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A perfluorocarbon-based oxygen delivery system to a membrane bioreactor

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A PERFLUOROCARBON-BASED OXYGEN DELIVERY SYSTEM TO A MEMBRANE BIOREACTOR

BY

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"A saint is a sinner who keeps on trying" – N.R. Mandela

DECLARATION

I, **Seteno Karabo Obed Ntwampe**, hereby declare that the contents of this thesis represent my own unaided work and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily that of the National Research Foundation of South Africa, DAAD, Carl and Emily Fuchs Foundation or that of the Cape Peninsula University of Technology and their sponsors.

All intellectual concepts, theories, methodologies and mathematical derivations and model developments used in this thesis and published in various scientific journals (except those that were used for review articles) were derived solely by the candidate and first author of the published manuscripts. Where appropriate, intellectual property of others was acknowledged by using appropriate references. The contribution of co-authors, for conference and published manuscripts, was in a training capacity (Mr F. Chowdhury, in-service training), research assistance (Mrs. C.C. Williams, laboratory research assistant to Prof. M.S. Sheldon) and supervisory capacity (Prof. M.S. Sheldon and Dr. H. Volschenk) to meet the requirements for the doctoral degree award.

Signature: Date:

ABSTRACT

The white rot fungus, *Phanerochaete chrysosporium* strain BKMF-1767 (ATCC 24725), produces the extracellular enzymes, Lignin peroxidase (LiP) and Manganese peroxidase (MnP), that constitute the major route for lignin degradation by this organism. LiP and MnP have also been shown to play a major role in aromatic pollutant degradation. Due to the need for continuous production of LiP and MnP, a fixed-film bioreactor, classified as a membrane gradostat reactor (MGR), was developed. The implementation of batch-reactor operational parameters to the MGR system was found to be ineffective, thus creating the need for further research to improve the operational aspects of the MGR system to optimise its capabilities for continuous and industrial-scale operations. The research undertaken in this study, provides information that can be used to classify the dissolved oxygen (DO) transport kinetics into immobilised fixed-films of *P. chrysosporium*. Operational limitations of the MGR relating to environmental stresses in the bioreactor during operation and to biofilm deterioration, including limitations of DO mass transport, oxidative stress, trace element accumulation and polysaccharide storage in the fungal biomass, were evaluated in single capillary MGR systems (SCMGRs). These conditions were identified as existing in the continuous MGR systems.

From DO profiles, the oxygen consumption and flux into the biofilms, including the distribution of DO, was determined to be dependent on the immobilised biofilm's age. Younger biofilms showed higher DO distribution than older biofilms even when aeration was directed to the extracapillary space (ECS) of the reactor against the biofilm's surface. An increase in anaerobic zone thickness was observed to be increasing with an increase in biofilm thickness. Although, DO kinetic parameters were comparable with those obtained in submerged mycelia pellets, higher oxygen consumption values were observed in biofilms grown in the SCMGRs. The limitations of MGR were identified as: 1) poor DO distribution in immobilised biofilms because of β-glucan production and storage in the immobilised

biomass, resulting in ethanol production; 2) the peroxidation of lipids of the biofilms, which in turn will affect the long-term performance of the biomass caused by oxygenation and 3) trace element ion accumulation enhanced by β-glucan production. Furthermore, trace element ion accumulation was higher in the MGRs than in batch cultures using the same nutrient medium.

The development of a perfluorocarbon (PFC) emulsion for the MGRs to counteract these limitations was investigated. The compatibility of the emulsion with oxygen-carrying capacity was shown with an improvement in biomass generation, LiP/MnP production and overall consumption of primary substrates, mainly glucose and ammonium tartrate, in batch cultures. The emulsions investigated were based on the addition of oxygen carriers: Perfluorooctyl bromide (PFOB), Bis-(Perfluorobutyl) ethene (PFBE) and Perfluoropropylamine (PFPA), using Pluronic F 68 (PF 68) as the surfactant. Concentrations of 10 to 30% (w/v) PFC and 8.5% (w/v) PF 68 were tested successfully in batch cultures. The emulsions containing 10% (w/v) PFCs resulted in improved biomass performance as opposed to emulsions with higher PFC oil concentrations. An emulsion containing 10% (w/v) PFOB was used to evaluate its efficacy in the SCMGRs, as the biomass yield and overall enzyme production were superior to PFPA and PFBE-based emulsions with similar oil concentrations. After successfully applying PFOB and PF 68 to the SCMGRs, the following results were obtained: 1) reduced ethanol production; 2) reduced trace element accumulation; 3) lower β-glucan production and 4) improved DO-penetration ratio in immobilised biofilms.

DEDICATION

To my parents

Rudolph Selelepoo (Bauba) and Peggy Ramatsobane (Hunadi)

Ngwetši ya Dinoko

Phathiswa Muriel Swaartbooi-Ntwampe (Mošitadi)

BIOGRAPHICAL SKETCH

Seteno Karabo Obed Ntwampe was born at Thabamoopo, Ga-Mphahlele and raised at Moroke, Magakala-a-Ntwampe in Sekhukhuneland, Limpopo Province. He attended St. Mark College at Jane Furse where he matriculated in 1997. He registered for a National Diploma and Bachelor of Technology degree in Chemical Engineering from 1999 to 2003. He was awarded a Master of Technology degree in Chemical Engineering *cum laude* in 2005. He also completed a certificate in project management at the University of Cape Town in 2005. During this period, he worked as a student tutor, part-time lecturer and co-supervisor for several Chemical Engineering in-service training students and Bachelor of Technology degree research projects at the Cape Peninsula University of Technology, Cape Town campus (formerly known as Cape Technikon). He enrolled for his doctoral degree in Chemical Engineering in 2006 under the supervision of Prof. Marshall Sheldon and Dr. Heinrich Volschenk. His research was based on the use of patented membrane technology for the production of secondary metabolites used in the bioremediation and pharmaceutical industries. He published several peer-reviewed scientific papers in international journals and presented his work at local and international conferences. He was awarded several merit scholarships during his tenure as a postgraduate student from the National Research Foundation of South Africa, DAAD (German Academic Exchange program), Canon Collins Foundation and the Carl and Emily Fuchs Foundation. An international paper award was conferred to him at the IWA Biofilms Technology conference held in Singapore in January 2008. He is currently lecturing Fermentation Technology, Process Technology Management and Bioprocessing in the Department of Agricultural and Food Science at the Cape Peninsula University of Technology.

LIST OF OUTPUTS

The following outputs are contributions by the candidate to scientific knowledge and development during his doctoral candidacy (2006 to 2009):

Peer Reviewed Publications

Ntwampe S.K.O. and Sheldon M.S. 2006. Quantifying growth kinetics of *Phanerochaete chrysosporium* immobilised on a vertically orientated polysulphone capillary membrane: Biofilm development and substrate consumption. *Biochemical Engineering Journal*, 30: 147 - 151.

Ntwampe S.K.O., Sheldon M.S. and Volschenk H. 2007. The membrane gradostat reactor: Secondary metabolite production, bioremediation and commercial potential. *African Journal of Biotechnology*, 6 (10): 1164 - 1170.

Sheldon M.S., Mohammed K. and **Ntwampe S.K.O.** 2008. An investigation of biphasic growth kinetics for *Phanerochaete chrysosporium* (BKMF-1767) immobilised in a membrane gradostat reactor using flow-cells. *Enzyme and Microbial Technology*, 42: 353 - 361.

Ntwampe S.K.O., Sheldon M.S. and Volschenk H. 2008. Oxygen mass transfer for an immobilised biofilm of *Phanerochaete chrysosporium* in a membrane gradostat reactor. *Brazilian Journal of Chemical Engineering*, 25 (4): 649 - 664.

Ntwampe S.K.O., Sheldon M.S. and Volschenk H. 2008. Limitations of a membrane gradostat bioreactor designed for enzyme production by biofilms of *Phanerochaete chrysosporium*. *Water Science and Technology,* 58 (11): 2259 - 2270.

Ntwampe S.K.O., Chowdhury F., Sheldon M.S. and Volschenk, H. 2008. Overview of parameters influencing biomass and bioreactor performance used for extracellular ligninase production from *Phanerochaete chrysosporium*. *Brazilian Archives of Biology and Technology,* (*Accepted for publication on 14th August 2008*).

Ntwampe S.K.O. and Sheldon M.S. 2009. Effect of a perfluorocarbon-Pluronic F68-based emulsion for a *Phanerochaete chrysosporium* biofilm immobilised in a membrane gradostat bioreactor. *Asia Pacific Journal of Chemical Engineering,* (*Accepted for publication on 21st January 2009*).

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Limitations of a membrane gradostat bioreactor (MGR) designed for continuous lignin and manganese peroxidase production by biofilms of *Phanerochaete chrysosporium*, IWA Biofilm Technologies Conference, 8 - 10 January 2008, Singapore.

Oxygen mass transfer for an immobilised biofilm of *Phanerochaete chrysosporium* in a membrane gradostat reactor. 7th WISA MTD Symposium and Workshop, 18 - 20 March 2007, Mabalingwe Nature Reserve, Limpopo Province, South Africa.

Research Awards

Paper Award: Limitations of a membrane gradostat bioreactor (MGR) designed for continuous lignin and manganese peroxidase production by biofilms of *Phanerochaete chrysosporium*. IWA Biofilm Technologies Conference, 8 - 10 January 2008, Singapore.

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PREFACE (THESIS ORGANISATION)

The overall aim of the study was to determine the effectiveness of a perfluorocarbon-based emulsion as an oxygen carrier in *Phanerochaete chrysosporium*-based membrane gradostat bioreactors (MGRs). The thesis is written in article format. The references for each article are listed at the end of each chapter according to the referencing style for the specific journal to which the paper was submitted for publication. For all other chapters, the Harvard referencing style was used. The materials and methods for each part of the study are explained in the relevant chapters. The thesis is subdivided into the following chapters:

- Chapter 1 serves as an overall introduction, providing background information on the operational parameters and limitations of *P. chrysosporium*-based MGRs. The benefits of perfluorocarbons used as oxygen carriers in microorganism-based bioreactors are highlighted. This chapter presents the overall objectives for the study and also delineates the research that was done.
- Chapter 2 is the first of three literature review chapters, and is a presentation of the literature reviewed on the preparation and the scaling-up of batch-type reactor systems to continuous reactors used for extracellular enzyme production from *P. chrysosporium*. Several factors that influence the biomass growth, ligninase production and possible limitations of *P. chrysosporium* biomass performance are reviewed.
- Chapter 3, the second literature review chapter, focuses solely on the MGR and its development. The membrane gradostat concept as well as prior research into this technology are explicated in this chapter.
- Chapter 4, the third literature review chapter, focuses on the effective use and benefits of perfluorocarbons (PFCs) and Pluronic F 68 (PF 68) for microbial cultures.
- Chapter 5 is the first results chapter. In this chapter, the dissolved oxygen mass transfer parameters in the MGR system are investigated and compared to those obtained in submerged *P. chrysosporium* batch cultures.

- Chapter 6, the second results chapter, evaluates the limitations of MGRs with regard to dissolved oxygen transport in biofilms of *P. chrysosporium* and explores the use of technical-grade oxygen in the SCMGR systems. The production of ethanol due to limited oxygen availability, the oxidative stress induced by the use of technical-grade oxygen and the accumulation of trace elements when basal (III) medium ionic strength was increased in the nutrient medium, is determined. The presence, storage and accumulation of the carbohydrate glucan in *P. chrysosporium* biomass is also evaluated.
- Chapter 7, the third results chapter, deals with the evaluation of *P. chrysosporium* growth in batch bioreactors in the presence of emulsified perfluorocarbons. The perfluorocarbons used were perfluorooctyl bromide (PFOB), perfluoropropylamine (PFPA) and bis-(perfluorobutyl) ethene (PFBE). Biomass development, enzyme production and consumption of essential substrates were monitored. The results in this chapter are then used to identify a suitable PFC and its concentration for application in MGRs.
- In Chapter 8, the fourth results chapter, the identified perfluorocarbon emulsion, including the optimum perfluorocarbon concentration (identified in Chapter 7), was used in single capillary MGRs, to evaluate whether it reduces the limitations identified in Chapter 6, which are related to the performance of *P. chrysosporium* biomass.
- In Chapter 9, the results obtained in chapters 5 to 8, including operational issues encountered during the study, are discussed and summarised. A comparison with other studies and further suggestions on the improvement of the MGR concept is also discussed. Research questions listed in Chapter 1 are also answered and discussed in this chapter. Furthermore, overall conclusions from the study are highlighted in this chapter and recommendations for future research are also listed.
- All analytical assays, biofilm antioxidation techniques, nutrient medium and maintenance techniques for *P. chrysosporium* are listed in the Appendices.

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SYMBOL		UNITS
$a,\ b,\ c$	- Experimental coefficients for Taylor's expansion method	-
β	- Nutrient film coefficient	m^{-1}
β_a	- Experimental coefficient in the aerial mycelia	m^{-1}
C	- Substrate (oxygen) concentration	$g.m^{-3}$
C_b	- Oxygen concentration in air	$g.m^{-3}$
C_s	- Oxygen concentration at the biofilm surface	$g.m^{-3}$
C_{O_2}	- Bulk dissolved oxygen concentartion	$g.m^{-3}$
$C_{O_2}^*$	- Saturation dissolved oxygen concentration	$g.m^{-3}$
$D_{a,f}$	- Oxygen diffusion coefficient in the aerial mycelia	$m^2.h^{-1}$
D_f	- Oxygen diffusion coefficient in the biofilm	$m^2.h^{-1}$
D_w	- Oxygen diffusion coefficient in water at incubation temperature	$m^2.h^{-1}$
E	- Biomass enhancement factor linked to perfluorocarbon use	-
J	- Substrate flux	$g.m^{-2}.h^{-1}$
$J_{a,m}$	- Averaged oxygen flux in the aerial mycelia	$g.m^{-2}.h^{-1}$
$J_{f,xs}$	- Oxygen flux at the biofilm surface	$g.m^{-2}.h^{-1}$
$J_{w,xs}$	- Oxygen flux to the biofilm surface from the nutrient film layer	$g.m^{-3}.h^{-1}$
K_m	- Half-saturation coefficient	$g.m^{-3}$
r_m	- Maximum uptake rate of oxygen	$g.m^{-3}.h^{-1}$
x	- Biofilm thickness	m
x_s	- Biofilm surface	m
$(x - x_s)$	- Biofilm penetration depth	m

Subscripts

f, x_s - Biofilm surface

a, f, x_s - Aerial mycelia region

w, x_s - Nutrient film layer

LIST OF ABBREVIATIONS

ABTS - 2.2-azino bis-3-ethyl-benzothiazoline-6-sulfonic acid

AF/F - Air filter/0.22 µm filter

AP - Air pump

BHT - Butylated hydroxyl toluene

CV - Closed valve

DO - Dissolved oxygen

ECS - Extracapillary space

H - Humidifier

HLB - Hydrophilic-Lipophilic balance

ICP-MS - Inductively coupled plasma mass spectroscopy

IPS - Institute of Polymer Science

KLa - Volumetric oxygen transfer coefficient

L - Lumen

LiP - Lignin peroxidase

MCMGR - Multi-capillary membrane bioreactor

MBR(s) - Membrane bioreactor(s)

MDA - Malondialdehyde

MGR(s) - Membrane gradostat reactor(s)

MnP - Manganese peroxidase

Mn-SOD - Manganese superoxide dismutase

MYGP - Malt extract-yeast extract-glucose-peptone

NF - Nutrient flask

NP - Nutrient pump

OTR - Oxygen transfer rate

P. chrysosporium - *Phanerochaete chrysosporium*

PF - Permeate flask

PF 68 - Pluronic F 68, also known as Poloxamer 188

PFBE - bis-(Perfluorobutyl) ethene

PFC(s) - Perfluorocarbon(s)

PFCSF - Perfluorocarbon storage flask

PFCP - Perfluorocarbon pump

PM - Polysulphone membrane

PFOB - Perfluorooctyl bromide

PFPA - Perfluoropropylamine

R^2 - Correlation coefficient between experimental and modelled data

R.H.S - Right hand side

ROS - Reactive oxygen species

SEM - Scanning electron microscope

SCMGR(s) - Single-capillary membrane gradostat reactor(s)

SdH_2O - Sterile distilled water

SIM - Spore inducing medium

STR - Stirred tank reactor

TM - Teflon mould

Tris-HCL - Hydroxymethyl amino methane-hydrochloride

VA - Veratryl alcohol

VOCs - Volatile organic compounds

WRF - White rot fungi

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Since the discovery of a group of extracellular ligninolytic enzymes, lignin peroxidase (LiP) and manganese peroxidase (MnP) in *Phanerochaete chrysosporium,* several research reports have detailed the development of efficient techniques to produce these enzymes in different bioreactors, using unique operational conditions. Attempts to produce these enzymes have been made in: 1) submerged flasks (Ürek & Pazarlioğlu, 2005; Levin *et al.*, 2004); 2) rotating drums (Domínguez *et al.*, 2001); 3) pneumatically operated immersion bioreactors (Rodríguez-Couto *et al.*, 2001); and 4) membrane gradostat bioreactors (MGRs) (Govender *et al.*, 2003; Govender, 2000; Leukes, 1999). Overall, the production of LiP and MnP was shown to be higher in biofilms immobilised on support matrices. The support matrices ranged from: 1) polysulphone membranes (Ntwampe, 2005; Sheldon & Small, 2005; Govender *et al.*, 2003; Garcin, 2002; Solomon & Petersen, 2002; Solomon, 2001; Govender, 2000; Leukes, 1999; Leukes *et al.*, 1999; Venkatadri & Irvine, 1993); 2) silicone membranes (Venkatadri *et al.*, 1992); 3) polystyrene foam (Rodríguez-Couto *et al.*, 2000; Kirkpatrick & Palmer, 1987); to 4) ceramic membranes (Sheldon, 2008; Sheldon & Small, 2005). A detailed review of the processes currently used for LiP and MnP production, including the carriers used to immobilise *P. chrysosporium* will be given in Chapter 2. A variety of limitations related to the use of these bioprocesses are listed in this chapter.

The design of a continuous fixed-film bioreactor, classified as the membrane gradostat reactor (MGR), has been found to have superior performance compared to batch cultures for LiP and MnP production (Ntwampe, 2005; Sheldon & Small, 2005; Govender *et al.*, 2003; Garcin, 2002; Solomon & Petersen, 2002; Solomon, 2001; Govender, 2000; Leukes, 1999; Leukes *et al.*, 1999). In a MGR, biofilms are immobilised on the external surface of a capillary membrane, and continuously supplied with liquid-based nutrients from the membrane lumen

side, while being exposed directly to an oxygen source in the extracapillary space (ECS). This provides for primary mycelial growth near the nutrient source, while older mycelia are located further away from the membrane, causing nutritional gradients in the immobilised biofilms. Due to carbon and nitrogen limitations and to enhance LiP and MnP production, the older biofilm further away from the membrane is kept in the idiophase, while, when the bioreactor is operated in the dead-end filtration mode, the permeate is continuously recovered. The older, productive biomass is directly exposed to the gaseous phase supplied in the ECS. The design, operation and the application of the MGR are reviewed in Chapter 3 to highlight its effectiveness in terms of LiP and MnP production.

The continuous production of the LiP and MnP enzymes is of great significance because these enzymes have been shown to be able to metabolise a variety of organic chemicals, many of which are pollutants both in liquid effluents and in soils (Ceribasi & Yetis, 2001; Walsh, 1998; Grifoll & Hammel, 1997; Griselda & Eduardo, 1990; Bumpus & Aust, 1987). Important external factors affecting the production of these enzymes from *P. chrysosporium* biofilms are temperature, pH, dissolved oxygen (DO) concentration and a fixed nitrogen concentration. Temperature, pH and a fixed nitrogen source have previously been optimised (Jeffries *et al.*, 1981). The optimum pH for lignin degradation was determined to be 4 to 4.5, while lignin degradation was suppressed at a pH above 5.5 and below 3.5. Lignin degradation was nonexistent under a 5% oxygen level, while its decomposition was 2 to 3 times better at a 100% oxygen level when compared to air (Kirk *et al.*, 1978). The source of nitrogen had limited influence, with its composition determined to be effective at a concentration of 24 mM achieving a 25 to 35% lignin decomposition (Kirk *et al.*, 1978). The optimum growth temperature for the fungus was determined to be 39°C, while the optimum enzyme production temperature was determined to be 30°C (Asther *et al.*, 1988).

These external factors, including the trace element requirements, had initially been designed and optimised for batch cultures. While the requirements for batch bioreactor systems are

well defined, several questions remained unanswered with regard to the operational requirements for continuously operated MGR systems. The MGR operational aspects in question were the following:

- The use of 100% oxygen in *P. chrysosporium* biofilms in the MGR only improves ligninolytic enzyme production for limited periods (Garcin, 2002; Solomon, 2001; Govender, 2000; Leukes, 1999). Prolonged exposure to high partial oxygen pressures in any cell culture can lead to oxygen toxicity (hyperoxia) from an increased generation of reactive oxygen species (ROS). When cultured cells, including biofilms, are used to determine oxygen toxicity, modest increments in oxygen partial pressure is sufficient to cause cell damage and death (Li *et al.*, 2004). For example, when *P. chrysosporium,* maintained on glucose, is exposed to a high partial pressures of oxygen, the fungal mycelia exhibit impaired mitochondrial functions (Zacchi *et al.*, 2000) as a result of the loss of both succinate dehydrogenase and cytochrome oxidase activities. Although this type of biomass has properties that induce LiP production in an oxygen atmosphere, the accumulation of ROS can damage the biomass. This will affect *P. chrysosporium* biofilm performance in bioreactors operated for prolonged periods. Furthermore, *P. chrysosporium* produces glucan as an oxidative stress response when high partial pressures of oxygen are used in bioreactors (Miura *et al.*, 2004).

  From this information, the question arises as to what method of oxygenation would be suitable for the MGR system to provide enough oxygen, yet not be limited by polysaccharide production and storage?

- The following trace elements were determined to be suitable for batch cultures of *P. chrysosporium* by Kirk *et al.* (1978)and Tien and Kirk (1988): Mg, Mn, Na, Fe, Co, Ca, Zn, Cu, Al and Ca. When the Tien and Kirk (1988) nutrient medium was designed, batch cultures were the preferred choice for ligninase production. As *P. chrysosporium* has a

capacity to absorb and accumulate metal ions, including Cu, Mn and Co (Baldrian, 2003; Falih, 1997), there is a potential risk of metal accumulation in continuous cultures supplied with this growth medium. Malonate and oxalate are chelators excreted by *P. chrysosporium* (Wesenberg *et al.*, 2003; Goodwin *et al.*, 1994; Khindaria *et al.*, 1994) and provide immobilisation of soluble ions or complexes to form insoluble oxalates.

From this information, it is clear that in a continuous process, trace-metal ion accumulation will be elevated compared to batch cultures. This raises the question: As *P. chrysosporium* produces chelators, will the rate at which the chelators are produced be sustained with the continuous supply of trace elements to the biomass? Will the polysaccharide mucilage developed in the biofilms entrap trace elements and thus exacerbate metal accumulation?

- For the MGR to be efficient and to be developed on pilot scale and eventually on an industrial scale for prolonged operation, the following question need to be answered: Which nutrient medium additives can be used to prolong the regeneration of *P. chrysosporium* biomass during nutrient starvation, exposure to trace metal ions, hyperthermia and death from shearing forces caused by aeration in the ECS of the MGR? These additives should be able to provide DO requirements and remove metabolic gas produced in the mycelia near the substratum, where primary mycelial growth is maintained, while maintaining a sufficient gradostat to keep the immobilised biofilms in the idiophase for continuous enzyme production.

A number of features make perfluorocarbon-based emulsions an attractive option for biotechnological purposes (especially in bioreactors where there is a deficiency in oxygen), these features include: high stability and inertness, a gas-dissolving capacity, hydrophobicity and lipophobicity; fluidity and the absence of metabolism (Riess, 2002). Perfluorocarbons (PFCs) also have much higher solubilities for carbon dioxide; therefore, they can potentially

remove toxic gaseous end-products; i.e. PFCs can be used to regulate both gas supply and gas removal from cultured biofilms (Lowe *et al.*, 1998; Ju *et al.*, 1991). Due to their remarkable ability to accumulate DO, PFC-derived materials have been widely used as blood substitutes and oxygen transporters in animals and humans (Lowe, 2003; Wang *et al.*, 2000). Some PFC oils are low molecular weight polymers of chlorotrifluoroethylene – with the formula $(CF_2CFCl)_n$, with n varying from 2 to 10 units (Wang *et al.*, 2000). PFCs are chemically inert compounds consisting of fluorine-substituted hydrocarbons. PFCs have the following advantages: 1) PFCs do not react with oxygen or other gasses; 2) the DO is not subject to the effects of microbial culture pH and 3) PFCs have been determined to facilitate effortless transfer of oxygen. The desirable characteristics in the second generation of PFCs are: 1) large DO carrying capacity and 2) large-scale production and availability (Goorha *et al.*, 2003). Oxygen solubility in PFCs increases as temperature decreases, making them effective for supplying oxygen to cells even at low temperatures (Richardson *et al.*, 2002). The application of emulsified PFCs in bioprocesses and their benefits to microbial cultures are highlighted in Chapter 4.

PFC-based emulsions have been tested and used previously in clinical trials for medical purposes (Inayat *et al.*, 2006; Goorha *et al.*, 2003; Lowe, 2003; Riess, 2002) and in several bioprocess engineering studies, but not in the fixed-film membrane bioreactors (MBRs) used for extracellular production of LiP and MnP. For the purposes of this study, it was hypothesised that by introducing a PFC-based emulsion into the MGR system, the DO availability in the system, along with the simultaneous removal of metabolic carbon dioxide, will greatly improve the functionality of the biofilm.

1.2 RESEARCH QUESTIONS

Many of the factors that have been listed and described in section 1.1 can influence the performance of *P. chrysosporium*-based continuous cultures. The following list of questions was used to acquire the information used to meet the objectives of the study:

- Does anaerobic zone formation occur in biofilms immobilised in single-capillary MGRs (SCMGRs)?
- What is the relationship between DO distribution and polysaccharide production in SCMGRs?
- Does significant metal accumulation occur in biofilms immobilised in SCMGRs?
- Is there any difference in *P. chrysosporium* biomass performance (oxidative stress) between aerated and oxygenated cultures?
- What are the effects of PFCs on *P. chrysosporium* growth and the production of ligninolytic enzymes when in direct contact?
- When formulating a PFC-based emulsion, what kind of surfactant will be compatible with both the PFCs and *P. chrysosporium*?

1.3 OBJECTIVES OF THE STUDY

The objectives for this study were to:

- Quantify the oxygen mass transfer kinetics, including the oxygen penetration depth, oxygen consumption, oxygen flux and the formation of anaerobic zones in biofilms, of *P. chrysosporium* immobilised on a polysulphone capillary membrane in the MGR system.
- Determine the levels of ethanol production as a result of oxygen limitations in the SCMGR system.
- Determine the oxidative stress when higher partial pressures of oxygen are used to flush the ECS in order to improve LiP and MnP production in the SCMGRs.
- Evaluate the effectiveness and compatibility of three different water immiscible DO carriers (perfluorocarbons) on *P. chrysosporium* growth and ligninolytic enzyme production in batch cultures and to select a suitable perfluorocarbon and concentration for application within the MGR.

- Apply the proposed emulsion to the MGR system and evaluate its effectiveness to enhance *P. chrysosporium* biomass performance in terms of ligninolytic enzyme production, while alleviating overall biomass performance limitations such as trace element accumulation, oxidative stress and polysaccharide storage.
- Evaluate the improvement biomass growth in the SCMGR system using a DO carrying perfluorocarbon emulsion.

1.4 SIGNIFICANCE OF THE STUDY

A number of studies have shown that pure oxygen improves ligninolytic enzyme production in bioreactors where *P. chrysosporium* is immobilised on different carriers. However, the use of pure oxygen is limited to short periods only, as determined by various researchers. This study will be of significance in the development of MGRs where there are limitations in terms of oxygen delivery. This research study will contribute towards developing efficient, cost-effective and prolonged production of enzymes using the MGR system. The use of PFC-based emulsions provides a way to supply a consistent supply of non-toxic oxygen into the bioreactor systems without using technical-grade oxygen.

This study further contributes to MBR development, as PFC-based emulsions have not been evaluated in: 1) a MBR in which continuous extracellular secondary metabolites were produced and 2) cultures of *P. chrysosporium* BKMF 1767, which are used in this study. The DO kinetics in biofilms of *P. chrysosporium*, where the biofilm was directly aerated, has never been investigated in a fixed-film bioreactor such as the MGR to ascertain whether DO mass transport is improved in this type of reactor. The use of an additive, Pluronic F 68, to improve biofilm growth of *P. chrysosporium* and the nutritional gradostat in immobilised biofilms is introduced to improve the MGR technology for pilot-scale studies.

1.5 DELINEATION OF THE STUDY

This research will be limited to oxygen optimisation in the bioreactors only, in order to maximise ligninolytic enzyme production. The following parameters and conditions will not be covered during this study:

- purification of the enzymes
- construction of a pilot plant-sized or commercial-sized MGR unit
- perfluorocarbon emulsion stability
- enzyme kinetic calculations
- the use of other poloxamers (F88, F108, L35)

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CHAPTER 2

LITERATURE REVIEW

Overview of parameters influencing biomass and bioreactor performance used for extracellular ligninase production by *Phanerochaete chrysosporium*

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CHAPTER 2

Overview of parameters influencing biomass and bioreactor performance used for extracellular ligninase production by *Phanerochaete chrysosporium*

Abstract

The industrial production of extracellular enzymes is gaining momentum as commercial interests seek alternative ways to improve productivity in the biotechnology and pharmaceutical industries. Early research studies looked at improving batch bioreactor operational challenges; however, the use of continuous cultures was subsequently shown to be favourable. This led to a new approach developed to produce extracellular enzymes continuously using fixed-film bioreactors that consist of biofilms immobilised on organic and inorganic membranes. In this review, the performance of *P. chrysosporium* biomass in producing ligninase using different bioreactor operating conditions is highlighted. Furthermore, limitations to the implementation of optimised batch-culture conditions to continuous fixed-film bioreactors are discussed. The role played by supplements used in the nutrient medium to enhance *P. chrysosporium* biomass performance in batch cultures will be addressed. Dissolved oxygen transportation, trace element toxicity and the effects of lipid peroxidation on *P. chrysosporium* biomass in fixed-film bioreactors operated for prolonged periods are discussed.

Keywords: Biofilm; Dissolved oxygen; Lignin peroxidase; Manganese peroxidase; Membrane bioreactors; *Phanerochaete chrysosporium*.

2.1 INTRODUCTION

The white-rot fungus (WRF), *Phanerochaete chrysosporium*, is well known for its capability to degrade lignin to carbon dioxide (CO_2), water (H_2O) and hydrogen peroxide (H_2O_2). Apart from the fact that the fungus exhibits high rates of lignin degradation, it is also characterised

by rapid growth in liquid cultures and rapid sporulation when immobilised on carriers (Gold & Alic, 1993; Buswell & Odier, 1987; Kirk *et al.*, 1978). The fungus is classified as a white-rot fungus, as it causes a form of wood decay that results in the bleaching of wood (Eaton & Hale, 1993). WRF are members of the *Enmycota*, subdivision *Basidiomycotina* (Bumpus & Aust, 1987). The *P. chrysosporium* BKMF 1767 (ATCC 24725) wild-type strain was originally isolated by T.I. Novobranova from the plant species *Vitis vinifera* in Kazakhstan and was initially classified as *Sporotrichum pulverulentum* by Novobranova (Kirk *et al.*, 1986). Of all the different WRF species studied, *P. chrysosporium* BKMF 1767 (ATCC 24725) was shown to produce the highest ligninase activity compared to other WRF strains (Linko, 1992; Kirk *et al.*, 1986).

The mechanisms involved in the degradation of lignin by the WRF are complex. As lignin is a three dimensional polymer, degradation by *P. chrysosporium* must take place as an extracellular process, using highly oxidative mechanisms (Breen & Singleton, 1999) induced by nitrogen and carbohydrate starvation (Bumpus & Aust, 1987). The oxidative process is facilitated by the production of extracellular enzymes whose catalytic activities have been studied using model lignin compounds (Kirk *et al.*, 1986; Kirk *et al.*, 1978; Kirk *et al.*, 1975). Under ligninolytic conditions, *P. chrysosporium* produces two families of peroxidases – lignin peroxidases (LiPs) and manganese peroxidases (MnPs) – in a H_2O_2-veratryl alcohol-O_2 generating system. *P. chrysosporium* BKMF 1767 belongs to a minority of WRF that produce no detectable levels of laccase activity (Kirk & Farrell, 1987). Although, Srinivasan *et al.* (1995) demonstrated that *P. chrysosporium* BKMF 1767 produces laccase in a culture medium containing cellulose and a culture medium which was designed by Tien and Kirk (1988) (Rodríguez *et al.*, 1999). However, Podgornik *et al.* (2001) refuted these research findings by determining that the presence of Mn^{3+} ions during assaying caused false positive laccase reactions.

The ligninolytic enzymes of *P. chrysosporium* are produced during secondary metabolism, when the biofilm is in the idiophase, an aspect identified as a major limitation to producing the enzymes in batch cultures (Bonnarme *et al*., 1993). The performance of the biomass to produce the required enzymes in liquid cultures is dependent on the presence of readily metabolisable substrates, such as glucose. Increasing the levels of glucose in liquid cultures has a strong activating effect on the rate of enzyme production, which is also influenced by the presence of manganese which overcomes the hydrogen peroxide-dependent inactivation of MnP by completing the catalytic cycle of the enzyme (Perie & Gold, 1991).

Several reviews and book chapters published by Hofrichter (2002), Have and Teunissen (2001), Leonowicz *et al*. (1999), Highley and Dashek (1998), and Eaton and Hale (1993), have covered the physiology, biochemistry and molecular biology aspects of *P. chrysosporium,* as well as the oxidative mechanisms of LiP and MnP. Furthermore, Linko (1992) reviewed bioprocessing aspects and other operational parameters used for the production of LiP. However, at present, the following parameters have been inadequately researched in continuous fixed-film membrane bioreactors (MBRs): 1) oxidative stress and lipid peroxidation of *P. chrysosporium* biomass during enzyme production; 2) the production of by-products under anaerobic operational conditions; 3) the effect of extracellular glucan on the dissolved oxygen (DO) mass transfer; 4) the possibility of trace metal accumulation in continuously operated bioreactors and 5) the effects of environmental stress on the development and performance of immobilised *P. chrysosporium* biomass.

This review provides a bioprocessing perspective on the improvements made thus far in the development of batch reactor systems for the efficient production of LiP and MnP enzymes by means of biofilms of *P. chrysosporium*. Various shortcomings of using optimised batch reactor operational conditions and nutrient medium developed for *P. chrysosporium* for continuous fixed-film MBRs are discussed. Issues related to DO transfer in *P. chrysosporium* mycelium, the ligninase assay techniques used to evaluate *P. chrysosporium* biomass

performance, the growth/production medium used for batch cultures and the development of continuous fixed-film MBRs used to overcome the use of lab-scale flasks and batch systems are highlighted. Parameters related to oxidative stress and lipid peroxidation in *P. chrysosporium* biomass, including the production of ethanol due to insufficient DO transport in fixed-film MBRs, are highlighted as limitations to the development of continuous fixed-film MBRs using *P. chrysosporium* BKMF 1767. The role of extracellular glucan production on DO mass transfer and possible trace-metal accumulation in continuously operated bioreactors are illustrated.

2.2 CONDITIONS USED FOR ENHANCED LIGNINASE PRODUCTION IN SUBMERGED BATCH BIOREACTORS

2.2.1 Inoculum development and preparation for *P. chrysosporium* cultures

The preparation of an inoculum entails using conidia grown on malt agar slants, using a spore inducing medium (SIM), as described by Tien and Kirk (1988). Currently, an inoculum of *P. chrysosporium* can be prepared using two methods, as shown in Figure 2.1.

Method 1 (preparation of seed culture): *P. chrysosporium* conidia are inoculated into small volumes of the nutrient medium to cultivate mycelia. The spores are grown in 90 ml of a nutrient medium, described by Tien and Kirk (1988), at 37°C in complete darkness for 48 h. The cultures are incubated in a rotary shaker under an air atmosphere. After four days, the broth containing mycelia is homogenised in a blender for 1 minute, and 10% (v/v) of the homogenised mycelia is used as inoculum (Rivela *et al.*, 2000; Rodríguez-Couto *et al.*, 2000a; Linko, 1988; Jäger *et al.*, 1985).

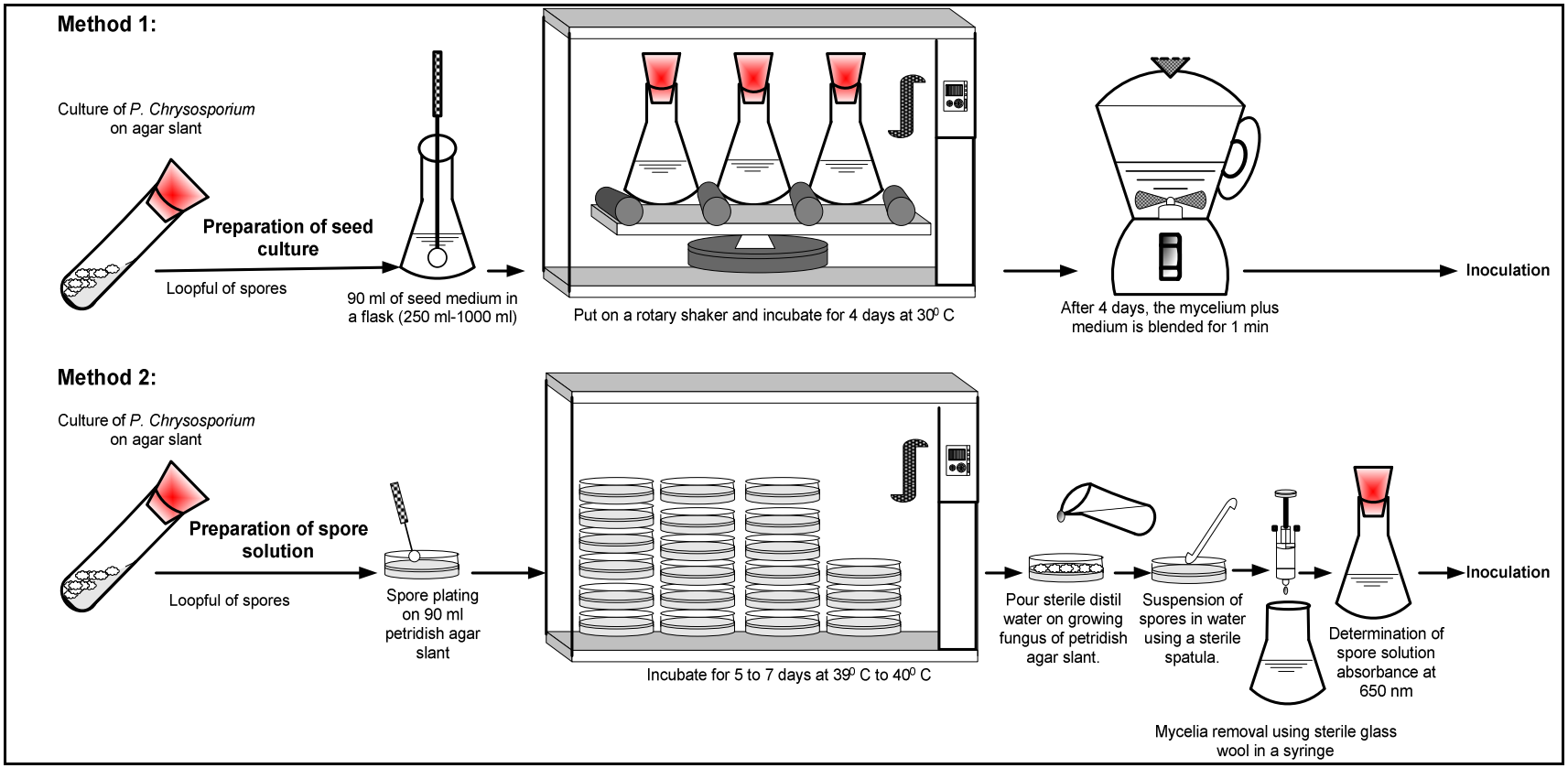

Figure 2.1: Inoculum development for *P. chrysosporium* cultures. *Method 1*: preparation of seed culture and *Method 2*: preparation of spore solution

Method 2 (preparation of spore solution): *P. chrysosporium* mycelia is cultivated on agar slants at 39℃ for 1 to 2 weeks (Kirk *et al*., 1986), or even up to 3 weeks (Lin *et al*., 1990). The spore suspensions are prepared from agar plates, using small volumes of sterile distilled water and a sterile spatula, to detach the spores and mycelia from the agar. Thereafter, suspensions are repeatedly passed through autoclaved glass wool, using syringes, to separate the mycelia from the spores. The amount of spores in the suspension is then determined spectrophotometrically at a wavelength of 650 nm (Tien & Kirk, 1988). The suspension is then used directly for inoculation without the addition of any nutrient medium. Alternatively, it can be stored at 4℃ for subsequent inoculations (Kay-Shoemake & Watwood, 1996; Griselda & Eduardo, 1990; Tien & Kirk, 1988). Stanbury *et al*. (1995) provide detailed guidelines on inoculum preparation and development.

2.2.2 Nutrient medium used for enhanced ligninase production with *P. chrysosporium*

The extracellular production of LiP and MnP has been studied using a synthetic medium based on the composition originally developed by Kirk *et al.* (1978). Other researchers modified this nutrient medium for use in ligninase production studies. A nitrogen limited (N-limited) nutrient medium was developed by Tien and Kirk (1988) and is the preferred nutrient medium for ligninase production studies. There are four types of nutrient medium compositions that have been used for LiP and MnP production: 1) carbon limited (C-limited) (Ruckenstein & Wang, 1994); 2) nitrogen limited (N-limited) (Tien & Kirk, 1988); 3) carbon excess (C-excess) (Datta *et al.,* 1991) and 4) nitrogen excess (N-excess) (Doseretz *et al*., 1993) growth medium. They are classified according to the ratio of the carbon-nitrogen (C/N) content in the medium, with glucose often being the carbon source, while different nitrogen sources have been used over time.

Table 2.1 shows the commonly used media for the production of extracellular enzymes from *P. chrysosporium* BKMF 1767. The C/N ratio can be used to selectively produce LiP and MnP. For example, when the ratio is low (C-limited), the production of LiP is sustainable without the production of extracellular polysaccharides. However, when the C/N ratio is high (N-limited), increased concentrations of polysaccharides are produced. LiP formation in this regard, is induced only when technical-grade (100%) oxygen is used. Under N-excess conditions, LiP activity above 1000 $U.L^{-1}$ can be achieved using air and 100% oxygen (Rothschild *et al*., 1995).

Table 2.1: Nutrient medium used in the production of LiP and MnP from *Phanerochaete chrysosporium*

	Carbon Source	Nitrogen Source	Surfactant	Buffer (pH)	Reference
C-limited	Glucose (2 $g.L^{-1}$)	NH_4Cl (0.12 $g.L^{-1}$)	-	Sodium tartrate pH 4.5 (20 mM)	(Ruckenstein & Wang, 1994)
N-limited	Glucose (10 $g.L^{-1}$)	$(NH_4)_2C_4H_4O_6$ (0.2 $g.L^{-1}$)	Tween 20/80 (0.05%)	2.2 Dimethyl succinate pH 4.2 (10 mM)	(Tien & Kirk, 1988)
C-excess	Glucose (25 $g.L^{-1}$)	L-Glutamic acid (0.25 $g.L^{-1}$)	-	KOH pH 4.0	(Datta *et al.,* 1991)
N-excess	Glucose (10 $g.L^{-1}$)	$(NH_4)_2C_4H_4O_6$ (11 $g.L^{-1}$)	-	Sodium acetate pH 4.5 (20 mM)	(Doseretz *et al.,* 1993)

Note: Quantities of reagents are listed at their final concentration in the nutrient medium

This implies that by decreasing the glucose content in the production medium, extracellular polysaccharide excretion and build-up in the mycelia can be limited, thus improving DO mass transfer through the biofilm. Moreira *et al.* (1997) designed a packed bed reactor where the C-limited medium was determined to have a positive effect on continuous extracellular LiP and MnP production.

The glucose content of the growth/production medium used by Moreira *et al.* (1997) contained 50 to 400 mg glucose.$L^{-1}h^{-1}$ and 0.1 to 1 mg ammonia.$L^{-1}h^{-1}$. The DO requirements were maintained above 25 mg.L^{-1} and MnP production of 150 to 300 U.L^{-1} was sustained. The production of MnP occurred only under C/N limitations, high O_2 tension and adequate Mn^{2+} concentrations. Protease production was linked to the use of air as an oxygen source. For the wild-type BKMF 1767, proteases were shown to be responsible for the loss of LiP in N-limited cultures (Dosoretz *et al.*, 1990). The disadvantages of the packed-bed reactor were high levels of protease production, which normally denature enzymes, as well as excessive mycelial growth, which eventually clogged the reactor bed leading to impaired DO transfer.

The basal medium contained the following (per litre of sterile distilled H_2O): 0.2 g of KH_2PO_4; 0.05 g of $MgSO_4.7H_2O$; 0.01 g of $CaCl_2$ and 1 ml of trace elements (Kirk *et al.*, 1978). Varying the ionic strength of the basal medium between 10- and 100-fold did not affect the onset, rate, or extent of lignin decomposition (Kirk *et al.*, 1978). The ionic strength of the basal medium used by Tien and Kirk (1988) was increased 100-fold. $CaCl_2$ was not incorporated into the trace element solution used by Tien and Kirk (1988). The nutrient medium was initially designed with batch cultures as the preferred choice for ligninase production. However, a high ionic strength for the basal medium can lead to metal accumulation in continuous fixed-film systems, where the immobilised biofilm is constantly supplied with the growth medium, which contains sources of heavy metals.

It was determined that a solution of Thiamine-HCl was suitable for *P. chrysosporium* growth and lignin metabolism during vitamin supplementation studies where a solution containing: 2 mg of biotin, 2 mg of folic acid, 5 mg of thiamin-HCl, 5 mg of riboflavin, 10 mg of pyridoxine-HCl, 0.1 mg of cyanocobalamine, 5 mg of nicotinic acid, 5 mg of calcium pantothenate, 5 mg of *p*-aminobenzoic acid and 5 mg of thioctic acid was used (Kirk *et al.*, 1978). Tien and Kirk (1988) used 1 mg of thiamine-HCl per litre of sterile distilled water. The growth and production medium designed for *P. chrysosporium* cultures and used by Tien and Kirk (1988) consisted of: 100 ml of basal medium, 100 ml of 10% glucose, 100 ml of 0.1 M 2,2-dimethyl succinate (pH 4.2), 10 ml of 0.1 $g.L^{-1}$ thiamine-HCl, 25 ml of 8 $g.L^{-1}$ ammonium tartrate, 100 ml of 0.4 M veratryl alcohol and 60 ml of trace element solution.

2.2.3 The role of surfactants on the performance of *P. chrysosporium* biomass

The use of surfactants such as Tween 80, Tween 20, polyoxyethylene oleate and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate in the growth medium used for *P. chrysosporium* cultures enhanced ligninase production by 200-fold after evaluating the surfactants as possible stimulators of ligninase activity (Leštan *et al.*, 1993; Jäger *et al.*, 1985). MnP was increased by 260-fold (Novotný *et al.*, 2004) after the addition of Tween 80 to submerged cultures. It was suggested that the extracellular enzymes of *P. chrysosporium* are weakly charged in their active state. This meant that ionic bonding was likely to be the retaining force in the production biomass. In the presence of a stronger binding force, such as extracellular polysaccharide-based mucilage, *P. chrysosporium* biomass will entrap some of the enzymes. It was determined that the enzymes were bound to the hyphae (Montgomery, 1982); thus the use of surfactants caused the release of the enzymes. Furthermore, surfactants were shown to prevent mechanical deactivation of ligninase as several surfactants were shown to protect purified ligninase against mechanical agitation (Venkatadri & Irvine, 1990). Tween 80, as used in the N-limited medium at a final concentration of 0.05% (v/v), had no inhibitory effects on fungal growth; whereas, some

surfactants like Tergitol NP-10 and Triton X-100 inhibited the growth rate of *P. chrysosporium* by 75% to 95% (Ruiz-Aguilar *et al*., 2002). Generally, non-ionic surfactants exert many biological effects on microorganisms. They have, however, been successfully exploited in some biotechnological processes, although some have been shown to have toxic effects. These surfactants readily bind to proteins and modify membrane phospholipids thus improving permeability (Cserháti, 1995). Optimising the surfactant concentrations is important, as high concentrations of cell membrane permeability can increase undesirable intracellular metal intake and accumulation. Various other studies that offer further reading and explanations on the improvement of enzyme production by supplementation of the basic growth medium with various additives (veratryl alcohol, Tween 20/80 and trace elements) or adaptation of the physical environments were reported by Asther *et al*. (1988), Bonnarme and Jeffries (1990) and Linko (1992).

2.2.4 Bioreactor configurations and operating conditions used for ligninase production

The production of LiP and MnP has been achieved in various types of reactors with different operating conditions, working volumes and operating times. The highest ligninase activities – greater than 1000 $U.L^{-1}$ (Moldes *et al*., 2003; Domínguez *et al*., 2001; Rodríguez-Couto *et al*., 2001; Bonnarme *et al*., 1993; Doseretz *et al*., 1993) – were achieved in bioreactor systems that used biofilm carriers/support and technical-grade oxygen (100%) or air to improve enzyme production. Previously reported bioreactor designs include: a rotating drum reactor, which achieved 1350 $U.L^{-1}$ of MnP and 1400 $U.L^{-1}$ of LiP production (Domínguez *et al*., 2001), and the immersion bioreactor, which achieved 1447 $U.L^{-1}$ of MnP and 277 $U.L^{-1}$ of LiP (Rodríguez-Couto *et al*., 2001). These two bioreactors used an aeration system during their operation, but both had a mobile mechanical device to move the biofilm in order to improve the oxygen transfer rate (OTR), while an N-limited nutrient medium was used. Another bioreactor design used for ligninase production consisted of a jacketed cylindrical glass

vessel with a round bottom, containing a wire basket filled with polypropylene cubes. The basket in the bioreactor was moved upwards and downwards using a pneumatic system. The basket was outside the medium for 90 s and inside the medium for 10 s (Rivela *et al.*, 2000).

When *P. chrysosporium* was cultured, using either cellulose or glucose as the carbon source, observations were made that the synthesis of extracellular polysaccharides in the cultures was similar (Zacchi *et al.*, 2000a). The production of extracellular polysaccharides restricts the DO transfer into the hyphae, which is necessary for LiP induction. It was suggested that agitation to improve DO transfer is of paramount importance, as LiP production is triggered by the response to oxidative stress or increased DO (Zacchi *et al.*, 2000b). This led to studies using stirred-tank reactors (STRs) and agitated-flask cultures, with agitation speeds of 30 (low) to 150 rpm (high), which were less effective for ligninase production (Janshekar & Fiechter, 1988).

Different types of reactors without agitation devices have also been evaluated for continuous MnP and/or LiP production. Examples of these reactors are: trickle/packed beds, airlifts and MBRs. Trickle-bed reactors with different packing materials were evaluated for enzyme production, as they have better DO mass transfer capabilities. The trickle-bed bioreactor design was such that the ligninase-containing supernatant was continuously removed and recycled. The operating conditions depended on the recycle ratio suitable for enhanced productivity. The reactor could be operated in semi-continuous- and continuous modes, while the biomass carriers were mobile as a result of liquid movement in the reactor, with some of the enzyme solution being recycled (Ruggeri & Sassi, 2003). However, the recycling of the supernatant can inhibit and delay the production of LiP and MnP. It was determined that when ligninolytic enzyme fluids were added to LiP containing solutions, activity decreased for both free-pellet cultures and immobilised cultures. LiP activity, obtained in cultures with recycled ligninolytic solution, was lower than in controlled cultures, without ligninolytic fluid addition. Enzyme activity was not significantly altered by the addition of ligninolytic solutions

containing only MnP (Feijoo *et al*., 1995a). The consequences of recycling ligninolytic enzyme fluids were less significant for submerged cultures, where a decrease of 10% was observed in LiP activity, than for immobilised cultures, where a decrease of 50% occurred (Moreira *et al*., 1997). However, other researchers have indicated that LiP production was highest in packed-bed reactors with lower recycling ratios (2:1) (Feijoo *et al*., 1994), while the airlift reactor provided a suitable low-shear environment for enzyme production using mycelial pellets. A simple method to produce LiP or MnP enzymes selectively was used by Bonnarme and Jefferies (1990), by increasing either the soluble Mn^{2+}/Mn^{3+} organic acid complexes to suppress LiP formation, or by using 100% O_2 in Mn-deficient cultures to induce LiP production while suppressing MnP as indicated by Perez and Jeffries (1992).

