Use of Filamentous Fungi for the Purification of Industrial Effluents

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Report to the Water Research Commission
by the
Department of Chemical Engineering
University of Pretoria

WRC Report No 535/1/98

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USE OF FILAMENTOUS FUNGI FOR THE PURIFICATION OF INDUSTRIAL EFFLUENTS

WRC Report

submitted to the Water Research Commission

by

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EXECUTIVE SUMMARY

Dissolved organic matter in industrial process effluents is normally converted to solid biological material so that it can be removed more easily from the water. Unfortunately sludge is created that has to be disposed of. A much better option will be to recover the organic material or convert the soluble COD to some saleable product, which will lead to some return on the treatment costs. A micro-screen-based selection process developed at the University of Pretoria showed promise for treating such effluents, while at the same time recovering good quality, saleable biomass. Through this process it is possible to sustain a filamentous fungus as a mono-culture under non-aseptic conditions.

This is a report on an investigation conducted to determine the potential of using the micro-screen process to convert industrial effluent COD into biomass that can be used for secondary purposes. The report describes the development of the process on one specific effluent, but the process is equally suitable for a large range of effluents in many of the organic industries world-wide. The effluent under discussion in this report is a typical low acetic acid containing effluent, but it also contains inhibiting substances that made conventional biological treatment difficult.

During the initial stages of the test work, specific objectives for the project were identified which were the following:

i) An organism had to be selected from nature that could be sustained in the reactor on the effluent, under the selection mechanisms applied.

ii) Nutrient requirements had to be determined.

iii) Various modes for operation of the system had to be investigated to solve the problems of unexpected shock loads and high oxygen requirements of the system.

iv) A system configuration had to be developed to handle high strength effluents that might contain inhibiting substances.

v) Since the process relies on dynamic selection principles to sustain the filamentous culture, any possible bacterial contamination had to be quantified.

vi) Biomass can be harvested from the process. Commercial applications for the biomass had to be found.

vii) It is known that the spores of this organism might cause *aspergilloses* in people with immuno-deficiencies when inhaled in large quantities. The safety of the process environment had therefore to be evaluated.

Aspergillus fumigatus, a thermotolerant organism able to grow well at 45 °C, was selected on this specific effluent. This organism could be sustained in the open system without any sterilisation procedures.

The organism had the following growth characteristics:

$\mu_{max} = 0{,}34\ h^{-1}$

$K_s = 465\ mg.l^{-1}$

$Y_g = 0{,}45\ gbiomass.g\ COD^{-1}$

$b = 0{,}009\ h^{-1}$

The nutrient composition has been quantified and optimum concentrations determined. Total costs of nutrient addition per day will be ± R 4000 to produce ± R18 000 of protein product.

Two alternative operational modes have been proposed (pH-controlled feed and oxygen-controlled feed) whereby addition of substrate (Sezela-effluent) is regulated by the assimilation rate of the organisms themselves. This would result in optimum utilisation of expensive nutrients, for example oxygen.

The pH controlled feed mode has been studied extensively and the influencing factors of the artificially controlled substrate concentration on mathematical modelling has been identified. The oxygen controlled feed mode still needs to be evaluated in more detail.

The process (Provisionally called the MyPro Water Treatment System) can be applied to high strength organic effluents. The problems with oxygen transfer and toxic loads were overcome by the metabolism-controlled feed mechanism. For such high strength effluents dilution is required. This can be supplied by recycling a part of the reactor effluent. The volume of the recycle stream could be reduced by oxygen enrichment and a slightly longer hydraulic retention time. The basic process performance with full recycle and pH-controlled feed rate is summarised in the following table:

Basic performance parameters of the MyPro Water Treatment System on a low acetic acid containing effluent

Parameter	Parameter value
COD_{in} ($mg.l^{-1}$)	15 000
COD_{out}	500
Reactor hydraulic retention time[1] (h)	3
Total system retention time (h)	12.3
Biomass production rate (mg biomass.$(l.h)^{-1}$	340
Treatment efficiency (%)	97

The total system retention time is the overall hydraulic retention time in the reactor and depends on the COD type and load in a particular effluent.

Bacterial contamination above a certain degree influences the dewatering characteristics and product quality of the biomass. This was quantified under various operating conditions in order to determine which conditions are conducive to

[1] Retention time resulting from F being the feed stream plus the recycle stream in the formula: $\tau = V/F$.

excessive growth. At a pH of 5,0 and 46 °C the reactor could safely be operated with a hydraulic retention time of 3,5 hours. When the temperature was increased to 50 °C, the hydraulic retention time could be increased to 5 hours.

Two possibilities for commercial use of the biomass have been investigated, namely use of the dried biomass as protein source, and secondary batch fermentation of the harvested biomass to produce cellulase enzymes.

The dried biomass contains 45 % protein, with a relatively good amino acid distribution, low fat content and high fibre content. Acceptability feeding trials for rats, fish and broilers were very good with up to 20 % of the biomass in feed mixes. No toxic effects have been detected in preliminary feeding trials. More scientific tests still needs to be done, as well as screening for specific toxins. From the available results the market value of the feed will be in the order of R 600 to R 1000 per tonne.

Initial tests showed promising results regarding the production of cellulase from the biomass produced in the process. Additional tests confirmed and improved on these results. A maximum enzyme activity of 7,2 IU was obtained which can be regarded as very good under the specific experimental conditions.

Because concern exists regarding the safety of process workers due to possible contamination of the air with spores or viable fungal material, tests have been conducted to quantify the amount of viable fungal material in the environment around the laboratory reactor. No significant amounts of fungal material could be detected in the working area of the laboratory scale process.

All the objectives of the report have been met. Further detailed experimentation on some of the aspects is necessary, and should be conducted in future.

The development of the process on a relatively "difficult" industrial effluent up to pilot plant scale, shows that the MyPro Water Treatment System has good potential for application in the water treatment industry. A comparison with conventional treatment processes can be found at the end of the report. The most important advantages of the MyPro System are as follows:

- Process effluents emerging at elevated temperatures (as high as 50 °C) can now be treated directly.
- No residual remains that has to be disposed of. A part or all of the treatment costs can be recovered by using the harvested biomass as protein source in animal feed or for the production of secondary metabolites, for example cellulase.
- Biomass separation, handling and drying is simple due to the filamentous nature of the fungi.

For future research on matters discussed in this report, the following is recommended:

i) Feeding trials should be conducted in more detail, including the associated toxicity tests.

ii) The dissolved oxygen controlled feed mechanism should be studied further since it has potential for application in in conventional aerobic treatment processes, as well as in the treatment of non-acidic effluents.

iii) Tertiary treatment of the process effluent might be investigated further if necessary. This would be determined by the downstream requirements of the user.

iv) The organism used here, *Aspergillus fumigatus* is also a producer of various antimicrobial substances. It also produces an important enzyme inhibitor for lowering blood cholesterol and an immuno-modulating substance that might be important in organ transplants. If such a substance could be extracted economically from the harvested biomass, it would add tremendous value to the MyPro System. Further research into the production and extraction of secondary metabolites is therefore recommended.

CONTENTS

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The Steering Committee responsible for this project, consisted of the following persons:

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Mr C. Klusener	:	Illovo Sugar Ltd.

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LIST OF SYMBOLS

Symbol	Definition	Units
Θ_c	Biomass residence time	h
τ	Theoretical hydraulic retention time	h
μ	Specific growth rate	h^{-1}
μ_{max}	Maximum specific growth	h^{-1}
b	Specific biomass decay rate	h^{-1}
BOD	Biological oxygen demand	$g.l^{-1}$
COD	Chemical oxygen demand	$g.l^{-1}$
DO	Dissolved oxygen concentration	$mg.l^{-1}$
F	Hydraulic flow rate	$l.h^{-1}$
P	Biomass production rate ($D_c.X$)	$g.(l.h)^{-1}$
Ro	Rate of oxygen requirement	$mg.(l.h)^{-1}$
S	Outflow substrate concentration	$g.(l\ COD)^{-1}$
V	Volume of reactor	l
X	Biomass concentration	$g.l^{-1}$
D_c	Biomass dilution rate ($1/\Theta_c$)	h^{-1}
K_s	Saturation constant	$g.l^{-1}$
S_o	Inflow substrate concentration	$g\ COD.l^{-1}$
Y_g	True growth yield	$g\ biomass.(g\ COD)^{-1}$
Y_{obs}	Observed growth yield	$g\ biomass.(g\ COD)^{-1}$
MLSS	Mixed liquor suspended solids	$g.l^{-1}$
CSTR	Continued stirred tank reactor	
MYEA	Malt yeast extract agar	
ACE	Azeotrope column effluent	
MyPro	Myco Protein	
SCP	Single cell protein	
F:M	Food : micro – organism ratio	

INTRODUCTION

This report describes the development of a new process for the treatment of organic effluents. The process is especially suitable for industries that produce process streams with a biochemical oxygen demand.

It is a known fact that organic industries world-wide are experiencing increased pressure from both the public and their customers to eliminate their effluent streams. Efforts to put pressure on industry to treat their effluents to an acceptable standard are sometimes met with considerable resistance. The reason being that expenditure on treatment plants are often perceived as money invested without any return. It is therefore a very sensitive aspect for most factories. Especially factories which produce effluents that do not have significant detrimental effects on the environment need to look carefully at this situation.

The effluents from these factories usually contain wasted products in dilute form. In order to reduce the outgoing pollutant loads, management basically have two options: They either have to go the recovery route whereby the dilute products are recovered or go the destruction/removal route where the products are oxidised or removed in a different form.

Conventional effluent treatment can be regarded as both a capital and running expense without return. The only return will be the possibility of recovered water. Recovery of products on the other hand, usually requires a large degree of development work and involves a certain risk. Both these options might be complicated further by the presence of other unknown products or toxic substances.

The newly developed MyPro Water Treatment System (Van der Westhuizen & Pretorius, 1996) might be an answer to this problem. A mono-culture of filamentous fungi is used to purify the water while simultaneously producing a saleable product

in the form of fungal protein. This ensures an additional return on the invested money.

Through a built-in selective mechanism (Pretorius, 1987), and by making use of specific selection pressure, a mono-culture of one filamentous microbial species is sustained in the reactor. The harvested biomass from the process could then be converted to a valuable by-product(s).

The initial objective of this project was to establish to what extent the micro-screen principle is applicable to the treatment of effluents in the sugar industry. A suitable effluent was identified and a process developed from initial selection of a suitable organism, up to the final evaluation of the process on pilot scale. The process is now known as the MyPro Water Treatment System (SA Patent 96/8217).

Although this report mainly describes the development on one effluent, the principles remain the same for other applications. The report is intended as an informative document without too much technical detail, aiming at introducing this new technology to the relevant industries. Results and experience from the laboratory and pilot plant reactors are discussed together, with a separate part specifically on the operation of the pilot plant. The report is divided into two parts, Part A describing the process aspects and Part B describing commercial utilisation of the fungal biomass. The various sections in each part are presented in logical order so that the reader will have a good understanding of the process at the end.

A brief resumé of the micro-screen process is given at first. Following this, a short chapter is included that gives an overview of the sugar industry and the potential of using the micro-screen process to treat any of the effluent streams in this particular industry.

PART A: PROCESS CONSIDERATIONS

1. Resumé of the MyPro Water Treatment System

The micro-screen process can be described as a chemostat (continuous flow bioreactor) with a specific modification, namely the introduction of a micro-screen in front of the reactor outlet. This has the effect of retaining organisms larger than the screen openings (i.e filamentous fungi). For these organisms the reactor simulates the model of a chemostat with cell recycle, with the screen serving as separation mechanism (The same role that a settling tank plays in the activated sludge system). Organisms smaller than the openings (i.e. bacteria) will be washed out from the reactor. In this way it is possible to let large, slow growing organisms (i.e. fungi) dominate over small, fast growing organisms (i.e. bacteria) because smaller organisms are continually washed out through the screen. A diagram of this concept is presented in Figure 1.1(A):

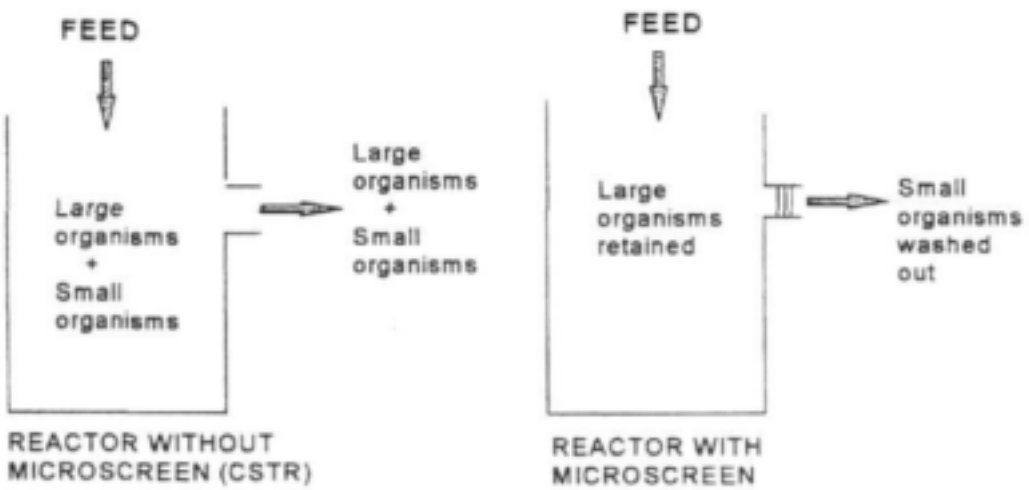

Figure 1.1(A) Illustration of the effect of a micro-screen on selection

By harvesting at a specific rate the filamentous organisms inside the reactor can be selected on basis of growth rate. The system can also be operated in different modes. These are the following:

- Mode I: By fixing the rate of substrate addition or incoming flow rate (F).
- Mode II: By linking the rate of substrate addition to the dynamic nature of the system.

For the purpose of the process resumé it is not necessary to go into further detail. The more technical aspects will be discussed in the appropriate chapters.

2. Applicability of the MyPro Water Treatment System in Industry (particularly the sugar industry)

For the micro-screen process to be used successfully to treat a certain effluent, such an effluent should:

- consist mainly of biodegradable organic substances that can include fatty acids, organic acids, sugars and other carbohydrates
- have a COD above 1000 $mg.l^{-1}$
- preferably be produced in volumes that yield at least more than two tons of COD per day
- preferably be produced as a constant stream with not too much change in its composition (although composition changes can be alleviated through equalisation)
- not be collected from diffuse sources which will influence the consistency of the effluent, i.e. drains, washings, etc. Pre-treatment might then be required.

Following is a summary of information gathered from literature sources and factory visits on the specific applicability of the MyPro Water Treatment System in the South African sugar industry. A short overview of the industry in South Africa and of the sugar manufacturing process is given in order to identify specific COD wastes that could possibly be useful for the micro-screen process.

Although only the sugar industry is covered here, this chapter can be used as a comparative example in order to evaluate the potential of the MyPro Water Treatment System for the purification of other organic effluents.

2.1 Types and location of sugar factories in south Africa

There are 16 sugar cane processing plants in South Africa and one stand-alone refinery. One of the 16 plants operates a furfural-from-bagasse plant in conjunction with their normal milling and refining operations (Purchase, 1995).

