Bioethanol Production from Waste Paper through Fungal Biotechnology

THESIS

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PAUL GEORGE VOIGT

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ABSTRACT

Bioethanol is likely to be a large contributor to the fuel sector of industry in the near future. Current research trends are geared towards utilizing food crops as substrate for bioethanol fermentation; however, this is the source of much controversy. Utilizing food crops for fuel purposes is anticipated to cause massive food shortages worldwide. Cellulose is the most abundant renewable resource on earth and is subject to a wide array of scientific study in order to utilize the glucose contained within it. Waste paper has a high degree of cellulose associated with it, which makes it an ideal target for cellulose biotechnology with the ultimate end goal of bioethanol production. This study focussed on producing the necessary enzymes to hydrolyse the cellulose found in waste paper and using the sugars produced to produce ethanol. The effects of various printing inks had on the production of sugars and the total environmental impact of the effluents produced during the production line were also examined. It was found that the fungus *Trichoderma longibrachiatum* DSM 769 grown in Mandel's medium with waste newspaper as the sole carbon source at 28 °C for 6 days produced extracellular cellulase enzymes with an activity of 0.203 ± 0.009 $FPU.ml^{-1}$, significantly higher activity as compared to other paper sources. This extracellular cellulase was used to hydrolyse waste newspaper and office paper, with office paper yielding the highest degree of sugar production with an end concentration of 5.80 ± 0.19 g/l at 40 °C. Analysis by HPLC showed that although glucose was the major product at 4.35 ± 0.12 g/l, cellobiose was also produced in appreciable amounts (1.97 ± 0.71 g/l). The sugar solution was used as a substrate for *Saccharomyces cerevisiae* DSM 1333 and ethanol was produced at a level of 1.79 ± 0.26 g/l, the presence of which was confirmed by a 600 MHz NMR spectrum. It was found that cellobiose was

not fermented by this strain of *S. cerevisiae*. Certain components of inks (the PAHs phenanthrene and naphthalene) were found to have a slight inhibitory effect (approximately15% decrease) on the cellulase enzymes at very high concentrations (approximately 600 μg/l in aqueous medium), while anthracene had no effect. Whole newsprint ink was shown not to sorb glucose. The environmental analysis of the effluents produced showed that in order for the effluents to be discharged into an aqueous ecosystem they would have to be diluted up to 200 times. They were also shown to have the potential to cause severe machinery damage if reused without proper treatment.

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%	percentage
<	less than
>	greater than
°C	degrees Celsius
ADH	alcohol dehydrogenase
ATP	adenosine triphosphate
CBH	exoglucanase
$CDCl_3$	deuterated chloroform
cm	centimetres
CO_2	carbon dioxide
COD	chemical oxygen demand
dB	decibels
DNA	deoxyribonucleic acid
DNS	3,5 - dinitrosalicylic acid
DO	dissolved oxygen
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DWAF	Department of Water Affairs and Forestry
EG	endogluconase
EMP	Embden–Meyerhof–Parnas Pathway
ENO	enolase

US EPA	United States Environmental Protection Agency
Eq.	equation
FAME	fatty acid methyl ester
FBPA	fructose bisphosphate aldolase
FlD	flame ionisation detector
FPU	filter Paperase units
g	grams
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GC	gas chromatography
GOD	glucose oxidase
H^+	hydrogen ions
H_2	hydrogen
H_2O	water
HK	hexokinase
HPLC	high performance liquid chromatography
IWR	Institute for Water Research
l	litres
LC_{50}	concentration of effluent in moderately hard water with a lethality of 50%
M	molar (moles per litre)
mg	milligrams
MHz	mega Hertz
ml	millilitres

mm	millimetres
mM	millimolar (millimoles per litre)
N_2	nitrogen
nm	nanometres
NMR	nuclear magnetic resonance
No.	number
NTU	nephelometric turbidity units
O_2	oxygen
OH^-	hydroxide ions
PAH	poly aromatic hydrocarbon
PDA	potatoe dextrose agar
PDC	pyruvate decarboxylase
PFK	phosphofructokinase
PGI	phosphoglucoisomerase
PGK	phosphoglycerate kinase
PGM	phosphoglyceromutase
pH	- log of the concentration of H^+
PLFA	phospholipid fatty acid
ppm	parts per million
PYK	pyruvate kinase
R	South African Rands
R.S.A.	Republic of South Africa
rpm	revolutions per minute
SAWiC	South African Waste information Centre
sp.	species

TDS	total dissolved solids
TPI	triose phosphate isomerase
UK	United Kingdom
US	United States
US$	United States Dollars
U.S.A.	United States of America
v/v	volume in volume
w/v	weight in volume
w/w	weight in weight
x g	relative centrifugal force
YPE	yeast peptone extract
µg	micrograms
µl	microlitres
µm	micrometres
µmol	micromolar (micromoles per litre)

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Chapter 1: Bioethanol from wastepaper, a literature review

1.1 - Introduction

Fuel production is one of the most important sectors in industry. The effect of fuel prices and availability reverberates throughout different industries in both the developing and developed worlds and is a major factor for sustainable and continued development in the 3rd world (Amigun *et al*, 2008). The realisation of the limited nature of the current fossil fuel reserves (Sheehan *et al*, 1998) and as a consequence the rise in fuel prices, together with the recent environmental awareness movement have led to the development of several alternative fuels such as bio-diesel, bio-hydrogen and bioethanol (Chen *et al*, 2007; van Wyk *et al*, 1999), collectively known as biofuels (Demirbas *et al*, 2007).

