Development and Characterisation of a Membrane Gradostat Bioreactor for the Bioremediation of Aromatic Pollutants using White Rot Fungi

Thesis

Submitted in Fulfilment of the Requirements for the Degree of

Doctor of Philosophy

of

Rhodes University

by

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January 1999

He that will not apply new remedies must expect new evils; for time is the greatest innovator.

- Francis Bacon

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ABSTRACT

Bioremediation of aromatic pollutants using the ligninolytic enzymes of the white rot fungi has been thoroughly researched and has been shown to have considerable potential for industrial application. However, little success in scale-up and industrialisation of this technology has been attained due to problems associated with the continuous production of the pollutant-degrading enzymes using conventional bioreactor systems. The low productivities reported result from the incompatibility of conventional submerged culture reactor techniques with the physiological requirements of these fungi which have evolved on a solid-air interface, viz. wood. The enzymes are also produced only during the stationary phase of growth and can therefore be regarded as secondary metabolites. This study reports the conceptualisation, characterisation and evaluation of a novel bioreactor system as a solution to the continuous production of idiophasic pollutant degrading enzymes by the white rot fungus *Phanerochaete chrysosporium.* The reactor concept evolved from observation of these fungi in their native state, i.e. the metabolism of lignocelluiosic material and involves the immobilisation of the organism onto a capillary ultrafiltration membrane. Nutrient gradients established across the biofilm, an inherent characteristic of fixed bed perfusion reactors, are exploited to provide both nutrient rich and nutrient poor zones across the biofilm. This allows growth or primary metabolism in the nutrient rich zone, pushing older biomass into the nutrient poor zone where secondary metabolism is induced by nutrient starvation. In effect, this represents a transformation of the events of a batch culture from a temporal to a spatial domain, allowing continuous production of secondary metabolites over time. Direct contact of the outer part of the biofilm with an air stream simulated the solid-air interface of the native state of the fungus.

In order to facilitate the practical application of the membrane gradostat reactor (MGR) concept, conventional capillary membranes and membrane bioreactor modules were first evaluated. These were found to be unsuitable for application of the MGR concept. However, critical analysis of the shortcomings of the conventional systems resulted in the formulation of a set of design criteria for the development of a suitable membrane and

module. These design criteria were satisfied by the development of a novel capillary membrane for membrane bioreactors, as well as a transverse flow membrane module, which is a novel approach in membrane bioreactor configuration.

For the physiological characterisation of the MGR concept, a single fibre bioreactor unit was designed, which allowed destructive sampling of the biofilm for analysis. Using this system, it was shown that distinct morphological zones could be observed radially across the mature biofilm obtained through MGR operation. That these morphotypes do represent the temporal events of a typical batch culture in a spatial domain was confirmed by following the morphological changes occurring during batch culture of the immobilised fungus where the onset of primary and secondary metabolic conditions were manipulated through control of the nutrient supply. The different morphotypes were correlated to distinct growth phases by comparison of the morphology to the secretion of known enzymatic markers for secondary metabolism, viz. succinate dehydrogenase and cytochrome C oxidoreductase. Detailed structure-function analysis of the biofilm using transmission electron microscopy and adapted enzyme cytochemical staining techniques showed that the biofilm appeared to operate as a co-ordinated unit, with primary and secondary metabolism apparently linked in one thallus through nutrient translocation. This study provided new insights into the physiology of *P. chrysosporium* and a detailed descriptive model was formulated which correlates well to existing models of wood degradation by the white rot fungi (WRF).

Evaluation of the process on a laboratory scale using a novel transverse flow membrane bioreactor showed that a volumetric productivity of 1916 U. $L.^{-1}day^{-1}$ for manganese peroxidase, one of the pollutant degrading enzymes, could be attained, corresponding to a final concentration of 2 361 U. $L.^{-1}$. This may be compared to the best reported system (Moreira *et al.* 1997), where a volumetric productivity of 202 U. $L.^{-1}day^{-1}$ was achieved with a final concentration of 250 U. $L.^{-1}$, However, MGR productivity is yet to be subjected to rigorous optimisation studies. The process could be operated continuously for 60 days. However, peak productivity could not be maintained for long periods. This was found to be due to physical phenomena relating to the fluid dynamics of the system

which caused fluid flow maldistribution, which would have to be resolved through engineering analysis. In evaluation of the MGR concept for aromatic pollutant removal, in this case *p*- cresol, from growth medium, good performance was also achieved. The calculated by linear regression for the MGR was 0.S (R^2 = 0.93), which compared favourably to that reported by Lewandowski *el aL* (1990), who obtained a of 0.34 for a packed bed reactor treating chlorophenol.

It was concluded that the MGR showed suitable potential to warrant further development, and that the descriptive characterisation of the biofilm physiology provided a sufficient basis for process analysis once engineering aspects of the system could be resolved.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people:

My supervisors, Peter Rose and Stephanie Burton. for not only indulging, but supporting, encouraging and investing time and material resources to enquiring minds and independent spirits.

The post graduate school of the Dept. of Biochemistry and Microbiology, for providing a stimulating, professional and comfortable environment for research. Special mention goes to my fellow research group member, Wade Edwards, for endless discussions and practical hints. The interaction with the rest of the group - Brenton, Trevor, Leslie, Raj, Kevin, Genevieve and Malcolme was also a major part of the educational process. Also, the students who are currently continuing this work for a most rewarding interaction,

To the Institute for Polymer Research, Stellenbosch - Ron Sanderson and Ed Jacobs, for being excellent partners in this work, especially Ed, who is both teacher and friend. The initiation of the development of the new capillary membrane in the pub in Johannesburg will always be my paradigm for effective collaboration. The work on the development of the membrane and bioreactor module by experts in the field shows the importance of multi-disciplinary research in bio-process development.

The WRC, for their financial investment into this work, and ESKOM, for their subsequent support thereof.

My colleagues in the Dept. of Biochemistry and Microbiology for their patience, support and stimulating discussions, especially Moira for superb organisation and necessary encouragement. The Rhodes University Electron Microscopy Unit, especially Shirley Pinchuck for first class technical assistance and Robin Cross for excellent advice and discussion.

The people of Grahamstown for providing the most stimulating environment for research, especially Cathy for pushing to get the thesis done.

My family, of course. Sorry you **had** to wait so long, other things kept on coming up.

CHAPTER 1

BIOREMEDIATION OF AROMATIC POLLUTANTS USING THE WHITE ROT FUNGI

1.1. BIOREMEDIATION

After the industrial revolution, the sudden introduction, over the last 100 years of the earth's history, of xenobiotic chemicals and the massive relocation of materials between ecosystems has resulted in the accumulation of pollutants in certain sites. These have reached harmful levels which exceed the self-cleaning capacity of the environment (Day 1993).

Bioremediation, the use of biological treatment systems to destroy or reduce the concentrations of hazardous waste from contaminated sites, has found widespread appeal in recent years. Applications include clean-up of ground water, soils, lagoons, process streams and even large stretches of oil-contaminated shoreline, as in the case of the Exxon Valdez spill onto the coast of Prince William Sound, Alaska (Caplan 1993; Young and Suk 1995).

Bioremediation currently comprises only a small fraction of the hazardous waste treatment market. With the increased public acceptance of "green technology" such as biotechnology, the potential for economic growth by replacing existing technologies is considerable. Hence, bioremediation has become one of the fastest-growing areas in the environmental management sector (Caplan 1993). In 1990, the U.S.A. bioremediation market was estimated at about $ 60 million (Caplan 1993) and is expected to expand at an annual rate of 16% (http://www.findsvp.com/tocs/ML0510.HTM). This is expected to be a worldwide trend due to increased legislative pressure.

Much industrial pollution can be traced to either waste-management practices that advocated disposal rather than treatment, or to both accidental or incidental spillages that were ignored in terms of non-existent, ineffective or un-enforced environmental

protection legislation (Hamer 1993). Present legislation, however, is increasingly designed both to ameliorate the adverse effects of past pollution, and to require immediate action to minimise the impact when industrial accidents producing new pollution do occur. A general consensus has emerged where the traditional "dilute, bury or burn" practise is no longer acceptable_

Industrial companies seek to operate within the requirement of the law, but to enable them to do so, the necessary technology for pollution control and pollutant elimination must be made available (Hamer 1993). The challenge to biotechnology is to generate efficient, cost-effective and environmentally safe bioremediation technologies to replace existing standard technologies, such as incineration, as well as to provide unique solutions for the remediation of contaminated sites (Liu and Suflita 1993).

1.2. AROMATIC POLLUTANTS

Aromatic compounds are generally released into the air, soil, and water bodies from coal gasification and liquefaction processes, waste incineration, coke, carbon black and resin manufacturers, foundries, paint-stripping operations and other petroleum-derived plants (Lanouette 1977; Klein and Lee 1978; Galli 1990; Boyd and Carlucci 1993). Plant-derived polyphenolic substances are also produced by processes such as wine manufacture, the paper and pulp industry and olive mill processing. These compounds can be toxic or have other unwanted effects when released into the environment. Of the monomeric phenols, as little as 0.005 mg. of phenol will impart objectionable tastes and odours to drinking water when it combines with chlorine to form chlorophenols (Lanouette 1977). Phenols are toxic to fish at levels above 2 mg. L. and can cause a taste in fish flesh at concentrations below the toxic level. They also have a relatively high oxygen demand, which depletes the oxygen of a receiving body of water (Lanouette 1977). The major aromatic pollutants persist in the environment because of their resistance to microbial attack. This is due partially to the chemical structure of some of these compounds, which may differ from those that occur naturally (Galli 1990),

Methods in current general use for the treatment of aromatic pollutants include recovery, incineration, adsorption, bioremediation and chemical oxidation (Lanouette 1977). Other technologies include solvent extraction and membrane processes such as reverse osmosis, ultrafiltration, and electrodialysis (Klein and Lee 1978).

1.2.1. Bioremediation of Aromatic Pollutants

The success of bioremediation in the treatment of hazardous, recalcitrant organics has received much attention since the 1980's after environmental catastrophes such as the Exxon Valdez and Mega Borg oil spills, and the Iraq-Kuwait war (Shannon and Unterman 1993). Promising and innovative research has been undertaken to address contamination by aromatic compounds using biotechnological solutions.

The potential advantages of biotechnological, rather than physico-chemical treatments are operation under milder, less corrosive conditions (pH, temperature and pressure); operation in a catalytic manner; execution of many sequential reaction steps; operation on trace level organic compounds and on organics not removed by existing physico-chemical processes; reduced consumption of oxidants; reduced amounts of adsorbent materials, such as charcoal, for disposal; greater efficiency of biocatalysts and biosorbents and hence greater yield and process efficiency; less expensive process equipment and consequently lower capital investment (Belfort 1989; Nicell *et al.* 1992).

Although biological processes have long been in use for the treatment of non-refractory wastes like sewage, their use for treating hazardous and refractory chemical wastes is more recent. Increased knowledge of biodegradation pathways and isolation and utilisation of newly identified microbes has contributed to this trend (Shannon and Unterman 1993; Boyd and Carlucci 1993).

Biological treatment systems may utilise enzyme-based- and/or whole cell (organism)-based systems (Nicell *et al_* 1992),

1.2.2. Enzyme-Based Systems

While the concept of using enzymes in waste treatment applications dates back to the 1930's, it was not until the 1970's that the selective removal of target chemicals in industrial waste waters using enzymes became a practical possibility (Klibanov *et al.* 1983). The potential advantages of the use of enzyme-based treatment over whole organism-based treatment systems include:

- Action on compounds toxic to microbes;
- Operation over wide temperature ranges;
- Operation over wide pH and salinity ranges;
- Operation over high and low concentration ranges of contaminants;
- Less disruptive effect of shock loading;
- No delays associated with shutdown and start-up (acclimatisation of biomass);
- Reduction in sludge volume (no biomass generation);
- A better defined system with simpler process control;
- Rapid reaction rates with well-characterised end products;

(Klibanov *et al.* 1983; Nicell *et al.* 1992).

The activity of the enzymes must, however, be well characterised, especially in terms of the fate of toxic organic compounds before application can be considered (Aitken *et al.* 1989). Another drawback of the enzymatic treatment of aromatic pollutants is that the complete degradation of the aromatic pollutants normally involves multiple enzyme reactions and may require several enzymes, and also co-factors which are difficult to re-generate. This is not a problem in the case of polyphenolase enzymes, which effect the polymerisation of small phenolic compounds into large water-insoluble complexes. However, these enzymes are limited to a narrow range of phenolic compounds. Another limitation of enzyme-based systems is the limited catalytic lifetime of enzymes. This affects the economic feasibility of such systems since production and purification of enzymes tend to be costly.

1.2.3. Whole Cell Treatments

Bioremediation of toxic aromatic pollutants by whole cell culture systems may involve processes using plants, algae, fungi and bacteria. However, most attention has been focussed on the development of fungal and bacterial processes. Bacteria have usually been the organisms of choice because the metabolic fate of aromatic pollutants in bacteria has been better studied. This is because bacteria are easier to culture and grow more quickly than fungi and they are amenable to straightforward genetic manipulation techniques (Higson and Focht 1992; Bouwer and Zehnder 1993).

Higher metabolic rates (Boyd and Carlucci 1993; Bouwer and Zehnder 1993) and the ability to metabolise chlorinated organics and certain other pollutants faster than fungi (Radehaus and Schmidt 1992; Bouwer and Zehnder 1993), as well as the ability of certain bacteria to degrade aromatic pollutants under anaerobic conditions, have contributed to the popularity of the use of bacteria as bioremediation agents.

Fungal systems have, however, been the subject of recent interest due to the inherent nature of the metabolism of complex organic compounds by fungi. These mechanisms are generally based on the secretion, by mostly filamentous fungi, of extracellular enzymes into complex solid matrices, This metabolic strategy makes the development of bioremediation technology using filamentous fungi advantageous over the use of bacteria under certain circumstances.

One of the problems pertaining to bacterial bioremediation is that of bioavailability. Bacteria are generally adapted to act upon soluble substrates in aqueous medium. However, many organo-pollutants (especially the aromatics) are poorly soluble in water and are likely to be adsorbed onto particulate matter, limiting their bioavailability. The pollutant-degrading systems of the fungi can still function under such conditions because the enzymes catalysing initial oxidation reactions are extracellular and their natural substrates tend to be insoluble polymers.

Many bacterial systems are not able to degrade low levels of organo-pollutants because the levels of the pollutant are too low to induce the biosynthesis of the enzymes required for their degradation. Fungal degradative enzymes tend to be induced by nutrient starvation, rather than the presence of the pollutant, which enables the degradation of pollutants to low concentration levels (Aust 1990).

Most bacteria are not able to degrade a broad spectrum of structurally diverse organo-pollutants due to the specific nature of their degradative mechanisms. This restricts their use to situations where only a limited number of pollutants are present or the use of hard-to-maintain consortia of bacteria (Babu *et at* 1995). The fungal enzymes are relatively non-specific with regard to aromatic pollutant structure, so a monoculture of fungi (especially the white rot fungi (WRF)) can degrade a very wide range of aromatic pollutants (Aust 1990). The wide substrate range of these fungal enzymes also allows the possibility to degrade newly synthesised compounds that end up in the environment, for which bacteria have not yet evolved degradative mechanisms. A good example of this is the dioxins, which are not easily transported into, and degraded by, bacteria (Bouwer and Zehnder 1993). Recombinant DNA techniques might be of use to engineer organisms capable of degrading newly developed compounds, but these bacteria tend not to be successful competitors where consortia of species are required. Also, regulations and public pressure against the use and release of genetically engineered organisms makes their use prohibitive (Hamer 1993; Miller 1997).

Thus, in general, the bacteria are simpler to use in bioremediation technology development than the fungi since a broad support base exists for the study of their metabolism and their utilisation in reactors. Nevertheless, certain characteristics of the fungi make them highly attractive bioremediation agents (as mentioned above). A group of fungi which have received much attention with regards to the development of bioremediation of aromatic compounds are the white rot fungi.

1.3. THE WHITE ROT FUNGI

White rot fungi are members of the Eumycota, subdivision Basidiomycotina, the class of fungi that are well known for their distinctive fruiting bodies commonly recognised as mushrooms, toadstools and puffballs (Bumpus and Aust 1987). These fungi are unique in that they are the only known organisms capable of completely degrading lignin (Gold and Alic 1993).

Lignin is a complex three-dimensional polymer that is responsible for providing structural support to woody plants. Biosynthesis of lignin in higher plants is mediated by a plant peroxidase system which catalyses the formation of phenoxy- free radicals from coniferyl, synapyl and p-coumaryl alcohols. These then polymerise in a seemingly random fashion to form the lignin polymer (Gold and Alic 1993; Bumpus and Aust 1987). This free-radical addition process results in the formation of a polymer with an irregular, non-repeating structure, which is non-stereospecific. Most of the bonds are 13-aryl ether linkages, but many other C-C and C-O bond types exist in lignin which are very stable. The structure of softwood lignin is represented in figure 1.1.

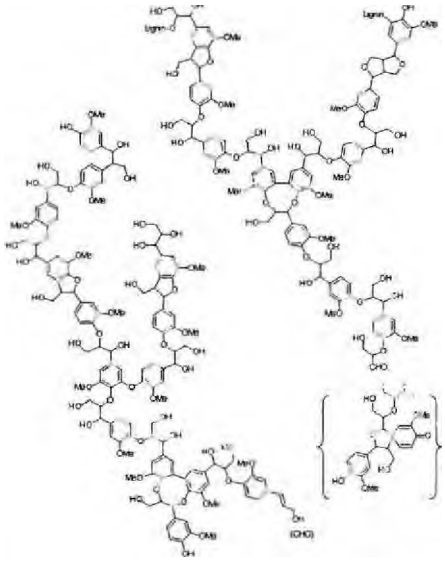


Fig. 1.1: Structure of softwood lignin.
(http://www.helsinki.fii—orgkin_ww/lignin_structure.html)

The above properties combine to make lignin highly recalcitrant to biodegradation because most organisms do not possess enzyme systems capable of degrading such a stable, structurally- and stereo - irregular compound (Bumpus and Aust 1987).

Lignin is the second most abundant renewable natural polymer compound on earth (making up to 30% of woody cell walls of gymnosperms (softwood), and angiosperms (hardwood)). Because relatively few organisms degrade lignin, it is thought that its biodegradation is the rate limiting step in the global carbon cycle (Bumpus and Aust 1987; Gold and Alic 1993). Although the subject of intense research for many years, details of the mechanisms of lignin biodegradation are only recently becoming clear (Higuchi 1990). These developments have had a major impact on the initiation of the development of technologies for the bioremediation of aromatic pollutants. This is because several aromatic pollutants (many of which are on the United States Environmental Protection Agency (USEPA) priority list) share structural similarity to lignin and its monomers.

Thus it was suggested that the ligninolytic system of the WRF could be used in the bioremediation of these otherwise recalcitrant pollutants. This was first shown to be feasible by Bumpus *et al.* (1985). Since then it has been shown that a wide range of these pollutants can be transformed, some partially and others completely mineralised, by these fungi (see table 1.1.).

The mechanism of lignin degradation by the WRF is complex and is briefly described here as it pertains to the biodegradation of aromatic pollutants. Basically, the ligninolytic system of the WRF is a non-specific, extracellular oxidative process initiated by nitrogen, carbohydrate or sulphur starvation (Bumpus and Aust 1987). It was initially shown that an active oxygen species was responsible for initial depolymerisation reactions, Subsequently, extracellular enzymes were discovered which were capable of the degradation of lignin model compounds *in vitro* (Tien and Kirk 1984).

Table 1.1: Partial listing of pollutants transformed by *P. chrysosporium.* (Aust 1990)

Pollutant Category	**Examples**
Aromatic Compounds	benzoic acid phenol o-cresol benzene toluene xylene
Lignin model compounds	veratrylglycerol-B-(0-methoxyphenyl) ether dehydrodiconiferyl alcohol dehydrovanillin
Biopolymers	lignin cellulose Kraft lignin
Chlorinated Aromatic Compounds	2,4,6-trichlorophenol
Polycyclic Aromatic Hydrocarbons	benzota]pyrene anthracene
Polycyclic chlorinated aromatic compounds	DDT(1,1,1-trichloro-2,2-bis-(p-chlorophenypethane polychlorinated biphenyls (e.g. Arochlor 1254)
Pesticides	atrazine
Explosives	TNT (Trinitrotoluene)
Non -aromatic xenobiotics	Cyanide

1.3.1. Lignin Peroxidase

The first enzyme capable of oxidative cleavage of the C-C bond of the non-phenolic propyl side chains of certain aromatic lignin model compounds was discovered by Kirk and co-workers in 1982 in the extracellular culture broth of the white rot fungus *Phanerochaete chrysosporium* Burdsall (Linko 1992). They called the enzyme ligninase, and published their discovery the following year (Tien and Kirk 1983). At about the same time Gold and co-workers (Glenn *et al.* 1983) published their independent discovery of a *P. chrysosporium* enzyme called hydrogen peroxide requiring diaryl propane oxygenase (Linko 1992). This enzyme turned out to be identical to ligninase and is more generally referred to now as lignin peroxidase (LiP) (EC 1.11.1.7 peroxidase donor: hydrogen peroxide oxidoreductase).

LiP has been purified by a combination of anion-exchange chromatography, gel filtration, Fast Protein Liquid Chromatography (FPLC) and isoelectric focussing (Gold and Alic 1993). The enzyme is present as a series of glycosylated isozymes with pI's ranging from 3.2 to 4.0 and molecular masses ranging from 38 to 43 kDa. Each isozyme contains 1 mol. of iron haeme per mol. of protein (Gold and Alic 1993). The mechanism of action of the LiP isozymes is similar to that of other peroxidases and is schematically depicted in figure 1.2. The ferric form of the enzyme, the resting form, is initially oxidised by two electrons from hydrogen peroxide to produce a form of peroxidase known as Compound I. Compound I can be reduced by one electron by chemicals having a suitable reduction potential, such as aromatic pollutants. The enzyme is reduced to a form called Compound II whereas the chemical (aromatic compound) is oxidised by one electron to form a radical. A second aromatic compound then donates a further electron to Compound II to return it to its resting state (Aust 1995). In the process, the aromatic reducing substrate is oxidised to an aryl cation radical (Gold and Alic 1993)_ The free radicals diffuse into solution where they can undergo spontaneous degradation reactions or polymerisation with other aromatic compounds (Nicell *et al.* 1993). LiP, therefore, exhibits Ping-Pong Bi Bi kinetics in that 1120_2 first oxidises the enzyme and the oxidised enzyme (Compound I) reacts with the substrate (Aust 1995).

LiP+1-1,02—>LiN +H_2O

LiP+ Ar—>LiPII+ Ar:

LiP17+ Ar---> LiP + Ar:+11,0

Fig.1.2: Catalytic cycle of LiP. Ar- Aromatic substrate.

LiP II can also react with $F1_202$ to form LiP III, an inactive form of LiP. It has been shown that veratryl alcohol, which is synthesised as a secondary metabolite by WRF (Shimada *et al.* 1981), plays a pivotal role in the activity of LiP. The veratryl alcohol produced is converted to a cation radical as depicted above. The VA^{-+} (veratryl alcohol cation radical) is considered an important intermediate in converting Compound III back to the ferric form of the enzyme and being a redox mediator for indirect oxidations of other compounds (Khindaria *et al.* 1995).

The lignin peroxidases are different from other peroxidases in that they have higher oxidation potentials (--1.35V) than do most other peroxidases such as horseradish peroxidase (-0.8V). In this way these enzymes have a somewhat greater range of chemicals that they can oxidise compared to other peroxidases. This does not, however, completely explain why so many chemicals are oxidised by these fungi. Aust (1995) assumes that other mechanisms or enzymes, yet to be discovered, are also involved. LiP has an extremely low pH optimum (-pH 2.5) for a peroxidase, and its pH dependence apparently is controlled by the pH reduction steps in the catalytic cycle (Gold and Alic 1993).

1.3.2. Manganese Peroxidase

Another enzyme that has been shown to play a major role in lignin degradation is manganese peroxidase (MnP). MnP has also been purified to electrophoretic homogeneity (Glenn and Gold 1985). This enzyme exists as a series of glycosylated isozymes with pI's ranging from 4.2 to 4.9, and with molecular masses ranging from 45 to 47 kDa. Each isozyme also contains I mol. of iron haeme per mol. of protein (Gold and Alic 1993). The oxidation of lignin and other phenols by MnP is dependent on free manganous ion. As shown in figure 1,3, the primary reducing substrate in the MnP catalytic cycle is the Mn (II), which efficiently reduces both Compound I (MnPI) and Compound II (MOH), generating Mn (III), which subsequently oxidises the organic substrate (Gold and Alic 1993).

MnP+11,0i—>MnPI +H2O

MnPI + Mn(II)--->MnPII +Mn(III)

MnPII + Mn(II)-+MnP+

Fig. 1.3: Catalytic cycle of MnP

1.3.3. Reactions Catalysed by LIP and MnP

LiP catalyses the 11_2O_2 dependent oxidation of a wide variety of non-phenolic lignin model compounds including synthetic lignin (Gold and Alic 1993). These reactions include benzylic alcohol oxidations, side chain cleavages, ring-opening reactions, demethoxylations and oxidative dechlorinations. All of these reactions are consistent with a mechanism involving the initial one-electron oxidation of susceptible aromatic nuclei by an oxidised enzyme intermediate to form a substrate aryl cation radical. This radical can then undergo a variety of non-enzymatic reactions to yield a wide range of final products (Hammel 1995). Redox potential, in part, determines whether an aromatic nucleus is a substrate for LiP. Strong electron withdrawing groups such as an a-carbonyl group tend to de-activate aromatic nuclei, whereas alkoxy groups, as are found in lignin, tend to activate them (Gold and Alic 1993; Hammel 1995).

The ability of LiP to oxidise lignin non-specifically, generating cation radicals which undergo a wide variety of reactions, accounts for the number of different metabolic products observed (Gold and Alic 1993).

MnP catalyses the H_2O_2-dependent oxidation of lignin and lignin derivatives and a variety of phenolic lignin model compounds. It has been shown that Mn(H) is the preferred substrate for MnP. Mn(1I) is oxidised to Mn(II) which diffuses from the enzyme surface and in turn oxidises the phenolic substrate. Organic acids, such as malonate and oxalate chelate Mn(III) to form stable complexes with high redox potentials (Zapanta and Tien 1997). Thus, Mn(III) ion participates in the reaction as a diffiisable redox couple rather than an enzyme binding activator. This is supported by the demonstration that chemically prepared Mn (III) complexed with organic acids, such as malonate, mimic the MnP reactions. The initial reaction of Mn(III) with a phenol is a one-electron oxidation to form a phenoxy radical intermediate. Subsequently, alkyl-phenyl cleavage, C_a—Cp cleavage, or benzylic carbinol oxidation yields the variety of products observed (Gold and Alic 1993). MnP also supports Mn-dependent lipid peroxidation, which in turn catalyses certain reduction and depolymerisation reactions in aromatic pollutants (Hammel 1995).

1.3.4. Examples of Pollutant Degradation by Fungal Peroxidases

Recent investigations show the direct involvement of *P. chrysosporium* LiP in the metabolism of anthracene, and of both LiP and MnP from P. *chrysosporium* in the metabolism of 2,4-diclorophenol (DCP) and 2,4-dinitrotoluene (DNT). Anthracene was oxidised to 9,10-anthraquinone (AQ) *in vitro* and mineralised *in vivo* by ligninolytic cultures of *P. chrysosporium.* Further evidence has shown that the pathway proceeds from anthracene to AQ to phthallic acid. Phthallic acid was shown to undergo ring cleavage before further metabolism to CO_2 (Aust 1990).

Both LiP and MnP from *P. chrysosporium* have been shown to catalyse the *in vitro* oxidation of DCP and several of its metabolites found in fungal cultures. A pathway was proposed for the degradation of DCP that includes several oxidations catalysed by LiP and MnP. Several DNT metabolites found in ligninolytic cultures of *P. chrysosporium* have also been shown to be oxidised *in vitro* by LIP and MnP. The pathway is similar to that proposed for the degradation of DCP in that it involves several oxidation, reduction and methylation reactions. A difference is that peroxidase activity in DNT metabolism must be preceded by reduction of one of the nitro groups to an amine (Lamar 1992).

Involvement of extracellular LiP and MnP in the metabolism of aromatic compounds in extracellular peroxidase-catalysed oxidations and intracellular reductions and methylations that regenerate peroxidase substrates would require metabolites to shuttle across the plasmalemma. Not much is known about such processes, or about the absorption and intracellular metabolism of the different intermediates produced. It is considered possible that peroxidases similar to LiP and MnP, and other oxidases, are produced intracellularly for metabolism of intermediates (Lamar 1992).

Substrate-specific differences have been observed between LiP and MnP. LiP has been shown to be capable of oxidising methoxybenzene congeners with high oxidation potentials, whereas MnP or chelated Mn^{3+} can only oxidise low potential congeners. LiP, but not MnP, was shown to catalyse the *in vitro* oxidation of nitrodimethoxybenzenes and chlorodimethoxybenzenes whereas MnP, but not LiP, catalysed the *in vitro* oxidation of

the DNT metabolites 2-amino-4-nitrotoluene and 4-nitrocatechol. These substrate-specific differences seem to indicate that the peroxidases work in synergy in the oxidation of aromatic compounds (Lamar 1992 and references therein).

1.3.5. Oxidases for the Production of H202

It was shown quite early in the elucidation of the biodegradative mechanism of the WRF that H202 was produced under ligninolytic conditions (Forney *et al.* 1982). The enzymes responsible for the formation of H_2O_2, a group of oxidases, reduce oxygen to form H202 using a variety of organic electron donors (Bumpus and Aust 1987). These oxidases include glyoxal oxidase, an extracellular, idiophasic copper containing enzyme (Kersten and Kirk 1987); glucose oxidase (Kelley and Reddy 1986); veratryl alcohol oxidase (Asada *et al.* 1995) and methanol oxidase (Eriksson and Nishida 1988).

An oxidase shown to be of particular significance is pyranose oxidase (POD). It catalyses the C-2 oxidation of several aldopyranoses, with the preferred substrate being glucose. It is a large flavin adenine dinucleotide glycoprotein (MW — 300 000) and has been identified in both mycelia' extracts and culture filtrates of laboratory cultures of several fungi, suggesting both intracellular and extra-cellular distributions (Daniel *et al.* 1994). A possible role for POD other than production of H_2O_2 for peroxidase oxidation has been postulated. It acts with laccase to maintain a glucose: quinone oxidoreductase cycle in order to prevent spontaneous repolymerisation of quinones formed during ligninolysis, thereby increasing the efficiency of lignin degradation (Szklarz and Leonowicz 1986).

Besides being a co-substrate for peroxidase enzymes, the H202 is an oxidant itself, and is also converted chemically to more powerful active oxygen species such as hydroxyl radicals and superoxide (Kremer and Wood 1992). These oxygen radicals are partially responsible for the non-specific cleavage of certain recalcitrant lignin and aromatic pollutant species.

1.3.6. Laccase

Many WRF produce an extracellular laccase (D' Souza *et al.* 1996). It was previously believed that *P. chtysosporium* was an exception to the above, but it was recently reported that laccase activity could be detected in this fungus under certain culture conditions (Srinavasan *et al.* 1995). Laccase is a blue copper protein which catalyses the one-electron oxidation of phenols to phenoxy radicals, Like MnP, laccase can catalyse the alkyl-phenyl and C- cleavage of phenolic lignin dimers and has a broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups (Higuchi 1990; Youn *et al.* 1995). It also catalyses the demethoxylation of several lignin compounds, but it has generally been accepted that laccase cannot degrade non-phenolic lignin model compounds (Thurston 1994; Youn *et al.* 1995). Several fungi produce laccase and MnP but not LiP, indicating that some WRF degrade lignin by a different mechanism to *P. chrysosporium* (Gold and Alic 1993; Thurston 1994).

Despite the amount of research expended on the elucidation of the different components of the ligninolytic system of the white rot fungi and their roles, it is believed that other uncharacterised substances and enzymes, not active or stable in crude culture supernatant, may also be of importance (Paice *et aL* 1993). The role of intracellular enzymes responsible for monomer degradation is also poorly understood, although several key enzymes have recently been isolated (Brock *et al.* 1995).

1.4. INDUSTRIAL APPLICATIONS OF THE LIGNINOLYTIC SYSTEM OF THE WRF

Since the discovery by Bumpus *et al.* (1985) that the ligninolytic system of the WRF was capable of the transformation of a range of aromatic compounds, and the subsequent elucidation of the enzymatic mechanism of ligninolysis by these fungi, much literature has appeared exploring the commercial application of these fungi and their enzymes. Most of the research activity was based on the bioremediation of aromatic pollutants, but other applications of the enzymes were also evaluated.

1.4.1. Bioaugmentation of Contaminated Soil and Groundwater

Contamination of ground water resources with pollutants like aromatic- and alkyl-group substituted aromatic hydrocarbons is considered a serious threat to human health, A major source of this pollution is leakage from underground petroleum storage tanks and distribution systems. Pump-and-treat systems, or *in situ* bioremediation strategies can be utilised as solutions.

Fungal enzymes have been applied to both such systems. **In the** case of pump-and-treat systems, the fungal peroxidases could be used to precipitate aromatic pollutants by polymerisation. They could also be used to de-toxify these pollutants prior to conventional biological treatment such as activated sludge systems. *In situ* fungal peroxidase-based treatment could be used to de-toxify the pollutants so that resident microbes can utilise the degradation products. It has been postulated that *P. chlysosporium* forms fairly close relationships with certain bacteria (Seigle-Murandi *et al.* 1996). Alternatively, fungal peroxidases could be used to link the pollutants to humic polymers, thereby rendering them inert. Various examples of this approach are listed by Sjoblad and Bollag (1981) and Shannon and Unterman (1993).

1.4.2. Bioremediation of Industrial Effluents

Besides the potential application of the WRF ligninolytic system for the bioremediation of soil, the same enzymatic systems can be applied to water contaminated with aromatic compounds.

Residual lignin in Kraft pulp is highly modified by alkaline condensation reactions during pulping and gives the pulp a characteristic brown colour. This residual lignin is commercially removed by bleaching with chlorine-based chemicals. It has been reported that chlorinated products derived from lignin during these bleaching procedures are mutagenic. These compounds also cause a waste treatment problem because of their toxicity and dark colour. Therefore, alternative ways to eliminate, or at least reduce, the use of chlorine-based chemicals in bleaching require development (Katagiri *et al.* 1995).

Given the lignin degrading capability of the WRF, a considerable body of research has been reported for the delignification and brightening of unbleached Kraft pulp using the WRF. Much progress has been made in the study of delignification by the WRF and the development of an economically feasible process (Katagiri *et al.* 1995). It was shown by various researchers that Me is the most important enzyme involved in the biochemical brightening of pulp (Katagiri *et al.* 1995), although this effect is not observed when using the enzyme alone (Paice *et al.* 1993). Tien (1987) has proposed that this might be considered one of the major potential industrial applications of fungal enzymes.

It has also been shown that the effluent from the first alkaline extraction stage of the Kraft process for wood pulping is a complex mixture of chlorinated lignin fragments, anisoles, phenols and some other low molecular weight chloro-organics. *P. chiysosporium* was shown to be capable of the removal of the above compounds (Hammel 1989).

1.4.3. Improving the Digestibility of Lignocellulosic Animal Feeds

Increasing the nutritional value of wood and agricultural by-products for ruminant animals has received much attention (Adaskaveg *et al.* 1995; Reid 1989a). The lignin in plant materials such as straw limits rumen digestibility of polysaccharides by blocking access by rumen bacteria and their enzymes to these polysaccharides. It has been shown that even partial de-lignification of animal feeds can give major increases in animal productivity. Fungal peroxidases could be used to selectively de-lignify these materials, leaving the polysaccharides exposed. These polysaccharides could, as an alternative, be further hydrolysed to sugars for fermentation to fuels, solvents, and other chemicals (Reid 1989a).

1.4.4. Other Applications

Fungal peroxidases are currently being evaluated for several other applications:

- In laundry detergents for bleaching of dyes in solution, preventing surplus dyes from the garment to deposit on and decolourise others (Schneider and Pederson 1995);
- For the enhancement of flavours in food (Schneider and Pederson 1995);
- A replacement for horseradish peroxidase if the fungal enzymes could be produced at a lower cost (Schneider and Pederson 1995);
- In the enhancement of polymerisation of lignin for the production of various composite materials, avoiding the use of artificial resins (Candeias 1995);
- Administration of peroxidases has recently been shown to promote regression of tumours in animals (Candeias 1995);
- Haloperoxidases are being formulated as antibacterial preparations given the bactericiodal action of hypohalous acids (Weyer 1995).

1.5. BIOTECHNOLOGY OF WRF ENZYME PRODUCTION

Large-scale industrial application of the fungal enzymes is constrained by the availability of effective production processes. Research in bioreactor design for ligninolytic enzyme production by WRF has basically followed two general directions. In early research, emphasis was placed on growth medium manipulation and isolation of novel strains in an attempt to manipulate the fungus to achieve good productivities in conventional tank reactor systems. These attempts are comprehensively covered in a review by Linko (1992), who sums up the developments towards industrial scale production of ligninolytic enzymes as an "exciting history of dreams and frustrations, failures and successes". More recent developments have focussed on novel reactor design to best suit the physiology of the fungus.

1.5.1. Culture Conditions

As already noted, ligninolysis and the production of ligninolytic enzymes occurs only during secondary (idiophasic) metabolism, the onset of which is triggered by the depletion in cultures of nutrient nitrogen, carbon or sulfur sources. This is believed to

occur because of nitrogen and/or carbon catabolite repression (Keyser *et al.* 1978) and regulation has been shown to take place at the level of LiP production (Faison and Kirk 1985; Gold and Alic 1993). Tien and Tu (1987) established that nitrogen regulation functions at the transcriptional level of the LiP genes.

1.5.2 Growth and Production Media

Most LiP production experiments have been carried out with completely synthetic media which are various modifications of the original nitrogen limited medium developed by Kirk *et al.* (1978) for studies on lignin degradation by *P. chtysosporium.* The medium contained mineral salts and vitamins with 10 g. $L.^{-1}$ of glucose as the C-source and 2.2 mM. of ammonium tartrate as the nitrogen source (Tien and Kirk 1988). The effect of N-limitation is not considered surprising given the low levels of nitrogen found in wood (Buswell and Odier 1987). In addition to lignin degradation, several other features of secondary metabolism in *P. cluysosporium* triggered by N-limitation have been studied. The formation of an extra-cellular glucan and synthesis of veratryl alcohol also occur as secondary metabolic events (Linko 1992).

Various studies have focussed on the improvement of peroxidase production by supplementation of the basic growth medium with various additives or adaptation of the physical environment (Asther *et al.* 1988; Bonnarme and Jeffries 1990; Linko 1992).

Additives which have been shown to improve ligninase production include:

- Veratryl alcohol (Kirk *et al.* 1986; Linko 1992);
- Surfactants such as Tween 20 and Tween 80 (Jager *et al.* 1985; Venkatadri and Irvine 1990; Legtan *et al.* 1993);
- Phospholipids and fatty acids (Asther and Corrieu 1987);
- Trace elements (Tien and Kirk 1988);
- Buffers and pH control (Kirk *et al.* 1978; Linko 1992; Haapala and Linko 1993).

1.5.3. Oxygen Requirement

The importance of having a pure oxygen environment for good ligninase production is well documented. The ligninolytic system of the WRF has been shown to be particularly active in cultures grown in high oxygen tension (Dosoretz *et aL* 1990). Lignin degradation was shown to be about 3-fold higher under 100% oxygen than under air (Kirk *et al.* 1978). Faison and Kirk (1985) reported that both ligninolysis and ligninase activities ofP. *chrysosporium* were increased in cultures initially supplied with air during their growth phase and then shifted to an oxygen atmosphere. Because of this, most laboratory-scale studies as well as scale-up attempts have employed the use of a pure oxygen environment for high productivities (Dosoretz *et al.* 1993). Dosoretz *et al.* (1990) also reported that different oxygenation conditions had a profound effect on the onset and decay of the peroxidative system, and the production of extra-cellular proteases and polysaccharides.

1.5.4. Production Strains

P. chrysosporium, the most commonly studied of the white rot fungi is an asexual, thereto-tolerant, sporulating imperfect fungus. The reason for the popularity of this species in studies of lignin degradation is due to the following:

- *P. chrysosporium* has an optimum growth temperature of 40°C and an optimum pH of 4.5, which is not common, making contamination of production cultures less of a problem;
- It has a relatively rapid growth rate compared to the other WRF;
- A thoroughly defined chemical growth medium has been developed for culture of this species making studies on the effects of its chemical environment easy;
- It is a potent lignin degrader;
- It produces asexual spores that can be used for mutagenesis. Also, a growth medium has been developed which allows colonial growth of the fungus. These features make genetic studies more convenient ;

- It was thought not to produce polyphenol oxidases which cause re-polymerisation reactions which would make studies on the depolymerisation of lignin difficult. However, it has recently been found that *P. chrysosporium* in fact contains a laccase (Gold and Cheng 1978; Linko 1992; Gold and Alic 1993; Jeffries T. 1995, pers. comm; Srinavasan *et al.* 1995)

Of the wild type strains of *P. chrysosporium* used, the strain ATCC 24725 (BKM-F-1767) has been reported to produce the highest ligninase activity (Linko 1992). The other well-studied wild type strain is ATCC 34541 (ME 446). Another well studied mutant is 1NA-12, which is nitrogen deregulated (Buswell *et al.* 1984) and produces LiP under high nitrogen and high carbon content. Several other *P. chrysosporium* mutants which produce LiP under nutrient sufficient conditions have also been reported (Linko 1992). One of the most noteworthy is PSBL-1, a mutant lysine auxotroph which produces high ligninase activities under high nitrogen conditions (Tien and Myer 1990).