With the exception of 2 plants which are situated in Mpumalanga, all of them are located in Kwazulu-Natal. Sugar cane is produced from April to December, the rest of the year the plants are being maintained (an important factor to be considered when evaluating the process).

Five of the processing plants are mills and refineries combined which, mainly for energy efficiency reasons, is the modern trend in the industry. Between twenty and twenty two million tons of cane are crushed each year, resulting in annual sugar production of approximately two million tons (Purchase, 1995).

2.2 Process resumé

2.2.1 Milling

The incoming cane is chopped and shredded by revolving shredders. The shredded cane is then crushed between rollers and counter-currently contacted with water to ensure maximum possible juice extraction. Most of the factories are now using the technique of percolating the water through a bed of moving shredded cane (Nunn, 1993). This water is called imbibition water. The bagasse is usually

used as boiler fuel but can also be used as raw material in the manufacture of other materials i.e. furfural (WRC, 1993c).

Lime is subsequently added to the juice to give a pH of about 8,5 and the solution is then heated to boiling point. This results in the precipitation of calcium phosphates, denatured organic complexes and insoluble matter like soil and fibre which are settled out in a clarifier. All associated juice is recovered from these solids by vacuum filtration.

The clarified juice is then concentrated to thick syrup in a multi-effect evaporator. A single-effect evaporator or vacuum pan is used to convert the syrup to a crystalline sucrose and a mother liquor. The crystals are recovered by centrifugation from the mother liquor. After a series of boiling operations the mother liquor is exhausted of sucrose and is known as final molasses. The recovered crystals are dried in drum driers before transport to the refinery (WRC, 1993c).

2.2.2 Refining

During refining the quality of the sugar is improved and all impurities are removed. This is done by re-dissolving the raw crystals in water and after screening, the melt liquor undergoes clarification through the addition of lime and phosphoric acid and subsequent flotation of impurities. The resulting sugar concentrate is crystallised in vacuum pans and sugar crystals are recovered by centrifugation and dried (Lewis, 1993; Nunn, 1993; WRC, 1993c).

2.3 Wastewater discharge

2.3.1 Effluent production

According to Purchase (1995) approximately 23 % by mass of total cane crushed result in effluent. This means that the sugar industry produces 5 million tons of effluent per year with a COD load of 1200 to 1500 mg.l^{-1}. Therefore the total amount of COD produced is in the order of 6000 to 7500 tons. If this COD could be converted to biomass (assuming a yield of 0,6 g.g COD^{-1} and a market price for SCP of R 1000 per tonne), a maximum of 3600 tons SCP can be produced per year with a potential market value of ± R 3,6 million per year.

These figures are however a gross overestimate of actual utilisable COD for the micro-screen process. In the first place all the available COD can not be used one hundred percent in such a bio-transformation process. Secondly, a large fraction of the COD in the wastewater originates from spills and washing of work areas and is therefore contaminated with soil, fibre, etc. which make it unsuitable to use readily in the process.

2.3.2 Wastewater origins

Visits to four sugar factories showed that effluent originates from mainly three areas (Lewis, 1993; Nunn, 1993), i.e.

- mills and cane yard
- factory drains
- smuts plant and cooling tower

During normal milling operations, wastewater only originates from washing of floors and equipment. Primary pollutants therefore consist mainly of sugars, spilled fibre and soil. Spillage from the production line is recycled as far as possible.

Wastewater flowing to the treatment units via factory drains usually consists of lost condensate, backwash and rinse water, and general washing water. Although it contains a certain amount of COD (± 600 - 800 $mg.l^{-1}$) it is also very much contaminated with fibres and soil.

Cooling water is re-circulated which inevitably leads to an accumulation of micro-organisms and organic pollutants, and blowdown of cooling water is required. This water is largely contaminated with entrained sugar from vacuum pans and evaporators and can have COD levels of 500 to 1000 $mg.l^{-1}$. As such it is the most useable wastewater fraction in the plant if the aim is treatment with the micro-screen process. The volumes of cooling water blowdown from the whole sugar industry relate to a maximum total of 4000 to 5000 tons of utilisable COD per year (for the micro-screen process).

On average each factory produces less than 500 tons of COD. According to these figures it does not seem that the normal sugar factories in South Africa produce enough utilisable COD in their effluents for the economical production of SCP with the micro-screen process.

As mentioned previously, one of the factories in Natal produces furfural from bagasse in conjunction with the normal sugar mill activities. The potential and composition of this factory's effluent are discussed next.

2.4 The Azeotrope column effluent (ACE)

2.4.1 Potential for COD conversion

Investigation showed that the furfural plant produces a steady stream of effluent with a COD of ± 18 000 $mg.l^{-1}$ at a rate of 3,1 Ml per day (Klusener, 1995). This relates to COD production of 10 000 to 12 000 tons or SCP of 5 000 to 6 000 tons per season with a potential market value of ± R 5 to 6 million per year.

2.4.2 Characteristics

The effluent is the remaining liquid from a process where furfural, furfuryl alcohol and diacetyl is extracted from bagasse obtained from the adjacent sugar mill. The factory's water demand is ± 36 000 m^3 per week of which 19 000 m^3 per week of process effluent is presently discharged via a surf zone pipeline. Although the environmental impact has been determined to be negligible (D'Aubrey-Whitehorn *et al*, 1992), negative public perception towards marine outfalls in general, is leading to increased pressure to cease the marine disposal of effluent. For the purpose of this report no detailed information will be given about the process itself that generates the effluent. The important characteristics of the effluent are summarised in Table 2.1(A):

Table 2.1(A) Characteristics of Azeotrope column effluent (ACE)

Property/constituent	Concentration ($mg.l^{-1}$ if units are not given)
Temperature	45 °C
PH	2,7
Total solids (dissolved and suspended)	800
Suspended solids	50
COD (filtered)	10 000 - 19 000
Acetic acid	9 000 - 18 000
Formic acid	900
Furfural	50 - 300
SOG (wax)	100 - 300

An ideal effluent for the micro-screen process would have all the characteristics described earlier, plus enough nutrients in the form of phosphorous, nitrogen and potassium and no substances that might be toxic to the micro-organisms.

As can be seen from Table 2.1(A), the effluent in its whole is suitable for treatment with the micro-screen process, except for the need for nutrients, possible toxic concentrations of furfural and high and variable COD. By using dilution or recycled water in some form the latter problem can be solved. The relative high temperature would not pose a problem if a thermotolerant organism could be selected.

2.5 Conclusions from the investigation

After taking into consideration all the information gathered it was concluded that it would not make sense at this stage trying to recover or convert the COD in the normal effluents of the sugar industry into biomass by using the micro-screen process. The most important reasons for this being that sugar mills do not produce a

process effluent as such but that the effluent is an assimilative stream of accidental spillage and wash water from different areas of the plant. Thus, a degree of pre-treatment would definitely be required.

It was subsequently decided that the ACE from the furfural plant at Sezela on the Kwazulu-Natal south coast would be ideally suited for this project. Although it was only one factory from the whole of the sugar industry, the following aspects enhanced the case for research on this effluent:

- The yearly production of COD from the Sezela plant (± 12 000 tons per annum) is more than double that achievable from the rest of the sugar industry (± 5 000 tons per annum) combined.
- The composition of the effluent was very similar to that of SASOL, an effluent on which considerable research has already been done.
- At present the particular factory is increasingly pressured to find alternative methods to sea disposal.
- The furfural plant produces a substantial amount of excess oxygen, which could improve the performance of the micro-screen process.
- The technological experience gained in the development of the MyPro System with this effluent could easily be extended to other industries with high BOD loads in their effluents. In this way an important and useful contribution could be made towards the development of a practical, reliable technology for such factories world-wide. The rest of the report describes in logical order the development of the system, tailored to the requirements of a specific site.

3. Selection of a new organism

3.1 Introduction

When an effluent is identified as suitable for treatment with the MyPro System, the first objective is to select a suitable organism that can be sustained on that particular effluent. Such a selection procedure takes more or less 4 weeks if it is necessary to select an organism from scratch. This is the recommended method because it is then certain that the selected organism can be sustained indefinitely as long as the conditions in the system stay constant.

The selective mechanism of the MyPro System is of such a nature that after initial inoculation, the filamentous organism which is best suited by the specific set of conditions that is created in the reactor, will dominate. These conditions can be one or combinations of the following:

- nature of substrate
- pH in reactor
- temperature
- nutrients and
- presence of toxic substances in the substrate.

The selected organism will dominate indefinitely, unless the operating conditions are changed or if some adverse condition causes its wash-out. The reactor will then have to be re-inoculated.

When choosing the controllable conditions in the experimental reactor, it is important to consider the set-up at the factory and what the properties of the effluent are.

The temperature at which the effluent emerges is of particular importance. Should an organism in the mesophyllic temperature range be selected, it would mean that the biological reactors would have to be cooled down in most cases. This would obviously have economic implications. The major aim was therefore to select an organism that could grow well at elevated temperatures, preferably as high as 45 °C (the ACE emerges at elevated temperature).

3.2 Methodology and selection

To select an organism, a batch reactor containing the effluent with some nutrients was used. Conditions in the reactor were kept as close to the envisaged effluent conditions as possible. A diverse inoculum of soil, compost, sewage, etc. was used.

After an acclimatisation period the reactor was changed to a fed-batch configuration. After another 5 days, a continuous flow-through of substrate was allowed, with a micro-screen in the outlet. The dilution rate was slowly increased in order to wash out non-filamentous organisms. After the tenth day harvesting slowly commenced at an increasing rate until one filamentous species remained. This was achieved after approximately 18 days. The organism that was selected on the ACE was identified as *Aspergillus fumigatus*, a well-known compost and soil fungus.

4. Optimisation and quantification of nutrient addition

4.1 Introduction

In some effluents, for example municipal biological treatment plants, no additional nutrients are required under normal conditions, since the effluent contains enough nutrients (i.e. phosphorous, nitrogen and trace elements). However, when industrial effluents (such as the Sezela-effluent) are treated, it is often required that the effluents be pre-treated (pH-neutralised or nutrients added) for biological treatment. The chemical composition of the Sezela-effluent is given in Table 2.1(A).

As can be seen from this table, the effluent does not contain any phosphorous, nitrogen or any of the required magnesium or potassium salts. The provision of sufficient nutrients is essential if efficient biological treatment is to be achieved because without it the organisms will not be able to perform their synthesis reactions. If excessive amounts are added it would have economic implications. It is therefore important to determine the exact amounts of nutrients. The theoretical and experimental methods that were used to determine the optimum nutrient amounts are described next.

4.2 Methodology

The amounts of nutrients (phosphorous and nitrogen) that had to be added were theoretically calculated by making use of a stoichiometric equation for microbial growth for this specific case with oxygen as electron donor and carbon in the form of acetate as electron acceptor (McCartney, 1975). By using this as a basis, the performance of the biological reactor was evaluated under 3 conditions, namely:

- with theoretical (calculated) amounts,
- with nutrients slightly in excess of the calculated amounts,

- with slightly less nutrients than calculated.

This was accomplished by measuring the nutrient concentration in the influent and effluent streams from the reactor.

As magnesium and potassium are not included in stoichiometric reactions for growth, the required amounts of these nutrients were determined by using literature sources as basis and evaluating performance as described in the former paragraph.

4.3 Results and discussion

In Table 4.1(A) the stoichiometric calculated nutrient amounts are shown. The amounts have been determined from the balanced stoichiometric equation for aerobic growth of the organism with acetate as electron donor:

$$CH_3COO^- + 0{,}792\ O_2 + 0{,}242\ NH_4^+ \longrightarrow 0{,}0946\ HCO_3^- + 0{,}7584\ H_2O + 0{,}0336\ CO_2 + 0{,}242\ C_5H_7O_2N$$

where $C_5H_7O_2N$ represents the estimated chemical formula for the fungal biomass.

The phosphate requirement has been taken as 20 % of the nitrogen requirement. The amounts for magnesium and potassium were obtained from a study by the Sugar Milling Research Institute (SMRI) and were not calculated.

Table 4.1(A) Theoretical nutrient requirements

Constituent	Requirement (mg nutrient per g of acetate COD used)	Commercial source used	Amount of commercial source (mg/g COD used)
Nitrogen (as N)	53.12	25% NH_3 as NH_4OH	212.5
Phosphorous (as P)	10.62	80% H_3PO_4	41.98 mg
Magnesium (as Mg)	1.1	$MgSO_4.7H_2O$	11.15 mg
Potassium (as K)	11.3	KH_2PO_4	39.4 mg

Table 4.2(A) on the next page shows the nutrient amounts required per gram of biomass formed as evaluated in the micro-screen process.

Table 4.2(A) Experimentally determined nutrient requirements

Constituent	Requirement (mg per g of actual ACE COD used)	Commercial source used	Amount of commercial source (mg/g COD used)
Nitrogen (as N)	25.7	25% NH_3 as NH_4OH	102.8
Phosphorous (as P)	3.92	80% H_3PO_4	17.2
Magnesium (asMg)	0.10	$MgSO_4.7H_2O$	1.01
Potassium (as K)	0.50	K_2HPO_4	1.12

The amounts given in Tables 4.1(A) and 4.2(A) are nutrients required per mass of utilised COD. The values have been determined for yields between 0,45 and 0,5. The amounts may change slightly with variations in the yield. It is interesting to note that the theoretical requirements were higher than the actual measured requirements. This is mainly due to the stoichiometric formula used for the biomass, $C_5H_7O_2N$, which has been calculated for bacteria and not fungi, and the fact that the actual ACE is composed not only of acetic acid, as is assumed in the calculations.

Much more salts like Mg and K are usually added than are needed by the organism. This is obvious from Table 4.1(A) and 4.2(A) when the amounts of Mg and K are compared. In fact, during further work it was established that KH_2PO_4 and $MgSO_4$ only needed to be added in minute amounts.

Although it is not shown in Table 4.1(A) or 4.2(A), no different levels of uptake of nutrients were observed when nutrients were supplied in excess (i.e. no prominent changes in the formula $\{S_{o(nutrient)} - S_{(nutrient)}\}/\{X\}$. The first time that an effect of nutrient concentration on biological growth was observed, was when no positive traces of nutrients could be measured in the reactor effluent. This means that for the specific system and organism used, no excess nutrients are needed. The micro-screen process can therefore be operated without adding any pollutants to the water in the form of nutrients or their related salts. The information from the tables may also be used to calculate the costs of nutrients for treating the whole stream of the particular factory's effluent:

Assumptions:
- *Observed yield* = 0,38
- $Q_{(Effluent)}$ = 120 m^3 per hour
- COD = 14 000 $mg.l^{-1}$.

From these assumptions it may be calculated that 1,7 tons of COD are produced per hour. With an observed yield of 0,38 $g.(g.h)^{-1}$, 646 kg of dried biomass can be produced per hour or 15.5 tons per day. Preliminary costs of nutrients per day are shown in Table 4.3(A).