Biofuels are defined as liquid or gaseous fuels for the transport sector that are predominantly produced from biomass (Demirbas *et al*, 2007; Robson, 2007). Biofuels have several advantages over conventional fossil fuels. They are renewable in nature and produce substantially lower sulphur and carbon dioxide emissions upon combustion (Demirbas *et al*, 2007).

There are several schools of thought with regard to the development of biofuels. Each has its own distinct advantages and drawbacks. Today, one of the most researched form of biofuel, already in main stream use, is bioethanol (Amigun *et al*, 2008; Robson, 2007; Demirbas *et al*, 2007). According to European standards, ethanol can be used as a petroleum fuel additive in amounts up to 5% for un-modified engines and up to 85% in modified engines (Demirbas *et al*, 2007). In Brazil all cars run on at least a 25% ethanol/petroleum mix, with 60%

of cars being referred to as "fuel flexible", i.e. these could run on up to 100% ethanol (Robson, 2007).

Bioethanol is generally produced by fermentation of different plant-based materials (Malca *et al.*, 2006). As the original plant grows, carbon dioxide is absorbed and processed during photosynthesis and later used to synthesize organic compounds such as cellulose (Cambell *et al*, 2003). When these plant tissues are used for bioethanol production the carbon fixed during photosynthesis is re-released as carbon dioxide during ethanol fermentation and successive bioethanol combustion in the engines of transport vehicles (McMillin, 1997; Quinteroa *et al*, 2008). The amount of carbon dioxide absorbed and then re-released is equal, making bioethanol a carbon-neutral fuel (Quinteroa *et al*, 2008; Pfeffer *et al*, 2007). Fossil fuels have a positive net carbon dioxide emission and are considered the biggest contribution by humans to climate change (Quadrelli *et al*, 2007). Bioethanol also has low toxicity, volatility and photochemical reactivity, resulting in less smog production as compared with conventional fossil fuels (Aristidou *et al*, 2000).

Bioethanol is produced by the fermentation of sugars such as glucose (Demirbas *et al*, 2007) which abound in all plant life. Many of these sugars are readily fermentable into ethanol e.g. D-glucose. However, a significant portion of these sugars, including glucose, is locked in lignocellulosic materials consisting of lignin, cellulose, and hemicellulose. Cellulose is a homogenous D-glucose polymer which accounts for 35-50% dry weight of agricultural lignocellulosic biomass (Agbogbo *et al*, 2006). The chemical and crystalline structures of lignin and cellulose make them highly resistant to biodegradation (reference), but they

still constitute a large pool of potential raw material for bioethanol production. . Thus the implementation of processes to hydrolyse the lignocellulosic materials is necessary in order to maximise yield while reducing costs (Demirbas *et al*, 2007; Aristidou *et al*, 2000; Chen *et al*, 2006). Already many industrial plants exist for the production of bioethanol in countries across the world, with Brazil currently being the world leader in the industry due to limited internal fuel resources and the fuel crisis of the 1970's (Demirbas *et al*, 2007; Robson 2007).

Development of successful biofuel production processes requires striking a balance between ecological preservation, practicality and acceptance of the alternative fuel by the general public, i.e. the ultimate end users (Demirbas *et al*, 2007; Charles *et al* 2007). In other words, a successful biofuel cannot compromise on performance, as compared to traditional fossil fuels, but must provide significant ecological advantages. Also, the energy balance must be positive in order for the production of biofuels to be feasible, i.e. the energy obtained from combustion of the biofuels must exceed the energy required for their manufacture..

1.2 - Common types of biofuels and production strategies in brief

1.2.1 - Bio-hydrogen

Bio-hydrogen is potentially a highly effective alternative fuel for the future (Kotay *et al* 2008; Jianzheng *et al* 2008, Manish *et al*, 2008). It can be used as a combustion fuel or in electrolytic energy producing fuel cells (Kotay *et al*, 2007; Yamanaka *et al*, 2008). It has the highest amount of energy out of any of the gaseous fuel sources at 143 GJ ton^{-1}, and does not produce any green house gases during combustion (Kotay *et al*, 2007). However, there are still issues with

regards to the production of hydrogen for use as a fuel, including the production of carbon dioxide and other green house gases at the production stage of the gas in both biological and chemical systems (Younesi *et al*, 2007) and the release of water vapour on combustion which has been shown to have climate change effects (Tabazadeh *et al*, 2004).