1.5.5. Culture Conditions

Stationary Cultures

Initial laboratory-scale experiments on LiP production were performed using static cultures of 10m1 of growth medium in 125m1 Erlenmeyer flasks using a spore suspension as inoculum (Tien and Kirk 1988). The fungus grows as a pellicle on the surface of the growth medium, and under typical conditions (N-limited medium, 39°C), and atmospheric oxygen, the ligninolytic activity appears after 3 to 4 days (Kirk and Nakatsubo 1983), Even before LiP was discovered, Keyser *et al.* (1978) reported a reproducible sequence of metabolic events of the culture following inoculation: From 0 to 24 hours germination of spores takes place followed by a period of linear growth and concomitant depletion of nutrient nitrogen. They found that primary growth started between 0 and 12 hours (as measured by DNA synthesis) and ceased after 30 hours. From 24 to 38 hours, cessation of linear growth and de-repression of ammonium permease activity (demonstrating nitrogen starvation) was observed. From 72 to 96 hours, ligninolytic activity was observed (determined by the appearance of $'^4CO_2$ from ^{14}C

synthetic lignin). This activity appeared between 2 and 3 days after the depletion of NH_4^+ and 2 days after maximum growth rate was achieved.

Agitated Cultures

One of the greatest hindrances to scale-up of the LiP production systems had been the limitation to use stationary cultures (Leisola and Fiechter 1985). It was initially observed by Kirk *et al.* (1978) that agitation, which leads to pellet formation, suppresses the ligninolytic system of *P. chrysosporturn* (Kirk *et aL* 1988; Linko 1992). This was believed to be due to oxygen limitation (Leisola and Fiechter 1985), but was subsequently shown to be due to mechanical inactivation of the ligninolytic system by shear stress (Linko 1992).

Jager *et al* (1985) reported that the addition of non-ionic detergents, such as Tween 80, to growth media of P. *chiysosporium* facilitated the production of ligninolytic enzymes in agitated flask cultures. Equal or higher LiP activities were obtained from cultures agitated at 200 rpm compared to stationary cultures, when the N-limited medium was supplemented with 0.02% Tween 80 and veratryl alcohol. A maximum enzyme activity of 179 U. $L.^{-1}$ was obtained from a 6 day-old culture. In cultures grown under agitation without a detergent only traces of ligninase could be detected (Linko 1992). These findings suggested new prospects for scale-up of the production of ligninolytic enzymes in stirred tank fermenters. One of the most successful systems involving conventional tank reactors was achieved by Bonnarme *et al.* (1993). They used the wild type production strain BKM-F 1767 with a patented glycerol-based growth medium (Anther *el al.* 1989).

Although techniques have been developed which will overcome the problem of sensitivity to agitation in stirred tank reactor systems, the scale-ability of freely suspended cultures in tank reactors is questionable since Janshekar and Fiechter (1988) failed to scale up a tank reactor from 42L to 300L using constant impeller tip speed, and constant gas flow rate as scale-up criteria. These authors suggested that adequate understanding of the relationships involved in the regulation of primary and secondary metabolic pathways is necessary for optimal design of enzyme production systems.

Immobilisation of *P. cluysosporium*

Various bioreactor geometries using immobilised biomass on different support matrices such as nylon web (e.g. Linko 1988) and polyurethane foam (e.g. Kirkpatrick and Palmer 1987) have been employed to produce LiP. Several recent reports of LiP production by immobilised *P. chtysasporium* have been summarised by Linko (1992),

In general, it was found that immobilisation of *P. chtysosporium* provided good LiP yields and improved the predictability of laboratory-scale cultures. Immobilisation of the biomass allowed for biocatalyst re-use in multiple repeated batch cultures (Kirkpatrick and Palmer 1987).

A good example of a reactor which showed potential for both semi-continuous LiP production (Venkatadri and Irvine 1993) and removal of PCP by immobilised *P. chrysosprium* is the silicone rubber membrane reactor. This reactor consisted of silicone rubber tubing wound into a stirred tank reactor which contained either a growth or production medium. The silicone rubber tubing was pressurised from its lumen side with pure oxygen. This arrangement provided excellent oxygen mass transfer because of solution-diffusion type transport of the oxygen through the silicone rubber. Alternative 3 day growth/production medium cycles yielded good batch productivities which could be repeated over several cycles. The silicone rubber tubing appeared to be an excellent support for fungal growth. A hollow fibre reactor was also used in a similar mode, but yielded inferior results (Venkatadri and Irvine 1993).

Good results were obtained with nylon web, polyurethane foam, sintered glass, silicone rubber tubing and poly (styrene-divinylbenzene) carriers (Ruckenstein and Wang 1994, and references therein), The most popular matrices for immobilisation of *P. chrysosporium,* probably due to availability, are polyurethane foam and nylon web materials (Moreira *et al.* 1997; Laugero *et al.* 1996). Thus, in summary, it can be stated that criteria now exist for good enzyme yields in batch- and semi-continuous enzyme production processes. The other major criterion for successful commercialisation is scale-

ability. Bosco *et al.* (1996), have identified some criteria for a scale-able reactor configuration for good ligninolytic enzyme production in *P. chrysosporium :*

- Supported biofilm;
- Low mechanical sheer stress on the biofilm due to gas and fluid dynamics;
- Large support area available for biofilm growth;
- Large liquid/gas/mycelial interfaces.

Moser (1991) stated that a bioprocess must be regarded as a highly complex network of interactions between the metabolism of cells and the environmental conditions determined by the reactor and properties of the medium. Productivities of bioprocesses, no matter what the application, are linked with productivities of bioreactors. Selecting and designing an appropriate bioreactor for a specific organism depends on the characteristics of this organism and requires an understanding of how the complex fluid-mechanical, nutritional and physico-chemical environment affects the cells (Papoutsakis 1991).

Continuous Culture

Continuous production of ligninolytic enzymes has not been widely reported for WRF. It has been difficult to achieve since these enzymes are produced as secondary metabolites. In several secondary metabolite producing species, the stationary phase may be prolonged for days by continuously or intermittently providing non-repressive and non-inhibitory levels of carbon source in a fed batch culture. In some organisms, the stationary phase is very short (approximately 4-20 hours) and in others such as *P. chrysosporium,* the stationary phase may extend to several days. Invariably, the rate of product formation declines and cessation of stationary phase occurs. This occurs for several reasons:

- Irreversible decay of one or more enzymes of a production pathway. These losses occur at primary and secondary metabolic level;
- Feedback effects of accumulated product lower productivity;

- Autolysis leading to loss of active biomass. This is particularly important in the case of *P. chrysosporium* since up to 40% of biomass can be lost due to lysis in the decline phase of typical batch cultures (Broda P. 1996, pers. comm.);
- Committed differentiation to end the life cycle.

(Demain *et all* 1983)

Continuous culture has been accepted as a means for dramatic bioprocess intensification (Shuler and Kargi 1992). If *P. chrysosporium is* to be applied to bioremediation by direct contact with effluents, it would have to compete with bacterial systems. The bioremediation capability of bacteria, although generally inducible, is a trophophasic function and is thus suited for continuous culture,

Continuous processes reported for ligninolytic enzyme production in *P.chrysosporium* have involved regeneration of mycelium in successive growth-production cycles (Feijoo *et aL* 1994). This type of system gave peaks of LiP activity lasting for 1 or 2 days during production phase before a regeneration of biomass phase of approximately 6 days was required. Activity was shown to decline after each successive cycle (Venkatadri and Irvine 1993; Feijoo *et al* 1994). This is clearly an inefficient approach.

The inability to sustain continuous ligninolytic activity in laboratory bioreactors is discrepant with the native state of the WRF, which are responsible for ligninolysis in the natural environment. Lignin degradation in nature appears to be highly productive and is certainly sustained continuously. This discrepancy might occur because the homogeneous environment of typical laboratory fermentors is clearly different from the natural environment in which WRF have evolved to cope best with, the solid-air interface of woody tissue. Some corroborative evidence was provided by Datta *et aL* (1991), who showed by immunoblotting and amino acid sequencing that the prominent ligninolytic iso-enzymes produced when *P. chrysosporium* was grown on wood pulp was different from that produced in cultures grown on a defined medium in liquid culture.

The effect of the physical environment can be explained by the fact that the growth habits of the fungus relate to its survival in nature. An important aspect of the natural external environment of the WRF is its heterogeneity, comprising of nutrient gradients.

1.6. NUTRIENT GRADIENTS

It has been shown that nutrient gradients are established in biofilms of immobilised organisms. Substrate gradients result in heterogeneous distributions of viable cells owing to growth and death of cells (see fig 1.4.). In fact, only for very small particles can non-uniform cell growth be prevented (Characklis 1990a).

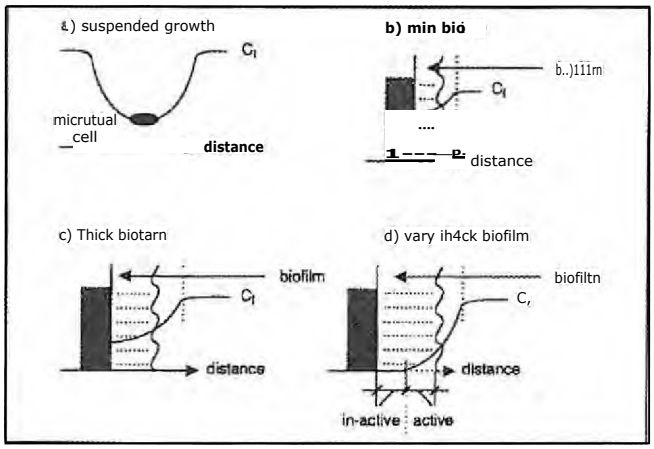


Fig. 1.4: Nutrient gradients in immobilised cell systems (Siebel 1992). C_1 represents substrate concentration.

The profile of substrate concentration versus distance from the surface of the biofilm is determined by the following:

- Microbial substrate uptake rate, which is a function of microorganism concentration and their affinities for the substrate;
- Substrate transport rate through the film which depends on substrate diffusivity through the biofilm;

- Substrate transport rate to the biofilm, which is a function of microbial substrate uptake rate, substrate diffusivity through the water and hydrodynamics near the biofilm surface.

(Siebel 1992).

Nutrient and physical gradients not only occur in immobilised cells but also in natural biofilms and other environments (Lovitt and Wimpenny 1981; Emerson *et al* 1994). Spatial heterogeneity and the general lack of nutrients in natural environments means that molecular diffusion is the dominant transport process for nutrients. For these reasons dynamic models have been developed describing simultaneous substrate diffusion, consumption and growth (Walsh and Malone 1995).

Similar research has been undertaken to characterise the structural heterogeneity of biofilms resulting from spatial gradients (Onuma and Omura 1982; Murga *et al.* 1995; Walsh and Malone 1995). This has shown that the structure, activities and composition of biofilms change with biofilm depth (Zhang and Bishop 1994).

In view of the above-mentioned phenomena, it was conceived that spatial concentration gradients could be exploited to continuously produce secondary metabolites as secondary metabolism is usually triggered by nutrient starvation. Thus, reactors were considered which operate on the basis of the maintenance of steady-state nutrient gradients. Such reactors are termed "gradostats" (Wimpenny 1990).

*1.*7. *GRADOSTAT SYSTEMS*

The term "gradostat" was first used by Lovitt and Wimpenny (1981) to describe a chamber where steady-state nutrient and oxygen gradients can be established and maintained at steady-state in order to study various ecological implications of similar environments in nature. In a review on the topic, Wimpenny (1990) describes three types of laboratory models to study diffusion of nutrients in heterogeneous environments and the effects of these gradients on microbial growth. These include:

- Open or steady-state gradient systems (Wimpenny 1990);

- Closed gel-stabilised diffusion models (Emerson *et al.*. 1994; and references therein; Wimpenny 1990);
- Bacterial colonies (Wimpenny 1990).

Gel-stabilised systems and bacterial colonies have been studied by microbiologists for several decades and warrant no further discussion here.

1.7.1. Open Gradient Systems

These are typically chemostat culture vessels linked together to form multistage continuous culture systems (Lovitt and Wimpenny 1981; Moser 1991). This provides uni-directional transfer of material, which is useful in modelling a system like the soil, where the net flow of materials is in one direction only (Wimpenny 1990).

Lovitt and Wimpenny (1981) describe a bi-directional open gradient system, which they called the "gradostat". This consisted of five laboratory fermentation units arranged on a series of steps. Culture was pumped up the array from vessel to vessel and was transferred in the opposite direction by gravity over weirs. An example of growth of a bacterium on two nutrients entering the system from different directions is described (Wimpenny 1990).

Various other formats of gradostats are described (Wimpenny 1990, and references therein) but none conform to the requirements for good ligninolytic performance by *P. chrysosporium* Attached growth perfusion systems were thus evaluated. One of the most effective of these are membrane bioreactors.

1.8. MEMBRANE B1OREACTORS

Membrane bioreactors evolved out of the use of membranes for filtration. Typical ultrafiltration membranes were adapted for immobilisation of microorganisms and mammalian cell lines (Belfort 1989; Prenosil and Hediger 1988).

Rony (1972) first published the use of membrane bioreactors and the successful use of hollow fibre bioreactors for the cultivation of mammalian whole cells was first reported by Knazec *et at* (1972). Subsequently, a number of applications have been reported in the literature with various bacteria, fungi and plant cells all being successfully grown (Belfort 1989). In the standard design, several or individual hollow fibres are potted together at each end and sealed in a housing (usually tubular) to separate the extra-capillary space (ECS) from the lumen space (Belfort 1989). Cells are normally confined to the shell side of a hollow fibre cartridge, although they have also been grown within and across the membrane fibres. Dissolved nutrients are normally supplied by transport from the lumen across the membrane to the cell mass by convection or diffusion (Belfort I989).

Many advantages relate to the use of membrane reactors for immobilisation of whole cells. They have high surface area to volume ratios, separation of cells from flow and high cell concentrations (Heath and Belfort 1992). The membrane properties provide a normally hydrophobic matrix which encourages cell attachment. With the advantage of high cell densities and hence, high volummetric productivities and sustained output resulting from a stabilised *in vitro* environment, immobilised cell membrane bioreactors are particularly attractive for difficult-to-culture organisms such as mammalian and plant cell cultures for production of complex bio-products (Belfort 1989). The biomass is retained in a low shear environment with continuous supply of nutrients and removal of metabolic wastes.

One of the major problems mentioned in literature concerning membrane bioreactors is that nutrient gradients have been shown to exist in hollow fibre bioreactors (Webster and Schuler 1979; Robertson and Kim 1985; Piret and Cooney 1990). When nutrients are supplied by diffusion, radial nutrient gradients are normally established as the cells closest to the membrane have first access to them while the cells furthest away from the membrane surface are normally starved of nutrients (Inloes *et at* 1983; Belfort 1989; Piret and Cooney 1990; Dall-Bauman *et at* 1990, Sardonini and DiBasio 1992). Further diffusional resistance is provided by extracellular product formation (Lawrence *et al.* 1994). This is depicted schematically in figure 1.5.

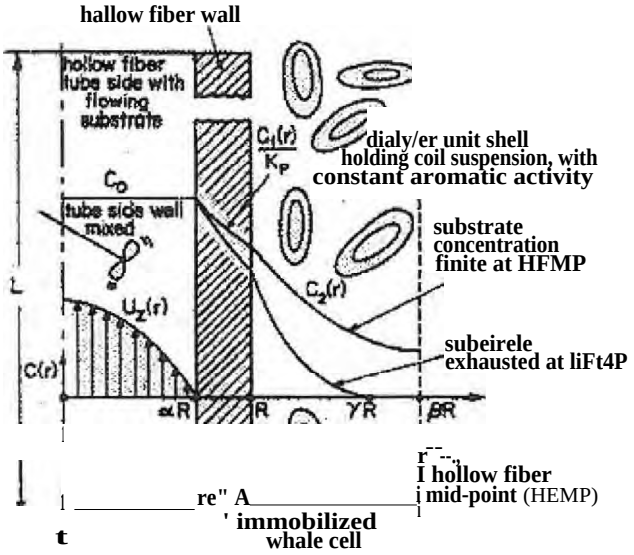


Fig. 1.5: Nutrient gradients typically encountered in membrane biofilms (Webster and Shuler 1979).

1.9. THE SOLUTION - CONCEPT OF THE "MEMBRANE GRADOSTAT REACTOR"

From a study of the requirements for bioreactor design for *P. einysasparium,* consideration of the native state of this organism and the characteristics of membrane bioreactors, a novel membrane gradostat reactor was conceptualised as a solution to continuous production of idiophasic enzymes and bioremediation using *P. chlysosporium,* It is based on the establishment of nutrient gradients in a perfusion biofilm reactor configuration.

Thus, the "problem" of nutrient gradients in membrane bioreactors and biofilms in general was formulated into a solution to the problem of continuous secondary metabolite production in filamentous fungi.

L9.I. The Membrane Gradostat

The term "membrane gradostat" is used to describe a biofilm reactor with good potential for industrial application, which uses a synthetic capillary ultrafiltration membrane as a support matrix for the biofilm. The term gradostat applies since although gas and liquid nutrient flow is uni-directional, bi-directional contact occurs between the primary and

secondary growth phases of the biomass. The essence of this system is described below and is depicted schematically in figure 1.6.

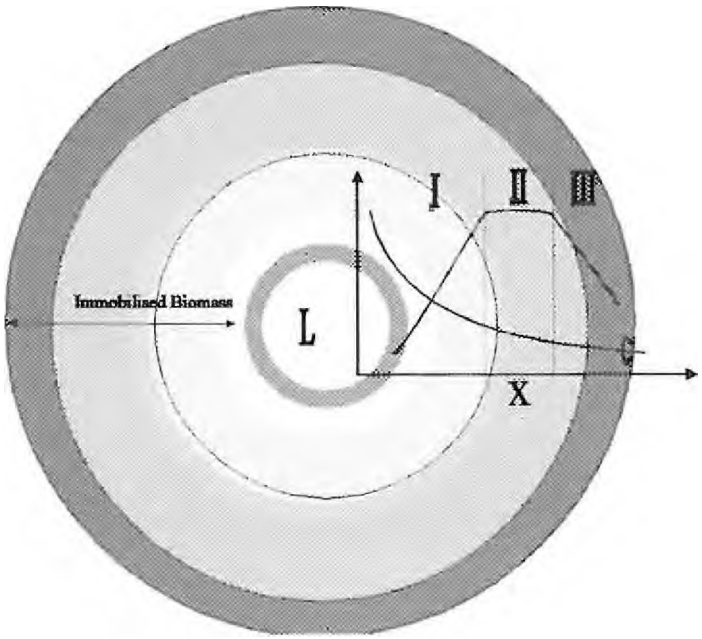


Fig. 1.6:Schematic representation of the membrane gradostat concept with superimposed batch culture growth curve. (I) Primary growth phase, (H) stationary phase, (M) decline phase. L is the lumen of the fibre from which the growth medium k supplied. X is radial distance from the lumen in the direction of nutrient flow. C is the concentration of growth-limiting substrate.

In practice the concept would involve immobilising fungal biomass onto the spongy layer of a capillary membrane and developing a biofilm of sufficient thickness, density and activity to establish a radial nutrient gradient across the biofilm. New biomass would then be produced continuously near the surface of the biofilm where nutrient rich conditions prevail. This biomass would be pushed outward by newly-formed biomass to an area of low nutrient concentration. Here the biomass passes into secondary metabolism activating its enzyme production system at the hyphal tips. Inactive biomass and spores produced are sloughed off at the outermost reaches of the biofilm by the turbulent air supply passing through the extra-capillary space (ECS). There would, therefore, be no need to regenerate biomass and force it into secondary metabolism by alternate growth medium/production medium cycles.

It was hypothesised, therefore, that if the life-cycle events of the fungus were to be transformed from a temporal domain to a spatial domain in this way (figure 1.6), then one could achieve continuous enzyme production over time in the bioreactor.

A logical research drive in the development of membrane biofilm systems was to increase the biofilm density and quantity in a reactor since the effectiveness of a biocatalytic system depends largely on the concentration of the biocatalyst. A major hindrance to the development of dense biofilms in membrane reactors was the establishment of radial nutrient gradients as explained above. Thus, many alternate reactor designs were directed at overcoming these nutrient gradients (Chresand *et aL* 1988; Heath and Belfort 1992; Prazares and Cabral 1994). The membrane gradostat concept utilises this "problematic" phenomenon of inherent nutrient gradients as an advantage and, therefore, challenges the above technological prejudice toward developments designed to overcome the nutrient gradients.

1.10. RESEARCH HYPOTHESIS

The ligninolytic state of WRF such as *P. chrysosporiurn* has promising commercial potential for bioremediation and/or ligninolytic enzyme production. The major hindrance to development of cost-effective processes to achieve the above is the inability to sustain continuous enzyme production for extended periods, and the shortcomings of conventional reactor systems, which relate to their incongruence with the heterogeneous native state of the organism.

It was postulated that sustained ligninolytic activity could be achieved by effecting spatio-temporal domain transformation in the development of the mycelium in a typical batch culture, ie. primary and secondary metabolism in the same thallus within the reactor. It was also postulated that this could accomplish high productivity due to the compliance of the system with the requirement of the fungi for a solid/air interface.

Testing this hypothesis required the development of the membrane gradostat reactor. The objective of this thesis was to test the following claims, to which the pragmatic considerations of the above hypothesis were reduced. These are:

1) That *P. chrysosporiurn* can be grown on membranes in gradostat operation mode and produce secondary metabolites, i.e. that the theoretical membrane gradostat concept holds empirically;
2) That the construction of laboratory-scale membrane bioreactors suitable for this application is technically feasible;
3) That the enzymes so produced are active, recoverable and can be used in bioremediation applications.

CHAPTER 2

THE MEMBRANE AND REACTOR MODULE

2.1. INTRODUCTION

The concept of the membrane gradostat bioreactor (MGR) was proposed as a possible system for the continuous production of fungal secondary metabolites. The transformation of this idea into a functional process required the selection of an appropriate membrane and reactor configuration for supporting differentiated fungal growth. The first component to be investigated was the membrane to be used as a support matrix for immobilisation of fungal growth.

Commercial membranes are available in 3 basic formats: *flat* sheet, tubular and capillary/hollow fibre membranes. Since capillaries offered the most favourable surface area to volume ratio, these were chosen as a support matrix for the MGR concept on the basis of recognition that the superior surface area to volume ratio would provide the best volumetric productivity (Cabasso 1980; Prenosil and Hediger 1988; Liu *et aL* 1991). Capillaries are also self-supporting, simplifying the hardware required for fabrication and operation (Cabasso 1980). Several attempts were made to use commercially available ultrafiltration (UF) membranes to evaluate the MGR concept. This represents a year's work with little success achieved. While results are not shown in detail, figure 2.1. shows the typical features of *P. chlysosporium* on a conventional membrane. A rigorous evaluation of these results led to the identification of the shortcomings of commercial UF membranes as matrices for the support of differentiated fungal growth as described below.

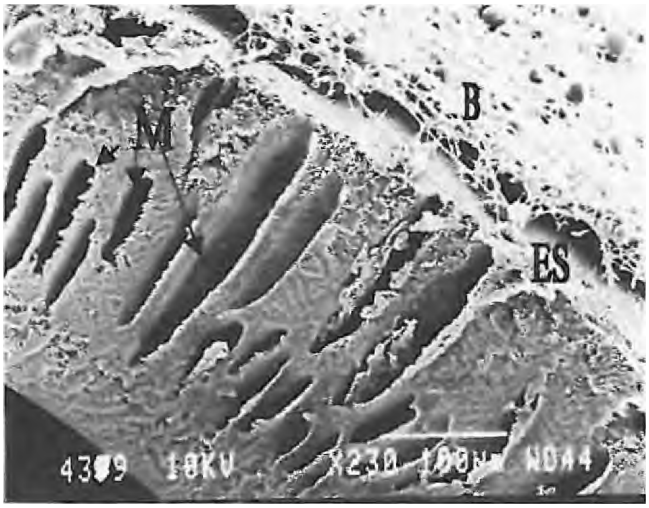


Fig. 2.1; Biofilm of P. chrysosporium immobilised on a conventional polyethersulphone ultrafiltration membrane. It can be observed here that the biofilm did not penetrate into the spongy wall of the membrane and appears not to be firmly anchored to the support matrix. B- biofilm, ES- external skin, M-macrovoid (Mag. = 230X).

Low Permeability

Slow filtration rate made the inoculation procedure tedious. Since this is a critical stage of biofilm development, the inoculation procedure would ideally involve the filtration of a spore suspension onto the outside spongy layer of the capillary membrane. The inoculation step then became a 4-day process, which was considered unacceptable. Flux during spore inoculation could be improved by increasing the hydraulic pressure of the inoculum feed. The increased pressure, however, created leaks in the system, occasionally leading to membrane bursts (especially at the potting material intersection) and contamination of the nutrient feed compartment.

Low Surface Area due to Blunt-Ended Macrovoids

Reduced wall space available for attachment of the fungal biomass resulted in inconsistency in establishment of stable, dense biofilms. Even when a biofilm was established, small increases in fluid flux resulted in slough-off of large chunks of the biofilm as it was not sufficiently well anchored.

It has been reported that biofilm removal results from shear forces acting parallel to the biofilm surface and also lift forces, resulting from fluid turbulence, acting normal to the substratum (Applegate and Bryers 1991). The problem of blunt-ended macrovoids

appears to be a general shortcoming of conventional anisotropic membranes as support matrices. Michaels (1980) has shown that these membranes are not optimally colonised by even small bacterial cells.

The Presence of an External Skin
The outer membrane layer made penetration of the fungal growth into the spongy wall of the membrane very difficult leading to poor anchorage of the biofilm.

Based on the above experience it was proposed that if the biofilm could be firmly entrapped within the macrovoids, sloughing of the biofilm by the above mechanisms could be avoided. If the macrovoids were not blunt-ended, the biofilm would be more firmly attached to a greater available wall surface, which in turn, would be more likely to sustain a differentiated fungal thallus.

The identification of above requirements for the system led to the development of a customised capillary membrane where the biofilm support matrix would be enhanced. The membrane development process has been reported by Jacobs and Leukes (1996).

2.2. MANUFACTURE OF A NOVEL MEMBRANE FOR ATTACHED FILM MEMBRANE BIOREACTORS

2.2.1. Introduction

Once some insight was gained into the shortcomings of conventional OF membranes for use as a support matrix for immobilisation, a rational approach could be taken for the development of an appropriate membrane for application in the MGR.

Most commercially available membranes are produced by the phase inversion technique, a versatile procedure for the fabrication of membranes (Mulder 1996). The technique is based on the transformation of a polymer from the liquid into the solid state under controlled conditions. The solidification process is initiated by the transition from one liquid state into two liquids (liquid-liquid de-mixing). At a certain stage during de-

mixing, the high polymer concentration liquid phase will solidify to form a solid matrix, which is the phase inversion (Mulder 1996). The role of polymer precipitation is determined by the progress of the concentration event, which is governed by phase interchange rates (Bottino *et al.* 1991). By controlling the initial stage of phase transition the membrane morphology can be controlled. In this way porous and non-porous membranes can be formed (Mulder 1996).

Various techniques for achieving phase transition are available. These include solvent evaporation, thermal precipitation, precipitation from the vapour phase and immersion precipitation. Most commercially available membranes are produced by the immersion precipitation technique, which involves the immersion of a polymer solution (polymer plus solvent) into a coagulation bath containing non-solvent. Precipitation occurs as a result of the exchange of solvent and non-solvent.

Hollow fibres and capillaries can be prepared via three methods, wet spinning (or dry-wet spinning), melt spinning, or dry spinning. The most common is dry-wet spinning, In this process the spinning dope, which is a viscous, degassed, filtered polymer solution containing a polymer, solvent and sometimes additives, is pumped through a spinneret. A non-solvent stream is passed through the inner tube of the spinneret, resulting in coagulation of polymer to give rise to the lumen of the membrane. After a short residence time in the air (dry stage), or a controlled atmosphere, the nascent hollow thread is immersed into a non-solvent bath (wet stage) where coagulation occurs. At this point the fibre has sufficient mechanical strength to pass over guides and rollers under moderate tension (Mulder 1996; Cabasso 1980).

A number of factors have been shown to affect the final morphology of the membrane wall. These are described below.

<u>The Choice of Solvent/Non-Solvent System.</u>
This is one of the main parameters in immersion precipitation systems. In order to produce a membrane by the phase-inversion technique, the polymer must be soluble in some solvent. Several solvents will be available for a particular type of polymer, but the solvent and non-solvent must be completely miscible. Water is the most common non-

solvent. In general, solvent and non-solvents with a high mutual affinity should be chosen. In this case, instantaneous de-mixing occurs in the water bath, resulting in porous membranes (Mulder 1996; Bottino *et al:* 1991). If the solvent and non-solvent are not completely miscible, delayed de-mixing occurs, which results in dense membranes (Mulder 1996; Reuvers 1987). Tsay and McHugh (1990) also suggest the use of a strong non-solvent like H_2O for the formation of a highly porous membrane, and it is reported by Cabasso (1980), that in employing a strong non-solvent, fibres displaying large macrovoids and cavities are obtained.

Choice of Polymer.

Polymer selection is important since it limits the solvents and non-solvents that can be used (Mulder 1996). Usually, however, the polymer is chosen first and then suitable solvents and non-solvents are found.

Polymer Concentration.

A high polymer concentration in the casting solution leads to high polymer concentration at the film inter-face, resulting in a less porous structure, even though instantaneous de-mixing occurs (Mulder 1996; Tsay and McHugh 1990). Low polymer concentrations should thus be used for the formation of highly porous membranes.

Composition of the Coagulation Bath

Addition of solvent to the coagulation bath is an important factor to consider in the determination of membrane structure. Some theory exists about this concept, but it is complex, since various phenomena are involved which must be considered in the context of the other casting and spinning parameters, In summary, delayed de-mixing tends to produce non-porous membranes with thick and dense skin layers, whereas low interfacial polymer concentration tends to produce more open top layers. Tsay and McHugh (1990) suggest the addition of high solvent concentration to the external coagulation medium for formation of thin skin layers, with sponge-like sublayers.

Presence of Additives

Small additions of low and high molecular weight non-solvent additives to the casting solution also result in more porous membranes. The example of PVP {poly-vinyl pyrrolidone) is described by Cabasso (1980). PVP is added to polysulphone spinning

dope to increase the viscosity to maintain the falling lumen configuration during spinning. In the coagulation bath the PVP, a water-soluble polymer, dissolves in the non-solvent leaving a residual porous, hydrophobic polysulphone matrix.

Spinning Parameters affecting Membrane Structure

Beside the composition of the spinning dope, certain fabrication parameters also influence the fabrication protocol. Liu *et al.* (1992) have reported that the following parameters influence the dimensions (inner diameter (ID), outer diameter (OD), wall thickness), morphology and performance of capillary membranes. These are extrusion rate and pressure of the polymer solution (Aptel *et al.* 1985; Mulder 1996; Liu *et al.* 1992; Miao *et al* 1996b), spinneret geometry (Mok *et al.* 1995), bore fluid flow rate (Cabasso 1980; Aptel *et al.* 1985; Mulder 1996), tearing rate (Mulder 1996) and viscosity of the spinning dope (Miao *et al.* 1996a), length of air gap (Aptel *et al.* 1985; Liu *et al.* 1992; Wienk *et al.* 1995; Miao *et at* 1996a) and spinning temperature (Cabasso 1980).

Changes in the above parameters are governed by the combined effects of desolvation, fibre swelling, and fibre stretching during fibre production (Miao *et al.* 1996a and b).

Factors affecting Macrovoid Formation

The formation of macrovoids is described fairly extensively in the literature. Their formation is normally considered unfavourable in OF membrane manufacture, because their presence could lead to weak spots or points of imperfection, causing fragility in the membrane, especially if high hydraulic pressures are to be used (Cabasso 1980; Mulder 1996; Miao *et al.* 1996b). Also, penetration of pinholes could occur at moderate pressures due to the presence of macroVoids (Cabasso *et al.* 1977).

The mechanisms which determine the type of membrane formed (porous or non-porous), tend to also determine whether or not macrovoids are formed. It has been shown, for many systems, that where instantaneous liquid-liquid de-mixing occurs, macrovoids are formed, and where there is delayed onset of de-mixing, they are absent (Cabasso *et aL* 1977; Mulder 1996). Low spinning dope viscosity, and low polymer concentration have been shown to enhance macrovoid formation, which supports the above description

(Cabasso 1980). Two phases in macrovoid formation play a role. These are initiation and growth (or propagation) of the macrovoid (Mulder 1996).

Macrovoid formation results from the liquid-liquid demixing, where nuclei of the polymer-poor phase are responsible for macrovoid formation. Growth takes place because of the diffusional flow of solvent from the surrounding polymer solution. A nucleus can only grow if a stable polymer/solvent/non-solvent composition is induced in front of it by diffusion. Growth will cease if a new stable nucleus is formed in front of it. Hence, the polymer solution in front of the nucleus must be kept stable and homogeneous if macrovoids are to be produced which extend from just outside the skin layer to the outer wall. If this is the case, solvent and non-solvent diffuse into the nuclei and the macrovoid grows until the polymer concentration at the macrovoid/solution interface becomes so high that solidification occurs. Clearly, a fine balance must be maintained, a difficult concept to engineer on a large scale.

2.2.2 Experimental Approach to Membrane Development

Casting Solution

Since skin formation on the lumen side generally results from contact with a strong non-solvent, pure water with no solvent or other additives was used as the internal coagulant to generate a thin-skinned membrane. For macrovoid formation, a casting solution and internal coagulant were chosen to sustain growth of the macrovoid from just below the skin layer all the way up to the membrane periphery.

For the formation of an open-porous surface on the membrane, gelation on contact with the external precipitation bath had to be suppressed. Gelation (skin-formation) was known to be suppressed if the non-solvent solution in the precipitation bath contains low concentrations of non-solvent (Smolders *et al.* 1992). It was therefore hypothesised that if the composition of the external coagulation tank mirrored that of the advancing polymer-poor phase front as it neared the membrane exterior, there should be no driving force for diffusion or heat of mixing (no concentration gradient), and that the phase-inversion process would cease. It was therefore decided, as a first approach, to use an

external coagulation bath with a solvent/non-solvent ratio near to the cloud-point of the casting solution. N-methyl-, 2-pyrrolidone (NMP) was used as solvent and a 20% aqueous solution of NMI' was chosen as an initial set of conditions.

Membrane Fabrication Protocol

A modified approach to membrane spinning was used. A conventional annular tube-within-tube extrusion die (spinneret) was used, but this was positioned at the bottom of the non-solvent coagulation tank, and the membrane was drawn vertically from the spinerette at a linear production rate of 4 m. min.$^{-1}$ As the external coagulant or contact bath was high in solvent content, the outside of the nascent membrane was still highly swollen, gel-like and soft when it was withdrawn from the external contact fluid, The membrane was therefore exposed to a non-solvent vapour atmosphere, humidified air in this case, to fix the structure once the membrane had been withdrawn from the bath, Phase separation by contacting the polymer solution with vapour of non-solvent is described in the literature (Wienk *et al.* 1995).

Once the phase-inversion membrane-formation process was completed, the membrane could be transferred to guide rollers in the rinse tanks without damage. The experimental membrane-formation equipment is illustrated in figure 2.2.

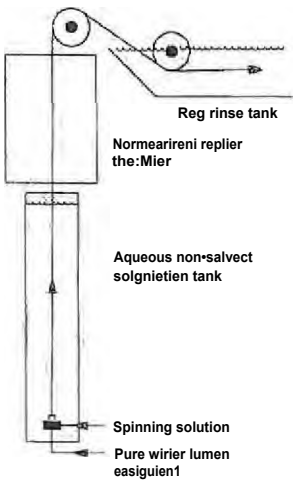


Fig 2.2: Schematic representation of the spinning line used (Jacobs and Leukes 1996).

The casting solution had the following components: -polysulphone (polymer), NMP (solvent), methyl cellusolve (MC, non-solvent additive), polyethylene glycol (PEG600,

low molecular weight polymer additive) and polyvinyl pyrrolidone (PVP 40 000 Da., high molecular weight additive). The compositions of some of the initial casting solutions are given in table 2.1.

Table 2.1: Casting solution formulations designed to produce membranes with finger-like voids (Jacobs and Leukes 1996).

Component	Membrane Code			
	PSF-1	PSF-2	PSF-3	PSF-4
	Concentration (% m/v)			
Ultrason S (PSI)	**26**	**24**	**24**	**24**
High-boiling point solvent (NMP)	**51**	**46**	**56**	**36**
Low-boiling point non-solvent (MC)	2	10	**10**	**10**
Low molecular mass polymer additive (PEG600)	**11**	**10**		**30**
High molecular mass polymer additive (PVP)	**10**	**10**	**10**	

2.2.3 Results and Discussion of the development of the new membrane

As a first approach the membranes were spun into an aqueous external contact bath with **an** NW solvent content of 80% (same solvent as that used in the spinning solution). Several membranes with different structures were produced, based on the various formulations used. These were shown in Jacobs and Leukes (1996). The most promising of these was PSf-4 (figure 2.3).

Although some of the features of membrane PSf-4 were what was required, it still needed further modification. The cavity walls of this membrane were skinned and not microporous. Therefore, the casting solution had to be modified further to promote greater porosity. The formulation was adjusted by decreasing the polysulphone concentration and increasing the PEG600 concentration. This formulation (PSf-5), which was subsequently used in all further experiments, is given in table 2.2.

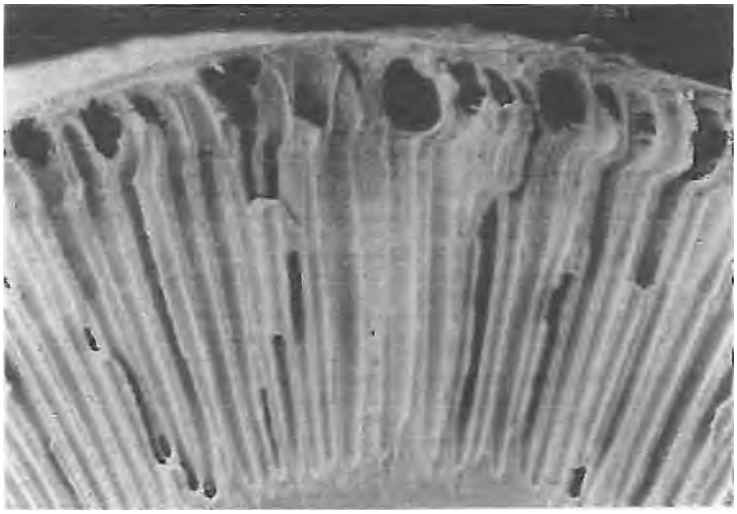


Fig. 2.3: Cross-section of PSf-4 membrane. The macrovoids are well developed to extend from outer to inner surface of the wall. An external skin can still be observed, however.

Table 2.2: Modified casting solution formulations to enhance porosity.

Component	Membrane Code	
	PSf-5	**PES-1**
	Concentration (% in/v)	
Ultrason S (PM)	22	
Ultrason E (PES)	22	
High-boiling point solvent (NMP)	36	36
Low-boiling point non-solvent additive (MC)	10	10
Low molecular mass polymer additive (PEG600)	32	32

It proved difficult to prevent the formation of a skin layer on the outside of the membrane, and in figure 2.4. an external skin layer is clearly visible on the micrograph, even though the external coagulant was high in solvent content and therefore had little precipitation potential. However, not all membranes coagulated in the 20% aqueous NMP-solution had well-defined external skin layers, as regularly spaced cavities were prominent in most of the membranes (figure 2.5).

Fig. 2.4: Electron micrograph of the cross—section of PSf-1 membrane. This clearly shows the presence of an external skin.

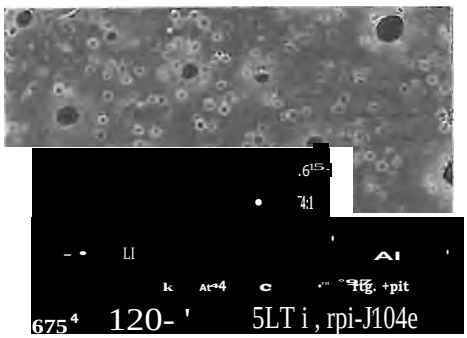


Fig. 2.5: External skin surface of a polysulphone membrane coagulated in a 20% aqueous solvent coagulant.

If the hypothesis regarding skin-formation was correct, the aqueous content of the external coagulant had to be reduced to below 20%, to prevent gelation, nucleation or phase-separation. The cloud point of the solution was empirically determined to be 9% and the coagulant and lower coagulant bath non-solvent concentrations were evaluated. The optimum was found to be 7.9%. The membrane produced in this way is shown in figure 2.6.