Table 4.3(A) Daily Nutrient costs for full scale plant (40 800 kg COD per day)

Nutrient	Amounts required per day	Costs per day
25% NH_3 as NH_4OH	3730 kg	@ R 0,70/kg = R 2 611
80% H_3PO_4	624 kg	@ R 1,80/kg = R 1 123
$MgSO_4.7H_2O$	41 kg	@ R 1,02/kg = R 41
KH_2PO_4	45 kg	@ R 5,00/kg = R 225
Total		**R 4 000**

The costs as given in Table 4.3(A) are for industrial grade chemicals as in July 1997, includes transport to site and excludes VAT.

The costs given are only an indication of nutrient costs and may vary if other nutrient sources are used. For example, if urea is used, the nitrogen cost will reduce by more than 50%. It has been determined that excess nutrients do not have any effect on growth as such. However, it is not known if the addition of excess nitrogen might improve the protein content of the biomass. Further research might be necessary in future. The role of micro-nutrients has not been studied either. Again, although it might be worthwhile, it was not considered as a priority research item.

5. Operational modes

5.1 Introduction

This section describes the modes in which the fungus can be grown in a continuous treatment process.

The micro-screen process may be operated in two modes, namely with constant flow rate (Mode I) or with the flow rate linked to some growth parameter (Mode II). The problem with having a fixed rate of substrate addition is that substrate can either be added too slow or too fast. When substrate is supplied too fast, it will have the effect of depleting the available oxygen in the reactor. Oxygen supply could be increased, but a point is reached where it is physically impossible to bring enough oxygen into contact with the organisms (because of the high viscosity of the suspension) and the rate of oxygen transfer thus becomes the limiting factor.

The subsequent oxygen shortage results in poor substrate utilisation since more substrate is supplied than the available oxygen needed to bio-oxidise it. In the case of the Sezela-effluent the substrate is mainly acetic acid and an over-supply of substrate will result in a drop in pH up to a point where the pH is too low for the organisms to grow well (pH < 3). The effect is break-up of the hyphae and subsequent loss through the screen, resulting in complete wash-out of biomass. The only way that the system can be operated with over-supply of substrate is when a part of the feed is neutralised. This would however create additional problems in that large amounts of alkali is needed (Van der Westhuizen, 1993) and substrate is not completely oxidised as explained.

On the other hand, oxygen can be supplied in excess (as is normally done in aerobic biological treatment plants). In other words, substrate is actually supplied at a lower rate that is required for optimum operation. The substrate utilisation will be

good, but a lot of oxygen will be wasted in the process. Oxygen is one of the most expensive nutrients that have to be supplied to the system and it will therefore be advantageous if the oxygen that is actually available for the organisms, could be utilised optimally. This may be achieved by linking the rate of feed supply to the assimilation rate of the organisms.

5.2 Alternative feeding modes

5.2.1 pH-controlled feed

The feeding rate to the reactor may be linked to the growth or assimilation rate of the organisms in two ways. The first is to make use of the effect of bio-oxidation of the acetic acid containing substrate. When this substrate is bio-oxidised, the nett effect is an increase in pH, mainly through the formation of HCO_3^- from CH_3COOH. A pH-controller unit linked to the feed pump is used in this case for the addition of new feed. When the pH rises to a selected set-point, the pump is switched on and feed is added until the pH drops below the set-point.

Due to the high COD (in the ACE) dilution of the feed was still required to keep the hydraulic retention time within limits. Recycling was used in this case to eliminate dilution water. If the system is operated under pH control and with a constant aeration rate, the readily biodegradable COD in the effluent (and in the reactor) is only governed by the degree of neutralisation. A larger degree of neutralisation of the incoming feed will therefore result in a higher COD in the effluent from the reactor.

5.2.2 Dissolved oxygen-controlled feed

Another way to link the growth rate of organism to the feeding rate, is through control of the dissolved oxygen concentration (DO) in the reactor. A dissolved oxygen meter is connected to the feed pump. If substrate is added, the available oxygen in the reactor is used up because oxygen is used as the primary electron acceptor in a stoichiometric relationship to the carbon source. The DO will drop to a certain pre-determined level and the pump will switch off. The DO level will rise again and the pump accordingly be switched on.

The advantage of this mode is that the system can be operated at the optimum oxygen concentration for the specific organism. Neither oxygen nor the carbon source will then be growth limiting and basically any other nutrient can be chosen as the limiting substrate.

In the subsequent paragraphs the performance of the pH controlled feed rate is discussed. Note that these experiments were done in 20 litre laboratory scale reactors and should only be read in comparison with each other. Due to scale-up factors, the figures should not be used for full-scale projections. Such data is provided later.

5.3 Performance of pH controlled feed addition

The laboratory reactor has been operated extensively at various combinations of pH, temperature and biomass retention times. In Table 5.1(A) and 5.2(A) it is shown how the process typically reacts to various temperatures and pH values. The experiments were conducted at temperatures of 44, 47 and 49 °C and pH values of 5,0; 5,5 and 6,0. Table 6.3 shows the effect of various biomass retention times. Values shown are average values determined from at least four sets of experiments.

Table 5.1(A) Effect of pH on process performance.

Temperature = 49 °C; Θ_c = 9,5 h.

Ph	X $g.l^{-1}$	S_o $mg.l^{-1}$	S $mg.l^{-1}$	Y_{Obs} $g.(gCOD)^{-1}$	P $g.(l.h)^{-1}$
5,0	3,3	3150	518	0,44	0,35
5,5	3,0	3124	484	0,39	0,33
6,1	2,9	3330	415	0,38	0,32

Table 5.2(A) Effect of temperature on process performance. pH = 5,5; Θ_c = 11,5 h.

Temp (°C)	X $g.l^{-1}$	τ (h)	Y_{Obs} $g.(gCOD)^{-1}$	P $g.(l.h)^{-1}$
44	3,8	3,27	0,39	0,31
47	4,1	3,32	0,52	0,33
49	4,1	3,35	0,50	0,35

Table 5.3(A) Effect of various biomass retention times on process performance.

Temperature = 49 °C, pH = 5,0

Θ_c	X	S_o	S	τ	Y_{Obs}	P
12	4,7	3074	652	3,35	0,50	0,37
9	3,7	3150	586	3,5	0,52	0,37
7	2,6	3270	545	3,8	0,53	0,35

From Tables 5.1(A) and 5.2(A) it can be seen that the optimum pH and temperature is 5,0 and 47 °C respectively. The COD values in Table 5.1(A) to 5.3(A) are not the lowest values attainable with the process. These values can be controlled to a degree and depends on the buffering capacity of the system, microbial products and non-biodegradable fractions.

The buffering capacity is caused by the action of four buffering systems, namely the carbonate, water (H^+, OH^-) and (if excess nutrients are present) phosphate and ammonia systems. The buffering capacity of each of these systems may be calculated through a 5-point titration method if the initial concentrations of PO_4 and NH_3 are known (Moosbrugger *et al*, 1992).

If the buffer capacity is high, more acidity will be required to maintain the pH of the reaction at 5,0 (the optimum pH for the system). In this case, the acidity is caused by the acetic acid in the effluent. The acetic acid has an oxygen demand. Therefore, the higher the buffer capacity, the higher the emerging COD (S). The average fraction of the remaining COD that can be attributed to this effect, is 150 mg.l.

The non-biodegradable fraction was determined by running a batch biodegradability study with an initial COD of 3500 $mg.l^{-1}$. An adapted *Aspergillus* culture was used as inoculum. After 150 hours the minimum COD was 205 $mg.l^{-1}$ which is about 7% of the initial COD. When glucose was used as substrate with the same inoculum, the minimum COD was 75 $mg.l^{-1}$, which is caused by microbial product formation (assuming that glucose is fully biodegradable). Therefore, the minimum COD without any buffering effects will be 205 $mg.l^{-1}$ of which 130 $mg.l^{-1}$ is attributable to the non-biodegradable fraction and 75 $mg.l^{-1}$ to microbial product formation.

Together with the COD caused by buffering, it can be seen that the minimum COD without any buffering effects will be in the region of 355 $mg.l^{-1}$. When the experiments were conducted that yielded the data in Table 5.1(A) to 5.3(A),

ammonia was added in large amounts, which caused a high degree of buffering, even if the buffering effect of ammonia is low. Values from these experiments are only from one effluent sample and will vary because of variations in the constituents of the azeotrope column effluent (ACE). It is however still a good indication of how the pH- controlled feed mode influences the final COD.

Since the amount of ammonia added to the process was decreased to give levels of < 5 $mg.l^{-1}$ as N in the reactor, an outflow substrate concentration (S) of below 400 mg.l could be sustained with ease. Table 5.4(A) shows typical results that were achieved with the laboratory reactor.

Table 5.4(A) Typical process performance

Θ_c (h)	X ($g.l^{-1}$)	S_o ($mg.l^{-1}$)	S ($mg.l^{-1}$)	P $g.(l.h)^{-1}$
9.4	3,14	3430	390	0,32
8.2	2,4	3310	426	0,33
7.6	2,1	3580	373	0,35
7.2	1,8	3580	368	0,35

From Table 5.4(A) it may be seen that the COD in the reactor can constantly be kept lower than 400 $mg.l^{-1}$. Again, this is only true if a certain amount of acidity is present in the effluent. If the acidity is low, it means that more residual effluent (i.e. COD) must be present to keep the pH value in the reactor constantly at 5,0.

6. Performance on high strength (high COD) effluents

6.1 Introduction

Without adaptations to the process configuration, the MyPro Water Treatment System can not handle high strength organic effluents. The reason for this being the same as that for conventional aerobic water treatment processes: A certain food : micro-organism (F:M) ratio is necessary for process operation, which is an inherent property of a particular organism-substrate combination. In other words, when the inflow substrate concentration increases, the concentration of biomass will also increase, to keep the F:M ratio constant. This increase in X can carry on as long as the nutrient requirements of the organisms (C, N, P and O_2) are satisfied. If the biomass concentration in the reactor keeps on increasing, a situation will be reached where the viscosity becomes so high that physical oxygen transfer is impaired. The oxygen requirements of the organisms can not be satisfied anymore and oxygen becomes the growth limiting substrate. The process will now either let through unutilised substrate, or the organisms will be poisoned if high substrate concentrations are toxic to the organisms.

Following is a discussion of the various approaches that have been investigated in order to solve this problem.

6.2 Using dilution water

The most obvious answer is to simply use an external source to dilute the incoming feed. The quality of this water does not need to be of potable standard. The main requirement is good bacterial quality because inoculation of the MyPro reactor with bacteria should be limited as far as possible. Other pollutants will either be converted by the micro-organisms or be unaffected. Of course the water should not contain any substances that inhibit the growth of the filamentous organisms.

In most cases, however, it does not make good sense to use water of a higher quality to dilute highly organically polluted water before treatment, especially in countries where water is scarce or expensive. A possible exception is when the process products are of such high value that the treatment costs can be justified.

6.3 Recycling of the MyPro Effluent

Instead of using an external source for dilution, a part of the water emerging from the MyPro reactor can be recycled and used as dilution water. The main requirements of such a recycle stream are again good bacterial quality and absence of substances that might affect the filamentous organisms adversely.

6.3.1 Bacterial quality

This water will usually have to be pasteurised in order to keep bacterial re-inoculation to the reactor as low as possible. When pasteurisation requirements are determined, the ultimate goal is to have no viable bacteria in the dilution stream. There are two reasons for it: Firstly the presence of bacteria tends to affect the filterability and de-watering characteristics of the biomass, resulting in more difficult and expensive downstream processing. Secondly, bacterial contamination influences the quality of the dried biomass by making it difficult to keep within specifications.

Tests on the processed ACE have shown that complete sterilisation is not necessary. The degree of pasteurisation depends on the prevailing selectivity in the system, i.e. temperature, pH, etc. The more effective these selective mechanisms are in suppressing the multiplication of bacteria, the less stringent the pasteurisation requirements.

Pasteurisation methods may include any sterilisation method that does not leave a residual. When such oxidising residuals are left, it will be recycled back to the reactor, where it might have a negative effect on the filamentous organisms. For the ACE the most effective method was ozonation. With effective ozone dosages of 3,5 to 4 mg/l, 99 % sterilisation could be achieved.

Heat pasteurisation was also evaluated, but it was found that heat changed the structure of other chemicals present in the ACE, causing adverse effects on the process. Although not evaluated in this study, other sterilisation or oxidation methods that could be used include ultra-violet light or catalysed H_2O_2 oxidation.

6.3.2 *Additional treatment*

In addition to removing viable bacteria from the recycling stream, further treatment might also be necessary in some cases. This would be due to the accumulation of substances (because of recycling) present in the industrial effluent stream which are either detrimental to the growth of the filamentous organisms themselves or that are undesirable for other downstream requirements.

Further treatment was necessary for the ACE because of the presence of inhibitory chemical substances. One of these substances was identified as furfural polymers that formed upon aeration of the furfural containing effluent. These polymers gave a brown colour to the water and increased in colour intensity with pH. In addition to this, it was also toxic for the organisms.

Experiments to determine the maximum ratio untreated (only pasteurised) reactor effluent that can be recycled (i.e. the maximum fresh water that can be replaced by reactor recycle), showed that only 30 % of the total dilution water stream can be replaced by reactor effluent that is only pasteurised. Beyond this ratio, the concentration of the inhibiting substance(s) was too high for the filamentous organisms to tolerate.

Laboratory tests showed that the larger portion of the polymers could be removed with $FeCl_3$ at a dosage of 50 - 60 $mg.l^{-1}$. The solids were then removed with dissolved air flotation (DAF). Figure 6.1(A) shows a flow diagram of the proposed full-scale plant.

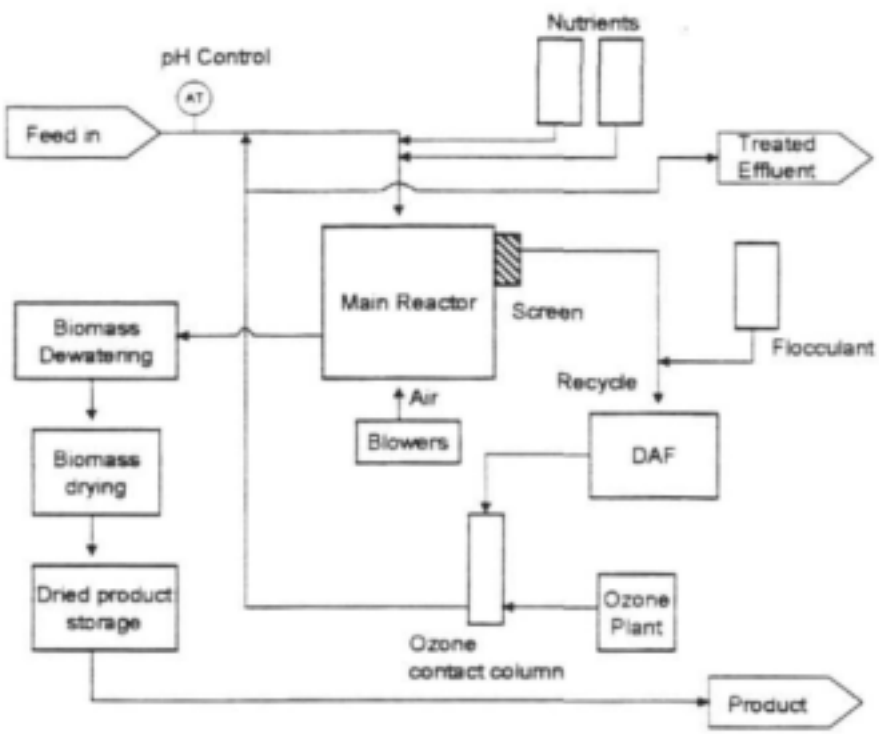

Figure 6.1(A) Flow diagram of the azeotrope column effluent (ACE) treatment plant

On this particular effluent (ACE) it was therefore possible to use recycling as a means for dilution, thereby effectively eliminating the need for external water. An additional cost factor, which should not always be necessary, is the inclusion of a solids removal step to stop the polymers from accumulating in the recycle to inhibiting levels. However, due to the nature and high concentration of the organic constituents, a relatively high dilution ratio of approximately 20 % raw effluent : 80 % dilution/recycling was necessary. This meant that the pasteurisation and solids removal units would have to handle 5 times the incoming flow, which could be a considerable cost. It should be mentioned however that this was achieved when the reactor was aerated with approximately 1 volume per volume per minute (v/v/min) of air from a membrane diffuser.

