary flask culture experiments were assayed using the ABTS oxidation method for MnP detection. However, during the course of this work *P. chrysosporium* was reported to produce the enzyme laccase, for which ABTS is also a substrate. Thus, in all further experiments MnP detection was subsequently quantified according to the MnP specific MBTH/DMAH method. While laccase is not produced under nitrogen-limiting conditions in flask culture, the impact of pollutants and immobilisation on its production were uncertain.

2.2.3.4. Statistics

Sample sizes for stationary culture and 96-well microtitre plates were $n = 3$ and $n = 6$, respectively. Statistical comparison of data was achieved by one-way ANOVA followed by the Bonferroni multiple comparisons post-test. Correlation between measurements was determined using two-tailed Pearson's correlation. For all statistical measurements, a confidence level of $P < 0.05$ was chosen as significant. Statistical analyses were performed using **GraphPad Prism**[®] version 3.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Cluster analysis of strains according to their growth measurements or peroxidase profiles was undertaken using **STATISTICA** (data analysis software system), version 6. StatSoft, Inc. (2002). www.statsoft.com.

RESULTS

2.3.1. Stationary culture

2.3.1.1. Growth rate

10 ml Stationary flask cultures were incubated for a period of ten days with daily sampling. From the growth curves generated for *P. chrysosporium* strains (Figure 2.1), the duration of lag phase, μ and the onset of stationary phase were seen to differ (Table 2.2). Strain BS 26 and BS 24 displayed a shorter lag period while the lag phase of strains BS 13 and BS 2.52 were the longest. Only the μ calculated for *P. chrysosporium* BS 2 was determined to be significantly greater than strains BS 24, ME446, BS 2.52 and BS 32 [$P < 0.05$]. The rank order for μ of each strain is reported in Table 2.2.

2.3.1.2. Substrate utilisation

The basal III medium (Appendix I:A-2) supporting mycelial growth in all *P. chrysosporium* cultures was a nitrogen-limiting, chemically defined medium. In each case, the NH_4^+ concentration of the extracellular culture fluid was rapidly depleted by the second day of incubation (Figure 2.2.a). In contrast, glucose in the extracellular medium was metabolised linearly over the ten-day sample period and depleted only by *P. chrysosporium* ME446 (Figure 2.2.a). Linear regression of glucose consumption [$R^2 > 0.95$] and the subsequent calculation of q showed differences in substrate utilisation by each strain (Table 2.2). Only *P. chrysosporium* ME446 showed a significantly higher q than all other strains examined [$P < 0.001$]. In addition to *P. chrysosporium* ME446, both strains BS 32 and BS 3 showed significantly higher q values than strains BS 17 [$P < 0.05$], BS 24 [$P < 0.01$, $P < 0.05$], BS 13 [$P < 0.001$, $P < 0.01$] and BS 26 [$P < 0.001$]. The rank order of *P. chrysosporium* strains with respect to q is reported in Table 2.2.

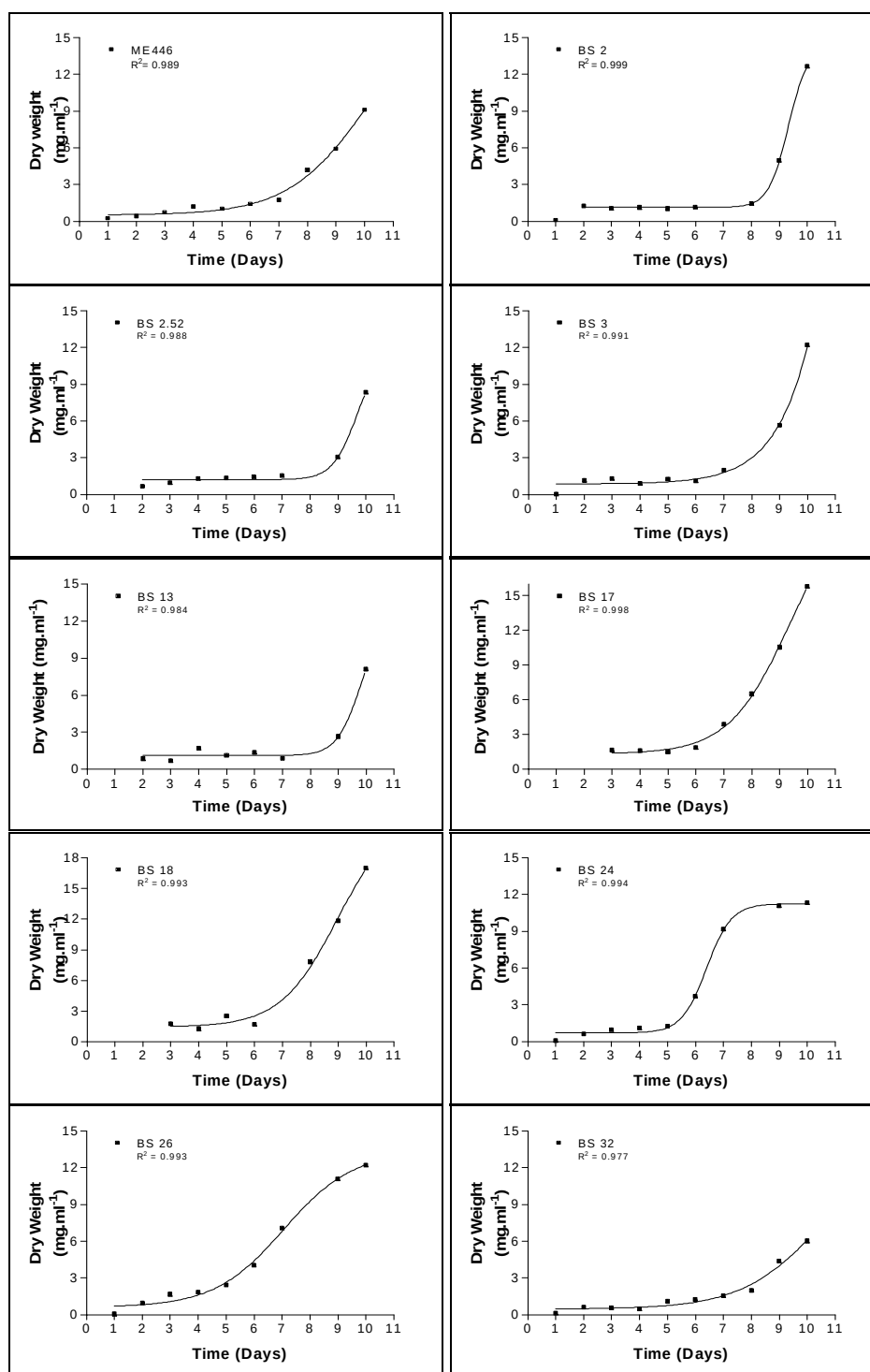


Figure 2.1 Time course of mycelial dry weight accumulation for each of the *P. chrysosporium* strains grown as 10 ml stationary flask cultures, showing sigmoidal, variable slope growth curves [$R^2 > 0.95$]. Data points represent the means $\pm$ SE of three replicates.

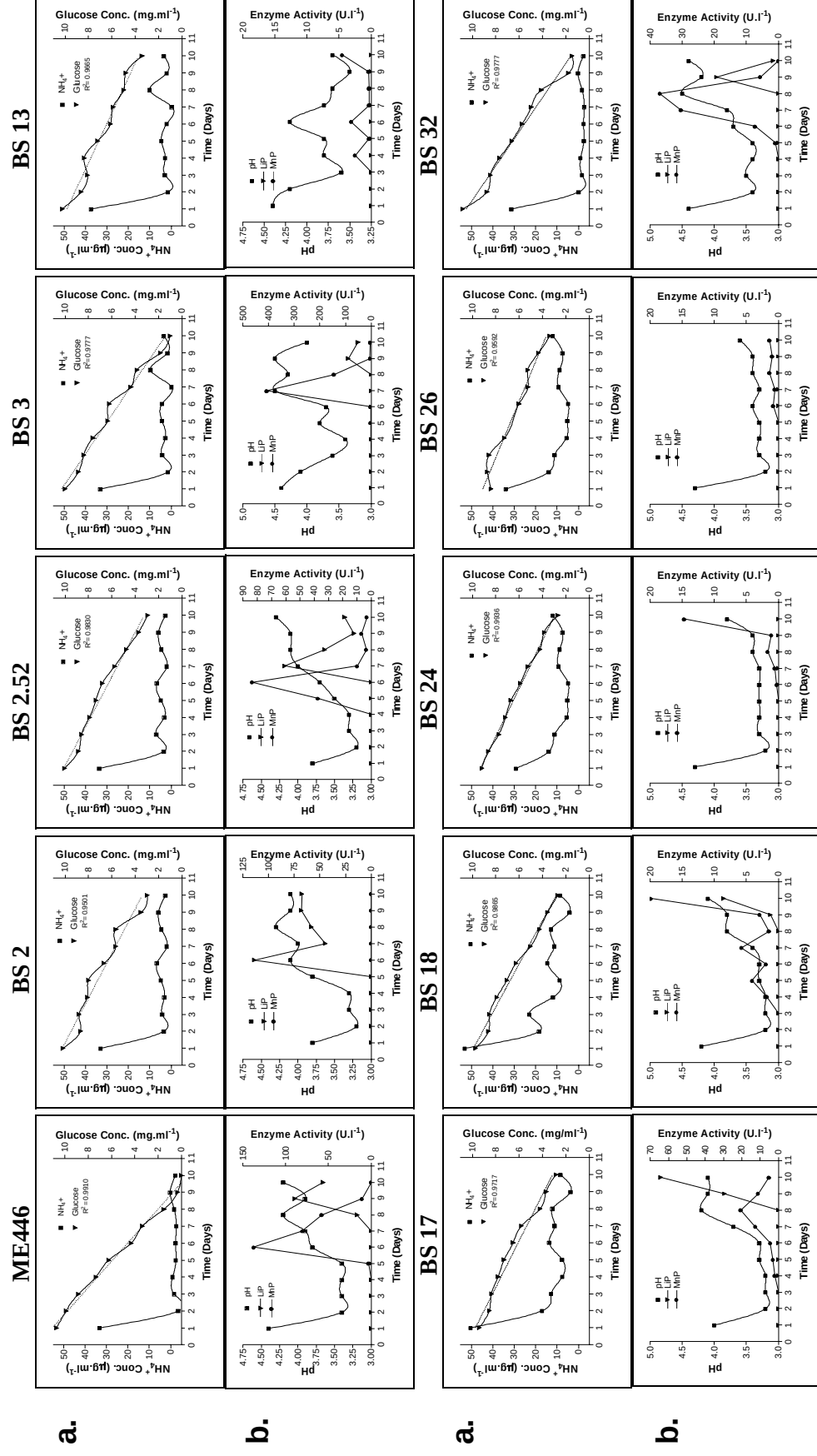


Figure 2.2: 10 ml Stationary flask cultures: (a.) showing substrate utilisation profiles for NH₄⁺ and glucose, (b.) time course of medium pH and extracellular LiP and MnP activities for the ten *P. chrysosporium* strains examined. Linear regression [$R^2 > 0.95$] of glucose consumption (dashed line) depicted. NH₄⁺ concentration in μg.ml⁻¹. Glucose concentration in mg.ml⁻¹. LiP and MnP activities in U.l⁻¹. In each case, data points represent the means ± SD of three replicates.

Table 2.2: Rank order for growth kinetics of *P. chrysosporium* strains grown as 10 ml stationary flask cultures. Highlighted cells indicate those strains showing significantly higher or lower growth kinetics than other strains.

Rank Order	Lag Phase (hrs)	Growth Rate [μ] (mg.day ⁻¹)	Rate of Glucose Utilisation [q] (mg.day ⁻¹)	Biomass Yield [Y] (mg dry weight. mg glucose ⁻¹)
1	BS 26 0-120	BS 2 5.60 ± 1.20	ME 446 1.298 ± 0.044	BS 2 7.29 ± 1.40
2	BS 24 0-120	BS 17 4.66 ± 0.35	BS 32 0.992 ± 0.053	BS 17 6.37 ± 0.14
3	BS 18 0-144	BS 18 4.59 ± 0.34	BS 3 0.983 ± 0.053	BS 18 5.83 ± 0.28
4	BS 17 0-144	BS 3 3.18 ± 1.17	BS 18 0.786 ± 0.032	BS 26 4.73 ± 1.96
5	ME446 0-168	BS 26 2.91 ± 1.06	BS 2.52 0.765 ± 0.036	BS 13 3.80 ± 2.98
6	BS 3 0-168	BS 13 2.67 ± 1.60	BS 2 0.760 ± 0.062	BS 24 3.40 ± 1.08
7	BS 2 0-192	BS 24 2.54 ± 1.21	BS 17 0.731 ± 0.044	BS 3 3.18 ± 1.43
8	BS 32 0-192	ME 446 2.38 ± 0.19	BS 24 0.704 ± 0.199	BS 2.52 2.62 ± 1.91
9	BS 13 0-216	BS 2.52 2.05 ± 1.13	BS 13 0.679 ± 0.045	BS 32 2.04 ± 0.16
10	BS 2.52 0-216	BS 32 2.03 ± 0.22	BS 26 0.601 ± 0.047	ME 446 1.83 ± 0.12

The yield coefficients (Y) of *P. chrysosporium* strains did not vary significantly from one another [$P > 0.05$], with the exception of *P. chrysosporium* BS 2 which showed significantly higher Y than BS 2.52 [$P < 0.05$], BS 32 [$P < 0.05$] and ME446 [$P < 0.01$]. Strain BS 2 can be said to use its resources more efficiently for biomass production, while biomass production in strains ME446, BS 32 and BS 2.52 appears to be more energy intensive. Table 2.2 lists the rank order for Y. There was no correlation between the extent of the lag phase observed for each culture and μ , q or Y values [$P > 0.05$] (Table 2.2).

2.3.1.3. Enzyme activity profiles

Uninoculated medium was buffered by a dimethyl succinate buffer at pH 4.2. In inoculated flasks, medium pH was routinely reduced by approximately 1 pH unit two days post-inoculation (Figure 2.2.b). The medium pH was maintained at pH 3.2-3.5 for an undefined period, its recovery to pH 4.0-4.5 coinciding with the detection of extracellular peroxidase activity. In those cultures where peroxidase activity was not detected the medium pH did not show this characteristic pH response during the ten-day sample period (Figure 2.2.b).

Differences in onset and extent of MnP and LiP activities were observed in stationary flask cultures (Figure 2.2.b, Table 2.3). MnP activity was detected in extracellular culture fluid three to five days before LiP activity was observed. Typically a single peak in MnP activity was detected on days 6-10 (144-240 hrs), however a second peak or prolonged MnP activity was detected in strains BS 2, BS 2.52, BS 13 and BS 18 (Figure 2.2.b). Peak MnP activities of *P. chrysosporium* strains ranged from 1.4 U.l⁻¹ (BS 26) to 407.7 U.l⁻¹ (BS 3), while the parent strain *P. chrysosporium* ME446 produced a maximum MnP activity of 137.6 U.l⁻¹, the second highest activity recorded amongst the ten strains examined (Table 2.3). Onset of MnP activity does not correlate with enzyme production (Table 2.3).

LiP activity was not detected in strains BS 24, BS 26 and BS 13 during the time course examined. Activity typically peaked on days 9-10 (216-240 hrs), suggesting a delay in onset of LiP in these strains beyond the ten-day period examined (Figure 2.2.b). Peak LiP activities of the different strains ranged from 8.6 U.l⁻¹ (BS 18) to 114.0 U.l⁻¹ (BS 2), with the parent strain, *P. chrysosporium* ME446, producing a maximum LiP activity of 89.4 U.l⁻¹, the third highest amongst the ten strains examined (Table 2.3).

Table 2.3: Maximum manganese peroxidase and lignin peroxidase activities, and time of onset of these activities, as detected in stationary flask cultures. Time in days at which peak activities were detected are recorded in brackets. Highlighted sections indicate *P. chrysosporium* strains showing significantly higher or lower ligninase activities or delayed onset times than other strains.

Rank Order	MnP activity (U.l ⁻¹)	MnP Onset (hrs)	LiP activity (U.l ⁻¹)	LiP onset (hrs)
1	BS 3 407.7 (168)	BS 2 48	BS 2 114.0 (144)	BS 2 144
2	ME 446 137.6 (144)	BS 18 96	BS 3 89.5 (216)	BS 2.52 168
3	BS 2.52 83.6 (144)	BS 17 96	ME 446 89.4 (216)	ME 446 192
4	BS 32 37.0 (192)	BS 13 96	BS 17 64.5 (240)	BS 3 216
5	BS 17 20.6 (192)	BS 3 120	BS 2.52 60.7 (168)	BS 32 216
6	BS 18 19.9 (240)	ME 446 120	BS 32 19.3 (216)	BS 17 216
7	BS 24 14.7 (240)	BS 2.52 120	BS 18 8.6 (240)	BS 18 216
8	BS 2 11.4 (144)	BS 24 144	BS 24 -	BS 24 -
9	BS 13 4.5 (216)	BS 26 144	BS 26 -	BS 26 -
10	BS 26 1.4 (240)	BS 32 192	BS 13 -	BS 13 -

2.3.2. Microtitre plate culture

2.3.2.1. Growth rate

96-well microtitre plate cultures were incubated for a period of six days, sampled at 12 hr intervals. From the growth curves generated for each *P. chrysosporium* strain examined (Figure 2.3) a more complete growth curve was generated in microtitre plates than was observed for ten-day stationary flask cultures. The lag phase of microtitre plate cultures was less pronounced and an extended stationary growth phase was observed, with several *P. chrysosporium* strains displaying diauxic growth 72-96 hrs post-inoculation. *P. chrysosporium* strains ME446, BS 3, and BS 26 all showed significantly higher μ than BS 32 [$P < 0.05$] (Table 2.4). ME446, BS 18 and BS 13 were shown to accumulate high maximum levels of biomass at stationary phase (x_f), while BS 32 was observed to produce far lower biomass levels [$P < 0.001$].

Time-course in microtitre plate cultures was limited by evaporation. The outer wells of 96-well microtitre plates were most noticeably affected by evaporation. These wells were excluded from sampling towards the end of a time-course study. Oxygen permeable parafilm seal, delayed, but did not prevent evaporation at the high incubation temperatures under which *P. chrysosporium* was cultured.

2.3.2.2. Substrate utilisation

As observed in stationary flask cultures, the NH_4^+ concentration of the nitrogen-limiting culture medium was rapidly depleted after 36-48 hrs incubation in microtitre plate cultures (Figure 2.3.a). The glucose concentration of extracellular medium was more variable than that of the flask cultures, due to evaporation of medium from microtitre wells, yet followed a linear trend as the glucose was utilised for growth (Figure 2.3.a). Nutrient carbon was not depleted within the six day culture period. Table 2.3 describes the rank order and categorical alignment of *P. chrysosporium* strains. Both *P. chrysosporium* BS 2 [$P < 0.001$] and BS 2.52 [$P < 0.05$] showed significantly lower q than all other strains examined except one another [$P > 0.05$]. BS 13 showed significantly higher q than BS 24 [$P < 0.05$], BS 26 [$P < 0.01$] and BS 3 [$P < 0.05$].

Similarly, *P. chrysosporium* BS 2 showed a significantly higher Y value than all other strains examined [$P < 0.001$]. Y for BS 2.52 was also greater than BS 18 [$P < 0.05$], BS 17 [$P < 0.01$], BS 13 [$P < 0.01$] and BS 32 [$P < 0.001$]. There appeared to be no correlation between the extent of lag phase in growth, μ , q or Y (Table 2.4)

Table 2.4: Rank order for growth kinetics of *P. chrysosporium* strains cultured in 96-well microtitre plates. Highlighted sections indicate those strains showing significantly higher or lower growth kinetics than other strains.

Rank Order	Lag Phase (hrs)	Growth Rate[μ] (mg.day ⁻¹)	Maximum Stationary Phase Growth [x_f] (mg.ml ⁻¹)	Rate of Glucose Utilisation [q] (mg.day ⁻¹)	Biomass Yield [Y] (mg dry weight per mg glucose)
1	BS 13 0-12	ME446 3.01 ± 0.67	ME446 4.50 ± 0.23	BS 13 1.60 ± 0.18	BS 2 6.26 ± 2.32
2	BS 2.52 0-12	BS 3 2.85 ± 0.44	BS 18 4.05 ± 0.11	BS 17 1.44 ± 0.32	BS 2.52 3.52 ± 0.97
3	ME446 0-24	BS 26 2.62 ± 0.53	BS 13 3.92 ± 0.26	ME446 1.43 ± 0.17	BS 3 2.66 ± 0.45
4	BS 3 0-24	BS 18 2.55 ± 0.90	BS 2.52 3.66 ± 0.14	BS 18 1.37 ± 0.22	BS 26 2.41 ± 0.06
5	BS 26 0-24	BS 24 2.54 ± 0.57	BS 26 3.62 ± 0.0.19	BS 32 1.35 ± 0.27	BS 24 2.26 ± 0.19
6	BS 18 0-24	BS 13 2.19 ± 0.10	BS 24 3.46 ± 0.15	BS 24 1.11 ± 0.19	ME446 2.07 ± 0.32
7	BS 2 0-24	BS 2.52 2.13 ± 0.10	BS 17 3.35 ± 0.10	BS 3 1.11 ± 0.30	BS 18 1.81 ± 0.53
8	BS 17 0-24	BS 17 2.11 ± 0.16	BS 3 3.40 ± 0.39	BS 26 1.08 ± 0.20	BS 17 1.52 ± 0.32
9	BS 24 0-36	BS 2 1.98 ± 0.50	BS 2 3.29 ± 0.13	BS 2.52 0.63 ± 0.15	BS 13 1.38 ± 0.13
10	BS 32 0-36	BS 32 1.51 ± 0.62	BS 32 2.62 ± 0.17	BS 2 0.36 ± 0.17	BS 32 1.07 ± 0.35

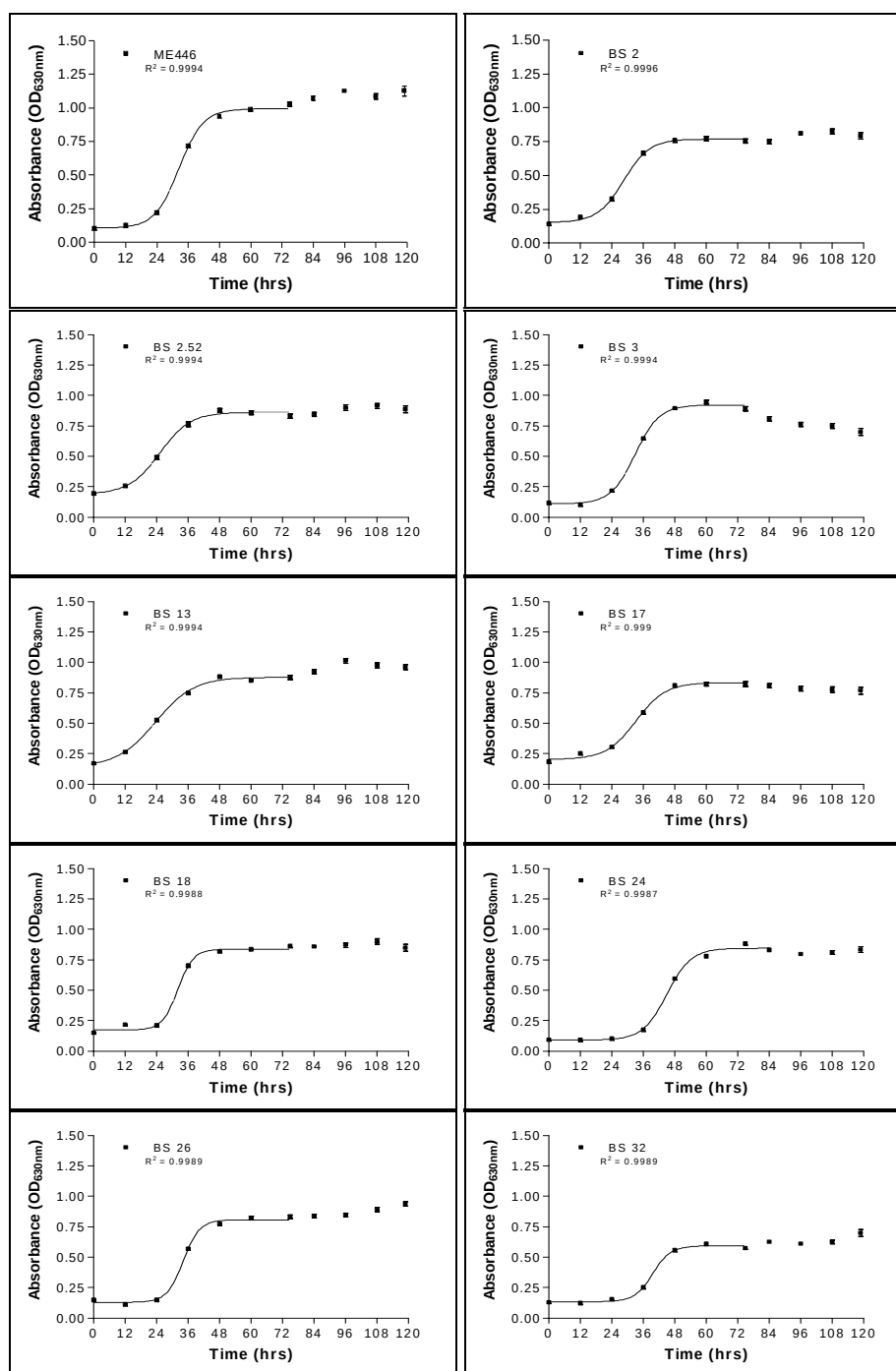


Figure 2.3: Sigmoidal, variable slope growth curves for *P. chrysosporium* strains cultured in 96-well microtitre plates, measured using a microplate reader (OD_{630 nm}) at 12 hr intervals [$R^2 > 0.99$]. Data points represent the means $\pm$ SE of six replicates.

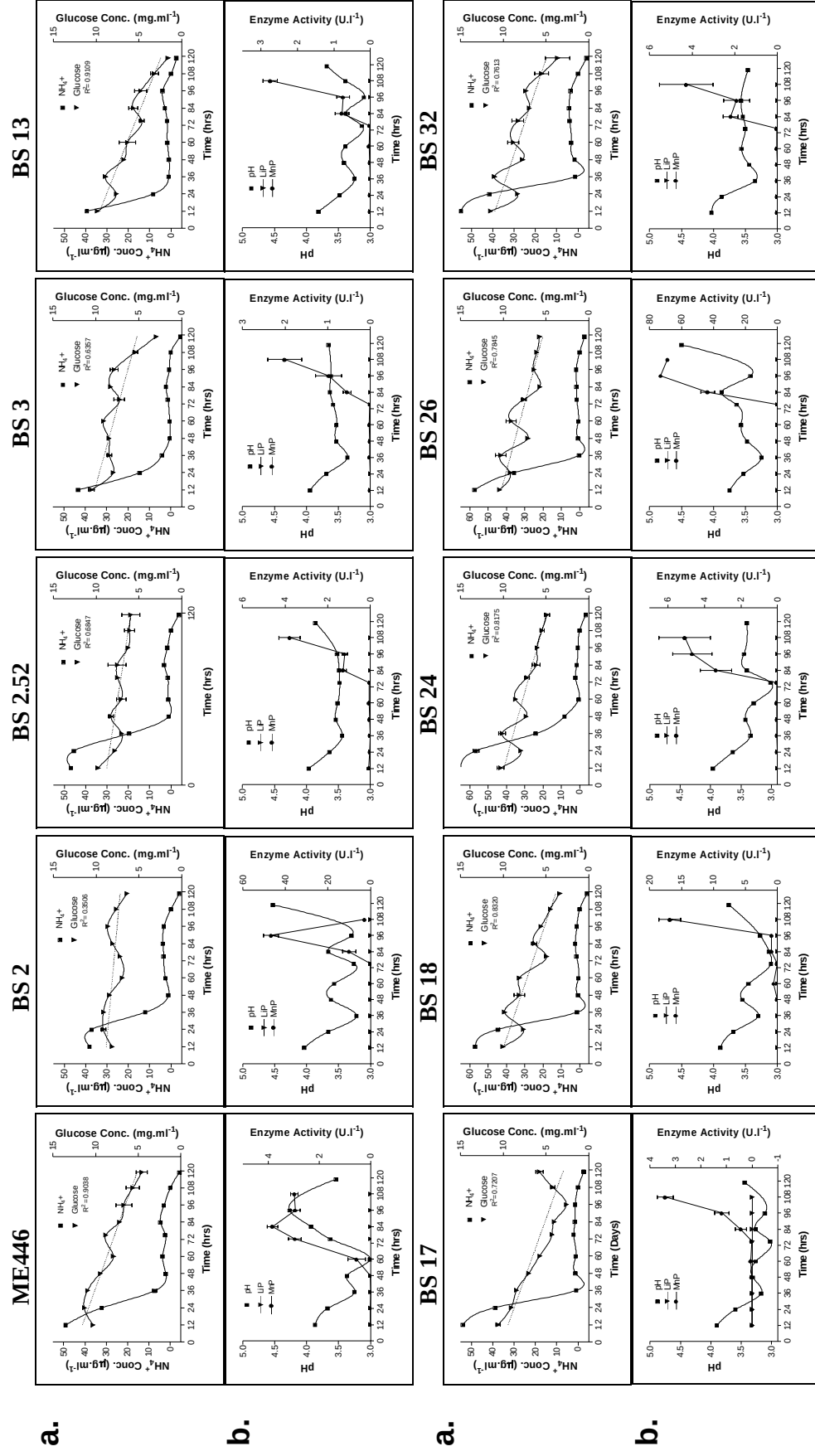


Figure 2.4: 96-well microtitre plate cultures: Showing substrate utilisation profiles (a), time course of medium pH and extracellular LiP and MnP activities (b) for each of the *P. chrysosporium* strains examined. Linear regression [$R^2 > 0.95$] of glucose consumption (dashed line) depicted. NH_4^+ concentration in $\mu\text{g.ml}^{-1}$. Glucose concentration in mg.ml^{-1} . LiP and MnP activities in U.l^{-1} . In each case, data points represent the means $\pm$ SD of six replicates.

2.3.2.3. Enzyme activity profiles

Enzyme activities and pH of the extracellular culture fluid were measured at 12 hr intervals. As in stationary flask cultures, the pH of extracellular medium sampled from microtitre plates decreased by approximately 1 pH value as a result of organic acid production (section 1.3.2.3), falling to pH 3.0-3.5 36 hrs post-inoculation. Recovery of pH to initial values did not correlate with peroxidase activity as well as was observed in stationary flask cultures, with MnP activity frequently detected prior to recovery of pH values to 4.0-4.5 (Figure 2.4.b).

The onset of MnP activity in microtitre plate cultures varied from 60-84 hrs (Table 2.5). Peak MnP activities in extracellular culture fluid occurred at 84-108 hrs post-inoculation. Typically a single peak in MnP activity was detected, however a second peak or prolonged MnP activity was detected in strains ME446, BS 2.52, BS 13, BS 24 and BS 26 (Figure 2.4.b). This was observed to a limited extent in stationary flask culture (Figure 2.2.b).

Table 2.5: Maximum manganese peroxidase and lignin peroxidase activities, and the onset of these activities in 96-well microtitre plate cultures. Time in hrs at which peak activities were detected is recorded in brackets. Highlighted sections indicate *P. chrysosporium* strains showing significantly higher or lower ligninase activities or delayed onset times than other strains.

Rank Order	MnP activity (U.l ⁻¹)	MnP onset (hrs)
1	BS 26 68.72 ± 1.96 (108)	ME 446 60
2	BS 2 46.84 ± 8.72 (96)	BS 18 60
3	BS 18 16.92 ± 4.14 (108)	BS 2 84
4	BS 24 5.07 ± 3.46 (108)	BS 26 84
5	BS 32 4.28 ± 3.07 (108)	BS 24 84
6	ME 446 3.85 ± 0.51 (84)	BS 32 84
7	BS 2.52 3.79 ± 1.22 (108)	BS 2.52 84
8	BS 17 3.41 ± 0.76 (108)	BS 17 84
9	BS 13 2.74 ± 0.47 (108)	BS 13 84
10	BS 3 2.01 ± 0.97 (108)	BS 3 84

Peak MnP activities of *P. chrysosporium* strains ranged from 2.01 U.l⁻¹ (BS 3) to 68.72 U.l⁻¹ (BS 26) while the parent strain, *P. chrysosporium* ME446, produced a maximum MnP activity of 3.85 U.l⁻¹, the sixth highest activity recorded amongst the ten strains examined. LiP activity was not detected in any of the cultures examined (Table 2.5). Only strains BS 26 and BS 2 displayed higher extracellular MnP activity than all other strains [$P < 0.05$]. No correlation between MnP onset and MnP production was apparent in microtitre plate cultures (Table 2.5).

2.3.2.4. Cluster analysis

Cluster analysis of *P. chrysosporium* strains was undertaken based on groups of dependent variables described as either growth characteristics (Table 2.4) or peroxidase related (Table 2.5). The peroxidase grouping included both MnP activities quantified in this work and LiP activities described by Raeder *et al.* (1989b). Joining (tree clustering) diagrams and K-means clustering data are reported as Appendix II.

In each case, grouping of *P. chrysosporium* strains according to their Euclidean distances showed three distinct clusters. The grouping of these clusters appeared to be weighted by variables such as lag phase and MnP onset, which showed fewer differences of greater values than the other variables within a group. Therefore, cluster analysis as a means of identifying variation amongst strains was believed to be less informative than the categorical description of individual variables.

2.4. DISCUSSION

The growth kinetics determined for *P. chrysosporium* strains were shown to follow all trends previously described for this organism. Firstly, a distinct lag period was observed, reflecting a period of spore germination and physiological adaptation. Although this lag period was more pronounced in shallow stationary flask cultures than previously reported, 96-well microtitre plate cultures exhibited a typical 0-24 hr lag (Keyser *et al.*, 1978). The longer lag phase observed in flask cultures appears to be a reflection of a lower sensitivity in recording the growth measurements by dry weight. Glucose and ammonium consumption (Figure 2.2.a) indicate that cultures were growing linearly through this lag period. Secondly, a brief acceleration phase was followed by a period of linear growth from which μ was calculated [$R^2 > 0.95$]. Linear growth was maintained over a period of three to four days in flask cultures or 12-24 hrs in 96-well microtitre plate cultures. The linear growth observed in microtitre plates coincided with that described for 10 ml stationary flask culture (Keyser *et al.*, 1978). Few *P. chrysosporium* strains achieved a stationary growth phase during the ten-day sample period examined in flask cultures. In contrast, 96-well microtitre plate cultures entered stationary growth phase 48-60

hrs post-inoculation, maintaining idiophasic growth up until 120 hrs post-inoculation, thus achieving a more complete growth cycle than was observed for flask cultures over a ten-day period.

Microplate cultures reached idiophasic growth at a far earlier point than was observed for flask cultures. It is possible that the surface area of the microtitre wells imposed an as yet undefined spatial constraint on mycelial extension not apparent until a later point in flask cultures. Alternatively, the indirect measurement of mycelial dry weights using absorbance at 630 nm was not as sensitive to further pellicle differentiation such as layered branching or aerial hyphae formation and EPS production, all factors contributing to dry weight measurements in flask cultures.

Intraspecific comparisons undertaken as early as 1986 showed *P. chrysosporium* BKMF-1767 and ME446 to have similar growth rates when grown on nitrogen-limiting medium (Kirk *et al.*, 1986a). Preliminary assessment of select *P. chrysosporium* strains showed that although some of these strains differ in the extent of the lag phase, time of onset of accelerated and decelerated growth and the maximum amount of biomass formed during stationary growth, little difference was detected in μ , q and Y values. The μ calculated for *P. chrysosporium* strains was similar for both flask and microtitre plate cultured organisms [$P > 0.95$]. Although intraspecific differences in μ were detected within either flask or 96-well microtitre plate cultures [$P < 0.95$], these differences were not consistent between culture methods. These differences may be a reflection of different culture conditions at the level of oxygen and nutrient transfer to the mycelial pellicle. The major concern in comparing microtitre plate cultures with flask cultures is in their differences in culture volume and aeration, factors essential to scale-down models of those growth conditions effecting secondary metabolism. Poor aeration concerns are less relevant in the screening of *P. chrysosporium* strains in stationary liquid culture, where mycelia grow as a pellicle upon the liquid surface and are thus not limited by the same oxygen diffusion rates of submerged cultures. Further, it has been reported that oxygen levels do not affect growth rate in *P. chrysosporium* cultures (Reid and Siefert, 1982). Thus the microtitre plate method does benefit high-throughput screening and the selection of organisms showing more distinct differences in stationary liquid culture. Indeed, this method appears to be more sensitive to detecting variability between *P. chrysosporium* strains than the conventional flask culture technique, particularly high/low MnP producing strains.

For all cultures, nutrient nitrogen was depleted 36-48 hrs post-inoculation, a reflection of the nitrogen-limiting growth conditions under which these organisms were cultured. This corresponds with previous observations on nutrient nitrogen depletion, as recorded by Keyser *et al.* (1978). Depletion of medium glucose levels to below 2 mg.ml⁻¹ was observed in BS 2 and BS 32 and ME446 in flask cultures only. q Calculated for each organism was predominantly consistent between culture methods.

While certain *P. chrysosporium* strains showed differences in the rate at which glucose was catabolised under either culture method [$P < 0.001$], only strain ME446 showed a significantly higher q value than all other strains examined.

Y did not differ significantly between culture methods, with the exception of strains BS 17 and BS 18. Only strain BS 2 showed a significantly different Y value from the other strains examined, irrespective of culture method. *P. chrysosporium* BS 2 was determined to be most efficient at utilising its energy resources for biomass formation, irrespective of the culture method used. In contrast, BS 32 was consistently the least efficient biomass-generating organism. The efficiency of growth observed was however not a reflection of their ability to produce either LiP or MnP. Lower MnP activities were reported in all *P. chrysosporium* strains when cultured using the 96-well microtitre plate method. This may be due to oxygen limitation in cultures grown under atmospheric conditions. In each instance, *P. chrysosporium* strains differed in the extent of either LiP or MnP activity detected in extracellular culture fluid. The rank order of MnP production also varied according to the culture method used. Intraspecific differences were detected in both the onset and duration of these activities in addition to the maximum enzyme activities detected. There appeared to be no correlation between LiP or MnP production and onset of enzyme production, length of lag phase, μ , q or Y [$P > 0.05$].

A notable distinction between the two culture methods was the apparent lack of LiP production in select *P. chrysosporium* strains when cultured in flask culture and in all microtitre plate cultures analysed. Microtitre plate cultures were grown under atmospheric conditions not favouring the production of LiP (Rothschild *et al.*, 1995). The absence of LiP activity in *P. chrysosporium* BS 24 stationary flask culture corresponded with previous reports that this isolate was a poor LiP producer (Raeder *et al.*, 1989b). In contrast strains BS 13 and BS 26 were previously described as high LiP producing isolates (Raeder *et al.*, 1989b). Thus, in oxygenated stationary flask cultures the absence of those LiP activities, previously reported by these authors, may have merely been a result of delayed onset in LiP production.

The relationship between pH and peroxidase activity is apparent in organisms grown in both flask and 96-well microtitre plate cultures. The results show an initial reduction in medium pH coinciding with NH_4^+ depletion. A drop in medium pH during primary growth under nitrogen-limiting conditions has been linked to organic acid production (Kuan and Tien, 1993), where the production of oxalic acid precedes peak MnP activity for which it acts as a mediator (Kuan and Tien, 1993). Peak MnP activity was detected three to five days or 36-72 hrs after NH_4^+ levels were first depleted and pH levels were reduced in flask or 96-well microtitre plate cultures, respectively. In each case this coincided with the depletion of medium glucose down to 5-6 mg.ml⁻¹.

In contrast, peak LiP activity (observed only in select flask cultures) was detected three to five days following the onset of MnP activity, coinciding with a reduction of medium glucose to below a concentration of 2 mg.ml^{-1} . The pH fluctuation previously reported for succinate-buffered cultures was attributed to an increase in pH as succinate was consumed towards the end of the fermentation when glucose was limited (Nagel *et al.*, 1999). The subsequent elevation of this pH by 0.5-1 units is subject to the uptake of glucose from the growth medium beyond $5\text{-}6 \text{ mg.ml}^{-1}$, suggesting that glucose limitation and succinate catabolism begins when nutrient carbon levels are depleted below a concentration of $5\text{-}6 \text{ mg.ml}^{-1}$. This is consistent with diauxic growth observed in microplate cultures 72-96 hrs post-inoculation (Doshi *et al.*, 1997). An increase in medium pH coincides with the cessation of mycelial biomass increase, a reduction of mitochondrial content and a decrease in intracellular pH at the onset of LiP activity (Leštan *et al.*, 1994, Bonnarme *et al.*, 1991). In microtitre plate cultures and those flask cultures in which LiP activity was not recorded, pH levels were not elevated during the time course examined and glucose levels remained above the 2 mg.ml^{-1} . Since this was consistent for all strains and all culture conditions examined, a threshold glucose value is proposed as a mechanism regulating the onset of LiP activity, mediated by cAMP signal transduction pathways (section 1.3.2.6). Extension of the time course for 96-well microtitre plate cultures beyond 120 hrs under conditions of elevated oxygen would inevitably allow for the detection of extracellular LiP activity.

Comparison of the *P. chrysosporium* strains examined in this chapter to previously published ligninase productivities (Raeder *et al.*, 1989b) was hampered by the inability to detect LiP in the cultures examined. This could be a reflection of their growth conditions and not their phenotype. However, classification of the *P. chrysosporium* strains according to MnP activity in 96-well microtitre plate cultures showed strains BS 26, BS 2 and BS 18 to have the highest MnP activities, and strains BS 3, BS 13, and BS 17 to have the lowest MnP activities. This compares favourably with the rank order for LiP production, described by Raeder *et al.*, 1989b (Table 2.1).

Categorical analysis (Appendix III, Table I) of *P. chrysosporium* strains appeared to be more useful than cluster analysis techniques. These results are described in Chapter 8 and form the basis of selecting distinctly different *P. chrysosporium* strains for future proteomic and genomic research. The identification of multivariate traits within genetically related strains, particularly *P. chrysosporium* strains, whose genome has been fully sequenced, enhances the probability of linking altered phenotypic traits with specific genes, alleles or chromosomes.

2.5. CONCLUSIONS

In evaluating the intraspecific differences observed between *P. chrysosporium* strains in liquid culture, the method used for this analysis was shown to be crucial. Growth kinetics generated using the microtitre plate technique were more consistent than those observed for 10 ml stationary flask cultures, thus the resolving power of this method was enhanced. Using the microtitre plate method, little intraspecific variation was detected in μ , q and Y with the exception of *P. chrysosporium* BS 2, which showed a significantly greater Y , suggesting improved metabolic efficiency in this strain.

Microtitre plate method derived intraspecific variation in the onset of MnP activity was attributed to medium glucose levels and not to differences in growth rates, which were similar. Variation detected in the maximum levels of peroxidase activity was however attributed to the organisms' phenotype. BS 26 and BS 2 recorded the highest MnP activity levels in microtitre plate culture, consistent with published data (Table 2.1). Similarly, results for strains BS 3 and BS 17, reported as lower producing strains, also compare favourably with published ligninase activities for these strains (Raeder *et al.*, 1989b).

The productivity of conventional stationary flask culture is adversely affected by changes in medium volumes or altered flask size, and scale-up is problematic (Linko, 1992). Similarly, scale-down of 10 ml flask cultures to microtitre plate format showed reduced ligninase productivity. Altered productivity in microtitre plate cultures is most likely due to growth under atmospheric culture conditions.

The effectiveness of the 96-well microtitre plate culture system for growth rate measurements and enzyme kinetics far outweighs its disadvantages, which stem mainly from high evaporation rates from peripheral microtitre wells when cultures are incubated at 37 °C. High incubation temperatures prevent prolonged time course studies. Maintaining cultures in an oxygenated, humidified environment may prevent evaporation and accommodate time course extension required for the detection of LiP activities.

CHAPTER 3

Variability in growth rate and sensitivity to aromatic pollutants among monokaryotic progeny of *P. chrysosporium* ME446

3.1. INTRODUCTION

The biodegradation capabilities of white rot fungi have been widely studied. The origin of such aromatic compounds is varied and includes both xenobiotics and natural aromatics such as lignin and humic substances. As these compounds often occur as complex mixtures in industrial effluents the toxic effects of the individual compounds play an important role in the extent of bioremediating such effluents. Agricultural waste and industrial effluents often contain a complex mixture of aromatic compounds of different molecular mass. In the presence of larger concentration of high molecular-mass polyphenols, fungal growth is inhibited and the efficiency of wastewater biotreatment is reduced (Sayadi *et al.*, 1996).

With respect to the detoxification of plant matter for use as a potential fodder source, limited research has been undertaken on the toxic effects of tannins on *P. chrysosporium* and their effect on biodegradation (Brand *et al.*, 2000, Makkar *et al.*, 1994). Information on *P. chrysosporium* sensitivity to xenobiotics is more readily available (Alleman *et al.*, 1992, Lamar *et al.*, 1990). Biodegradation is often favoured in the presence of biomass and influenced by culture conditions, physiological state, type and age of culture, all of which may affect the tolerance of an organism to a particular pollutant (Reddy and Gold, 2000, Lin and Wang, 1990).