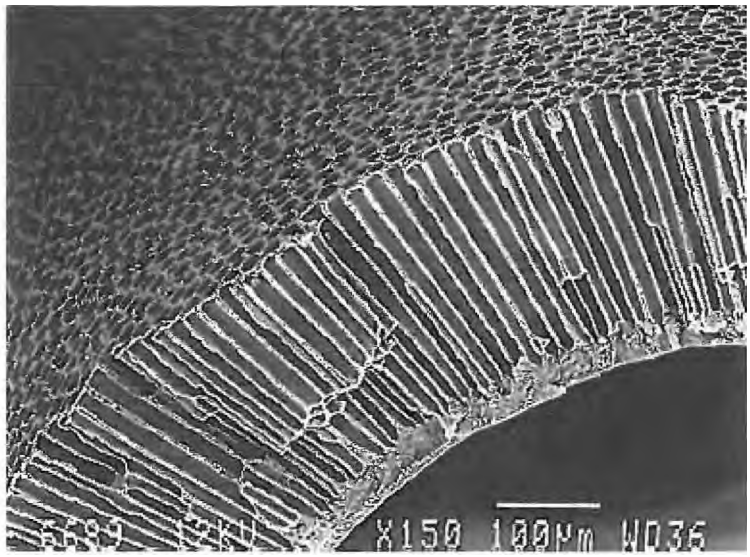


Fig. 2.6: Cross-section of PSf-5/3 showing the thin inner skin layer, porous macrovoid substructure and skinless exterior.

This membrane had a well-defined internal skin layer and the open-ended narrow-bore macrovoids which radiated from the internal skin layer were regularly spaced as shown in figure 2.7.

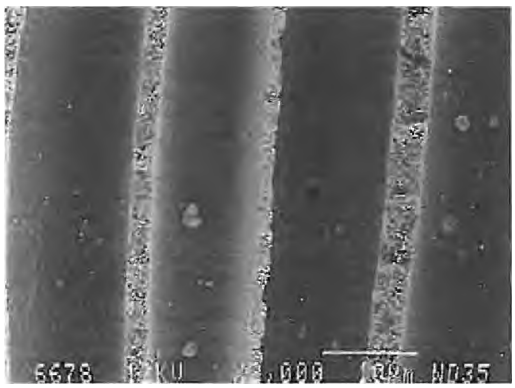


Fig. 2.7: Regular spacing of the finger-like macrovoids in membrane PSf-5/3.

The average diameter of the macrovoids was 20pm (figure 2.7). From the micrographs the cross-sectional diameter of the membrane was calculated to be 1.8 mm. and the diameter of a macrovoid opening to be 25pm (i.e. including one wall thickness). On the basis of these figures, it was estimated that there were more than $9x10^6$ macrovoids per metre-length of membrane (Jacobs and Leukes 1996). Figure 2.8 shows the skinless

external surface of the membrane wall. This membrane was coded IPS 763 and used in the evaluation of the MGR concept.

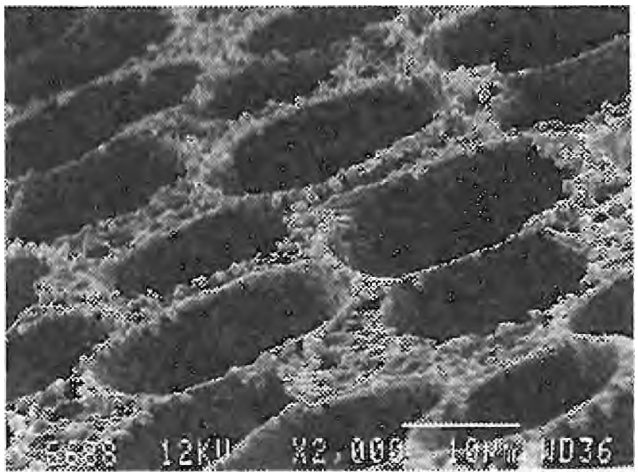


Fig. 2.8: The exterior surface of the PSf 5/3 membrane.

Mechanical Integrity of the Membrane

It was reported that the presence of macrovoids in the substructure of membranes decreases the mechanical integrity of the membrane (McKelvey and Koros 1996; Mulder 1996). It was also found to be necessary to reduce the thickness of the internal skin layer to stimulate macrovoid formation and maximise the void length. This resulted in a thinner skin-support layer, reducing the membrane resistance and hence the hydrostatic driving force requirements of the membrane. The membrane was still found to be quite robust with an instantaneous burst-pressure ranging from 2,3 MPa. for membrane PSf-1 to 1.8 Mpa. for membrane PSf-5. A membrane with high mechanical strength is important to prevent fibre disruption due to uncontrolled, excessive cell growth and to prevent membrane leakage during operation (Tharakan and Chau 1986; Linton *et al.* 1989). Given that the normal turgor pressure of cells is 200 —300 kPa. (Walsh and Malone 1995), the membrane should be strong enough to withstand pressure exerted by a biofilm. Reported burst pressure values for commercial membranes (Amicon) range from 20-200 kPa. These are clearly inadequate, being in the range of pressures that expanding cells can exert on their surroundings.

2.3. THE SINGLE FIBRE BIOREACTOR

With the successful production of a capillary membrane with the required characteristics, it was necessary to develop reactor designs appropriate for its evaluation. In order to characterise the immobilised fungal biofilm, destructive sampling for electron microscopy and biochemical tests was necessary. Due to the cost and closed nature of membrane reactors, this required the development of an inexpensive laboratory-scale membrane bioreactor with a reusable housing which could easily be disassembled for sampling. Single fibre reactors are very well suited for this type of work because of their convenience, simplicity and small amounts of chemicals needed for testing (Reiken and Briedis 1990) What was called the disposable membrane mini-reactor, shown in figure 2.9. was developed with the following dimensions and characteristics:

Housing length	14 cm.
Diameter	0.7 cm.
Effective membrane length	12 cm.
Membrane Diameter	0.2 cm.

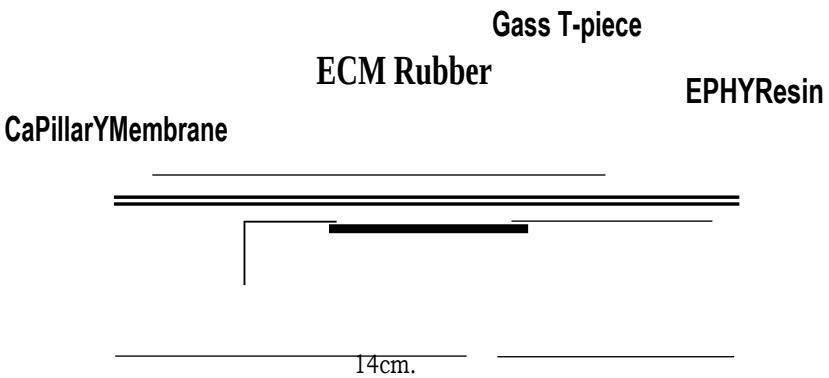


Fig. 2.9: Schematic diagram of a disposable mini-membrane reactor.

It was manufactured with glass T-pieces joined by silicone tubing. The potting material used was commercially available epoxy resin. Use of a single membrane fibre made characterisation, operation and assembly of the system less complicated.

In order to perform experiments that involved study of biofilm development over time, a system was required which would provide the same conditions to a set of several disposable mini-reactors, so that they could be sacrificed and analysed when required. This proved difficult in practical terms since uneven nutrient supply to reactors in the array was encountered. This is typical of in-parallel multiple channel units and is experienced in scale-up of cylindrical units to incorporate large numbers of fibres (Prenosil and Hediger 1988). Such a system was designed and built at relatively low cost (—R1 500) which was effective and durable. It is depicted schematically in figure 2.10. and has the following features:

- Pressure compensated flow regulators on the medium supply inlet of each bioreactor to ensure that each reactor was exposed to the same flow and pressure as the rest[1] These could be replaced with needle valves if different flow rates are required for each individual reactor.
- Heat-sterilisable quick connect/disconnect fittings (Bosch) allow for easy aseptic transfer of growth medium supply.
- Each reactor is connected to a non-return valve so that any reactor could be removed from the manifold without affecting the other reactors.

[1] A chance encounter with an irrigation specialist resulted in an efficient, inexpensive solution in the form of "drippers", which are used in long irrigation sprinkler-type irrigation pipes where pressure drop is a major problem. These drippers behave as diaphragm-based pressure-compensating flow regulators.

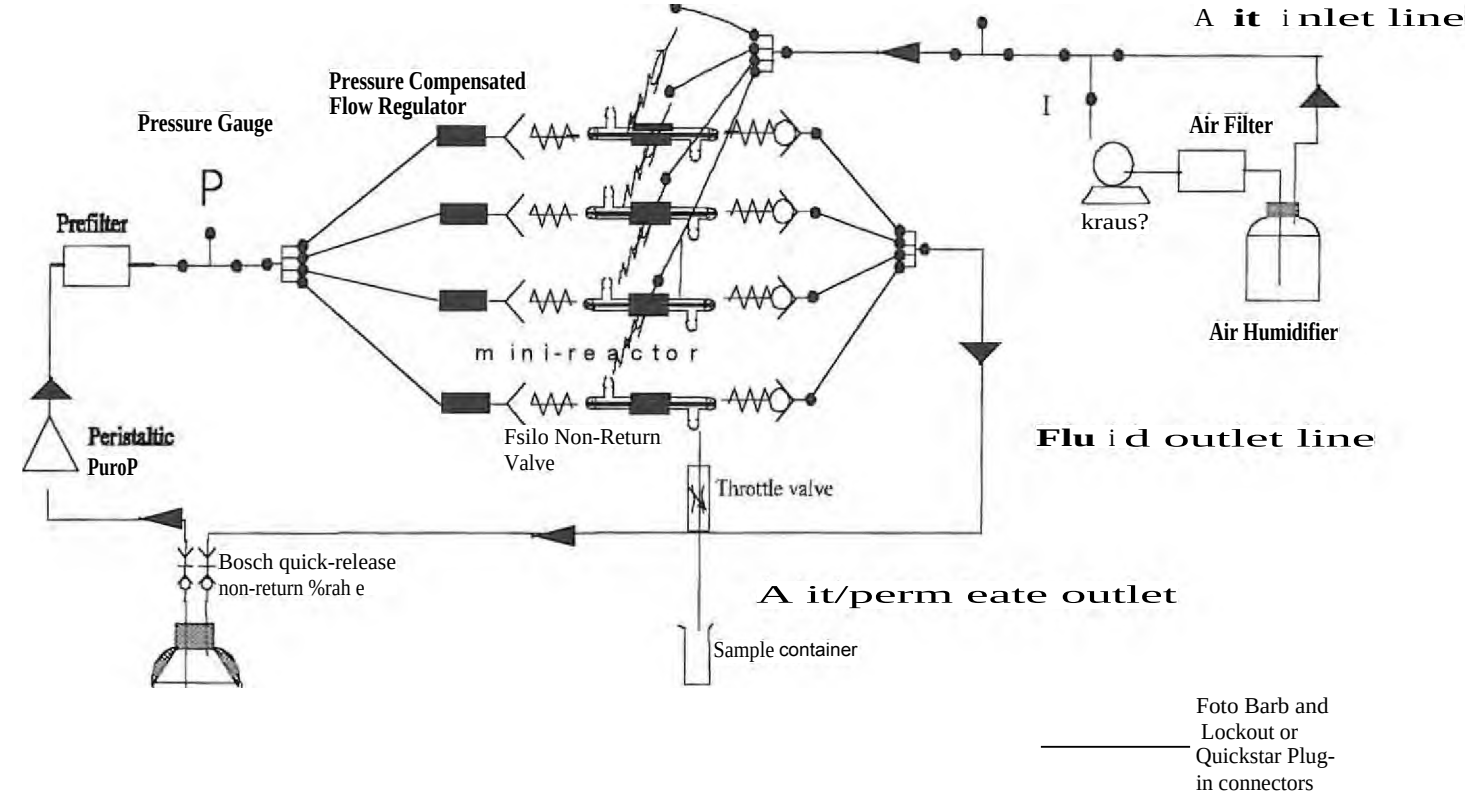


Fig. 2.10: Multiple mini-reactor rig. P-pressure gauge. 1-inoculation line

2.3.1. Operation of the Mini-Reactor System

Sterilisation

The bioreactor design and operational mode must preclude the accidental entry of contaminating microrganisms at entries, exits and sampling ports for liquids and gases. Sterility design is also crucial for regulatory reasons. This is relevant in the cultivation of *P. chrysosporium* in South Africa and the Plant Protection Research Institute stipulates complete containment, especially in the case of the more potent strain, BKM-F 1767. This caution is in consideration of the use of wooden railway sleepers in South Africa and *P. chrysosporium* being a potent lignin degrader. It is for this reason that initial studies and evaluation of the bioreactors was performed using the weaker strain ME - 446.

Thus, a suitable sterilisation procedure had to be developed. Formaldehyde was used as a sterilising agent due to its volatility so that it could sterilise the gas phase equally well. Since the reactor system lends itself to air bubble formation, this was necessary.

After incorporation of the mini-reactors into the system, the fluid outlet line was connected to the air inlet line behind the pressure gauge, using the Quickstar connections. The air filter to the pressure gauge section of the air supply circuit was autoclaved. To sterilise the rest of the reactor, the culture medium was replaced with a 4% formaldehyde solution in tap water. The air/inoculation/permeate outlets of each mini-reactor were connected to the reservoir return. The formaldehyde solution was then re-circulated through the reactor system for at least 24 hours. The formaldehyde was then rinsed out by replacing the formaldehyde reservoir with a reservoir of 5L of autoclaved distilled or tap water. The reactor outlets were disconnected and the fluid present allowed to drain to rinse out the formaldehyde solution. The reactor was then connected in inoculation mode by aseptic re-connection techniques. Plastic ends were drenched in 70% alcohol and steel connections were heated using a Bunsen burner, before detachment or attachment.

Inoculation

After sterilisation, an inoculum vessel was aseptically connected to the air supply line behind the pressure gauge. At the end of the rinsing procedure, water was forced to permeate the membrane walls under pressure and allowed to fill the ECS. This was to ensure minimal air bubble entrainment within the fibre macrovoids or at the membrane surface. It was found by Caldwell and Lawrence (1988) that it is necessary to pre-soak a system with the desired growth medium just before inoculation to define the chemistry of the surface. Thus, the ECS and lumenal space were soaked with growth medium for aproximately 30 minutes before inoculation.

The inoculum was then pumped into the ECS of the reactors via a peristaltic pump. Initially, the reactor outlets were left open, so that the reactors could be primed to relieve them of air bubbles. The outlets were then closed to allow the ECS to be pressurised to 150kPa. In this way, 10 mL. of inoculum. (mL. of reactor volume)" was presented to the external surface of the membrane by dead-end reverse filtration.

Spore Inoculum

Spore inocula of *P. chrysosporium* were prepared by inoculating a malt extract agar plate with the stock culture. This was allowed to grow to confluence. At this stage an —1 $cm.^2$ disc of agar plus growing fungus was transferred to a Roux bottle containing the sporulation medium of Tien and Kirk (1988) (Appendix A). After 10 days, the spores were suspended in 400mL. of water. This procedure yielded a spore suspension that was consistent from one experiment to the next.

Vegetative Inoculum

When vegetative inoculum was required, 2.8 L. Fernbach flasks containing 100 mL. of the growth medium described by Tien and Kirk (1988) (Appendix **B),** except that no veratryl alcohol was used, were inoculated with a spore suspension as described above After 3 days of growth the mycelia formed were homogenised using a Waring blender at high speed for thirty seconds. The homogenate was introduced to the ECS of the bioreactors via an inoculation port and recirculated by a peristaltic pump to allow

attachment of the vegetative mycelia to the membrane. The ECS was then pressurised by air to 150 kPa overnight to allow a biofilm to establish on the membrane surface

Table 2.3: Operational parameters for the multi — mini-fibre rig.

Reactor length	14 cm.
Shell side diameter	6 — 8 mm.
Active fibre length	12 cm.
No. of reactors	8
Fibre polymer material	Polysulphone
Fibre type	IPS 763
Tubing material-peristaltic pump	Silicone rubber
Tubing (rest of system)	Silicone rubber
Tubing diameter	4 rnm.
Fibre internal diameter	1 mm.
Fibre external diameter	2 mm.
Volumetric air flow rate	— 1 vol. vol.$^{-I}$ min.$^{-1}$
Air pressure	Atmospheric
Air flow regime	Longitudinal to fibre
Nutrient flow rate	I00 mL.11^{-1}
Nutrient flux	0.1 — 1 L. in^{-2}.h^{-1}
Nutrient back pressure	n. d.
Nutrient flow regime	Recirculation

Flow Conditions

Growth medium was recirculated through the capillary membrane lumen using a peristaltic pump. The pressure-compensated flow regulators were rated at 2 L. h."^{1}, but a flow rate of 100mL, h.$^{-I}$ was used in most cases since this gave approximately the desired flux for most experiments. The flow regulators served the purpose of ensuring that the flow was evenly split between the reactors attached. The mini-reactor rig was operated in recirculation mode only since no pump could be found which could supply sufficiently low rates to provide dead-end flow in the correct flux range. The air flow rate was chosen to be similar to that of a gassed CSTR vessel, so that easy comparisons could be made in terms of oxygen mass transfer. Thus, a volumetric air flow rate of 1 vol. (vol. ECS).$^{-1}$

min.$^{-1}$ (Shuler and Kargi 1992) was chosen. A summary of the operating conditions is given in table 2.3.

Adequate temperature control was achieved by housing the entire unit in a constant environment (C.E.) room set to 37 $^{\circ}$C. The lack of an outer skin and improved surface area of the new membrane allowed the formation of a dense biofilm several micrometers thick. It was found that sufficient spores could be filtered onto the membrane to make the inoculation time a total of 30 minutes long. This was a noticeable improvement over the 4—day procedure necessary for application of the conventional membrane.

2.4. CONCLUSION

The skinless membrane developed for the purpose of proving the utility of the membrane gradostat bioreactor concept can be considered as an important breakthrough in the field of membrane bioreactors in general and a crucial step in the development of the MGR concept. This membrane was the result of a very close collaboration with the Institute for Polymer Science (University of Stellenbosch), which shows the importance of collaboration in multi-disciplinary research.

The design and fabrication of the mini-reactor rig, and the formulation of an operational protocol met the requirements for proof of the MGR concept (described in the next chapter). Thus, the requirements for the development of suitable hardware were met.

CHAPTER 3

THE CONCEPT OF SPATIO-TEMPORAL DOMAIN TRANSFORMATION

3.1. INTRODUCTION

The principle hypothesis of this study proposes that true continuous production of secondary metabolites by the white rot fungus *P. chrysosporium* would require the simultaneous presence of different phases of growth within the same thallus. The MGR was designed to demonstrate that the temporally distinct phases of batch culture — primary, secondary and decline phases — may be observed spatially across the immobilised biofilm of this organism. The arrangement whereby temporal growth events in the fungi may be observed in the spatial domain is fundamental to the successful operation of the gradostat reactor. This chapter reports studies undertaken to evaluate this concept. Several means of demonstrating the growth phase and activity of a biofilm have been reported in the literature.

Direct Study of the Nutrient Gradients

The growth phases of batch cultures may be associated with the nutrient status of a culture (Shuler and Kargi 1992), Thus, if the concentration of the limiting nutrient could be determined as a function of radial distance across the biofilm, a precise indication of the nutrient status, and thus the physiological state of the various zones of the biofilm, could be determined. In practical terms this can be accomplished in two ways:

1) The use of published mathematical models to describe the nutrient gradient as a function of the growth and substrate utilisation kinetics of the organism and the operating conditions of the system (Siebel 1992). This is a very attractive option, since the only measurements required are growth medium substrate concentration, biofilm density, reactor outlet substrate concentration and dilution rate. Several models exist describing nutrient gradients in biofilms generally (Characklis 1990a), and in membrane bioreactor biofilms, specifically under diffusive transport as well as convective transport conditions (Salmon ***et al.*** 1988; Kelsey ***et al.*** 1990).

Unfortunately, the paucity of refined models for the growth of *P. chrysosporiurn* in solid and liquid culture conditions made this a venturesome approach.

2) Direct measurements of nutrients using micro-electrodes (Bungay 1969; Brune *et al.* 1995; Brune and Kuhl 1996). This would give the most precise empirical description of the nutrient gradients, but the unavailability of such equipment precluded its application.

Sectioning of Biofilms and Biochemical Study of the Sections

Techniques have been demonstrated for saggital sectioning of biofilms (Burrill *et aL* 1983, Fry 1990; Murga *et al.* 1995). These sections could then be assayed for viability or the presence of certain markers by published biochemical means (Fletcher 1990; Herbert 1990; Ladd and Costerton 1990). This would have been ideal for a rectangular biofilm, but when attempted in practise, the radial nature of the biofilm derived from the MGR system made its application difficult, since sharp, clearly defined zones were not obtainable. So-called "optical sectioning" of biofilms using scanning confocal laser microscopy or on-line epifluorescence microscopy (Suhr *et aL* 1995) seemed a useful alternative. Once again, equipment of this type was not available for use.

Morphological Studies

It has been shown that the morphology of fungal mycelia can be related to their physiological state (Rittmann *et al.* 1992). The rationale for testing biofilm differentiation as a function of nutrient concentration is based on the knowledge that although the differentiation and physiological states of fungi leading to secondary metabolite synthesis are still not well understood, growth (Rhigelato *et al.* 1968), metabolic activity, pathogenicity, pigmentation (Obert *et al.* 1990) and product formation (Megee *et al.* 1970) have been related to structural differentiation of the hyphae. Paul *et al.* (1993) have shown that secondary metabolite formation in the form of penicillin synthesis is associated with increased vacuolation in *Penicillium chtysogenurn,* and that this can easily be determined microscopically and quantified by automated image analysis techniques. The morphology of a filamentous fungus can be described in terms of microscopic morphology, which determines the shape and size of individual hyphal elements; and macroscopic morphology, which determines the shape and size of fungal

pellets (Nielsen and Krabben 1995) or biofilms. These morphologies relate to each other and to the process environment (Viniegra-Gonzalez *et al.* 1993).

The morphological approach was chosen for this study following the failure to acquire useful information from the bioreactor using the sectioning approach.

3.1.1. Research Objectives

To prove that changes in nutrient environment can be correlated to morphological changes in *P. chrysosporium* and that such morphological changes can be observed across a biofilm of *P. chrysosporium* as a function of distance from the fibre lumen, the following studies were undertaken:

- Development of the biofilm in the MGR;
- Characterisation of spatial differentiation in the mature biofilm;
- Correlation of the differentiated states mentioned above to temporal growth events in a typical batch culture of immobilised *P. chrysosporium.*

3.2. MATERIALS AND METHODS

3.2.1 Study of Biofilm Development and Demonstration of Morphological Differentiation across a Mature Biofilrn of P. chrysosporium ME446 *in both Spatial and Temporal Contexts,*

A set of 8 disposable membrane mini-reactors was operated for 10 days in continuous MGR mode. The conditions and apparatus used are described in chapter 2. Spore inoculum was used since this overcomes the need to prepare vegetative inoculum that would need to attach passively. Spore inoculation would therefore improve productivity of the system and would be used if industrialisation of this system were realised. Spore inoculation was made possible using the custom-designed IPS 763 membrane described in chapter 2, Thus, the IPS 763 membrane was used in the mini-reactors and spore inoculation of the multi-mini-reactor rig was performed according to the method described in chapter 2 (section 2.5.3).

Individual reactors were sacrificed at appropriate intervals for the study of biofilm development and flow rates were re-adjusted to maintain initial conditions in each reactor. Sections were made (2-4 mm. lengths of membrane) and prepared for Scanning Electron Microscopy (SEM) according to Appendix C. The permeate from each sacrificed reactor was analysed for LiP according to Appendix G.

3.2.2. Correlation of Morphological Characteristics with Growth Phases

For batch cultures, 8 disposable mini-reactors were assembled in a multiple mini-reactor rig as described in chapter 2. The reactors were operated in batch mode to determine the morphological changes observable in the biomass in response to substrate supply.

Conventional (with an outer skin) ultrafiltration capillary membranes were used so that growth only occurred around the outside skin of the capillaries in order to simplify the characterisation of the biofilm. Vegetative inoculum was used to achieve greater synchrony of growth phases since it is known that fungal spores do not germinate simultaneously (Bosch *et al.* 1995; Nielsen and Krabben 1995).

After inoculation the reactors were supplied with a nutrient-sufficient medium as used for pre-inoculation preparation. This was fed at a rate sufficient to prevent the establishment of nutrient gradient formation and thus enable exponential growth. After 5 days the nutrient supply was stopped to starve the culture and force it into stationary growth phase; and then decline or sporulation phase. Observations were made to determine morphological changes in response to nutrient limitation.

Intracellular marker assays were performed on other sections of the same membrane to correlate the morphological changes to growth phases (described below).

<u>Determination of the Onset of Secondary Metabolism</u>

This proved to be a challenge since the most obvious way of doing this would have been by direct measurement of LiP or MnP, but this approach was found to be problematic for the following reasons:

- The available enzyme assays were found to be insensitive based on personal experience and literature;
- No permeate was expected when the culture was starved, since starvation was achieved by stopping nutrient flow, thus it would have been difficult to establish extra-cellular activity.

Thus, some other marker of secondary metabolism was needed. A candidate for consideration was veratryl alcohol, a metabolite known to be produced *in situ* by *P. cluysosporium* under stationary growth phase conditions (Shimada *et al.* 1981). Due to problem number 2 (above), however, veratryl alcohol would have had to be separated from a cell lysate, requiring extensive sample preparation to enable reasonable analysis.

A specific enzyme marker was, therefore, thought preferable. Succinate Dehydrogenase, a marker for mitochondrial activity and Cytochrome C Oxidoreductase, a marker for endoplasmic reticulum activity (Bonnarme *et al.* 1991) were chosen on the basis that the specific activity of these enzymes has been shown to increase sharply at the onset of stationary phase in *P. chrysosporium* (Capdevila *et al.* 1990; Bonnarme *et al.* 1991; Bonnarme *et aL* 1993).

Intracellular Enzyme Marker Analysis

Intracellular marker enzymes, Succinate Dehydrogenase (a marker for mitochondria) activity) and Cytochrome C Oxidoreductase (a marker for endoplasmic reticulum activity) were determined as described in Appendix E. In order to calculate specific activity, intracellular protein was determined according to the method of Bradford (1976).

3.2.3. Strain and Medium

P. clnysasporiurn strain ME446 was used in this experiment and was maintained and cultured as described in Appendix A and B. This strain was used for the sake of safety due to the lack of experience at that stage with the operation of the newly-developed equipment. The growth of this strain is less aggressive than the typical production strain (BKM- F- 1767) used for LiP production.

3.2.4. Reproducibility and Validity of Results

The experimental protocol was tested and optimised through a series of 8 runs, by which time clear trends and suitable consistency resulting from sufficient experience with the new system were achieved. Lignin peroxidase (LiP) determinations, however, cannot be considered reproducible, since the module was not designed for product analysis. The permeate volume collected after 24 hours was too low to allow replicate analysis.

The results shown were obtained from a typical experimental result. Values shown are calculated means of duplicate measurements, except for dry mass determinations, which are single readings taken after constant mass was achieved through drying. Electron micrographs are typical examples of observations of 2- 5 specimens.

3.3. RESULTS AND DISCUSSION

The MGR concept was shown to be a practical reality. Continuous, non-intermittent LiP production at high levels was achieved after 3 days of operation (figure 3.1). Thus, it appeared to take 3 days for the biofilm to reach a sufficient thickness so that nutrient gradients could be established. These results, although typical, must be considered preliminary, since insufficient permeate could be collected from each mini-reactor per day for replicate determinations of enzyme activity. It does, however, indicate qualitatively that the MGR provides continuous enzyme production.

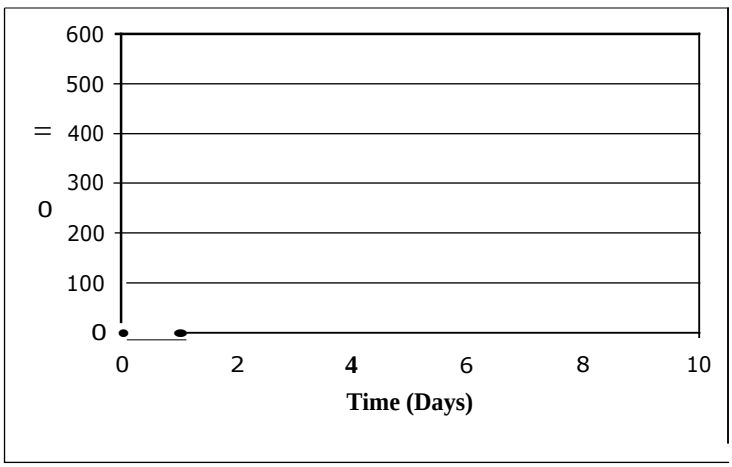


Fig. 3.1: Lignin peroxidase production in single fibre bioreactors over a 9 day period.

3.3.1. Biotilm Development

Daily samples taken of the biofilm (figures 3.2a and b) before it reached steady-state allowed the following descriptive model for biofilm development to be formulated. Biofi ims in general are accepted to develop in three stages, attachment, accumulation and dynamic equilibrium (Wanner *et al.* 1995; Ascon-Cabrera *et al.* 1995). Development of the *P. cinysosporiun2* biofilm in the MGR is, therefore, discussed in this context.

Attachment

Spores could be seen to attach to the surface of the membrane. This is probably due to hydrophobic interactions between the spore surface and the membrane polymer matrix.

According to literature, colonisation of a surface consists of a number of processes, some occurring in parallel and some in series. When a process consists purely of a number of steps in parallel, the slowest steps represent the rate-limiting steps, which control the rate of the process. It is generally believed that the rate-limiting step in biofilm formation is colonisation of a surface (Ramsden *et al* 1994). This was thought not to be the case in the use of spores with the newly developed IPS 763 membrane since the spores were driven into the macrovoids by convection (figure 3.2.a.), which offered a better transport efficiency than adhesion of spores to the surface of the membrane. From figure 3.2.b it can be seen that large amounts of spores were also to be found at the surface of the capillaries. This could be due to too high a concentration of spores in the inoculum.

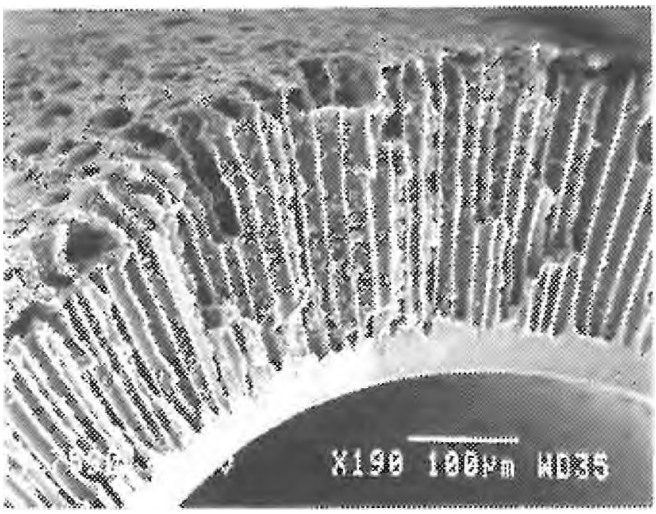


Fig. 3.2a: Colonisation of the membrane by spores upon inoculation (Mag. =190X).

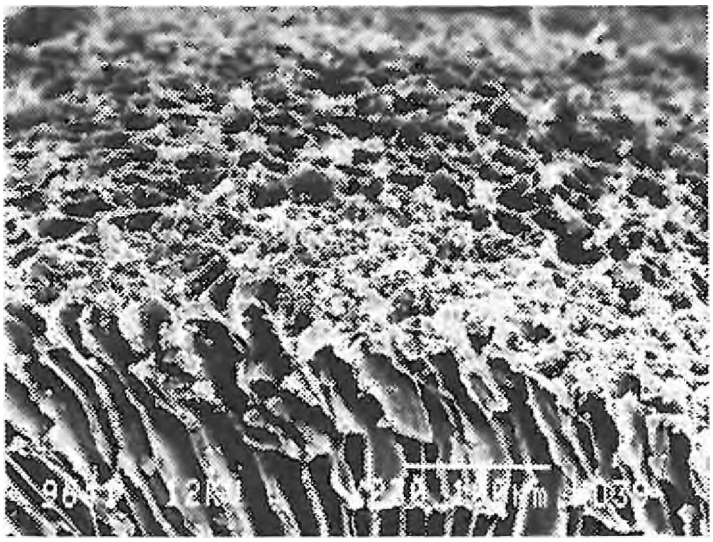


Fig. 3.2b: Biofilm of *P. chrysosporium* immediately after inoculation, showing the spores attached to the surface of the membrane (Mag. = 270X).

Since spore attachment was found to be a rapid process, spore germination was expected to be the rate limiting step. This would require careful attention in the development of an optimised process (Escher and Characklis 1990).

Germination and Biomass Accumulation

It was observed that, predominantly, spores at the surface of the capillary wall germinated (figure 3.3). Literature states that spore germination follows 2 distinct phases: The spore first undergoes enlargement of its size, during which it increases in diameter and biomass (spherical growth). In this phase new wall layers are formed and laid down

over the whole inner surface. After a given time, polarity is established and a germ tube grows out from the swollen spore (Bosch *et al.* 1995).

The spores on the membrane can be seen to have germinated and hyphae appeared to project downwards towards the lumen, possibly due to chemotropism.

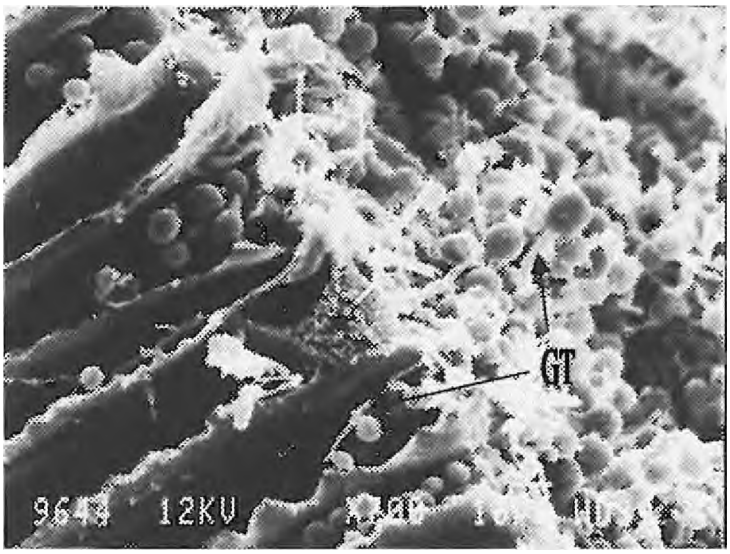


Fig. 3.3: Biofilm of *P. chrysosporium* after 1 day of operation, showing germ tube (GT) formation (Mag. = 700X).

Dynamic Equilibrium

As new biomass was laid down on the matrix closest to the nutrient source, the germinating (as well as ungerminated) spores were pushed upwards until a biofilm of sufficient thickness and density was established such that nutrient gradients were established across it. This required 3 days to occur. Under the operating conditions described in chapter 2, LiP production occurred after approximately 3 days (see figure 3.1). The steady-state biofilm is defined as a special case in which the gains in biofilm mass (due to new cell growth) are just balanced by the losses in biofilm (mostly from decay and shearing), i.e. none of its components or phases change with time (Characklis 1990a; Ascon-Cabrera *et al.* 1995). In practice, this condition is never expected to be fulfilled exactly, but is considered acceptable as long as deviations are small and occur locally and infrequently (Characklis 1990b, Characklis *et al.* 1990)

On day 6 the profile of the continuous biofilm around the outside of the capillary depicted in figure 3.4. was obtained. It was found that, as predicted, various distinct morphological zones could be distinguished.

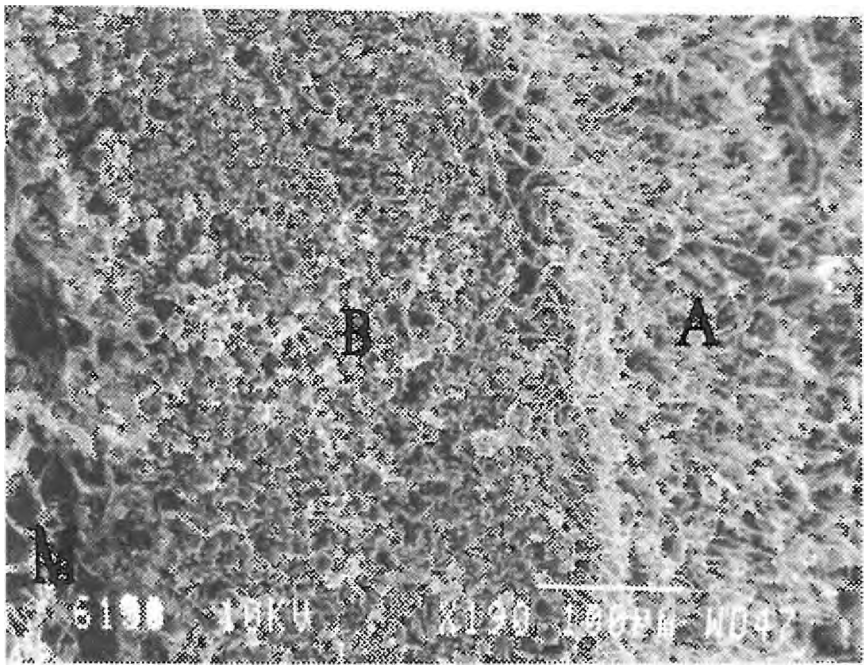
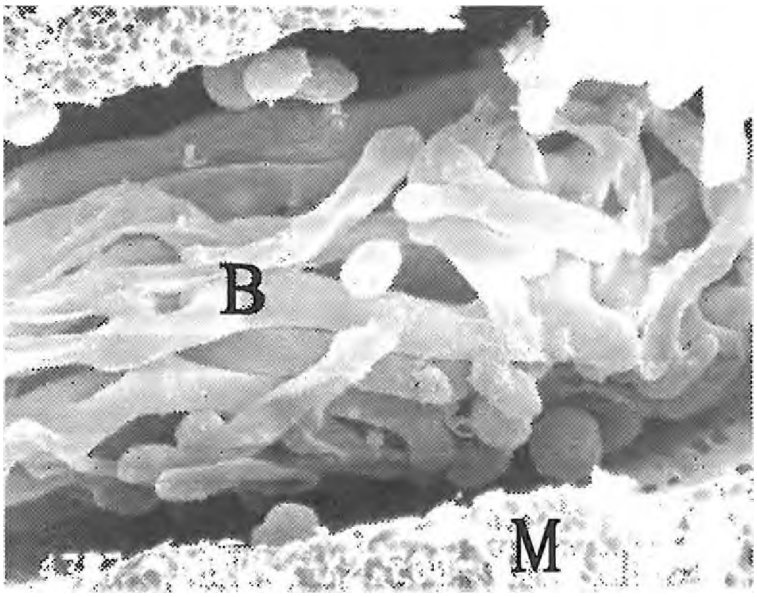


Fig. 3.4: Profile of the *P. chrysosporium* biofilm around the outer surface of the capillary in steady-state A-aerial mycelia, B-biofilm, M-membrane outer surface (Mag. = 190X).



Fig, 3.5: Cross section of the biofilm in the macrovoids of the membrane. B-biofilm, M-membrane (Mag. = 2 700X).

3.3.2. Morphological Differentiation in the Mature Biofilin

The observed biofilm was found to display noticeably distinct zones of differentiation. These are described below.

Zone 1: Biomass in the Macrovoids

The biomass penetrating into the macrovoid is shown in figure 3.5. This is a different type of growth from that observed in the biofilm surrounding the capillary. It appears similar to the leading hyphae described by Steele and Trinci (1975). These hyphae are found at the margins of differentiated mycelia on plates and represent growth of hyphae in a high nutrient environment. This is as expected from the gradostat concept. These hyphae are similar to the undifferentiated hyphae found in exponentially growing mycelia on solid culture and submerged cultures. Steele and Trinci (1975) do, however, point out distinguishable differences between leading and undifferentiated hyphae. The most important of these is that the maximum extension rates of the leading hyphae of differentiated mycelia were about 2-fold to 7-fold (depending on the strain) faster than the mycelia of undifferentiated hyphae. This is distinguishable morphologically as differences in the hyphal extension zones between the two hyphal types. Unfortunately, in this study the density of the fungal biomass was too high to objectively determine differences with statistical validity. If this could be achieved, some important information could be obtained about the specific growth rate of the hyphae, since the two parameters were shown to be related (Trinci 1974; Steele and Trinci 1975).

Zone 2: Biofilm Around the Capillary Wall

This biofilm had a thickness of approximately 300 pm. Significant mycelia] differentiation could also be observed in this film (figure 3.4.). This is more easily seen in the composite thin section of high magnification (figure 3.6.).The zones distinguishable at this level are numbered A-D.

Zone A:

This is the zone directly above, and attached to, the capillary wall. A large number of large round structures are observable, These are likely to be swollen, ungerminated spores (Gerin *et aL* 1995), since only a certain fraction of spores were expected to eventually germinate (Nielsen and Krabben 1995). Both the fraction of viable spores and the time interval for spore germination (time for initiation of spore germination and time

for termination of spore germination) have been shown to depend on the spore quality and medium composition (Nielsen and Krabben 1995 and references therein; Bosch *et al* 1995), i.e., the percentage viability is higher on a complex medium than a defined medium, and germination time is faster with fresh spores than old spores. Thus, another process parameter can be identified, viz. spore preparation and germination for optimisation of the process.