Several research groups have shown improved productivity using MBRs for the continuous production of LiP and MnP with immobilised biofilms of *P. chrysosporium* (Sheldon & Small, 2005; Govender *et al*., 2004; Govender *et al*., 2003; Solomon & Petersen, 2002; Leukes, 1999; Leukes *et al*., 1999; Venkatadri & Irvine, 1993). The use of continuous fixed-film MBRs by Leukes *et al*. (1999), Solomon and Petersen (2002), Govender *et al*., (2003) and Sheldon and Small (2005), where a biofilm of *P. chrysosporium* was immobilised on the external surface of a capillary membrane, showed potential for continuous extracellular enzyme production. This MBR system was classified as a membrane-gradostat reactor (MGR), where the nutrient medium was continuously supplied to the membrane lumen. The MGR was based on the establishment of nutritional gradients in the immobilised biofilm, while DO was supplied in the form of a gas to the extracapillary space (ECS) of the reactor. The industrial use of this type of reactor is hampered by DO mass transfer limitations in the immobilised biofilm. However, the distinctive advantage is that MBRs provide a suitable environment that mimics the living conditions of microorganisms in their natural environment. The microorganisms immobilised on the membranes find themselves in microenvironments containing nutrient gradients across the biofilm thickness.

As a result of nutrient gradients, *P. chrysosporium* produces extracellular secondary metabolites when its biomass is kept in the idiophase. MnP production levels of 2361 $U.L^{-1}$ were achieved by Leukes (1999), using the MGR system. Nutritional-gradient conditions in immobilised systems allow for quicker metabolite production than the submerged-culture type reactors, where the microorganisms are usually submerged in a liquid-based medium. This is because of existing nutritional gradients, some of the biomass will undergo starvation and be forced into the idiophase growth stage, thus producing valuable extracellular enzymes.

Since *P. chrysosporium* is a facultative aerobe, the fungus produces ethanol under anaerobic conditions (Kenealy & Dietrich, 2004), while the activity and the production of LiP and MnP are influenced by the availability of DO. The rate of lignin degradation by *P. chrysosporium* BKMF 1767 was reported to be 2 to 3 times greater when using technical-grade oxygen (100%) rather than air. This is in spite of better growth in air (Leisola *et al.*, 1984; Kirk *et al.*, 1978). The ligninolytic system of the WRF has been shown to be particularly active in cultures grown in high oxygen tension (Doseretz *et al.*, 1993). Faison and Kirk (1985) reported that ligninase production by *P. chrysosporium* was increased in cultures aerated during the primary growth phase of the fungus, before shifting to a technical-grade oxygen (100% O_2) supply. However, lignin degradation in 5 atm. of air was found to be similar to that in 1 atm. of 100% O_2 (Reid & Seifest, 1980). Agitation under 100% O_2 completely inhibited lignin metabolism (Kirk & Fenn, 1982), highlighting the sensitivity of ligninase to agitation. Agitation of the liquid cultures generally resulted in the formation of pellets, which greatly suppressed lignin decomposition (Kirk *et al.*, 1978). This was because pellet formation restricted the transportation of DO to inner parts of the mycelial pellets, further hampering nutritional support of active mycelia at the centre of the pellets. LiP- and MnP formation required DO in the catalytic cycles to produce H_2O_2, as this was the primary substrate in both LiP and MnP production cycles. Prolonged exposure to high oxygen partial pressures might lead to hyperoxia as a result of enhanced intracellular formation in reactive oxygen species

(ROS) (Li *et al.*, 2004), but the formation of limited quantities of ROS was shown to improve LiP induction (Belinky *et al.*, 2005). From this information, it is clear that a delicate balance is required to limit ROS formation, while improving the longevity of active biomass in fixed-film bioreactors.

General bioreactor operating conditions for LiP- and MnP production differ, and the operating parameters are generally optimised to ensure the highest ligninase production for a specific bioreactor. For example, periodical 100% O_2 purging was used to enhance enzyme production. The flasks were purged with 100% O_2 for 10 minutes at 0.02 $L.h^{-1}$ (Kirkpatrick & Palmer, 1987), and in some of the reactors the growth medium (N-limited, containing 10 $g.L^{-1}$ glucose) was later replaced with a production medium (C-limited, containing 2 $g.L^{-1}$ glucose). Ruggeri and Sassi (2003) also trickled the culture medium at different flow rates of 0.01 to 0.08 $cm.s^{-1}$ and 100% O_2 was flushed up-flow at 0.01 $v.v^{-1}.min.^{-1}$ (volume O_2 per volume liquid per minute).

During its primary growth phase, *P. chrysosporium* requires a source of fixed nitrogen, but is relatively inactive in terms of ligninase production during this stage. Furthermore, when the fungus undergoes nitrogen starvation, the production of oxidation enzymes is induced (Fenn *et al.*, 1981; Jeffries *et al.*, 1981). LiP production observed in the polysulphone-based MGR systems, was detected after two to three days of operation (Sheldon & Small, 2005), which was determined to be the start of the exponential growth phase as determined by Ntwampe and Sheldon (2006). The ammonium source, continuously supplied to the reactor in the form of a nutrient medium and measured in permeate samples recovered from the MGR systems, was exhausted after three days of bioreactor operation.

P. chrysosporium growth consists of multiple growth phases when grown in flasks using N-limited medium. It was observed that when *P. chrysosporium* was incubated at 35°C, two exponential growth phases occurred (Ceribasi & Yetis, 2001). The primary exponential phase

occurred after 10 to 65 h, while the secondary exponential phase occurred after 100 to 150 h, using submerged cultures. This phenomenon can be exploited in the immobilised fixed-film MGR systems where the idiophase zone in the biofilms can be increased, as the biomass can be kept in the idiophase as a result of limited nutrient concentrations and distribution.

Ruggeri and Sassi (2003) used a 5 cm internal diameter and 85 cm high borosilicate glass column filled with random packing. Liquid distribution was maintained to achieve either a continuous or a pulsed regime on the reactor bed. The pulsed regime had a frequency of 30 to 40 pulsations per minute. In some bioreactors, pH was allowed to change freely during the immobilisation, growth and production phases (Linko, 1988).

Conditions and operational parameters in different bioreactors used for the production of LiP and MnP from *P. chrysosporium* BKMF 1767 (ATCC 24725) in carbon/nitrogen-limited liquid cultures and the maximum production levels obtained during bioreactor operations are summarised in Table 2.2.

Table 2.2: Conditions and parameters associated with the production of extracellular enzymes from *Phanerochaete chrysosporium* BKMF-1767 (ATCC 24725) in carbon/nitrogen-limited liquid cultures, including assay substrates used to quantify extracellular enzymes and the maximum production levels obtained during bioreactor operations

	Bioreactor type	Surface carrier or support	Condition/gas	Size/time of operation	Ligninase assay (oxidation)		Maximum enzyme activity levels		Reference
					LiP Substrate	MnP Substrate	MnP	LiP	
C-limited	Erlenmeyer Flask, Agitated, 150 rpm reduced to 30 rpm	-	Submerged/Air/O_2	0.6 L/120 h	Veratryl alcohol	n/d	n/d	n/d	(Haemmerli *et al*., 1986a)
	Erlenmeyer Flask, Agitated, 30 rpm	-	Submerged/Air/O_2	0.6 L/72 h	Veratryl alcohol	n/d	n/d	n/d	(Haemmerli *et al*., 1986b)
	Flask, Rotary shaker (150 rpm, 2.5 cm)	-	Submerged/air	0.6 L/48 h	Veratryl alcohol	Phenol red	n/d	n/d	(Leisola *et al*., 1987)
	Stirred tank reactor, 150 rpm	-	Submerged/O_2	30 L/288 h	Veratryl alcohol	Phenol red	n/d	62 $U.L^{-}$	(Janshekar & Fiechter, 1988)
	Stirred tank reactor, 80 rpm	-	Submerged/O_2	200 L/288 h	Veratryl alcohol	Phenol red	n/d	0 $U.L^{-1}$	(Janshekar & Fiechter, 1988)
	Packed bed bioreactor	-	Submerged/O_2	7.6 L/132 h	Veratryl alcohol	n/d	n/d	730 $U.L^{-1}$	(Linko, 1988)

Table 2.2: *(Continued)*

	Bioreactor type	Surface carrier or support	Condition/gas	Size/time of operation	Ligninase assay (oxidation)		Maximum enzyme activity levels		Reference
					LiP Substrate	MnP Substrate	MnP	LiP	
N-limited	Polyethylene, 'carboy', 1rpm	-	Submerged/ O_2	20 L/ n/a	Veratryl alcohol	Vanillyacetone	n/d	n/d	(Paszczynski *et al.*, 1985)
	Erlenmeyer Flask, Agitated, 150 rpm	-	Submerged/Air/O_2	600 ml/72 h	Veratryl alcohol	n/d	n/d	370 $U.L^{-1}$	(Haemmerli *et al.*, 1986b)
	Erlenmeyer Flask, stationary	-	Submerged/O_2	10 ml/240 h	Veratryl alcohol	n/d	n/d	23 $U.L^{-1}$	(Kirk *et a*l., 1986)
	Erlenmeyer Flask, stationary *Strain SC26	-	Submerged/O_2	10 ml/240 h	Veratryl alcohol	n/d	n/d	34 $U.L^{-1}$	(Kirk *et al.*, 1986)
	Flask, Agitated, 125 rpm, 2.5 cm	Polyurethane foam	Submerged/ O_2	600 ml/288 h	Veratryl alcohol	n/d	n/d	95 $U.L^{-1}$	(Kirkpatrick & Palmer, 1987)
	Flask, stationary	-	Submerged/O_2	20 ml/120 h	Veratryl alcohol	Phenol red	11 $U.mg^{-1}$	7 $U.mg^{-1}$	(Leisola *et al.*, 1987)
	Flask, Agitated	-	Submerged/air	-	Veratryl alcohol	Vanillyacetone	n/d	n/d	(Farrell *et al.*, 1989)
	Airlift bioreactor	-	Submerged/Air/O_2	5.0 L/150 h	Veratryl alcohol	Vanillyacetone	130 $U.L^{-1}$	760 $U.L^{-1}$	(Bonnarme & Jeffries, 1990)
	Stirred tank reactor, 100 rpm	-	Submerged/ O_2	500 ml/192 h	Veratryl alcohol	n/d	n/d	180 $U.L^{-1}$	(Michel *et al.*, 1990)
	Erlenmeyer Flask, Agitated, 200 rpm	-	Submerged/Air	150 ml/ n/a	Veratryl alcohol	n/d	n/d	100 $U.L^{-1}$	(Tuisel *et al.*, 1990)
	Fernbach Flask, Agitated, 200 rpm	-	Submerged/Air	850 ml/ n/a	Veratryl alcohol	n/d	n/d	100 $U.L^{-1}$	(Tuisel *et al.*, 1990)
	Flask, Agitated, 120 rpm, 2.5 cm	-	Submerged/O_2	100 ml/192 h	Veratryl alcohol	Vanillyacetone	100 $U.L^{-1}$	650 $U.L^{-1}$	(Bonnarme *et al.*, 1991)

Table 2.2: *(Continued)*

	Bioreactor type	Surface carrier or support	Condition/gas	Size/time of operation	Ligninase assay (oxidation)		Maximum enzyme activity levels		Reference
					LiP Substrate	MnP Substrate	MnP	LiP	
N-limited	Flask	Polyurethane foam	Submerged/O_2	100 ml/192 h	Veratryl alcohol	Vanillyacetone	250 $U.L^{-1}$	1300 $U.L^{-1}$	(Bonnarme *et al.*, 1991)
	Solid-state reactor, using polypropylene	-	Submerged/Air	1.95L/72 h	Veratryl alcohol	Vanillyacetone	n/d	450 $U.L^{-1}$	(Datta *et al.*, 1991)
	Fluidised bed reactor	Sintered glass, shaped as Raschig rings	Submerged/ Immobilised/Air/ O_2	350 ml/12 h	Veratryl alcohol	n/d	n/d	9.6 nkat/ml	(Rogalski *et al.*, 1992)
	Stirred tank reactor, batch	Silicone membrane wrapped around stainless steel rods	Submerged/Air/ O_2	2.5 L/192 h	Veratryl alcohol	n/d	n/d	195 $U.L^{-1}$	(Venkatadri *et al.*, 1992)
	Airlift reactor, Batch, ID = 8cm	-	Submerged/Air/ O_2	0.5 L/100 h	Veratryl alcohol	Vanillyacetone	1812 $U.L^{-1}$	4500 $U.L^{-1}$	(Bonnarme *et al.*, 1993)
	Erlenmeyer Flask, Agitated, 200 rpm	-	Submerged/Air/O_2	45 ml/72 h	Veratryl alcohol	n/d	n/d	n/d	(Boominathan *et al.*, 1993)
	Flask, stationary	-	Submerged/O_2	10 ml/144 h	Veratryl alcohol	Phenol red	421 $U.L^{-1}$	270 $U.L^{-1}$	(Cancel *et al.*, 1993)
	Erlenmeyer Flask, Agitated, 140 rpm	-	Submerged/O_2	90 ml/240 h	Veratryl alcohol	Phenol red	3500 $U.L^{-1}$	800 $U.L^{-1}$	(Dosoretz *et al.*, 1993)
	Hollow fibre reactor	Polysulphone	Immobilised/air	45 ml ECS/216 h	Veratryl alcohol	n/d	n/d	190 $U.L^{-1}$	(Venkatadri & Irvine, 1993)
	Stirred tank reactor	Silicone Tubing (mesh)	Submerged/ Immobilised/O_2	1.5 L/ 840 h	Veratryl alcohol	n/d	n/d	230 $U.L^{-1}$	(Venkatadri & Irvine, 1993)
	Erlenmeyer Flask, Agitated, 30 rpm	Poly(styrene-divinylbenzene)	Immobilised/O_2	75 ml/960 h	Veratryl alcohol	n/d	n/d	602 $U.L^{-1}$	(Ruckenstein & Wang, 1994)

Table 2.2: *(Continued)*

	Bioreactor type	Surface carrier or support	Condition/gas	Size/time of operation	Ligninase assay (oxidation)		Maximum enzyme activity levels		Reference
					LiP Substrate	MnP Substrate	MnP	LiP	
N-limited	Packed bed reactor	Polyurethane	Submerged/ Immobilised/O_2	167 ml/600 h with 8 h HRT	Veratryl alcohol	n/d	n/d	200 $U.L^{-1}$	(Feijoo *et al*., 1995a)
	Erlenmeyer Flask	-	Submerged/O_2	10 ml/600 h	Veratryl alcohol	2.6 Dimethoxy phenol	22.3 $U.L^{-1}$	89.4 $U.L^{-1}$	(Kaal *et al*., 1995)
	Serum bottles	-	Submerged/air	5 ml/270 h	Veratryl alcohol	2.6 Dimethoxy phenol	-	525 $U.L^{-1}$	(Mester *et al*., 1995)
	Erlenmeyer Flask	Raschig rings	Submerged/O_2	30 ml/500 h	n/d	n/d	0.03 $U.ml^{-1}$	600 $U.L^{-1}$	(Bosco *et al*., 1996)
	Fernbach Flask	Raschig rings	Submerged/O_2	30 ml/400 h	n/d	n/d	n/d	700 $U.L^{-1}$	(Bosco *et al*., 1996)
	Trickle Fixed Bed Reactor, ID = 5 cm	Berl saddles, Ca-alginate encapsulated conidia	Immobilised/O_2	250 m at 0.6 $cm.min^{-1}$/400 h	n/d	n/d	n/d	1200 $U.L^{-1}$	(Bosco *et al*., 1996)
	Packed bed reactor	Polyurethane	Submerged/ Immobilised/Air/O_2	167 ml/3360 h	n/d	2.6 Dimethoxy phenol	250 $U.L^{-1}$	n/d	(Moreira *et al*., 1997)
	Flask, Agitated, 150 rpm, 2.5 cm	-	Submerged/O_2	90 ml/96 h	n/d	2.6 Dimethoxy phenol	n/d	n/d	(Palma *et al*., 1997)
	Trickle bed reactor, ID = 5cm	Berl saddles, immersed in Ca-Alginate solution	Submerged/O_2	250 ml/450 h	Veratryl alcohol	n/d	n/d	1200 $U.L^{-1}$	(Bosco *et a*l., 1999)
	Membrane bioreactor (MGR)	Polysulphone membrane	Immobilised/ O_2	Continuous flow	-	2.6 Dimethoxy phenol	2361 $U.L^{-1}$	n/d	(Leukes, 1999)
	Erlenmeyer Flask	-	Submerged/Air	20 ml/264 h	Veratryl alcohol	Phenol red	220 $U.L^{-1}$	120 $U.L^{-1}$	(Rothschild *et al*., 1999)
	Immersion Bioreactor (pneumatic system)	Nylon sponge	Submerged/ Immobilised/Air	2500 ml/480 h	Veratryl alcohol	2.6 Dimethoxy phenol	987 $U.L^{-1}$	356 $U.L^{-1}$	(Rivela *et al*., 2000)

Table 2.2: *(Continued)*

	Bioreactor type	Surface carrier or support	Condition/gas	Size/time of operation	Ligninase assay (oxidation)		Maximum enzyme activity levels		Reference
					LiP Substrate	MnP Substrate	MnP	LiP	
N-limited	Erlenmeyer Flask	Polyurethane foam	Submerged/air	12 ml/216 h	Veratryl alcohol	2.6 Dimethoxy phenol	200 $U.L^{-1}$	20 $U.L^{-1}$	(Rodríguez-Couto *et al.*, 2000a)
	Erlenmeyer Flask	Polyurethane foam, nylon sponge	Submerged/air	300 ml/288 h	Veratryl alcohol	2.6 Dimethoxy phenol	1371 $U.L^{-1}$	197 $U.L^{-1}$	(Rodríguez-Couto *et al.*, 2000b)
	Rotating drum reactor, 3 rpm, batch	Wire mesh, with cubes of fibrous nylon sponge	Partly submerged/Air	-/456 h	Veratryl alcohol	2.6 Dimethoxy phenol	1350 $U.L^{-1}$	1400 $U.L^{-1}$	(Domínguez *et al.*, 2001)
	Static bed reactor	Polypropylene sponge	Submerged/air	1.0 L/288 h	Veratryl alcohol	2.6 Dimethoxy phenol	154 $U.L^{-1}$	194 $U.L^{-1}$	(Rodríguez-Couto *et al.*, 2001)
	Immersion Bioreactor (pneumatic system)	Polypropylene sponge	Submerged/air	1.0 L/288 h	Veratryl alcohol	2.6 Dimethoxy phenol	1447 $U.L^{-1}$	277 $U.L^{-1}$	(Rodríguez-Couto *et al.*, 2001)
	Erlenmeyer Flask	Fibrous nylon sponge	Submerged/Air	12 ml/240 h	Veratryl alcohol	2.6 Dimethoxy phenol	510 $U.L^{-1}$	0 $U.L^{-1}$	(Cabaleiro *et al.*, 2002)
	Erlenmeyer Flask	Corncob cubes	Submerged/Air	12 ml/240 h	Veratryl alcohol	2.6 Dimethoxy phenol	662 $U.L^{-1}$	0 $U.L^{-1}$	(Cabaleiro *et al.*, 2002)
	Fixed-bed reactor	Fibrous nylon sponge	Submerged/Air	0.25 L/600 h	Veratryl alcohol	2.6 Dimethoxy phenol	1300 $U.L^{-1}$	1100 $U.L^{-1}$	(Moldes *et al.*, 2003)
	Trickle Fixed Bed Reactor, ID = 5 cm	Berl saddles	Immobilised /O_2	250 ml at 4.8 $cm.min^{-1}$/400 h	Veratryl alcohol	n/d	n/d	3430 $U.L^{-1}$	(Ruggeri & Sassi, 2003)
	Erlenmeyer Flask	-	Submerged/-	25 ml/432 h	n/d	Phenol red	n/d	n/d	(Levin *et al.*, 2004)

Table 2.2: *(Continued)*

	Bioreactor type	Surface carrier or support	Condition/gas	Size/time of operation	Ligninase assay (oxidation)		Maximum enzyme activity levels		Reference
					LiP Substrate	MnP Substrate	MnP	LiP	
N-limited									
	Erlenmeyer Flask	Polyurethane foam	Submerged/Air/O_2	100 ml/240 h	-	Guaiacol	1000 $U.L^{-1}$	n/d	(Chung *et al.*, 2005)
	Fixed-bed tubular reactor, Batch	$ZrOCl_2$-activated pumice	Submerged/Air	-/168 h	Veratryl alcohol	Guaiacol	60 $U.L^{-1}$	0 $U.L^{-1}$	(Pazarlioğlu *et al.*, 2005)
	Erlenmeyer Flask	Polystyrene foam	Submerged/Air	-/192 h	n/d	2.6 Dimethoxy phenol	380 $U.L^{-1}$	n/d	(Ürek & Pazarlioğlu, 2005)
	Stirred tank reactor, 300 rpm, batch	Plastic composite support	Submerged/Air/O_2	1000 ml/144 h	Veratryl alcohol	Vanillyacetone	63.0 $U.L^{-1}$	50 $U.L^{-1}$	(Khiyami *et al.*, 2006)

HRT – hydraulic retention time
n/d – not determined or negligible enzyme activity
**Strain SC26 is a mutant derived from BKM-F 1767*

2.2.5 Carriers used in bioreactors to enhance immobilised biomass performance

Biomass in the form of conidia or mycelia can be entrapped by physical or chemical means, depending on the process parameters selected. Immobilisation of *P. chrysosporium* mycelia or conidia was used for the improvement of ligninase production and to simulate the natural habitat of the fungus, where submerged conditions are non-existent. Improved biomass concentrations were obtained in immobilised cultures where the biofilm were grown on matrices with cavities over that of biomass grown in submerged conditions. Polyurethane foam is commonly used to immobilise *P. chrysosporium* because of its biocompatibility and consistent microporous structure (Romaškevič *et al.*, 2006). This material was mostly used in semi-submerged flask cultures for LiP or MnP production (Chung *et al.*, 2005; Rodríguez-Couto *et al.*, 2000a; Rodríguez-Couto *et al.*, 2000b; Moreira *et al.*, 1997; Feijoo *et al.*, 1995b; Bonnarme *et al.*, 1991; Kirkpatrick & Palmer, 1987).

Other types of carriers that are commonly used are made of: 1) nylon (Rivela *et al.*, 2000; Rodríguez-Couto *et al.*, 2000b); 2) polystyrene (Ürek & Pazarlioğlu, 2005) and 3) polypropylene (Rodríguez-Couto *et al.*, 2001). Innovative mycelium supports are: 1) corncob cubes, which also provide nutritional supplements to the fungus (Cabaleiro *et al.*, 2002); 2) activated pumice treated with $ZrOCl_2$ (Pazarlioğlu *et al.*, 2005); 3) Berl saddles treated with calcium alginate (Bosco *et al.*, 1999) and 4) sintered glass shaped like Raschig rings (Rogalski *et al.*, 1992). The studies conducted using these types of support materials were all performed on laboratory batch-scale. However, this necessitated the use of other materials suitable for pilot scale bioreactors, and hence capillary tubes and membranes were introduced to avoid submersion and to provide continuous removal of ligninolytic solution from the bioreactors. Examples of these types of reactors using silicone, polysulphone and ceramic capillary membranes, were described by Venkatadri and Irvine (1993), as well as Sheldon and Small (2005). The carriers were autoclaved or boiled (nylon sponge) prior to use, while the polyurethane foam was washed in methanol and rinsed three times with

distilled water. Some of the carriers were inert and were used only to immobilise mycelia, while the corncob acted as a nutrient source (Rivela *et al*., 2000). Ruggeri and Sassi (2003) used ceramic Berl saddles immersed in a solution of 2.0 $g.L^{-1}$ Na-alginate, while Bosco *et al.* (1996) used ceramic Raschig rings with a size of 6.35 mm and ceramic Berl saddles of 12.7 mm. The immobilisation of biomass has a number of advantages, as is shown in Table 2.3 (Dörnenburg & Knorr, 1995).

Table 2.3: Some advantages of biomass immobilisation in bioreactors, adapted from Dornenburg and Knorr (1995)

	Advantages		Reason/Consequence
1.	Continuous use of biomass and improved recovery of products.	1.	Entrapment of biomass.
2.	Maintenance of stable and active biocatalyst in the ligninolytic stage for longer periods.	2.	Elimination of unproductive growth phases and reduced risk of contamination.
3.	Employment of a continuous process.	3.	Continuous removal of metabolic inhibitors; improved process control and product recovery.
4.	Increased productivity; reduced costs.	4.	Increased density and elimination of lag phase.
5.	Reduced labour for medium preparation.	5.	Production and growth medium are identical.

2.2.6 Analytical assays used to evaluate ligninase activity from *P. chrysosporium* biomass

The performance of a culture in which enzymes are produced is evaluated by the activity of the enzymes produced. Before any assays can be performed, fluid cultures are purified to separate mycelia, the supernatant and enzymes. This can be done simply by centrifuging at 4800 rpm for 10 minutes using 10 kDa cut-off tubes, and then diluting the enzymes using a buffer solution or water before the assaying procedure (Tien & Kirk, 1988). The activity of the enzymes has been spectrophotometrically determined using a substrate, which oxidatively converts into another product. To do this, a 1.0 cm glass cuvette is normally used within a temperature-controlled spectrophotometer. The product formed is measured at the wavelength at which it most strongly absorbs light. The enzymes catalyse an oxidation

reaction in a buffered solution. Enzyme activity is normally measured in units per litre ($U.L^{-1}$). One unit (U) is defined as the amount of enzyme that oxidises 1 μmol of substrate in 1 minute. Tien and Kirk (1988) described the preferred method, which is used to quantify LiP. This method is universal and has been used by several research groups. However, MnP is measured using several different substrates such as phenol red, vanillyacetone and other suitable substrates. Some assays use oxidative coupling techniques for the detection of MnP to prevent LiP and laccase interference, which occurs even when MnP concentrations are low (Castillo *et al.*, 1994). Table 2.4 lists substrates suitable for MnP assays with their extinction coefficients and the wavelength at which substrate oxidation is normally monitored. Table 2.5 lists reagents at their final concentrations and parameters used for determining LiP and MnP.

Table 2.4: Substrates for manganese peroxidase assays (Paszczynski *et al.*, 1985)

Substrate	Wavelength (nm)	Extinction coefficient ($M^{-1}.cm^{-1}$)
TMPD	610	11600
Syringic acid	260	8050
Curcumin	430	23100
Syringaldazine	525	65000
Coniferyl alcohol	263	13400
o-Dianisidine(-2HCl)	460	29400

TMPD = N,N,N,N-Tetramethyl-1.4-phenylenediamine

Table 2.5: Final concentration of reagents and parameters used in ligninase assays used to quantify lignin and manganese peroxidase

	lignin peroxidase		Manganese peroxidase	
Reagent/Parameter	**(Tien & Kirk, 1988)**	**(Archibald, 1992)**	**(Castillo *et al.*, 1994)**	**(Pazarlioğlu *et al.*, 2005)**
Substrate	2 mM veratryl alcohol	32 µM Azure B	0.07 mM MBTH 0.99 mM DMAB	1 mM Guaiacol
Buffer(s)	50 mM tartaric acid, pH 2.5	50 mM Na tartrate, pH 4.5	100 mM Na succinate, pH 4.5 100 mM Na lactate, pH 4.5	500 mM Na tartrate, pH 4.5
Initiator	0.4 mM H_2O_2	0.1 mM H_2O_2	0.05 mM H_2O_2 0.3 mM $MnSO_4$	1 mM H_2O_2 1 mM $MnSO_4$
Volume of enzyme	100 µL	100 µL	100 µL	300 µL
Temperature	25 ℃	25 ℃	25 ℃	25 ℃
Wavelength	310 nm	651 nm	590 nm	465 nm
Extinction coefficient	9300 $M^{-1}.cm^{-1}$	48800 $M^{-1}.cm^{-1}$	53000 $M^{-1}.cm^{-1}$	12000 $M^{-1}.cm^{-1}$

Table 2.5: *(Continued)*

Manganese peroxidase *(Continued)*

Reagent/Parameter	(Paszczynski *et al.*, 1985)	(Kuwahara *et al.*, 1984)	(Glenn & Gold, 1988)	(Wariishi *et al.*, 1992)
Substrate	0.1 mM Vanillyacetone	0.01% phenol red	40 $\mu g.ml^{-1}$ ABTS	1 mM 2.6 Dimethoxyphenol
		3 $\mu g.ml^{-1}$ egg albumin	3 $\mu g.ml^{-1}$ egg albumin	
Buffer (s)	100 mM Na tartrate, pH 5	20 mM Na succinate, pH 4.5	50 mM Na succinate, pH 4.5	50 mM Na malonate, pH 4.5
			50 mM Na lactate, pH 4.5	
Initiator	0.1 mM H_2O_2	0.1 mM H_2O_2	0.05 mM H_2O_2	0.1 mM H_2O_2
	0.1 mM $MnSO_4$	0.1 mM $MnSO_4$	0.1 mM $MnSO_4$	0.5 mM $MnSO_4$
Volume of enzyme	100 µL	**10 to 40 µL	1 to 10 µL	20 to 100 µL
Temperature	22 ℃	30 ℃	25 ℃	30 ℃
Wavelength	336 nm	610 nm	415 nm	469 nm
Extinction coefficient	18300 $M^{-1}.cm^{-1}$	*4460 $M^{-1}.cm^{-1}$	36000 $M^{-1}.cm^{-1}$	49600 $M^{-1}.cm^{-1}$

MBTH = 3-methyl-2-benzothiazolinone hydrazone
DMAB = 3-(dimethylamino) benzoic acid
ABTS = 2.2-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid)
Vanillyacetone = 4-(4-Hydroxy-3-methoxyphenyl)-3-buten-2-one
* ε=4460 $M^{-1}.cm^{-1}$ (Michel *et al.*, 1991)

2.3 LIMITATIONS RELATED TO *P. CHRYSOSPORIUM* BIOMASS PERFORMANCE IN CONTINUOUS FIXED-FILM BIOREACTORS

2.3.1 DO mass transport limitations in biofilms of *P. chrysosporium*

DO transport in *P. chrysosporium* biofilms is a limiting parameter for the production of both LiP and MnP. It was shown that DO limitation is the main reason for slow and incomplete lignin degradation (Kirk *et al.*, 1978). When an Clark-type DO microsensor was used to quantify DO in mycelial mats, the DO penetration depth was less than 1000 µm even under cultures incubated under 100% O_2 at 1 atm. (Leisola *et al.*, 1983). DO penetration in pellets equilibrated in air-saturated culture fluid was ~400 µm (Michel *et al.*, 1992). Oxygen limitation was also shown to be the main reason for slow, incomplete degradation of lignin in non-agitated cultures (Leisola *et al.*, 1983). As DO transfer is limited to a depth of less than 1000 µm, oxygen limitation can result in the production of ethanol (Kenealy & Dietrich, 2004). Furthermore, DO concentration levels decreased during the growth periods in aerated cultures, and the oxygen uptake rate was highest after one to three days. Venkatadri *et al.* (1992) determined that thinner biofilms are more efficient in LiP production, and thus this effect can be directly link to improved transportation of oxygen in thinner biofilms. The oxygen uptake rate for *P. chrysosporium* improved when Tween 80 was used in bioreactors operated for periods of more than 20 days. However, it was shown that the oxygen uptake rate between 0 to 10 days was higher in cultures without Tween 80, while cultures with Tween 80 showed increased oxygen uptake rates (4 times higher) from 20 to 40 days (Hodgson *et al.*, 2000).

P. chrysosporium produced ethanol from glucose, mannose, cellobiose, maltose and sucrose when grown with limited oxygen (Kenealy & Dietrich, 2004). Under oxygen-limited growth on glucose, low levels of acetate were also produced when the nitrogen content was limited or in excess. *P. chrysosporium* does not grow fermentatively, but survives transient oxygen-

limitation by fermentation (Kenealy & Dietrich, 2004). LiP was about two to three times less tolerant to these solvents than MnP (Field *et al*., 1996).

Proposed solutions for improving gaseous oxygen transport were to: 1) limit biofilm thickness and 2) increase pressure in the bioreactors where the biofilms were immobilised. However, when the biofilm thickness was restricted to the determined limiting thickness, the *P. chrysosporium* mycelial mats grown in air degraded lignin at the same rate than that measured in 100% O_2 (Leisola *et al*., 1983). This again implies that the transport of DO is limited in fixed-film bioreactors where sufficient biofilm thickness is required to establish areas with limited nutrients.

The use of pressurised bioreactors to improve DO transport in mycelial mats of *P. chrysosporium* was shown to be effective, as lignin degradation in 5 atm. of air was similar to that of oxygen in 1 atm. (Reid & Seifest, 1980). It was also determined that lignin was degraded sufficiently in 1 atm. and 2 atm. of 100% O_2, but degradation under 3 atm. pressure was limited; whereas, under 4 atm. of 100% O_2, growth was restricted completely. This indicated that high oxygen concentration was the inhibiting factor in achieving sufficient growth (Reid & Seifest, 1980). However, the use of pressurised bioreactor systems to improve DO transfer might not be effective for prolonged production periods because of an increase in free and mycelial attached polysaccharides sheath in cultures where air and 100% O_2 are used (Doseretz *et al*., 1993). Leisola *et al.* (1982) suggested that DO limitations are caused by the production of excess polysaccharides, identified as mucilaginous material made of β-glucans (Buchala & Leisola, 1987). When the atmosphere in a bioreactor of immobilised *P. chrysosporium* was changed from air to 100% O_2, the thickening of an extracellular glucan layer was observed after two to three days of bioreactor operation (Miura *et al*., 2004). The production of glucan is induced as an oxidative stress response and a defensive mechanism, i.e. the fungus produces extracellular glucan to limit DO diffusion to protect the cells from oxidative stress (Miura *et al*., 2004).

Glucans are synthesised by a membrane-bound glucan synthase complex using glucose as a substrate (Latge & Calderone, 2006) and the glucans are used as a storage carbohydrate that facilitates growth by providing energy and carbon skeletons for protein synthesis during nutrient limitation periods (van Oijen *et al*., 2005). This suggests that during continuous LiP and MnP production, using a production medium containing high concentrations of glucose, glucan formation is possible as a result of environmental stress experienced by the fungus.

The fungus also synthesises the polysaccharides in order to have available nutritional reserves, which can be used under starvation conditions (Bes *et al*., 1987), because extracellular glucan-based mucilaginous hyphael sheaths facilitate the movement of enzymes involved in the degradation of lignin (Gutievves *et al*., 1995). Several researchers have provided hypotheses as to why glucans are produced in *P. chrysosporium* cultures under certain conditions. The association of peroxidase oxidation and glucan mucilage production is that: 1) it provides support and transportation for H_2O_2 and ligninolytic enzymes to the surface of the wood cells (Ruel & Joseleau, 1991; Bes *et al*., 1987); 2) it retains water because of its complex gel formation, which helps to maintain an environment suited for enzymatic catalysis (Ruel & Joseleau, 1991); 3) it binds the mycelium onto the cellulose surfaces (Igarashi *et al*., 2003) and 4) it provides 40% protection against *Rhizoctonia* canker and 60% protection against dry rot (Wolski *et al*., 2006).

When glucose concentrations fall below the level necessary for adequate H_2O_2 production, a glucan-hydrolyzing enzyme degrades the glucan back to glucose. Glucan was produced to a maximum of 215 $mg.L^{-1}$ after nine days in batch-submerged cultures (Bes *et al*., 1987), and glucan production was highest during days six to ten, while production started around four to five days (96 to 120 h).

2.3.2 Metal accumulation and toxicity for immobilised *P. chrysosporium* biomass

WRF, including *P. chrysosporium*-BKMF 1767, require trace amounts of essential heavy metals such as Cu, Mn and Fe for their growth, as well as other trace elements as listed in Kirk *et al*. (1978); however, these metals are toxic when present in excess. WRFs have the capacity to adsorb and accumulate metal ions including Cd, Pb, Ni, and Co (Baldrian, 2003; Falih, 1997). There is a risk of metal accumulation in continuous cultures even when the culture is supplied only with the growth medium. The growth rate of *P. chrysosporium* is inhibited by Mn^{2+} concentrations from 150 to 300 $mg.L^{-1}$ (Falih, 1998). When Mn^{2+} was not added to the production medium for LiP production, the enzymes were formed in both N-limited and N-excess liquid culture when exposed to air, but suppressed in cultures containing >13 $mg.L^{-1}$ Mn^{2+} concentrations. However, this was overcome in oxygen flushed, N-excess cultures with Mn^{2+} concentrations in the range of 3 to 1500 $mg.L^{-1}$ (Rothschild *et al*., 1999). Higher MnP activity was shown in cultures induced by heat shock and Mn^{2+} compared to cultures induced by Mn^{2+} alone (Brown *et al*., 1993), while complexes of Cu and Fe enhanced oxygen radical damage. Mn^{2+} in various forms inhibit ROS damage (Cheton & Archibald, 1988).

However, in some studies, Mn^{2+} was shown to have a beneficial effect on immobilised biofilms of *P. chrysosporium*. Concentrations of Mn^{2+} above 50 µM prevented chemical-supported lipid peroxidation measurable by the production of malondialdehyde (MDA) (Tampo & Yonaha, 1992). The prevention of lipid peroxidation by Mn^{2+} suggests that in fixed-film continuous bioreactors Mn^{2+} accumulation might regulate excessive ROS production and limit LiP production. It was determined that in continuous MGRs, LiP production was limited for one to five days of bioreactor operation, while MnP took precedence thereafter (Garcin, 2002; Govender, 2000; Leukes, 1999). This effect might be caused by polysaccharide production, restricted DO transport and Mn^{2+} accumulation. Cu accumulation was found to cause oxidative damage to proteins by the induction of oxidative stress associated with the presence of ROS (Stohs & Bagchi, 1995), even though low Cu concentrations (0.0004 to

1.2 µM) in the nutrient medium increased the activity of LiP and MnP of *P. chrysosporium*. Organic chelators, such as malonate and oxalate, are also metabolic bioproducts excreted by *P. chrysosporium* (Wesenberg *et al.*, 2003; Goodwin *et al.*, 1994; Khindaria *et al.*, 1994). The chelators immobilise soluble ions or complexes to form insoluble oxalates. This also provides a tolerance mechanism for the accumulation of metals in *P. chrysosporium*. However, the effect of metal accumulation with increasing ionic strength in the basal medium should be studied in more detail in continuous fixed-film bioreactors. The function of the nitrilotriacetate added to the trace element solution used for nutrient medium preparation was to provide chelation therapy in case of metal accumulation in the *P. chrysosporium* biomass (Kirk *et al.*, 1978).

2.3.3 Long term effects of MnP-exposed *P. chrysosporium* biomass

It is well known that oxygen radical-mediated processes are involved in the peroxidation of lipids (Forney *et al.*, 1982). Thus, it is unsurprising that MnP can oxidise unsaturated lipids, causing the formation of lipoxyradicals intermediates (Breen & Singleton, 1999). These lipoxyradicals can cause increased lipid peroxidation in biomass depleted of nutrients in bioreactors that operate for prolonged periods. The supernatant of WRF contains a pro-oxidant or lipid peroxidation-promoting activity, which is dependent on the presence of MnP (Kapich *et al.*, 2005). It was postulated that lipid peroxidation (a sign of oxidative stress) of highly unsaturated fungal membrane lipids may be linked to lignin degradation in wood decaying *Basidiomycetes* fungi (Kapich, 1990). The entrapment of MnP in immobilised biomass initiates the formation of peroxyl and other lipid radicals (Kapich *et al.*, 1999; Bao *et al.*, 1994; Kapich *et al.*, 1990). A high degree of lipid oxidative damage was detected in Mn-deficient LiP producing cultures of *P. chrysosporium*. This indicates intensive exposure to high concentrations of ROS, which are typically found in oxygenated LiP-producing cultures. These observations reconfirm the hypothesis that the induction of LiP expression is, at least

partially, mediated by the intracellular formulation of ROS (Belinky *et al.*, 2005), while Mn deficiency increases the production of lipoxyradicals.

However, when Mn^{2+} is available as a free metal ion; it has the ability to act in the reverse mode of Fenton reactions, thus destroying free radicals. Furthermore, the Mn^{2+} ion acts as a cofactor in the formation of manganese-superoxide dismutase (Mn-SOD), which plays a role in the protection of cells against phospholipid peroxidation (Have & Teunissen, 2001; Tampo & Yonaha, 1992), by converting superoxide radicals into H_2O_2. Thus, Mn plays a regulatory role in LiP and MnP formation (Rothschild *et al.*, 1999), limiting the level of oxygen radicals that were thought to play a major role in LiP formation, while acting as a substrate in the MnP cycle. When high levels of Mn^{2+} and Mn^{3+} ions are in the presence of a suitable chelator, MnP production is stimulated, while LiP production is suppressed (Perez & Jeffries, 1992).

Whatever the mechanisms of H_2O_2 production, intracellular catalase- and Mn-SOD activities decrease during the secondary growth phase. *P. chrysosporium* biomass will be more susceptible to oxidative stress during this growth phase. It has been shown that catalase levels inside the cell decrease at the end of the primary growth phase, while the peroxidase levels increase extracellularly (Morpeth, 1987). However, it was noted that primary and secondary growth phases were indistinguishable in wood grown mycelia, when compared to an artificial liquid medium (Highley & Dashek, 1998), making it difficult to link catalase production levels with lignin degradation.

2.4 CONCLUSIONS AND RECOMMENDATIONS

Bioprocess engineering considerations and challenges surrounding production of extracellular ligninase remain daunting. Effective measures are required to phase out batch/semi-batch type processes and to promote the development of fixed-film continuous extracellular enzyme processes and bioreactors. This literature review shows that progress

was made in describing the bioprocessing aspects related to bioreactor development for continuous enzyme production and assays used to quantify the activity of produced ligninase. However, some aspects studied and optimised for submerged batch cultures can lead to limitations in continuous cultures. This led to a number of general conclusions and recommendations for further research, especially in the development of fixed-film continuous reactors. The following are factors that require further research to realise the prospects of continuous production of both LiP and MnP from *P. chrysosporium* BKMF 1767:

1) The use of pressurised sources of oxygen proved to be effective in overcoming DO transport in immobilised biofilm. The DO driving force can be maintained and controlled by limiting mycelia thickness. The limitations of biofilm thickness will impede the efficiency of nutritional gradients existing in immobilised fungal mycelia. In polymeric-based MGRs where the biofilm thickness is not controlled, the flux of liquid nutrients supplied through the lumen of the membrane will decrease over time. This is because greater amounts of pressure will be required with increasing biofilm thickness, while luminal pressure will be constant during bioreactor operation. Surprisingly, the use of liquid oxygen carriers has not yet been investigated to establish their suitability for addressing DO deficiencies in immobilised fixed-film or submerged cultures of *P. chrysosporium*. Liquid oxygen carriers as an alternative to the use of technical grade oxygen will be the focus of this thesis. The different oxygen carriers are discussed in Chapter 4 and their suitability for use with *P. chrysosporium* cultures is evaluated and discussed in Chapters 7 and 8.

2) It was determined that by increasing trace elements up to 100-fold in batch cultures negligible limitations to enzyme production were detected, thus leading to the development of a nutrient medium with 100-fold increase in trace element ions. This nutrient medium was used in studies for fixed-film bioreactors. As *P. chrysosporium* is known to absorb heavy metals, further research is required to establish the scale of metal accumulation from the

nutrient medium in fixed-film reactors. This is an important parameter for Mn, which prevents ROS, while Cu increases ROS. This is evaluated and discussed in Chapter 6.

3) The role of extracellular glucan and its physiological effects during enzyme production has previously been misunderstood. Several studies indicated its role as a storage carbohydrate moisture provider, while others indicated that it provided a way for the mycelia to become attached to the lignin, shielding the fungus from oxidative stress. However, its role during metal accumulation is unclear. In fixed-film bioreactors, where the biofilm is kept in the idiophase for long periods of time during the operation of the bioreactor, available glucan will be consumed within short periods. Other strategies to protect the fungus from environmental stress need investigation. This is evaluated and discussed in more detail in Chapter 4 and 7.

As the development of continuous bioreactors becomes a necessity for LiP and MnP production, it is recommended that the following parameters be further investigated in fixed-film bioreactors: 1) determining the relationship between the amounts of glucan produced extracellularly and DO kinetics in fixed-film bioreactors; 2) investigating metal accumulation in the nutrient medium used by Tien and Kirk (1988) in continuous fixed-film reactors; 3) evaluating the use of liquid oxygen carriers in *P. chrysosporium* cultures and 4) evaluating oxidative stress in biofilms directly exposed to gaseous oxygen sources in fixed-film reactors, even at low oxygen partial pressures.

2.5 SUMMARY

Phanerochaete chrysosporium, a filamentous fungus produces delignifying enzymes LiP and MnP. Different conditions and bioreactor systems are used to enhance the production of these extracellular enzymes. However, these bioreactor systems were found to be inefficient, as the achieved production rates of LiP and MnP were low. A major limitation to the bioreactors identified from the reviewed literature was the transport of dissolved oxygen. When oxygen is used glucan production becomes prevalent, leading to increased metal

entrapment in the biofilms. Overall, the performance of the biofilm becomes limited, leading to restrictions in the use of the bioreactors over long periods. This led to the design and commissioning of a bioreactor described as a membrane gradostat reactor (MGR) in which aeration is directed against the immobilised biofilm in the ECS. Furthermore, the alleviation of limitations related to biofilm performance identified in batch reactors were never proven to have been resolved in the MGR. This led to the following questions (listed in Chapter 1), which are the subject of this thesis:

- Are there any anaerobic zone formations in biofilms immobilised in the MGR?
- Is there a relationship between aeration, oxygenation and polysaccharide production in the MGR?
- Does significant metal accumulation occur in biofilms immobilised in the MGR?

To answer these questions, the quantification of the oxygen mass transfer kinetics, including the oxygen penetration depth, oxygen consumption, oxygen flux and the formation of anaerobic zones in biofilms of *P. chrysosporium* immobilised in the MGR systems was necessary, and the findings are reported in Chapter 5. The findings on polysaccharide storage and metal accumulation in immobilised biofilms in the MGR are reported in Chapter 6.

The conceptualisation and operation of the MGR bioreactor, which was shown to be suitable for continuous LiP and MnP production in this chapter (Chapter 2), will be explained and further discussed in the following chapter (Chapter 3).

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CHAPTER 3

LITERATURE REVIEW

The membrane gradostat reactor: Secondary metabolite production, bioremediation and commercial potential

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CHAPTER 3

The membrane gradostat reactor: Secondary metabolite production, bioremediation and commercial potential

Abstract

The use of membrane bioreactor (MBR) technology is rapidly advancing in the wastewater treatment industries. However, this is not the case in the biopharmaceutical manufacturing industries. This chapter focuses on the membrane gradostat reactor (MGR) as an entirely different concept than that of submerged hollow fibre modules. The MGR has shown great potential and versatility in terms of industrial applications. It can be used in both wastewater treatment and biopharmaceutical manufacturing, using different modes of operation to meet any predetermined process requirements. The MGR concept uses polysulphone capillary membranes that contain microvoids in the substructure to immobilise microbial cells or enzymes, depending on the bioreactor's application. The operational requirements of the MGR and its commercial potential are discussed from a bioprocess-engineering perspective in this chapter.

Keywords: Biofilm; Gradostat reactor; Membrane bioreactor; Secondary metabolite production; Wastewater treatment

3.1 INTRODUCTION

Membrane bioreactors (MBRs) provide an environment for increased biomass density and improved productivity. The biomass immobilised in these systems is retained through a membrane barrier or support. Many membrane configurations have been tested, and the hollow fibre configuration is noteworthy (Charcosset, 2006). The production of secondary metabolites from immobilised biomass in the biotechnology and pharmaceuticals industries by means of MBR systems has not been widely used for the production of low-volume, high-value bio-products. Novel developments and refinements in membrane technology continue

to be prevalent research themes. The broad range of applications for membrane technologies suggests that these technologies are now well accepted and cost effective (Wiesner & Chellam, 1999). In South Africa, membrane technology development tends to focus on water-related applications, although other membrane process applications have been commercialised or are on the verge of being commercialised (Offringa, 2002). However, the use of membrane technology for biotechnological applications is still under-exploited. By using this technology, alternative approaches to continuously producing high-value secondary metabolites, can be made a reality. Experiments indicate that most microbial biofilms are shear-sensitive and would easily detach and slough off from membranes with relatively smooth exterior surfaces (Jacobs & Sanderson, 1997). Researchers have also established that some microbial cultures perform poorly in shear environments where a shaker or aeration by means of bubbling is used.

This clearly complicates the immobilisation of biofilms on external surfaces of membranes. Until recently, extracellular enzymes of *P. chrysosporium*, LiP and MnP, were produced in stationary- or shaken flasks and the supernatant was concentrated by centrifugation to recover the enzymes. Methods to continuously produce these secondary metabolites were developed by immobilising biofilms of *P. chrysosporium* in MBRs (Venkatadri & Irvine, 1993, Leukes, 1999; Leukes *et al*., 1999; Solomon, 2001; Garcin, 2002; Ntwampe, 2005; Sheldon & Small, 2005). Generally, in these systems, microbial biomass was grown in the extra-capillary space (ECS) with the nutrient medium flowing through the fibres. Recently, another geometry was proposed, where a hollow fibre was inserted into another to grow cells in the annulus between the two fibres (Yang *et al*., 2006). However, the concept of a membrane gradostat reactor (MGR) is not well understood. It is often confused with submerged hollow fibre membrane modules, which are commonly used in wastewater treatment applications. In this chapter, the structure and workings of the MGR will be explained in detail. Other studies related to the use of the MGR will also be reviewed.

3.2 THE MEMBRANE GRADOSTAT REACTOR CONCEPT

The original bi-directional compound gradostat reactor was described by Lovitt & Wimpenny (1981a,b). They simplified the reactor by making a more compact apparatus in which substrate gradients are established by diffusion between adjacent culture chambers (Lovitt & Wimpenny, 1981a; Lovitt & Wimpenny, 1981b). The devised gradostat had neighbouring reactors connected together with pumps in one direction and overflowing weirs in another. In the system, opposing chemical gradients were readily established. Wimpenny *et al*. (1992) designed a directly coupled gradostat consisting of different compartments, unlike the conventional gradostat, which used pumps and weirs to transfer material between neighbouring vessels. The directly coupled vessels relied on open transfer ports. However, the use of this type of gradostat proved to be costly, yielding low quality products; it also did not mimic natural living conditions of most microbial biofilms.

A novel MGR was developed by Leukes *et al*. (1999) and other researchers which immobilised *P. chrysosporium* biofilms to produce LiP and MnP using a polysulphone membrane (Solomon, 2001; Leukes *et al*., 1999; Jacobs & Sanderson, 1997; Jacobs & Leukes, 1996). Sheldon and Small (2005) showed that capillary ceramic membranes were also suitable for immobilising *P. chrysosporium* biofilms in MGR systems. The term MGR is used to describe a biofilm reactor that uses a synthetic capillary ultrafiltration membrane as a support matrix for the biofilm. The internally skinned and externally unskinned polysulphone membranes (Figure 3.1A) that were used in this study provided a unique substructure matrix within which a fungus of a filamentous nature could be immobilised on its external surface, as shown in Figure 3.1C. Immobilising other microbial cell types might be easier because of the microvoids in the polysulphone membranes. The substructure contained closely packed narrow-bore microvoids (Figure 3.1B) that extended all the way from just below the internal skin layer to the membrane's external unskinned region (Figure 3.1A).

As a theoretical concept the term membrane gradostat is appropriate, since the dissolved oxygen (DO) and liquid nutrient gradients flow is bi-directional and contact occurs between the primary (I), stationary (II) and decline (III) growth phases of the biomass (Figure 3.2). The essence of this system is schematically illustrated in Figure 3.2A and 3.2B. When nutrients are supplied to the biofilm, which is immobilised on the external wall of a capillary membrane, bi-directional radial nutrient gradients are established across the biofilm. The biomass closest to the membrane has first access to the nutrients, while the biomass furthest away from the membrane surface is starved, therefore, undergoing a ligninolytic stage, a requirement for secondary metabolite production. Active biomass grows in the nutrient-rich zones, and the nutrient-stressed biomass, which is highly productive in terms of secondary metabolites, is in a nutrient poor zone.

