PENINSULA TECHNIKON
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Design of a Packed-Bed Fungal Bioreactor

The application of enzymes in the bioremediation of organo-pollutants present in soils and industrial effluent

Thesis submitted in fulfilment of the requirements for the degree of Masters of Technology in Chemical Engineering

By:

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"Research is to see what everybody else sees, and to think what nobody else has thought."

Albert Szent-Gyorgyi

In loving memory of my parents...
To my family and friends, thanks for your endless support and encouragement. Sorry for waiting so long...

STATEMENT

I, the undersigned, hereby declare that the work contained in this thesis is my own original work.

Data collected by B Tech students working under my supervision is appropriately referenced and acknowledged.

I have not previously, in its entirety or in part, submitted it at any other institution for a degree.

Signature: Date:
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PREFACE

This thesis is a report that covers an investigation into fungal bioreactors, especially the development of a packed-bed bioreactor.

This project is an offspring of research that started four years ago at Rhodes University, funded by Eskom, during which bioreactor systems were proposed to produce ligninolytic enzymes called Lignin and Manganese Peroxidase from *Phanerochaete chrysosporium*. Eskom experienced problems with soil contamination of polychlorinated biphenyl's (PCBs) and due to the high cost and other problems associated with clean-up technologies such as incineration, bioremediation seemed to be an economical alternative.

Membrane technology was the initial idea to implement in the development of fungal bioreactors. Collaborators such as Rhodes University, ML Sultan, Cape Technikon and The University of Stellenbosch worked closely to propose different membrane fungal bioreactors. The transverse-flow membrane gradostat bioreactor was developed as a bench scale operation but has not yet been implemented on a larger scale due to some operational problems. Alternative reactor designs, such as the packed-bed bioreactor, were then further investigated at Peninsula Technikon to assist in the development of fungal bioreactors.

ABSTRACT

Certain fungi have been shown to excrete extracellular enzymes, including peroxidases, laccases, etc. These enzymes are useful for bioremediation of aromatic pollutants present in industrial effluents (Leukes, 1999; Navotny *et al*, 1999).

Leukes (1999) made recent significant development in the form of a capillary membrane gradostat (fungal) bioreactor that offers optimal conditions for the production of these enzymes in high concentrations. This system also offers the possibility for the polluted effluent to be treated directly in the bioreactor. Some operating problems relating to continuous production of the enzymes and scale-up of the capillary modules, were, however, indentified.

In an attempt to solve the above-mentioned identified problems the research group at Peninsula Technikon considered a number of alternative bioreactor configurations. A pulsed packed bed bioreactor concept suggested by Moreira *et al.* (1997) was selected for further study. Their reactor used polyurethane pellets as the support medium for the fungal biofilm and relied upon pulsing of the oxygen supply and *recycle* of nutrient solution in order to control biomass accumulation. These authors reported accumulation due to the recycle of proteases that were believed to destroy the desired ligninases. We experimented with a similar concept *without recycle* to avoid backmixing and thereby overcome protease accumulation. In our work, a maximum enzyme productivity of 456 Units.$L^{1}day^{-1}$ was attained. Since this was significantly greater than the maximum reported by Moreira *et al*, 1997 (202 Units.$L^{-1}day^{-1}$) it appeared that the elimination of recycle had significant benefits.

In addition to eliminating recycle we also used a length / diameter (L / D) ratio of 14:1 (compared with 2.5:1 used by Moreira *et al*, 1997) in order to further reduce backmixing. Residence time distributions were investigated to gain insight into mechanisms of dispersion in the reactor.

It was found that the pulsed packed bed concept presented problems with regard to blockage by excess biomass. This led us to consider the advantages of a fluidized bed using resin beads. Accordingly, growth of fungi on resin beads in shake flasks was investigated with favorable results. An experimental program is proposed to further investigate the fluidized bed concept with a view to extending the operation time of the bioreactor.

From our literature survey to date, packed bed fungal bioreactors are still the best reactor configuration for continuous production of ligninolytic enzymes.

An interesting study of the application of laccases to the degradation of naphthalene and MTBE is described in an addendum to this thesis.

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LIST OF SYMBOLS & ABBREVIATIONS

Symbol	Definition	Units
A	Cross sectional area of bioreactor	m^2
c_a	Ammonium concentration	mg / l
c_{ai}	Inlet ammonium concentration	mg / l
c_{ao}	Outlet ammonium concentration	mg / l
C_g	Glucose concentration	mmol / l
C_{gi}	Inlet glucose concentration	mmol / l
C_{go}	Outlet glucose concentration	mmol / l
C	Conductivity	µS / cm
C1	Upstream conductivity	µS / cm
C2	Downstream conductivity	µS / cm
C	Concentration	mmol / l [units / l]
C_e	Concentration of enzyme in the reactor effluent	Units / l
D	Dilution rate (F/V)	day^{-1}
F	Total flowrate into live bioreactor	ml / min
G	Rate of glucose generation	mmol / l.min
L:D	Length to diameter ratio ("Aspect Ratio")	[-]
P	Productivity ($D * C_E$)	$units.l^{-1}.day^{-1}$
L	Total depth of bed	m
N_{Re}	Reynolds number	[-]
R_g	Rate of disappearance of glucose (species g)	mmol / l.min
$-r_a$	Rate of disappearance of ammonium (species a)	mg / l.min
V	Working volume of reactor vessel	ml
Δz	Thickness of differential section	mm

Greek symbol	Definition	Units
ε	Molar extinction coefficient	$M^{-1}cm^{-1}$
τ	Transit time	s^{-1}
ρ	Density	kg / m^3
µ	Viscosity	$N.s / m^2$

Abreviations	
ECS	Extra capillary space
LiP	Lignin peroxidase
PBB	Packed bed bioreactor
P.chrysosporium	*Phanerochaete chrysosporium*
MTBE	Methyl tert-butyl-ether
MnP	Manganese peroxidase
S/O/ATT	Stirred oygenated attached flask cultures
S/O/S	Stirred oygenated suspended flask cultures
S/A/S	Stirred aerated suspended flask cultures
S/A/ATT	Stirred aerated attached flask cultures
SEM	Scanning Electron Microscope
U/A/S	Unstirred aerated suspended flask cultures
U/A/ATT	Unstirred aerated attached flask cultures
U/O/S	Unstirred oxygenated suspended flask cultures
U/O/ATT	Unstirred oxygenated attached flask cultures

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Flask culture work

CHAPTER 1

SCOPE OF INVESTIGATION AND LITERATURE STUDY

1.1 INTRODUCTION AND SCOPE OF INVESTIGATION

Xenobiotics – a global problem and conventional solutions.

Industries worldwide are experiencing increased pressure from both the public and their customers to reduce discharges of toxic organic pollutants into the environment. Understanding the fate of foreign compounds (xenobiotics) in the environment is a natural outgrowth of man's curiosity about how their actions affect the environment. Xenobiotic pollutants refer to any non-natural (man-made) compounds. The term "xenobiotic" was adopted to refer to all organic compounds that are foreign to microorganisms (Brodie, 2000). These pollutants inevitably result in contaminated soil, groundwater, lakes and other valuable supplies of freshwater. One of the most disturbing aspects of groundwater contamination by xenobiotics is that it is an irreversible act (Sampat, 2000).

Conventional soil remediation treatments such as soil ventilation, pump-and-treat systems and soil incineration incur large capital and running expenditures but provide very little financial returns (Van der Westhuizen *et al*, 1998). Some remedial technologies cannot effectively treat contaminated soil due to the difficulties involved in accessing the subsurface systems (Azadpour-Keeley *et al*, 1999).

In South Africa polychlorinated biphenyls (PCBs) are a specific class of xenobiotic pollutants that are of major concern to some industries such as ESKOM (SA). PCB-containing products in electrical transformers and capacitors for example were manufactured by ESKOM for applications demanding stable, fire resistant heat transfer properties (http://www.eskom.co.za, 21/11/01). In 1979, ESKOM discontinued the use of PCBs. In 2000, approximately 70 tons of these PCB-containing capacitor cans were

transported to a European incineration facility for disposal (http://www.eskom.co.za/enviroreport01, 21/11/01). A large number of these transformers, however, still remain in use in the electrical industry. These transformers need to be managed safely (http://www.eskom.co.za/, 7/09/2001). ESKOM required solutions for the safe disposal of residual polychlorinated biphenyl (PCB) stocks as well as methods for treatment of contaminated soil and groundwater. The research group at Peninsula Technikon decided that one possible solution to the above problem was the use of biological techniques.

Bioremediation solutions:

Biological treatment offers an effective, method for the treatment of PCBs and a number of other hazardous complex chemicals including explosives residues in the mining industry (Nordwick *et al*, 1999), phenolic effluents in the chemical process industries, DDT, polycyclic aromatic hydrocarbons and other pollutants present in industrial effluents. Bioremediation is therefore being investigated by many countries and organisations. It is currently being used, in various modes, as a feasible and potentially cost-effective treatment strategy for contaminated soils.

A list of some hazardous compounds is given in Table 1.1 below.

Compound group	Examples
Aromatic compounds	Ferulic acid Syringic acid 2,6 Dihydroxybenzoic acid 7-Hydroxycoumarin Catechol Benzoic acid Veratryl alcohol Phenol toluene xylene
Lignin model compounds	Veratrylglycerol-β-(*O*-methoxyphenyl)- ether Guaiacylglycerol-β–coniferyl alcohol ether Dehydrodiconiferyl alcohol Dehydrodivanillin
Polycyclic aromatic compounds	Benzo[a]pyrene Anthracene
Chlorinated aromatic compounds	2,4,6-trichlorophenol 4-Chlorobenzoic acid 4,5-Dichloroguaiacol 5-Chlorovanillin 3-chloroaniline
Polycyclic chlorinated aromatic compounds	DDT(1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane polychlorinated biphenyl's (e.g. Arochlor 1254) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin 3,4,3′,4′-Tertrachlorobiphenyl 1,2,3,4,5,6-Hexachlorocyclohexane(Lindane)
Non-aromatic chorinated compounds	Lignin Cellulose Kraft lignin 3,4-Dichloroaniline-lignin conjugate
Biopolymers	atrazine
Pesticides	TNT (Trinitrotoluene)
Explosives	Cyanide
Non-aromatic xenobiotics	

Table 1.1: Partial listing of compounds degraded by *P.chrysosporium* (Bumpus *et al.*, 1985; Aust 1995 and Leukes, 1999).

These compounds tend to be resistant to biodegradation by most microorganisms (Bumpus *et al*, 1985). They are typically insoluble or very sparingly soluble in water and tend to be partitioned to soil and humic particles rather than to the soil moisture. Their insolubility also ensures that they are not easily transported into the cells of microorganisms that may have intracellular enzymes that can degrade the molecules. Organisms that produce extracellular enzymes capable of degrading the xenobiotics are thus of great interest.

In particular, the ligninolytic enzymes of the White Rot fungus, *Phanerochaete chrysosporium* (*P.chrysosporium*), have been shown to have the ability to degrade many of the above aromatic compounds (Leukes *et al*, 1996).

The ability of these enzymes to degrade complex molecules has been extensively studied by researchers with a view to bioremediation of environmental pollutants. The main enzymes excreted by *P.chrysosporium*, are called Lignin and Manganese Peroxidase. Laccase is also produced by *P.chrysosporium*. This enzyme has been found to be effective for the degradation of polycyclic aromatic hydrocarbons.

In order to utilize the degradative ability of enzymes for bioremediation, two general approaches can be considered:

One approach is to introduce enzymes or whole organism directly to the contaminated sites. It is envisaged that when using this approach either purified enzymes will be produced in a production facility and applied to the site or strains of organisms (bacteria or fungi) would be cultivated and inoculated into the soil environment. The enzymes could be produced in a fungal bioreactor in which optimal conditions for fungal growth and enzyme secretion can be maintained and controlled.

An alternative approach is to extract the pollutants from the soil and to treat the derived stream in a bioreactor. This could involve the production of purified

enzymes (as outlined above), and then a treatment step or it may involve introducing the polluted solution (or an effluent) directly into an immobilized enzyme bioreactor. The bioreactor could be of the type where the fungi are maintained in a state that optimally produces the enzymes and simultaneously effectively allows the enzymes to degrade the pollutant.

In either approach, the pollutant may previously be separated from contaminated water or soil via the use of another process such as extraction, volatilization, a membrane step, adsorption, recovery etc. Whichever approach is adopted (according to the demands of a given situation or treatment system) an efficient bioreactor in which the enzymes can be produced is required. In an effort to identify practical alternatives for either approach, the following types of fungal bioreactors were considered:

- *Airlift Bioreactors (Bonnarme and Jeffries, 1990)*
- *Bubble Column Bioreactors (Laugero et al, 1996)*
- *Fluidized-bed Bioreactors (Moreira et al, 1996)*
- *Biofilms on Flat Sheets of Various Materials (Fillis, 1997)*
- *Packed Bed Bioreactors (Moreira et al, 1997)*
- *Suspended and Attached Growth in Flask Cultures (Dayile and Martin, 1998)*
- *Transverse-flow Membrane Gradostat Bioreactors (Leukes, 1999)*

In the above systems fungal spores can be inoculated either into the suspending medium or onto carrier support material e.g. polyurethane foam pellets and membrane surfaces using suitable methods. Viable fungal biofilms can then be maintained in these systems. The ligninolytic enzymes (including the laccases) are excreted during secondary metabolism when the carbon or nitrogen supply becomes limiting. The role of mass transfer of the required oxygen and nutrients in determining the ideal conditions in these reactor configurations is consequently a very important factor that has a bearing on their relative effectiveness and on the selection of configurations that will have optimal performance.

The growing implementation of membrane technology in various industrial applications has encouraged researchers in South Africa to recently propose that fungal membrane bioreactors could successfully be scaled up for industrial use. Although a number of experimental fungal membrane bioreactors are currently in operation the author is not aware of the existence of any industrial scale fungal membrane bioreactors. In order to scale up a bioreactor it is necessary to understand the interaction of the fungal biofilm and the characteristics of the support, whether this be a membrane or other device. The primary objective of this investigation was to obtain a better understanding of this interaction and enabling a comparison between any proposed larger scale designs with alternatives.

A secondary objective was to construct a laboratory scale bioreactor in order to understand the growth and enzyme kinetics of the fungus with a view to optimizing efficiency of a practical, functioning reactor. It is hoped to use the information gleaned from the laboratory scale bioreactor to build a prototype large-scale reactor.

One of the problems encountered with all bioreactors is the control of the biomass produced. In this regard washout, cell retention, cell recycle and waste are all operating variables peculiar to a given type of reactor or operating configuration. The nutrients must be effectively and/or efficiently utilised and secondary pollution or avoidance post treatment needs to be obviated as far as possible. Plugging of the reactor components or active support device is a major consideration when control of biomass is dealt with in the suspended culture type reactors by cell recovery and recycle using settlers or ultra- or micro-filtration steps. In the case of the attached or immobilised cell systems it is necessary to provide some form of shear or possibly scraping or other mechanism to release excess biomass to prevent plugging. A successful bioreactor system would be one in which this aspect is adequately managed by the process operators.

A Transverse-flow Membrane Gradostat Bioreactor concept (using capillary membranes) described by Leukes (1999) offered continuous production of the pollutant-degrading enzymes and simultaneous control of the fungal growth. Their laboratory scale reactor

configuration, however, presented operational problems related to pressure loss in the lumen which caused variation of the volume flux along the length of the capillaries. This caused the desirable gradostat effect to be compromised. This problem appears to be one of the overall design problems for scaling up the laboratory reactor modules to industrial units. The collaboration project (see acknowledgements) had previously identified the need for engineering design and development of the Transverse-flow Membrane Gradostat Bioreactor.

The research group at Peninsula Technikon considered it wise to gain a practical working knowledge of fungal growth on membranes before attempting any modeling or scale-up. Early work therefore included flask cultures and flat sheet membrane cultures. The next step would have been single capillary membrane cultures leading to a larger scale Transverse-flow Membrane Gradostat Bioreactor. Work performed by Mokrani (2000) on gas transfer through capillary membranes provided a parallel opportunity to learn about the characteristics of capillary membrane bioreactors. Alternative ways of employing membranes in bioreactor systems, such as bubble-free aeration of suspended cultures, removal of carbon dioxide waste gases, pH regulation, etc. were also of interest and therefore gas transfer in membranes was considered important in the research program.

The "Packed Bed Bioreactor" suggested by Moreira *et al.* (1997) is an alternative fungal reactor that could allow control of fungal biomass accumulation. The system proposed by Moreira *et al.* (1997) is also capable of controlling secondary metabolic conditions necessary for maximum enzyme production for longer periods of time. It was felt that an investigation of this packed bed concept would be an appropriate intermediate step to attaining the ultimate goal of an industrial scale fungal bioreactor. This system could also provide a benchmark against which to measure the feasibility and performance of more advanced devices such as the Membrane Gradostat Bioreactor.

The scope of the work therefore consisted of developing a packed bed bioreactor for the continuous production of enzymes using polyurethane foam pellets as carrier material.

1.2 LITERATURE SURVEY ON BIOREMEDIATION OF XENOBIOTIC POLLUTANTS

1.2.1 Xenobiotic Pollutants

Xenobiotic compounds are released into air, soil, and water environments from coal gasification, natural gas liquefaction processes and from other industrial operations that utilize petroleum-derived products (Lanouette (1977); Klein and Lee (1978); Galli (1990); Leukes (1999)). Aromatic compounds used in products such as refrigerants, fire retardants, paints, solvents, herbicides and pesticides could be toxic or have harmful effects when released into the environment. Polycyclic aromatic hydrocarbons occur as constituents and combustion products of fossil fuels and are widespread environmental contaminants (Heitkamp *et al*, 1988). Substituted polyaromatics eg. PCBs are used in electrical tranformer oils, lubricants and other products where their chemical stability is of value. They enter the environment through leakages and unsafe disposal practices.

Aromatic pollutants persist in the environment because of their resistance to microbial attack is conferred by their chemical structure that differs from naturally occurring microbial substrates (Galli, 1990 and Leukes, 1999). Further, many industrial chemicals are halogenated and halogenation often is implicated as a reason for their resistance to microbial attack (Chaudhry *et al*, 1991). By the 1980`s waste treatment and environmental remediation had become a serious financial burden for U.S companies that had failed to adequately address their waste disposal practices in the past. Biological treatment and bioremediation in the treatment of hazardous, recalcitrant organics has received much attention in the past few decades.

A listing of some compounds, including xenobiotic pollutants, degraded by *P. chrysosporium* is contained in table 1.1.

1.2.2 Bioremediation Technologies

Conventional treatment of xenobiotic pollutants includes recovery, incineration, adsorption, and chemical oxidation (Lanouette 1977; Leukes, 1999). Biological treatment of industrial wastewater has become standard practice while bioremediation offers an attractive and viable alternative tool for restoration of contaminated soil, subsurface and groundwater environments (Fang *et al*, 1997).

There are several reports concerning the biodegradation of chlorinated polycyclic aromatic hydrocarbons by bacteria and fungi (Barton *et al*, 1988; Bedard *et al*, 1987; Kreis *et al*, 1981; Subbo-Rau and Alexander, 1985).

Extracellular enzymes that are produced by White Rot fungi are the objects of great interest for the development of biotechnological processes. Purified forms of these enzymes have been found to directly oxidize recalcitrant xenobiotics such as polycyclic aromatic hydrocarbons, chlorophenols and azo dyes (Collins *et al*, 1997).

White Rot fungi, such as *Phanerochaete chrysosporium* and *Trametes versicolor* cause mineralization of polycyclic aromatic hydrocarbons (PAHs), indicating that complete breakdown of PAHs occurs (Pickard *et al*, 1999). Pyrene, a polycyclic aromatic hydrocarbon with four fused aromatic rings, has been detected in environmental samples (Grossler *et al*, 1991; Kucklick *et al*, 1997 as referenced by Ravelet *et al*, 2000). It has been used as an indicator for PAH-contamination monitoring (Gschwend and Hites, 1988). Bacterial degradation of pyrene has been studied by Grossler *et al* (1991) and Kucklick *et al* (1997). A very limited number of studies with fungi have, however been published (Ravelet *et al*, 2000). A number of white rot fungi including *Phanerochaete chrysosporium* (Hammel *et al*, 1981) and *Pleurotus ostreatus* (Bezalel *et al*, 1997) have been shown to degrade pyrene.

Potential advantages (Belfort 1989; Nicell *et al*, 1992) of using biotechnological treatments are:

- The treatments can operate under milder, less corrosive conditions (pH, temperature and pressure).
- The technique can operate in a catalytic manner.
- Execution occurs as many sequential reaction steps.
- The treatments operate on organics not removed by existing physico-chemical processes.
- Lower consumption of oxidants.
- Reduce amounts of adsorbent materials, such as charcoal, for disposal.
- Greater efficiency of biocatalysts and biosorbants.
- The treatments enhances greater yield and process efficiency.
- The method uses less expensive process equipment.

1.2.3 The White Rot Fungi

White rot fungi have received attention over the past two decades because of their ability to degrade lignin (Buchanan, 1999). These fungi belong to the group of Basidomycetes that naturally colonize wood. They decompose lignin in lignocellulosic materials to cause the white rotting of wood. Under starvation conditions the white rot fungi produce extracellular ligninolytic enzymes composed of peroxidases and laccases (Kirk and Farell, 1987). The physiological role of lignin biodegradation is in the destruction of the lignin matrix so that the fungus can gain better access to its preferred substrates (Field *et al*, 1992).

Phanerochaete chrysosporium is a potentially useful filamentous fungus because it is able to degrade a broad spectrum of structurally diverse organic compounds (Bumpus and Aust, 1985; Ruckenstein *et al*, 1994).

The following conclusions are relevant:

- Most microbial systems are adapted to act upon water-soluble substrates. However, many organopollutants, DDT for example, are poorly soluble in water, thus restricting their availability for attack by microorganisms because the microorganisms produce intracellular enzymes requiring transport of the pollutant across the cell wall (Bumpus and Aust, 1985)

- Lignin, is an insoluble macromolecule. In white rot fungi, the lignin degrading system can still function under such conditions whereby the substrates are poorly soluble in water because of the extracellular enzymes produced by the fungi (Leukes 1999).

- White Rot fungi produce enzymes under starvation conditions rather than in the presence of substrate as in the case of many bacterial systems (Leukes 1999).

- Most microorganisms are not able to degrade a broad spectrum of structurally diverse organopollutants. This restricts their use for bioremediation to situations where only a limited number of pollutants are present (Aust, 1995).

Biosynthesis and Biodegradation of Lignin

Lignin is an aromatic complex three-dimensional polymer, which can be regarded as nature's plastic (see figure 1.1)

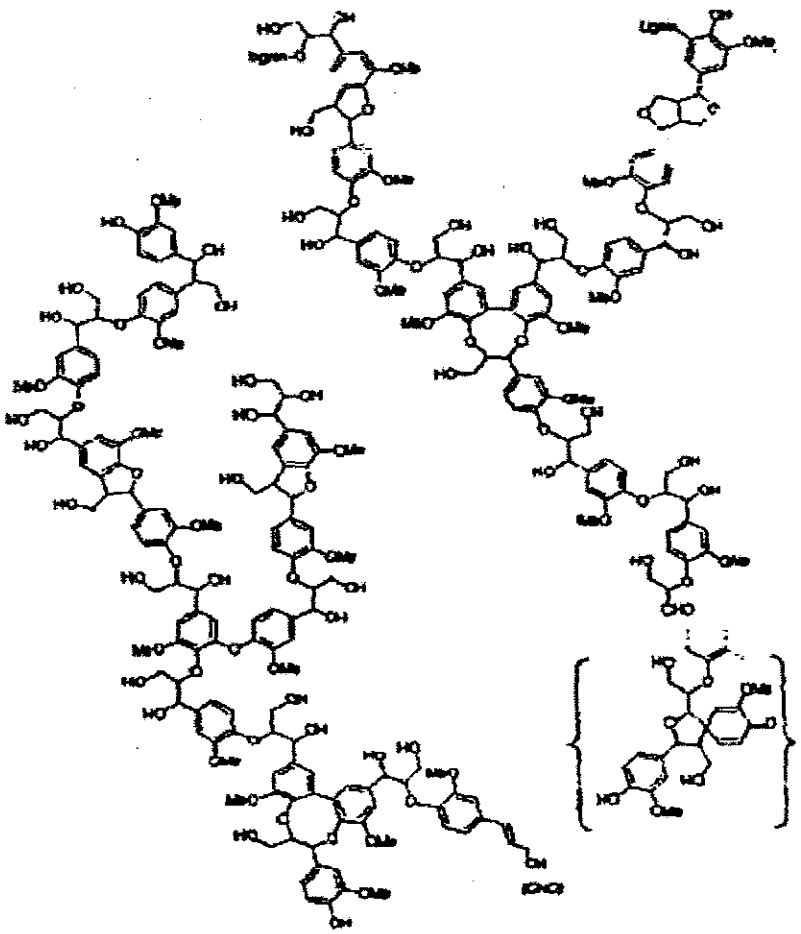


Figure 1.1: Lignin Structure
(Leukes, 1999)

Lignin imparts structural rigidity to woody tissue and protects it from most forms of microbial attack. However, certain filamentous fungi are capable of degrading it to the level of CO_2 (Tien, 1993).

Biosynthesis of lignin is achieved through plant enzyme systems which catalyses the formation of phenoxyl free radicals (from precursors such as *p*-hydroxycinnamyl

alcohols). These then polymerize in a random fashion to form the lignin polymer. Most of the bonds in lignin are β -aryl ether linkages (see figure 1.1). The structure of lignin is irregular due to the existence of other carbon-carbon and carbon-oxygen bonds that occur in lignin in various random proportions (depending on the particular plant species). These properties combine to make lignin very resistant to biodegradation because most microorganisms do not possess an enzyme system that is capable of degrading molecules that lack structural and stereo regularity.

1.2.4 Ligninolytic Enzyme Production Systems

Both extra- and intracellular enzymes have been mainly used in the food, beverage, biotechnical industries and in household products although a great potential also exists in the forest, chemical, pharmaceutical industries and in waste treatment. Many problems are, however, associated with the economical production of enzymes on a large scale i.e. Lignin and Manganese Peroxidase are produced in the secondary metabolic phase which is accomplished by starving the fungus (*Phanerocheate chrysosporium*) of a carbon or nitrogen source. The quantities of these enzymes produced are therefore dependant on factors such as controlling the nutrient concentrations (co-factor concentrations), keeping the secondary metabolic phase active for as long as possible, etc.

Maintaining sufficient and optimal aeration and mixing rate is a key operating condition for ligninolytic enzyme production. Feijoo *et al.* (1995) reported that by introducing oxygen directly into bioreactors (instead of introducing it in the feeding stream) allows the Lignin Peroxidase synthesis to be favoured. He also reported that a feeding strategy of maintaining a residual glucose concentration in the bioreactor during the production phase enhanced enzyme stability as the production of enzyme inhibitors was minimized.

1.2.4.1 Improving ligninolytic enzyme production of White Rot Fungi (WRF)

Much attention has focussed on methods of improving the production of peroxidases (Bonnarme *et al,* 1993). Most of these methods have, however, not addressed possible

modifications to the bioreactor itself. In particular little attention has been given to mixing and aeration factors mentioned above.

Jager *et al.* (1985) reported that the production of lignin peroxidase (LiP) markedly increased with the addition of nonionic surfactants, such as sorbitan polyoxyethylene monooleate (Tween-80) and sorbitan polyoxyethylene monolaurate (Tween-20) in agitated submerged cultures. Asther and Corrieu (1987) reported that LiP production could be markedly increased and the fermentation time for maximum activity reduced when the culture was supplemented with Tween-80. Addittion of oleic acid, with or without Tween-80 as an emulsifier, also gave similar effects. Tien and Kirk (1988) reported that LiP production showed a high temperature dependence and that the rate approximately doubles with every 7°C increase. Bonnarme *et al.* (1990) reported that production of LiP was enhanced when the growth medium was supplemented with various phospholipid sources. According to these workers, one could also improve LiP production by selecting mutants of *P. chrysosporium.* Palma *et al.* (1997) reported that manganese peroxidase activity is greatly dependent on the Mn^{+2} concentrations.

By adding veratryl alcohol to the final growth medium the lignin peroxidase production could be enhanced (Ruckenstein and Wang, 1994). Addition of tryptophan to various cultures could have a large stimulatory effect on lignin peroxidase activity levels (Collins *et al*, 1997). The enhancement was greater than that observed in the presence of veratryl alcohol.

Bonnarme, *et al.* (1993) also reported that the selection of a carrier support material played an important role. Kirkpatrick *et al.* (1987) found that the immobilization of the fungus on hydrophobic polyurethane foam permitted an increase in LiP production in agitated submerged cultures.