Limited research has been undertaken with regards to intraspecific variation in pollutant degradation (Wyatt and Broda, 1995, Lamar *et al.*, 1990). The detection of differences in sensitivity to and/or degradation of toxic compounds within the group of closely related fungal strains may help define the mechanism of tolerance to such compounds. Through the use of model pollutants it may be possible to elucidate the mechanisms of tolerance to toxic pollutants and their biodegradation by lignolytic organisms. If phenotypic differences relating to lignin or pollutant degradation can be ultimately correlated to differences at the genome, transcriptome or proteome level, then mechanistic inferences and gene targets for genetic manipulation could be obtained.

Under the direction of a *P. chrysosporium* breeding programme, Raeder *et al.* (1989a, 1989b) and Wyatt and Broda (1995) attempted to correlate restriction fragment length polymorphism (RFLP) analysis with ligninase activity and ¹⁴C-labelled synthetic lignin (DHP) degradation, respectively.

This information was to be used in the generation of a more selective approach to improved biodegradation technologies through the selection of a more robust lignin degrading *P. chrysosporium* strain. Further, the linkage of performance to specific alleles would allow for strain improvement using genetic engineering.

Natural variation among sexual and asexual progenies of wildtype dikaryons has been used for determining the genetic control of variation. Most variation is continuous and highly sensitive to environmental conditions, and it is thus difficult to isolate a gene effect. The adaptation of filamentous fungi to growth on solid substrates, representing a more natural environment, is proposed to allow for a more accurate assessment of the mechanism involved in the control and regulation of mycelial growth. Agar surface cultures offer a simple rapid means of determining the toxic effects of various compounds on growth rate in solid culture. Colony radial growth rate (Kr) is used as a measure of growth (Prosser and Tough, 1991).

P. chrysosporium is adapted to growth on solid substrates such as wood. Pellicle formation upon liquid culture surfaces, as in the microtitre plate method, is subject to disturbance and thus the solid surface agar culture method was proposed to provide a more stable and sensitive analysis system. However, biodegradation is influenced by environmental parameters, the physicochemical characteristics of the pollutant and its microbial toxicity (Section 1.2.2). This infers that biodegradation differs in solid state and liquid culture systems. The water content of culture media, namely agar or liquid media, alters solubility and diffusion of nutrients, oxygen and the pollutant itself, all factors that may also influence the tolerance of an organism to a specific pollutant.

Kr values and μ calculated from agar surface cultures and 96-well microtitre plate cultures, respectively, were used to generate pollutant sensitivity profiles for *P. chrysosporium* strains. Kr or μ values obtained for a series of pollutant concentrations were normalised against controls to give percentage inhibition curves used for the calculation of an IC50 (inhibitory concentration, 50 %) value for each of the strains examined. Intraspecific differences in pollutant sensitivity are based on the statistical comparison of these IC50 values.

3.1.1. Aims

This chapter examines the tolerance of ten *P. chrysosporium* strains to model aromatic pollutants found in various industrial effluents. The toxicity of PCP, *p*-cresol, tannic acid (hydrolysable tannin) and mimosa ME (condensed tannin) on mycelial growth were determined. Two distinct methods, a solid-state, agar surface method and the stationary liquid culture, 96-well microtitre plate method were employed for this purpose.

3.2. MATERIALS & METHODS

3.2.1. Organisms

Refer to section 2.2.1

3.2.2. Inoculum

For agar surface cultures, inoculum plates were prepared by aseptically transferring pieces of fungal mycelium from slants to 2 % malt extract agar (MEA) plates (Appendix I:A-3.1). The fungi were incubated at 37 °C until confluent growth was attained.

For microtitre plate cultures, spore inoculums were prepared as described in Appendix I:B. Spore suspensions were stored at 4 °C and a standard concentration of approximately 1×10^5 spores was routinely used per ml culture fluid.

3.2.3. Agar surface cultures

Solid surface cultures were examined using both complete (MEA) (Appendix I:A-3.1) and nitrogen-limited (NLM) agar medium (Appendix I:A-3.2). Medium-dependent differences in an organism's tolerance to a pollutant were easily assessed using the agar surface method. Petri plates containing 15 ml aliquots of either 2 % MEA or 2 % nitrogen-limiting medium (NLM) were prepared for the determination of inherent intraspecific differences in growth rates (Appendix I:A-3). Each plate was inoculated in its centre with a 3 mm² plug of mycelium taken from the margin of pre-inoculum plates, and incubated at 37 °C for up to five days or until confluence. Colony radial extension was recorded at 12 hr intervals as the average increase in colony radii (mm), measured on perpendicular axes (Figure 3.1.a). Five replicates, prepared for each strain on each medium, were used to plot growth curves.

The Radial Growth Rate (K_r), defined as: $K_r = (R_1 - R_0)/(t_1 - t_0)$, where R_0 and R_1 are the colony radii at time t_0 and t_1 , respectively, was calculated during exponential growth (Figure 3.1.b) (Trinci, 1971).

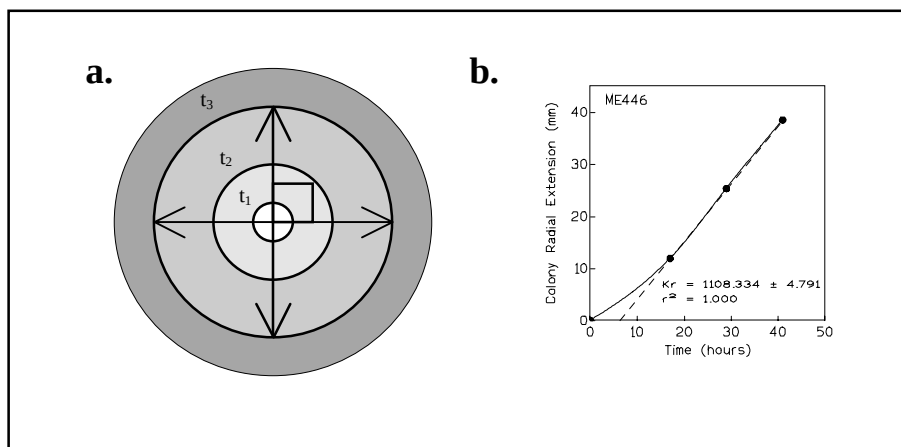


Figure 3.1: (a.) Colony radial extension was measured in perpendicular directions at set time intervals. Average radial extension values ($n = 5$) were used to plot a growth curve for each of the strains examined. (b.) The growth curve generated for *P. chrysosporium* ME446 as cultured on NLM agar (solid). Also shown is the linear regression of the growth phase (dashed) used to calculate the maximum radial growth rate ($K_r \pm SE$ ($\mu\text{m} \cdot \text{hr}^{-1}$)).

3.2.4. 96-well microtitre plate cultures

Microtitre cultures containing nitrogen-limiting basal III medium (Appendix I:A-2) were inoculated with 2.5×10^4 spores in a final volume of 300 μl per well. The 96-well microtitre plate format is comprised of twelve columns (1-12) and eight rows (A-H) of wells. Using a single 96-well microtitre plate, rows A and B were maintained as uninoculated controls. Row B was spiked with pollutant at the highest level assayed. Rows C through H were inoculated with the relevant *P. chrysosporium* strain and spiked with a given pollutant at successive concentration levels 12 hrs post inoculation, prior to the onset of linear growth. Plates were incubated in the dark at 37 °C.

Growth curves from microtitre plate cultures were generated by measuring biomass absorbance at 630 nm, using a microplate reader (Biotek Powerwave) as described by Langvad (1999). Absorbance measurements were taken at 12 hr intervals for a period of 6 to 10 days. For each strain, a time course profile reflecting the mean $\text{OD}_{630 \text{ nm}}$ was generated for different pollutant levels. Assuming a standard of 4.2 $\text{mg} \cdot \text{ml}^{-1}$ dry weight per absorbance unit for filamentous fungi (Langvad, 1999) the linear slope of each curve [$R^2 > 0.95$] was used to calculate the linear growth rate (μ) in mg dry weight per ml per day.

3.2.5. Pollutants

The sensitivity of each strain to PCP (Sigma), *p*-cresol (Sigma), tannic acid (Sigma) or Mimosa ME (Minchen) in agar surface cultures was determined by calculating K_r from inoculated Petri plates containing 15 ml aliquots of either MEA or NLM spiked with the relevant pollutant. PCP was tested at 0, 5, 10, 15, 20, and 25 mg.l⁻¹; *p*-cresol at 0, 50, 100, 150, 200, 250, and 300 mg.l⁻¹; while tannic acid and mimosa ME were tested at 0, 0.25, 0.5, 1.0, 1.5, 2.5 and 5.0 g.l⁻¹. *n* = 5 Agar plates for each medium type and concentration level.

Pollutant sensitivity in microplate cultures was determined from μ , calculated at each concentration level. PCP was tested at 0, 5, 10, 15, 20, and 25 mg.l⁻¹; *p*-cresol at 0, 100, 150, 200, 250, and 350 mg.l⁻¹; while both tannic acid and mimosa ME were tested at 0, 0.1, 0.15, 0.2, 0.3, 0.5 and 1.0 g.l⁻¹. *n* = 12 Wells per concentration level.

PCP was dissolved in acetone to aid its solution in both agar and liquid medium. The final volume of acetone (1 %) was constant for each PCP concentration, including the 0 mg.l⁻¹ control.

IC50 values, representing the pollutant concentration at which K_r or μ were reduced by 50 %, were calculated for each strain. IC50 was derived from logIC50 using a sigmoidal dose-response curve, equation: $Y = B + [T - B] / [1 + 10^{[(\log IC_{50} - X) * Hill\ slope]}]$, where *B* (minimum *Y*) and *T* (maximum *Y*) were fixed at 0 % and 100 % inhibition, respectively, and Hill slope was variable (Black and Leff, 1983). Statistical comparison of IC50 values is detailed below.

3.2.6. Statistical comparison

Inherent variation in K_r (μm.hr⁻¹) or μ (mg.day⁻¹) ± standard deviations, as calculated for each *P. chrysosporium* strain grown under pollutant free conditions, was compared by one-way ANOVA followed by the Bonferroni multiple comparisons test. A confidence level of *P* < 0.05 was chosen as significant. Similarly, the IC50 values calculated for each *P. chrysosporium* strain cultured in the presence of PCP, *p*-cresol, tannic acid or mimosa ME were analysed as above. Statistical analyses were performed using **GraphPad Prism**® version 3.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Cluster analysis of strains according to their growth measurements or peroxidase profiles was undertaken using **STATISTICA** (data analysis software system), version 6. StatSoft, Inc. (2002). www.statsoft.com.

3.3. RESULTS

3.3.1. Control growth rates

Kr values calculated from solid surface cultures of each strain appeared similar when grown on either MEA or NLM agar plates [$P > 0.05$]. This similarity in Kr may represent a maximum mycelial extension rate independent of the medium used (Figure 3.2.a). Statistical comparison of Kr for *P. chrysosporium* strains showed variability in the significance of these differences depending on the medium used. No significant differences were detected between strains grown on NLM [$P > 0.05$], while differences in the Kr of each strain were highly significant when cultured on MEA [$P < 0.001$], with the exception of strain ME446 vs. BS 32 [$P > 0.05$]. Medium-dependent differences in the ability to detect intraspecific variation were a function of the larger standard deviations recorded for growth on NLM. Similarly, *P. chrysosporium* strains cultured on NLM in 96-well microtitre plates did not differ significantly from one another in terms of μ [$P > 0.05$] (Figure 3.2.b).

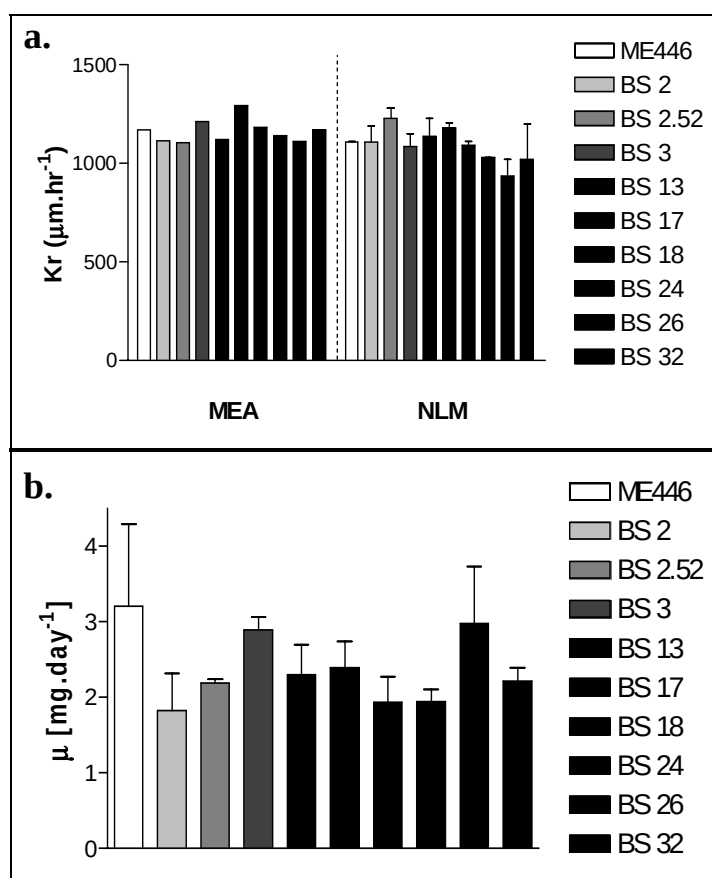


Figure 3.2: (a.) Control radial growth rates (Kr) calculated for each *P. chrysosporium* strain, cultured using either nutrient-sufficient (MEA) or nitrogen-limiting (NLM) agar medium. (b.) μ Calculated from stationary liquid, microtitre plate cultures grown on nitrogen-limiting medium (NLM).

Slight variation in the rank order reported for μ in this and the previous chapter was observed. However, *P. chrysosporium* ME446, BS 26 and BS 3 showed consistently higher μ [$P < 0.05$], while a consistently low μ was observed for strain BS 2 [$P < 0.05$]. Little significant difference was recorded between other strains, in terms of μ [$P > 0.05$].

In those cultures spiked with 1 % acetone (to aid the solution of PCP), neither K_r nor μ values obtained for fungal growth were inhibited by the acetone [$P > 0.05$].

3.3.2. Pentachlorophenol sensitivity (PCP)

Differences in response to PCP, dependent on the growth medium used, were apparent (Figure 3.3). The dose-response curves generated for each strain conformed to the variable-slope sigmoidal equation, defined previously [$R^2 > 0.97$], with distinctly different Hill slope values when cultured on either MEA or NLM agar plates. These curves reveal medium-dependant differences in the extent of inhibition of radial growth. In addition to the lag phase of the response being far shorter under nutrient sufficient conditions, colony extension was completely inhibited on MEA plates containing 20 mg.l⁻¹ PCP, whereas marginal growth was still apparent on NLM plates containing up to 25 mg.l⁻¹ PCP. Higher doses were not examined due to the solubility constraints of this pollutant.

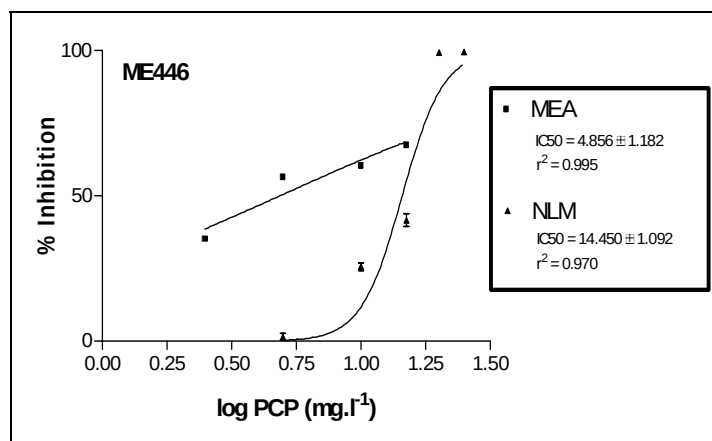


Figure 3.3: Agar surface culture dose response curves generated for *P. chrysosporium* ME446 exposed to various concentrations of PCP. The goodness of fit (R^2) of the data to the variable slope sigmoidal equation (section 3.2.3) is presented in each Figure. Curves like this were used to determine the IC50 value of PCP for each *P. chrysosporium* strain.

In general IC₅₀ values for strains cultured on MEA agar plates were approximately half those calculated for NLM agar cultures [$P < 0.001$]. This confirms an increase in tolerance to PCP when grown under nitrogen-limiting conditions. A greater degree of variation in the PCP dose required to inhibit mycelial growth by 50 % was seen when *P. chrysosporium* strains were cultured on MEA (Figure 3.4.a). This is consistent with the greater degree of variation observed in control growth rates when MEA is used as a culture medium. This variation in IC₅₀ values was, however, not statistically significant [$P > 0.05$].

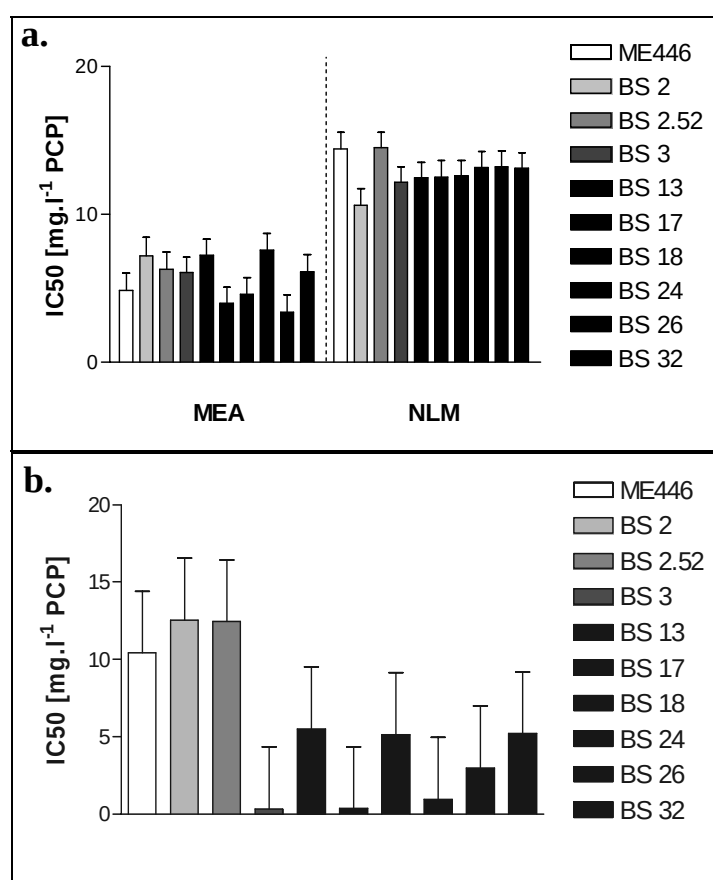


Figure 3.4: Tolerance of *P. chrysosporium* strains to PCP is recorded as the concentrations (mean $\pm$ SD) inhibiting growth by 50 % (IC₅₀). (a.) IC₅₀ values calculated from colony radial extension rates (Kr) on either nutrient-sufficient (MEA) or nitrogen-limited (NLM) agar medium. (b.) IC₅₀ values calculated from the mean growth rate (μ) from nitrogen-limited microtitre plate cultures.

The sensitivity of each *P. chrysosporium* strain to PCP was more variable in liquid culture, as determined from 96-well microtitre plate assays (Figure 3.4.b). This variability masked the detection of intraspecific differences due to high standard deviations recorded [$P > 0.05$].

3.3.3. *p*-Cresol sensitivity

The differences detected in toxic response to *p*-cresol when cultured on each medium type are not as apparent as that for PCP (Figure 3.5). However, a greater degree of variability was observed in the growth response of *P. chrysosporium* strains to *p*-cresol. Due to the higher solubility and lower toxicity of *p*-cresol in comparison to PCP, the concentration range examined was far greater. This larger range allowed for a more sensitive evaluation of differences in the toxic response between *P. chrysosporium* strains.

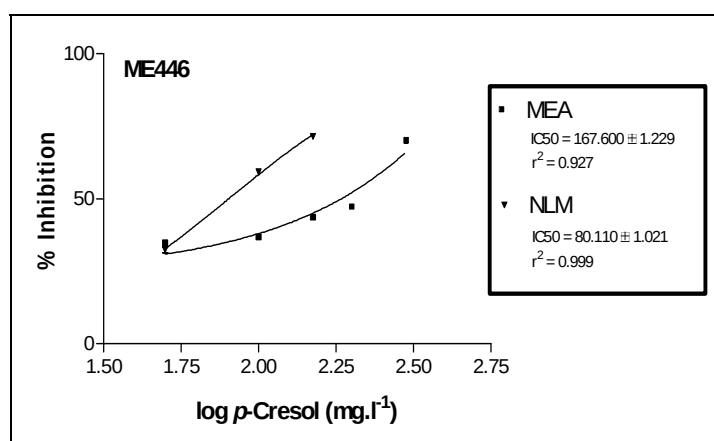


Figure 3.5: Agar surface culture dose response curves generated for *P. chrysosporium* ME446 exposed to various concentrations of *p*-cresol. The goodness of fit (R^2) of the data to the variable slope sigmoidal equation (section 3.2.3) is presented in each Figure. Curves like this were used to determine the IC₅₀ value of *p*-cresol for each *P. chrysosporium* strain

Figure 3.6.a represents the IC₅₀ values calculated from dose-response curves [$R^2 > 0.97$] using the agar surface method. These values range from 29.1 mg.l⁻¹ (BS 2.52) to 167.6 mg.l⁻¹ (ME446) in MEA grown cultures and 12.5 mg.l⁻¹ (BS 26) to 80.3 mg.l⁻¹ (BS 3) for NLM grown cultures. ME446 showed consistently high tolerance to *p*-cresol when cultured using the solid surface method (Table 3.2). Interestingly, the other dikaryon examined in this work exhibited a similarly low tolerance to *p*-cresol in solid surface cultures, irrespective of the culture medium used (Table 3.2). Intraspecific differences in IC₅₀ values calculated for each strain grown on either MEA or NLM agar plates were apparent. These differences were mostly highly significant [$P < 0.001$] (Table 3.1). There also appeared to be an inverse relationship in a particular strains' tolerance to *p*-cresol and the culture medium used (Figure 3.6.a).

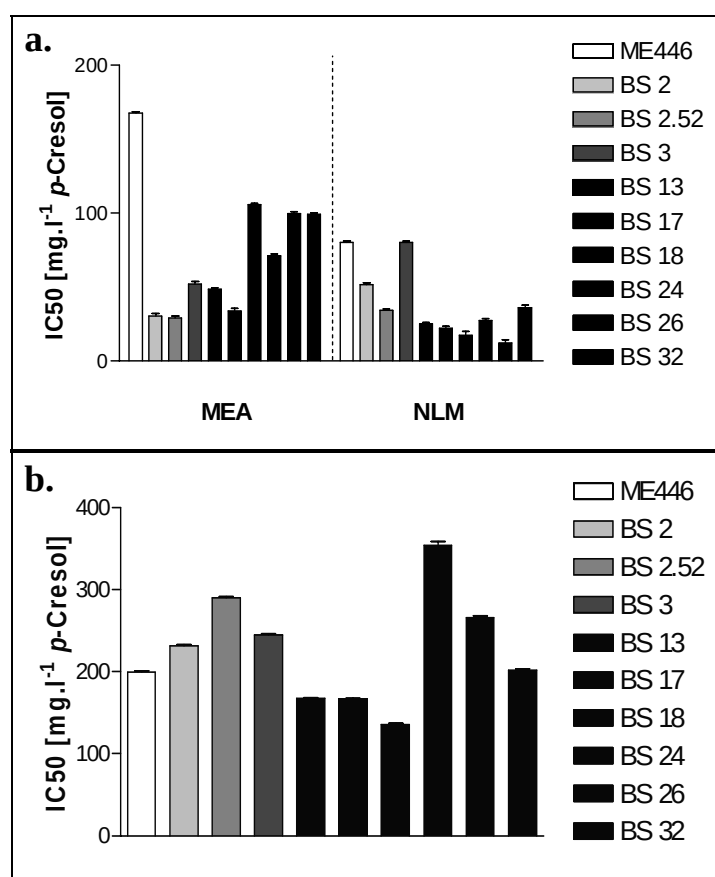


Figure 3.6: Tolerance of *P. chrysosporium* strains to *p*-cresol is recorded as the concentrations (mean $\pm$ SD) inhibiting growth by 50 % (IC₅₀). (a.) IC₅₀ values calculated from colony radial extension rates (Kr) on either nutrient-sufficient (MEA) or nitrogen-limited (NLM) agar medium. (b.) IC₅₀ values calculated from the mean growth rate (μ) from nitrogen-limited microtitre plate cultures.

Table 3.1: Intraspecific statistical comparison of IC₅₀ values generated for *p*-cresol treated MEA or NLM grown cultures.

MEA										
	ME446	BS 2	BS 2.52	BS 3	BS 13	BS 17	BS 18	BS 24	BS 26	BS 32
ME446		***	***	***	***	***	***	***	***	***
BS 2	***		ns	***	***	ns	***	***	***	***
BS 2.52	***	***		***	***	ns	***	***	***	***
BS 3	ns	***	***		ns	***	***	***	***	***
BS 13	***	***	***	***		***	***	***	***	***
BS 17	***	***	***	***	ns		***	***	***	***
BS 18	***	***	***	***	*	ns		***	ns	*
BS 24	***	***	ns	***	ns	ns	***		***	***
BS 26	***	***	***	***	***	**	ns	***		ns
BS 32	***	***	ns	***	***	***	***	**	***	
NLM										

The levels of significance are presented as [P < 0.001]*** [P < 0.01]** [P < 0.05]* and [P > 0.05]^{ns}.

p-Cresol sensitivity in 96-well microtitre plate assays were also determined to be highly significant [$P < 0.001$] (Figure 3.6.b). The exceptions were strains ME446 versus BS 32 [$P > 0.5$] and BS 17 versus BS 18 [$P > 0.05$]. Surprisingly, *P. chrysosporium* strains appeared to be less sensitive to *p*-cresol when grown in liquid culture using the 96-well microtitre plate method.

Table 3.2: Rank order for medium- and method-dependent *p*-cresol sensitivity, based on mean $\pm$ SD IC50 values (mg.l^{-1} *p*-cresol), calculated for each *P. chrysosporium* strain under the respective culture conditions. Highlighted cells indicate *P. chrysosporium* strains showing significantly higher or lower *p*-cresol IC50 values than other strains.

Rank Order	<i>p</i> -cresol IC50 values Solid Substrate Method (n = 5) (Agar Surface Cultures)		<i>p</i> -cresol IC50 values Stationary Liquid Method (n = 6) (Microtitre Plate Cultures)
	MEA	NLM	NLM
1	ME446 167.6 $\pm$ 1.3	BS 3 80.3 $\pm$ 1.0	BS 24 354.0 $\pm$ 4.4
2	BS 18 105.7 $\pm$ 1.1	ME446 80.1 $\pm$ 1.0	BS 2.52 290.3 $\pm$ 0.9
3	BS 26 99.7 $\pm$ 1.2	BS 2 51.5 $\pm$ 1.3	BS 26 266.1 $\pm$ 1.8
4	BS 32 99.1 $\pm$ 1.1	BS 32 36.0 $\pm$ 1.6	BS 3 244.9 $\pm$ 1.6
5	BS 24 71.0 $\pm$ 1.4	BS 2.52 34.2 $\pm$ 1.0	BS 2 231.6 $\pm$ 1.6
6	BS 3 52.3 $\pm$ 1.2	BS 24 27.4 $\pm$ 1.3	BS 32 201.7 $\pm$ 1.3
7	BS 13 48.5 $\pm$ 1.2	BS 13 25.1 $\pm$ 1.1	ME446 199.7 $\pm$ 1.2
8	BS 17 34.1 $\pm$ 1.3	BS 17 22.1 $\pm$ 1.4	BS 13 167.5 $\pm$ 0.1
9	BS 2 30.6 $\pm$ 1.5	BS 18 17.2 $\pm$ 2.7	BS 17 166.7 $\pm$ 1.0
10	BS 2.52 29.1 $\pm$ 1.5	BS 26 12.5 $\pm$ 1.7	BS 18 135.6 $\pm$ 1.1

3.3.4. Tannin sensitivity

Tannins have an inhibitory effect on mycelial growth. Using the solid surface method, all *P. chrysosporium* strains appeared to be more tolerant to tannic acid when cultured under nutrient sufficient culture conditions (MEA) than under nitrogen-limited culture conditions (Figure 3.7 and 3.8). Marginal colony extension of *P. chrysosporium* cultures grown on NLM plates was still observed in the presence of 0.15 g.l⁻¹ tannic acid. However, on MEA agar plates growth was still apparent in the presence of 2.5 g.l⁻¹ tannic acid. Medium-dependent differences were not significant [$P > 0.05$], a function of the large standard errors obtained for growth in the presence of this

compound. Similarly, intraspecific comparison of IC₅₀ values calculated using the agar surface method showed no significant variation in tannic acid sensitivity [$P > 0.05$].

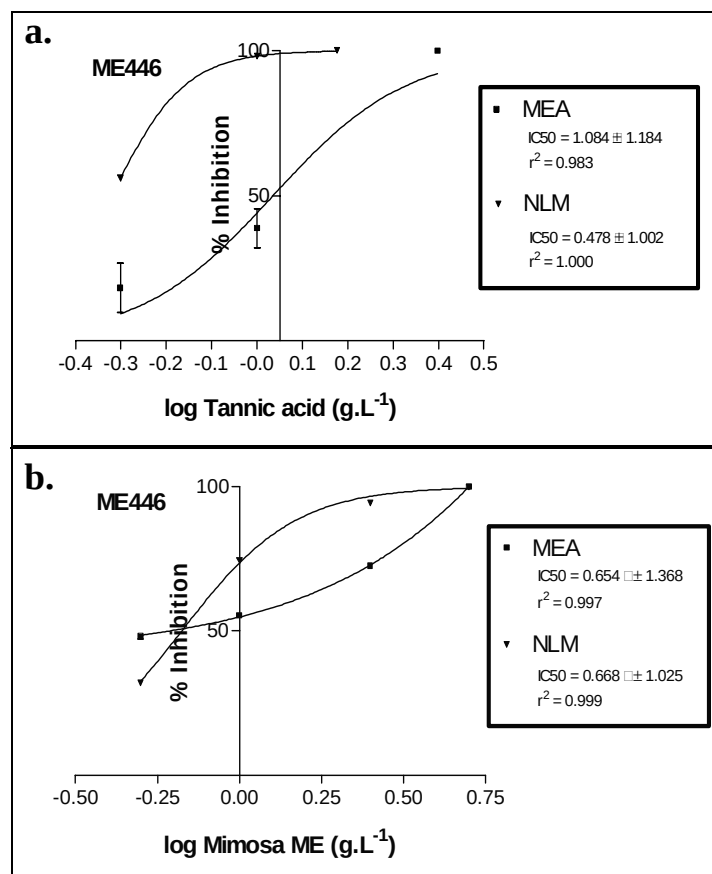


Figure 3.7: Agar surface culture dose response curves generated for *P. chrysosporium* ME446 exposed to various concentrations of tannic acid (a.) and mimosa ME (b.). The goodness of fit (R^2) of the data to the variable slope sigmoidal equation (section 3.2.3) is presented in each Figure. Curves like these were used to determine the IC₅₀ value of these two tannins for each *P. chrysosporium* strain.

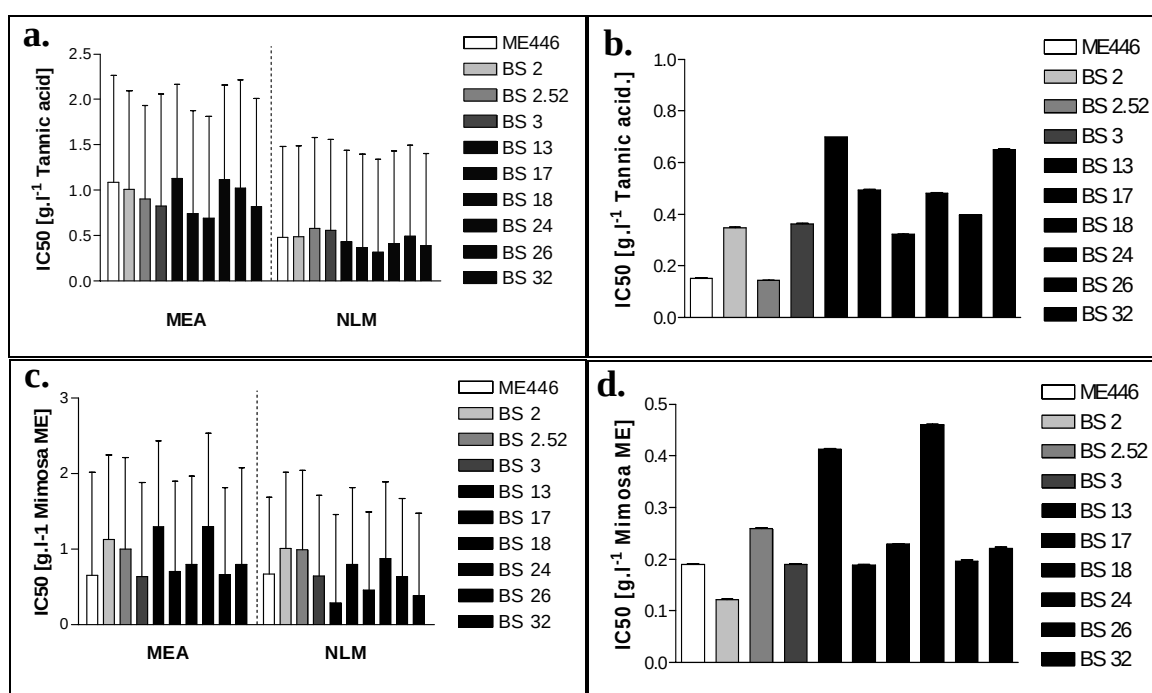


Figure 3.8: Tolerance of *P. chrysosporium* strains to tannic acid [T.A] (a. and b.) or mimosa ME (c. and d.) are recorded as the concentrations (mean $\pm$ SE) inhibiting growth by 50 % (IC50). (a. and c.) IC50 values calculated from colony radial extension rates (Kr) on either nutrient-sufficient (MEA) or nitrogen-limited (NLM) agar medium. (b. and d.) IC50 values calculated from the mean growth rate (μ) from nitrogen-limited microtitre plate cultures.

P. chrysosporium strains were shown to have similar tolerance levels to both mimosa ME (condensed tannin) and the lower MW tannic acid. The dose-response curves generated for mimosa ME [$R^2 > 0.97$] represented a more variable, medium-independent, intraspecific response to this compound, similar to that of *p*-cresol (Figure 3.7.b). Marginal growth was still apparent at a concentration of 5.0 g.l⁻¹ mimosa ME, independent of the type of culture medium used [$P > 0.05$] (Figure 3.8.c). The high standard deviations recorded for Kr derived IC50 values rendered intraspecific comparisons not significant [$P > 0.05$] (Figure 3.8.c). As complete inhibition did not occur at the concentration levels tested, the sigmoidal dose-response curves did not fully converge [$R^2 < 0.7$] and the IC50 values for certain strains may differ slightly from values reported. The addition of higher concentrations of this tannin to agar medium prevented the polymerisation of agar, through the formation agar-tannin complexes.

Using the 96-well microtitre plate assay, the large standard errors recorded for growth and thus tannic acid and mimosa ME IC₅₀ values was reduced. Not only were the observed intraspecific differences in IC₅₀ values determined as significant in microtitre plate cultures, but these responses were also more variable than implicated from agar surface data. *P. chrysosporium* strains growth appeared to be more sensitive to tannins when grown in liquid culture than on using the solid surface method. Using the microtitre plate method *P. chrysosporium* strains BS 13 and BS 32 were less sensitive to tannic acid at the concentrations tested, while ME446 and BS 2.52 were inhibited to a greater extent than all other strains [$P < 0.001$] (Figure 3.8.b and Table 3.3). Similarly, significant variation was also observed in the tolerance of *P. chrysosporium* strains to mimosa ME when cultured using the microtitre plate method (Figure 3.8.d and Table 3.3). Strains BS 24 and BS 13 showed significantly higher tolerance to mimosa ME in contrast to strain BS 2 which was approximately three-fold more sensitive (Table 3.3). The rank order for *P. chrysosporium* strain tolerance to tannic acid and mimosa ME differed suggesting different mechanisms are involved in tolerance to these two tannins.

Table 3.3: Rank order for μ derived (using the microtitre plate method) IC₅₀ values (mean $\pm$ SD) in g.l⁻¹, for tannic acid and mimosa ME, calculated for each *P. chrysosporium* strain under the respective culture conditions. Highlighted cells indicate *P. chrysosporium* strains showing significantly higher or lower tolerance to tannins (IC₅₀ values) than other strains.

Rank Order	Tannic Acid IC ₅₀ values	Mimosa ME IC ₅₀ values
	[Microtitre Plate Cultures] (n = 6)	[Microtitre Plate Cultures] (n = 6)
	NLM	NLM
1	*BS 13	BS 24
	> 0.700	0.461 $\pm$ 0.001
2	BS 32	BS 13
	0.651 $\pm$ 0.002	0.413 $\pm$ 0.001
3	BS 17	BS 2.52
	0.495 $\pm$ 0.001	0.260 $\pm$ 0.001
4	BS 24	BS 18
	0.482 $\pm$ 0.001	0.229 $\pm$ 0.000
5	BS 26	BS 32
	0.396 $\pm$ 0.002	0.221 $\pm$ 0.003
6	BS 3	BS 26
	0.363 $\pm$ 0.001	0.197 $\pm$ 0.002
7	BS 2	ME446
	0.348 $\pm$ 0.001	0.190 $\pm$ 0.002
8	BS 18	BS 3
	0.323 $\pm$ 0.001	0.190 $\pm$ 0.001
9	ME446	BS 17
	0.152 $\pm$ 0.001	0.189 $\pm$ 0.001
10	BS 2.52	BS 2
	0.145 $\pm$ 0.001	0.122 $\pm$ 0.001

The sigmoidal dose-response curve for BS 13 did not converge as this strain showed a greater tolerance to tannic acid than all other strains examined.

3.3.5. Cluster analysis

Cluster analysis for *P. chrysosporium* strains according to their tannic acid, mimosa ME and *p*-cresol IC50 values is recorded in Appendix II. Three clusters or groupings of strains are apparent. Yet, these differences do not accurately represent the complex response of *P. chrysosporium* strains to different pollutants. Instead, *P. chrysosporium* strains were categorically subdivided into high (H), medium (M) and low (L) tolerance to *p*-cresol, tannic acid or mimosa ME (Appendix III, Table I), more comprehensively described in Chapter 8.

3.4. DISCUSSION

3.4.1. Culture methods

Both solid state (agar surface) and stationary liquid (96-well microtitre plate) methods were able to distinguish between the different responses of *P. chrysosporium* strains to each pollutant examined. Differences in both the extent and the mechanisms of toxicity of tannins, PCP and *p*-cresol were apparent. For example, *P. chrysosporium* strains were most sensitive to PCP and showed an increased tolerance to this pollutant in nitrogen-limited (NLM) solid surface cultures. PCP tolerance remained low in nitrogen-limited liquid culture, suggesting that the improved tolerance observed in solid substrate cultures may be related to differences in hyphal morphologies in liquid and solid surface cultures. *P. chrysosporium* strains appeared to have an inverse response to *p*-cresol when cultured under nutrient sufficient and nitrogen-limited culture conditions using the agar surface method.

Both Kr (agar surface cultures) and μ (stationary liquid cultures) derived IC50 values could be used for determining intraspecific variation in genetically related *P. chrysosporium* strains. However, the extent to which each method was able to do this appeared to be dependent on the nature of the pollutant tested.

The agar surface culture method simulates solid-state conditions such as those used in the delignification of agricultural waste, bioaugmentation of hazardous wastes in soil or immobilised systems for wastewater treatment; and offers the advantages of a simple, rapid, cost effective method for the measurement of radial growth in response to pollutants. Its discriminatory power was however, limited by the high standard deviations obtained for growth measurements in the presence of compounds interfering with hyphal growth patterns, and thus Kr determinations. The high standard deviations observed for agar cultures spiked with tannins are real, a reflection of the erratic pattern of mycelial extension observed with growth on this substrate. Tannins are astringent, complexing with proteins, carbohydrates and other microbial metabolites (Bhat *et al.*, 1998). The altered patterns of

colony extension in the presence of tannins may be related to the complexation of tannins with hyphal growth factors. Compounds secreted by advancing hyphae were shown to regulate radiating hyphal extension in filamentous fungi (Bottone *et al.*, 1998). Complexation of such messengers with tannins in the medium may deregulate colony growth. In such cases the mycelial front is thinner, with hyphal extension occurring in an unbalanced pattern as foraging hyphae attempt to locate a less toxic nutrient source.

The 96-well microtitre plate method overcame those problems observed with the agar surface method. Growth measurements based on the absorbance of biomass accommodated erratic hyphal extension of the peripheral growth zone in response to the tannins and reduced standard deviations of calculated IC₅₀ values. Growth measurements based on the absorbance of fungal biomass, enabled the detection of absolute biomass production irrespective of altered branching, hyphal thickening or accumulation of EPS in response to pollutants, all of which contribute to absolute biomass (dry weight) values. Thus, the microtitre plate method was found to be more robust and strain-dependent toxic response to growth altering tannic acid and mimosa ME were found to differ significantly between *P. chrysosporium* strains [$P < 0.001$].

3.4.2. Tannins

The effect of tannic acid and mimosa ME on mycelial growth in stationary liquid cultures of *P. chrysosporium* strains was distinctive (Figure 3.8). Certain strains showed no sensitivity to tannic acid (BS 13, BS 32). BS 13 also showed high levels of tolerance to mimosa ME (Table 3.3). Interestingly, the two dikaryons examined in this work, strains ME446 and BS 2.52 appeared to be highly sensitive to tannic acid (Table 3.3). IC₅₀ values derived from μ showed *P. chrysosporium* strains to be less tolerant of both tannic acid and mimosa ME in liquid culture than was recorded for solid-state cultures (Table 3.3).

These differences warrant closer examination, where extreme response of *P. chrysosporium* strains to tannic acid may provide an ideal model system for future proteome, transcriptome and genetic linkage studies.

3.4.3. Pentachlorophenol (PCP)

Despite the increased sensitivity of the 96-well microtitre plate method, this liquid culture system was unable to distinguish between altered growth response to PCP [$P > 0.05$]. The inability to detect significant differences between *P. chrysosporium* strains, using either solid surface or liquid culture

methods, was attributed to the low solubility of this phenolic compound (Mileski *et al.*, 1988). From visual inspection of Kr derived IC50 values, *P. chrysosporium* strains exhibited a medium-dependent response, where strains were approximately 50 % more tolerant to PCP when cultured on NLM. While PCP has been shown to be degraded under both nutrient-sufficient and nutrient-limiting culture conditions, degradation is enhanced under nutrient-limiting conditions (Armenante *et al.*, 1994, Mileski *et al.*, 1988). Degradation of PCP may facilitate an increased tolerance to PCP when cultured on NLM. A converse relationship was observed for tannic acid, suggesting that inhibition of growth occurs via different mechanisms for PCP and tannic acid.

3.4.4. *p*-Cresol

Unlike PCP, no noticeable increase in tolerance to *p*-cresol occurred when *P. chrysosporium* strains were cultured under nitrogen-limiting conditions using the agar surface method. Under lignolytic conditions, *P. chrysosporium* has been reported to degrade *p*-cresol concentrations of up to 150 mg.L⁻¹ without inhibiting mycelial growth (Kennedy and Lema, 1994, Aitken *et al.*, 1994). Our results show and increased sensitivity to *p*-cresol (Table 3.2) in comparison to Kennedy and Lema (1994), attributed to the different genomic complement of the strains used in these experiments.

P. chrysosporium strains were able to tolerate ten-fold higher concentrations of *p*-cresol than PCP. This reduced toxicity and the wider range of concentrations that could be tested decreased the variation in Kr and μ measurements and hence the standard deviations calculated for each value. Further, *p*-cresol inhibition of *P. chrysosporium* growth did not influence concentric growth, so either method could be used to elucidate the toxicity of this compound. Thus, intraspecific variation in *p*-cresol sensitivity could be determined using either culture method [$P < 0.001$]. Table 3.2 describes the rank order of *p*-cresol tolerance for each of the *P. chrysosporium* strains examined. Under nitrogen-limiting conditions only strains BS 13, BS 17 and BS 18 recorded a consistently low tolerance to *p*-cresol.

In comparing the rank orders for *P. chrysosporium* strain tolerance to *p*-cresol, tannic acid and mimosa ME, only strain BS 24 showed consistently high tolerance to each pollutant, when using the 96-well microtitre plate method. Medium-, method- and pollutant-dependent responses in the growth rates of each *P. chrysosporium* strain suggest a complex interplay of metabolic pathways dependent on nutrient and environmental conditions. In fact, the relationship between *p*-cresol tolerance and growth medium appears to be inverse for solid-state cultures of certain strains. *P. chrysosporium* ME446 shows a high tolerance to *p*-cresol not observed within its monokaryotic derivatives. This may

be attributed to the double genomic complement of the ME446 dikaryon. This contrasts with findings for tannic acid where dikaryons appeared to be more sensitive to growth inhibitions. These findings warrant further study, and highlight both the potential of polyploidy as a means of strain improvement (Dhawan and Lavania, 1996), and a need for process specific strain improvement strategies.