Alternatively, these could be the swollen, yeast-like cells, described by Pirt and Callow (1959) cited in Puszhtahelyi *et al.* (1997), found in *Penicillium chrysogeman* mycelia. They found that at the optimum pH for penicillin production, short, highly branched hyphae containing a large number of swollen cells could be observed before pellet formation in continuous chemostat cultures. Pusztahelyi *et al.* (1997), observed the dominance of this growth form in advanced stages of growth. They report the observation by other authors of similar growth forms under various growth conditions, e.g. at alkaline pH, in unusually acidic media, during oxygen limitation, and after exposure to high concentrations of CO_2. Pusztahelyi *et al.* (1997) refer to this growth form as a specialised survival form of *Penicillium chlysogenum.*

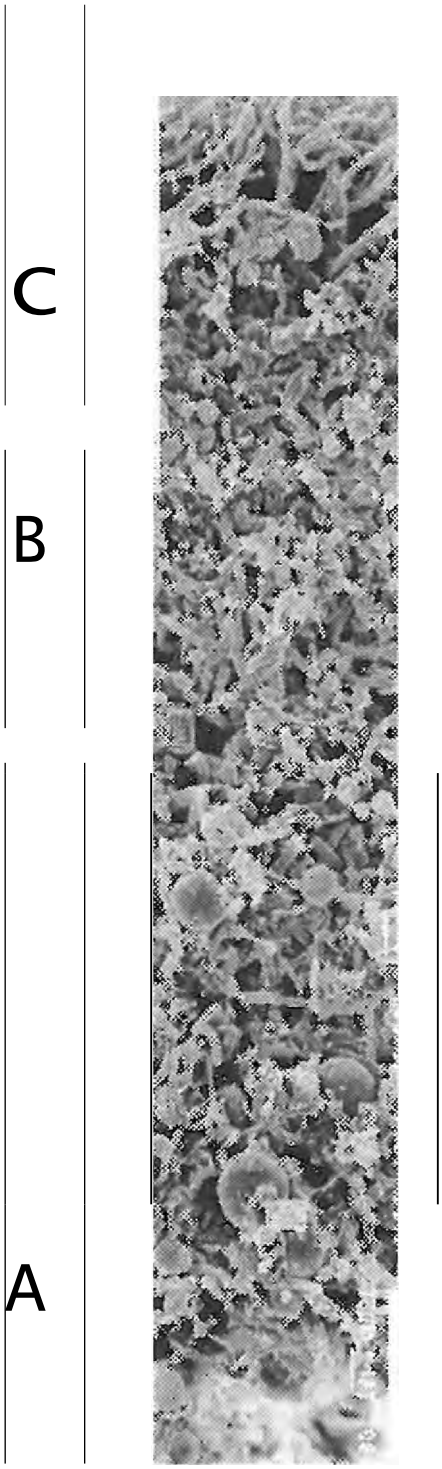


Fig. 3.6: Composite picture of the biofilm profile. The arrow indicates direction of growth medium flow. The letters A-D show the perceived differentiated zones. Zone A would be closest to the nutrient supply (Mag. = 900X).

Zone B

This is the zone of mostly highly branched and fragmented mycelia. These are likely to be the highly branched mycelia typical of stationary phase as described in the batch culture experiment (described later). As mentioned above, the high level of fragmentation is probably due to increased vacuolation. This agrees well with the demonstration by Paul *et al.* (1994), that fragmentation of hyphae was not due primarily to mechanical shear, as was previously thought, but rather due to physiological state as affected by vacuolation, since this region in the gradostat biofilm is not expected to experience much shear. Puszhtahelyi *et at* (1997), propose an intricate link between branching, vacuolation, fragmentation and I3-lactam antibiotic production in *Penicillium chtysogenum* and *Acremonium chrysogenum.* Based on this, and other data in this thesis, a similar prediction can be made for ligninolytic enzyme production of *P. chrysosporium.*

Zone C

This zone is a thin layer of profusely conidiating biomass, with large necrotic spaces indicating an advanced stage of growth and autolysis. This is possibly due to the limiting nutrient(s) concentration reaching a critically low level to initiate sporulation.

Zone **D**

Above the layer of nutrient limited biomass, an area of biofilm of much lower density was observed. The lower density could have been due to detachment and sloughing off of dead biomass and spores since this is expected to be an area of relatively high turbulence and shear. It has been shown from previous experiments that dead *P. elyysosparium* biomass detaches from biofilms very easily. Alternatively, the lower density could have been due to differentiation of the thallus to maximise oxygen mass-transfer. The fact that the mycelia in this zone appeared to be viable, with narrowed hyphae, with distinct alignment parallel to the radial axis of the fibre (perpendicular to the flow of air) supports the differentiation hypothesis. These are typical examples of aerial hyphae commonly observed on solid surface cultures of filamentous organisms. Physiologically, they are a form of specialised hypha for oxygen uptake. Their formation is linked to secondary metabolism in various filamentous fungi and bacteria (Trinci 1974). Physically, they provide the biofilm with a macroscopic roughness, the impact of which is described below.

The above-mentioned zones agree well with the first approximation description of the membrane gradostat concept. Zone **D,** however, was not anticipated.

A dynamic equilibrium, therefore, seems to have been established between the generation of new biomass at the lumen side of the biofilm and the sloughing off of biomass on the shell side of the biofilm, with secondary metabolism occurring continuously in between.

3.3.3. Macroscopic Characteristics of the Biofilm

Biofilm Thickness

The thickness of a biofilm is an important control parameter for the performance of a fixed film reactor (Freitas dos Santos and Livingston 1995; Pavasant *et al.* 1996). A balance must be struck between maximum biomass concentration and penetration depth of oxygen. A biofilm of approximately 500 p.m was obtained using a flux of 1 $L.m^{-2}.h^{-1}$. (figure 3.4). A subjective comparison with other systems for the cultivation of *P. chrysosporium* shows that no other reported system reaches this thickness. This is encouraging since, if it can be shown that adequate oxygen supply is attained, then this could be the basis of a competitive system. Future research will require the determination of oxygen penetration into the biofilm, however, such equipment was not available for such studies.

The biofilm thickness correlates well with the maximum penetration depth of oxygen into fungal biofilms. Lejeune and Baron (1997, and references therein) show a penetration range of 70 to 350 gm., depending on the density, based on mathematical models and microprobe measurements of mycelia! pellets.

Biofilm Uniformity

Most mathematical models of biofilms assume or predict a homogeneous distribution of cells and extra-cellular polymeric substances (EPS). These are, however, based on simple populations of bacteria (Ascon-Cabrera *et al.* 1995), which are not expected to show complex differentiation behaviour, as is the case of the filamentous fungi.

The distinct differentiation, mentioned above, occurred on a microscopic level. On a macroscopic level, the biofilm appeared homogeneously distributed along the length of the single membrane fibre (see figure 3.7). However, it could be seen that the thickness of the biofilm tapered from the inlet to outlet side (left to right in the photograph). This was due to pressure drop along the length of the fibre, which led to diminishing wall permeate flux. This phenomenon is an important design criterion for consideration in the development of larger modules.

It has been shown in bacterial films (Aston-Cabrera *et al.* 1995) that special channels form in stagnant biofilms which aid in mass transport. This has not been found to be the case in this system. Initially it was thought that this was due to the nature of the system, which entails perfusion of growth medium through the biofilm rather than flow of nutrients over the surface. Subsequent work with another fungus, *Trametes versicolor,* seemed to have shown the presence of such channels in a membrane gradostat system, implying that the formation of channels could be species-, rather than process-specific (results not shown). This notion was confirmed by Murga *et al.* (1995). The absence of such channels simplifies process models, but the impact on productivity cannot be predicted.

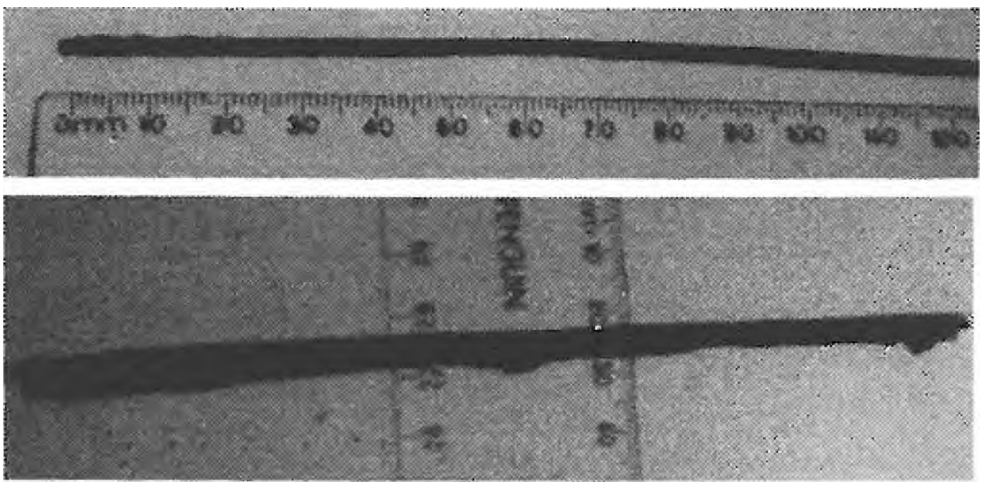


Figure 3.7: Biofilm distribution along the length of a single capillary fibre (Top). The photograph below shows the biofilm obtained with a 5X higher flux, providing further proof that the biofilm size is nutrient limited, since a thicker biofilm is observed with increased nutrient supply. Photographs were taken by magnifying the biofilm with an overhead transparency projector.

Surface Roughness

Biofilm accumulation and metabolic activity are controlled by momentum-, energy- and mass-transport processes. Biofilms, in turn, have significant influence on these transport processes. This is because biofilms increase hydrodynamic frictional resistance, and in so doing, increase advective heat and mass transfer. Biofilm roughness is related to its thickness (macro-roughness). It is therefore important to quantify hydrodynamic roughness before interfacial transfer phenomena can be described (Characklis *et al.* 1990, Ascon-Cabrera *et al.* 1995).

Biofilm accumulation may constrict conduits for airflow in the reactor, so that more energy is required to transport the same quantity of fluid through the reactor at the same rate. This resistance to momentum transport is often referred to as pressure drop (Characklis I990b). Alternatively, the biofilm roughness may cause eddy currents, which dissipate some of the energy available for flow. The eddy currents increase the rate of heat and mass transport from the liquid to the biofilm by advective heat- and mass-transport. The currents enhance the transport of mass to and from the biofilm surface. Thus, nutrients are transported to the biofilm surface faster. Mass transfer at a rough surface may be as much as three times as high as at a smooth surface. (Characklis *et aL* 1990). At the same time, the biofilm is subjected to a greater shear force, resulting in more biofilm detachment.

By visual inspection, it could be seen that the outer surface of the *P. chtysosporium* biofilm was noticeably roughened by the presence of aerial hyphae. The impact of this phenomenon can presently only be speculated about, but will be the subject of further study.

Presence of Zones

The presence of visually distinct zones makes the process of modeling biofilm physical characteristics convenient, since each zone, once its metabolic characteristics are known can be modeled as an individual plug-flow reactor (finite space elements approach), for which a considerable literature base exists. Alternatively, each zone can be treated as an

individual "species", each with unique characteristics. Multi-specific biofilm models with inter-specific interactions can then be applied (Ascon-Cabrera *et aL* 1995).

3.3.4. Physiological Characterisation of Batch Culture Progression

This experiment was performed to determine whether similar differentiation patterns to that observed in a cross section of the biofilm under the continuous culture conditions, described according to the MGR concept, could observed at different times during the batch culture described (section 3.2.2),

Characterisation of Growth Phases during Batch Culture.

The growth of *P. chtysosporium* on the membrane (figure 3.8.) seemed to follow a typical trend for this organism where initial exponential growth was followed by a very short stationary phase, followed by a decline phase. The extent of the decline phase is typical for *P. chtysosporium,* which can show up to 40% loss of dry weight due to autolysis upon nutrient starvation (Broda **P.,** 1996, pers. comm.).

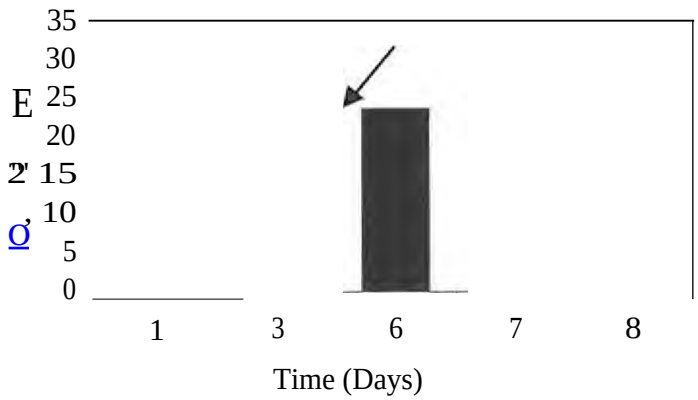


Fig 3.8: Change in biofilm dry mass over time. Dry mass shown is that of a 30mm length of membrane with immobilised fungus. The arrow indicates when nutrient supply was discontinued. Section 3.2.4 describes the reproducibility of the data.

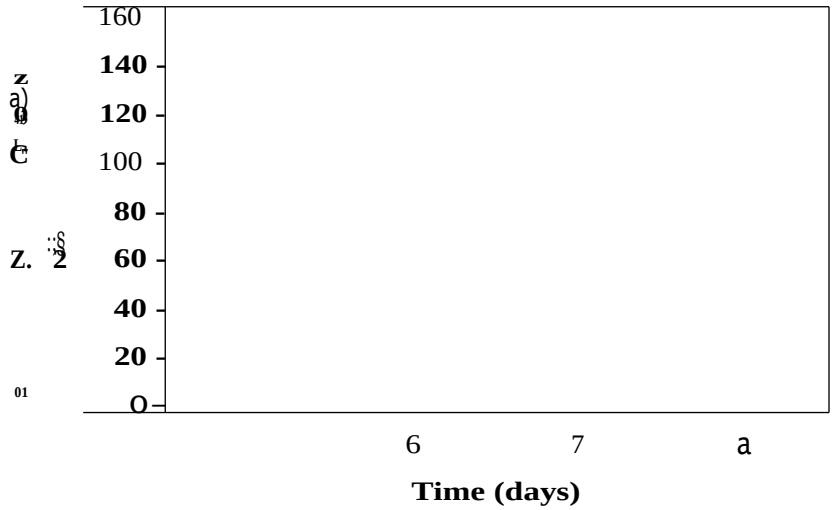


Fig. 3.9: Succinate Dehydrogenase activity, a marker for mitochondrial function. The arrow indicates when nutrient supply was stopped to starve the culture. The increase in activity on days 7 indicates the onset of stationary phase.

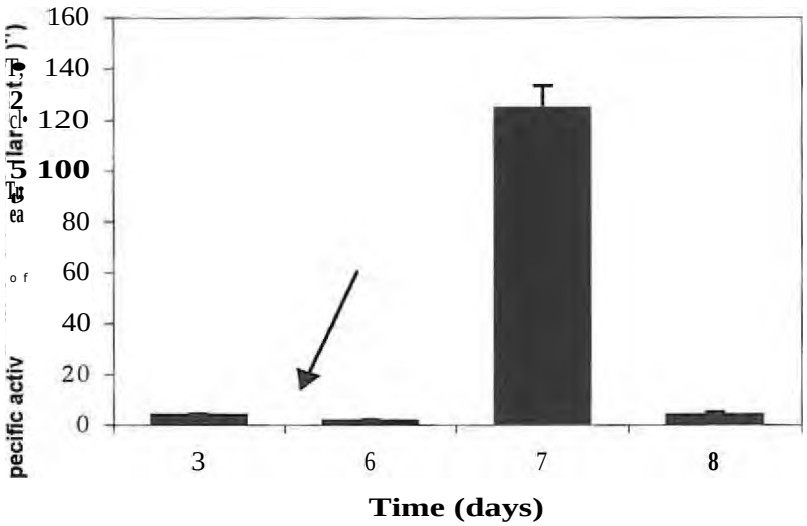


Fig. 3.10: Cytochrome C Oxidoreductase activity, a marker for Endoplasmic Reticulum activity. A similar increase in activity to that in figure 3.9. shows the onset of stationary phase on day 7.

From figure 3.9. it can be seen that after the growth medium supply was stopped, noticeable changes in mitochondria! activity occurred as indicated by Succinate Dehydrogenase activity. An increase in activity was observed 12 hours after starvation, peaking the next day (day 7) and indicating a switch to the secondary metabolic phase. On day 8 Succinate Dehydrogenase activity was significantly reduced, indicating that the senescent or decline phase was reached.

Endoplasmic reticulum activity as indicated by Cytochrome C Oxidoreductase activity peaked very suddenly on day 7 and disappeared just as suddenly on day 8 (figure 3.10). These phase changes correspond to those predicted by biofilm dry mass changes (figure 3.8). Thus, based on 3 criteria, it could be stated with some confidence that stationary phase extended through day 7, and decline phase commenced on day 8.

Morphological Characterisation

Figure 3.11. shows the morphological changes corresponding to the physiological events marked by the intracellular organelle activity changes. The mycelia in primary growth phase are elongated filaments of regular shape (figure 3.11. A and B). This compared well with published accounts of undifferentiated hyphae found in submerged, and exponentially growing mycelia (Trinci 1974). Mycelia in secondary metabolic phase tended to be more branched, fragmented and of irregular shape (figure 3.11. C). Fragmentation of mycelia and increased frequency of hyphal branching have been shown to be indicative of filamentous fungi in stationary growth phase (Pusztahelyi *et al.* 1997, and references therein). The fragility of starved mycelia has been shown to be partially due to increased vacuolation in *Penicillium chiysogenum* (Nielsen and Krabben 1995), Increased vacuolation is also indicative of idiophasic mycelia. Figure 3.11. D shows the mycelia in the decline phase. These are mostly dead cells, cell debris and spores.

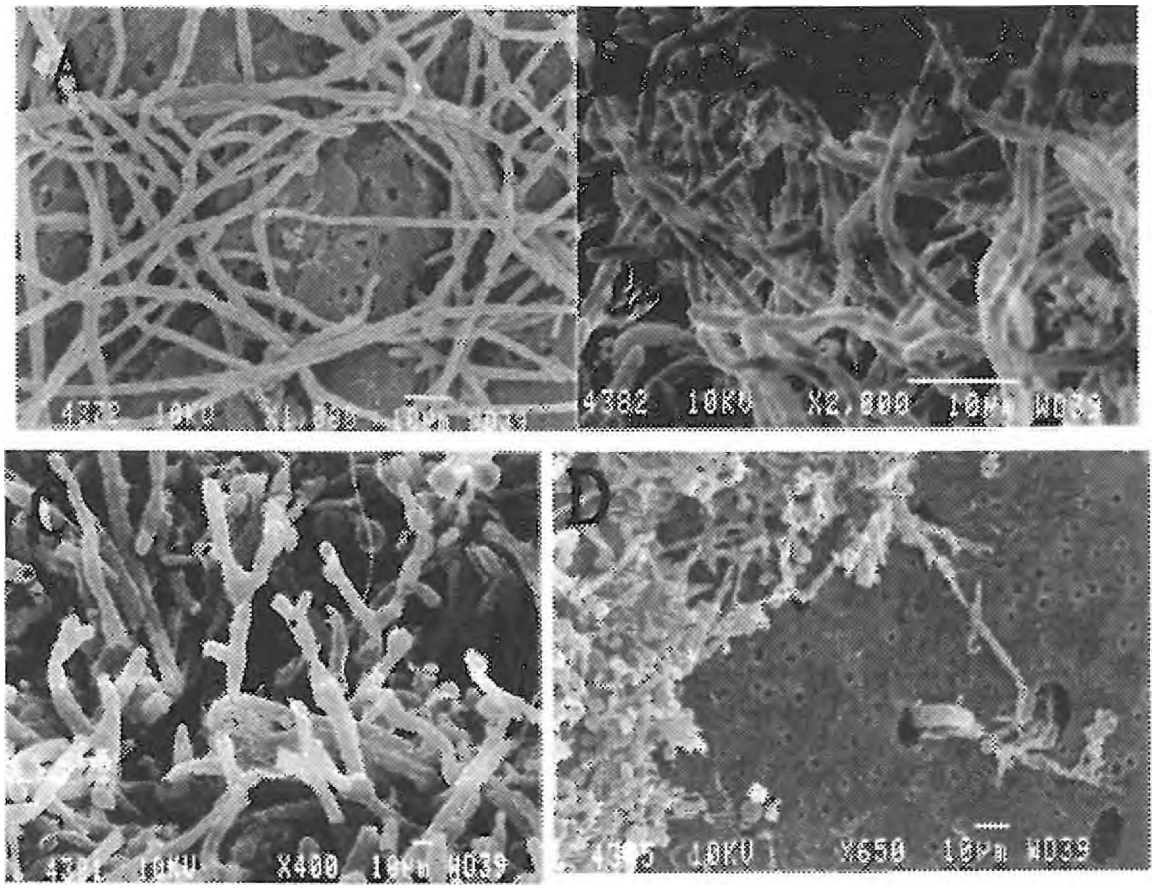


Fig.3.11: Morphological changes through the batch experiment. (A) day 1. (B) day 3. (C) day 6 (12 hours after nutrient supply was stopped). (D) day 8 (mostly spores and dead cells). A and B show mycelia in primary growth phase, C shows mycelia in stationary phase and D shows mycelia in decline phase. Magnifications are shown on the micrographs.

Figure 3.12. shows biofilm development over the same time course. The initial growth appeared to be the production of exploratory mycelia (figure 3.12. A). The biofilm then underwent an accumulation phase during which biomass accumulated (figure 3.12. B). This would correspond to the exponential growth phase in submerged culture. Figure 3.12. C shows the biofilm in late stationary phase at which time sporulation takes place. Figure 3.12. D shows the biofilm in the decline phase. From this electron micrograph and from observations of wall growth of this fungus in a laboratory CSTR, it is evident that dead biomass detaches readily from the support matrix.

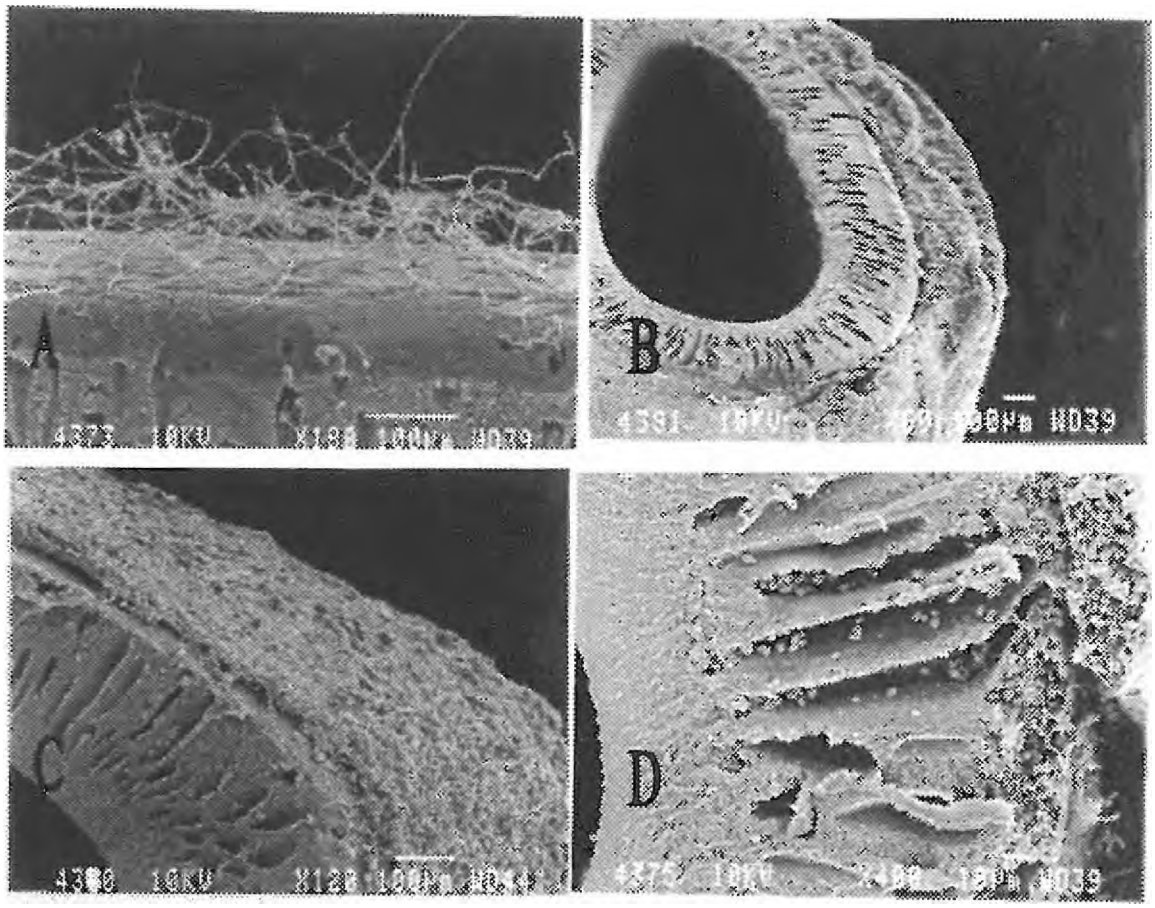


Fig. 3.12: Biofilm development over the same period as described in figure 3.11. Initially sparse exploratory hyphae were produced (A). Thereafter, rapid biofilm accumulation was visisble (B), followed by autolysis (C) and sporulation (D). Magnifications are shown on the micrographs.

3.3.5. Summary of Observations

Correlation of morphological changes of the biofilm to physiologically determined growth phases indicated distinguishable differences. Thus, it could be concluded that hyphal differentiation could be used as a tool to distinguish the growth phase of a whole or part of a biofilm of P. *chrysosporium.*

The primary growth phase was characterised by the presence of elongated, turgid, sparsely-branched hyphae. Similar structures were observable of the biofilm in the macrovoids of the membrane under MGR operational conditions. Entry of the culture into stationary phase, triggered by nutrient starvation through discontinuation of nutrient supply, was confirmed by the observation of increased specific activity of Succinate

Dehydrogenase and Cytochrome C Oxidoreductase, known intracellular markers for secondary metabolism in *P. chrysasporium* (Bonnarme *et al.* 1991). The morphological changes corresponding to the change from primary to secondary metabolism were marked. Hyphae became highly branched, short, vacuolated and fragmented. This was observed in Zone B of the biofilm surrounding the capillary (figure 3.6.) in the MGR biofilm.

The decline phase, indicated by a decrease in culture biomass (figure 3.8.), was correlated to excessive cell lysis and sporulation. Cell debris, fragmented hyphae and asexual spores were the predominant components of the biofilm. The above morphological description corresponded well to that observed in Zone C of the MGR biofilm, which is expected, since Zone C resides in a nutrient poor environment.

3.4. CONCLUSION

In batch cultures it was shown that morphological differentiation occurred as a function of state of growth. The state of growth (primary, stationary or decline phase) was a function of the nutrient supply and was indicated by the changes in activity of the intracellular marker enzymes. It was also shown that mycelial differentiation occurred as a function of spatial nutrient gradients radially across the biofilm of immobilised fungus in the continuous MGR operational mode.

This is a manifestation of the paradigm shift in which growth cycle events in artificial culture conditions are converted from the time domain to the space domain within the same thallus so that secondary metabolism occurs continuously over time. This proves that the membrane gradostat concept, in principle, appears to hold empirically.

It was, however, observed that the morphological changes occurring across the *P. chlysosporiurn* biofilm, in the context of membrane gradostat operation, are more complex than the simple model of superimposition of a normal microbial growth curve across space instead of time. Two morphotypes relating to Zones A and D of the biofilm (figure 3.6.) could not be accounted for in batch culture. This is because in the biofilm of

the MGR the different morphotypes are linked together in one thallus. The key to the understanding of the nature of this link is the well-known phenomenon of differentiation in the filamentous fungi.

Thus, further detail in terms of ligninolytic enzyme localisation in the biofilm and ultra-structural differentiation was required for a more accurate description of the workings of this biofilm. This is explored in the next chapter.

CHAPTER 4

DIFFERENTIATION WITHIN THE BIOFILM

4.1. INTRODUCTION

Based on the results in Chapter 3, it was concluded that a more detailed description of the physiology of differentiation in the biofilm of *P. chrysosporium* in the context of the MGR was required. The differentiation observed in response to nutrient gradients is not surprising since it is known that wood-decay fungi operate within a spatially and temporally dynamic environment (Barrasa *et al.* 1995). They, therefore, exhibit spatial and temporal differences in metabolic functioning, which are directly influenced by their developmental state. White and Boddy (1992) suggest a "developmental plasticity", the ability to adopt and switch between a variety of distinct "functional modes", i.e. programmed morphogenetic cycles which confer versatility on the fungus. This allows these fungi to adapt their functioning to a local environment which is in constant flux (White and Boddy 1992). Some of these mycelial polymorphs include yeast-mycelial transitions, slow-dense or fast-effuse morphs, and aerial vs. appressed or submerged growth. Evidence has been found for differences in extra-cellular enzyme production related to different modes of growth (see table 4.1),

Different morphogenetic modes are also sometimes considered to be consistently exhibited in an exact spatio-temporal pattern within individual thalli (White and Boddy 1992). In a comprehensive study on the ultra-structural localisation of the enzyme pyranose oxidase (POD) using immunocytochemical localisation techniques, Daniel *et al* (1992) described the temporal development of the hyphae grown in agitated liquid cultures and found significant differentiation over time as a function of the chemical environment.

Table 4.1: Some examples of differentiation of fungi as they degrade wood (White and Boddy **1992, and references therein). n/d — not determined**

Species	**Colony Morph**	**Function**	**Enzymes produced**
Hymenoehaete corrugata	**1)Woolly** white form with extensive aerial mycelium **2)Appressed,** pigmented form	1) n/d 2)Associated with more decayed regions	**1)** n/d 2)Laccase and tyrosinase
Phellinus tremulae	1)"Aerial" form **2)** Appressed pigmented form which extends slowly and grows at higher temperatures	n/d	1)Peroxidase but not phenol-oxidase 2)Phenoloxidase only
Rigidasparus microporus	**1)Form which** produces mycelial cords. 2)Mycelial form tolerant of poor aeration	1)Responsible for ectotrophic spread 2)May be responsible for development within wood	1) No laccase 2) Laccase
Phlebia radiate and *Phlebia rufa*	1)CoIonics have peripheral growing front composed of sparsely branched, rapidly extending coenocytic, appressed, noanastomosing mycelia 2)The above are followed by a mycelial system of highly branched, septate, aerial and submerged hyphae, with clamps if mating has occurred	1)Adapted to rapid extension which in nature may lead to early establishment on wood (primary resource capture). 2)Adapted to replacing other fungi, exploitation of resource and defense of territory.	Enzymology not studied but differences are expected due to differences in functionality.

It has been proposed in this study that the MGR, by simulating the natural environment of the fungus, would provide valuable insights into the metabolism of the fungus. Due to establishment of steady-state nutrient and oxygen gradients, the organism resides in an environment similar to that occurring in wood. The hydrophobic nature of the synthetic polymer is similar to that of wood, and the finger-like macrovoids of the membrane are similar to the fibrous voids in the lignin substructure. Also, in this reactor, the fungus grows as a biofilm on a solid-air interface, as is the case in wood, but here, a defined chemical environment can be presented to the fungus in the form of liquid perfusate. It is also easier to harvest the resulting extracellular products than in wood. Hence, it is probable that descriptive models for wood degradation by WRF could be used to best describe the growth of *P. chrysosporium* in the membrane gradostat environment. Once a more thorough understanding of the physiology of the fungus could be obtained, this knowledge could then be used for a rational approach to strain improvement (Demain 1991) and reactor design.

4.1.1. Objectives

The objective of this chapter was to provide a detailed description of biofilm differentiation and the ultra-structural localisation of peroxidase enzyme production. The reasons for this were two-fold:

- Based on the biofilm dynamics described in the previous chapter, further research seemed necessary to refine the first approximation of the functioning of the MGR concept;
- It was considered useful to analyse the metabolic differentiation in the context of wood degradation models. These models, in conjunction with other models for secondary metabolite production and differentiation could be utilised to enhance the understanding of the biofilm.

To facilitate the above study, it was necessary to first develop a method to visualise the presence and localisation of active ligninolytic peroxidases by transmission electron microscopy (TEM) within a biofilm of *P. chlysosporium* immobilised on the skinless membrane (IPS 763).

4.2. MATERIALS AND METHODS

4.2.1. Strain

Strain DSM 1556 (ME 446) of *P. chrysosporium* was used for this experiment and prepared and maintained as described in Appendix A.

4.2.2. Reactor System and Operation

Eight disposable mini reactors were arranged in a manifold system and operated as described in chapter 2 (section 2.5.3). Reactors were operated in gradostat mode and sacrificed after 24 h., 42 h., 56 h., 6 days and 8 days. Once a reactor was removed, it was quickly dismantled and the membrane sectioned. Specimens were then immediately immersed in glutaraldehyde fixative so that the biofilm observed was representative of

the actual state of the fungus in the bioreactor. The mature, differentiated biofilm obtained after day 6 was used for the descriptions presented in this chapter.

After day 8, the biofilm was starved by stopping nutrient supply to the remaining mini-reactors and then again sampled. This was done to show the physiological differences between the biofilm obtained through MGR operation and that obtained when the whole culture was starved, the purpose being to determine the impact of nutrient gradients on culture differentiation.

4.2.3. Cytological Staining of Peroxidases

A procedure had to be developed to visualise the presence of the ligninolytic peroxidases in the biofilm, so that enzyme production could be linked to the responsible morpho-types.

Diaminobenzidine (DAB) is a substrate for general peroxidase activity. As a substrate it has the advantage that the oxidised product from the reaction of DAB with H_2O_2, which is catalysed by peroxidases, reacts with osmium tetroxide to form osmium black, a water-insoluble deposit which can be visualised by TEM as darkly stained deposits. This gives an indication of the location of the peroxidase enzymes in whatever tissue is investigated (Sexton and Hall 1991).

Previously, H202 produced by *P. chrysosporium* was localised by utilising the ubiquity of catalase (which has peroxidase activity at high pH (7-9)) as well as other intracellular peroxidases in fungal tissue to catalyse the DAB reaction (Forney *et al.* 1982). In the case of Forney *et al.* (1982), DAB without H_2O_2 was used for the staining.

Controls

The interfering action of catalase was determined by the addition of aminotriazole, a specific inhibitor of catalase, to the control reactions (Forney *et al.* 1982). The effect of peroxidases other than LiP was minimised by carrying out the staining reactions at a pH below that at which the intracellular peroxidases are active. Intracellular peroxidases normally have an activity range of pH 7-9 (Sexton and Hall 1991), while LiP has a range

of pH 2-5 (Tien and Kirk 1988). Hence, all reactions were carried out at pH 4. To ascertain whether the black deposits observed were due to peroxidase activity, control samples were pre-incubated with KCN, which is a known inhibitor of haeme-containing enzymes (Sexton and Hall 1991). The procedure is described in detail in Appendix F.

The staining procedure was carried out on a starved biofilm to ensure that LiP production had occurred.

4.2,4. Transmission Electron Microscopy (TEM)

The protocol used for TEM is described in Appendix **D.** Some microtome sections were not stained with uranyl acetate and lead citrate as controls in order to determine whether the stains interfered with the cytochemical staining procedure.

4.2.5_ Reproducibility of Results

Since interpretation of trends observed by transmission electron microscopy are often subjective, this experiment was performed several times to ensure reproducibility. Numerous subsequent studies over the period of 1995 to 1998 have all shown identical trends. Results presented here are from one experiment to ensure consistency.

4.3. RESULTS AND DISCUSSION

*4.3.1. Development and Evaluation of a Cytochemical Procedure for the Ultrastructural Localisation **of** Ligninolytic Enzymes in the P. chrysosporium Biofilm.*

On the micrographs depicting mycelia treated with DAB and H_2O_2, distinct black deposits could be observed around the cell wall of the fungal filament (figure 4.2). **It is** evident that these deposits resulted from DAB and H_2O_2 staining of samples in that these deposits were absent ***in*** untreated mycelia (figure 4.1). It can be inferred that the deposits were produced by a peroxidase-catalysed reaction because where KCN, a peroxidase inhibitor, was added to the reaction mixture, no dark deposits were observed (figure 4.3),

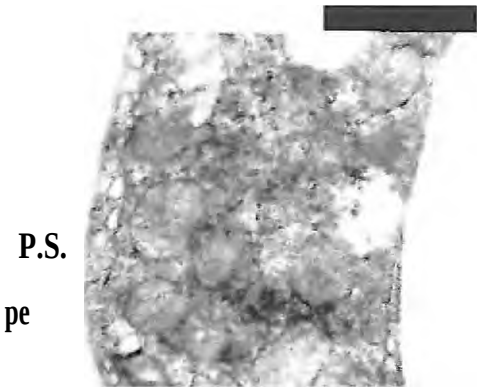


Fig. 4.1: TEM of a longitudinal section of mycelia without DAB staining. The outer wall does not contain any dark deposits. Neither does the polysaccharide sheath (P.S.) of an adjacent cell (Mag. = 12 000X).

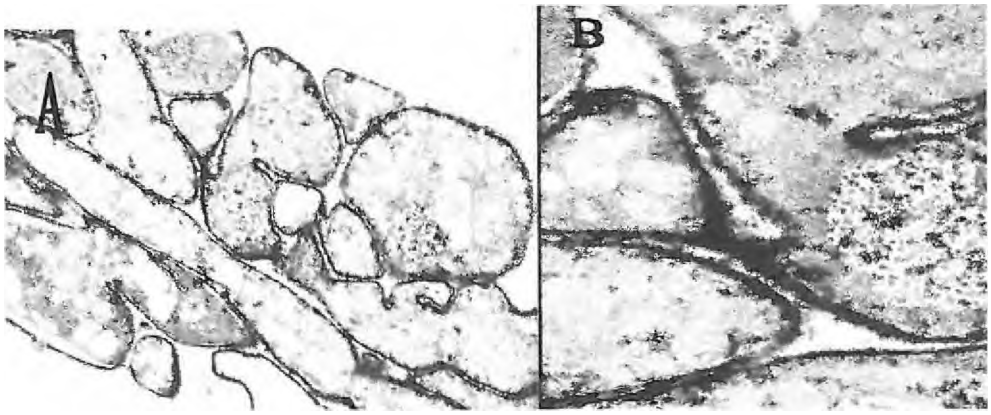


Fig.4.2: (A) Cross section of mycelia after treatment with H_2O_2 and DAB. The black deposits in the polysaccharide sheath around the cell wall represent the products of DAB + H202 catalysed by LIP (Mag. = 4 800X). (B) Higher magnification of the same mycelia as (A). DAB located on EPS sheath and membrane, and on what appears to be rough ER. A multitude of mitochondria could also be observed (Mag. = 14 000X).

Fig. 4.3: Cross section of a fungal cell wall showing much reduced stain deposition. This sample was treated with DAB + H_2O_2 + KCN (a peroxidase inhibitor) (Mag. = 7 200X).

In order to determine whether LiP was responsible for the staining, aminotriazole, a catalase inhibitor, was added to the DAB staining mixture in one experiment since catalase, an enzyme distributed throughout the fungal mycelia, has peroxidase activity and could have been responsible for formation of the black deposits and not LiP. The black deposits observed with sections pre-treated with aminotriazole were similar to those obtained when aminotriazole was excluded (figure 4.4), indicating that the effect of catalase was negligible. Figure 4.5. shows that the black deposits are not due to uranyl acetate and lead citrate post-staining.

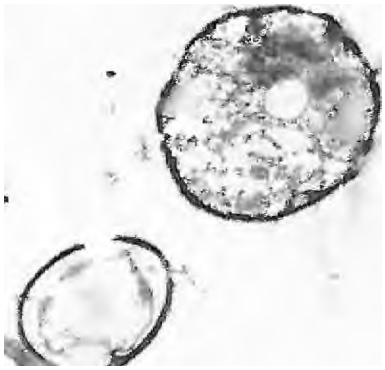


Fig. 4.4: Cross section of mycelia after treatment with DAB, H_2O_2 and aminotriazole, a catalase inhibitor. The similar black deposits obtained show that catalase is not responsible for this reaction. Loss of cell contents seemed to occur because of osmotic shock during the pre-incubation of the mycelia with aminotriazole (Mag. = 10 000X).

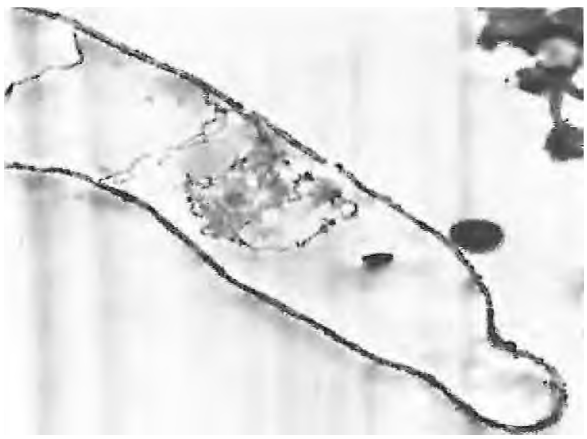


Fig. 4.5: Longitudinal section of mycelium treated with DAB + H_2O_2 +aminotriazolc, but not post-stained with uranyl acetate and lead citrate. The black deposits show that the post-stains do not give false positive results (Mag. = 7 200X).