1.2.4.2 Harvest of the enzyme

Tien and Kirk (1988) described the process for harvesting of enzymes from *Phanerocheate chrysosporium* cultures. Shallow stationary as well as agitated methods were used to grow enough culture to produce enzyme supernatant.

Shallow stationary cultures (10ml) were grown in rubber-stoppered, 125-ml Erlenmyer flasks at 39°C under 100% oxygen. The utilization of 400 flask cultures typically yielded about 3.8 liters of ligninase-containing culture supernatant. Agitated cultures, 45 and 750 ml, were grown in 125-ml Erlenmeyer flasks and 2-liter Erlenmeyer flasks respectively. The cultures were grown at 39°C on a rotary shaker and flushed with 100% oxygen at the time of inoculation and daily thereafter. The shaking speed was adjusted for the small flasks to 200 rpm, and the larger ones to 125 rpm. Enough cultures (about 4.2 liters of cultures) were grown to yield approximately 3.8 liters of culture supernatant.

Mycelial growth stopped on day 2 under nitrogen limited conditions. On day 4 ligninase activity appeared in the supernatant. On days 5 and 6 the activity reached a maximum for both stationary and shaken cultures. The supernatant was obtained by centrifugation at 10 000 g for 5 minutes at 4°C. The yellow supernatant (3.3 liters), which contained all the activity (0.075 U/ml), was then concentrated by ultrafiltration using a 10-kDa cut-off membrane. After concentration to approximately 40 ml, the preparation was filtered (0.45-µm) to remove precipitated mycelial slime and then further concentrated (Amicon, 10 kDa cut-off) to a final volume of 13.5 ml. The sample was then dialyzed overnight against 4 liters of 10-mM sodium acetate, pH 6, for Mono-Q chromatography. The concentration and dialysis step resulted in very little loss in total activity.

It may be assumed that similar steps in downstream processing would be appropriate for commercial scale harvesting of enzymes produced in any of the bioreactor systems contemplated in this thesis. The cost of such downstream processing may be a significant factor to take into account when selecting between the two alternatives of direct degradation of a pollutant within a fungal bioreactor or an external enzyme contacting system.

1.2.5 The Degradation Process (*Process Chemistry*)

Lignin Peroxidase

Many wood degrading fungi produce lignin peroxidase. Two important factors distinguish the wood degrading fungal peroxidases from plant peroxidases such as horseradish peroxidase: they display very low pH optima and much higher redox potential optima.

Activity and stability of the enzyme (LiP) of *Phanerocheate chrysoporium*

Maximal LiP activity has been determined at pH 2.5. However, the enzyme is found to be unstable at that pH. Activity decreases as pH increases. No activity is observed above pH 6.5. The enzyme is stable at pH 3.5. Lignin peroxidase is found to be active over a wide range of temperatures, with an upper limit of approximately 60 °C and maximum activity under standard conditions in the range of 45°C-50°C. (http://www.tienzyme.co.za, 10/11/2000).

Degradation process

Leukes (1999) described the mechanism of lignin degradation by the white rot fungi (WRF) as complex. The ligninolytic enzyme system of the WRF is an extracellular oxidative process initiated by nitrogen, carbohydrate or sulphur starvation (Bumpus and Aust 1987; Leukes, 1999). It was discovered that the extracellular enzymes are capable of the degradation of lignin model compounds in vitro (Tien and Kirk 1983; Leukes, 1999). The mechanism of action of the LiP isozymes is similar to that of other peroxidases and is proposed to occur according to equations (1) to (3) below. (Leukes, 1999).

$$LiP + H_2O_2 \longrightarrow LiP(I) + H_2O \qquad (1)$$

$$LiP + Ar \longrightarrow LiP(II) + Ar^{+}. \qquad (2)$$

$$LiP(II) + Ar \longrightarrow LiP + Ar^{+}\cdot + H_2O \qquad (3)$$

Isozymes are multiple molecular forms of the same enzyme (Ferscht, 1984). Using two electrons from hydrogen peroxide to produce a form of peroxidase known as Compound (I) initially oxidizes the enzyme. Chemicals having a suitable reduction potential, such as aromatic pollutants can reduce this Compound (I) by one electron further to form Compound (II). The aromatic compound is then oxidized to form a radical. Compound (II) returns to its resting state when a second aromatic compound donates a further electron (Aust 1995; Leukes, 1999). The free radicals diffuse into solution where they undergo further degradation reactions or polymerization with other aromatic compounds (Nicell *et al,* 1992; Leukes, 1999). Lignin peroxidase exhibits a "Ping-Pong Bi Bi" kinetics which means that H_2O_2 first oxidizes the enzyme and the oxidized enzyme [Compound (I)] reacts with the substrate (Aust, 1995). Ping pong refers to reactions in which one or more products are released before all the substrates are consumed (Fersht, 1984).

Manganese Peroxidase

Manganese peroxidase (MnP) is another enzyme which plays a major role in lignin degradation (Leukes, 1999). It exists as a series of glycosylated isozymes with an isoelecric point ranging from 4.2 to 4.9, and with molecular masses ranging from 45 to 47 kDa. Manganese peroxidase is unique among peroxidases in that its substrates are organic acid chelates (i.e. oxalate, malonate, lactate) of Mn (II). These chelates become oxidized to the corresponding Mn (III) complexes that diffuse away from the enzymes active site and oxidise secondary substrates.

Activity and stability of the enzyme of *Phanerocheate chrysoporium*

The pH optimum for Manganese peroxidase isolated from *P.chrysosprium* is 4.8 with approximately linear losses of activity down to pH 4.0 (~10% activity remaining) or up to pH 6.0 (~10% activity remaining). Manganese peroxidase was found to be active over a

wide range of temperatures, with an upper limit of approximately 60°C and maximum activity under standard conditions in the range of 40°C-50°C (http://www.tienzyme.co.za, 10/11/2000).

Degradation process

The oxidation step by MnP is dependent on free manganous ion [Mn (II)]. The primary reducing substrate in the MnP catalytic cycle is the Mn (II), which reduces both Compound (1) [MnP(I)] and Compound (II) [MnP(II)] and generates Mn (III) which oxidizes the organic substrate as shown in equations (4-6) (Gold and Alic 1988; Leukes,1999):

$$MnP + H_2O_2 \rightarrow MnP(I) + H_2O_2 \qquad (4)$$

$$MnP(I) + Mn(II) \rightarrow MnP(II) + Mn(III) \qquad (5)$$

$$MnP(II) + Mn(II) \rightarrow MnP + Mn\ (III) + H_2O \qquad (6)$$

Significance

The significance of understanding the above degradation processes of Lignin and Manganese peroxidase lies in the application of these enzymes to a particular effluent stream or pollutant and substrate. It does not contribute directly to the design of a fungal bioreactor but rather to how the enzymes produced from the bioreactor can be useful in biodegradation.

1.2.6 Potential Industrial Applications of the Ligninolytic Enzymes of *P. chrysosporium*.

Most research to date on ligninolytic enzymes relates to bioremediation of aromatic pollutants. Recently, however, other applications of the enzymes were also evaluated (Leukes, 1999):

- Fungal peroxidases could be used in cases such as pump-and-treat systems to precipitate aromatic pollutants by polymerization, especially in cases such as leakages from underground petroleum storage tanks (Shannon and Unterman, 1993).

- The peroxidases could also be used to link the pollutants to humic polymers to make them inert (Shannon and Unterman, 1993).

- One of the major applications of the ligninolytic system is the bioremediation of soil. The same enzyme system could be applied to water contaminated with aromatic compounds (Leukes *et al*, 1996).

- Fungal peroxidases could be used in the enhancement of polymerization of lignin for the production of various composite materials as an alternate source of resins (Candeias 1995).

- Manganese peroxidase is used for bleaching pulp and paper i.e. removal of coloured compounds from the pulp and paper effluents (Gerin *et al*, 1997).

1.2.7 Conclusions on Potential Regarding Bioremediation

The literature survey had enabled the author to pinpoint the most crucial and important factors to consider when designing the bioreactor. A clearer understanding of the importance of the ligninolytic enzyme degrading system of *Phanerochaete chrysosporium* had been gained. Several reactor configurations on a laboratory scale have been suggested by gaining insight first through experimental flask culture work. This is done in order to optimize the fungal growth and enzyme production with a view to production scale operations. There is a need to maintain secondary metabolic conditions (necessary for peak enzymatic production of the fungus) stable for longer periods than grown in a batch reactor. The preference of having the fungus in a better controllable environment (by allowing it to form a carrier-immobilized biofilm) led to the

development of various models of continuous reactors. It was therefore important to explore the different types of fungal bioreactors.

1.3 LITERATURE STUDY ON TYPES OF BIOREACTORS

1.3.1 Suspended and Attached Growth in Batch Reactors

Previous experimental work on flat sheet membranes and in flasks (Fillis, 1997; Dayile and Martin, 1998) led to the following observations:

- The use of air or oxygen as well as the type of carrier support material were important factors to consider.
- The fungi display a strong tendency to attach to any available surface (polyurethane foam).
- Observations such as the appearance of a brownish colour could be related to higher productivity of enzymes during periods of nutrient starvation.

These observations led the researchers to conclude that a continuous reactor (rather then flask cultures) immobilized with polyurethane foam pellets would be a suitable reactor configuration for the continuous production of the enzymes.
See Appendix A for detailed observations on suspended and attached growth in flask cultures.

1.3.2 Packed Bed Bioreactors

In chemical process industries packed bed reactors are mostly used for absorption operations (to enhance gas-liquid contact). This is usually achieved by randomly oriented packing material that has the purpose of spreading the liquid flows over the packing surface. The increased surface area may also be created by regularly positioned packing (Perry *et al*, 1984).

A packed bed bioreactor would utilize similar principles except that a biofilm exists on the surface of the packing. A well-known example of this is the trickling bed filter used

in wastewater treatment. The packing serves to distribute air or oxygen so that it can reach the biofilm. The gas phase can be introduced intermittently between flooding of the packing or continuously in either a co-current or countercurrent fashion. Alternatively the packing may be submerged in a continuum of liquid phase with the gas phase being dispersed as bubbles. The bubble dispersion may be introduced continuously or intermittently in a pulsing mode.

Feijoo *et al.* (1995) reported that fungal packed bed bioreactors operated in semi-continuous mode could provide a better solution to scaling-up enzyme production than using batch cultures. Moreira *et al.* (1997), in their efforts to produce Lignin and Manganese peroxidase for extended periods of time, operated a packed bed bioreactor inoculated with *Phanerocheate chrysosporium*, (BKM-F-1767). They made use of oxygen pulses in the input stream to shear away excess mycelium growth. A schematic of the system used by Moriera *et al.* (1997) is shown in Figure 1.2 below.

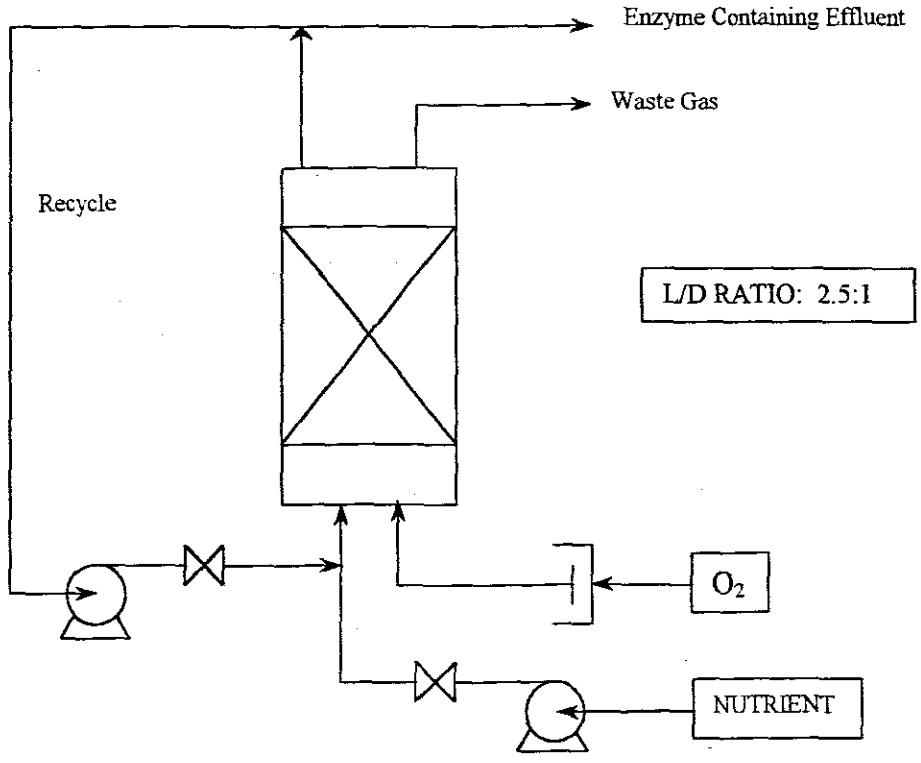


Figure 1.2 Packed bed bioreactor with gas pulsing and recycle proposed by Moreira *et al*, 1997

The system shown above was operated under the following conditions:

- The temperature was maintained at 37°C.
- Oxygen was supplied to the reactor in a *pulsing mode and controlled* at 1.5 cm^3/min and 4 cm^3/min.
- The single bed consisted of randomly pack polyurethane carrier pellets (0.5 cm^3)
- Surface area of polyurethane carrier pellets was 414 ± 10 m^2/m^3
- Medium fed to the bioreactor:

  N-Limited medium with glucose (2-5.7 g/l)

  Ammonium tartrate (20-60 mg/l) in 20 Mm sodium acetate buffer (4.5)

  B(III) mineral medium (Tien and Kirk. 1988)

Moreira *et al,* 1997 achieved steady-state conditions for up to 140 days during which time the fungi sustained secretion of Manganese Peroxidase and displayed a maximum activity of 250 Units/L (see table 1.2 below).

Type of Bioreactor / Process	Operation Time (days)	Max. Activity (UL^{-1})	Productivity (P) ($U.L^{-1}d^{-1}$)
Packed bed / Continuous	140	250	202

Table 1.2. Packed bed/continuous bioreactor productivity of MnP (Moreira *et al,* 1997)

From our the study of their work four fundamental problems relating to development of a packed bed bioreactor system for our intended application were identified:

- Proteases produced by the same fungus destroy Lignin and Manganese Peroxidase enzyme activity.
- Extracellular protease accumulates in Moreira's packed bed bioreactor through recycling of the nutrient.
- Complete mixing inside the bioreactor favors distribution of any protease formation.
- A better understanding of the fungal growth and biofilm morphology and behaviour would be beneficial.

To eliminate the sources of backmixing and to reduce the protease effect it was decided in the current study not to use recycle. The L:D ratio was also increased to 14:1 to further reduce mixing effects that might result from pulsation of the oxygen stream. A detailed description of the experimental rig used in the current study is given in Chapter 3, Section 3.2.

1.3.3 Fluidized-bed Bioreactors

When a fluid is passed upwards at low flow-rates through a bed of particulate material the pressure drop across the bed increases in proportion to the flowrate. By increasing the superficial up-flow velocity a point is reached when the frictional drag on particles equals their apparent weight (this is the onset of incipient fluidization) and the particles move apart. They offer less resistance to flow of fluid and the pressure drop increases less rapidly. As the flowrate is increased still further the bed continues to expand until individual particles separate from one another entirely and become freely supported in the fluid. The bed is then said to be fluidized (Perry *et al*, 1984). Gas bubbles can be passed through such a fluidized bed resulting in a system closely resembling a boiling liquid.

If we introduce this "fluidized bed" concept to bioreactors we can achieve aeration and control of excess biomass simultaneously. For the bioreactor it is desirable to employ carrier support material that is durable and of an appropriate size range. Resin beads offer such a carrier material for immobilized fungi. Typically, resin beads have densities in the range 1.03-1.08 kg/m^3 and diameters in the range 0.3 – 1.3 mm (Helfferich, 1962). These provide suitable bed expansion at the low solution feedrates appropriate to fungal reactors (Hendry *et al*, 1999). No literature has been found by this author which refers to fluidized bed fungal bioreactors incorporating resin beads as the support material for attached growth. It was envisaged that these systems would offer some potential and consequently research has been initiated on studying attachment of fungi on resin beads.

1.3.4 Airlift Bioreactors

The airlift pump is a device for raising liquid by means of compressed air. It is operated by introducing air into the liquid near the bottom of a draft tube submerged in the liquid. The consequent difference in densities between the liquid in the draft tube and annular section causes the liquid to circulate. The principle can be applied to achieve circulation

and aeration simultaneously in a bioreactor. Such devices are sometimes referred to as draft tube or airlift bioreactors. The advantage of this system lies in the fact that mixing can be achieved with low shear and no moving parts in the reactor. Bonnarme and Jeffries (1990) and Bonnarme *et al*, 1993 employed airlift fungal bioreactors (*P.chrysosporium*) operated in batch mode. Using this system they successfully produced the enzyme, Manganese Peroxidase. They were, however, only able to produce the enzyme for limited periods of time. Refer to table 1.3.

Reference	Type of Bioreactor/ Process	Operation Time (days)	Max. Activity (UL^{-1})	Productivity (P) ($U.L^{-1}d^{-1}$)
Bonnarme and Jeffries (1990)	Airlift / Batch	3.9	192	49
Bonnarme et al, (1993)	Airlift / Batch	3.8	365	95

Table 1.3. Airlift/ batch bioreactor productivity of MnP

1.3.5 Bubble-column Bioreactors

In these types of continuous or batch reactors, gas is sparged to distibute it as bubbles into the liquid phase (Kastanel *et al*, 1992).

Laugero *et al* (1996) proposed a bubble-column bioreactor operating in batch mode to cultivate *Phanerocheate chrysosporium* (BKM-F-1767). They reported significantly greater productivity of Manganese Peroxidase after four days than did Bonnarme *et al* (1993). See Table 1.4.

Type of Bioreactor / Process	Operation Time (day)	Max. Activity (UL^{-1})	Productivity (P) ($UL^{-1}d^{-1}$)
Bubble-column/ Batch	4	726	181

Table 1.4. Bubble-column/ batch bioreactor productivity of MnP (Laugero *et al*, 1996)

1.3.6 Transverse -flow Membrane Gradostat Bioreactor.

The term "membrane gradostat" (Leukes *et al*, 1996 and Leukes, 1999) is used to describe a type of biofilm reactor which uses a synthetic capillary ultrafiltration membrane as a support matrix for the biofilm.

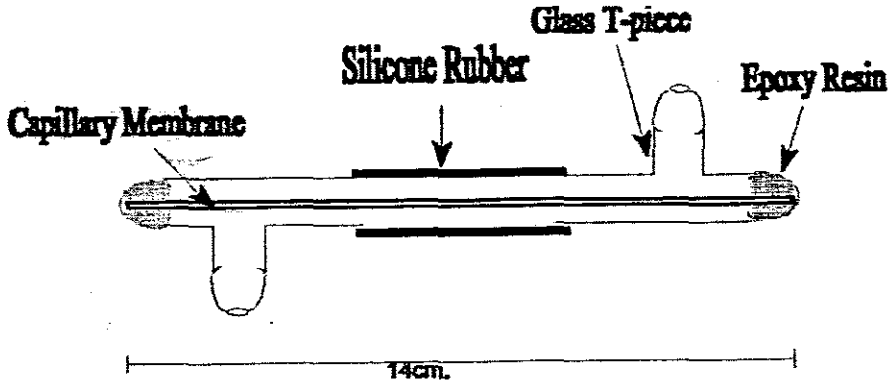


Fig. 1.3.1: A schematic diagram of a mini-membrane reactor

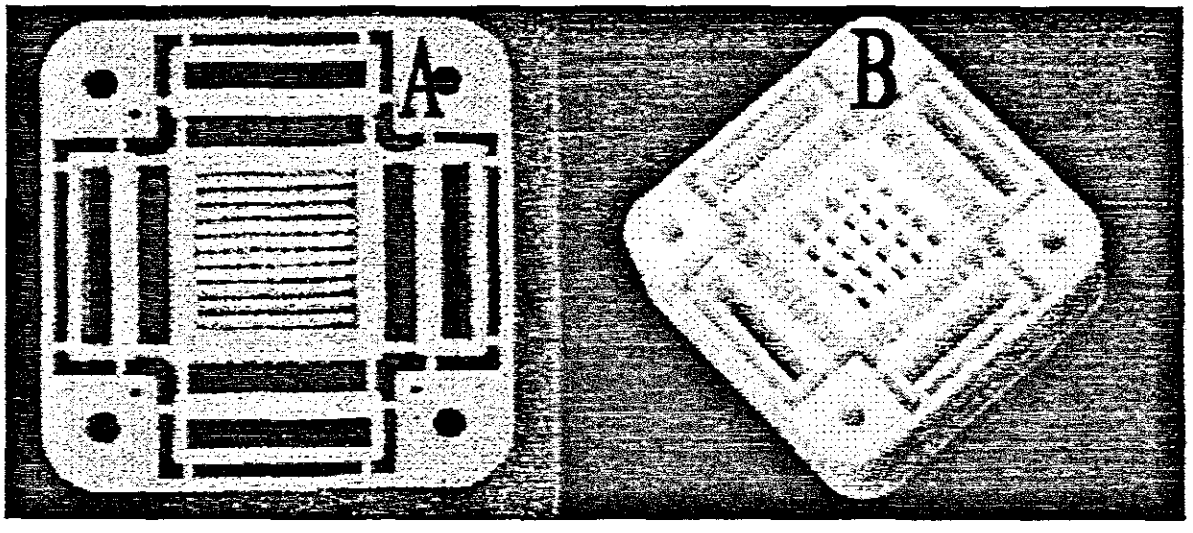


Figure 1.3.2: (A) A transverse flow module with normal fibre spacing. (B) Fibres in alternate slots giving larger interfibrillar distance (Leukes, 1999).

In its operation, fungal biomass would be immobilized onto the spongy layer of a capillary membrane. A biofilm of sufficient thickness, density and activity to establish a radial nutrient gradient across the biofilm would then be established. Biomass would then be produced in the region where nutrient rich conditions prevail. This growth

would continuously push biomass radially outwards to an area of low nutrient concentration. This nutrient starvation would stimulate enzyme production through secondary metabolism at the hyphal tips. Inactive biomass and spores produced would be sloughed off by the turbulent air supply passing through the extra-capillary space.

High surface area to volume ratio offered by capillary membranes is a favorable feature of this design as this would provide the best volumetric productivity. The Transverse-flow Membrane Gradostat Bioreactor offers control of the fungal growth kinetics and continuous production of the pollutant-degrading enzymes. The laboratory scale reactor configuration, however, presented the researchers with operational problems. The main problem being how to obtain an even transmembrane flux at all points along the length of the capillaries. Sterilization and plugging of the reactor would also need to be considered when scaling up the laboratory reactor modules to industrial units (Leukes, 1999).

The Transverse-flow Membrane Gradostat Bioreactor concept proved to be superior or at least comparable with the best reported systems, even without optimization. This bioreactor had been capable of operating continuously for 60 days.

Type of Bioreactor / Process	Operation Time (day)	Max. Activity (UL^{-1})	Productivity (P) ($UL^{-1}d^{-1}$)
Capillary membrane module/ Continuous	60	2 361	1 916

Table 1.5. Transverse flow capillary membrane bioreactor productivity of MnP (Leukes, 1999)

One of the most obvious shortcomings, however was the inability to sustain steady-state operations. This had been found to be due to fluid flow maldistribution, a hydrodynamic problem inherent to the operating conditions used.

Leukes (1999) tested the device for bioremediation applications by injecting p-cresol in the nutrient feeding line. It was concluded that the Membrane Gradostat Bioreactor would be most suitable only for producing the ligninolytic enzymes (over a certain time period) and not for direct applications to toxic effluents. This is due to the fouling of the ultrafiltration layer.

1.4 OVERALL CONCLUSIONS FOLLOWING FROM THE LITERATURE SURVEY

A summary comparison of the different types of bioreactors is shown in table 1.6 below.

Type of Bioreactor / Process	Operation Time (day)	Max. Activity (UL^{-1})
Airlift / batch	4	365
Bubble column / batch	4	726
Packed bed / continuous	140	250
Capillary membrane / Continuous	60	2 361

Table 1.6. A comparison between different fungal bioreactors in terms of operation time and maximum enzyme activity as reported in literature (Bonnarme and Jeffries, 1990; Laugero *et al*, 1996; Moreira *et al*, 1997 and Leukes, 1999)

From the literature survey on different fungal bioreactor configurations, packed bed fungal bioreactors proved to be able to sustain the longest period of process operation (140 days). From an economical point of view, operating these types of reactors on a commercial scale seems to be more cost effective than operating capillary membrane bioreactors. Packed bed bioreactors would need to be shutdown only three times a year (reinocculation on inexpensive, replaceable polyurethane foam cubes) as compared to capillary membrane bioreactors which needs to be shutdown six times a year (reinocculation and, probably, replacement of membranes). Further, although capillary membrane bioreactors offer greater enzyme activity, its operational costs are higher than that of packed bed bioreactors and they are therefore less feasible to run commercially.

Short operational times of four days in stirred-tank bioreactors or low-shear stress bioreactors (airlift, bubble column) were reported. The excessive growth of mycelia provoked operational problems such as fouling of fermentor probes and growth back along nutrient feed and sampling lines. This gave rise to practical and technical difficulties in culturing fungi and consequently difficulties with regard to the control and regulation of hyphal extension and pellet size.

In the current study packed bed systems were selected for further study mainly because they appear to offer control of the fungal growth. The uniqueness of Moreira's packed bed bioreactor amongst others (see Chapter 1, section 1.3.2) was the application of a pulsation in a packed-bed bioreactor that permitted the maintenance of continuous secretion of Manganese peroxidase. It also showed promise with respect to enabling control of clogging and limiting mycelium growth. The literature confirmed that the "pulsed packed bed reactor" suggested by Moreira *et al,* 1997 had potential for further development.

CHAPTER 2

FORMAL STATEMENT OF PROBLEMS, OBJECTIVES AND ASSUMPTIONS GUIDING THE INVESTIGATION.

2.1 PROBLEM STATEMENT

Moreira *et al.* (1997) adopted recycling for three reasons:

- It allowed glucose concentrations to be kept high throughout the packed bed to reduce protease production that was unwanted because it destroys peroxidases.
- To maximize the rate of mass transfer of oxygen into the biofilm.
- Recycling would provide increased fluid velocities to control (shear away) excess fungal biomass that would be a consequence of having excess glucose.

They demonstrated that recycling caused Manganese peroxidase product destruction because of the proteases being recycled.

They later adopted oxygen pulsation to:

- Substitute for high liquid velocities i.e. to reduce the recycle rate (decrease fluid velocity).
- Further improve oxygen mass transfer into the active biofilm (pellet center is oxygen deprived) while high peroxidase production needs high oxygen concentration).

2.2 STATEMENTS OF SUB-PROBLEMS

2.2.1 Recycle Problem

Can we run the packed bed bioreactor without recycle and still control excess biomass clogging?

Moreira *et al.* (1997) implemented recycling of the nutrient in their design in order to wash out excess biomass and to avoid oxygen limitation in the pellet. Through this mechanism, however, extracellular protease was also being recycled back to the bioreactor.

2.2.2 Length/diameter (L/D) Effect on Mixing

Does increasing the L/D sufficiently reduce the backmixing?
By how much should the L/D be increased to gain benefits?

The bioreactor proposed by Moreira *et al.* had a relatively small L:D ratio (2.5:1). This allowed an almost completely mixed behavior inside the bioreactor. This could be overcome by increasing the L/D ratio and by avoiding recycle.

2.2.3 Biomass Control /Fungal Growth and Productivity Model

What do we need to analyze for and what methods do we need to use, to collect data that can be useful to feed into a process model that would help in the design of a scale-up reactor?

A satisfactory process model would provide insight about the growth, steady state and death phase of the fungal biofilm and assist in the design of a full-scale reactor system.

2.3 RESEARCH PROJECT OBJECTIVES:

Specific objectives:

- To establish whether a packed bed bioreactor using oxygen pulsing could be successfully operated without recycle.
- To establish whether increasing the L/D ratio will result in an operable system.

Secondary objectives:

- To propose an improved packed bed bioreactor design, which can be used without serious operational problems.
- To obtain more insight about the growth of the fungi in various types of bioreactors.
- To measure nutrient consumption rates and obtain mass balances for glucose consumption.
- To attempt to model the production rates for the enzymes (Manganese Peroxidase and Lignin Peroxidase) or at least the glucose/substrate consumption rates in a modified packed bed bioreactor so as to establish a tentative design basis for a commercial scale bioreactor.