3.5. CONCLUSION

P. chrysosporium *P. chrysosporium* strains have been shown to differ in their response to a particular pollutant by either medium-dependent (PCP and tannic acid) or -independent (*p*-cresol and mimosa ME) means, depending on the nature of the pollutant. It is, however, apparent that consistency in response to a particular pollutant is essential for the detection of intraspecific variability. While nutrient composition and pH play an important role in mycelial growth and activity, the most important factor remains the nature of the pollutant itself. Using the solid-state agar surface method, a uniform growth pattern is essential for the calculation of K_r values with low error. For pollutants such as the tannins, which appear to affect the hyphal growth zone to a larger extent and hence the standard error of K_r , alternative methods such as the 96-well microtitre plate technique introduced in this work would be more valuable in determining intraspecific differences. Highly toxic compounds render the concentration range over which a dose-response curve is to be generated too narrow e.g. PCP. A wider range of concentrations allows for a more sensitive analysis of variability in response to that pollutant e.g. *p*-cresol. The biochemical nature of these responses is, however, unknown.

Pollutant degradation is frequently favoured in the presence of biomass, thus toxicity is potentially a rate-limiting factor in biological waste treatment. These results show that different pollutants elicit different toxic responses in the *P. chrysosporium* strains studied. Further examination of the mechanisms and pathways facilitating these different toxic responses and their correlation at the level of proteome, transcriptome and genome of *P. chrysosporium* strains would allow for the informed selection and/or engineering of strains with improved biodegradation capabilities. A larger selection of pollutants eliciting a greater variety of toxic responses would benefit this approach.

CHAPTER 4

Biodegradation capabilities of monokaryotic progeny of *P. chrysosporium* ME446, assessed using a 96-well microtitre based method

4.1. INTRODUCTION

The biodegradation capabilities of *P. chrysosporium* have been reviewed (section 1.2). The mineralisation of toxic pollutants is attributed to lignolytic enzymes and is frequently favoured in the presence of biomass. The *P. chrysosporium* strains examined in this work differ from one another in the extent of their LiP (Raeder *et al.*, 1989b) and MnP production (Chapter 2). Intraspecific differences were also detected in the toxic response of each organism to *p*-cresol, tannic acid and mimosa ME (Chapter 3). Further, Wyatt and Broda (1995) showed these same strains to differ in their ability to degrade synthetic lignin (Table 2.1), suggesting that *P. chrysosporium* strains may also vary in their ability to degrade different recalcitrant pollutants.

The stationary liquid culture, microtitre plate method has effectively allowed for the comparison of control and pollutant-altered growth in *P. chrysosporium* strains. Miniaturisation of the stationary liquid culture system to 96-well microtitre plates also offers an attractive solution to accommodating the comparison of the degradation capabilities of a large number of organisms simultaneously under the same conditions.

The polymeric anthraquinone dye, Poly-R478, has routinely been used as a quick, reproducible and inexpensive screening method for lignolytic activity (de Jong *et al.*, 1992, Field *et al.*, 1992). This preliminary screening method has been used in conjunction with liquid culture techniques for the correlation of enzyme activities with the lignin degradation capabilities of different isolates (Tekere *et al.*, 2001). In this chapter, the potential of a 96-well microtitre plate method in the investigation of intraspecific differences in *P. chrysosporium*, through the *in vivo* decolourisation of Poly-R478, was assessed.

Similarly, the biodegradation capabilities of *P. chrysosporium* strains were explored through the removal of *p*-cresol from culture media. The mechanism of mineralisation of *p*-cresol has been reported (Aitken *et al.*, 1994) therefore this phenolic compound serves as a model pollutant for further evaluating pollutant degradation using the 96-well microtitre plate method. *p*-Cresol mineralisation

has been shown to occur in the presence of both LiP and MnP, is favored in the presence of biomass and is completely mineralised within five days, irrespective of initial concentration levels of this pollutant (Kennes and Lema, 1994).

Further analysis of the biodegradation capabilities of *P. chrysosporium* strains, in relation to previously defined growth kinetics, ligninase activities and pollutant sensitivity may enable the correlation of certain growth characteristics to biodegradation. Categorical analysis of *P. chrysosporium* strains according to pollutant sensitivity and biodegradation may enable the selection of strains best adapted to those effluents, generating an informed approach to improved biodegradation technologies.

4.1.1. Aims

This chapter aims to develop and evaluate a convenient 96-well microtitre plate-based Poly-R478 biodegradation assay for the screening of lignolytic activity. This method was further assessed as a means of differentiating between the biodegradation capabilities of *P. chrysosporium* strains, using a model pollutant, *p*-cresol.

4.2. MATERIALS & METHODS

4.2.1. Organisms

Refer to section 2.2.1

4.2.2. Culture conditions

P. chrysosporium strains were cultured in sterile 96-well microtitre plates with lids (Greiner), containing nitrogen-limiting basal III medium (Appendix I:A-2) inoculated with 2.5×10^4 spores in a final volume of 300 μ l per well. Plates were sealed with strips of parafilm and incubated in the dark at 37 °C, under atmospheric conditions i.e. darkened incubator with air circulation.

4.2.3. Growth measurements

Mycelial growth was monitored by measuring the absorbance of biomass at 630 nm using a microplate reader (BioTek Powerwave) as described by Langvad (1999). Absorbance measurements were taken at 12 hr intervals. Assuming a standard of 4.2 mg.ml⁻¹ dry weight per absorbance unit for filamentous fungi (Langvad, 1999) maximum biomass (x_t) formed at stationary phase growth was calculated.

4.2.4. Poly-R478 decolourisation

Microtitre wells were spiked with 0.02 % Poly-R478 36 hrs post inoculation, coinciding with the onset of stationary phase growth, as determined from absorbance at 630 nm (Langvad, 1999). Growth measurement based on absorbance provided a convenient method for determining the growth conditions of each *P. chrysosporium* strain and for determination of the onset of stationary phase.

For each organism, three separate experimental controls were included: (1) Uninoculated controls, either plain media or spiked with the appropriate recalcitrant compound, serving as absorbance blanks and the autoxidation control. (2) Inoculated control, representing normal growth by each strain. (3) One hour prior to the addition of 0.2 % Poly-R478, biomass was killed by the addition of 10 mM sodium azide to each well, serving as an absorption control.

Following the addition of dye, a single column was sampled from each microtitre plate, including two uninoculated wells and six sample wells ($n = 6$), at set time intervals. Samples were combined into a single microfuge tube and centrifuged at 10 000 rpm for 2 minutes. 300 µl aliquots of supernatant were transferred to a clean 96-well UV microtitre plate in triplicate and both a spectral scan and absorbance at 520 nm (Moreira *et al.*, 2001) was recorded using a microplate reader (BioTek Powerwave). Production of metabolites by the organism under normal growth conditions, autoxidation and adsorption were quantified in addition to dye decolourisation by each organism ($n = 3$).

4.2.5. *p*-Cresol removal

Investigation of *p*-cresol degradation was undertaken using the same methods developed for Poly-R478 decolourisation. 96-well microtitre plate cultures were spiked with 150 mg.l⁻¹ *p*-cresol 36 hrs post-inoculation. The concentration level of *p*-cresol assayed was selected from previous sensitivity studies (Chapter 3) and was below IC50 values calculated for each *P. chrysosporium* strain.

p-Cresol disappearance was monitored by HPLC (Beckman System Gold) at 254 nm using a Waters ODSI5 C18 column, with a mobile phase of 60:40 % methanol:water at 1 ml.min⁻¹ (Aitken *et al.*, 1994).

4.2.6. Statistical analysis

Degradation values (at 228 hrs) reported as a percentage of uninoculated controls and normalised for adsorption, were compared by one-way ANOVA and Bonferonni post-test. Statistical analysis was undertaken using GraphPad Prism[®] version 3.03 for Windows (www.graphpad.com). A level of $P < 0.05$ was chosen as significant.

Cluster analysis of strains according to their growth measurements or peroxidase profiles was undertaken using STATISTICA (data analysis software system), version 6. StatSoft, Inc. (2002). www.statsoft.com.

Correlation analysis based on the means of each dependent variable was undertaken using STATISTICA (data analysis software system), version 6. (www.statsoft.com). A level of $P < 0.05$ was chosen as significant. Correlations were undertaken using casewise deletion of missing data these values were not available or alternatively were not determined.

4.3. RESULTS

4.3.1. Poly-R478 decolourisation

P. chrysosporium decolourisation of Poly-R478 in microtitre plate cultures was initiated approximately 84 hrs (120 hrs post-inoculation) following the addition of the dye at the onset of stationary phase (36 hrs post-inoculation) (Figure 4.1).

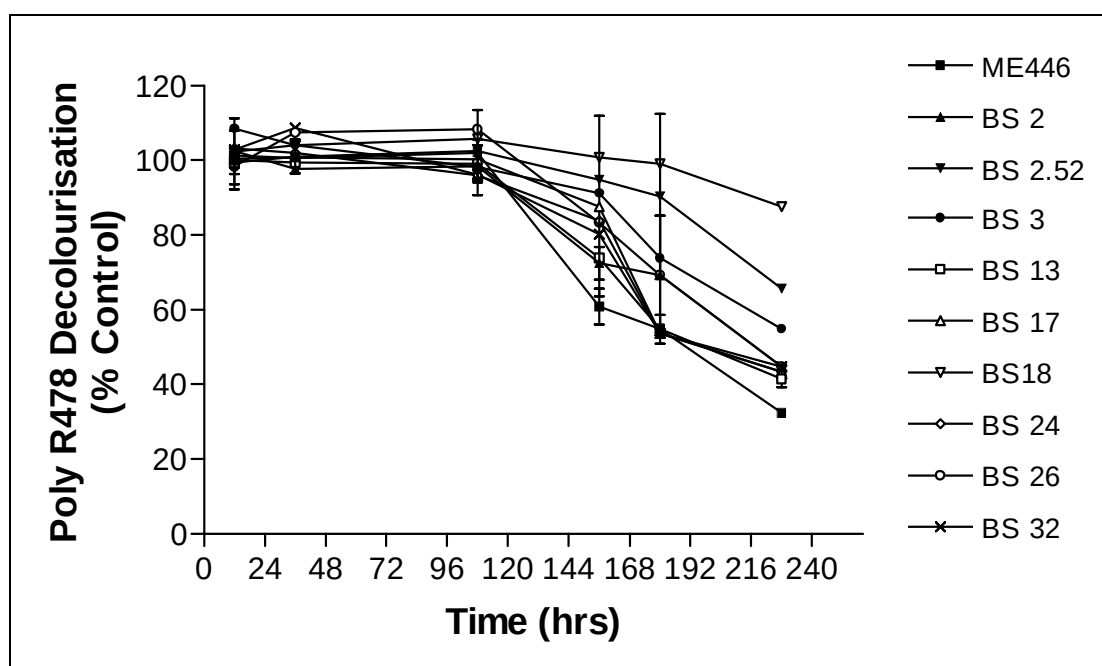


Figure 4.1: Time course showing the decolourisation (mean $\pm$ SD) of Poly-R478 by each of the *P. chrysosporium* strains examined. $t = 0$ is point at which Poly-R478 was added to cultures (36 hrs post-inoculation). Percentage decolourisation of Poly-R478 by each strain was done at $t = 228$ hrs (264 hrs post-inoculation). Data, calculated from $OD_{520\text{ nm}}$, was corrected for adsorption.

Poly-R478 decolourisation was observed as the degradation of chromophores absorbing around 540 nm and 360 nm, as depicted in Figure 4.2. This is observed as a change in medium colour from red to yellow. Complete mineralisation does not occur. Instead, following 180 hrs incubation with the dye, the accumulation of degradation products formed from the disappearance of the chromophore at 540 nm are seen to increase absorbance at lower wavelengths (280-480 nm), even increasing the peak at 360 nm initially shown to be reduced. This observation was not due to the accumulation of metabolites produced by *P. chrysosporium*, nor due to autoxidation or adsorption of Poly-R478, as assessed from controls.

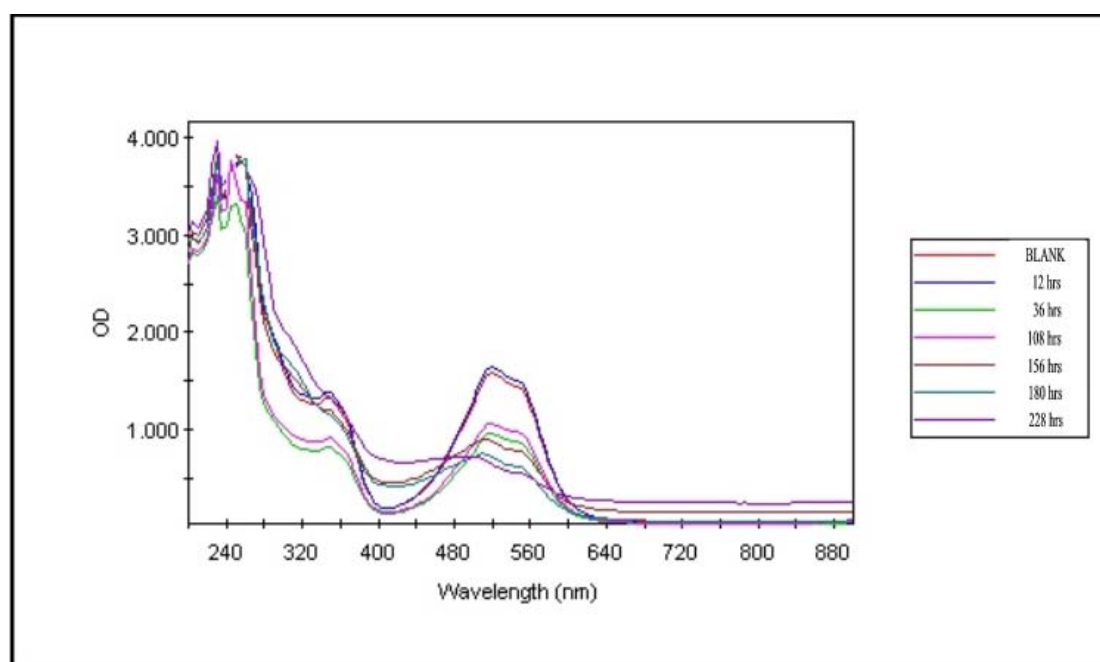


Figure 4.2: Spectral scan showing Poly-R478 degradation by *P. chrysosporium* ME446 at 12-72 hr intervals, following the addition of the dye to microtitre plate cultures.

In each experiment, dye or pollutant was added to cultures at the onset of the stationary growth phase, 36 hrs post-inoculation. Stationary phase biomass yields for each experiment are recorded in Table 4.2. In the presence of Poly-R478, no significant differences were detected in maximum stationary phase growth (120 hrs post-inoculation) recorded for *P. chrysosporium* strains [$P > 0.05$]. These values were all slightly lower than those described under control conditions (Chapter 2). These differences were however not significant and may be attributed to marginal, and as yet unquantified differential effects of Poly-R478 on growth in *P. chrysosporium* strains.

Dye decolourisation by autoxidation was negligible. Dye decolourisation by adsorption was approximately 0-15 % of uninoculated controls. Within a sample period, adsorption values of strains were within 1-3 % of one another. Only strains BS 3 and BS 26 showed significantly higher adsorption values than other strains [$P > 0.05$]. Although biomass levels were found not to be significantly different, *P. chrysosporium* strains BS 13 and BS 26 did show higher biomass levels than other strains, an observation that may be linked to increased absorption in these organisms.

Intraspecific comparison of percentage Poly-R478 decolourisation at 228 hrs (264 hrs post-inoculation) (Table 4.2) shows *P. chrysosporium* strains to differ significantly in their dye decolourising capabilities. *P. chrysosporium* strains ME446 and BS 13 showed significantly higher

Poly-R478 decolourisation [$P < 0.001$], while BS 18, BS 2.52 and BS 3 showed significantly lower dye decolourisation [$P < 0.001$]. Strains BS 17, BS 24, BS 26, BS 32 and BS 2 were similar in their ability to decolourise Poly-R478 by 55-57 % of controls (Table 4.2) [$P > 0.05$].

No correlation was detected between the ability of each strain to decolourise Poly-R478 and the amount of biomass present [$r = 0.026$, $P > 0.05$].

Table 4.1: Maximum biomass and percentage decolourisation of Poly-R478, detected in 96-well microtitre plate cultures ($n = 6$). Highlighted cells indicate *P. chrysosporium* strains showing significantly higher or lower growth kinetics than other strains.

Rank Order	^a Max. Stationary phase growth ($\times_{120 \text{ hrs}}$) (mg.ml ⁻¹)	^b Percentage Poly-R478 decolourisation ($t = 228$)
1	BS 3 3.38 $\pm$ 0.87	ME446 67.7 $\pm$ 0.1
2	BS 32 3.23 $\pm$ 1.05	BS13 58.6 $\pm$ 0.9
3	BS 26 3.13 $\pm$ 1.15	BS 17 56.6 $\pm$ 0.7
4	BS13 2.99 $\pm$ 0.70	BS 24 56.6 $\pm$ 0.7
5	BS 18 2.78 $\pm$ 0.86	BS 26 55.2 $\pm$ 0.2
6	BS 2 2.70 $\pm$ 0.41	BS 32 55.2 $\pm$ 0.2
7	ME446 2.42 $\pm$ 0.64	BS 2 55.2 $\pm$ 0.2
8	BS 17 2.38 $\pm$ 0.86	BS 3 45.1 $\pm$ 0.0
9	BS 24 2.27 $\pm$ 0.91	BS 2.52 34.4 $\pm$ 0.1
10	BS 2.52 2.04 $\pm$ 0.40	BS 18 12.3 $\pm$ 0.1

^a Maximum biomass concentrations were measured at 120 hrs post-inoculation. ^b Maximum Poly-R478 decolourisation was quantified at 228 hrs.

4.3.2. *p*-Cresol removal

Figure 4.3 demonstrates a typical growth curve generated for *p*-cresol inoculated microtitre plate cultures. Treatment of microtitre plate cultures with sodium azide prior to the addition of pollutant inhibited further *P. chrysosporium* growth, as observed in controls for pollutant loss due to adsorption (Figure 4.3). *p*-Cresol was added to cultures at the onset of stationary growth phase to minimise growth inhibition and maximise *p*-cresol degradation. The *p*-cresol concentration selected for degradation studies were based on the IC₅₀ values previously calculated for *P. chrysosporium* strains

(section 3.3.3). In each case, 150 mg.l⁻¹ *p*-cresol caused less than 50 % inhibition of growth (except in BS 18 whose IC₅₀ for *p*-cresol = 135.6 ± 1.1). Although inhibitory to all *P. chrysosporium* strains *p*-cresol at this level was not lethal. The inhibitory effects of 150 mg.l⁻¹ *p*-cresol on actively growing *P. chrysosporium* ME446 are illustrated in Figure 4.3. However, differences in biomass levels in the presence or absence of *p*-cresol were found to be not significant [*P* > 0.05].

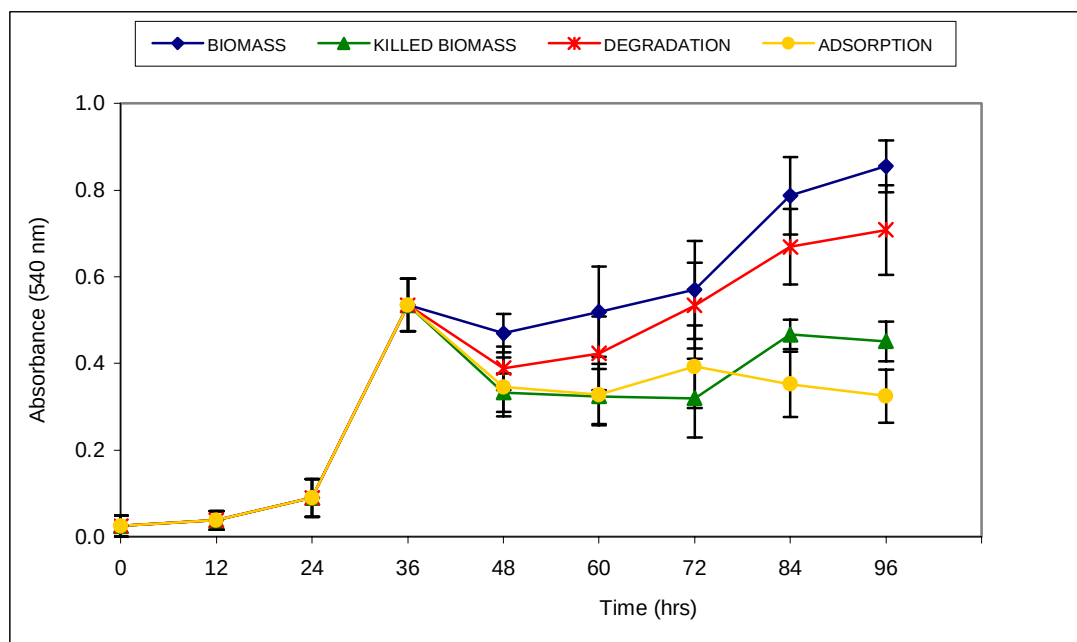


Figure 4.3: Growth curve demonstrating the effects of the addition of 150 mg.l⁻¹ *p*-cresol on *P. chrysosporium* ME446 control growth (biomass – no *p*-cresol), control killed culture (killed biomass – no *p*-cresol), adsorption control (adsorption – killed biomass with *p*-cresol) and degradation experiment (degradation – biomass with *p*-cresol). Pollutant was added to cultures 36 hrs post-inoculation and samples were assessed 96 hrs post-inoculation (60 hrs following the addition of *p*-cresol).

Complete removal of *p*-cresol by *P. chrysosporium* ME446 occurred within 60 hrs of the addition of this pollutant to microtitre cultures (96 hrs post-inoculation). *p*-Cresol removal does not infer complete mineralisation of this product. In this work, degradation products of *p*-cresol as a result of incubation with *P. chrysosporium* were not identified.

Using *P. chrysosporium* as a marker, evaluation of pollutant removal by *P. chrysosporium* strains cultured in 96-well microtitre plates was assessed 60 hrs after the addition of *p*-cresol to each culture. Distinct differences in the extent of *p*-cresol removal by each strain were detected (Table 4.2).

Table 4.2: Maximum biomass and percentage decolourisation of *p*-cresol removal, detected in 96-well microtitre plate cultures. Highlighted cells indicate *P. chrysosporium* strains showing significantly higher or lower growth kinetics than other strains.

Rank Order	Stationary phase growth ($\times 96$ hrs) in mg.ml^{-1}	Percentage <i>p</i> -cresol removal ($t = 60$ hrs)
1	BS 2 3.51 $\pm$ 0.81	ME446 100.0
2	BS 17 3.22 $\pm$ 0.58	BS 32 100.0
3	BS 2.52 3.05 $\pm$ 0.54	BS 24 93.1
4	ME446 2.97 $\pm$ 0.43	BS 2 70.5
5	BS 3 2.92 $\pm$ 0.82	BS 3 57.9
6	BS 24 2.53 $\pm$ 0.72	BS 26 45.7
7	BS 18 1.96 $\pm$ 0.67	BS 17 25.9
8	BS13 1.76 $\pm$ 0.65	BS 2.52 20.5
9	BS 26 1.57 $\pm$ 0.70	BS 13 0.0
10	BS 32 1.17 $\pm$ 0.25	BS 18 0.0

$t = 60$ hrs refers to time following the addition of pollutant to cultures (i.e. 96 hrs post-inoculation).

Greater variability in biomass formation by each *P. chrysosporium* strain was observed with *p*-cresol treated cultures than was reported for Poly-R478 decolourisation experiments (Table 4.2). Differences in biomass levels may be attributed a combination of differences in the time course that these measurements represent, and the differential inhibitory effects of 150 mg.l^{-1} *p*-cresol on *P. chrysosporium* strain growth, as reported in Chapter 3.

P. chrysosporium strains ME446, BS 32 and BS 24 appeared to have significantly higher *p*-cresol degradation rates than other *P. chrysosporium* strains, while strains BS 13 and BS 18 showed no removal of *p*-cresol from culture fluid (Table 4.2).

No correlation was detected between the ability of each strain to decolourise *p*-cresol and the amount of biomass present [$r = 0.083$, $p > 0.05$].

Differences in the degradation of *p*-cresol by the *P. chrysosporium* strains examined remained highly significant [$P < 0.001$]. Strains ME446 and BS 32 were most successful, removing 100 % of *p*-cresol from culture fluid within 60 hrs of its addition. In contrast, strains BS 13 and BS 18 showed no degradation of *p*-cresol within this time course (Table 4.2). The rank order for *p*-cresol degradation differs from that reported for synthetic lignin mineralisation (Table 4.1).

4.3.3. Cluster analysis

Cluster analysis of *P. chrysosporium* strains according to the degradative capabilities of *P. chrysosporium* strains is reported in Appendix II. Strains are clearly grouped into three clusters. But, once again, these clusters do not accurately reflect the complex response of *P. chrysosporium* strains to pollutants and their ability to degrade these pollutants. Categorical analysis was therefore undertaken as the means by which all parameters recorded for *P. chrysosporium* strains are assessed and the most significantly variable of these strains are recommended for future analysis.

4.3.4. Correlation

The measure of the relation between growth kinetics (Chapter 2), pollutant sensitivity (Chapter 3) and the degradative capabilities of *P. chrysosporium* strains, as assessed using the 96-well microtitre plate culture method, are recorded in Appendix II (Table I). Significant correlations (linear relationship) were primarily related to growth, and are described below:

1. The correlation between substrate utilisation rate (q) and the biomass yield (Y) [$r = 0.925$, $P < 0.001$] was to be expected, as the former was in fact used to calculate the latter values (Chapter 2).
2. Maximum stationary phase biomass levels were positively correlated to μ [$r = 0.7629$, $P < 0.05$].
3. Tolerance to tannic acid (high IC₅₀) was correlated to a shorter lag phase [$r = 0.7698$, $P < 0.05$] and lower stationary phase biomass levels [$r = 0.8177$, $P < 0.05$].
4. Percentage *p*-cresol removal was correlated with a shorter lag phase [$r = 0.7584$, $P < 0.05$] and lower stationary phase biomass levels [$r = 0.7803$, $P < 0.05$].
5. Percentage Poly-R478 decolourisation was correlated with earlier MnP onset [$r = 0.9689$, $P < 0.001$].
6. No correlation was observed between MnP activity and either Poly-R478 decolourisation or *p*-cresol degradation [$P > 0.95$].

4.4. DISCUSSION

Poly-R478 is a high molecular weight anthraquinone whose chemical structure includes aromatic rings, quinones, and a side chain of amines with sulphonated groups (Dawson, 1981). Poly-R478 is resistant to degradation, remaining coloured for long periods of time (Banat *et al.*, 1996, Zollinger, 1991). The overlaid spectral scans shown in Figure 4.1 demonstrate a shift in the absorbance profile of Poly-R478 during the course of the nine-day degradation experiment. The spectral scans indicate a change in the chromophore observed as colour change, but not the complete mineralisation of this compound within the time frame examined (Figure 3.2).

The inability to correlate Poly-R478 decolourisation with the amount of biomass produced by each strain suggests that dye degradation by these organisms is a function of their metabolism. The positive linear relationship described for Poly-R478 and MnP onset is misleading. While Poly-R478 decolourisation showed distinct intraspecific differences, only two MnP onset times were recorded at 60 hrs and 84 hrs (Chapter 2). Early onset (60 hrs) was observed in two strains only, ME446 and BS 18. These strains also displayed the highest and lowest Poly-R478 decolourisation, respectively (Table 4.2). No relationship was observed between dye decolourisation and MnP activities, suggesting further mechanisms and pathways are involved in its degradation. The presence of low levels of LiP facilitating degradation under atmospheric conditions cannot be excluded at this later stage of culture growth. However, no correlation was found previously between LiP and synthetic lignin degradation (Raeder *et al.*, 1989b). The rank order for dye decolourisation does not correspond with that reported for synthetic lignin degradation (Table 4.1 and 4.2).

It was previously noted that one of the main limitations of the 96-well microtitre plate method was the extent of evaporation of culture fluid from peripheral wells (Chapter 2). Thus, the eleven-day experimental period that seemed necessary to detect the decolourisation of 0.02 % Poly-R478 was disadvantageous.

The use of *p*-cresol as a model pollutant in examining the degradation capabilities of *P. chrysosporium* strains overcame this problem. *p*-Cresol degradation occurs in the presence of both LiP and MnP within five days of its addition to cultures (Kennedy and Lema, 1994). Our results showed certain strains removed *p*-cresol from microtitre plate cultures within 60 hrs of its addition. Intraspecific variability in the degradation capabilities of *P. chrysosporium* strains was distributed over a broad range, with certain strains facilitating 100 % removal while others had no effect on the *p*-cresol concentration in culture fluid. No correlation was found between *p*-cresol removal and an organism's sensitivity to this pollutant. Interestingly, *p*-cresol degradation was correlated with shorter

lag phase and lower levels of stationary phase biomass. Tolerance to tannic acid was also correlated with shorter lag phase and lower levels of stationary phase biomass, suggesting the mechanisms of tolerance to tannic acid and degradation of *p*-cresol might be linked.

The rank order for *p*-cresol degradation differs from that for Poly-R478 decolourisation (Table 4.1 and 4.2). This observation is supported by Tekere *et al.*, (2002) who showed that good lignin degradation does not always coincide with good dye degradation. Further, the rank order for *p*-cresol degradation does not correlate with the rank order previously reported for synthetic lignin degradation (Wyatt and Broda, 1995).

4.5. CONCLUSION

Microtitre-based culture methods introduced in Chapters 2 and 3 were successfully applied to the analysis of the degradation capabilities of *P. chrysosporium* strains. The standard deviations observed for replicate samples in both Poly-R478 decolourisation and *p*-cresol removal experiments, when using the microtitre plate method, was far lower than the 65 % variation previously reported for synthetic lignin (DHP) degradation by *P. chrysosporium* strains (Wyatt and Broda, 1995). These lower standard deviations allowed the quantitative assessment of *P. chrysosporium* strains.

Late onset in the decolourisation of the recalcitrant, high molecular weight Poly-R478 was unfavourable. While significant intraspecific differences in Poly-R478 decolourisation were detected between *P. chrysosporium* strains, evaporation at the 37 °C incubation temperature did not allow for the extension of time course studies. In contrast, the rapid removal of *p*-cresol from culture fluid and the broad range observed in intraspecific degradation of this compound support its use as a model compound for further elucidating the mechanisms of biodegradation by these *P. chrysosporium* strains.

Significant correlations were detected between tannic acid sensitivity and *p*-cresol degradation and *P. chrysosporium* strain growth. However, no correlation was found between enzyme production and pollutant tolerance or the biodegradation capabilities of *P. chrysosporium* strains. Only strains ME446 and BS 18 showed consistently high and low, respectively, dye decolourisation and *p*-cresol degradation capabilities. Categorical assessment of the degradation capabilities of *P. chrysosporium* strains (see Chapter 8) recommends these strains for future characterisation and genetic linkage studies.

SECTION B:

NOVEL MICROBIOLOGICAL TOOLS.

**INTRODUCING AND EVALUATING THE POTENTIAL OF MEMBRANE
BIOREACTOR TECHNOLOGIES IN LINKING MYCELIAL
DIFFERENTIATION AND SECONDARY METABOLITE PRODUCTION IN
PHANEROCHAETE CHRYSOSPORIUM.**

CHAPTER 5

Using the Gradostat Concept as a Tool for Biofilm Analysis

5.1. INTRODUCTION

The 96-well microtitre plate liquid culture system has proven advantageous in assessing the growth kinetics and biodegradation capabilities of sibling strains (Chapters 2-4). The inability to correlate physiological traits such as enzyme activity with the degradation capabilities of sibling strains; and the correlation of pollutant sensitivity and degradation with growth; implies the need for alternative systems that can be used in further assessing the role of morphological and metabolic traits associated with biodegradation in these organisms.

The immobilisation of *P. chrysosporium* using solid substrate fermentations and in various bioreactor configurations has proven beneficial in maximising both lignolytic enzyme production and the biodegradation capabilities of this organism. The membrane gradostat bioreactor, introduced in section 1.3.3.8 has been suggested as a novel tool in characterising intraspecific differences in biofilm formation and in describing the role of mycelial differentiation in pollutant and lignin biodegradation under, controlled conditions.

Unpublished data has shown the gradostat principle of the aforementioned bioreactor to be suited to idiophasic growth and complex biofilm formation by *P. chrysosporium* (Leukes, 1999). The regulation of lignolytic enzyme biosynthesis is itself a complex process, which is not co-ordinately regulated in response to stress. Promoters of the different peroxidase isoenzyme genes have been found to respond differently to internal signals (section 1.3.2.7). By maintaining lignolytic fungi such as *P. chrysosporium* in a state of complex morphological development, namely the biofilm obtained under gradostat conditions, it is hypothesised that a greater degree of complex signals could be achieved and maintained, allowing for the production of a larger suite of peroxidase isoenzymes with more efficient biodegradation potential.

Previous experiments undertaken with *P. chrysosporium* showed enhanced mycelial differentiation of this organism when immobilised in a gradostat membrane bioreactor system. Biofilm growth within the macrovoids of the membrane and around the capillary wall is distinct and can be further subdivided into zones whose hyphal morphology differs in appearance (Leukes, 1999).

Penetrative hyphae have been likened to leading hyphae, as described by Steele and Trinci (1975), while the biofilm around the capillary wall showed characteristics visualised during mycelial growth

in liquid culture (Leukes, 1999). The nature of the mycelia penetrating the macrovoids has since become of interest, being likened to mycelial cords or rhizomorphs observed in filamentous fungi (Figure 5.1). What appear to be chlamydospore-like fruiting structures (section 1.3.1) have also been observed in these penetrative “cords” (Figure 5.1.b and c) (Walsh, 1998).

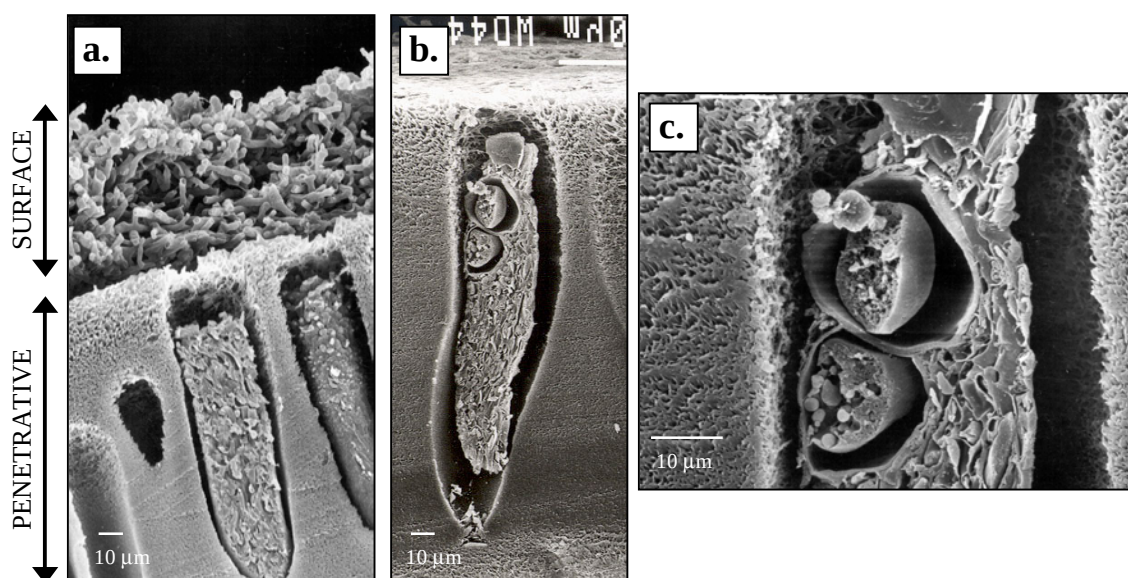


Figure 5.1: Scanning electron micrographs of a *P. chrysosporium* biofilm immobilised on a capillary membrane, showing (a.) penetrative and covering/surface biofilm zones and (b.) chlamydospore-like fruiting structure observed within the differentiated, rhizomorph-like penetrative growth zone. (c.) A closer view of the same fruiting structure revealing its densely granular contents (Walsh, 1998).

5.1.1. Using the gradostat concept as a tool for examining the role of mycelial differentiation in secondary metabolism

The nature of the biofilm generated by *P. chrysosporium* when cultured using the gradostat membrane bioreactor system is regulated by the oxygen and/or nutrient gradients imposed on the mycelia across the membrane (Figure 5.2).

In light of the increased productivity of this system in comparison to alternative fermentation technologies (section 1.3.3.7.3) it was proposed that this differentiation plays an integral role in the production and/or secretion of secondary metabolites. The peroxidase enzymes produced by *P. chrysosporium* as secondary metabolites are implicated in lignin and pollutant degradation. It was proposed that by culturing *P. chrysosporium* as a differentiated biofilm, resembling saprophytic growth, distinct hyphal morphologies might be linked to peroxidase production.

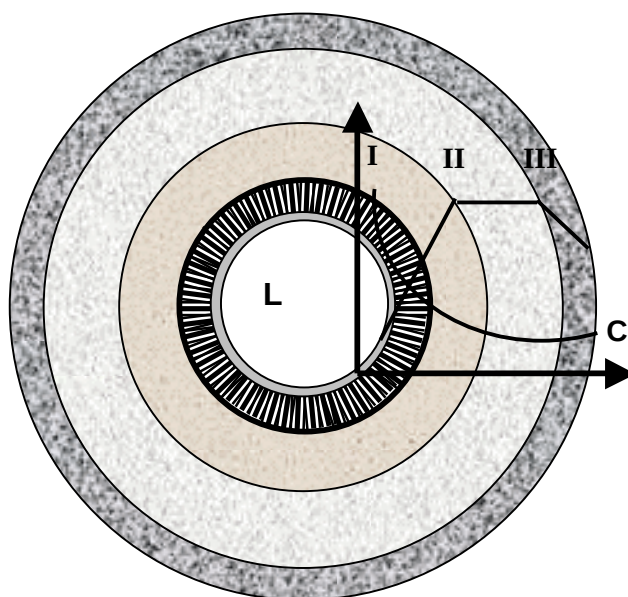


Figure 5.2: Schematic representation of the membrane gradostat concept with superimposed batch culture growth curve. (I) Primary growth phase, (II) stationary phase, (III) decline phase (blue). L is the lumen of the fibre from which the growth medium is supplied. X is radial distance from the lumen in the direction of nutrient flow. C is the concentration of growth-limiting substrate (Leukes, 1999).

Fungal differentiation is closely correlated to metabolism and forms an important role in foraging or resource location and utilisation. Observations into the extent of differentiation observed in gradostat bioreactors introduces the concept of using this system as a tool for examining those factors inducing mycelial differentiation within biofilms and the correlation of this differentiation with productivity.

A mutant strain, *P. chrysosporium* mutant strain INA-12, was isolated through mutation selection programmes (Buswell *et al.*, 1984). This organism is reported to be better suited to immobilisation than the wildtype strain BKMF-1767 (Buswell *et al.*, 1984, Capdevila *et al.*, 1989, Bonnarme and Jeffries, 1990, Asther *et al.*, 1988a, 1988b). The inclusion of differentiation and biofilm formation capabilities into an informed strain improvement programme would enable the screening and selection of strains better suited to immobilisation.

5.1.2. Single-fibre membrane gradostat bioreactor

The principal undertaking of this thesis was the comparison of genetically related *P. chrysosporium* strains as a strain improvement strategy. Due to the high variability observed with *P. chrysosporium* growth in liquid culture systems, the use of the gradostat system as a tool for the identification of intraspecific differences in the morphological differentiation and its influence on physiological activity was evaluated.

The feasibility of the single-fibre membrane bioreactor system as a tool is dependent on its reliability. A number of operational conditions were therefore evaluated in order to determine the most robust system for *P. chrysosporium* ME446 growth. Optimal conditions were regarded as those providing the least variability in biofilm formation in addition to high yield secondary metabolite production, in the form of peroxidase enzymes, under nitrogen-limiting conditions. Selected parameters were thus applied to intraspecific comparison of *P. chrysosporium* strains.

5.1.3. Aims

The potential of the capillary membrane gradostat bioreactor for the analysis of morphological and biochemical differentiation of *P. chrysosporium* biofilms is investigated. This chapter serves to:

1. Establish stable, reproducible operational conditions for the single-fibre membrane gradostat bioreactor.
2. Evaluate tissue formation and mycelial differentiation in *P. chrysosporium* biofilms.
3. Compare the extent of mycelial differentiation and biofilm formation in *P. chrysosporium* sibling strains using single-fibre membrane gradostat bioreactor modules.

5.2. MATERIALS & METHODS

5.2.1. Single-fibre membrane gradostat bioreactor design

The single-fibre reactor (SFR) (manufactured by Glass Chem C.C., Cape Town) was designed for ease of both inserting the membrane length into the glass manifold following capillary testing and the intact removal of the biofilm-coated membrane for dissection and analysis.

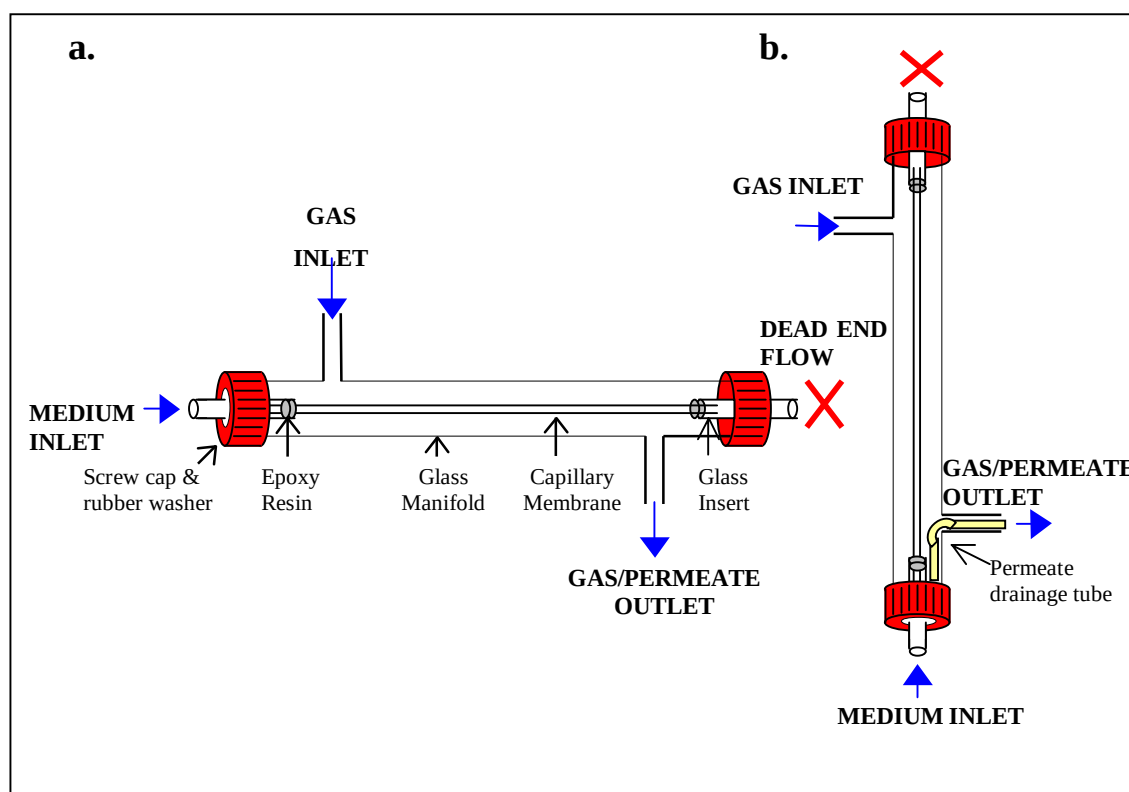


Figure 5.3: Single fibre bioreactor design in (a.) horizontal and (b.) vertical orientation.

Each reactor consists of a cylindrical glass manifold (180 mm x 20 mm o.d. x 16 mm i.d.) with gas inlet and outlet vents (40 mm x 8 mm o.d. x 5 mm i.d.) positioned 30 mm from each end, on opposite sides of the manifold (Figure 5.3). Each end of the manifold is threaded and sealed with a polypropylene screw cap. A 160 mm-long capillary membrane was sealed at each end within a cylindrical glass insert, providing an “active” membrane length of 130 mm, such that fluid may flow through the glass insert and membrane lumen unchallenged. The membrane was suspended within the glass manifold as each of the inserts fed through and was supported by a rubber washer creating an airtight seal when the cap was tightened (Figure 5.3). These washers also allowed the membrane to be maintained taught and central to the manifold even when saturated or weighed down with biomass.

When SFRs were orientated in a horizontal position, permeate drainage occurred simply through the gas outlet vent (Figure 5.3.a). However, when in vertical orientation bioreactors were modified by the addition of a narrow tube extending to the base of the manifold, thus facilitating drainage of any accumulating permeate due to the positive pressure of airflow through the manifold (Figure 5.3.b).

5.2.2. Capillary membrane testing

The externally skinned polysulphone capillary membranes with which gradostat bioreactors are constructed, were supplied by the Institute for Polymer Research, Stellenbosch University, Stellenbosch. Previous experiments showed that the variability observed between sets of bioreactors was largely due to differences in membrane flux. In order to standardise this variable, all membranes were screened using permeability and integrity tests described below. For a given experiment, membranes of similar flux were selected for each of the bioreactor modules.

5.2.2.1. Membrane permeability

In order to determine whether the flux distribution along the capillary length and between membranes was consistent, membranes were secured across a compartmentalised permeate collecting tray and operated under dead-end flow using a Watson Marlow peristaltic pump. Tests were undertaken at room temperature, using water at a standardised flow rate over a period of 30 min. Permeate from discrete sections of the membrane were simply measured and local membrane flux expressed in $\text{ml.cm}^{-2}.\text{min}^{-1}$.