The localisation of the LiP on the cell wall and polysaccharide sheath corresponds well with previous reports using immunocytochemistry (Daniel *et al* 1989; Garcia *et al.* 1987). These reports involved the use of immuno-gold labeling to provide information on the localisation of the enzyme, but not on activity. Kurek and Odier (1990) have used biochemical methods to prove the association of active enzyme with the fungal cell wall. The technique described here provides information on both localisation and activity of ligninolytic peroxidases.

From the results obtained above it can be concluded that the method developed for the determination of active LiP within a biofilm of *P. chrysosporium* was successful. This technique, although less sensitive and specific than comparable immunocytochemical methods, (Forney *et al.* 1982) has the advantage that it obviates the need for the time-consuming antibody production process and tedious controls (Evans *et al.* 1991). Also it only reflects the presence of active peroxidase.

Using this technique, it was found that a noticeable amount of LiP excreted by the fungus was retained in the polysaccharide sheath surrounding the cell wall of the mycelia. This technique was then used to provide further characterisation of the biofilm.

4.3.2. Study of Biofilm Differentiation of P. chrysosporium and Ligninolytic Enzyme Localisation in a Membrane Gradostai Reactor.

LiP activity was observed in the reactor permeate after 3 days. At this stage complete differentiation of the biofilm could be observed by TEM. This was maintained until day 8, indicating "steady-state" conditions. Details given below are for the biofilm of the bioreactor membrane sacrificed on day 6.

Biofilm in the Macrovoids — Close to the Lumen

In the nutrient rich zone, represented by mycelia in macrovoids close to the membrane lumen, hyphae with a relatively large diameter were observed (figure 4.6 and figure 4,7.). These hyphae had the following features:

They appeared to have a large proportion of their total volume occupied, by what was identified as rough endoplasmic reticulum (ER) and smooth ER (Cross, R. 1995 pers. comm.). It is known that LiP and MnP are transported from the ER via Golgi apparatus to the cell surface, (Bonnarme *et al.* 1991; Bonnarme *et al.* 1993). These authors also suggested that the amount of ER present is a limiting factor for protein secretion by *P. chrysosporium.* Few mitochondria could be observed. These mycelia, therefore, probably have a fermentative mode of respiration. The mycelia had relatively thin walls with an extensive polysaccharide sheath. Lignin peroxidase activity was visible in the polysaccharide sheath and cell wall by the DAB reaction products. Hyphae were turgid, un-vacuolated, un-branched, densely packed and spirally wound around each other.

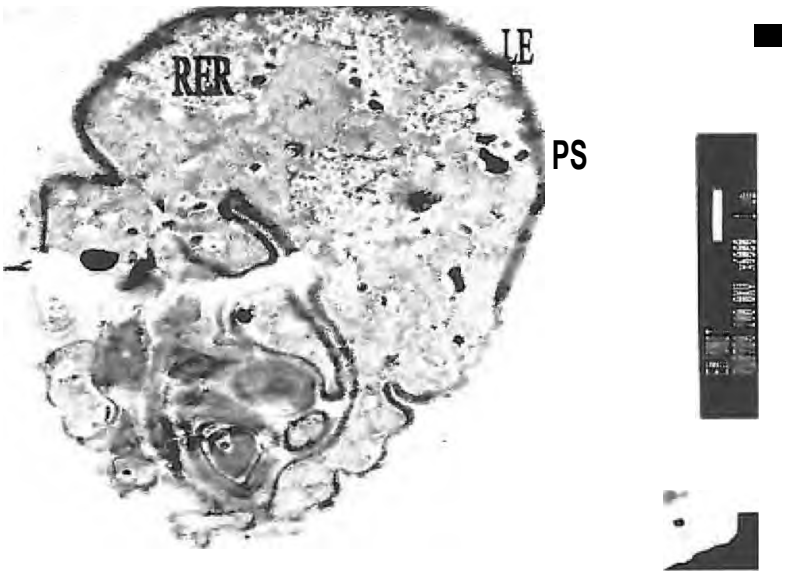


Fig. 4.6. Oblique section through a macrovoid containing P. chrysosporium showing biomass close to the fibre lumen. (RER-rough endoplasmic reticulum, LE- ligninolytic enzymes, PS-polysaccharide sheath) (Mag.= 6 000X).

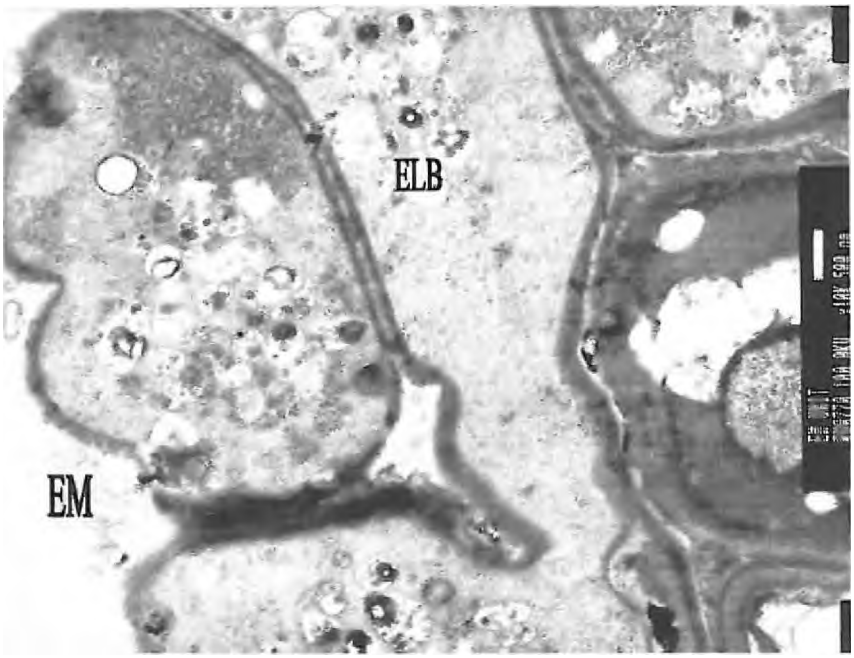


Fig. 4.7: Higher magnfication of the hyphae depicted in Fig. 4.6., showing the presence of multiple electron lucent bodies (ELB), the extracellular matrix (EM) which is only found between the hyphae and the membrane and not in between hyphae (Mag. = 10 000X).

A multitude of multi-vesicular bodies (MvB) similar to those described by Daniel *et al.* (1992) were also visible (figure 4.8.), but these were not always associated with cell membranes as reported by Daniel *et al.* (1992). These could be responsible for secretion of ligninolytic enzymes or materials for cell wall synthesis.

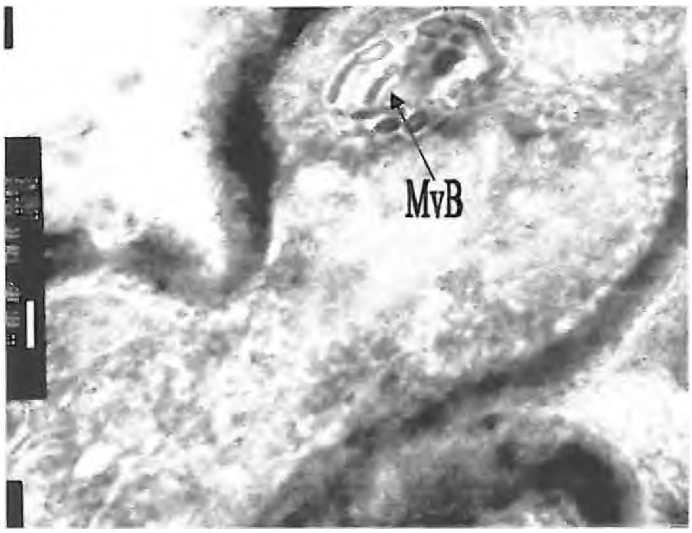


Fig. 4.8: Cross section of a mycelium showing multi-vesicular bodies (MvB) as described by Daniel et al. (1992) (Mag. = 60 000X).

Micro-hyphae could be seen to penetrate into the wall of the capillary (figure 4.9.). It is not known whether *P. chrysosporium* is capable of degrading the polysulphone polymer from which these capillaries were fabricated, but it may have been possible that the hyphae exerted sufficient mechanical force to penetrate weak spots. Barrasa *et al.* (1995) report that the attack on lignin by *P. chrysosporium* is characterised by the formation of erosions and fissures, with minimal lignin removal, and that contact with cell walls seemed necessary.

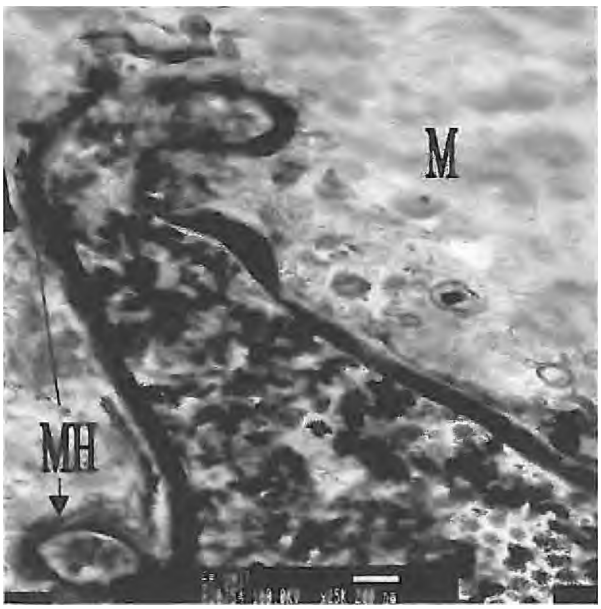


Fig. 4.9: Oblique section of a macrovoid showing a hyphal tip and microhypha (MH) penetrating the capillary wall (M - Membrane) (Mag. = 25 000X).

Two types of mycelia appeared to be present (figure 4.10), the large hyphae containing large amounts of ER described above, which surround a highly convoluted type with which intra-hyphal growth was associated. The above features would correlate well with a penetrative function and the morphological structures associated with decayed wood described in table 4.1.

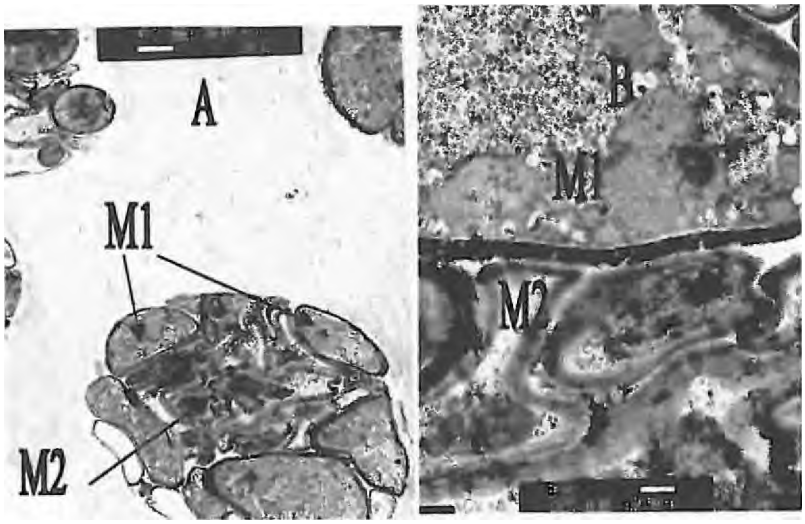


Fig. 4.10: (A) Two major mycelial morphotypes (M1 and M2). MI is a turgid, morphologically active form, while M2 is a membranous, convoluted matrix (Mag. = 3 000X). (B) Higher magnification of A (Mag. = 12 000X)

As mentioned previously, a dense polysaccharide sheath could be seen around the hyphae (figure 4.6). It was reported by Ruel and Joseleau (1991) that in the context of lignin degradation, the glucan sheath forms a bridge between the fungus and the plant cell wall, providing a point of attachment. It also behaves as an immobilisation matrix for enzymes. This sheath was shown to have some affinity to ligninolytic enzymes and was also shown to be accumulated at the hyphal apex (Ruel and Joseleau 1991).

In this work, polysaccharide deposits were found to be sparse or absent from hyphae radially distant from the lumen. Patches of the polysaccharide sheath were also shown to be separated from the hyphae. The absence of the polysaccharide sheath in this part of the biofilm could have been due to hydrolysis to provide sugars for H202 production and the separated patches could allow peroxidases and oxidases to be held in close contact and attached to their substrate (Ruel and Joseleau 1991).

Biofilm in the Macrovoids Distant from the Lumen

In the macrovoids more radially distant from the lumen and nutrient source, the mycelial structure was noticably different. Here the hyphae were sparsely packed and highly vacuolated (figure 4.11). These vacuoles contained several deposits, most of which were darkly stained. A noticeable amount of lysed mycelia and cell debris was also visible.

These hyphae tended not to have much rough ER, but most hyphae observed had several rounded mitochondria especially visible in highly vacuolated hyphae. Here one can distinguish between the electron lucent bodies described by Daniel *et al.* 1992, and the mitochondria observed here (figure 4.12). These two structures appeared similar initially. In figure 4.12, multi-vesicular bodies were associated with the cell membrane, as described by Daniel *et al.* (1992). However, this was found not to be typical. The hyphal membranes and surrounding polysaccharide sheath did not appear as heavily stained with the DAB reaction products.

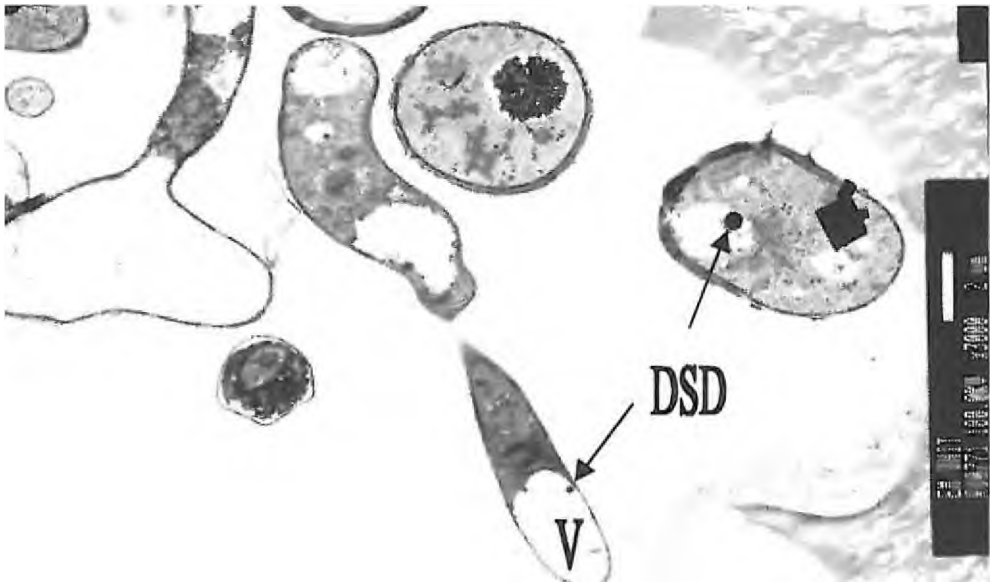


Fig. 4.11: Oblique section of a macrovoid showing the biomass found in macrovoids close to the ECS side, (ie. radially distant from the lumen). DSD- darkly stained deposits (Mag. = 3 000X).

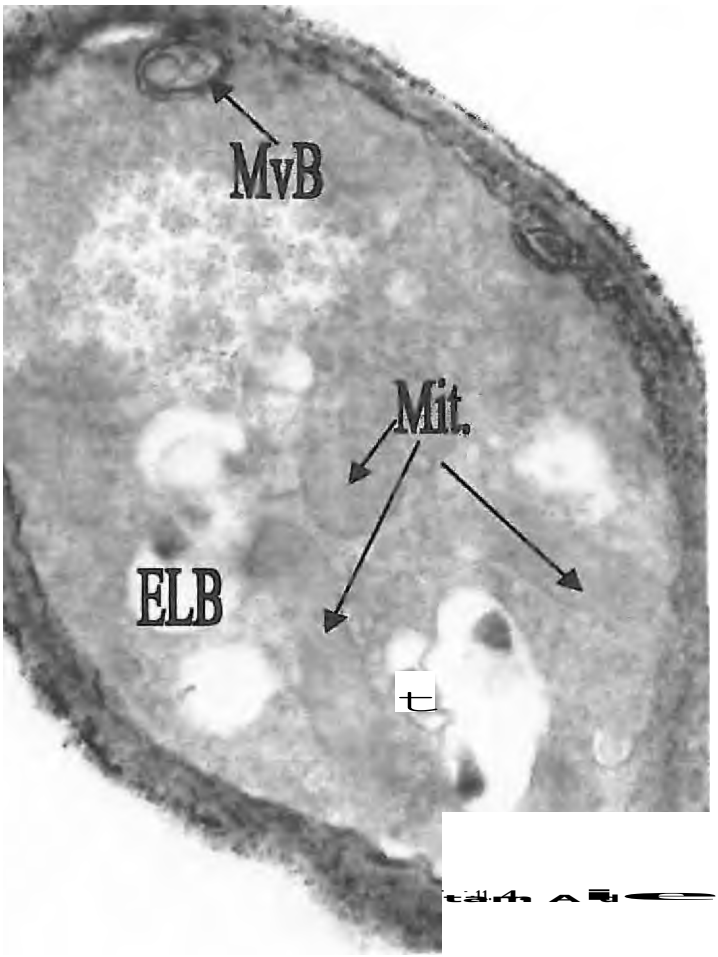


Fig. 4.12: Cross section of a hypha (not particularly typical of this section of the biufilm, showing the differences between electron lucent bodies (ELB) and mitochondria (Mit.) Multi-vesicular bodies (MvB) can be seen to be associated with the cell membrane and electron dense bodies can be seen to be in a state of decay. These are all typical structures described by Daniel et al. (1992) in 6 -to 10 -day old flask cultures (Mag. = 20 000X).

Biofilm Surrounding the Capillary

The biomass surrounding the outside of the capillary formed a biofilm of several hundred micrometers thick (see figure 16). Here a mycelial morphotype was observed which was entirely different from that observed in the biomass entrapped in the macrovoids (figure 4. 1 3).

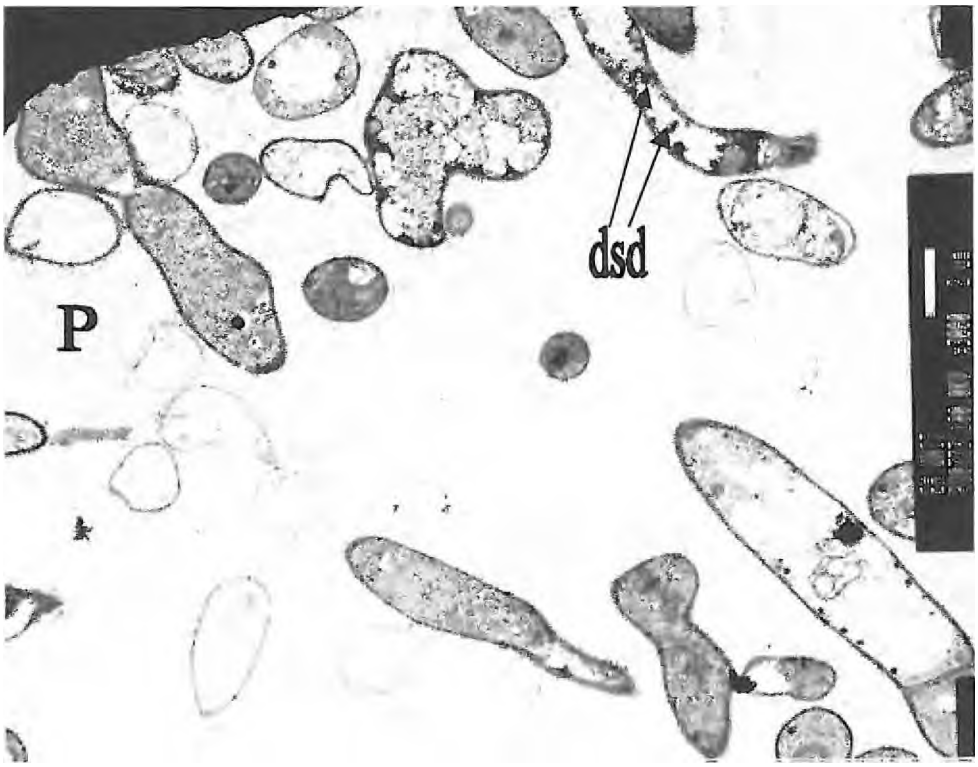


Fig. 4.13: Cross section of the biofilm surrounding the capillary tube. Darkly stained deposits (dsd) can be observed in the vacuoles, indicating intracellular localisation of LiP. Extensive cell lysis is noticeable. The presence of faint dark deposits interspersed between *the* hyphae could indicate the presence of released polysaccharide (P) with entrapped LIP (Mag. = 3 000 X).

Firstly, the orientation of the hyphae was far more random, which complies well with the mycelia differentiated for cover of the substrate, linear spread and defence of territory described in table 4.1. Most of the hyphae were lysed or highly vacuolated. Little evidence of ER, mitochondria, multivesicular bodies or electron lucent bodies were observable. Several darkly stained deposits (dsd), were, however, clearly visible, associated with the internal membranes of the vacuoles. The absence of these structures in sections which were not DAB stained indicated that they could be intracellular stores of ligninases. Several reports of intracellular localisation of LiP have been made in the literature (Evans *et al.* 1991 and references therein). Evans *et al.* (1991) stated that there is little biochemical data to support the intra- or extra-cellular localisation of LiP in solid state culture, since most studies have concentrated on submerged cultures.

Although it appears that peroxidases could be stored in vacuoles, further confirmation will be required using immunocytochemical techniques. However, in support of the observation, Dornenberg and Knorr (1995) state that synthesis and storage of secondary compounds in plant cells often occurs in vacuoles. Also, 14_2O_2 production capability and catalase are said to be compartmentalised internally by vacuolation to protect the intracellular environment from OH radicals (Forney *et al* 1982). Vacuole-encased peroxidases are also said to protect organs from toxins. This was shown to be the case in plant cells (Wink 1997). It is in this zone where most of the conidiation was shown to take place (see figure 3.6.). It is not clear how much ligninolytic peroxidase was associated with the extra-cellular polysaccharide layer relative to the other forms, possibly because of release of the ligninolytic peroxidase enzymes. The space in-between the hyphae was speckled with dark spots and this could be released enzyme entangled in a polysaccharide matrix.

Aerial Mycelia

Another, highly distinctive form, the aerial mycelia, was noticeable in the biofilm most distant from the lumen and closest to the air supply (figs. 4.14 and 4.15). These hyphae were relatively narrow in diameter, un-vacuolated, un-lysed and had a high surface area to volume ratio. The hyphae were electron-dense, even when not stained with DAB, so no conclusions could be drawn about LiP localisation. Abundant, rounded mitochondria could be observed. These were not typical of mitochondria observed in other zones, but rounding of mitochondria has been reported in idiophasic yeast cells (Werner-Washburne *et al* 1993). Highly organised ER was visible, as well as electron dense bodies in various stages of decay. These hyphae may be specialised to provide oxygen and/or energy to the rest of the thallus and are normally involved in differentiation to form reproductive structures.

Fig. 4.14: Cross section of the biofilm surrounding the capillary showing aerial mycelia at the outermost edge of the biofilm (Mag. = 3 000X).

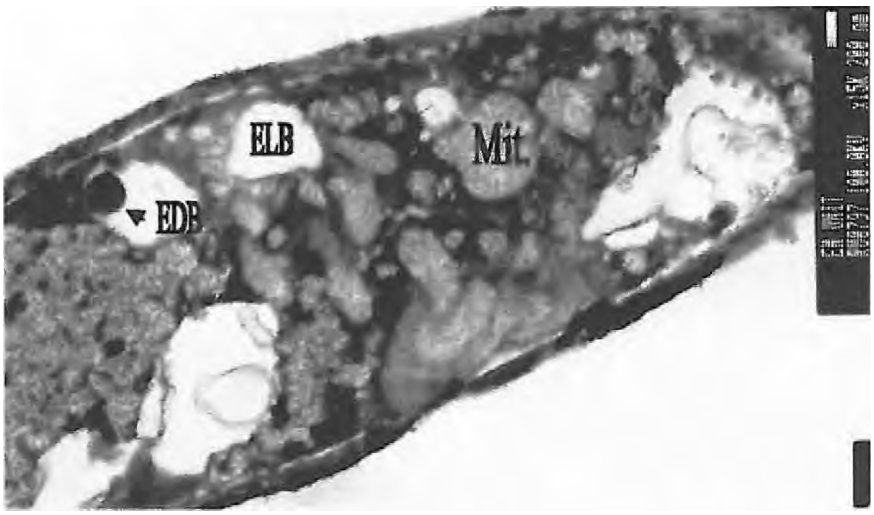


Fig. 4.15: Higher magnification of the hyphae depicted in figure 4.14. Mil-mitochondria, EDB are electron dense bodies located inside electron lucent bodies (ELB) described by Daniel et al. (1992) (Mag. = 15 000X).

Peroxidase Secretion

Ligninolytic peroxidase production, indicated by DAB reaction products in the extra-cellular sheath and cell membrane seemed (by inspection) to be concentrated around the hyphal tips close to the capillary lumen (figure 4.6). This was not unexpected since it is known that the hyphal tips are the most metabolically active parts of the thallus and that this is where most of the protein secretion occurs (Carlile 1995). The paradox arose from

the fact that the hyphal tips resided in a zone of nutrient sufficiency in the MGR, and it is known that LiP is produced as a secondary metabolite triggered by conditions of carbon, nitrogen or sulphur limitation only. This paradox could have arisen because the initial work on ligninolytic enzyme production was performed under submerged liquid culture conditions, which is a significantly dissimilar environment from the MGR. This ties in well, though, with the work of Moukha *et al* (1993) who studied the localisation of LiP in colonies of *P. chlysosporium* sandwiched in-between two polycarbonate membranes. They found that, in a differentiated thallus (which occurred once confluent growth on Petri plates was attained), most of the secretion of the enzyme occured at the colony margin, although the whole colony released enzyme. They concluded that the peroxidases were secreted by the invasive hyphae, but that there is a time delay before release of the enzymes, giving the appearance of secretion by the whole colony, Thus, the results of this study in 3 dimensions, supports the findings of others in a 2-dimensional system.

The above would indicate that the part of the thallus experiencing nutrient limiting conditions signals the metabolically active regions of the thallus to produce secondary metabolites. Translocation of nutrients is a well-known phenomenon in filamentous fungi (Gray *et al* 1995). It has been shown by Bonnarme *et al.* (1993) that mycelia] extracts of *P. chrysosporium* in stationary phase significantly enhance LiP production when added to cultures of the same strain. Further research should be undertaken to determine how this occurs.

Starved Biofilm

In order to simulate what occurs in conventional culture systems when the culture is starved of nutrients, and to determine whether nutrient gradients play a role in the differentiation described above, as opposed to the possibility that the whole biofilm was starved, the biofilm was purposefully starved after 8 days of continuous culture by stopping the nutrient supply to the capillaries. Figures 4.16 — 4.19 show the cytological observations made.

From these electron micrographs it could clearly be seen that when the thallus as a whole was starved, a completely new set of responses took place. The biomass closest to the capillary lumen then contained abundant, rounded mitochondria (figure 4.16). As mentioned previously, these are typical of stationary phase yeast cells in submerged culture (Werner-Washburne *et al.* 1993). The larger hyphae appeared to contain proportionately more ER (figure 4.17) and the electron lucent bodies containing electron dense bodies, precisely the same as those described by Daniel *et al.* (1992), became abundant.

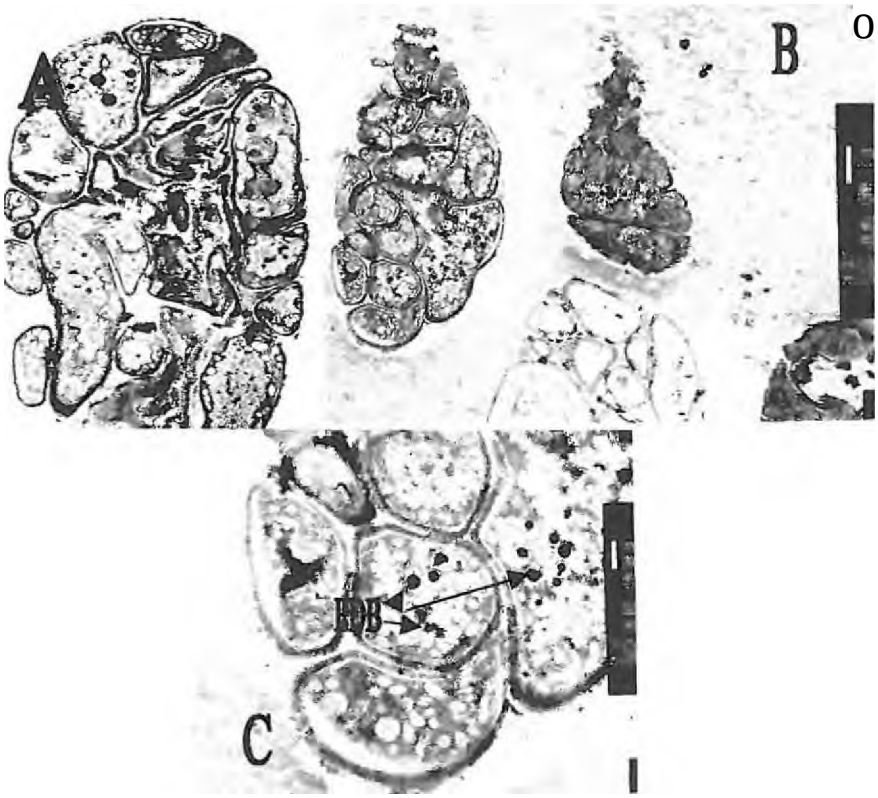


Fig. 4.16: Oblique section of a macrovoid showing starved mycelia close to the fibre lumen. (A) and (B) show the formation of abundant mitochondria and ER (Mag. = 3 000X). (C) shows the same section at a higher magnification (Mag. = 10 000X). Electron dense bodies (EDB) could be observed within electron lucent bodies.

Fig. 4.17: Oblique section showing mycelia at the outermost reaches of the macrovoids. These contained fewer mitochondria than the mycelia observed in fig. 4.16 (Mag. = 8 000X).

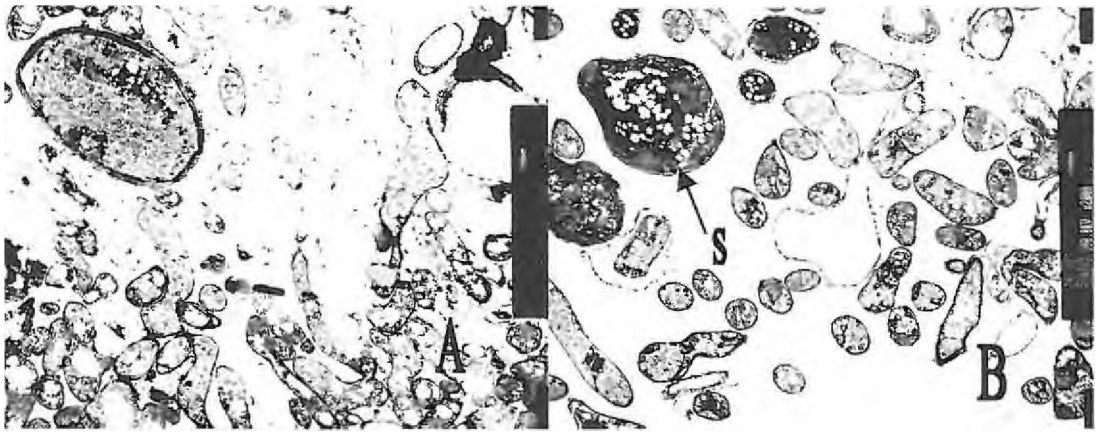


Fig. 4.18: Cross section of the biofilm surrounding the capillaries. (A) biomass close to the capillary side. (B) biomass close to the outer edge of the biofilm The presence of asexual spores (S) can be observed here (Mag. = 2 000X).

Fig. 4.19: Cross section showing (A) aerial mycelia at the outermost reaches of the biofilm (Mag. = 2 000X) (B) higher magnification of A (Mag, = 18 000X)

These electron dense bodies were clearly different from those described earlier for the non-starved cultures residing in this zone. The increase in mitochondria] content could be said to be induced by nutrient starvation (see figure 3.9; Bonnarme *et al.* 1993). It could also be seen from figure 4.16 that the cytosol appeared to be far less turgid, indicating that hyphal extension would have ceased. Figure 4,17 shows hyphae in the macrovoids radially distant from the capillary lumen. These hyphae appeared not to have an increased amount of mitochondria, but did show proportionately more ER. Figure 4.18 shows the hyphae in the biofilm surrounding the capillary. These showed an increase in ER compared to the steady-state thallus obtained with MGR operation, but although mitochondria' content seemed to have increased, this was not as dramatic as is the case with the mycelia close to the fibre lumen (figure 4.16). Differences in mitochondria' content could be seen between the biomass nearest the fibre lumen (figure 4.18A) and that closer to the ECS (figure 4.18B). Also, more spores could be seen to have been produced near the ECS end of the biofilm. Extensive hyphal lysis could be observed and the vacuoles no longer contained what was presumed to be LiP-indicating deposits observed in the steady-state biofilm. These could presumably have been released. Unfortunately, LiP activity in the reactor outlet was not monitored so this could not be verified.

Figure 4.19A shows the aerial mycelia of the starved thallus which appeared to have lost their mitochondria and ER. The hyphal membrane was highly involuted (figure 4.19B) and the whole mycelium appeared to be in a state of decay, which could signify autolysis.

4.4. CONCLUSION

A detailed physiological understanding of the biofilm was necessary for process development so that observed phenomena could be described within the correct context. This has been, in general, a serious flaw in the approach of some other reports in the field of reactor development for ligninolytic enzyme production by WRF.

It was shown in Chapter 3 that the biofilm responds to a nutrient gradient. It appeared, however, that although the model of the superimposition of a microbial growth curve

over space was adequate as a first approximation to describe the MGR biofilm, it was not sufficiently accurate, since the various growth forms were co-ordinated in one thallus. The work presented in this chapter confirmed this notion. The detailed cytological and enzyme cytochemical study undertaken showed the following:

- Ligninolytic enzymes and a polysaccharide sheath, considered in literature to be secondary metabolites, were shown to be associated with the biofilm component in the macrovoids close to the lumen. This is an area of high nutrient concentration, where secondary metabolites were not expected to be observed;
- Biomass residing in the nutrient poor zone showed internal stores of peroxidase. This confirms the presence of intracellular stores of LiP, an enzyme considered to be extracellular;
- Starvation of the entire biofilm showed distinct morphological changes when compared to the biofilm in the presence of nutrient gradients, verifying the difference between batch and MGR physiology.

Based on the above observations and the detailed study of the intracellular structures of different components of the biofilm, a different approach was chosen for the description of this biofilm. This approach relates more to the behaviour of the organism in its native state than on microbial batch growth kinetics.

Thus, instead of description of the observed biofilm zones in terms of primary, stationary and decline phase, this descriptive model involves the presence of:

- Hyphae differentiated for penetrative growth. These hyphae are adapted for rapid assimilation of easily utilisable nutrients and for penetration of complex polymers;
- Hyphae differentiated for ectotrophic spread and substrate cover. These hyphae show little indication of metabolic activity and appear to contain intracellular stores of peroxidase enzymes, which could be released upon cell lysis;
- Aerial mycelia. These hyphae show indications of high metabolic activity, as would be expected of mycelia differentiated for oxygen uptake for maximum respiratory activity.

The above morphotypes are integrated into one thallus, and it is expected that intracellular translocation of metabolites, both trophophasic and idiophasic, occurs between them. The role of the nutrient gradient would, therefore, be to trigger and provide way-points for this differentiation.

The results obtained in this chapter provide further evidence that the dogma of lignin degradation is perhaps simplistic. The dogma of ligninolysis in the WRF is being challenged as more information is being gathered about the metabolism, physiology and the effect of different culture conditions of the WRF. The original hypotheses about the role of primary and secondary metabolites in lignin degradation were also based on the general assumption is that the growth kinetics of the WRF follow that of bacteria in homogeneous environments.

The results of this work suggest the operation of a co-ordinated biofilm with distribution of metabolic labour between differentiated mycelia' forms interconnected within one thallus, as if in the case of proto-tissue. Modern trends in biofilm research show that such complexity exists even in bacterial biofilms, with the discovery of quorum-sensing genes and their products, and the presence of nutrient transport channels. The body of literature on physiological models of filamentous fungal growth provides further backing to these observations. Detailed studies on the mechanisms of differentiation will subsequently commence

CHAPTER 5

BIOREACTOR SCALE-UP AND LIGNINOLYTIC ENZYME PRODUCTION

5.1. INTRODUCTION

The single-fibre mini-reactor had served its purpose adequately in that detailed studies of the biofilm's response to nutrient gradients could be performed to show that the MGR concept holds empirically. Scale—up to a laboratory-scale multi-fibre module then had to be accomplished to ascertain the performance and scalability of the MGR concept.

5.1.1. Reactor Scale-Up

As a system is scaled up, a shift occurs from a stage where the micro-kinetics of cellular reaction control the system's response at a small-scale to transport limitations controlling the system's response at a large-scale (Shuler and Kargi 1992). When a change in the controlling regime takes place, the results of small-scale experiments become unreliable with respect to predicting large-scale performance. This difficulty arises from the complexity of bioprocesses. Moser (1991) described a bioprocess as a complex network of interactions between biology (kinetics, stoichiometry, thermodynamics) and physics (transport of mass, momentum and heat).

Information is, therefore, required of some of the above-mentioned physical phenomena on a laboratory scale that will affect the process on industrial scale. Principal issues to be considered in the development of a suitable module for application of the MGR included membrane bioreactor geometry, the fluid regime and module design.

5.1.2. Consideration of Membrane Bioreactor Geometry

Membranes are usually assembled and associated in a module with a determined geometry offering distinct flow zones for the feed and permeate streams. A variety of

spatial arrangements of biomass and fibres in combination with a multiplicity of solute flows give rise to a wide range of reactor configurations and virtually every shape and membrane structure can be used, depending on the specific application (Robertson and Kim 1985; Belfort 1989; Heath and Belfort 1992). A complex dependence of fluid flow (and hence mass-transfer) on membrane properties, operating conditions and the module geometry has been shown (Kelsey *et al.* 1990). This must be taken into account in the design and analysis of membrane bioreactors.

Various commercially manufactured units are available. These are all based on the shell-and-tube heat-exchanger configuration. In this configuration a bundle of capillaries are potted (i.e. glued) into a cylindrical vessel as depicted in figure 5.1. This arrangement segregates a lumenal fluid compartment from an extracapillary compartment.

Fig 5.1: Cylindrical axial flow fibre bundle membrane bioreactor.

Despite the reported sub-optimal geometries of cylindrical systems, they are the most prevalent in industry, largely because of ease of construction and operation. Availability is also a major factor and commercially available dialysis and UF units can be used as bioreactors with very little modification (Selfort 1989). As a first approach, such a system was evaluated as a model bioreactor, since a reasonable amount of experience and literature on the utilisation of such systems exists. The scale-up criteria are also well established for such systems based on the experience gained with UF (Prenosil and Hediger 1988).

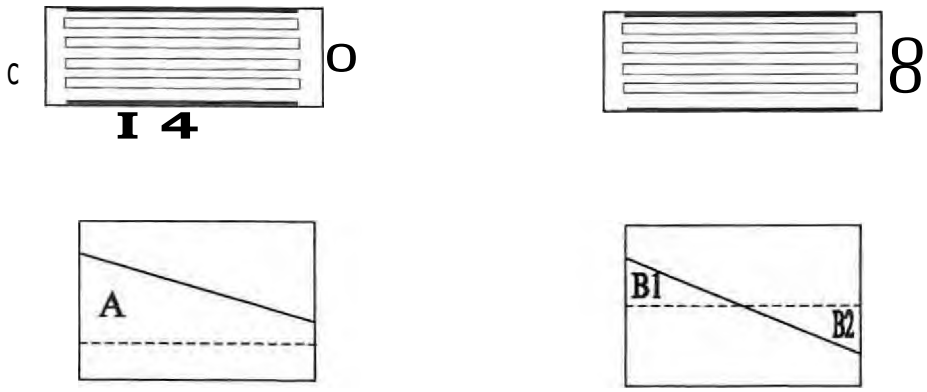
Other, more sophisticated module geometries (Robertson and Kim 1985; Chung and Chang 1988) were developed to overcome some of the shortcomings of the conventional units, though, and this is becoming an interesting area of membrane bioreactor research.