2.4 ASSUMPTIONS MADE:

- All spores will be effectively immobilized on the carrier material to provide even inoculation of the packed bed of polyurethane foam pellets.
- The cylindrical polyurethane pellets used (0.6 cm ϕ x 1 cm long) would provide a packed bed of similar total surface area and voidage (0.02 m^2 and 0.56 respectively) to that of Moreira (0.04 m^2 and 0.46 respectively).
- The reduction of axial velocity resulting from elimination of recycle would result in reduced wash out of excess biomass but the pulsation of oxygen supply would compensate and provide adequate control of plugging.

By having a clear layout of the problem statement, sub-problems and objectives for this thesis the author then investigate in chapter 3 to find solutions to the mentioned problems through experimental work. A systematic approach following the objectives provided a valuable guide that contributed well to the knowledge and understanding of this research field.

CHAPTER 3:

EXPERIMENTAL DETAIL: PACKED BED BIOREACTOR (PBB) DESCRIPTION AND HYDRODYNAMICS, START-UP AND RESULTS

3.1 INTRODUCTION:

In spite of the problems experienced by Moreira *et al.* (1997) it was nevertheless concluded that their reported reactor performance would serve as a suitable benchmark for the current study. Modifications to the bioreactor configuration proposed by Moreira *et al.* were, however, made in order to overcome their operational problems (Chapter 1, Section 1.3.2 and Chapter 2).

A modified bioreactor system, based on the reactor system of Moreira *et al*, was designed for the current study. The bioreactor system designed was used to determine the Residence Time Distribution (RTD) of nutrients within the reactor as well as to study the kinetics of enzyme production. A large number of experimental runs were initially performed on this bioreactor. The large number of runs was required due to two reasons. Firstly, the author needed to develop competency and relative facility with the analytical methods required. Secondly, this was done so as to become familiar with, and overcome, the many unforeseen and seemingly intractable problems that constantly presented themselves.

While these early runs were underway a program of flask culture work was simultaneously. The flask work was conducted to determine alternative support materials (polyurethane foam and resin beads) for use as packing material. Owing to the exploratory nature of these runs only data for the most recent two successful runs are presented and discussed.

The bioreactor was loaded with 80 polyurethane foam pellets (6x10mm), attached to 5 strings (nylon thread) carrying 15 pellets with a 5mm spacing between one another. The reason for attaching the pellets to strings was the fact that they would otherwise float at the top of the reactor due to the density difference between the liquid and the pellets. While these runs were successful from the point of view of our experimental objectives, they also were limited in duration due to bed clogging and plugging of lines. It became clear that the oxygen pulsation technique as implemented in our work did not adequately control the accumulation of biomass. Nevertheless, we concluded that although it is difficult to maintain the operation for extended periods, packed bed bioreactors could be successfully operated to provide repeatable results.

Dealing with operational problems the author experienced with the modified packed bed bioreactor.

- To avoid contamination, such as growth of the fungi on the inside of the nutrient feeding lines we autoclaved all the glassware and tubing at 121°C for 15 minutes
- Separate feeds for trace elements, glucose and ammonium, each with independently adjustable feed rates which allowed easy variation of the main nutrient parameters and concentrations in the feed to the reactor.
- The ammonium, glucose and trace elements solutions were fed to the reactor with separate feeding lines from 5 liter autoclaved Schott bottles.

1. If any of those bottles would become contaminated, it could be easily replaced without stopping the experimental run.
2. The carbon to nitrogen ratio (C:N) could also be easily altered without any inconvenience (simply by altering the pump setting).
3. It avoided the problem that, if all the nutrients were supplied in one Schott bottle, growth of unwanted organisms and contamination would easily take place since all the necessary nutrients would be present.

- We incorporate millipor filters (0.22 μm) as a further precaution to avoid clogging up of nutrient feeding lines. An alternative filter (using glass wool) was used later because the disposable millipor devices plugged frequently.

3.2 APPARATUS DESCRIPTION

A schematic of the bioreactor system used is given in Figure 3.1 below:

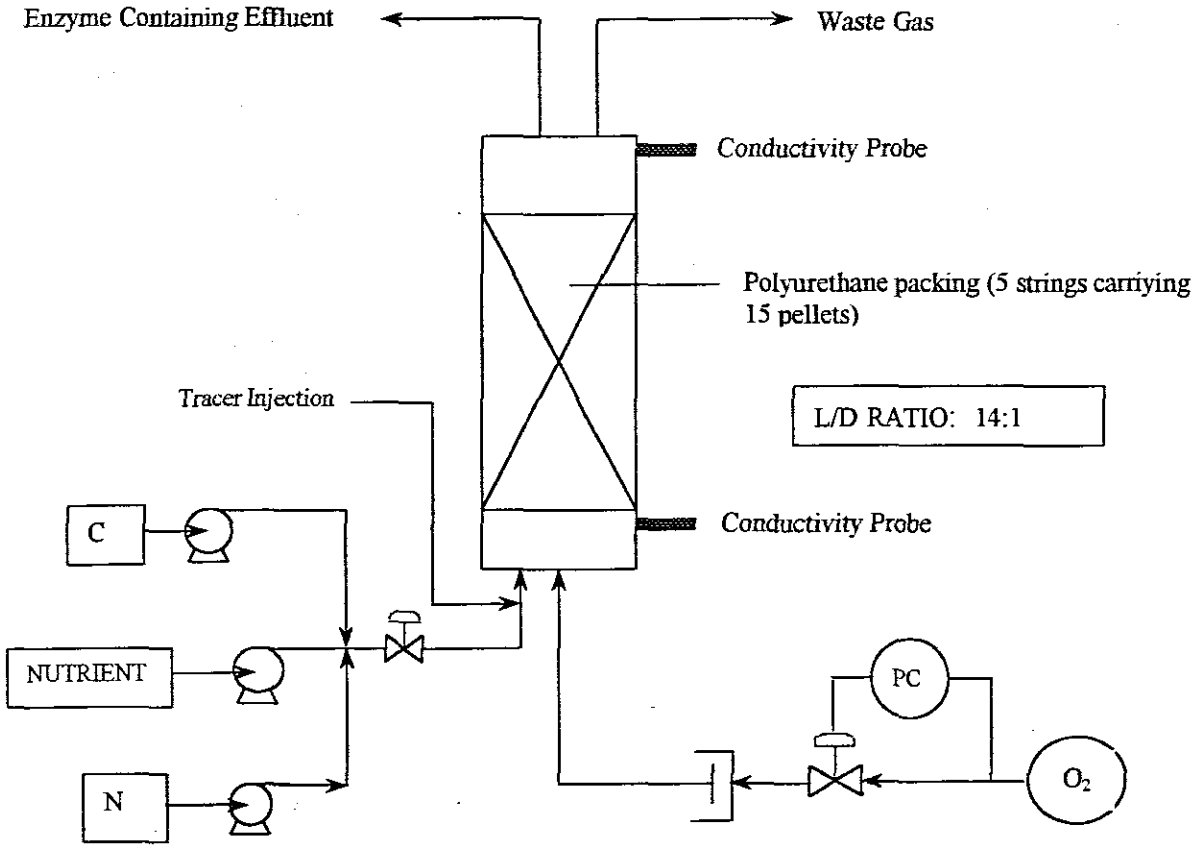


Figure 3.1: Modified packed bed bioreactor (Pentech design)

Operating conditions: (for all PBB experiments)

We intended to vary some of these parameters but the time frame for the experimental program did not allow it (interupted by a study visit to The University of Michigan).

Glucose solution flowrate	0.7 ml / min (100g/l)
Ammoniumtartrate solution flowrate	0.3 ml / min (8 g/l)

Nutrient flowrate	0.76 ml / min
Total solution flowrate (feed) into bioreactor	1.76 ml / min
Oxygen flowrate (pulse frequency - 8 s)	20 ml/min

The bioreactor was constructed out of clear PVC tube.

ID	= 36mm
OD	= 40 mm
Cross sectional area	= 10.2 cm^2
L: D ratio	= 14:1
Length of tube	= 51 cm
Volume of packing	= 519 cm^3

Time constraints,however, did not permits an investigation of all parameters (interupted by a study visit to The University of Michigan).

See section 3.4 for experimental details for live PBB runs.

3.3 STUDY OF HYDRODYNAMICS OF THE MODIFIED PACKED BED BIOREACTOR

3.3.1 Introduction:

The performance of a chemical reactor depends not only on the relevant intrinsic kinetics of the reaction processes, but also on the physical processes occurring in the reactor. The physical processes such as inter-phase, inter-particle and intra-particle mass transfer occurring within a multiphase reactor depend very significantly upon the mixing characteristics of the various phases involved (Kastanel, 1992)

A duplicate reactor to that of the live bioreactor was constructed and an experimental project carried out by undergraduate chemical engineering students, whereby a technique was established to use two conductivity meters in the inlet and outlet stream of the reactor to measure the residence time distribution.. Although the polyurethane foam pellets were not inoculated with fungal spores, the positioning of the conductivity meters

as well as the point of tracer injection is shown in figure 3.1. This technique could be used for future experimental work regarding residence time distribution for bioreactor development. All the information obtained from this study was therefore transferable to the live bioreactor.

3.3.2 Residence Time Distribution (RTD)

In general, in reactors there may be significant deviation from ideal plug flow and mixed flow. The following may cause this:

- A non-uniform velocity profile
- Short-cicuiting, bypassing, and channelling of fluid
- The presence of stagnant regions of fluid caused by the reactor shape and internals
- Backflow of a fluid within the reactor as a result of relative velocity differences between two mixing fluid phases
- The recycling of fluid within the reactor as a result of agitation

However, we only need to know how long will the individual molecule spend in the reactor and this can be determined by step input or pulse input experiments. These determine the distribution of residence times of the flowing fluid (Kastanel, 1992).

3.3.3 Experimental measurement of RTD in PBB (Tracer tests)

The RTD is determined by injecting an inert with an easily measured chemical (coloured, conductive or radioactive), called a tracer at the inlet stream or at some point within the reactor and observing the corresponding response at the exit stream or at some other downstream point within the reactor. To illustrate the RTD of actual reacting fluids, a proper choice of tracer in a given reacting system is very important. The basic requirements for a satisfactory tracer experiment can be outlined as follows (Kastanel, 1992).

- The tracer should be miscible and it should have physical properties closely resembling the fluid stream under investigation

- The tracer should be accurately detectable in small concentrations, so that the introduction of a tracer does not affect the flow pattern of the main fluid stream. The small concentration should also allow approximately linear response, so that prior calibration of the tracer detection equipment can be kept to a minimum.
- Normally, the tracer should be non-reactive (inert), so that the analysis of the RTD curve can be kept simple.
- During an experiment in a multiphase system, the tracer should not be transferred from one phase to another. For example, a gaseous tracer used in a gas-liquid reactor should not be absorbed by liquid; and a liquid tracer used to measure the liquid phase RTD curve should not be volatile.
- For measuring the RTD of a very fast-moving phase, the sensitivity and the response time of the tracer concentration recording equipment may be a problem. (A radioactive tracer offers an advantage in that the scintillation detection counter can be interfaced with very rapid recording systems or multichannel analysers).
- The tracer itself (particularly for large industrial-scale systems) and its detection device and other auxiliary equipment should be relatively cheap.

It was concluded that a salt tracer (NaCl) and two conductivity probes would be suitable for system in this study.

The two most used methods of injection are pulse and step input.

The Pulse input

An amount of tracer is suddenly injected in one shot to the feedstream entering the reactor in as short a time as possible. The outlet concentration is then measured as a function of time.

The Step input

Step test is normally easier to carry out than the pulse test. At the start of the experiment, a constant amount of tracer is continuously injected into the system. The

test is continued until the concentration in the exit stream is the same as that concentration in the feed.

Experimental Details:

The reactor used in this study, simulates the reactor dimensions, packing material and operating conditions as that of the live bioreactor.

Packing material	Shape	Size (cm^3)	Length (cm)	Diameter (mm)
Polyurethane foam	cylindrical	0.5	51	36

Five millilitres of NaCl solution (0.1M) was injected as a tracer into the space below the bed in as short a time as possible. The tracer concentration in downstream and upstream positions was measured as a function of time by two conductivity cells. Distilled water at room temperature was employed as the test fluid at a flowrate of 1.85 ml / s which simulates the feed flowrate (1.76 ml / min) of the live bioreactor. Oxygen was sparged (25 ml / min), which simulates the pulsed oxygen (20 ml / min) to the live bioreactor.

Experimental results at flowrate 1.85 ml/s.

Note: C1= Upstream conductivity

C2 = Downstream conductivity, both measured in (μS/cm)

Time (min)	C1 (μS/cm)	C2 (μS/cm)
0	21.4	23.2
1	22.5	23.2
2	143.6	23.2
3	76.8	86.2
4	50.1	98.2
5	36.2	86.8
6	29.9	62.4
7	26.2	53.1
8	24.3	45.8
9	23.2	35.4
10	22.6	33.3
11	22.2	30.7
12	22	28.4
13	21.9	26.7
14	21.8	25.4
15	21.7	24.6
16	21.7	24
17	21.7	23.6
18	21.6	23.4
19	21.6	23.2

By referring to figures (3.2.1 and 3.2.2) below, one would get a rough estimate of the residence time of the tracer molecules inside the reactor at a flow rate of 1.85 ml / s

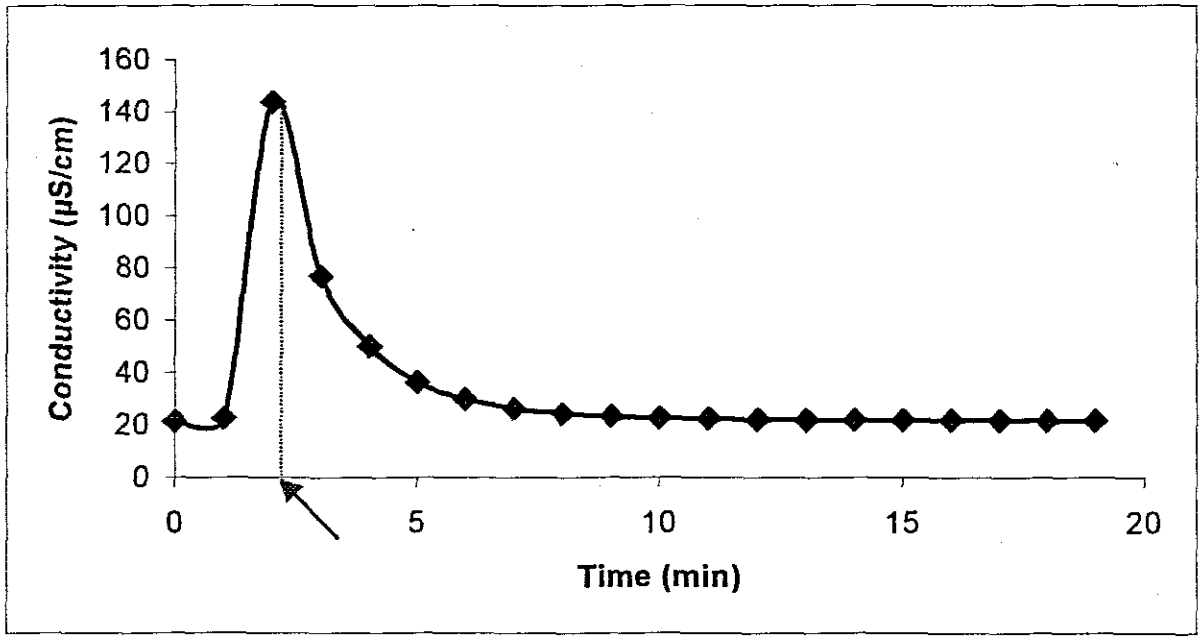


Figure 3.2.1: Upstream (C1), flowrate (1.85 ml / s) [Packing]

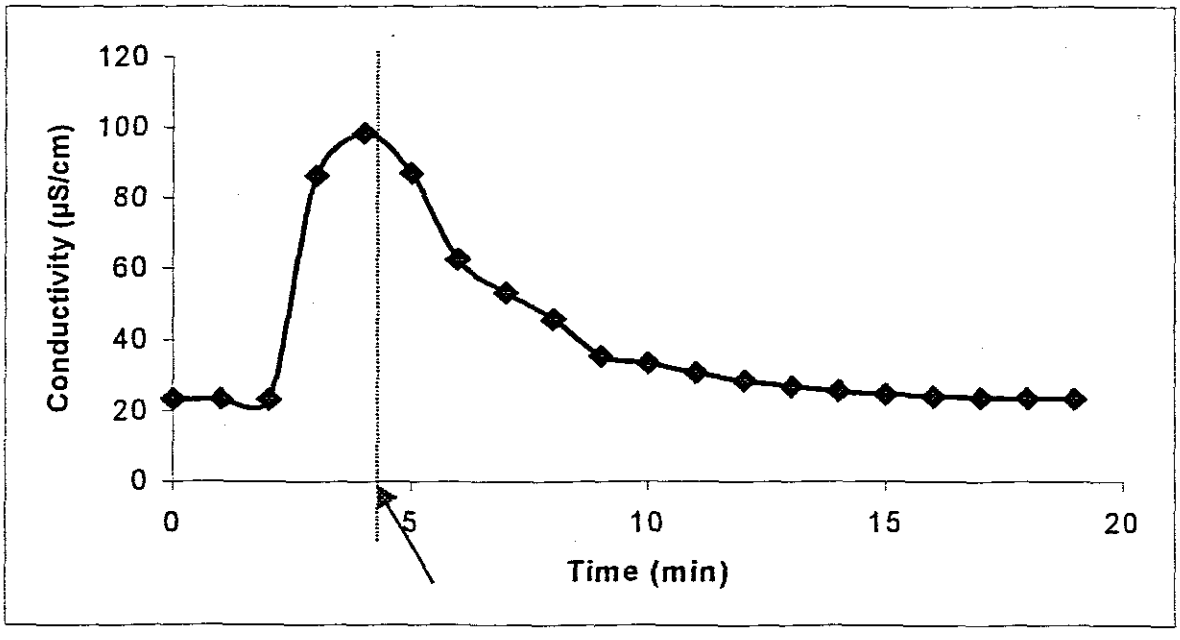


Figure 3.2.2: Downstream (C2), flowrate (1.85 ml / s) [Packing]

From the tracer tests above (figures 3.2.1 and 3.2.2) it can be seen that it took approximately 2 minutes to reach the upstream conductivity cell from the point of injection, and approximately 4 minutes to reach the downstream conductivity cell.

The tracer tests also showed that the reactor showed significant plug flow behaviour due to the following observations:

- The liquid is displaced mostly as a plug of liquid (figure 3.2.1 and 3.2.2)
- The slope in both figures are similar.
- The broadening of the peak downstream (figure 3.2.2) is most likely due to turbulent eddies within the packing created by oxygen sparging.
- It should be noted that this mixing resulted in only a 30 % reduction of the peak height (figure 3.2.2).

3.3.4 Conclusions

The tracer tests confirmed that plug flow prevails predominantly inside the reactor and that the residence time for the tracer molecules was 2 minutes.
A more thorough investigation should also include variation of the L:D ratio as well as the flowrates.

3.4 EXPERIMENTAL DETAILS FOR LIVE PACKED BED BIOREACTOR RUNS

3.4.1 Laboratory Facilities that were available

All of the live work was carried out in the Microbiology laboratory at Peninsula Technikon. The homemade bioreactor was operated in a 37°C walk-in incubation room. The feed was supplied by pumping it from outside the incubation room. Amongst the main instruments situated in this laboratory for analytical analyses were:

- Nikon (OPTIPHOT) microscopes.
- Laminarflow hood, with UV light.
- Beckman Model TJ-6 centrifuge.
- DMS 100 UV visible spectrophotometer

- 4°C walk-in fridge room
- 37°C walk-in incubation room
- ultra-low freezer (-80°C)
- MCP 4 channel peristaltic pump
- Cooling pump
- Timer/relay/solenoid valve system for oxygen control
- Pure oxygen supply cylinder and pressure regulator

The UWC Physics department provided the service for using the Electron microscope.

3.4.2 Materials and Methods

Microorganism

The following materials and methods were used in all of the experiments. The Department of Microbiology, Rhodes University, provided the organism used in this study. *P. chrysosporium* BKM-F-1767 was originally plated and cultivated at The University of the Western Cape in the Department of Microbiology under supervision of Dr. Volker Brozel. The BKM-F-1767 was a pure strain and the inoculated plates were kept in a 37°C room for optimal growth. It was a normal laboratory procedure to prepare fresh inoculated plates every six to eight weeks.

Culture conditions

The spores were maintained on malt agar grown at 37°C.

The malt agar contained (1L):

Agar	20g
Glucose	10g
Malt	10g
Peptone	2g
yeast extract	2g
KH_2PO_4	2g

$MgSO_4*7H_2O$	1g
thiamin-HCL	1mg.

Spores were harvested and the filtrate containing the spores was kept at 4°C until use. By using the light microscope the spores were observed to be uniformly spread and oval shaped. (width, 1.5 µm; length, 3µm). These observations confirmed that there was no contamination present such as different spore shapes and colors or any bacterial growth in our spore solution. These tests were always carried out in the presence of a Microbiology assistant for guidance and bacterial identification. If the sample showed any traces of bacterial contamination, it was discarded immediately.

Spore concentration

The spore concentration is determined by measuring absorbance at 650 nm with a spectrophotometer. According to Tien and Kirk (1988) an absorbance of 1.0 cm^{-1} is approximately 5 x 10^6 spores/ml.

3.4.3 Inoculation of Polyurethane Foam Pellets with Fungal Spores of *Phanerochaete chrysosporium (P. chrysosporium)*

Sterilization

The bioreactor packed with pellets was sterilized by circulating 4% formaldehyde for 24 hours. It was then rinsed with 5 liters of autoclaved water (leaching of pellets). The pellets then remain filled with autoclaved water. The bioreactor was placed under the laminar flow hood before it was dismantled only at the top bolted flange where 180 ml of nutrient solution (as described by Tien and Kirk, 1988) was poured in from an autoclaved measuring cylinder still under sterile conditions. The surface of the laminar flow hood was cleaned with 60% ethanol as disinfectant. The outlet of the measuring cylinder was flamed before the nutrient was transferred to the bioreactor. All outlets of the bioreactor were cleaned with ethanol before being closed-off with autoclaved cotton wool and foil.

Nutrient medium composition (1L):

Basal (III) medium	100ml
0.1 M Dimethylsuccinate	100ml
Ammoniumtartrate (8g/l stock)	25ml
(0.02 M) Veratrylalcohol	100ml
Trace elements	60ml
H_2O dist	563ml
10% glucose	50ml
Thiamin	2ml

Inoculation:

The spore solution (20-ml) was injected at the top of the bioreactor with a sterile pipette. The reactor was placed into a shaker (4 hours) to ensure an even spread of spores onto the polyurethane foam pellets. The shaker was adjusted to 80% oscillation at 37°C to give a satisfactory degree of mixing. Oxygen was filtered through 0.45 µm filters before it was introduced to the inoculated medium.

3.4.4 Bioreactor Start-up

The bioreactor with inoculated polyurethane foam pellets was removed from the shaker after 4 hours and connected with the oxygen and feeding lines (with in-line glass wool filters). This was regarded as Day 1 of the experimental run since the author experimented with a continuous-fed bioreactor which started when the feeding lines were connected and formed the basis for any calculations during that experimental run.

3.4.5 *Analytical Methods:*

- Manganese peroxidase activity was measured according to the method of Gold *et al.* (1988). The assay is based on the spectrophotometric determination of the oxidation of ABTS (Diammonium 2,2`-Azinobis (3-ethyl-6-benzothiazoline sulfonate)) by MnP

in the presence of lactate, Mn^{+2} and H_2O_2. The molar extinction coefficient(ε) for the oxidation product at 415 nm is 3600 $M^{-1}.cm^{-1}$. Activity was expressed as $U.L^{-1}$.

1U = 1 micromole/minute

[Appendix B provided further details]

- Ammonium concentration was measured by making use of the Spectroquant 14752, kit of Merck Ltd (1989). The Berthelot's reaction was based on the formation of an indophenol blue dye by ammonia, in an alkaline solution.

  [Appendix B provided further details]

- Glucose concentration was measured by making use of the Ciba Corning Express Analyzer [Appendix B provided further details], which used the glucose oxidase method for clinical diagnostic analyses. Glucose oxidase catalyzes the oxidation of glucose to gluconolactone and hydrogen peroxide. We tried alternative methods such as the glucose assay from experimental Biochemistry (Clark and Switzer, 1976) which gave us results that were not reliable since it gave us difficulty in reproducing it.

3.5 PBB EXPERIMENTAL DATA, RESULTS AND DISCUSSION

3.5.1 Tentative Process Model

3.5.1.1 Introduction

A complete mass balance involving the production of enzymes in the packed bed bioreactor cannot be easily calculated due to the complexity of enzyme metabolism. Overall component mass balances and a glucose mole balance (formulated on a differential element) was however, attempted.

From overall component mass balances done on the bioreactor system during the first 30 hours of experimental run 2 in the packed bed, consumption rates of glucose (species C_g) and ammonium (species c_a) were calculated (Note, in this thesis capital symbols were adopted for molar flowrates and small letter symbols for mass flowrates). From figures

3.3 and 3.4, it can be seen that the consumption rates (both glucose and ammonium, respectively) varied and the data presented was scattered.

Having varying data, a representative approximation for an average value for glucose consumption (R_g) in the packed bed bioreactor was needed (see equation 3.15). The reactor is divided up into "thin slices" and each thin slice is treated as a batch reactor. A tentative process model (equation 3.17) is described for glucose consumption (see section 3.5.1.2). Alternatively the model can also be applied to ammonium consumption. The model is based on a plug flow approach. The purpose of the balance is to attempt to predict outlet glucose concentrations over each thin slice of the bioreactor. From the model, the inlet glucose concentration to the reactor is known (222 mmol / l) and the glucose consumption rate at that concentration is obtained from flask culture experimental data. Therefore the outlet glucose concentration can be calculated and used as the inlet concentration to the consecutive slice etc. This approach would then lead to the calculation for an average value of glucose consumption in the bioreactor.

It was observed, however from flask culture experiments (see figures 3.5.1-3.6.2) that pass a certain glucose concentration the biomass become saturated with glucose and that the relationship between rates of disappearance of glucose to glucose concentration assumes the form of saturation kinetics. The feed glucose concentration (222 mmol / l, see appendix C for calculation) to the bioreactor were well above the saturation concentration (60 mmol / l). The model was therefore not applicable to the conditions under which the packed bed bioreactor was run.

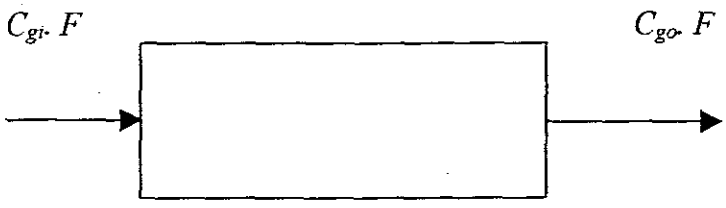
This model would have been applicable if the packed bed bioreactor was run at lower feed concentrations or if a much longer reactor was used to deplete the glucose to much lower concentrations.

3.5.1.2 Theory

This section covers theory used in the interpretation of results of the experiments relating to flow regimes, overall component mole and mass balances, process models and discusses the concept of reactor enzyme productivity.

The empirical overall component mole balance for glucose
(the same approach can be used for ammonium)

Overall mole and mass balances done on the bioreactor system during the first 30 hours of experimental run 2 for glucose.



Molar flowrate in - Molar flowrate out + Generation = Accumulation of species g

$$C_{gi} \bullet F - C_{go} \bullet F + G = 0 \qquad (3.1)$$

Where:

C_g = glucose concentration (mmol / l)

C_{gi} = inlet glucose concentration (mmol / l)

C_{go} = outlet glucose concentration (mmol / l)

G = rate of glucose generation (mmol / l.min)

[no glucose is generated in reactor but rather consumed

= $R_g . V$

R_g = rate of disappearance of glucose (mmol / l.min)

V = total reactor volume (ml)

F = total flowrate into bioreactor (ml / min)

[For constant volume (V), inlet flowrate and outlet flowrate remain the same]

Equation (3.1) becomes,

$$C_{gi} \bullet F - C_{go} \bullet F + R_g \bullet V = 0 \qquad (3.2)$$

By deviding by V:

$$\frac{F}{V}[C_{gi} - C_{go}] + R_g = 0 \qquad (3.3)$$

By rearranging equation (3.3)

$$-R_g = \frac{F}{V}[C_{gi} - C_{go}] \qquad (3.4)$$

and

$$\left(R_g = \frac{dC_g}{dt} \right)$$

Therefore

$$-\frac{dC_g}{dt} = \frac{F}{V}[C_{gi} - C_{go}] \qquad (3.5)$$

Similarly,
For ammonia:

$$-r_a = \frac{F}{V}[c_{ai} - c_{ao}] \qquad (3.6)$$

or

$$-\frac{dc_a}{dt} = \frac{F}{V}[c_{ai} - c_{ao}] \qquad (3.7)$$

[where species a refer to ammonia and the small letter symbols refer to mass rather then moles as for glucose]

$-r_a$ = Rate of disappearance of species a (mgl / l.min)

c_a = ammonium concentration (mg / l)

c_{ai} = inlet ammonium concentration (mg / l)

c_{ao} = outlet ammonium concentration (mg / l)

Equations 3.5 and 3.7 were used later in section 3.5.1.3 to calculate the overall rates of disappearance of glucose and ammonium respectively in the packed bed bioreactor.