5.2.2.2. Membrane integrity

Membrane integrity testing was undertaken using the Bubble Point Test (Noble and Stern, 1995). The process involves the forcing of gas through the lumen of a completely wetted membrane, under pressure, to a point such that when the membrane is submerged, a continuous stream of bubbles appears which corresponds to the largest pore in the membrane.

Following membrane permeability tests, the water-saturated membranes were attached to a nitrogen gas cylinder and submerged in a basin of water. The remaining water in the membrane lumen was forced out and the gas pressure was gradually increased to approximately 150 kPa at which point the membranes were screened for bubble streams. The pressure was monitored using a digital manometer. The presence of bubbles at 150 kPa is indicative of the presence of pinholes or pore sizes greater than 1 μm diameter in the membrane wall. Such membranes when found were discarded, as pinholes would allow for the transfer of inoculum into the membrane lumen.

5.2.3. Sterilisation and inoculation

Bioreactors were sterilised overnight with a 4 % solution of formaldehyde. Formaldehyde was thoroughly rinsed from the membranes and manifolds using sterile distilled water prior to inoculation. Humidifiers were autoclaved, as were permeate collection vessels.

Optimisation of membrane bioreactor conditions was undertaken with *P. chrysosporium* ME446. SFRs were inoculated with a spore suspension (Appendix I:B) at a concentration of 1×10^5 spores per cm^2 membrane.

5.2.4. Operating conditions

5.2.4.1. Experiment 1: Orientation and flow rate

The purpose of these experiments was to evaluate those operational conditions inducing both the most consistent *P. chrysosporium* biofilm structure and extracellular peroxidase production. Major growth altering factors such as medium flow rate and oxygenation conditions were examined in addition to the role of reactor orientation, direction of feed supply in vertical reactors and length of adhesion period during inoculation. Medium was supplied with a Watson Marlow peristaltic pump using 0.015 ml.mm^{-1} accurated PVC tubing (AEC-Amersham).

Sixteen SFR modules were used to examine the effects of low ($\pm 0.01 \text{ ml.cm}^{-2}$ membrane) or high ($\pm 1 \text{ ml.cm}^{-2}$ membrane) medium flow rates and vertical or horizontal orientations (see Figure 5.3) on biofilm formation and enzyme production. Four sets of four modules were constructed in parallel and operated under VERTICAL/LOW, VERTICAL/HIGH, HORIZONTAL/LOW or HORIZONTAL/HIGH operating conditions. This SFR rig is pictured in Figure 5.4.

SFR modules were each inoculated with *P. chrysosporium* ME446. Vertical SFRs were fed with basal III medium (Appendix I:A-2) from below and all modules were aerated at approximately 20 kPa by diaphragm pumps (Marltons). Oxygen supply to SFR reactor sets was regulated by needle flow control valves (Festo, GR-M5B) equilibrated with a digital manometer. All SFR modules were operated at low medium supply rates for the first three days of growth phase in order to facilitate spore germination and attachment, preventing biomass washout.

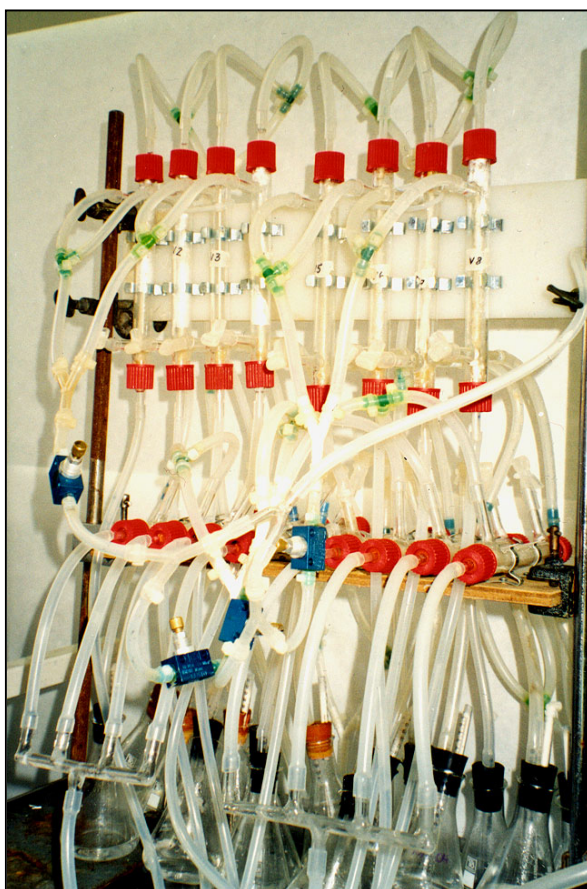


Figure 5.4: Showing the SFR rig as used for orientation and flow rate experiments. Reactor modules, in quadruplicate, were run as: horizontal/slow flow rate, horizontal/fast flow rate, vertical/slow flow rate or vertical/fast flow rate. Air was distributed to each reactor set through needle valves regulating the airflow rate.

5.2.4.2. Experiment 2: Aeration and direction of medium flow

SFR modules were configured as above. Vertical SFRs were fed with basal III medium (Appendix I:A-2) from above. All modules were aerated at approximately 20 kPa by diaphragm pumps (Marltons) for the first three days after which they were supplied with pure oxygen at the same flow rate. Air/oxygen supply to SFR modules were regulated by needle flow control valves (Festo GR-M5B) equilibrated with a digital manometer. All SFR modules were operated at low medium supply rates for the first three days of growth phase in order to facilitate spore germination and attachment, preventing biomass washout.

5.2.4.3. Tissue formation and mycelial differentiation

The operational conditions selected for biofilm analysis using SFRs were optimised as described in sections 5.2.4.1 and 5.2.4.2. Those operational criteria selected for subsequent experiments were as follows:

1. Reactors were configured in a vertical orientation.
2. Constant medium supply was from below.
3. Greater MnP activity was detected at lower flow rates.
4. Constant aeration stimulated a more constant and healthier biofilm.

SFRs operated for a ten-day period were dissected and processed for SEM and TEM or stored at -70°C for subsequent analysis.

5.2.4.4. Intraspecific comparison of biofilms

SFRs were inoculated in duplicate with standardised spore inocula (Appendix I:B) prepared for each *P. chrysosporium* strain and cultured according to operational conditions described in section 5.2.4.3.

5.2.5. Flow rate

Membrane flux was recorded daily by measuring the permeate volume obtained overnight from each SFR module. Medium flow rate was calculated in $\text{ml}\cdot\text{min}^{-1}$ ($n = 4$). The percentage variation of the standard deviation from the mean flow rate was used to evaluate the consistency of each operational condition.

5.2.6. Substrate utilisation

Glucose and NH_4^+ concentrations of permeate collected daily from each SFR module ($n = 4$) were assayed using the Nelson-Somogyi method (Appendix I:C-1) and a modified Nessler reaction (Appendix I:C-2), respectively.

5.2.7. Enzyme activities

The pH and extracellular LiP and MnP activities of permeate collected from SFRs were recorded daily ($n = 4$). LiP and MnP activities were determined using veratryl alcohol oxidation (Appendix I:D-1) and DMAB/MBTH oxidation methods (Appendix I:D-2.2) using a microplate reader.

5.2.8. Electron microscopy

Twelve days post-inoculation, reactors were sacrificed. Sections at 4 cm intervals along the reactor length were fixed overnight in phosphate-buffered glutaraldehyde and prepared for SEM (Appendix I:E-1) or TEM (Appendix I:E-2).

5.2.9. Image analysis

Electron micrograph images were scanned and saved as TIF files. These images were analysed using the image processing and analysis software package ImageJ, downloaded from the internet (<http://rsb.info.nih.gov/ij/index.html>). Total biofilm area was quantified for each SFR module, from cross-section images of membrane biofilms sectioned 1 cm, 6 cm and 10 cm from medium feed, using the area calculator tool (<http://rsb.info.nih.gov/ij/plugins/area.html>). Mean biofilm radii for each section were calculated by measuring biofilm radius in perpendicular quadrants ($n = 4$).

RESULTS

5.3.1. Membrane integrity and permeability

Those membranes selected for use in SFR experiments were not compromised by pinholes or defects as determined by the Bubble Point Test (section 5.2.2.2).

Capillary membrane permeability was found to differ both between and within manufactured batches. Variability within the single batch used for these experiments was determined to be approximately 10 % ($n = 50$). Flux variability in SFR operating conditions was reduced by selecting those membrane inserts with similar flux characteristics (Figure 5.5).

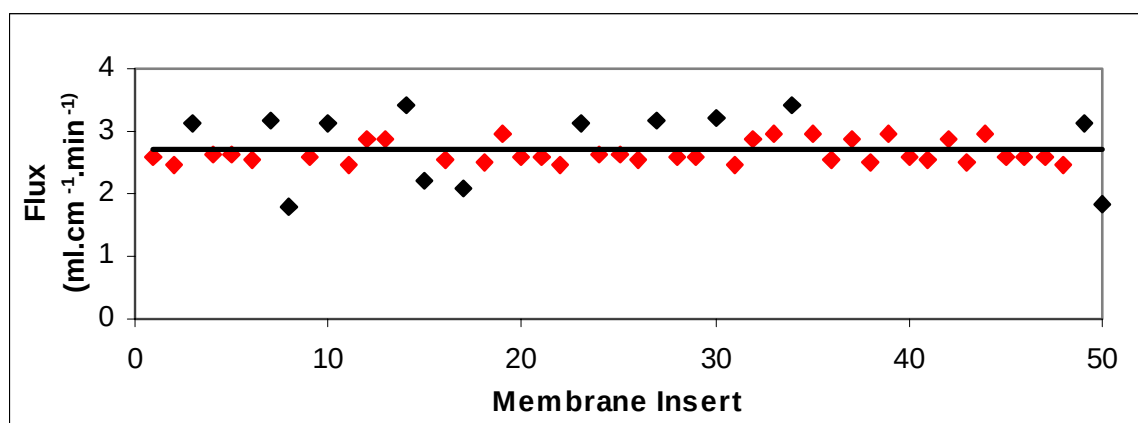


Figure 5.5: Scatter-plot showing membrane permeability measurements obtained while screening SFR inserts for variability. SFR inserts tested showed 9.81 % variability in flux. ($n = 50$). Membranes corresponding with outlying points were discarded.

5.3.2. Experiment 1: Orientation and flow rate

The daily flow rate measurements for each experimental condition operated in parallel ($n = 4$), namely VERTICAL/LOW, VERTICAL/HIGH, HORIZONTAL/LOW and HORIZONTAL/HIGH, are recorded in Figure 5.6.a. Mean flow rates for each sample group were calculated over the seven day period during which high/low medium flow rate conditions were operated (Table 5.1; Figure 5.6.a). There appears to be no correlation between the high percentage variation observed within a sample group and its operating conditions.

Table 5.1: Mean flow rates ($\pm$ SD) as calculated for each SFR operating condition from days 3-10 under high/low operating conditions ($n = 4$).

Operating Condition	Flux (ml.min^{-1})	% Variation
VERTICAL/LOW	4.77 ± 1.67	35.09
VERTICAL/HIGH	25.85 ± 5.26	20.35
HORIZONTAL/LOW	6.68 ± 1.33	19.94
HORIZONTAL/HIGH	23.63 ± 7.26	30.73

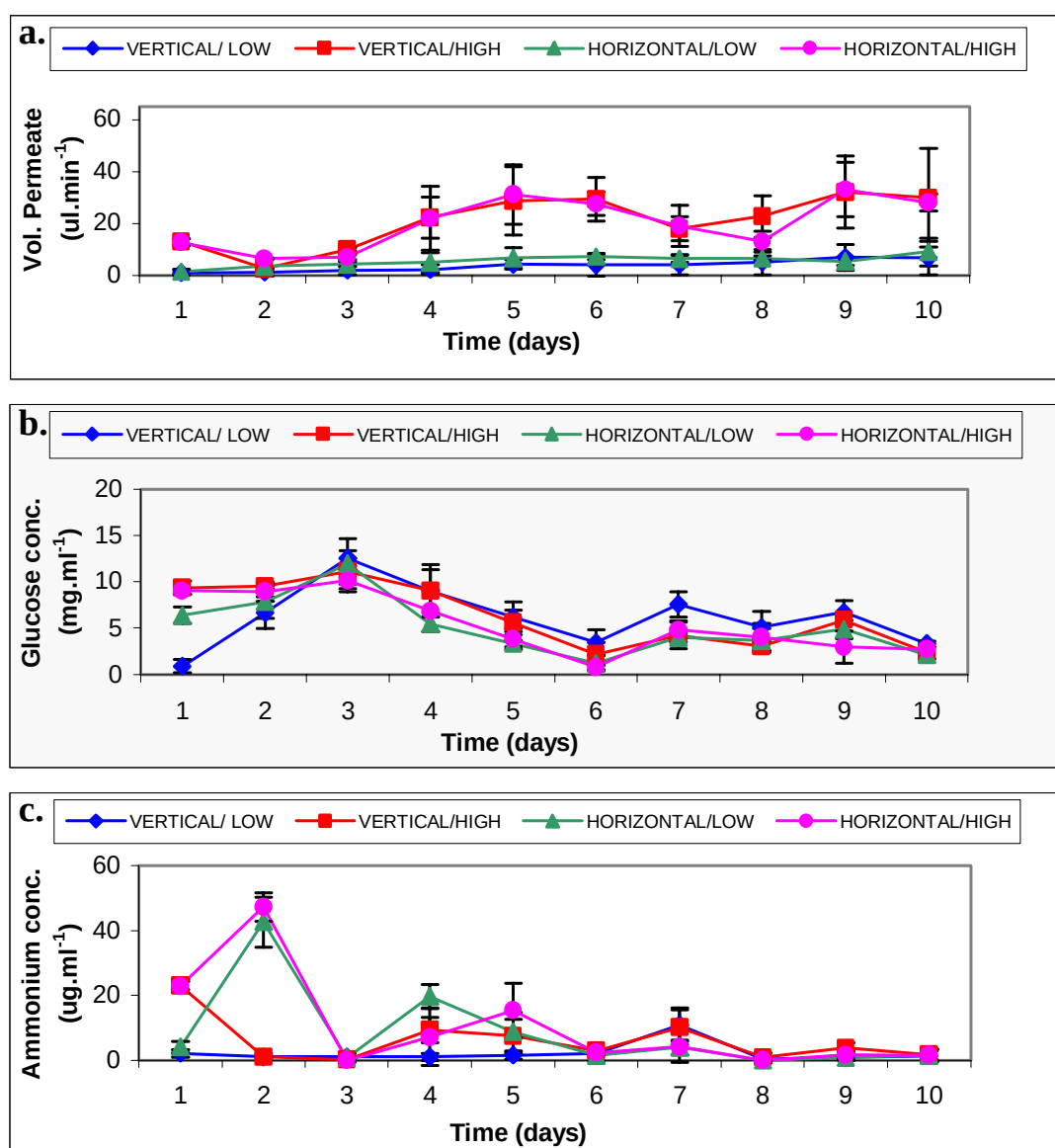


Figure 5.6: Time course showing the mean ($\pm$ SD) flow rates (a.) glucose concentrations (b.) and NH_4^+ concentrations (c.) measured in the permeate collected from membrane reactor modules ($n = 4$) when operated at low or high flux in either vertical or horizontal orientation

5.3.2.1. Influence on substrate utilisation

Glucose (Figure 5.6.b) and NH_4^+ (Figure 5.6.c) levels remaining in permeate collected from each SFR module were analysed and time course profiles generated. Differences in glucose and NH_4^+ levels detected between SFR operational conditions on days 1-2 were exaggerated by the differential dilution of feed medium with residual sterile distilled water remaining from the sterilisation procedure. Upon the induction of high flow rates from day 3, substrate utilisation profiles followed similar trends and did not differ significantly between operating conditions. Only nutrient nitrogen became growth limiting, with the lowest NH_4^+ levels consistently detected in the VERTICAL/LOW SFR modules (Figure 5.6.c).

5.3.2.2. Effect on extracellular enzyme production

Daily pH levels of permeate collected from each SFR operational condition ($n = 4$) are shown in Figure 5.7.b. Supernatant pH profiles were similar for all sample groups, showing a reduction of 0.5-1 pH units two to three days post-inoculation, followed by the onset of MnP activity.

Maximum MnP activities were detected on days 5-6 in VERTICAL/LOW SFR modules. MnP activity in HORIZONTAL/LOW SFR modules peaked on days 6-7 and did not achieve the same high level as seen with vertical modules. The onset of MnP activities in high flow rate modules was delayed by three to four days and did not achieve levels as high as low flow rate modules. LiP activity was not detected under any of the SFR operational conditions examined.

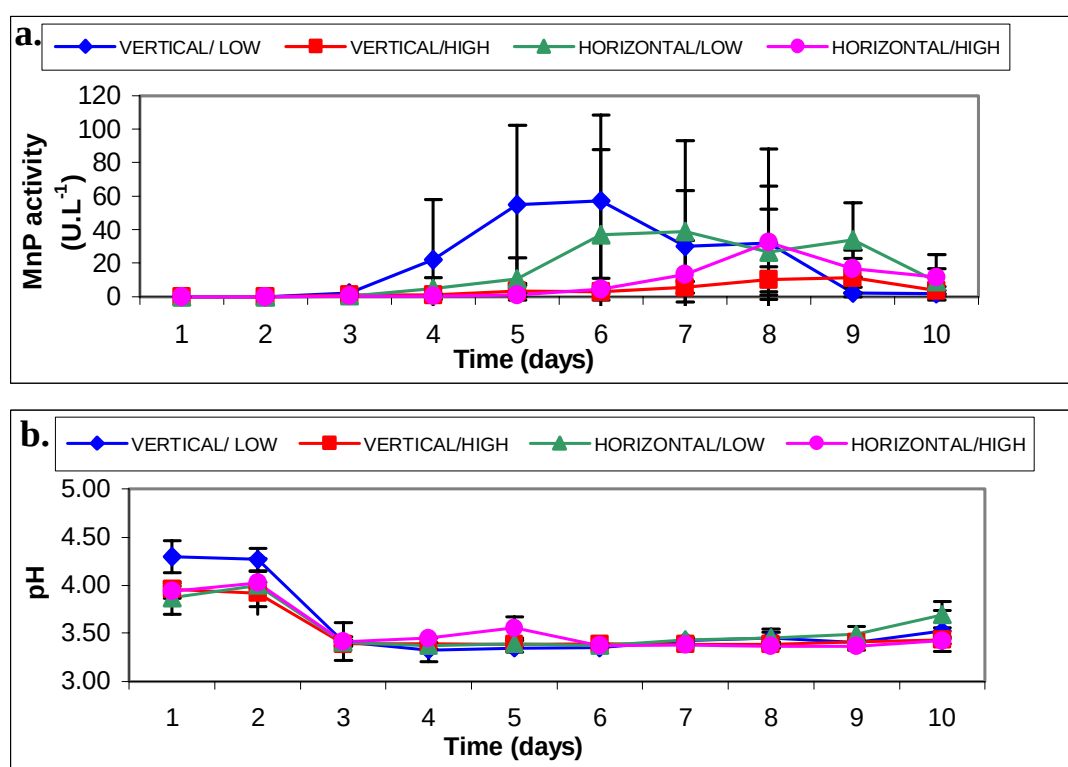


Figure 5.7: Time course showing manganese peroxidase activities (a.) and pH profiles (b.) of extracellular culture fluid collected from membrane reactor modules ($n = 4$) when operated at low or high flux in either vertical or horizontal orientation

5.3.2.3. Influence on biofilm formation.

Differences in SFR operational condition regulated biofilm formation were easily observed (Figure 5.8). High flow rates sustained the development of a thicker biofilm than observed at lower flow rates, while horizontal orientation sustained biofilm formation along the entire membrane length. However, horizontal biofilms were irregularly formed while vertical modules showed more uniform, cylindrical biofilm formation (Figure 5.8). Vertical orientation induced tapering biofilm growth away from medium fed from below. These characteristics were confirmed by electron micrograph image analysis (Figure 5.9).

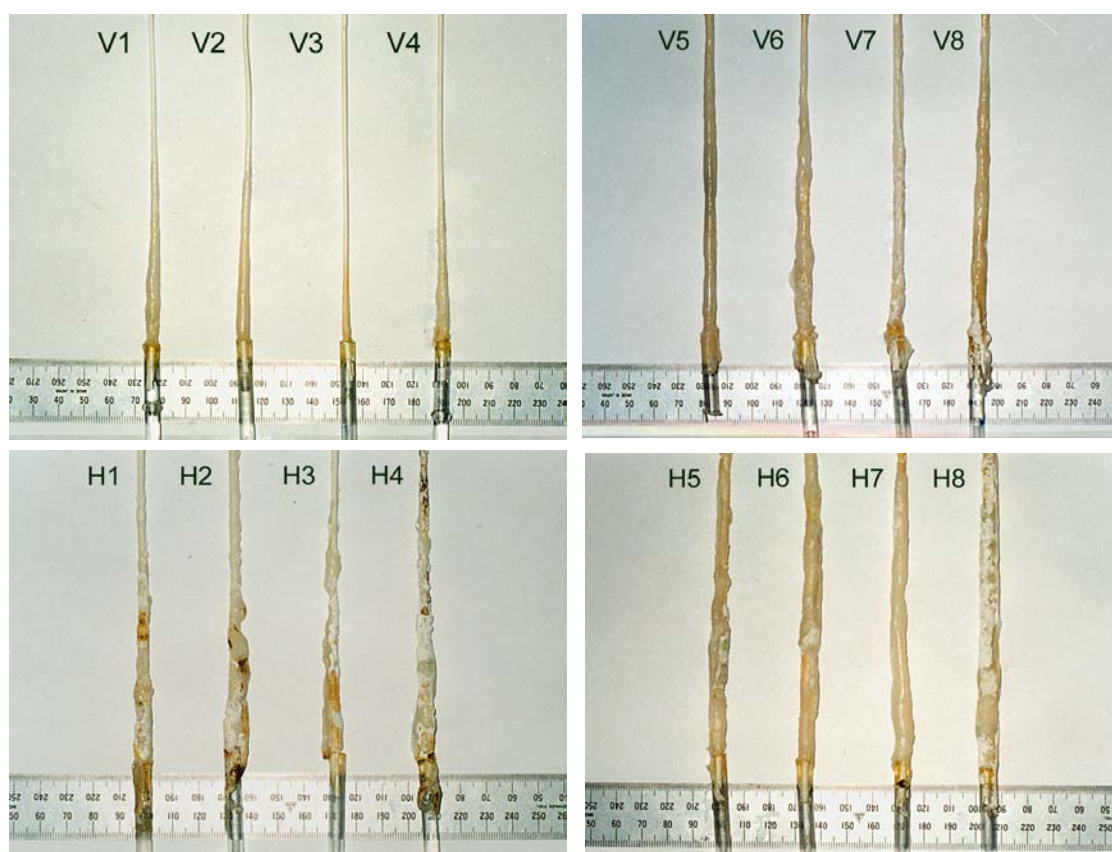


Figure 5.8: Biofilm formation visualised using the single-fibre rig pictured in Figure 5.4, operated in either vertical [V] or horizontal [H] orientation, under low (1-4) or high (5-8) flow rates. Air was distributed to each reactor using a single diaphragm air pump at approximately 20 kPa.

Significant differences in the extent of biofilm formation was observed under different SFR operational conditions (Figure 5.10). While all sample groups showed a progressive reduction in total biofilm when moving away from the nutrient inlet this was less apparent in horizontal modules.

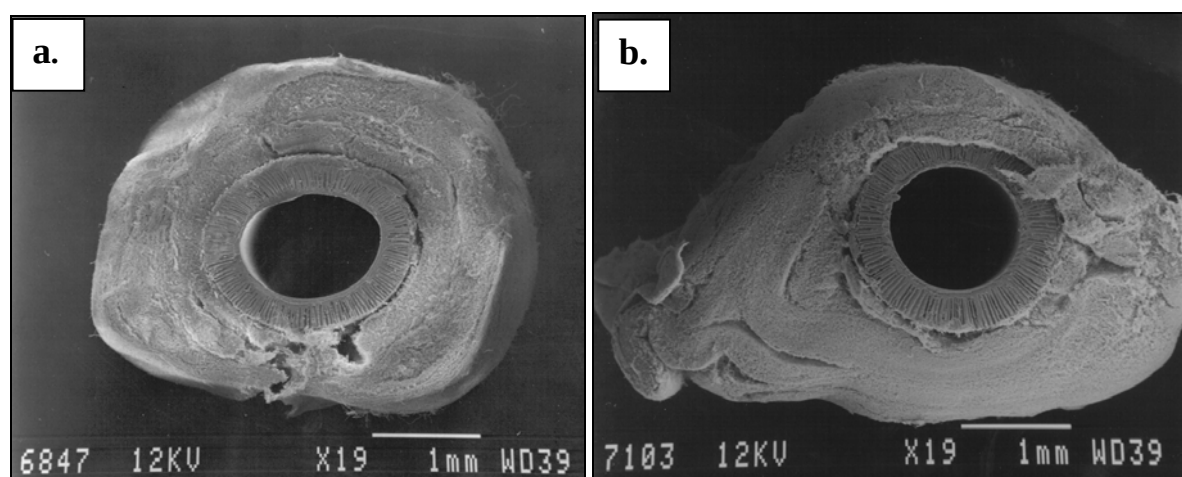


Figure 5.9: Typical biofilm formation in SFR modules grown under vertical or horizontal orientations. Electron micrograph showing cross section of (a.) vertical SFR V7, and (b.) horizontal SFR H5, sectioned 6 cm from medium inlet. Both SFR modules were cultured under high flow rate conditions. Magnification X19.

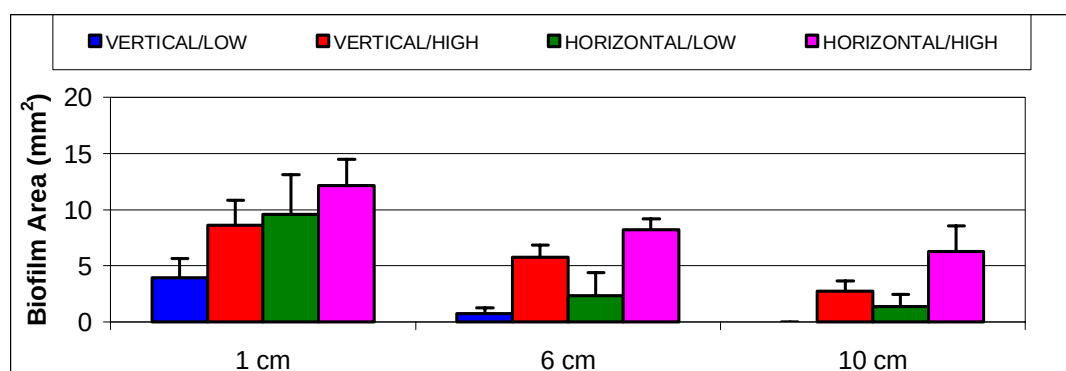


Figure 5.10: Mean biofilm area (mean $\pm$ SD) was calculated from electron micrograph images using ImageJ. Cross-sections of membrane reactor biofilm were analysed at distances of 1 cm, 6 cm and 10 cm away from the nutrient feed, for each module ($n = 4$) operated under low or high flux in either vertical or horizontal orientation.

5.3.3. Experiment 2: Medium supply and aeration

Time course profiles of flow rate measurements for each experimental condition are recorded in Figure 5.11.a. Mean flow rates for each condition, calculated from days 2-10 during high/low flow rate operational conditions, showed greater variation between sample groups than was seen for Experiment 1 (Table 5.2, Figure 5.11.a). This is primarily due to a reduction in flow rates on days 4–5

(Figure 5.11.a). As observed previously (section 5.3.2) there seems to be no correlation between percentage variation and operational condition. However, the extent of variation did appear to be consistent between experiments (Table 5.1 and 5.2).

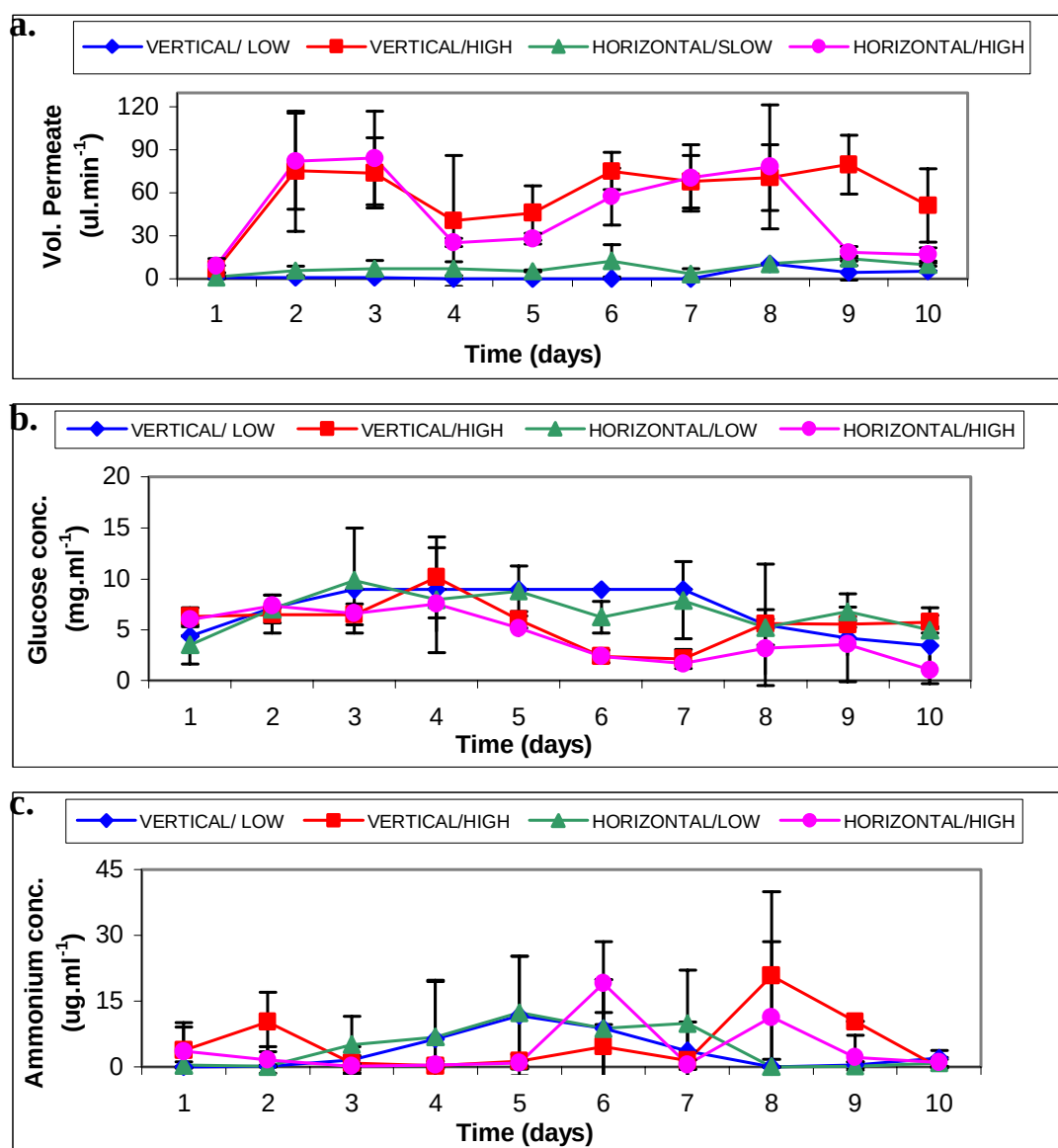


Figure 5.11: Time course showing the mean ($\pm$ SD) flow rate (a.) glucose concentration (b.) and NH_4^+ concentration (c.) measured in the permeate collected from membrane reactor modules ($n = 4$) when operated at low or high flux in either vertical or horizontal orientation

Table 5.2: Mean flow rates ($\pm$ SD) as calculated for each SFR operating condition (n = 4).

Operating Condition	Flux (ml.min⁻¹)	% Variation
VERTICAL/LOW	0.58 $\pm$ 0.82	141.1
VERTICAL/HIGH	42.10 $\pm$ 10.33	24.54
HORIZONTAL/LOW	3.70 $\pm$ 1.24	33.49
HORIZONTAL/HIGH	33.84 $\pm$ 20.22	59.74

5.3.3.1. Influence on substrate utilisation

Time course profiles showing glucose and NH_4^+ levels in permeate collected from each SFR module are shown in Figure 5.11.b and c, respectively. The low glucose and NH_4^+ levels detected in VERTICAL/LOW modules are a reflection of the very low flow rates observed in these modules (Figure 5.11, Table 5.2). Glucose consumption was more apparent in high flow rate sample groups than in the low flow rate modules (Figure 5.11.b). NH_4^+ levels were more variable (Figure 5.11.c).

5.3.3.2. Effect on extracellular enzyme production

Daily pH levels of permeate collected from each SFR operational condition (n = 4) are shown in Figure 5.12.b. Supernatant pH profiles were variable and showed no distinct trend.

While the onset of MnP activity was detected from day 4 in all SFR modules, an increase in MnP levels was detected only in high flow rate modules (Figure 5.12.a). Elevated MnP activity levels were detected in HORIZONTAL/HIGH and VERTICAL/HIGH SFR modules from days 7 and 10, respectively. While the onset of MnP activities in high flow rate modules paralleled those observed in Experiment 1, MnP activities recorded in low flow rate SFR modules were maintained at minimum levels. Further, in comparison to those MnP levels recorded under similar operational conditions in Experiment 1, high flow rate modules showed basal MnP activities. LiP activity was not detected under any of the SFR operational conditions examined.

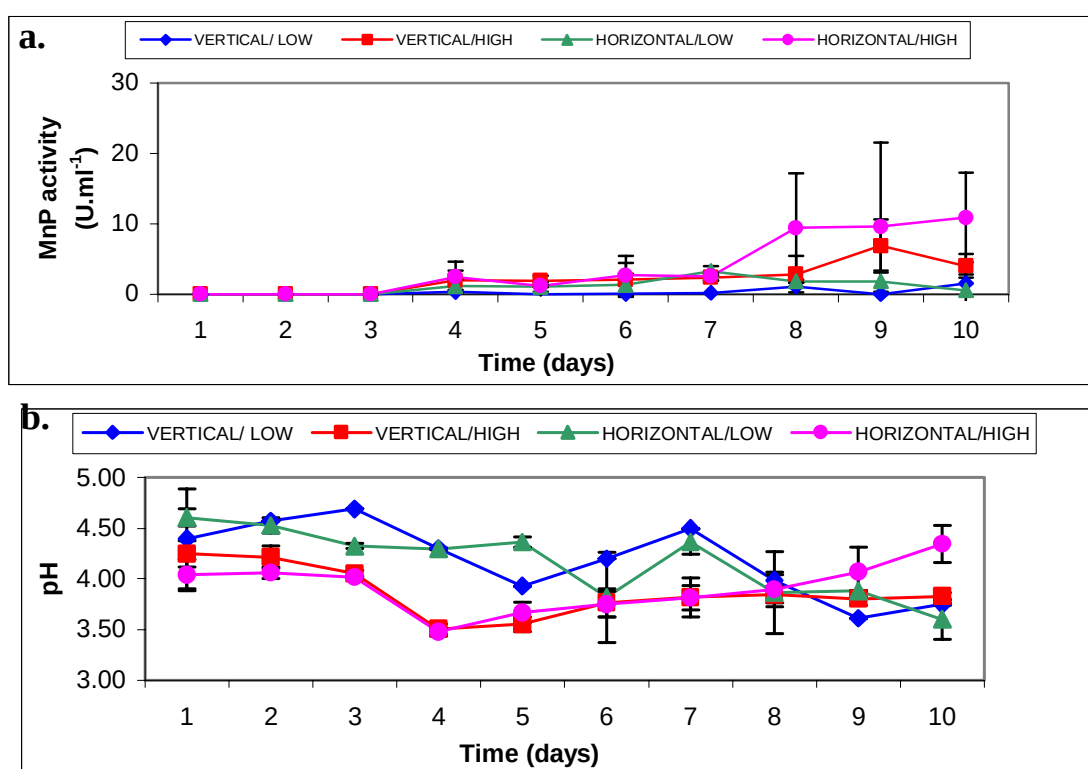


Figure 5.12: Time course showing (a.) pH profile and (b.) extracellular manganese peroxidase (MnP) activity detected in permeate collected from membrane reactor modules ($n = 4$) when operated at low or high flux in either vertical or horizontal orientation.

5.3.3.3. Influence on biofilm formation

Biofilm formation was similar to that described in section 5.3.2.3. High flow rates sustained the development of a thicker biofilm on the outer surface of capillary membranes while biofilm formation under low flow rate conditions was minimal. This was attributed to the interruption of flow (Figure 5.11.a) at low flow rates by the accumulation of air bubbles within medium inlet tubes. Under the operational conditions examined, oxygenation of cultures did not appear to have a stimulatory effect on enzyme production, possibly also a feature of poorer biofilm formation under hypervariable flow rates.

5.3.4. Tissue formation and mycelial differentiation in SFRs

Biofilm formation in *P. chrysosporium* begins with the germination of spores distributed across the membrane surface and within macrovoids following inoculation by reverse flow procedures. Exploratory mycelia produced by germinating spores extend across the membrane surface forming a network of interlinking hyphae that firmly attaches the organism onto the capillary membrane (Figure 5.13).

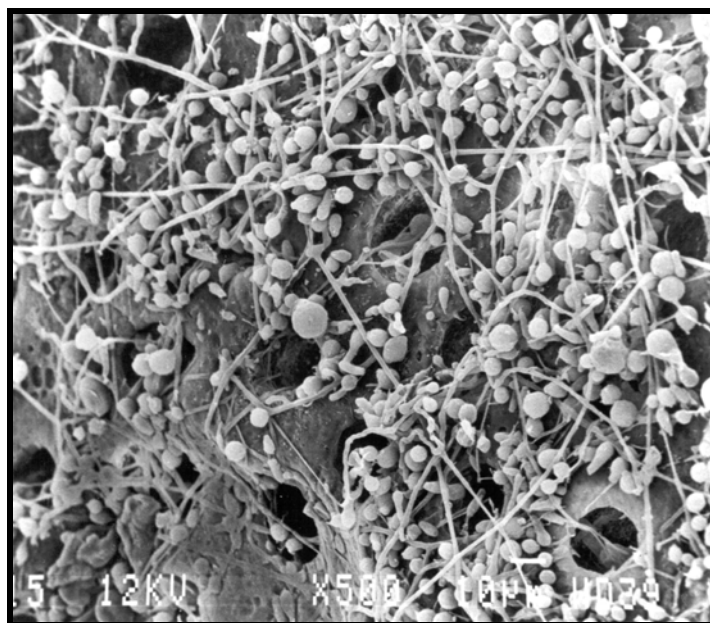


Figure 5.13: Germination of spores on SFR polysulphone membrane surface. Mycelia extending from germinating spores form a network across the membrane surface and within macrovoids. Magnification X500.

Biofilm formation progresses, limited by nutrient supply from the membrane surface. A faster flow rate generates a thicker biofilm, which is not suited to ligninase production due to oxygen starvation of hyphae furthest from the air interface. Nutrient and oxygen limitation arise from concentration gradients (see Figure 5.2) created by the dilution of substrates across the biofilm and by the utilisation of substrates by the biofilm.

Complex biofilm differentiation, observed under the nutrient and oxygen gradients of the gradostat system and depicted in Figure 5.14, is described below.

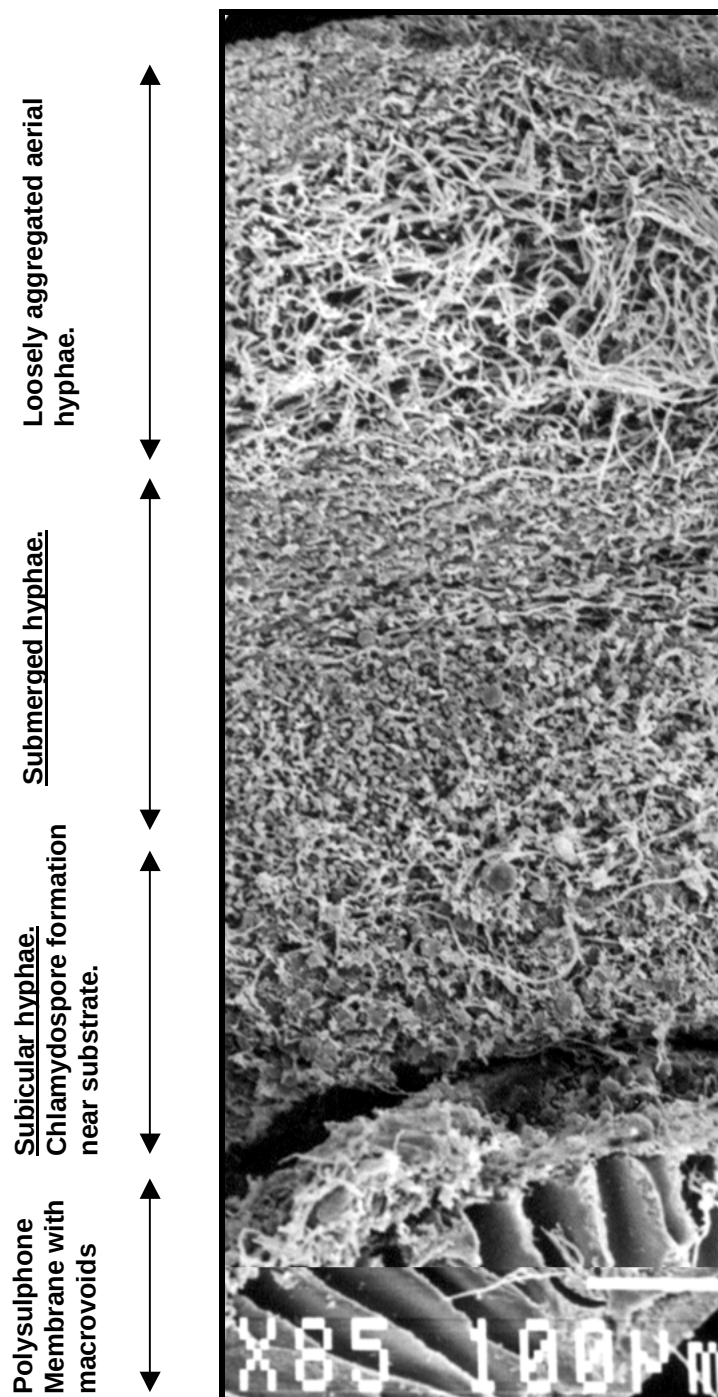


Figure 5.14: Radial differentiation of a *P. chrysosporium* ME446 biofilm from the membrane surface, where nutrient concentrations are highest, to the air interface, where oxygen levels are highest. Magnification X85.

Loosely aggregated aerial hyphae (schematic shown in Figure 1.1.5) were observed closer to the air interface. These hyphae were frequently seen to form conidia (schematic shown in Figure 1.1.7), particularly under higher air/oxygen flow rates. TEM images show an increased number of mitochondria within aerial hyphae in addition to small vacuoles and secretory vesicles associated with hyphal tips (Figure 5.15). Similar subcellular localisation has been observed for mycelial pellets (Jiménez-Tobon *et al.*, 2003).

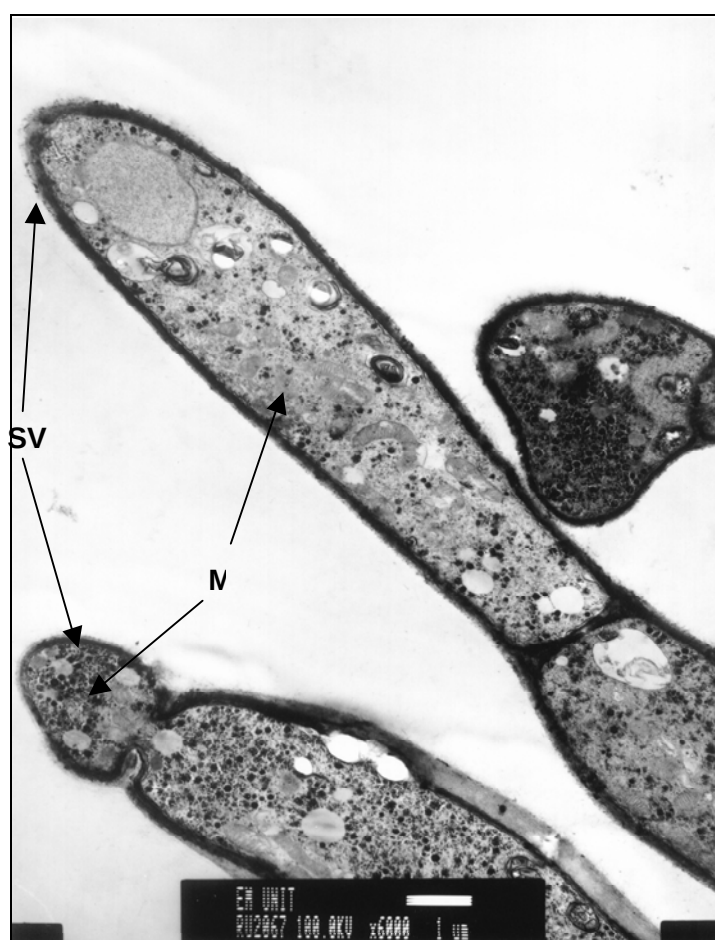


Figure 5.15: TEM image showing loosely aggregated, septate aerial hyphae. Ultrastructure shows a large number of mitochondria (M) and secretory vesicles (SV) associated with hyphal tips. Magnification X6000.