5.1.3. Consideration of Fluid Flow Regime

Various operational modes involving different fluid flow regimes have been reported and characterised. The two relevant modes are:

- closed shell (recycle mode);
- open shell (UF mode);

These are depicted schematically in figure 5.2.



open shell closed shell

Fig. 5.2: Schematic representation of the open- and closed-shell configurations. Upper diagrams indicate fluid flow. Open arrows indicate inlet streams, while closed arrows indicate effluent streams. Lower diagrams indicate pressure distributions. Dashed lines represent shell side (ECS) **pressure, while solid lines represent tube side (lumenal) pressures. A-open shell pressure difference. B1 and B2-closed shell pressure differences. Note that B2 is negative. Redrawn from Tharakan and Chau (1986).**

Each of the above configurations has its own flow patterns which have been modeled (Kelsey *et al.* 1990). Most work to date has focussed on the closed shell configuration, in which there is no net convective flow from lumen to shell. The axial pressure drop due to flow through the lumen causes lumen pressure to be greater than that in the shell in the upstream part of the fibres (B1 in figure 5.2.). Then, as the lumen hydraulic pressure declines axially, and the ECS pressure increases due to permeation from the fibre lumen, flow is reversed in the downstream part of the device due to a reverse in the pressure gradient (B2) (Patkar *et al.* 1993; Kelsey *et al.* 1990). This is called convective

recirculative flow or Starling flow since it is analogous to the flow in capillary-tissue systems *in vivo* (Starling 1896). This phenomenon was demonstrated empirically and quantified by direct pressure measurement (Tharakan and Chau 1986) and Nuclear Magnetic Resonance Imaging (Heath *et al.* 1990).

In practical terms, this operational mode is obtained when the shell is closed. The growth medium circulates through the lumen of the fibres and substrate diffuses across the hollow fibre membranes and is then converted by the cell suspension into an extracelluiar product, which then diffuses back into the lumen (Webster and Shuler 1978; Prenosil and Hediger 1988, Shuler and Kargi 1992). This is clearly not suitable for the purposes of the MGR since uni-directional gradients were a priority which would be difficult to attain given the torroidal flow fields typically encountered in such systems (Kelsey *et aL* 1990). Also, air flow through the ECS is a pre-requisite of the system described in chapter 1 (section 1.9.1). This would be impossible if the shell was closed.

The OF mode was therefore decided upon for practical application of the gradostat concept.

Oxygen Delivery

An oxygen delivery system had to be developed so that good mass transfer would be attained at low cost and sufficient shear could be introduced to erode off dead biomass. However, the shear had to be low enough to avoid mechanical damage and destabilisation of the whole biofilm. Another function of the oxygen supply was to enable bulk flow of the reactor permeate through the module outlet.

Based on the experience of other researchers, as reported in literature on membrane bioreactors and cultivation of *P. chrysosporitun* (Venkatadri and Irvine 1993), axial and radial oxygen depletion is believed to be the critical scale-limiting factor in the design of aerobic (particularly mammalian) cell culture hollow fibre bioreactors (Piret and Cooney 1990).

It was reported by Ahmed *et al.* (1996) and Ahmed and Semmens (1996) that mass transfer of oxygen is largely influenced by construction geometry. They also proved that mass transfer of oxygen was much better when flow of oxygen occurred transverse to hollow fibres as compared to when flow occurred parallel to the fibres. This is most likely due to turbulence promotion by the fibres themselves (Futselaar *et al.* 1993). The term "transverse flow" indicates that the airflow is perpendicular to the fibre axis. This is to be distinguished from "cross flow" which has a different meaning in membrane technology (Futselaar *et aL* 1993). Yang and Cussler (1986), on the other hand, show that in the design of hollow fibre gas-liquid contactors, the key parameter to maximisation of performance is surface area to volume ratio, i.e. effective packing density. Wickramasinghe *et aL* (1992) reported on the impact of a combination of these effects and included the advantages of high flow velocities and the negative impact of polydispersed fibres vs. regular packing of fibres.

Uniform distribution of air through the reactor is also important, and the ease of achieving this depends on the packing configuration of the reactor and the growth of the fungus (Reid 1989b). Optimising packing density is a problem often encountered in solid substrate fermentations (Reid 1989b). In the context of lignocellulose degradation by WRF, the optimum support matrix particle, wood chip volume, is determined by a balance between air diffusion into particles and circulation between them. Thus, in a membrane bioreactor, having a small inter-fibre space would facilitate aeration by shortening the distance of gas diffusion from bulk into biofllm, and also increase the packing density (and thereby volumetric productivity). However, a small inter-fibre distance restricts air circulation between these membranes and could result in blockage of the air flow path_ Blockage of support media is a well-documented shortcoming of fixed-bed reactors. Lasarova *et al.* (1997) used air injection as a solution to blockage of their system, so the concept of using gas flow to erode unwanted dead biomass (as described in chapter 1, section 1.9.1) seemed reasonable.

Liquid Nutrient Delivery Rate

The liquid nutrient feed needed to be sterile, quantifiable (in terms of supply rate and composition) and supplied in a manner that produces the most uniform permeate flow field possible; minimises axial gradients; has a high enough permeation rate to provide a thick biofilm, but low enough so that nutrient gradients could be established and biofilm washout would not occur.

The biofilm produced was required to be axially homogeneous (for ease of characterisation) and thick enough so that significant nutrient gradients could be established by diffusion and reaction. A large amount of biomass needed to be produced so that good enzyme production could be attained and gas stripping of substrate and pollutants (in bioremediation studies) was minimised (Freitas dos Santos and Livingston 1995). With dense biofilms the chemical dose (mg. of pollutant. (mg of biomass)$^{-1}$) would be minimised for a given concentration of pollutant for bioremediation studies. However, the biofilm should not be too thick that oxygen limitation would occur.

The permeate was required to exit the reactor as quickly as possible (after perfusion of the biofilm), minimising blockage, and making product (ligninolytic enzymes produced, or pollutants transformed) analysis simple.

Two factors were expected to affect mass transfer rates from the bulk growth medium fluid to the biofilm: fluid velocity and liquid phase concentration of the material being transported. To keep the process of characterisation as simple as possible, the concentration was kept constant and equal to the standard growth medium described for *P. chrysasporium* by Tien and Kirk (1988).

As mentioned earlier, the strategy chosen was to evaluate a modified conventional unit (a cylindrical shell-and tube configuration) for its ability to meet the necessary physical requirements (as discussed above). If this was satisfactory, then further development would not be necessary and scale-up could be accomplished according to guidelines in

the literature. If it was not good enough, then modifications would be made based on the experience gained and observations made.

5.1.4. Conventional Cylindrical Membrane Module

Modified conventional systems were the first choice for application of the MGR concept. The modifications that needed to be made to the geometry were to improve oxygen mass transfer to the biofilm; to minimise fibre contact and to minimise axial gradients. Several schemes and models have been developed to serve this purpose (Chresand *et al.* 1988). These generally involve optimisation of the length to diameter ratio of the fibres to minimise axial gradients and optimisation of fibre spacing to improve fibre-fibre interaction, but will not be discussed here due to the failure of these systems for application to the MGR concept. From several experiments with these units (results not shown), the following shortcomings of cylindrical membrane bioreactors were identified:

- The reactor geometry needed to be changed in order to decrease the fibre length, improve oxygen mass transfer while maintaining a similar fibre packing density, and reduce fibre contact;
- The liquid nutrient delivery mode needed to be changed to minimise torroidal flow, pressure drop and trans-compartmental contamination.

5.1.5. Development of a Novel Transverse Flow Module

Thus, as was the case with the development of a suitable membrane (Chapter 2), a multiple-fibre module had to be developed which would fit the design criteria established for the MGR concept. Due to the shortcomings of the cylindrical systems, it became clear that a completely different reactor configuration had to be applied. Based on the inherently good oxygen mass transfer, and regularity of fibre spacing of these modules, a transverse flow module was considered the option with the best potential. The dimensons chosen were to minimise the effect of axial lumenal pressure drop.

Thus, a novel reactor was developed in collaboration with the Institute for Polymer Science of the University of Stellenbosch and was used to test the system on a small scale (the mini-reactor can be considered as very small). This reactor, termed a Transverse Flow Bioreactor (TVFBR) is depicted in figure 5.3. and has the following features:

- A large number of membranes, completely separate from each other, could be configured into a small volume reactor. This provides a membrane reactor with a large membrane surface area to volume ratio. The fact that the membranes are not in contact with each other is important for proper biofilm differentiation on each fibre;
- The membranes are arranged perpendicularly with separate fluid supply channels for each direction allowing flexibility in terms of feed supply and product extraction;
- Despite the large amount of membrane surface area made available, the length of the individual fibres is sufficiently short to prevent Starling flow conditions (Tharakan and Chau 1986) which would adversely affect the system;
- Air can be supplied transversely to the membranes containing the biofilm, providing good mass transfer without having to resort to high pressures or high flow velocities as would be the case with axial flow systems (Ahmed *et al.* 1996).

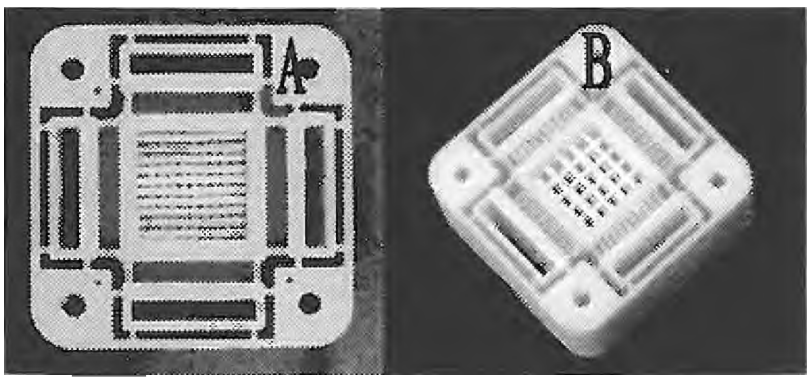


Fig. 5.3: (A) A transverse flow module with normal fibre spacing. **(B) Fibres in alternate slots giving larger interfibrillar distance.**

5.1.6. Objectives

Since the MGR concept was proven (Chapters 3 and 4), and a fundamental physiological description of the metabolic state of the biofilm obtained, the aim of this chapter, was to determine the feasibility of its practical application to secondary metabolite production, in

this case, the production of peroxidases with the view to eventually meeting the discussed requirements of scalability, productivity and extended use. The objectives are listed below:

- To prove that enzyme production can be sustained for extended periods of time;
- To evaluate the TVFBR geometry as a feasible production system from a biochemical engineering perspective, and to suggest refinements at an early stage, with the view to achieving the above mentioned criteria for a successful system;
- To compare the efficiency of the MGR system to other reactor systems reported in literature.

5.2. MATERIALS AND METHODS

5.2.1. Experimental Approach

An empirical approach was chosen to finding a set of operating conditions for enzyme production. This was not theoretically-based, nor by any means was it optimal. The conditions chosen were judgement-based and fell within the possibilities of the equipment available. Several attempts were to be made, in anticipation of unforeseen process upsets, to attain a goal of 60 days continuous production. A more detailed study was then performed to determine the productivity of the systems for purposes of comparison.

The approach to use for comparison was a daunting choice to make due to the variety of strains, media and operating conditions applied to ligninolytic enzyme production by *P. chiysosporium* in the literature. Thus, in order to evaluate only the reactor geometry, immobilisation support and operational mode (MGR), a standard production strain was used, with a standard production medium.

The reactor performance was determined according to:

- Volumetric productivity, which is a true indication of the production capacity of a reactor system. This is reported as the amount of product produced (in Units of enzyme activity). (litre reactor volume (the entire volume of the TVFBR was used here)).$^{-1}$ day"'.

- Concentration of product in the permeate. Although this measure gives no indication of the role of productivity it is the one most commonly used in literature and strongly influences the purification cost, which is a major consideration in process design.

5.2.2. Strains

The production strain *P. chtysosporiurn* BKM-F 1767 was used for this study for the sake of comparison with other reports, where this strain is most commonly used. The culture was stored and sub-cultured on malt extract agar plates. Spores for inoculation of the reactor were prepared according to Appendix A.

5.2.3. Growth Medium

The medium was the standard medium of Tien and Kirk (1988) except that sodium acetate buffer (20mM) was used instead of dimethyl succinate buffer and veratryl alcohol was excluded. This was due to the expense of dimethyl succinate and to evaluate the system without any expensive additives such as veratryl alcohol.

5.2.4. Reactors

TVFBRs were evaluated here due to their high surface area to volume ratio while maintaining good oxygen mass transfer. A total of 0.013 m^2 of membrane surface area (based on the internal diameter of the membranes) was available in the unit used.

The TVFBR module was produced at the Institute for Polymer Science, University of Stellenbosch. A template was designed and produced by injection molding. The material used was high-density polyethylene because of cost and ease of molding. Capillary membranes were cut to the required length (slightly longer than the length of the template) and clipped into place on grooves molded into the plastic template. The templates (containing the membranes in place) were then stacked with the membranes running perpendicular to those of the adjacent template until a reactor of sufficient size was built. Epoxy resin was then injected under pressure to pot the membranes and to seal the reactor. The extending ends of the membrane capillaries were then trimmed to size when the epoxy had set. Various packing configurations were possible. These are demonstrated in figure 5.4. The "crossed in-line" configuration (figure 5.4. (c)) was

chosen intuitively, since it was presumed that it would give good mass transfer with the least chance of blocking.

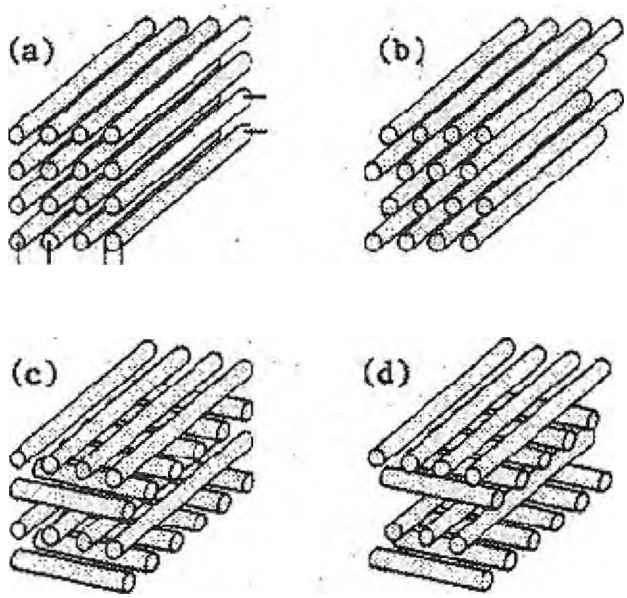


Fig. 5.4: Various fibre packing configurations for TVFBRs. (a) parallel-in-line, (b) parallel staggered, (c) crossed in-line (d) crossed staggered. (Futselaar *et aL* 1993).

To determine the effect of packing density of the reactors, two reactor formats were used:

- A densely packed module was used with an inter-capillary distance of I nun. (figure 5.30. This system gave a high surface area to volume ratio of 0.325 m^2. m^3. This reactor will be referred to hereafter as Reactor 1;
- A more sparsely spaced configuration was produced using alternative fibres removed (figure 5.3b). Two of these reactors were stacked on top of each other, which provided a reactor with the same surface area, but double the volume of reactor 1, giving a surface area to volume ratio of 0.1625 m^2. ni^a. This reactor will hereafter be referred to as Reactor 2.

Design of the Reactor Manifold

The design of the manifolding system proved very important for the proper functioning of the reactor. The material chosen was polypropylene since this was the least expensive material which satisfied the desired materials requirements. For long term operation, the manifold had to be designed to take into account the following:

- Air velocity profiles, because the air supplied was passed through a narrow channel. This caused the biomass in the centre of the membrane to experience high shear and was sloughed off while the biomass near the edges of the membranes might have been oxygen starved;
- Good sealing characteristics were important to prevent leakage and contamination;
- Biomass accumulation at the bottom of the reactor caused clogging of the reactor outlet and inconsistencies in results.

Thus, a manifold was designed with long funnel-shaped air supply. This minimised radial air velocity profiles leading to more equal coverage of the membranes by the gas supply. The same air supply funnel shape was used for permeate and sloughed-off biomass collection which reduced biomass accumulation in the reactor outlet. The manifold was designed with sufficient physical strength and rigidity to be threaded to provide leak-free joints with the supply tubing.

Reticulation and Operation of the Transverse Flow Reactor

The reticulation system for the transverse flow reactor is shown in figure 5.5. Slight variations in some experiments were made and these are described in the relevant sections. This system is sterilised and inoculated in the same way as the mini-reactor system (Chapter 2, section 2.5.3).

Convective flow was used since it minimises diffusive mass transport limitations (Kelsey *et al.* 1990). This is because the pressure drop within the lumen between the inlet and stagnant point is much lower than the pressure drop down the length of an axial flow open tube. Hence, a more uniform transmembrane flux along the length axis of the bioreactor could be expected (Brotherton and Chau 1995).

Operation was similar to that of the mini-reactor with the exception that dead-end filtration mode of nutrient supply was used to give convective-dominant flow. This provided a constant flux regime as opposed to a constant re-circulation rate used in the

mini-reactor system. In the case of the constant flux regime, the capillary lumena were first primed with growth medium.

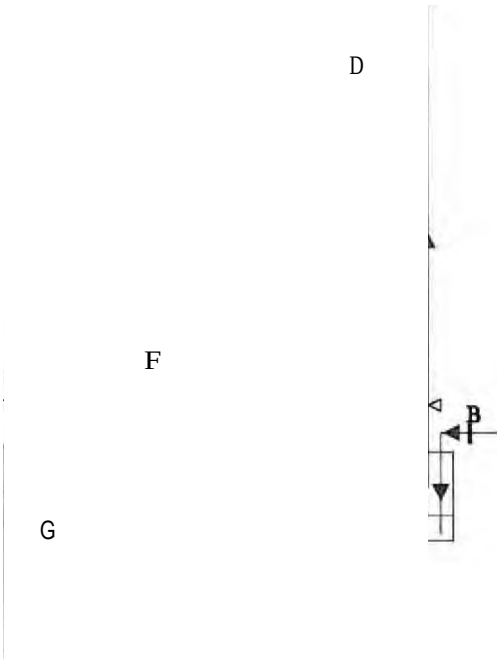


Fig.S.5: Schematic depiction of theTVFBR. A- oxygen supply. Where air was used an aquarium pump was attached and if pure oxygen was used, an oxygen cylinder was attached. B- air filter. C- humidifier vessel —gas was bubbled through distilled water for humidification. D- Hoffmann clamp. E- Inoculation vessel. F- peristaltic pump. G— growth medium reservoir vessel. BI- permeate collection vessel. The inlet to this vessel was sealed with a cotton wool bung to allow spent air to escape while retaining spores within the vessel. 1- growth medium inlet channel within the TVFBR J- the actual TVFBR. This configuration shows 2 units with 2mm. inter-capillary space (see fig. 5.3b) stacked on top of each other. K-represents the membranes. The perfusate of these ends up as the permeate in H. L-prime line. This was used to allow air to be flushed out of the membrane capillaries and to ensure that they were all filled with growth medium. This line exits to waste during priming and was closed off during dead-end operational mode. Arrows show directions of flow.

The recirculation line was then closed using a Hoffman clamp. Medium was then supplied via a small liquid chromatography peristaltic pump threaded with 0.1 mm. i.d. tubing. The medium supply rate therefore defines the transmembrane flux (which is the same as biofilm perfusion rate or dilution rate), With this method, more precise control over the biofilm perfusion rate was expected to be established. The operational parameters of this reactor are described in table 5.1.

Table 5.1. Operational parameters for the TVFBR.

Parameter	*Value*
Oxygen supply rate	1 vol. (vol. reactor space).$^{-1}$ min.'"
Growth medium supply regime	Constant flux (by dead-end filtration).
Biofilm perfusion rate (flux)	0.1 L. m.'.11^{-1}
Fibre type	IPS 763
Air flow regime	Transverse to fibres
Temperature control	Ambient (C.E. room set to 37°C)
Active fibre length	3 cm.

5.2.5. LIP Assay

LiP activity was determined by the oxidation of veratryl alcohol to veratryl aldehyde as described in the Appendix G.

5.2.6. MnP Assay

MnP activity was determined spectrophotometrically by following the oxidation of ARTS at 420 nm., as in Appendix G.

Normalisation of Enzyme Activity

All assays were performed at 18 °C. When direct comparisons with other systems were made, activities should be normalised to approximately that which would be attained at 37°C, which is most commonly used. It has been reported that LiP activity doubles for every 7°C increment up to 40°C (Venkatadri and Irvine 1993). Thus enzyme activities at 18°C should be multiplied by 4 to approximate that at 37°C. Also, MnP activities can be measured using various substrates, the most common being Phenol Red, ABTS, and di-methoxy phenol (DMP). Moreira *el al.* (1997), normalised the results of Bonnarme *et al.* (1993) by dividing the results of activity by Phenol Red oxidation (as used by Bonnarme *et al.* 1993) by a conversion factor based on the ratio of extinction coefficient between the oxidation products of the 2 substrates, Thus activity results from Bonnarme *et al.* (1993) were divided by a factor of 4.96. This appeared invalid since activity is not based on absolute values of absorbance, but rather rates of change therein. Also, reaction rates for different substrates should play a more important role (assuming they are in excess - if not, enzyme affinity for the substrate (K_m) would also play a role)

In addition, factors such as presence of certain ions, inhibitors, pH and various other factors affect the sensitivity of different assays (del Pilar Castillo *el al.* 1997). Thus, the emprical comparison of del Pilar Castillo *et al.* (1997) using high concentrations of pure, recombinant MO was considered the preferred approach. This provided conversion factors of 2.82: 1 for Phenol Red : DMP activities and 1.71 1 for ABTS : DMP-based activities (del Pilar Castillo *et* al1997).

5.2.7. Productivity

Productivity is defined as the amount of product formed per unit time, per unit reactor volume, and methods for its calculation abound in textbooks on biotechnology and chemical engineering. For the purpose of this study, productivity was calculated as:

$$P = D.\ C. \qquad 5.1.$$

Where P = productivity (Units. (litre reactor volume).$^{-1}$'day^{-1})

C = concentration of enzyme in the reactor permeate (Units.litre^{-1}, where 1 unit is described as catalytic ability to transform luinol. of substrate in one minute.).

D = Dilution rate = Flow rate . Volume^{-1}.

For the measurement of D, the definition of reactor volume required careful consideration. By definition, V is the working volume of the reactor. This is easy to define in tank-type reactors, but not so in the case of the MGR. Here, the volume of the biofilm constitutes the actual volume in which the desired reaction takes place. This is supported by Trilli (1990), who states that productivity in secondary metabolite formation can only be compared if it is defined as a function of biomass content. On the other hand, it can be argued that the air space around the biofllm influences productivity through mass transfer. Hence the entire ECS volume was used for calculations.

5.2.8, Analysis of Nutrients.

The reactor permeate was analysed for the concentration of glucose and NH_4^+. Glucose was determined according to the Somogyi Nelson method as described by Clark and Switzer (1976). Ammonium was determined using the Spectroquant® Ammonium kit (Merck).

5.2.9. Validity and Reproducibility of Results

Because this research involved initial development of a membrane, module and operating system, a multitude of parameters had to be empirically determined and then controlled. Due to this complexity and the requirement to perform experiments over extended periods, process upsets were frequent. Hence, considerable data was obtained to validate the findings presented. However, data from incomplete experiments will not be shown. Although changes in performance were observed between experiments, trends of the progression of the process were consistent and reproducible. For the sake of consistency, all the data presented in this chapter were derived from one experiment, except for figure 5.6, which was a different run.

5.3. ***RESULTS AND DISCUSSION***

Traces of both LiP and MnP produced during MGR operation were detected after 2 days of operation following inoculation in all experiments with the TVFBR. It is presumed that this is the time required for the biofilm to reach a sufficient thickness for nutrient gradients to be established.

5.3.1. The Effect of Fibre Packing Density

Similar final product concentrations were obtained with both systems (reactors 1 and 2) in the early stage of operation, but it was clear that reactor 1 (with high packing density) could not sustain production for more than 10 days. Inspection of the reactor showed that fungal growth had occluded the inter-capillary spaces, This condition could not be remedied with strong bursts of the air supply although large chunks of biomass were seen exiting the reactor. This biomass appeared to emanate from the top layer of the membranes, where the air velocity was highest, resulting in sloughing off of active biomass, rather than the

clearance of occluding biofilm. Work with Reactor 1 was thus discontinued despite good performance.

5.3.2. Extended Operation of Reactor 2

Reactor systems with the configuration described as Reactor 2 performed adequately over extended periods. Continuous production was maintained for the target 60 days, at which time the process was stopped. This was the first report of true continuous production of ligninolytic enzymes using an attached growth reactor (Leukes *et al.* 1996). Results for the first month are shown in figure 5.6. Only very recently has another group reported true continuous production of MnP (M.oreira *et al* 1997), who operated their system for 140 days.

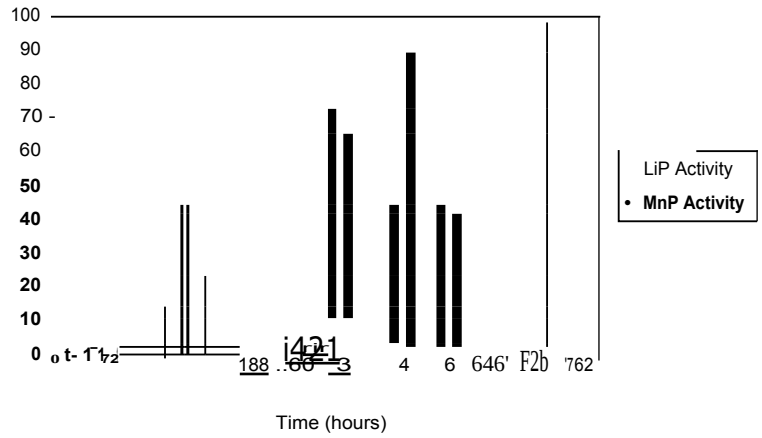


Fig. 5.6: Long term enzyme production in the MGR. LiP levels in the spent broth *were* measured daily for the first 568 hours. MnP activity was determined from samples stored at — 20° C for several weeks. Sample for the first 400 hours showed no activity, presumably through damage due to storage. These results were used, nevertheless, since this graph serves only to demonstrate extended operation.

5.3.3. Uniformity of Operation

During long term operation of the MGR using the Reactor 2 configuration, a serious design flaw of this configuration was encountered. Constant fluid supply throughout the reactor was found to be difficult to maintain. This was found to be due to the static pressure applied by the column of fluid in the liquid supply compartment and tubing above (figure 5.5. I and L). This pressure varied along the height of the reactor, which caused unequal flux through the capillaries. The impact of this flow maldistribution was

that the capillaries at the bottom of the reactor operated at a higher flux than the capillaries at the top of the reactor, with the capillaries at the top of the reactor eventually drying up. The level of fluid in the prime line could clearly be seen to be gradually dropping.

This problem, in terms of continuation of operation, could be partially overcome by priming the lines at a high flow rate for approximately 30 minutes. The unit had to be primed at high flow rates so that air (sucked into the system through the drop in fluid level) could be flushed out. This, however, affected steady-state operation, since it caused periods of high flux, washing off of chunks of biomass, and disturbing the steady-state of the biofilm. This can best be seen by the changes in the reactor effluent concentration in glucose (figure 5.8.). The increases in glucose concentration due to priming were due to higher flux resulting from this activity.

The observable outcome in terms of productivity was an oscillation of enzyme production, which is observable in figure 5.6. The effect of the flow maldistribution on the biofilm can be observed on photographs of biofilm thickness differences between the top and bottom of the reactor (figures 5.12 — 5.14, to be discussed subsequently).

5.3.4. Productivity of the System

In the previous section, the MnP production levels described were from samples stored at minus 20°C for up to several weeks. To determine the productivity more accurately, samples were collected from the reactor outlets over 3 hours, to give more accurate productivity results. Since more permeate was required, a slightly higher flux was used (0.188 L. m^{-2}. h^{-1}.). All the results presented below are from one experiment (different from the experiment from which figure 5.6. was generated) using a TVFBR in Reactor 2 configuration.

The enzyme production profiles are given for MnP in figure 5.9, and LiP in figure 5.11. These are discussed individually in subsequent sections. The outlet NI-1_4^+ and glucose concentrations were also measured to determine the extent of the nutrient gradients. Figure 5.7. shows NH_4^+ concentrations and figure 5.8. shows glucose concentrations.

From figure 5.7. it can be seen that NH_4^+ -limitation occurred , since the permeate NH_4^+ concentration ranged from 0.02 — 0.04 mg. I-[1]. This represents an 80 —90% depletion of the growth medium NH_4^+ which was 0.2 mg. $L.^{-1}$. It is not clear whether the NH_4^+ measured in the permeate was simply unassimilated NH_4^+or released NH_4^+ since it has been shown that NI-1_4^+ is released into the growth medium by lysed mycelia (Bonnarme *et al.* 1993). Glucose appeared not to be growth limiting, except when pure oxygen was supplied at a high flow rate.

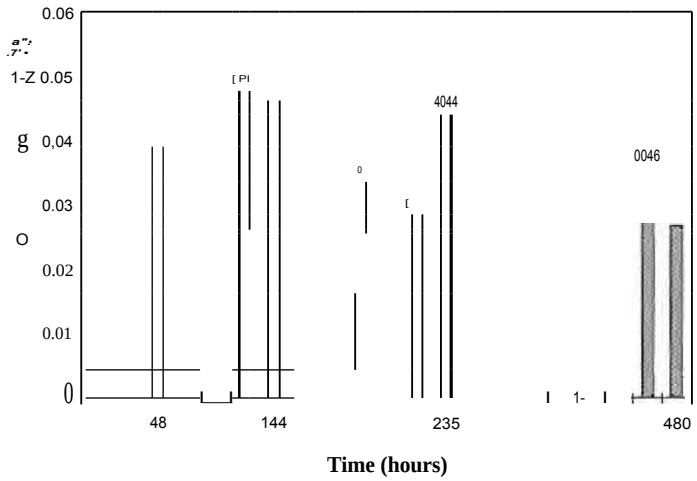


Fig. 5.7: NH_4^+ concentration in the reactor outlet. Inlet concentration was 0.2 mg.$L.^{4}$

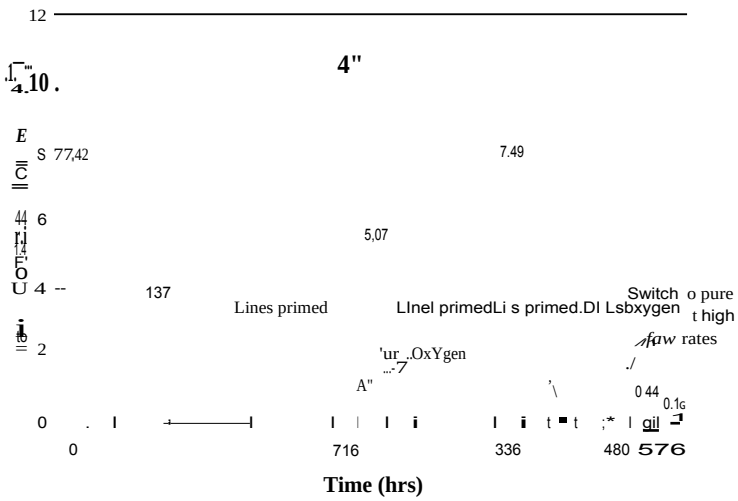


Fig. 5.8: Glucose concentration in the reactor outlet. Inlet concentration was 10 $mg.L.^{-1}$ Au increase in glucose concentration can be seen after the lines were primed because of an increase in flux. "Pure Oxygen" indicates a switch to pure oxygen instead of air as the oxygen source.

5.3.5. MnP Production in Air

Daily production of MnP for the first week of operation with air supplied as the 0_2 source is shown in figure 5.9. Despite the arbitrarily chosen flux and air flow rates, the amount of MnP produced was surprisingly high, especially considering that air was used and not pure O_2, which is used in virtually every other production reported. It was reported that the production of peroxidases in bioreactors using air is low or undetectable (Laugero *el al* 1996, and references therein).

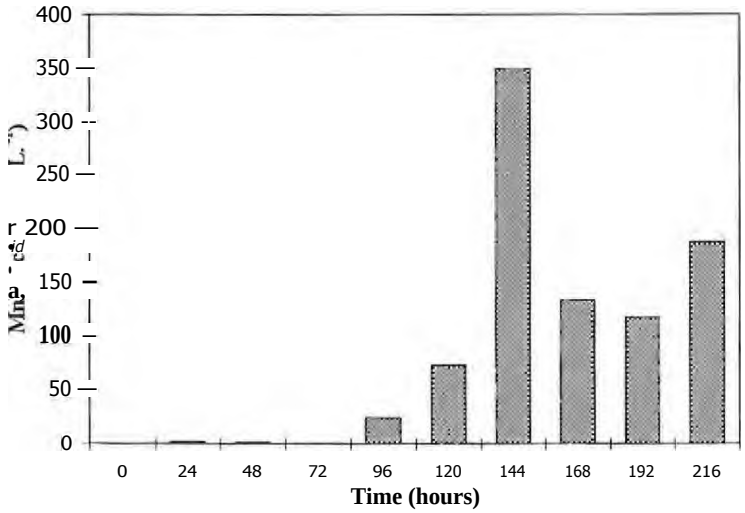


Figure 5.9: MnP production over the first 10 days of operation

5.3.6. MnP Production with Pure 02

To determine the response of the system to oxygen, pure **02** was used to replace air at the same approximate flow rate. After several days of continuous operation with air as the 0_2 source, the aquarium pump, providing air to the system, was replaced with a pure Or containing gas cylinder. The improvement in enzyme yield can clearly be seen from figure 5.10. The enzyme concentration rose to 900 U. $L.^{-1}$, which is competitive with other systems, even those with improved expensive medium additives and optimised operational systems. A comparison with the most competitive systems thus far reported is shown in table 5.2.

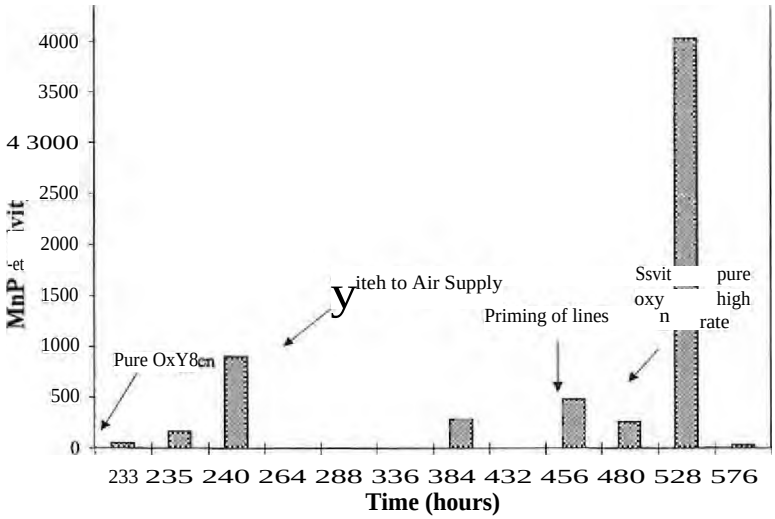


Figure 5.10: Impact of oxygen supply on MnP production.

The aim of the experiment using pure 0_2 supply was a characterisation exercise to determine whether the difference in productivity due to 0_2 in air supply was based on physiological criteria (as described in Dosoretz *et al.* 1990; Zitomer and Lowry 1992) or on an engineering component affecting o_2 mass transfer rates.

Based on the speed of the response, it was concluded that the effect was due to 0_2 mass-transfer improvement. To extend this hypothesis, the o_2 supply was again swapped with air supply, resulting in a sudden drop in productivity, providing further support for this conclusion. Since the drop in productivity was most probably also strongly influenced by the fluid level drop effect, the growth medium line was primed and pure 0_2 was again supplied, but this time at a much higher flow rate (approximately 4-fold). The MnP concentration then rose to a very high level of 4 000 U. $L.^{-1}$, making this the highest transient productivity thus far reported (see table 5.2.). Although this is very encouraging, it needs to be seen whether productivity in this range can be maintained for extended periods, and it is anticipated that a considerable effort needs to be exerted to accomplish this due to fluid flow maldistribution problems.

A comparison of MnP average productivity and peak productivity and other reported systems using the standard Tien and Kirk (1988) media is given in table 5.2. This system out-performs conventional flask cultures and several other systems. The consideration that this system is un-optimised, uses air as an 0_2 supply as opposed to the standard pure $O_{2,}$ excludes the use of VA or any other expensive additives and allows continuous operation

shows its potential. Moreira *et al.* (1997) claimed that their system showed the highest productivity for MnP yet reported. These authors used essentially the same growth medium, except that this was optimised in terms of Mn^2 concentration (5000 µM), 0_2 supply, and operational characterisation (flow regime etc.). Hence, this is a fair comparison in terms of peak production (their system did maintain a steady-state, but showed inexplicable peak behaviour, probably also due to fluid flow irregularities).

Table 5.2: Comparison of the performance peaks of the MGR with the best literature reported productivities

Description	MnP Concentration (LI/L.)	Productivity (U.C.day^{-1})	Reference
Bubble column/ batch	**726[d] (1 277)[2]**	**181[d] (318)[2]**	**Laugero** *et al.* **(1996)**
Packed bed /continuous	**250**	202	**Moreira** *et al.* (1997)
Air (peak)	**205[b] (350 ± 23.3)[2]**	**166[b] (283)[2]**	**This work**
Pure 0_2 (peak)	**526[b] (900 ± 141.4)[b]**	**427 (731)[2]**	**This work**
Pure 0_2 . high flow (peak)	**2 361[b] (4038 ± 35.4)[2]**	**1916 (3278)[2]**	**This work**

'values re-normalised to DMP assay from phenol red according to section 5.2.6. ARTS assay values normalised to DMP assay values. `Original ABTS values (recorded at 18°C). [d]productivities normalised according to Moreira *et al.* **(1997) as described in section 5.2.6.**

5.3. *7. LIP Production*

Figure 5.**11 shows LiP production to be disappointingly low, especially when compared** to that obtained for the single **fibre mini-reactors.**

When LiP production in pure 0_2 was compared, the difference between LiP and MnP **productivity was clearly noticeable, and it can be said that the MGR system favours the production of MnP. Unfortunately, MnP** was not measured on the single fibre reactor, but the difference in LiP productivity between the **2** systems was most probably due to differences in 0_2 mass transfer between the single fibre and multi-fibre units, and the exclusion of veratryl alcohol from the growth medium in the **TVFBR. It is known** that veratryl alcohol not only stimulates production of **UP, but also stabilises it by controlling**

oxidation of LiP to LiP 111, an inactive form, by $}1_2O_2$. Zhao *et al.* (1996) observed that no LiP production occurs on solid substrates without the addition of veratryl alcohol.

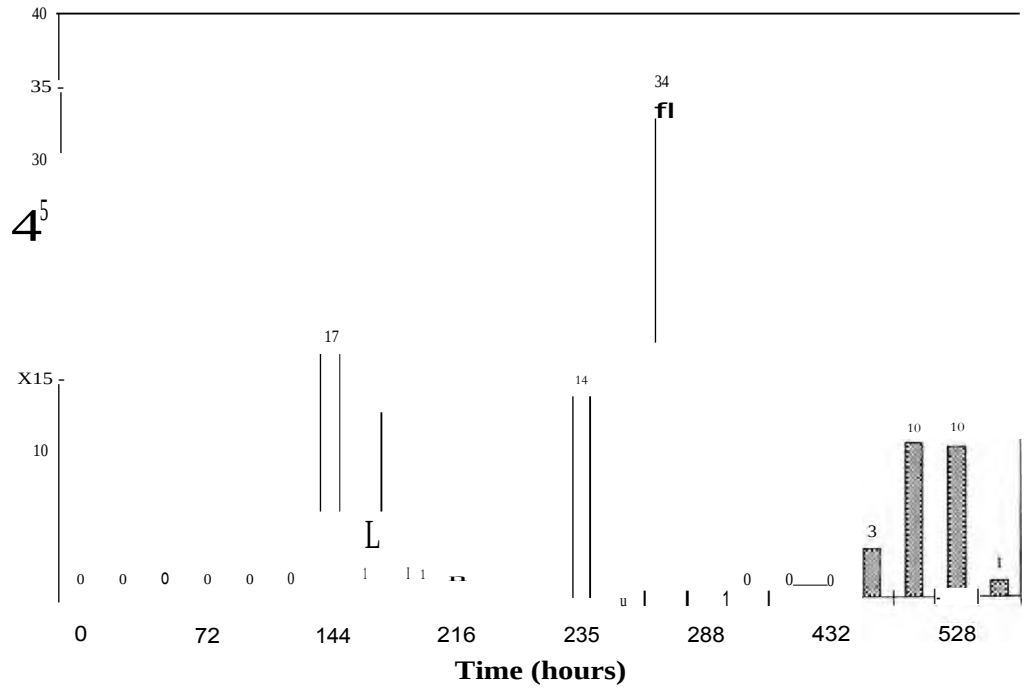


Figure 5.11: LiP production during over the same period as described above for MnP (same experiment). Values reported are rounded off to the nearest integer value. Hence, it can be seen again that LiP activity appeared on day 2. The cyclical nature of the production was due to the flow maldistribution problem.