Two main branches of hydrogen production exist, namely chemical and biological. Biological production is currently receiving the most attention as it is regarded as the most environmentally friendly approach due to a substantially lower energy demand for production and fewer toxic waste products (Younesi *et al*, 2007; Kotay *et al*, 2007; Pan *et al*, 2008). There two approaches within the biological production of biohydrogen, namely light-driven and anaerobic processes. The first type comprises of the light-driven processes, which involve the photosynthetic breakdown of H_2O into H_2 and O_2 by certain species of blue-green algae (Jianzheng *et al* 2008). Problems with this method exist in that the design of an efficient bioreactor with ample sun-light absorbing properties is relatively difficult from the engineering point of view and presents scale-up issues (Liu *et al*, 2008).

The second biological approach is anaerobic fermentation, utilizing organic hydrogen sources (Jianzheng *et al*, 2008). The process involves the enzymatic breakdown of organic materials by means of the hydrogenase enzymes, with H_2 being produced during oxidation of organic substrates by a fermenting organism (Kotay *et al*, 2008), such as *Ruminococcus albus*, a bacterium found in the rumen of ruminants (Ntaikou *et al*, 2007). Substrates used include wastewaters, cellulosic materials, animal by-products and sugars (Kotay *et al*, 2008). The

design and practicality of bioreactors focusing on this means of biohydrogen production is an improvement in comparison to light-drive reactors, due to the limitations presented with light-drive reactors on an industrial scale (Pan *et al.*, 2008; Liu *et al*, 2007).

There is currently a significant amount of research being conducted in biohydrogen production, focussing on the microbiology and genetics involved with the end goal of a reliable hydrogen fuel industry (Liu *et al*, 2007). Also from a practicality stand point, a lot of development in efficient technology to store and run off hydrogen fuel is taking place (Kotay *et al*, 2007). Several problems with the production of a commercially viable hydrogen fuel still exist; a highly energy intensive, high costs, and potentially environmentally detrimental processes need attention (Kotay *et al*, 2007). In a microbiological process, the development of large scale hydrogen plants still presents problems, with current technology still far better suited to small, de-centralised systems (Kotay *et al*, 2007). A major concern with biohydrogen production is the highly inflammable nature of hydrogen gas, the handling of which requires specialised equipment. Also, these processes are largely anaerobic where aerobic processes are always preferable (Krupp and Widmann, 2009).

1.2.2 - Bio-diesel

Biodiesel has gained an enormous amount of publicity and social acceptance over the last few years. The follow is a commonly applied definition of biodiesel, but other, more stringent definitions can always be found in the literature. Biodiesel is commonly defined as fuel of high enough quality to be used in diesel engines, consisting of fatty acid methyl and ethyl esters, the

sources being either animal fats or vegetable oils (Lapuerta, Rodríguez-Fernández, Agudelo, 2008). This is largely due to the relative ease at which used cooking oil can be converted into a suitable bio-diesel, and hence the cheapness as compared to fossil fuels and the perceived environmental advantages (Lapuerta, Rodríguez-Fernández, Agudelo, 2008). There are many home made bio-diesel recipes, common place on the internet (Utah Biodiesel, 2005; Journey to Forever, 2008). Also, if prepared correctly, conventional diesel engines need no modification to run on bio-diesel (Demirbas *et al*, 2007). However, as a sustainable, efficient fuel there are some underlying problems.

Three main options are available for the production of bio-diesel. These are unmodified vegetable oil, a mixture between vegetable oil and fossil fuel, and the conversion of vegetable oil into the fatty acid methyl esters. The latter is considered "pure" bio-diesel, the use of which requires absolutely no modification to an existing diesel engine. Unmodified vegetable oil has been shown to fuel engines with slight engine modification; however there are several drawbacks. Used cooking oil is a popular starting point for biodiesel users due to its low cost (Lapuerta, Rodríguez-Fernández, Agudelo, 2008), however, this contains high levels of impurities, introduced through the cooking of food, and the viscosity of such oils is very high, ranging from 27.2 to 53.6 mm^2/s (Demirbas *et al*, 2007), thus causing ignition/starter problems at lower temperatures. At the same time, the combination of high impurities and high viscosity run the risk of engine clogging and result in poorer performance from the engine. However, on the positive side, it is extremely cheap to run (Lapuerta, Rodríguez-Fernández, Agudelo, 2008), but with the costs of repairing a clogged engine, the cost benefit is highly debateable.

The second option is mixing of the vegetable oil with petroleum products, such as conventional diesel fuel, or other organic fuel sources, such as bioethanol (Lu *et al*, 2008). Although this doesn't eliminate the need for fossil fuels, it will certainly lower the pollution, and extend the viability of existing fossil fuel reserves (Demirbas *et al*, 2007). However, this still facilitates the need to rely on fossil fuels, which is not the desired outcome for biofuel production.