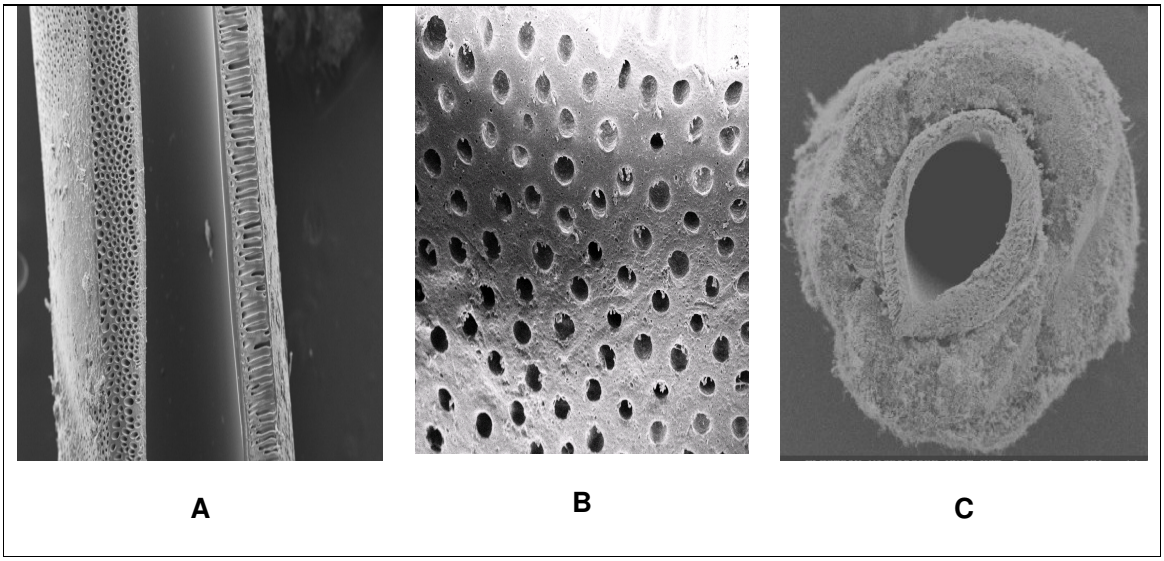

Figure 3.1: (A) SEM of a longitudinal section of the polysulphone membrane. (B) The external surface of the polysulphone membrane showing cavities where spores can be immobilised. (C) *P. chrysosporium* biofilm immobilised on the external surface of the polysulphone membrane

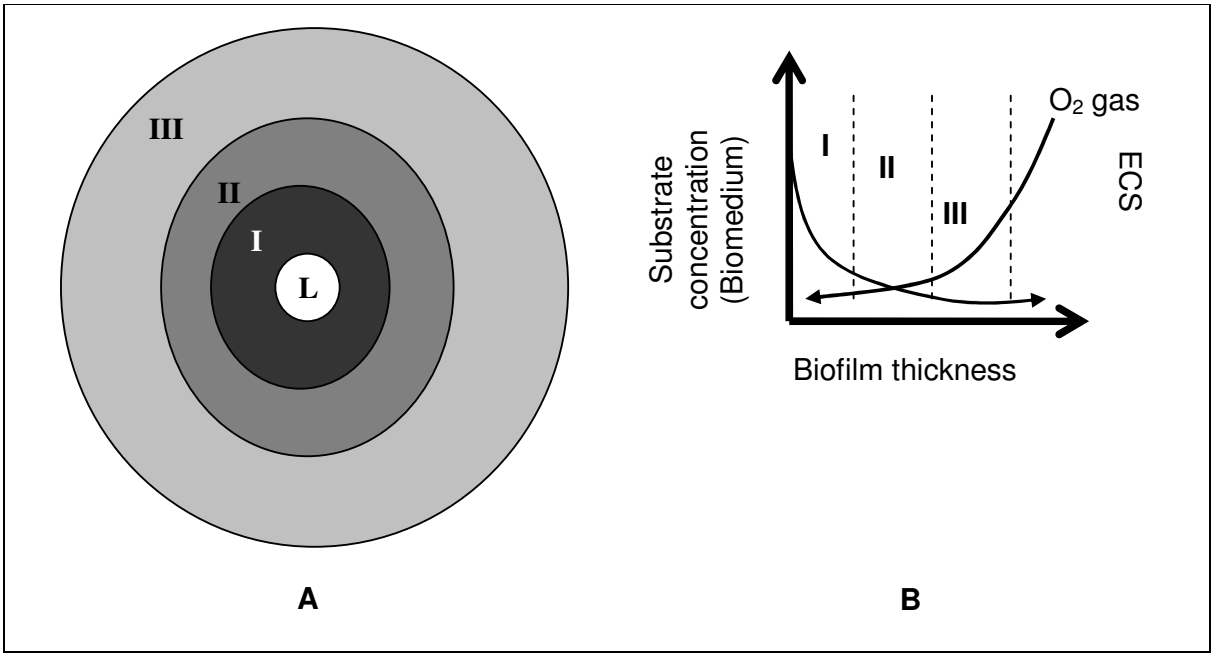

Figure 3.2: (A) Schematic representation of the membrane gradostat concept; I is the primary growth phase; II is the stationary growth phase; III is the decline phase; L- Lumen of the capillary membrane from which the nutrients are supplied. (B) Illustration of substrate concentration distribution and radial flow at different biofilm growth phases and thickness. Nutrient medium convective flow is opposite to the direction of oxygen diffusion, supplied in gaseous form on the shell side (ECS) of the MGR

3.3 MEMBRANES SUITABLE FOR THE MGR SYSTEM

The important feature of the polysulphone membrane is the regularity of the microvoids present in the substructure and the complete absence of an external layer. This feature allows the microvoids to be inoculated with fungal spores or mycelial cells by reverse filtration. Membrane morphology can be classified into symmetric and asymmetric structures. The thickness of symmetric membranes (porous and nonporous) ranges roughly between 10 to 200 µm. Asymmetric membranes consist of a dense top layer (skin) with a thickness of 0.1 to 0.5 µm. These membranes combine the high selectivity of a dense membrane with the high permeation rate of a thin membranes (Howell *et al.*, 1993). According to Jacobs and Sanderson (1997), capillary membranes are by definition narrow-bore tubular-type membranes, typically with an outside diameter ranging from 0.4 to 2.4 mm. Unlike the large-bore tubular membrane types, capillary membranes are self-supporting because of their small diameters. The capillary membranes used by Leukes *et al.* (1999) fall in the category of

integrally-skinned asymmetric membranes. Integrally skinned refers to the fact that the skin layer of the membrane is an integrated part of the membrane substructure. Asymmetric refers to the graded porosity of the membrane's substructure, which is most dense just below the skin layer, but increasingly porous as the distance from the skin layer increases. The substructure is sponge-like, containing finger-like microvoids (Jacobs & Leukes, 1996). Internally-skinned capillary polysulphone (coded IPS 763) and ceramic membranes allowed for better attachment and immobilisation of *P. chrysosporium* spores than tubular membranes did. Average biofilm thicknesses across the length of the membrane exceeding 1000 μm were achieved on capillary ceramic membranes, 830 μm on capillary polysulphone membranes and 450 μm on tubular membranes. The characteristics of capillary membranes suitable for use in the MGR systems are listed in Table 3.1.

Table 3.1: Characteristics of capillary membranes successfully used in the MGR system (Sheldon & Small, 2005)

Material / Parameter	Polysulphone capillary membrane	TiO_2-αAl_2O_3 capillary membrane
Outer diameter (m)	0.0012	0.0029
Inner diameter (m)	0.0009	0.0019
Wall thickness (m)	$\pm$ 200 x 10^{-6}	0.001
External average pore size (μm)	11	3
Internal average pore size (nm)	-	0.9
Burst pressure (kPa)	1400	8000
Operating pH	4 - 11	0 - 14
Maximum operating temperature (°C)	50	350

It has been determined that capillary ceramic membranes are suitable for the industrial production of secondary metabolites because: 1) microbial stresses in the immobilised biofilms will be higher due to greater biofilm thickness; 2) they are more rigid (mechanically stable) and 3) they can be sterilised chemically and with steam (Sheldon & Small, 2005).

3.4 PRODUCTION OF SECONDARY METABOLITES: SCMGR VS. MCMGR

To improve productivity, the scaling-up of a bioprocess is normally evaluated for economical purposes, and it is for this reason, Govender *et al.* (2003) evaluated the scalability of a *P. chrysosporium*-based MGR. The scale-up process of the MGR was performed using *P. chrysosporium* ME446. A 10-fold scale-up from a single capillary MGR to a 2.4 L multi-capillary MGR resulted in a 7-fold increase in MnP production with a peak enzyme activity at 209 $U.L^{-1}.d^{-1}$ (Govender *et al.*, 2003). A 0.5 L, 15-membrane multi-capillary MGR, with nutrient capsules at the top and bottom of the reactor, was designed by Ntwampe (2005) for continuous secondary metabolite production from biofilms of *P. chrysosporium* BKMF 1767. A LiP activity peak of 40 $U.L^{-1}.d^{-1}$ was achieved without the use of technical-grade oxygen (~100%) to improve ligninolytic activity in the biofilms (Ntwampe, 2005). Even at a developmental stage, the MGR showed great potential as a secondary metabolite-producing system. Comparisons of MnP concentration and productivity with continuous systems are shown in Table 3.2.

Table 3.2: Comparison of MnP concentrations and productivity with other continuous bioreactor systems

Reactor and process description	MnP concentration ($U.L^{-1}$)	Productivity ($U.L^{-1}.day^{-1}$)	Reference
Packed bed (continuous)	250	202	Moreira *et al*., 1997
MGR (continuous)	2361	1916	Leukes, 1999
MGR (continuous)	16171	428	Garcin, 2002

3.5 GROWTH KINETICS AND SUBSTRATE CONSUMPTION IN THE MGR

Growth Kinetics of Phanerochaete chrysosporium: Research on the growth kinetics and consumption of essential nutrients in the MGR using a well-researched microbe, *P. chrysosporium* BKMF 1767 (ATCC 24725), was completed using the nutrient medium of Tien and Kirk (1988) which was developed for this fungus (Ntwampe & Sheldon, 2006; Ntwampe, 2005). The nutrient medium contained 55 mM glucose and 1.1 mM ammonium

tartrate as the ammonium source (Tien & Kirk, 1988). In membrane gradostat reactor systems in which *P. chrysosporium* was immobilised on polysulphone membranes, the logistic curve showed primary and secondary biofilm growth phases as the regeneration of mycelium occurred in successive growth cycles (Ceribasi & Yetis, 2001; Yetis *et al*., 2000; Kirk *et al*., 1978). The biomass generated on the membranes increased during the first 168 h (7 days) after which it stabilised until 216 h (day 9), with the secondary phase occurring thereafter. The results obtained were similar to those obtained by Kirk *et al.* (1978), where the secondary growth phase occurred after 10 days for mycelia cultured at 39°C, in a 125 ml flask with air. Furthermore, the following growth phases were identified by Ntwampe (2005) in the MGR biofilms: 1) lag phase (0 to 48 h); 2) exponential growth phase (72 to 120 h); 3) stationary phase (144 to 216 h) and 4) secondary growth phase (>216 h). The stationary phase was classified where the harvested biofilms mass did not change for a period of 48 h. The dry biomass density was physically measured using a helium pycnometer. The representative growth-rate constant for *P. chrysosporium* biofilms in the MGR was determined as 0.035 h^{-1} (Ntwampe & Sheldon, 2006; Ntwampe, 2005).

Substrate consumption in the MGR: Substrate consumption by the immobilised biofilms was determined by measuring the difference between carbon and ammonium sources in the feed and in the permeates recovered. The increase in carbon source (glucose) consumption was determined at different periods during the MGR operation and was observed to increase with increases in dry density. The rate of glucose consumption in the MGRs was 94.7 $g.m^{-3}.h^{-1}$ over a period of 264 h, compared to 43 $g.m^{3}.h^{-1}$ obtained in a Trickle Fixed Bed reactor operated for a similar period of time (Ntwampe & Sheldon, 2006; Bosco *et al.,* 1996). The basis for comparison was that both systems were continuous enzyme production systems where the nutrient medium was designed by Tien and Kirk (1988), and a similar microbial strain, *P. chrysosporium* BKMF 1767, was used in both. Ntwampe (2005) reported a maintenance coefficient of 0.028 h^{-1}. After the lag phase (2 days), 90% of all the ammonia tartrate, supplied to the MGR daily, at a rate of 1.68 $ml.h^{-1}$ was consumed. Overall, the

growth kinetics obtained in the MGR were similar to those determined for submerged batch cultures operated for a similar period of time (Ntwampe, 2005).

3.6 MGRs' OPERATIONAL REQUIREMENTS

3.6.1 Bioreactor orientation: Horizontal vs. Vertical

Consistent radial substrate distribution in the immobilised biofilms is a requirement for an efficient MGR. The biofilms produced are required to be axially homogenous across the length of the membrane and thick enough for a nutrient gradostat to be attained across the biofilms (Leukes, 1999; Leukes *et al.*, 1999). The optimal conditions for biomass accumulation and development in MBR systems are not necessarily identical to those for secondary metabolite production. Permeate flux resulting from pressure effects were evaluated on a horizontally placed bioreactor system using a dead-end filtration mode. A considerable flux variation was established in clean fibre lengths without biofilm attachment, as well as between different fibres of the same length (Garcin, 2002). Theoretically, horizontal bioreactors will minimise radial substrate gradients in the biofilms as a result of the gravitational forces acting on the nutrient medium.

Vertically-orientated MGR systems have the advantage that the permeate will proceed down the length of the membrane and establish the required gradients. This also ensures the more efficient use of essential nutrients. In multi-capillary MGR systems, droplet formation from other fibres will not interfere with biofilm growth on other membranes when reactors are placed vertically. Biofilms grow on reactor walls in horizontally placed MGR systems as a result of biofilm detachment caused by gravitational forces. This caused mycelial growth in the recovered permeates. However, vertically placed reactors have: 1) consistent biofilm development and thickness across the length of the membrane and 2) can be operated longer without permeate contamination. It was concluded that horizontally placed MGR systems would not be suitable for an efficient membrane gradostat because of: 1) uneven

biofilm growth on the membranes, because of flux maldistribution; 2) biofilm contamination in the recovered permeate samples and 3) sagging of polysulphone membranes due to heavy biofilms in the bioreactor (Ntwampe, 2005; Garcin, 2002). The proposed MGR operational-set setup is shown in Figure 3.3.

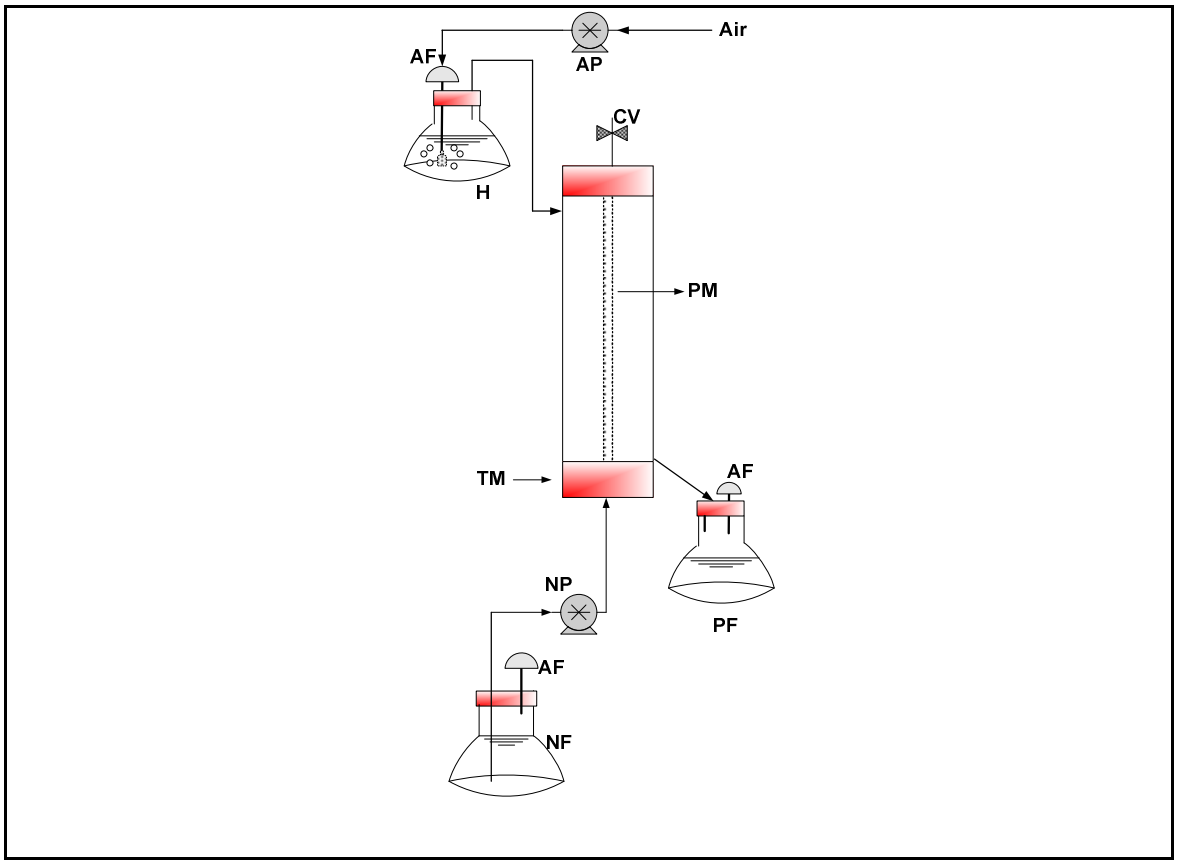

Figure 3.3: Schematic illustration of a vertical single capillary membrane gradostat reactor set-up. AF: air filter; AP: air pump; CV: closed valve (dead-end filtration mode applied); H: humidifier; NF: nutrient flask; NP: nutrient pump; PF: permeate flask; M: polysulphone or ceramic capillary membrane TM: Teflon mould. Note: The air was supplied from the shell side and it helped with the flow of permeate to the permeate flask (PF)

3.6.2 Nutrient and air/oxygen supply

Peristaltic pumps are generally used to maintain and to achieve low flux across the length of the membrane. Low pumping rates of the nutrient medium are used so that appropriate radial nutrient gradients in the biofilm can be established. Liquid-medium flow rates of less than 3 $ml.hr^{-1}$ per 120 mm of polysulphone membranes are used for secondary metabolite

production. Air/oxygen is supplied at approximately 1 vol.ECS^{-1}.min^{-1} or less, to avoid the sloughing off of the biofilm. The air/oxygen is passed through a humidifier into the ECS using an air pump so that the aerial biomass in the MGR would not dry out. However, other operational parameters, such as nutrient mass transfer kinetics, in the biofilms immobilised in the MGR system had not yet been determined. The parameters listed above were reported in Leukes *et al.* (1999) and Leukes (1999).

3.6.3 Conidia (spore) inoculation and germination

The inoculum for MGR systems can be prepared by suspending conidia or mycelia in sterile distilled water (Tien & Kirk, 1988). Inoculation took place by reverse filtration where a suspended conidia/mycelia solution was pumped through the shell side via the permeate port. The water passed through the external surface of the membrane to the lumen side, leaving the conidia entrapped in the cavities on the externally unskinned polysulphone membrane (Govender *et al.*, 2004). The backpressure was monitored to ensure that the pressure limit (150 kPa) was not exceeded. The inoculation solution had to be passed through the system to ensure that quantified numbers of conidia (quantified using spectrophotometry techniques) were immobilised. To allow for conidial germination and mycelial attachment, a small amount of nutrient medium was passed through the system and then stopped for approximately 24 h. As the MGR is operated in dead-end filtration mode, this precaution prevents conidia from detaching from the cavities into the permeate flask in the initial stages of bioreactor operation (Luke & Burton, 2001). It was determined that using smaller volumes of the inoculation solution, not exceeding twice the ECS volume of the MGR, prevents a prolonged inoculation procedure and increases immobilisation without compromising the integrity of the membranes or the bioreactor system (Ntwampe, 2005).

3.7 BIOREMEDIATION APPLICATIONS USING THE MGR

To evaluate the MGR's versatility, Edward *et al.* (1999) used it as a wastewater treatment application using *Neurospora crassa*, which produces laccase. The production of laccase with the MGR system was sustainable for a period of 30 to 40 days at 10 $U.ml^{-1}$ using the fungus *Neurospora crassa*. The phenolic compounds phenol and *p*-cresol were effectively removed from 5 mM solutions when the solutions were fed through the lumen of the MGR to the immobilised *Neurospora crassa* (Edwards *et al.*, 1999). The biofilms immobilised in the MGR continuously sustained this removal efficiency for a four-month period; whereas batch cultures remained active for 8 to 15 days, after which cultures were no longer viable (Luke & Burton, 2001). Polyphenol oxidase was immobilised on the externally unskinned polysulphone membrane, and 949 µmol phenolics were removed from a solution containing 4 mM total phenolics which was continuously supplied to the reactor system (Edwards *et al.*, 1999). The average phenol content from olive wastewater was reduced from 30 to 10 $mg.L^{-1}$ over a period of 24 h, using permeate from a multi-capillary MGR system where biofilms of *P. chrysosporium* were immobilised (Ntwampe, 2005). These applications showed the versatility of the MGR system.

3.8 COMMERCIAL POTENTIAL OF THE MGR

Synexa Life Sciences (Bellville, South Africa) currently uses MGRs for the production of commercial secondary metabolites from various species of microorganisms. The MGR was shown to increase production compared to submerged fermentation systems. The design targets the biotechnological, bioprocessing and biopharmaceutical manufacturing industries. Although, the research and development costs are relatively high for pilot plant MBRs, the use of submerged cultures is outdated, as they: 1) are inefficient in terms of increased product development lead-time; 2) are anaerobic; and 3) operate in high shear environments, as a result of aeration and stirring. Currently, the use of genetically modified organisms (GMOs) is on the increase, but this causes high development costs and an increase in the

allocation of human capital. The MGR is advantageous because: 1) it closely mimics the natural environment in which most microbial biofilms live; 2) it allows for extended and continuous production of secondary metabolites; 3) it generates little waste, thus reducing waste-disposal costs and 4) it works well with wild type strains, thus reducing the need for genetic engineering.

3.9 CONCLUDING REMARKS

The use of the MGR or any other membrane reactor technology systems in the biotechnological and biopharmaceutical production of secondary metabolites is limited. Many of these membrane bioreactors are currently used in the wastewater treatment industries. The concept of the MGR was addressed and discussed in this chapter. Capillary membranes suitable for use in the MGR and their characteristics were listed. The membranes have an external unskinned porous structure with an internal skin. The membrane structure had previously been found to be suitable for biofilm attachment. Operational conditions were highlighted, which included the choice to operate the MGR vertically. The use of multi-capillary membranes for the production of secondary metabolites was compared to the single capillary MGR systems. The versatility of the MGR system and its commercial and industrial potential was highlighted.

3.10 SUMMARY

The viability and versatility of the MGR (as reviewed in this chapter) has proved to be advantageous when compared to batch reactors. However, the limitations related to aeration, oxygenation and environmental stress experienced by immobilised biofilms discussed in Chapter 2 have yet to be resolved for the MGR system.

This led to the hypothesis that the use of emulsified synthetic oil-based oxygen carriers can improve biofilm functionality and reduce the limitations identified. The use of oil-based

oxygen was initially investigated in batch cultures (Chapter 7) and in the MGR system (Chapter 8). In Chapter 4, the application of oxygen carriers in different bioprocesses is reviewed. Even though the application of perfluorocarbons has not previously been used within the MGR, it was clear that when oxygen carriers are used properly, they enhance biomass generation and bioproduct formation. As biofilms in MBRs are also susceptible to environmental stresses, the use of a protective emulsifier for the oxygen carriers is necessary. The following chapter (Chapter 4) discusses the advantages and disadvantages of using perfluorinated oxygen carriers.

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CHAPTER 4

LITERATURE REVIEW

Water-immiscible dissolved oxygen carriers in combination with Pluronic F 68 in bioreactors

CHAPTER 4

Water-immiscible dissolved oxygen carriers in combination with Pluronic F 68 in bioreactors

Abstract

The supply and availability of dissolved oxygen (DO) in aerobic bioprocesses is often a limiting factor for the scaling up, improvement and general performance of these bioprocesses. The use of different DO carriers, particularly the use of perfluorocarbons as oxygen carriers, is discussed in this review. It also highlights interactions of microbial cultures with the surfactant, Pluronic F 68. Although oxygen carriers have been used extensively in the medical field, this review only focuses on their use in microbial bioprocess used for the production of high-value bioproducts. The use of water-immiscible compounds in combination with Pluronic F 68 in bioprocesses is discussed with the intention of analysing their combined effect where bioreactor and biomass performance is affected by DO limitations, nutrient starvation, high concentrations of trace element ions, oxidative stress and cell death from mechanical stress.

Keywords: Dissolved oxygen; Oxygen carriers; Perfluorocarbon; Pluronic F 68; Surfactant

4.1 DISSOLVED OXYGEN TRANSPORT LIMITATIONS IN BIOREACTORS

In aerobic bioprocesses, dissolved oxygen (DO) transport from the gas phase into the liquid medium is one of the critical parameters for effective bioprocess operations. In bioreactors such as membrane gradostat reactors (MGRs), where biofilms are attached to membrane surfaces, low shear aeration conditions are employed in order to reduce biofilm sloughing. This particular bioreactor uses a biofilms system so that the different parts of the biofilm can experience different nutrient concentrations, such that a gradient can be established. This cannot occur if the biofilm sloughs off. However, under these conditions, the overall DO mass transfer into the immobilised biofilms is restricted and controlled by a liquid film at the gas-

liquid-biomass interface (Ju *et al.*, 1991a). This adversely affects overall biofilm- and reactor performance. Furthermore, DO transfer is hampered by extracellular polymeric substances (EPS) produced or stored by microbial biomass during fermentation. In fungal bioprocess systems where high glucose concentrations are used in the nutrient medium, the production of EPS as storage carbohydrates further hampers DO transport as a result of the availability of excess glucose, necessitating the use of pressurised bioreactor systems. Although the use of pressurised bioreactors might improve DO transport, this is also likely to increase the rate of biofilm metabolism and the generation of carbon dioxide. Furthermore, the possibility of carbon dioxide entrapment in the fermenting biomass will increase as a result of EPS production associated with high DO transport into the biofilms. EPS production is also reported to be promoted by the high partial pressures of oxygen used during aeration, limited nitrogen availability and low pH conditions that are prevalent in bioreactors used to produce secondary metabolites, such as the MGR system (Ahimou *et al.*, 2007; Ryu & Beuchat, 2004; Damiano & Wang, 1985; Jarman *et al.*, 1978).

As oxygen has a low solubility in aqueous medium, DO transfer becomes an important design aspect for any aerobic bioprocess design. Inefficient DO transfer influences the scale-up and economic value of the fermentation processes, as it increases operational costs and reduces recoveries related to the aerobic biosynthesis of high-value bioproducts (Cascaval *et al.*, 2006). Furthermore, respiratory activity of the immobilised microorganism can be inhibited by bioproducts when the biomass is suspended in the product solution. DO cannot be increased by heating the water-based medium, as it has been found that the oxygen-transfer rate (OTR) remained constant between 20℃ and 55℃ (Vogelaar *et al.*, 2000). The presence of dissolved salts in most fermentation media has also resulted in decreased solubility and transfer of DO into microbial cells during the course of bioreactor operations (Anke & Weber, 2006). For example, an increase in the salinity of freshwater from 0% to 8% resulted in the reduction of DO from 200.4 to 192.3 μmol $O_2.L^{-1}$ (Unisense, 2008).

Spargers, agitators and technical-grade oxygen (pressurised ~100% O_2) have been used to improve the availability of DO and the performance of biomass in fermentation broths hampered by DO transfer (Murhammer & Goochee, 1990). However, the use of these devices and aeration sources can result in cell rupture as a result of the mechanical stress caused by mixing and continuous aeration. Conditions that can also limit biomass performance include: hyperoxia resulting from the availability and the use of high partial pressures of oxygen, and the generation of reactive oxygen species (ROS), which cause lipid peroxidation and unwanted by-product formation as the biomass tries to protect itself from environmental stressors in the bioreactors.

DO carriers are used to alleviate DO limitations in different bioprocesses. They have been used extensively in the medical field (Goorha *et al.*, 2003; Lowe, 2003; Riess, 2002) with limited applications within membrane-based bioreactors. To alleviate DO limitations, the use of water-immiscible DO carriers becomes necessary to optimise biomass performance. Some of these water-immiscible organic compounds have a greater solubilisation capacity for oxygen than aqueous media (Junker *et al.*, 1990). These materials include haemoglobin derivatives (polymerised, polymer conjugated, intramolecular cross-linked, recombinant and lipid-based vesicle haemoglobin) and organic oils based on synthetic, highly fluorinated organic compounds named perfluorocarbons (PFCs) (Goorha *et al.*, 2003; Lowe, 2003). The advantage of these PFCs over haemoglobin-based oxygen carriers is that haemoglobin is slowly oxidised by oxygen into methaemoglobin (Adlercreutz & Mattiasson, 1982), thus limiting their potential to be recovered and recycled in highly oxidative processes. The presence of trace element ions can further increase the oxidation of haemoglobin-based DO carriers. The presence of a DO carrier in the liquid phase can have an improved effect on the rate of oxygen transfer in fermentation processes, thus improving the performance of the biomass and bioproduct yield.

4.2 WATER-IMMISCIBLE GAS CARRIERS: THEIR APPLICATION AND BENEFITS TO DIFFERENT CELL CULTURES

Gas saturated water-immiscible DO carrier droplets can be used to enhance oxygen supply by liquid-liquid and liquid-biomass contact. Some are heavier than, and immiscible in, aqueous medium and can be collected at the base of the vessel for reuse in subsequent fermentations (Richardson *et al*., 2002; Lowe *et al*., 1998; Kabalnov *et al*., 1990). Some DO carriers, such as PFCs, have higher solubilities for carbon dioxide than ordinary aqueous media. Therefore, they can also carry metabolically produced carbon dioxide, removing it from the fermentation broth and thus improving overall biomass and bioreactor performance, especially for continuous bioprocess systems. One such example was the inclusion of 50% (v/v) perfluorodecalin in the fermentation medium using *Streptomyces coelicolor* A3(2). This resulted in a five-fold increase in the maximum actinorhodin production. The use of water-immiscible DO carriers can also improve the maximum specific growth rates of microorganisms with increasing concentrations (Amaral *et al*., 2007), while the specific death rate decreases with DO availability. These phenomena clearly suggest that the additional oxygen supplied by inclusion of water-immiscible gas carriers could be readily utilised by aerobic submerged cultures resulting in the improved performance of the fermentation system (Elibol & Mavituna, 1999). Examples of other water-immiscible DO carriers are: hexanol (Koide *et al*., 1985); olive and lard oil (Liu *et al*., 1994) and soybean and silicone oil (Morão *et al*., 1999).

The benefits of using gas carriers in microbial culture systems include: (1) a reduction in mechanical damage to biomass by eliminating the effects of conventional aeration through sparging or continuous stirring; (2) the provision of a multi-phased interface for effective DO transfer; (3) prolonged survival rates of microorganisms; (4) ease of sterilisation (by means of autoclaving or filtering);and (5) ease of recovery and recycling (Lowe *et al*., 1998). Microbial cultivation in these dispersions is attractive because maximum DO transfer rates in some cases were increased by over 400% when using oxygen carriers (McMillan & Wang, 1988).

In the majority of these cases, the emulsions have not been used in volume fractions exceeding 40% (v/v) (Junker *et al.*, 1990), thus limiting fermentation broth viscosity close to that of water. Table 4.1 lists examples of applications and benefits for different water-immiscible gas carriers in different submerged microbial cultures:

Table 4.1: Application and benefits of water-immiscible oxygen carriers in submerged cultures

Process	Oxygen carrier Surfactant	Microorganism	Consequence	Reference
Dihydroxyacetone production	0.85 mM oxyhaemoglobin	*Gluconobacter oxydans*	>4.0 mM dihydroxyacetone production	(Adlercreutz & Mattiasson, 1982)
Dihydroxyacetone production	32.4 % (v/v) FC-72	*Gluconobacter oxydans*	4 - 5 x dihydroxyacetone production	(Adlercreutz & Mattiasson, 1982)
Dihydroxyacetone production	20 - 80 mM *p*-benzoquinone	*Gluconobacter oxydans*	70% increase in dihydroxyacetone production	(Adlercreutz & Mattiasson, 1984)
IgG production	Fluorinert FC-40	*Mouse-mouse hybridoma, 4C10B6*	High cell density attained	(Hamamoto *et al*., 1987)
E. coli and *S. cerevisiae* culture	20% Fluosol-DA (F-DA) Perfluorodecalin	*Escherichia coli HB101* *Saccharomyces cerevisiae*	*E. coli* and *S. cerevisiae* inhibited by F-DA *E. coli* not affected by 15 to 30% PFC	(Chandler *et al*., 1987)
Hybridoma cell culture	perfluoromethyldecalin	*Mouse-mouse hybridoma, cell line (#824)*	Increased oxygen transfer Increased cell density when compared to batch culture	(Cho & Wang, 1988)
Penicillin fermentation	0. 5% , 2%, 5% *n*-hexadecane	*Penicillium chrysosgenum, Wis. 54 to 1255*	Increased cell growth Increased penicillin production	(Ho *et al*., 1990)
A. aerogenes culture	54.9 mg.L^{-1} n-dodecane and 118 mg.L^{-1} F66E 12.5 mg/ml Pluronic F 68	*Aerobacter aerogenes*	3.5 x K_La achieved	(Rols *et al*., 1990)
E. coli culture	15% (v/v) FlurO_2	*Escherichia coli K-12*	2.55 x enhance in biomass concentration	(Ju *et al*., 1991a)
Protoplast culture	Perfluorodecalin 0.01% (w/v) Pluronic F 68	*Petunia hybrida*	52% plating efficiency than control	(Anthony *et al*., 1994)

Table 4.1: *(continued)*

Process	Oxygen carrier Surfactant	Microorganism	Consequence	Reference
E. coli culture	50% (v/v) Foralkyl 10 mg/ml Pluronic F 68	*Escherichia coli*	0.17 g $O_2.L^{-1}h^{-1}$ supply and 0.23 g $CO_2.L^{-1}h^{-1}$ extraction	(Martin *et al*., 1995)
C. acetobutylicum culture	18.5% Forane F66E	*Clostridium acetobutylicum*	9% extraction of total CO_2 produced	(Percheron *et al*., 1995)
Anthraquinone production	0.11% (w/v) n-hexadecane	*Morinda citrifolia*	2 x Anthraquinone production than control	(Bassetti & Tramper, 1995)
Actinorhodin production	10% (v/v) Perfluorodecalin 4% (w/v) Pluronic F 68	*Streptomyces coelicolor A3(2)*	2 x increase in actinorhodin production compared to 10% PFC (/v) without Pluronic	(Elibol & Mavituna, 1996)
M. parafortuitum culture	26% (v/v) FC-40 18.2% (v/v) FC-40	*Mycobacteria parafortuitum*	1.8 x K_La achieved 1.2 x K_La achieved	(Cesário *et al*., 1996)
Post thaw culture	Perfluorodecalin 0.01% (w/v) Pluronic F 68	*Oryza sativa cv.*	21% increase in post thaw viability	(Anthony *et al*., 1997)
Actinorhodin production	50% (v/v) Perfluorodecalin	*Streptomyces coelicolor A3(2)*	5 x increase actinorhodin production	(Elibol & Mavituna, 1997)
Hybridoma antibody production	Natural bovine haemoglobin Erythrogen™-1 Formula-1™ Polyethylene glycol cross-linked haemoglobin perfluorocarbon	*Hybridoma 3C11*	No antibody production increase for natural bovine haemoglobin 104% antibody production increase using Erythrogen™-1 20% antibody production increase using glycol cross-linked haemoglobin 78% antibody production increase using perfluorocarbon	(Shi *et al*., 1998)

Table 4.1: *(continued)*

Process	Oxygen carrier Surfactant	Microorganism	Consequence	Reference
S. cerevisiae culture	Perfluorodecalin 4% (w/v) Pluronic F 68	*Saccharomyces cerevisiae*	Significant increases in K_La values	(Elibol, 1999)
Actinorhodin production	50% (v/v) Perfluorodecalin	*Streptomyces coelicolor A3(2)*	5 x increase in actinorhodin production	(Elibol & Mavituna, 1999)
Lactate production	Fluorinert FC-40	*Spodoptera frugiperda sf9*	Increased cell density, growth yield and lactate yield	(Gotoh *et al*., 2001a)
Virus infected *S. frugiperda* culture	Fluorinert FC-40	*Spodoptera frugiperda; sf9*	Cell density achieved was higher than that in surface aeration Recombinant protein yield increased 1.6 x K_La achieved	(Gotoh *et al*., 2001b)
Actinorhodin production	10% Perfluorodecalin	*Streptomyces coelicolor A3(2)*	3.0 x K_La achieved	(Elibol, 2001)
A. terreus culture	n-dodecane	*Aspergillus terreus ATCC 20542*	1.4 x increase in lovastatin production	(Lai *et al*., 2002)
Egg hatching after storage	Fluorinert-77	*Oncorhynchus mykiss*	>75.1% hatching/ compared to 14.3 % in control	(Richardson *et al*., 2002)
P. shermanii culture	5 - 20% (v/v) *n*-dodecane	*Propionibacterium shermanii*	3.5 - 5 x K_La achieved	(Cascaval *et al*., 2006)
S. cerevisiae culture	5 - 20% (v/v) *n*-dodecane	*Saccharomyces cerevisiae*	3.5 -5 x K_La achieved	(Cascaval *et al*., 2006)
A. chroococcum culture	5% (v/v) perfluorodecalin	*Azotobacter chroococcum ACB 121*	>5 x increase in cell concentration 3.4 x nitrogenase activity 4.5 x increase in nitrogen content	(Bakulin *et al*., 2007)

Enhanced oxygen supply in bioreactors through the addition of oil-in-water emulsions can be calculated using the enhancement factor, E, as shown in Eq. 4.1. The factor is a ratio between the biomass generated in bioreactors with and without the PFC emulsion (Ju *et al.*, 1991a).

$$E = \frac{\text{Biomass generated (PFC emulsion)}}{\text{Biomass generated (control)}} \qquad \text{Eq. 4.1}$$

The effects of enhancing DO supply to bioreactors, quantified as the oxygen transfer enhancement factor, can be assessed by the volumetric oxygen transfer coefficient, $K_L a$ measurements. The oxygen transfer rate (OTR) is shown in Eq. 4.2. It is expressed as the ratio between experimental OTR and the difference between saturation DO concentration at the gas-liquid interface and DO in the continuous bulk phase (Ju *et al.*, 1991a).

$$\frac{OTR}{C^*_{O_2} - C_{O_2}} = K_L a \qquad \text{Eq. 4.2}$$

Where $C^*_{O_2}$ is the saturation concentration of DO transfer at the gas-liquid interface, C_{O_2} is the DO concentration in the bulk phase and $K_L a$ is the volumetric oxygen transfer coefficient (Shuler & Kargi, 1992).

4.3 PERFLUOROCARBONS AS DISSOLVED OXYGEN CARRIERS

The use of perfluorinated organic oils as oxygen carriers was initially investigated using mice. The oils were shown to assist the animals with liquid breathing (Clark & Gollan, 1966). Over the years, they have also been shown to be effective as DO carriers in bioprocesses using different microorganisms. Recently, Amaral *et al.* (2007) demonstrated the effects of concentrations of 10 to 20% (v/v) perfluorodecalin in *Yarrowia lipolytica* under agitation

conditions. The result was an increased glucose consumption and specific growth rate for the culture. As PFCs are noncorrosive, odourless and colourless (Lowe, 2002), they were also shown to be suitable for the development of synthetic blood (Inayat *et al.*, 2006; Goorha *et al.*, 2003; Lowe, 2003; Riess, 2002). Other examples of PFC-emulsion applications are listed in Table 4.1.

PFCs are chemically inert compounds consisting of fluorine-substituted hydrocarbons in which most or all of the hydrogen atoms have been replaced by fluorine. A progressive substitution of fluorine for hydrogen leads to an increase in molecular mass, resulting in liquids that are much heavier than other hydrocarbon oils such as mineral oil. The oils typically have specific gravities, approximately twice that of water (Lowe *et al.*, 1998). They are stable, non-toxic and can store and release oxygen at a greater rate (Goorha *et al.*, 2003; Lowe, 2003; Richardson *et al.*, 2002; Riess, 2002). Oxygen solubility in these oils is related to the molecular volume of the dissolving gas and decreases in the following order: $CO_2 > O_2 > N_2$ (Lowe *et al.*, 1998), making them suitable for oxygen delivery and metabolic CO_2 removal from fermentation systems. PFCs have the following advantages: 1) they do not react chemically with oxygen or other gasses; 2) oxygen solubility is not subject to the effects of pH; 3) they are not susceptible to dissolved salts in the fermentation medium and 4) they facilitate an effortless transfer of oxygen. Oxygen solubility here is inversely proportional to the molecular weight and directly proportional to the number of fluorine atoms in the oils (Goorha *et al.*, 2003). When comparing traditional bioprocesses with PFC-supplemented bioprocesses, it can be seen that the OTR is enhanced without the need for supplementary energy consumption for mixing. However, the use of these oxygen carriers needs to be further analysed to determine their compatibility with microbial strains that are chosen for a defined bioprocess (Cascaval *et al.*, 2006). The characteristics of commonly used PFC liquids at standard pressure (1 atm.) and temperature (25°C) are listed in Table 4.2.

Table 4.2: Characteristics of PFC liquids compared to those of water at standard pressure and temperature (Lowe *et al.*, 1998; Ju *et al.*, 1991a)

Liquid	Oxygen	Carbon dioxide	Density[c]	Boiling point[d]	Molecular weight[e]
Water	0.2[a]	57[a]	1.0	100	18
FC-40	37[b]	142[b]	1.87	155	650
FC-43	36[b]	140[b]	1.88	174	670
FC-77	56[b]	214[b]	1.78	97	415
FC-84	59[b]	224[b]	1.73	80	388
Bis-(Perfluorobutyl) ethene	44.0[a]	203[a]	1.41	60	464
Perfluorobutyl tetrahydrofuran	51[b]	209[b]	1.77	102	416
Perfluorodecalin	35.5[a]	125[a]	1.92	142	462
Bis-Perfluorohexyl ethene	37.9[a]	159[a]	1.77	195	664
Perfluoro-n-hexane	65[b]	248[b]	1.68	59	340
Perfluorooctyl bromide	44.0[a]	185[a]	1.93	142	499
Perfluorotributylamine	35.2[a]	123[a]	1.85	155	671
Perfluorotripentylamine	31[b]	117[b]	1.94	215	821
Perfluorotripropylamine	39.6[a]	146[a]	1.82	130	521

a- gas dissolving capacity in mM at 25°C (Lowe et al., 1998)
b- gas dissolving capacity in ml gas/100 ml PFC at 37°C (Ju et al., 1991a)
c- Density in g.cm^{-3}
d-Boiling point in °C
e-Molecular weight in g/mol
FC 40/43/77/84 – are Fluorinert Electronic Liquids, products of 3M Company (USA)

There are several disadvantages related to applying pure PFCs in cell cultures, such as: 1) a reduction in biomass generated and 2) increased lag phases in the fermentation (Amaral *et al.*, 2007). In order to use PFCs as oxygen carriers, their effects on the physiological state and performance of microorganisms need to be determined. The consensus is that PFCs have different effects on prokaryotic and eukaryotic cells. PFCs in their emulsion form have no apparent effect on prokaryotic cells, whilst some eukaryotic cells show ultrastructural changes after treatment with pure PFCs (Elibol, 2001; Chandler *et al.*, 1987). Problems may also occur in batch PFC-mediated aeration cultures, where ventilation is only carried out through PFCs, as volatile organic compounds (VOCs) can be produced and thus hamper the metabolic activity of microorganisms (Gotoh *et al.*, 2001b). VOCs are organic chemical compounds, mostly carbon-based molecules such as aldehydes and ketones, with high pressures, which enables them to rapidly vaporise and enter the atmosphere. PFCs cannot dissolve electrolytes and other organic compounds, except for fluorine-substituted

compounds. The VOCs can , therefore, accumulate in the medium without being removed, especially in batch cultures (Gotoh *et al.*, 2001b).

4.4 PREPARATION OF PERFLUOROCARBON EMULSIONS

Being virtually insoluble in water, PFCs are usually formulated as submicron emulsions. PFC emulsions can be prepared using different types and concentrations of surfactants as nano- or micro-emulsions; nano-emulsions (droplets covering the size range of 100 to 600 nm) are preferable, as they have increased stability (Bouchemal *et al.*, 2004). The formulation of an emulsion requires a surfactant capable of ensuring adequate dispersion, homogeneity, reproducibility, stability and biocompatibility (Riess, 2002).

The droplet size of the dispersed PFC phase can be controlled with the concentration of the surfactant. After preparation, the initial oil-particle size decreases with increasing surfactant concentration. PFC emulsions containing 10% (w/w) PFC were determined to be stable at surfactant concentrations as low as 0.5% (w/w) (Magdassi & Siman-Tov, 1990). In the preparation of PFC emulsions, dispersions with surfactant-PFC ratios of <2% were determined to be surfactant limited, whereas those with a ratio of >5% were energy-input limited (De Vleeschauwer & Van der Meeren, 1998). Although, several disadvantages of supplying pure PFCs in cell cultures are evident, the use of PFC emulsions with lower oil concentrations outweigh any disadvantages determined when cells are cultured in pure PFC dispersions (Ju *et al.*, 1991b).

Emulsions are usually prepared with a mixture of surfactants with different hydrophobicity. The concept of hydrophilic-lipophilic balance (HLB) was introduced as an indication of the relative strength of the hydrophilic-lipophilic portions of the surfactant molecule and can be characterised by the relative affinity of surfactants for the aqueous and organic parts of the surfactant molecule (Griffin, 1949). The HLB arbitrary scale range is 1 to 30. As a result, surfactants with high HLB values (>15) tend to stabilise oil-in-water (O/W) emulsions, while

surfactants with low HLB values (<10) tend to stabilise water-in-oil emulsions. As most PFC emulsions used in bioprocesses are O/W emulsions, surfactants with high HLB values are preferred in the preparation of these emulsions (Sajjadi, 2006). Examples of the HLB values of several PFCs and commonly-used surfactants are listed in Table 4.3, indicating that PF 68 with an HLB value of 24 to 29, is suitable for O/W emulsions.

Table 4.3: Hydrophilic-lipophilic balance of perfluorochemicals and surfactant (Weers, 1993)

Perfluorochemical	HLB value	Surfactant	HLB value
Perfluorodecalin	9.5	Lecithin	8.0
Perfluorooctyl bromide	6.0	Potassium oleate	18.0
Perfluorotributylamine	10.3	Pluronic F 68	24.0 - 29.0

HLB value = 3 to 6 (water-in oil surfactant)
HLB value = 8 to 15 (oil-in-water surfactant)
HLB value = >15 (solubiliser)

4.5 PLURONIC F 68 AS A SURFACTANT AND AS A BIOMASS PROTECTOR

Polymeric surfactants have been extensively used in various applications ranging from personal care products to pharmaceutical and industrial use (Plucktaveesak *et al.*, 2000). Pluronic F 68 (Poloxamer 188/ PF 68) is a non-ionic block copolymer of polyethylene glycol and polypropylene glycol (Elibol, 1999), which has been used to emulsify PFCs for use in oxygen-transport fluids (Johnson *et al.*, 1990). This surfactant has been used as a growth-promoting additive to animal and microbial cultures (Lowe *et al.*, 1994). When a PF 68 concentration of 10 $mg.ml^{-1}$ was used in Perfluorooctyl bromide (PFOB) emulsions, the emulsion was extremely stable and remained in this state for more than 7 days. This was advantageous for cultures, as the emulsions were homogeneous, but led to great difficulty in PFOB recovery. The total recovery percentage was low (78%) after centrifugation. When a PF 68 concentration of 5 $mg.L^{-1}$ was used, destabilisation occurred rather rapidly, but the recovery of PFOB was easier, resulting in a 91% recovery rate (Martin *et al.*, 1995).

The mass transfer characteristics of PF 68 were investigated in a MYGP (malt extract-yeast extract-glucose-peptone) medium using *Saccharomyces cerevisiae* (NCYC 239) and was found to reduce the volumetric oxygen transfer coefficient ($K_L a$). This was not the case in the presence of PFC oils, where final biomass concentrations were unaffected (Elibol, 1999). Typical usage levels of surfactants in an emulsion system are: 1) 1 to 5% (w/v) for water-in-oil emulsions and 2) 5 to 10% (w/v) for oil-in-water emulsions (Floyd, 1999). Table 4.4 shows concentration levels for some of the surfactants most commonly used in PFC-based emulsion formulations, as compared to PF 68.

Table 4.4: Surfactants most commonly used in PFC-based emulsions (Floyd, 1999)

Surfactants and/or their combinations	Range/ratio
Glycerol/propylene glycol	30 - 70% w/w individually
Egg lecithin	1 - 3% (w/w)
Pluronic F 68, 88, 108	1.5 - 10% (w/v)
Polysorbate 80	0.4% (w/w)

During PFC-based emulsion preparation, the particle size of the oils decreased when a combination of PF 68 and Tween 80 were used as hydrophilic surfactants. It was concluded that PF 68 greatly influenced the particle-size distribution profile in the PFC-based dispersions (Bouchemal *et al*., 2004). Emulsions prepared with PF 68 have a lower probability of stability loss due to coalescence resulting in homogeneous emulsions with an even distribution of DO. When coalescence and ripening are suppressed, the emulsion might remain stable for years (Mason, 1999).

PF 68 has also been used as a cytoprotectant and growth promoting additive to animal cell and microbial cultures (Lowe *et al*., 1994). Microbial cell damage arising from gas sparging is considered to be a major obstacle in the operational longevity of large-scale bioprocesses (Wu, 1995), thus requiring the application of protective additives in the culture medium. PF 68 is one of the most recognised and commonly used additives, as it has been shown to

have strong protective effects in microbial cultures (Wu, 1995). Several other poloxamers (F88, F108, L35) have also shown varying degrees of protective effects in agitated and aerated fermentation systems (Wu, 1995).

PF 68 has been determined to protect cells by coating the membranes of microorganisms, thus directly altering the cell membrane and resulting in the reorganisation of membrane lipids. The surfactant affects lipid-lipid and lipid-protein interactions, thus improving the survival rate of microorganisms by inhibiting damaging interactions between the cell membrane, fermentation broth and the air-liquid interface (King *et al.*, 1991; Murhammer & Goochee, 1990). PF 68 was also shown to protect and prolong the survival of low concentrations of cell suspensions during nutrient starvation. Furthermore, the surfactant prevented death caused by concentrations of Ca^{2+}, prolonging the survival of cells exposed to higher ion concentrations of Ca^{2+}, Na^{+} and K^{+}. It was effective in the postponement of death caused by trace element ions like Zn^{2+}, Fe^{3+} and Cu^{2+} and death caused by shearing forces, while prolonging the survival of cells exposed to hyperthermia (Hellung-Larsen *et al.*, 2000). The surfactant protects cells by regulating the permeability and loss of ions from the affected cells (Laouar *et al.*, 1996). Table 4.5 summarises examples of microbial cultures and the effects caused by the addition of PF 68 to these cultures, showing that PF 68 has mostly positive effects on microbial cultures. In addition, no quantitative results were reported in literature as to whether PF 68 improves microbial growth when used as an additive to synthetic media. This was therefore evaluated as part of this study and the results are reported in Chapter 7.

Table 4.5: Examples of different applications of Pluronic F 68

Surfactant	Microorganism	Consequence	Reference
0.2% (w/v) Pluronic F 68	*Spodoptera frugiperda Sf9*	Cell protection	(Murhammer & Goochee, 1990)
20% (w/v) Pluronic F 68	*Saccharomyces cerevisiae*	Improved fluorescein diacetate uptake	(King *et al*., 1991)
0.1 - 1.0% (w/v) Pluronic F 68	*Saccharomyces cerevisiae (X 2180 1B)*	No adverse effect on growth kinetics	(Laouar *et al*., 1996)
0.01% (w/v) Pluronic F 68	*Tetrahymena thermophila*	Protection against chemical/physical stress	(Hellung-Larsen *et al*., 2000)

Note: Quantities of reagents are listed at their final concentration in the nutrient medium

4.6 CONCLUSIONS

As new technologies are developed to continuously produce high-value biopharmaceuticals in order to meet increasing demand, the effective use of water-immiscible DO carriers and additives to improve bioreactor operational efficiency and longevity remains overlooked. This review clearly shows the positive effects of adding DO carriers to different microbial systems.

PFCs have been shown to be effective in carrying and delivering oxygen to biological cells in storage or culture systems. The general consensus was that PFCs, especially those in emulsions, can have different effects on prokaryotic and eukaryotic cells, but their use in many instances showed that they increased the overall biomass performance, and, in some systems, they were shown to increase the yields of commercially important cellular products such as antibiotics. However, their application in bioprocessing systems needs to be evaluated in order to avoid harmful effects; therefore, determining adequate concentration levels for a predetermined bioprocesses is important.

The promising capabilities of PFC and PF 68-based emulsions for providing culturing conditions suitable for a general improvement in microbial biomass performance and extended product formation have been illustrated. PF 68's ability to improve the functioning of individual cells in fermentation systems, while protecting the cells against trace element toxicity, shearing effects, hypothermia and product inhibition, will provide for increased biofilm and bioreactor performance in fixed-film bioreactors, thus improving the economic viability of these continuous systems. The effects of PF 68 and PFC-based emulsions in *P. chrysosporium* cultures will be evaluated and discussed in Chapters 7 and 8.