Due to the high viscosity the oxygen requirements of the organisms could not be satisfied and the reactor was operated at a zero dissolved oxygen (DO) concentration. The rate limiting nutrient was now oxygen and not carbon anymore. All efforts to reduce the recycle ratio should therefore be concentrated on improving the oxygen transfer.

Experiments on the laboratory reactor showed that when the oxygen requirement of the system is satisfied, i.e. the reactor operated at a positive DO, the raw effluent : recycle water ratio could be increased from 18 - 20 % to 25 - 30 %.

6.4 Enrichment with oxygen

Another way to reduce the volume of the recycle stream is to enrich the aeration air with oxygen. Due to the higher concentration gradient of oxygen in the bubbles diffusing through the medium, more oxygen will be physically transferred for utilisation by the organism. The maximum biomass concentration at which oxygen transfer becomes impaired, is increased. This will have the effect of increasing the reaction rate leading to a reduced retention time (τ). Alternatively, by fixing τ, a

higher substrate concentration can be introduced, resulting in a lower dilution water requirement.

Before deciding on oxygen enrichment, the operating cost of pure oxygenshould be weighed against the higher capital cost for the larger recycle stream. Table 6.1(A) shows the effect of increasing the oxygen concentration by only 10 % from 20% in normal air to 30 %.

The following assumptions were made: μ_{max} = 0,34 h^{-1}; K_s = 469 $mg.l^{-1}$; b = 0,009 h^{-1}; Y = 0,45 $g.g^{-1}$; ß = 1,2; Raw effluent COD = 15 $g.l^{-1}$

Table 6.1(A) Effect of oxygen enrichment on the recycle volume

Parameter	20 % O_2 (Normal air)	30 % O_2 (10% enriched)
Biomass age (h)	10.5	10.5
Hydraulic retention time (h)	3.0	3.0
Growth rate (h^{-1})	0.34	0.34
Substrate out ($mg.l^{-1}$)	206	206
Biomass concentration ($g.l^{-1}$)	5.09	7.4
Production rate $(g.(l.h)^{-1})$	0.485	0.630
ro $(mg\ O_2.(l.h)^{-1})$	501	693
Inflow substrate ($mg.l^{-1}$)	3456	4700
Recycle stream (fraction of total flow (recycle + feed) into reactor)	76 %	67%

Although the addition of more oxygen only makes a difference of approximately 9 % on the volume of the recycle stream, this will have an appreciable effect on capital

cost when the recycle stream has to be pre-treated before going back to the reactor again. Calculations have only been done for 10 % oxygen enrichment due to excessive costs of oxygen at the site.

7. Bacterial contamination

7.1 Introduction

Probably the most unique feature of the MyPro Water Treatment System is that a mono-culture of one filamentous organism could be sustained in an open system without any need for sterilisation. By stating this, it should be realised that although no bacteria are visible under the microscope if a reactor sample is studied, there will always be some bacteria present inside the reactor. The number of bacteria will depend on the size of bacterial inoculum, i.e. viable cells that come in with the feed or from the atmosphere, their growth rate under a specific set of pH and temperature conditions, and the wash-out rate, i.e. the hydraulic retention time in the reactor.

There are mainly four reasons why it is necessary to quantify any bacterial species that might be present in the reactor at various times. They are the following:

- If the biomass is used as animal feed, it is a prerequisite that all micro-organisms in such a process be quantified and identified.

- It should be established whether the occurring bacteria are not pathogenic organisms that might be harmful to humans.

- It has been observed in the laboratory that excessive bacterial growth in the reactor leads to operating problems, especially blocking of the screen. Therefore, it should be determined what conditions are conducive to excessive growth.

- Bacterial growth in the micro-screen process is reduced or eliminated by a relatively short hydraulic retention time. How long or short this time is, depends on the effluent, the pH and

the temperature. If the bacterial contamination could be quantified, the critical hydraulic retention time can be established.

In this chapter the effect of various combinations of pH and temperatures on bacterial growth is described. Individual bacterial species were isolated and identified.

7.2 Methodology

The reactor was operated at a temperature of 46 °C in the pH controlled mode at pH values of 5,0; 5,5 and 6,2 respectively. Three days were allowed between each change so that the microbial population could stabilise. At pH 5,0 the reactor was also operated at a temperature of 50 °C to see if this would have any effect on bacterial counts.

Samples from the reactor were filtered and diluted. The dilutions were streaked out in triplicate on plate count agar. The plates were incubated at 44 °C for 48 hours after which colony forming units (CFU) were counted.

Pure cultures were prepared from the colonies on the 10^{-4} dilution plate. The pure cultures were identified by the Department of Microbiology at the University of Pretoria with the API identification system.

7.3 Results and discussion

Figure 7.1(A) shows the bacterial growth at various combinations of pH, temperature and hydraulic retention times.

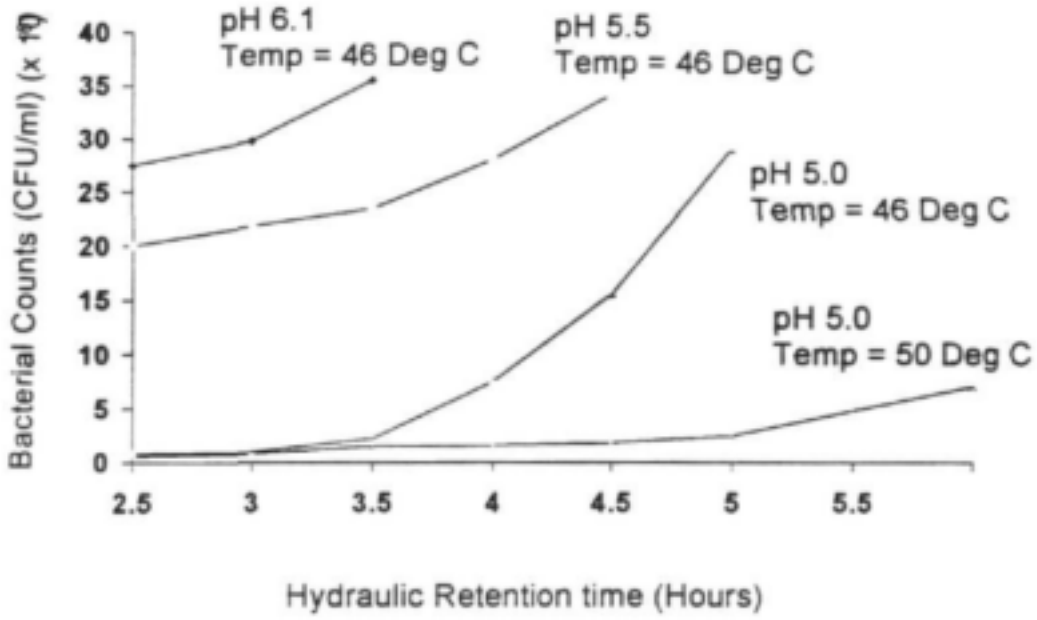

Figure 7.1(A) Effect of pH, temperature and hydraulic retention time on bacterial contamination in the MyPro laboratory reactor.

From Figure 7.1(A) it may be seen that by far the most bacterial growth occurs at pH 6.2, which is not unexpected. Even at a hydraulic retention time of lower than 3 hours bacterial counts are relatively high.

With pH 5.0 a dramatic decrease in colony forming units can be seen. If the temperature is increased to 50 °C, the reactor can be operated with a hydraulic retention time of up to 5 hours, without excessive bacterial contamination.

When the bacteria were isolated, it became clear that from the 10^{-4} dilution upwards, there were only two species of bacteria that completely dominated. They were identified as *Pseudomonas stutzeri* and *Bacillus macerans*. *P. stutzeri* was the dominating species by approximately 80%. This organism formed small, cream

coloured colonies. *B. macerans* is a spore forming organism and colonies are large and yellow.

The complete dominance of only two organisms can be expected when the conditions that they have to prevail in are taken into consideration, namely high dilution rate, low pH and high temperature. During the six months that plate counts were done, no abnormal growth or dominance by other bacteria occurred.

When these bacteria did multiply to unacceptable numbers in the reactor it was mainly due to altered process conditions caused by equipment failure. In the laboratory reactor it was fairly easy to get rid of the bacteria by decreasing the pH or increasing the temperature slightly. After severe contamination the reactor could be fully operational again within 24 hours. When such contamination occurred, the treatment efficiency of the process was not influenced, but the water emerging from the reactor contained bacterial cells, visible as turbidity. If bacterial quality of the purified effluent is important, this aspect will have to be considered. On the envisaged full scale plant the occurrence of such a problem will be solved by using a DAF unit after ozonation in the recycle stream.

The organism that might cause operational problems is the *Bacillus*. When this organism is allowed to multiply for a prolonged period in the reactor (i.e. when the cell age for this organism increases to about 12 hours, filamentous sheaths were formed. When this occurred, the micro-screen served as selector for both the filamentous fungus and the *Bacillus*, making it more difficult to wash out. When wall-growth did occur in the pilot plant, microscopic examinations showed that this was by far the dominating organism in the biofilm. The growth rate of this bacterium can be suppressed sufficiently to allow it to be washed out with 3 hours hydraulic retention time. This was done by operating the reactor at a pH of 4 - 4.3 and an increased temperature of 47 °C.

The presence of a high concentration of bacterial cells influences the downstream processing and the quality of the product. During the time that the reactor is contaminated, a product of inferior quality will be produced. It is therefore recommended that plant design should be aimed at minimising failure of the critical equipment.

During the six month period that plate counts were done, no abnormal growth or dominance by other bacteria occurred.

8. Operational experience: Pilot plant

8.1 Introduction

A 5000 litre pilot plant reactor has been in operation at the Sezela site for eight months during 1995. Following is a general discussion on the process and practical aspects of the daily operation of a larger plant. The objective of the pilot plant work was to see if there were any scale-up effects, to produce biomass for feeding trials and to gain experience in the practical operation and application of various process units in the process.

8.2 Plant lay-out

The reactor was designed on the airlift principle, with diffusers at the bottom. Air was supplied via a positive displacement blower. The working volume of the reactor was 5000 litres with a height of 4 meters. Tap water was used for dilution, instead of recycling a part of the reactor effluent. Therefore, temperature was controlled with heat exchangers, instead of regulating it with the flow as would be the case on full-scale plant.

Feed addition was controlled by the pH-feed-on-demand system as described in Section 5. The feed was equalised in a 18 000 litre equalisation tank, resulting in 30 hour retention time. In order to simplify sampling and determinations of COD, nutrients and other constituents, batches were made up individually in 2000 litre tanks, from where it was fed to the reactor. Dilution water was controlled manually in order to sustain a certain volume in the reactor, i.e. keeping the retention time constant.

Harvesting was usually done with a separate positive displacement pump. The harvested biomass was dewatered in a plate and frame filter-press.

8.3 Comparable pilot plant performance

To determine any scale-up effects the pilot plant was operated in exactly the same way as the laboratory reactor of 22 litres. Air was supplied at a rate of approximately 1 volume air per reactor volume per minute. The feed was controlled in the same way, i.e. constant level control and pH-controlled feed rate. Table 8.1(A) shows a typical comparison between the pilot plant and the lab units.

Table 8.1(A) Comparison of performance between pilot plant and laboratory reactors. Parameters are specified in the text.

Parameter	Lab. Reactor	Pilot reactor
Biomass age (h)	11,5	12,2
Biomass conc. ($g.l^{-1}$)	3,7	4,0
COD in ($mg.l^{-1}$)	2655	3100
COD out ($mg.l^{-1}$)	330	415
$Yield_{obs}$ ($g.g^{-1}$)	0,40	0,37
Productivity $(g.(l.h)^{-1})$	0,32	0,33
Treatment efficiency (%)	88 %	87 %

The performance of the laboratory and pilot plant reactors were very similar as can be seen from Table 8.1(A), if similar conditions were applied.

It must be noted that both systems were operating under oxygen limited conditions. With an aeration rate of 1 v/v/min and the aeration equipment that was used, the oxygen utilisation rate of the organism culture could not be satisfied. The pH of the system will only increase when bio-oxidation occurs, but for this to occur, there should also be enough oxygen. If oxygen supply is limited, the process rate will be impaired accordingly, since the organisms cannot utilise the carbon source as fast as their kinetic characteristics determine. The result of oxygen limitation is a

decrease in carbon utilisation rate and subsequently a higher ratio of dilution water will be required to maintain the chosen hydraulic retention time.

Although it is not shown here, a slight improvement in oxygen transfer efficiency was noticed in the pilot plant because of the longer contact time and slightly higher pressure due to the deeper reactor. The effect of this would be an increase in the carbon utilisation rate as explained before.

The pilot plant again emphasised the fact that one of the key areas of optimal operation of the MyPro system, is efficient supply and utilisation of oxygen.

8.4 Bacterial contamination in the pilot reactor

Bacterial contamination in the pilot plant was not quantified as was done with the lab reactor. Plate cultures were only made to check on the bacterial species present in the reactor. The same two species that dominated in the lab reactor, also dominated here.

An aspect that should be mentioned here is that the *Bacillus* species might cause operational problems in the reactor if it is allowed to establish itself in the system. This happened when the selector screen did not work well. It was noticed that if the cell age of this organism was allowed to increase to more than approximately 8 hours, the individual rods formed chains within a sheath, which is typical of some *Bacillus* species. Since the system selects for filamentous growth, once this stage is reached it was not possible to wash out the organism through the screen.

A method had therefore to be developed through which this organism could be taken out of the system. The best way to get rid of this or any other bacteria that established itself in the reactor, was to reduce their growth rate to lower than the harvest rate of the filamentous fungus, thereby harvesting out the unwanted

organism. This was achieved by changing the conditions in the reactor, i.e. varying the temperature or set pH of the system.

No significant bacterial wall-growth occurred if the reactor was operated with a short retention time, right from start-up.

8.5 Practical aspects: Start-up and shut-down

Referring to Section 2 where the selection of filamentous organisms is described, the question might have arisen if such a procedure had to be adhered to every time the MyPro system was started up. This would not be necessary once a suitable organism had been identified for a specific effluent and the fungus could be stored in large enough quantities. The only concern would then be to find a storage method for the biomass. This was even more important at the Sezela site where the plant is shut down for 4 months of the year, as well as during periods of heavy seasonal rains.