The ideal approach to bio-diesel is by performing trans-esterification and methylation of the fatty acid chains to form fatty acid methyl esters (FAMEs; Lapuerta, Rodríguez-Fernández, Agudelo, 2008, Demirbas *et al*, 2007). The viscosity is considerably lower than that of the un-methylated oil, ranging from 3.59 to 4.63 mm^2/s and has a higher and more predictable rate of energy release during combustion (Demirbas *et al*, 2007). However, for optimal quality, virgin vegetable oil or fat is required as used cooking oil often contains large amounts of free fatty acids, which are difficult to convert to bio-diesel. In 2007, the production of bio-diesel from fats and oils was more expensive than fossil fuels, estimated at US$ 0.50 per litre while those produced from waste oil is estimated to US$ 0.34 per litre, versus current fossil fuel prices of approximately US$ 0.20 per litre (Demirbas *et al*, 2007). However, in mid 2008 the price of diesel has risen to US$ 1.19 per litre in the United States (Washington Post, 2008), and US$0.89 per litre in South Africa for April (Department of Minerals and Energy, 2008). Sources for these oils include palm, soya, canola and other food crops, potentially causing social problems (Zheng *et al*, 2007), and more recently microalgae, which shows much promise (Christi, 2007).

Although suitably prepared bio-diesel is efficient and more environmentally friendly than conventional fossil fuels, there is still much work to be done on biodiesel to overcome certain issues, such as a lower heating energy and the formation of potential engine clogging materials (Lapuerta, Armas, Rodrîguez-Fernàndez, 2008). Currently, there is significant research in the area of bio-diesel to eliminate some of the drawbacks, while maintaining renewability of the production process, and it is likely to be one of the alternative fuels to find mainstream use in the future.

1.3 - Bioethanol

1.3.1 - Bioethanol production

Bioethanol is produced by the conversion of organic polysaccharides and monosaccharides, such as cellulose and glucose respectively, into ethanol (Kim *et al*, 2004). The starting substrate is generally food crops, such as wheat, maize or sugar cane, or other plant materials with a high content of fermentable sugars (Kim *et al*, 2004; Charles *et al*, 2007; Demirbas *et al*, 2007). This is known as first generation biofuel production (Charles *et al*, 2007). However, using food crops as a substrate has its own controversies and drawbacks. There is much speculation that an increase in the use of food crops for the production of bioethanol could result in a worldwide increase in food prices and ultimately lead to a global food shortage (Charles *et al,* 2007). There are also questions arising as to the validity of the proposed economic benefits of growing food crops for bioethanol production, as this impedes on food production which would have long term detrimental effects (Charles *et al*, 2007). Second generation biofuels, i.e. those produced from non-food crops; create less concern with regards to undermining food production (Charles *et al*, 2007). There are

also environmental challenges that need to be met, including degradation due to agricultural run-off and a loss of bio-diversity surrounding the agricultural land (Charles *et al*, 2007; Rowe *et al*, 2007). The growth of lignocellulosic crops for energy use potentially has better environmental impact characteristics as compared to arable farming; however there is still an environmental effect in relation to undisturbed land (Rowe *et al*, 2007). In spite of these issues, scientific, as well as public opinion is in favour of continued development in bioethanol to produce a renewable, cleaner fuel while minimising the environmental impacts of fuel crop farming and eliminating the need to utilize food crops (Rowe *et al*, 2007; Demirbas *et al*, 2007; McMillan, 1997).

The starch and cellulosic materials in plant matter can be converted into fermentable sugars, such as glucose and xylose (Demirbas, 2007). This is achieved either by enzymatic degradation or chemical processes, namely acid hydrolysis (Spano *et al*, 1976). These sugars are then fermented into ethanol through enzymatic processes which are illustrated in figure 1.1. The result is bioethanol, a clean burning, carbon neutral, organic molecule with a high energy content suitable for energy related needs (Shapouri *et al*, 1995; McMillan, 1997).

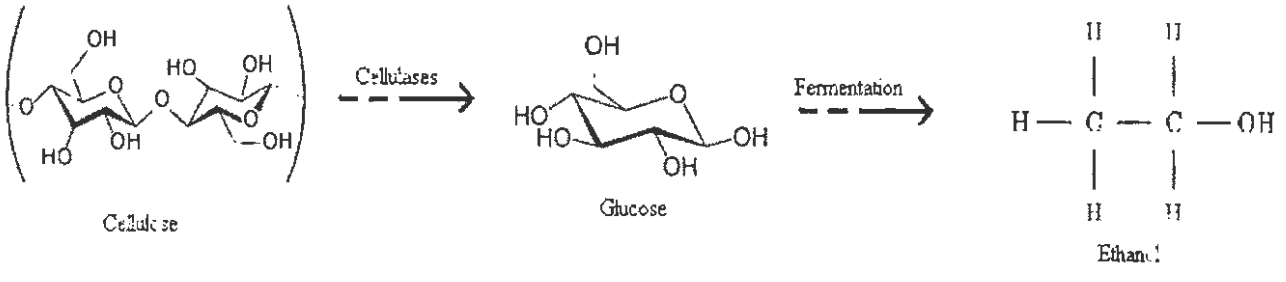


Figure 1.1: Schematic breakdown of cellulose into ethanol.