4.7 SUMMARY

The use of PFCs to overcome DO transport deficiencies was demonstrated to be a suitable intervention to improve biomass generation and bioproduct formation in different microbial cultures. The use of Pluronic F 68 has been shown to have protective effects. It was demonstrated that the surfactant limits metal accumulation, provides protection against shear stress and delays biomass lysis, which can result in the prolonged use of immobilised biomass.

Since *P. chrysosporium* biomass performance is related to the limitations listed in this chapter and in Chapter 2, it was deemed appropriate to investigate the use of perfluorocarbons with this fungus using the MGR. The following questions resulted from the review and were addressed in Chapter 7:

- What are the effects of PFCs on fungal growth and the production of ligninolytic enzymes when in direct contact with *P. chrysosporium*?
- When formulating a PFC-based emulsion, what kind of surfactant will be compatible with both the PFCs and *P. chrysosporium*?

Objectives with regard to the evaluation of PFCs were as follows:

- To evaluate the effectiveness and compatibility of three different PFCs on *P. chrysosporium* growth and ligninolytic enzyme production and select a suitable PFC and concentration for application in the MGR.
- To apply the proposed emulsion to the MGR system and evaluate its effectiveness in enhancing *P. chrysosporium* biomass performance in terms of ligninolytic enzyme production, while improving overall biomass performance, thus curbing limitations such as trace-element accumulation, oxidative stress and polysaccharide storage.

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CHAPTER 5

RESULTS

Oxygen mass transfer for an immobilised biofilm of *Phanerochaete chrysosporium* in a membrane gradostat reactor

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CHAPTER 5

Oxygen mass transfer for an immobilised biofilm of *Phanerochaete chrysosporium* in a membrane gradostat reactor

Abstract

A novel system, the membrane gradostat reactor (MGR), designed for the continuous production of secondary metabolites, has been shown to have higher production per reactor volume than batch culture systems. The MGR system mimics the natural environment in which native microbial biofilms flourish. The biofilms are immobilised on the external surface of an ultrafiltration membrane where substrate distribution gradients are established across the biofilm. The hypothesis that dissolved oxygen (DO) mass transfer parameters obtained in submerged pellets can be used to describe and model DO mass transfer parameters in the MGR was refuted. *Phanerochaete chrysosporium* biofilms, immobilised on ultrafiltration capillary membranes in the MGR system, were used to quantify DO distribution using a Clark-type microsensor. The DO penetration depth decreased with increasing biofilm thickness, which resulted in the formation of anaerobic zones in the biofilms. Oxygen flux values of 0.27 to 0.7 $g.m^{-2}.h^{-1}$ were obtained during the MGR operation. The consumption of oxygen and the Monod saturation constants used in the modelling of oxygen distribution in immobilised biofilms were in the range of 894.53 to 2739.70 $g.m^{-3}.h^{-1}$ and 0.041 to 0.999 $g.m^{-3}$, respectively.

Keywords: Extracellular enzyme production; Membrane bioreactor; Oxygen mass transfer; *Phanerochaete chrysosporium*

5.1 INTRODUCTION

Since the discovery of extracellular enzymes, such as manganese peroxidase (MnP) and lignin peroxidase (LiP), in *Phanerochaete chrysosporium* biofilms (Kuwahara *et al*., 1984;

Tien & Kirk, 1984; Kirk *et al*., 1978), efficient methods for continuous production of these enzymes have been developed. The use of membrane bioreactors (MBRs) where *P. chrysosporium* biofilms are immobilised on the external surface of support matrices has been shown to result in higher enzyme production per reactor volume than submerged batch cultures (Sheldon & Small, 2005; Govender *et al*., 2003; Leukes, 1999; Venkatadri & Irvine, 1993; Linko, 1988; Kirkpatrick & Palmer, 1987). The continuous production of these enzymes is of great economic importance, as it has been demonstrated that they are able to metabolise a variety of organic compounds, many of which are pollutants in both liquid effluents and soils (Walsh, 1998; Griselda & Eduardo, 1990; Livernoche *et al*., 1981). The membrane gradostat reactor (MGR) was conceptualised as an alternative to batch culture systems and has been proven to be more effective than submerged cultures and other MBRs in terms of LiP and MnP production (Ntwampe & Sheldon, 2006; Sheldon & Small, 2005; Govender *et al*., 2004; Govender *et al*., 2003; Leukes, 1999; Leukes *et al*., 1999).

The most important external factors affecting the production and activity of enzymes produced by *P. chrysosporium* are temperature, pH, dissolved oxygen (DO) concentration and a fixed nitrogen concentration (Leisola *et al*., 1984; Fenn *et al*., 1981; Fenn & Kirk, 1981; Jeffries *et al*., 1981). The morphology of immobilised biofilms is arranged according to the conditions at which they are grown, and this affects substrate mass transfer rates and microbial activity (Beyenal & Lewandowski, 2002). DO mass transfer is an essential component of the microbial activity of aerobic biofilms, such as that of *P. chrysosporium* (Bishop & Yu, 1999). Measured substrate-concentration profiles for immobilised biofilms can be used to quantify mass transfer parameters in the identified biofilm (Lewandowski & Beyenal, 2003b). Any substrate's mass transfer in biofilm systems is characterised by three steps: (a) transfer from the bulk medium to the biofilm surface; (b) diffusion and (c) consumption within the biofilm (Zhang & Bishop, 1994). The rate of substrate transport in a biofilm is determined by linking the convective mass transfer rate to the diffusive mass transport rate across the biofilm surface (Beyenal & Lewandowski, 2002). As there is no

substrate consumption in the bulk medium, the flux of substrates across the biofilm surface must be conserved, which requires the rate of external (J_f) and internal $(D_f(dC/dx))$ oxygen mass transfer, to be equal, as described by Eq. 5.1 (Hibiya *et al.*, 2003; Lewandowski & Beyenal, 2003b; Beyenal & Lewandowski, 2002). To solve Eq. 5.1, the oxygen diffusivity coefficient in the biofilm (D_f) and the oxygen diffusivity coefficient in water (D_w) at the incubation temperature need to be determined.

$$J_f = D_w \beta (C_b - C_s) = D_f \left(dC / dx \right)_{f,x_x} \qquad \text{Eq. 5.1}$$

Several published papers that relate the diffusion of oxygen in biofilms to variables such as biofilm dry density and thickness are available. However, these experiments were performed using microbial biofilms immobilised on flat surfaces (in plate open channel reactors), and the biofilms were submerged in a nutrient medium (e.g. submerged MBRs). These biofilms were not immobilised on the external surface of a capillary membrane or anything that resembles the MGR used in this study. In submerged MBRs, the transfer of DO is from the bulk nutrient medium into the biofilm matrix, or oxygen is supplied through the lumen of the membrane to the biofilm. The biofilms immobilised were not of a fungal nature and were not continuously exposed to the gaseous oxygen source. In the present study, oxygen was continuously supplied in the extracapillary space (ECS/shell side) of the MGR system. Biofilms positioned horizontally are still commonly used to study kinetic parameters. However, in this study, the MGRs were in a vertical position to enhance radial distribution of substrates (including DO) across the immobilised biofilm, which is a requirement for an effective MGR system (Leukes *et al.*, 1999).

5.2 OBJECTIVES

The objectives of the study were to: 1) quantify oxygen mass transfer parameters using measured DO profiles obtained from *P. chrysosporium* biofilms and 2) use the mass transfer

parameters obtained in a mathematical model to reproduce the DO distribution. The study also aimed to determine any limitations to oxygen supply within the immobilised biofilms. This would contribute to the understanding of DO transfer in biofilms immobilised in the MGR and on asymmetric capillary membrane systems. As the MGR system is currently being evaluated for its application in the commercial production of secondary metabolites, it was important to establish the parameters directly linked to the production of the metabolites, in order to optimise the system. The DO mass transfer parameters obtained were compared with those obtained in submerged, batch cultures where mycelia pellets of *P. chrysosporium* were removed, allowed to equilibrate with air and probed with an oxygen microelectrode to determine the mass transfer parameters (Michel *et al.*, 1992). The study evaluated the hypotheses that DO mass transfer parameters determined in batch cultures could be used to estimate the DO mass transfer parameters for immobilised microorganisms in the MGR. Other parameters established were biofilm thickness (related to growth rate of the fungus) and the DO penetration ratio (the ratio between the DO penetration depth into the immobilised biofilms and the increase in biofilm thickness). *P. chrysosporium* BKMF 1767 produces ethanol from glucose under limited-oxygen conditions. The ethanol in cultures of *P. chrysosporium* deactivates the production of LiP and MnP (Kenealy & Dietrich, 2004). Therefore, it was important to identify anaerobic zones in the MGR biofilms over the entire duration of the study. LiP and MnP activity were not quantified as part of this study, as other researchers had quantified and established patterns of enzyme production using the MGR system (Sheldon & Small, 2005; Govender *et al.*, 2004; Govender *et al.*, 2003; Solomon & Petersen, 2002; Leukes, 1999).

5.3 MATERIALS AND METHODS

5.3.1 Bioreactor setup and operation

Biofilms of *P. chrysosporium* strain BKMF 1767 (ATCC 24725) were grown at 39℃ in single-capillary MGR systems. A schematic illustration of a vertically-positioned single capillary MGR (SCMGR) is shown in Figure 5.1.

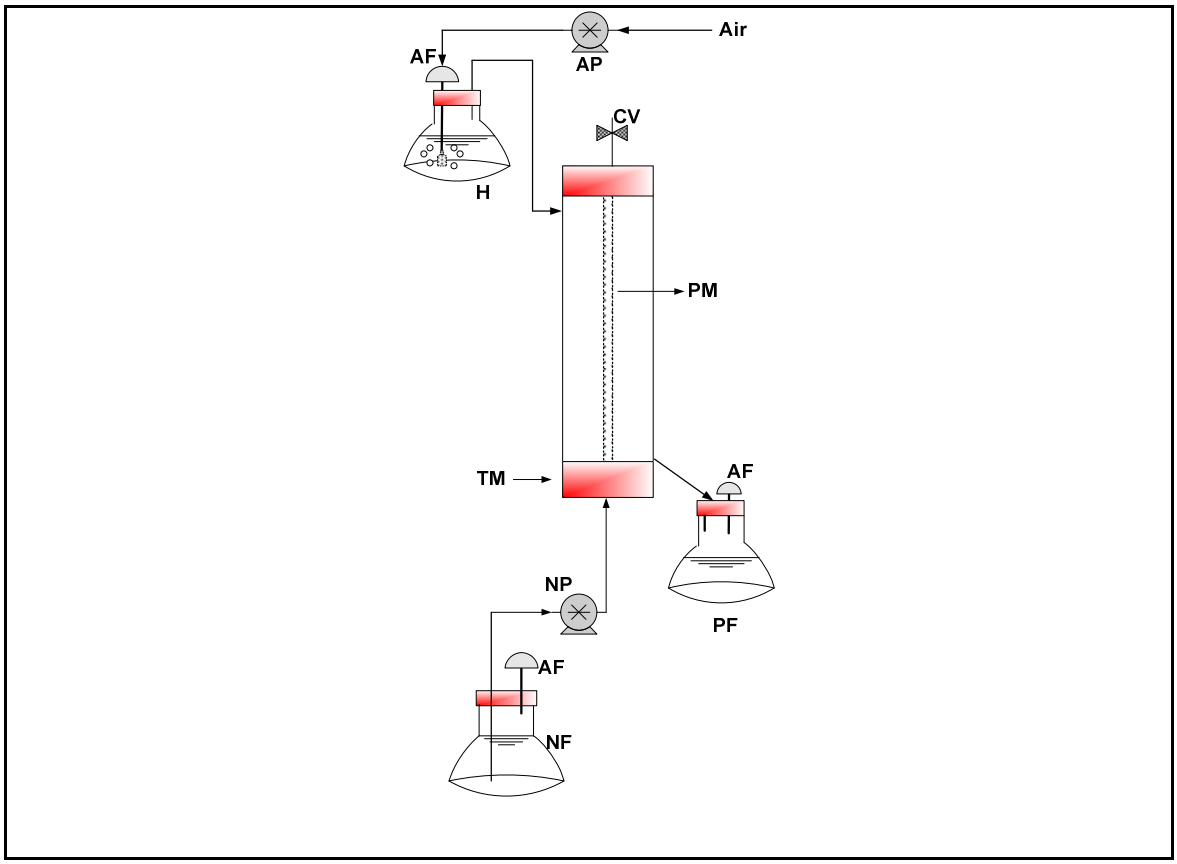

Figure 5.1: Schematic illustration of a vertical single-capillary MGR. AF: air filter; AP: air pump; CV: closed valve (dead-end filtration mode applied); H: humidifier; NF: nutrient flask; NP: nutrient pump; PF: permeate flask; PM: polysulphone membrane TM: Teflon mould. Note: The air was supplied on the shell side; it helps with the flow of permeate to the permeate flask (PF)

The single capillary MGR (SCMGR) dimensions and operational conditions are listed in Table 5.1. SCMGRs with capillary polysulphone membranes fixed to the centre of the glass modules were used. The polysulphone capillary membrane used was specifically developed for use in the MGR system. The membranes were manufactured at the Institute of Polymer Science, Stellenbosch University (Stellenbosch, South Africa) and were coded as IPS 763 (Jacobs & Leukes, 1996). A Teflon mould was used at the bottom of the bioreactor to prevent

accumulation of the permeate. The experimental setup was chemically sterilised with a 4% (v/v) formaldehyde solution and rinsed with sterile distilled water before the spore-inoculation process.

Table 5.1: Dimensions and operating conditions of a vertically-orientated single capillary MGR system

Parameter	Details
Module housing	Glass
Module length	230 mm
Module diameter	12 mm
Active internal volume	20.4 ml
Type of membrane	Polysulphone (IPS 763)
Active membrane length	160 mm
Capillary membrane outer diameter	~1.7 - 1.9 mm
Capillary membrane inner diameter	~ 1.3 mm
Membrane Surface area – Polysulphone	985 mm^2
Operating conditions:	
Nutrient supply rate to the bioreactor	1.68 $ml.h^{-1}$
Airflow rate	1 $vol.ECS^{-1}.min^{-1}$
*Amount of spores per membrane length	3 million
ECS air pressure	Atmospheric

**Prepared using a spectrophotometer at 650 nm; 1 Abs = 5 million spores per ml (Tien & Kirk, 1988).*

A spore solution containing an estimated 3 million spores was used for inoculation. The spores were harvested from mycelia grown on malt agar plates for 5 days at 39°C. Preparation of the spore solution was carried out by suspending spores containing mycelia in sterile distilled water, followed by passage through a sterile glass wool so as to free spores from contaminating mycelia (Tien & Kirk, 1988), as described in Appendix A. The spores were immobilised in the cavities of the membrane by reverse filtration using a 40 ml spore solution pumped through the shell side of the MGR (Govender *et al.*, 2004). The nutrient medium used for the growth of *P. chrysosporium* biofilms in the SCMGR was similar to that used by Tien and Kirk (1988) to culture *P. chrysosporium* in batch cultures. The nutrient medium contained 56 mM glucose; 1.1 mM ammonium tartrate and was supplemented with

Veratryl alcohol; Dimethyl succinate; Thiamin and trace elements. All bioreactor operating conditions, including spore concentration, nutrient and air flow rates, are listed in Table 5.1.

The nutrient medium was supplied to the membrane lumen using a multi-channel Watson-Marlow 505S pump (Dune Engineering, Germany). Humidified air was supplied to the MGR to provide DO to the immobilised biofilms. Air was filter sterilised before being passed through the ECS of the MGR, as shown in Figure 5.1. Air was supplied using a multi-port air pump (HAILIPAI, ACO-9620 aquarium air pump) with flow control to ensure that the flow was consistent throughout all the bioreactor modules. Air entered through the top of each SCMGR system and exited at the bottom, through the permeate port, without being pressurised in the ECS. The MGRs were operated in the dead-end filtration mode for 264 h. The MGRs were operated simultaneously and were independent of one another.

The permeate pH and redox potential were monitored on a 24 h basis using a Hanna HI 8314 (Hanna Instruments, Portugal) pH meter to determine whether the bioreactors were biochemically similar as shown in Figure 5.2. The increase in redox potential in the recovered permeate samples showed that the solution had high oxidative capabilities, a sign of extracellular enzyme production. The high variation in pH- and redox potential observed at the beginning of the experiments was attributed to the spore's acclimatisation to the conditions in the SCMGR system. The pH and redox potential values stabilised after 72 h of bioreactor operation.

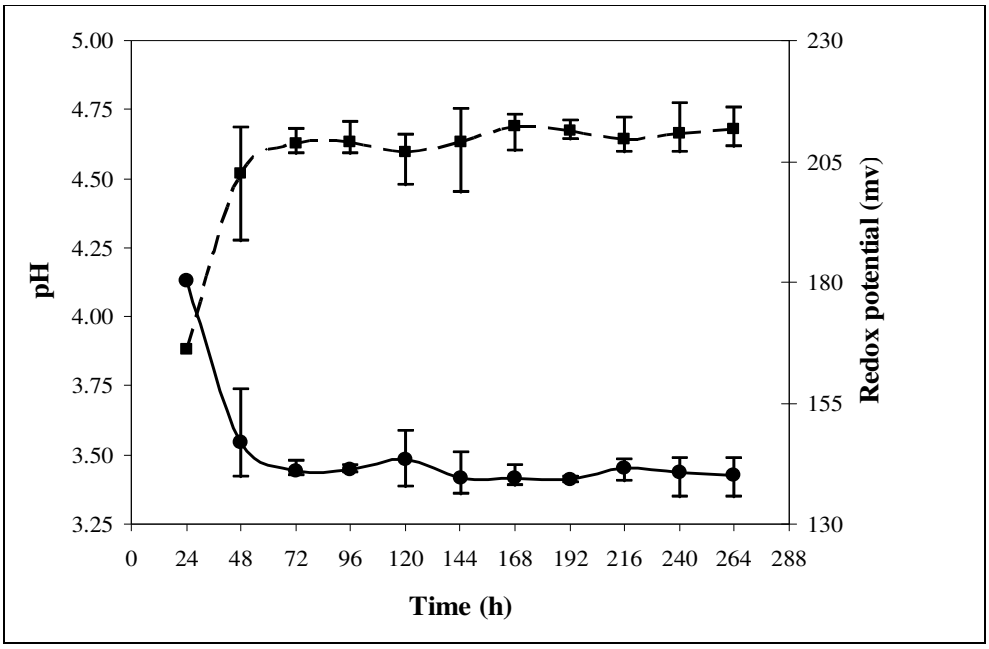

Figure 5.2: pH reduction (-●-) and increasing redox potential (-■-) in permeate samples collected from single-capillary MGR systems. Error bars represent variation of individual values from pH- and redox potential values

5.3.2 Dissolved oxygen measurements

A Clark-type oxygen microsensor (OX 10, outer-tip diameter less than 20 µm) supplied by Unisense (Denmark) was used to measure DO across the biofilms at intervals of 10 µm. The setup consisted of a high-sensitivity picoammeter connected to the microsensor, which was fixed to a micromanipulator which, in turn, was used to move the microsensor into the biofilm. The picoammeter was connected to a computer loaded with *Profix v1.0* software for data capturing. The microsensors were used to measure the DO concentration in the immobilised biofilms in triplicate at time intervals of 72, 120, 168, 216 and 264 h. The DO was measured at atmospheric pressure in three independent experiments. The MGRs were dismantled by removing the glass manifold and exposing the membrane-attached biofilm for easy microsensor measurement. Oxygen microsensor preparation and calibration are described in Appendix G.

The computational methods used to interpret the mass transfer parameters from the DO profiles are explained in the analytical methods section of this chapter. Available

computational procedures for determining the mass transfer parameters from the substrate concentration profiles required the assumption that the biofilms were homogeneous (uniform), not heterogeneous. For current conceptual models of biofilms and the use of mathematical models available to interpret microsensor measurements, the researcher can minimise the effects of biofilm heterogeneity by selecting the locations of microsensor measurements (De Beer & Schramn, 1999; Lewandowski & Beyenal, 2001; Lewandowski & Beyenal, 2003a). Although the biofilms in the SCMGRs were of a similar thickness across the active membrane length, an area closer to the bottom of the bioreactor where sporulation was not evident (~ 2 cm above the Teflon mould), was chosen as suitable for measurement of the oxygen profiles. Sporulation was evident towards the top of the bioreactors. Biofilm thicknesses were determined at this point of measurement. During microsensor calibration, the DO concentration in water was measured as ~ 6.5 ± 0.2 $g.m^{-3}$ at room temperature.

5.3.3 Biofilm thickness, oxygen penetration depth and ratio

The biofilm thickness was determined by using a Carl Zeiss Axiovision light-microscope digital imaging system equipped with measuring software. The objective used for the measurements was a 2.5x magnification. The calibrated microscope objectives took real-size measurements from biofilms attached to the polysulphone membrane surface. The average thickness of a clean membrane was determined to be 1.73 mm, which falls within the manufacturing range of 1.7 to 1.9 mm, as shown in Table 5.1. The average biofilm thickness was measured by determining the overall thickness of the membrane-attached biofilm and then subtracting the thickness of the clean membrane and dividing the result by two to obtain overall biofilm thickness. The oxygen penetration ratio was calculated by dividing the oxygen penetration depth by the biofilm thickness. The DO penetration depth can be described as the depth at which the DO concentration measured in the biofilm is below a concentration of 0.01 g/m^3.

5.4 ANALYTICAL METHODS

5.4.1 Model assumptions

a. Rates of DO mass transfer in the biofilm are proportional to the concentration difference across the boundary layer and biofilm thickness, while proportionality factors are directly related to DO mass transfer constants. Furthermore, it was assumed that the aerial mycelia metabolic activity is very low due to nutrient starvation.

b. The DO mass transfer was assumed to be one-dimensional from the gaseous ECS across the biofilm thickness towards the substratum (polysulphone membrane). Reproducible DO profiles (three profiles per location) were measured several minutes apart showing minimal magnitude of error between the profiles. Figure 5.3 shows examples of reproduced profiles from three independent experiments measured at ~2 cm above the Teflon mould after 120 h of SCMGR operation. This showed that: 1) no DO radial flows (steady or unsteady) occurred parallel to the membrane surface.

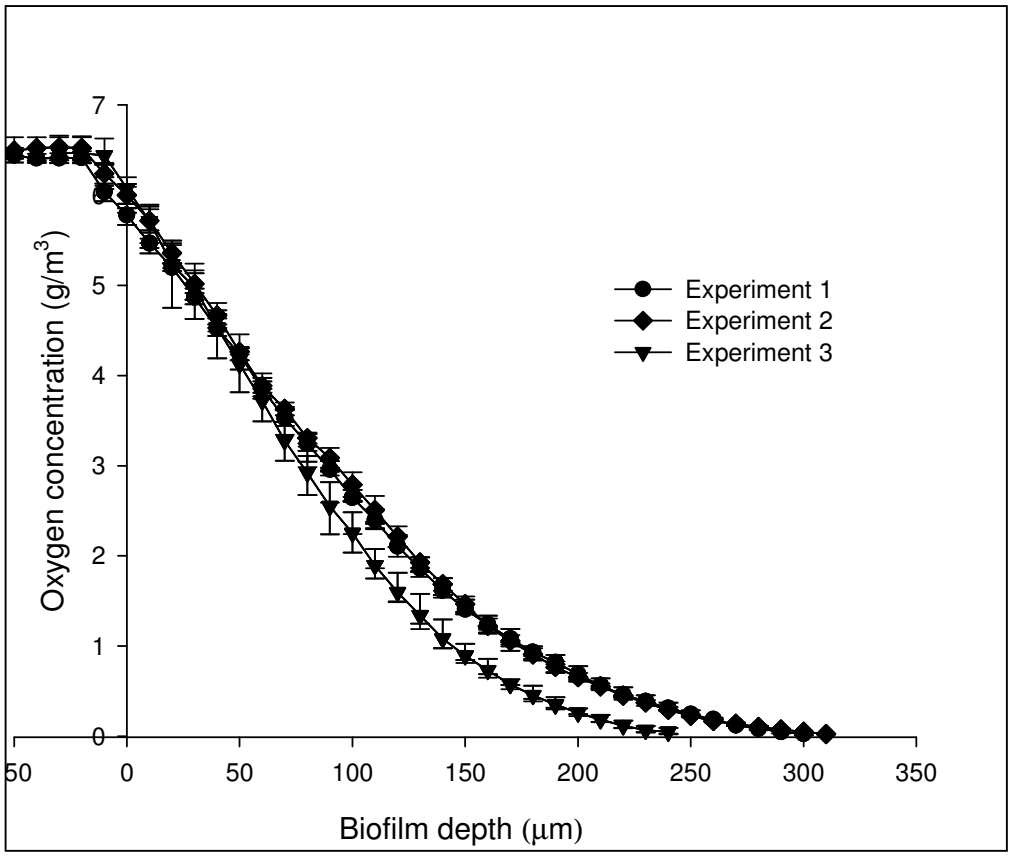

Figure 5.3: DO profiles for immobilised *P. chrysosporium* biofilms measured in three independent experiments after 120 h of single-capillary MGR operation

c. As *P. chrysosporium* is an aerobic microorganism, oxygen consumption through biological reaction was assumed to be described by the Monod equation.

d. The immobilised biofilms were at a pseudo-steady state with respect to the transport of DO into the inner mycelia, as shown in Figure 5.4. A pseudo-steady state $\left(dC_s/dt = 0\right)$ was defined as a condition where oxygen concentration does not change for a period of time at the biofilm surface and aerial mycelia when oxygen is transported from the gaseous phase into the mycelia. The distribution of DO in the aerial mycelia, where the biofilm thickness was 0 to 40 μm, was determined to be similar across all three experiments over a period of 264 h, as shown in Figure 5.4.

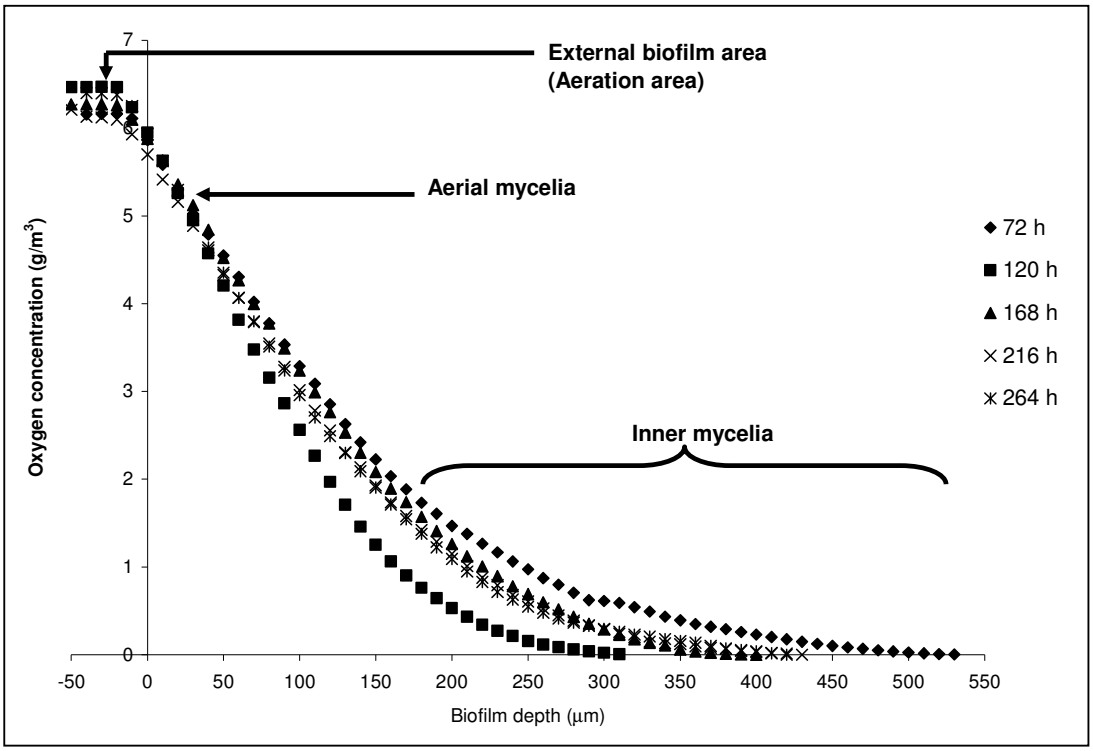

Figure 5.4: A combination of averaged DO profiles for biofilms of *P. chrysosporium* determined at different times during single-capillary MGR operation

5.4.2 Model validation

It was important to determine whether DO transport into the biofilm took place through convection or diffusion. This parameter was important, as it determined the type of model to be used in determining the DO mass transport parameters in the immobilised biofilms. The experimental coefficient, β_a, was determined in the aerial mycelia, 0 to 40 μm, from the biofilm surface using an averaged profile from the five average DO profiles (shown in Figure 5.4) measured at different times, as DO transport in the region was similar. This was achieved by plotting an empirical exponential function $\text{Ln}[1-(C - C_s)/(C_b - C_s)]$ against DO penetration depth $(x - x_s)$ in the aerial mycelia region using $C_b = 6.5 g/m^3$. This technique was reported in Lewandowski and Beyenal (2001). The experimental coefficient (β_a) was determined from the slope of the graph ($R^2 = 0.98$). By using Eq. 5.1 with known values of the oxygen diffusivity coefficient in water with 3% salinity (D_w) and the DO concentration

gradient in the aerial mycelia $(dC/dx)_{a,f,xs}$, parameters such as DO flux, including the diffusion coefficient of oxygen in the aerial mycelia region, could be determined. The parameters that were obtained are shown in Table 5.2. The ratio between the oxygen diffusivity coefficient determined in the nutrient medium and oxygen diffusivity coefficient in the aerial mycelia was equivalent to 0.97. This showed that DO transfer into the biofilm was diffusional as the ratio was closer to 1. Previously, it was determined that aerial mycelia were fragmented compared to the compact vegetative mycelia closer to the membrane surface (Ntwampe, 2005).

Table 5.2: Parameters determined in the aerial mycelia (0 to 40 µm)

Parameter	Value
Experimental coefficient (β_a)	25889 m^{-1}
$\beta_a(C_s-C)$	30421.18 $g.m^{-4}$
D_w (3% salinity)	1.161E-05 $m^2.h^{-1}$
$J_{a,m}$ (averaged flux in the aerial mycelia)	0.35 $g.m^{-2}.h^{-1}$
$(dC/dx)_{a,f,xs}$	29376.55 $g.m^{-4}$
$D_{a,f}$	1.202E-05 $m^2.h^{-1}$
$D_w/D_{a,f}$	0.97

**Nutrient medium contained 3% salinity; measured using a hydrometer.*

5.4.3 Computing mass transfer parameters from DO profiles

By considering all the assumptions in section 5.4.1, Eq. 5.2 can be used to model the mass transport and reaction kinetics in biofilms (De Beer & Schramn, 1999; Lewandowski & Beyenal, 2001; Hibiya *et al.*, 2003; Lewandowski & Beyenal, 2003a).

$$D_f\left(d^2C/dx^2\right)_f = \frac{r_m C}{K_m + C} \qquad \text{Eq. 5.2}$$

The mass transfer parameters were determined using Taylor's expansion method (explained in section 5.4.3.1), as demonstrated by Lewandowski and Beyenal (2003b). The mass transfer parameters obtained were tested using the mass balance equation in porous materials to reproduce the distribution of dissolved profiles in the biofilms of *P. chrysosporium* (Frank-Kamenetskii, 1969), as explained in section 5.4.3.2. Hibiya *et al.* (2003) also demonstrated the use of this method.

5.4.3.1 Taylor's expansion (Lewandowski & Beyenal, 2003b)

Using Taylor's expansion of the function describing the concentration profiles around the point, $x = x_s$, positioned at the biofilm surface, the substrate concentration profiles were obtained near this point and are described by Eq. 5.3.

$$C = C_s + \left(\frac{dC}{dx}\right)_{xs}(x - x_s) + \frac{1}{2!}\left(\frac{d^2C}{dx^2}\right)_{xs}(x - x_s)^2 + \frac{1}{3!}\left(\frac{d^3C}{dx^3}\right)_{xs}(x - x_s)^3 + ... + \frac{1}{n!}\left(\frac{d^nC}{dx^n}\right)_{xs}(x - x_s)^n \qquad \text{Eq. 5.3}$$

Since there are three unknown parameters in Eq. 5.2, D_f, r_m and K_m, calculating the first three derivatives, in Eq. 5.3, was sufficient as only three equations are required to calculate the parameters. As the flux of the substrate across the biofilm has to be continuous, the flux described by Eq. 5.1 and represented as $J = J_{w,xs} = J_{f,xs}$ will hold. Eq. 5.4 and Eq. 5.5 describe the flux at the biofilm interface and nutrient-film, respectively.

On the biofilm surface side:

$$J_{f,xs} = D_f\left(dC/dx\right)_{f,xs} \qquad \text{Eq. 5.4}$$

On the nutrient-film side:

$$J_{w,xs} = D_w \left(dC/dx\right)_{w,xs} \qquad \text{Eq. 5.5}$$

Therefore, $\left(dC/dx\right)_{f,xs}$ can be estimated using the oxygen flux across the nutrient-film layer and the diffusion coefficient in the biofilm as shown in Eq. 5.6, which is the solution to the first derivative in Eq. 5.3.

$$\frac{J_{w,xs}}{D_f} = \left(dC/dx\right)_{f,xs} \qquad \text{Eq. 5.6}$$

As the derivatives were evaluated from the biofilm surface, the higher derivatives for Eq. 5.3 are:

2nd order derivative: $$\left(\frac{d^2C}{dx^2}\right) = \frac{r_m}{D_f}\frac{C_s}{\left(K_m + C_s\right)} \qquad \text{Eq. 5.7}$$

3rd order derivative: $$\left(\frac{d^3C}{dx^3}\right) = \frac{J_{w,xs}}{D_f}\frac{r_m K_m}{D_f}\frac{1}{\left(K_m + C_s\right)^2} \qquad \text{Eq. 5.8}$$

By using the least-square method to fit the experimental data to a third-order polynomial equation, the experimental constants *a*, *b*, and *c*, as shown in Eq. 5.9, can be obtained to simplify Eq. 5.3.

$$C = C_s + a\left(x - x_s\right) + b\left(x - x_s\right)^2 + c\left(x - x_s\right)^3 \qquad \text{Eq. 5.9}$$

From Eq. 5.9, representing 1st, 2nd and 3rd order derivatives, the following will be valid:

$$a = \frac{J_{w,xs}}{D_f}$$ Eq. 5.10

$$b = \frac{1}{2}\frac{r_m}{D_f}\frac{C_s}{K_m + C_s}$$ Eq. 5.11

$$c = \frac{1}{6}\frac{J_{w,xs}}{D_f}\frac{r_m K_m}{D_f}\frac{1}{(K_m + C_s)^2}$$ Eq. 5.12

Eq. 5.10, 5.11 and 5.12 can then be solved by substituting the numerical values for C_s and $J_{w,xs}$ to determine the parameters, D_f, r_m and K_m. As the shape of the DO profile above the biofilm surface (described as the nutrient-film layer) is exponential, it can be described by a function shown in Eq. 5.13 (Lewandowski & Beyenal, 2003b).

$$\frac{C - C_s}{C_b - C_s} = 1 - \exp[-\beta(x - x_s)]$$ Eq. 5.13

The nutrient-film coefficient, β, can be determined when representing graphical coordinates Ln[1-(C - C_s)/(C_b – C_s)] and $(x - x_s)$. In addition, Eq. 5.13 was differentiated across the nutrient-film to obtain Eq. 5.14.

$$(dC/dx)_{w,xs} = \beta(C_b - C_s)$$ Eq. 5.14

$J_{w,xs}$ and $(dC/dx)_{w,xs}$ can be calculated by using the oxygen diffusivity coefficient, 1.161E-05 $m^2.h^{-1}$, in water at 39°C with a salinity content of 3% (determined for this nutrient medium using a hydrometer). The method assumed that the oxygen diffusion transfer was constant across the biofilm thickness. The flux, as described in Eq. 5.1, with other mass transfers could, therefore, be calculated.

5.4.3.2 Diffusion kinetics in porous surfaces (Frank-Kamenetskii, 1969)

The overall rate of reaction on a porous material is the integral sum of the rates for the differential parts of the surface, which are characterised by different accessibilities with regard to diffusion. The overall rate depends on the shape, diameter, thickness and geometrical shape of the pores at different depths of the layer of material. To analyse the problem, regardless of the shape and diameter of the pores themselves, diffusion within a mass of porous material can be described with a diffusion coefficient (D_f), defined in such a way that the diffusion equation in the mass of material has the form shown on the R.H.S of Eq. 5.2. Provided that $r_m C/(K_m + C)$ is only a function of the substrate concentration, C, the R.H.S of Eq. 5.2 can be integrated as a quadrate by using substitutions and thus obtaining Eq. 5.15 (Frank-Kamenetskii, 1969).

$$\frac{dC}{dx} = \sqrt{\frac{2}{D_f}\int \frac{r_m C}{K_m + C} dC} \qquad \text{Eq. 5.15}$$

The mass balance equation in Eq. 5.15 can then be transformed into Eq. 5.16 (Ntwampe, 2005).

$$\frac{dC}{dx} = -\sqrt{2\frac{r_m}{D_f}\left(C - K_m Ln\left(\frac{K_m + C}{K_m}\right)\right)} \qquad \text{Eq. 5.16}$$

Using the parameters determined from Taylor's expansion, the values of (r_m / D_f) and K_m could then be used in an ordinary differential equation solver to determine the first derivative of oxygen concentration distribution across the biofilm using Eq. 5.16. The modelled parabolas obtained were compared to the experimental data. This method had previously been used to delineate the substrate mass transfer in microbial biofilms (Nielsen *et al.*, 1990; Lewandowski & Beyenal, 2003a).

5.5 RESULTS AND DISCUSSION

5.5.1 Biofilm development and DO distribution in the immobilised biofilms

Biofilms were first noticed on the external surface of the membranes after 48 h of MGR operation. The biofilm thickness increased with time, as shown in Figure 5.5. The patterns of growth were similar to those determined in other studies of SCMGR (Ntwampe & Sheldon, 2006). Measurements of biofilm thickness obtained with the Carl Zeiss microscope were shown to be consistent across the three experiments. The biofilm thickness increased from an average of 912 µm after 72 h to 2246 µm after 264 h. From literature, the average diameter of *P. chrysosporium* pellets in submerged cultures was 2100 µm after 240 h in sealed shake-flasks (Michel *et al.*, 1992). For this study, the second decelerated growth phase occurred in the period 216 to 240 h (9 to 10 days). From literature, the secondary growth phase for *P. chrysosporium* obtained by Kirk *et al.* (1978) occurred after 10 days (mycelia cultured at 39°C, in a 125 ml flask with 21% O_2), which is in agreement with the results obtained in this study.

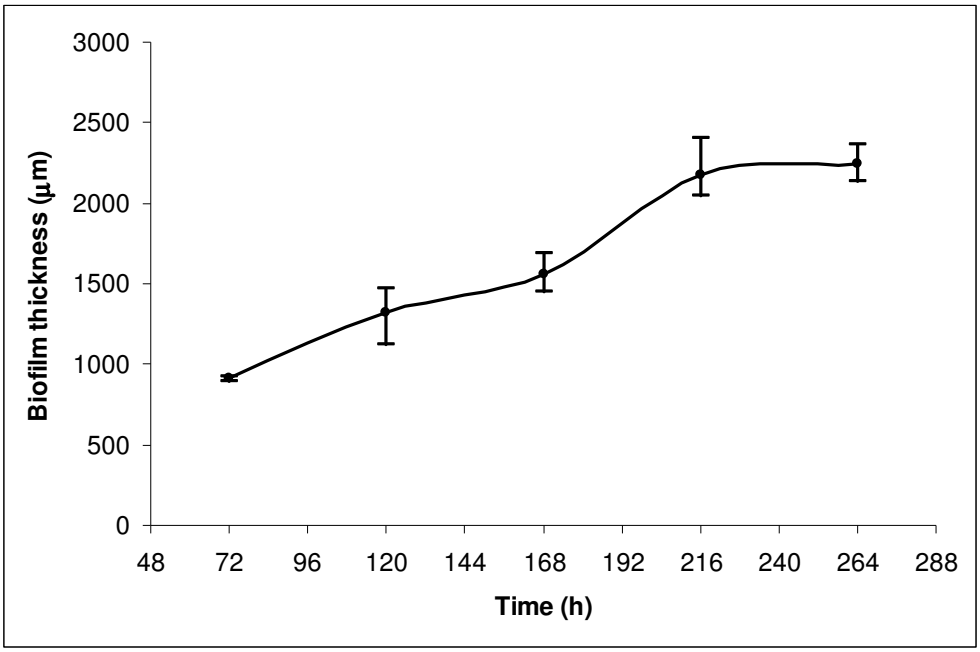

Figure 5.5: Biofilm thickness of *P. chrysosporium* grown in single-capillary MGR systems over a period of 264 h. The thickness was determined at ~2 cm above the Teflon mould from the bottom of the bioreactor systems, where the DO profiles were measured

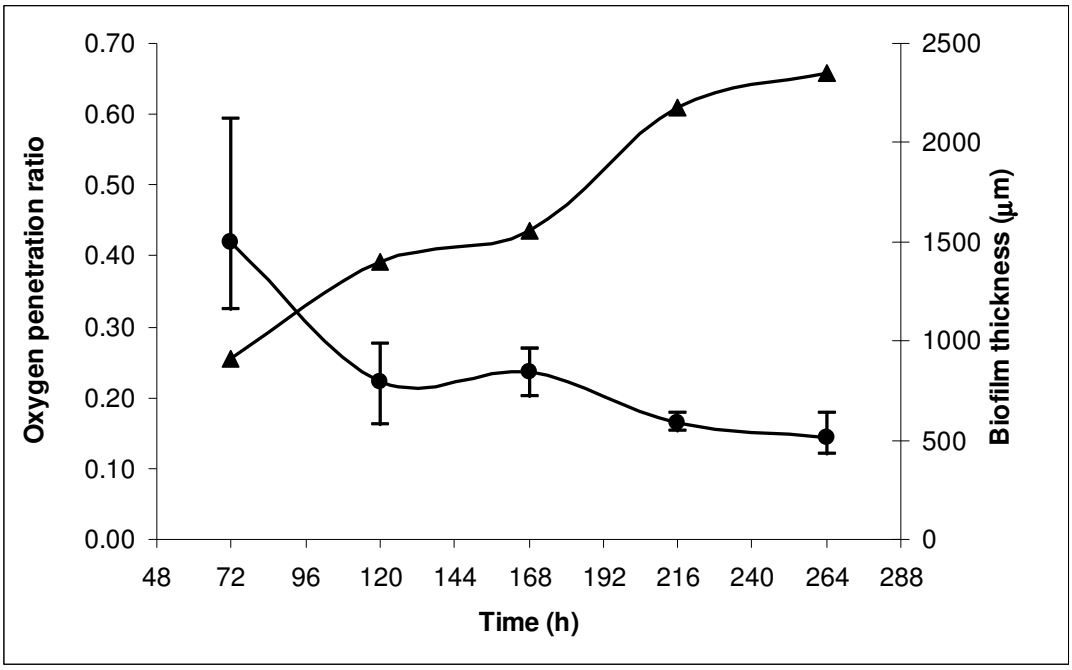

Figure 5.6: Dissolved oxygen penetration ratio determined as the ratio between DO penetration depth and biofilm thickness. Penetration ratio (-●-) over the duration of the study compared to average biofilm thickness (-▲-)

The oxygen penetration depth was determined from the measured DO profiles and the penetration ratio was calculated and compared to the biofilm thickness as shown in Figure 5.6. The DO penetration ratio decreased with an increase in biofilm thickness. The DO penetration depth was higher in younger biofilms when the biofilm thickness was at 912.10 µm after 72 h. The lowest penetration depth of 310 µm was obtained after 120 h in the immobilised biofilms. The average DO penetration depth in the biofilms of three experiments after 72 to 264 h was determined to be in the range of 306 to 530 µm. The averaged DO penetration depth obtained in this study was similar to that of Lejeune and Baron (1997), which was in the range of 310 to 390 µm, where the growth of a filamentous fungus and the DO penetration depth was simulated in three dimensions. From the DO profiles obtained in this study, significant changes in terms of DO distribution and penetration depth with biofilm age in cultures of *P. chrysosporium* immobilised in the SCMGR systems, were observed. Furthermore, similar DO penetration depth in the range of 400 to 430 µm was seen for periods of 168 to 264 h for the SCMGR biofilms. This was comparable to the

oxygen penetration depth obtained in batch cultures by Michel *et al.* (1992), in which an oxygen penetration depth of ~400 µm was achieved after 96 h.

Furthermore, DO distribution in the aerial mycelia of *P. chrysosporium* pellets was observed to have similar patterns of distribution obtained towards the centre of the pellets (Michel *et al.*, 1992). However, patterns of DO penetration depth and distribution obtained in this study were inconsistent with those observed in submerged cultures. In the SCMGRs, the penetration depth was 530 µm after 72 h, thereafter decreasing to 310 µm after 120 h. The distribution of oxygen from the bulk phase, on the shell side, through the aerial mycelia (0 to 40 µm) was similar across biofilms of different ages (See Figure 5.4). Additionally, distinguishable DO distribution in the biofilms closest to the membrane surface (vegetative mycelia) was observed. This pattern was attributed to different oxygen consumption rates as new mycelia were generated in the nutrient-rich zones near the substratum.

The distribution of DO in the biofilms was consistent with the concept of the MGR, whereby substrate gradients in the biofilms should occur. The mycelium close to the membrane was expected to be highly active, as it grew in a nutrient-rich zone. This obviously resulted in an increased consumption of DO, while the aerial mycelia were expected to show decelerated growth further away from the nutrient-rich zone and hence a limited usage of DO.

As was shown in Figure 5.6, the implications of DO penetration ratio decreases in the biofilms from an average of 0.42 after 72 h to 0.12 after 264 h are that the anaerobic areas closer to the membrane surface, where high glucose concentrations were prevalent, increased over time. This is illustrated in Figure 5.7. A DO penetration ratio of 0.22 had previously been determined in submerged cultures after 96 h (Michel *et al.*, 1992). However, a similar penetration ratio in the SCMGR was only achieved after 120 h of SCMGR operation.

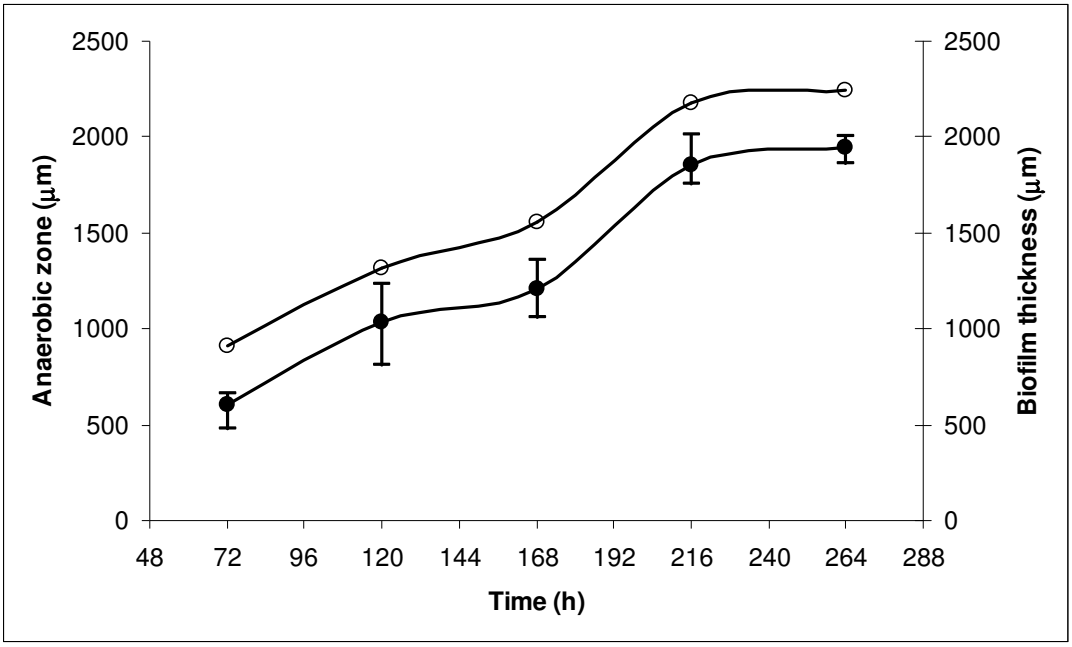

Figure 5.7: The thickness of the anaerobic zones (-●-) in immobilised biofilms of *P. chrysosporium* quantified over the 264 h period in the MGR systems as biofilm thickness (-○-) increased

The anaerobic zone thickness was measured as the difference between the biofilm thickness and the DO penetration depth. Figure 5.7 shows that anaerobic zones increased from 602 µm after 72 h to 1940 µm after 264 h of SCMGR operation. The zones were closer to the membrane, where active mycelia were expected to grow. The reasons for the decrease in DO transfer through the biofilm will be further discussed as part of Chapters 6 and 8. This was clearly identified as one of the limitations of the MGR system, as *P. chrysosporium* is known to produce ethanol under anaerobic conditions. Ethanol production was not determined during the course of this section of the study but it was quantified and discussed as part of Chapters 6 and 8.

5.5.2 Dissolved oxygen parameters

The parameters, D_f, r_m and K_m, were determined using the Taylor's expansion series. A third-order polynomial fit was used to determine the experimental constants, explained in section 5.4.3.1 and shown in Eq. 5.9. The averaged oxygen profiles from the MGR systems were used to quantify these parameters. From a graph represented by coordinates:

1) experimental DO concentration (C) and 2) oxygen penetration depth $(x - x_s)$, a third-order polynomial fit was used to determine experimental constants (a, b, c) in Eq. 5.9. The values of the experimental constants and a correlation coefficient (R^2) of 0.99, obtained from the fit, are shown in Table 5.3.

Table 5.3: Experimental coefficients obtained from the third-order polynomial fit, using the least square method with correlation coefficients obtained from the fit

Time (h)	a x 10^4	b x 10^7	c x10^{10}	R^2
72	3.19	5.93	3.76	0.99
120	4.01	6.85	0.271	0.99
168	3.25	4.61	0.518	0.99
216	3.20	5.07	1.51	0.99
264	3.64	7.01	3.91	0.99

The values that were obtained for *a*, *b*, and *c* were then used to quantify the DO mass transfer parameters, using Eq. 5.10, 5.11 and 5.12. To reproduce the average DO profiles, using Eq. 5.16, the following parameters were considered in the model: 1) the concentration of oxygen used in the model at the biofilm surface, C_s ; 2) the Monod saturation constant, K_m and 3) the ratio between the substrate consumption and oxygen diffusivity coefficient in the biofilm, r_m / D_f . The parameters that were calculated from the experimental constants are listed in Table 5.4.

Table 5.4: Analysis of modelled profiles from averaged DO profiles using parameters determined using an ordinary differential equation solver

Time (h)	C_s (g.m^{-3})	$2 r_m / D_f$ x 10^8 (g.m^{-5})	K_m (g.m^{-3})
72	5.863	3.03	0.999
120	5.950	2.77	0.041
168	5.880	1.92	0.164
216	5.699	2.25	0.398
264	5.935	3.27	0.512

Monod's saturation constants (K_m) in the range of 0.041 to 0.999 $g.m^{-3}$ were obtained in the MGR systems over 264 h. There was no clear indication of an increase or a decrease in the saturation constants obtained from the averaged DO results. From literature, values of 0.5 ± 0.3 $g.m^{-3}$ for the Monod's saturation constant (K_m) and 0.76 ± 0.1 $g.m^{-3}.h^{-1}$ for oxygen consumption were obtained by others in mycelia pellets of a submerged system (Michel *et al.*, 1992).

Table 5.5 lists the following set of mass transfer parameters: 1) oxygen consumption (r_m); 2) oxygen diffusion into the biofilm (D_f) and 3) oxygen flux across the biofilm (J_f). The consumption of oxygen (r_m) obtained in the MGR was in the order of 10^2 to 10^3 $g.m^{-3}.h^{-1}$, which was much higher than that obtained from submerged cultures. The oxygen consumption was determined to be in the range of 894.53 to 2739.70 $g.m^{-3}.h^{-1}$. Higher oxygen consumption values, above 1000 $g.m^{-3}$ were determined after 72 h, 120 h and 264 h, while values lower than 1000 $g.m^{-3}$ were obtained at 168 h and 216 h. DO diffusion coefficients in the range of 7.94E-06 to 1.750E-05 $m^2.h^{-1}$ were obtained. The effective oxygen diffusivity coefficients in the biofilms were higher than that of water (7.2E-06 $m^2.h^{-1}$) and that calculated for the nutrient feed at 39°C (1.161E-05 $m^2.h^{-1}$). A distinctive pattern was established from the DO effective diffusion coefficients obtained during this study. During decelerated growth phases (at 72 h and 264 h) in the MGR, higher diffusion coefficients were obtained as described by Ntwampe and Sheldon (2006). During other periods, oxygen diffusion coefficients obtained were much closer to 1.161E-05 $m^2.h^{-1}$. DO flux in the range 0.27 to 0.7 $g.m^{-2}.h^{-1}$ was determined in the MGR systems over the operation period of 264 h.