There are two ways in which the biomass could be stored, namely as de-watered filaments with a 30 % solids content or as air-dried sheets. The first option requires cooling facilities (2 - 4 °C). The second option requires space to dry the biomass or a low-temperature (< 50 °C) dryer. In both cases enough biomass should be dried to start up with a concentration of not less than 0,1 $g.l^{-1}$. For a 500 m^3 reactor this would relate to a minimum of 150 kg wet material or 50 kg dry material. Experience has shown that the wet material is more amenable to bacterial contamination.

After initially using wet material to start up the reactor, we changed to using only dry material. By the end of the testing period, the pilot plant could be in steady-state within 48 hours after starting with an inoculum of 500 g dry material. This short start-up period proves to be one of the system's major advantages, especially in the treatment of seasonal effluents.

8.6 Safety Aspects

Safety aspects in a production facility should mainly involve unwanted production of spores or the intake of viable fungal material. Due to the non-ideal set-up in the pilot plant, spillage occurred on a daily basis on the floors, etc. It was observed that the fungus grew in quiet places, for example crevices and corners where it is not washed. Growth occurred as a white fluff, but surprisingly very little or none of the characteristic green-grey spores were visible, even after two weeks of growth. Considering the information given in section 4(B), this should not pose a significant risk to the health of process workers.

The atmosphere above and around the pilot reactor was also screened regularly for viable spores or hyphae. The incidence of viable pieces was never higher than the control, measured outside the plant.

Nevertheless, it is still recommended that care must be taken when working with the living biomass product against unnecessary exposure. Secondly, the sterilisation procedure must have a safety factor and sterilisation effectivity should be checked on a regular basis in order to maintain a high kill rate.

8.7 Concluding remarks

Apart from the engineering aspects regarding equipment, for example the selector screen, the only significant difference between the pilot plant and lab reactors was the improved oxygen transfer in the pilot plant.

Of all the equipment, the most important unit is the selector screen used to select the filamentous organisms. Wrong selection or ineffective operation of the screen will result in loss of biomass or selection of unwanted bacterial species.

When such unwanted growth did occur, it could be eliminated by variations in the temperature and pH settings.

The pH-controlled feed method proved to have various advantages in the treatment of industrial effluents. Apart from simplifying the operation of the plant, it also optimises the utilisation of available oxygen, and protects the biological culture against shock-loads.

Plant operation overall seemed to be relatively easy. By having the process rate linked to the feed supply, the rest of the plant, i.e. harvest rate, volumes, recycle ratios, etc, can easily be linked up as well. The MyPro Water Treatment System is therefore fully amenable to manual or PLC controlled modes, depending on the requirements of the customer.

A method was found through which the biomass could be stored in a viable state for a relatively long period without significant deterioration. Start-up and shut-down could therefore be simplified.

PART B: COMMERCIAL UTILISATION OF *ASPERGILLUS FUMIGATUS* BIOMASS

1. Introduction

Aspergillus fumigatus has not yet been exploited commercially. Published information on this aspect is also relatively scarce. This is probably due to the negative publicity the fungus has received from early work being done on it. Because of the implication of the fungus in various cases of *Aspergilloses* (see section 4(B), almost all the earlier work concentrated on identification and isolation of possible toxins or antigens from this organism (Glister & Williams, 1944; Parrish *et al*, 1965; Yokota *et al*, 1984).

In the process, research into actual utilisation of the organism in novel applications tended to be missed to a large degree. The first major study into the possible commercial exploitation of the fungus, was actually during 1975 when researchers in Canada evaluated the organism as protein source when grown on cassava extract (Reade & Gregory, 1975). After this, various antibiotics discovered earlier, have been studied in more detail, and recently, a special enzyme inhibitor was identified that decreases the uptake of cholesterol in the human body (Tae-Sook *et al*, 1994). It has also been known for years that *A. fumigatus* is a producer of two enzymes, namely cellulase and A-amylase.

This part of the report investigates the products that are produced by *A. fumigatus*. Some of the products are discussed briefly, but our research was mainly concentrated on two areas. These were firstly the utilisation of the organism as a protein source and secondly, the production of cellulase enzymes. The work presented on these two aspects is within the objectives of this project, and although more specific research will be required, the initial groundwork has been done with

positive results. Because of questions that might arise regarding health aspects involved, most of the available scientific information on working with the organism and the production of toxins has been reviewed and is also discussed briefly. Of course it is not within the scope of this report to deal in depth with these issues, but more information can be obtained from detailed reviews by the authors and the relevant references.

2. Utilisation as protein source

2.1 Introduction

The reason why this option was explored is firstly that conversion of the biomass to a protein product is relatively easy and involves little downstream processing. Secondly, there is a large market for feed protein in South Africa. Detailed records of the country's requirements and imports are available from the Protein Research Trust of the Agricultural Research Council (ARC). For instance, during the 94/95 season South Africa imported 168 000 tons of fish meal and 256 000 tons of oil cake at a cost of over R 600 million.

Any competitively priced protein source could therefore be sold easily on the local market. It can be added that since this is actually the ideal protein source for human consumption (high fibre, high protein and low fat content), it might show the direction for future upgrading of the biomass protein for human consumption.

The idea of using a thermotolerant fungus to purify water and produce a utilisable protein source of high quality is relatively new and it solves the problem of heat generation in similar SCP processes. At the same time effluents emerging at elevated temperatures can be treated without cooling requirements. As explained earlier, a balance must however be obtained between the objectives of a high yield and a low COD in the reactor effluent (S). This aspect as well as optimisation for each objective is discussed in Section 4. The purpose of this section is to look at the various aspects that determine the marketability of the biomass as protein source.

The aspects that will be covered are stability of the organism in the system, nutritional value of the fungal material and toxicology. These are the most important aspects of SCP product quality.

2.2 Nutritional value of the organism

Very little research information exists with which to compare test results obtained from our studies on the nutritional value of the organism. Apart from organism stability and toxicity there are broadly three factors that determine the food value of the product (Atkinson & Mavituna, 1991). Firstly, since it is a protein source, it should have an acceptable amino acid profile. It is especially important that the lysine and methionine content should be sufficient. Secondly, the protein should be utilisable. i.e. the fungal material has to be digestible enough to allow the uptake of the amino acids by the test animal. Thirdly, and most importantly, there should be a market for such a product.

Table 2.1(B) shows the amino acid profile of *Aspergillus fumigatus* in comparison to that of other protein and SCP sources.

Table 2.1(B) Crude protein and amino acid profile of *Aspergillus fumigatus* compared to that of other sources (Pretorius & Lempert, 1993).

Amino Acid (g/100g)	*Aspergillus fumigatus*	*Geotrichum candidum**	*Candida. Utilis*	Soybean meal	Fish meal
Alanine	2,8	4,7	4,1	2,1	4,2
Argenine	2,8	6,2	3,5	3,5	3,7
Aspartic acid	3,6	2,2	7,0	5,4	6,2
Cystine	ND	0,3	0,6	0,6	0,7
Glutamic acid	5,4	5,0	8,5	8,7	8,9
Glycine	2,1	4,0	3,2	2,1	3,9
Histidine	0,9	0,6	1,5	1,4	1,5
Isoleucine	1,9	2,1	3,7	2,1	3,2
Leucine	3,1	3,1	5,5	3,6	5,0
Lysine	2,6	4,9	5,5	2,9	4,9
Methionine	0,7	0,2	1,1	0,6	1,9
Phenylalanine	1,7	1,5	3,4	2,4	2,9
Proline	2,1	1,8	2,6	2,2	2,9
Serine	1,9	2,6	3,6	2,6	3,0
Threonine	1,6	2,5	3,8	2,5	3,0
Tyrosine	1,2	1,0	2,8	1,9	2,3
Valine	2,2	2,7	4,1	2,1	3,7
% N	7,9	8,8			
% Crude protein	49,4	55,0			
% True protein	> 36,5	45,6	65,0	47,0	66,0

ND - not determined
* Kühn and Pretorius [1989]

From Table 2.1(B) it may be seen that the amino acid profile compares well with other sources, although the true protein content (i.e. total protein - RNA-associated protein) is lower. Table 2.2(B) will perhaps give the reader a better idea of how *A. fumigatus* protein compares to its direct competition in South Africa. Results for *A. fumigatus* in Table 2.3(B) was generated by the Department of Agricultural sciences, University of Stellenbosch. Broilers were fed over a four day period with various inclusion rates, after which the metabolised energy values were measured.

The inclusion rate is the maximum percentage at which a protein source can be included in an animal diet. This percentage is limited by other characteristics of the foodstuff, for example fibre and fat content, taste and energy value. The inclusion rate of *Aspergillus* protein is for example limited by the high fibre content and the low energy value.

Table 2.2(B) Nutritional value of *A. fumigatus* compared to other protein and energy sources.

Feed	ME* (MJ/kg)	% Prot	% Fat	% Fibre	Inclusion rate (%)	Price** R/tonne
Maize	14,43	8,5	4,0	2,6	-	490-600
Hominy chop	-	-	-	-	-	500
Sorghum	13,75	10,0	3,4	3,4	-	475
Pollard	-	-	-	-	-	400
Fish meal	13,27	65,0	10,0	1,0	< 18	2100
Blood meal	-	-	-	-	< 2	1930
Carcass meal	9,90	50,0	12,0	1,0	< 5	1547
Soya oil cake	10,0	47,0	9,0	-	< 15	1200
Sunfl. oil cake	10,2	-	-	8,0	< 5	900
Full fat soya		47,0	10,0	-	-	1058
A. fumigatus	8,38	49,4	2,7	9,6	< 12	-

- \- No values available
- * Metabolisable Energy
- ** 1995 Prices

It may be seen that the SCP from *A. fumigatus* compares favourable with especially carcass meal and soya oil cake. The fat content of 2,7 % compares well with other published data on fungal protein (Litchfield, 1983). The determination on the specific

sample showed a crude protein value of 46,4 % which is slightly lower than the 49 % when the organism was grown on spent sulphite liquor (Pretorius & Lempert, 1993).

When the various protein sources in the table are compared, an idea might be formed of the potential value of the dried biomass, but it should be emphasised that additional tests still have to be performed before a market value could be ascribed to it.

Although preliminary feeding tests have been done already, conclusive results will only be available once more intensive tests are completed. Further information required includes toxicology (see section 2.4(B)) and performance results. Regarding performance as protein there are three basic parameters that are commonly used to compare protein sources, namely:

i) Digestibility (D): The percentage of the total nitrogen that is absorbed from the alimentary tract.

ii) Biological value (BV): The percentage of the total nitrogen assimilated that is retained by the body, taking into account the simultaneous loss of endogenous nitrogen through urinary excretion.

iii) Protein efficiency ratio (PER): The proportion of nitrogen retained by animals fed the test protein, compared to nitrogen retained when a reference protein, such as egg albumin, is fed to the animals.

None of these have yet been determined, but results will be available soon. Further informal tests have however been done in order to determine the acceptability and initial safety of the dried SCP biomass. Although not of much scientific value it provided a positive indication that the biomass can be evaluated further in more refined tests. Broilers were fed a standard poultry feed, supplemented with 12 %

Aspergillus biomass. After 14 days the chicks showed no visible signs of any disorder and were on or above their target weights. They seemed to accept the SCP without any problem. The dried biomass was also fed to koi fish for four weeks as only food to determine its acceptability and acute toxic effects. Again no mortalities occurred and the granules were well accepted.

2.3 Stability of the organism

In SCP processes over the years, concern has been expressed by regulating authorities as to the stability of the organisms used. The fear is that the proven non-toxic strain used during the testing period would one day mutate to a toxin producer that might have great potential for harm.

It is probably true that no organism in a continuous culture system running for a long time, is exactly the same at the end as at the beginning. This is because of the spontaneous genetic instability, an inherent characteristic of all genetic material (Lindegren *et al*, 1954). In a steady state continuous culture, described by the Monod equation,

$$\mu = \frac{\mu_{max} \cdot S}{K_s + S}$$

the concentration of the growth limiting substrate exerts a selection pressure, so that any mutation or variation which can confer upon the organism the ability to reduce the steady-state substrate concentration will possess a competitive advantage.

Reduction of S can be brought about by increasing μ_{max} or decreasing the value of K_s. Selective advantage will also be conferred on a mutation or variation by changes which provide a more efficient conversion of substrate to biomass, i.e. improved yield. This effect has been described by Harder *et al* (1977) and Van der Westhuizen (1993).

It follows that unless a mutation can bring about improvements in u_{max}, Y or K_s, or produces a toxin that kills the rest of the population, it will not affect the reactor population. Therefore in a process where selection pressure is for biomass production (for example the MyPro system) a mutation which exhibits secondary metabolite production, will always have impaired values for u_{max}, Y or K_s and will be washed out of the reactor in preference to the faster growing organisms.

So, if the reactor conditions are kept within reasonable limits, the chance for a toxin producing strain to "take over" the reactor are slim. Any mutation will be towards a faster growing strain. Over the two years that the specific strain of *Aspergillus fumigatus* has been used, a slight improvement of the kinetic parameters has indeed been noticed. No difference has however been noticed in cell structure or growth form in the reactor broth or on agar plates.

2.4 Toxicology

The toxicology of *A. fumigatus* has two components to it. The first one is toxicity originating from ingestion of toxic material. It is known that the organism produces certain toxins in the form of intra- or extracellular metabolites (Dubeaubuis & Lafont, 1978). The second component is the toxicity associated with spore formation. The spores of the organism can be toxic when inhaled. The objective of Section 2.4 is firstly directed towards the establishment of the health aspects of using *Aspergillus fumigatus* as a source of single cell protein (SCP) in feed mixtures. In a later section the spore-forming aspects will be discussed as a safety consideration in the production environment.

A list of the known metabolites excreted by *A. fumigatus* that have been shown to have either antimicrobial, phagocytic or toxic effects are summarised in Table 2.3(B). Note that no enzymes are included here.

Table 2.3(B) Metabolites produced by *Aspergillus fumigatus*

Metabolite	Function/Description	Reference
Helvolic acid/fumigacin	Toxin/antibacterial	Glister & Williams (1944); Waksman, (1944)
Gliotoxins	toxin/immunomodulating	Mullbacher *et al* (1985); Waring *et al* (1986)
Fumigatin	antibacterial	Glister & Williams (1944)
Fumagillin	antibacterial	Okuyama & Yamazaki (1984)
Kojic acid	Antimicrobial	Parrish *et al* (1966)
calvine alkaloids	toxins	Dorner *et al* (1984)
tremorgens	toxins	Dorner *et al* (1984)
Geri-BP001	ACAT-inhibitor	Tae-Sook *et al* (1994)
Fumitoxins	Toxins	Dubeaupuis & Lafont (1978)

As can be seen from Table 2.3(B), not all of the metabolites produced by *Aspergillus fumigatus* are proven toxins. Probably the most published incidents involving this fungus, were cases where the fungus grew for extended periods in animal feed. The fungus has been implicated in naturally occurring toxic syndromes of beef and dairy cattle in which animals suffered from irritability, poor co-ordination, behavioural changes and death (Haller & Suter, 1974). A typical case is described by Dorner *et al* (1984) where 120 beef cattle on a farm in Michigan were intoxicated as a direct result of *A. fumigatus* growth on corn that was being used as feed. Clinical signs were evident 10 - 14 days after consumption. No mention was however made of whether the toxicity was due to intake of fungal hyphae or spores.