Reynolds number (N_{Re})

A N_{Re} was calculated to determine if the bioreactor could be expected to operate under plug flow conditions (see appendix C).

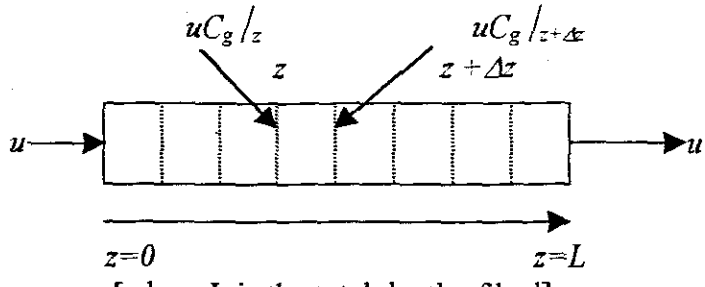
The calculated N_{Re} (0.4) indicated that plug flow might not be expected. Tracer tests as discussed in section 3.3.3, prove, however, that a significant plug flow effect was present inside the bioreactor.

Glucose differential mole balance

A glucose mole balance was formulated on a differential element by using the differential-section approach (Bailey *et al*, 1986).

Assumptions made:

- The reactor behaves predominantly plug flow
- The reactor operates at steady state and feed concentration is constant
- The role of the gas pulses can be neglected
- The axial velocity remains constant since there will be no change in flowrate.
- There will be no mass transfer between the slices.
- There is no accumulation of glucose



[where L is the total depth of bed]

A mole balance on a thin section as indicated above can be written as:

$$A\,u\,C_g\big|_z - A\,u\,C_g\big|_{z+\Delta z} - A\,\Delta z\,R_g\big|_z = 0 \qquad (3.8)$$

Where A = Cross sectional area of bioreactor (m^2)

Δz = thickness of differential section (mm)

u = axial velocity (m / s)

R_g = rate of disappearance of species g (mmol / l.min)

C_g = glucose concentration (mmol / l)

L = total depth of bed (m)

By rearranging:

$$A\,\Delta z\,R_g\big|_z = A\,u\,C_g\big|_z - A\,u\,C_g\big|_{z+\Delta z} \qquad (3.9)$$

By dividing equation (3.9) by AΔz (volume):

$$R_g\big|_z = \frac{u\,C_g\big|_z - u\,C_g\big|_{z+\Delta z}}{\Delta z} \qquad (3.10)$$

Taking limits as Δz tends to zero

$$\frac{d}{dz}(u\,C_g) = R_g \qquad (3.11)$$

Equation 3.11 becomes:

$$C_g\,\frac{d}{dz}(u) + u\,\frac{dC_g}{dz} = R_g$$

where u is constant and dC_g is the change in glucose concentration

$$u\frac{dC_g}{dz}=R_g \qquad (3.12)$$

The quantity, L/u, when $z=L$ is equal to the time required for a small slice of fluid to move from the reactor entrance, when $t = z/u$ at $z=0$ to the axial position L

Total transit time for an element of fluid is defined as:

$$\tau=\frac{L}{u} \qquad (3.13)$$

and

$z = tu$

$dz = d(tu)$

By substituting dz, we introduce a new independent variable from equation (3.12) and equation (3.12) can be written:

$$u\frac{dC_g}{d(tu)}=R_g$$

Since u is constant: $d(tu)$ = udt

$$\frac{dC_g}{dt}=R_g \qquad (3.14)$$

Each thin slice behaves as a batch reactor. Equation (3.14) follow the same form for a constant-volume batch reactor

in our case (for glucose):

$$R_g=\int_0^L\frac{dC_g}{dt} \qquad (3.15)$$

Integration on one differential slice:

$$\left(C_{gi} - C_{go}\right) = R_g \left(t_i - t_{out}\right) \tag{3.16}$$

Therefore,

$$C_{go} = C_{gi} - R_g \left(t_i - t_{out}\right) \tag{3.17}$$

From equation (3.17) outlet glucose concentrations over each consecutive thin slice can then be calculated as mentioned in section 3.5.1.1.

3.5.1.3 Experimental results and discussion

In this section the results obtained from the various experimental programs are utilized in order to draw conclusions about reactor performance.

The empirical overall component mole balance for glucose

(For complete set of experimental data, see Appendix C)

By applying (equations 3.5 and 3.7), figures (3.3 and 3.4) below were obtained.

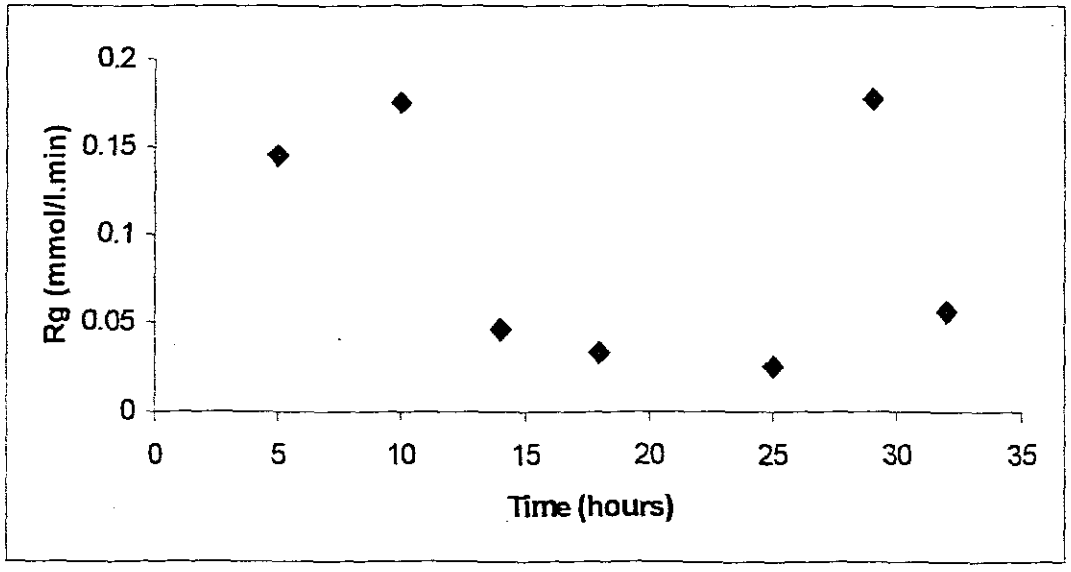


Figure 3.3: Run 2, PBB: Rates of disappearance of glucose during the first 30 hours in the packed bed bioreactor

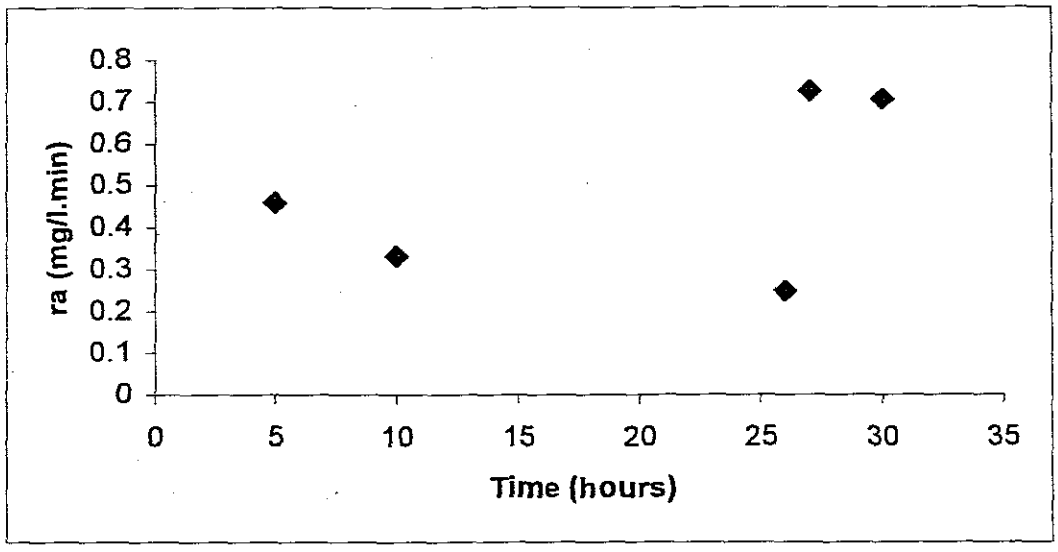


Figure 3.4: Run 2, PBB: Rates of disappearance of ammonium during the first 30 hours in the packed bed bioreactor

From figures 3.3 and 3.4, it can be seen that the consumption rates (both glucose and ammonium, respectively) varied and the data that was presented was scattered (no error analysis was done on the data presented above).

These observations gave us an insight of what is really happening inside the bioreactor. Instead of having biomass only on the polyurethane foam pellets, there could also be variable but significant amounts of biomass in the nutrient solution phase. Biomass in the nutrient could be the reason why there are sudden drops and increases in the rates of disappearance of these species (glucose and ammonium), since the fungal growth activity is not concentrated in the polyurethane pellets alone.

Having varying data, a representative approximation for an average value for glucose consumption (R_g) in the packed bed bioreactor was needed (see equation 3.15).

Glucose differential mole balance

The data from batch flask cultures were used to determine rates of glucose consumption per unit volume of solution versus glucose concentration in the solution at the start. Glucose consumption rates for the packed bed bioreator can be obtained from these batch flask culture data by reading it off from corresponding glucose concentrations. By

having these rates, the model (equation 3.17) can be used to calculate the outlet glucose concentrations on each individual differential slice. The outlet glucose concentration from the first slice will then be used as the inlet glucose concentration for the next slice. By so doing an average value for glucose consumption can then be obtained.

The following figures represent flask culture work over a period of 10 days where (S/O/ATT) refers to stirred oxygenated attached culture and (S/O/S), refers to stirred oxygenated suspended culture. A B-Tech student (Siko, 1999) did this work under the author's supervision.

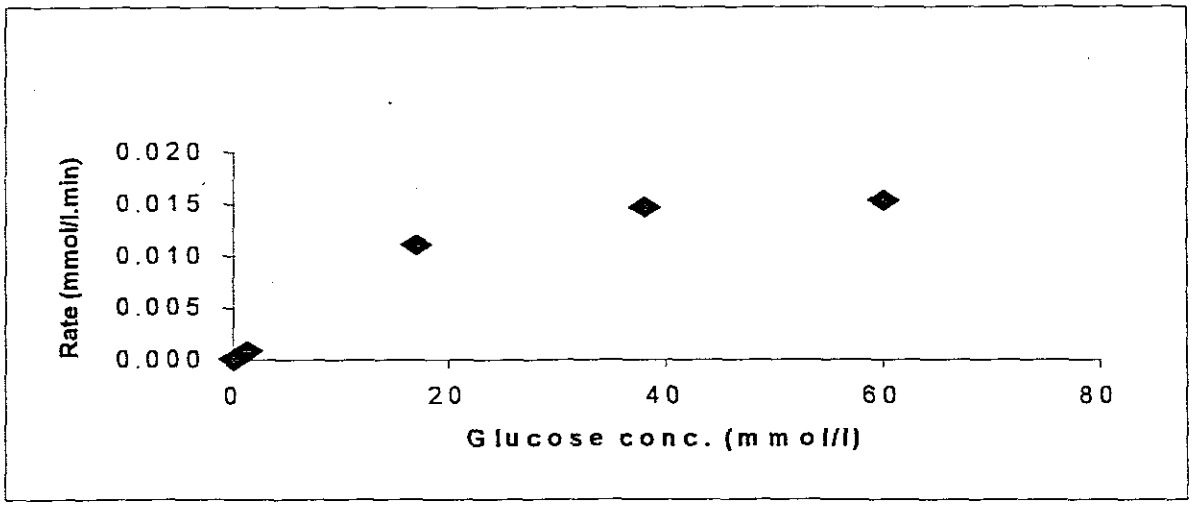


Figure 3.5.1: (S/O/ATT): Run 1- Rates of disappearance of glucose vs glucose concentration

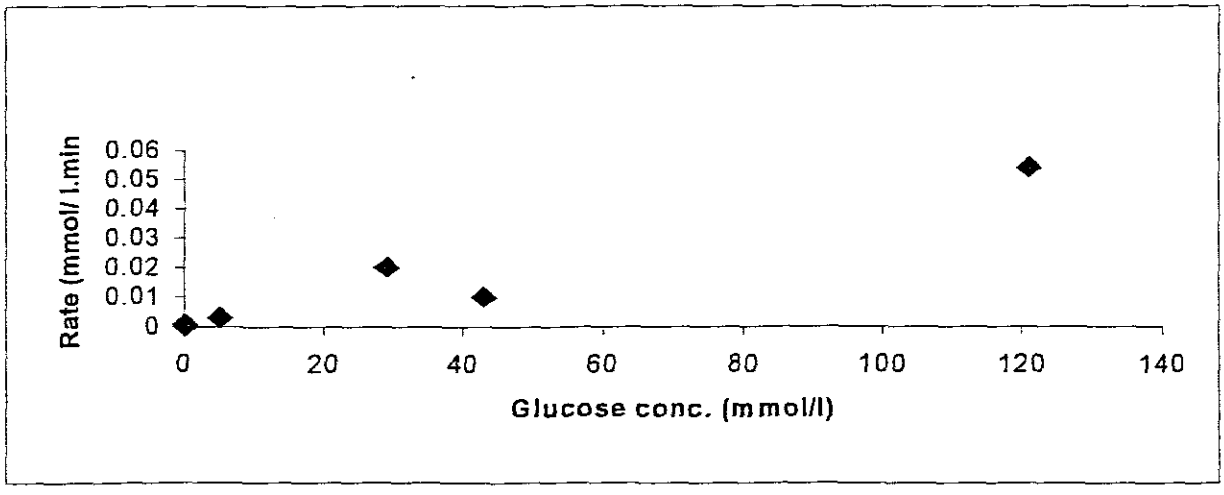


Figure 3.5.2: (S/O/ATT): Run 2- Rates of disappearance of glucose vs glucose concentration.

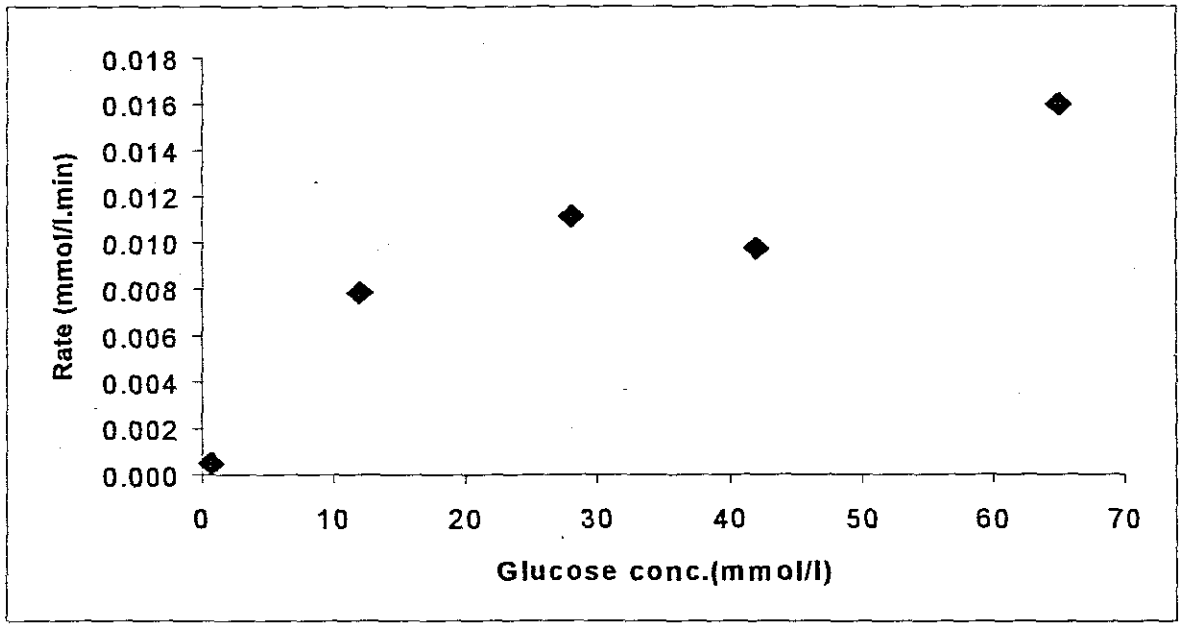


Figure 3.6.1: (S/O/S): Run 1- Rates of disappearance of glucose vs glucose concentration

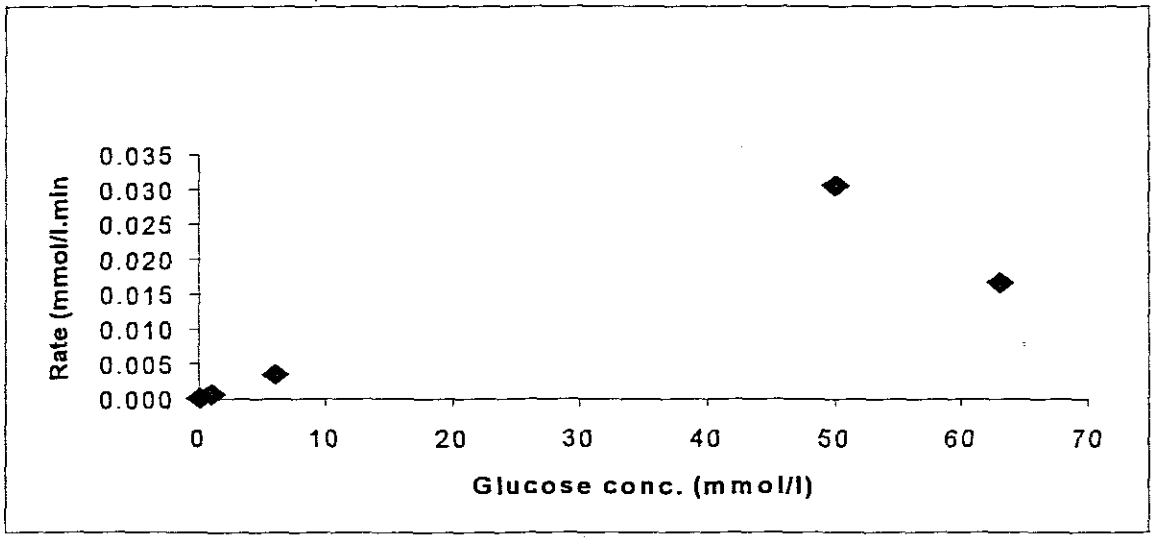


Figure 3.6.2: (S/O/S): Run 2-Rates of disappearance of glucose vs glucose concentration

As shown from figures (3.5.1-3.6.2), the relationship between glucose consumption rates to glucose concentration assumes the form of saturation kinetics above glucose concentrations of 60 mmol / l to 120 mmol / l. From these observations it can be suggested that the packed bed bioreactor was also operated under saturation conditions since the feed (222 mmol / l) and outlet glucose concentrations were well above these saturation concentrations.

It was not possible to measure active biomass concentrations in the bioreactor but if it is assumed that saturation conditions prevailed then the model would not be applicable because glucose consumption rate would not have varied from the inlet to the outlet of the bioreactor.

Reactor Enzyme Productivity:

Enzyme productivity is defined on the basis of the quantity of enzyme produced by a reactor of a given volume per day (hour).

Reactor volume is also incorporated in the concept of dilution rate, D, defined as F/V, where

F = total flowrate to the bioreactor (ml / min)

V = working volume of reactor vessel (ml), hence for the measurement of D, the definition of reactor volume required careful consideration.

Therefore enzyme productivity can be calculated as:

$$P = D \bullet C_e \qquad (3.18)$$

Where:

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

C_e = concentration of enzyme in the reactor effluent (enzyme activity) (Units.l^{-1}, where 1 unit is described as the catalytic ability to transform 1μmol of substrate in one minute

(Leukes, 1999)

To calculate the bioreactor enzyme productivity, enzyme activity (enzyme concentration) data was obtained from experimental work.

Note: one unit of activity is defined as one micromole of substrate 2,2-Azino-Bis (3-ethylbenzthiazoline-6-sulfonicacid) (ABTS) converted per minute and the molar extinction coefficient E used, was 3 600 M^{-1}

The data on enzyme activity was obtained during two experimental runs of 15 and 21 days respectively on polyurethane foam pellets in a packed bed bioreactor. The experimental runs followed a similar trend in terms of enzyme activity.

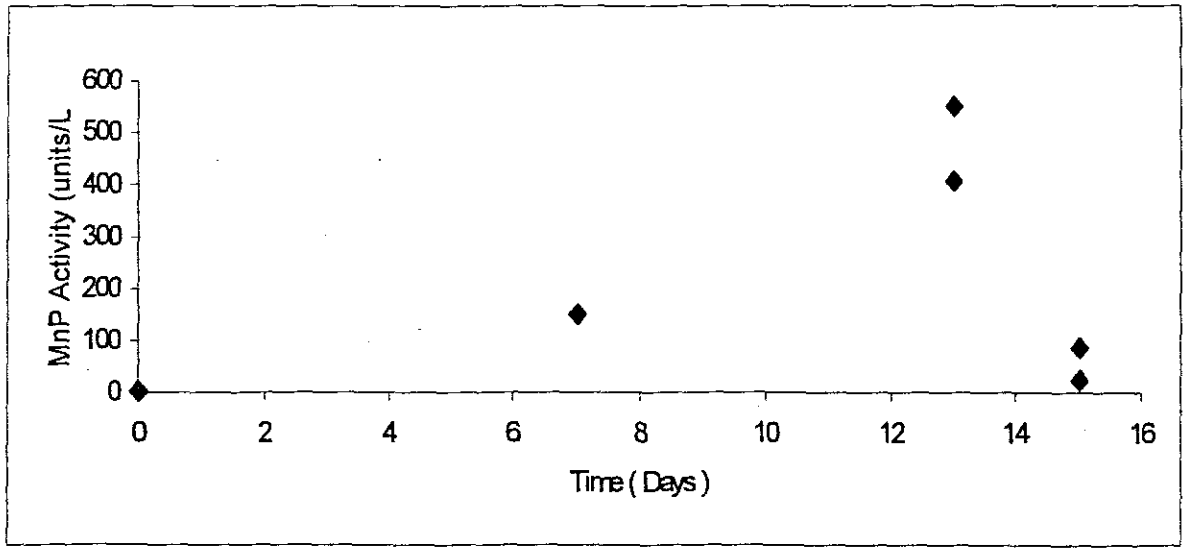


Figure 3.7.1: Run1, PBB: MnP Measurement (Units/l) over a period of 15 days in effluent

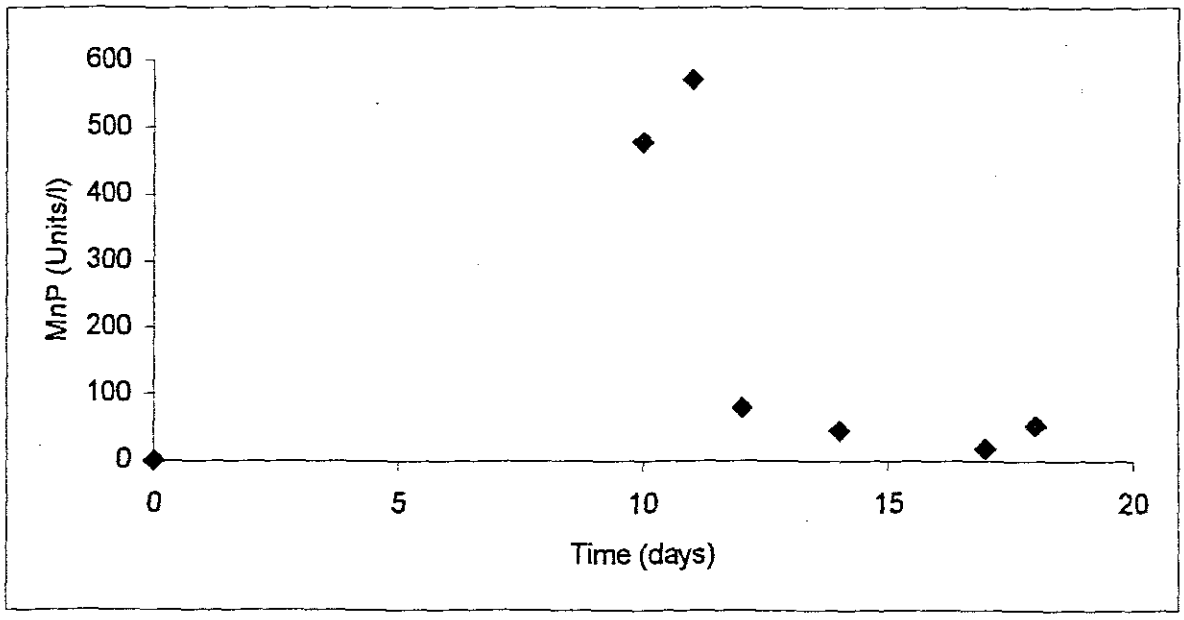


Figure 3.7.2: Run 2, PBB: MnP Measurement (Units/l) over a period of 21 days in the effluent

From the data it can be seen that the maximum activity of Run1 (Figure 3.7.1) was reached on day 13 (550 Units/L), and dropped to 23 Units/L on day 15. Similarly, the maximum activity of Run 2 (Figure 3.7.2) was reached on day 11 (570 Units/L), and dropped to 17.3 Units/L on day 17.

By using these maximum activities from both the experimental runs, we calculated our maximum reactor productivity for the two respective runs by applying equation (3.18).

Run 1

Dilution rate, D (day^{-1}) (Leukes, 1999 and Moreira *et al*, 1997)	Activity, C_E (Units / l)	Productivity, P (Units / l.day^{-1})
0.8	550	440

Run 2

Dilution rate, D (day^{-1}) (Leukes, 1999 and Moreira *et al*, 1997)	Activity, C_E (Units / l)	Productivity, P (Units / l.day^{-1})
0.8	570	456

Appendix C provide the full experimental data:

3.5.1.4 Conclusions

The empirical overall component mole balance for glucose

From the overall component mole balances for glucose and ammonium, it showed that the consumption rates of these species varied inside the packed bed bioreactor. This variation was due to the presence of biomass in the nutrient which was assumed to be the result of shear forces, which were applied on the polyurethane foam pellets through pulsation by oxygen. This was not a mode that was considered originally, but the experiments showed that it was the case.

Reactor Enzyme Productivity:

Evaluation of the process on a laboratory scale using a packed bed bioreactor showed that a maximum volumetric productivity of 456 Units.$L^{-1}day^{-1}$ for manganese peroxidase. correspond to a final concentration of 570 Units. L^{-1}. This can be compared to the system of Moreira *et al*, 1997, where a volumetric productivity of 202 Units.$L^{-1}day^{-1}$ was achieved with a final concentration of 250 Units.L^{-1}.

3.5.2 Experimental Studies (PBB and Flask culture work)

3.5.2.1 Introduction

Our experimental studies consisted of evaluating polyurethane foam pellets as carrier support material for fungal spore immobilization in the Packed Bed Fungal Bioreactor as well as in flask culture work. Our aim was to determine its effectiveness in terms of enzyme activity and process operation stability. We also carried out flask culture experiments using resin beads as an alternative carrier support material. Resin beads were not used in a continuous bioreactor operation but we do include a proposed future work plan whereby resin beads would be used in a fluidized-bed bioreactor (see section 4.2).

PBB studies

Interesting observations were made when the pH and redox potential of samples were measured in the effluent over a period of 30 hours in the packed bed bioreactor (see figures 3.8 and 3.9 below). The pH drops gradually from 6.11 to 4.34 over a period of 30 hours. An increase in biomass was observed which could have released more CO_2 into the nutrient to make it more acidic. Anaerobes may also have been present that produced acid.