Currently the price of standard, wholesale gasoline fuel is approximately US$ 0.71 per litre as of April 2008 (Department of Minerals and Energy, 2008), which is considerably cheaper than bioethanol, still estimated at approximately US$ 1.00 per litre for 2008 (US Department of Energy, 2008). However, future incremental improvements aim to produce bioethanol, in the midterm, for as low as US$ 0.43, with long term estimations at US$ 0.21 per litre (US Department of Energy, 2008). At present, the major obstacles in the production of low cost bioethanol are the relatively high costs of enzyme production, half of which is dictated by costs of suitable substrates with which to form enzymes to degrade lignocellulosic materials, which are, themselves, very cheap (Sassner *et al*, 2008).

While there are definite drawbacks to all the biofuels currently being researched, and there will continue to be debates as to the most viable replacement of fossil fuels, it is still important to be researching all possible avenues of providing sustainable energy (Faaij, 2006). Ultimately the more routes explored, the more experience and knowledge there is to draw on when the time comes to completely replace fossil fuels. However, to produce a sustainable energy supply, a renewable starting material is needed; starch and cellulosic materials could be the answer. There are many research groups and approaches to utilizing cellulosic material. Some of these can be found below.

1.3.2 - Cellulose exploitation

Cellulose is the most abundant renewable resource on Earth (Aristidou *et al*, 2000) and approximately 100 billion tonnes are produced globally each year

(Spano *et al.*, 1976). Cellulose consists of glucose units linked through β-1, 4-glycosidic bonds (Cambell *et al*, 2003), shown in figure 1.2, and is a major constituent of plant cell walls (Williamson *et al*, 2002). Cellulosic materials contribute a massive proportion to household and industrial wastes annually (Bayer *et al*, 2007).

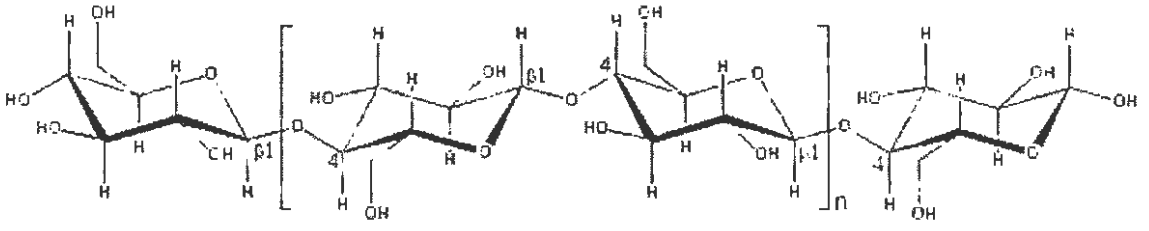


Figure 1.2: Cellulose structure

The conversion of cellulose into other utilizable molecules, such as pharmaceutical compounds and animal feeds, is already a fairly common practice (Pandey *et al*, 2007). It is used as a practical experiment for undergraduate university students as a demonstration in bioconversion (van Wyk *et al*, 1999), the production of pharmaceutical components, and of course in many organisms such as termites and cows, whereby microorganisms contribute in the production of cellulase enzymes (Mauldin *et al*, 1972).

In the quest for an economical and efficient utilization of cellulose, two distinct approaches have been investigated; that of a chemical nature, i.e. acid hydrolysis, and that of an enzymatic nature (Spano *et al*, 1976). Acid hydrolysis makes used of, often, concentrated acids and reaction temperatures of 120 °C to 250 °C (Wei *et al*, 2009). As can be seen, acid hydrolysis requires expensive

corrosion proof reaction vessels, a higher reaction temperature as compared with an enzymatic approach, which can result in the degradation of the desired product, and results in a higher volume of industrial effluent (Spano *et al*, 1976). The enzymatic approach is better suited for industrial use based on the following argument. The enzymes responsible for cellulose degradation are known as cellulases and are split into two major groups, endoglucanases and cellobiohydrolases (or exoglucanases), both groups attack the β-1,4-glycosidic bonds of the cellulose macromolecule, with a minor group, the β-glucosidases, each group consists of several smaller groups (Crennell *et al*, 2002). Using an enzymatic approach, the potential for fermentation cultures presents itself, and hence renewability and reduced cost. Ultimately, this method is what is used in the brewing industry. The enzymatic hydrolysis has clear advantages with respect to industrial application/production.