The most important aspect in all these studies is the condition under which all these metabolites are produced. Various substrates and incubation temperatures were employed, but the common factor in all the work was that the culture had to be incubated for at least 3 days, and in most cases 5 or more days before metabolites were formed in appreciable amounts. This means that the metabolites are secondary products of the fungus which are excreted only after the logarithmic growth phase. All of these tests were done in batches. The only toxicology tests that were done on continuous culture or on young batch cultures (< 20 hours) are described by Reade & Gregory (1975). No toxicity was detected in rats fed for 90 days. Analysis of the mycelia for a variety of specific toxins also showed negative results. This fits in with the principle of the selection mechanism that favours cell growth rather than product formation, as was discussed earlier.

Therefore, when taking an unbiased look at the toxicity aspect, it can be concluded without question that toxic metabolites are formed. However, all indicators show that *A. fumigatus* does not produce metabolites in toxic amounts during its logarithmic growth phase, the phase in which the organism is constantly kept in the MyPro system. Evidence for this have been discussed previously but can be summarised as follows:

- The selective pressure of the continuous culture MyPro system exerts pressure towards biomass production and away from product formation

- Preliminary feeding trials showed no toxicity. This was substantiated by the findings of Reade & Gregory (1975).

- Toxin research showed that metabolites only formed after at least 3 days. The age of biomass that is produced in the MyPro System is not more that 24 hours and usually less than 12 hours.

The next step will be to do specific tests that will satisfy the regulating authorities in order to get the product certified as animal feed. The Council of European Communities issued a directive for such tests which is acceptable to the authorities in South Africa. The tests will be done over a period of 1 year.

Although not important in this context, it might be worthwhile to mention that some of the substances in Table 2.3(B) might have possible medicinal applications. Helvolic acid and fumigacin (which Waksman (1944), suspects to be the same substance), is active against gram-positive bacteria. It is only slightly toxic to animals and might find an application in years to come as a new antibiotic. There are also four variations of gliotoxin, some of which have immunomodulating effects. This substance can be used to suppress the immune system locally, which will have applications in organ transplants. The discovery of an inhibitor of the enzyme responsible for cholesterol absorption in the body, was widely publicised (Tae-Sook *et al*, 1994) and might present a way of decreasing cholesterol levels in humans. These possibilities will be pursued further.

3. Utilisation of *Aspergillus fumigatus* for production of cellulase enzymes

3.1 Introduction

Apart from the possibility of using the biomass as SCP, it is also possible to produce other substances like enzymes from *Aspergillus fumigatus*. This may be seriously considered, especially since *A. fumigatus* is a known producer of a variety of enzymes of which cellulase is the most important. Cellulase catalyses the conversion of cellulose to glucose. *A. fumigatus* is for instance one of only a few species that produces the complete cellulase complex, meaning that the organism is able to degrade the lignin structures of ligno-cellulosic materials (i.e. grass) as well.

However, the idea of producing extra-cellular enzymes in the micro-screen process was initially dropped after it was discovered that no economically feasible method exists by which the enzymes can be separated from the bulk water mass.

During the study of literature on this subject, it became clear that one of the reasons why cellulases are so expensive to produce, is because of the costs involved to grow large quantities of the cellulase producing fungi on a suitable carbon source. By means of the MyPro System it is possible to grow large quantities of biomass. This idea prompted some basic experiments to see whether cellulases can be produced from the biomass harvested from the MyPro System reactor. Initial studies showed maximum enzyme yields of 3 IU.

Following is a report on further experiments done on this subject. Relevant information on the enzyme and factors that influence its production was extracted from the literature and are presented first in condensed form. Following the literature review, further experiments done on this subject are described. The experimental

section is presented in a simplified manner, although certain minimum detail had to be given to allow for comparisons and further research.

3.2 Literature review

3.2.1 The cellulase complex

The cellulase enzyme from *Aspergillus fumigatus* is actually a complex of three enzymes. Although cellulase is found in a variety of organisms, there are only a few that possesses the complete complex. These three enzyme activities (with their international codes) are the following (Stewart & Heptinstall, 1988):

- exo-cellobiohydrolase (1,4-ß-D-glucan cellobiohydrolase, exo-glucanase, E.C. 3.2.1.91),
- endo-glucanase (1,4-ß-D-glucan 4-glucanohydrolase, E.C. 3.2.1.4)
- ß-glucosidase. (ß-D-glucoside glucohydrolase, E.C. 3.2.1.21)

Several reports stated that *A. fumigatus* produced a xylanase (E.C. 3.2.1.8, endo-1,4-ß-xylanase) activity when grown on cellulose and lignocellulose substrates. This activity is needed for the complete enzymatic hydrolysis of plant material to glucose.

In Table 3.1(B) the size, substrates and inhibitors of the *A. fumigatus* cellulase complex are shown.

Table 3.1(B) Important characteristics of *A. fumigatus* cellulase complex

Property	Enzyme		
	Exo-glucanase	Endo-glucanase	ß-glucosidase
Standard substrate	Avicel	Carboxy-methyl-cellulose	Cellobiose
Molecular mass	± 46 000	± 12 500	± 40 800
Inhibitor	Glucose	Cellobiose	Glucose ?

In Figure 3.1(B) the cellulase enzyme system is presented schematically. Substrate, products and feedback inhibition are shown. Each step shows typical Michaelis-Menten kinetics with strict competitive or uncompetitive inhibition.

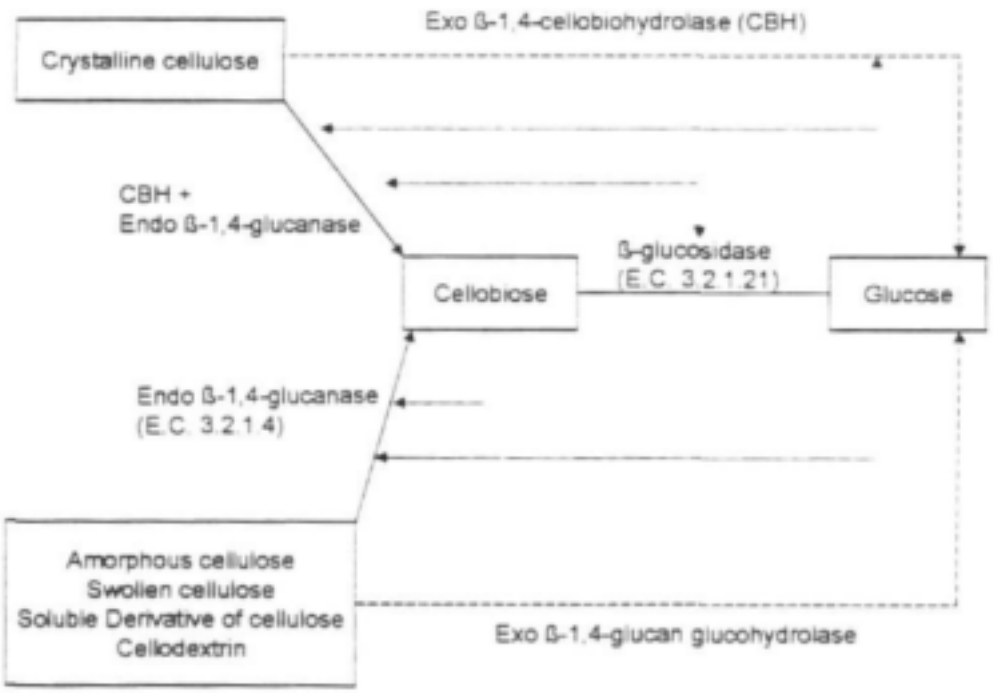

Figure 3.1(B) Schematic diagram of substrate, enzyme and products in cellulose hydrolyses (Bailey & Ollis, 1986)

3.2.2 Factors influencing cellulase production

There are various important factors that must be taken into consideration when cellulase is produced. Again, only the most important aspects are covered.

a) Induction factors

No specific reference is made in the literature to a specific inducer for the organism to produce cellulase. It is most likely that cellulose itself serve as inducer (Stewart & Parry, 1981; Wase *et al*, 1985). Hence, a cellulose source must be present to activate the enzyme complex. According to Stewart & Parry (1981), more cellulase enzyme is produced when an insoluble cellulose substrate is used. Although lactose was found to be an inducer for the cellulase complex of *Trichoderma reesei*, this was not the case for *A. fumigatus*.

b) Nutrients

As mentioned in the previous paragraph, cellulose apparently has to be present as the only carbon source, otherwise the organism will not have any incentive to produce the enzymes which requires more energy.

The nitrogen source that showed the best results is ammonium salts, especially NH_4NO_3, followed by $(NH_4)_2SO_4$.

Good growth of the organism was observed at a pH of 3,0 to 6,0, with the optimum at 4,5 to 5,5. Maximum production of cellulase occurred at maximum growth rates.

c) Physical factors

Maximum enzyme production occurred at a temperature of 45 °C (Stewart & Parry, 1981).

It has been shown by Aust (1994) that the production of ligno-cellulosic enzymes in white-rot fungi are severely impeded by high shearing forces. Wase *et al* (1984) noticed the same effect in a study on reactor design for the production of cellulase. They proposed an airlift reactor where the minimum shear is experienced by the organisms. Additionally, they also found that the rate of enzyme production is directly proportional to the amount of available oxygen, i.e. a positive concentration of dissolved oxygen is required for optimum enzyme production.

d) Time

Although it was showed that the cellulase enzymes are produced during the growth phase of the organism (Stewart & Parry, 1981), in most of the studies maximum enzyme production only occurred after 3 to 10 and even after 15 days.

Preliminary results obtained from batch shake flask experiments showed that it might be worthwhile to further investigate the possibility of commercial cellulase production from *Aspergillus fumigatus*. Through consideration of the above abstract from literature, it was possible to prioritise the important factors that influence enzyme production. These factors were evaluated in the experimental work that is described below.

3.3 Experimental work

At first, the optimum culture conditions for cellulase production from this particular strain of *Aspergillus fumigatus* were determined. This was followed by tests with a pure cellulase substrate to verify the initial results (Progress report, 1994). The effects of the following variables on cellulase production were finally evaluated to give a perspective on the technical viability of cellulase production by this organism:

i) Use of a different (pure) cellulose source
ii) Total exclusion of a cellulose carbon source to determine if enzyme formation is induced or not.
iii) Use of an industrial waste product, hydrolysed bagasse, as cellulose carbon source.
iv) Use of various concentrations of bagasse and biomass inoculum.

3.4 Materials and Methods

3.4.1 Production of cellulase

All the experiments were conducted in shake flasks at a temperature of 45 °C, except where indicated otherwise. Due to changes in pH experienced during the initial trials, the medium was buffered in all cases with sodium citrate buffer so that pH changes in the short term could be resisted.

Three different media compositions, varying only in its nitrogen source, were evaluated to find the best one to use in subsequent experiments. The substrate broths were all based on Czapek's basal medium (Couts & Smith, 1976).

The inoculum consisted of various amounts of the dewatered biomass. For pH studies the medium was prepared in concentrated form and mixed with sterile citrate buffer of the required pH to give the correct concentration of nutrients and 0,05 M buffer solution. Studies were conducted at pH ranging from 4 to 8.

3.4.2 Determination of enzyme activity

After incubation, the culture broth with biomass was filtered to separate the remaining cellulose and mycelial growth from the broth. All enzyme activity was measured as total filter paper (FP) activity, or exo-glucanase activity as it is also known (It is accepted that if a species of *A. fumigatus* shows FP activity, it will most probably also have endo-glucanase and ß-glucosidase activities).

A small volume of culture filtrate was incubated with filter paper in a citrate buffer for 30 minutes at 60 °C. After incubation, the amount of soluble sugar formed from the filter paper was determined spectrophotometrically with the phenol/H_2SO_4 method of Dubois *et al* (1956).

Enzyme activity is expressed as international enzyme units (IU), i.e. µmole glucose liberated by one millilitre of enzyme broth per minute.

3.5 Results and discussion

3.5.1 Determination of optimum culture conditions for cellulase production

Each flask contained 100 ml medium and 1% Avicel and was shaken continuously for 5 days. Each value represents the mean of determinations on triplicate flasks. The effect of the various nitrogen sources on cellulase production is shown in Figure 3.2(B).

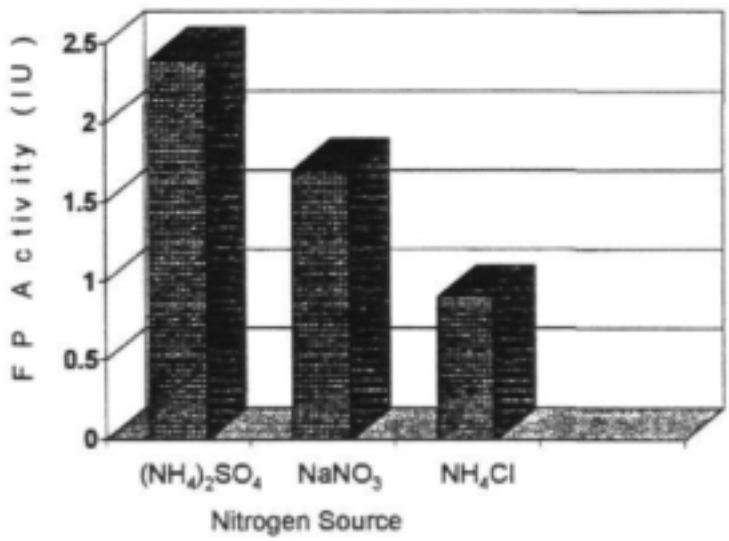

Figure 3.2(B) Effect of nitrogen source on cellulase production (FP Activity = filter paper activity)

Ammonium sulphate constantly gave the highest cellulase production. This is in accordance with the findings of other workers (Ghose & Sahai, 1980; Stewart & Parry, 1981). This ammonium salt was accordingly used in all subsequent experiments. The effect of the temperature and pH of the enzyme production medium can be seen in Figure 3.3(B) and 3.4(B) on the next page.