3.5.2.2 Results

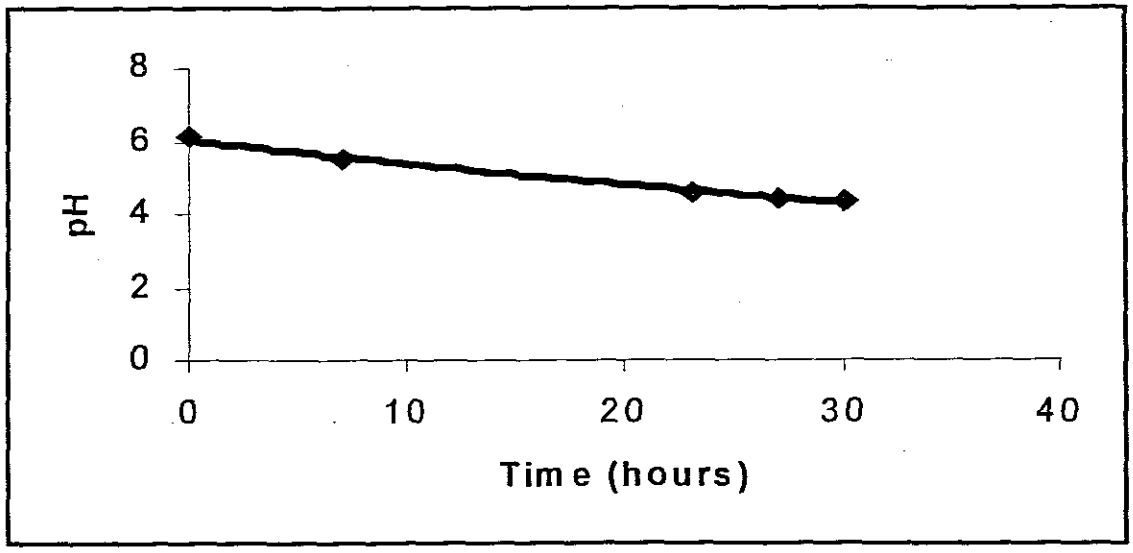


Figure 3.8: Run 2, PBB: pH vs time in the effluent over a period of 30 hours in PPB

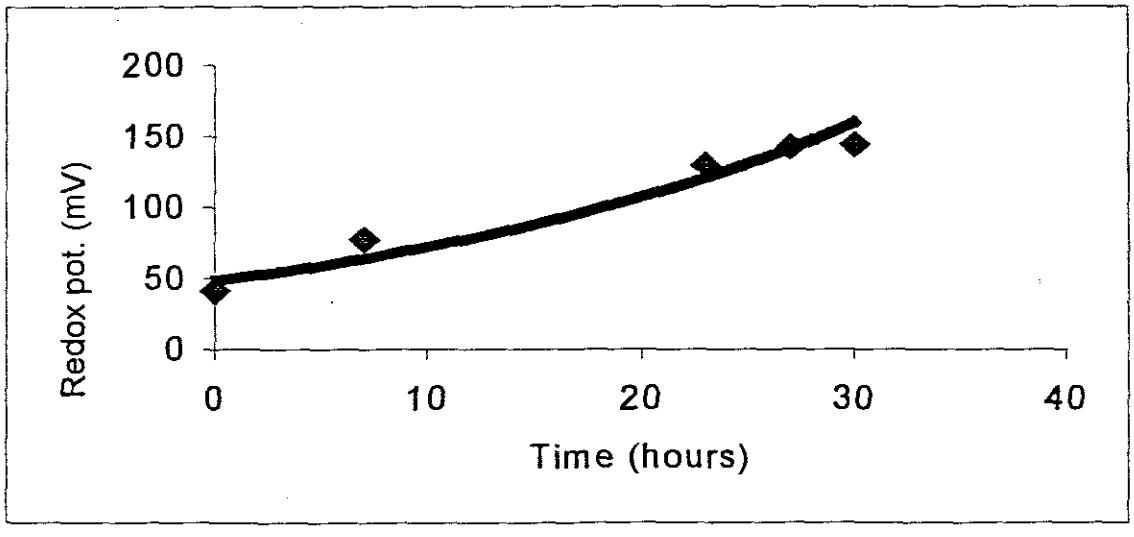


Figure 3.9: Run 2, PBB: Redox potential vs time in the effluent over a period of 30 hours

There is an increase in redox potential from 41 mV to 144 mV over a period of 30 hours.

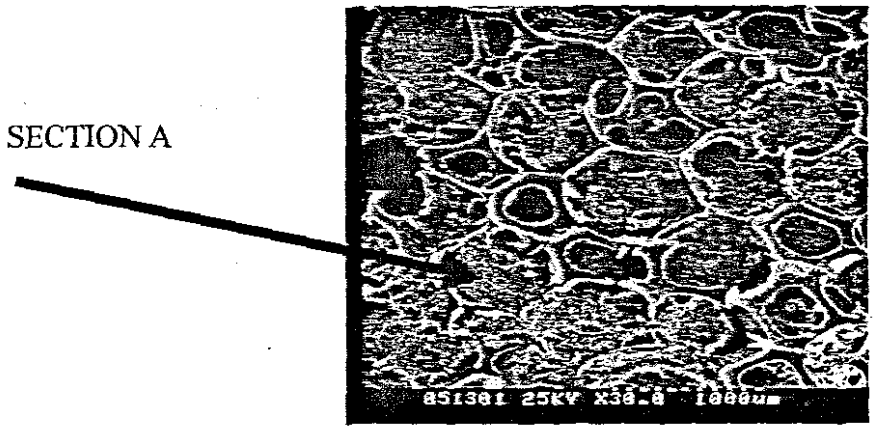


Figure 3.10.1: Mycelial growth inside polyurethane foam pellet

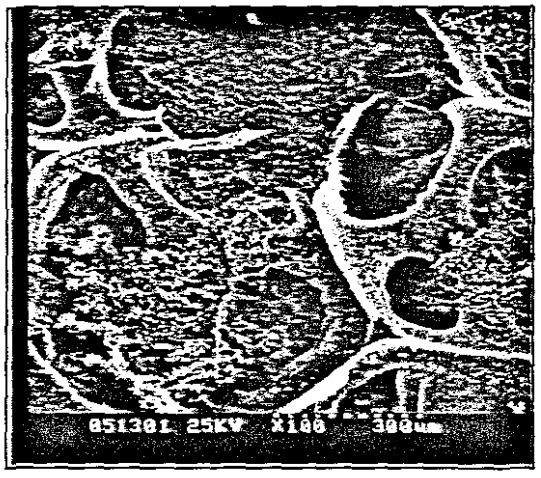


Figure 3.10.2: Magnification of mycelium biomass in pores

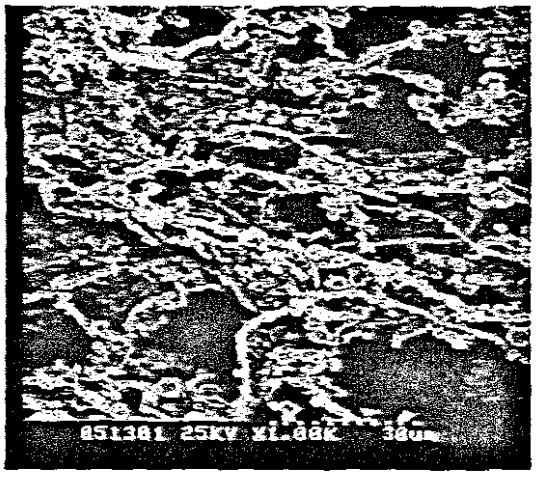


Figure 3.10.3: Magnification of section A [Intertwined cord formation of hyphal growth]

Flask/batch culture work

Considering the scope of investigation, sophisticated designs such as factorial designs were not considered to be appropriate at this stage, duplicates or triplicates were used where appropriate.

The aims for the Flask/batch culture experimental program:

Aims:

- To observe the morphology changes over the experimental period of 10 days (subjected to extension)
- To investigate fungal growth behavior
- To measure enzyme activity (Manganese peroxidase)

Using polyurethane foam pellets as carrier support material in batch/flask cultures

Two experimental set-ups were considered to assist in the understanding of fungal growth attachment and control on carrier support material.

Experimental set-up description:

Eight 250 ml Erlenmeyer flasks were autoclaved with rubber stoppers carrying 15 polyurethane foam pellets (5x5mm) each. The rubber stoppers were drilled with three holes and fitted with stainless steel tubes for an air/oxygenation inlet, air and CO_2 outlet and one for sampling. After the flasks were autoclaved they were filled with 100 ml of nutrient solution under the laminar flow hood and injected with 5 ml of spore solution with a sterile pipette. The flasks were connected to a shaker, which was adjusted to a desired degree for mixing (150 rpm). The first four flasks were supplied with filtered air (from air pump) daily for three minutes and the remaining four flasks with oxygen (from oxygen cylinder). Samples were taken from each flask daily to test for manganese peroxidase activity.

A) Stirred Oxygenated Attached flask cultures

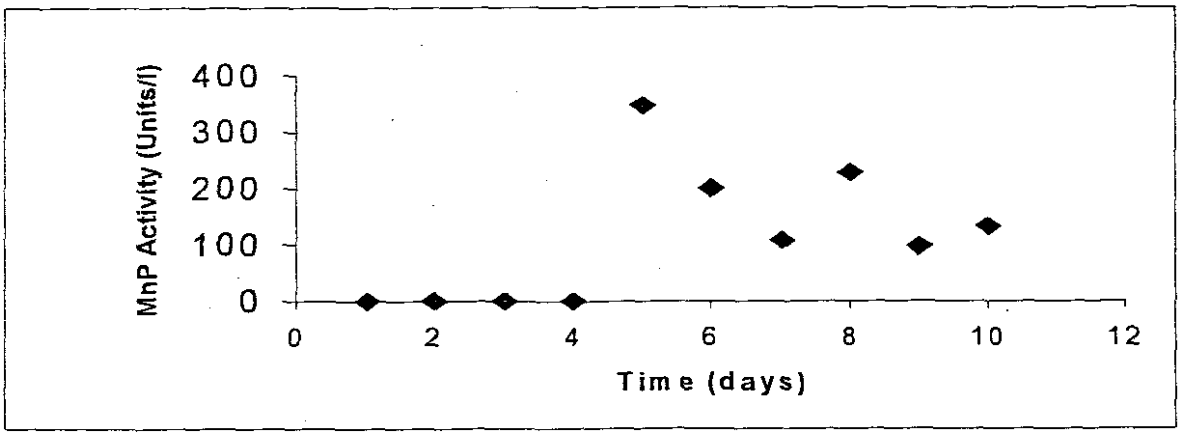


Figure 3.11.1: MnP Activity (measurement) using polyurethane foam in flask oxygenated cultures

B) Stirred Aerated Attached flask cultures

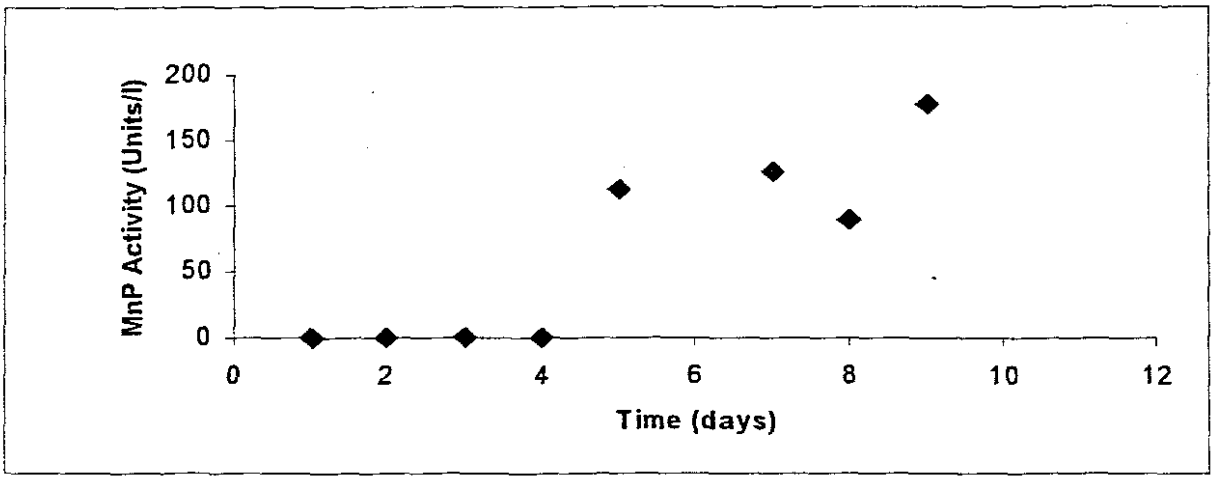


Figure 3.11.2: MnP Activity (measurement) using polyurethane foam in aerated flask cultures

From figure 3.11.1 maximum MnP activity was obtained on day 5 (347 Units/l) and dropped to 96 Units/l on day 9 but showed an increase again on day 10 (133 Units/l). From figure 3.11.2 the highest activity was obtained on day 9 (177 Units/l) and although it dropped on day 8 to 89 Units/l it increased again on day 9. The oxygenated flask cultures using polyurethane foam pellets as carrier material produced much higher levels of MnP activity than aerated systems, since there is a lower oxygen content in air than for

pure oxygen. From figure 3.11.2 much lower levels of MnP activity was obtained. It showed a steady increase in MnP activity after 4 days.

Using resin beads as carrier support material in flask cultures

Resin beads have been conducted as an alternative carrier support material for immobilising fungi.

A) Stirred Aerated Attached flask cultures

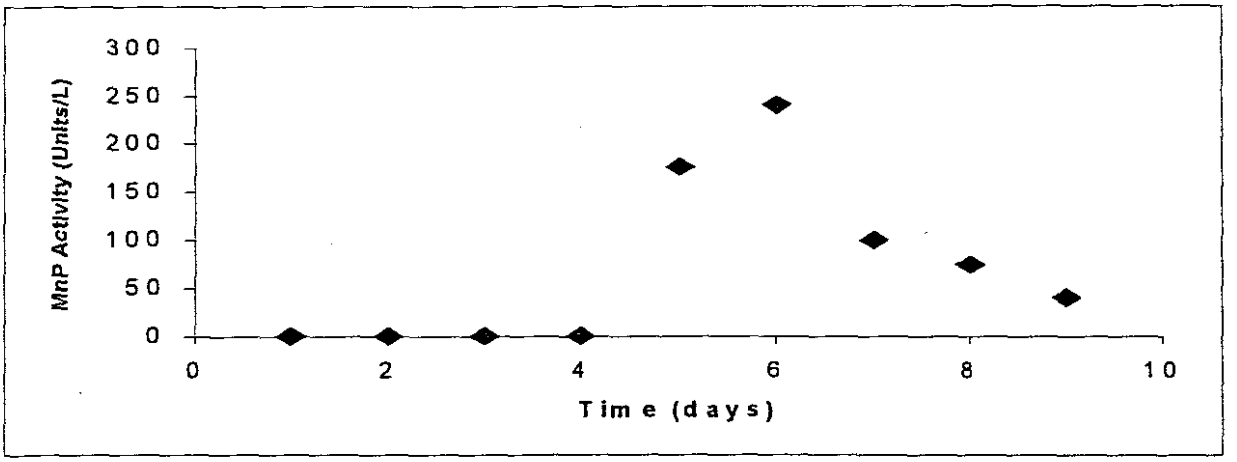


Figure 3.12.1: MnP Activity (measurement over a period of 9 days) for stirred aerated attached flask cultures using resin beads

From figure 3.12.1 the highest activity was reached on Day 6 (240 Units/L) and drop to 40 Units/L on Day 9

B) Stirred Oxygenated Attached flask cultures

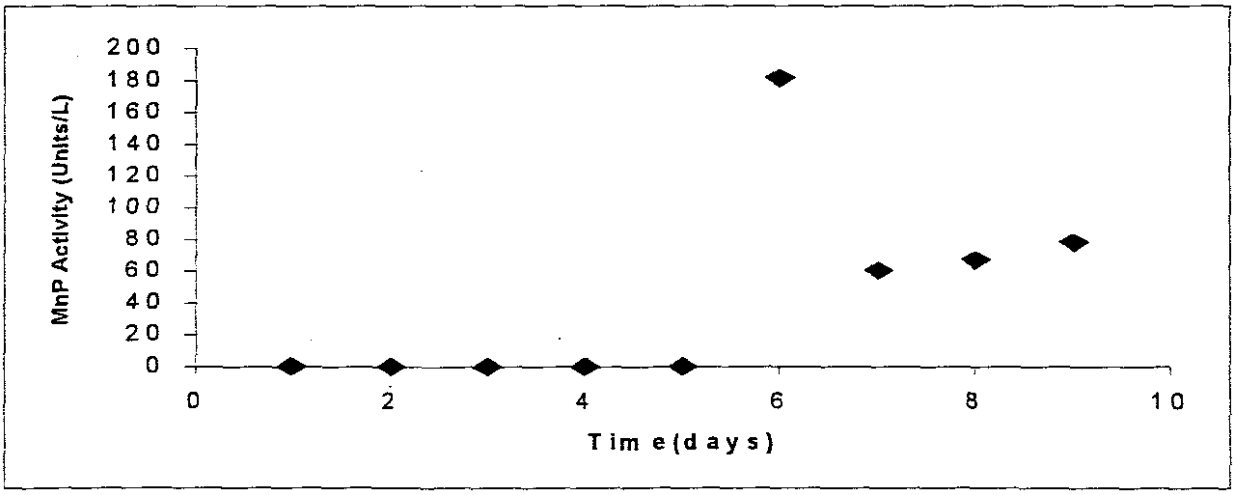


Figure 3.12.2: MnP Activity (measurement over a period of 9 days) in stirred oxygenated attached flask cultures using resin beads

From figure 3.12.2 above, the highest activity was reached on Day 6 (181.3 Units/L) and drop to 60 Units/l on Day 7 and increase again to 77.6 Units/L on Day 9.

3.5.2.3 Conclusions (PBB and Flask culture work)

The gradual decrease in pH indicated that CO_2 had been produced which made the nutrient much more acidic. The CO_2 released in the nutrient could be linked to the observed increase in biomass.

By using polyurethane foam pellets as carrier support material in a continuous bioreactor set-up, the fungi produced higher enzyme activities (double) than in the case of batch/flask cultures. By comparing enzyme activity between the two carrier material in flask cultures, in both cases, aerated and oxygenated flasks, polyurethane foam pellets seemed to be a more effective carrier in terms of Manganese Peroxidase activity. The highest enzyme activity using polyurethane foam pellets in flask cultures is 350 Units / l, compare to resin beads (250 Units / l). It should also be recommended that the pH is monitored in future experiments.

CHAPTER 4:

CONCLUSIONS AND RECOMMENDATIONS

4.1 CONCLUSIONS

The flask culture work could be regarded as the core or basis of understanding and gaining insight in the fungal morphology of *Phanerochaete Chrysosporium.* The preliminary flask culture experiments which were carried out parallel to that of the packed bed bioreactor did make valuable contributions in the decision making steps when the parameters were considered, such as carrier support material, aeration/ oxygenation and reactor hydrodynamics. A summary of the experimental results of both PBB and flask culture work are given below (table 4.1).

Bioreactor and Process	Carrier support material	Oxygenated/ Aerated	Operation time (d)	Maximum.act. ($U.L^{-1}$)	Minimum.act. ($U.L^{-1}$)
Packed Bed/ Continuous	Polyurethane foam pellets	Oxygenated	21	570	17.3
Packed Bed/ Continuous	Polyurethane foam pellets	Oxygenated	15	550	23
Flask culture/Batch	Polyurethane foam pellets	Oxygenated	10	177	89
Flask culture/Batch	Polyurethane foam pellets	Aerated	10	347	96
Flask culture/Batch	Resin Beads	Oxygenated	10	181.3	60
Flask culture/Batch	Resin Beads	Aerated	10	240	40

Table 4.1: Summary of enzyme activities of the packed bed bioreactor and flask culture work, using polyurethane foam and resin beads respectively as carrier material.

Reference	Bioreactor and Process	Operation Time (d)	Max. Act. (UL^{-1})
Bonnarme et al, 1993	Airlift/Batch	3.8	365
Laugero et al, 1996	Bubble-column/ Batch	4	726
Moreira *et al*, 1997	**Packed bed/ Continuous**	**140**	**250**
Leukes (1999)	Capillary membrane module/ Continuous	60	2361
Solomons (5/11/2001)	Single fibre capillary module/Continuous	14	90
Fillis (2001)	**Packed bed/Continuous**	**21**	**570**

Table 4.2: Overall comparison between different fungal bioreactors in terms of operation time and maximum enzyme activity

The packed bed bioreactor proposed by Moreira *et al*, 1997 was not an effective benchmark to use for our experimental work. We improved the design of Moreira significantly, but the consumption rates of glucose and ammonium (Chapter 3, figures 3.3 and 3.4) inside the packed bed bioreactor were variable. Our modified bioreactor was successful in terms of manganese peroxidase enzyme production, much higher activities were obtained than that of Moreira but over much shorter time periods. The expectation was that we would observe a higher productivity than was reported by Moreira but no provision was made in our program to operate with recycle or to vary the L/D ratio as a comparison. The highest enzyme activity (570 Units/l) was reached on day 11 over an experimental run of 21 days. Although the experimental runs were repeatable, the rate expressions for glucose varied significantly.

We concluded that, while our model assumed attached growth on polyurethane pellets only, we had to (artificially) allow for an equal significant suspended biomass activity. Biomass in the solution appeared to be responsible for this variation. This pointed us to curing the bioreactor stability problem by looking to fluidized beds as the answer (see recommendations, section 4.2). One of the main operational problems we were facing was bed clogging. Pulsation did not seem to be a very successful mycelial growth

control mechanism probably due to the fact that we operated the bioreactor at a much higher L/D ratio that resulted in accumulation of a significant portion of active suspended biomass.

Further investigation into packed bed bioreactors should be done to further contribute to the ultimate aim of an industrial scale bioreactor to produce enzymes for extended periods. The methods in this thesis are, however recommended for further investigations to find ways to produce enzymes .

It can be seen from table 4.2 that packed bed fungal bioreactors still show promise for larger scale reactor development because of its operation time, which is still the best reported in literature (Moreira *et al*, 1997).

4.2 RECOMMENDATIONS

- Attention in future should focus on different ways to control the mycelium growth inside packed bed bioreactors.
- Practical sterilization techniques are another key factor, which need to be focussed on strongly for future industrial scale applicability.
- Resin beads, as alternative carrier material should be further investigated.

Proposed experimental program

- Testing of a fluidized-bed bioreactor system with resin beads used as carrier material for the immobilization of fungi.
- The fluidized-bed system should be optimized to secure sustained enzyme production.
- Feed the reactor with an effluent stream containing a known concentration of pollutants to evaluate the reactor's process performance in terms of pollutant degradation efficiency.

- It should be noted that there is scope for development of simple and cost effective downstream recovery and purification of enzymes produced for remediation in external reactor systems. The use of ultrafiltration may be suggested as a simpler and cheaper recovery and purification method, replacing both the centrifugation step and the dialysis step as discussed in Chapter 1 (section 1.2.4.2).

APPENDICES

APPENDIX A
OBSERVATIONS OF FLASK CULTURE WORK OVER A 10 DAY PERIOD

Polyurethane foam pellets was used as carrier material in the flask cultures

Please note the following abbreviations:
Stirred/aerated/suspended systems-S/A/S
Stirred/aerated/attached systems-S/A/ATT
Stirred/oxygenated/suspended systems-S/O/S
Stirred/oxygenated/attached systems-S/O/ATT
Unstirred/aerated/suspended systems-U/A/S
Unstirred/aerated/attached systems-U/A/ATT
Unstirred/oxygenated/suspended systems-U/O/S/
Unstirred/oxygenated/attached systems-U/O/ATT

Results:

Observations: [From B-Tech Project, (Dayile and Martin, 1998), supervised by Mr. V. Fillis]

Day 1

S/A/S	S/A/ATT	S/O/S	S/O/ATT
Fungus grows as regular shaped pellets of 2-8 mm diameter	Clear solution	Bigger pellets than in aerated system	Clear solution
U/A/S	**U/A/ATT**	**U/O/S**	**U/O/ATT**
Hazy solution	Threads attached from one material to the other	Fungus formed on the sides of the flask and and a thin layer on the surface	Threads attached from one material to the other A thin layer for formed on the surface of the flask

Day 2

S/A/S	S/A/ATT	S/O/S	S/O/ATT
Increased size of pellets, 2-10mm diameter. 75% of bottom covered with mycelium	No mycelium visible in the nutrient solution Carrier material sunk to the bottom	5mm diameter pellets were observed 15mm diameter lump at the hose tip was observed	Mycelia visible inside carrier material as white dots Clear nutrient solution
U/A/S	**U/A/ATT**	**U/O/S**	**U/O/ATT**
Increased fungal growth	Layer at the bottom was	A layer at the surface of	Growth observed

At the hose tip Turbidity increased	thicker than in day 1	the solution started to form. Solution is more Hazy than in aerated systems	at the surface of the solution above carrier material Growth at the bottom of carrier increased
Day 3			
S/A/S	**S/A/ATT**	**S/O/S**	**S/O/ATT**
Increased size of pellets 2-10mm diameter 75% of pellets covered with mycelium	No mycelium visible on the solution Carrier material sunk to the bottom	5mm diameter pellets were observed 15mm diameter lump at the hose tip was observed	Mycelia visible in carrier support material as white dots Clear nutrient solution
U/A/S	**U/A/ATT**	**U/O/S**	**U/O/ATT**
Increased fungal growth at the hose tip Turbidity increased	Layer at the bottom was thicker than in day1	A layer at the surface of the solution started to form Solution is more hazy than in aerated system	Growth observed at the surface of the solution above carrier material Growth at bottom of carrier increased
Day 4			
S/A/S	**S/A/ATT**	**S/O/S**	**S/O/ATT**
Increased size of pellets 2-15mm diameter 90% of bottom covered with mycelium	No mycelium visible in the solution Solution is slightly turning brown spots were observed inside the carrier material which is an indication of enzyme production	10-20mm-diameter lump at the hose tip was observed	No mycelium visible in the nutrient solution More spots (brown) were observed inside the carrier material which is an indication of enzyme production
U/A/S	**U/A/ATT**	**U/O/S**	**U/O/ATT**
Increased fungal growth At the hose tip Turbidity increased	Thick layer (4mm) was observed at the surface	A layer at the surface is thicker (3mm) Fungi grows from the bottom upwards	A thicker layer was observed (5mm) on the surface. Fungi grows from the bottom upwards and attaching to

			to the carrier material. Brown color was observed at the rim and it covered 40% of the rim.

Day 5

S/A/S	S/A/ATT	S/O/S	S/O/ATT
95% of bottom covered with mycelium	No mycelium visible in nutrient solution Brown spots increased were observed inside the carrier material	Pellets cover 55% of the bottom	No mycelium visible in the nutrient solution All the carrier material was covered with brown spots which is an indication of increased enzyme activity
U/A/S	**U/A/ATT**	**U/O/S**	**U/O/ATT**
Increased fungal growth at the hose and at the surface Turbidity increased	Increase in mycelial mat Solution turn to light brown	A layer at the surface was thicker (4mm) Fungi grows from the bottom upwards	A thicker layer was observed (5mm) Fungi grows From the bottom Upwards. Brown Color covers the interface. Spores At the hose above The solution were also observed.

Day 6

S/A/S	S/A/ATT	S/O/S	S/O/ATT
95% of bottom covered with mycelium	No mycelium visible in the nutrient solution. Brown spots increased inside the carrier material	Pellets cover 55% of the bottom	No mycelium visible in the nutrient solution All carrier material was Covered with Brown spots Which is an Indication of Increased enzyme

activity

U/A/S	U/A/ATT	U/O/S	U/O/ATT
Increased fungal growth At the hose and at the Surface Turbidity increased	Thicker layer Brown color has formed at the rim and it covers 50% of the rim	A layer at the surface was thicker (4mm) Fungi grows from the bottom upwards	A thicker layer was observed (5mm). Fungi attaching to the Carrier material. Brown color Covers the Interface. Spores Were observed as White patches Here and there.