1.3.3 Enzyme systems

There are several enzymes and enzyme families needed to produce bioethanol from cellulosic materials in an economically viable way. Of first consideration is the degradation of the cellulose into fermentable sugars, such as glucose. In an enzymatic system this is achieved through the water soluble cellulase enzymes (Spano *et al.*, 1976; Demirbas, 2007; Bayer *et al*, 2007). This first step is vital as the direct conversion of cellulose into ethanol is not necessarily the most economic approach due to the rigidity of the cellulose molecule and the specificity of the enzymes required to hydrolyse the cellulose molecule.

Within the family of cellulase enzymes several groups exist, each with its own distinct role in the bioconversion process. Several groups of cellulase enzymes

are often found within one organism, but most organisms do not provide a viable solution for industrial scale production (Solovyeva *et al*, 1997; Shen *et al*, 2004). For instance, it is noted that although *Trichoderma longibrachiatum* is reported as having very high endo and exo gluconase activities, and the enzyme system produced is the most efficient, single organism cellulase system in use (Ahamed *et al*, 2008), the overall hydrolysis of lignocellulosic material is increased when supplemented with β-glucosidases from other species (Maheshwari *et al*, 1994). When this is the case, a cocktail of different enzymes or organisms can be used, where appropriate as illustrated by Maheshwari *et al* (1994) and Gutierrez-Correa *et al* (1999).

There are several choices presented to the biotechnologist when deciding on which organism's cellulase system to use. Several highly researched cellulase producing organisms, both bacterial and fungal, include; *Rhodothermus marinus* (Crennel *et al*, 2002), *Penicillium decumbens* (Mo *et al*, 2004) *Fusarium solani* (Wood *et al*, 1977), *Aspergillus niger* (Solovyeva *et al*, 1997; Lee *et al*, 2007), *and Trichoderma longibrachiatum* (Esterbauer *et al*, 1991; Liming *et al*, 2004; Ahamed *et al* 2008), previously known as *T. reesei* (Simmons, 1977). Each species produces subtly different variations of the enzyme systems. For example, *Aspergillus niger* is reported as having a high cellobiase activity (Shen *et al*, 2004), whereas *Trichoderma longibrachiatum* is reported as having high endogluconase and exogluconase activity (Sternberg *et al*, 1982).

In industrial processes, the cellulase system from *Trichoderma longibrachiatum,* a non-pathogenic filamentous white rot fungus, has emerged as the preferred standard (Margolles-Clark *et al*, 1997; Lee *et al*, 2007; Ahamed *et*

al 2008), with applications varying from textile manufacture to biofuel production (Ahamed *et al,* 2008). This is due to the high quality and efficiency of the cellulase complexes produced by *Trichoderma longibrachiatum*. Genetically modified strains of this fungus produce extremely high levels of extracellular cellulase enzymes.

1.3.4 - Mode of action of the cellulase enzymes

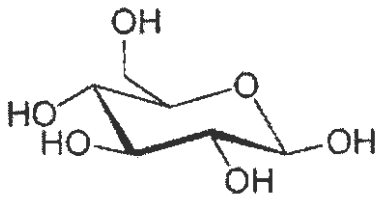
Cellulases are members of the glycoside hydrolase family of enzymes (Hirvonen *et al*, 2003). This family of enzymes is responsible for hydrolyzing oligosaccharides and polysaccharides. Within the family there are three major components of cellulase enzymes, namely endogluconases (EG), exogluconases (CBH) and β-glucosidases, such as cellobiase (Martins *et al* 2008; Ahamed *et al*, 2008), which work in synergy to complete the hydrolysis of cellulose into glucose (Shen *et al*, 2004; Solovyeva *et al*, 1997).

The cellulase enzymes, as a whole, work by cleaving the β-1,4 glycosidic bonds between the glucose units that comprise the cellulose macromolecule. Each different cellulase enzyme acts through subtly different modes of action. For example, the exogluconase, CBH 1, from *Trichoderma longibrachiatum* acts by removing distinct sections of cellulose along the entire length, whereas the endogluconase, EG II , also from *Trichoderma longibrachiatum* has a "smoothing effect" on the cellulose strands by hydrolysing loose chains (Lee *et al*., 1999). It is noted that the EGs act randomly and increase the number of free cellulose chain ends, opening up the availability for the more specific exogluconases (Ramos *et al*, 1999). It has been noted that the CBH enzymes penetrate the cellulose surface and separates the microfibrils creating exposed

ends for the EG enzymes to attack.