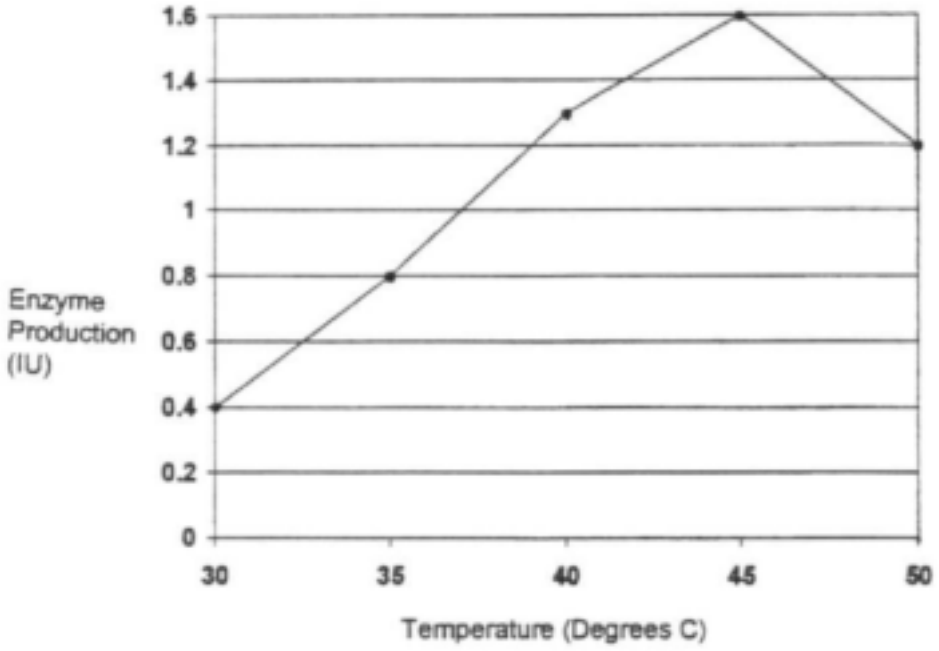

Figure 3.3(B) Effect of temperature of production medium on cellulase production

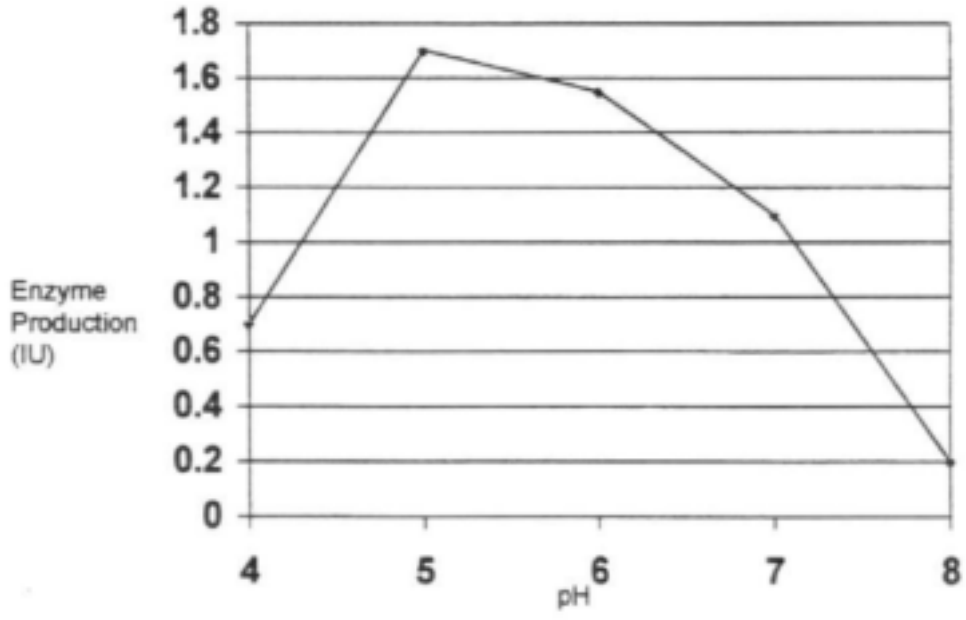

Figure 3.4(B) Effect of pH of production medium on cellulase production

Maximum production of cellulase (measured as total filter paper activity) was found to be at pH of ± 5,0 and temperature of 40 - 45 °C. The results compare favourably with those obtained by Stewart & Parry (1981) and Trivedi & Rao (1979) who found pH and temperature optima for *Aspergillus fumigatus* in the ranges of 5,0 to 6,0 and 35 to 45 °C respectively.

3.5.2 Determination of the effect of various carbon sources

The effect of using various carbon sources on cellulase production is shown in Figure3.5(B), on the next page. CF1 Cellulose is long-fibred, CF11 medium-fibred and Avicel crystalline cellulose. The bagasse source is so-called hydrolysed bagasse which is the fine bagasse residue left after steam-extraction of furfural.

The hydrolysed bagasse was washed with warm water beforehand until no further colour was released into the water. This step was necessary because any colour present in the substrate influences the colorimetric determination of liberated sugar.

Cellulase production on Avicel seemed to be slightly higher than on fibred cellulose, with hydrolysed bagasse more or less in the same region. This is contradictory to the results of Stewart & Parry (1981) who found increased cellulase production from more amorphous cellulose sources. The enzyme activity obtained during these experiments compare very well with values obtained from the literature. A comparison of filter paper activity as obtained by other workers is given in Table 3.2(B).

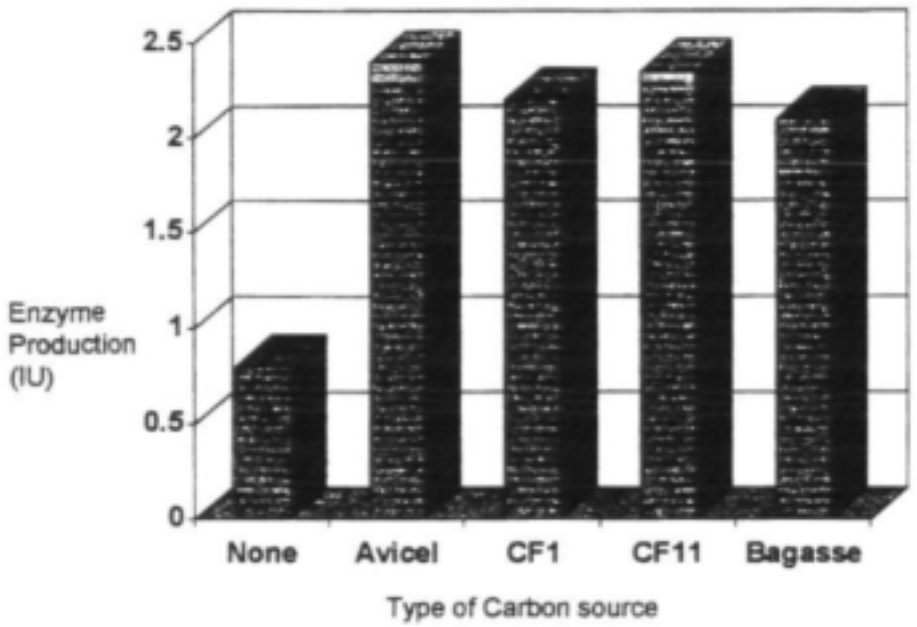

Figure 3.5(B) Effect of carbon source on cellulase production

Table 3.2(B) Comparison of cellulase yields

Enzyme activity (IU)	Carbon source	Fermenter type	Organism	Reference
0,2 – 2,2	Wheat straw (treated)	Shake flasks	*A. fumigatus*	Shaker *et al* (1984)
1,0 – 2,4	Solka floc (wood cellulose)	Shake flasks	*Sporotrichum*	Coutts & Smith (1976)
1,0 – 3,5	Wood cellulose	Shake flasks	*Trichoderma reesei*	Watson, *et al* (1984)
12 – 57	Wood cellulose	Fed batch	*T. reesei*	Watson *et al* (1980)

It is important to note that increased cellulase production can be expected when a fermenter is used where optimum conditions can prevail. The highest published yield of enzyme production is the 57 IU achieved by Watson *et al* (1984) who made use of a fed-batch culture.

An interesting observation from Figure 3.5(B) is the production of cellulase (although in relatively low quantities) in the absence of any carbon source. This would mean that the formation of cellulase in this particular organism is not induced. Such a situation is not impossible, but rather suspect since all research done so far on the fungal cellulase enzyme complex (although not specifically on *A. fumigatus*) showed that cellulase enzymes are induced, probably by the presence of cellulose.

A possible explanation might be that formation of the enzymes is induced by nutrient limitations. The way the biomass is grown separately on an industrial effluent, washed, and then added as inoculum in the form of active filamentous growth is different from all other cellulase production studies so far, in the sense that the growth phase takes place on another carbon source. This would mean that the production of cellulase by *A. fumigatus* is a function of secondary metabolism.

3.5.3 Effects of time, substrate concentration and inoculum size

The promising results obtained with this specific strain of *Aspergillus* prompted us to further investigate the production of cellulase from an industrial source. The obvious substrate being the hydrolysed bagasse residue which is a waste product after furfural extraction at the Sezela factory. It is currently used as boiler fuel.

Figure 3.6(B) shows the effect of time on cellulase production from bagasse.

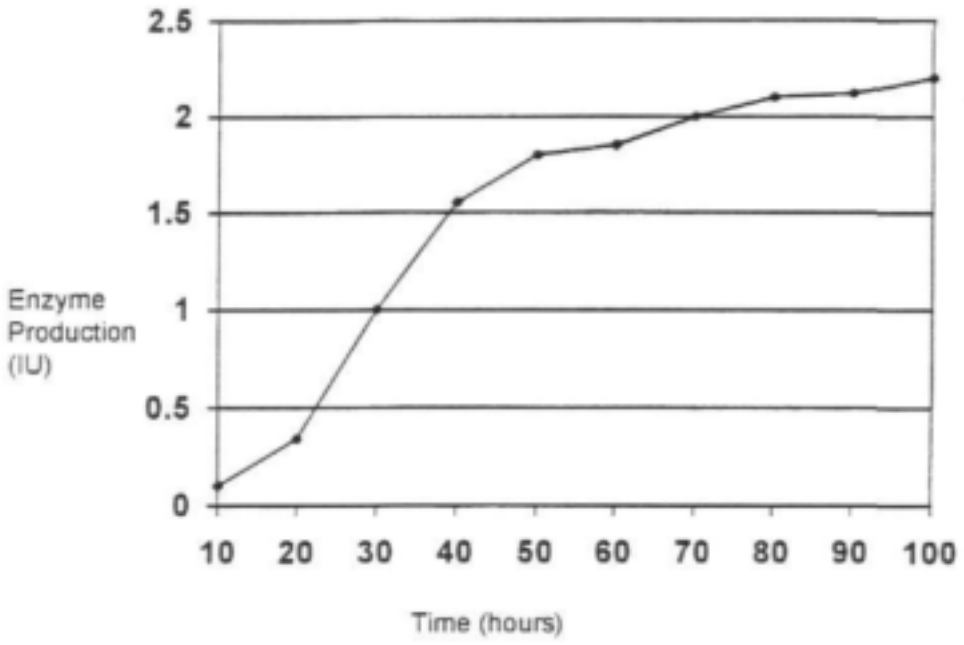

Figure 3.6(B) Effect of time on *cellulase* production from bagasse

For two known cellulase producers, *T. reesei* and *T. viride*, the maximum ezyme concentration was reached after approximately 120 to 200 hours (Montenecourt, 1983; Watson *et al*, 1989). If this is compared to Figure 3.6(B), it may be seen that a plateau (maximum enzyme production) is reached in a shorter time with *Aspergillus fumigatus*. This is in accordance with the results obtained by Coutts & Smith (1976) who also found faster production of cellulase with the thermophilic fungus *Sporotrichum thermophilum* (grown at a temperature of 45 °C) than with mesophylic fungi.

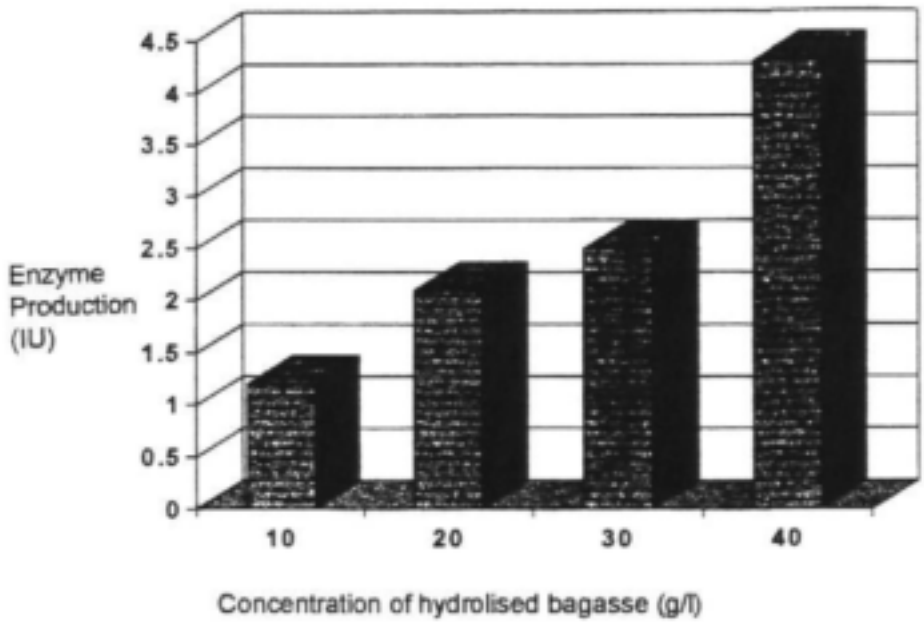

Figure 3.7(B) Effect of increased concentrations of substrate (hydrolysed bagasse) on celllulase production.

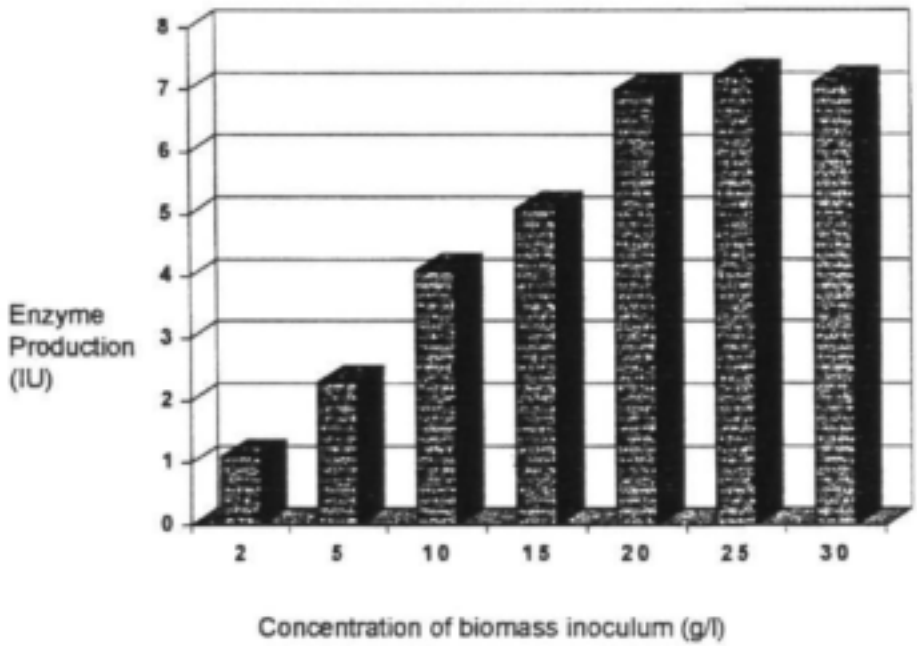

Figure 3.8(B) Effect of increased inoculum on cellulase production from hydrolysed baggase (40 g/l bagasse was used)

From Figure 3.7(B) it may be seen that maximum enzyme production only occurred at relatively high bagasse concentrations of 40 to 50 $g.l^{-1}$. Coutts & Smith (1979) and Watson et al (1989) also found maximum production at these or even higher levels of cellulose. Though no higher concentrations were evaluated, it might be worthwhile to repeat the experiment with even higher cellulose concentrations.