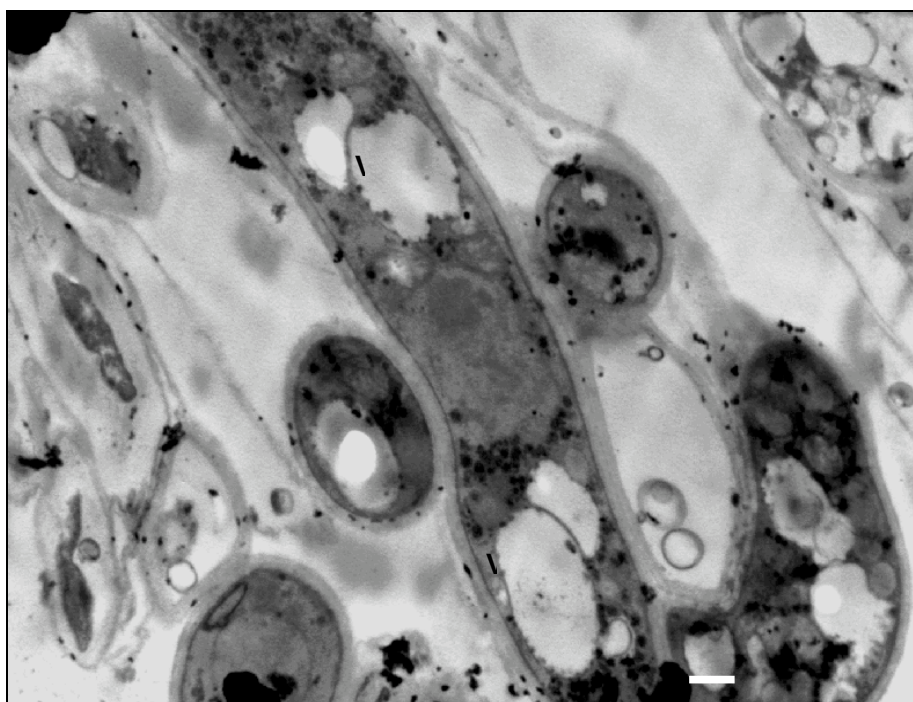


Figure 5.16: TEM image showing frequently branched, densely packed subicular hyphae, with granular cytoplasm and large vacuoles (V). Magnification X6000.

Below aerial hyphae is a zone of submerged hyphae (Figure 5.14). These hyphae are thick walled and frequently branched and thus more densely aggregated than aerial hyphae (schematic shown in Figure 1.1.6). Subicular hyphae (schematic shown in Figure 1.1.4) are thin walled, smooth and branched at right angles. SEM images of subicular hyphae show large vacuoles and granular cytoplasm (Figure 5.16). In differentiated mycelial pellets increased vacuoles are associated with ageing cells (Jiménez-Tobon *et al.*, 2003).

Subicular hyphae reported to be located nearer the substrate where they are sometimes associated with oval to spherical chlamydospore-like cells (Burdshall and Eslyn, 1974). Figure 5.17 presents a spherical, intercalary chlamydospore-like cell as observed within the subicular hyphal zone depicted in Figure 5.13 (schematic shown in Figure 1.1.9). Dissections of these cells reveal a densely granular cytoplasm (Figure 5.1.c). Chlamydospore-like cells associated with the subicular zone are also associated with penetrative hyphae (Figure 5.18.c and 5.1). Jiménez-Tobon *et al.* (2003) describe chlamydospore-like cells, formed in mycelial pellets, which appear to act as reservoirs of peroxidase.

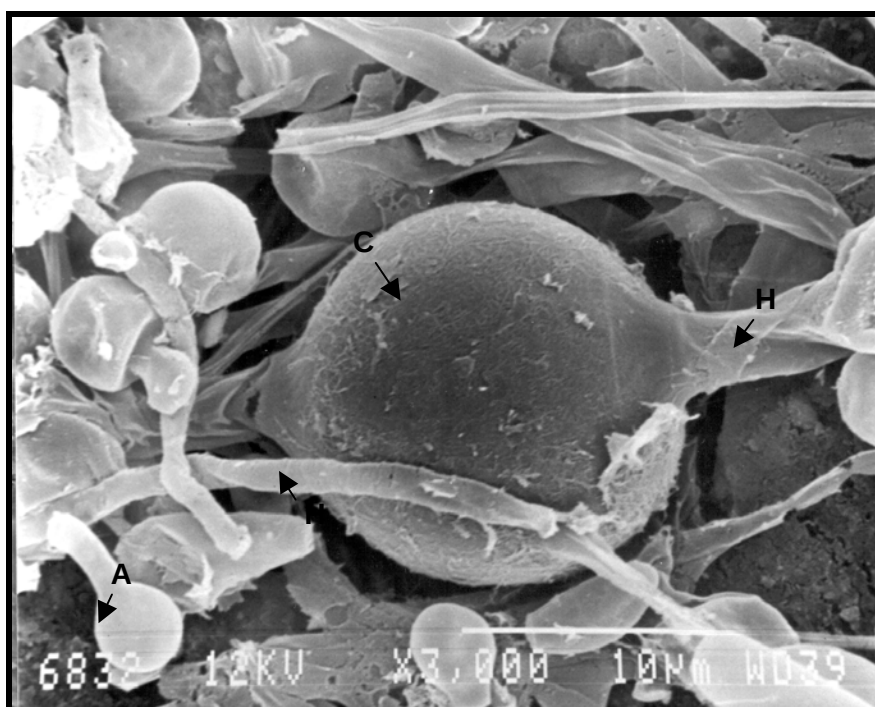


Figure 5.17: SEM image showing a single large (14 μm) intercalary chlamydospore-like fruiting structure [C] produced by hyphae [H] within the subicular growth zone of a *P. chrysosporium* biofilm. Also shown are terminal arthrospores [A] (3-5 μm). Magnification X3000.

Penetrative hyphal growth extends into the macrovoids of capillary membranes (Figure 5.18). These hyphal aggregates have a rhizomorph-like appearance (Figure 5.18). TEM sections of penetrative hyphae show distinct differences in the ultrastructure of penetrative hyphae at different levels within the macrovoids (Figure 5.19). Penetrative hyphae located closer to the subicular zone have a high vacuole and secretory vesicle content (Figure 5.19.a). Closer to the nutrient feed, penetrative hyphae are shown to have a higher mitochondrial content (Figure 5.19.b).

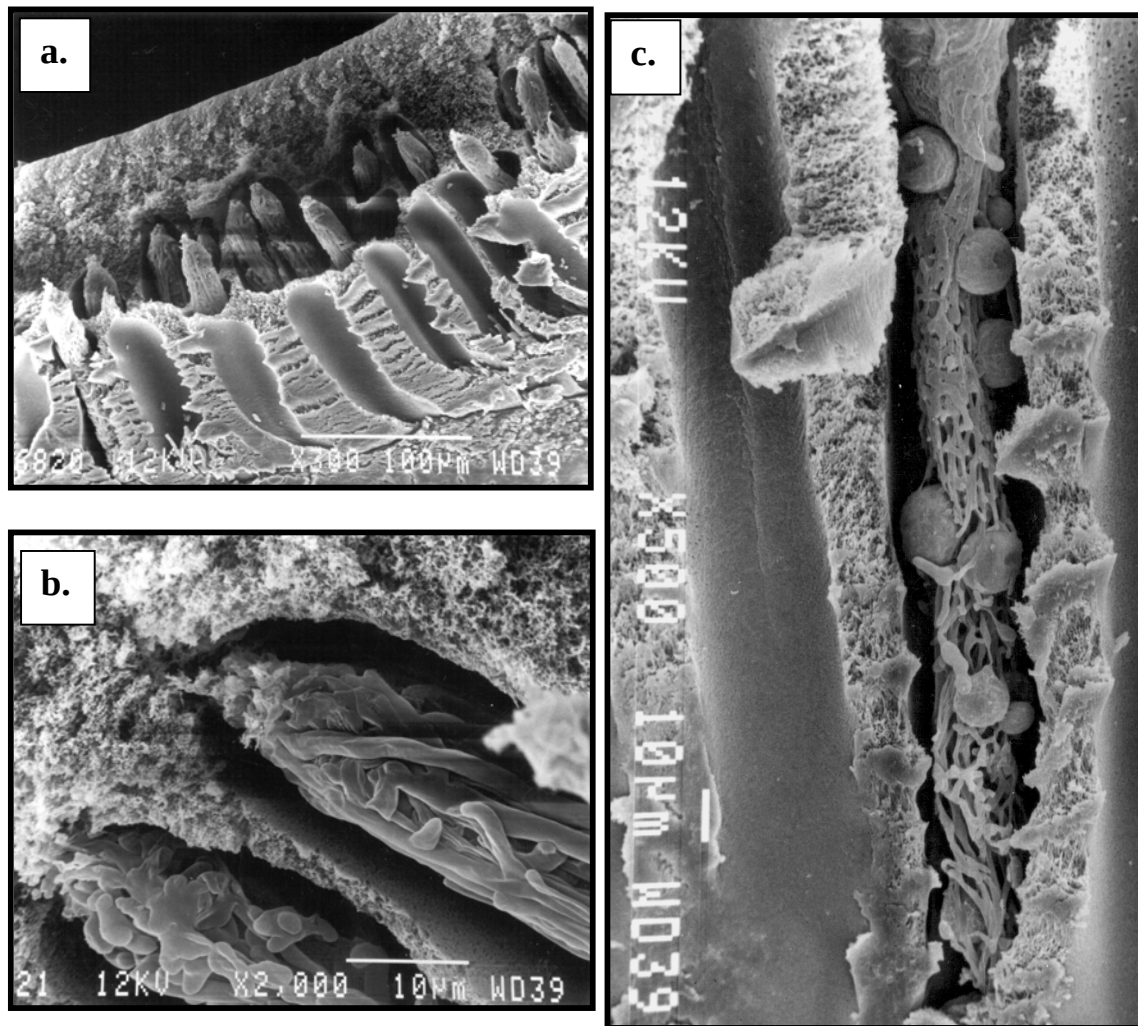


Figure 5.18: (a.) Mycelia penetrating the macrovoids of capillary membrane have a rhizomorph-like appearance. Magnification X300. (b.) These extending hyphal aggregates are seen to interact with macrovoid walls in much the same way as penetrative hyphae would interact with woody tissue. Magnification X2000. (c.) Chlamydospore-like fruiting structures, 10-12 μm in size, were identified in association with penetrative growth of *P. chrysosporium* cultures. Magnification X500.

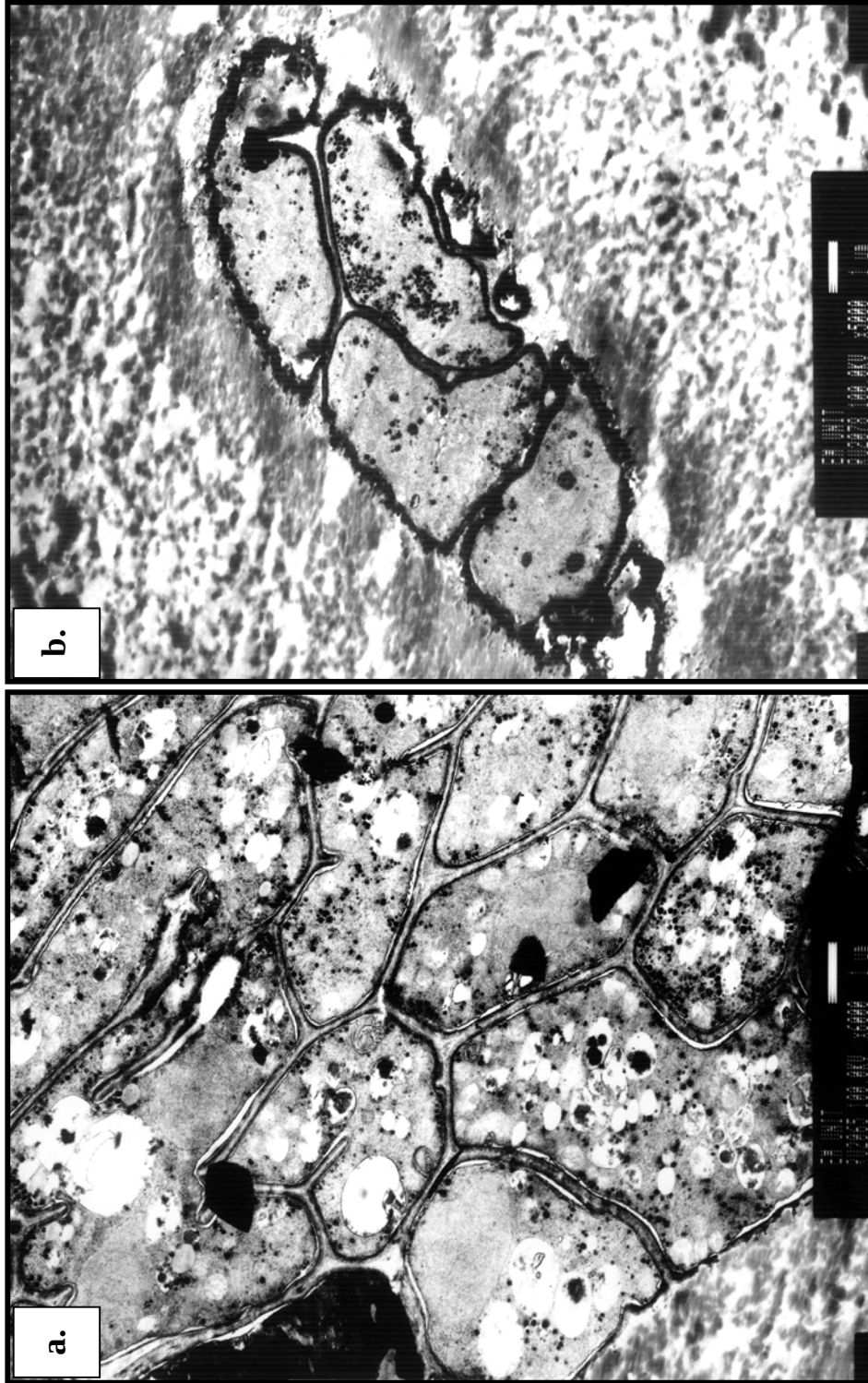


Figure 5.19: TEM images of penetrative hyphae showing densely aggregated, linear hyphal strains penetrating macrovoids. (a.) The cytoplasm of penetrative hyphae closest to the subicular zone show extensive vacuoles and secretory vesicles within the cell cytoplasm. Magnification X6000. (b.) Nearer the substrate, where macrovoids are narrower, the number of penetrative hyphae are reduced. The cytoplasm of these cells appears to have a greater mitochondrial content associated with hyphal extension and increased nutrient uptake. Magnification X5000.

Penetrative hyphae or rhizomorphs were shown to interact with the macrovoid walls (Figure 5.18.b and 5.20) in a manner that simulates its interaction with wood (Ruel and Joseleau, 1991, Daniel *et al.*, 1994, 1990).

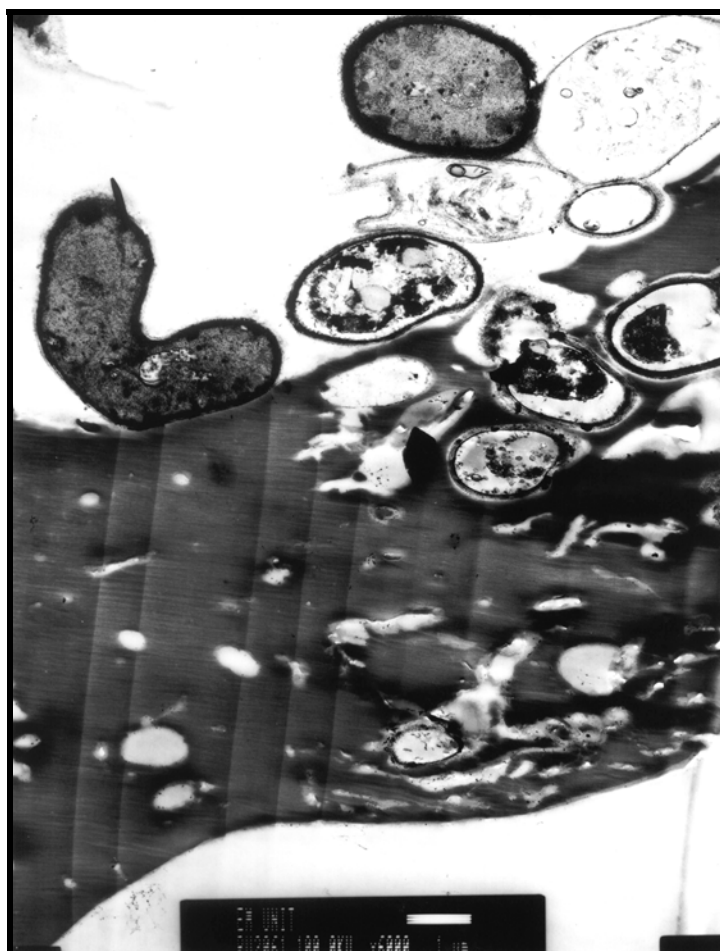


Figure 5.220: TEM image showing the association of exploratory hyphae with polysulphone capillary walls and the association of these hyphae with weakness in the macrovoid walls as would occur when cultured on a woody substrate. Magnification X6000.

5.3.5. Intraspecific comparison of *P. chrysosporium* biofilms

Morphological differentiation of *P. chrysosporium* ME446 is described extensively above. The detection of intraspecific variability in the morphological characteristics of biofilm formation in sibling strains was limited by variation in the flow rates of SFRs. Only the most notable differences in biofilm development are thus recorded below. In each case, similar low levels of MnP activity (3.2-6.2 U.l⁻¹) were detected in sibling strain inoculated SFRs.

5.3.5.1. *P. chrysosporium* BS 13

SEM images of BS 13 biofilms differed from that observed for ME446 in section 5.3.4 in that the aerial hyphal zone was not well developed. Instead, this strain showed an extensive subicular zone with large aggregates of chlamydospore-like cells (5.21.a). The subicular zone formed a distinct layer below a relatively thick, densely packed zone of submerged hyphae (Figure 5.21).

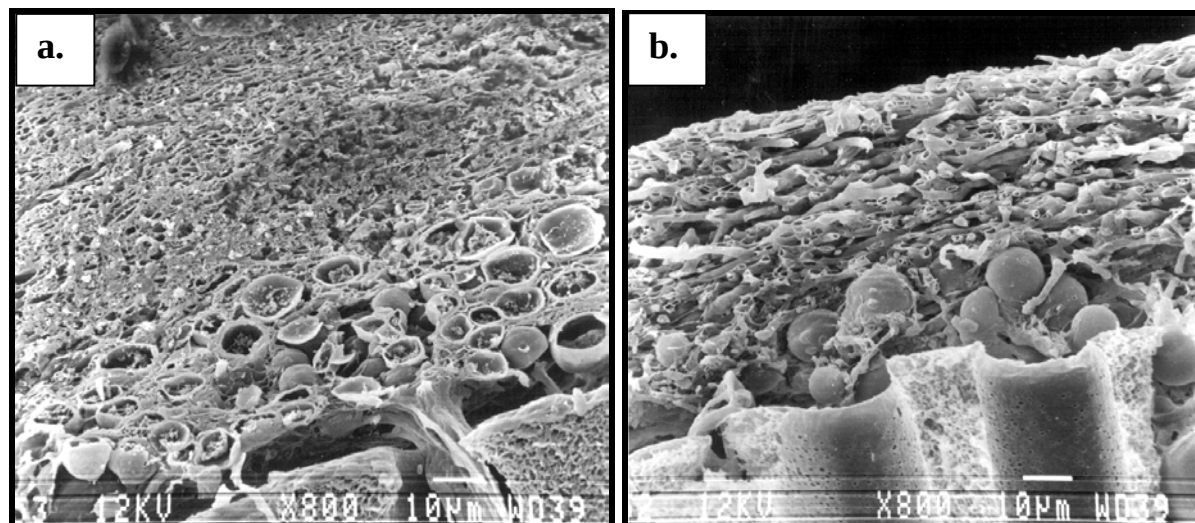


Figure 5.231: Scanning electron micrographs showing tissue differentiation *P. chrysosporium* BS 13 biofilm immobilised on the surface of an SFR polysulphone capillary membrane. (a.) SFR 1 and (b.) SFR 2 operation showed different flow rates, yet similar biofilm structure. Magnification X800.

5.3.5.2. *P. chrysosporium* BS 26

In contrast to strain BS 13, strain BS 26 showed little hyphal differentiation. The biofilm appeared to be comprised only of aerial hyphae (Figure 5.22.a) producing conidiospores (Figure 5.22.b).

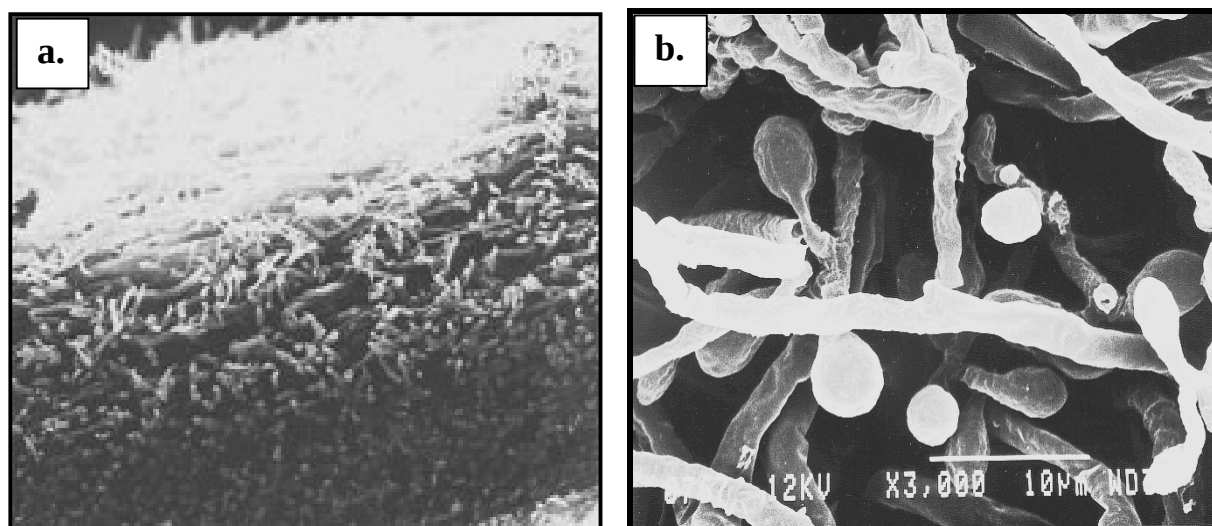


Figure 5.242: Scanning electron micrographs of *Phanerochaete chrysosporium* BS26. (a.) Cross-section of biofilm located upon the surface of a capillary membrane. (b.) Closer view of biofilm cross-section showing less compact hyphae with swollen hyphal tips. Magnification for A and B are X500 and X3000, respectively.

This observation could not be attributed to differences in biofilm thickness as differentiation was observed in BS 13 biofilm of 55-60 μm thickness (Figure 5.21.b) while the BS 26 biofilm was approximately 70 μm thick (Figure 5.22.a).

5.3.5.3. *P. chrysosporium* BS 17

P. chrysosporium BS 17 showed poor biofilm formation (Figure 5.23) whose surface growth appeared to be comprised solely of submerged hyphae. Figure 5.23.b shows a closer view of these densely packed, highly branched hyphae.

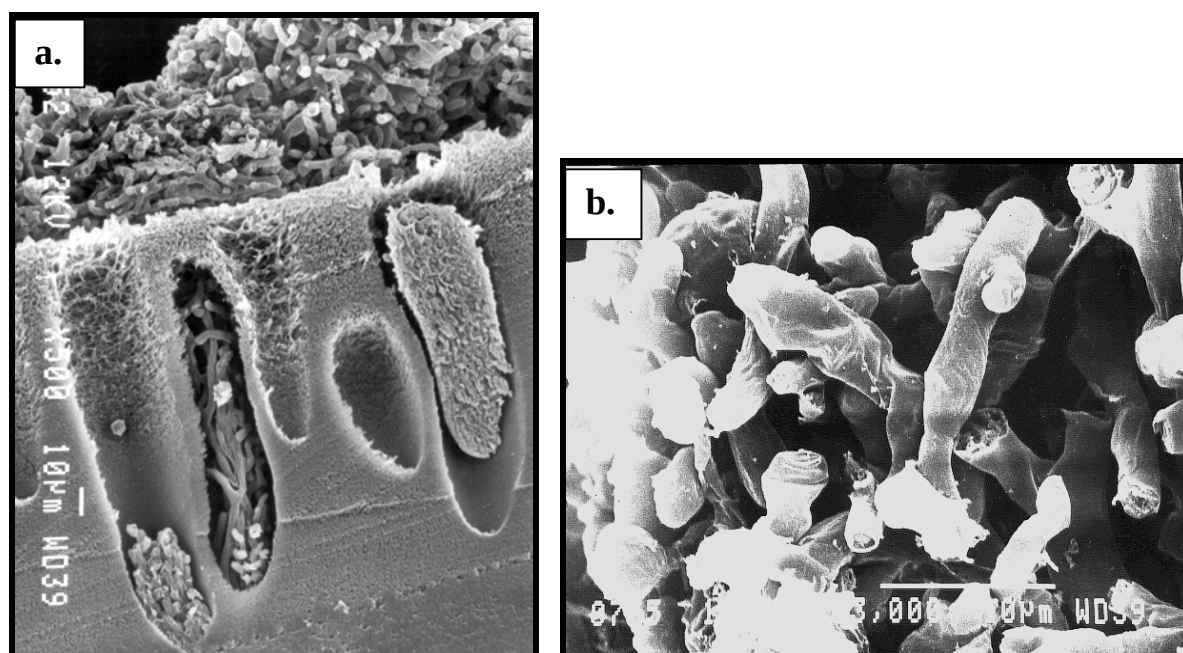


Figure 5.253: Scanning electron micrographs of *Phanerochaete chrysosporium* BS 17. (a.) Cross-section of biofilm located upon the surface of a capillary membrane. (b.) Closer view of biofilm cross-section showing more compact highly branched hyphae with swollen hyphal tips. Magnification for A and B are X1000 and X3000, respectively.

5.4. DISCUSSION

5.4.1. SFR optimisation

Those SFR operational conditions providing the least variability in biofilm formation and extracellular enzyme production were evaluated using factorial design. Orientation and inoculum were maintained as experimental constants and flow rate, oxygenation and medium supply were varied between experiments.

The results from Experiments 1 and 2 showed clearly that membrane orientation plays a crucial role in the development of the biofilm upon the membrane surface. When in horizontal orientation, permeate accumulates in droplets along the membrane length causing a misdistribution of nutrients and thus inconsistent biofilm formation. At higher flow rates, this biofilm growth tends to accumulate along the bottom of the glass manifold, further metabolising permeate and thus influencing permeate substrate levels and enzyme activities. In contrast, when reactors were operated in vertical orientation more cylindrical biofilm growth was obtained. Permeate runoff was favoured and no droplet formation was observed. However, at high flow rates biofilm detachment was more apparent as permeate was channelled down the length of the membrane between the biofilm and the membrane surface.

At lower flow rates, vertical biofilms showed tapering growth due to a pressure drop in the medium supplied from the bottom, emphasised by permeate runoff. A thicker biofilm was formed at the base of SFR module due to an increased nutrient supply. By changing the medium supply route from below (Experiment 1) to above (Experiment 2) it was thought that the pressure drop in medium supply across the length of the membrane would counteract medium supply from run-off, thus reducing the tapering growth observed in vertical modules. While the supply of medium from above did indeed influence tapering growth, flow rate in these modules was more variable, particularly under low flow rate conditions. This was attributed to the differential accumulation of air bubbles at medium inlets, thus preventing medium supply to those membranes. Air bubbles were released in medium lines due to pressure changes as medium was passed through narrow-bore peristaltic tubing.

All SFR modules were inoculated with a standardised *P. chrysosporium* ME446 spore inoculum that was allowed to adhere to the membrane surface and macrovoids therein using reverse filtration overnight. The germination of this inoculum and its continued attachment and growth is dependent on nutrient supply rates and oxygen availability. Attachment periods of one (Experiment 2) or three (Experiment 1) days, at low flow rates, were examined. Further, SFR modules examined in Experiment 1 were cultured under continuous air while those operated in Experiment 2 were cultured under air for the first three days, after which biofilms were supplied with pure oxygen. These results show that in SFR modules switched to high flow rate conditions, a period of three days attachment at a low flow rate was more favourable for optimal growth and metabolism. Similarly, SFR modules cultured under constant air produced healthier biofilms. At early stages of biofilm growth, pure oxygen may have an inhibitory effect on growth.

Maximum MnP activities were detected in vertical orientation, low flow rate and continuous air cultures supplied with medium from below. Although maximum MnP activity was not the overwhelming criterion by which the operational conditions for reduced variability and maximum efficiency of SFRs was selected, the correlation between optimal conditions for biofilm formation and MnP activities would benefit intraspecific comparisons correlating mycelial differentiation with extracellular MnP production.

5.4.2. Mycelial differentiation

Biofilm formation in the membrane gradostat bioreactor is self-limiting. The specific growth rate of the culture (correlated with maximum biomass levels in Chapter 4) is dependant on the concentration of the growth-limiting substrate. This means that growth rate is regulated by nutrient gradients established across the biofilm (Figure 5.2). Due to the translocatory nature of mycelia, hyphae colonising the nutrient rich area closer to the membrane surface are able to support the differentiation and metabolism of hyphae farther away from the membrane, where the growth-limiting substrate is depleted. Biofilm thickness thus reaches a maximum, where further growth cannot be supported and the biofilm exists in a equilibrium, sustained by the constant flow of nutrients.

Biofilm thickness and the onset of stationary growth phase in membrane gradostat bioreactors are therefore influenced by flow rate. The morphology and branching pattern of hyphae changes with specific growth rate, suggesting that morphology is co-ordinately regulated by the surrounding substrate (Müller *et al.*, 2000). Oxygen levels influence mycelial differentiation (Blackstone, 2000). No oxygen is measured in a mycelial mat deeper than 1mm thick (Bonnarme *et al.*, 1991).

Extensive differentiation and tissue formation was observed in *P. chrysosporium* ME446 biofilms. Different growth zones were identified within both surface and penetrative growth forms. Surface biofilm was distinctly separated in three zones whose hyphal morphologies were dominated by (1) aerial hyphae, (2) submerged hyphae, and (3) subicular hyphae types.

The aerial hyphal zone was loosely aggregated and showed a greater subcellular localisation of mitochondria, required for respiration. Smooth, submerged hyphae were highly branched, forming a more closely aggregated hyphal zone than observed with aerial hyphae. Frequency of hyphal branching has been correlated with pH in *Penicillium chrysogenum* (Pirt and Callow, 1959). pH In turn is affected by oxygen limitation and organic acid production and plays an integral role in cAMP regulation, an important biological mediator in mycelial differentiation (Calvo *et al.*, 2002, Gold and

Cheng, 1979). pH is also implicated in the production of specialised, swollen, survival cells in *Penicillium chrysogenum* produced in submerged cultures (Pusztahelyi *et al.*, 1997). These cells may be likened to the chlamydospore-like cells observed with the subicular zone. Subicular hyphae are generally produced closest to the substrate.

Fruit-body formation in *P. chrysosporium* occurs under strong catabolite repression by glucose in the presence of physiological levels of nitrogen (Gold and Cheng, 1979). Glucose repression is however reversed under nitrogen limitation and in the presence of cAMP (Gold and Cheng, 1979). This infers that the subicular zone may be under either nitrogen-limited and/or oxygen starved, where reduced pH has stimulated cAMP production, potentially inducing the formation of chlamydospore-like cells. Furthermore, in *P. chrysosporium*, nitrogen and glucose catabolite repression act independently in both fruiting and ligninase production (section 1.3.2.7). Similar chlamydospore-like cells have been identified in mycelial pellets (Jiménez-Tobon *et al.*, 2003). These authors describe the appearance of intercalary swollen cells on day's two to three and their disappearance coinciding with increased supernatant peroxidase levels, thus proposing a role for chlamydospore-like cells as enzyme reservoirs (Jiménez-Tobon *et al.*, 2003).

Increased vacuolation and sporulation, as observed in subicular hyphae and to a greater extent in upper levels of penetrative hyphae, have been correlated with secondary metabolite production in *Penicillium chrysogenum* cultures (Pusztahelyi *et al.*, 1997). LiP and MnP are associated with golgi vesicles, endoplasmic reticulum and light density vesicles (Bonnarme *et al.*, 1994, 1991) and concentrated within chlamydospore-like cells (Jiménez-Tobon *et al.*, 2003). High mitochondrial content is also detected in lignolytic tissue, in accordance with stimulation of these enzymes by molecular oxygen (Bonnarme *et al.*, 1991). The association of LiP or MnP production with specific zones of hyphal "tissue" zones such as subicular or penetrative growth may enable the modification of bioreactor design or operation in order to maximise these growth forms, or alternatively the selection of fungal strains predominately exhibiting those "tissue" types.

Preliminary intraspecific comparison of biofilms produced by different *P. chrysosporium* strains showed certain strains to differ in both the extent of biofilm formation and in the nature of the zones of differentiation observed within surface growth. Strain BS 13 showed a well developed subicular zone while strains BS 26 and BS 17 biofilms were dominated by what were identified as aerial hyphal growth and submerged hyphal growth, respectively. In order to fully characterise these biofilm types, it may be useful to classify zones of growth according to their hydrophobin content or a similar

marker. Mycelial hydrophobins are associated with aerial hyphae and fruit-body formation but not submerged hyphae (de Vries *et al.*, 1993).

5.5. CONCLUSIONS

The membrane gradostat bioreactor offers an ideal tool for examining the effects of substrate concentration and oxygen levels on hyphal differentiation unhindered by problems originating from substrate diffusion in a solid environment (Müller *et al.*, 2000).

Using the membrane gradostat bioreactor, mycelial differentiation into distinct tissue types including rhizomorph-like penetrative growth and fruiting structures was shown. Differentiated hyphae revealed different subcellular distribution of organelles, inferring that different metabolic pathways are undertaken in these tissue types.

The link between secondary metabolism and morphological differentiation has been established in better studied fungi such as *Aspergillus* spp. and *Penicillium* spp. The gradostat membrane bioreactor offers an ideal, controlled environment for closer examination of the interaction of signal transduction pathways, sporulation or mycelial differentiation and secondary metabolism in *P. chrysosporium*. Comparison of sibling strains showing altered biofilm characteristics would enable the elucidation of genetic markers to be used as future strain selection criteria.

Furthermore, our results showed that tolerance to tannic acid and *p*-cresol degradation are correlated to *P. chrysosporium* growth (Chapter 4). Strains showing different pollutant sensitivity and degradation profiles were also shown to differ in the nature and extent of mycelial differentiation in immobilised biofilms (discussed further in Chapter 8). Neither LiP nor MnP have been correlated with lignin or pollutant degradation (Chapter 4). The ability to correlate degradation capabilities with specific morphological traits may allow for a more robust screening method for the selection of strains showing improved degradation. Improved tolerance and degradation of toxic pollutants may be better correlated to hyphal type i.e. the different cell wall components of aerial, submerged or subicular hyphae, for example, might influence pollutant uptake; and ultrastructure i.e. organelle content, a reflection of hyphal metabolism, might implicate further mechanisms or pathways responsible for improved degradation or tolerance. While the correlation of organelle content with peroxidase levels has been established, this has not been undertaken for pollutant degradation in *P. chrysosporium*.

CHAPTER 6

The design of a novel plug-flow membrane bioreactor system for the study of mycelial differentiation in filamentous fungi

6.1. INTRODUCTION

Hyphal organisation may account for differences in lignin degradation, pollutant degradation or the ability of an organism to perform well in liquid, solid or immobilised culture systems. The extent of differentiation observed when culturing *P. chrysosporium* using the membrane gradostat bioreactor (Chapter 5) and the organisation of mycelia penetrating the macrovoids of an unskinned capillary membrane into rhizomorph-like growth units prompted further study into the factors regulating hyphal organisation.

In their natural growth environment, saprophytic fungi occur as an extensive thallus, whose hyphal morphology is differentiated in order to locate and utilise available resources. Linear “organs of translocation” described as mycelial cords or rhizomorphs have been shown to mediate the partitioning of [^{14}C] glucose (Wells *et al.*, 1995), [^{32}P]phosphorus (Wells *et al.*, 1998; Wells and Boddy, 1990) and [^{137}Cs] (Gray *et al.*, 1995) between resources, in addition to the relocation of biomass (Wells and Boddy, 1990). Further, in *Phanerochaete velutina*, extracellular oxidase, cytochrome oxidase and peroxidase activities have been associated with mycelial cords (Dyer *et al.*, 1992), suggesting a further role in foraging.

Neither the mechanism nor the significance of translocation in mycelial differentiation and its effect on bioremediation are clearly understood. Those criteria thought to stimulate cord formation in saprophytic fungi include reduced water potential, low nutrients, copper, calcium, wood extractives and the presence of antagonistic fungi (Dowson *et al.*, 1986). Studies using *P. velutina* have shown mycelial distribution is not affected by NH_4NO_3 , potassium, phosphorous or glutamine limitation in soil (Donnelly and Boddy, 1998). The composition of the soil substrate used in these experiments not only complicates the chemistry influencing differentiation, but sterilisation of the soil substrate was shown to inhibit mycelial cord formation. Cords are more readily formed under non-sterile conditions (Thompson and Rayner, 1983).

A new plug-flow membrane system was introduced as a means of controlling and extending the nutrient gradients imposed upon hyphae in an attempt to enhance gradient-induced mycelial differentiation under monoculture conditions. The identification of those criteria that influence fungal differentiation is of both fundamental interest and biotechnological importance. It may be possible to predict the role of differentiation in lignin degradation and soil bioremediation and in so doing, use such predictions as selective criteria when applying an organism to a particular biotechnological system.

6.1.1. Aims

This chapter reports on the design of a plug-flow membrane bioreactor to be used as a potential tool in examining the effects of nutrient and/or oxygen gradients on mycelial differentiation. The effectiveness of this tool is demonstrated by the differentiation of mycelia within the plug flow bioreactor to the level of organised hyphal units identified as mycelial cords.

6.2. MATERIALS & METHODS

6.2.1. Fungal strain and inoculum

Stock cultures of *P. chrysosporium* ME446 were maintained on spore and maintenance agar slants at 4 °C (Appendix I:A-1). Two-day old agitated cultures of *P. chrysosporium*, grown in 20 ml nitrogen-limiting basal III medium (Appendix I:A-2) and fragmented using a Sorval blender, was used to inoculate each bioreactor.

6.2.2. Plug-flow bioreactor configuration

The plug-flow membrane bioreactor (Figure 6.1.a) comprised two 440 mm long polysulphone, non-porous gas separation (G.S) membranes (0.25 mm i.d.) both inserted and sealed within a 25 mm long polypropylene microfiltration (M.F) membrane (2 mm i.d.) at one end, while individually feeding into a length of silicon rubber tube (3 mm i.d.) at the opposite end. The entire membrane length was sealed within a glass manifold. Under normal operating conditions, growth medium was fed from the M.F end, filtering into the polypropylene membrane lumen, through the G.S membrane lumens, and into collection vessels A and B through the silicon rubber tubing.

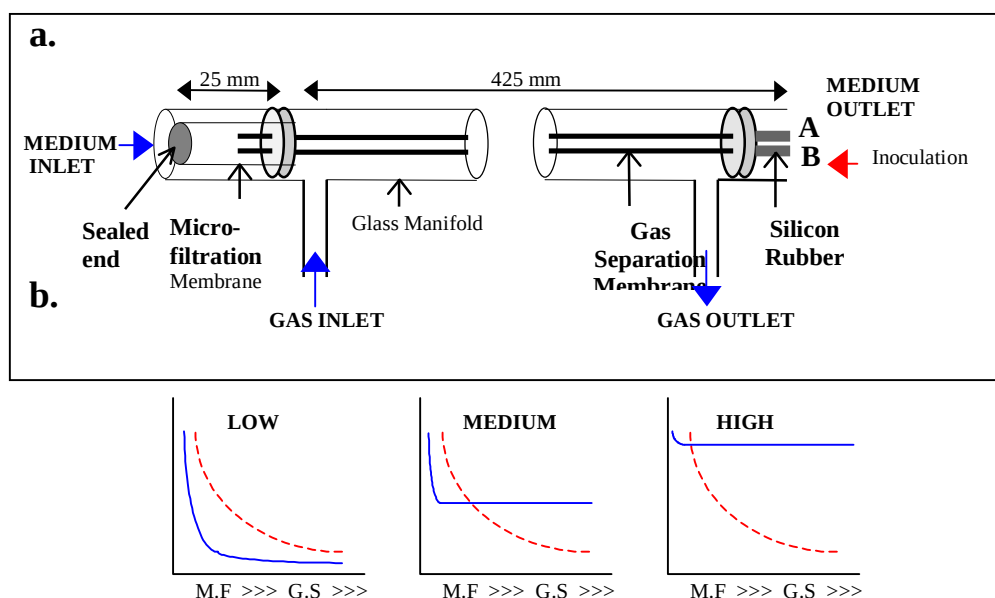


Figure 6.1: (a.) Design of plug-flow membrane bioreactor. (b.) Theoretical nutrient (dashed line) and oxygen gradients (solid line) imposed along the G.S membrane length of each plug-flow membrane reactor. Low (reactor I), Medium (reactor II) and High (reactor III) oxygenation conditions were applied.

6.2.3. Inoculation of reactor

Each reactor was inoculated by reverse flow by injecting the mycelial inoculum directly into the lumen of the G.S membranes. The inoculum was then flushed towards the M.F membrane at the reactor head (Figure 6.1.a) using sterile distilled water at a flow rate of 1 ml.min^{-1} for 10 min. Distilled water was then replaced with nitrogen-limiting growth medium at a flow rate of 0.1 ml.min^{-1} for a two day incubation period. This allowed for the attachment and immobilisation of the mycelial inoculum within the reactor.

6.2.4. Culture conditions

Following the incubation period, normal operating conditions were introduced. Medium was supplied from the M.F end at a total initial flow rate of 0.1 ml.min^{-1} . The flow of medium through the plug-flow bioreactor was stabilised at $0.002 \pm 0.002 \text{ ml.min}^{-1}$ by day 12. This generated a nutrient-rich reserve within the M.F lumen and biomass-induced nutrient depletion along the G.S membrane length (Figure 6.1.b) Feed medium was aerated, preventing oxygen-limitation within the M.F lumen. Furthermore, three different oxygen regimes (low, medium and high) were introduced along the G.S membrane length (Figure 6.1.b). In reactor type I (low oxygen) feed medium was aerated and nitrogen

gas was supplied across the external surface of the G.S membranes within the glass manifold, generating an oxygen gradient from the M.F lumen along the G.S membrane length as seen with the nutrient supply. In addition to aerating the feed medium, either pure oxygen (reactor type III – high oxygen) or air (reactor type II – medium oxygen) was supplied across the external surface of the G.S membranes within the glass manifold (Figure 6.1.b). The oxygen content of the permeate, as a measure of the efficiency of oxygen transfer across the G.S membrane, was monitored using a YSI Model 57 Dissolved Oxygen Meter.

6.2.5. Substrate utilisation.

The pH, glucose (Appendix I:C-1) and NH_4^+ (Appendix I:C-2) concentration of the feed medium and permeate collected from each sample port was monitored daily.

6.2.6. Enzyme activities

The permeate collected from sample ports A and B was assayed for LiP (Appendix I:D-1) and MnP (Appendix I:D-2.1) activity daily.

6.2.7. Electron microscopy

Plug-flow reactors were dissected twelve days post-inoculation. Sections at 10 cm intervals along the reactor length were fixed overnight in phosphate-buffered gluteraldehyde and prepared for SEM (Appendix I:E-1) or TEM (Appendix I:E-2).

6.3. RESULTS

6.3.1. Reactor conditions

Irrespective of oxygenation conditions applied to the plug-flow bioreactor, substrate utilisation profiles for each reactor were similar. By day 12, a reduction in glucose levels from $8.84 \pm 0.10 \text{ mg.ml}^{-1}$ (inlet) to $2.16 \pm 0.15 \text{ mg.ml}^{-1}$ (outlet A) or $4.38 \pm 2.43 \text{ mg.ml}^{-1}$ (outlet B) and medium NH_4^+ levels from $30.26 \pm 4.43 \text{ }\mu\text{g.ml}^{-1}$ (inlet) to $1.99 \pm 0.65 \text{ }\mu\text{g.ml}^{-1}$ (outlet A) $7.81 \pm 1.78 \text{ }\mu\text{g.ml}^{-1}$ (outlet B) was observed, indicating the formation of a nutrient gradient along the reactor length. Neither LiP nor MnP activities were detected. The pH of the nutrient medium dropped from pH 4.5 to pH 3.4-3.8 as it flowed through the plug-flow reactor from inlet to outlet.

6.3.2. Biomass differentiation

The membrane bioreactor system described above was developed with the initial goal of inducing fungal differentiation along the axial dimension of the G.S capillary by exposing *P. chrysosporium* to extended nutrient and/or oxygen gradients established within a plug-flow reactor. It was hypothesised that a dense plug of biomass would be formed, differentiating at both the metabolic and structural levels, in response to the nutrient and/or oxygen gradients.

Three distinct types of mycelial growth were identified in each plug-flow reactor: (1) covering growth (Figure 6.2); (2) parallel, longitudinally aligned hyphae forming differentiated rhizomorph-like mycelial plugs (Figure 6.3 and 6.4); and (3) the tapering and extension of mycelial cords embedded in a polysaccharide sheath (Figure 6.5).