Table 5.5: Mass transfer parameters determined from averaged oxygen profiles

Time (h)	r_m (g.m^{-3}.h^{-1})	D_f x 10^{-5} (m^2.h^{-1})	J_f (g.m^{-2}.h^{-1})
72	1298.68	0.858	0.27
120	2423.41	1.75	0.7
168	958.78	0.998	0.32
216	894.53	0.794	0.25
264	2739.70	1.68	0.61

The hypothesis that submerged cultures can be used to model substrate transport in biofilms immobilised in the MGR system was shown to be misleading, as the patterns of substrate distribution in aerial mycelia and inner mycelia (near the membrane) as well as the substrate consumption values were found to be different from those obtained in MGR systems. Similarities were found with studies performed by Michel *et al.* (1992) in which the oxygen penetration depth, penetration ratio, mycelia growth rate and the Monod saturation constant were similar. This might be a result of biofilm density changes, thus affecting overall DO kinetic parameters determined for immobilised biofilms.

As the biofilms of *P. chrysosporium* were placed in direct contact with the gaseous phase, it was assumed that the active mycelia in the nutrient-rich zones experienced decelerated growth at certain times during a MGR operation, resulting in reduced oxygen consumption in the biofilms. It was concluded that this was the reason for higher effective diffusion coefficients. In the MGR, aerial mycelia in the MGR system were expected to be in the decelerated or stationary growth phase compared to the mycelia in the nutrient-rich zones. This meant that aerial mycelia in the MGR was expected to provide negligible growth in terms of biofilm thickness achieved when compared to the active mycelia in the nutrient-rich area. Further evidence of microbial activity was seen in the differentiated distribution of DO in the inner mycelia when compared to DO distribution in the aerial mycelia. The mass transfer parameters determined in Table 5.4 and 5.5 were used to reproduce the experimental profiles with Eq. 5.16, as shown in Figure 5.8.

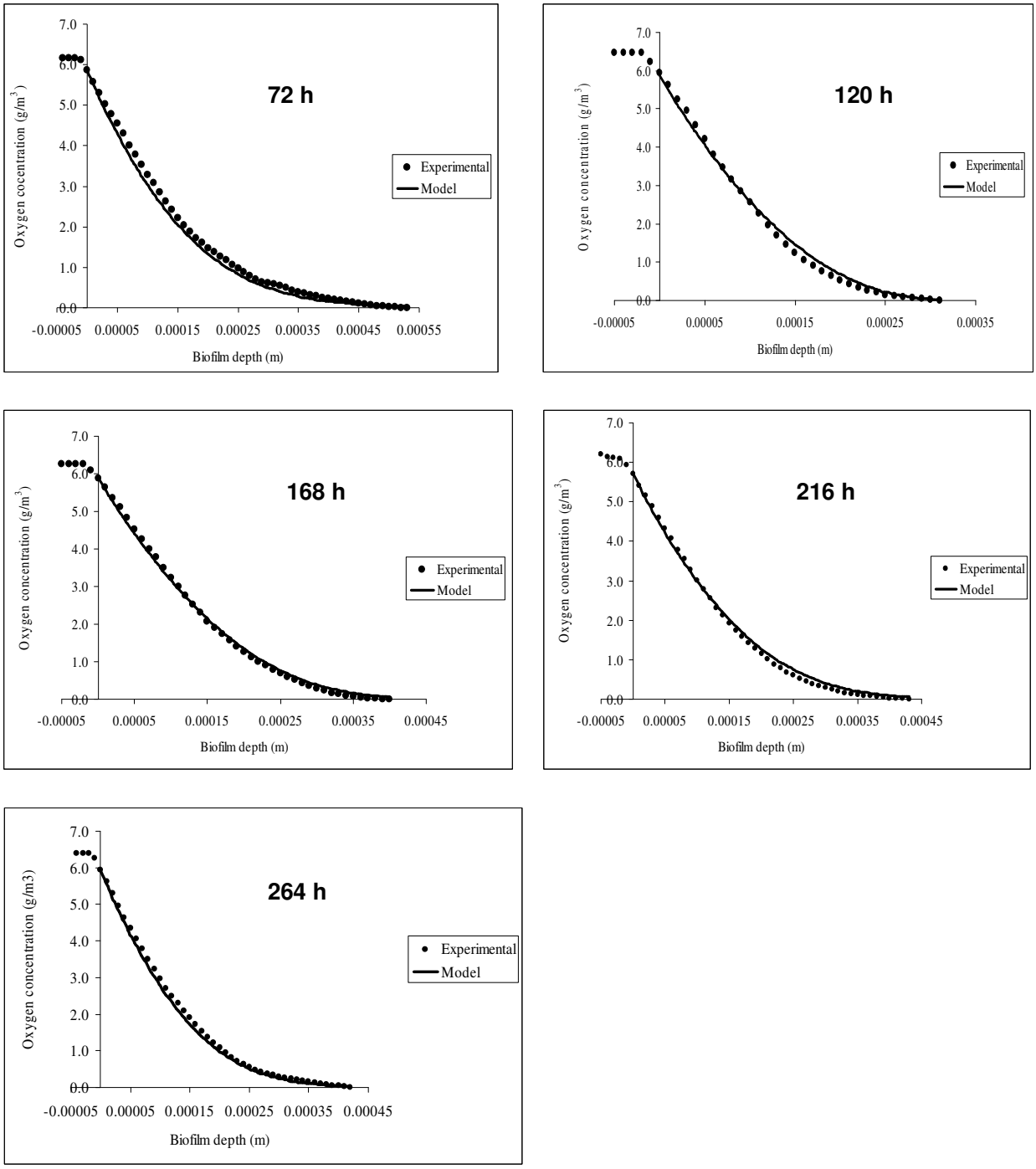

Figure 5.8: A comparison between experimental average DO profiles and modelled profiles using parameters in Tables 5.4 and 5.5

In this study, oxygen diffusivity coefficient values of up to 150% of that of oxygen in water were determined in the immobilised biofilms where aeration was directed against the biofilm

surface. Siegrist and Gujer (1985) found higher effective diffusivities, which varied from a low 40% up to 140% of the corresponding value in pure water. In this study, it was determined that high effective diffusivity: 1) increased with biofilm thickness and 2) was higher for biofilms grown on the synthetic medium than for those grown on industrial effluent. In addition, their biofilms grew on a permeable membrane and the substrate diffused across the biofilm thickness as in this study. Furthermore, it was concluded that an increase in substrate diffusion could be described by *eddy diffusion* in the inner mycelia. This means that eddy currents occurred as a result of microbial activity and growth, as the biofilms used limited substrates. Emanuelsson and Livingston (2004), and Larsen and Harremoes (1994), also reported substrate diffusion coefficients greater that 100% using a synthetic medium, and this was attributed to eddy flow or currents in the biofilms.

5.5.3 Practical uses of the findings

Parameters determined during this part of the study can be used to monitor and estimate the DO distribution during MGR operation, as the MGR is used to immobilise aerobic microbes to produce low-volume, high-value bioproducts. Anaerobic zones were evident in the immobilised biofilms, and therefore other aeration techniques, such as the use of technical-grade oxygen (~100%) or DO carriers need to be investigated to reduce these zones in the biofilms. Production of unwanted by-products in the MGR, which deactivates high-value bioproducts should also be investigated in future studies.

5.6 CONCLUSIONS

The methods used in this study provided a clear understanding of oxygen mass transfer in *P. chrysosporium* biofilms grown in continuous vertical SCMGRs over a period of 264 h. Oxygen transfer parameters in the MGR biofilms were found to differ from those obtained in submerged pellets. It was found that:

- The biofilm thickness of immobilised *P. chrysosporium* increased during the bioreactor operation reaching 2246 µm after 264 h in the SCMGR systems.
- The DO penetration ratio in the immobilised biofilms decreased with an increase in anaerobic zone thickness in the biofilms, increasing from ~66% to ~86% of biofilm thickness after 72 h and 264 h respectively, showing DO transport difficulty in older biofilms.
- High diffusion coefficients attributed to *eddy diffusion* were associated with higher oxygen consumption and flux into the biofilms.
- Oxygen distribution in aerial mycelia was determined to be similar, with varying distribution in the mycelia closer to the substratum of the bioreactor system due to oxygen consumption and active transport of DO to sustain inner mycelia growth.
- Results on biofilm thickness, oxygen penetration, oxygen penetration ratio and Monod saturation constants were found to be similar to those obtained in submerged mycelia pellets of *P. chrysosporium*, with higher oxygen consumption values achieved in biofilms immobilised in the MGR.
- Oxygen diffusion coefficients in the range of 7.94E-06 to 1.750E-05 $m^2.h^{-1}$ and DO flux values of 0.27 to 0.7 $g.m^{-2}.h^{-1}$ were determined during the operation of the SCMGR systems.

5.7 SUMMARY

A major finding in this chapter, related to the application of aeration in MGR-based immobilised cultures of *P. chrysosporium*, was the formation of anaerobic zones where DO penetration achieved was less than 600 µm. The following chapter (Chapter 6) investigates the limitations related to anaerobic zone formation and the consequence of oxygenation as a possible solution. Furthermore, trace metal accumulation and glucan storage were investigated. This is related to the following objectives:

- Determining the ethanol production due to oxygen limitations in the MGR system.

- Determining levels of oxidative stress when higher partial pressures of oxygen are used to flush the ECS to improve LiP and MnP production in the MGRs.

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CHAPTER 6

RESULTS

Limitations of a membrane gradostat bioreactor designed for enzyme production by biofilms of *Phanerochaete chrysosporium*

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CHAPTER 6

Limitations of a membrane gradostat bioreactor designed for enzyme production by biofilms of *Phanerochaete chrysosporium*

Abstract

Growing interest has been shown in the continuous production of high-value products such as extracellular secondary metabolites used in the biotechnology, bioremediation and pharmaceutical industries. These high-value extracellular secondary metabolites are traditionally produced in submerged fermentations. However, the application of continuous membrane bioreactors (MBRs) has been determined to be a highly productive alternative. A novel MBR, classified as a membrane gradostat reactor (MGR), has previously been used to immobilise biofilms on an externally unskinned and internally skinned membrane to continuously produce extracellular secondary metabolites. However, anaerobic zones were previously identified in the MGR system when air was used for aeration; these are potentially detrimental to large-scale secondary metabolite production. To improve the MGR system, this study set out to determine the limitations related to the performance of the MGR bioreactor using *Phanerochaete chrysosporium* immobilised in single and multi-capillary MGRs (SCMGRs and MCMGRs). A DO penetration depth of ± 450 µm was measured after 264 h, with the anaerobic zone thickness reaching ~1943 µm in the immobilised biofilms. The penetration ratio, decreased from 0.42 after 72 h to 0.14 after 264 h. This led to the production of ethanol in the range of 10 to 56 $mg.L^{-1}$ in the MCMGR and 7 to 54 $mg.L^{-1}$ in SCMGR systems. Ethanol production could potentially be attributed to the increase in β-glucan production within immobilised *P. chrysosporium* biofilms where an oxygen-enriched aeration source was used. Furthermore, increased lipid peroxidation and trace-element accumulation was observed with the use of an oxygen-enriched source.

Keywords: Biofilms; Lignin peroxidase; Manganese peroxidase; Membrane bioreactor; *Phanerochaete chrysosporium*

6.1 INTRODUCTION

A white-rot fungus, *Phanerochaete chrysosporium* has been identified as having the potential to degrade various compounds found in industrial effluents. It produces extracellular enzymes, manganese peroxidase (MnP) and lignin peroxidase (LiP), that can degrade aromatic compounds and remove colour compounds from effluents. In recent publications, the fungus has been used: 1) as a biosorbant for removing 2,4 dichlorophenol (2,4-DCP) (Wu & Yu, 2007); 2) to degrade azo dye *in-vitro* using its extracellular enzymes (Yu *et al.*, 2006) and 3) to degrade a middle fraction diesel fuel residue (Kanuly & Hur, 2006).

As a possible solution to continuously producing LiP and MnP, various researchers have shown that it is possible to immobilise biofilms of *P. chrysosporium* on capillary membrane surfaces. In the membrane bioreactor (MBR) setup, a section of the biofilm is maintained in the idiophase for continuous enzyme production (Sheldon & Small, 2005; Govender *et al.*, 2003; Leukes *et al.*, 1999; Venkatadri & Irvine, 1993). When a fixed-film is immobilised on the external surface of a membrane and nutrients are supplied through the lumen of the membrane, nutritional gradients form within the biofilm. When air or oxygen comes in direct contact with the external surface of the biofilm, radial dissolved oxygen (DO) diffusion occurs across the immobilised biofilm structure towards the membrane. This forms a bi-directional and radial flow of essential nutrients, thus forming a gradostat (Lovitt & Wimpenny, 1981a; Lovitt & Wimpenny, 1981b). The biomass closest to the membrane has access to high nutrient concentrations, supporting primary growth, while the biomass furthest away from the membrane surface is starved of nutrients, thus undergoing secondary metabolism. This was proposed as an advance in the continuous production of enzymes, and the bioreactors were classified as membrane gradostat reactors (MGRs) (Leukes, 1999). Figure 6.1 shows a representation of the MGR concept, illustrated with a *P. chrysosporium* biofilm immobilised

on the external surface of a polysulphone membrane (Figure 6.1A), with a superimposed substrate concentration profile (Figures 6.1B and 6.1C).

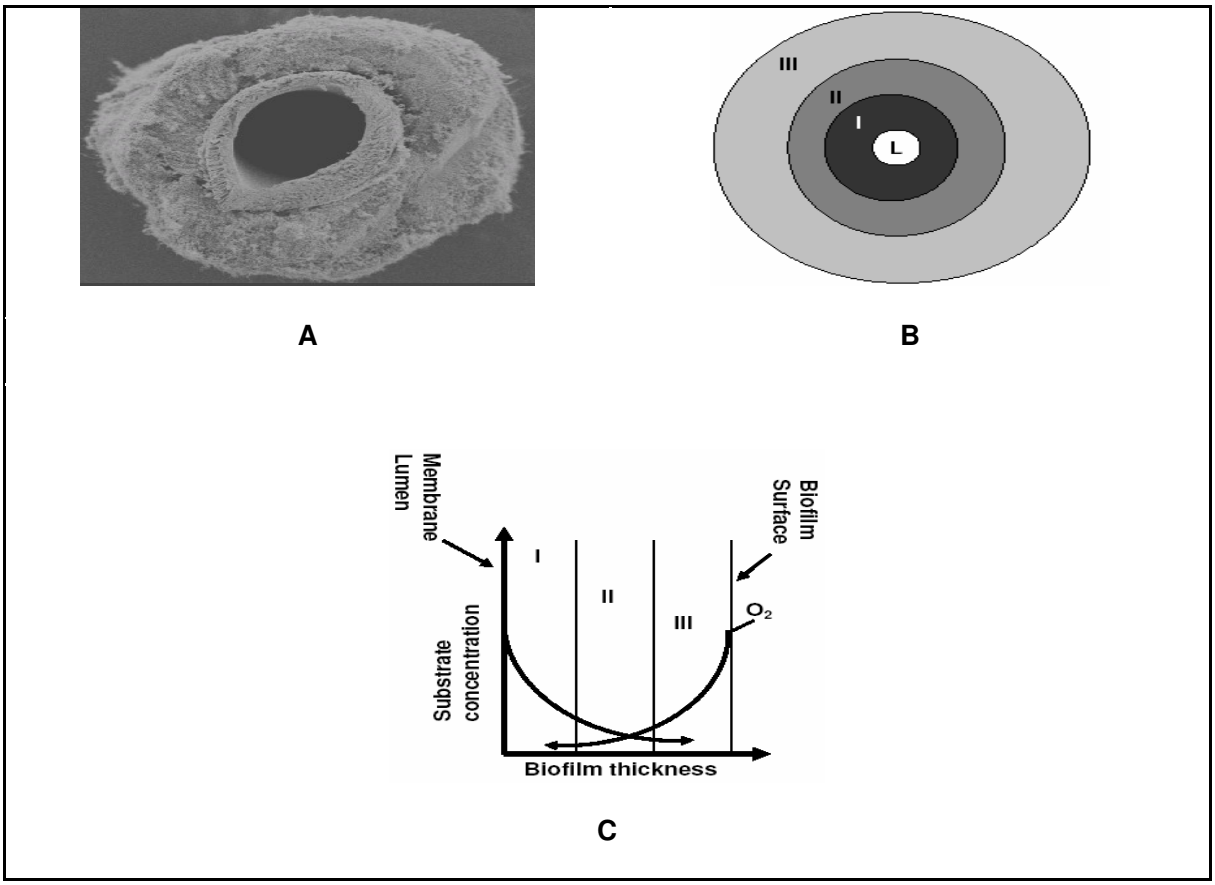

Figure 6.1: (A) *P. chrysosporium* biofilm immobilised on the external surface of a polysulphone membrane. (B) Schematic representation of the membrane gradostat concept; I is the primary growth phase; II is the stationary growth phase; III is the decline phase; L- Lumen of the capillary membrane from which the nutrients are supplied. (C) Illustration of substrate concentration distribution and flow at different biofilm thicknesses. Nutrient medium convection flow is opposite to the direction of dissolved oxygen diffusion, supplied on the shell side of the membrane gradostat bioreactor

As a potential new tool for secondary metabolite production, the MGR mimics the natural environment of microbial cultures in their natural habitat better, compared to submerged batch cultures. *P. chrysosporium* as well as the shortcomings related to enzyme productivity in batch culture systems have been studied extensively. However, not much research has been conducted using continuous fixed-film reactor systems, such as the SCMGR and MCMGR systems described in this study.

It was shown by Michel *et al.* (1992) that biofilms of *P. chrysosporium* have poor DO transport capabilities. *P. chrysosporium* was shown to produce ethanol from glucose when grown in conditions of limited DO (Kenealy & Dietrich, 2004). In preliminary MGR studies, DO profiles using a Clark oxygen microsensor showed that biofilms closer to the capillary membranes had limited oxygen levels (Ntwampe *et al*., 2008a) in an area with a high glucose concentration, conditions that favour ethanol production. However, the determination of actual ethanol production under such conditions has not previously been determined. *P. chrysosporium* enzymes were observed to be deactivated by alcohols (mainly ethanol) and LiP was more sensitive to ethanol than MnP, as it generally increased the rate of enzyme deactivation (Field *et al*., 1996).

Leisola *et al.* (1982) suggested that DO transport limitations in *P. chrysosporium* biofilms could be caused by excess exopolysaccharide production, identified as mucilaginous material made of β-glucans (Buchala & Leisola, 1987) as a result of the availability of excess glucose in cultures. The fungus produces the polysaccharide as a storage carbohydrate in order to have nutritional reserves available that can be used under nutrient-limiting conditions (Bes *et al*., 1987). Furthermore, the use of technical-grade oxygen (~100%) in *P. chrysosporium* biofilms improved extracellular enzyme production only for limited periods. The use of pure oxygen was ineffective as the DO penetration depth was less than 1000 µm in mycelial pellets of the fungus even under cultures incubated under 100% O_2 at 1 atm. (Leisola *et al*., 1983). These results showed that polysaccharide production limited DO transport in immobilised biofilms of *P. chrysosporium*. Similarly, it was shown that DO penetration in *P. chrysosporium* pellets, equilibrated in an air saturated culture fluid, was ~400 µm (Michel *et al*., 1992). The growth of *P. chrysosporium* was determined to be better in air, while it was also determined that 40 to 60% oxygen is superior to 100% oxygen for lignin metabolism (Kirk & Fenn, 1982). High partial oxygen pressures in the cell culture medium can lead to hyperoxia and lipid peroxidation from increased generation of free radicals (Li *et al*., 2004). The peroxidation of lipids in fixed-film bioreactors, where the biofilm

is directly exposed to the aeration source in the extracapillary space (ECS) and under lower oxygen partial pressures (~50%), has not previously been quantified and will be investigated in this part of the study.

Cultures of *P. chrysosporium* BKMF-1767 require trace amounts of essential trace metals such as Cu, Mn and Fe for its growth (Kirk *et al.*, 1978), but these metals are toxic when present in excessive amounts. The fungus has the capacity to adsorb and accumulate metal ions, including Cd, Pb, Ni, and Co (Baldrian, 2003; Falih, 1997). In earlier studies, the ionic strength of the basal medium used by Tien and Kirk (1988) was increased by 100-fold from that used by Kirk *et al.* (1978). However, varying the ionic strength of the basal medium from 10- to 100-fold did not affect the onset, rate or extent of lignin decomposition (Kirk *et al.*, 1978). A higher ionic strength basal medium can result in the accumulation of metals in continuous fixed-film systems, while some of the complexes of Cu and Fe enhance oxygen radical damage (Baldrian, 2003). Metal accumulation can also cause the oxidative stress of proteins by the induction of oxidative stress associated with the production of reactive oxygen species (ROS) (Stohs & Bagchi, 1995). When the nutrient medium was designed, batch bioreactors were the preferred choice for ligninase production. The risk of metal accumulation in the MGR supplied with the growth medium containing 10- and 100-fold basal medium in the nutrient solution was investigated in this part of the study. While complexes of Cu and Fe enhance oxygen radical damage, Mn in various forms inhibits ROS damage (Cheton & Archibald, 1988). Concentrations of Mn above 50 µM prevent chemical-supported lipid peroxidation measured by the production of malondialdehyde (MDA) (Tampo & Yonaha, 1992).

6.2 OBJECTIVES

The objectives for this part of the study were to monitor ethanol production caused by limited DO transport during the MGR operation, while also monitoring the production of glucan in the biofilms. The formation of MDA in the biofilms was also quantified to evaluate oxidative

stress in the biofilms using aeration and oxygenation in the bioreactor. The accumulation of Cu and Mn in the biofilms was evaluated with the variation of basal medium composition in the nutrient medium. This was carried out to determine the risk of metal accumulation during prolonged bioreactor operation. As the MGR is under development as a possible replacement for batch bioreactors and as an alternative reactor for the continuous production of high-value extracellular secondary metabolites, limitations identified thus far have to be addressed in order to allow for its use in the biotechnology, wastewater and pharmaceutical industries.

6.3 MATERIALS AND METHODS

6.3.1 Microbial strain and culture conditions

P. chrysosporium strain BKMF-1767 (ATCC 24725) was used to culture biofilms at 39°C in single- and multi-capillary MGRs (SCMGRs and MCMGRs), using a spore inoculum prepared according to the method described by Tien and Kirk (1988). The nutrient medium used was the standard medium for *P. chrysosporium,* as previously described by Tien and Kirk (1988). The nutrient medium classified as nitrogen-limited, contained 10 $g.L^{-1}$ of glucose and 200 $mg.L^{-1}$ ammonium tartrate (~39 $mg.L^{-1}$ NH_4^+). The volume of the basal medium component of the nutrient medium varied from 10 to 100-fold using 10 ml and 100 ml of the solution during the preparation of the nutrient medium. This was carried out to evaluate the Mn and Cu accumulation in the immobilised biofilms during MGR operations when the ionic strength of the basal medium was varied. Refer to Appendices A and B for spore solution and nutrient medium preparation.

6.3.2 Polysulphone membranes

The externally unskinned polysulphone membranes provided a unique substructure matrix within which a fungus of a filamentous nature could be immobilised. The substructure

contained closely-packed, narrow-bore microvoids that extended all the way from just below the skin layer to the membrane periphery. The polysulphone membranes used in this study were manufactured by the Institute of Polymer Science (Stellenbosch University, R.S.A.) using a wet-phase inversion process according to the fabrication protocol of Jacobs and Leukes (1996). SEM photographs of the polysulphone used in this study are shown in Figures 6.2A and 6.2B.

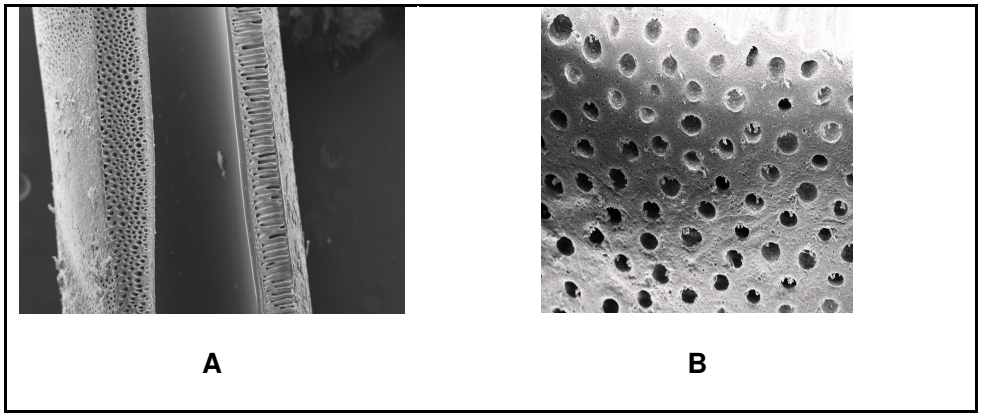

Figure 6.2: (A) SEM of a longitudinal section of the polysulphone membrane. (B) The external surface of the polysulphone membrane showing cavities in which conidia or mycelia can be immobilised. The polysulphone membrane's outer diameter is ~1.7 to 1.9 mm

An important feature of the polysulphone membrane was the regularity of the microvoids present in the substructure and the complete absence of an external layer, which allowed the microvoids to be inoculated with fungal spores through reverse filtration. These membranes were found to be well-suited for fungal immobilisation (Ryan *et al.*, 1998).

6.3.3 Bioreactor setup and operation

Bioreactor sterilisation: All the materials (tubing, bioreactor and flasks) were autoclaved at 121℃ for 20 minutes before use. Sterile deionised water was used in the humidifiers and in the nutrient-medium make-up. After assembling the MGR modules, the bioreactors were further chemically sterilised using a 4% (v/v) formaldehyde solution followed by rinsing with sterile distilled water before inoculation.

Preparation of spore solution and immobilisation: An estimated 3 X 10^6 spores per SCMGR and 45 X 10^6 spores per MCMGR were used by measuring the spore concentration using a spectrophotometer at 650 nm, where the absorbency of 1 is equivalent to 5 million spores per ml (Tien & Kirk, 1988). The fungal spores were inoculated through the permeate port and were forced onto the membrane using reverse filtration as explained in Govender *et al.* (2004).

Single and multicapillary membrane gradostat reactor design and operation: Multiple SCMGR systems with working volumes of 20.4 ml each and an active membrane length of 160 mm were used to cultivate *P. chrysosporium* biofilms. For the MCMGR, the working volume was 622 ml, while an active membrane length of 210 mm was used. These bioreactors were positioned vertically, as shown in Figure 6.3. The SCMGR and MCMGR systems were developed with dimensions as indicated in Table 6.1.

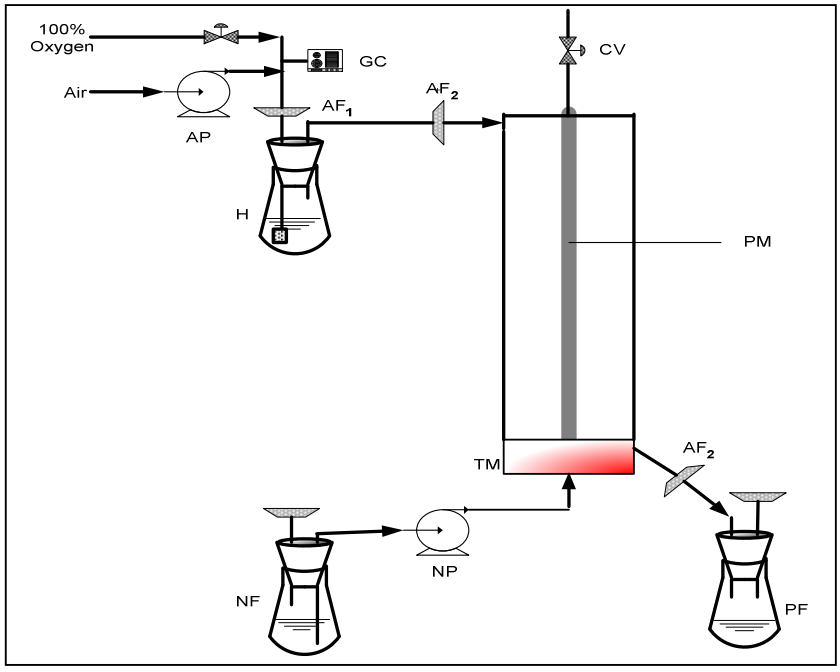

Figure 6.3: Schematic illustration of a single capillary membrane gradostat reactor (SCMGR) set-up. AF_1/AF_2: air filter; AP: air pump; CV: control valve; H: humidifier; NF: nutrient flask; NP: nutrient pump; PF: permeate flask (collection/sampling point); PM: polysulphone membrane; TM: Teflon mould; GC: gas controller. A similar bioreactor set-up was used for multicapillary membrane gradostat reactor (MCMGR)

Table 6.1: Dimensions of vertically orientated SCMGR and MCMGR systems

Parameter	Details	
	SCMGR	**MCMGR**
Module housing	Glass	PVC
Module length	230 mm	220 mm
Module diameter	12 mm	60 mm
Active internal volume	~20.4 ml	~622 ml
Nutrient capsule length	-	67 mm
Nutrient capsule volume	-	~189.4 ml
Active membrane length	160 mm	210 mm
Number of membranes	1	15
Pitch	-	Triangular pitch
Membrane spacing	-	6 mm
Membrane surface area – Polysulphone	985 mm^2	~18 800 mm^2

A Teflon mould was used at the bottom of each SCMGR system in order for the permeate to exit the bioreactors without accumulating at the bottom of the bioreactors. Fifteen SCMGRs were operated simultaneously. Three SCMGR systems were disconnected simultaneously at time intervals of 72, 120, 168, 216 and 264 h, in order to determine different biofilm-associated parameters, such as DO distribution penetration, biofilm thickness, oxidative stress and metal accumulation over time. Permeate samples were also collected daily from different bioreactor systems for analysis of ethanol production in SCMGRs (40 $ml.day^{-1}$) and MCMGRs (300 $ml.day^{-1}$). The bioreactors were operated vertically (SCMGR and MCMGR), because in horizontally placed bioreactors the polysulphone membranes sagged due to biofilm accumulation, while droplet formation on the biofilm caused the biofilm to detach and grow on the bioreactor walls.

The MCMGR systems were used to monitor ethanol production in a larger system when air was used in the ECS. The shell and nutrient capsules in the MCMGR encased the membrane cartridge. The membrane cartridge consisted of an epoxy mould, to which the membranes were attached. The nutrient medium was supplied from the bottom capsule

throughout the membrane to the top capsule. Air was supplied directly into the ECS of the bioreactors and exited at the permeate port at the bottom of the bioreactor. In the MCMGR design, provisions were made for the shape of the bottom side mould to ensure that there was no liquid build-up in the bioreactor that could affect growth at the bottom of the bioreactor. An assembled MCMGR system is shown in Figure 6.4.

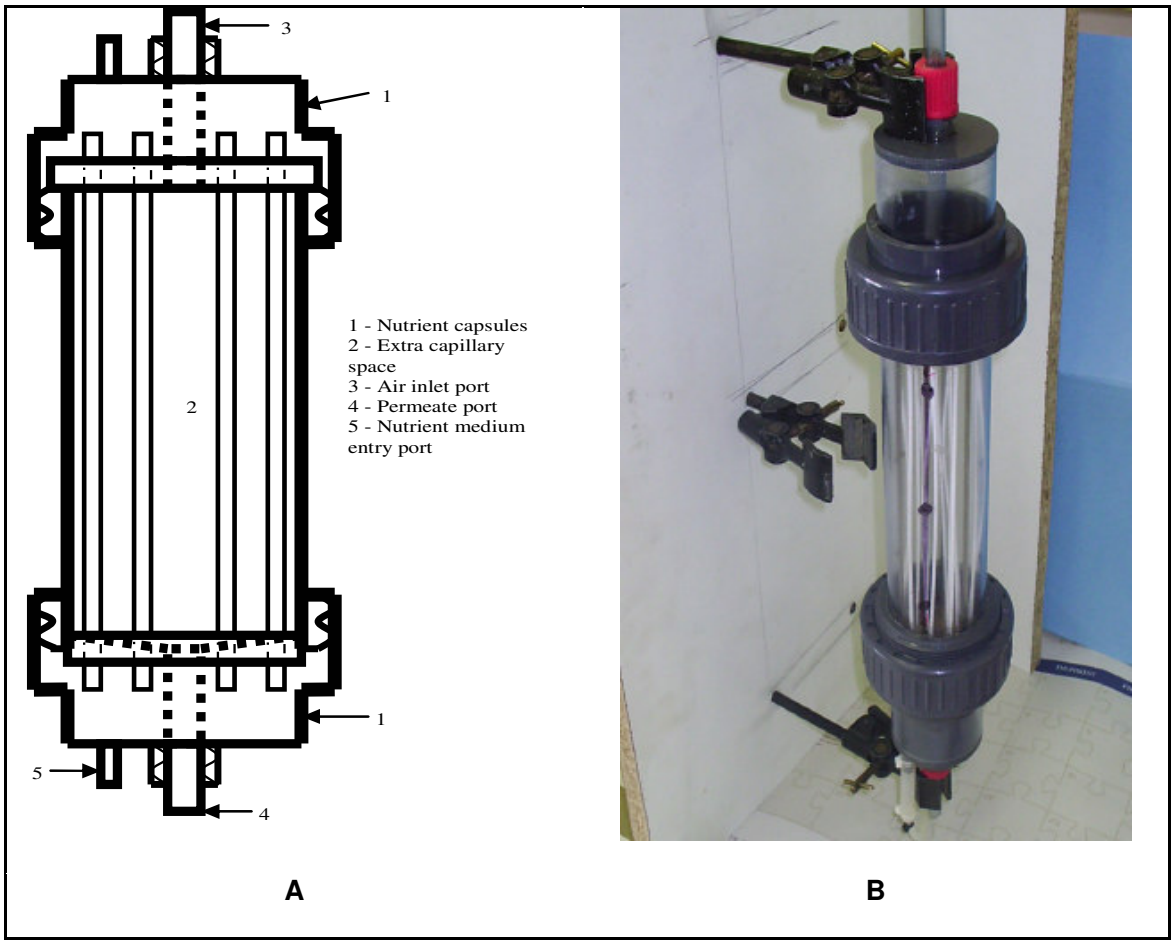

Figure 6.4: (A) Schematic diagram and (B) a photograph of the complete MCMGR assembly operated in a vertical position

In the SCMGR systems, the nutrient medium was pumped to each reactor from individual nutrient flasks at a rate of 1.67 $ml.h^{-1}$ through the lumen of the polysulphone membrane by using a multi-channel Watson-Marlow 505S pump (Germany). In the MCMGR systems, the nutrient pumping rate was adjusted to 12.5 $ml.h^{-1}$. All experiments were run in the dead-end filtration mode. Air or a combination of air and oxygen (~50% air-O_2 v/v), was supplied to

SCMGR systems at a rate of 100 ml.min^{-1} to study oxidative stress, while others were supplied with only air at a flow rate of 100 ml.min^{-1}. Multi-port air pumps with flow control and an oxygen gas controller were used to ensure that the flow was consistent throughout all the bioreactor modules. The airflow rate was 622 ml.min^{-1} for the MCMGR system. Air and oxygen supplied to the bioreactor systems were filter-sterilised (using a 0.22 μm, acetate, non-pyrogenic filter). Both air and oxygen were humidified before being passed through the ECS of the bioreactors.

6.4 ANALYTICAL METHODS

6.4.1 Determination of DO penetration depth and biofilm thickness

The SCMGR systems were dismantled by removing the glass manifold and exposing the membrane-attached biofilm for easy microsensor measurement. The SCMGRs were disconnected in triplicate at time intervals of 72, 120, 168, 216 and 264 h. A Clark-type oxygen microsensor (OX 10, outer-tip diameter less than 20 μm), supplied by Unisense (Denmark), was used to measure the DO across the biofilms at interval depths of 10 μm. The setup consisted of a high-sensitivity picoammeter connected to the microsensor, which was fixed to a micromanipulator used to move the microsensor into the biofilm. The picoammeter was connected to a computer loaded with *Profix v1.0* software for data capturing. The microsensors were used to measure DO concentration in the immobilised biofilms in triplicate. Refer to Appendix G for microsensor setup and calibration. Although biofilms in the SCMGRs were of a similar thickness across the active membrane length, an area closer to the bottom of the bioreactor (~2 cm above the Teflon mould), was chosen as suitable for measurement of the DO profiles. Biofilm thicknesses were determined at this point of measurement. The biofilm thickness was determined by using a Carl Zeiss light-microscope and Axiovision digital imaging system equipped with measuring software. The objective used for the measurements was a 2.5x magnification. The calibrated microscope objectives acquired real-size measurements from biofilms attached to the polysulphone membrane

surface. The biofilm thickness was calculated by subtracting the clean membrane's outer diameter from the membranes with biofilm growth and then dividing by two. The values obtained were averaged to establish actual biofilm thickness. The penetration ratio was calculated by dividing the oxygen penetration depth by the biofilm thickness.

6.4.2 Preservation of biofilms from further oxidation

After DO measurements, a section of the biofilm was surgically separated and rinsed in a 5 mM butylated hydroxyl toluene (BHT) solution supplemented with a 20 mM hydroxymethyl amino methane–hydrochloride (Tris-HCl), before storing at -80℃ in the Tris-HCl solution. This was done to prevent new lipid peroxidation during biofilm storage. This solution was also used in the homogenisation and biomass lysis process for MDA and glucan determinations.

6.4.3 Biofilm drying

After the DO oxygen measurements were taken, the biofilm was detached from the membrane and was rinsed in distilled water before drying in an oven at 30℃ for a period of 24 h to remove any nutrient solution and excess water. The dried biofilm samples were used to determine metal accumulation.

6.4.4 Accumulation of trace elements in biofilms

Dried fungal biomass of 0.014 to 0.117 g was weighed into digestion vessels and decomposed in a mixture of 4 ml of a 65% HNO_3 solution and 1 ml of a 30% H_2O_2 solution (Čurdová *et al.*, 2004). Digestion was accomplished using the following microwave program: 7 minutes at 120 W, 2 minutes at 460 W and 1 minute at 700 W. The samples were cooled for 1 h and the digests were filtered using glass wool and a 0.22 µm filter. The volume was made up to 30 ml with deionised water. The digests were analysed at the University of

Stellenbosch (R.S.A), using an ICP-MS for the determination of copper (Cu) and manganese (Mn) in the digested filtrate. Negative control samples contained only solutions of HNO_3 and H_2O_2.

6.4.5 Ethanol production

The ethanol concentration was determined by using a Roche® ethanol test kit. The overall ethanol concentration produced was determined daily by determining the ethanol concentration in the permeate samples for each SCMGR and MCMGR system for 264 h. The ethanol production was measured at different stages of biofilm development, both with and without the use of a higher oxygen concentration (~50% v/v air and oxygen) on the ECS. As ethanol can also be produced by contaminants, mostly yeast, contaminants in the permeate samples were monitored through microscopic observation. Refer to Appendix D for the complete assay protocol.

6.4.6 Measurement of oxidative stress (Malondialdehyde formation)

Lipid peroxidation is a well-established mechanism of cellular injury. Lipid peroxidation intermediates and end-products derived from polyunsaturated fatty acids are unstable and decompose to form a complex series of compounds including malondialdehyde (MDA). The formation of MDA was determined with an MDA assay kit (Northwest Life Sciences Specialties, Vancouver, WA) following the manufacturer's directions (See Appendix E). Preserved biofilms stored at -80℃ were homogenised with glass beads in 2 ml Eppendorf tubes using a vortex mixer until the mixture was well homogenised. The biofilm homogenates were centrifuged at 10 000 x g for 10 minutes to obtain a clear supernatant before being used in the assay. The MDA in homogenates was determined by following the assay protocol by absorption at 532 nm.

After the DO measurements, a section of the biofilm was surgically separated and rinsed in a 5 mM butylated hydroxyl toluene (BHT) solution supplemented with a 20 mM hydroxymethyl amino methane–hydrochloride (Tris-HCl), before storing at -80℃ in the Tris-HCl solution. This was done to prevent new lipid peroxidation during biofilm storage. This solution was also used in the homogenisation and biomass lysis process for MDA and glucan determinations. See Appendix F for the preparation of the solutions used for antioxidation preservation.

6.4.7 Glucan determination

Glucan concentration in homogenates was measured using a mixed-linkage β-Glucan assay kit purchased from Megazyme (Ireland). The supernatant used for MDA determination was also used for glucan determination. The concentration of β-glucans in homogenates was determined by following the assay protocol by absorption at 510 nm using 100 µl of the supernatant. Refer to Appendix E for the complete assay protocol.

6.5 RESULTS AND DISCUSSION

6.5.1 DO penetration depth and anaerobic zone formation

DO limitation was shown to be the main reason for slow, incomplete degradation of lignin in non-agitated cultures (Leisola *et al.*, 1983), while in other studies, the limitation of oxygen resulted in the formation of ethanol by *P. chrysosporium* grown on glucose (Kenealy & Dietrich, 2004). Although it was determined that thinner biofilms were more efficient for LiP production (Venkatadri *et al.*, 1992), the development of biofilms with sufficient thickness is a prerequisite for the fixed-film MGR concept. This is required for the establishment of sufficient nutritional gradients in order to maintain parts of the biofilm in the idiophase. However, an increase in biofilm thickness in the MGR resulted in the formation of anaerobic

zones in SCMGR systems, as seen in Figure 6.5 and as previously demonstrated in Chapter 5.

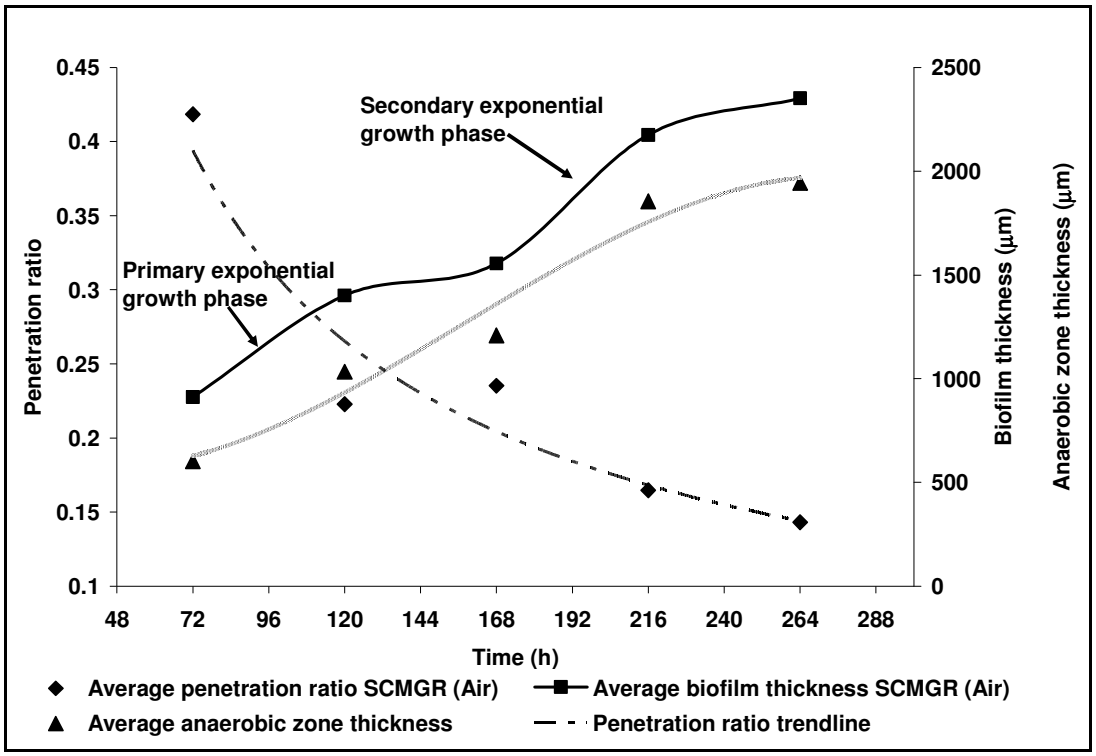

Figure 6.5: A relation between DO penetration ratio, anaerobic zone thickness and biofilm thickness determined in aerated SCMGR systems

Anaerobic zones were classified as areas of the biofilm where the DO concentration was <0.001 $g.m^{-3}$, measured using a DO microsensor. Figure 6.5 shows that the anaerobic zone thickness increased with increasing biofilm thickness, reaching an average of 1943 µm when the average biofilm thickness was 2352 µm after 264 h. This resulted in a decrease in the DO penetration ratio, which is the ratio between DO penetration depth and biofilm thickness. Thinner biofilms (600 µm, obtained after 72 h of bioreactor operation) indicated higher DO penetration (540 µm) compared to thicker biofilms (Ntwampe *et al.*, 2008). A DO penetration depth averaging at 400 µm was determined in biofilms after 168 h, which had also been also observed by Michel *et al.* (1992) in batch systems. This indicated poor DO mass transport in the immobilised biofilms in the MGR systems. Leisola *et al.* (1982) suggested that DO limitations were caused by excessive polysaccharides production (Buchala & Leisola, 1987).

6.5.2 Ethanol production

Further investigation into the relationship between the formation of anaerobic zones in immobilised biofilms and ethanol production was done by simply measuring the ethanol production in permeate samples collected daily from the SCMGR and MCMGR systems. The ECS on the SCMGR was supplied with air (21% O_2 v/v) as well as 50% O_2 (v/v), while only aeration (21% O_2 v/v) was used for the MCMGR, as shown in Figure 6.6.

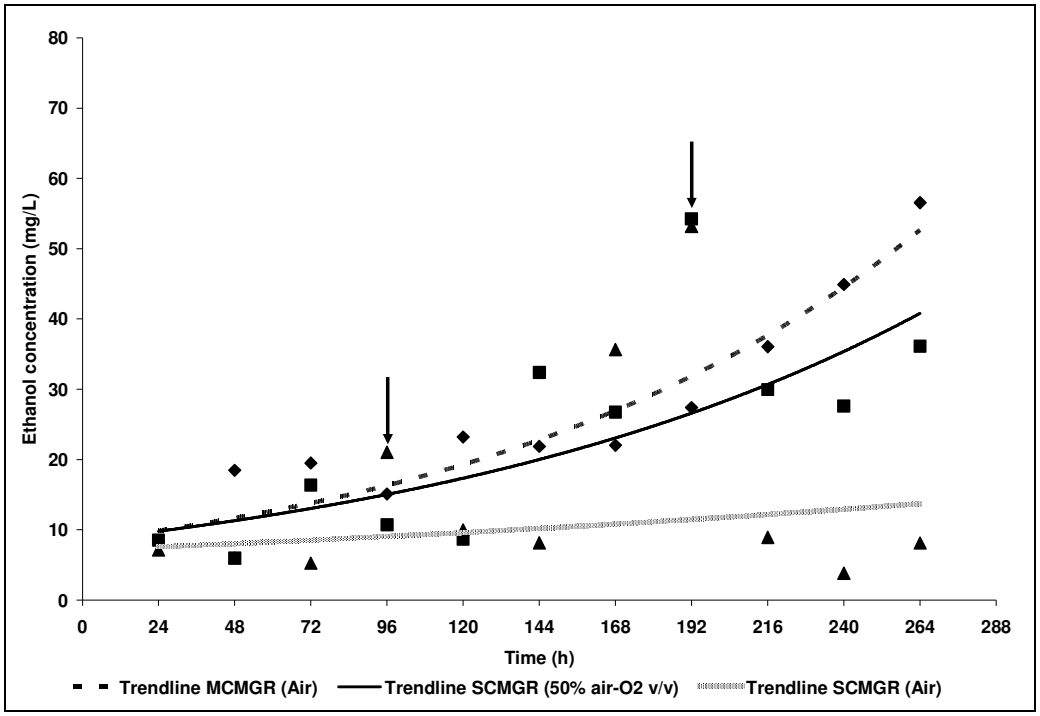

Figure 6.6: Trendline representation of ethanol production from immobilised *P. chrysosporium* in SCMGRs and MCMGRs as a result of DO limitations for aerated and oxygenated (50% v/v air:O_2) bioreactor systems. The arrows indicate periods were biomass development in the MGR was in the deceleration phase

Ethanol production in the MGR systems varied during the period under investigation. The production of ethanol (Figure 6.6) was higher in aerated biofilms in the MCMGR systems compared to biofilms in the SCMGR that were aerated (21% O_2 v/v) and oxygenated (~50% v/v air:O_2). Ethanol production was the lowest in cultures grown in aerated SCMGR systems. Higher ethanol production was observed in the SCMGR systems during the period of 96 to 192 h, which was observed as a period of decelerated *P. chrysosporium* growth and where a minimal increase in mycelial thickness was observed. During this period, ethanol

concentration peaked at >50 $mg.L^{-1}$ in SCMGRs, as shown in Figure 6.6. Surprisingly, in oxygenated SCMGR systems, higher ethanol production compared to aerated SCMGRs was observed, which was not significantly different from that obtained in the MCMGRs. It was postulated that the higher ethanol production was caused by the formation of glucan because of oxidative stress.

6.5.3 Oxidative stress (Malondialdehyde formation)

MDA formation was measured in the homogenates obtained from aerated and oxygenated biofilms from SCMGRs and the results are shown in Figure 6.7. The formation of MDA was higher (14 to 34 μM $MDA.ml^{-1}$ of BHT homogenates) in oxygen-enriched *P. chrysosporium* biofilms compared to aerated systems (4 to 12 μM $MDA.ml^{-1}$ of biomass extracts). These results suggested elevated oxidative stress levels in the biofilms exposed to higher concentrations of oxygen. The use of oxygenation to eliminate anaerobic zones and the consequent ethanol production demonstrate a limitation in the MGR system, which can affect biomass performance in bioreactors operated for extended periods of time. In a previous study, the amount of DO levels in the biofilms decreased during periods of accelerated growth in aerated cultures and the oxygen-uptake rate was determined to be high during the period of 1 to 3 days in flask cultures (Venkatadri *et al.*, 1992). This was previously attributed to an increase in free- and mycelial-attached polysaccharides sheath in cultures where air and 100% O_2 was used (Dosoretz *et al.*, 1993). Others (Miura *et al.*, 2004), concluded that the production of glucan was induced as an oxidative stress response and a defensive mechanism, i.e. the fungus produces extracellular glucan to limit DO diffusion to protect the cell from oxidative stress, leading to the formation of anaerobic zones in biofilms and thus increasing ethanol production as demonstrated in Figure 6.6.

Figure 6.7 shows accumulative oxidative stress between aerated and oxygenated biofilms harvested from the SCMGR systems. Cumulative oxidative damage gives a better indication

of the existence of stress (Belinky *et al.*, 2005) as a result of stress factors such as the presence of ROS, accumulation of metal ions or redox-recycling agents (Jamieson, 1995).