This results in the formation of, mostly, glucose, xylose and cellobiose, shown in figure 1.3, with glucose being the major product (van Wyk *et al*, 1999; Spano *et al*, 1976). Xylose, a pentose sugar, which originates from a relatively small quantity of hemicellulose associated with cellulose, and glucose, a hexose sugar, the β(1-4) linking of which forms the cellulose macro structure, are fermentable sugars, and hence can be converted to ethanol with further enzymatic activity. However, cellobiose, a glucose diamer, is also produced in appreciable quantities and a build up of this compound in the cellulose fermentation media results in an inhibitory effect on the cellulase enzymes, resulting in a poorer yield of the fermentable sugars (Sadana *et al*, 1983; Shen *et al*, 2004; Ahamed *et al*, 2008). The enzyme group responsible for the hydrolysis of cellobiose into glucose is the β-glucosidase group. These enzymes act by hydrolyzing β-linkages between D-gluco configured molecules. They have no action against highly ordered celluloses; however they do hydrolyze non-ordered cellulose, such as cellulose pre-treated with inorganic acids (Sadana *et al*, 1983).

a)



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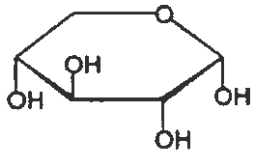

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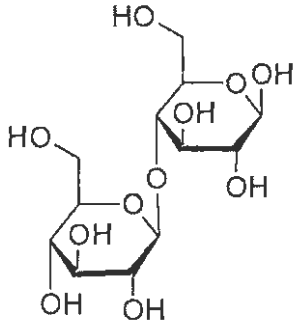


Figure 1.3: Products of cellulose hydrolysis. a) glucose, b) xylose, c) cellobiose

As the cellulose molecules cannot pass into the fungal or bacterial cells, the cellulase enzymes are secreted extracellularly. In the extracellular environment, the cellulases hydrolyse the cellulose into smaller carbohydrate units which may pass into the cells to provide nutritional requirements of the organism in question.

Although *Trichoderma longibrachiatum* is the preferred industry standard for cellulase production, the β-glucosidase makes up approximately 1% of this organisms total cellulase system. Although this is sufficient to satisfy the nutritional requirements of *Trichoderma longibrachiatum* (Sternberg *et al*, 1982), in a bioreator system this can result in a build up of cellobiose, potentially

resulting in a less than ideal glucose production level and rate due to the inhibition characteristics of cellobiose on the cellulase enzymes (Shen *et al*, 2004). In order to compensate for this, several researchers have experimented with introducing either β-glucosidase from another organism (Ortega *et al*, 2001) or by running a mixed culture (Maheshwari *et al*, 1994; Gutierrez-Correa *et al*, 1999).

If mixed cultures are under consideration, they need to be carefully analysed before being implemented. Of noted importance is the determination of strain compatibility and synergism between the organisms in question. The β-glucosidase from *Aspergillus niger* is often used as a supplementation to the cellulases from *Trichoderma longibrachiatum* in order to increase the efficiency of a desired process (Ortega *et al*, 2001; Berlin *et al*, 2005; Chen *et al*, 2007). A mixed culture of *Aspergillus niger* and *Trichoderma longibrachiatum* has been reported as successful (Gutierrez-Correa *et al*, 1999) not only improving the overall activity of the cellulase system, but also by improving the overall economics of the cellulase production process on nutritionally poor substrates by increasing the quantity of glucose produced (Gutierrez-Correa *et al*, 1999). A similar result was reported by Maheshwari *et al* (1994). This is an interesting avenue to explore when trying to maximise the activity of the industrial cellulase system in question.

All components of the cellulase system work in synergy and optimising the balance between the different components is of great significance in the attempts at optimising the efficiency of the produced enzyme system (Gutierrez-Correa *et al*, 1999). Depending on the economic profiles desired, this could entail using

natural, high enzyme producing organisms, genetically modified organisms, mixed cultures or mixed enzyme broths.

1.3.5 - *Trichoderma* sp. physiology:

The *Trichoderma* genus belongs to the fungal group *Deuteromyces* (Montenecourt *et al*, 1977). It is a prototrophic, eukaryotic fungus and is involved in the decay of organic compounds. *Trichoderma longibrachiatum* is known as a white-rot fungus and its extracellular excretion of highly efficient cellulase enzymes has led to significant research in feed stock, paper and pharmaceutical industries (Ahamed *et al*, 2008). More recently attention is being paid to this fungus and its cellulase enzymes for its potential in the biofuel industry (Ahamed *et al*, 2008, Liming *et al*, 2004).

Growth takes place at the apex, creating long hyphae with branching occurring at the side. Septa divide the heterokaryotic cells (Montenecourt *et al*, 1977). Reproduction occurs through heavily branched conidiophores; as yet no sexual reproductive cycle has been reported. Phialides are produced individually or in clusters (Montenecourt *et al*, 1977). Green, single celled conidia are associated in small terminal lumps. (Montenecourt *et al*, 1977).

The reason *Trichoderma longibrachiatum* has received so much attention is due to the production of a highly efficient cellulase system. In the presence of cellulosic materials and certain other inducers, cellulase enzymes are produced; with relevant gene expression being regulated by catabolic repression and end product inhibition (Esterbauer *et al*, 1991). The purpose of this enzyme system is to produce glucose to fulfil the fungus's nutritional carbon requirements. Much

research has been conducted into the optimal conditions for cellulase production with variables ranging from temperature and pH to carbon source, carbon-nitrogen balance and the addition of excipients to enhance the production and activity of the enzymes (Ahamed *et al*, 2008).