No aerial hyphae or spores were observed within each reactor, consistent with growth in a submerged environment without a liquid/air interface. Morphological differences between the three reactor types were most notably the extent of cord formation and extracellular polysaccharide production. These differences are best visualised in images showing covering growth found to line the lumen of microfiltration membranes (Figure 6.2). The secretion of extracellular polymeric substances (EPS) was directly related to the amount of oxygen to which the biomass was exposed. The EPS levels associated with mycelium were far greater under elevated oxygen levels. Figure 6.2 shows the progressive increase in EPS visualised when reactors were operated under low (Figure 6.2.a), medium (Figure 6.2.b) or high (Figure 6.2.c) oxygen regimes. While large amounts of EPS were seen in reactor II (Figure 6.2.b), the fungus was not completely embedded in polysaccharide as in reactor III (Figure 6.2.c), but formed isolated “colonies” of polysaccharide-bound hyphae.

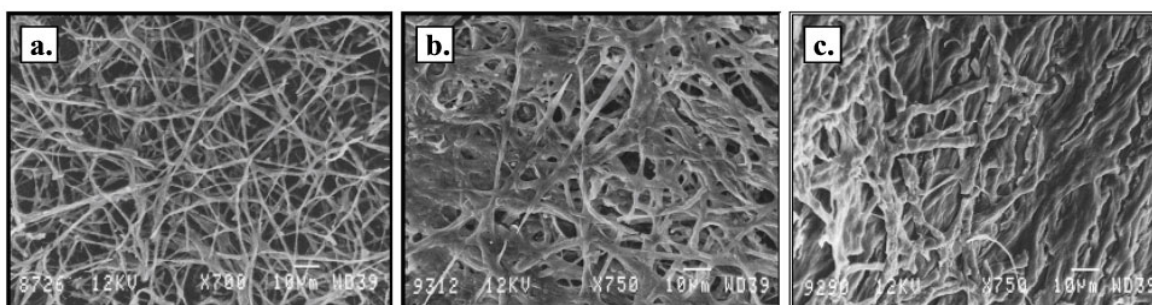


Figure 6.2: *P. chrysosporium* mycelial growth, as observed within the lumen of M.F. membranes in bioreactor types I (a), II (b) and III (c), following twelve days incubation at 37 °C. Note the increase of extracellular polysaccharide in Figures (a) through (c)

Hyphal extension through gas separation membranes showed the organisation of longitudinally aligned hyphae into a rhizomorph-like plug filling the lumen of the membrane (Figure 6.3.a and b). Cross section of this cylindrical plug showed distinct tissue differentiation (Figure 6.3.c). Zones of tissue identified as the internal medullar region and the outer crust (Figure 6.3.c), typical of rhizomorphs (Moore, 1995), were identified. Transmission electron micrograph images showed these zones to differ in number and orientation of hyphae, where hyphae within the compact outer crust appeared to be predominately longitudinally aligned (Figure 6.4.a). The cytoplasm of these hyphae was dominated by large vacuoles and a dense granular cytoplasm (Figure 6.4.a). In contrast, hyphae situated in the medullar region were loosely intertwined (Figure 6.4.b). The cytoplasm of these hyphae contained a variety of vacuoles and electron dense storage granules (Figure 6.4.b).

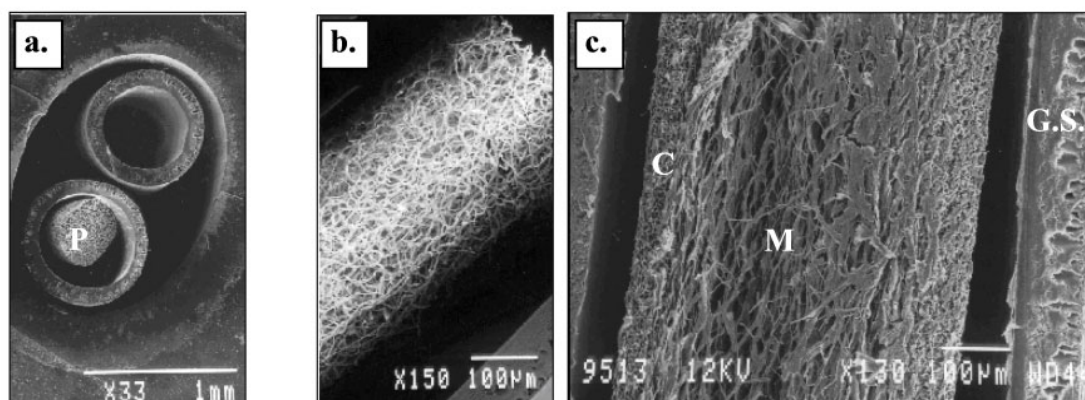


Figure 6.3: Electron micrographs showing *P. chrysosporium* plug formation within the G.S./M.F. junction of a typical plug-flow reactor. This junction includes approximately 5 mm of G.S membrane embedded within an M.F membrane, adjacent to the nutrient source. In cross-section a cylindrical plug [P] of mycelial growth is observed (a). While a longitudinal view of the mycelial plug (b), also seen in cross-section (c), reveals differentiation typical of a rhizomorph, where a less dense, medullar region [M] is encapsulated by a more dense outer crust [C].

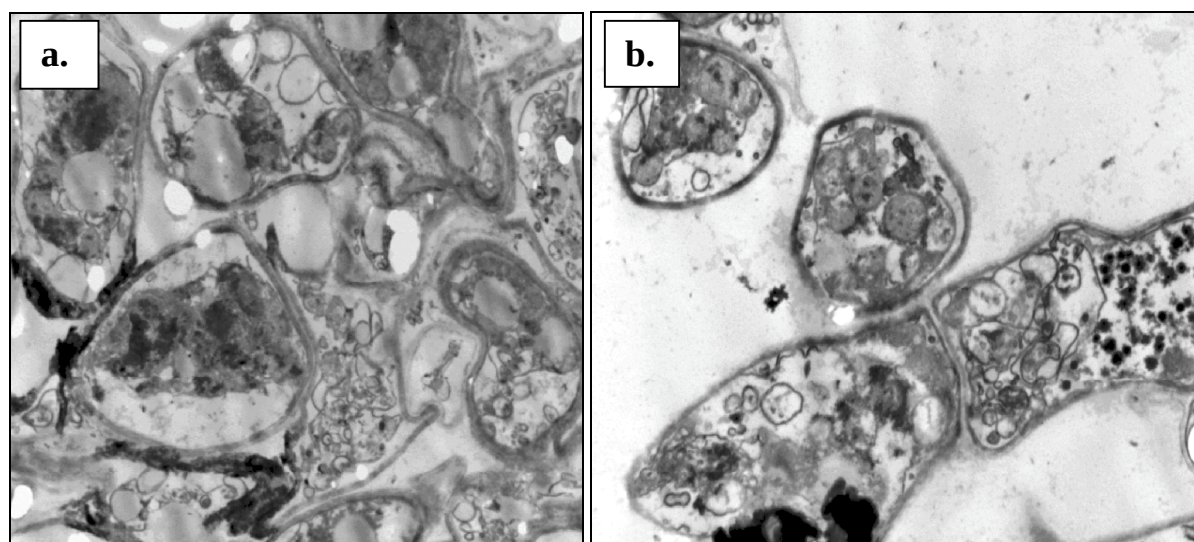


Figure 6.4: Show transmission electron micrograph images of (a.) densely aggregated, predominately longitudinally aligned hyphae sectioned through the outer crust of a rhizomorph-like mycelial plug. Cells are dominated by large vacuoles. Magnification X8000. (b.) Loosely intertwined hyphae located within the medullar region of a rhizomorph-like mycelial plug. Cell cytoplasm is filled with smaller secretory vesicles and electron dense storage granules. Magnification X8000.

Mycelial cords were identified within the G.S membranes of all three reactor types (Figure 6.5). The morphology of the cords observed varied from narrow cylindrical ropes (Figure 6.5.a) to almost planar extensions of hyphae (Figure 6.5.b).

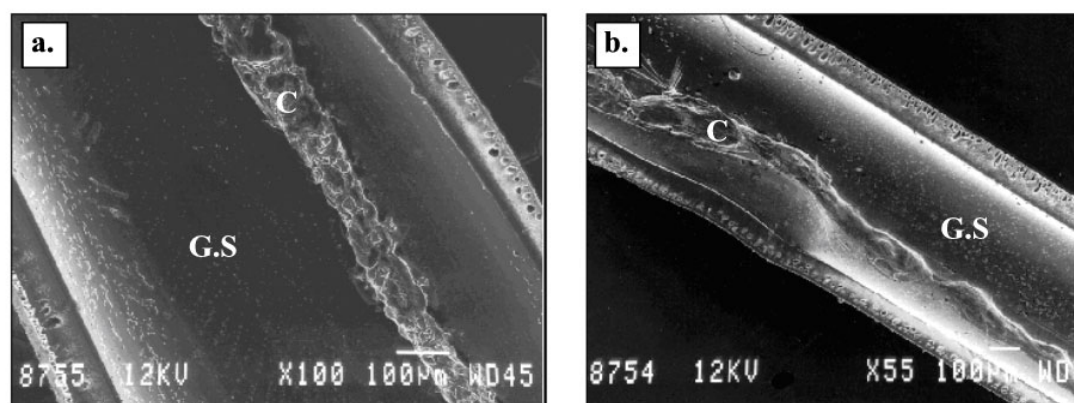


Figure 6.5: Electron micrographs showing the two different forms of mycelial extensions identified as cords [C], as observed within the lumen of G.S membranes. (a.) Was more cylindrical, while (b.) was more planar. Both were encrusted in a sheath of extracellular polysaccharide.

Cord formation was only observed in the M.F membrane of reactor I (Figure 6.6). The surface, covering growth lining the lumen of the M.F membrane in reactor I had little extracellular polysaccharide, enabling the direction of growth of individual hyphae to be identified. Figure 6.6.a shows the progressive, coordinated organisation of individual hyphae into longitudinally aligned linear strands. Upon closer examination a cylindrical “cord” of interwoven hyphae is seen to extend away from the nutrient source. At a certain point along the length of the mycelial cord the induction of secondary metabolite production became apparent as extracellular polysaccharide begins to be secreted, forming a “sheath” around the extending cord (Figure 6.6.b).

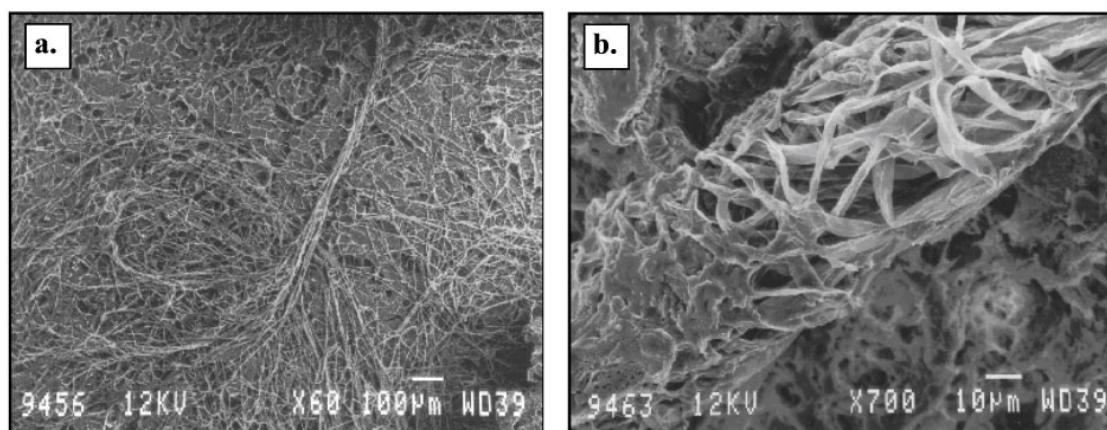


Figure 6.6: The progressive formation of a mycelial cord by *P. chrysosporium*, identified within the lumen of the M.F membrane of reactor II. (a.) Hyphae aggregate, becoming longitudinally aligned, intertwining to form a linear “mycelial cord”. (b.) This cylindrical linear organ extends away from the nutrient source, and gradually becomes encrusted in a sheath of extracellular polysaccharide.

6.4. DISCUSSION

Morphogenesis or cellular differentiation is attributed to the timely induction of growth related genes, which influence cellular organisation at both the micro- and macroscopic levels (Harold, 1995). Such genes are often regulated by environmental factors in response to the growth requirements of the organism.

The plug-flow reactor system, as described here, has been introduced as a potential tool for the analysis of mycelial differentiation in filamentous fungi. The isolation of those factors influencing differentiation is of fundamental interest, while contributing to the pool of knowledge required for optimal bioreactor performance and design where filamentous fungi, particularly those with sophisticated life cycles such as the Basidiomycetes, are used.

The formation of mycelial cords within the plug-flow membrane system was of particular interest. Mycelial cords are generated for the efficient exploration, acquisition and use of available resources and are responsible for nutrient exchange between resource bases and the regression of mycelium from depleted resources (Dowson *et al.*, 1986). Cords showing a greater degree of internal “tissue” differentiation are also referred to as rhizomorphs due to their root-like structure and functions, and are described as “organs” of translocation (Moore, 1995). The direction in which the mycelial cords extended within the reactor appeared to be away from the nutrient feed.

Although *P. chrysosporium* has routinely been studied using a variety of culturing techniques (including batch and continuous liquid fermentation systems, immobilised bioreactors, solid substrate fermentation, and as soil or agar grown cultures), cord-formation has not been described before in *P. chrysosporium*. Initial characterisation of this organism by Burdsall and Eslyn (1974) described *P. chrysosporium* as being non-rhizomorphic. This work provides evidence to the contrary. Further, cord-formation was induced in relatively young cultures (reactors were examined twelve days post inoculation) under sterile conditions; whereas in most saprophytic fungi, cord formation is primarily induced under non-sterile conditions (Thompson and Rayner, 1983) and often only in older cultures (Dowson *et al.*, 1986).

Differentiation of the mycelial plug (Figure 6.3.c) into tissue types resembling the inner medullar region and outer crust as observed in rhizomorphs (Moore, 1995) occurred within the plug-flow reactor in a submerged, aqueous environment. Tissue differentiation requires a surface, oxygen and a supply of nutrients (Moore, 1995). As mycelial cords were identified in all three reactors under different oxygen regimes, nutrient supply appears to play the major role in the organisation of hyphae into linear strands. The regulatory factors responsible for the initiation of this growth form are, however, unknown. It is possible that induction of cord-formation occurs indirectly, in response to environmental signals, as with the organism *Stereum hirsutum* which produces rhizomorphs in response to a highly mobile cytoplasmic transmitted factor (Coates and Rayner, 1985). Further, hyphal morphogenesis has also been shown to be influenced by cAMP levels (germination in *Uromyces appendiculatus*) (Epstein *et al.*, 1989) or the presence of prostaglandins (branching) (Herman and Herman, 1985).

The morphological differentiation observed along the length of the plug-flow reactor was observed under nutrient-reduced conditions. Glucose and NH_4^+ concentrations within the permeate were significantly lower than the feed medium. The nature of the axial gradient established within the membrane lumen was not quantified. While it cannot be assumed that this gradient is linear, its quantification is hampered by the translocatory nature of filamentous fungi.

The inability to detect LiP or MnP activities could imply that LiP and MnP activity are low in cords of *P. chrysosporium*. Alternatively, it may be due to adsorption of these proteins to the membrane surface.

The role of oxygen availability on the biofilm distribution and differentiation within the plug-flow reactor is unclear. Oxygen availability appears to play a less significant role in the gross distribution and differentiation of the mycelial mass than nutrients. Oxygen does however influence the biofilm structure at the level of extracellular polymeric substance (EPS) production. EPS are produced during stationary phase growth, where secretion is influenced by substrate concentration, oxygen availability, and shear force (Linko, 1992). Comparison of the different reactors operated under the same nutrient conditions but different oxygen supply regimes suggest that oxygen availability has the most marked effect on EPS production (Figure 6.2).

6.5. CONCLUSION

By establishing nutrient and/or oxygen gradients within a plug-flow membrane reactor system, morphological differentiation was enhanced. While cord formation has been demonstrated in a number of saprophytic fungi including *P. velutina*, it has not been reported for *P. chrysosporium*. Further manipulation of these gradients in the plug-flow reactor described in this work may allow for greater control of this differentiation.

Most research on fungal differentiation has focused on the chemical conditions influencing secondary metabolite production and fungal differentiation (Dowson *et al.*, 1986); while the impact of physical forces on mycelial differentiation has only been established for agitated cultures (Bonnarme *et al.*, 1993). This plug-flow system may be useful for further characterisation of the impact of both the chemical and physical environment on differentiation. Research into the metabolic events involved in cord formation utilising transcriptome analysis is currently being undertaken to determine the role of cord formation in lignin degradation.

Through further development, the plug-flow membrane bioreactor may prove to be a useful tool in describing extended spatio-temporal relationships between nutrient or oxygen gradients and secondary metabolism or hyphal differentiation. The more accurately the nutrient or oxygen gradients can be manipulated within the plug-flow reactor and membrane gradostat bioreactor, the greater the resolving power of these tools in detecting physiological and morphological differences between genetically related strains.

CHAPTER 7

High yield peroxidase production using a vertical, multiple-fibre membrane gradostat bioreactor

7.1. INTRODUCTION

Liquid culture techniques and solid substrate fermentations have been readily applied to the assessment of LiP and MnP activity and the biodegradation capabilities of *P. chrysosporium*. Ligninase production using liquid culture techniques is highly variable. Solid substrate fermentations offer a more stable growth environment for filamentous organisms, however separation and purification of lignolytic enzymes from the substrate introduces complications. Similarly, the complexity of solid substrates interferes with fundamental analysis into the mechanisms and pathways influencing enzyme production.

Integrated processes for the production, isolation and recovery of high yield secondary metabolites are increasingly being applied to bioreactor design and operation. Immobilisation has proven advantageous in the production of peroxidase enzymes by *P. chrysosporium* and other white rot fungi (section 1.3.3.7). The membrane gradostat bioreactor system offers both competitive yields and the advantages of simple and cost effective isolation and extraction of extracellular secondary metabolites (section 1.3.3.8). Furthermore, immobilisation of biomass on an inert surface provides an ideal environment for the manipulation of growth parameters and the assessment of these factors on biofilm productivity.

7.1.1. Multiple Membrane Gradostat Bioreactor (MMGB)

Scale-up of the membrane gradostat system has been the focus of several research projects. Simplicity of design appears to be the key to success. Most recently, a vertical MMGB was developed by Garcin (2002).

This vertical MMGB maintains previously recorded advantages of the gradostat system (section 1.3.3.8) in that it provides good oxygen transfer in a low shear environment. Extracellular product is continuously removed from the system, thus limiting product inhibition and enabling continued removal of product in its crude form.

Optimal parameters for uniform biofilm growth were evaluated in Chapter 5. In the design of the vertical MMGB, Garcin (2002) addresses the problems of uneven growth affected by axial nutrient gradients and pressure drop along the membrane fibre length, droplet formation and excessive growth and fouling.

P. chrysosporium readily forms a complex biofilm when cultured using gradostat bioreactors. Sustained production of peroxidase enzymes under these conditions has been reported (Garcin, 2002, Leukes, 1999, Walsh, 1998).

7.1.2. Crude enzymatic suites

LiP and MnP are produced as families of differentially regulated isoenzymes varying in their catalytic properties (section 1.3.2.7). The composition and ratio of these peroxidase suites is affected by both nutrient composition and culture conditions (section 1.3.3). Their stability is influenced by the presence of proteases (section 1.3.2.4) and inactivation by excessive amounts of H₂O₂.

Different isoenzymes are produced in stationary, agitated or immobilised production systems. Isoenzyme production in batch culture is transient. The gradostat membrane bioreactor potentially offers the continuous production and isolation of a stable enzymatic suite dependent on the nutrient and oxygen gradients imposed on a specific organism. Operation of the gradostat membrane bioreactor under different conditions may, in theory, render altered crude enzymatic suites with different catalytic properties. Similarly, different strains selected for their superior ability to degrade different pollutants may be cultured to produce enzymatic suites tailor-made for a specific process.

Furthermore, the continuous production of high concentration extracellular peroxidase, using the membrane gradostat bioreactor, introduces the possibility of a more reliable means of comparing enzyme production between sibling strains than in liquid culture systems or when using a single fibre membrane bioreactor system. Laboratory scale bioreactors are generally produced with a greater number of membranes, effectively increasing both the number of replicates (although combined) and the amount of enzyme produced. The improvement of the discriminatory power of methods used to describe differences between sibling strains is an ongoing process allowing for more accurate assessment of physiological and morphological differences.

The extraction and purification of lignolytic enzymes usually includes a dialysis step in order to minimise media components. Dialysed enzyme samples have inferior degradation capabilities in comparison to undialysed enzymes. It has been suggested that certain low molecular weight mediators enhancing enzyme activity and thus degradation are lost during this process. Crude extracellular enzymatic suites would maintain such mediators.

In linking fundamental and applied research, immobilisation of biomass on an inert membrane surface using the membrane gradostat bioreactor would allow for the biochemistry of immobilised biofilms to be explored in a controlled environment. This continuous system offers the easy manipulation of growth conditions allowing for the assessment of their impact on the production of crude enzymatic suites in a number of filamentous organisms. However, a reproducible production system is essential for such applications.

7.1.3. Aims

The membrane gradostat bioreactor effectively combines liquid and solid-surface culture techniques into a stable immobilisation system. Using a laboratory scale membrane gradostat bioreactor, the continuous production of peroxidase by *P. chrysosporium* biofilms, produced under a liquid-solid-air interface (closely resembling its natural saprophytic environment), is explored.

This chapter serves to:

4. Establish stable, reproducible operational conditions for the production of high titre peroxidase enzymes using a vertical, multiple-fibre membrane gradostat bioreactor.
5. Comparison of peroxidase production by *P. chrysosporium* strains using the multiple-fibre membrane gradostat bioreactor.

7.2.MATERIALS & METHODS

7.2.1. Multiple membrane bioreactor design

The multiple membrane gradostat bioreactor (MMGB) was designed as a pilot-scale reusable vertical reactor (Figure 7.1). Each reactor consisted of a transparent PVC tube acting as a shell, sandwiched between two polypropylene manifolds. The manifold and shell encased a membrane cartridge comprised of 24 capillary membranes suspended between and sealed within two polypropylene rods. A stainless steel tube, penetrating each polypropylene rod, protruded from the outer surface, central to the respective rod, served as inlet and outlet ports (Figure 7.1.b). Four galvanised steel threaded rods were used to support the MMGB module, compressing the segments together such that they were tension sealed against O-rings (Figure 7.1.a).

MMGB modules were assembled such that the o-rings provided an air- and liquid-tight seal separating the extra-capillary space within the PVC shell from medium reservoirs at each end. Reservoirs were positioned between polypropylene manifolds and the membrane cartridge. Air and medium flow were counter current. In vertical orientation, medium flow was channelled from the lower reservoir through capillary membrane lumens into the upper reservoir displacing any pockets within (Figure 7.1.a). Airflow was through the upper stainless steel tube into the extra-capillary space and out through the lower stainless steel tube, together with any permeate (Figure 7.1.b)

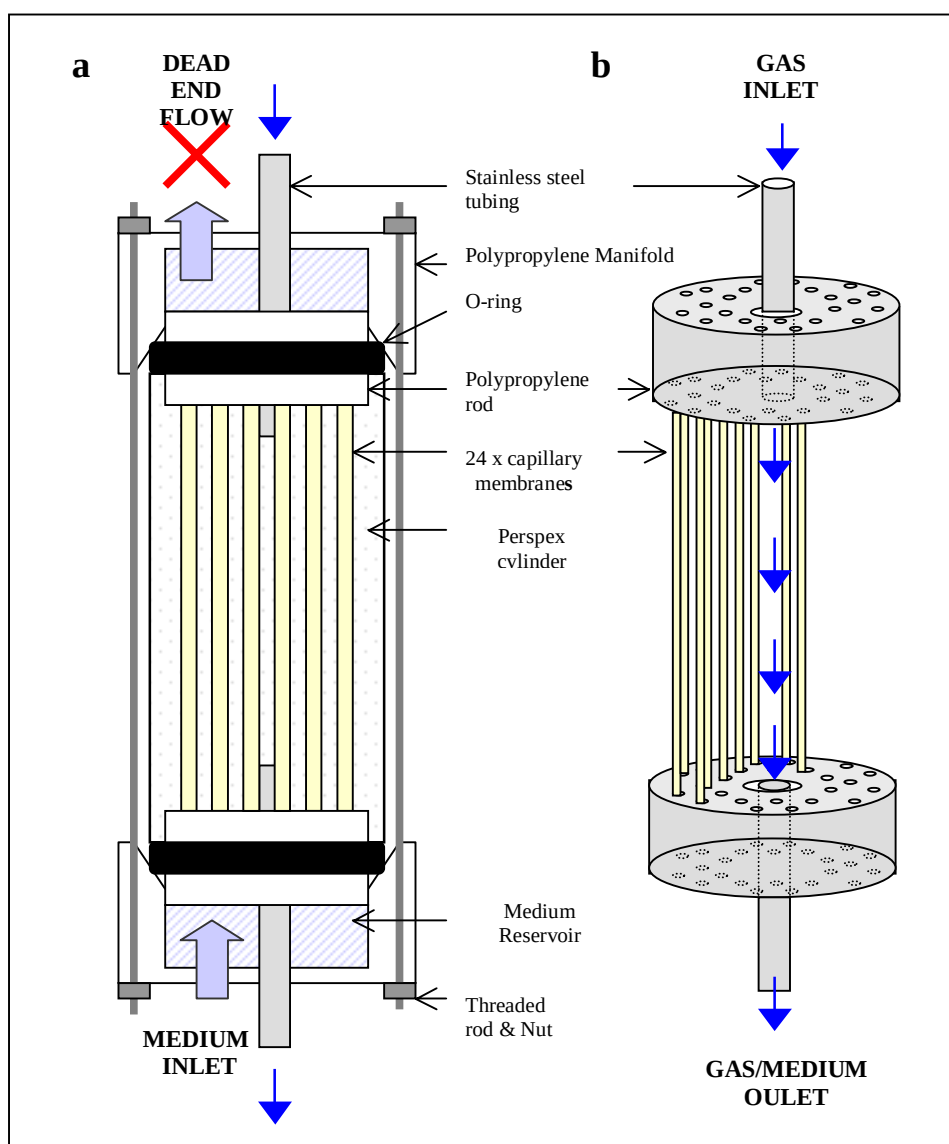


Figure 7.1: Design of a pilot-scale MMGB in vertical orientation. (a.) MMGB module showing medium reservoirs and direction of flow. (b.) Showing membrane cartridge and airflow inlet and outlet.

7.2.2. Sterilisation and inoculation

Bioreactors were sterilised with a 4 % solution of formaldehyde overnight. Formaldehyde was then thoroughly rinsed from the membranes and manifolds using sterile distilled water prior to inoculation. Humidifiers were autoclaved as were permeate collection vessels.

Evaluation of MMGBs for the production quantities of extracellular peroxidase was undertaken with *P. chrysosporium* ME446 (section 7.2.3.1). Preliminary experiments into the production of extracellular enzyme suites using different *P. chrysosporium* strains (section 7.2.3.2). MMGBs were inoculated with a standardised spore suspension of 1×10^5 spores per cm^3 membrane (Appendix I: C).

7.2.3. Operating conditions

7.2.3.1. Effect of growth parameters on extracellular enzyme production

Immobilised *P. chrysosporium* ME446 was cultured under constant, dead-end medium flow, but with altered temperature and oxygenation cycles. MMGBs were supplied with basal III medium (Appendix I:A-2) at approximately $0.1 \text{ l.m}^{-2}.\text{min}^{-1}$, using a Watson Marlow peristaltic pump with 0.1 ml.mm^{-1} accurated PVC tubing (AEC-Amersham). Medium flow was from above, reducing axial gravitational effects. The accumulation of air bubbles within the system (Chapter 5) was reduced under the higher flow rates at which MMGBs were operated.

All MMGBs were aerated with humidified air, supplied at approximately 20 kPa by diaphragm pumps (Marlton), for the first fifteen days of growth. MMGB cultures were incubated at 37 °C for the first five days followed by a drop in incubation temperature to 28 °C for a further ten-day period. Fifteen days post-inoculation cultures were returned to 37 °C but still operated under air. On day 25, MMGBs were supplied with humidified oxygen at approximately 20 kPa. Flow rates were equilibrated using a digital manometer.

7.2.3.2. Comparison of *P. chrysosporium* strains

Ten different *P. chrysosporium* strains, including ME446 were cultured in parallel under constant, dead end medium flow, at 37 °C. MMGBs were supplied with basal III medium (Appendix I:A-2) at approximately $0.1 \text{ l.m}^{-2}.\text{min}^{-1}$, using a Watson Marlow peristaltic pump with 0.1 ml.mm^{-1} accurated PVC tubing (AEC-Amersham).

All MMGBs were aerated with humidified air, supplied at approximately 20 kPa by aquarium pumps (Marlton), for the first three days of growth, at which point cultures were supplied with humidified oxygen at the same flow rate, equilibrated using a digital manometer.

7.2.4. Flow rate

Membrane flux was recorded daily by measuring the permeate volume obtained overnight from each MMGB. Medium flow rate was calculated in ml per minute

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7.2.5. Substrate utilisation

Glucose and NH_4^+ concentrations of permeate collected daily from each MMGB module were assayed using the Nelson-Somogyi method (Appendix I:C-1) and modified Nessler reaction (Appendix I:C-2), respectively.

Assuming a constant glucose concentration in feed medium, the specific rate of substrate utilisation (q) was calculated in mg glucose consumed per day.

7.2.6. Enzyme activities

The pH and extracellular LiP and MnP activities of permeate collected from MMGBs were recorded daily. LiP and MnP activity was determined using veratryl alcohol oxidation (Appendix I:D-1) and DMAB/MBTH oxidation (Appendix I:D-2.2) methods, using a microplate reader.

7.3. RESULTS

7.3.1. MMGB growth parameters

Figure 7.2 represents the time course profiles generated for each of the eight MMGBs inoculated with *P. chrysosporium* ME446. Standard observations for reactors I through VIII are described below, highlighting only significant differences between the replicates.

7.3.1.1. Flow Rate

Permeate volumes collected daily were used to generate a time course profile of transmembrane flux for each MMGB. Observations into flux variability were made within designated timeframes for each of the experimental parameters under which MMGBs were operated (Table 7.1). The mean fluxes ($n = 8$) recorded within each timeframe or experimental parameter examined did not differ significantly from one another [$P > 0.05$], remaining relatively constant throughout the experimental run, although standard deviations became progressively more variable, particularly for days 25-30. This reflects differences in flux observed for each of the replicates, specifically Reactor VI, which showed an unacceptable 69.73 % deviation from the mean flow rate (Table 7.2).

Table 7.1: Mean ($\pm$ SD) flux and specific rate of glucose utilisation for ME446 under each of the operational conditions that MMGBs were exposed to.

Time (days)	Operational Conditions	Mean Flux (ml.day ⁻¹)	Mean Substrate Utilisation Rate [q] (mg.day ⁻¹)
1-5	37 °C; Air	24.8 $\pm$ 6.6	0.635 $\pm$ 0.226
6-15	28 °C; Air	37.6 $\pm$ 8.4	0.058 $\pm$ 0.203
16-25	37 °C; Air	31.1 $\pm$ 8.3	0.141 $\pm$ 0.154
26-35	37 °C; Oxygen	30.5 $\pm$ 12.9	0.148 $\pm$ 0.093

7.3.1.2. Substrate utilisation

NH₄⁺ levels in MMGB permeate were depleted three to five days post-inoculation as the biofilm was established within MMGBs. Biofilm growth is self-regulating: nutrient depletion triggers idiophasic growth, while constant medium supply maintains nutrient nitrogen levels at reduced levels (Figure 7.2). Thus, secondary metabolism is maintained throughout the experimental run in this continuous system. In contrast, glucose levels in MMGB permeate was more readily influenced by fluctuating permeate levels and what appeared to be operational condition linked biofilm growth.

Table 7.2: Mean ($\pm$ SD) permeate volumes, specific rate of glucose utilisation and maximum MnP activities recorded for each MMGB replicate inoculated with *P. chrysosporium* ME446.

MMGB replicate	Mean Flux (ml.day ⁻¹)	Mean Substrate Utilisation Rate [q] (mg.day ⁻¹)	^a Max MnP activity (U.l ⁻¹)
I	37.2 $\pm$ 4.9	0.158 $\pm$ 0.026	125 (29)
II	29.7 $\pm$ 11.1	0.181 $\pm$ 0.024	379 (25)
III	35.9 $\pm$ 7.6	0.203 $\pm$ 0.025	409 (27)
IV	35.9 $\pm$ 8.7	0.172 $\pm$ 0.028	668 (28)
V	33.1 $\pm$ 7.7	0.188 $\pm$ 0.027	1108 (26)
VI	23.4 $\pm$ 16.3	0.189 $\pm$ 0.025	506 (26)
VII	32.2 $\pm$ 9.9	0.129 $\pm$ 0.031	450 (27)
VIII	27.3 $\pm$ 7.3	0.225 $\pm$ 0.025	1479 (26)

^a Day on which maximum enzyme activities were detected are shown in brackets.

Table 7.1 describes the effects of altered operational conditions on substrate utilisation by *P. chrysosporium* ME446 biofilms. The greatest q values were determined within the first five days of growth at 37 °C under air. q Was rapidly reduced by altering the incubation temperature to 28 °C, below that required for optimum growth. Returning of cultures to an incubation temperature of 37 °C increased q . Exposing immobilised cultures to pure oxygen instead of air had a marginal effect on q . At this point, glucose levels detected in the MMGB permeate became depleted.

No significant differences were detected in q calculated for each MMGB over the entire time course, with the exception of MMGB VII versus VIII (Table 7.2).

7.3.1.3. pH values

Permeate pH levels were reduced from approximately 5.37 pH units to a minimum of 3.17 pH units by day 14. pH levels were gradually increased over a period of ten days and were seen to stabilise at approximately pH 4.4 for the remainder of the experiment, coinciding with maximum MnP activities.

7.3.1.4. Enzyme activities

MnP activities were first detected on days 3-4, with peak activities of 6 U.l⁻¹ (reactor III) to 226 U.l⁻¹ (reactor VI) detected on days 5-8, before returning to basal activity levels. Exposing established *P. chrysosporium* ME446 biofilms to pure oxygen on day 25 induced maximum MnP activities of 125 U.l⁻¹ (Reactor I) to 1479 U.l⁻¹ (Reactor VIII), detected on days 25-29 (Figure 7.2). Those MMGBs recording high activity levels within the first ten days did not necessarily record the highest levels following induction with oxygen.

Extracellular peroxidase levels recorded for *P. chrysosporium* ME446 differed by ten-fold between replicate MMGBs (Table 7.2). Maximum MnP levels recorded were extremely high, comparing favourably with yields reported for more potent *P. chrysosporium* strains such as BKMF-1767 (section 1.3.3.7).

Low levels of LiP activity were detected throughout the operation of MMGBs, were not ubiquitous amongst replicate reactors and did not exceed 2 U.l⁻¹.

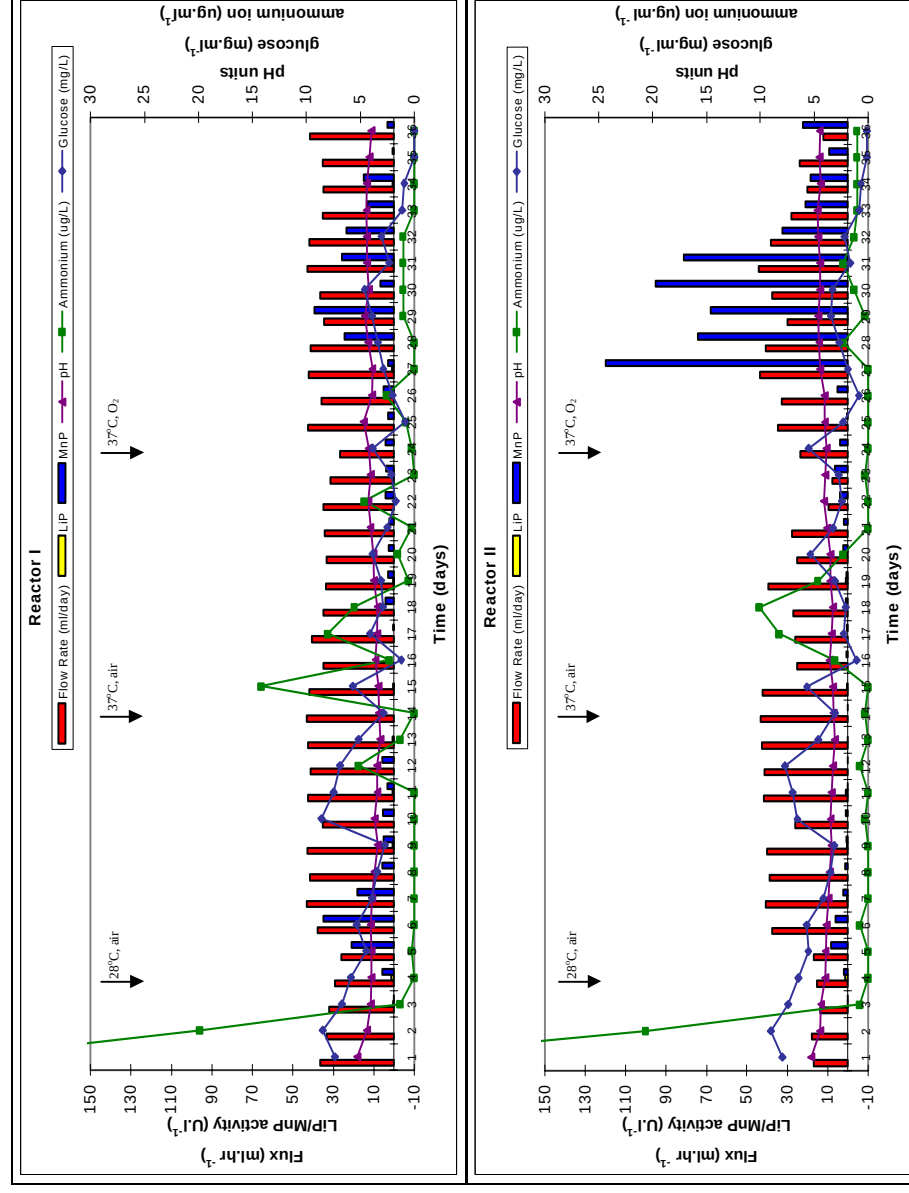


Figure 7.2: Time course showing glucose concentration (mg.ml^{-1}), NH_4^+ concentration ($\mu\text{g.ml}^{-1}$), NH_4^+ concentration ($\mu\text{g.ml}^{-1}$), NH_4^+ concentration ($\mu\text{g.ml}^{-1}$), mean flow rate, pH level and extracellular LIP and MnP activities (U.l^{-1}) of vertical, multiple-fibre membrane bioreactors ($\pm$ SD). Each MMGB (reactors I-VII) were incubated under air at 37 °C for the first five days followed by ten days at 28 °C. The temperature was increased to 37 °C on day 15. Air was replaced with pure oxygen 25 days post-inoculation ($n = 8$).

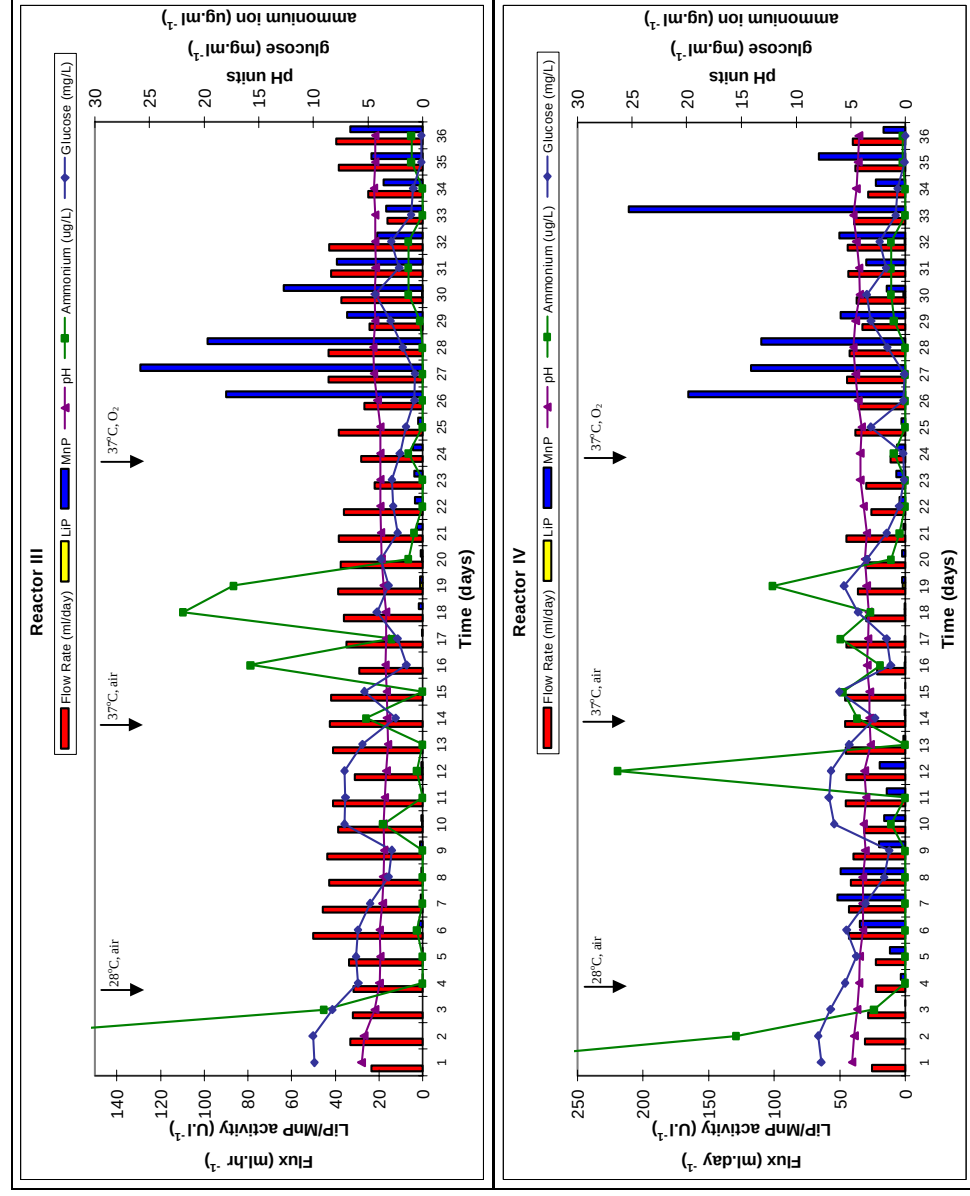


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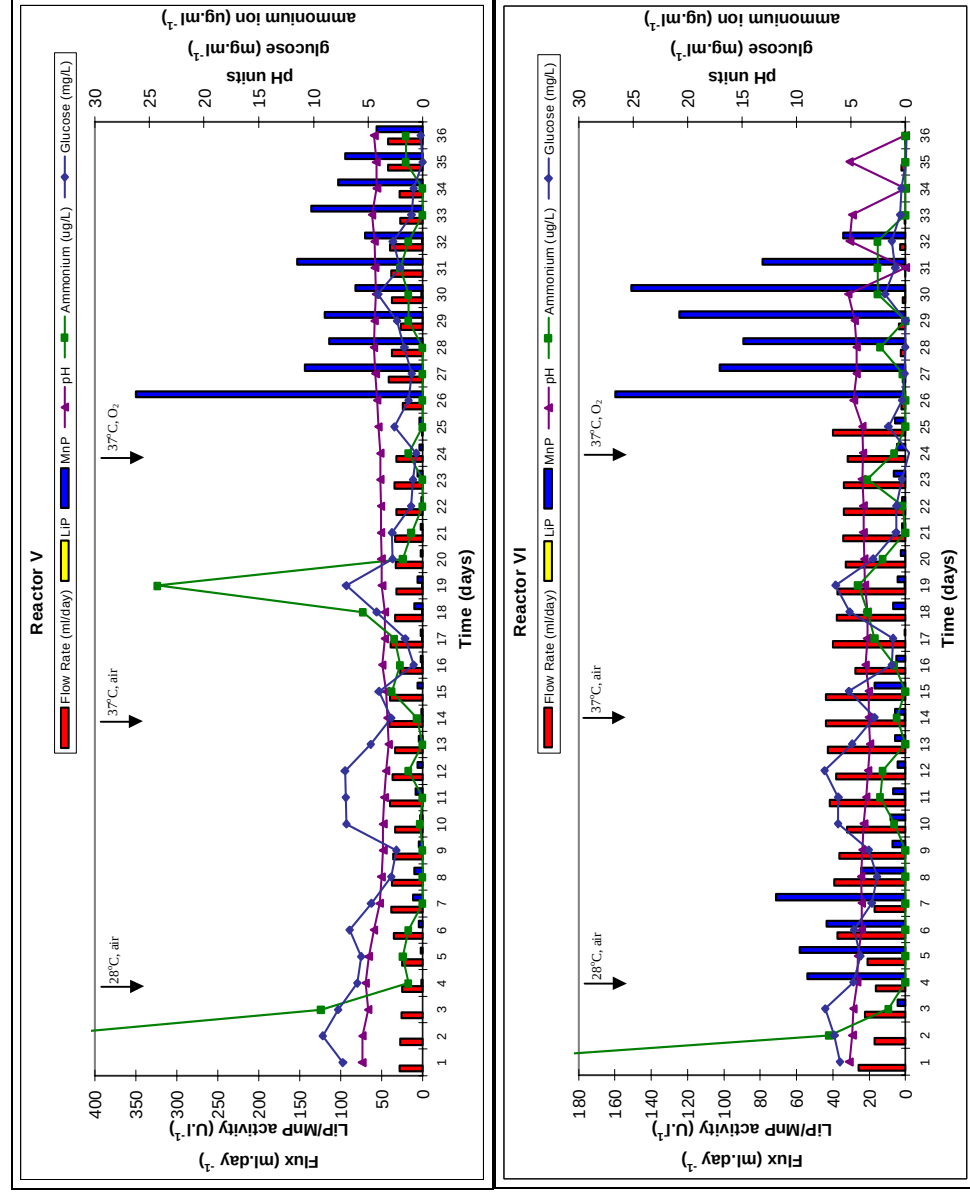


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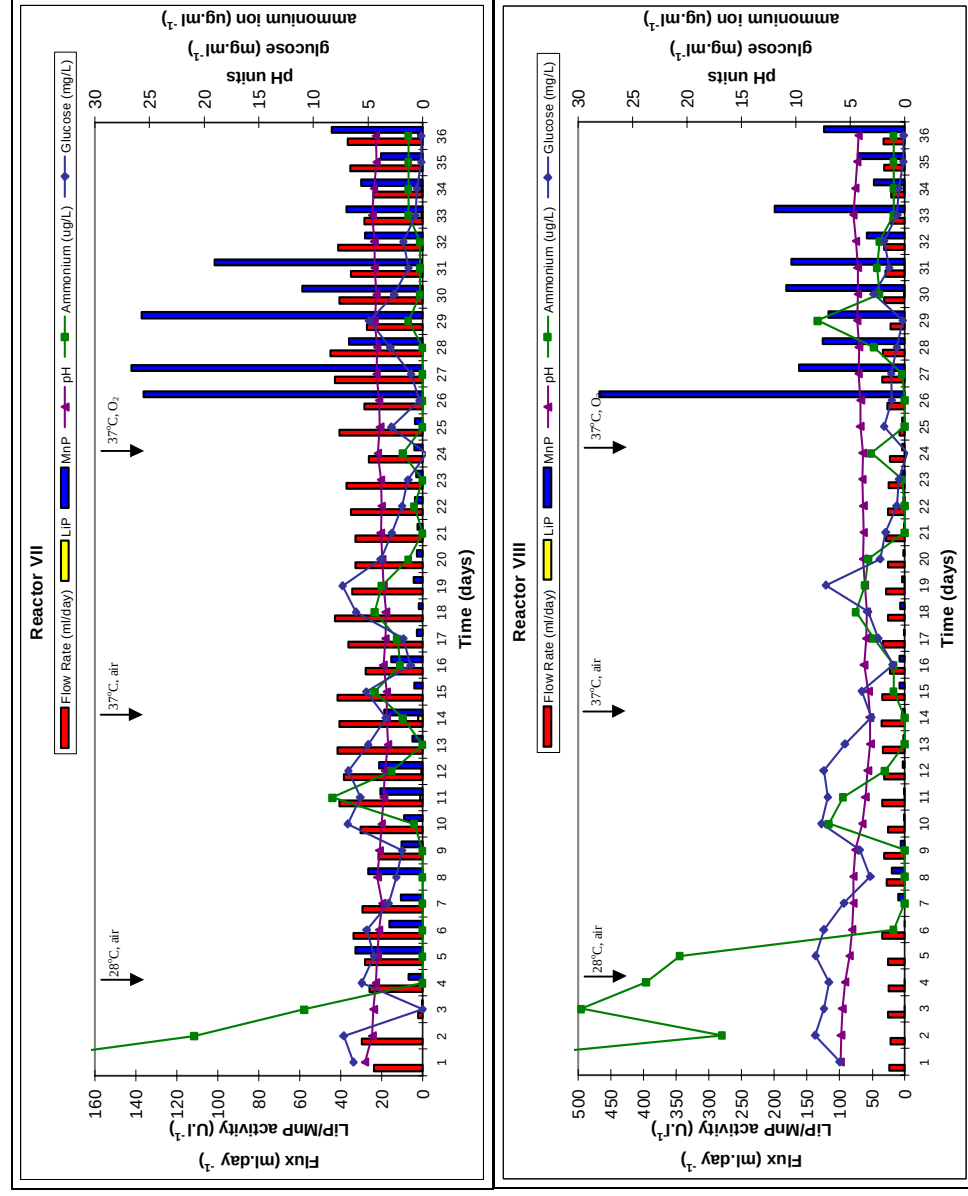


Figure 7.2 continued.