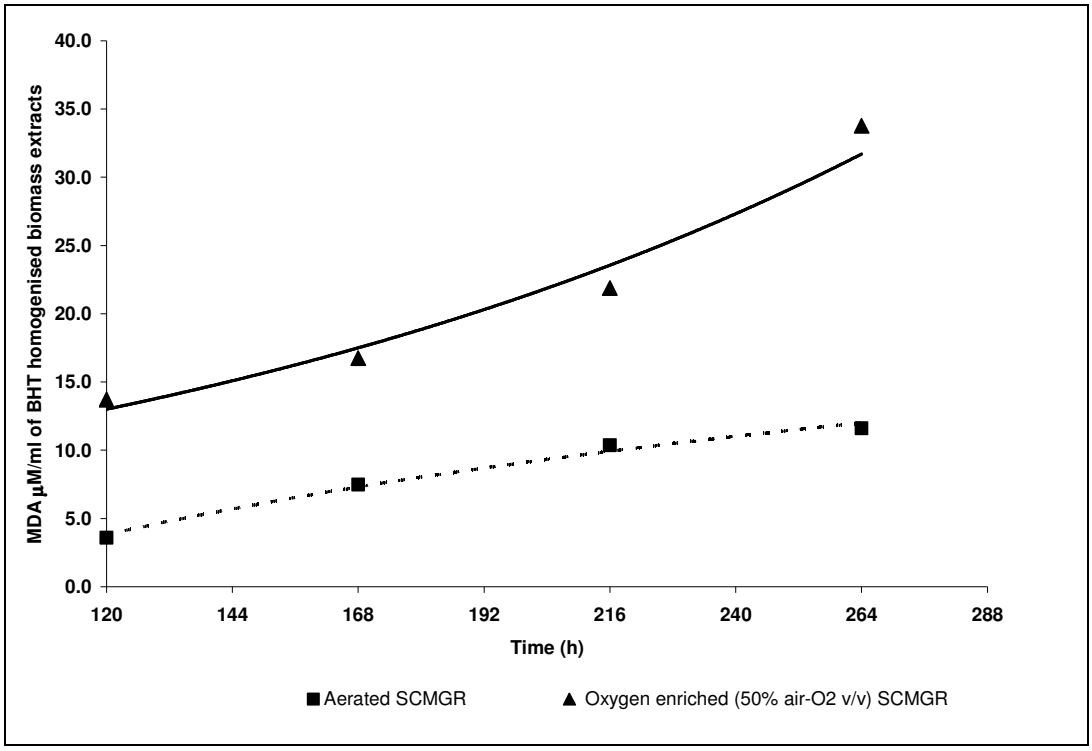

Figure 6.7: Malondialdehyde formation in air and oxygen in the ECS for *P. chrysosporium* in SCMGR systems which was used as a measure of lipid peroxidation in the biofilms

Since the ROS are generally highly reactive with lipids, modified lipids were determined to be a suitable option for establishing oxidative stress in biofilms obtained from the bioreactors. The results indicated that oxidative stress damage occurred in both aerated and oxygenated bioreactors, as the biofilms were directly exposed to both the aeration and oxygenation sources. *P. chrysosporium* produced enzymes, such as catalase (Kwon & Anderson, 2001), for detoxifying excess ROS. However, the levels of ROS can increase at a rate that is beyond the neutralisation capability of the fungus.

6.5.4 Accumulation of trace elements in biofilms

As *P. chrysosporium* has been shown to accumulate considerable amounts of heavy metals from the growth medium (Falih, 1997). It seemed that the metal ions were transported and

stored in the hyphae when they were in high concentrations, thus inhibiting mycelial growth. Falih (1997) further reported that a reduction in mycelia dry weight of up to 60% occurred in cultures containing 0.4 $g.L^{-1}$ Mn ions compared to cultures without Mn ions in the nutrient medium. As the nutrient medium for this study contained Mn and Cu, it was important to determine Mn and Cu accumulation in the biofilms growing in the MGR system, because high concentrations of Mn ions were determined to inhibit LiP production (Rothschild *et al.*, 1999), while Cu accumulation was shown to contribute to oxidation of proteins (Stohs & Bagchi, 1995). Although *P. chrysosporium* was shown to be able to survive, grow and adapt to elevated concentrations of Mn and Cu, the ligninolytic system of the fungus might be compromised at such concentrations.

Aerated and oxygenated biofilms from SCMGRs were tested for metal accumulation by using two different Cu/Mn concentrations in the nutrient medium (see Table 6.2). The results are summarised in Table 6.2. Biofilms cultivated in oxygenated SCMGR systems showed an average increase of ~32% for Mn present in the biomass compared to the aerated systems when supplied with the same concentration of Mn in the nutrient medium. Similarly, the average Cu increase was ~35% in the biomass when oxygenated. Aerated biofilms supplied with 10% (10 ml) of the required trace-element solution in the basal medium showed a lower presence of both Mn and Cu in the *P. chrysosporium* biofilm. Furthermore, the MGR biofilms supplied with much lower concentrations of Mn and Cu, showed higher accumulation of Mn and Cu in *P. chrysosporium* biomass when compared to previously published results of biofilms grown at higher Mn and Cu concentrations in batch cultures.

Table 6.2: A comparison of metal accumulation in biomass of *P. chrysosporium* using nutrient medium containing different concentrations of Mn and Cu for batch cultures and continuous SCMGR cultures and compared to batch cultures of Falih (1997)

Metal concentration (µg/ml) in nutrient medium	Metal accumulation	
	Cu (mg/g dry biomass)	Mn (mg/g dry biomass)
***Falih, 1997 (batch culture)**		
100 µg/ml of Cu/Mn (aerated)	1.0	0.9
200 µg/ml of Cu/Mn (aerated)	2.0	2.1
†This study (Continuous culture)		
2.99/13.6 µg/ml of Cu/Mn (aerated)[A]	7.1±1.0[C]	27.2±4.1[C]
2.63/12.0 µg/ml of Cu/Mn (aerated)[B]	3.1±1.7[C]	5.35±1.5[C]
2.99/13.6 µg/ml of Cu/Mn (oxygenated)[A]	11.0±3.0[C]	40.0±10.1[C]

**Batch cultures*
†Continuous SCMGR using Tien & Kirk (1988) nutrient medium
A-100 ml of basal (III) medium in nutrient solution
B-10 ml of basal (III) medium in nutrient solution
C-Averaged metal concentration in harvested biofilms (120 to 264 h)

It has previously been shown that a high presence of metals in *P. chrysosporium* decreased the overall fungal growth rate, resulting in an increased lag phase (Baldrian, 2003). The production of metal chelators, oxalate, malonate, etc. (Wesenberg *et al.*, 2003; Goodwin *et al.*, 1994; Khindaria *et al.*, 1994), might be ineffective if a nutrient medium containing heavy metals is continuously supplied to a fixed biofilm.

6.5.5 The role of glucan formation on DO restriction and metal accumulation

Glucan formation in aerated and oxygenated biofilms was evaluated using nutrient medium containing 10 and 100-fold concentrations of basal medium. Glucan presence was more prevalent in oxygenated homogenised biomass than the aerated biomass, as shown in Figure 6.8. This might explain the increased ethanol production and Mn/Cu accumulation in the oxygenated biofilms. Cultures that contained 10 ml of basal medium in the nutrient medium and that were aerated showed lower glucan concentrations. The presence of higher metal concentrations might have exacerbated glucan formation. In previous studies, glucan

had been produced to a maximum of 0.215 mg/ml after 9 days in batch-submerged cultures (Bes *et al.*, 1987) and had been at its highest during the period of 144 to 240 h, while production had started around 4 to 5 days (96 to 120 h).

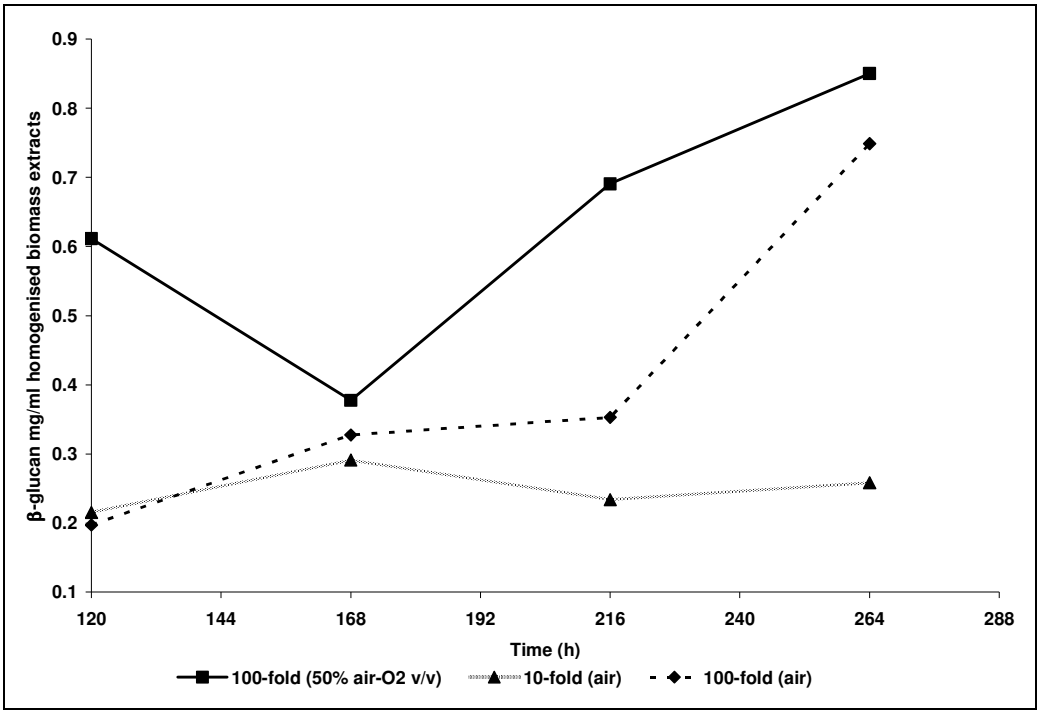

Figure 6.8: β-glucan presence in biofilms immobilised in the SCMGR systems

Belinky *et al.* (2003) had determined that ROS levels were high during the logarithmic growth phase, but decreased during the transition to idiophase in aerated and oxygenated cultures. In SCMGR systems, the idiophase was determined to occur during the period 120 to 168 h (Figure 6.5). In this time, glucan presence in the SCMGR biofilms showed to decrease in oxygenated biofilms (Figure 6.8).

6.6 CONCLUSION

The anaerobic zone thickness in aerated systems increased with increasing biofilm thickness, thus leading to increased ethanol production. Furthermore, oxygenation led to the formation of glucan, which further inhibited DO mass transfer resulting in higher ethanol

production, albeit smaller than the production observed in a MCMGR system. Oxygenation led to higher concentrations of MDA formation, a sign of lipid peroxidation and oxidative stress. The high concentration of glucan in some biofilms was attributed to increased metal accumulation from the nutrient medium. Biofilms supplied with nutrient medium containing 10% of the required basal medium solution exhibited less accumulation of Mn and Cu, although the values obtained were higher than those observed in batch cultures where higher concentrations of Cu and Mn were used.

6.7 RECOMMENDATIONS FOR FUTURE STUDIES

1) Continuous fixed-film bioreactors are considered suitable for continuous enzyme production, they provide a new challenge to optimise nutrient medium for batch cultures and refine it for use in continuous systems. As the nutrient medium designed for *P. chrysosporium* by Tien and Kirk (1988) also contained other metals such as Mg, Na, Fe, Co, Ca and Zn, the analyses of these metals in fixed-film bioreactors is required.

2) The effects of metal accumulation in the MGR on the production of LiP and MnP should be investigated.

3) Other supplements and DO carriers need to be investigated that might prolong the survival of *P. chrysosporium* biomass during: 1) nutrient starvation; 2) exposure to higher concentrations of trace metal ions; 3) hyperthermia (optimal fungal temperature is 39℃) and 4) death caused by shearing forces, conditions identified as prevalent in continuous MGR systems.

5) To reduce glucan production, a carbon-limited nutrient medium should be used in continuous cultures, as excess glucose can readily be stored as glucan by *P. chrysosporium*.

6.8 SUMMARY

Major findings for this chapter are as follows: 1) aeration can lead to ethanol production, as anaerobic zones were formed in immobilised biofilms; 2) the use of MCMGRs further increased ethanol production; 3) oxygenation initially used to improve DO transport further hampered biofilm performance by increasing the formation and storage of glucan in the biofilms and 4) Lipid peroxidation and increased trace element accumulation were also observed.

The following chapter (Chapter 7) evaluates the use of different perfluorocarbons in batch cultures. This was a proposed solution identified to alleviate some of the limitations that were identified in this chapter (Chapter 6). The objective in Chapter 7 is to:

- Evaluate the effectiveness and compatibility of three different water immiscible DO carriers (perfluorocarbons) on *P. chrysosporium* growth and ligninolytic enzyme production in batch cultures and to select a suitable perfluorocarbon and concentration for application in SCMGR (Chapter 8).

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CHAPTER 7

RESULTS

Influence of perfluorocarbons on *Phanerochaete chrysosporium* biomass development, substrate consumption and enzyme production

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CHAPTER 7

Influence of perfluorocarbons on *Phanerochaete chrysosporium* biomass development, substrate consumption and enzyme production

Abstract

Limitations placed on aerobic cultures by dissolved oxygen can be alleviated by using oxygen-vectors such as perfluorinated organic oils. In this study, *Phanerochaete chrysosporium* was tested in batch cultures to evaluate the efficacy of perfluorinated oils in improving biomass development, nutrient consumption and extracellular enzyme production. The oils used in an emulsified form in this study were perfluorotripropylamine, perfluorooctyl bromide and bis-(perfluorobutyl) ethene, as they dissolve higher quantities of oxygen compared to others. The surfactant used to stabilise the emulsion was Pluronic F 68, which was used at a concentration of 8.5% (w/v). The addition of only the surfactant to the medium resulted in a 6.9-fold increase (1.38 $mg.ml^{-1}$) in biomass concentration of *P. chrysosporium*, while a biomass concentration of 0.82 to 1.53 $mg.ml^{-1}$ (4.1 to 7.6-fold increase) was observed in the PFC emulsions with an oil-phase fraction in the range of 0.1 to 0.3 (w/v), i.e. 10 to 30% (w/v). The highest enzyme activity from crude samples was 307 and 410 $U.L^{-1}$ for LiP and MnP, respectively, compared to <100 and <50 $U.L^{-1}$ enzyme activity for LiP and MnP obtained in control cultures, respectively. Ammonium and glucose utilisation was higher in emulsions with PFC concentrations of 10% (w/v) for all the perfluorocarbons evaluated than at concentrations of 20% and 30% (w/v). It was concluded that the use of the perfluorocarbon emulsions improved the performance of *P. chrysosporium* BKMF 1767 in terms of biomass development, nutrient consumption and enzyme production. Furthermore, PF 68 where found to have growth-promoting abilities for *P. chrysosporium*.

Keywords: Lignin peroxidase; Manganese peroxidase; Perfluorocarbon; *Phanerochaete chrysosporium*

7.1 INTRODUCTION

Researchers have screened a large number of *Basidiomycetes* for their ability to degrade lignin and various pollutants in industrial wastewater. The ligninolytic system of the white-rot Basidiomycete, *Phanerochaete chrysosporium*, consists of an extracellular oxidative process initiated by nitrogen, carbohydrate and sulphur limitation (Bumpus & Aust, 1987). Under ligninolytic conditions the fungus produces extracellular enzymes, lignin peroxidase (LiP) and manganese peroxidase (MnP), which were found to exhibit high rates of lignin degradation (Buswell & Odier, 1987; Kirk *et al*., 1978). Other critical external factors conducive to the improved production of LiP and MnP are temperature, pH and dissolved oxygen (DO) concentration (Buswell & Odier, 1987; Leisola *et al*., 1984; Fenn *et al*., 1981; Fenn & Kirk, 1981; Jeffries *et al*., 1981; Kirk *et al*., 1978).

The conditions necessary for the production of LiP and MnP, can be better exploited in continuous fixed-film membrane bioreactors (MBRs), as a section of the biofilm is kept in the idiophase, while the products can be continuously recovered (Sheldon & Small, 2005; Govender *et al*., 2003; Solomon & Petersen, 2002; Leukes, 1999; Leukes *et al*., 1999). However, in fixed-film MBRs conditions related to nutrient starvation, continuous exposure to trace element ions from the nutrient medium, hyperthermia and shearing forces caused by continuous aeration, prevail. These conditions are unfavourable for prolonged bioreactor- and biomass performance. Furthermore, *P. chrysosporium* biomass has been determined to have poor DO transfer capabilities (Leisola *et al*., 1983). This is due to glucan production and storage by *P. chrysosporium* biomass (Buchala & Leisola, 1987) as a defence mechanism to protect itself against oxidative stress (Miura *et al*., 2004) resulting from the use of high partial pressures of oxygen and continuous aeration. In addition, the ability of the fungus to adsorb and accumulate metal ions can exacerbate and limit overall biomass performance in fixed-film MBRs that are operated for prolonged periods (Falih, 1997). To overcome these limitations, the use of DO-carrying perfluorinated organic oils (perfluorocarbons/PFCs)

emulsified in a poloxamer, Pluronic F 68 (PF 68), to promote growth and enhance the performance of *P. chrysosporium* biofilms, was investigated in this study.

The use of PFCs decreased mechanical damage to biomass caused by conventional aeration through sparging or continuous stirring in the batch cultures. Controlled concentrations of DO can be used in the PFC emulsions, thus reducing oxidative stress to the immobilised biomass (Richardson *et al*., 2002; Lowe *et al*., 1998). The use of PF 68 as the surfactant was prompted by evidence suggesting that it prolonged the survival of *Tetrahymena* cells during nutrient starvation, exposure to higher concentrations of trace elements and hyperthermia (Hellung-Larsen *et al*., 2000), conditions identified to be prevalent in continuous fixed-film MBRs (Leukes, 1999). Typical surfactant levels used in a PFC-based emulsion had been 1 to 5% (w/v) for water-in-oil emulsions and 5 to 10% (w/v) for oil-in-water emulsions (Floyd, 1999). A surfactant (PF 68) concentration in the nutrient medium of ~8.5% (w/v) was therefore selected for this study.

7.2 OBJECTIVE

The primary objective of this part of the study was to evaluate the compatibility of three PFCs, perfluorotripropylamine (PFPA), bis-(perfluorobutyl) ethene (PFBE) and perfluorooctyl bromide (PFOB) with *P. chrysosporium*. Furthermore, the effect of the emulsions on biomass development, substrate consumption and enzyme production was assessed.

7.3 MATERIALS AND METHODS

7.3.1 Microbial strain and inoculum preparation

P. chrysosporium strain BKMF 1767 (ATCC 24725) was grown at 39°C on malt agar slants using the maintenance and spore inducing medium (SIM) described by Tien and Kirk (1988). The spores from agar plates were harvested after 7 to 10 days and prepared by suspension

in sterile water. This was followed by passage through sterile glass wool to entrap suspended mycelia. The inoculum suspension used in each flask consisted of approximately 3 x 10^6 spores. Refer to Appendix A for information on the growth and maintenance of *P. chrysosporium* and inoculum development.

7.3.2 Nutrient medium (continuous phase)

The nutrient medium contained 100 ml of a basal medium solution, 100 ml of 0.1 M 2,2-dimethylsuccinate, 100 ml of 55.5 mM glucose, 100 ml of 0.4 M veratryl alcohol, 60 ml trace element solution, 25 ml of 1.1 mM ammonium tartrate, 10 ml of 10 $mg.L^{-1}$ thiamin-HCl, 376 ml of 22.5% (w/v) PF 68 and 129 ml autoclaved distilled water (Tien & Kirk, 1988). The final concentration of PF 68 in the nutrient medium was ~8.5% (v/v). For the control experiments, PF 68 was excluded from the nutrient medium and replaced with 376 ml of autoclaved distilled water. Refer to Appendix B for the nutrient-medium development for *P. chrysosporium*.

7.3.3 Pluronic F 68 preparation

Pluronic F 68 (PF 68) has been widely used as a cytoprotectant and growth promoting additive to animal cell and microbial cultures (Lowe *et al*., 1994). Hangzhou Onicon Chemical Company LTD (China) supplied the surfactant. The concentration chosen for the preparation of the emulsions was 8.5% (w/v).

7.3.4 Perfluorocarbon preparation

Perfluorocarbons, perfluorotripropylamine (Keensun Trade Company LTD, China), perfluorooctyl bromide (Exfluor Research Corp., USA) and bis-(perfluorobutyl) ethene (Oakwood Products Inc., USA) were chosen for the experiments as they exhibit high solubilities for oxygen compared to water, as shown in Table 7.1. PFC fractions of 0.1 to

0.3 (w/v), i.e. 10 to 30% (w/v), were used for PFPA and PFOB, while fractions of 0.1 to 0.2 (w/v) were used for PFBE. All PFC emulsions were prepared in an ~8.5% (w/v) PF 68-based nutrient medium. Appropriate quantities of PFCs were filter sterilised using a 0.22 µm filter, before adding them to the nutrient medium (continuous phase). The PFC emulsions were oxygenated for 10 min before use. The PFCs were recovered at the end of the experiment by centrifugation at 4000 rpm for 10 min, as explained by Elibol and Mavituna (1996).

Table 7.1: Properties of PFC liquids compared to those of water at standard pressure and temperature (Lowe *et al.*, 1998)

Liquid	Dissolved oxygen (mM)	Density (25°C)	Boiling Point
Water	0.2	1.0 $g.ml^{-1}$	100°C
Perfluorotripropylamine	39.6	1.82 $g.ml^{-1}$	130°C
Perfluorooctyl bromide	44.0	1.93 $g.ml^{-1}$	142°C
Bis-(Perfluorobutyl) ethene	44.0	1.41 $g.ml^{-1}$	60°C

7.3.5 Culture conditions

Twelve stationary 250 ml Erlenmeyer flasks containing 15 ml of the emulsions were used for each experiment. The emulsions were oxygenated with ~100% technical-grade oxygen for 10 min. The flasks were sealed with rubber stoppers and incubated at 39°C; the only source of DO was from the PFCs. Mechanical agitation and shear stress have been shown by researchers to deactivate the ligninolytic system (Leisola *et al.*, 1984; Kirk *et al.*, 1978); therefore, the flasks were not agitated for 192 h. Thereafter, the flasks were shifted to a shearing environment for 24 h using a shaking incubator at 150 rpm, in order to monitor the protective effects of the emulsions on the extracellular enzymes produced. During sampling, 1.5 ml samples were taken daily from three different flask for two consecutive days (48 h), with subsequent flasks used thereafter.

7.4 ANALYTICAL TECHNIQUES

7.4.1 Enzyme activity

A determination of the enzyme activity was made at 25℃ using crude permeate samples; i.e. the extracellular fluid used in the assay was not concentrated through ultrafiltration. Enzyme activities were measured in control cultures and compared with activities obtained in surfactant and PFC emulsion-based experimental runs, respectively.

7.4.1.1 Lignin peroxidase activity

LiP activity was measured according to the method of used by Tien and Kirk (1988). The oxidation of veratryl alcohol to veratryl aldehyde was recorded at 310 nm for 60 seconds, with one unit being defined as 1 µmol of veratryl alcohol oxidised to veratryl aldehyde per minute in 1 ml of reaction volume $\left(\varepsilon_{310nm} = 9300 M^{-1}.cm^{-1}\right)$. The reaction mixture contained 0.2 ml of veratryl alcohol (0.01 M), 0.2 ml of tartaric acid (0.25 M), 0.3 ml of enzyme solution, 0.22 ml of distilled water and 0.08 ml of H_2O_2 (0.005 M). Refer to Appendix C for a complete LiP protocol.

7.4.1.2 Manganese peroxidase activity

MnP activity was measured by monitoring the change in the oxidation state of Mn^{2+} to Mn^{3+} at 420 nm for 60s $\left(\varepsilon_{420nm} = 36000 M^{-1}.cm^{-1}\right)$. The reaction solution contained 350 µl of reagent A and reagent B, with 300 µl of enzyme supernatant in a total reaction mixture of 1.0 ml. Reagent A contained sodium succinate, sodium lactate buffers, each at 100 mM and pH 4.5, 6 $mg.L^{-1}$ of egg albumin, 200 µl of $MnSO_4$ (0.95 $mg.ml^{-1}$) and ABTS at 80 $\mu g.ml^{-1}$. Reagent B contained 100 µM of H_2O_2 (Gold & Glenn, 1988). Refer to Appendix C for a complete MnP protocol.

7.4.2 Determination of ammonium and glucose concentrations in crude samples

A Spectroquant Ammonium Test Kit (Merck, Germany) was used to measure the amount of ammonium present in the broth at different times. Because the suites of ligninolytic enzymes from *P. chrysosporium* are only produced under conditions of nutrient limitation, in this case nitrogen limitation, it was necessary to monitor ammonium consumption. The concentration of glucose concentration in the samples was determined by using a Roche® D-glucose test kit (AEC Amersham, R.S.A). Glucose consumption was evaluated to monitor different consumption rates in flasks. The concentration of ammonium and glucose obtained at different times in the broth was subtracted from the initial concentration in the nutrient medium to yield the corresponding consumption. Refer to Appendix D for the complete protocols used.

7.4.3 Dry weight

The generated mycelium's dry weight was determined by washing the mycelium twice with sterile distilled water. The mycelium was dried at 60°C until the dry weight was constant.

7.4.4 Biomass enhancement factor

In any study of enhancing oxygen supply in bioreactors through the addition of PFC emulsions, the effects of the emulsion can be calculated using the enhancement factor, E, which is a ratio between biomass generated in bioreactors with and without the PFC emulsion (Ju *et al.*, 1991).

$$E = \frac{\text{Biomass generated (PFC emulsion)}}{\text{Biomass generated (control without PF 68)}} \qquad \text{Eq. 7.1}$$

7.5 RESULTS AND DISCUSSION

P. chrysosporium cultures were studied in batch flasks for two major reasons: Firstly, to evaluate the compatibility of the fungus with PF 68 and the DO carrying perfluorinated water-immiscible oils (PFPA; PFBE; PFOB). This was because *P. chrysosporium* mycelia were identified to have poor DO transport capabilities even when high partial pressures of oxygen are used. Secondly, to evaluate the overall performance of the *P. chrysosporium* biomass in terms of actual biomass development, nutrient consumption and extracellular LiP and MnP production in order to identify a suitable PFC and concentration.

P. chrysosporium biomasses were grown in three types of nutrient medium: Type 1, was the traditional nutrient medium designed by Tien and Kirk (1988), without any surfactants or PFCs, Type 2 was the same medium supplemented with ~8.5% (w/v) PF 68 and Type 3 was the nutrient medium supplemented with different fractions of PFCs using PF 68 as a surfactant. The first two types, without the PFCs, served as the control experiments. All the results of all the assays were calculated as an average of the three parallel flasks.

7.5.1 Effects of Pluronic F 68 on *P. chrysosporium* biomass

PF 68 is a neutral polymer and does not have any nutritional functions for *P. chrysosporium*. However, Figure 7.1 shows that the addition of the polymer increased the biomass from 3 mg to above 19.5 mg in the batch cultures for all three PFC emulsions. The biomass enhancement factor (*E*), in terms of biomass generated in the PF 68-supplemented medium without PFCs, was determined as 6.9 (Table 7.2). PF 68 has been identified as a surface-active poloxamer that modifies cellular membranes, thus modifying cellular metabolism events (Wu, 1995). Although low conidia concentrations of the fungus were used as an inoculum in this study, PF 68 was found to increase the rate at which essential nutrients are utilised by the fungus by modifying the cellular membranes of *P. chrysosporium* mycelia and thus increasing the rate of nutrient usage and biomass development.

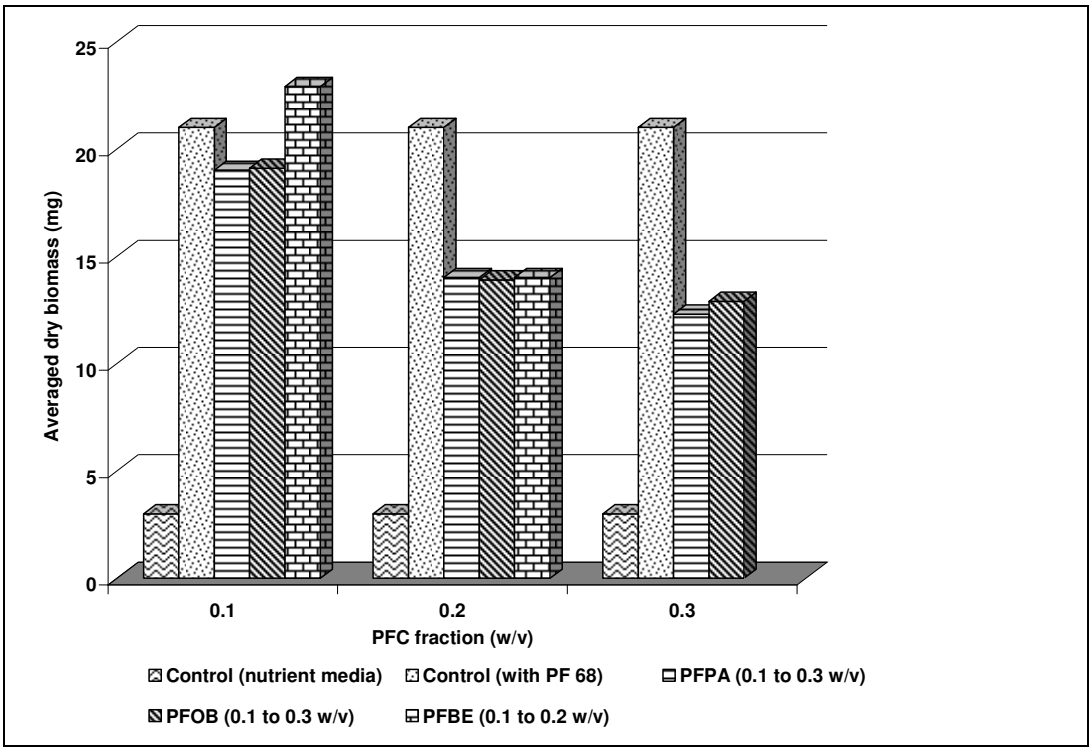


Figure 7.1: Comparison of *P. chrysosporium* biomass development in 15 ml batch cultures: control medium with no PF 68; control medium supplemented with PF 68 only; and nutrient medium with PF 68 combined with different fractions of PFPA, PFOB and PFBE. The biomass was harvested after 216 h of reactor operation

Furthermore, it had previously been shown that surfactants with high hydrophilic-lipophilic balance (HLB) values provided significant protection to biomass against shearing effects, while those with low HLB values promoted cell lysis and growth inhibition (Wu, 1995). Previously, Tween 80 had been shown to have protective effects on LiP and MnP by reducing enzyme deactivation in agitated cultures of *P. chrysosporium*. However, Tween 80 was shown to have only short-term protective effects (Tween has an HLB value of 15, while PF 68 has an HLB value of 24.0 to 29.0). As researchers try to develop continuous processes for prolonged LiP and MnP production, non-ionic surfactants such as Tween 80 are normally used as supplements in the nutrient medium. However, they have not been proven to have the capacity to protect immobilised biomass. The enhancement of biomass performance by Tween 80, during conditions where *P. chrysosporium* biomass was continuously exposed to high concentrations of trace element ions, hyperthermia, and nutrient starvation, was not evaluated when studies of Tween 80 were performed in order to

evaluate the surfactants effectiveness and suitability for prolonged continuous bioreactor operation.

7.5.2 *P. chrysosporium* biomass development in emulsified perfluorocarbons

Figure 7.1 shows a representation of dry biomass achieved in the various media during this study. Overall, biomass development decreased with increasing PFC fractions in the emulsions. A biomass reduction of >45% was observed as PFC fractions were increased from 0.1 to 0.3 (w/v). Several researchers reported the same phenomena when they increased the fraction of PFCs in the fermentation medium used in their experiments. One example was that of Actinorhodin production, where *Streptomyces coelicolor* biomass decreased as the fraction of perfluorodecalin was increased from 0.1 to 0.5 (w/v) (Elibol & Mavituna, 1996). In this study, low concentrations of *P. chrysosporium* conidia were shown to have poor growth capabilities with increased biomass achieved using PFC emulsions, although PF 68 was identified as the main growth promoter for the cultures. Higher biomass growth was achieved using a fraction of 0.1 (w/v) compared to 0.2 and 0.3 (w/v) for all three PFCs. For a fraction of 0.1 (w/v), PFPA and PFOB produced similar biomass, with the highest biomass achieved using PFBE. Although the increase in PFC fractions decreased the biomass actually formed, the biomass formed in the 0.3 (w/v) emulsions still represented an excess biomass of 400% compared to control cultures without ~8.5% (w/v) PF 68.

Values of the enhancement factor, (*E*), using the different media and emulsions are listed in Table 7.2. The values of biomass generation decreased with an increase in the PFC fraction in the medium. The highest *E* value of 7.6 was achieved with 0.1 (w/v) PFBE. It was observed that, after mechanical agitation for 24 h, the PFC phase attached to the formed biomass, leaving a clear continuous phase compared to the milky emulsions observed before agitation.

Table 7.2: Enhancement factor of *P. chrysosporium* biomass in different culture media supplemented with perfluorocarbons

Culture medium	Dry biomass formed (mg)	Enhancement factor, *E*
Control (pure medium)	2.5 ± 0.5	-
Control (PF 68)	21.5 ± 3.5	6.9
0.1 (w/v) PFPA	18.5 ± 2.5	6.3
0.2 (w/v) PFPA	13.5 ± 2.5	4.7
0.3 (w/v) PFPA	12.5 ± 3.5	4.1
0.1 (w/v) PFOB	20.5 ± 3.5	6.4
0.2 (w/v) PFOB	14.0 ± 2.0	4.6
0.3 (w/v) PFOB	12.5 ± 3.5	4.3
0.1 (w/v) PFBE	23.5 ± 4.5	7.6
0.2 (w/v) PFBE	13.5 ± 2.5	4.7

7.5.3 Ammonium and glucose consumption

The control cultures supplemented with PF 68 only also performed better in terms of glucose consumption compared to control cultures without the surfactant during 144 to 216 h, with similarities from 0 to 144 h. This was expected, as an increase in the biomass formed was significant in PF 68-based control cultures. The ammonium concentration in the control cultures was above 30 $mg.L^{-1}$, even after 216 h of biomass incubation, further supporting evidence that poor DO availability led to poor overall nutrient consumption and biomass development. Significantly, 0.2 (w/v) PFPA cultures had a rapid ammonium consumption rate, whereby >80% of the ammonium was consumed after 48 h even though the biomass enhancement factor was lower than that of 0.1 (w/v) PFPA emulsion.

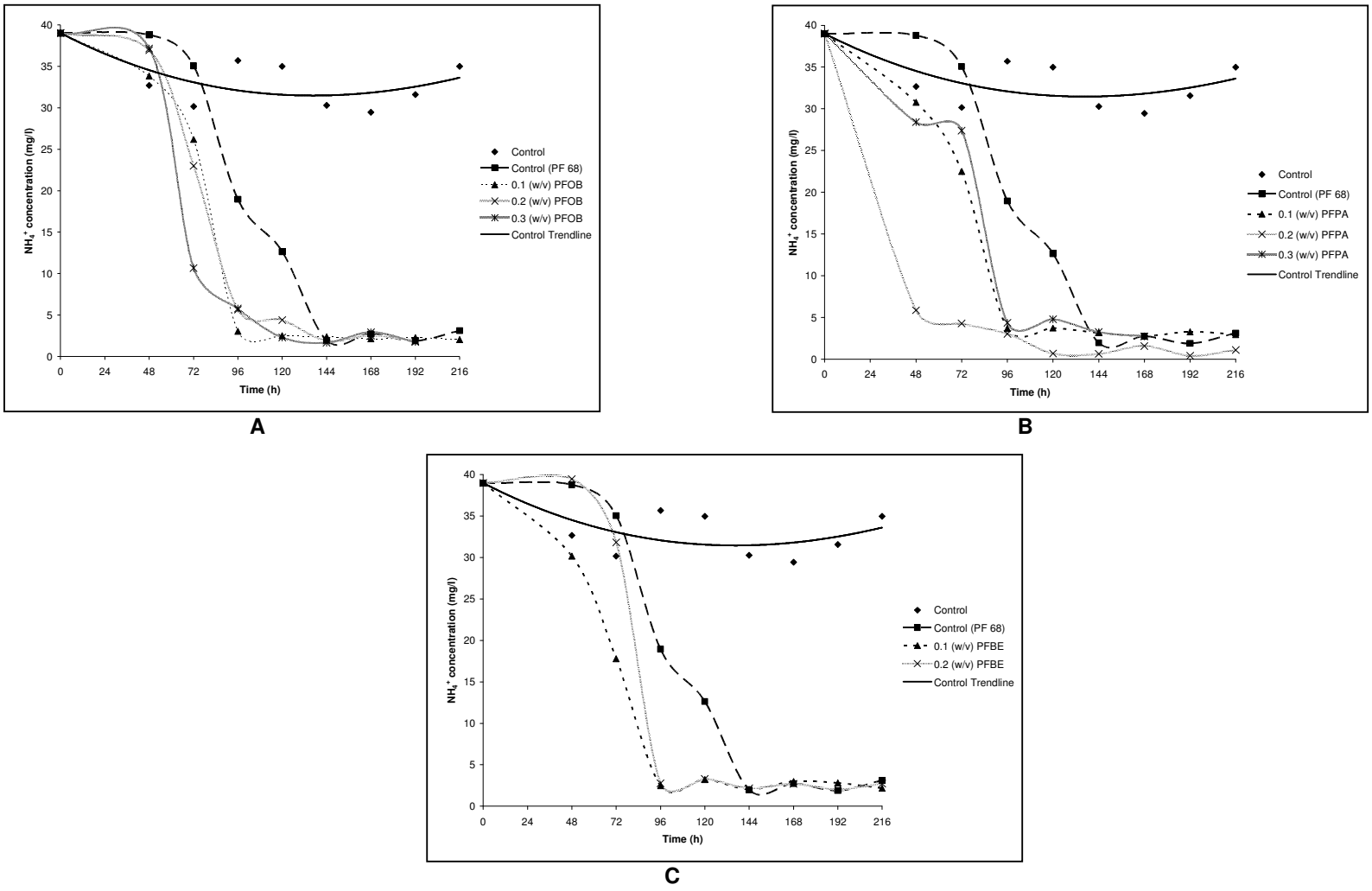
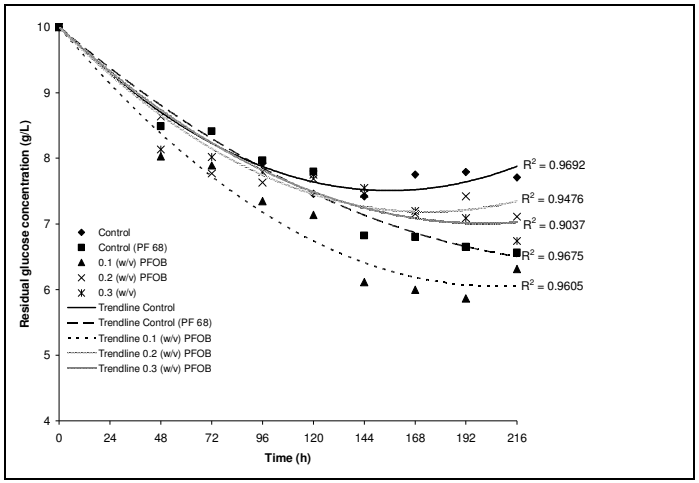


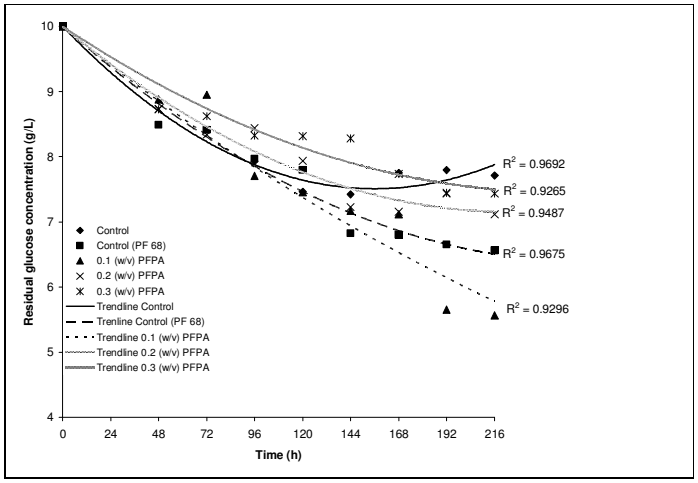
Figure 7.2: Residual Ammonium (NH_4^+) concentrations in batch cultures over 216 h of operation. A comparison was made using different PFCs and their fractions to quantify the depletion of the ammonium source in the broth: A – PFOB, B – PFPA and C – PFBE cultures

Figure 7.2 shows that the residual ammonium concentration in the PFC emulsions was below 10 mg.L^{-1} after 96 h for all emulsion fractions investigated, showing an improved rate of consumption for the ammonium source when compared to the control cultures. The delay in the ammonium source consumption in the emulsions was attributed to the effect of PF 68 on the physiological state and germination of *P. chrysosporium* conidia in the cultures, as the conidia adjusts to the presence of the surfactants and PFCs in the nutrient medium. This was evident for the 0.1 to 0.3 (w/v) PFOB and 0.2 (w/v) PFBE emulsions. However, no evident lag phase was observed for the 0.1 to 0.3 (w/v) PFPA emulsions and 0.1 (w/v) PFBE emulsions. As PF 68 was shown to increase fluorescence uptake by *Saccharomyces cerevisiae* (King *et al*., 1991), the uptake rate during this study of the ammonium source and glucose in the control cultures supplemented with PF 68 was lower compared to cultures supplemented with PFC emulsions. This was attributed to the low availability of DO in the control cultures, as the PFC emulsions had greater oxygen availability.

Figure 7.3 illustrates residual glucose concentrations in the batch cultures studied. The rapid rate of glucose consumption in the emulsions was similar to that of the ammonium consumption. The PFC emulsions with lower oil fractions, 0.1 (w/v), performed better than those with a higher oil fraction. After 168 h, the residual glucose concentration in the cultures was observed to be below 6.5 g.L^{-1}, compared to the control cultures where the residual concentration was above 7.5 g.L^{-1} after 216 h of biomass incubation. The fungus grown in 0.1 (w/v) PFPA emulsion showed poor ability to use the carbon source, as the residual glucose concentration was high during the 0 to 192 h period, when compared to 0.1 (w/v) of PFOB and PFBE emulsions. This was further corroborated by the enhancement factor as the increments were in the order of PFPA < PFOB < PFBE for an emulsion fraction of 0.1 (w/v).



A



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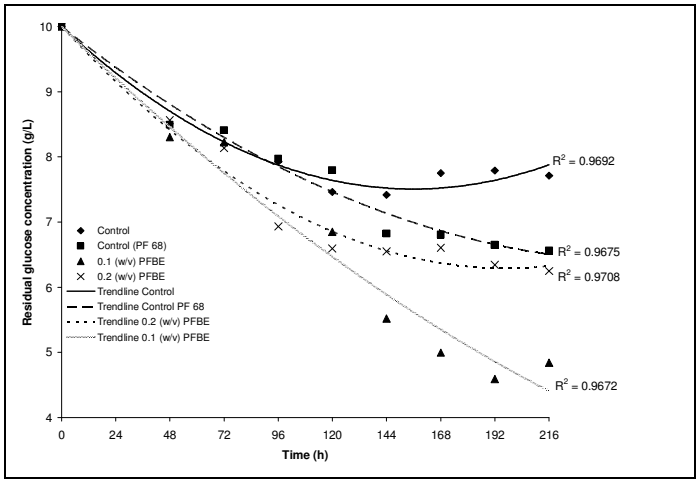

C

Figure 7.3: Residual glucose concentrations in batch cultures over 216 h. A comparison was made using different PFCs and their fractions to quantify the depletion of the ammonia source in the broth. A – PFOB, B – PFPA and C – PFBE cultures

7.5.4 Extracellular secondary metabolite production

LiP and MnP production were measured for different emulsions at different concentrations, and the results are presented in Figures 7.4 and 7.5, respectively. Previously, the addition of PF 68 to the fermentation medium without DO carrying water-immiscible oils was shown to decrease the DO transfer coefficient, even under fermentation broth agitation speeds exceeding 500 rpm (Elibol, 1999). That was why the LiP production was negligible in the control experiment for this study, where the medium was supplemented with ~8.5% (w/v) PF 68 only. Negligible LiP was detected in the control, with PF 68; 0.1 and 0.2 (w/v) fractions of PFBE, respectively. This could have been caused by the production and interaction of volatile compounds with the enzyme, attributable to the PFCs' low boiling point (60°C) and culture-incubation temperature (39°C). However, at this point, further experimentation is required to verify this hypothesis. Furthermore, the presence of PFPA had a positive effect, as higher activity rates were determined when compared to the control cultures.

It was observed that moderate agitation (150 rpm) had negligible effects on enzyme activity deactivation in the presence of PFC emulsions. In the control cultures, average LiP and MnP rates were 77 and 12.6 $U.L^{-1}$, respectively, after 192 h. After 24 h of broth agitation, the enzymes were reduced to an average of 55.8 $U.L^{-1}$ for LiP, while MnP was reduced to 0.8 $U.L^{-1}$ in the cultures. However, no significant changes in MnP activity were observed in the different fractions of the PFOB- and PFPA emulsions. Increases in MnP activity was observed in all fractions of the PFBE emulsions. All PFPA emulsions contributed significantly to higher LiP increases after the broth agitation, with 0.1 and 0.2 (w/v) PFOB emulsions showing decreasing activity, while the 0.3 (w/v) PFOB emulsion showed an increase. The highest activity obtained from crude samples during the study was 307 and 410 $U.L^{-1}$ for LiP and MnP respectively, as shown in Figures 7.4 and 7.5.

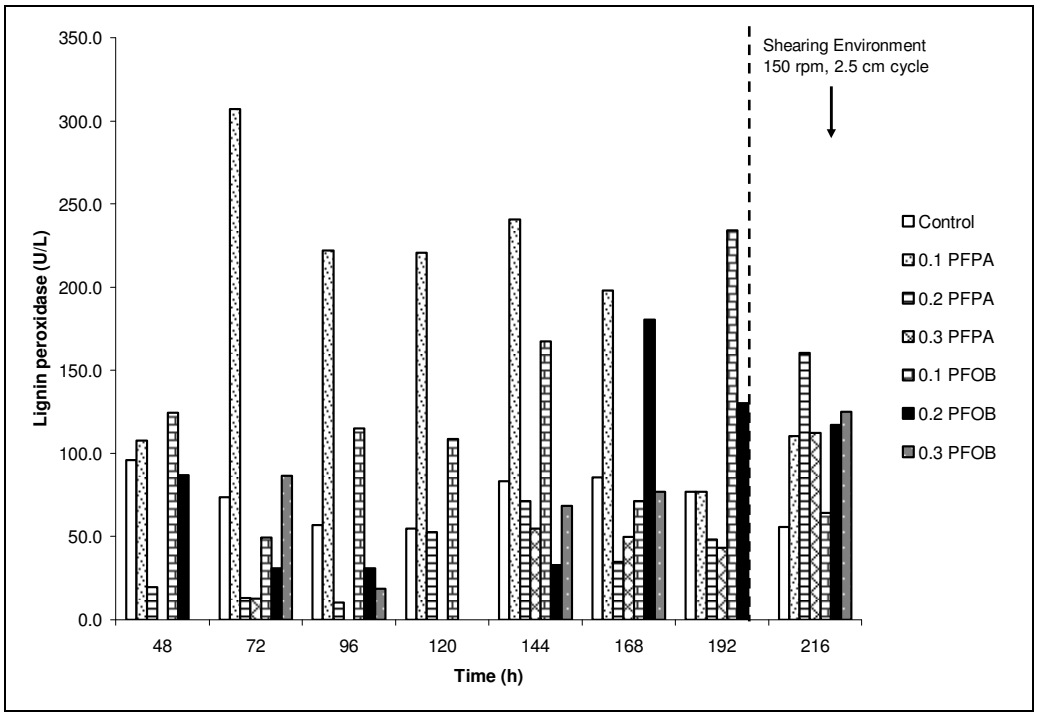

Figure 7.4: Lignin peroxidase activity obtained using: 1) pure medium, 2) medium supplemented with PF 68 and 3) with emulsified PFCs. Negligible LiP activity was found in 1) control (PF 68), 2) 0.1 PFBE and 3) 0.2 PFBE experiments

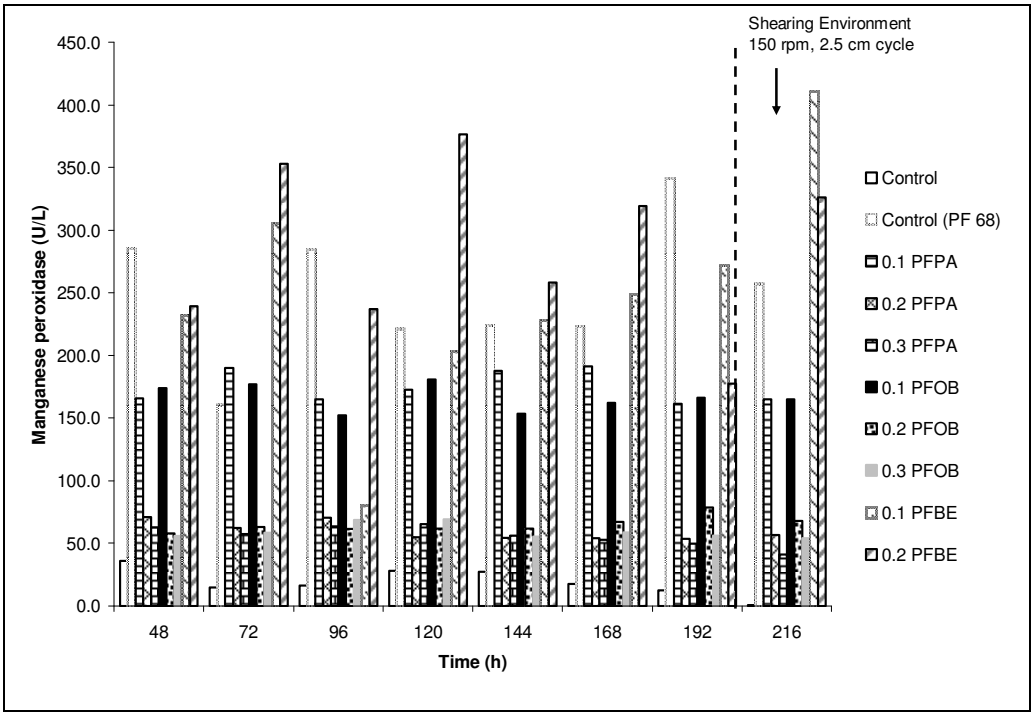

Figure 7.5: Manganese peroxidase activity obtained using: 1) pure medium; 2) medium supplemented with PF 68; and 3) with emulsified PFCs. The activity was determined in crude samples

It was difficult to observe a clear enzyme production trend over time. However, there was a noteworthy difference in enzyme productivity observed for cultures containing PFC emulsions when they were compared to the control cultures. For LiP, maximum enzyme activity was observed after 72 h. This was attributed to easier DO transport in younger biofilms of *P. chrysosporium*, which was shown to influence LiP production (reported in Chapter 2) Furthermore, this coincided with the depletion of the ammonium source in the culture broth. However, this pattern was not evident with the presence of MnP in the cultures, in which maximum activity was observed at different time intervals during the course of this part of the study.

7.6 CONCLUSION

In this part of the study, the possibility of developing sufficient biomass and enhancing extracellular enzyme production from low inoculum concentrations of *P. chrysosporium* was experimentally analysed using DO carrying oils and a poloxamer, PF 68, as the emulsifier. PFBE, PFOB and PFPA were successfully used as oxygen carriers for *P. chrysosporium*. The PFCs tested improved overall biomass formation, substrate consumption and enzyme production compared to the control cultures.

The use of PFC emulsions reduced the effects of agitation on enzyme deactivation, as no significant decrease in enzyme activity was observed after agitation. Although some of the PFCs performed better than others, sufficient amounts of biomass were achieved when compared to the cultures grown without the emulsions and PF 68 surfactant. LiP production rates of >200 $U.L^{-1}$ were observed over the period of 72 to 168 h, with 0.1 (w/v) PFPA. Overall, a shearing environment resulted in reduced LiP activity in cultures with different PFC concentrations. Higher fractions of 0.3 (w/v) PFC showed greater enzyme protection under shearing conditions. However, the increase in PFC concentration resulted in lower MnP production (<60 $U.L^{-1}$). It was demonstrated that for effective enzyme production, biofilm immobilisation and supplementation of the nutrient medium with essential additives is a

viable option for improving biomass performance. Despite the fact that PF 68 had not previously been observed to enhance biomass growth, it was shown in this part of the study that it significantly enhances *P. chrysosporium* biomass generation.

7.7 SUMMARY

In this study, the hypothesis that emulsified PFCs can enhance the performance of *P. chrysosporium* was evaluated. However, a higher PFC concentration was determined to decrease biomass concentration. Lower PFC concentrations of 10% (w/v) were shown to have lower growth-inhibiting properties. Perfluorooctyl bromide was shown to be the most advantageous emulsion for biofilms *P. chrysosporium*.

In the following chapter (Chapter 8), an emulsion containing ~10% (w/v) PFOB, will be evaluated within SCMGRs. The following objectives relate to Chapter 8:

- To apply the proposed emulsion to a SCMGR system, and evaluate its effectiveness in enhancing *P. chrysosporium* biomass performance while alleviating overall biomass performance limitations, trace element accumulation, oxidative stress and polysaccharide storage; and
- To evaluate the enhancement of the overall biomass in the SCMGR system using a DO carrying PFC emulsion in terms of growth kinetics.