The role of oxygen supply in the determination of enzyme profiles could also be mediated through glucose metabolism. MnP is produced earlier than LiP in a typical batch culture. Thus, depending on the extent of the nutrient gradient, LiP production could be limited. This view is supported by the observation that at lower dilution rate or longer hydraulic retention times in the biofilm (when flux is lower as in the case of the experiment from which figure 5.6. was derived), LiP production was improved. This gave a peak LiP concentration of 42 U. L.^{4} (figure 5.6.) with air supply while the higher flux operation (figure 5.11) gave 16 U. L.$^{-1}$ with air supply and 34 U. L.$^{-'}$ with pure oxygen supply. A reason for this is provided by Feijoo *et aL* (1995), who, in optimising the nutrient supply of their system, found that a residual glucose concentration in their reactor outlet of 1 g. L.$^{-1}$ shifted metabolism towards growth, inhibiting LiP activity. 0 g. L. of residual glucose led to LiP instability due to induced protease activity, thus a compromise of 0.5 g. L.$^{-1}$ was used. The range of glucose concentrations in the reactor outlet (figure 5.8.) showed that the former

(high glucose concentration in the permeate) was the case in the TVFBR system. Since glucose metabolism is strongly influenced by oxygen availability, this might account for the profound differences between the single-fibre reactor and the TVFBR.

The difference between MnP production and LiP production can be also explained by published observations. In initial flask studies it was shown that LiP is the dominant enzyme produced, later studies have shown that in its natural environment, i.e. in wood, *P. chtysosporium* produces more MnP than LiP. It is also becoming evident that MnP plays a more vital role in the initial events of wood degradation than was originally thought (Evans *et al* 1991). Thus, if the MGR truly resembles the natural environment of the fungus, then it is not surprising that the enzyme production profile would be similar. It has also been shown in studies of LiP localisation in wood that LiP was retained within the biofilm (Evans *et al.* 1991). MnP is believed to be responsible for degradation of wood over a long range and LiP is proposed to deal with primary degradation fragments (Ruel and Joseleau 1991). It would be expected that MnP is secreted and LiP is retained by the biofilm, presumably by the polysaccharide matrix. This supports cytochemical observations of the MGR biofilm (chapter 4).

5.3.8. Effect of Nutrient Sufficiency

Dosoretz *et al* (1993) initially showed that dramatic improvements in LiP production could be obtained by using excess NH_4^- (45 to 60mM as opposed to 2.4mM in N-deficient cultures). This led to more rapid carbon depletion. They showed 6 to 8 times higher LiP production and 2 to 3 times lower MnP activity under excess nutrient conditions compared with limiting nutrient conditions. To determine whether this was the case in the MGR, the medium was changed to a concentration of 24 mM ammonium tartrate. When this was done, excess biomass was produced due to the increased nutrient load This led to rapid clogging of the system, as can be seen in figure 5.14, and 5.15.

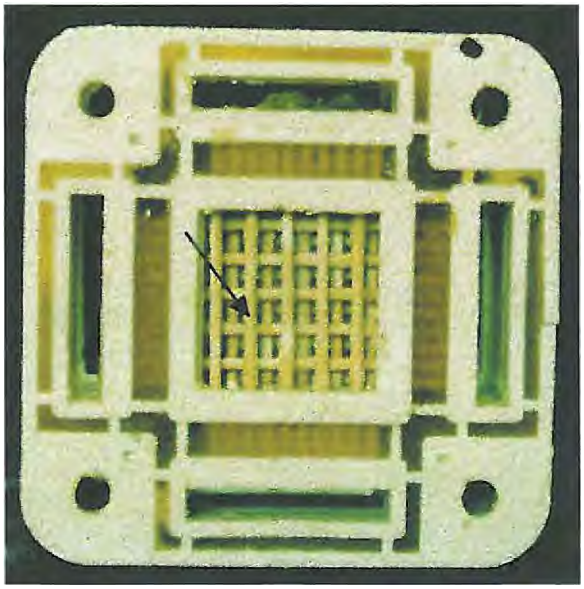


Figure 5.12: Top of the uppermost TVF module showing a thin biofilm surrounding the membranes. Arrow indicates the biofilm.

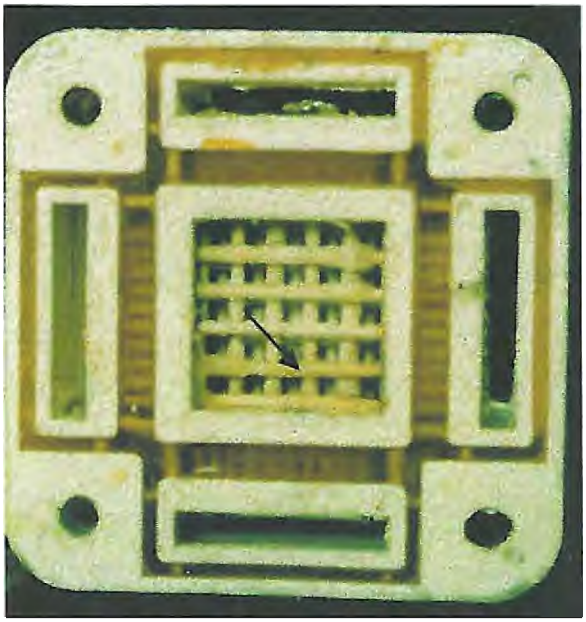


Figure 5.13: Bottom of the uppermost TVF module showing a thicker biofilm.

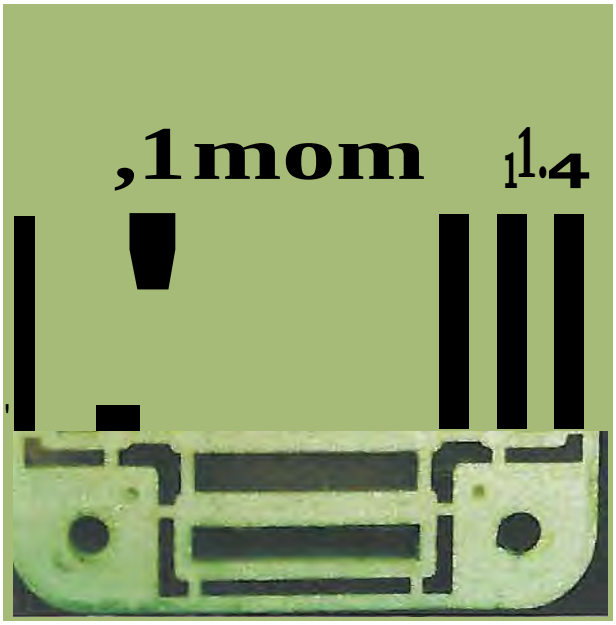


Figure 5.14: Top of the lower TVF module showing an even thicker biofilm due to the increased nutrient flux experienced.

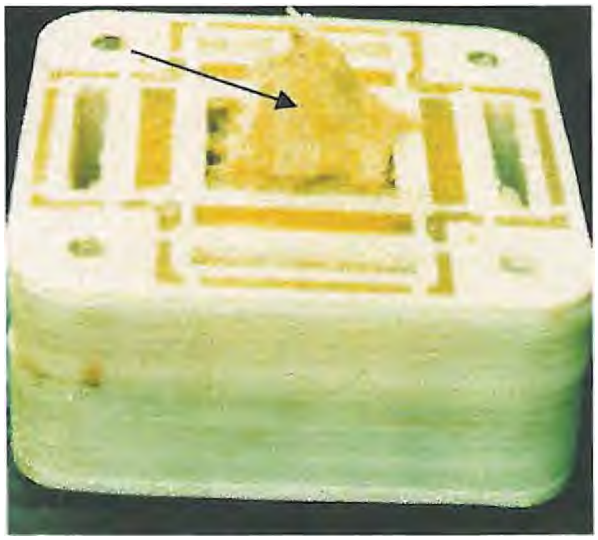


Figure 5.15: Bottom of the lower module showing accumulated dead biomass.

This result shows that the biofilm thickness is controlled by nutrient supply and not shear, and proves that the biofilm was N-limited, since glucose concentration was not changed. It was also observed that the growth medium changed to a dark brown colour. This interfered with enzyme assays. It was expected, though, that little or no enzyme production would occur during the transition period (Dosoretz *et al.* 1993) until re-differentiation occured. Bacterial contamination in the biomass compartment was also observed at this stage for the

first time. This supports the model of a defensive growth form around the capillary wall, in nutrient-limited conditions (chapter 4).

5.4. ***CONCLUSION***

The results in this chapter show that ligninolytic enzyme production could be obtained by the establishment of nutrient gradients across a biofilm of P. *chrysosporium* according to the MGR concept.

The production of enzymes could be maintained for extended periods, and the productivity was surprisingly high, considering the arbitrarily chosen operational parameters. However, due to the problem of static pressure, flow maldistribution occurred, preventing true steady-state operation, which is considered to be an important requirement of this system. Several attempts to improve this by experimenting with different operating conditions did not provide significant improvement, or any new insights about the process.

At this stage, improvements in the fluid dynamics of the system were the paramount requirement for the full development of the potential of this system. Hence, an engineering approach to module re-design was considered the best strategy for further development of the process. The initial indication of the superior performance of this system presented in this chapter has justified this further development. The MnP productivity of this system already exceeds the enzyme production range of the best systems available at present. However, engineering practise normally dictates that costs and benefits of different reactor systems can only be compared from pilot scale studies. Laboratory-scale studies tend only to show the potentials of a system, which was the objective of this chapter.

CHAPTER 6

APPLICATION OF THE MEMBRANE GRADOSTAT REACTOR TO BIOREMEDIATION

6.1. INTRODUCTION

Two approaches can be followed in bioremediation using the ligninolytic systems of *P. chtysosporium:* either whole organism treatments, or use of the enzymes produced.

6.1.1. Use of Ligninolytic Enzymes for Bioremediation of Aromatic Pollutants

There are two major advantages in the use of enzyme treatments: high concentrations of toxic pollutants (above the lethal dose for the fungus) can be dealt with, and the ability of the enzymes to function in organic solvents, with which several phenolic effluents are associated (Popp *et al.* 1991; Vasquez - Duhalt 1994; Yoshida *et al.* 1996).

Extracts of lignin degrading enzymes have been proposed for commercial applications such as biomechanical pulping of wood and waste treatment. Advances have been made in the production of these enzymes, and since they are an extra-cellular product, purification is not expected to be difficult, if at all necessary (Hu *et al.* 1993). Ligninolytic enzymes have been shown to be applicable to de-toxification of phenolic effluents (Bollag *et al.* 1988). Such treatments were shown to be potentially economically feasible with the use of the similar plant peroxidase, BRP, in the case of certain industrial effluents (Nicell *et al.* 1992; Cooper and Nicell 1996). HRP has similar degradation properties to LiP and MnP, but is more stable. The technology for the use of this enzyme is well established (Buchanan and Nicell 1997) and could easily be applied to the fungal peroxidases.

The use of fungal enzyme extracts for degradation, transformation, and/or detoxification of aromatic pollutants have been demonstrated on laboratory-scale only (Schmall *et al.*

1989; Tuisel *et al.* 1990; Valli *et aL* 1992; Koduri *et al.* 1996). These enzymes are best suited to applications where they either remove pollutants by polymerisation, or detoxify them by humification or transformation, since they are not capable of complete mineralisation (Popp *et al.* 1991; Valli *et al.* 1992;). The transformation of pollutant molecules to less toxic compounds (Stahl and Aust 1993a) as a pre-treatment to anaerobic digestion seems a very attractive approach (Carberry and Kovach 1992). Pump-and-treat systems for soil bioremediation also appears to have potential (Britto *et al.* 1996) since the use of *P. chrysosporium* in soil is hampered by various soil factors such as pH, temperature, high moisture content (Okeke *et at.* 1996), competition for resources with autochthonous organisms (Ali and Wainwright 1994; Tucker *et al.* 1995), lack of oxygen (Legtan *et al.* 1996; Pfender *et al.* 1997) and toxic levels of pollutants (Spiker *et al.* 1992; Sayadi and Ellouz 1992; Tucker *et al.* 1995; Bogan *et al.* 1996b).

The de-toxified soil washout can then be returned to the soil for further mineralisation by autochthonous bacteria (Brodkorb and Legge 1993), or anaerobic digestion and activated sludge systems (Sayadi and Ellouz 1992; Carberry and Kovach 1992).

There are, however, severe limitations in the use of enzymes, which makes the investigation of whole organism treatments feasible. These are: instability of the enzyme (Tuisel *el al.* 1990; Hu *et al.* 1993; Chung and Aust 1995); requirements for co-factors for activity, viz. H_2O_2, Mn^{2+} and chelating agents such as oxalate for MnP action (Zapanta and Tien 1997) and inactivation of the enzyme by certain substances (Koduri *et al.* 1996).

6.1.2. Whole Organism Bioremediation Using the WRF

Mineralisation of certain compounds occurs under ligninolytic and non-ligninolytic conditions. It was initially shown that the ligninolytic enzymes are responsible for the initial transformation of aromatic pollutants, and that these pollutants are then internalised by the biomass, where they are mineralised (Chang and Bumpus 1993; Sayadi and Odier 1995; Brock *et al.* 1995). Armenante *et al.* (1994), stated that both

ligninolytic enzymes and fungal biomass are required for the degradation of certain pollutants. Subsequent work has shown the non-involvement of enzymes in the degradation of certain pollutants (Bumpus and Brock 1988; Yadav and Reddy 1992; Yadav and Reddy 1993; Yadav *et al.* 1995). This was shown to be the case since degradation of these compounds occurred in nutrient sufficient cultures, where the ligninolytic enzymes were not induced (Dhawale *et al.* 1992; Yadav *et al.* 1995). Further evidence came from bioremediation studies with mutants which lacked LiP and MnP activity entirely (Yadav and Reddy 1992).

The biomass-based degradation has been shown to be due to reduction of compounds by establishment of trans-membrane redox potentials due to proton export by the fungus. This has been shown to be the case in the reduction of highly oxidised compounds such as TNT and its impact is directly proportional to the amount of active biomass present (Stahl and Aust 1993a, b; Stahl and Aust 1995), This shows that other mechanisms for detoxification of aromatic pollutants by the biomass of *P. elvysosporiurn* exist. Other possibilities include the presence of as yet unknown enzymes (Thomas *et al.* 1992; Brock *et at* 1995; Badkoubi *et al.* 1996) or the use of intra-cellular membrane-bound enzymes (Forney *et aL* 1982; Thomas *et aL* 1992; Barclay *et at* 1995; Brock *et at* 1995).

Initial degradation of certain compounds has also been shown to be catalysed by lipid peroxides in the membrane wall (Bogan and Lamar 1995). This peroxidation is initiated by MnP action (Bogan *et aL* 1996a), It has been shown that adsorption of the aromatic compounds to biomass accounts for the initial rapid removal of these pollutants from solution and plays an important role in removal efficiency (Dietrich *et aL* 1995; Badkoubi *et al.* 1996; Jaspers and Pennickx 1996). This results from an increase in concentration of the pollutant in the vicinity of the biomass-associated MnP and LiP as well as other degradative mechanisms (Wolfaardt *et aL* 1995; Barclay *et al.* 1995). In fact, adsorption to the support matrix would further enhance this phenomenon. Lin *et al.* (1991) showed the superiority of a system on which the degradative biofilm and enzymes were co-immobilised onto an adsorbent support matrix (in their case, activated charcoal).

6.1.3. Reactor Systems for Continuous Treatment of Aromatic Pollutants by WRF

Few studies have reported the use of *P. chrysosporium-based* reactor systems for waste treatment (Venkatadri and Irvine 1993). A typical example is the rotating biological contactor (RBC) described by Joyce (1984, cited in Venkatadri and Irvine 1993) for the treatment of bleach plant effluents. The process involved a growth period followed by a decolourisation period, which lasted only a limited time (5-7 days). After treatment, the whole process needed to be restarted.

Since then several more sophisticated systems have been developed, involving mostly RBCs or packed bed reactors with various support materials. As is the case for enzyme production, polyurethane foam provides a good support for pollutant degradation (Ruckenstein and Wang 1994 and references therein). Lewandowski *et aL* (1990) tested various nutrient media and reactor configurations and subjected these to engineering analysis to provide reactor design criteria for bioremediation. Pal *et al.* (1995) have provided an excellent process model to optimise the amount of enzyme and biomass required in a system, and what the co-substrate requirements would be for a required performance. In summary, reactor systems for bioremediation using the WRF have been found to lack :

- Consistent production of high levels of ligninolytic enzymes;
- Steady-state continuous enzyme production and fungal activity over extended periods of time (Pal *et aL* 1995). Sufficient nutrients must be supplied to sustain viability;
- Controlled growth of the fungus. A high biomass density with good viability needs to be maintained if good throughputs are to be expected. This requirement arises out of the adsorption surface, and lower chemical dose (g pollutant. g. mycelium^{-}) since pollutant toxicity and degradation capacity are more tightly correlated to chemical dose than to solution concentration of the pollutant (Alleman *et al.* 1995);
- Criteria for convenient scale-up to large-scale waste water treatment;
- A low shear stress environment.

The membrane gradostat reactor system has the potential to satisfy all of the above criteria (see chapters land 5). The biofilm reactor of Venkatadri *et al.* (1992) appeared to fulfil these requirements as well, strengthening the case for the MGR.

6.1.4. Biorernediation Using the Membrane Gradostat

It was considered worthwhile to investigate the potential of the membrane gradostat reactor as a bioremediation system for potential use either in effluent treatment or in pump-and-treat systems for soil remediation, based on the advantages of using biomass and useful features inherent to the membrane gradostat system.

The membrane support matrix used in the MGR can be considered a good adsorbent for the aromatic compounds. The utility of membranes for extraction of such pollutants is well researched (Pakhania *et al.* 1994; Brindle and Stephenson 1996). The spatial differentiation could also play a role, since it has been shown by Jaspers and Penninckx (1996) that bleach plant effluent is most effectively treated when *P. ehlysosporium* was present in the form of a "fluffy, pelleted material".

The presence of both "primary" and "secondary" growth phase biomass could be an advantage. Different metabolic pathways are utilised for the degradation of phenanthrene under ligninolytic and non-ligninolytic conditions (Tatarko and Bumpus 1993; and references therein). LiP was shown to transform intermediates from the one pathway to intermediates utilisable by the other, thereby linking the two pathways (Tatarko and Bumpus 1993). The MGR, therefore, provides interesting possibilities since it was shown (chapter 4) that the biofilm consists of biomass which is in both primary and stationary phase, and that the thallus behaves as a co-ordinated unit, with linkage between primary and secondary metabolic pathways.

Thus, it was considered worthwhile to obtain a preliminary evaluation of the MGR applied to the treatment of aromatic pollutants. Its possible unique advantages over conventional systems could be summarised as a high density biofilm consisting of actively growing, idiophasic and otherwise differentiated biomass associated with a conglomerate of degradative enzymes and a support matrix with good adsorbent properties. Other attributes are high oxygen mass-transfer due to direct contact with air

streams and relatively low moisture content in comparison to submerged cultures. Both of these parameters have been shown to be an advantage to the bioremediation capacity of *P. chrysosporium* (Okeke *et al.* 1996).

6.1,5. Objectives

The MGR concept needed to be evaluated for its potential application to bioremediation of aromatic pollutants at an early stage of its development, since this is the anticipated primary application of the technology. Experiments with single fibre mini-reactors (results not shown) indicated that transmembrane flux was the main factor affecting performance of the system.

The aim of this experiment was to operate the reactor in recirculation mode (the anticipated mode of operation in an industrial situation, for this application) using growth medium spiked with 100 mg. $L.^{-1}$ of p-cresol. The effect of the changes in flux which normally occur with this type of operational mode were correlated to reactor performance. This transient state experimental approach was chosen above the steady-state approach since this would avoid long term biofilm changes (such as changes in biofilm density and thickness) in response to changes in flux.

6.2. MATERIALS AND METHODS

6.2.1. Culture and Growth Medium.

P. chrysosporium DSM 1556 (equivalent to ATCC 34541) was used for this study and prepared as in Appendix A.

6.2.2. Reactor Operation

The membranes used were externally unskinned polysulphone capillaries (IPS 763) as described in chapter 2. Using these membranes an internal surface area (for ultrafiltration) of 0.0113 m^2 was available in a reactor volume of 36cm^3. This is the module with the densely packed membranes (figure 5.3.a). The reticulation system is depicted in figure 6.1.

Before use the reactors were sterilised and inoculated as described in section 5.2.4. Growth medium was recirculated through the reactor for four days until LiP production was observed. Thereafter p-cresol was added to fresh growth medium and recirculated through two different reactors used. The two reactors were operated as follows:

- Reactor 1: High flux. Growth medium was recirculated to provide a flux range of 0.007 to —2 $L.m^{-2}.11^{-1}$
- Reactor 2: Low Flux. Here the flux ranged from —0,01 to —0.1 $L.m^{-2}.h^{-1}$

Samples were collected from the emerging effluent as well as from the recirculating medium as a control sample from an in-line sample port (see figure 6.1.). The difference in concentration between the two samples indicated the removal of p-cresol in a single pass through the biofilm, The samples were assayed for p-cresol concentration as well as pH. In order to determine the residual effect of the enzymes produced in the reactor, samples were also taken from stored effluent of the reactor 24 hours after emergence from the reactor and assayed for p-cresol concentration. Enzyme production was not measured since it has been shown that phenols inhibit the oxidation of veratryl alcohol to veratraldehyde (Aitken *et aL* 1989).

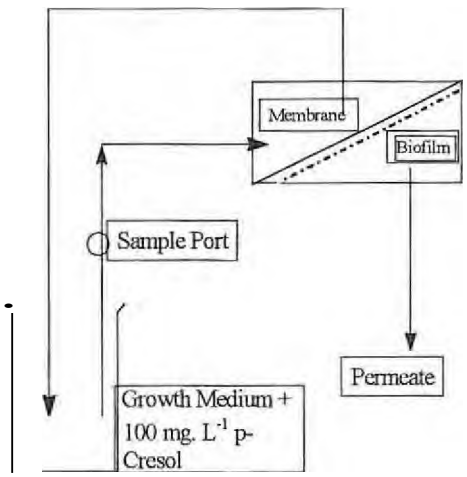


Fig. 6.1: Reticulation system of the TVFBR used for the study of the degradation of p-cresol. The reactor was operated in recirculative mode. p-Cresol concentration was measured in the permeate and from the in-line sample port.

6.2.3. Analyses

To challenge the system p-cresol was chosen because it is degraded only under ligninolytic conditions by *P. chlysosporium* (Kennes and Lema 1994). Phenols do not stabilise LiP against inactivation by H_2O_2 (Chung and Aust 1995). This is due to the inability of phenols to revert compound HI back to the ferric enzyme. This ensures the requirement for biomass which is actively producing ligninolytic enzymes in the system since the turnover of enzymes would be expected to be rapid. p-Cresol concentrations in the re-circulating medium and the treated effluent were determined by HPLC as in Appendix **H.**

Removal rates were calculated as follows:

$$\%\text{A removal} = \frac{S_{in} - S_{out}}{S_{in}} \times 100 \qquad 6,1.$$

Where Si_n = cresol concentration in the medium entering the reactor (as measured from an in-line sampling port).

S_{oot} = cresol concentration in the reactor permeate.

% Removal indicates the efficiency of the removal of the pollutant from the perfusate in a single pass through the reactor. Reactor performance was also expressed in terms of productivity, which is defined as the amount. (in mg of cresol removed) time:' membrane surface area."[1] and was calculated as follows:

$$Q.(Sout-Sin) = P \qquad 6.2.$$

Where P = the productivity of cresol removed [mg. cresol removed. (m^2 of membrane surface areal'. $hour^{-1}$].

Q = volumetric trans-membrane flux rate [$L.m^{-2}.h^{-1}$].

6.2.4. Statistical Validity of Results.

p- Cresol measurements were performed in duplicate and reported values are averages. In each case standard error was not more than 1% and so is not reported. In terms of the

validity of the trends reported, a third reactor was subsequently run to show that the ranges and trends were typical.

The reactors were operated for a total of 160 hours. p-Cresol degradation trials commenced after 96 hours of operation by which time a suitably differentiated biofilm would have established. The expected flux changes would cause transient state behaviour, so the experiment was executed over a relatively short time so that large changes in biofilm thickness could be avoided.

*6.3. **RESULTS AND DISCUSSION***

6.3.1. Reactor Performance

The efficiency of the two reactors is shown in figure 6.2A and B.

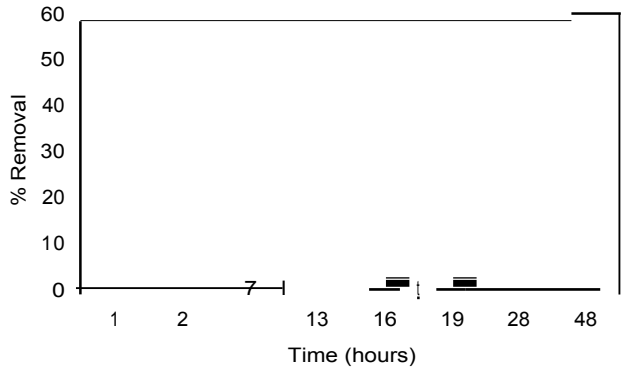


Fig. 6.2 A: History of Reactor I (high flux) in terms of efficiency, which is the % of pollutant removed in a single pass through the reactor. The arrow indicates when the growth medium reservoir was changed.

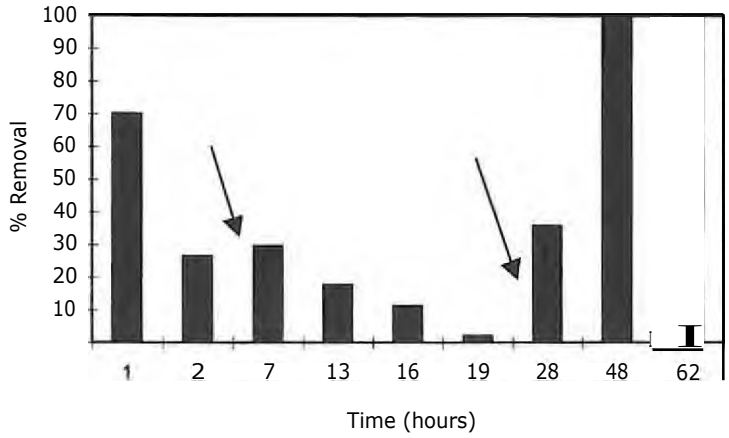


Fig. 6.2 B:History of Reactor 2 (Low flux) in terms of efficiency for the duration of the experiment. Arrows indicate when the growth medium reservoir was changed.

The maximum efficiency ofp-cresol degradation achieved in Reactor 1 (figure 6.2A) was 52 % removal in a single pass through the reactor. This efficiency was maintained for approximately 6 hours before a sharp decrease in efficiency was observed. It was also observed that the maximum efficiency was achieved shortly after fresh growth medium was supplied. Similar trends could be observed in Reactor 2 (figure 6.2B). Maximum cresol removal efficiency of 100% was maintained for approximately 20 hours in this case, was achieved shortly after a medium change. Reactor productivities over the duration of the experiment are depicted in figures 6.3. and 6.4.

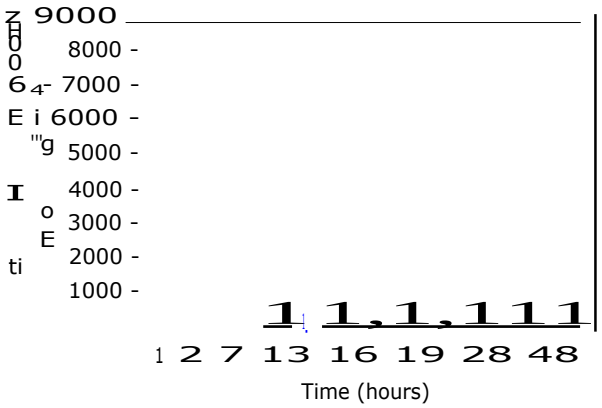


Fig. 6.3: The productivity history of Reactor 1. Productivity relates to the rate of pollutant **removal and** was calculated according to equation 6.2.

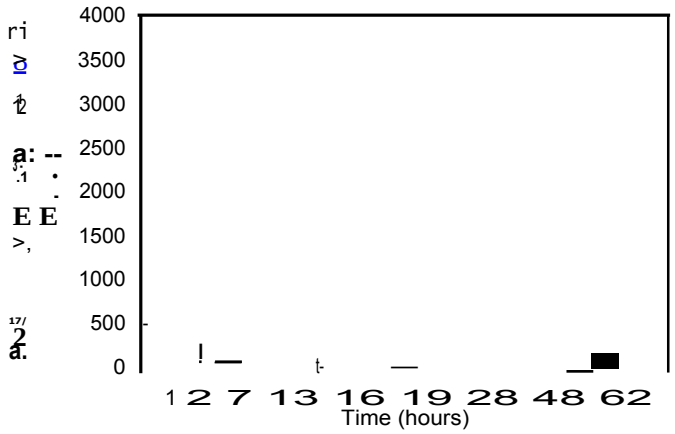


Fig. 6.4:Reactor 2 productivity history. The productivity of this reactor, operated at lower flux, was found to be generally poorer than that of Reactor 1.

An initial lag period in productivity could be observed in figure 6.3. This was followed by a period of rapid increase in productivity over time which contrasts with the bioreactor efficiency results. A similar trend was observable in Reactor 2 (figure 6.4). In this case the bioreactor productivity showed a similar trend to bioreactor efficiency in that the supply of fresh medium had an enhancing effect on reactor performance. Since these responses were transient one can conclude that the biofilm was not in steady-state, but was constantly changing as a result of changes in the feed medium. The changes in the feed medium most likely occurred through back diffusion of waste products into the recirculating medium.

6.3.2. Removal of p-Cresol by Residual Enzyme Activity in the Reactor Effluent

Samples of the effluent from Reactor 2 which were allowed to stand for 24 h. showed no trace of p-cresol indicating that the ligninolytic enzymes and/or the biomass washed out of the reactor were still active in the removal of p-cresol. This suggests the possibility of use of the reactor permeate for contact with an effluent, rather than direct contact between harsh effluents and the biomass.

Further reductions in cresol concentrations of 20-30% were observed in the effluent of Reactor 1 after 24hrs. Although this still leaves a significant amount of cresol remaining, this is a high throughput reactor, so the reactor effluent could easily be recycled **until** complete removal was achieved. Since p-cresol has been shown to be completely

removed by, and only in the presence of, the ligninolytic enzymes of *P. chrysosporium* (Kennes and Lema 1994), it could be postulated that a greater ligninolytic enzyme concentration could have been present in the low flux reactor,

6.3.3. Factors Affecting Bioreactor Performance.

Effect of Flux

The medium re-circulation rates and pressures of both reactors were not changed but the flux was allowed to change on its own throughout the experiment so that the effects of different trans-membrane flux rates could be determined. This constantly changing flux was an inherent fault in the operating procedure of these reactors and is amplified in changes in biofilm density resulting from changes in flux. This, in turn, affects the flux, providing a complex system. Figure 6.5. shows the flux changes observed over time during the experiment

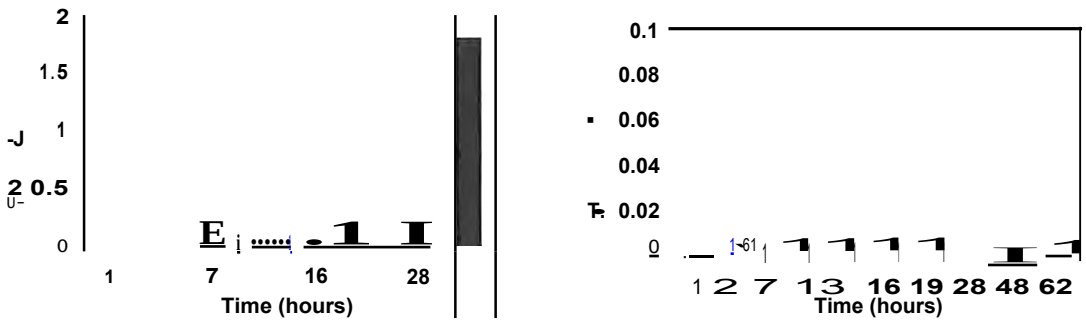


Fig. 6.5:Flux history of Reactor 1 (6.5A) and Reactor 2 (6.5B). The flux was allowed to change during the experiment so that the effects of the flux changes could be quantified.

From figure 6.5, noticeable flux increases were observed in both reactors. The reason for these flux changes are not known. The effect of different flux rates on bioreactor performance is shown in figure 6.6. for Reactorsl and 2.

Figure 6.6.(A) shows a trend in reactor efficiency for Reactor 1 which increased with increasing flux up to ± 0.21 $L.rri^{-2}.hr^{-1}$. Thereafter a non-linear drop in p-cresol removal was observed with increasing flux. This trend was not observable in Reactor 2 (figure. 6.6.B). This implies that some operating parameter other than flux had an influence on degradation efficiency.

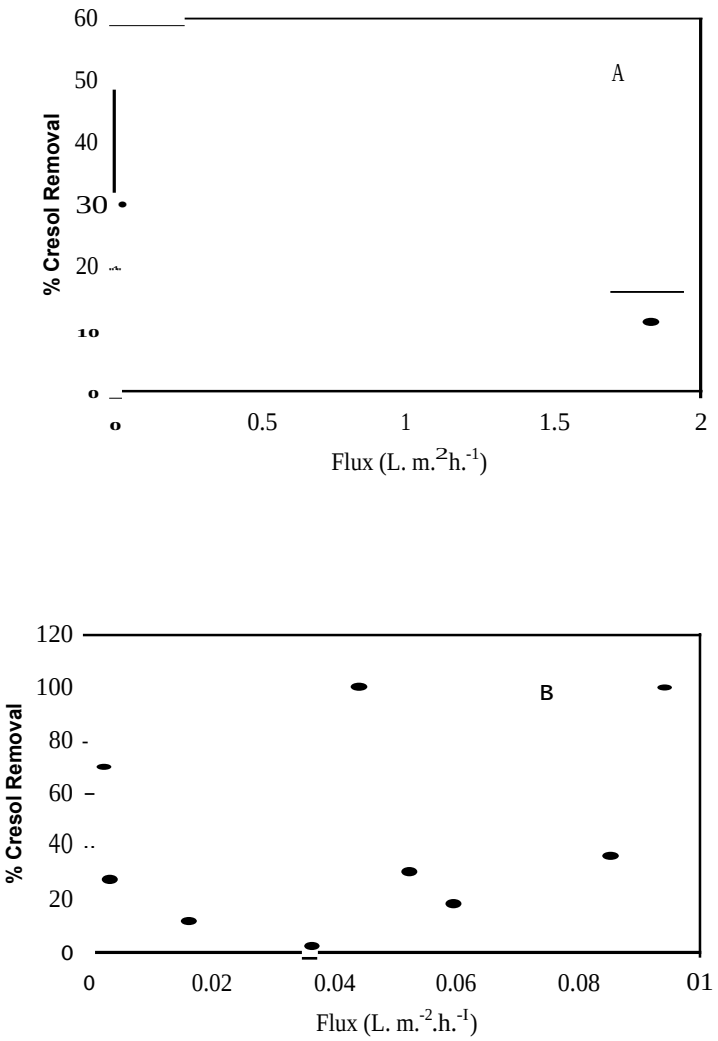


Fig. 6.6. A: Effect of flux on Reactor I efficiency. A trend can be seen here in that efficiency increases with flux up to 0.21 L tn.$^{-2}$.h.$^{-1}$. Thereafter a decrease in efficiency was observed. B- effect of flux on Reactor 2 efficiency. No clear trends could he distinguished here.

Figure 6.7. shows the effect of flux on bioreactor productivity.

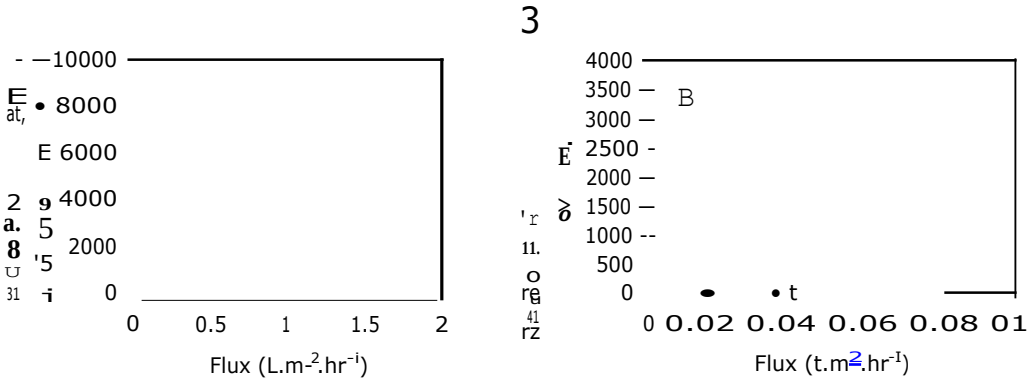


Fig. 6.7: Relationship between flux and reactor productivity. A = Reactor 1. B = Reactor 2. Clear but different trends can be observed indicating that the dominant factors affected by flux which influence reactor productivity change under different operating conditions. No attempt was made to fit the data to mathematical trends.

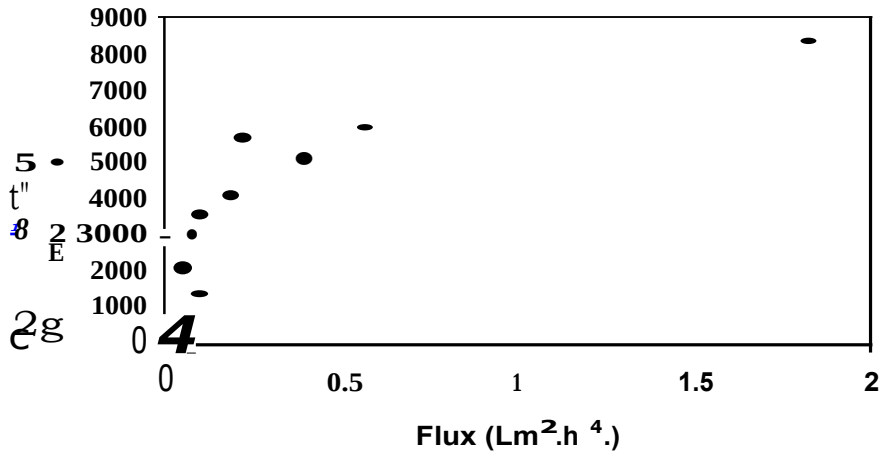


Fig. 6.8: Combined data from Reactors 1 and 2 approximates an S-shaped curve. This provides some information on the mechanism of degradation (described in text).

Combining the results from Reactors 1 and 2 (figure 6.8.) provides a clearer demonstration of the relationship between flux and reactor productivity. In Reactor 2, there appears to be an exponential increase in reactor productivity with flux, while at higher fluxes (reactor 1), this becomes a saturation curve.

The saturation-type kinetics could arise from the degradation mechanism. Several researchers have shown that in *P. chtysosporium,* for certain pollutants, the reaction mechanism is a two-stage process. The first stage was shown to be a rapid removal of the pollutant from solution by adsorption to the fungal mycelia and EPS (Cripps *et al.* 1990; Barclay *et al.* 1995; Badkoubi *et aL* 1996) and the second stage, a subsequent degradation of the adsorbed pollutants by the hyphal-associated ligninolytic systems (including peroxidase enzymes and other systems) as is the case for bacterial biofilms (Wolfaardt *et al.* 1995).

Due to the above, the importance of surface area to volume ratio of the biofilm becomes an important parameter in removal rate. Several models have been suggested for the nature of this adsorption (Barclay *et al.* 1995).

Very low productivities are attained at extremely low fluxes (< 0.05 L.m $^{-2}$.h.$^{-1}$). Peak productivity seems to be in the range of 8 000 mg.ti $^{-2}$.h.$^{-1}$ attained at flux rates of above 1.5 L.m^{-2}.h."^{1}. The relationship between flux and productivity provides valuable information on operating conditions in terms of flux and membrane surface area required to achieve a given productivity ofp-cresol removal.

Influence of Nutrient Status

It can be seen for both reactors that productivity and efficiency improve with a change to fresh growth medium. This is not suprising since it has been shown that WRF require a co-substrate for lignin and pollutant degradation (Kirk *et al.* 1976; Fernando *et al.* 1989). Kirby *et al.* (1995) report that certain aromatic compounds can be used as a sole carbon source by *P. elnysasporium* although degradation is limited in these cases. However, excessive nutrient supply would (as in the case with high flux) be expected to suppress ligninolytic activity. The impact of this can only be speculated about in the case of pollutant degradation, since such a wide array of physical and metabolic processes influence degradation concurrently. Empirical studies showed that for the batch treatment of 250 mg. L.$^{-1}$ chlorophenol over 15 days, 10 g. L.$^{-1}$ glucose and 0.2 g. L^{-1} of ammonium tartrate were required for its complete removal, with nutrient starvation leading to inferior

performance (Perez *et al.* 1997). Beside providing energy and metabolic intermediates for the biomass, glucose is also required for the production of H_2O_2, which is vital for the transformation of the pollutant.

The above is based on the assumption that the re-circulating medium changes in composition over time, which can be expected in re-circulative flow, where the membrane could perform an extractive function. It is also probable that metabolic wastes and inhibitory degradation products could be expected to diffuse into the lumen due to Starling flow phenomena resulting from the re-circulative nutrient supply regime (Patkar *et al.* 1993; Kelsey *et al.* 1990).

It was also realised that operating the reactor in constant nutrient supply mode (constant recirculation rate) would not be the ideal way to study the reactor under steady-state conditions since very little control over nutrient supply to the biofilm is possible. This is because hydraulic transport of the growth medium is mostly diffusional and, therefore, trans-membrane flux is more dependent on resistance of the membrane wall and the biofilm. Hence membrane flux under diffusional transport conditions would be more dependent on biofilm density which is affected by a number of factors.
Table 6.1. shows a comparison of the constant nutrient supply rate vs. constant flux (as used in chapter 5) operational modes.