Comparison of peroxidase production by *P. chrysosporium* strains using MMGB modules

Figure 7.3 represents the time course profiles generated for each *P. chrysosporium* strain examined using MMGBs. Standard observations for each strain are described below, highlighting only significant differences.

7.3.2.1. Flow rate

Permeate volumes collected daily were used to generate a time course profile of flux for each MMGB. The mean fluxes detected for each reactor, calculated over the twenty-day time course, did not differ significantly from one another. MMGBs showed high standard deviations in flux (Table 7.3) comparable to that observed in section 7.3.1.1 over the same twenty-day period.

Table 7.3: Mean permeate volumes, specific rate of glucose utilisation and maximum MnP activities recorded for each *P. chrysosporium* strain examined.

<i>P. chrysosporium</i> strain	Mean Flux (ml.day ⁻¹)	Mean Substrate Utilisation Rate [q] (mg.day ⁻¹)	^a Maximum MnP activity (U.l ⁻¹)
ME446	39.3 ± 13.7	0.354 ± 0.064	190 (20)
BS 2	32.3 ± 20.2	0.312 ± 0.068	149 (20)
BS 2.52	48.5 ± 17.2	0.222 ± 0.063	16 (20)
BS 3	23.8 ± 15.7	0.305 ± 0.063	85 (20)
BS 13	42.9 ± 18.3	0.405 ± 0.070	51 (20)
BS17	35.2 ± 11.2	0.415 ± 0.069	21 (20)
BS 18	45.9 ± 12.2	0.404 ± 0.069	236 (19)
BS 24	50.7 ± 16.3	0.150 ± 0.080	46 (20)
BS 26	45.7 ± 12.7	0.362 ± 0.064	236 (19)
BS 32	34.5 ± 13.9	0.230 ± 0.066	175 (20)

^a Day on which maximum enzyme activities were detected are shown in brackets

7.3.2.2. Substrate utilisation

Nutrient nitrogen utilisation profiles in MMGBs inoculated with the different *P. chrysosporium* strains proceeded as before, with the depletion of NH_4^+ levels in MMGB permeate three to six days post-inoculation. These levels were subsequently maintained at reduced concentration throughout the experimental run (Figure 7.3). As before, glucose levels in MMGB permeate were influenced by fluctuating permeate levels. Table 7.3 shows differences observed in glucose utilisation rates [q] by biofilms of the different *P. chrysosporium* strains. Only strain BS 24 showed a significantly lower q than strains BS 13, BS 17, BS 18 and BS 26 [$P < 0.01$].

7.3.2.3. Biofilm development

Visual observation of each MMGB showed the strains to differ in the extent of biofilm formation (Table 7.4). Biofilm growth was generally found on the upper section of membranes, closer to the nutrient feed.

Table 7.4: The extent of biofilm formation observed on MMGB's inoculated with different *P. chrysosporium* strains.

<i>P. chrysosporium</i> strain	Biofilm Description
ME446	50 % coverage.
BS 2	10 % coverage. Thin biofilm. Complete growth on two membranes.
BS 2.52	20-30 % coverage. Complete growth apparent on only one membrane.
BS 3	40 % coverage. Predominantly at top of membranes. Good biofilm.
BS 13	Complete growth on only six membranes, along entire length.
BS 17	Poor growth, biofilm very thin.
BS 18	70-80 % growth covering membranes. Good growth, particularly at top of membranes. Biomass not uniform, occurs as clumps.
BS 24	Mycelial growth apparent on only two membranes. Does however coat entire membrane length.
BS 26	40 % growth covering membranes, isolated to membranes section at the top of the reactor. Biofilm smooth.
BS 32	50 % growth covering membranes. Biomass not uniform, growth in clumps showing spore formation.

While nutrient consumption may be linked to the amount of biomass present, the differences detected in glucose utilisation rates (Table 7.3) do not correlate with visual observations on the extent of biofilm formation in each MMGB (Table 7.4).

7.3.2.4. Enzyme activities

It is difficult to correlate maximum MnP activities with the extent of biofilm formation recorded in these experiments for each MMGB. Estimation of biofilm coverage was subjective and not an absolute measure of biofilm growth, i.e. thickness, density and coverage. Nor does the extent of biofilm coverage describe the mycelial differentiation hypothesised to influence secondary metabolite production.

Permeate pH levels were more variable than those reported in section 7.3.1.3. A distinct phase showing the reduction of permeate pH to a minimum was not clear, although a trend towards stabilisation of pH around pH 4.4 coinciding with the onset of MnP activity was apparent.

Low levels of MnP activity were first detected on days 3-4, varying from 2.6 U.l⁻¹ (BS 26) to 10.5 U.l⁻¹ (BS 3). Within the twenty-day time course, maximum MnP levels of 15.8 U.l⁻¹ (BS 2.52) to 236.4 U.l⁻¹ (BS 18 and BS 26) were detected on days 19-20, coinciding with the depletion of glucose in MMGB permeate (Table 7.3). The production of high levels of MnP earlier in the fermentation did not necessarily coincide with high production at a later point. LiP activity was negligible.

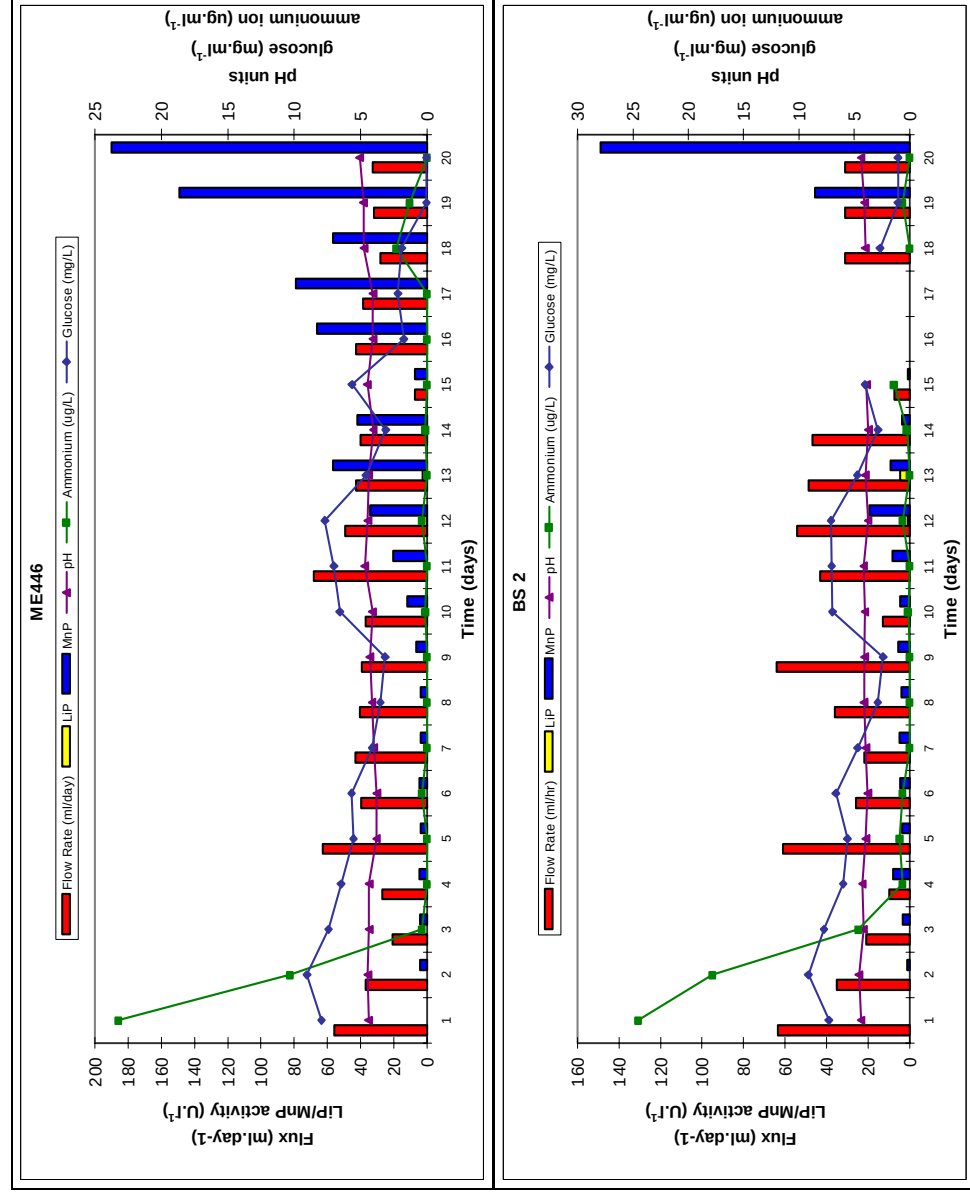


Figure 7.3: Time course showing flow rate (ml.hr⁻¹), glucose (mg.l⁻¹) and NH₄⁺ (ug.ml⁻¹) concentrations, medium pH and MnP activities (U.l⁻¹) for each *P. chrysosporium* strain cultured using vertical, multiple-fibre membrane gradient bioreactors.

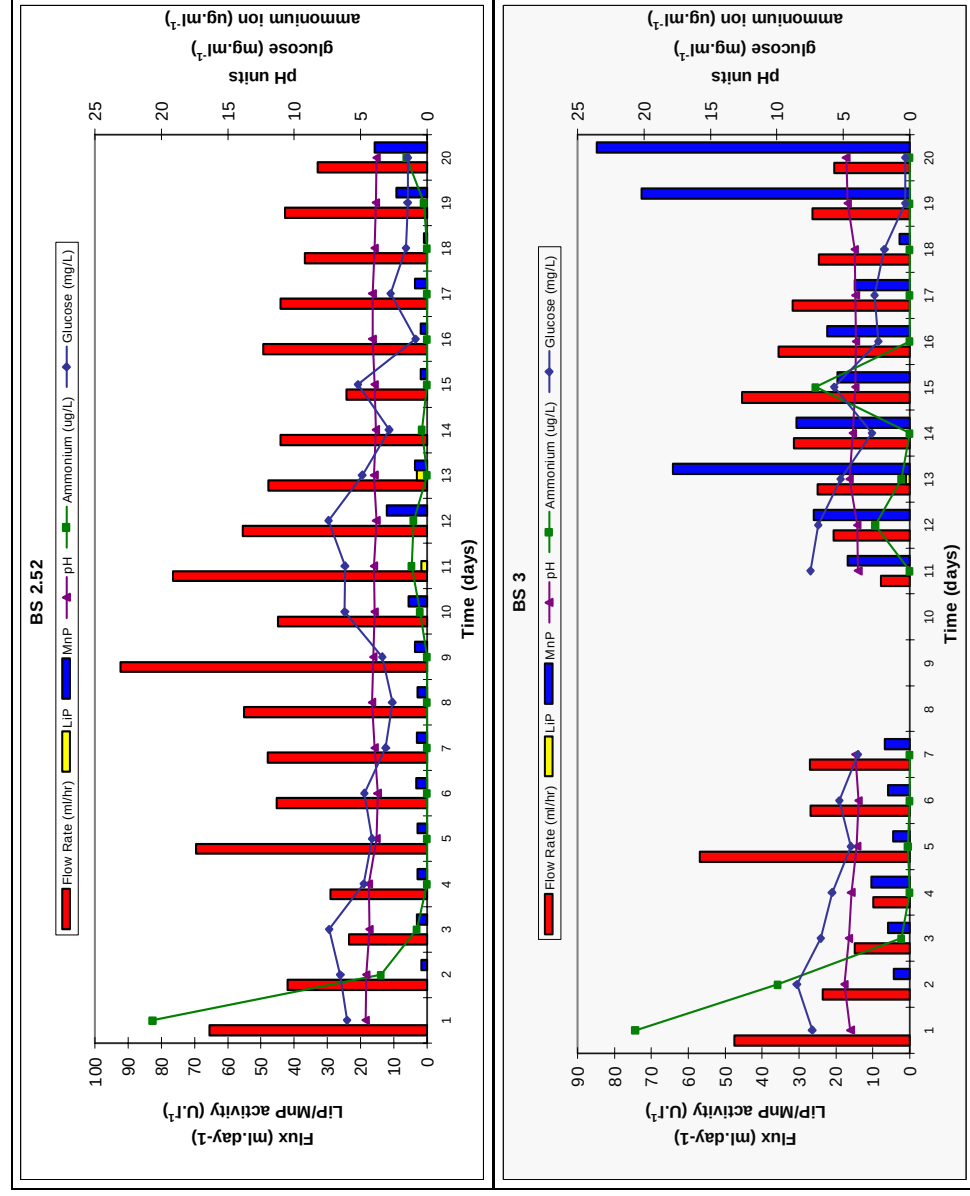


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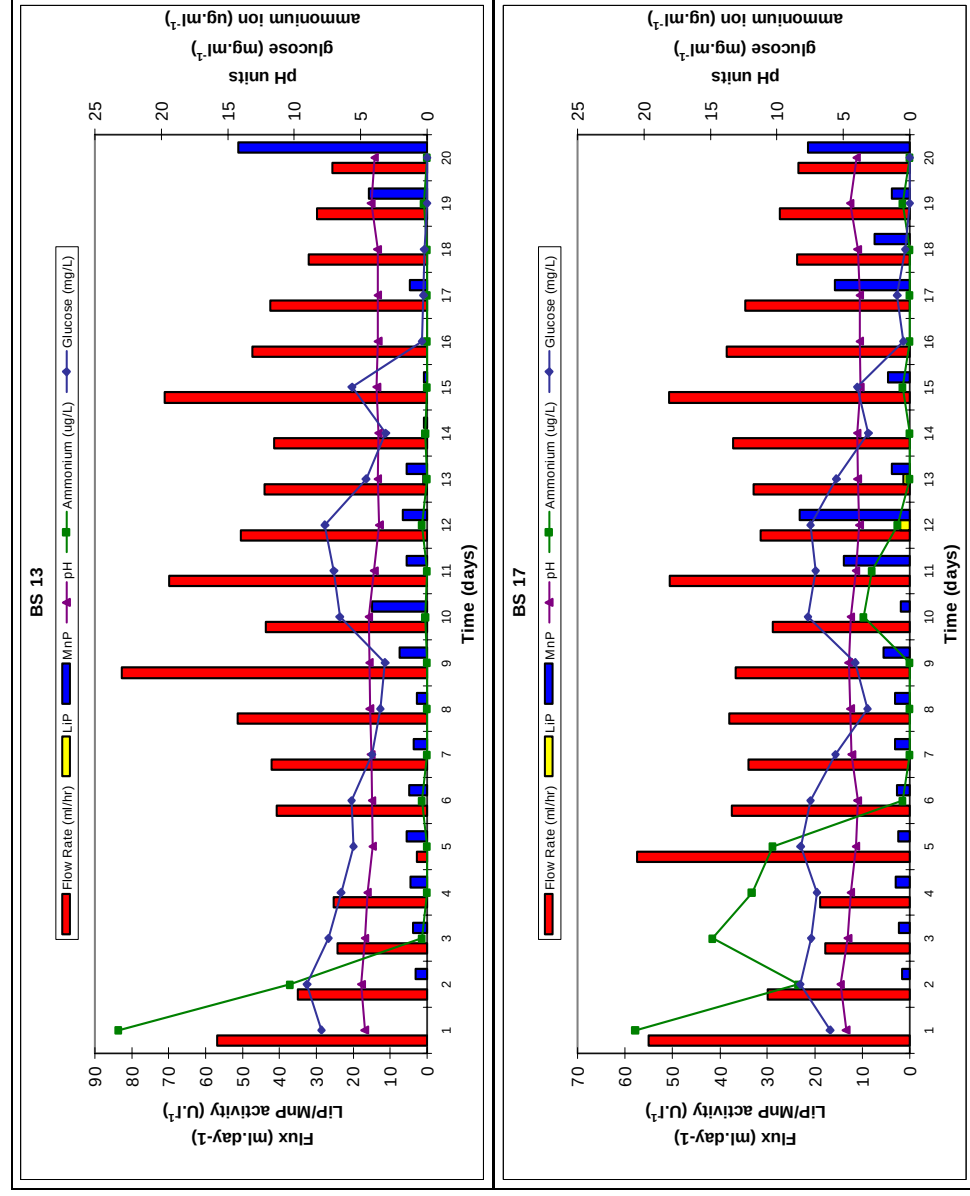


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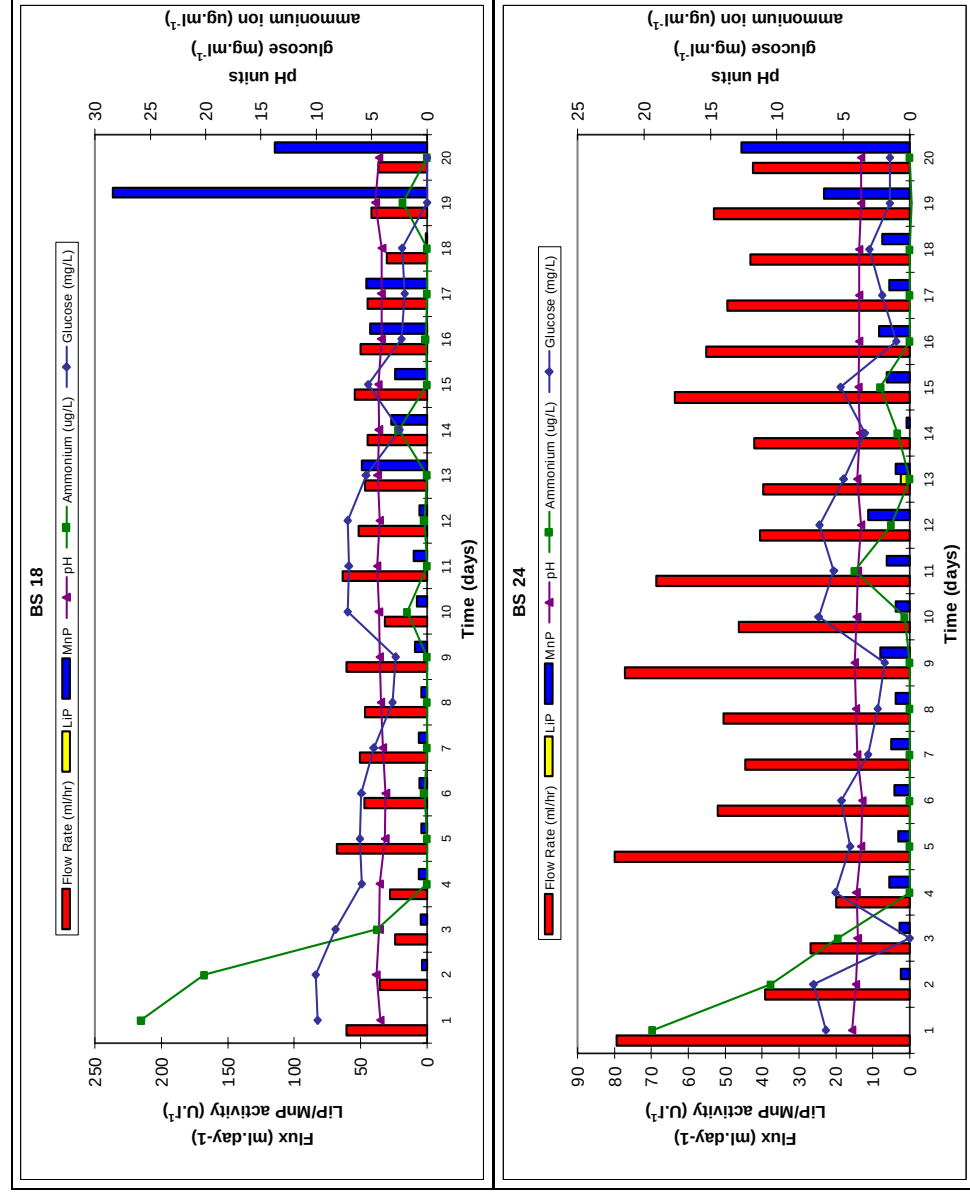


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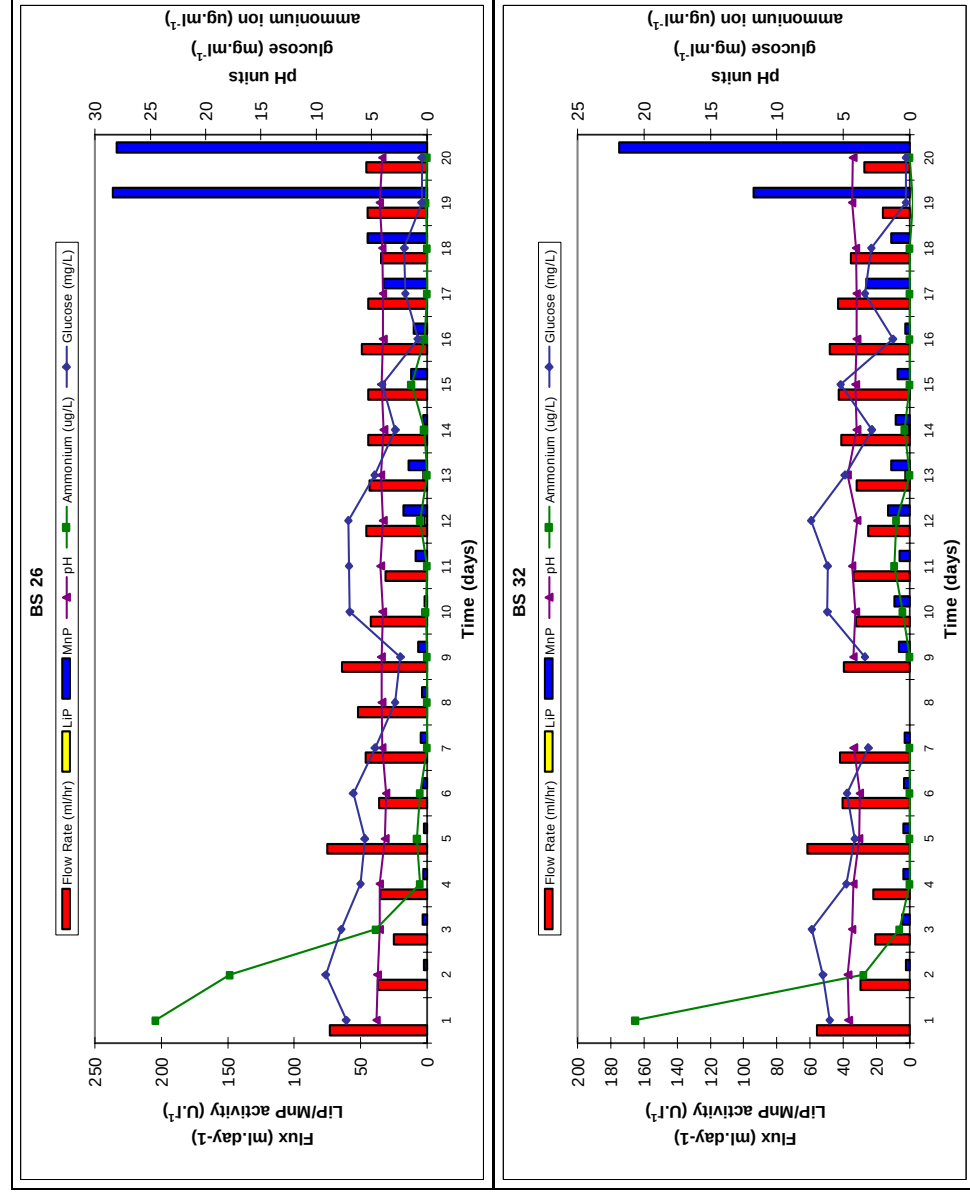


Figure 7.3 continued.

7.4. DISCUSSION

The nature of the gradostat membrane bioreactor is such that biofilm growth would initially follow the pattern for growth kinetics observed in liquid culture systems, where the lag phase in development corresponds to inoculum germination and attachment to capillary membranes, followed by an exponential growth phase, and finally a stationary phase during which secondary metabolites (coinciding with the detection of MnP activity) are produced. Due to the continuous supply of nutrients, these growth phases are continuous in the mature biofilm. The nutrient gradients influencing secondary metabolism are self-sustaining, modulated by the extent of biofilm formation.

Altered temperature cycles had a distinct effect on substrate utilisation (Table 7.1), which was markedly reduced during the 28 °C incubation cycle. This lower temperature had less of an effect on peroxidase production than expected (section 1.3.3.1). The rate-limiting step in secondary metabolite production, including peroxidase production, is reported to be oxygen supply (section 1.3.3.5). The correspondingly low levels of MnP activity detected in MMGB permeate when operated under air versus that detected following the supply of pure oxygen at the same rate support this theory. Hyperoxidative conditions affect a number of pathways influencing peroxidase production, including regulatory mechanisms at a transcriptional level (Zacchi *et al.*, 2000, Miura *et al.*, 2004, Belinky *et al.*, 2003).

The dikaryon, *P. chrysosporium* strain ME446, is a less potent lignin degrader than strain BKMF-1767, which produces nine-fold the peroxidase activity levels of ME446 and is most frequently used in production studies (Kirk *et al.*, 1986b, Faison and Kirk, 1985). Despite this, continuous production of high levels of MnP production using the MMGB has been shown. A maximum activity of 1479 U.l⁻¹ was recorded, comparable with those higher levels reported for BKMF-1767 cultured using other immobilisation systems (section 1.3.3.7). These MnP activities are ten-fold greater than ligninase activities produced by BKMF-1767 in other membrane bioreactor systems (Table 1.5). The crude enzyme produced by *P. chrysosporium* ME446 immobilised MMGBs was freeze dried and kept for further analysis (see Chapter 8).

Within optimisation experiments using *P. chrysosporium* ME446, all bioreactors (n = 8) were inoculated and operated simultaneously under the same conditions. Variability in the data between replicates must therefore be attributed to inconsistencies in the MMGB modules. The polysulphone capillary membranes from which each module is constructed have been shown to vary from batch to batch and within batches. This variability accounts for a minimum 10 % inconsistency within a given experiment.

Similarly, daily fluctuations in transmembrane flux are compounded by the medium supply where flow rate is influenced by both the peristaltic pressure and biomass accumulation within capillary macrovoids, creating backpressure. The operation of bioreactors at slow flow rates for long periods of time tends to degrade the narrow bore accurated tubing required for these slow flow rates, introducing further variability at the level of medium supply.

The vertical MMGB module eliminates biofilm maldistribution cause by droplet formation created by local variation in membrane permeability (Chapter 5). However, this vertical orientation of MMGBs promotes greater medium flow through the bottom capillaries (and thus biofilm growth) due to hydrostatic pressure effects (gravity) when medium is supplied from below. This is alleviated somewhat by medium supply from above, provided air bubbles (which tend to accumulate and interrupt medium flow) are eliminated or periodically flushed from the system. Bubble accumulation occurs as dissolved air comes out of solution due to a pressure drop as medium flows from thin to thicker lumen tubing. Air bubbles interrupted medium flow, although to a lesser extent than observed in Chapter 5 due to the higher flow rates each MMGB module was operated under.

In comparing MnP production by *P. chrysosporium* ME446 during the operational parameter experiment (section 7.3.1) with those results obtained for intraspecific comparison experiments (section 7.3.2), it is clear that the induction of MnP activity by molecular oxygen (Figure 7.1) is more effective in established, nutrient-limiting biofilms than those cultured under high oxygen tension throughout the fermentation (Figure 7.2: ME446). This is consistent with liquid culture in that stationary flasks are routinely grown under air for the first three days, promoting mycelial growth. Oxygenation was found to be most effective when combined with nutrient depletion and the onset of secondary metabolism.

Preliminary intraspecific comparisons of MMGB immobilised *P. chrysosporium* strains (examined throughout this project) were inconclusive. Crude extracellular enzyme suites generated from these reactors were freeze-dried for future proteomic work. However, more replicates using a more stable system are required before any conclusions can be drawn regarding the nature of their composition and degradative activities.

7.5. CONCLUSIONS

The MMGB has been used to produce competitively high yields of extracellular peroxidase using a continuous process in which hypersecretion of MnP was induced by exposing established *P. chrysosporium* ME446 biofilms to hyper-oxidative conditions.

Further optimisation of MMGB operational conditions, stimulating higher levels of activity in MMGBs, may minimise the variability observed in low productivity systems and therefore improve the resolution power of this system. At this point the variability observed between MMGB replicates inoculated with *P. chrysosporium* ME446 does not allow for conclusive insight into the use of MMGBs as a tool for developing crude enzymatic suites for intraspecific comparison. The experimental variability of this system precludes the attribution of significant differences in peroxidase production to genetic differences rather than operational differences.

CHAPTER 8

Conclusion

8.1. INTRODUCTION

Until recently, most of the research into enzyme production and bioremediation using *P. chrysosporium* has been focused on process outcomes and fermentor design, treating the organism involved as a black box. Extensive information has been gathered on the effects of culture parameters on ligninase production and isoenzyme composition. The first results of molecular research already show distinct differences in the level of gene expression and regulation of lignolytic isoenzymes in response to nutritional composition, stationary or agitated liquid culture, and submerged or solid substrate fermentations.

The optimisation of bioremediation technologies would benefit from a detailed understanding of the organism involved and the mechanisms by which that organism facilitates biodegradation. Only a combination of strain selection, strain development, molecular genetic approaches and production-process development will result in the comprehensive understanding of those factors regulating and enhancing the bioremediation potential of an organism.

The basis for various strain improvement strategies lies in the development of sensitive experimental procedures, enabling the screening, detection and subsequent isolation of a stable variant displaying phenotypic traits that differ from the wildtype strain. Inter- and intraspecific comparison of *P. chrysosporium* has primarily been undertaken as stationary flask cultures. Lignolytic enzyme production by basidiomycetes is hyper-variable under these conditions (Baldrian and Gabriel, 2002, Linko, 1992, Raeder *et al.*, 1989b). This is attributed to their preferential growth as biofilms on solid substrates such as wood, in accordance with their saprophytic role in the environment.

8.2.OVERVIEW

This thesis has undertaken the intraspecific comparison of ten genetically related *P. chrysosporium* strains, with the aim of describing differences in their pollutant degradation capabilities and attributing those differences to physiological and morphological characteristics in these strains. The examination of sibling strains for this purpose allows for those differences observed in the phenotypic traits of these organisms to be linked to a small number of genes.

P. chrysosporium is sensitive to small changes in its environment and experimental variability was determined to be a major factor influencing the detection of significant differences between sibling strains. It was hypothesised that a 96-well microtitre plate based liquid culture system would improve the power of detection of liquid culture methods through the simultaneous comparison of all organisms and an increased number of replicates.

Differences in toxic response to pollutants were introduced as a selective criterion for strain improvement. Medium, method and pollutant-dependent differences in the toxic response of sibling strains alluded to different mechanisms and pathways for pollutant tolerance and degradation in these organisms. It was proposed that physiological and morphological characteristics of sibling strains would differ in solid surface culture and that the experimental variability observed in liquid culture might be reduced through immobilisation of *P. chrysosporium* sibling strains. The membrane gradostat bioreactor offered a blending of liquid culture and solid surface culture methods under controlled conditions. Biomass was stabilised through its immobilisation onto an inert, porous capillary membrane, mimicking biofilm formation on a natural woody substrate, without the complication of an alternative nutrient source in the wood itself. The membrane gradostat bioreactor was evaluated in parallel to liquid culture techniques as a tool for the intraspecific comparison of sibling strains.

A second plug-flow membrane bioreactor was also evaluated as an experimental tool hypothesised to extend the nutrient and oxygen gradients observed to effect mycelial differentiation in the membrane gradostat bioreactor.

The close genetic relationship of the ten sibling strains examined in this thesis required the establishment of operational procedures whose sensitivity and reproducibility enabled the phenotypic differences between these strains to be distinguished from one another. The advantages, shortcomings and potential applications of both conventional and novel methodologies used in the comparison of sibling strains are described below. Using these methods, a selection of sibling strains displaying the

most variable combination of ligninase production, pollutant sensitivity, pollutant degradation and morphological differentiation were identified and recommended for future study as a means of describing the different molecular and metabolic mechanisms observed in these organisms.

8.3. COMPARISON OF CULTURE METHODS

8.3.1. Liquid culture systems

8.3.1.1. Applicability

Previous experiments involving the *P. chrysosporium* sibling strains, and further evaluated in this work, were undertaken in liquid culture (Wyatt and Broda, 1995, Raeder *et al.*, 1989a, 1989b, Raeder and Broda, 1986). Thus, stationary liquid culture using nitrogen-limiting medium was selected as the basis for further characterisation of these same organisms.

It became apparent that *P. chrysosporium* growth and peroxidase production was highly sensitive to small changes in culture conditions, and was subject to increased variability amongst replicates even with standardisation of experimental procedures. A 96-well microtitre procedure that effectively miniaturized the 10 ml stationary flask culture system was therefore explored as a convenient means of increasing the number of samples within a given experiment; allowing for more replicates.

8.3.1.2. Reproducibility

The production of lignolytic enzymes by Basidiomycetes in liquid culture is acknowledged as being inconsistent (Baldrian and Gabriel, 2002, Raeder *et al.*, 1989b, Linko *et al.*, 1987). This may be attributed to liquid culture being an unnatural growth environment for Basidiomycetes. The introduction of 96-well microtitre plate based methods for the growth and characterisation of *P. chrysosporium* sibling strains in liquid culture proved advantageous, in that these methods were easily able to accommodate the simultaneous analysis of increased replicates previously found to be laborious and logistically difficult in flask cultures.

By increasing the number of replicates it was envisaged that the discriminatory power of liquid culture methods would be enhanced, allowing improved power of detection of inherent differences in the sibling strains to be examined. Further, pellicle formation in small microtitre plate wells was less likely to be disturbed by external factors thus improving reproducibility amongst replicates.

Using the 96-well microtitre plate method accurate growth curves and consistency in growth kinetics was observed between replicate wells for a given organism. However, as observed in 10 ml stationary flask cultures, extracellular peroxidase levels of replicate samples remained highly variable. Increasing the sample size even further may further enhance the power of detection of this method.

8.3.2. Pollutant sensitivity and degradation

Pollutant sensitivity studies were undertaken with the view of classifying each of the sibling strains examined according to their tolerance of a series of pollutants, and in the selection of a model pollutant and concentration level for further biodegradation study.

The two methods selected for assessing pollutant sensitivity of each of the sibling strains were the agar surface method and a 96-well microtitre plate, liquid culture system. *P. chrysosporium* is saprophytic, adapted to biofilm growth on wood or soil. Yet, through this organism's ability to degrade toxic pollutants it is frequently applied to the bioremediation of aqueous effluents. While the agar plate method represented a more natural, solid surface, growth environment for the isolates being examined, the 96-well microtitre plate method proved advantageous in that growth measurements based on culture absorbance proved to be a more sensitive reflection of filamentous growth in the presence of growth interfering compounds.

The 96-well microtitre plate method was subsequently shown to be capable of elucidating intraspecific differences in the biodegradation capabilities of sibling strains. Due to the number of strains examined in this work, the experimental controls and the number of microtitre plates required in each case, the number of replicate wells sacrificed in a time course study was limited. Improved power of detection will be better served through the analysis of fewer isolates with a greater number of replicates. Through categorical analysis of the ten sibling strains examined in this thesis, those sibling strains showing distinctly different characteristics (resolved by using the microtitre plate technique) have been identified for this purpose (Section 8.3).

8.3.3. Membrane bioreactors

8.3.3.1. Multiple membrane gradostat bioreactor (MMGB)

The advent of a bioreactor system that reflected the natural growth environment of the saprophytic *P. chrysosporium* and whose design encompassed the operational criteria of a continuous fermentation system was met in the membrane gradostat bioreactor. Continuous production of peroxidase by *P.*

chrysosporium was achieved by sustaining primary, secondary and decline growth phases within a biofilm, self-regulated by nutrient and oxygen gradients established across the biofilm, through a process referred to as the membrane gradostat concept.

In this and previous work the potential of the membrane gradostat bioreactor was established, with high yield peroxidase production exceeding that of enzyme production in liquid cultures of *P. chrysosporium* ME446. However, scale up of this system remains problematic. In the design of the vertical multiple membrane bioreactor the major factors impacting on the reproducibility of this system were attributed to fluctuations in medium supply due to inconsistencies in the capillary membranes from which membrane modules were constructed, the accumulation of air bubbles within medium lines and the degradation of accurated tubing through prolonged use.

Those criteria allowing for the continuous operation of MMGBs, namely; reuse of biomass; removal of inhibitory waste products; and harvest of secondary metabolites of interest also provide a flexible system for determining the effects of medium composition on secondary metabolite production.

8.3.3.2. Single-fibre membrane bioreactor (SFR)

Those same criteria influencing the reproducibility of the MMGB were apparent to a far greater extent in SFR modules that were explored as a tool for biofilm analysis. Although the polysulphone membrane inserts were more easily screened for inconsistencies, variability in transmembrane flux remained high at the slower flow rates required for the operation of SFRs. This variability may be counteracted in future by degassing growth medium (may be detrimental to biofilm development) and through the use of less permeable membranes that allow for the constant supply of medium at low flow rates under pressure. Although medium supply using pressure vessels has been explored, the low pressures required to operate SFRs under low flow rates could not be accurately or consistently maintained with the current, highly permeable membranes. Low flow rates are essential for peroxidase production in *P. chrysosporium* as the flow rate determines the biofilm thickness. Thicker biofilms become oxygen starved and are therefore poorly adapted to maximal peroxidase production where oxygen is a rate-limiting factor.

Using vertical SFRs operated under air at low flow rates, enhanced mycelial differentiation was observed within *P. chrysosporium* biofilms. *P. chrysosporium* ME446 showed a high level of differentiation in its biofilm structure, where both surface and penetrative growth regions showed distinct tissue differentiation and subcellular distribution of organelles. Surface growth was

subdivided into three zones dominated by aerial hyphae (closer to air interface), submerged hyphae (middle) or subicular hyphae (closer to membrane surface). Chlamydospore-like fruit bodies were associated with subicular hyphae and rhizomorphs like mycelial aggregates penetrating macrovoids.

The membrane gradostat bioreactor offers a unique environment where biofilm growth on inert polysulphone capillaries was able to simulate the colonisation of wood by this filamentous organism, in a controlled environment, without the complications of the woody substrate itself. The membrane gradostat bioreactor promises to be a useful tool in evaluating the effects of nutrient and oxygen levels on mycelial differentiation at both morphological and physiological levels.

Further, intraspecific differences in the nature of the biofilm developed, and the extent of differentiation were observed between sibling strains. These results substantiate the potential of the membrane gradostat bioreactor as a valuable tool in future characterisation of the multigenic traits between *P. chrysosporium* sibling strains.

Linkage of lignolytic enzyme production with morphological traits through the study of sibling strains would allow for the identification of genetic markers and the informed selection of organisms better suited to growth in liquid, solid substrate or immobilised culture systems (see section 8.3).

Peroxidase production is regulated at the level of transcription by both medium composition and molecular oxygen. Mycelial differentiation and multicellularity has also been linked to nutritional factors and oxygen gradients (Blackstone, 2000). Several links between differentiation and peroxidase production are implicated in the literature (section 9.5.3). Using the gradostat principle it may be possible to correlate peroxidase activity and hyphal differentiation of sibling strains with nutrient and/or oxygen levels within a biofilm.

8.3.3.3. Novel plug-flow membrane bioreactor

The plug-flow membrane bioreactor was introduced to test the hypothesis that nutrient and/or oxygen gradients did indeed influence intercellular communication and induce complex tissue formation, particularly aggregation of submerged hyphae to form mycelial cords, as observed for penetrative growth in the membrane gradostat bioreactor. This theory was vindicated by the observation of cord-formation in submerged hyphae within plug-flow reactors, in response to nutrient depletion.

The link between differentiation of filamentous organisms into organised hyphal units or specialised tissues and secondary metabolite formation is pioneering, as are the tools for elucidating the complex mechanisms of intercellular communication. Development of sensitive and consistent technologies for this purpose would facilitate new correlations describing lignolytic enzyme production and/or pollutant degradation in *P. chrysosporium*.

8.4. STRAIN IMPROVEMENT

The *P. chrysosporium* sibling strains examined in this thesis were selected for further characterisation as part of a breeding programme aimed at improving the lignin degradation capabilities of this organism (Wyatt and Broda, 1995, Raeder *et al.*, 1989a, 1989b, Raeder and Broda, 1986). Stationary liquid culture, using the 96-well microtitre plate method was selected as the method of choice in the assessment of intraspecific differences in these sibling strains.

Sibling strains, cultured using the 96-well microtitre plate method, were used to determine the relationship between growth kinetics, peroxidase production, pollutant sensitivity and pollutant degradation. The following variables were used for correlation analysis:

1. length of lag phase (hrs);
2. μ ($\text{mg}\cdot\text{day}^{-1}$);
3. maximum stationary phase biomass ($\text{mg}\cdot\text{ml}^{-1}$);
4. q ($\text{mg}\cdot\text{day}^{-1}$);
5. Y ($\text{mg}\cdot\text{mg}^{-1}$);
6. MnP activity ($\text{U}\cdot\text{l}^{-1}$);
7. time of MnP onset (hrs);
8. tannic acid IC50 ($\text{mg}\cdot\text{l}^{-1}$);
9. mimosa ME IC50 ($\text{mg}\cdot\text{l}^{-1}$);
10. *p*-cresol IC50 ($\text{mg}\cdot\text{l}^{-1}$);
11. percentage *p*-cresol removal; and
12. percentage Poly-R478 decolourisation.