Day 7

S/A/S	S/A/ATT	S/O/S	S/O/ATT
95% of bottom covered with mycelium 10-25mm diameter pellets were observed	No mycelium visible in the nutrient solution. Brown spots increased the carrier material	Pellets cover 55% of the bottom	No mycelium in the nutrient solution. Brown color started to disappear

U/A/S	U/A/ATT	U/O/S	U/O/ATT
Increased fungal growth At the hose and at the Surface. Turbidity increased	Thicker layer Brown color has formed at the rim and it covers 50% of the rim. Spores At the surface were observed.	A layer at the surface was thicker (4mm) Fungi grows from the bottom upwards. Brown color at the rim was Observed. Spores were Observed close to the hose	A thicker layer was observed (5mm). Fungi attaching to the carrier material. Brown color covers the interface. Increase spores at the surface and at hose

Day 8

S/A/S	S/A/ATT	S/O/S	S/O/ATT
95% of bottom covered with mycelium 10-25mm diameter pellets were observed	No mycelium visible in the nutrient solution. Brown spots increased the carrier material	Pellets cover 55% of the bottom	No mycelium in the nutrient solution. Brown color started to disappear

U/A/S	U/A/ATT	U/O/S	U/O/ATT
Increased fungal growth	Thicker layer	A layer at the surface	A thicker layer

At the hose and at the Surface. Turbidity increased	Brown color has formed at the rim and it covers 50% of the rim. Spores At the surface were observed.	was thicker (4mm) Fungi grows from the bottom upwards. Brown color at the rim was Observed. Spores were Observed close to the hose	was observed (5mm). Fungi attaching to the carrier material. Brown color covers the interface. Increase spores at the surface and at hose

Day 9

S/A/S	S/A/ATT	S/O/S	S/O/ATT
95% of bottom covered with mycelium 10-25mm diameter pellets were observed	No mycelium visible in the nutrient solution. Brown spots increased the carrier material	Pellets cover 55% of the bottom	No mycelium in the nutrient solution. White spots were observed and the nutrient solution was clear

U/A/S	U/A/ATT	U/O/S	U/O/ATT
Increased fungal growth At the hose and at the Surface. Turbidity increased	Thicker layer Brown color has formed at the rim and it covers 50% of the rim. Spores At the surface were observed.	A layer at the surface was thicker (4mm) Fungi grows from the bottom upwards. Brown color at the rim was Observed. Spores were Observed close to the hose	A thicker layer was observed (5mm). Fungi attaching to the carrier material. Brown color covers the interface. Increase spores at the surface and at hose

Day 10

S/A/S	S/A/ATT	S/O/S	S/O/ATT
95% of bottom covered with mycelium 10-25mm diameter pellets were observed	No mycelium visible in the nutrient solution. Brown spots increased the carrier material	Pellets cover 55% of the bottom	No mycelium in the nutrient solution. White spots were observed and the nutrient solution was clear

U/A/S	U/A/ATT	U/O/S	U/O/ATT
Increased fungal growth At the hose and at the Surface. Turbidity increased	Thicker layer Brown color has formed at the rim and it covers 50% of the rim. Spores At the surface were	A layer at the surface was thicker (4mm) Fungi grows from the bottom upwards. Brown color at the rim was	A thicker layer was observed (5mm). Fungi attaching to the carrier material.

	observed.	Observed. Spores were Observed close to the hose	Brown color covers the interface. Increase spores at the surface and at hose

APPENDIX B
ENZYME ASSAYS AND COLORIMETRIC METHODS

MnP Enzyme activity

Reagents:

1 M sodium lactate buffer

Weigh 10.22 g lactic acid into a 100 ml beaker. Add 80 ml distilled water and adjust pH to 4.5 with 1 M NaOH (40g / l). Make up to volume with sterile water

1 M sodium succinate buffer

Weigh 11.81g succinic acid into a 100 ml beaker. Add 80 ml distilled water and adjust pH to 4.5 with NaOH. Make up to volume with sterile water.

$MnSO_4$ solution

Weigh 95 mg of $MnSO_4*H_2O$ into a 100 ml volumetric flask. Make up to volume with water.

ABTS solution

Weigh 50 mg of ABTS into a 25 ml volumetric flask. Make up to volume with water

Reagent A

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

Egg albumin
150 mg (6 mg / L)

Sodium lactate buffer
2.5 ml (100 mM)

The stirring speed must be very slow as the entire albumin has to dissolve but without any physical damage that could cause denaturation of the protein.

After the protein has dissolved completely add carefully the other reagents:

Sodium succinate buffer
2.5 ml (100 mM)
$MnSO_4$
1.0 ml (200 mM)

ABTS solution
1.0 ml (80µg / ml)

Pour mixture in a second flask to avoid a volumetric error resulting from the magnetic stirres in the flask.

Make reagent up to exactly 25 ml with distilled water and mix (invert bottle)

"Reagent A" can be stored up to week at 4°C but if it turns blue discard immediately

Reagent B=100 µM H_2O_2

Place 1.0 µl (pipettor) of 30% H_2O_2 into a 100 ml volumetric flask and make up to volume with water. Keep at 4^0 C and make up fresh daily.

Assay

Into a cuvette (normal 3 ml plastic cuvette for the DMS 100 spectrophotometer), place:

Reagent A	900 µl
Reagent B	900 µl
Enzyme sample	200 µl

Invert, mix and assay immediately

An alternative MnP and a LiP method are described by Tienenzyme, 1998

Operation instructions for Spectrophotometer DMS 100
(read also in the DMS 100 manual, page 13ff and 21ff)

Regulate the volume that gets sucked in by the sample hose to ≈ 800-900 µl

On the operation panel:

- Ensure that double beam modus has been selected (DB) and that MULTI λ, SCAN MODE and REPEAT MODE are all switched **off.**

- Select the required operation mode (ABS).

- Choose time constant (5 sec).

- Set spectral bandwidth (0.1).

- Enter observation wavelength (420 nm) with numerical keyboard and press the MAX λ key.

- Press the START-STOP key to drive the monochromator to the right wavelength.

- Wash cuvette inside the spec by letting the hose 3-4 times suck some blank (as enzyme assay mix but instead of the enzyme solution use 200 µl dist.water). After each suction press the ZERO button and see how the absorbance goes towards the 0.000 reading. Repeat until you have a stable zero reading for your blank material.

- For the sample mix Reagent A, Reagent B and the enzyme containing sample (which is either the sample coming directly out of the flask culture (for days where high enzymes activity are expected) or the residue collected above the membrane of the cut-off tube (at the start and the end of a batch experiment).

- Let sample hose suck twice to ensure a complete (air bubble free) sample filling of the cuvette inside the spectrophotometer.

- Monitor the absorbance change (in 5 seconds intervals) until the absorbance values stay the same or decrease.

Evaluation and calculation of the enzyme activity

Evaluation and calculation has been done according to guidelines of Leukes (1999)

- The objective is to find a reading in Units per Liter, where 1 Unit = 1micromol substrate converted per min.

- Take your absorbance reading and plot a graph showing the absorbance vs. time.

- The steepest slope (dA / dt) will give you the value for "initial rate of reaction."

- To convert this to "d concentration /dt" use Lambert-Beer and divide by the (substratespecific) extinction coefficient E which is 3 600 M^{-1}. This gives a value in "mol/(L·min).

- Moles are now changed to micromole by multiplying by 10^6. This gives micromoles / (minute·L)
  micromoles / min. = 1 Unit. So your answer is now in Units per liter.

- Now you have to consider sample handling. First, you need to account for dilution in your assay. If you've followed our protocol and used 200 microlitres 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 cutoff tubes initially with 3ml and you have 0.5 ml concentrated enzyme solution left your factor would be (6).

- As a guideline, if you have not concentrated your sample, then multiply you dA/dt readings (in minutes) by 277.8 and you get the correct answer for the enzyme activity.

Glucose analysis (Autoanalyzer method)

- The purified filtrate of the "Cut off" tube is used for the glucose determination.

- Collect about 1 ml of filtrated (enzyme and debris-free) sample in an Eppendorf tube and forward it to the "Paramedical Science" lab for the "Autoanalyzer."

Glucose (Hexokinase) Reagent set:

Principle

Glucose + ATP $\xrightarrow{HK}$ G-6-P + ADP

G-6-P + NAD $\xrightarrow{G6PDH}$ 6-Phosphogluconate + NADH

The enzymatic hexokinase (HK) catalyzes the reaction between glucose and adenosine triphosphate (ATP) to form glucose-6-phosphate and adenosine diphosphate (ADP). In the presence of NAD, the enzyme glucose-6-phosphate dehydrogenase (G6PDH), oxidizes glucose-6-phosphate to 6-phosphogluconate. The increase in NADH concentration is directly proportional to the glucose concentration and can be measured spectrophotometrically at 340 nm.

Reagent composition

When reconstituted with distilled water as directed, the reagent contains the following:

Hexokinase	1000 U / L
G6PDH	1000 U / L
ATP	1.0 nm
NAD	1.0 mM
Buffer	100 mM pH = 7.5 ± 0.1 (30°C)

Nonreactive stabilizers and preservatives have been added

Precautions

Reagent is for " in vitro" diagnostic use only

Reagent preparation

Reconstitute reagent vials with volume of distilled water stated on vial label. Swirl gently to dissolve.

Reagent storage

1. The dry reagent and standard should be stored refrigerated at 2-8°C.
2. Reconstituted reagent is stable for 48 hours at room temperature and for 30 days refrigerated at 2-8°C.

Reagent deterioration

Do not use if:

1. Reagent has an absorbance greater than 0.30 when measured against water at 340 nm.
2. The reagent fails to recover stated control values or meet stated linearity.
3. The reconstituted reagent reagent develops turbidity, indicating contamination.

Procedure for operating the Glucose autoanalyzer:

Checks

- Check the level of the distill water supply in the container
- Empty the waste container
- Empty the cuvette waste container
- Fill the cuvette supply tray

1. Make sure the samples to be analyze have been diluted to fall within the specified calibration range (5.5-16.5 mmol / l)

Load the samples and reagents in the following order:

2. Put in your sample tray: Blank: Distilled water
   Calibration std's: TCAL1;TCAL2

Controls: TC1;TC2

Samples: ID numbers: 1a,1b; 2a,2b etc.

3. Put in your reagent tray: Glucose hexokinase reagent bottle

Reagent glucose: Position 1

Go to main menu - Worklist F1 - F5 - F1 - Yes [Enter]

CUP - Sample Type - Sample ID - Test - Glucose - Select - F1

Last position empty - Acc Worklist

Press start - Move curser down to position 1 in reagent bar code - Type in GLUC - screen should show - test T-GLUC

Samples will now be automatically analyzed

Wait 30 minutes- 1hour to view results

APPENDIX C
EXPERIMENTAL DATA FOR BOTH PACKED BED AND FLASK CULTURE WORK

Relate to the tentative process model:
Calculating the Reynolds number inside the packed bed bioreactor

$N_{RE} = (ID * v * \rho) / \mu$

ID = 0.036 m
V = $1.6 * 10^{-5}$ m/s
ρ = 1 000 kg/m^3
μ = $1.5 * 10^{-3}$ N.s/m^2

N_{RE} = 0.4

Rates of disappearance of species g (glucose) (mmol/l.h), and species A (ammonium) (mg/l.h) over a period of 30 hours. (figures 3.3 and 3.4)

Calculated glucose concentration into live bioreactor:

Glucose flowrate : 0.7 mmol / l
Molar weight of glucose : 180 g / mol
Total flowrate into bioreactor: 1.76 ml / min

1.76 (mmol / l) / 0.7 (mmol / l) = 2.5

Dilution factor therefore 2.5

Glucose stock concentration: 100 g / l
Therefore glucose concentration entering the bioreactor = 100 / 2.5
= 40 g / l

Glucose molar concentration entering the reactor:

40 (g / l) / (180 g / mol) * 1000 = 222 mmol / l

The calculated inlet glucose molar concentration (220 mmol / l) compares well to the measured glucose concentrations (see table below)

Rates of disappearance of species g
(figure 3.3 ; experimental run 2) (glucose)

$R_g = (C_{gi} - C_{go}) F / V$

Time	*Cgi* (measured)	*Cgo* (measured)	*F*	*V*	*Cgi - Cgo*	*(Cgi - Cgo)*F*
(hours)	(mmol/l)	(mmol/l)	(l/min)	(l)	(mmol/l)	(mmol/min)
5	167.2	129.6	0.002	0.519	37.6	0.075
10	231	185.4	0.002	0.519	45.6	0.091
14	218.4	206.4	0.002	0.519	12	0.024
18	236.4	228	0.002	0.519	8.4	0.017
25	205.8	199.2	0.002	0.519	6.6	0.013
29	213.6	167.4	0.002	0.519	46.2	0.092
32	186	171.6	0.002	0.519	14.4	0.029

Time	R_g
(hours)	(mmol/l.min)
5	0.145
10	0.175
14	0.046
18	0.033
25	0.025
29	0.177
32	0.056

Rates of disappearance of species a **(figure 3.4; experimental run 2)** (ammonium)

$r_a = (c_{ai} - c_{ao}) F / V$

Time	C_{ai}	C_{ao}	*F*	*V*	$C_{ai} - C_{ao}$	$(C_{ai} - C_{ao})*F$
(hours)	(mg/l) (measured)	(mg/l)	(l/min)	(l)	(mg/l)	(mg/min)
5	196.8	78.03	0.002	0.519	118.77	0.238
10	196.8	111.03	0.002	0.519	85.77	0.172
26	196.8	133.03	0.002	0.519	63.77	0.128
27	196.8	8.9	0.002	0.519	187.9	0.376
30	196.8	13.65	0.002	0.519	183.15	0.366

Time (hours)	r_a (mg/l.min)
5	0.459
10	0.331
26	0.247
27	0.724
30	0.705

Relate to the tentative process model, rates of disappearance used from flask/batch cultures. Data obtained from experimental work done by a B-Tech student, (Siko, unpublished work, 1999, under the author`s supervision).

(S/O/ATT)-RUN 1 (figure 3.5.1)		
Time	Glucose conc.	Rates of disappearance
(Days)	(mmol/l)	(mmol/l.min)
1	60	0.015
2	38	0.015
3	17	0.011
4	1.2	0.001
5	0	0.000
6		0.000
7		0.000
8		0.000
9		0.000
10		0.000
(S/O/ATT)-RUN2 (figure 3.5.2)		
Time	Rates of disappearance	Glucose conc.
(Days)	(mmol/l.min)	(mmol/l)
1	0.054	121
2	0.01	43
3	0.02	29
4	0.003	5
5	0.0006	1
6	0	0.1
7	0	0.1
8	0	0.1
9	0	0.1
10	0	0.1

(S/O/SUS)-RUN 1 (figure 3.6.1)		
Time	Glucose conc.	Rates of disappearance
(Days)	(mmol/l)	(mmol/l.min)
1	65	0.016
2	42	0.010
3	28	0.011
4	12	0.008
5	0.7	0.000
6		0.000
7		0.000
8		0.000
9		0.000
10		0.000

(S/O/SUS)-RUN2 (figure 3.6.2)		
Time	Glucose conc.	Rates of disappearance
(Days)	(mmol/l)	(mmol/l.min)
1	63	0.017
2	39	#N/A
3	50	0.031
4	6	0.003
5	1	0.001
6	0.1	0.000
7	0.1	0.000
8	0.1	0.000
9	0.1	0.000
10	0.1	0.000

pH vs time in the effluent over a period of 30 hours (Figure 3.8)

Time(h)	pH
0	6.11
7	5.47
23	4.56
27	4.37

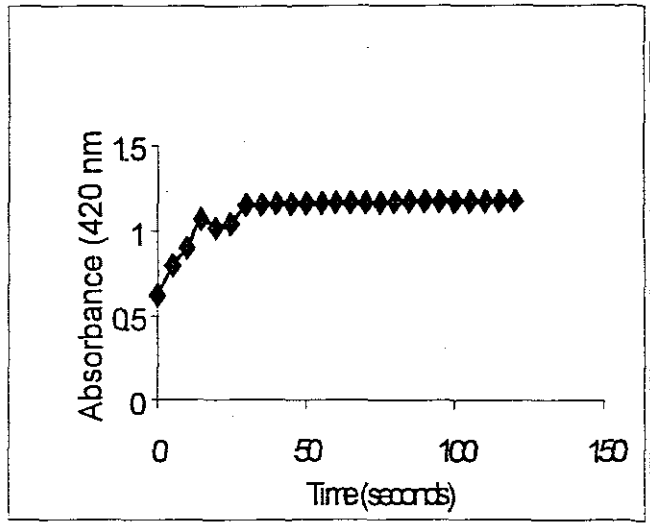
Redox potential vs time in the effluent over a period of 30 hours (Figure 3.9)

Time(h)	mV
0	41
7	77
23	129
27	142

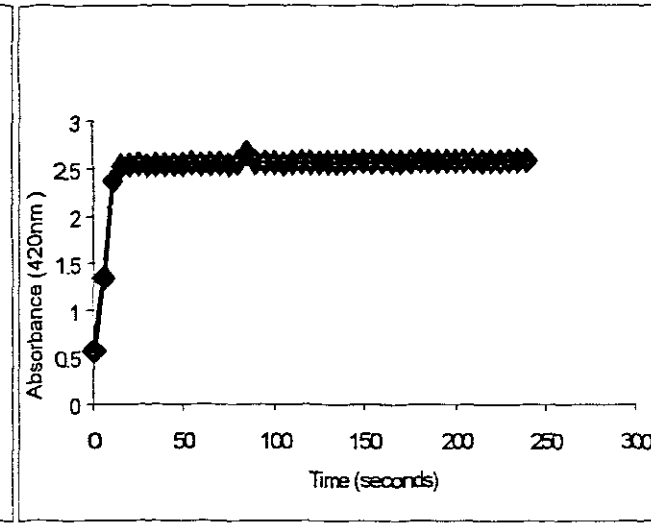
Continuous production of MnP in Packed Bed Bioreactor

***Run 1 MnP measurement* (Units/L) over a period of 15 days, figure 3.7.1**

Time (days)	MnP Activity (Units/L)
0	0
7	150
13	550
13	408
15	86.7
15	23.3



Day 7



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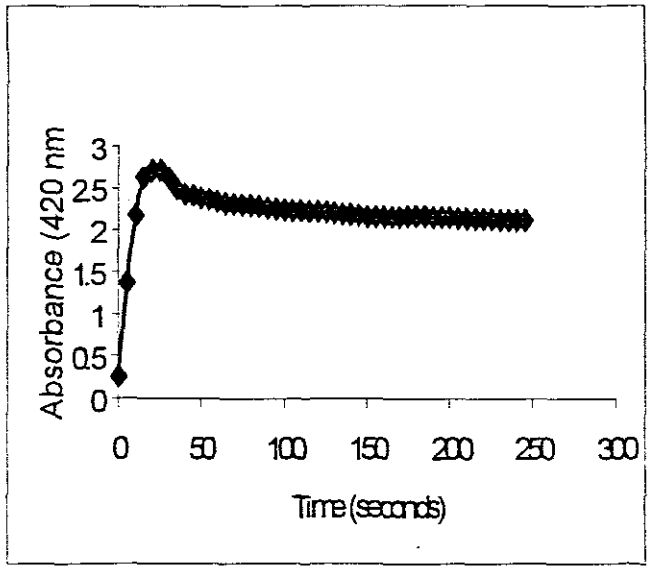

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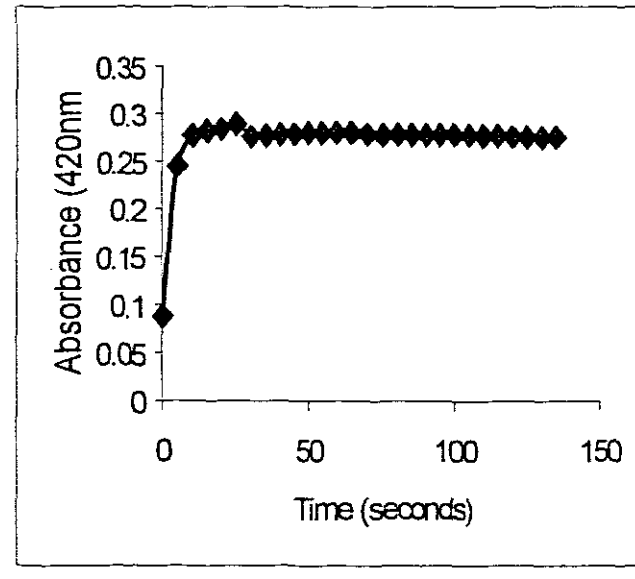

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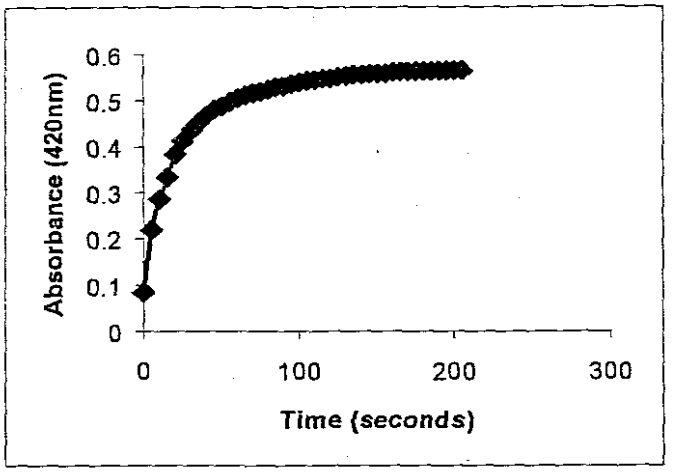


Day 15

Sample calculation of MnP Activity of day 7

dA/dT = 0.2/4.45

= 0.045 Abs/s

*60) = 2.70 Abs/min

/M^{-1}) = 7.49 *10^{-4} mole/min.L[
[where M^{-1} = molar extinction coefficient
= 3 600]

*10^{6}) = 749.1 micromoles/min.L

/CF=5) = 150 micromoles/min.L

MnP Activity = 150 Units/L
[where 1 micromole/min = 1 Unit ; CF= concentration factor]

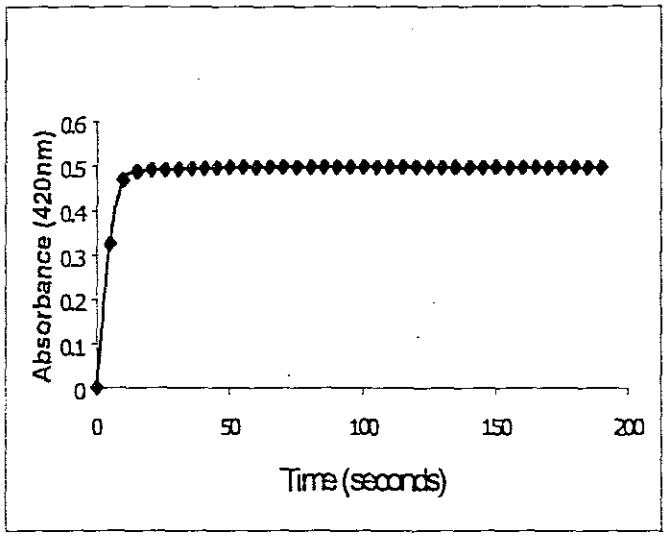
Run 2 MnP measurement (Units/L) over a period of 21 days (Packed Bed Bioreactor), figure 3.7.2

Time (days)	MnP Activity (Units/L)
0	0
10	191.4
11	570
12	78.4
14	44.6
17	17.3
18	52.2
21	43.4

MnP activity using polyurethane foam pellets as carrier support material in batch/flask cultures

A) Stirred Aerated Attached flask cultures, **figure 3.11.1**

Time (days)	MnP Activity (Units/L)
0	0
5	175.2
6	240
7	99.04
8	73.7
9	40



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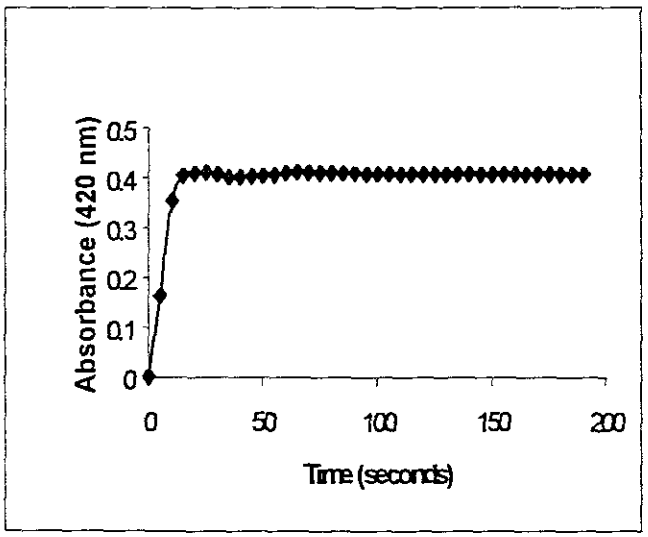

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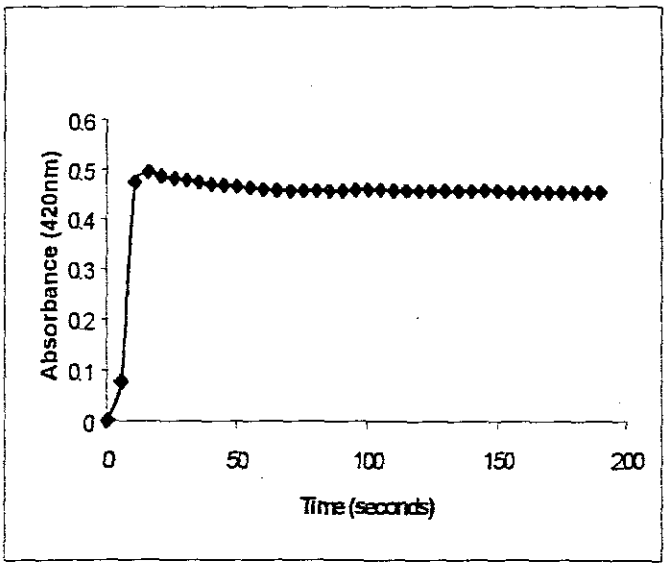

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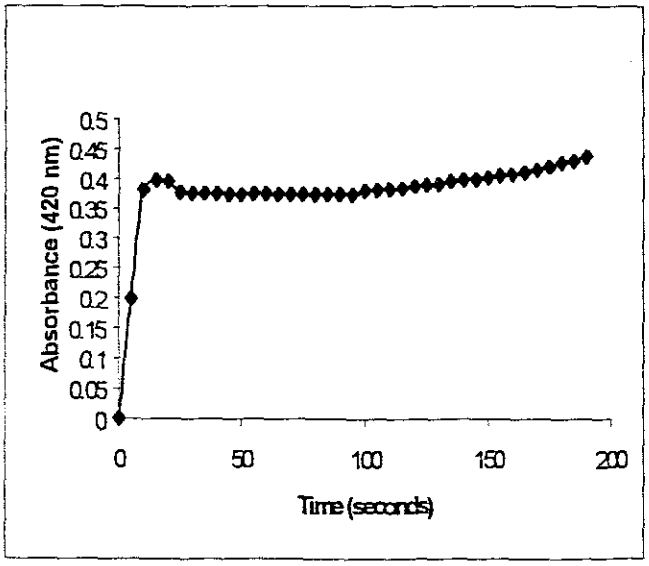

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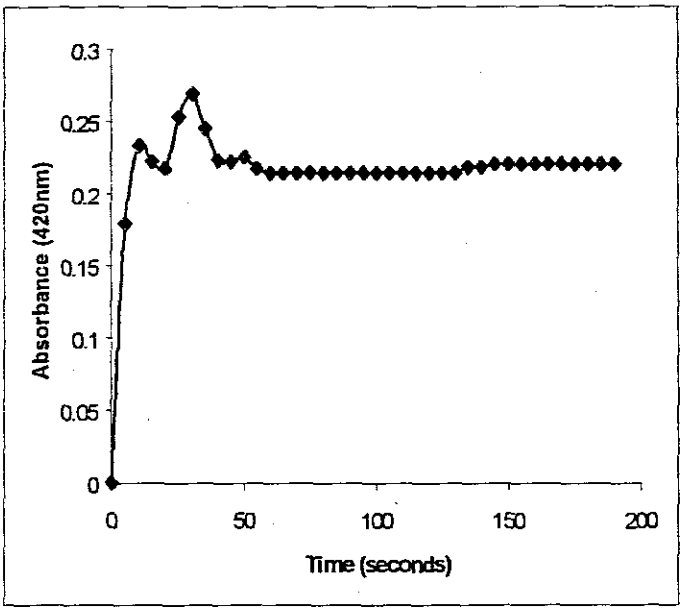
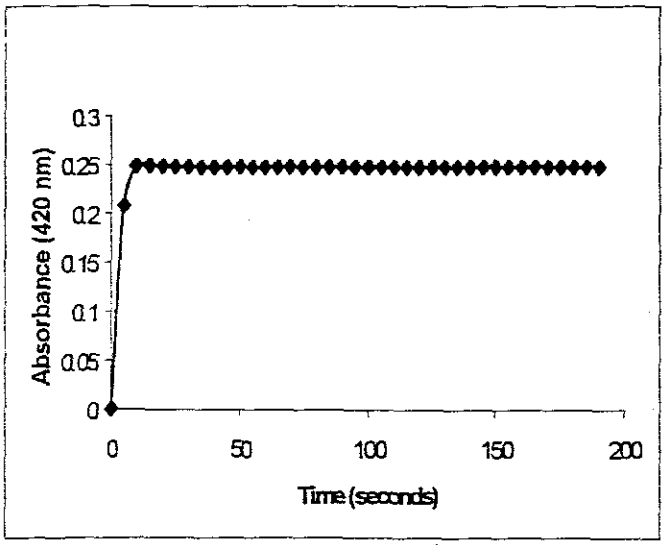