Increasing concentrations of original inoculum also resulted in increased cellulase concentrations. A maximum yield of cellulase of 7,2 IU was achieved with 40 $g.l^{-1}$ bagasse and 25 $g.l^{-1}$ biomass concentrations. It therefore seems that the size of inoculum plays a major role in incubation time. These results also explain the observation by previous researchers that maximum production occurred after a relatively long incubation time (See 3.2.2(d)). Because of the small inoculum used by them, enough biomass had to be formed first. The yield of 7,2 IU was the highest yield that could be obtained so far in our experiments. When compared to values in literature, it is indeed a very high yield, taking into consideration the fact that the fermentation has not been fully optimised on the process side, i.e. by using a specialised fermenter.

3.6 Concluding remarks

It was proved that the specific strain of *Aspergillus fumigatus* that is continuously selected in the micro-screen process, is a potent producer of cellulase, when used in a secondary batch process. It is important to note that our research conducted so far was not directed towards characterising of the enzyme complex. Data on the effect of further increases of cellulose concentrations on enzyme production are also lacking, but for the purpose of this report enough information has been generated.

The market for cellulase is relatively new and is expanding quite rapidly. Cellulase enzymes can be used in food applications, but for this application strenuous testing and certification is required. The enzyme also found applications in the fruit processing industries where it is used to improve juice recovery. Good growth is expected in this area.

Industrial grade cellulase is used mainly in two industrial sectors, i.e. the detergent and textile industries. However, a specific enzyme of the cellulase complex, xylanase, may have exceptional potential in the pulp and paper industry to break down xylan structures. Research is currently concentrated on finding a cost-effective method to separate the xylanase from the other enzymes.

Dilute mixtures of cellulase are often included in detergent recipes, especially in Europe. The most important functions of the enzyme in detergent formulations are to prevent fluffyness and to improve light reflection. Both of these objectives are obtained by the enzyme acting on any protruding cellulose fibres and thus preventing the formation of fluffs.

The biggest application for cellulase in the world today is in the textile industry. Cellulase is the main agent responsible for the faded look of modern denim garments. It is also used extensively for bio-polishing and the prevention of pilling. The market in South Africa (estimated at ± R 5 million annually) is only a fraction of the total world market.

By producing cellulase in a secondary step after the water purification step, the enzyme can be produced at reduced cost. Since the production of cellulase would result in higher financial returns from the treatment process, it is recommended that this aspect be investigated further. The next logical step would be to characterise the specific enzyme complex further, and then to optimise the production process, probably using a fed-batch system as proposed by Watson *et al* (1989).

4. Safety of the production environment

4.1 Introduction

One of the main reasons why *Aspergillus fumigatus* has not been commercially exploited to a great deal was because of concern about the implication of this organism in *aspergilloses* (Marsh *et al*, 1979). This disease is caused by inhalation of the spores and the issue is therefore discussed in this section. However, it forms part of the toxicology of the organism and should be read in conjunction with Section 2.4. Following is a summary from literature sources to determine precisely what the current status is towards *aspergilloses*

4.2 Literature review

Various sources confirm the pathogenicity of *A. fumigatus* spores on a wide scale especially for people that are already ill (Land *et al*, 1989; Marsh, 1979). In medical terms the organism is therefore known as an opportunistic pathogen. Natural intake of the organism occurs mainly through the respiratory ways. The spores are 2-3 μm in diameter (Domsch *et al*, 1980) and are small enough to invade the small respiratory tracts and alveoli.

Growth in the human body is almost always limited to the respiratory tracts. Instances have however been recorded where lung fibre has been affected. In one or two cases the organism has also spread to the hart, kidneys and liver (Reijula, 1991). Colonisation of internal body cavities is usually chronic and the presence of "fungus balls" in the mucus is common when infection occurred. The extent of the disease varies from patient to patient, but it is seldom deadly (Marsh, 1979). No specific demographic pattern of the disease exists except for higher incidence in older people which are suffering from other maladies. When *aspergilloses* was diagnosed in patients, it was usually the secondary disease in the following

ailments: asma, tuberculosis, sarcoidosis, histoplasmosis, chronic bronchitis, emphysema and cancer (Marsh, 1979). Other potential victims are people with depressed immune systems. It has also been shown that inhaling massive amounts of spores (> 10^8) can overwhelm the immune system and cause infection in healthy people (Land *et al*, 1989).

Aspergilloses is also known as "Farmer's Lung" or "Wood-trimmer's disease". This disease, although usually chronic, can also be acute. The acute reaction is characterised by fever, tremors, coughing and impaired respiration. Symptoms appear within 4-8 hours after exposure. The symptoms usually disappear within 24 hours (Land *et al*, 1989). The more common, chronic form of the disease is called chronic alveolitis and is caused by continuous exposure. The result is progressive lung fibrosis.

According to De Lomas *et al* (1985) and Singh *et al* (1989), the appearance of keratitis (infection of the cornea) in rural agricultural areas is increasing. This is due to fungal growth on the cornea following injury to the eye. The incidence is however still very small.

For the purpose of this study it was also deemed necessary to determine whether there were existing ways of determining risks and applying control measures. The first thing that should be determined is where spores are typically produced and in what concentrations. Secondly the toxic dose should be established. Spores are typically produced in organic composting plants and in sawmills. Spore concentrations in these areas are typically in the order of 10^4 to 10^8 spores per m^3 (Passman, 1983). According to Land *et al* (1987), spore concentrations above 10^6 are potentially dangerous.

Taking all the information available into consideration, it is possible to place the pathogenicity of *Aspergillus fumigatus* spores in perspective. According to Marsh *et*

al (1979) it should be remembered that there are thousands of other micro-organisms on earth which are also opportunistic pathogens. If activities are being planned where spores might be produced, it is, however, wise to include preventative measures. The site's position and wind patterns towards hospitals, blood banks, clinics, medical offices and any other potential "soft spots" should be ascertained. The distribution of spores to these areas should be prevented as far as possible.

Regarding workers in such production facilities, should spores be produced, normal dust masks (as are compulsory in dusty mining areas) will prevent inhalation of spores. Care should be taken to prevent oral intake of viable fungus material. Additionally, workers with open wounds or injuries should not be allowed to continue working in such areas.

The situation with the production of *A. fumigatus* in the micro-screen reactor is different. No spore formation was observed during either of the previous project or this current one. The reason is that the organism is continuously cultivated near its maximum growth rate and spore formation is usually a function of the secondary growth phase. It is therefore questionable if any spores are released into the environment at all. It could however be possible that small pieces of hyphae were released.

4.3 Research work

It has already been established that no spores are formed during limitation in any of the nutrients. The only time when spores might be formed, was when conditions in the reactor deteriorated so much that the biomass age increased above 40 hours (i.e. a continuous high pH, toxic substances, etc). However, due to the importance of toxicity aspects, it was decided to do extensive tests in order to determine beyond any doubt where and how much spores were formed during operation of the

process. The formation of spores inside and outside of the reactor was therefore investigated.

4.3.1 Spores released into the environment

To determine whether any viable fungal material is released into the environment surrounding the process, extensive air sampling was done in the vicinity of the laboratory reactors. Sampling was done twice a day with a PBI Air Sampler (incubation on potato dextrose agar with chloramphenicol) over a period of 30 days. Samples were taken from three areas around the reactors, i.e. directly above the reactors, in a 2 meter zone at chest level, and in a 5 meter zone at chest level. For control, a sample was taken outside the buildings once a day. The results are shown in Table 4.1(B):

Table 4.1(B) Viable spore counts in process surroundings

Zone	Viable fungal matter count (CFU/m^3 air)
Above reactor	0 – 2
2 meter zone	0 – 13
5 meter zone	0 – 9
Outside Laboratory	0 – 23

The results show that there were very little viable fractions released into the surroundings of the reactor. The counts were well inside the level of 10^8 spores per m^3 as cited by Land *et al* (1980) where it might become a health hazard.

4.3.2 Spore formation inside the reactor

It has already been mentioned that no spore formation was detected in the liquid culture itself. When the reactor was operated with a biomass concentration of higher than 2 $g.l^{-1}$, a "rim" of biomass was formed just above the liquid level inside the reactor. This "rim" was studied under a microscope to determine if any spores were formed.

No spores could be seen when the layer was only a couple of hours old, but when it was left for more than 24 hours, spore formation could clearly be seen. It is important to note that, although the spores were not quantified, they occurred very sparsely. The amount of spores in such a layer would be minuscule if the total volume of a full-scale or even pilot scale reactor is taken into consideration. Nonetheless, to ensure that no problems arise due to spore formation, it would be advisable to include a mechanism in the final design that would prevent the formation of such a layer.

It has been shown in this study that no unusual health hazards were created in the operating environment of the process by the release of viable fungal material from the reactor, or from accidental spillage in the area.

SUMMARY

The MyPro Water Treatment System is a new and exiting development in the field of water treatment. The technology is not yet fully commercialised, but soon the process could be a technically and commercially superior alternative to traditional processes like activated sludge and anaerobic treatment. Like most treatment processes, it is not suited for all effluents, but can be very effective in the right application.

In general, the MyPro system is applicable to any organic process effluent that produces easily to medium biodegradable soluble organic matter. There is no fixed limit on the COD concentration of effluents for which the MyPro System is considered. However, the total COD load of the effluent should be in excess of approximately 2000 kg per day (A minimum amount of biomass has to be produced to justify the costs of the drying equipment). Typical examples of effluents amenable to treatment with the MyPro system are:

- acetic acid containing effluents,e.g. petrochemical and furfural industries
- xylose containing effluents, e.g. black liquor effluent from the pulp and paper industry
- starch containing effluents, e.g. glucose manufacturing industry
- sugar and other carbohydrate containing effluents.

Although the work for this project was concentrated on one effluent only, all the principles are equally applicable to most of the above effluent types. Following are some general comparisons between the MyPro System and more conventional processes like activated sludge and anaerobic processes.

- The first important advantage of the MyPro system is its higher resistance to inhibiting substances in the feed (e.g. furfural and organic acids).

- Aeration requirements are similar to that of the conventional activated sludge process. Although the MyPro system requires theoretically less oxygen due to the shorter sludge ages, more energy is required in practice since the fungal suspension has a higher viscosity than activated sludge.

- Hydraulic retention time of the MyPro System is generally shorter than that of activated sludge processes, although it depends on the rate of oxygen transfer and the presence of inhibitors. However, there is a difference in sludge age. The sludge age of the MyPro system is markedly shorter than that of the activated sludge process. Typically, a sludge age of between 6 and 10 hours will be used. In activated sludge systems, sludge ages of 20 to 48 hours and higher are common.

- No cooling is necessary for the treatment of most industrial process streams with the MyPro System. The organism, *Aspergillus fumigatus* is thermo-tolerant and is still very active at temperatures as high as 51 °C. At temperatures above 35 °C, poor settling is often observed in conventional activated sludge plants treating industrial effluents. The maximum practical operating temperature for anaerobic systems is seldom higher than 38 °C.

- Solids separation is simply achieved through a micro-screen (size-based separation). The biomass in the MyPro system consists of filamentous fungi with relatively large hyphae, making retention of the biomass easy. In both aerobic and anaerobic conventional systems, separation is mostly achieved by gravity (mass-based). Poor aeration, introduction of easily biodegradable organic material (i.e. sugars), and other process variations, often results in settling problems in the activated sludge process.

- In addition, wash-out is also prevented by the micro-screen which means that one of the biggest problems of suspended growth systems is eliminated.

- Unlike in the activated sludge process, no external sludge recycling is necessary in the MyPro system.

- Sludge handling with the MyPro system comprises direct harvesting from the reactor, dewatering through a filter press and drying in an oven. Due to the filamentous nature of the fungi, unusually high filtration rates are possible. The dried biomass is then ready for selling as protein source (or extraction of metabolites), depending on the form the client prefers the material to be in. Sludge from an activated sludge plant has to be thickened, digested, dewatered and composted. Alternatively, it can be dewatered, and landfilled, dried or incinerated.

- The dewatering and drying characteristics of the fungal biomass is far superior to that of activated sludge due to the fungi's filamentous nature.

- The biggest advantage if the MyPro system when compared to activated sludge or anaerobic systems, is that no residue is formed that have to be disposed of. Instead, by selling the biomass, part or all of the treatment costs could be paid for.

Summarised below are the more technical aspects of the process:

- It was possible to select a thermotolerant fungus, *Aspergillus fumigatus*, on the specific effluent used in this study. The organism is able to grow well at 45 °C in suspension, resulting in elimination of any further cooling

of the effluent. This organism has not yet been exploited elsewhere in the world in such an application.

- The nutrient composition has been quantified and optimum concentrations determined. Total costs of nutrient addition per day will be ± R 4000. The value of the protein containing biomass produced daily is approximately R 17 000.

- The control system that was developed for the process, solved various problems: Two alternative operational control modes have been proposed (pH-controlled feed and oxygen-controlled feed) whereby addition of substrate (Sezela-effluent) is regulated by the assimilation rate of the organisms themselves. This resulted in optimum utilisation of expensive nutrients, for example oxygen. The pH controlled feed mode has been studied extensively and the influencing factors of the artificially controlled substrate concentration on mathematical modelling has been identified. The oxygen controlled feed mode has been tested, but its is recommended that further work be done.

- An additional advantage of the controlled feed rate is that the system will "automatically" detect toxic loads and concentrations. The organisms will not be killed as is often the case in conventional treatment plants, but the process rate will only slow down in order to handle the extra load.

- When the MyPro System is applied to high COD concentration effluents, dilution of the incoming feed is necessary. The need for external water for dilution has been eliminated through recycling of the reactor effluent. The volume of the recycling stream could be reduced by enhancing oxygen transfer through injecting pure oxygen. The reactor could also be operated at a slightly higher hydraulic retention time. For some effluents which contain inhibiting substances (i.e the ACE), additional treatment

steps for the recycle stream might be required before it can be used for dilution.

- Bacterial contamination of the reactor content could result in operational problems. The extent of such contamination under various operating conditions was quantified in order to determine which conditions are conducive to excessive bacterial growth. At a pH of 5,0 and 46 °C the reactor could safely be operated with a hydraulic retention time of 3,5 hours. When the temperature was increased to 50 °C, the hydraulic retention time could be increased to 5 hours.

- Feeding studies are being conducted at the University of Pretoria with chicks to determine the nutritional value and possible toxic effects of the biomass when used as feed supplement. Results received so far are promising and indicate a potential value of the biomass between R 800 and R 1000 per tonne. A strict feeding and toxicity testing programme should be implemented since the *Aspergillus* biomass will be subject to public concern over its use as feed.

- Since concern exists regarding the safety of process workers due to possible contamination of the air with spores or viable fungal material, tests have been conducted to quantify the amount of viable fungal material in the environment around the laboratory reactor. No significant amounts of fungal material could be detected in the working area of the laboratory scale process.

- Initial tests showed promising results regarding the production of cellulase from the biomass produced in the process. Additional tests confirmed and improved on the previous results. A maximum enzyme concentration of

7,2 IU was obtained which can be regarded as very good under the specific experimental circumstances.

- The only two possibilities that were explored for commercial exploitation are SCP and cellulase. However, there are strong indications that the biomass can be used in secondary steps for the production and extraction of various other metabolites.

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