The correlations detected were predominately associated with *P. chrysosporium* growth, either through differences in lag phase, μ or the maximum biomass levels reported for sibling strains in microtitre plate culture [$P < 0.05$]. Percentage *p*-cresol removal and tannic acid tolerance were both correlated with a shorter lag phase [tannic acid: $r = 0.0769$, $P < 0.05$; *p*-cresol: $r = 0.7584$, $P < 0.05$] and lower stationary phase biomass levels [tannic acid: $r = 0.8177$, $P < 0.05$; *p*-cresol: $r = 0.7803$, $P <$

0.05] (Chapter 4, Appendix II: Table I). One of the more robust traits associated with heritable variation is growth rate (Gay *et al.*, 1993, Simchen, 1966a, 1966b, Croft and Simchen, 1965). These results imply that differences in the biosynthetic pathways for biomass accumulation in sibling strains play a significant role in the variation observed in pollutant sensitivity and degradation between strains; and that primary growth events have a significant impact on secondary metabolism and stationary phase performance.

A significant correlation (linear relationship) was also detected between percentage Poly-R478 decolourisation and time of onset of MnP [$r = 0.9689$, $P < 0.001$]. The value of this correlation is however uncertain as only two MnP onset times were recorded for sibling strains. Early onset (60 hrs) was observed only in ME446 and BS 18. These strains also displayed the highest and lowest Poly-R478 decolourisation, respectively (Chapter 4). Thus, it is unlikely that time of onset of MnP has a significant relationship to Poly-R478 decolourisation. This observation highlights the importance of the critical assessment of statistical data.

No relationship was observed between dye decolourisation, *p*-cresol degradation, lignin degradation and LiP or MnP activities, suggesting alternative mechanisms and pathways are involved the degradation of these compounds [$P > 0.05$]. Thus, no new selective criteria for improved performance have been identified.

8.4.1. Intraspecific comparison of *P. chrysosporium* sibling strains using the microtitre plate culture method.

8.4.1.1. Categorical analysis

One-way ANOVA and multiple comparison post-tests of each of the variables listed above, revealed significant differences between sibling strains (Chapters 2-4). Statistical comparison of strains was undertaken with the aim of describing and defining the variability between the sibling strains in relation to their growth characteristics, extracellular enzyme activities and degradation capabilities, with the view of selecting distinctly different, yet genetically related *P. chrysosporium* strains for more comprehensive assessment. By examining the rank orders generated for each dependent variable in combination with intraspecific comparisons by one-way ANOVA and post-tests, sibling strains were categorised into three different ratings, showing high (H), medium (M) or low (L) values for each variable (Appendix II: Table II).

Due to the close genetic relationship between sibling strains and limited resolving power of liquid culture methods, only those organisms showing the greatest variation were recommended for future study. Strain selection was initially undertaken within the following groups of variables.

Peroxidase activities. (LiP and MnP activities)

Based on the extracellular LiP and MnP activities reported in Chapter 2, the following sibling strains were recommended for future analysis:

1. BS 2 and BS 26 produced significantly higher levels of MnP and LiP.
2. BS 3 BS 17 and BS 24 produced consistently lower levels of MnP and LiP.
3. BS 13, previously reported to produce high levels of LiP was found to produce low levels of MnP.
4. ME446 and BS 18 showed an earlier onset in MnP activity than all other strains.

Growth characteristics. (lag phase, μ , maximum biomass produced, q and Y).

Correlation analysis undertaken in Chapter 4 demonstrated the significance of growth in pollutant tolerance and degradation particularly lag phase and stationary phase biomass levels. Those sibling strains demonstrating the most variable growth characteristics are listed below:

1. ME446 scored consistently high values for growth rate, biomass production and glucose consumption.
2. BS 32 scored consistently low values for growth rate and biomass production.
3. BS 2 and BS 2.52 showed significantly lower substrate utilization rates and hence higher biomass yields (Y), conversely, BS 13, BS 17 and BS 18 showed higher substrate utilization rates and lower biomass yields (Y).
4. BS 2.52 and BS 13 showed shorter lag phases than all other strains.

Tolerance of toxic pollutants. (tannic acid IC50, mimosa ME IC50 and *p*-cresol IC50)

Sibling strains showing significantly different responses to toxic pollutants were suggested for further exploring the mechanisms of tolerance or sensitivity to pollutants:

1. BS 24 showed consistently high tolerance to all toxic compounds evaluated.
2. BS 13, BS 17 and BS 18 showed significantly lower tolerance to *p*-cresol.
3. BS 13 showed consistently high tolerance to tannins.
4. The dikaryons ME446 and BS 2.52 showed significantly lower tolerance to tannic acid than all other haploid strains.

Degradation capabilities. (% Poly-R478 decolourisation, % *p*-cresol removal and DHP-degradation)

No correlation was detected between Poly-R478 decolourisation, *p*-cresol removal and synthetic lignin degradation in the sibling strains examined (Chapter 4). However, MnP onset was correlated to Poly-R478 decolourisation [$r = 0.9689$, $P < 0.001$]. Sibling strains showing different degradation capabilities and MnP onsets are listed below:

1. ME446 and BS 24 showed consistently high degradation rates for all pollutants assessed.
2. BS 2.52 was found to have consistently low degradation capabilities.
3. BS 18 was reported to be the most efficient at synthetic lignin degradation, yet showed poor *p*-cresol and Poly-R478 degradation capabilities.
4. While other strains showed some consistency in their ability to degrade Poly-R478, *p*-cresol or synthetic lignin (based on categorical assessment), BS 13 and BS 32 showed no relation in their ability to degrade these compounds.

In summary, those strains recommended for future analysis are recorded in Table 8.1. Sibling strains demonstrating variation overlapping in at least three of the experimental groupings assessed in this work were limited to strains BS17, BS 13, ME446, BS 24, BS 2.52 and BS 18.

Table 8.1: List of *P. chrysosporium* sibling strains showing distinctly different characteristics and recommended for further analysis.

Peroxidase Production	Growth Characteristics	Pollutant Tolerance	Lignin/Pollutant Degradation
BS 26			
BS 3			
BS 2	BS 2		
BS 17	BS 17	BS 17	
BS 13	BS 13	BS 13	BS 13
ME446	ME446	ME446	ME446
BS 18	BS 18	BS 18	BS 18
BS 24		BS 24	BS 24
	BS 2.52	BS 2.52	BS 2.52
	BS 32		BS 32

8.4.1.2. Cluster analysis

Cluster analysis (joining tree clustering) of sibling strains was based on previously reported LiP activities (Raeder *et al.*, 1989b) and synthetic lignin (DHP) degradation, and the experimental means for length of lag phase (hrs); μ ($\text{mg}\cdot\text{day}^{-1}$); maximum stationary phase biomass ($\text{mg}\cdot\text{ml}^{-1}$); q ($\text{mg}\cdot\text{day}^{-1}$); Y ($\text{mg}\cdot\text{mg}^{-1}$); MnP activity ($\text{U}\cdot\text{l}^{-1}$); time of MnP onset (hrs); tannic acid IC50 ($\text{mg}\cdot\text{l}^{-1}$); mimosa ME IC50 ($\text{mg}\cdot\text{l}^{-1}$); p -cresol IC50 ($\text{mg}\cdot\text{l}^{-1}$); percentage p -cresol removal; and percentage Poly-R478 removal, assessed in microtitre plate cultures (Chapters 2-4). The joining tree diagram in Figure 8.1 shows the relationship between the sibling strains assessed in this thesis, based on those dependent variables listed above.

Linkage based on Euclidean distances show three clusters of strains:

Cluster 1. ME446 and BS 32

Cluster 2. BS 13, BS 17 and BS 18

Cluster 3. BS 2, BS 26, BS 3, BS 2.52 and BS 24.

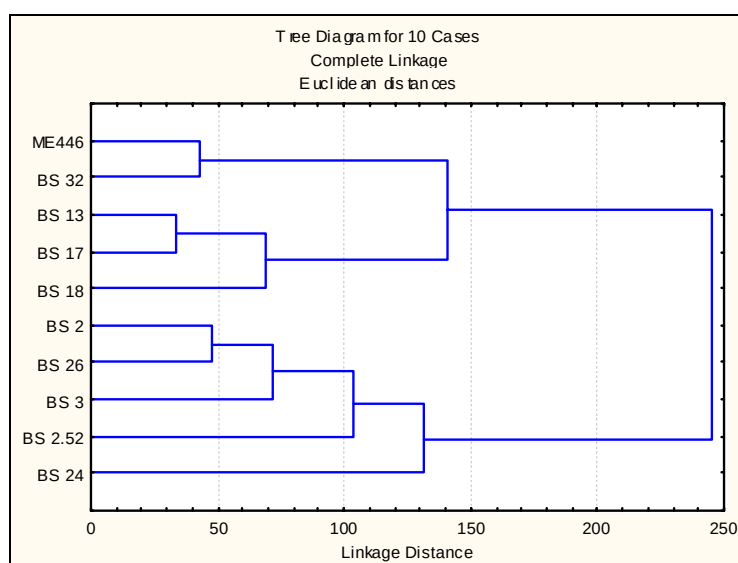


Figure 8.1: Joining tree clustering of sibling strains based on LiP activities from Raeder *et al.* (1989b), DHP degradation values from Wyatt and Broda (1995), and microtitre plate data, including: lag phase (hrs); μ ($\text{mg}\cdot\text{day}^{-1}$); maximum stationary phase biomass ($\text{mg}\cdot\text{ml}^{-1}$); q ($\text{mg}\cdot\text{day}^{-1}$); Y ($\text{mg}\cdot\text{mg}^{-1}$); MnP activity ($\text{U}\cdot\text{l}^{-1}$); time of MnP onset (hrs); tannic acid IC50 ($\text{mg}\cdot\text{l}^{-1}$); mimosa ME IC50 ($\text{mg}\cdot\text{l}^{-1}$); p -cresol IC50 ($\text{mg}\cdot\text{l}^{-1}$); percentage p -cresol removal; and percentage Poly-R478 decolourisation.

The sibling strains recommended for future analysis (Table 8.1) did not appear to group into any particular cluster. As the strains identified through categorical analysis (Section 8.3.1.1) showed distinct and significant differences to one another, it would appear that evaluation and selection of strains categorically was more valuable than if strains had been selected on the basis of their grouping into different clusters.

8.4.2. Biofilm formation

The significance of *P. chrysosporium* growth in intraspecific comparisons has been discussed. Interestingly, the *P. chrysosporium* strains ME446, BS 13, BS 17 and BS 26, identified as showing some of the more variable physiological characteristics in stationary liquid culture (section 8.3.1), were also shown to differ significantly from one another in the extent and nature of biofilm formation, and extracellular enzyme production, when immobilised in membrane gradostat bioreactors (Chapters 5 and 7).

While the role of mycelial differentiation in secondary metabolism has been suggested, the implication of differentiation on the bioremediation potential of *P. chrysosporium* has not been explored. Strain selection based on morphology and differentiation would certainly be valuable in the identification of organisms better suited to soil bioremediation or wastewater treatment, for example. Furthermore, the identification of strains better suited to immobilisation and hence more efficient commercial production of extracellular peroxidases was illustrated in the identification of *P. chrysosporium* INA-12 (Buswell *et al.*, 1984, Capdevila *et al.*, 1989, Bonnarne and Jeffries, 1990, Asther *et al.*, 1988a, 1988b).

Immobilisation of sibling strains using the membrane gradostat bioreactor has proven a valuable tool in the identifying morphological differentiation in sibling strains. Improved reproducibility of this technique may allow for the characterisation of more subtle differences in mycelial differentiation not readily observed in other strains examined. The potential applications of the membrane gradostat bioreactor as an experimental tool are discussed in section 8.4

The results reported above suggest the complexity of *P. chrysosporium*'s lignolytic system, where the synergistic nature and regulatory mechanisms of biodegradation are systematically being described through proteomic and genomic research (Chapter 1). By further examining inherent variation among sibling strains such as those characterised in this work, any information gained would contribute towards the understanding of lignin degradation by white-rot fungi. More comprehensive experimentation of distinctly different sibling strains, using novel and robust techniques such as the membrane gradostat bioreactor and microtitre plate culture methods, respectively, may facilitate the correlation of physiological or morphological characteristics with the lignin and/or pollutant degradation capabilities of these strains. Genetic linkage studies correlating physiological and/or morphological traits with specific alleles would allow for the identification of genetic markers for future strain selection and improvement programmes.

8.5. FUTURE PERSPECTIVES.

The recent release of a full genome sequence for the basidiomycete *Phanerochaete chrysosporium* BKMF-1767 has provided an invaluable platform for future technologies (Martinez *et al.*, 2004, <http://www.jgi.doe.gov/whiterot>). These technologies incorporate the analysis of gene expression (transcriptomics), protein (proteomics) and metabolite (metabolomics) production, at the level of the complete organism. However, these technologies rely heavily on sophisticated laboratory equipment and the development of data analysis and pattern-recognition tools (bioinformatics). This research will undoubtedly allow for a more holistic insight into the lignolytic system, the complexity of which has frustrated many researchers.

The identification and selection of genetically related *P. chrysosporium* strains displaying distinctly different physiological and morphological traits and the advent of microbiological culture systems supporting the detection of intraspecific differences between these strains, has laid the groundwork for future molecular genetic characterisation and linkage studies in these organisms. The techniques required for proteome and transcriptome characterisation have been established for *P. chrysosporium* ME446. These methods may facilitate future studies into the relatedness of physiology and differentiation in sibling strains.

8.5.1. Proteomics

The field of proteomics has arisen from the convergence of three primary disciplines, 2-D gel electrophoresis, protein chemistry and bioinformatics.

Due to the complexity of the technology required for the separation, analysis and identification of the thousands of proteins transcribed by a typical genome, proteomics has progressed far slower than DNA and mRNA approaches. The *P. chrysosporium* genome has recently been fully sequenced, establishing a platform for convergence of this genome with proteome analysis.

8.5.1.1. Isoenzyme separation

The production, secretion, activity and stability of peroxidases of *P. chrysosporium* have routinely been explored in liquid culture. The influence of medium composition and culture conditions on LiP and MnP isoenzyme profiles have been assessed by basic isoelectric focusing and more routinely FPLC and HPLC.

HPLC using both a cation exchange (Toyopearl SuperQ) and convective interactive media (CIM) technologies (BioSeparation CIM DEAE and MonoQ) for ligninase separation and quantification were evaluated during the course of this research project (Podgornik *et al.*, 1999, 2001). The high throughput separation of isoenzymes is difficult. While CIM methods successfully reduced the resolving time of isoenzymes from approximately 1 hr to as little as five minutes, CIM methods were found to be less sensitive and better suited to larger volume samples. This data has been excluded from discussion but remains the basis of future collaborative work with Dr. Sims at UMIST (described below).

8.5.1.2. Proteomics & Genomics

The *P. chrysosporium* lignolytic system is an ideal system for establishing novel methods and approaches in the study of the proteome. The lignolytic system is advantageous in that while the complexity of this system is undoubted, lignolytic enzymes are primarily extracellular, offering several advantages:

1. Ease of separation of proteins of interest from thousands of intracellular proteins;
2. In *P. chrysosporium* several LiP and MnP isoenzymes have been identified, but not all of these proteins have been linked to gene sequences; and
3. Several other enzymes and unidentified low molecular weight polypeptides have been implicated in ligninase activity and stability.

The effect of membrane immobilisation on peroxidase isoenzyme profiles has not been established. Through a collaborative scheme exploring novel technologies the isoenzyme content of crude extracellular peroxidase produced by *P. chrysosporium* ME446 cultured using replicate vertical MMGB's (Chapter 6) is being assessed by 2-D technologies, linking 2-D gel electrophoresis, liquid chromatography and mass spectroscopy. The outcomes of these experiments aims to establish a set of techniques capable of linking protein sequence data generated from tryptic digests of a crude enzyme suite to putative gene sequences identified within the *P. chrysosporium* genome database. Through this process we hope to:

1. Confirm the composition of crude extracellular enzymes produced using the MMGB;
2. Identify and sequence peroxidase isoenzymes and other proteins produced by membrane immobilised *P. chrysosporium*; and
3. Correlate these proteins with putative and known gene sequences within the *P. chrysosporium* genome database.

This information, in conjunction with molecular screening and database mining of unknown sequences, would benefit further genetic linkage studies with those *P. chrysosporium* ME446 derived sibling strains examined in this report.

Further, these methods have the potential to identify low copy number enzymes and polypeptides influencing the activity, stability and turnover of lignolytic activity in crude extracellular extracts.

8.5.2. Transcriptome analysis

The type, amount and ratio of different isoenzymes are differentially regulated by nutrient composition and culture conditions at the level of transcription, translation and post-translational modification. Through the quantification of mRNA within lignolytic tissue and the correlation of these values with isoenzyme profiles generated from the same samples, the level at which different isoenzymes are regulated may be inferred and further examined.

The tools required for transcriptome analysis were explored through a student exchange programme at UMIST under the supervision of Dr PFG Sims. The procedures for competitive RT-PCR of lignolytic tissue from *P. chrysosporium* ME446, using several primer pairs targeting mRNA encoding *LIG1-5* and *mnp1-3* were established. Transcriptome analysis initiated in this work forms the basis for continued post-graduate and/or post-doctoral research programmes.

8.5.3. Strain selection and strain improvement strategies

In this work, the continued examination of those *P. chrysosporium* strains generated through Wyatt and Broda (1995) breeding programme has contributed experimental evidence to be added to the pool of knowledge on the lignolytic system of this organism. From this and previous research it is apparent that under those conditions examined neither pollutant nor synthetic lignin degradation appear to be directly correlated with extracellular LiP or MnP activities.

The 96-well microtitre plate method described in this work offers an attractive solution to future high throughput characterisation of these or other *P. chrysosporium* strains with the view of establishing correlations between biodegradation capabilities, tolerance to various toxic chemicals and phenotypic traits of these organisms. High throughput characterisation may also enable further multifactorial analysis of signal transduction regulators and cofactors, such as cAMP and molecular oxygen, and their influence on strain productivity and differentiation. Seven sibling strains showing distinct variation in growth, physiology and morphology have been recommended for closer examination. Insight into the relationship between differentiation and mechanisms of secondary metabolite production, differentiation and morphogenesis is of both fundamental interest and would be invaluable to strain improvement and selection, and bioprocess engineering programmes.

The linkage of phenotypic traits to molecular markers, as Wyatt and Broda (1995) initially set about doing, would be advantageous in the screening of organisms for those phenotypic traits facilitating improved suitability to different bioprocesses. These may include new molecular markers identified through proteomics and genomics programmes, as described above; gene knockouts such as cellulase negative variants better suited to biobleaching and animal feedstock processes; gene duplication resulting in the hyperproduction of rate-limiting enzymes; altered differentiation effecting an organisms suitability to alternative bioprocesses such as altered immobilisation or shear sensitivity.

8.5.4. Membrane bioreactors as experimental tools

The localisation of peroxidase enzymes in lignolytic tissue has previously been described. The application of techniques such as immunogold-labelling and *in situ* RT-PCR in defining the role of differentiation in transcription, translation and secretion of peroxidase and other enzymes would be best served by an experimental system that promotes primary, secondary and decline growth phases simultaneously. The membrane gradostat bioreactor is unique in its ability to support highly differentiated biofilm development in a controlled environment.

Further development of both the membrane gradostat bioreactor and plug flow membrane bioreactor described in this work is necessary prior to establishing stable experimental systems that can be used as experimental tools (section 8.2.3). Such tools may be used in describing the link between mycelial differentiation and secondary metabolism in *P. chrysosporium* and other organisms. However, correlation of the *in situ* distribution of protein and mRNA's with nutrient and oxygen gradient induced morphogenesis, as hypothesised in gradostat biofilms such as immobilised *P. chrysosporium*, is complicated by the translocatory nature of filamentous organisms. Sensitive miniaturised probes

would benefit the establishment of a kinetic model describing biofilm formation under controlled gradostat conditions.

8.5.5. Secondary metabolism and mycelial differentiation, avenues for further research.

Recently Calvo *et al.* (2002) reviewed the relationship between secondary metabolism and fungal development in well studied mycotoxin producing *Aspergillus* spp. Several links between signal transduction pathways, sporogenic molecules and the regulation of aflatoxin production have been established.

A link between fungal development or mycelial differentiation and ligninase production has been suspected for some time. However, a comprehensive overview linking the pool of information available on lignin and manganese peroxidase, signal transduction and differentiation has not been undertaken, nor has this outlook been explored. Furthermore, this thesis has shown that enzyme activity is not directly correlated to either pollutant tolerance or biodegradation capability. Correlation analyses suggest that differences in sibling strain growth and differentiation may play a more significant role than the lignolytic enzymes themselves. A few avenues influencing mycelial differentiation that warrant further study are described briefly below.

1. cAMP plays a crucial role in differentially regulating LiP and MnP activities, at the level of transcription, in response to pH (section 1.3.2.6). cAMP plays a key role in fungal development (Calvo *et al.*, 2002). The effects of pH and cAMP on biofilm development and differentiation in *P. chrysosporium* have however not been explored.
2. The role of organic acids in the regulation of LiP and MnP has been implicated (section 1.3.2.3). The biosynthesis of organic acids may be linked to cAMP regulation through pH control. Redox signalling systems may be linked to the origin of multicellularity (Blackstone, 2000). In *Aspergillus* spp. organic acids serve as precursors for a series of sporogenic molecules called psi factors (Calvo *et al.*, 2002). The presence of psi factors in *P. chrysosporium* has not been established, nor have organic acids been implicated in morphogenesis in this fungus.

For example, through the comparison of sibling strains, using microtitre plate methods, correlations between pH, cAMP, possible psi factors and growth may be explored. Using the membrane gradostat bioreactor or plug-flow reactor as tools, the correlation of these factors with specific zones of differentiated hyphae within different biofilms might be accomplished by immunogold labelling or *in situ* RT-PCR techniques. These techniques can also be applied to lignolytic enzymes such as the peroxidases, where a number of key links between mycelial differentiation and the coregulation of peroxidase production may be explored.

The improvement of the control and robustness of the membrane gradostat bioreactor and plug-flow membrane bioreactor notwithstanding, these experimental tools would be revolutionised by the insertion of miniaturised probes into membrane biofilms. Such probes may monitor biofilm pH, dissolved oxygen concentrations or substrate concentration and even electric charge. In this way, the conditions under which differentiated hyphae are formed could be monitored.

8.6. FINAL CONCLUSIONS

This work describes a number of experimental tools that were used to explore the fundamental aspects of *P. chrysosporium* metabolism. Classification of sibling strains was undertaken as part of an ongoing process of strain improvement in *P. chrysosporium*. Several sibling strains showing distinct physiological differences were identified, these include strains BS 17, BS 13, ME446, BS 32, BS 2.52 and BS18.

The potential of the microtitre plate method in the high throughput characterisation was established. The power of detection of this method would however benefit from the processing of a greater number of replicates; made possible by examining a fewer number of strains showing more variable characteristics (as recommended above).

Using the microtitre plate method, the biodegradation performance of sibling strains could not be correlated to extracellular peroxidase levels in these organisms, thus disproving the first of the hypotheses described in Chapter 1. Instead, sensitivity to toxic pollutants and the biodegradation performance of sibling strains appeared to be growth related, supporting the second hypothesis described in Chapter 1.

The membrane gradostat bioreactor and plug-flow bioreactor are promising experimental tools with which the role of differentiation in ligninase production, pollutant tolerance and biodegradation performance can be explored. In both the membrane gradostat bioreactor and plug-flow bioreactor,

enhanced mycelial differentiation was observed. Further, sibling strains were shown to differ in the extent and nature of mycelial differentiation in immobilised biofilms, using the membrane gradostat bioreactor. These strains also showed variable phenotypic traits, as identified through categorical analysis of stationary liquid microtitre plate cultures.

A laboratory scale, vertical MMGB was successfully applied to the continuous production of high-level MnP production. Using this system maximum MnP levels of 1479 U.l^{-1} were recorded for *P. chrysosporium* ME446.

The most rewarding part of studying a system as complex as the lignolytic system is in successfully establishing a set of techniques and a broad base of knowledge that provides a foundation for future research.

APPENDIX I: Standard Procedures

A. CULTURE CONDITIONS

A-1. Spore and Maintenance Medium

A nutrient rich medium described by Tien and Kirk (1988) was used in maintenance of the different *P. chrysosporium* isolates, and in the generation of basidiospores for inoculation purposes.

Composition of agar for maintenance and spore production:

Glucose	10 g
Malt extract	10 g
Peptone	2 g
Yeast extract	2 g
L-Asparagine	1 g
KH ₂ PO ₄	2 g
MgSO ₄ ·7H ₂ O	1 g
Thiamin-Hcl	1 mg
Agar	<u>20 g</u>
Make up to 1 L with distilled water.	

Dispense media and autoclave.

A-2. Basal III nitrogen-limiting medium

In all experiments the standard culture medium composition used was the nitrogen-limiting basal III medium, as described by Tien and Kirk (1988).

Basal III medium (filter sterilized)	100 ml
10% Glucose (autoclaved)	100 ml
0.1 M 2,2-dimethylsuccinate, pH 4.2	100 ml
(autoclaved)	
Thiamin (500 mg/L stock, filter sterilized, 4°C)	2 ml
Ammonium tartrate (8 g/L stock, autoclaved)	25 ml
Veratryl alcohol(0.2 M stock, filter sterilized)	100 ml
Trace elements	60 ml
spores (Absorbance at 650 nm = 0.5)	100 ml
make up to 1 L with distilled water.	

Basal III medium stock	
KH ₂ PO ₄	20 g
MgSO ₄	5 g
CaCl ₂	1 g
Trace Element solution.	100 ml
make up to 1 L with distilled water	

Trace Element Solution	
MgSO ₄	3.0 g
MnSO ₄	0.5 g
NaCl	1.0 g
FeSO ₄ .7H ₂ O	0.1 g
CoCl ₂	0.1 g
ZnSO ₄ .7H ₂ O	0.1 g
CuSO ₄	0.1 g
AlK(SO ₄) ₂ .12H ₂ O	10 mg
H ₃ BO ₃	10 mg
*Nitrilotriacetate	1.5 g
make up to 1 L with distilled water	

*Dissolve nitrilotriacetate in 4/5 water, adjust pH to 6.5 with 1M KOH

A-3. Agar's

For solid substrate cultures defined and undefined medium containing 2% w/v agar was used. These were nitrogen-limiting basal III (NLM) and malt extract (MEA), respectively.

A-3-1. % w/v Malt Extract Agar (MEA)

Malt Extract	30 g
Peptone (soymeal)	3 g
Agar	<u>2 g</u>

Make up to 1 L with distilled water.

A-3-2. % w/v Nitrogen Limiting Medium (NLM)

Basal III medium	1 L
Agar	<u>20 g</u>

B. SPORE INOCULUM

For each *P. chrysosporium* strain spores were isolated from 7-12 day old Roux flask cultures by suspending them in sterile water. This spore suspension was filtered through sterile glass wool to remove contaminating mycelia and the conidiospores harvested by centrifugation at 4000 rpm for 10 min. The resulting pellet was washed with sterile distilled water, re-centrifuged and suspended in an appropriate volume of sterile distilled water.

Conidiospore concentration was determined spectrophotometrically as described by Tien and Kirk (1988) were: $A_{650\text{ nm}} (1.0\text{ cm}^{-1}) \approx 5 \times 10^6$ spores/ml. Spore preparations were stored at 4°C. A standard concentration of 1×10^5 spores per ml of culture fluid was used as an inoculum for all liquid culture experiments.

C. SUBSTRATE ASSAYS

C-1. Ammonium

Nitrogen ammonium was measured using a modification of the Nessler method (NH_4^+ spectroquant kit, Merck). This method was adapted for quantification on a spectrophotometer by using ammonium tartrate as a standard.

C-2. Glucose

Reducing sugars were measured according to the Somogyi-Nelson Method (Nelson, 1944). D-glucose was used as a standard.

D. ENZYME ACTIVITY

Enzyme activities were expressed in international units, calculated using the following equation:

$$\text{Volume Activity (U.L}^{-1}\text{)} = V(\Delta\text{OD}/\text{min})/e.d.v$$

V = total volume in assay mixture (ml)

v = volume of enzyme in assay mixture (ml)

e = extinction coefficient of substrate or product
(L/μM/cm)

d = pathlength (cm)

D-1. Lignin Peroxidase

Lignin Peroxidase activity was measured according to the oxidation of veratryl alcohol (VA) to veratryl aldehyde (VA^o), as described by Tien and Kirk (1988). Samples were concentrated 10-fold using a 10 kDa cut-off centrifuge filter. 1 unit represents 1 mM of VA oxidised to VA^o per minute. This method was modified for assay using a microplate reader (Powerwave).

REAGENT A

10 mM Veratryl alcohol	200 μl
250 mM Sodium tartrate buffer, pH 2.5	200 μl
Distilled water	420 μl

REAGENT B (fresh)

H ₂ O ₂	5 mM
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Shimadzu Spectrophotometer

Enzyme solution	100 μl	$E_{310\text{ nm}}(\text{VA}^{\circ})$	$= 9300\text{ M}^{-1}.\text{cm}^{-1}$
Reagent A	820 μl	Pathlength	$= 1\text{ cm}$
Reagent B (starts reaction)	<u>80 μl</u> 1000 μl	Wavelength	$= 310\text{ nm}$

Powerwave Microplate reader

Enzyme solution	30 μl	$E(\text{VA}^{\circ})$	$= 9300\text{ M}^{-1}.\text{cm}^{-1}$
Reagent A	246 μl	Pathlength	$= 0.8\text{ cm}$
Reagent B (starts reaction)	<u>24 μl</u> 300 μl	Wavelength	$= 310\text{ nm}$

D-2. Manganese Peroxidase

Initial experiments used the ABTS [diammonium 2,2'-azinobis(3-ethyl-6-benzothiazoline sulfonate)] method (Gold and Glenn, 1988) for measuring Mnp activity in extracellular culture fluid. More recent publications refuting the consensus that *P. chrysosporium* did not produce a laccase (Srinivasan *et al.*, 1995, Rodríguez Couto *et al.*, 1997) prompted a change in the methodologies used for assaying for Mnp activity as ABTS can be used as a substrate by both Mnp and laccase. Thus, the MBTH/DMAB method was used to assay for Mnp activity in subsequent experiments (Castillo *et al.*, 1994).

D-2-1. Oxidation of ABTS

Manganese Peroxidase activity was originally determined using the ABTS oxidation method (Gold and Glenn, 1988).

REAGENT A (fresh)

Egg albumin	150 mg
1 M Sodium lactate buffer, pH 4.5	2.5 ml
1 M Sodium succinate buffer, pH 4.5	2.5 ml
MnSO ₄	1.0 ml
ABTS	<u>1.0 ml</u>
Distilled water	25 ml

REAGENT B (fresh)

H ₂ O ₂	100 µM
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Spectrophotometer

Enzyme solution	100 µl	$E_{420\text{ nm}}(\text{ABTS})$	= 36 000 M ⁻¹ .cm ⁻¹
Reagent A	450 µl	Pathlength =	1 cm
Reagent B (starts reaction)	<u>450 µl</u>	Wavelength =	420 nm
	1000 µl		

D-2.2. Oxidation of MBTH/DMAB

Manganese peroxidase activity was assayed using a method recording the oxidation of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino)benzoic acid (DMAB), as described by Castillo *et al.* (1994)

REAGENT A

0.7 mM MBTH	100 μ l
9.9 mM DMAB	100 μ l
3 mM MnSO ₄	100 μ l
1M succinate-lactate buffer, pH 4.5	<u>100 μl</u>
Distilled water	100 μ l

REAGENT B (fresh)

0.125 mM (Shimadzu)	or 0.5 mM (Powerwave) H ₂ O ₂
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Spectrophotometer

Enzyme solution	100 μ l	$E_{590\text{ nm}}$ (MBTH/DMAB)	=53 000 M ⁻¹ .cm ⁻¹
Reagent A	500 μ l	Pathlength	= 1 cm
Reagent B (starts reaction)	<u>400 μl</u>	Wavelength	= 590 nm
	1000 μ l		

Microplate reader

Enzyme solution	30 μ l	$E_{590\text{ nm}}$ (MBTH/DMAB)	=53 000 M ⁻¹ .cm ⁻¹
Reagent A	150 μ l	Pathlength	= 0.8 cm
Reagent B (starts reaction)	<u>120 μl</u>	Wavelength	= 590 nm
	300 μ l		

E. ELECTRON MICROSCOPY

E-1. Scanning Electron Microscopy (SEM)

Samples were sectioned and fixed overnight at 4 °C in 2.5%, phosphate buffered glutaraldehyde, pH 7.3. Samples were rinsed of excess glutaraldehyde, dehydrated with ethanol of increasing percentage and critical point dried. Dried sections were carefully mounted and gold coated (Cross, 2001). Coated samples were visualized using Jeol and images recorded photographically.

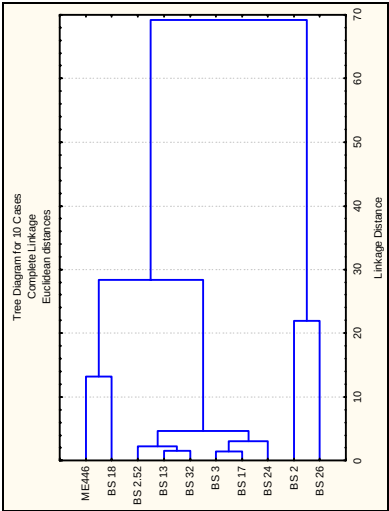
E-2. Transmission Electron Microscopy (TEM)

Glutaraldehyde fixed (as above) sections were treated for 60 min with 1% buffered osmium tetroxide, pH 7.3 before stepwise dehydration with ethanol of increasing percentage. Dehydrated samples were infiltrated with propylene oxide dissolved Araldite resin (of increasing concentrations) and maintained in 100% resin overnight prior to incubation at 60°C for 36 hrs (Cross, 2001). Thin sections were cut using an ultramicrotome and observed using Jeol and images recorded photographically.

PEROXIDASE CLUSTERING

Based on LiP activities, MnP activities and time of MnP onset.

Joining (tree clustering)



K-Means Clustering

Cluster no. 1: Medium-high enzyme activities

	ME446	BS 18
distance	3.8169	3.8169

Cluster no. 2: Low enzyme activities.

	BS 3	BS 17	BS 24	BS 2.52	BS 13	BS 32
distance	0.8594	0.0502	0.9096	0.7772	0.6282	0.5477

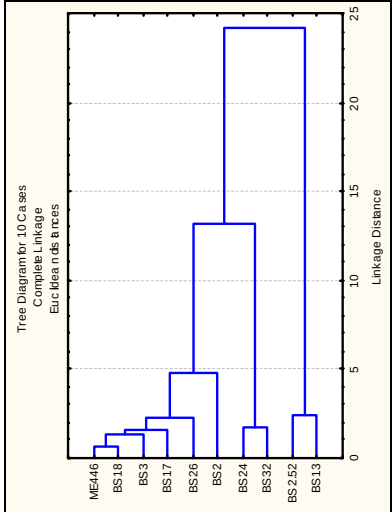
Cluster no. 3: High enzyme activities

	BS 2	BS 26
distance	6.3171	6.3171

GROWTH CHARACTERISTICS CLUSTERING.

Based on lag phase, μ , q , maximum biomass values and Y .

Joining (tree clustering)



K-Means Clustering

Cluster no. 1: Medium-high growth.

	ME446	BS 2	BS 3	BS 17	BS 18	BS 26
distance	0.5639	1.6241	0.2557	0.6488	0.4999	0.7664

Cluster no. 2: Medium-high growth with short lag phase.

	BS 2.52	BS 13
distance	0.5280	0.5280

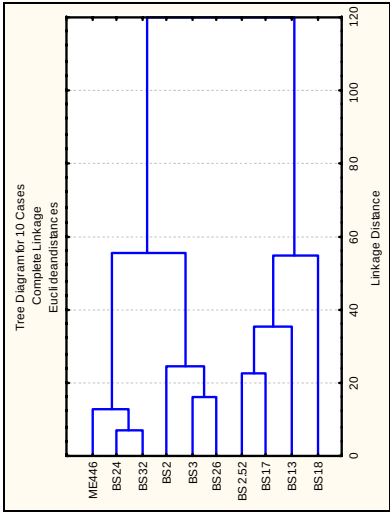
Cluster no. 3: Medium-low growth characteristics with longer lag phase.

	BS 24	BS 32
distance	0.4016	0.4016

POLLUTANT DEGRADATION CLUSTERING:

Based on % Poly-R478 removal, *p*-cresol removal and DHP degradation.

Joining (tree clustering)



K-Means Clustering

Cluster no. 1: Medium *p*-cresol removal.

	BS 2	BS 3	BS 26
distance	9.1391	4.7668	9.0283

Cluster no. 2: Low *p*-cresol removal, medium-high DHP degradation.

	BS 2.52	BS 13	BS 17	BS 18
distance	9.4167	16.2917	16.7029	22.5136

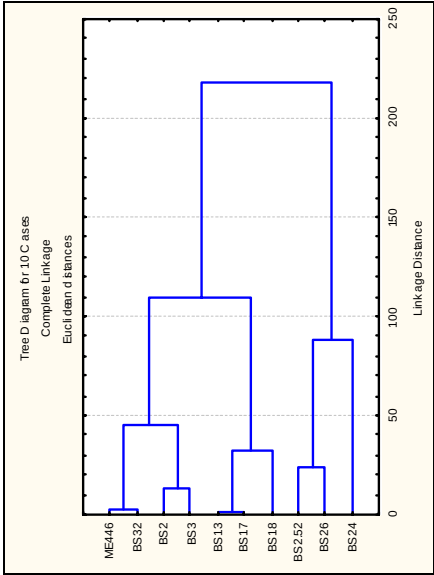
Cluster no. 3: High *p*-cresol removal, medium Poly-R478 removal.

	ME446	BS 24	BS 32
distance	5.7579	3.9599	3.6385

TOXICITY CLUSTERING.

Based on T_A, mimosin ME and *p*-cresol IC50 values

Joining (tree clustering)



K-Means Clustering

Cluster no. 1: High *p*-cresol tolerance.

	BS 2.52	BS 24
distance	6.2946	5.8315

Cluster no. 2: Medium-high *p*-cresol tolerance

	ME446	BS 2	BS 3	BS 26	BS 32
distance	16.8014	1.6171	9.2953	21.535	15.6469

Cluster no. 3: Low tolerance to *p*-cresol, medium to high tolerance of tannins.

	BS 13	BS 17	BS 18
distance	6.2946	5.8315	12.1249

APPENDIX III: Correlation Analysis

Table I: Showing correlations for microtitre plate growth, MnP activities, pollutant sensitivity and pollutant degradation capabilities; in addition to previously reported LIP activities (Raeder *et al.* 1989) and synthetic lignin degradation capabilities (Wyatt and Broda, 1995) for *P. chrysosporium* sibling strains.

A-1-MICROTITRE PLATE CULTURE CULTURES														
													B.A	C.B
	LAG PHASE	μ	MAX STAT BIOMASS	q	Y	MNP ACTIVITY	MNP ONSET	TANNIC ACID IC50	MIMOSA ME IC50	p-CRESOL IC50	% CRESOL REMOVAL	% POLY-R478 DECOLOURISATION	LIP activity	DHP degradation
LAG PHASE	1.0000	-3892	-5599	2158	-3748	-3295	2870	768	7061	5133	7584	3650	0584	-0556
	p=---	p=.388	p=.191	p=.642	p=.408	p=.470	p=.533	p=.043	p=.076	p=.239	p=.048	p=.421	p=.901	p=.906
μ	-3892	1.0000	7629	0430	-0146	1128	-2290	-6972	2423	3020	-4095	-3479	-5013	.4178
	p=.388	p=---	p=.046	p=.927	p=.975	p=.810	p=.621	p=.082	p=.601	p=.510	p=.362	p=.444	p=.252	p=.351
MAX STAT BIOMASS	-5599	7629	1.0000	0392	0648	-2679	-6728	-8177	0935	-1163	-7803	-6711	-2618	.7401
	p=.191	p=.046	p=---	p=.933	p=.890	p=.561	p=.098	p=.025	p=.842	p=.804	p=.038	p=.099	p=.571	p=.057
q	.2158	.0430	.0392	1.0000	-9781	-5481	-3034	4361	2850	-3439	-3151	-2870	-3965	-0053
	p=.642	p=.927	p=.933	p=---	p=.000	p=.203	p=.508	p=.328	p=.536	p=.450	p=.491	p=.533	p=.379	p=.991
Y	-3748	-0146	.0648	-9781	1.0000	5079	1961	-5386	-3710	1961	1475	1787	3209	.0195
	p=.408	p=.975	p=.890	p=.000	p=---	p=.245	p=.674	p=.212	p=.413	p=.673	p=.752	p=.701	p=.483	p=.967
MNP ACTIVITY	-3295	1128	-2679	-5481	5079	1.0000	0690	-4238	-3768	1310	-1176	1253	6917	.4457
	p=.470	p=.810	p=.561	p=.203	p=.245	p=---	p=.883	p=.343	p=.405	p=.780	p=.802	p=.789	p=.085	p=.316
MNP ONSET	2870	-2290	-6728	-3034	1961	0690	1.0000	4368	0035	5745	7240	9689	0000	-4201
	p=.533	p=.621	p=.098	p=.508	p=.674	p=.883	p=---	p=.327	p=.994	p=.177	p=.066	p=.000	p=1.00	p=.348
TANNIC ACID IC50	768	-6972	-8177	4361	-5386	-4238	4368	1.0000	2713	0789	5977	5150	0798	-4027
	p=.043	p=.082	p=.025	p=.328	p=.212	p=.343	p=.327	p=---	p=.556	p=.866	p=.156	p=.237	p=.865	p=.370
MIMOSA ME IC50	7061	2423	0935	2850	-3710	-3768	0035	2713	1.0000	6424	3383	0672	-4646	.4237
	p=.076	p=.601	p=.842	p=.536	p=.413	p=.405	p=.994	p=.556	p=---	p=.120	p=.458	p=.886	p=.294	p=.343
CRESOL IC50	5133	3020	-1163	-3439	1961	1310	5745	0789	6424	1.0000	6737	5621	-1545	.2086
	p=.239	p=.510	p=.804	p=.450	p=.673	p=.780	p=.177	p=.866	p=.120	p=---	p=.097	p=.189	p=.741	p=.653
% CRESOL REMOVAL	7584	-4095	-7803	-3151	1475	-1176	7240	5977	3383	6737	1.0000	7181	1752	-3984
	p=.048	p=.362	p=.038	p=.491	p=.752	p=.802	p=.066	p=.156	p=.458	p=.097	p=---	p=.069	p=.707	p=.376
% POLY-R478 DECOLOURISATION	3650	-3479	-6711	-2870	1787	1253	9689	5150	0672	5621	7181	1.0000	0749	-2760
	p=.421	p=.444	p=.099	p=.533	p=.701	p=.789	p=.000	p=.237	p=.886	p=.189	p=.069	p=---	p=.873	p=.549
LIP activity	0584	-5013	-2618	-3965	3209	6917	0000	0798	-4646	-1545	1752	0749	1.0000	.0000
	p=.901	p=.252	p=.571	p=.379	p=.483	p=.085	p=1.00	p=.865	p=.294	p=.741	p=.707	p=.873	p=---	p=1.00
DHP degradation	-0556	.4178	.7401	-0053	.0195	.4457	-4201	-4027	.4237	.2086	-3984	-2760	.0000	1.0000
	p=.906	p=.351	p=.057	p=.991	p=.967	p=.316	p=.348	p=.370	p=.343	p=.653	p=.376	p=.549	p=1.00	p=---

A. Lip activities as reported by Raeder *et al.* (1989).
B. Synthetic lignin (DHP) degradation as reported by Wyatt and Broda (1995)
Highlighted correlations were significant (P < 0.05).

Table II: Showing categorical description of *P. chrysosporium* sibling strains. Growth, MnP activities, pollutant sensitivity and pollutant degradation capabilities; in addition to previously reported LiP activities (Raeder *et al.*, 1989) and synthetic lignin degradation capabilities (Wyatt and Broda, 1995); are categorised as high (H), medium (M) or low (L) based on rank orders and statistical differences between strains [P < 0.05].

	P. CHRYSOSPORIUM STRAIN											
	ME446	BS 2	BS 2.52	BS 3	BS 13	BS 17	BS 18	BS 24	BS 26	BS 32		
B	-	H	-	L	H	L	M	L	H	H		
	M	H	M	L	L	L	H	M	H	M		
	H	L	L	L	L	L	H	L	L	L		
	M	M	H	M	H	M	M	L	M	L		
	H	L	M	H	M	M	M	M	H	L		
	H	M	M	M	H	M	H	M	M	L		
	H	L	L	M	H	H	M	M	M	M		
	M	H	H	M	L	L	M	M	M	L		
	L	M	L	M	H	H	M	H	M	H		
	M	L	M	M	H	M	M	H	M	M		
	M	M	H	M	L	L	L	H	H	M		
	H	M	L	M	L	L	L	H	M	H		
	H	M	-	L	M	M	H	H	H	L		
	DHP DEGRADATION											

A. Synthetic lignin (DHP) degradation as reported by Wyatt and Broda (1995)
B. Lip activities as reported by Raeder *et al.* (1989).
*For lag phase and time of MnP onset, a high rank order was attributed to shorter lag or earlier onset.
†For IC50 values a high ranking was attributed to increased tolerance of a pollutant.

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