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CHAPTER 8

RESULTS

Application of perfluorooctyl bromide: Evaluation in a fixed-film polysulphone-based membrane gradostat reactor for continuous enzyme production

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CHAPTER 8

Application of perfluorooctyl bromide: Evaluation in a fixed-film polysulphone-based membrane gradostat reactor for continuous enzyme production

Abstract

The present study highlights the application of perfluorooctyl bromide (PFOB), an oxygen carrier, in fixed-film membrane gradostat reactors (MGRs) in which biofilms of *Phanerochaete chrysosporium* BKMF-1767 (ATCC 24725) were immobilised. The nutrient medium used in the MGRs was supplemented with the surfactant Pluronic F 68 (PF 68). Overall, the results showed that the supply of nutrient medium from the top of the MGRs resulted in higher glucan storage than in MGRs in which nutrient medium was fed from the bottom of the bioreactors. Reduced ethanol production was determined using a combination of nutrient medium aeration and PFOB/PF 68 supply. Lower LiP production in PFOB/PF 68 cultures was observed compared to MnP production. This was comparable to MnP production obtained in batch cultures in which perfluorocarbons were used as oxygen carriers, suggesting insignificant generation of reactive oxygen species. Lipid peroxidation, quantified as the formation of malondialdehyde, was slightly lower in these systems.

Keywords: Lignin peroxidase; Manganese peroxidase; Membrane bioreactor, Perfluorocarbon, *Phanerochaete chrysosporium*

8.1 INTRODUCTION

Membrane bioreactors (MBRs) have been conceptualised as a solution for the continuous production of extracellular secondary metabolites. Several MBR designs have been proposed using different support matrices to immobilise biofilms and are used in bioremediation applications. The concept of a membrane gradostat reactor (MGR) described by Leukes (1999) and Leukes *et al.* (1999) was found to be highly effective for the

continuous production of lignin peroxidase (LiP) and manganese peroxidase (MnP) from *Phanerochaete chrysosporium* (Ntwampe & Sheldon, 2006; Sheldon & Small, 2005; Govender *et al.*, 2003; Solomon & Petersen, 2002; Govender, 2000; Leukes, 1999; Leukes *et al.*, 1999). A detailed explanation and application of the concept was described in Ntwampe *et al.* (2007). However, the use of the MGR as explained by Leukes *et al.* (1999), with *P. chrysosporium* biofilms, has some limitations as: 1) dissolved oxygen (DO) transport is restricted, 2) oxidative stress is prevalent due to the use of aeration and oxygenation in immobilised biofilms, 3) trace element accumulation occurs and 4) ethanol is produced as the immobilised biofilm forms anaerobic regimes.

When the MGR was designed and patented, the inventors overlooked certain parameters related to biomass performance during bioreactor operation. The following parameters were used and overlooked: 1) the casual use of nutrient medium designed for batch cultures was adopted without modification, 2) DO mass transport limitation and anoxia, 3) trace element ion accumulation, 4) hyperoxia/oxidative stress due to the use of high partial pressures of oxygen to improve secondary metabolite production, and 5) poorly established nutritional gradostat because of gravitational influence on the distribution of nutrients as the bioreactor was operated in the dead-end filtration mode. These limitations were quantified and discussed in Chapter 6, where a combination of aeration and oxygenation was used to address them.

The use of multi-capillary MGRs (MCMGRs), led to limitations similar to those experienced in single-membrane MGRs (SCMGRs). Others (Feijoo *et al.*, 1995) have suggested the recycling of the ligninolytic solutions containing secondary metabolites (LiP and MnP) to improve the overall performance of the MGR. However, this technique had been shown to inhibit and delay extracellular enzyme production when the recovered supernatant was used in freshly inoculated reactors. It is likely that the recycled supernatant had sufficient quantities of DO, to support enzyme production.

The production of LiP and MnP had previously been studied mostly in batch cultures, and more recently in continuous MGR systems. The use of oxygen carriers such as perfluorocarbons (PFCs) in the MGR system to overcome DO limitations and improve biomass performance has never been explored. As PFCs are prepared in an emulsion form using Pluronic F 68 (PF 68), the PFC/PF 68-based emulsions were deemed suitable to be used in the MGR. PFC emulsions were initially evaluated in batch cultures in which an addition of PF 68 to the medium resulted in a 6.9-fold increase in biomass generation of *P. chrysosporium* (Chapter 7). This represented a 4.1- to 7.6-fold increase, with the oil phase concentration fraction in the range of 0.1 to 0.3 (w/v), respectively.

8.2 OBJECTIVE

The objective for this part of the study was to evaluate bioreactor and biofilm performance using a PFOB and PF 68 emulsion in SCMGRs, and to evaluate the efficacy of this mixture in the system. Furthermore, the transfer and distribution of DO in the immobilised biofilms were evaluated.

8.3 MATERIALS AND METHODS

8.3.1 Microbial strain and inoculum preparation

P. chrysosporium strain BKMF 1767 (ATCC 24725) was grown at 39°C on malt agar slants using a spore-inducing medium (SIM) described by Tien & Kirk (1988). The spores from the agar plates were harvested after 7 to 10 days, by suspending them in sterile water. This was followed by passage through sterile glass wool to entrap suspended mycelia. The inoculum consisted of an estimated 3×10^6 spores for each reactor. The fungal spores were inoculated through the permeate port and were forced onto the membrane using reverse filtration. Refer to Appendix A for growth and maintenance procedures used for the fungus and inoculum preparation.

8.3.2 Nutrient medium

The nutrient medium contained 100 ml of a basal medium solution: 100 ml of 0.1 M 2,2-dimethylsuccinate, 100 ml of 55.5 mM glucose, 100 ml of 0.02 M veratryl alcohol, 60 ml trace element solution, 25 ml of 1.1 mM ammonium tartrate, 10 ml of 10 mg.L^{-1} Thiamin-HCl, 1 ml of 10% (w/v) PF 68 and 504 ml autoclaved distilled water (Tien & Kirk, 1988). Refer to Appendix B for the preparation of stock solutions used in the preparation of the nutrient medium. For the control experiments, PF 68 was excluded from the nutrient medium and replaced with 1 ml of autoclaved distilled water.

Emulsions containing high concentrations of PF 68 were found to rapidly lose moisture at 39℃, an optimum temperature used for *P. chrysosporium* growth. When high concentrations of PF 68 were used in the SCMGRs, it resulted in a cake formation on the external surface of the capillary membranes, which hampered fungal growth. This necessitated the reduction of PF 68 amounts initially evaluated in batch cultures from ~8.5% (v/v) to 0.01% (v/v). A solution of 10% (w/v) Pluronic F 68 (Hangzhou Onicon Chemical Company LTD, China) was prepared separately. Furthermore, 1 ml of this solution was added per litre of nutrient medium, thus contributing 0.01% (w/v) of PF 68 in the nutrient medium. As perfluorooctyl bromide (PFOB) is denser than water, it settled at the bottom of the nutrient flasks due to insufficient surfactant levels. This resulted in the oil being pumped to the MGRs while the water-based nutrient medium remained in the flask even when rigorous agitation was used. Therefore, the pumping configuration was modified such that the PFOB was supplied to the top of the MGRs, concurrently with the 0.01% (w/v) PF 68-based nutrient medium. The PFOB and PF 68-based nutrient medium were pre-mixed, using an inline T-connection prior to entering the reactor at the top of the SCMGRs, as shown in Figure 8.1.

8.3.3 Perfluorooctyl bromide oil

The PFC oil, perfluorooctyl bromide (PFOB) (Exfluor Research Corp., USA), was used and supplied separately from the nutrient medium to the reactors. The supply rate of the oil prior to premixing with the nutrient medium was 1.62 ml.day$^{-1,}$ and it was such that it composed a ~0.1 (w/v) concentration fraction of the total suspension (nutrient medium and PFOB oil) supplied to the MGRs. The oil was maintained in a reservoir and aerated daily for 10 minutes. The PFC was filter-sterilised before being added to the reservoir.

8.3.4 Bioreactor sterilisation

All the materials (tubing, bioreactor and flasks) were autoclaved for 20 min before use. Sterile deionised water was used in the humidifiers and in the nutrient medium make-up. After assembling the MGR modules, the bioreactors were further chemically sterilised using a 4% (v/v) formaldehyde solution followed by rinsing with sterile distilled water before inoculation.

8.3.5 Nutrient flow rate, airflow rate and bioreactor setup

Prior to premixing with the PFOB, the nutrient medium, was pumped to the SCMGRs for the PFOB/PF-68 experiment at a rate of ~1.5 ml.h^{-1}; while for the control experiment, the nutrient flow rate was 1.68 ml.h^{-1}. The nutrient medium combined with the PFOB was supplied through the lumen of the capillary membrane by using a multi-channel Watson-Marlow 205S pump (Germany) to each MGR system with a combined flow rate of ~1.68 ml.h^{-1}. To avoid the sloughing off of the biofilm, an airflow rate of 1.88 ml.h^{-1} was supplied to the extra capillary space (ECS) of the MGR systems by means of multi-port air pumps with flow control to ensure that the flow was consistent through all the reactor modules. Air was also supplied to continuously-stirred nutrient flasks to improve DO in the nutrient for the control and PFOB/PF 68 experiments. The air supplied to the reactor systems and nutrient flasks was filter sterilised (using a 0.22 µm, acetate non-pyrogenic filter). The air was humidified before

being passed through the shell side of the reactors. Nine MGRs were used for this study. The SCMGR design and setup were as shown in Figure 8.1. The membranes used for the MGRs during this study were polysulphone membranes and are similar to those used when the MGR was commissioned and patented. The membrane characteristics have already been discussed in Section 3.3 and their characteristics listed in Table 3.1. To avoid PFOB settling in the silicone tubing used for the nutrient feed, and to improve PFOB distribution in the immobilised biofilms, the nutrient feed configuration was changed so that the premixed feed was fed from the top of the SCMGRs.

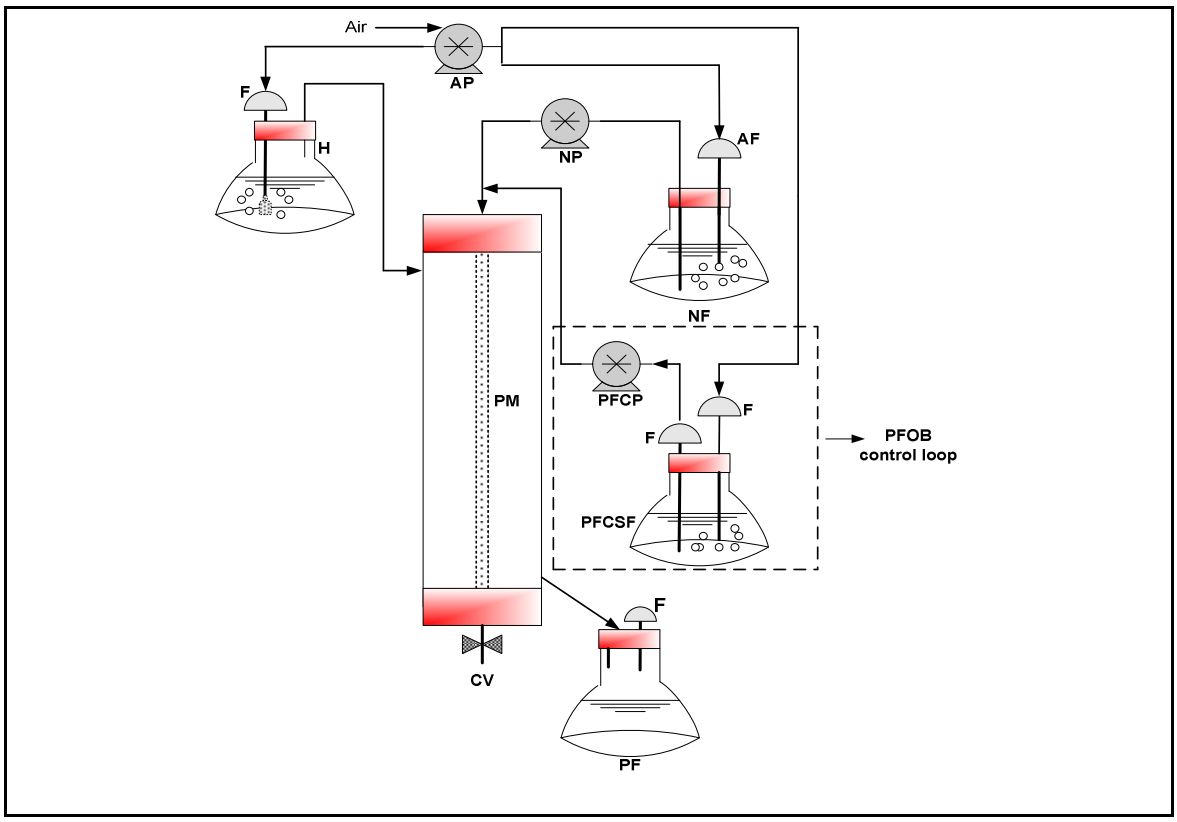

Figure 8.1: The MGR setup used for application of Perfluorooctyl bromide and Pluronic F 68. AF/F: air filter/ 0.22µm filter; AP: air pump; CV: closed valve (dead filtration mode applied); H: humidifier; NF; nutrient flask; PF: permeate flask; PFCP: perfluorocarbon pump; PFCSF: perfluorocarbon storage flask; PM: polysulphone membrane

Permeate samples were collected daily and were used to measure pH, redox potential, residual ammonium/glucose concentration, ethanol production, enzymes LiP/MnP and the volume of samples.

8.4 ANALYTIC METHODS

8.4.1 Ammonium tartrate and glucose concentration assays

Ammonium tartrate assays were determined using a Spectroquant Ammonium Test Kit (Merck, Germany) to measure the amount of ammonium tartrate present in the permeate samples collected. Because the suite of ligninolytic enzymes from *P. chrysosporium* are only produced under conditions of nutrient limitation, in this case nitrogen limitation, it was necessary to monitor the effective nitrogen source consumption in relation to operation time.

The glucose concentration in the permeate samples was established by using a Roche® D-glucose test kit (AEC Amersham, R.S.A.). The glucose concentration assays were performed in permeates of each MGR system to monitor the usage of glucose during biofilm development. Refer to Appendix D for a complete protocol of the ammonium and glucose assays.

8.4.2 Lignin peroxidase and Manganese production assays

LiP activity was measured according to the method of Tien and Kirk (1984) The oxidation of veratryl alcohol to veratryl aldehyde was recorded at 310 nm for 60 seconds, with one unit defined as 1 µmol of veratryl alcohol oxidised to veratryl aldehyde per 1 $\min\left(\varepsilon_{310nm} = 9300\ M^{-1}.cm^{-1}\right)$ in 1 ml of reaction volume. The reaction mixture contained 0.2 ml of veratryl alcohol (0.01 M); 0.2 ml of tartaric acid (0.25 M); 0.52 ml of enzyme solution and 0.08 ml of H_2O_2 (0.005 M).

MnP activity was measured by monitoring the change in oxidation state of Mn^{2+} to Mn^{3+} at 420 nm for 60 s $\left(\varepsilon_{420nm} = 36000\ M^{-1}.cm^{-1}\right)$. The reaction solution contained 0.35 ml of reagent A and reagent B, with 0.3 ml of enzyme supernatant in a total reaction mixture of 1.0 ml. Reagent A contained sodium succinate and sodium lactate buffers, each at 100 mM

with a pH of 4.5; 6 $mg.l^{-1}$ of egg albumin, 0.2 ml of $MnSO_4$ (0.95 $mg.ml^{-1}$) and 80 $\mu g.ml^{-1}$ of ABTS (2.2-azino bis-3-ethyl-benzothiazoline-6-sulfonic acid). Reagent B contained 100 µM of H_2O_2 (Gold & Glenn, 1988). All reagents and their concentrations used in the ligninolytic assays are listed in Appendix C.

8.4.3 Ethanol assays

The ethanol concentration in the permeate samples was determined by using a Roche® (Germany) ethanol test kit. The overall ethanol concentration produced was determined using permeate samples from each SCMGR. The ethanol concentration was determined by following the assay protocol by absorption at 340 nm. Refer to Appendix D for a complete protocol of an ethanol assay.

8.4.4 Determination of DO penetration depth and biofilm thickness

The SCMGR systems were dismantled by removing the glass manifold and exposing the membrane-attached biofilm for easy microsensor measurement. The bioreactors were disconnected in triplicate at time intervals of 192 h and 264 h. A Clark-type oxygen microsensor (OX 10, outer-tip diameter less than 20 µm) supplied by Unisense (Denmark) was used to measure the DO across the biofilms at interval depths of 50 µm. The setup consisted of a high-sensitivity picoammeter connected to the microsensor, which was fixed to a micromanipulator that was used to move the microsensor into the biofilm. The picoammeter was connected to a computer with *Profix v1.0* software for data capturing. The microsensors were used to measure the DO concentrations in duplicate from three different MGRs. Measurements were performed in biofilms at the top and bottom of the active membrane length. The microsensor set-up and calibration are discussed in Appendix G.

Biofilm thicknesses were determined at the top and bottom of the active membrane length, at the same position where the DO profiles were measured. The biofilm thickness was

determined by using a Carl Zeiss light-microscope and Axiovision digital imaging system equipped with measuring software. The objective used for the measurements was a 2.5x magnification. The calibrated microscope objectives acquired real-size measurements from biofilms attached to the polysulphone membrane surface. The biofilm thickness was calculated by subtracting the clean membrane outer diameter from the membranes with biofilm growth. The value obtained was divided by two and averaged to obtain the actual biofilm thickness.

8.4.5 Accumulation of trace elements in biofilms

Dried fungal biomass, between 37.9 to 80.1 mg, was weighed into digestion vessels and decomposed in a mixture of 4 ml of a 65% HNO_3 solution and 1 ml of a 30% H_2O_2 solution (Čurdová *et al.*, 2004). Digestion was accomplished using the following microwave program: 7 minutes at 120 W, 2 minutes at 460 W and 1 minute at 700 W. The samples were cooled for 1 h, and the digests were filtered using glass wool and a 0.22 µm filter. The volume was made up to 30 ml with deionised water. The digests were analysed at the University of Stellenbosch (R.S.A), using an ICP-MS to determine copper (Cu) and Manganese (Mn) in the digested filtrate. Negative control samples contained only solutions of HNO_3 and H_2O_2.

8.4.6 Measurement of oxidative stress (Malondialdehyde formation)

After the DO measurements, a section of the biofilm was surgically separated and rinsed in a 5 mM butylated hydroxyl toluene (BHT) solution supplemented with a 20 mM hydroxymethyl amino methane–hydrochloride (Tris-HCl), before storage at -80℃ in the Tris-HCl solution. This was done to prevent new lipid peroxidation during biofilm storage. This solution was also used in the homogenisation and biomass lysis process for MDA and glucan determinations. See Appendix F for the preparation of the solutions used for antioxidation preservation.

The formation of MDA was determined with an MDA assay kit (Northwest Life Sciences Specialties, Vancouver, WA) following the manufacturer directions. Preserved biofilms stored at -80℃ were homogenised with glass beads in 2 ml Eppendorf tubes using a vortex mixer until the mixture was well homogenised. The biofilm homogenates were centrifuged at 10 000 x g for 10 min to obtain a clear supernatant before being used in the assay. The MDA in homogenates was determined by following the assay protocol by absorption at 532 nm. A complete protocol for the measurement of MDA is listed in Appendix E.

8.4.7 Glucan determination

Glucan concentration in homogenates was measured using a mixed-linkage β-Glucan assay kit acquired from Megazyme (Ireland). The supernatant used for MDA determination was also used for glucan determination. The concentration of β-glucans in homogenates was determined by following the assay protocol (Refer to Appendix E) by absorption at 510 nm using 100 µl of the homogenised biofilms.

8.5 RESULTS AND DISCUSSION

This part of the study focused on the evaluation of ligninolytic activity and biomass performance of *P. chrysosporium* using PFOB oil in combination with PF 68-supplemented nutrient medium within the SCMGR system. The application of the emulsion to the MGRs was conceptualised to increase and improve the DO in the immobilised biofilms in order to improve enzyme production from the biofilm during the secondary metabolic stage.

8.5.1 General bioreactor operation and performance

Figure 8.2 shows that a similar trend in the reduction of pH was observed in the permeate collected from the MGRs for control experiments and experiments in which PFOB and PF 68 were utilised. A steady decline in the permeate pH for PFOB experiments was observed after

48 h of MGR operation. The pH for both the control and PFC experiments stabilised after 72 h at ± 3.5. The results obtained were similar to those obtained in Chapter 5 (Figure 5.2) for aerated SCMGR systems. This was expected, as the fungus adjusted to the presence of the PFC oil and surfactant in nutrient medium. It had previously been determined that MnP and LiP had stable pH ranges of 2.6 to 4.5 and 2.0 to 5.0, respectively (Wesenberg *et al.*, 2003). Overall, the bioreactors operated within the range suitable for MnP and LiP production, and the pH range in which LiP and MnP are stable.

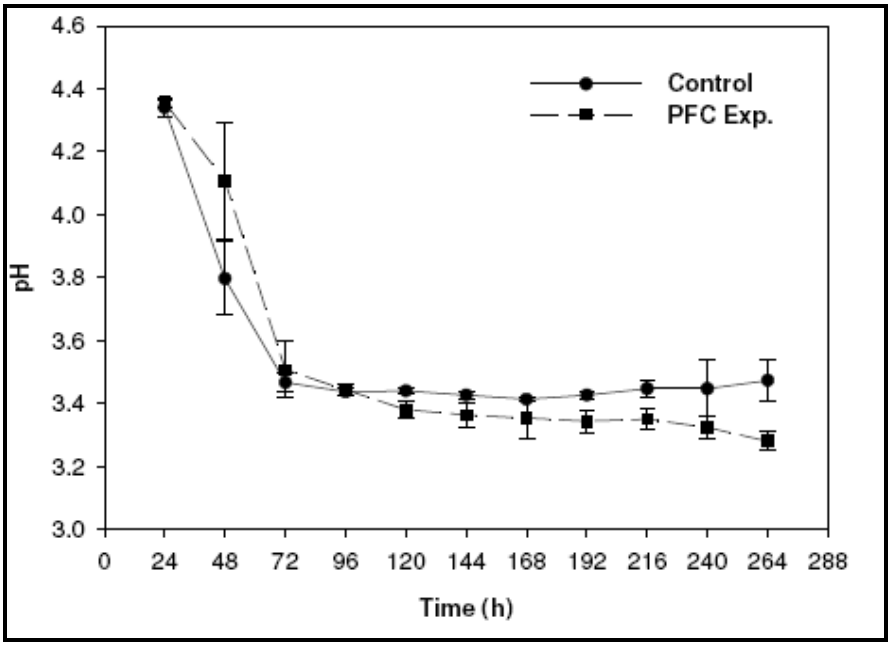

Figure 8.2: pH profile observed for permeate collected from MGRs in which PFOB and PF 68 were used

By monitoring the redox potential of recovered permeate, the presence of ligninolytic enzymes could be forecasted with the increase in the redox potential of the permeate. An increase in the redox potential indicated enzyme production, while a decrease or stagnant redox potential showed inefficient enzyme production. Figure 8.3 shows the redox potential of the control and PFOB/PF 68 emulsion experiments of the MGRs. The redox potential of the control and PFC-based experiments was greater than 200 mV after 72 h of MGR operation. Slightly improved redox potentials were obtained during this part of the study

compared to those obtained in aerated SCMGRs (Chapter 5, Figure 5.2). The performance of the PFOB/PF 68 bioreactors showed a higher redox potential than control experiments from 216 to 264 h. During the same period, the redox potential of control experiments declined. This suggested that the PFOB/PF 68-based MGRs might be more suitable for prolonged operation as a steady trend of increasing redox potential was observed.

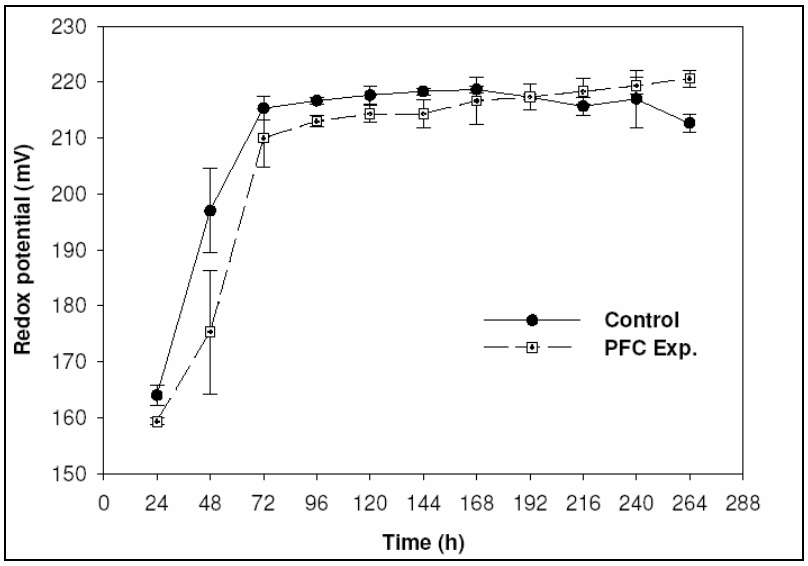

Figure 8.3: Redox potential of permeate samples collected from the MGRs showing active enzyme production by immobilised biofilms

The average volume of permeate collected from control and PFOB MGRs were dissimilar and inconsistent during the study, as is shown in Figure 8.4. Since, the nutrient flow rate was 1.68 ml.h^{-1} for control experiments, the expected permeate volume was 40.32 ml.day^{-1}. Similarly, for the PFOB experiment, the nutrient flow rate was 1.5 ml.h^{-1}; together with ~0.18 ml.h^{-1} PFOB, resulting in an theoretical overall permeate volume of ~38 ml.day^{-1}. For both the control and PFOB-based experiments, a reduction in the averaged permeate volumes reduction was observed during the MGRs' operation. This was expected, as fungal biomass grew in the porous structure of the membranes, decreasing membrane permeability. Furthermore, it was assumed that membrane permeability was also affected by the presence of PF 68 in the nutrient medium, which resulted in membrane fouling. The average permeate

volume for control experiment during the MGRs operation was ~38 ml.day^{-1}, while an average of 26 ml.day^{-1} for the PFOB experiment was observed.

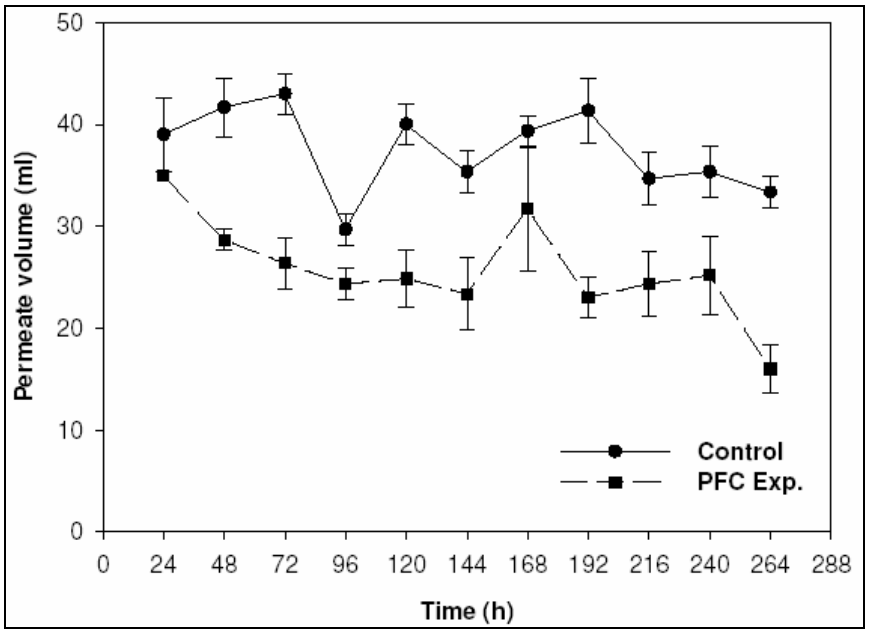

Figure 8.4: Averaged permeate volume collected at 24 h intervals for perfluorooctyl bromide MGRs experiment compared to control experiments

8.5.2 Biofilm thickness

As biofilm thickness can be used to monitor growth kinetics of microorganisms in MGRs, the thickness of *P. chrysosporium* was monitored to determine the effect of PFOB and PF 68 on the immobilised fungus. As the operation of the MGRs was changed to improved nutrient medium and PFOB hydraulic residence time, the growth of the fungus was evenly distributed in the control cultures. However, the introduction of PFOB and PF 68 resulted in the biofilm being thicker at the top of the bioreactor compared to the biofilm at the bottom of the reactors, as illustrated in Table 8.1. In control the MGRs, the averaged biofilm thickness at the bottom was ~2250 μm. This was more than the biofilm thickness at the bottom of the PFOB and PF 68-based bioreactors, which was at an average of 1100 ± 308 μm. The observed biofilm reduction at the bottom of the MGRs was attributed to the settling of PFOB at the bottom of reactor, as high concentrations of PFCs were observed to reduce biomass

production in batch cultures. This reasoning was further validated by the fact that: 1) PFOB has a density of 1.93 $g.ml^{-1}$, 2) insufficient quantities of PF 68 were used and 3) PFOB was not introduced into the MGRs in an emulsified form.

Table 8.1: Biofilm thickness determined for MGRs supplied with PFOB

	Control cultures*	PFOB cultures▪	
Time (h)	Bottom	Top	Bottom
192 h	n/d	2516 ± 141 μm	833 ± 216 μm
264 h	2250 ± 110 μm	3104 ± 504 μm	1100 ± 308 μm

n/d – not determined
∗ - Nutrient medium fed from the bottom
▪ - Nutrient medium fed from the top

8.5.3 Glucose and Ammonium consumption

The average glucose concentration measured in the nutrient solution using the assay kit was 10.5 $g.L^{-1}$. The ammonium (NH_4^+) concentration in the nutrient feed was determined as 39.7 $mg.L^{-1}$ compared to the theoretical 39 $mg.L^{-1}$. The production of peroxidases by *P. chrysosporium*, took place as an extracellular process using mechanisms dependant on nutrient availability (Breen & Singleton, 1999).

The production of LiP and MnP enzymes was induced by nitrogen and carbohydrate (carbon source) limitation (Bumpus & Aust, 1987). As shown in Figure 8.5, the ammonium (ammonium tartrate) source was rapidly depleted after 72 to 96 h of MGR operation, representing a 95% depletion.

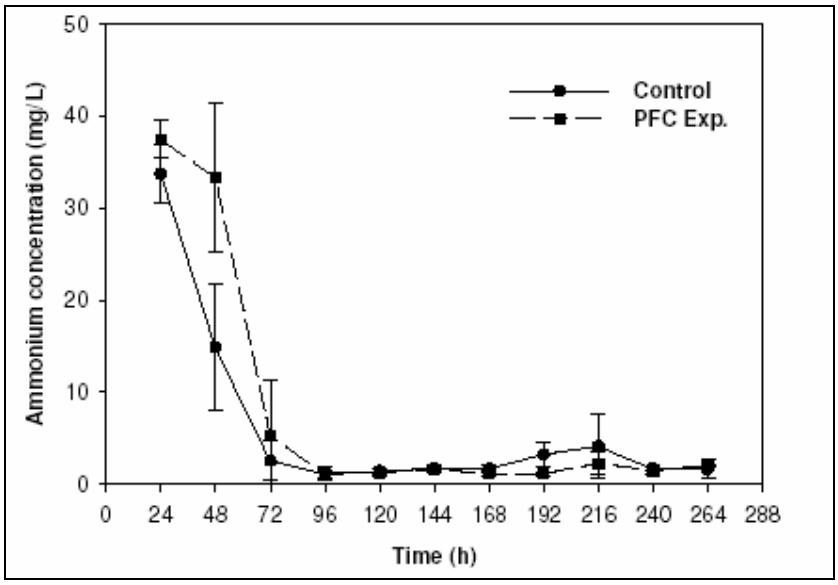

Figure 8.5: Ammonium (NH_4^+) concentration in the permeate samples collected from MGRs operated for 264 h

The rapid consumption of the ammonium source observed in this study was similar to those measured in batch cultures by Rothschild*et al.* (1995) and Keyser*et al.* (1978). The ammonium depletion after 72 to 96 h coincided with the increase in redox potential, which was above 200 mV after 72 h, indicating the production of enzymes under nitrogen limitation for this fungus.

The addition of PFOB to the MGRs had an effect on the consumption of glucose, as shown in Figure 8.6, when comparing the control to the PFOB/PF 68 MGRs. A difference was observed in the periods of 24 to 144 h, where control cultures showed slightly higher glucose consumption compared that of the to PFOB MGR culture. Glucose consumption averaged at 0.7 $g.day^{-1}$ for PFOB MGRs compared to 1.27 $g.day^{-1}$ for the control cultures, representing a 55% difference. These results were in contradiction with results obtained from batch cultures, where the addition of PFOB resulted in an increase in glucose consumption. This was attributed to the continuous PFOB supply to the MGRs, which reduced the amount of biomass generation at the bottom of the reactor.

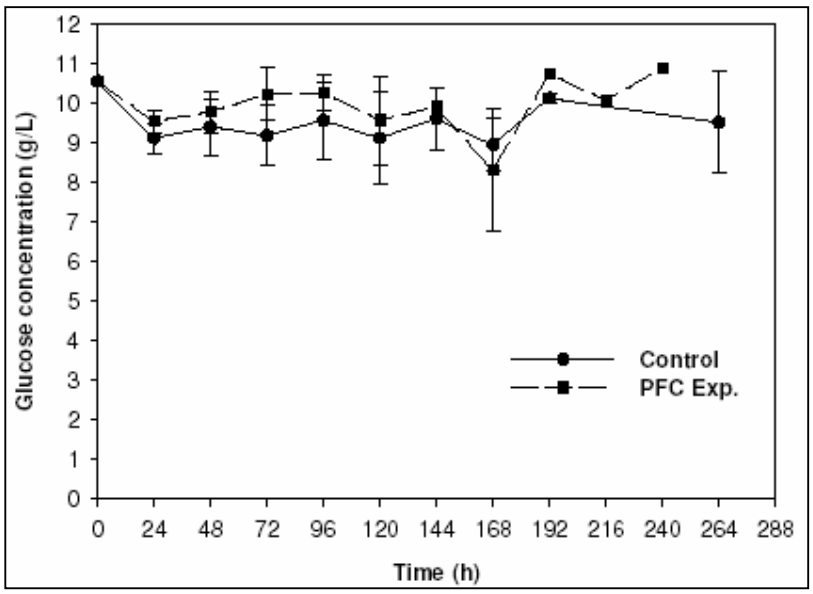

Figure 8.6: A comparison of averaged glucose consumption for PFOB-based MGRs and control cultures

8.5.4 Dissolved oxygen distribution in immobilised biofilms and related parameters

The DO in the PFOB and PF 68-based immobilised biofilms was measured at 192 h and 264 h, both at the top (thicker biofilm) and at the bottom (thinner biofilms) of the SCMGRs, as shown in Figure 8.7. The averaged DO penetration depth for biofilms at the top of the reactor was 250 μm and 400 μm at time intervals of 192 h and 264 h, respectively. From the DO penetration depth (Figure 8.7) and biofilm thickness obtained (Table 8.1), it was clear that the presence of anaerobic zones was still irrefutable. Similar DO penetration results were observed in biofilms in which the DO was measured for periods 120 to 264 h in Chapter 5, Figure 5.4. Previously, the oxygen penetration ratio, which is the ratio between DO penetration depth and biofilm thickness obtained after 264 h of MGR operation (Figure 5.6) was determined as ~0.15. However, the introduction of PFOB resulted in improved DO penetration ratio of 0.36 for biofilms at the bottom of the SCMGRs (thinner biofilms), while penetration ratio of 0.21 was determined in thicker biofilms at the top of the SCMGRs in this part of the study. This represented an average of ~40% increased DO penetration depth

between that was obtained in Chapter 5 (0.15) and that obtained for PFOB-PF 68 MGR cultures (0.21).

The high variability of DO profiles obtained at 192 h and 264 h at the top of the bioreactors was attributed to biofilm heterogeneity. The profile shapes also indicated limited oxygen consumption and it was assumed that *eddy* currents were the driving force of DO in this part of the biofilms as the area was an entry point for the humidified air. However, at the bottom of the bioreactors, a higher DO penetration depth, in excess of 650 μm, was observed, which was an improvement on the maximum DO penetration depth of 530 μm determined in thinner biofilms after 72 h of aerated SCMGR operation previously shown in Figure 5.4. In this study, unconventional DO profiles were obtained at the bottom of the reactor, where DO concentration started increasing at penetration depths exceeding 650 μm, as depicted in Figure 8.7A. This further ratified the assumption that PFOB settled in the lumen of the SCMGRs. At 264 h (Figure 8.7B), similar DO penetration was observed as illustrated in Chapter 5, although increasing DO concentrations towards the membrane surface were not observed previously. Overall, there was poor distribution of PFOB in the lumen of the capillary membranes was evident, but it is apparent that PFC application in MGRs can be utilised to increase DO availability.

When comparing the profiles at the bottom of the reactors between 192 h and 264 h, in Figure 8.7, it was evident that the DO in the biofilm was becoming exhausted, with DO concentration being less than 0.1 $g.m^{-3}$ at a depth of 650 μm after 264 h of operation.

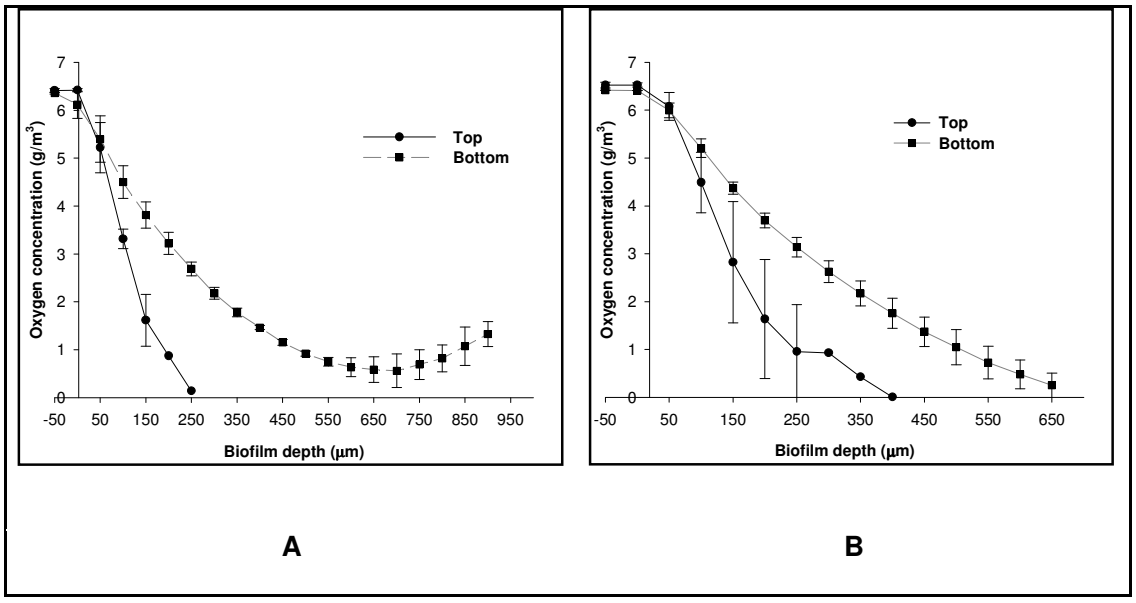

Figure 8.7: Dissolved oxygen distribution in PFOB-based MGR at time intervals of: a) 192 h and b) 264 h

As DO limitation in *P. chrysosporium* cultures leads to ethanol production, the determination of ethanol production was of paramount importance, as illustrated in Figure 8.8.

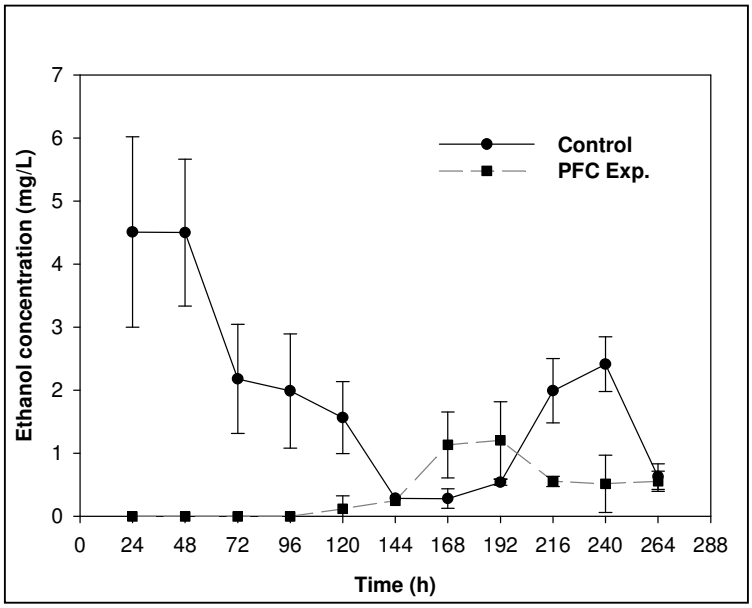

Figure 8.8: Ethanol production in MGRs systems supplied with a PFOB and PF 68-based emulsion

As a further improvement to the MGR design and to limit ethanol production, the nutrient medium for both the control and PFOB/PF 68 was aerated continuously. Lower ethanol production, less than 1 $mg.L^{-1}$, was observed during 144 h and 192 h, which is a period previously identified as a phase characterised by decelerated and stagnating growth for *P. chrysosporium* immobilised in the MGR (Ntwampe & Sheldon, 2006; Sheldon *et al.*, 2008). Very low ethanol production was determined for PFOB experiments. Surprisingly, during the deceleration and stationary growth phases (144 to 196 h), increased ethanol presence in the permeate was observed for the control experiment. The observed trend was previously seen as illustrated in Chapter 6, Figure 6.6, in which higher ethanol production was observed during the period of 96 to 192 h for SCMGRs and MCMGRs. Overall ethanol production was lower in the reactors supplied with PFOB and PF 68 than in the control experiments. In the control cultures, the ethanol detected in the permeate samples was less than 10 $mg.L^{-1}$, as was previously observed (Chapter 6, Figure 6.6), and decreased with time for the PFOB/PF 68 results.

8.5.5 Continuous ligninolytic enzyme production in perfluorooctyl bromide MGRs

Generally lower extracellular enzymes LiP and MnP (shown in Figures 8.9A and 8.9B), production was observed. There was a high degree of variability between individual reactors. Figure 8.9A shows that LiP production was higher in control cultures to when PFOB was used in the MGR. The application of PFOB in the MGR clearly needs to be investigated further. Figure 8.9B shows that the MnP production was higher in the PFOB/PF 68 experiment than the control experiments. Also, MnP production was higher in PFOB/PF 68 SCMGRs than LiP. As LiP was determined to be influenced and induced by reactive oxygen species (ROS) (Belinky *et al.*, 2005), the production of LiP might have been hampered by the negligible presence of ROS. Comparing this study to earlier results in Chapter 7, the batch-based PFC cultures performed better than the MGR system.

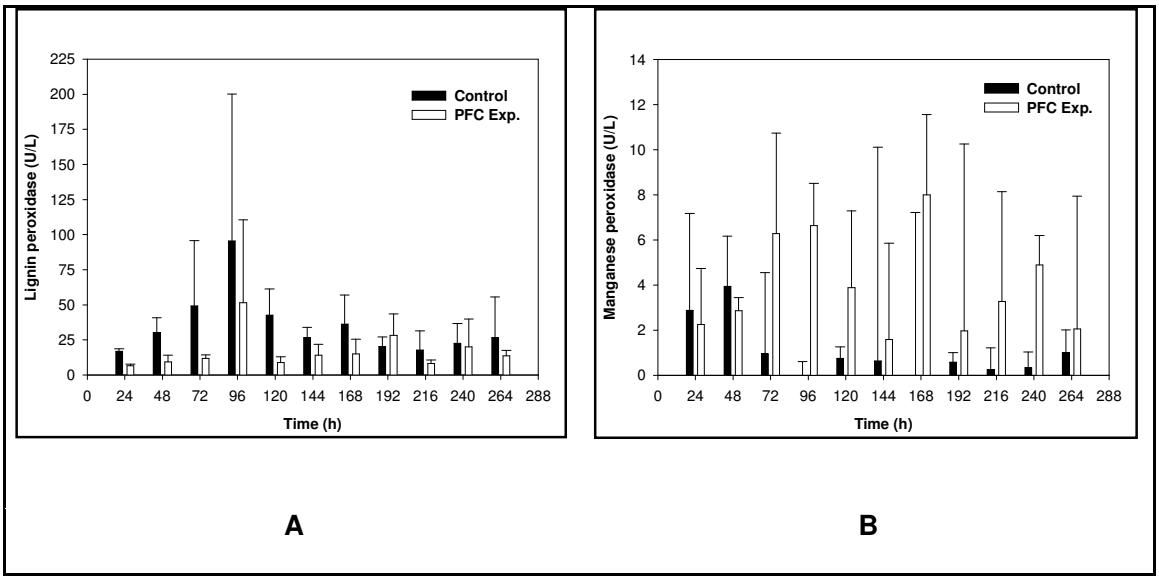

Figure 8.9: A) Lignin peroxide and B) Manganese peroxidase production from reactors supplied with PFOB/PF 68

8.5.6 Biofilm associated performance and parameters

Table 8.2 illustrates the comparison of glucan storage in *P. chrysosporium* biofilms using the nutrient medium of Tien and Kirk (1988) to the PFOB/PF 68 medium used in this part of the study. Comparable values of glucan storage were observed between both sets of studies, with glucan storage reaching 1.71 mg.ml^{-1} after 264 h of MGR operation. The presence of glucan and the values obtained in this study after homogenate analysis were higher than those obtained in Figure 6.8 (Chapter 6), where the nutrient medium was supplied from the bottom of the bioreactor. The values changed from a range of ~0.2 to 0.9 mg.ml^{-1} to the current ~1.64 to 1.99 mg.ml^{-1}. The availability of PFOB and PF 68 did not contribute to a reduction in the storage of glucan. As glucan restricts DO transport in mycelia, the availability of PFOB as an oxygen carrier in the immobilised biofilms should counteract the resistance to transfer of DO.

Table 8.2: An assessment of β-glucan storage in biofilms exposed to PFOB/PF 68 in SCMGRs

Time (h)	Control cultures ($mg.ml^{-1}$)*	PFOB and PF 68 cultures ($mg.ml^{-1}$)*
96 h	1.64 ± 0.05	n/d
192 h	1.99 ± 0.06	1.18 ± 0.01
264 h	1.71 ± 0.08	1.71 ± 0.07

n/d – not determined
* - The units are described as mg/ml of homogenised biomass extracts

Increased polysaccharide storage was earlier shown to entrap trace-element ions (Chapter 6, Table 6.2). Manganese (Mn) and copper (Cu) accumulation were measured during this study and the results are listed in Table 8.3. Reduced amounts of Mn and Cu were observed in cultures where a PFOB-based medium was supplied. One reason for the reduced metal accumulation was that flux values in these cultures were generally lower than that of the control cultures. Therefore, the reduced presence of Mn and Cu in biofilms was assumed not to be directly linked to the presence of PFOB/PF 68. As expected, by increasing the nutrient hydraulic residence time in the biofilms, there was a noticeable increase in Mn and Cu accumulation compared to cultures studied in Chapter 6 (Table 6.2).

Table 8.3: Metal accumulation in biofilms supplied with PFOB compared to control cultures

Time (h)	Control cultures (mg/g dry biomass)		PFOB cultures (mg/g dry biomass)	
	Mn	Cu	Mn	Cu
96 h	121 ± 0.0	32.0 ± 0.0	52.4 ± 0.0	19.3 ± 0.0
192 h	67.6 ± 2.25	12.8 ± 0.26	29.7 ± 1.26	9.2 ± 1.63
264 h	34.7 ± 1.75	6.1 ± 1.34	28.8 ± 1.11	5.8 ± 0.44

The presence of lipid peroxidation was evident, as measured by the formation of malondialdehyde (MDA), also previously determined in Chapter 6 (Figure 6.7). As shown in Table 8.4, the application of 0.01% (w/v) PF 68 to the MGRs showed a slight improvement in terms of MDA presence in the harvested biofilms. After 264 h, MDA formation in the control

cultures averaged at ~31.06 $\mu M.ml^{-1}$ compared to the averaged concentration of 30.75 $\mu M.ml^{-1}$ of homogenised biomass. Similar results of 28.26 $\mu M.ml^{-1}$ of homogenised biomass in control cultures and 26.34 $\mu M.ml^{-1}$ of homogenised biomass in PFOB/PF 68 cultures were observed after 192 h of operation. In the case of MDA formation, the orientation of the nutrient supply line had a negligible effect on lipid peroxidation in the MGRs.

Table 8.4: Malondialdehyde (MDA) formation in biofilms exposed to PFOB/PF 68 in MGRs

	Control cultures	PFOB and PF 68 cultures
Time (h)	**($\mu M.ml^{-1}$)***	**($\mu M.ml^{-1}$)***
96 h	14.75 ± 0.09	n/d
192 h	28.26 ± 0.45	26.37 ± 0.11
264 h	31.06 ± 2.17	30.75 ± 0.95

n/d – not determined
* - The units are described as $\mu M.ml^{-1}$ of BHT homogenised biomass extracts

8.6 CONCLUSIONS

The application of PFOB and PF 68 to the MGRs contributed a small but significant difference into the performance of the bioreactors. The reduction in pH and an increase in the redox potential were similar for the control experiments in which a defined medium was used. A reduction in the flux of the PFOB/PF 68 bioreactors was attributed to possible caking in the membrane lumen. Therefore, it was concluded that the presence of PF 68 in the nutrient medium contributed to the low fluxes obtained.

Glucose and ammonium consumption was higher in the control experiments during the initial stages of the bioreactor operation, as the *P. chrysosporium* adjusted to the presence of PFOB in the PFC experiment. DO distribution was better at the bottom of the reactor, where increased DO availability was seen to improve towards the membrane surface, with anaerobic zones observed in those biofilms immobilised at the top of the membranes in the reactors. Although anaerobic formation was evident, the DO penetration and distribution was

improved, resulting in significant ethanol production reduction and improved MnP production. LiP presence in control cultures was high compared to PFC/PF 68 cultures. This was attributed to a low generation of ROS corroborated by less MDA formation in PFOB/PF 68 cultures, for which MDA quantification was used as a measure of lipid peroxidation for the cultures.

An increase in glucan presence and, subsequently, in its storage in the harvested biomass were higher in this study compared to those bioreactors in which the nutrient medium was fed from the bottom of the MGRs. However, the prevalence of glucan was comparable between control and PFOB/PF 68 cultures. Younger biofilms were observed to have higher a trace elements presence with a decrease in metal-ion presence in older biofilms. From the results, it is clear that the application of PFOB and PF 68 to the immobilised biofilms resulted in metal ion presence.

8.7 SUMMARY

Significant contributions of the PFOB/PF 68-based nutrient medium to the performance of immobilised *P. chrysosporium* biofilms in the MGRs were:

- Reduced ethanol production detected in permeate samples collected, with ± 1 $mg.L^{-1}$ ethanol production during 168 h and 192 h.
- Reduced lipid peroxidation in younger biofilms.
- Reduced β-glucan in younger biofilms.

The subsequent chapter (Chapter 9: Overall discussion and conclusions) discusses the overall results obtained over the course of the research. The discussion includes difficulties experienced during the study and solutions developed to complete the project.

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CHAPTER 9

OVERALL DISCUSSION AND CONCLUSIONS

CHAPTER 9

OVERALL DISCUSSION AND CONCLUSIONS

9.1 INTRODUCTION

In aerobic bioprocesses, dissolved oxygen (DO) plays a critical role in the use of nutrients to produce high-value bioproducts. The supply of oxygen in these bioprocesses determine the product yields and recovery costs, making DO transport, including regulation, a paramount parameter for effective biomass and bioreactor performance. As a coping mechanism, many aerobic microorganisms respond to anoxia by producing bioproducts of less value. The membrane gradostat reactor (MGR) and concept that were used in this study were invented to provide a method and technology for the continuous production of secondary metabolites. When the MGR was designed and patented, the inventors overlooked certain parameters related to biomass performance during bioreactor operation, which included:

- The use of a nutrient medium designed for batch cultures.
- DO mass transport limitation.
- Trace-element ion accumulation
- Hyperoxia/oxidative stress due to the use of high partial pressures of oxygen to improve secondary metabolite production
- Poorly established nutritional gradostats due to gravitational influences on the distribution of nutrients

The focus of this study was to improve the operational capabilities of the MGR system and the performance of immobilised biomass for aerobic cultures, particularly oxygen delivery to the system. This thesis described the parameters that limit *P. chrysosporium* biomass performance in relation to the MGR concept. Besides monitoring *P. chrysosporium* biomass

performance during this study, the concepts developed can potentially be adopted and adapted for use with any aerobic microorganism.

9.2 OVERALL DISCUSSION

9.2.1 General aspects of dissolved oxygen in biofilms

During fungal bioprocesses in batch cultures, the broth of filamentous mycelia can become viscous. This limits the gas to liquid mass transfer of DO leading to low internal DO transfer in the fungal biomass. This was also observed for *P. chrysosporium* biofilms, where the DO penetration depth was limited to less than 600 μm in aerated biofilms immobilised in the MGR. The DO penetration depth represented less than 30% of the actual biofilm thickness achieved in the MGRs operated for 264 h. However, younger biofilms showed higher DO penetration depth compared to older biofilms. The consequence for this was the production of an unwanted by-product, ethanol, which was shown to deactivate enzymes produced by *P. chrysosporium*.