Day 9

B) Stirred Oxygenated Attached flask cultures, **figure 3.11.2**

Time (days)	MnP Activity (Units/L)
0	0
5	181.3
7	60
8	66.7
9	77.6



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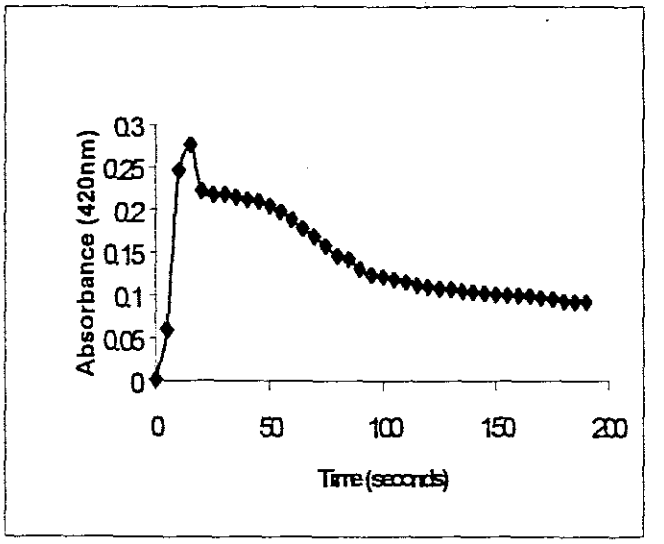

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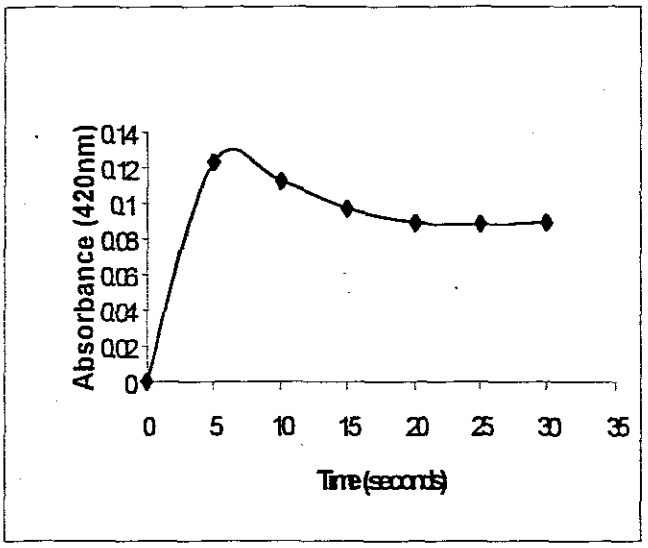

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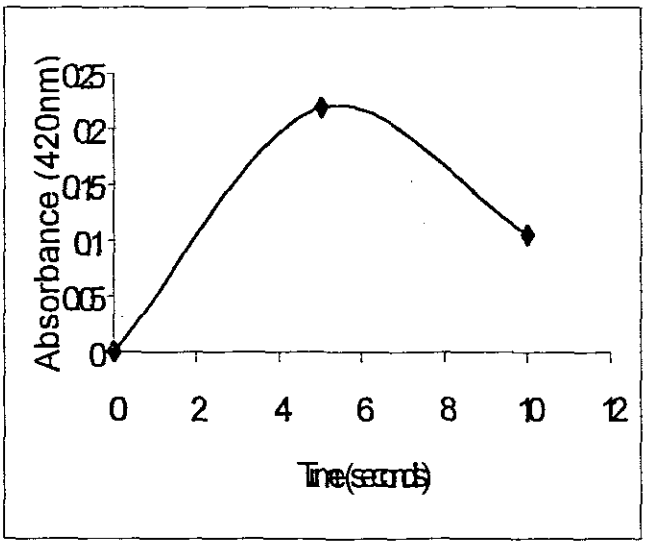


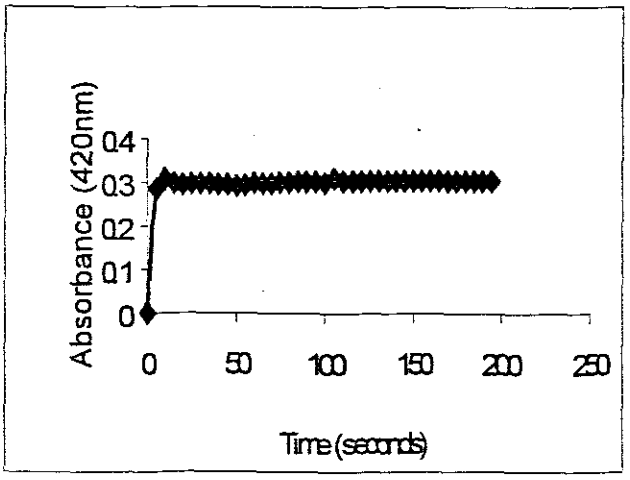
Day 9

MnP activity using resin beads as carrier support material in batch/flask cultures

A) Stirred Aerated Attached flask cultures, **figure 3.12.1**

Time (days)	MnP Activity (Units/l)
1	0
2	0
3	0
4	0
5	175.2
6	240
7	99.04
8	73.7
9	40

Day 5



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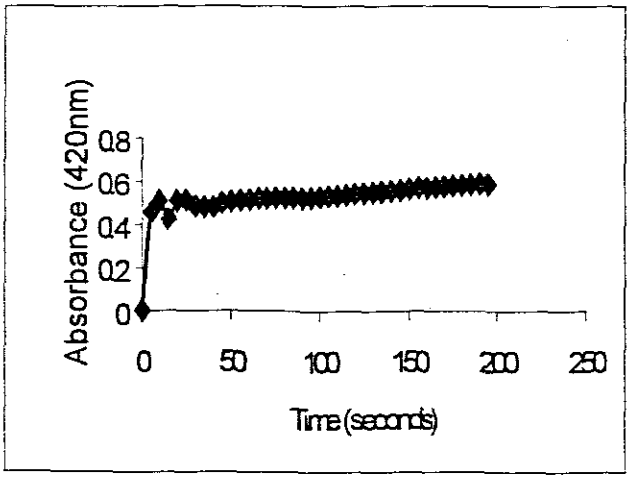

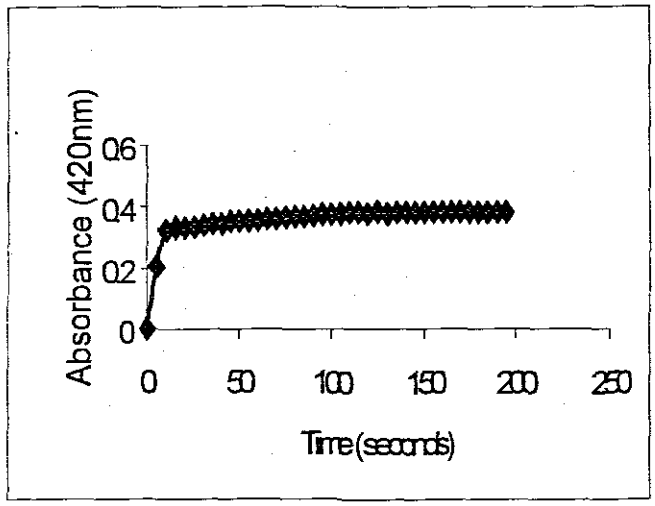

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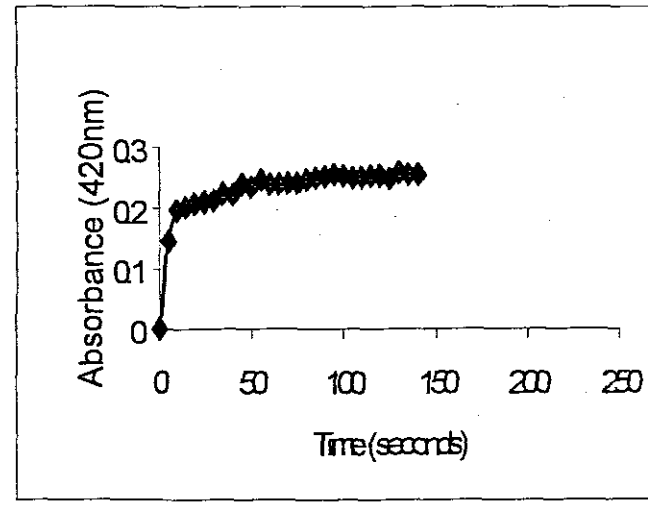

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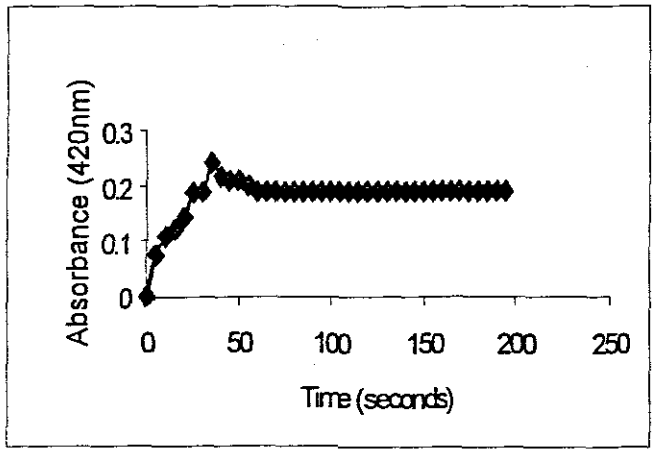
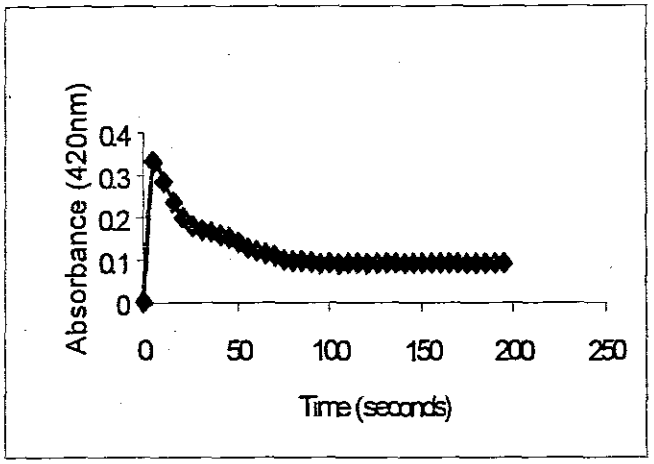


Day 9

B) Stirred Oxygenated Attached flask cultures, **figure 3.12.2**

Time (days)	MnP Activity (Units/l)
1	0
2	0
3	0
4	0
5	0
6	181.3
7	60
8	66.7
9	77.6



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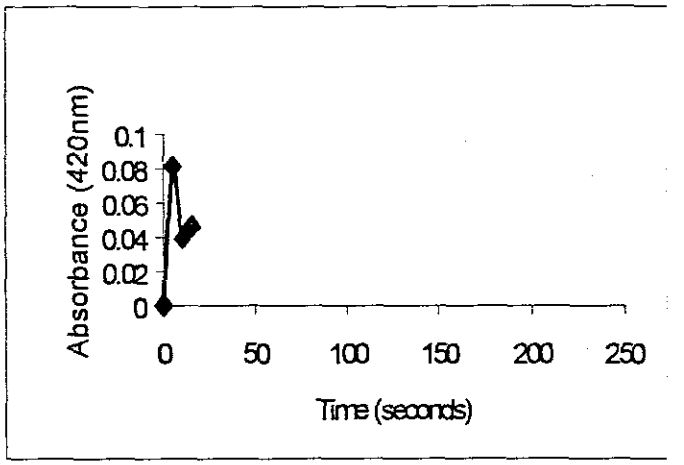

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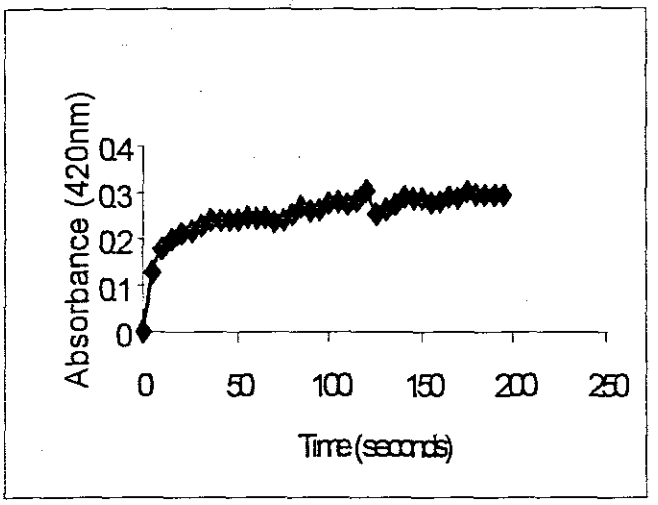

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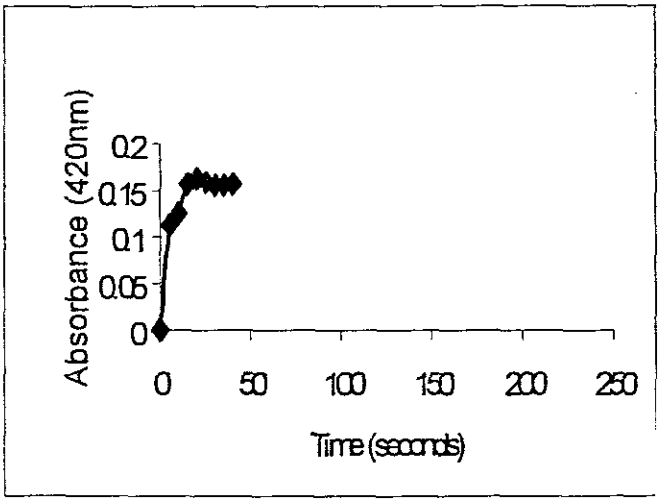

Day 9

ADDENDUM

Project Title:

Degradation of Polycyclic Aromatic Hydrocarbon (PAH) and Methyl t-butyl Ether (MTBE) by a Partially Purified Laccase Enzyme

ACKNOWLEDGEMENTS:

The author is grateful to Professor Michael Barcelona of the Department of Civil and Environmental Engineering for his encouragement and valuable ideas and for promoting this project. A special mention goes to Mr. Tim Baker and Ms Lynn Homola for excellent technical laboratory assistance. Mrs. Vicki Earl is thanked for her outstanding administrative assistance. I thank Professor Stuart Batterman for his helpful discussions. I would also like to express my thanks to the research group of the National Center for Integrated Bioremediation Research and Development (NCIBRD) for providing expert guidance and support.

PREFACE

This project deals with the application of enzymes to biodegrade pollutants found present in soils and groundwater.

The work reported in this project was carried out during a six-month study visit to The Department of Civil and Environmental Engineering at The University of Michigan.

This study visit was supported by a grant from the Tertiary Education Linkage Program (TELP), between Peninsula Technikon, South Africa and The University of Michigan, Ann Arbor, USA, sponsored by the United States Agency for International Development.

ABSTRACT

The application of the fungal enzymes for degradation of organics has relevance for on going soil and groundwater remediation work. The use of enzymes either in pump- and-treat technologies or directly on contaminated soil requires knowledge of their effectiveness on targeted organic compounds (Fang *et al*, 1997).

During a study visit to the University of Michigan, Ann Arbor, USA, the author had the opportunity to conduct bench-scale experiments to determine the degrading ability of a partially purified Laccase enzyme expressed by *Rhus vernificera* on naphthalene and methyl t-butyl ether (MTBE). These two target compounds were selected for study because they are among the most common and persistent organic contaminants present in affected groundwater in the USA.

Three different enzyme concentrations (36 Units/l, 72 Units/l and 144 Units/l) were tested respectively using syringaldazine as the mediating substrate. The gas chromatography results showed that enzyme concentration had little effect on naphthalene degradation but a marked effect on MTBE degradation. Mass spectrometer results indicated incomplete breakdown of the substrate. This would suggest that enzyme "cocktails" produced directly by live fungi in a bioreactor might be more effective than one particular enzyme in isolation.
Temperature and mediating substrate are variables that should be investigated with a view to optimizing degradation effectiveness. Degradation effectiveness of immobilized enzymes and whole organism contacting methods should also be compared. It is furthermore recommended that a comparison with pure enzyme should be included as a control in future experimental work.

It was also concluded that the experimental methods and procedures employed were satisfactory and they are recommended for further work involving other Polycyclic Aromatic Hydrocarbon (PAHs).

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1. RESEARCH PROBLEM AND ITS BACKGROUND

Work perform in this project describe the effective use of enzymes for degradation of pollutants. The focus of the experimental work done at Michigan was on groundwater and soil pollutants (naphthalene and methyl t-butyl ether) using Laccase derived from plant matter.

The widespread prevalence and toxicity of polycyclic aromatic hydrocarbons (PAHs) made these compounds one of the most important groups of environmental pollutants in the USA. Accidental disposal of distillation wastes from the chemical industry resulted in severe, long-lasting contamination of the soil in many areas (Braun-Lullemann *et al*, 1999). The contaminants of greatest concern to hydrogeologists and public health officials are those which are simultaneously toxic, mobile and persistent in the subsurface (Jackson, 1990).

Polycyclic aromatic hydrocarbons such as naphthalene, benzo (a) pyrene, anthracene ect. were among the most common and persistent organic contaminants found present in groundwater contamination and terrestrial environments (Pickard *et al.* 1999). MTBE (methyl tert-butyl ether), was the second most frequently detected VOC in groundwater sampled from 210 shallow wells and springs in 8 urban areas in the United States alone (Delzer *et al.*1999). A large group of xenobiotic pollutants occurs as common constituents of petroleum, coal tar and shale oil, which were important pollutants found in soil and sediments due to their potential health hazards to humans (Ravelet *et al.* 2000). Some of these PAHs and/or their metabolites were carcinogenic (Song, 1999).

White Rot fungi belong to the group of Basidiomycetes that colonize wood in nature and decompose lignin in lignocellulosic materials that cause white rotting of wood (Novotny *et al.* 1999). Extracellular ligninolytic systems composed of peroxidases, H_2O_2-producing oxidases and laccase was specialized for the decomposition of lignin (Kirk & Farrell, 1987). The white rot fungi are likely to be involved in the biodegradation of PAH pollutants in soil and their use in PAH bioremediation had been proposed (Lesten

and Lamar, 1996). In addition, they were considered promising for application to the clean- up of contaminated soils, but little information was known about their ability to colonize various soils and their biochemical activities (Lamar *et al*, 1987).

Contamination of soil and groundwater resulted from infiltration of crude oil spills, leakages from underground storage tanks and other related improperly managed industrial effluents. Site remediation strategies for managing groundwater contamination, such as pump and treat technologies involve high cost with low efficiency (Fang *et al*. 1997). Bioremediation remained a growing research area due to the effectiveness of the microorganisms and associated enzymes to degrade a variety of relatively recalcitrant environmental pollutants (Song, 1999; Bumpus and Aust, 1985). Extracellular laccase excreted from wood rotting fungi (*Phanerochaete Chrysosporium, Coriolopsis gallica, Trametes Versicolor* and others) have shown good potential in degrading these contaminants (Pickard *et al*, 1999). Therefore, it was important to conduct bench-scale experiments to analyze the degrading ability of these enzymes, especially laccase for future field scale implementation.

1.1 AIM:

To monitor the extent of biodegradation by using laccase expressed by *Rhus Vernificera*, on these target compounds individually under the same experimental conditions. The target compounds included MTBE (methyl tert-buthyl-ether) and naphthalene.

1.2 RESEARCH PROJECT OBJECTIVES:

Specific objective:

- To identify the parameters which need to be optimized for the complete degradation of naphthalene and MTBE.

Secondary objective:

- To identify the transformation products from mass spectrometer analyses

1.3 RESEARCH WORK EXPECTATIONS AND ASSOCIATED BENEFITS:

It was expected that both the target compounds will be degraded to a certain extent and that the extent of degradation would assist us to evaluate experimental conditions for complete degradation. Among the major benefits however would be the use of the data to extend enzymes to groundwater contamination mitigation.

2. LITERATURE REVIEW

2.1 ORIGIN OF THE POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) AND METHYL TERT-BUTYL ETHER (MTBE)

Polycyclic aromatic hydrocarbons (PAHs) were distributed in the USA environment as a result of the use of petroleum and its refinery products. They were also associated with incomplete combustion in forest and prairie fires, automobile exhausts and fossil fuels (Cerniglia, 1992; Gramss *et al*, 1999). They were also formed in biological conversions from biogenic precursors (Aizenshtat, 1973; Gramss *et al*, 1999).

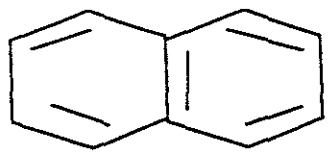
Naphthalene: ($C_{10}H_8$)

Naphthalene is among the most abundant constituents of coal tar. Dry coal tar contains about 11% naphthalene. It is volatilized appreciably at room temperature. Solubility in methanol or ethanol is ~ 8g/100ml.

Uses:

- Manufacturing of phthalic and anthranilic acids, which was used in making indigo, indanthrene, and triphenylmethane dyes.
- Manufacturing of hydronaphthalenes (Tetralin, Decalin) which was used as solvents, in lubricants, and in motor fuels
- Has also been used in dusting powders, as an insecticide and internally as an intestinal antiseptic and vermicide.
  (Merck Index, 1989)

Chemical structure of naphthalene



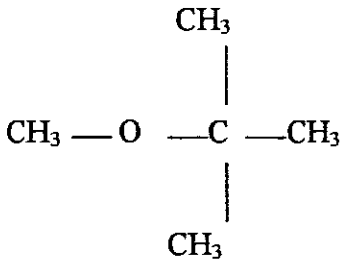
Methyl tert-butyl-ether (MTBE): ($C_5H_{12}O$)

Methyl-tert-butyl-ether is a liquid at room temperature. It has a vapor pressure of 245-mm Hg at 25° C and 4.8 g MTBE dissolved in 100 g of water. It disperses rapidly in water, and is far less biodegradable than common gasoline constituents, such as benzene, toluene, ethylbenzene, and total xylene (BTEX) (Delzer *et al.*1999). MTBE partitions strongly from the gas phase into the water phase if contaminated air is brought into contact with uncontaminated water, due to its great water solubility, which was probably the most important chemical property. This affected the partitioning of organic compounds between water and subsurface solids (Squillace *et al,* 1998).

Uses:

- Methyl tert-butyl ether (MTBE) is a gasoline oxygenate. MTBE, when added to gasoline, increase the gasoline's oxygen level and decrease vehicular carbon monoxide emissions and ozone levels in the atmosphere (Delzer *et al,* 1995).

Chemical structure of Methyl-tert-butyl-ether (MTBE):



2.2 PROCESS CHEMISTRY OF LACCASE AND ITS APPLICATION

Laccase is a unique enzyme because it differs from peroxidases in that it does not require hydrogen peroxide to oxidize substrates. Laccase store four electrons, and does not release intermediates in the O_2 reduction pathway. Similar to that of peroxidases, the substrate radicals produced by laccase catalysis undergo various polymerizations, cleavage, and other reactions. The redox potential of laccase (0.8-1.0 eV), is lower than that of horseradish peroxidase. The capabilities of laccase can be enhanced via the inclusion of a mediating substrate such as ABTS and syringaldazine. In this particular

study we use syringaldazine as mediating substrate, since the substrates (target pollutants), namely naphthalene and MTBE are supplied in small concentrations and we need to keep the substrate concentration high in order to keep the reaction rate of Laccase on these target pollutants high. The expected transformation products for the two pollutants are given below:

Microbial degradation of naphthalene

Metabolites from microbial degradation kinetics at 20° C of naphthalene were identified as 1-methyl-naphthalene, 2-methylnaphthalene, acenaphthene and acenaphthylene (Squillace *et al*, 1998).

Microbial degradation of MTBE

MTBE could be degraded in the atmosphere by various processes including photolysis and reactions with the hydroxyl radical, ozone, and nitrate radicals and recent research indicates that MTBE reaction with the hydroxyl radical was the most prevalent. Complete mineralization of an organic compound in water and soil was almost always a consequence of microbial activity. The major degradation product of MTBE in the atmosphere was tert-butyl formate. Degradation products in water included methyl acetate (acetic acid), acetone, tert-butyl alcohol, and formaldehyde (Squillace *et al*, 1998).

2.3 OTHER POTENTIAL APPLICATIONS OF LACCASE, BESIDES THE APPLICATION FOR THIS PARTICULAR STUDY.

- The most heavily studied uses for laccase have been in the pulp and paper industry, to lower the kappa number of pulp in the pulping processes.
- Immobilized laccases have been studied for removal of xenobiotics from aqueous waste streams, and oxidation of phenolics in wine.
- Biosensor applications used laccase (in the form of immobilized-enzyme electrodes) to various stages, which included the measuring of phenolic contents of aqueous samples, in more specific applications, fruit juices, tea and other beverages.

- Laccase has also been suggested as a replacement for horseradish peroxidase (HRP) as the marker enzyme in enzyme-linked immunoassays, due to the tendencies for HRP to exhibit high background staining and occasional formation of unproductive complexes with H_2O_2.
  (http://www.tienzyme.com/)

3. EXPERIMENTAL DETAILS

3.1 EXPERIMENTAL AND ANALYTICAL METHODS AND PROCEDURES

Samples were prepared in duplicate along with their associated blanks and controls in 42-ml vials, which form the reaction vessels. Each target compound was monitored at three different enzyme concentrations with associated mediating substrate. Tienzyme Inc described the ratio of enzyme to mediating substrate concentration used (http:///www.tienzyme.com, 17/10/2000). The author analyzed samples by using the Tekmar 7000 Headspace autosampler interfaced with a HP 5890 II GC and HP 5972 MSD.

Experimental Procedures:

An MTBE standard curve was prepared from a stock solution of 1000 ppm. The highest concentration used was 1000 ppb and the lowest concentration used was 50 ppb. Refer to Appendix 1 for calibration curves for MTBE and naphthalene respectively.

From the calibration data the percentage relative standard deviation for MTBE and naphthalene was very small, 7.3 and 4.8 respectively, which was far less than 20%. If the RSD of the calibration or response factors was less than or equal to 20% over the calibration range, then the linearity through the origin may be assumed, and the average calibration or response factor may be used to determine sample concentrations.

Partially purified Laccase from *Rhus Vernificera* was obtained from Sigma-Aldrich, at 1mg of solid equals 180 Units. Fresh Laccase solution for each experimental run was prepared in 50mM potassium phosphate buffer (pH~7.0) to give an activity of 360 Units/liter. The prepared solution was kept in an ice jacket until further use.

Syringaldazine (1.0 mM) was used as the mediating substrate and was prepared in methanol. The volume ratio (1:1) of enzyme to mediating substrate volume was used as described by Tienzyme Inc; with reference to their Laccase enzyme assay. The chemical

syringaldazine was obtained from Sigma-Aldrich. The prepared solution was kept in a fridge until further use.

Analytical procedures:

Enzyme Assay:

Reagents

100mM Potassium Phosphate, pH 5.5 (buffer)

1.0 mM Syringaldazine in Methanol

Laccase enzyme [Storage buffer: 50mM Potassium Phosphate, pH 7.0; store at -20° C]

Procedure:

In a 4-ml cuvette, add:

3.2 ml buffer

400µl enzyme solution (may required dilution)

400µl syringaldazine solution

Blank against buffer/enzyme solution; follow absorbance change at 530 nM for one minute after addition of the syringaldazine to the reaction vessel. One unit produce an absorbance change of 0.001/min, at pH 5.5 at 25° C.

Sample preparation: (MTBE & Naphthalene as target compounds, syringaldazine as mediating-substrate)

Each experimental batch consisted of 20 vials (42ml volume)

Vial composition:

4.2ml of Laccase solution; concentration (360 Units/l; 720 Units/l; 1440 Units/l))

4.2ml of syringaldazine solution; concentration (1.0 mM)

4.2 µl of MTBE; from stock solution (10,000 ppm)

42 µl of naphthalene; from stock solution (1000 ppm)

Fill up to 42ml sample volume, and avoid any headspace.