Table 6.1: Differences between constant flux and constant **flow conditions** as **MGR** operational modes.

Criterion	Constant flow	Constant flux
Hydraulic transport mode	Mostly diffusional	Mostly convectional
Filtration mode	Cross-flow	Dead-end
Application to simultaneous ultrafiltration and transformation	Good potential (because of cross-flow)	Poor potential (vulnerable to fouling)
Biofflm dynamics	Transient state (due to changes in flux)	Mostly steady-state (because of constant flux-depends on nutrient consistency)
Application to continuous secondary metabolite production	Poor- not as predictable	Good- steady-state operation should be more easily attainable.
Application to bioremediation	Good because simultaneous ultrafiltration is possible. Effluents are inconsistent so transient state required,	Good because it allows for good control of one of the most important operational parameters viz. flux, but subject to clogging if effluent contains colloidal particles.

Effect of pH

Changes in efficiency and productivity due to changes in the environment caused by fungal metabolism was also investigated. The fungus has been shown to decrease the medium pH and raise the redox potential of the medium in the MGR (results not shown). This contrasts with several reports in literature which show that culture pH tends to increase to about pH 6 or 7 after 7 days of cultivation (Linko 1992 and references therein). In typical batch culture, the pH of the culture medium initially drops due to fungal growth and metabolism. The effect of changing medium pH was found to be complex (see figures 6.9 and 6.10).

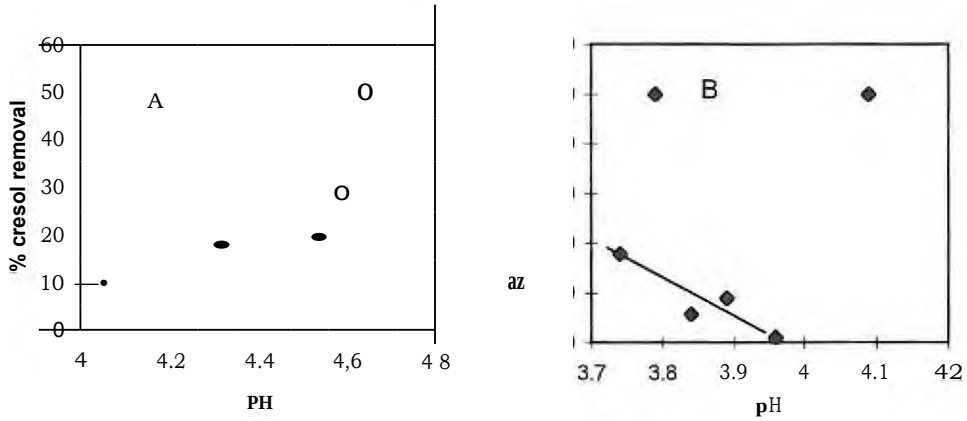


Fig. 6.9: Relationship between the pH of the re-circulating growth medium and reactor efficiency. A = Reactor 1 and B = Reactor 2. In Reactor 1 increase in pH can be correlated to an increase in efficiency. In Reactor 2, if the data from the last growth medium batch is ignored, an inverse relationship between pH and efficiency of removal can be seen (see trend line).

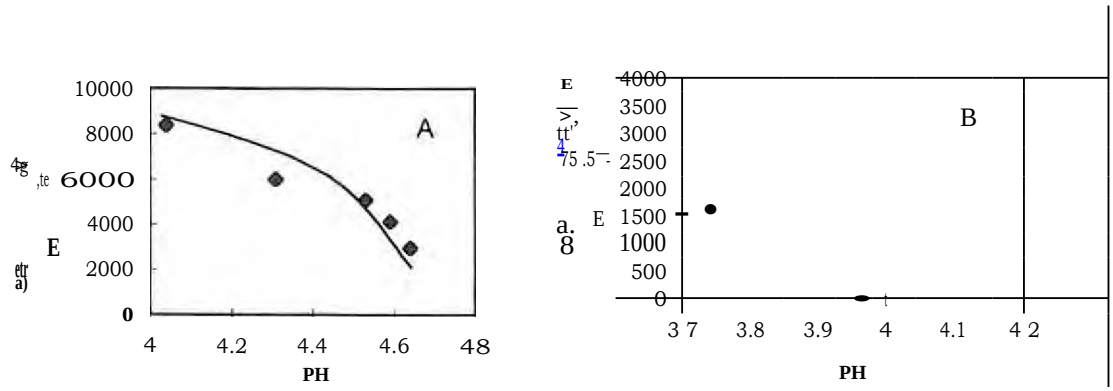


Fig. 6.10: Relationship between growth medium pH and reactor productivity. A = reactor 1. B = reactor 2. An inverse relationship between pH and productivity can be clearly seen for Reactor 1. Careful consideration of the data presented for Reactor 2 indicates an inverse relationship between pH and productivity if the data from different nutrient batches are considered separately.

It can be seen from the productivity studies for Reactors 1 and 2 that the productivity was inversely related to pH of the reactors over the range measured. If the change in productivity is a result of the change in pH, the results can be described in terms of the pH optima of the ligninolytic enzymes and the biomass. The pH optimum for LiP activity is 2.5 and MnP is 3.5, while that of the biomass is 4.5 (Tapia and Vicuna 1995). Hence, at lower pH, the enzymes would be active and the biomass less active, while the inverse would apply at higher pH. The system would be expected to be more complex, though, since it has been shown that higher phenol degradation rates occur at pH 4.75 than at pH

3, while 2-chlorophenol showed greater removal at pH 3 than at 4.75. This occurs because although initial rates of target compound removal might be higher at pH 3, overall removal depends on the useful life of the enzyme (Aitken *et al.* 1989), these being more stable at higher pH. Also, adsorption to biomass by aromatic pollutants is more rapid at low pH due to the lower solubility and greater affinity for biomass (Badkoubi *et al.* 1996),

6.3.4. Mathematical Description of Process Performance

Few models for the description of biodegradative processes using *P. chiy.sosporium* have been reported with the objective of process description for improved performance and economics (Lin *et al.* 1990).

A structured model with few lumped parameters is provided by Lin *et al. (1990)* for the degradation of PCP. This model takes into account 2 pathways for degradation: a pathway which includes the initial degradation of pollutants by extra-cellular enzymes to certain intermediates, followed by mineralisation of these intermediates by the biofilm. The second pathway involves direct mineralisation of the PCP by hyphal-associated processes. The model includes the parameters and conditions influencing each pathway, including co-factor requirements, oxygen demand and enzyme concentrations. Such a model is unachievable for the MGR process at this stage.

A "black box" approach using unstructured models according to Pal *et al* (1995), Alleman *et at* (1995) and Lewandowski *et al.* (1990) is more appropriate at this stage, since the MGR system is highly complex, unknown and approximates steady-state.

Two criteria were used to evaluate the performance of this reactor as a function of certain operational parameters. These are efficiency and productivity. These criteria are, however, not suitable for comparison of different reactor configurations. Lewandowski *et al* (1990) provided a convenient steady-state performance model which they claimed was the first reported Michaelis-Menten kinetics-based design parameters available in

literature for treating hazardous waste. The model is based on a plug-flow packed bed reactor, operated at steady-state with a 2-phase production/regeneration cycle. They claim steady-state conditions since no growth was expected during the enzyme induction phase. A standard mass balance was written for the plug-flow reactor as :

$$Q.S.\ Q.(S+dS) + rAV — 0 \qquad 6.3.$$

Michaelis-Menten kinetics were reported as:

$$r_e = \frac{V_{max} \cdot S}{K1+s} \qquad 6.4.$$

On integration:

$$\frac{K r_a}{max} \quad S1 \quad 1 \qquad 6.5.$$

Where r, = reaction rate.

Q = volumetric flow rate.

S = pollutant concentration.

V = reactor volume.

V_{nia},, and lc, = Michaelis-Menten parameters.

This approach was adapted for use in the MGR, since plug-flow conditions prevail and steady-state biofilm thickness and enzyme concentration are assumed. In addition re-differentiation of the biomass was assumed to be minimal over the time period of operation and the flux range for each reactor was small enough to prevent major changes.

The regression was, however, performed differently for the MGR data. Lewandowski *et al.* (1990), assumed a constant Si,,, plotted flow rate vs. S_{out} and regressed the data to include the limiting point of $S_{ow} = Si_n$ at an infinite Q. In regressing their data, Si. was allowed to float, allowing them to determine the accuracy of their results by the regressed value for Si_n. In the case at hand, Si_n could not be held constant since re-circulative flow caused changes to the growth medium. It could, however, be determined accurately via an inline sampling port. Thus, the procedure by Lewandowski *et al.* (1990) would yield meaningless results. Instead, In Si_n/S_{oot} was plotted against V/Q. Since Si,, could not be held constant, the numerical value of the intercepts would have been doubtful. However,

the slope, Krn/Vmax could be used as a valuable parameter in estimating reactor performance (the accuracy of the trend was determined by the linear correlation co-efficient, R^2). This parameter can be related to the catalytic efficiency of an enzyme, since it also has the dimensions [concentration^{-1}. time'"]. A high value for this parameter would indicate a high V_{max} or low K_m, both of which are advantageous in a process.

Data from one nutrient batch was used for the sake of consistency. Furthermore, only the data of Reactor 1 could be treated since insufficient data points were obtained for one nutrient batch for Reactor 2. Also, more consistent trends were observed with Reactor 1. The resultant plot and linear regression data are depicted in figure 6.11. The R^2 value shows a good linear fit (0.99). The value of V,,,$_m$1K,, for Reactor I was found to be 0.8. This compared very well with the values of 0.34 obtained for a 400cm^3 packed bed reactor and 0.195 for a 1800cm^3 packed bed reactor treating chiorophenol (Lewandowski *et al.* 1990). However, differences in K_n, for p-cresol and 2-chlorophenol need to be taken into account to compare reactors.

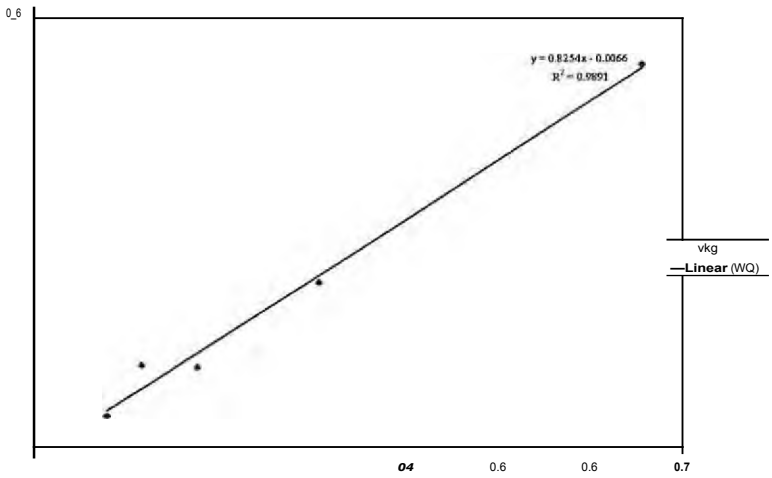


Fig. 6.11: Determination of V./K. for Reactor 1. The performance parameter V_m/K_m for the reactor was determined from the slope of this graph and relates to the catalytic efficiency of the reactor.

6.4. CONCLUSION

In an attempt to assess the factors which play a role in the performance of the MGR for the degradation ofp-cresol, the problem of inability to sustain steady-state behaviour was overcome, to a certain extent, by setting up two reactors with different initial flux ranges. This gave a high flux reactor with a thick, active biofilm and a low flux reactor with a thinner biofilm, with the greater concentration of ligninolytic enzymes due to nutrient limitation of more of the biofilm. The effect of flux on biofilm thickness can be observed in figure 3.7. These reactors were operated for a limited time only, to minimise changes in biofilm composition caused by changes in operating conditions.

For each reactor, two performance parameters were chosen viz. efficiency and productivity. The following factors were shown to influence performance.

Flux appeared to be the dominating factor. This is not surprising since the following characteristics are strongly influenced by flux:

- Biofilm thickness;
- Amount of enzyme produced;
- Oxygen mass transfer (related to biofilm thickness);
- Shear stress;
- Residence time of compounds in biofilm;
- Adsorption of pollutants to mycelia (related to residence time and biofilm thickness)

It was shown that with a thick biofilm and high flux ranges, efficiency increased as flux increased up to approximately 0.1 $L.n1^{2}.h.^{-1}$. Presumably adequate supply of nutrients and a residence time within the adsorptive capacity of the system was the reason for this effect. When flux reached levels above 0.1 $L.m^{-2}.11.^{-1}$ loss of efficiency was observed, probably due to saturation of the biofilm with pollutant, and too slow a degradation rate to clear adsorptive sites. No clear trends could be observed in the low flux reactor.

In terms of productivity, an apparently exponential improvement in productivity with flux is observed in the low flux reactor, while a saturation curve was observed in a plot of flux

vs. productivity with the high flux reactor. Combining the results of Reactor 1 and 2 provided a more complete picture of the relationship between flux and productivity. This showed that:

- The data are reliable since the combination of data from two very different reactors fit a common trend indicating reasonable reproducibility;
- This curve could form the basis of a simple design curve for a rational approach to scale-up, costing or application;
- Productivity is a more robust measure of reactor performance than efficiency, since such clear trends were not shown for efficiency. This saturation curve is typical of continuous reactors without cell washout. Results imply that adsorption to the membrane and mycelia might be the rapid, initial step in removal due to the high dilution rates (short residence times) at the high fluxes tested.

Superimposed on the effect of flux, other parameters could be shown to play a role:

- Nutrient status of the biofilm. Addition of fresh nutrients caused a noticeable improvement in performance (efficiency and productivity) in both reactors. The improvement could also be due to removal of metabolic wastes, which are expected to back-diffuse into the lumen due to re-circulative flow operation;
- Growth medium pH was shown to either effect - or be correlated with - changes in reactor performance. In terms of efficiency, an inverse relationship was shown between the efficiency of the low flux reactor and flux, while a direct relationship was shown between efficiency and pH in the high flux reactor. In the case of productivity, an inverse relationship was found between reactor productivity and medium pH in both high and low flux reactors. This difference in trends between efficiency and productivity in the high flux reactor is an interesting result and alludes to the complexity of the system.

An interesting result was the discovery that p-cresol removal continued in the reactor effluent due to residual enzyme and biomass reaction. This implied that the reactor could be used to indirectly contact toxic effluents as a pre-treatment for detoxification purposes.

The reaction products need to be analysed before further assumptions about its usefulness can be made.

In terms of quantitative analyses, the adapted method of Lewandowski *et al.* 1990, proved useful, Their reactor performance features can be lumped into a useful Michaelis — Menten-based kinetic parameter, here called catalytic efficiency for convenience. This parameter is robust enough for treatment of the data obtained for these experiments. It accounts for reactor efficiency as well as productivity. This parameter can be used to directly compare continuous flow reactor configurations and operational parameters and will be a vital optimisation parameter and means of comparison with competing technologies.

It will be endeavoured to develop a more structured model for the description of this highly complex, but potentially competitive system. This requires the ability to operate the system under steady-state conditions so that the effects of the individual factors can be fully explained.

Thus, it can be concluded that the use of the MGR concept, scaled up to a multi-fibre device in the TVFBR format, has sufficient potential to warrant further development. Despite the complexity of interactions of physical operational parameters, the system is competitive in terms of performance despite being un-optimised.

Further research needs now to be directed towards characterisation of the physical parameters to facilitate optimisation and scale-up of this system.

CHAPTER 7

CONCLUSION

A novel bio-process approach, the MGR, was conceptualised for the continuous production of idiophasic enzymes by the WRF which involved the use of membrane bioreactor technology and the exploitation of the inherent characteristic of nutrient gradient establishment in biofilms (Leukes *et* a/.1996). Development of a new bio-process typically follows distinct phases: proof of concept, characterisation, optimisation and then scale-up. The objective of this work was to formulate the solution to a process problem from a biological perspective, prove the concept and derive a descriptive characterisation of the process.

Z*1. MEMBRANE AND MEMBRANE BIOREACTOR DESIGN*

The field of membrane bioreactor technology is an active area of research, with a variety of developments by way of membrane types and assembled systems. These have thusfar mainly been applied to mammalian cell culture. It was thus considered probable that such systems could be conveniently applied to the development of the MGR concept. However, after several attempts, conventional OF membranes and cylindrical axial flow systems were abandoned due to the inability to establish a uniform biofilm with the use of these systems. Following a critical analysis of the reasons for the failure of these, appropriate design criteria for a membrane with suitable characteristics to suit the MGR requirements were identified. These criteria included the absence of an external skin; an anisotropic wall structure with macrovoids which penetrate from the skin to the external boundary of the membrane; good permeability for rapid immobilisation and good mechanical strength to avoid rupture by the growing biofilm.

Several possibilities for achieving the above were explored. These included abrasion of conventional membranes by sand blasting to erode away the external skin, nuclear track-etching for extended macrovoid formation, or the use of an open-structured microporous membrane. It was eventually decided to attempt the development of an entirely new

membrane by manipulation of wet-phase inversion spinning technique parameters. This culminated in the development of the IPS 763 membrane (Jacobs and Leukes 1996) which fulfilled all of the theoretical requirements. The membrane market, at present, shows a distinct paucity of membranes designed specifically for membrane bioreactors. Thus, the development of this specialised membrane can be considered to be of considerable significance in the field of membrane bioreactor research.

Practical demonstration of the performance of this membrane then followed. Other than providing a crucial solution in the development of the MGR, this novel membrane development has already found application in the immobilisation of enzymes for biotransformations and bioremediation (Edwards *et al* 1999), the production of enzymes and bioremediation by another WRF, *Trametes versicolor* (Ryan *et al.* 1998) and the continuous production of spores from *Neurospora crassa* (Ryan *et al.* 1998).

A suitable bioreactor module was then required for sequential destructive sampling of the membrane-associated biofilm. This requirement was met by the development of a disposable single-fibre mini reactor. Also, a multi-fibre module, fitting pre-determined performance criteria had to be developed, which could be used to test the sensitivity to scale-up of the MGR concept and which could provide sufficient amounts of enzyme product for process analysis. This was achieved through the development of a transverse flow module, as originally described by Futselaar *et al.* (1993). To the knowledge of the author, this is the first reported application of such a transverse flow membrane module as a fixed-film bioreactor. A set of empirically-based operational conditions were identified in order to undertake the evaluation of these systems. These proved adequate, but not ideal. This component of the research was perhaps the most difficult, since expertise in membrane manufacture, reactor engineering and module design had to be combined with understanding of the physiological requirements the fungus. The success of this venture was therefore entirely due to a close and highly interactive research collaboration with a polymer scientist and chemical engineer.

7.2. PROOF OF THE MGR CONCEPT

The MGR concept was then demonstrated empirically with the use of the IPS 763 membrane. Complete proof of the MGR hypothesis was based on several observations and criteria, listed below:

- Morphology

It was shown that morphological changes occurred radially across the mature biofilm, indicating that the temporal events occurring in typical batch cultures can be observed in a spatial domain. This is the cornerstone of the membrane gradostat concept. These morphological differences were related to typical growth phases experienced during batch culture on conventional OF membranes and verified using internal metabolic markers for different growth phases.

- Enzyme Production

It was shown that enzyme production could be attained without switching between growth and production cycles. This provided the functional proof that the MGR concept not only holds, but can be applied.

- Nutrient Gradient Establishment

In the originally formulated operational parameters, zero ammonium in the reactor permeate was sometimes observed. In the process which used double the flux for closer process observation (Chapter 5, section 5.3.4.), typically 75-90% of the NW originally present in the growth medium was removed by the biofilm even though NH_4^+ re-release was known to occur. Thus, nutrient gradients must exist in the biofilm, providing further evidence that the differentiation and secondary metabolite production could result from these gradients. Further proof was obtained from the observation that the biofilm thickness increased when the concentration of NI-I_4^+ was increased (Chapter 5, section 5.3.8.). The same was observed when the flux was raised (Chapter 3, figure 3.7.). This showed that the biofilm was NI-I_4^+ limited. That enzyme production ceased when the concentration of NH_4^+ was increased indicated that secondary metabolite formation is related to the establishment of nutrient gradients.

7.3. CHARACTERISATION OF THE BIOFILM

Based on observations of the biofilm at the level of ultra-structural localisation of ligninolytic enzymes and of details of cellular structure and organelle distribution, it could be concluded that the structural complexity observed in the biofilm of *P. cluysosporium* in the MGR more closely resembles the behaviour of the fungus in its natural environment, than flask cultures with liquid medium.

The above observations and descriptions revolve around provisional evidence for co-ordination and possible proto-tissue type organisational behaviour. Elucidation of the control and regulatory mechanisms of this organisation is also a prospect for future application of the MGR, since the structural observations can be put into the context of chemical and physical micro-environments. The nature of these micro-environments can be established theoretically by existing mathematical models and also empirically using micro-electrodes. Also, since the different morphological forms are segregated in space, molecular genetic mechanisms can be elucidated by the use of *in situ* hybridisation techniques. This will be the focus of future research.

7.4. PERFORMANCE OF THE MGR

Evaluation of the TVFBR showed it to be a good model reactor for process analysis, since good productivity was obtained for MnP production, comparable with the best reported systems available, even without optimisation (see table 5.2). This indicates that the concept of the MGR is worthy of further development for enzyme production. The performance of the MGR system, as described by its catalytic efficiency, also shows that it warrants further consideration as a useful technology for direct bioremediation of aromatic pollutants. This system has also been evaluated for application to bioremediation of cresylic effluents by *T. versicolor,* another basidiomycete and *N. crassa,* a zygomycete (Ryan *et al.* 1998). A new procedure, based on single fibre reactors has recently been tested to elucidate degradative mechanisms and kinetics without interference from pollutant adsorption to reactor components and gas stripping (Walsh. C.G. (1998)— BSc (Eons). thesis).

Compared to other reactors, the MGR compares well in terms of productivity and the ability to operate continuously, ie. no intermittent growth and production cycles are required. This is of particular relevance to the application of bioremediation, since a biomass regeneration stage would be costly, both in terms of time and growth medium requirements.

However, since development of the MGR is in its infancy, it does not compare well with other reactor systems in certain aspects due to inexperience with the use of this system. The most obvious shortcoming is the inability to sustain steady-state operation. This has been found to be due to fluid flow maldistribution, a hydrodynamic problem inherent to the operating conditions used. The obvious next step in development is to re-design the hardware associated with the membrane gradostat so that true steady-state operation can be achieved. This will require re-development of the process on a rational basis, using the information presented here. It is typical of bio-process research that both engineering and scientific development occur simultaneously, In his treatise on the development of nanotechnology, Eric Drexler stated that " Science and technology intertwine. Technologists use knowledge produced by scientists, and scientists use tools designed by engineers..." (Drexler 1990).

In order to facilitate progress in the engineering of the MGR concept, a collaborative research directive was established in 1997 between the Department of Biotechnology of Rhodes University, the Institute for Polymer Science, Stellenbosch University, and the Chemical Engineering Departments of the M.L. Sultan, Cape, and Peninsula Technikons, The priorities of this program are:

- Re-design of the transverse flow module design or formulation of operating conditions so that the problem of fluid flow maldistribution can be overcome.
- Design of a reticulation system, process inoculation and operation technique to prevent contamination over long-term operation, and to formulate CIP procedures for either *in situ* cleaning, or at least to allow re-use of the system if contaminated, which is not presently possible.

Several new modules have already been designed and are being evaluated. Once the above have been achieved, a process model can be tested, and regime analysis on the current concept can be performed.

7.4. FUTURE RESEARCH

The next phase of characterisation would be macro-scale process parameter identification to perform regime analysis. This requires systems where precise control of process parameters, particularly physical phenomena, can be maintained to accurately determine the effects of these phenomena. Thus, future development of this work has commenced in two directions, the consolidation of fundamental aspects of the biology and engineering of the MGR into a robust, effective process, and further exploration of the practical application of this technology.

Recent work has also shown that continuous penicillin production can be achieved using the MGR (Purohit, N.(1998) BSc (ions). thesis). Presently, applications of secondary metabolites compose a major part of the pharmaceutical industry, especially in the case of antibiotics. With original patents on novel antibiotics beginning to expire, the need for competitive production technologies will soon have a major impact on the industry. Evidence already exists that the MGR could become a technology that might provide a competitive advantage in this area.

PUBLICATIONS ARISING FROM THIS WORK

PATENTS

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REPORT

Burton, S.G., Boshoff, A., Edwards, W., Leukes, W.D., Russell, A.K., Russell, 1., Ryan, D., Rose, P.D. and Jacobs, E.P. (1998) Membrane-based Biotechnological Systems for the Treatment of Organic Pollutants. *Water Research Commission,* **South Africa.**

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Ryan, D.R., Russell, A.K., Leukes, **W.D., Rose, P.D. and Burton, S.G. (1998). Suitability of a modified capillary** membrane **for growth of fungal biofilms. Desalination 115: 303-306.**

INTERNATIONAL CONFERENCES

Leukes W., Burton S., Jacobs E., and Rose, P.(1994). Monitoring of hollow- fibre membrane immobilised fungal enzyme production. First International Workshop on Catalytic Membranes, Lyon-Villeurbanne, France.

Leukes, W, Edwards, W., Burton, S., Jacobs, E, Sanderson, R. and Rose P.(1995). A critical investigation into the use of *Phanerochaete chrysosporium* **in a hollow fibre bioreactor for the degradation of cresol. Society for General Microbiology first Joint Meeting with the American Society for Microbiology on Bioremediation, Aberdeen, Scotland.**

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Leukes, W., Burton, S. and Rose, P (1995). A cytochemical staining procedure for the detection of active lignin peroxidase in a biofilm of *Phanerochaete chrysa5porim.* 21st Congress of the South African Biochemical Society, Bloemfontein, S.A.

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Jacobs, E.P., Koen, Leukes, W.D., Edwards, W., Pinchuck S.C., Cross, R.H.M. (1997) Effect of factor manipulation during the wet-phase inversion process on the morphology of capillary membranes. MS SA '97. Microscopy Society of South Africa '97. Cape Town.

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ASSOCIATED PUBLICATIONS

The following are selected publications which have arisen from work involving the use of the IPS 763 membrane, the reactor modules and/or the MGR concept developed for this study:

Edwards, W., Bownes, R., Leukes, W.D., Jacobs, E.P., Sanderson, R., Rose, P.D., Burton, S.G. (1998) A capillary membrane bioreactor using immobilised polyphenol oxidase for the removal of phenols from industrial effluents. Enzyme Microb. Tech. 24(3-4): 209-217

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

Edwards, W., Leukes, W.D., Rose, P.D., Burton, S.G. (1998) Membrane-mediated effects on enzyme-substrate contact and conversion in a capillary-membrane bioreactor using immobilised polyphenol oxidase for conversion of phenols. Biotechnol. Bioeng. (submitted)

Edwards, W., Leukes, W.D., Rose, P.D., Burton, S.G.(1998) Immobilisation of polyphenol oxidase on chitosan-coated polysulphone capillary membranes for improved phenolic effluent bioremediation. Enzyme Microb. Tech. (submitted)

Edwards, W., Leukes, W.D., Jacobs, E.P., Sanderson, R.D., Rose P.D., Burton, S.G. (1998) Upscale of a transverse-flow capillary membrane module to a demonstration size unit for the treatment of phenol-containing industrial effluent. WISA '98. Cape Town.

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

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membranes in a transverse flow module. Biotech SA '97. Second Grahamstown Conference. Biotechnology and Development in South Africa. Grahamstown

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Edwards, W., Leukes, W.D., Burton, S.G., Jacobs, E.P., Sanderson R.D., Rose. P.D. (1996) Dephenolisation of wastewater using immobilised polyphenol oxidase in a hollow-fibre membrane transverse-flow biological contactor. WISA '96. Port Elizabeth.

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polyphenol oxidase. Society for General Microbiology - First Joint Meeting with the American Society for Microbiology on Bioremediation. Aberdeen, Scotland.

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Edwards, W., Leukes, W.D., Lalloo, R., Burton, S.G., Jacobs, E.P., Rose, P.D. (1994) Removal of phenols from wastewater using soluble and immobilised fungal polyphenol oxidase. First International Workshop on Catalytic Membranes. Lyon-Villeurbanne. France.

APPENDICES

Chemicals were obtained from Saarchem, Merck, BDH or Sigma unless otherwise stated,

APPENDIX A: CULTURE MAINTAINENANCE AND SPORE INOCULUM DEVELOPMENT

Cultures were stored on malt extract agar (2%) plates and sub-cultured every six months. Spore inocula were prepared by transfer of stock cultures to Roux bottles containing the sporulation medium of Tien and Kirk (1988), described below:

Glucose	10g.
Malt Extract	10g,
Peptone	2g.
Yeast extract	2g.
Asparagine	lg.
KH_2PO_4	2g.
$MgSO_4.7H_2O$	1g.
Thiamin.HCL	1 mg.
Agar	20g.

The components above, with the exception of Thiamin.HCI, were autoclaved in 1 L. of distilled water. A stock solution of Thiamin. HC1. was thereafter filter sterilised and added to the growth medium. Roux bottles were autoclaved separately. Approximately 250 mL. of the above medium was introduced per Roux bottle.

APPENDIX B: GROWTH MEDIUM

The growth medium used for all experiments was essentially that of Tien and Kirk (1988). This medium was used since it is the one most often cited in the literature, allowing direct comparisons to be made with other systems where the same medium was used. This medium uses glucose as a carbon source and ammonium (in the form of ammonium tartrate) as a nitrogen source. This is an unbalanced medium which is N-deficient in order to trigger secondary metabolism as soon as the available nitrogen is used up for biomass formation. This medium also contains excess mineral salts which have been shown to enhance ligninolytic enzyme production.

MATERIALS:

Trace Element solution (per litre):

$MgSO_4.7H_2O$	6g.
$MnSO_4,H_2O$	0.5g.
NaCL	lg.
$FeSO_4.7H_2O$	0.1g.
$CoC1_2.6H_2O$	0.183g.
$ZnSO_4.711_2O$	0.1g.
$CuSO_4.5H_2O$	0,156g.
$AlK(SO4)2.12H_2O$	10mg.
H_3B0_3	10mg.
$Na_2Mo\ 0_4.211_2O$	10mg.
Nitrilotriacetate	1.5g.

Basal Ill Medium (per litre)

KH_2PO_4	20g.
$MgSO_4.7H_2O$	10g.
$CaC1_2.2H_2O$	1.32g.
Trace Element Solution	100mL.

Other stock solutions:

10% glucose solution (C-source)

Thiamin-HCI stock solution 100mg. U^{1}

Ammonium Tartrate solution (8g. U^{1}) (N-source)

Buffer 20mM Dimethyl Succinate pH 4.2.

Although this is the recommended buffer, sodium acetate buffer was sometimes used as an alternative where large amounts of medium was required due to the cost of the Dimethyl Succinate buffer.

Preparation of Trace Element Solution:

The Nitrilotriacetate (a chelating agent) was first dissolved in 800mL. of water. The pH was then adjusted to pH to —6.5 with 1N KOH. Each component was then added in order and make up to 1L.

Preparation of Growth medium:

The trace element solution, Basal III medium, ammonium tartrate solution and buffer with the required amount of dilution water were autoclaved together. The glucose solution was autoclaved separately to prevent caramelisation and added subsequently and the Thiamin. HCl solution was filter sterilised.

Quantities of Reagents (per litre of growth medium):

Glucose solution	100mL.
Thiamin.HC1 solution	10mL.
Ammonium tartrate solution	25mL.
Basal I1T Medium	100mL.
Buffer solution	100mL.
Veratryl Alcohol (Aldrich) Solution	100mL.
Water	565mL.

APPENDIX C: SCANNING ELECTRON MICROSCOPY

Sections were fixed for at least 12 hours in 2.5% glutaraldehyde in potassium phosphate buffer (p1-1 7.0), washed with phosphate buffer (pH 7.0) and then dehydrated through a series of ethanol concentrations (30%, 50%, 70%, 80%, 90%, 100%),

The standard procedure for sample preparation would then have involved gradual transfer to an amyl acetate concentration series (Cross, R. 1994, pers. comm.). The possibility that the amyl acetate solution might dissolve the membranes was considered, hence the procedure was stopped after two washes with 100% ethanol before Critical Point Drying from CO_2. The samples were then gold coated and viewed in a JEOL JSM-840 SEM.

APPENDIX **D:** TRANSMISSION ELECTRON MICROSCOPY

Sections fixed and stained with DAB were washed in phosphate buffer (pH 7.4), stained and secondary fixed in 1% osmium tetroxide and dehydrated with alcohol as above. Then the samples were prepared for embedding by replacing the alcohol with propylene oxide using a gradual increase in propylene oxide: alcohol concentrations and then a series of embedding mixture: propylene oxide mixtures. The samples were embedded in pure resin which was allowed to polymerise for 36 hours at 60 °C. The resin used was a mixture of Taab 812 and Araldite CY212.

1-2 gm sections were then made for light microscopy using an LKB Ultratome ultramicrotome. 80 -100 nm sections were cut for TEM, post-stained with uranyl acetate and lead citrate and viewed using a JEOL 1210 TEM.

APPENDIX E: INTRACELLULAR MARKER ASSAYS FOR THE ONSET OF STATIONARY PHASE.

The assay for the onset of stationary phase was based on the observation of Bonnarme *et al.* (1991) that a significant increase in the specific activity of the intracellular enzymes Cytochrome C Oxidoreductase and Succinate Dehydrogenase upon entry of a culture of *P. chrysosporium* into stationary phase. Therefore, the activity of these enzymes was followed to determine the onset of stationary phase in membrane-immobilised cultures.

MATERIALS

Protein Extraction Mixture (soln A) (1L.)

Sorbitol	182.2 g.
HEPES	2.383g.
EDTA	0.372g.

Adjusted to pH 7.4

Succinate Dehydrogenase assay (stock solutions in 100mL.)

KCN stock (10X)	0.938g.
MTT stock (10X)	20mg.
Na-succinate stock (10X)	4.32g.
$K_2HPO_4.3H_2O$	18.26g (p11 7.5)
Phenazine Methosulphate (10X)	80mg.

Cytochrome C Oxidoreductase Assay (stock solutions in 10OrnL.)

13-NADPH (Boehringer) stock (10X)	0.238g.
KCN stock (10X)	0.466g.
$K_2HPO_4.3H_2O$	9.13g.
Cytochrome C (type II) (100X)	0,45g.

All stock solutions were prepared freshly before use, except for the extraction buffer (solution A), which was stored at 4°C.

PROCEDURE

Protein Extraction

Membrane sections containing immobilised fungus were frozen in liquid nitrogen and and stored at — 70 °C. Samples were ground in a mortar and pestle in the presence of liquid nitrogen. Sufficient amounts of filter sterilised solution A was added to obtain a homogeneous suspension. The suspensions were centrifuged at 5 000 *g* for 10 min. (Bonnarme *et al.* 1991). The supernatant was collected and used for intracellular marker enzyme assays and total intracellular protein analysis by the method of Bradford (1976) using Bovine Serum Albumin as standard.

Determination of Succinate Dehydrogenase Activity

Succinate Dehydrogenase, a marker for mitochondria! activity was determined spectrophotometrically by measuring the rate of reduction of tetrazolium salt (MTT) resulting from the oxidation of succinate to finnarate as described by Bonnarme *et aL* (1991).

For 100mL. of reaction mixture (solution B), the following were added:

KCN stock	20mL.
MTT stock	10mL.
Sodium succinate stock	10mL.
K_2HP**04 so**lution	10mL.
Distilled water	balance

The pH of this solution was adjusted to p1-17.5 and then incubated at 30 °C before use.

The assay was performed in 3mL. cuvettes and contained, per reaction:

Solution B	1.25 mL.
Enzyme sample	0.1 mL.

Water	0.5 mL.

This was allowed to incubate at 30°C before 0,25 mL, of phenazine methosulphate stock was added to start the reaction. The rate of change in absorbance at 570 nm. was determined as a measure of enzyme activity.

Cytochrome C Oxidoreductase

Cytochrome C oxidoreductase, a marker for Endoplasmic Reticulum activity, was determined spectrophotometrically by measuring the reduction of cytochrome C by the method of Bonnarme et al. (1991) except that type II cytochrome C was used instead of type IV and 220mM 13-NADPH was used instead of 286 mM.

Solution C:

KCN stock	10 mL.
K_2HPO_4 stock	10mL.

The assay contained the following:

Solution C	0. 5m.L.
Enzyme sample	0.1mL.
NADPH stock	1.9mL,

This solution was allowed to incubate at 30°C.

0.025mL. of the Cytochrome C stock solution was added to start the reaction, which was monitored at 550 nm.

Activities for both enzymes were expressed as $U.g^{-I}$ of intracellular protein, where I Unit — the conversion of 1p.M of substrate per minute.

APPENDIX F: CYTOCHEMICAL STAINING PROCEDURE FOR THE ULTRASTRUCTURAL LOCALISATION OF LIP IN A BIOFILM

A procedure for the determination of the LiP in biofilm sections was adapted from methods described by Forney *et al.* (1982) and Sexton and Hall (1991). The basic principle was that the peroxidase substrate DAB formed electron-dense osmophilic end products after transformation by peroxidases, These deposits react with the secondary fixative, osmium tetroxide, to form black depostis, observable by TEM. Control experiments were performed to determine the interference of catalase, which is ubiquitous and has peroxidase activity at high pH. This can be accomplished by pre-incubation of the sample with amino-triazole, a catalase inhibitor, to which peroxidases are insensitive. Controls for false positives were performed by pre-incubation of samples with KCN, a peroxidase inhibitor.

PROCEDURE

Sample Pre-Fixation

Sections were fixed for 3.5 hours in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. Glutaraldehyde cross-links the proteins to hold them in place as well as enhancing the activity of peroxidases (Forney *et al.* 1982; Sexton and Hall 1991).

Enzyme Reaction

The sections were washed with sodium acetate buffer (70 mM. pH 4) and then incubated in an aerated solution of 2mg. $mL.^{-1}$ DAB tetrahydrochloride (Sigma) and 10mg. $mL.^{-1}$ glucose in the above buffer for twenty minutes for the DAB to penetrate the sample after which H_2O_2 was added to a final concentration of 5mM. After 15 min. this solution was replaced with the above solution containing 1-1_2O_2 and left to incubate for 35 minutes, The last step was repeated. All incubations were carried out at 39°C.

In the control experiments, the samples were pre-incubated for 30 min. in sodium acetate buffer (pH 4) containing 50 nM. 3-amino-1,2,4,- triazole (Sigma) or 50 mM. KCN. The

DAB staining was then followed except that the solutions also contained KCN or aminotriazole. All reactions were performed in a dark environment.

Samples were then incubated with the secondary fixative, osmium tetroxide, and prepared for TEM according to Appendix D.

APPENDIX G: LIGNINOLYTIC ENZYME ASSAYS

CONCENTRATION OF SAMPLES

Extracellular fluid was typically used for enzyme determinations. Mycelia were first removed by filtration through glass wool. Then 5inL. of the extracellular fluid was concentrated to 750 [IL. using a 10 000 MWCO centrifuge ultrafiltration device (MSI Scientific). The retentate was used for enzyme assays.

LIGNIN PEROXIDASE ASSAY

Lignin peroxidase activity was determined spectrophotometrically at 18°C by the method of Tien and Kirk (1988) using veratryl alcohol as substrate. The rate of oxidation of veratryl alcohol to veratraldehyde was monitored at 310nm. The extinction coefficient used for veratraldehyde was 9 300 114-'cm", Activity is expressed in U,L ^{1}. (1U. — lumol.min^{-1}).

The assay solution contained the following:

10 rnM. Veratryl alcohol (Aldrich) solution	200p.L.
250 mM. Tartaric acid solution	2041.
Distilled water	420RL.
Enzyme solution	100µL.
5 mM. H_2O_2 solution	80µL.

Veratryl alcohol stock solutions were stored in a dark container. All other solutions were prepared fresh.

MANGANESE PEROXIDASE ASSAY

MnP activity was performed according to the method of Glenn and Gold (1988). The assay is based on the spectrophotometric determination of the oxidation of ABTS (Diammonium 2,2'-Azinobis(3-ethyl-6-benzothiazoline sulfonate)) by Mn? in the the presence of lactate, $Mn^{2'}$ and H_2O_2. The reaction was followed spectrophotometrically by the measurement of product formed. The extinction coefficient for the oxidation product at 415 nm. is 3 600 $M.'^{i}cm^{-1}$. Activity was expressed as $U. L.^{-1}$

Reagents:

Reagent A:

Egg albumin	150mg.
1 M. sodium lactate buffer (pH 4.5)	2.5mL.
1 M. sodium succinate buffer (pH 4.5)	2.5mL.
$MnSO_4$ solution	95mg.
ABTS solution (80 rig. $rnL.^{-11}$)	

Reagent **B:**

$\mathbf{H_2O_2}$	100p.M.

Assay

Reagent A	450µL.
Reagent B	450p.L.
Enzyme sample	100pL

APPENDIX H: DETERMINATION OF P-CRESOL CONCENTRATION BY HPLC

p-Cresol was determined using a Beckman System Gold HPLC unit with Beckman System Gold software for chromatographic analysis. A reverse-phase Machery-Nagel Nucleosil 5 11 column was used with water:acetonitrile (6:4) as the mobile phase at a flow rate of ImL.min."[1] p-Cresol was detected on a Diode Array UV-Detector at 254nm. Assays were performed in duplicate and the averages were used.

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