At 25°C, in air-saturated water, the DO concentration is 8.4 $g.m^{-3}$ at 1 atm. The DO concentration achieved at the biofilm surface of *P. chrysosporium* biofilms immobilised in the MGR's was 6.5 $g.m^{-3}$ when the ECS was aerated. This represented a 23% reduction of DO concentration attributed to the liquid-film interface and mucilage on the biofilm surface. Moreira *et al.* (1997) demonstrated that the continuous production of MnP was achieved at DO greater than 25 $g.m^{-3}$, with protease activity less than 5 $U.L^{-1}$. In other studies, the recycling of the ligninolytic supernatant reduced MnP production, while protease activity increased with the recycling ratio. This was associated with the DO depletion in the recycled supernatant. Sheldon (2008) determined that a more porous layer existed in the aerial mycelia. From these observations and conclusions, convective oxygen transport should have been prevalent. However, in this study, after using averaged profiles obtained after 72 , 120 , 168 , 216 and 264 h of SCMGR operation, the mass transport of oxygen into the biofilm was

determined to be diffusive with an average flux of 0.35 $g.m^{-2}.h^{-1}$ with a liquid-film hydrodynamic layer of less than 20 µm. Although the use of pressurised bioreactors to improve DO transport in bioreactors of *P. chrysosporium* is advisable, Venkatadri *et al.* (1992) determined that thinner biofilms had increased effective oxygen transport when compared to biofilms in high-pressured bioreactors. This was also confirmed in this study, where a penetration depth of ~550 µm was accomplished after 72 h of MGR operation when compared to other periods in which DO measurements in the biofilms were performed.

The resultant flux determined in this study for young and thinner biofilms was surprisingly lower than that of older biofilms, although older biofilms had a lower DO penetration ratio. Controlling and reducing the thickness of the biofilms in MGR systems will effectively render the bioreactor design redundant, as an effective nutritional gradostat in immobilised biofilms will not be established, thus limiting the capacity of biofilms kept in the idiophase. In this study, it was seen that the DO flux in *P. chrysosporium* biofilms immobilised in MGRs was independent of biofilm thickness but was dependent on different biofilm growth phases. During active mycelia growth cycles, higher DO fluxes occurred more often than during stationary phases. During these phases, higher DO consumption rates were determined when air was used. The consequence of low DO transport was ethanol production, which increased with increasing biofilm- and anaerobic zone thickness.

When technical-grade oxygen was used, ethanol production increased because of an increase in glucan production in the immobilised fungus. Furthermore, by using this technique, lipid peroxidation was evident and reduced the effectiveness and performance of the immobilised biomass. DO transport is the most studied area of aerobic bioprocesses; surprisingly, however, DO and efficient gradostat development for the MGR have been neglected and overlooked in the process of developing this technology.

9.2.2 Other factors influencing MGR immobilised biofilms

Since the MGR was conceptualised as a bioreactor to replace submerged batch fermentation systems for the production of pharmaceuticals, there are other factors that obviously influence the successful use of the system with different microorganisms, as discussed in previous chapters of this thesis.

From this study, it was evident that trace element accumulation in the MGR system can be problematic for microorganisms with a high affinity for trace-element ion accumulation. As the MGR was designed as a generic bioreactor system for microorganisms that continuously produce high-value extracellular secondary metabolites, it is imperative that the microorganism used in the systems could cope with a continuous supply of trace element ions present in the specified nutrient medium. Cell lysis is common in fermentation systems in which a high content of ions is prevalent; therefore, the ability of a microorganism to assimilate accumulated trace element at a higher rate than the rate at which they are supplied to the MGR needs investigation. This preliminary evaluation was paramount for the success and viability of the culture in the bioreactor, particularly the MGR, for prolonged bioreactor performance and effective biomass development.

During the formation and assimilation of biofilms, the role played by exopolysaccharides during this process is paramount, as they act as binding agents. Their production and storage is common in biofilm development, and more so in fermentation systems where high microbial concentrations and densities are required for effective bioproduct production. In this study, glucan was identified as a polysaccharide that is stored by *P. chrysosporium*, resulting from the availability of excess glucose and as a defence mechanism. Although microorganisms can use the polysaccharides during nutrient starvation, they can also limit their transport, thus leading to the entrapment of essential and nonessential micronutrients in the cultures. The resultant effect is the production of unwanted by-products as the microorganisms try to cope with environmental influences in bioreactors. In this study, the

storage of glucan led to DO transport limitations and a consequent increase in ethanol production. The use of additives to overcome the production and storage of polysaccharides is common. This will be the case with the use of the MGR, especially when the microorganism used within the bioreactor stores or produces excessive quantities of polysaccharides.

9.2.3 Application of perfluorocarbon emulsion for use in a MGR

In Chapter 7, *P. chrysosporium* performance was evaluated in PFC emulsions using batch bioreactors. The role of PFC emulsions on the generation of biomass, the rate of substrate consumption (ammonium source and glucose) and enzyme production was determined using PFOB, PFPA and PFBE. The results obtained are summarised in Table 9.1. The compatibility of PFCs with *P. chrysosporium* had never before been explored, with limited information available on the use of PF 68 in the studies of this fungus. The surfactant commonly used in *P. chrysosporium* studies, Tween 80, was shown to protect enzyme deactivation. Its role in terms of protection against trace metal ion, hyperthermia, and prolonged biomass protection in bioreactors remains unclear. As the development of high densities of biomass are required in the development of the MGR, the results represented in Table 9.1 were based on the consumption of glucose, which was the main carbon source influencing biomass generation and maintenance for both batch and continuous bioreactor systems.

9.2.4 Adjustment of nutrient supply configurations for the MGR

The MGR system was designed such that nutrient feed is supplied from the bottom (inlet) of the reactor, while the top (outlet) remained closed (dead-end filtration mode). This led to uneven biofilm development as observed by Sheldon and Small (2005). The biofilm thickness achieved at the top of the reactor was 49% less than that achieved at the bottom of reactor. Fredericks (2007) confirmed these results, where a 59% difference was observed,

with thicker biofilms developing at the bottom of the reactor. This was attributed to the dead-end filtration mode used for the reactors. Garcin (2002) suggested the reason for this was that nutrient distribution and permeation was uneven along the length of the membrane .

To improve the biomass development and the hydraulic residence time of the nutrient medium in the immobilised biofilms, the design of the MGR was changed so that the nutrient medium was fed from the top of the reactors in Chapter 8 instead of from the bottom of the reactors (Chapters 5 and 6). The mode of the medium supply was kept as a dead-end filtration mode. The change in the supply configuration was done in order to develop evenly distributed biofilms on the membrane surface and improve the residence time of nutritional sources for the fungus. From physical observations, it was clear that the biofilm growth pattern was similar to that in which the nutrient medium was supplied from the bottom of the reactor without any DO carriers. Previously, the nutrient medium supplied to the MGR biofilms was not aerated. Instead, high partial pressures of oxygen were used in the ECS of the bioreactor to induce higher secondary metabolite production. In Chapter 8, the medium supplied to the MGR was agitated and aerated with sterile air.

Table 9.1: The determination of biological constants related to perfluorocarbon and *P. chrysosporium* studies

Experiment	Biomass generated (mg)	Biomass concentration ($mg.ml^{-1}$)	Glucose consumed ($g.L^{-1}$)	Growth yield coefficient, $Y_{x/s}$ (g biomass/g glucose)	Glucose consumption rate, dS/dt ($g.L^{-1}.h^{-1}$)	Enzyme activity LiP/MnP
Control	3	0.20	2.29	0.09	0.011	+/+
Control (PF 68)	20.8	1.39	3.44	0.40	0.016	-/+
0.1 (w/v) PFPA	19	1.27	4.44	0.29	0.021	+/+
0.2 (w/v) PFPA	14	0.93	2.89	0.32	0.013	+/+
0.3 (w/v) PFPA	12.3	0.82	2.57	0.32	0.012	+/+
0.1 (w/v) PFOB	19.1	1.27	4.14	0.31	0.019	+/+
0.2 (w/v) PFOB	13.9	0.93	2.89	0.32	0.013	+/+
0.3 (w/v) PFOB	12.9	0.86	3.26	0.26	0.015	+/+
0.1 (w/v) PFBE	22.9	1.53	5.16	0.30	0.024	-/+
0.2 (w/v) PFBE	14	0.93	3.75	0.25	0.017	-/+

+ Positive enzyme detection
- Negligible enzyme detection

The growth-yield coefficients for the cultures grown in batch bioreactors supplemented with PFCs were in the range of 0.25 to 0.32 g biomass/g glucose compared to 0.09 g biomass/g glucose achieved in control experiments, with glucose consumption rates in the range of 0.013 to 0.024 $g.L^{-1}h^{-1}$. However, it was observed from the experimental data that PFBE inhibited LiP production at concentration fractions of 0.1 and 0.2 (w/v). Emulsions of PFOB and PFPA showed positive results for LiP and MnP production (Chapter 7). An emulsion with a fraction of 0.1 (w/v) PFOB showed better performance in terms of the growth-yield coefficient, glucose based consumption rate and enzyme-activity detection achieved in batch cultures. From these results, it was concluded that 0.1 (w/v) PFOB emulsion should be suitable for the MGR system, and therefore its efficacy and effectiveness was studied in Chapter 8.

As it was observed that a higher concentration of PFCs oils in the emulsions resulted in the reduction of *P. chrysosporium* biomass produced, meaning that the continuous supply of the emulsions to the MGR could lead to detrimental effects on the immobilised biomass, rendering the development of the biomass gradostat ineffective. Furthermore, as the capillary membranes used in most MBRs were prone to clogging, because of gel formation on the lumen side of the membranes as a result of particulates present in the nutrient medium, the use of an ~8.5% (w/v) PF 68-based emulsion for the MGR proved problematic.

In initial experiments, an emulsion consisting of ~8.5% (w/v) PF 68 (as used in batch cultures) was used in the MGRs, with indifferent results. Cake formation in the fittings of the nutrient supply line was evident during the MGRs operation. Some of the silicone tubing burst because of blockages and the emulsion dried on the outside of the capillary membranes, where fungal conidia was immobilised, thus preventing fungal growth. Although, it was determined that a concentration of 5 to 10% (w/v) of PF 68 for PFC emulsions created stable emulsions, it was evident that, for the MGR, a lower concentration of the surfactant in the emulsion had to be used. Initially, an investigation was performed in which different

concentrations of the surfactant ranging between 1 to 5% (w/v) were used in the emulsions. Then, 1 ml of the emulsions were left in petri dishes for 24 h at the MGR operating temperature of 30℃ to evaluate cake formation after evaporation.

These concentrations were demonstrated to be unsuitable; therefore, concentrations below 1% (w/v) were investigated (see Table 9.2 for experimental data). From the literature reviewed, it was reported that even at concentrations below 1% (w/v), PF 68 was still able to provide sufficient protection against chemical and physical stress (Hellung-Larsen *et al.*, 2000). A concentration of 0.01% (w/v) was therefore used, as it also showed to have no cake formation when the liquid phase evaporated in the petri dishes.

Although a lower concentration of PF 68 for the emulsions promoted emulsion instability, a separate PFOB control loop was designed so that the PFC could be supplied directly and premixed with a nutrient medium containing 0.01% (w/v) PF 68 before entering the lumen of the membranes. The control loop contained a stirring mechanism and the oil was aerated for 10 min every 24 h.

Table 9.2: Pluronic F 68 cake formation after evaporation in petri dishes

PF 68 %(w/v)	Results	PF 68 %(w/v)	Results	PF 68 %(w/v)	Results
5%	+++	0.8%	++	0.07%	++
4%	+++	0.6%	++	0.05%	++
3%	+++	0.4%	++	0.03%	++
2%	+++	0.2%	++	0.01%	+
1%	++	0.1%	++	0.00%	control

+++ High cake formation
++ Cake formation
+ Negligible cake formation
Control – pure nutrient medium

9.3 OVERALL CONCLUSIONS

9.3.1 DO mass transport and application of high oxygen partial pressures

In aerated immobilised biofilms, the formation of anaerobic zones was observed. An increase in biofilm thickness resulted in an increase in anaerobic zone formation. These anaerobic zones resulted in ethanol production. Younger and thinner biofilms showed greater DO mass transport capabilities. The highest DO penetration depth of less than 550 μm was determined in aerated biofilms. Young biofilms that were less than 1000 μm had a high DO flux, and DO consumption rates were high during accelerated growth phases. Extracellular glucan formation, lipid peroxidation and trace-metal ion accumulation increased when oxygenation was used. This exacerbated ethanol production.

9.3.2 Trace element ion accumulation in immobilised biofilms

Generally, there was higher trace element accumulation in aerated MGRs compared to aerated batch cultures. The identification and assessment of the overall bioreactor performance indicated the need for a modified nutrient media.

A reduction in trace-element ions accumulation in biofilms was observed when the concentration of trace-element ions present in the nutrient medium used were reduced. Additionally, oxygenation resulted in increased trace-element entrapment in the biofilms. Although the reduction of trace-element ions in the nutrient medium reduced their accumulation in the MGR biofilms, biomass protection and reduction of biomass lysis is required for sustainable and enhanced immobilised biomass performance. This required the addition of a biomass protector when developing the improved nutrient medium, namely Pluronic F 68, which was used as emulsifier in the perfluorocarbon studies.

9.3.3 Choice of the emulsifier for the perfluorocarbon studies

For batch cultures, increased *P. chrysosporium* biomass formation was observed using PF 68 as an emulsifier. However, negligible LiP presence was observed in cultures supplemented with PF 68 in batch cultures. This was also observed in continuous cultures even with a reduced PF 68 concentration of 0.01% (w/v).

9.3.4 Application of perfluorocarbon emulsions in batch cultures and membrane gradostat bioreactors

The application of PFC emulsions in batch cultures resulted in increased biomass formation. However, an increase in the PFC concentration from 10 to 30% (w/v) resulted in decreasing *P. chrysosporium* biomass formation, leading to poor nutrient uptake rates, specifically glucose and ammonium usage. A lag phase period was observed for ammonium consumption with increased PFC concentrations. Overall enzyme production improved with the use of PFCs, with predominantly higher MnP production compared to LiP production.

The application of PFOB and PF 68 resulted in reduced ethanol production. Although anaerobic zones were found in biofilms immobilised towards the top of the reactor, DO penetration was similar to that obtained from the bottom in reactors . An increasing DO availability was seen towards the surface of membranes in biofilms at the bottom of the reactor, which resulted with improved overall DO penetration ratio compared to aerated cultures. Furthermore, a reduction in β-glucan accumulation in younger biofilms (192 h) was seen. As lipid peroxidation was identified as a major performance inhibitor for immobilised biofilms, decreased lipid peroxidation, which is an indication of reduced oxidative stress, was seen in *P. chrysosporium* biofilms supplied with the PFOB/PF 68 nutrient medium. The production of MnP was higher compared to LiP; a result which was similar to those in batch cultures. Overall trace element reduction for Cu and Mn was observed in the biofilms.

9.4 OVERVIEW OF CONCLUSIONS

An assessment of the microbial biofilm activity studied in this thesis suggests that slower growing mycelia initially lead to larger aerobic zones in which sufficient DO levels are present. However, as the mycelium grows, the supply of DO becomes insufficient and the available DO is consumed before it can penetrate >600 μm of the immobilised biofilms. It was observed that the addition of oxygen carriers improved enzyme production both in batch and MGR biofilms. The limitations associated with related trace element accumulation, glucan formation, ethanol production and oxidative stress were alleviated using a perfluorooctyl bromide and Pluronic F 68 emulsion.

9.5 RECOMMENDATIONS FOR FUTURE STUDIES

This research project reported on the application of emulsified perfluorocarbons in continuous MGRs in order to enhance biomass performance and proved protection for prolonged MGR operation. In order to improve the bioreactor further, the following parameters using the MGR design with immobilised *P. chrysosporium* need to be further addressed:

- Evaluation of the effects of veratryl alcohol concentrations on growth kinetics.
- Profiling of all trace-metal ions present in the nutrient medium in the immobilised biofilms
- Quantification of the production of chelators from the biofilms.
- Evaluation of Pluronic F 68's ability to protect biomass under strenuous conditions and comparison with the commonly used Tween 80 with *P. chrysosporium*.
- Investigation of protease production in the MGR using oxygenation and aeration, and in pressurised MGR systems.
- Evaluation of other surfactants for their compatibility with PFCs and *P. chrysosporium* BKMF 1767.
- Evaluation of the stability of PFCs at different oil concentrations.

- Investigation into fouling effects of PFC emulsion on asymmetric membranes for different surfactant concentrations

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AUTHOR INDEX (IN-TEXT REFERENCES)

APPENDIX A: GROWTH AND MAINTENANCE OF *PHANEROCHAETE CHRYSOSPORIUM*

Preparation of the growth medium (spore-inducing medium)

The method for preparation of the spore inducing medium was taken from Tien and Kirk (1988).

Add the following ingredients in order into a 1000 ml Schott bottle.

Glucose	10 g	
Malt extract	10 g	
Peptone	2 g	
Yeast extracts	2 g	
Asparagine	1 g	
KH_2PO_4	2 g	
$MgSO_4.7H_2O$	1 g	($MgSO_4$ = 2.05 g)
Agar-agar	20 g	
Thiamin-HCl	1 mg	

Fill up to the 1000 ml mark with distilled water, close the cap and shake to dissolve powder. Cover with tinfoil to prevent contamination, and put a piece of heat-detecting tape (Autoclave tape) on the foil to prepare for autoclavation. If no cap is available, cover the opening with non-absorbent cotton wool and then wrap it with tinfoil. Label the bottle with the present date, your name and the description of the contents and place into the autoclave for 20 min at 121℃ to ensure sufficient sterilisation. The water level in the autoclave chamber must cover the bottom. After removing from the autoclave, allow the content to cool down to a workable temperature before casting into the Petri dishes or Roux bottles.

Casting of the agar slants

Petri dishes:

Working under a laminar flow hood and using the flaming technique (flame after opening and before closing the bottle), fill each petri dish with the hot agar by opening the lid only as far as necessary and pouring agar evenly into it until the bottom of the petri dish is covered by about 1 cm. Flame the agar flask outlet before and after the agar is poured onto a Petri dish to avoid any possible transfer contamination. Leave the lids slightly open for a while to prevent moisture forming (condensation) underneath the lids. After the agar has cooled down sufficiently, it will become stiff with the consistency of a jelly. Now you can proceed with the inoculation of *Phanerochaete chrysosporium* spores.

Roux bottles:

Working under an operating laminar flow hood and using the flaming technique, fill each Roux bottle with about 250 ml of still-hot agar. Cover the tops with non-adsorbent cotton wool and leave the bottles slightly elevated for air circulation. Allow the agar to cool down for a few hours; it will become stiff as jelly and ready for inoculation.

Inoculation *Phanerochaete chrysosporium* on the agar slants

Petri dishes:

Work under an operating laminar flow hood and clean the surface thoroughly with 70% ethanol to avoid contamination. Use a platinum rod as a tool to transfer spores. Flame the rod under a Bunsen burner and cool it down by dipping it into the fresh agar located on the rim of the petri dish. Cut out a square piece (about 1 cm) of the inoculation culture, place it in the middle of the fresh petri dish and cover the lid. Continue until all the petri dishes are inoculated. Store the Petri dish upside down and incubate at 37℃ for approximately seven days until the spores are ready. Label with your name and present date.

Roux bottles:

Working under an operating laminar flow and using the flaming technique, cut out a square piece (about 1 cm) with a flamed object and place in the middle of the Roux bottle and cover with cotton wool. Store the Roux bottle, lying on its side with the top slightly elevated, and incubate at 37℃ for approximately seven days until the spores are ready.

Spore solution preparation

Autoclave the following equipment:

- 1000 ml distilled water in a Schott bottle.
- 20 ml syringe with glass wool.
- 250 ml flask.

Under sterile conditions, pour 5 ml cooled sterile distilled water into each Petri dish containing spores and mycelium, close the lid and shake the water in rotational movements for one minute, to ensure that all spores are in the solution. Transfer the washing solution from the Petri dishes into a sterile 100 ml Schott bottle; repeat this three to four times on one agar slant. Continue with this procedure until you have enough spore solution. The bottle now contains a spore/mycelium solution.

Separation of spores from mycelium

Heat sterilise (autoclave) a syringe with glass wool for 15 minutes. Filter the solution through the syringe with glass wool into a 250 ml flask.

Determination of spore concentration

Spore concentration

The spore-solution concentration is determined by measuring absorbance at 650 nm with a spectrophotometer. The spectrophotometer should be switched on in a fixed wavelength mode at least 30 min before it is used. The UV and visible lights should be switched on.

Ordinary quartz cuvettes are used for the spore-solution concentration measurement. According to Tien and Kirk (1988) an absorbance of 1.0 cm^{-1} is approximately, 5 x 10^6 $spores.ml^{-1}$.

- Pour distilled water into a plastic corvette and place into the spectrophotometer.
- Blank the spectrophotometer using clean water, until the absorbance reads close to 0.
- Replace the blank with a quartz corvette filled with spore solution.
- Read off the absorbance and write it down.

For example, to determine the number of spores:

An absorbance of, $1 = 5 * 10^6$ $spores.ml^{-1}$

i.e. $0.0741 = x$

$\therefore$ $$x = \frac{0.0741 * (5 * 10^6)}{1} = 3.7 * 10^5 \text{ spores.ml}^{-1}$$

APPENDIX B: PREPARATION OF NUTRIENT MEDIUM USED FOR *PHANEROCHAETE CHRYSOSPORIUM*

The development of the nutrient-medium stock solutions was taken from Tien and Kirk (1988).

Trace element stock solution

Dissolve 1.5 g Nitrilotriacetate in 800 ml distilled water. After dissolving the nitrilotriacetate completely, adjust the pH to 6.5 with 1 M KOH (8 g/500 ml). Add each component sequentially.

$MgSO_4$	3 g	($MgSO_4.7H_2O$ = 6.14 g)
$MnSO_4$	0.5 g	($MnSO_4.H_2O$ = 0.56 g)
NaCl	1 g	
$FeSO_4.7H_20$	0.1 g	
$CoCl_2$	0.1g	($CoCl_2.6H_2O$ = 0.187 g)
$ZnSO_4.7H_2O$	0.1 g	
$CuSO_4$	0.1 g	
$AlK(SO_4)_2.12H_2O$	0.01 g	
H_3BO_3	0.01 g	
$Na_2MoO_4.2H_2O$	0.01 g	

Fill the solution up to 1000 ml with autoclaved distilled water. Filter sterilise the solution into an autoclaved bottled using a 0.22 µm filter and store at 4℃. This solution should be light yellow.

Basal III medium stock solution

KH_2PO_4	20 g	
$MgSO_4$	5 g	
$CaCl_2$	1 g	($CaCl_2.2H_2O$ = 1.32 g)
Trace element solution	100 ml	

Fill the solution up to 1000 ml with autoclaved distilled water. Filter sterilize through 0.22 µm filter into sterile bottle and store at 4°C.

10% Glucose stock solution

Glucose	100 g

Fill the solution up to 1 L with autoclaved distilled water. Autoclave for 20 min and store at 4°C.

0.1 M 2, 2-dimethylsuccinate stock solution (pH 4.2)

2, 2-dimethylsuccinate 13.045 ml in 1000 ml autoclaved distilled water.

Calculated as follows:

For a powder:

Mw = 146 $g.mol^{-1}$

Moles = mass/Mw

0.1 M = mass/146.1

Mass = $0.1 * 146.1 = 14.61$ g (put into 1 L)

If liquid:

density = 1.12 $g.mol^{-1}$

= Mass/volume

Volume = 14.61/1.12

= 13.045 ml (put into 1 L)

Autoclave for 20 minutes and store at 4°C.

Thiamin-HCL (Do not autoclave)

Thiamin-HCl 100 $mg.L^{-1}$ stock

Filter sterilize through 0.22 µm filter into sterile bottle and store at 4°C.

Ammonium tartrate

Ammonium tartrate 8 g

Fill the solution up to 1 L with autoclaved distilled water. Autoclave for 20 minutes and store at 4°C.

0.02 M Veratryl alcohol (light sensitive)

Veratryl alcohol 2.907 ml in 1 L

Calculated as follows:

If powder:

Mw = 168.19 $g.mol^{-1}$

Moles = mass/Mw

0.02M = mass/168.19

Mass = 3.36 g (put into 1 L bottle)

If Liquid:

If density = 1.15 $g.mol^{-1}$

= mass/volume

Volume = 2.91 $g.ml^{-1}$ (put into 1 L bottle)

Filter sterilize through 0.22 µm filter into sterile bottle and store at 4℃ (Store in a dark place when not in use).

Nutrient medium composition

Basal III medium	100 ml
10% glucose stock solution	100 ml (Carbon source)
0.1 M 2, 2-dimethylsuccinate	100 ml (Growth enhancer)
Thiamin-HCL	10 ml (Vitamin)
Ammonium tartrate	25 ml (Nitrogen source)
0.4 M Veratryl alcohol	100 ml (Growth enhancer)
Trace elements	60 ml

Fill the solution up to 1000 ml with autoclaved distilled water.

Points to remember:

- When dispensing a culture medium, always use sterile measuring cylinders and dispense into sterile containers.
- Always use autoclaved-distilled water.
- All stock solutions should be sterilised beforehand. It is, therefore, not necessary to autoclave the nutrient medium again after it is made up.
- Nutrient mediums must be kept at 4℃ when not in use.

APPENDIX C: LIGNINOLYTIC ENZYME ASSAYS FOR LIGNIN AND MANGANESE PEROXIDASES

The following method for preparing the growth medium was taken from Tien and Kirk (1988).

Lignin peroxidase assay

Lignin peroxidase activity was determined spectrophotometrically at 25℃ using veratryl alcohol as the substrate (Tien & Kirk, 1998). The activity was measured by determining the rate of oxidation of veratryl alcohol to veratraldehyde. This was monitored at 310 nm. The alcohol exhibits no absorbance at 310 nm, whereas the aldehyde absorbs strongly. The extinction coefficient used for veratraldehyde was 9300 $M^{-1}.cm^{-1}$. Activity is then expressed in $U.L^{-1}$, where 1 unit = 1 $\mu mol.min^{-1}$.

Prepare the following stock solutions:

10 mM Veratryl Alcohol

- Transfer 1.454 ml into 1000 ml distilled H_2O.
- Store at 4℃ in a dark container when not in use.

250 mM Tartaric acid

- Add 9.43 g to 80 ml distilled H_2O.
- Using 1 M NaOH, adjust pH to 2.5 (i.e. add 40 $g.L^{-1}$ NaOH to distilled water to make 1 M NaOH).
- Make up to 250 ml volume.
- Store at 4℃.

Calculated as follows:

Mw	=	150 $g.mol^{-1}$
0.25 M	=	0.250 $mol.L^{-1}$
(150 g/mol*0.25 mol/L)	=	37.725 $g.L^{-1}$
Required	=	9.43 g/ 250 ml

5 mM Hydrogen peroxidase (H_2O_2)

- Made up fresh every time.
- Hydrogen peroxide is light sensitive; do not leave standing in direct sunlight for extended periods.
- Transfer 50 µL of the 30% H_2O_2 in a 100 ml flask and make up to volume.

Assay reagents and method

	Blank	Sample
10 mM Veratryl alcohol	200 µL	200 µL
250 mM Tartaric acid	200 µL	200 µL
Distilled water	520 µL	220 µL
Enzyme solution		300 µL
5 mM H_2O_2	80 µL	80 µL
Total	**1000 µL**	**1000 µL**

Note: Add everything into the Eppendorf tube except the H_2O_2, which is added just before assaying.

Spectrophotometer settings:

- Kinetics mode.
- UV light switched on.
- Read at 310 nm for 1 minute at 10 seconds intervals.
- Factor: 1075.3.
- Temperature: 25℃.
- Extinction coefficient: 9300 $M^{-1}.cm^{-1}$.

Calculation of activity

- The objective is to get a reading in units per litre, where 1 unit = 1 μmol substrate converted per minute.
- Take your absorbance readings and plot a graph showing absorbance versus time.
- The initial slope, $\frac{dA}{dt}$ will give you the value for initial rate of reaction.
- To convert this into $\frac{dC}{dt}$, use Lambert-Beer method and divide by the extinction coefficient E, which is 9300 $M^{-1}.cm^{-1}$. This gives a value in $mol.min.^{-1}L^{-1}$.
- Moles are now changed to micromoles by multiplying by 10^6. This gives micro-$moles.min^{-1}.L^{-1}$, which equals 1 unit; therefore, the answer is now in units per litre.
- Now you have to consider sample handling. First, you need to account for dilution in your assay. If you used 200 μl of enzyme sample solution in a 2 ml reaction volume, this constitutes a dilution of 1 in 10. You therefore have to multiply your answer by 10.
- If you have concentrated your sample by using cut-off tubes, you have to divide by your concentration factor. If you fill your cut off tubes initially with 3 ml and you have 0.5 ml of concentration enzyme solution left, your factor would be 6.
- As a guideline, if you have not concentrated your sample, then multiply your $\frac{dA}{dt}$, readings in minutes by 1075.3.

Therefore: $$\text{activity} \quad (U.L^{-1}) = \left[\frac{\frac{dA}{dt} * (\text{dilution} \quad \text{factor})}{\text{extinction} \quad \text{coefficient}} \right] * 60 * 10^6$$

Manganese Peroxidase (Oxidation of ABTS)

The activity is based on the spectrophotometry determination of oxidation of ABTS by MnP in the presence of lactate, Mn^{2+} and H_2O_2. Mn^{2+} is oxidised to Mn^{3+} that in turn oxidises organic compounds such as ABTS. ABTS oxidation is dependent on ∝-hydroxy acids such as lactate.

Preparation of stock solutions

1 M Na Lactate buffer

- Add 10.22 g lactic acids ($C_3H_6O_3$ to M_w 90.08 g/mol) into a 100 ml beaker.
- Add 80 ml distilled water.
- Adjust to pH 4.5 using 1 M NaOH and make up to 100 ml volume

  If the volume is 100 ml and the pH is not at 4.5 yet, add NaOH until the pH is correct.
- Store at 4℃.

1 M Na Succinate buffer

- Add 11.81 g succinic acid ($C_6H_{10}O_{4\ to}$ M_w 146.14 g/mol) into a 100 ml beaker.
- Add 80 ml distilled water.
- Adjust pH to 4.5 using 1 M NaOH and make up the volume with distilled water. If the volume is 100 ml and the pH is not at 4.5 yet, add NaOH until the pH is correct.
- Store at 4℃.

$MnSO_4$ solution

- Add 95 mg $MnSO_4$*H_2O to 100 ml distilled water ($MnSO_4 4H_2O$ = 125 mg).

ABTS solution (light green in colour; turns blue when oxidized)

- Add 50 mg ABTS into 25 ml volumetric flask and make up to volume with distilled H_2O.
- Store at 4℃.

Preparation of Reagent A

Add the ingredients in the following order into a 25 ml volumetric flask and mix with a magnetic stirrer:

- Egg albumin 150 mg
- 1M Na lactate buffer 2.5 ml

The stirring speed must be slow, as the entire albumin must dissolve without any physical damage that could cause the denaturation of the protein. After the protein has dissolved completely, add the other reagents:

- 1M Na succinate buffer 2.5 ml
- $MnSO_4$ solution 1.0 ml
- ABTS solution 1.0 ml

Fill up with distilled water to 25 ml. Centrifuge the solution before use to give a clear supernatant.

Preparation of Reagent B

- Add 100 µM H_2O_2 solution, 1 µl of a 30% (m/v) H_2O_2 solution to a 100 ml volumetric flask.
- Make up fresh and store in a dark place. Hydrogen peroxide is light sensitive; do not leave standing in direct sunlight for extended periods of time.

Assay material and method

	Blank	**Sample**
Reagent A	350 µl	350 µl
Reagent B	350 µl	350 µl
Enzyme solution	----	300 µl
Distilled water	300 µl	----
Total	**1000 µl**	**1000 µl**

- A clear supernatant of Reagent A is used and is added together with Reagent B into an Eppendorf tube.
- Add everything into the Eppendorf tube, except the enzyme solution; this is added just before assaying.

Spectrophotometer settings:

- Kinetics mode.
- UV and visible light switched on.
- Temperature 25℃.
- Read at 420 nm for 1 minute at 5 seconds interval.

Calculation of activity

Activity of enzymes calculated similarly to that of LiP.

APPENDIX D: GLUCOSE, ETHANOL AND AMMONIUM ASSAYS

Glucose assay using Roche ® D-Glucose kit (Cat No: 10 716 251 035)

Dissolve contents of one bottle 1 (ATP) with 45 ml sterile distilled water. Store in a fridge at 4℃. Warm up to 20 to 25℃ before use.

Procedure for assay

Pipette into cuvettes	Blank	Sample
Solution 1	1.0 ml	1.000 ml
Sample solution	----	0.100 ml
distilled water	2.000 ml	1.900 ml
Mix**, read absorbance's of the solutions (A_1) after approximately 3 min and start reaction by addition of:		
Suspension 2	0.020 ml	0.020 ml
Mix **, wait for the end of the reaction (approximately 10 - 15 min) and read absorbance's of the solutions (A_2).		

- Pipette 1000 µl (1 ml) of bottle 1 solution into two plastic cuvettes, namely B (blank) and S (sample).
- Dilute the permeate sample, by adding 10 µl of permeate to 990 µl of sterile distilled water (Dilution factor = 100).
- Add 100 µl of diluted permeate into the S (sample) cuvettes. To test nutrient solution, add 100 µl of diluted nutrient into the S (sample cuvettes).
- Add 1900 µl of sterile distilled water to S (sample) and 2000 µl of sterile distilled water into B (blank). Close and mix. Bring samples to 20 to 25℃ before use.
- Read absorbency (A1) of the blank and samples against water in a 1 cm (10 mm) light path at 340 nm.

- Add 20 µl (0.02 ml) of bottle 2 (solution of enzyme, Hexokinase) into both cuvettes (B and S) and mix. Wait until the reaction has stopped (10 to 15 minutes) and read off absorbencies (A2).
- Determine the absorbance differences (A2 to A1) of the blank and sample.
- Use the equation below to evaluate the glucose concentration.

NB: If the sample has been diluted during the preparation, the result must be multiplied by the dilution factor F.

$$c(g.L^{-1}) = \frac{V \times Mr \times F}{\varepsilon \times d \times v \times 1000} \times \Delta A \qquad \text{Where: } \Delta A = (A_2 - A_1)_{sample} - (A_2 - A_1)_{blank}$$

Dilution table

Estimated amount of D-glucose per litre measurements at different absorbencies		Dilution with water	Dilution factor
340 or 334 nm	365 nm		
<0.5 g	<1 g	--	1
0.5-5g	1-10 g	1 + 9	10
5-50 g	10-100g	1 + 99	100
>50 g	>100 g	1 + 999	1000

Ethanol assay using Roche ® D-Glucose kit (Cat No: 10 176 290 035)

Preparation of solutions

- Use contents of bottle 1 undiluted.
- Dissolve a tablet in bottle 2 with 3 ml solution of bottle 1. This is reaction mixture 2.
- Use bottle 3 undiluted.

Procedure for assay

Pipette into cuvettes	Blank	Sample
Reaction mixture 2	3.0 ml	3.0 ml
Sample solution	----	0.100 ml
Redist. water	0.100 ml	----
Mix**, read absorbances of the solutions (A_1) after approximately 3 min and start reaction by addition of:		
Suspension 3 (bottle 3 solution)	0.050 ml	0.050 ml
Mix **, wait for the end of the reaction (approximately 10-15 min) and read absorbances of the solutions (A_2).		

- Pipette 3000 µl (1 ml) of reaction mixture 2 into plastic cuvettes, for blanks and samples.
- Add 100 µl of permeate into the sample cuvettes. Do not dilute the permeate sample, i.e. use permeate sample as is. Use sterile distilled water for blank cuvettes.
- Read absorbency (A1) of the blank and samples against water in a 1 cm (10 mm) light path at 340 nm.
- Add 50 µl (0.05 ml) of bottle 3 (solution of enzyme, alcohol dehydrogenase) into both Blank and Sample cuvettes and mix. Wait until the reaction has stopped (10 - 15 min) and read absorbencies (A2).
- Determine the absorbance differences (A2 - A1) of the blank and sample.
- Use the equation below to evaluate the glucose concentration.

NB: If the sample has been diluted during the preparation, the result must be multiplied by the dilution factor F.

$$c(g.L^{-1}) = \frac{3.150 \times 46.07}{6.3 \times 1.0 \times 0.1 \times 2 \times 1000} \times \Delta A \qquad \text{where: } \Delta A = (A_2 - A_1)_{sample} - (A_2 - A_1)_{blank}$$

Ammonia Assay using Merck Spectroquant NH_4+ kit (Cat No: 1.00683.0001)

Method

Ammonium nitrogen NH_4-N occurs partly in the form of ammonium ions and partly as ammonia. A pH-dependent equilibrium exists between the two forms. In strong alkaline solutions, NH_4-N is present almost entirely as ammonia. This reacts with hypochlorite ions to form monochloramine, which in turn reacts with a substituted phenol to form a blue indophenol. This is then determined photometrically.

Measuring range

Wavelength	Measuring range	Number of determinations
712 nm	2.0 - 150 mg.L^{-1} NH_4-N 2.6 - 193 mg.L^{-1} NH_4^+	95

Preparation

- Analyse immediately after sampling.
- Check the ammonium content with the spectroquant Ammonium Test. Samples containing more than 193 mg.L^{-1} NH_4^+ must be diluted with distilled water.
- Filter turbid samples.
- Decompose or extract solid sample materials by an appropriate method.

Procedure

Preparation of measurement sample for measuring range of 2 – 75 mg.L^{-1} NH_4-N (2.6-96.6 mg.L^{-1} NH_4^+).

Reagent	Quantity	Procedure/action
Reagent NH_4-1	5 ml	Pipette into a test tube
Pre-treated sample (20-30°C)	0.2 ml	Add using pipette and mix
Reagent NH_4-2	1 level blue micro spoon in cap of bottle	Add and shake vigorously until the reagent is completely dissolved
Leave to stand for 15 min, then fill the measurement sample into a 10-mm cell and measure in the spectroquant or in a photometer at 712 nm		

Calculation

$$NH_4^+(mg.L^{-1}) = 1.2877(NH_4 - N) + 0.0247$$

APPENDIX E: β-GLUCAN AND MALONDIALDEHYDE ASSAYS

MEGAZYME Mixed-linkage β-glucan assay:

Procedure

- Homogenise harvested biomass vigorously (20 - 30 count), in an Eppendorf tube using glass beads.
- Centrifuge at 10 000 x g for 6 minutes and use clear supernatant for assaying.

Preparation of buffers

Sodium acetate buffer (50 mM, pH 4.0)

- Add 2.9 mL of glacial acetic acid to 900 mL of sterile distilled H_2O and adjust the pH to 4.0 using 1 M NaOH solution.
- Adjust the volume to 1.0 L using sterile distilled H_2O.
- Add 0.2 g Sodium azide.

Sodium acetate buffer (200 mM, pH 4.0)

- Add 11.6 mL of glacial acetic acid to 900 mL of SdH_2O and adjust the pH to 4.0 using 1M NaOH solution.
- Adjust the volume to 1.0 L using sterile distilled H_2O.
- Add 0.2 g Sodium azide.

Preparation of GOPOD reagent

- Dilute the contents of bottle 3 (GOPOD reagent buffer) to 1.0 L with sterile distilled H_2O. This is solution 3.
- Dissolve the contents of bottle 4 (GOPOD reagent enzymes) in 20 mL of solution 3.

- Transfer the mixture to the GOPOD reagent buffer (solution 3).
- Cover the bottle with aluminium foil to prevent reagents from light.

Preparation of β-glucosidase solution

- Dilute the entire contents of bottle 2 (β-glucosidase, 40 $U.ml^{-1}$) to 20.0 mL with 50 mM sodium acetate buffer (pH 4.0).

Prepare test tubes and transfer the following:

Solution	Blank	Sample	Glucose standard
Filtrate (Homogenates)	-	100 µL	-
Glucose standard solution (bottle 5)	-	-	100 µL
H_2O	100 µL	-	-
Na acetate buffer (50 mM, pH 4.0)	100 µL	100 µL	100 µL
β-glucosidase- acetate buffer solution (50 mM, pH 4.0)	100 µL	100 µL	100 µL
Incubate the tubes at 40 °C for 15 min			
GOPOD reagent	3000 µL	3000 µL	3000 µL
Incubate at 40 °C for 20 min Measure absorbance at 510 nm Use water as a reference (blank)			

β-glucan ($mg.L^{-1}$) = ΔA x F x 3.6 where F = 100/ $\Delta A_{Glucose\ standard}$

Where ΔA = Is the Absorbance after β-glucosidase treatment (reaction) minus reaction blank Absorbance

NWLSS^{TM} Malondialdehyde Assay (Product NWK-MDA01)

Introduction

Malondialdehyde (MDA) is one of many low molecular weight end-products of lipid products of lipid peroxidation. However, the use of MDA as a marker for lipid peroxidation using the NWLSS^{TM} assay conditions has been designed to minimise errors in the determination of MDA concentration.

Test principle

The NWLSS^{TM} assay is based on the reaction of MDA with thiobarbituric acid (TBA) forming an MDA-TBA_2 adduct that absorbs strongly at 532 nm. Butylated hydroxytoulene (BHT) and EDTA are added to the sample and reaction mixtures to minimise oxidation of Lipids that contribute artificially during sampling, and sample processing and the TBA reaction. Because MDA is protein bound, mostly as a Schiff base, the pH of the reaction has been optimised to facilitate hydrolysis of the MDA.

Reagent preparation

TBA reagents

- Add 10.5 mL sterile distilled H_2O to the TBA bottle.
- Using a magnetic stirrer, and mix until TBA has dissolved.
- Store at room temperature; do not refrigerate.

Other reagents

The Acid reagent, BHT reagent, Assay buffer and calibrators are supplied ready to use.

Sample handling/Preparation

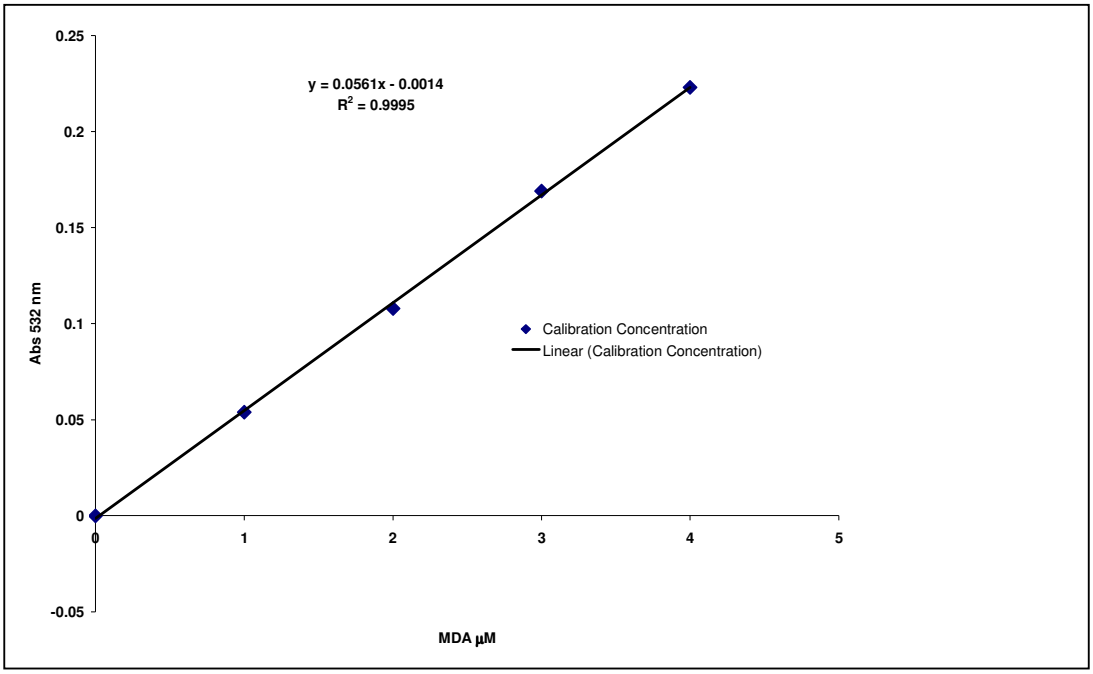
Tissue culture

Prepare 10% w/v homogenate in cold assay buffer or other buffer. Clarify the homogenate by centrifugation and store the supernatant on ice. The MDA concentration can be normalised to the wet weight of the tissue sample or to the protein concentration of the homogenate. In this study, the determinations were performed per mL of homogenate.

Procedure for assay

Pipette into cuvettes	Sample
BHT reagent	10 µL
Sample/Filtrate/Homogenates	250 µL
Acid reagent	250 µL
TBA reagent	250 µL
• Mix**, vortex vigorously (5-count) and incubate for 60 min (1 h) at 60 °C. • Centrifuge at 10 000 *x* g for 2 to 3 min. • Transfer reaction mixture to cuvette. • Read absorbances of the solutions at wavelength of 532 nm.	

MDA calibration curve



0.0561 (MDA) -0.0014 = Abs_{532nm}

Therefore: (Abs_{532nm} - 0.0014)/0.0561 = MDA ($\mu M.mL^{-1}$ of BHT Homogenised biomass extracts)

APPENDIX F: ANTIOXIDATION PRESERVATION ASSAY

Preparation of 20 mM Tris-HCL buffer (pH 7.4)

Tris – Hydroxymethyl amino methane

MW = 121.14 $g.mol^{-1}$ [$C_4H_{11}NO_3$]

Required concentration = 5.43 $g.L^{-1}$ Tris in sterile distilled H_2O

Adjust pH to 7.4 using diluted HCl

Autoclave at 121℃ for 20 minutes.

Preparation of 0.5 M Butylated Hydroxy Toluene (BHT)

MW = 220 $g.mol^{-1}$

Required concentration = 0.11 $g.ml^{-1}$ BHT in technical grade acetonitrile

Preservation of *P. chrysosporium* to prevent biofilm oxidation

1) Dilute 0.5 M BHT to a final concentration of 5 mM in 20 mM Tris-HCL buffer. i.e. transport 10 µl of BHT solution per ml of Tris-HCL buffer.

Note: If no antioxidant is added to the samples, new peroxidation can occur during storage and homogenisation. If not assayed immediately, samples should be stored at -80℃ to prevent sample oxidation.

2) Section biofilms at 0.2/0.9/1.5 cm where biofilm thickness measurements were taken, using a Carl Zeiss microscope. Spray BHT/Tris-HCl buffer solution onto the external surface of the biofilms before cutting.

3) Detach remaining biofilm from membranes and rinse in 5 mM BHT/20mM Tris-HCL buffer to get rid of excess nutrients in the biofilm. Preserve the biofilm in a 1 ml of 5 mM BHT/20 mM Tris-HCL buffer and store at -80℃ until analysis.

4) After measuring the biofilm thickness, remove biofilms from the membrane and rinse in sdH_2O and dry at 60℃. The dry biofilms were used for trace-element ion determinations.

Preparation of Oxidative and Nitrosative stress Assays

1) Remove biofilm from -80℃ and thaw in a water bath to room temperature (25℃).

2) Remove biofilm mycelia and dry using a sterile cotton wool (Autoclaved) and measure 1 g (wet weight) of mycelia.

3) Sonicate the mycelia in a 2 ml of 5 mM BHT/Tris-HCL buffer for 30 minutes. A precipitate is expected to form. This will not affect the outcome of the assay.

4) After homogenising, centrifuge the homogenate at 3000 x g at 4℃ for 10 minutes. However, longer may be required to remove turbidity from the homogenate.

5) The clear supernatant is used for the assays. The supernatant should be kept on ice and if not analysed immediately after preparation, must be kept frozen at -80℃ for longer storage.

APPENDIX G: OXYGEN MICROSENSOR OPERATION

Polarising the picoammeter:

Channel 1 of the Unisense pA 2000 picoammeter was used. The 'Display' selector was turned to 'Pol 1' and adjusted to -0.80 V using the polarisation control. The 'Display' selector was turned to 'signal 1' and then the 'Gain' screw was completely turned counter-clockwise. The display signal was then adjusted to zero on the 'offset' dial and left for 24 h to stabilise.

Connecting the oxygen microsensor:

The BNC of the microsensor was connected, which contains connections for both the reference and sensing cathodes, to the input terminal of the picoammeter, and then the yellow cathode was connected to the guard terminal first and the signal on the display was monitored.

Pre-polarisation of the microsensor:

Before the sensor is used, it must be was polarised, as the electrolyte in the sensor will contain oxygen. The sensing cathode must consume this oxygen and the guard cathode before stable operation of the sensor is possible. The sensor was secured and adjusted such that the tip was immersed in continuously aerated water (obtained by bubbling air from and aquarium pump through the water). The sensor was polarised until it has exhibited a stable signal for 10 minutes.

Calibration of the microsensor:

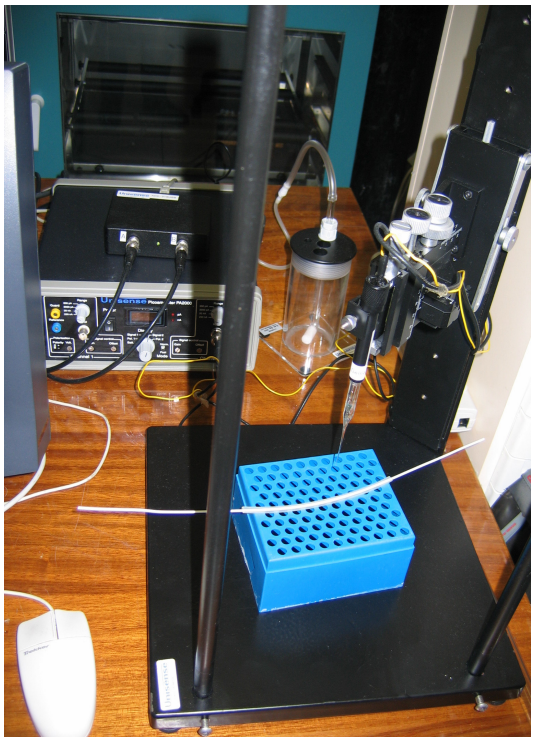
- **Concentration reading (0%)** – The oxygen microsensor was placed in an anoxic solution (Sodium ascorbate and NaOH, both to final concentrations of 0.1 mol.L^{-1}), which acts as a strong oxygen reducing agent. The tip of the microsensor was placed in this

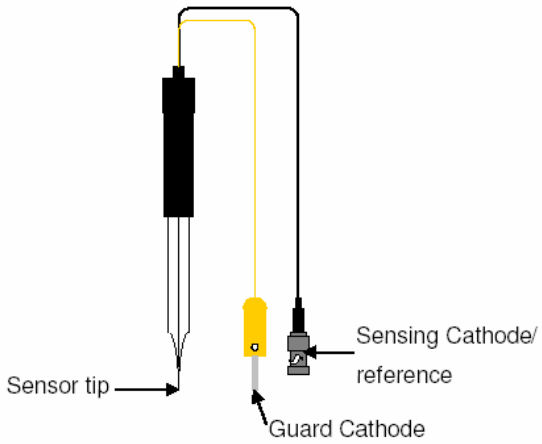
solution for 5 minutes, and the signal obtained was the zero setting for the oxygen concentration.

- **Concentration reading (100%)** – The sensor tip was placed in the calibration solution (nutrient medium), which was aerated by bubbling industrial oxygen. After 5 minutes of vigorous bubbling, the oxygen was turned off and the signal obtained was noted as a calibration value for 100% oxygen. Then the sensor was ready for use.

General use: Oxygen microsensor

- Turn on the power switch on the picoammeter.
- Check that the "GAIN" screw is turned fully counter clockwise.
- Check polarisation voltage. The value should be -0.80 V. If not this value, correct using "VOLT" screw and "POLARITY" switch.
- Put the "DISPLAY" knob to "SIGNAL". The reading on the LCD display should be 0.00 before connecting the sensor, if not adjust to zero with the "OFFSET" screw at "200 nA" range.
- Connect the sensor leads to the meter in the following order: 1) Connect black wire to "INPUT". 2) Connect yellow wire to "GUARD".
- Polarise the sensor by immersing in continuously aerated water. When polarisation is initiated, the signal will be very high and then drop rapidly over the first few minutes. There after the signal will drop slowly.
- Turn the "RANGE" to "2000 pA". The signal should stabilise (positive on the LCD display)
- Perform calibration according to protocol.
- Sensor is ready for use.





A **B**

Photograph of the microsensor setup; (a) the microsensor connected to a picoammeter and fixed on a micromanipulator with a visible calibration chamber in the background, (b) the OX 10 microsensor with a glass tip of less than 20 µm.