The vials were placed inside an incubated horizontal shaker at 25° C and 150 rpm.

Autoclaved distilled water was used in all experiments and the control samples were first autoclaved before it were spiked with the target compounds. 100 µl of aminobenzoic acid (10mM) was also added after autoclavation as a final precaution to stop all activity.

Experimental program design:

Five identical sets of microcosms were prepared, each with duplicate, control and blank as shown in the example below:

Vial no.	Enzyme volume (ml)	Mediating substrate volume (ml)	MTBE volume (µl)	Naphthalene volume (µl)	Total sample volume made up with freshly autoclaved distilled water (ml)
1a	4.2	4.2	4.2	42	42
1b (duplicate)	4.2	4.2	4.2	42	42
1c (control)	4.2	4.2	4.2	42	42
1d (blank)	4.2	4.2	-	-	42

A similar experimental set was prepared for each enzyme concentration studied (36, 72 and 144 Units/l), varying only the concentration of the enzyme stock solution added to the vial. These enzyme concentration stock solutions were prepared every second day while the naphthalene and MTBE stock solutions were kept in the fridge at 4°C (were not made-up daily).

3.2 EXPERIMENTAL DATA, RESULTS AND DISCUSSION

Degradation of these target compounds (MTBE and Naphthalene) was monitored over 5-days at three different enzyme concentrations. The displayed results were found to be reproducible. Please refer to Appendix 2, which displays the tables for the experimental data with standard deviations. The following figures display the results graphically.

The following three graphs represent the percentage degradation on naphthalene and MTBE at 36 Units/l; 72 Units/l and 144 Units/l respectively:

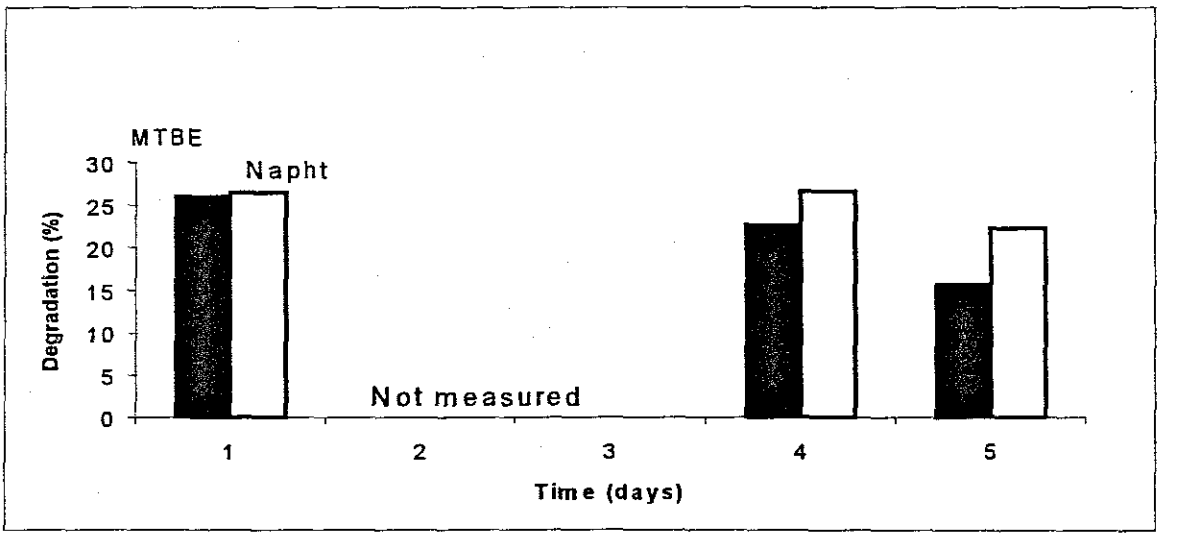


Figure 3.1.1: 36 Units/L: % MTBE and Naphthalene degradation over time

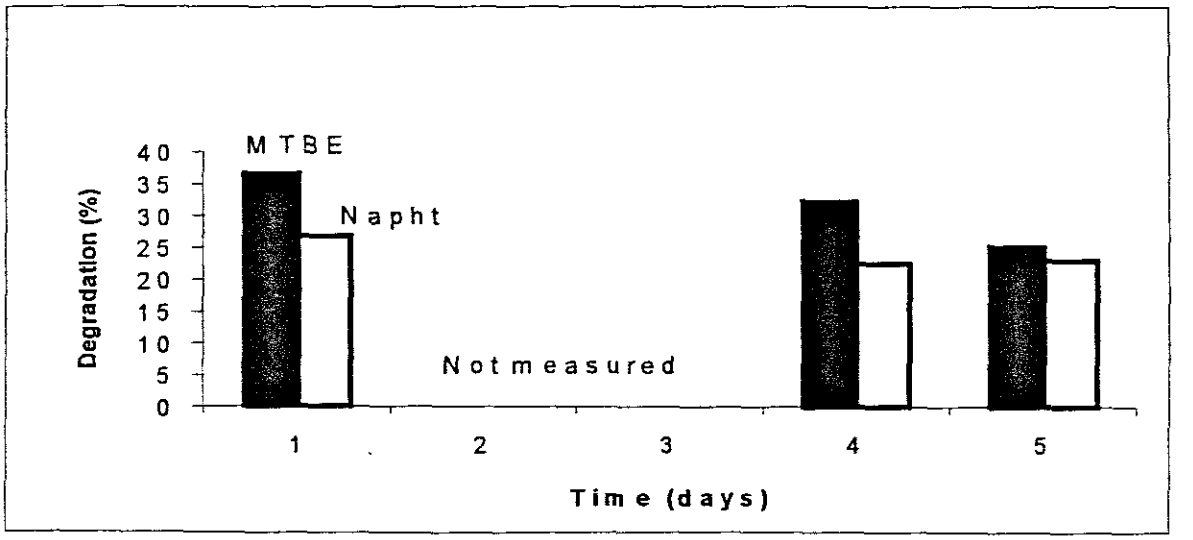


Figure 3.1.2: 72 Units/L: % MTBE and Naphthalene degradation over time

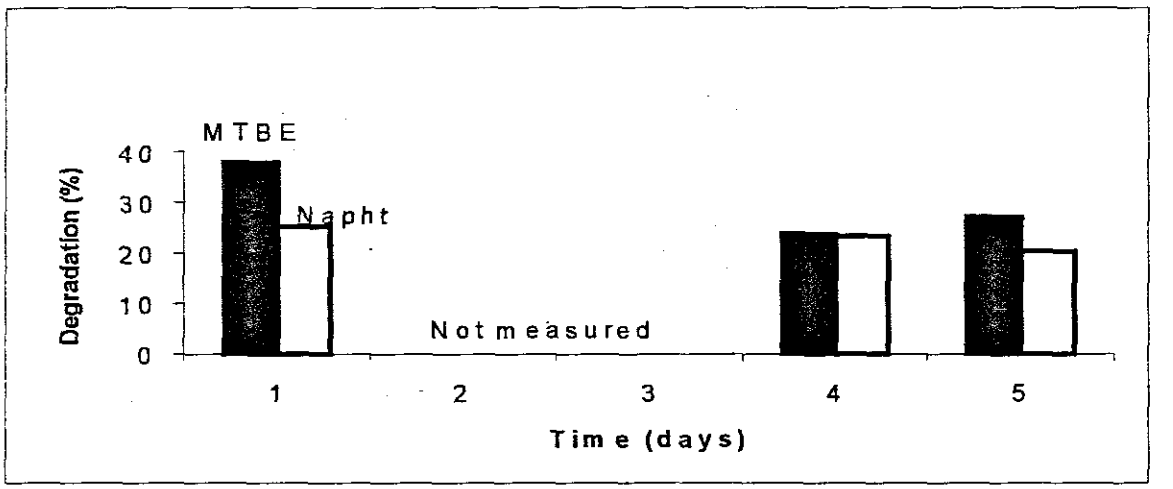


Figure 3.1.3: 144 Units/L: % MTBE and Naphthalene degradation over time

Discussion:

Figure 3.1.1 represent MTBE and naphthalene degradation at an enzyme concentration of (36 Units/l). The maximum extent of both MTBE and naphthalene degradation at this specific enzyme concentration were found to be 26 %.

Figure 3.1.2 shows MTBE and naphthalene degradation at an enzyme concentration of 72 Units/l. The maximum extent of both MTBE and Naphthalene degradation at this specific enzyme concentration were found to be 38 % and 25 % respectively.

Figure 3.1.3 shows MTBE and naphthalene degradation at an enzyme concentration of 144 Units/l. The maximum extent of both MTBE and Naphthalene degradation at this specific enzyme concentration was found to be 37% and 27% respectively.

The following two graphs represent the percentage degradation of naphthalene and MTBE at different enzyme concentrations:

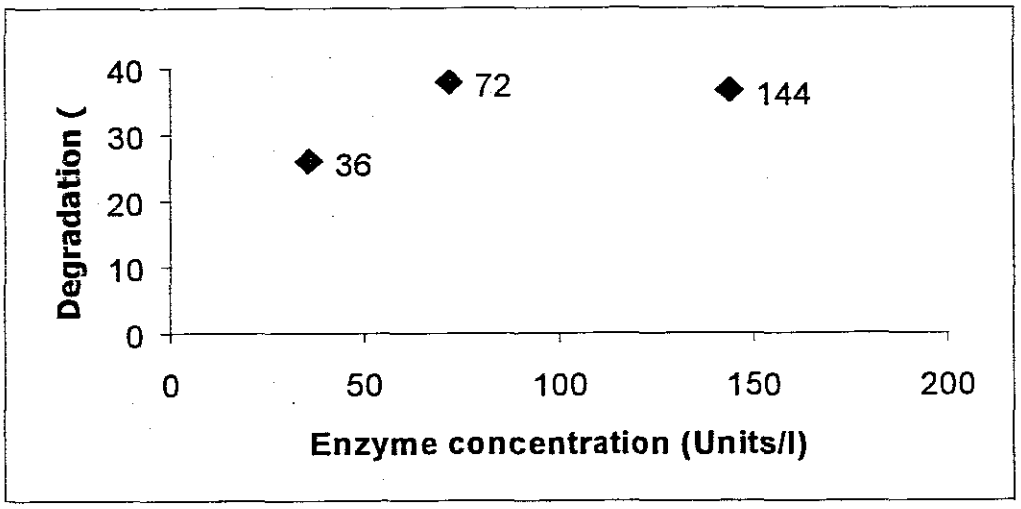


Figure 3.2: **% MTBE degradation** vs Enzyme concentration

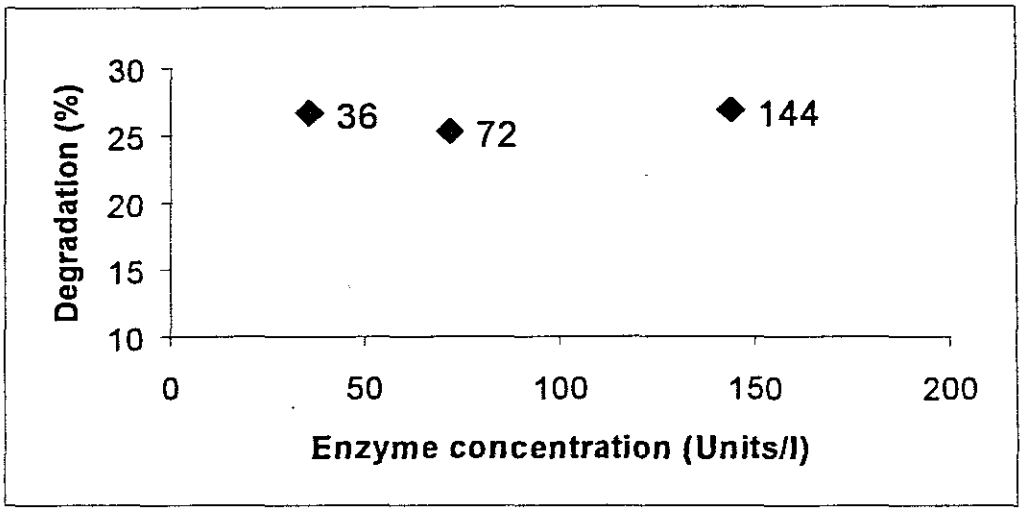


Figure 3.3: **% Naphthalene degradation** vs Enzyme concentration

Discussion

From figure 3.2 it seems that an increase in enzyme concentration did increase MTBE degradation, but a further increase from 72 to 144 (Units/l), does not increase MTBE degradation. This latter observation raises questions about possible enzyme inhibition.

From figure 3.3 it could be seen that an increase in enzyme concentration had little effect on naphthalene degradation. Some other factors such as temperature and variation of

mediating substrate [2,2- Azino-Bis (3-ethylbenzthiazoline-6-sulfonicacid)] should be looked at to enhance naphthalene degradation.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1 CONCLUSIONS

Little is known about the degrading ability of Laccase from Rhus Vernificera on these polycyclic aromatic hydrocarbons, but these experiments had proved its potential use as an alternative in bioremediation applications. Optimum conditions have not yet been determined for complete degradation, but important parameters have been identified such as enzyme concentration, mediating substrates and temperature that shown to have an effect in the biodegradation process. It seemed that most of the degradation took place during the first day, and that more complete degradation may be expected if the enzyme concentration is increased. Transformation products were visible from the mass spectrometer analyses but could not be identified. The sample preparation and incubation procedure worked quite well. 5-10% loss of enzyme activity was determined by performing an enzyme assay on the samples after gas chromatography analyses. This, however proves that the enzyme was indeed recoverable. Further experimental work and study needs to be done on this particular enzyme, but the data obtained from this project could be used as a basis for future work.

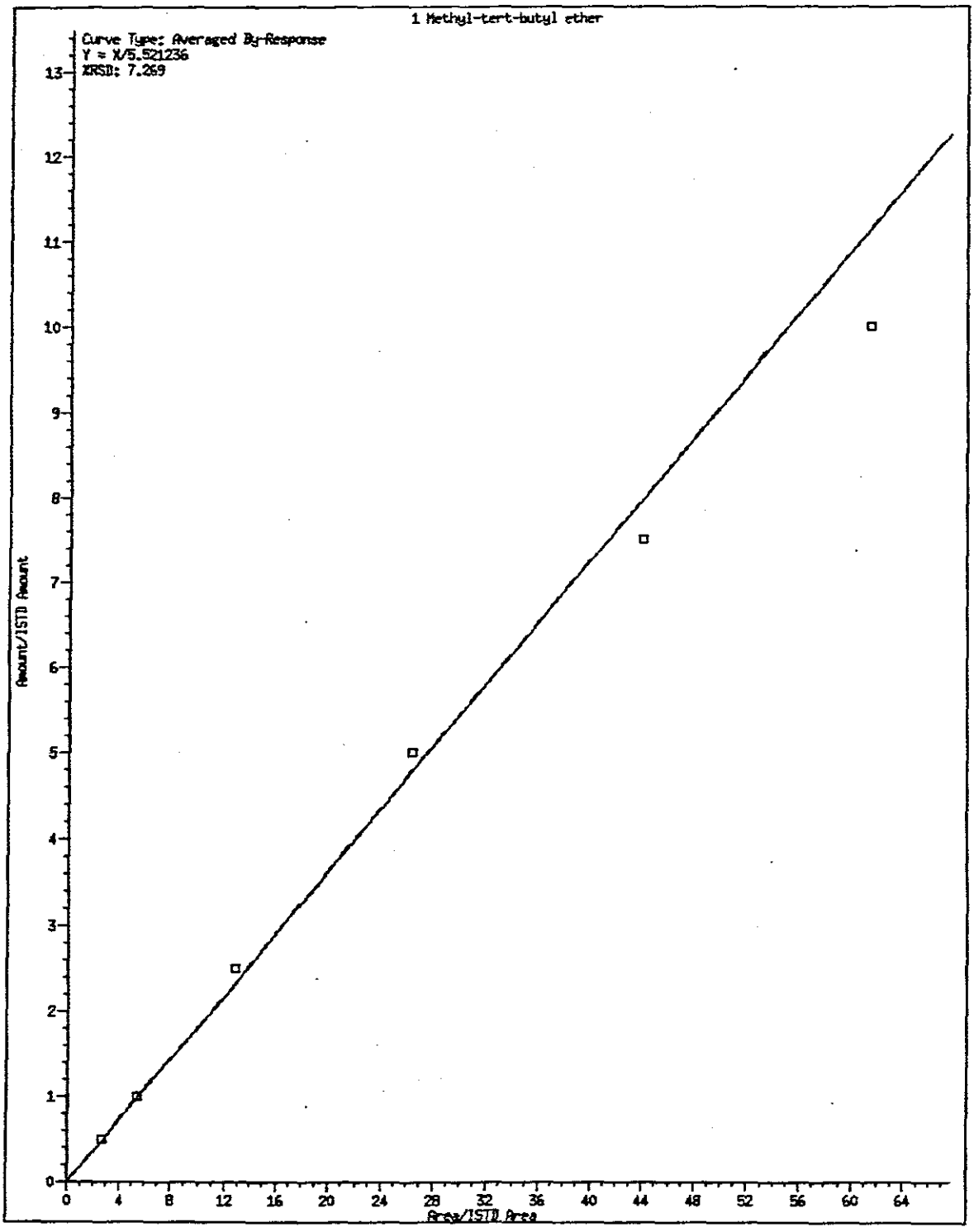
4.2 RECOMMENDATIONS:

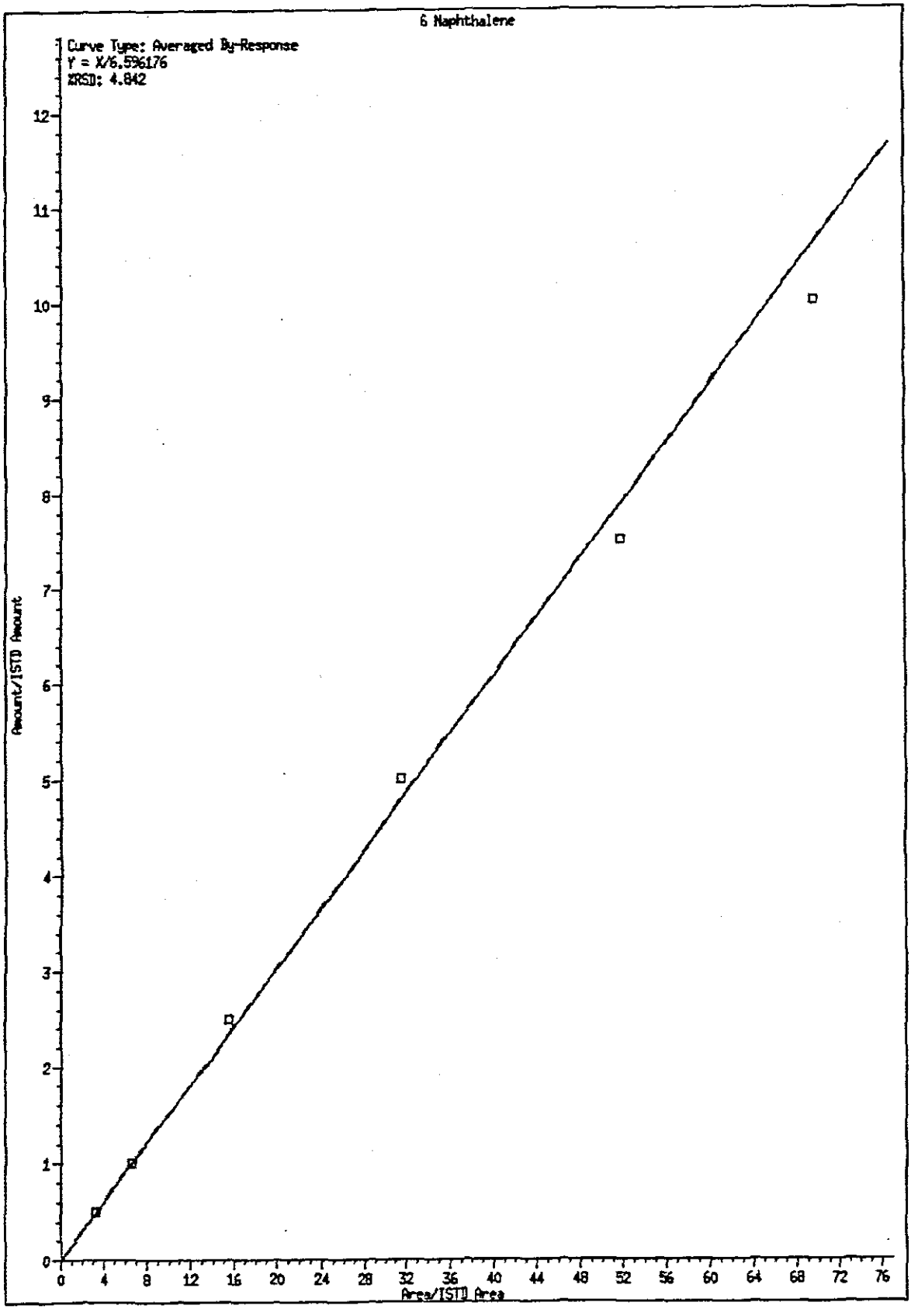
- Care should be taken when storing the enzyme, especially when determining the enzyme activity in the sample (store enzyme in fridge at -20 ° C).
- The experimental methods and procedures in this study are satisfactory and can be used involving other Polycyclic aromatic hydrocarbons (PAH's), such as anthracene, benzo-a-pyrene etc.
- Enzyme concentration range should be extended to assist in the development of optimum conditions for complete biodegradation.
- Comparison of the effects of different mediating substrates used such as syringaldazine and ABTS [2,2-Azino-Bis (3-ethylbenzthiazoline-6-sulfonicacid)] will assist in the development of an improved biodegradation system.

- Mass spectrometer chromatographs would help to explain the steps in the degradation of naphthalene and MTBE observations.
- Pure enzymes should be compared against partially purified laccase (Sigma Aldrich product) and temperature effects should be investigated.

APPENDIX 1
GAS CHROMATOGRAPHY CALIBRATION FOR MTBE AND NAPHTHALENE

	1	5	10	50	100	200	__	
Compound	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6	RRF	% RSD
1 Methyl-tert-butyl ether	5.33601	5.39197	5.09946	5.28080	5.86518	6.15399	5.52124	7.269
2 tert-Butyl alcohol	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++ <-
6 Naphthalene	6.58720	6.62017	6.18241	6.29568	6.90427	6.98732	6.59618	4.842
$ 3 1,4-Difluorobenzene	1.21803	1.21377	1.20438	1.21980	1.19172	1.22052	1.21137	0.934
$ 5 4-Bromofluorobenzene	0.62085	0.61440	0.62068	0.61531	0.60039	0.61040	0.61367	1.243


1 Methyl-tert-butyl ether
Curve Type: Averaged By-Response
Y = X/5.521236
%RSD: 7.269
Amount/ISTD Amount
Area/ISTD Area
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64


6 Naphthalene
Curve Type: Averaged By-Response
Y = X/6.596176
%RSD: 4.842
Amount/ISTD Amount
Area/ISTD Area
0
1
2
3
4
5
6
7
8
9
10
11
12
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76

APPENDIX 2
EXPERIMENTAL DATA

% Degradation of MTBE and Naphthalene

All figures presented in this report are based on the average control values

The data in bold are used in the experimental analysis:

@ 36 Units/L

Time (days)	MTBE (%)	NAPHT. (%)	Time (days)	MTBE (%)	NAPHT. (%)
0	**25.94**	**26.46**	0	20.35	23.58
3	**22.65**	**26.59**	1	20.22	22.35
4	**15.67**	**22.19**	2	19.11	22.12

@ 72 Units/L

Time (days)	MTBE (%)	NAPHT. (%)	Time (days)	MTBE (%)	NAPHT. (%)
0	**37.86**	**25.23**	0	40.26	27.38
3	**23.63**	**23.31**	1	34.43	25.51
4	**27.08**	**20.29**	2	27.76	21.18
			4	33.13	19.86

@ 144 Units/L

Time (days)	MTBE (%)	NAPHT. (%)	Time (days)	MTBE (%)	NAPHT. (%)
0	**36.56**	**26.77**	1	15.40	24.40
3	**32.30**	**22.63**	2	32.66	22.79
4	**25.37**	**23.21**	5	37.57	21.67

MTBE & Naphthalene samples [36 Units/L]

Day 0 Individual Controls

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
1a	948	951.7	96.55	922	941.33	16.77
1b	1 050			950		
1ab	857			952		
1c	1 290			1 280		
1d						
% degrad.	**26.23**			**26.46**		

Day 3

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
2a	978	994	22.72	942	939.67	5.86
2b	984			944		
2ab	1 020			933		
2c	1 280			1 280		
2d						
% degrad.	**22.34**			**26.59**		

Day 4

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
3a	1 190	1 083.67	100.20	992	996	5.66
3b	991					
3ab	1 070			1 000		
3c						
3d						
% degrad.	**#DIV/0!**			**#DIV/0!**		

MTBE & Naphthalene samples [36 Units/L]

Day 0 Average Controls

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
1a	948	951.6	96.55	922	941.33	16.77
1b	1 050			950		
1ab	857			952		
1c	1 285			1 280		
1d						
% degrad.	**25.94**			**26.46**		

Day 3

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
2a	978	994	22.72	942	939.67	5.86
2b	984			944		
2ab	1 020			933		
2c	1 285			1 280		
2d						
% degrad.	**22.65**			**26.59**		

Day 4

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
3a	1 190	1 083.67	100.20	992	996	5.66
3b	991					
3ab	1 070			1 000		
3c	1 285			1 280		
3d						
% degrad.	**15.67**			**22.19**		

MTBE & Naphthalene samples [72 Units/L]

Day 0 Individual Controls

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
1a	817	804.67	67.35	935	934.67	15.50
1b	732			950		
1ab	865			919		
1c	1 210			1 220		
1d						
% degrad.	**33.50**			**23.39**		

Day 3

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
2a	1 010	989	18.19	970	958.67	25.01
2b	979			976		
2ab	978			930		
2c	1 380			1 280		
2d						
% degrad.	**28.33**			**25.10**		

Day 4

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
3a	920	944.33	164.85	981	996.33	14.57
3b	793			1 010		
3ab	1 120			998		
3c						
3d						
% degrad.	**#DIV/0!**			**#DIV/0!**		

MTBE & Naphthalene samples [72 Units/L]

Day 0 Average Controls

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
1a	817	804.67	67.35	935	934.67	15.50
1b	732			950		
1ab	865			919		
1c	1 295			1 250		
1d						
% degrad.	**37.86**			**25.23**		

Day 3

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
2a	1 010	989	18.19	970	958.67	25.01
2b	979			976		
2ab	978			930		
2c	1 295			1 250		
2d						
% degrad.	**23.63**			**23.31**		

Day 4

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
3a	920	944.33	164.85	981	996.33	14.57
3b	793			1 010		
3ab	1 120			998		
3c	1 295			1 250		
3d						
% degrad.	**27.08**			**20.29**		

MTBE & Naphthalene samples [144 Units/L]

Day 0 Individual Controls

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
1a	897	878.67	68.37	910	919	13.89
1b	803			912		
1ab	936			935		
1c	1 350			1 240		
1d						
% degrad.	**34.91**			**25.89**		

Day 3

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
2a	964	937.67	24.01	939	971	30.61
2b	932			1 000		
2ab	917			974		
2c	1 420			1 270		
2d						
% degrad.	**33.97**			**23.54**		

Day 4

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
3a	991	1 033.67	101.93	965	963.67	16.042
3b	1 150			947		
3ab	960			979		
3c						
3d						
% degrad.	**#DIV/0!**			**#DIV/0!**		

MTBE & Naphthalene samples [144 Units/L]

Day 0 Average Controls

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
1a	897	878.67	68.37	910	919	13.89
1b	803			912		
1ab	936			935		
1c	1 385			1 255		
1d						
% degrad.	**36.56**			**26.77**		

Day 3

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
2a	964	937.67	24.01	939	971	30.61
2b	932			1 000		
2ab	917			974		
2c	1 385			1 255		
2d						
% degrad.	**32.30**			**22.63**		

Day 4

Sample	**MTBE (ppb)**	Ave (ppb)	Stand.dev.	**Napht. (ppb)**	Ave. (ppb)	Stand.dev.
3a	991	1 033.67	101.93	965	963.67	16.042
3b	1 150			947		
3ab	960			979		
3c	1 385			1 255		
3d						
% degrad.	**25.37**			**23.21**		

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