1.3.6 - Ethanol fermentation

Humans have been performing this biotechnological process for centuries through brewing of alcoholic beverages and medicaments (Yun *et al*, 2007; Katahira *et al*, 2008). The production of ethanol requires a fermenting organism; the most commonly used is the yeast *Saccharomyces cerevisiae* (McMillan, 1997). Glucose, the major product of cellulose hydrolysis is a fermentable sugar; however, various other minor products which originate from the hemicellulose portion of lignocellulosic materials, such as xylose, are not as easily fermented. In order to increase the overall production of ethanol fermentation, various genetic modifications have been performed on strains of *Saccharomyces cerevisiae* (Katahira *et al*, 2008). The mechanism of ethanol production by *Saccharomyces cerevisiae* is a glycolysis reaction, following the Embden–Meyerhof–Parnas Pathway (EMP). One molecule of glucose is converted into two molecules of pyruvate. In anaerobic conditions, the pyruvate is further metabolised to yield ethanol and CO_2. This process is illustrated in figure 1.4. The ethanol production is a by-product of the production of ATP required for cell growth. As such, there is a close relationship to biomass growth and ethanol production. (Bai *et al*., 2008).

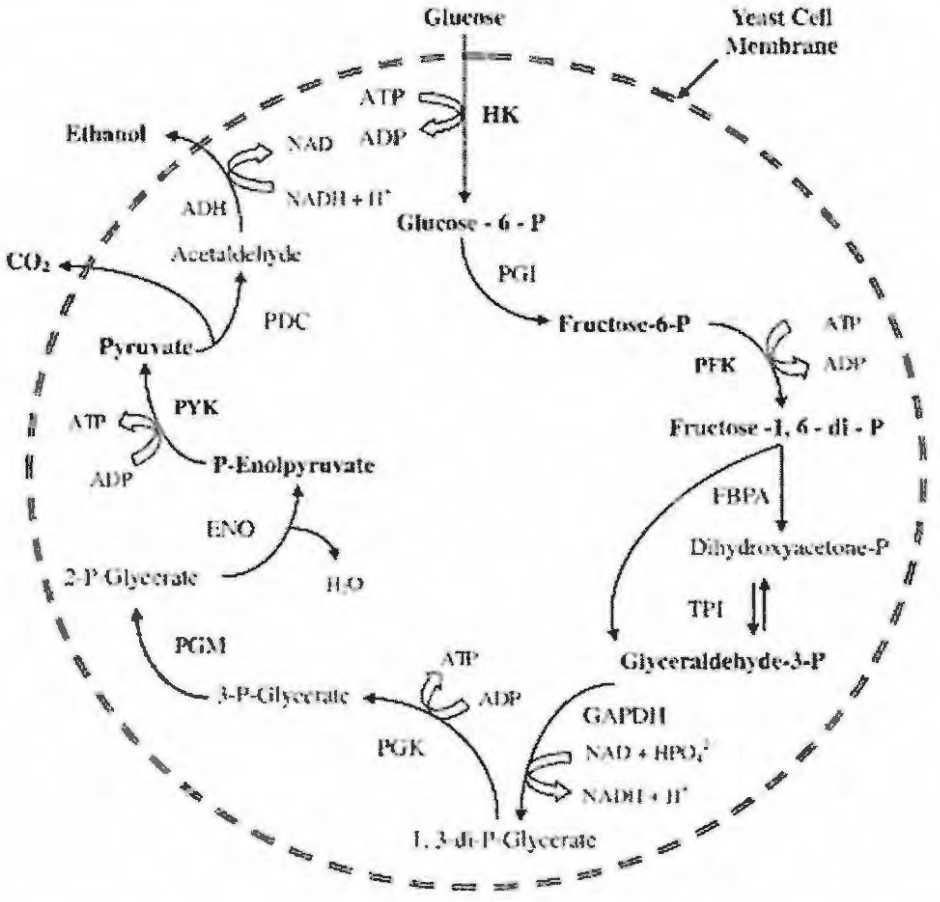


Figure 1.4: Metabolic pathway of ethanol fermentation in *S. cerevisiae*. Abbreviations: HK: hexokinase, PGI: phosphoglucoisomerase, PFK: phosphofructokinase, FBPA: fructose bisphosphate aldolase, TPI: triose phosphate isomerase, GAPDH: glyceraldehydes-3-phosphate dehydrogenase, PGK: phosphoglycerate kinase, PGM: phosphoglyceromutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase. Obtained from Bai *et al* (2008)

1.3.7 - *Saccharomyces* sp. physiology

Saccharomyces sp. is a yeast, capable of producing fuel grade ethanol (Katahira *et al*, 2008). In the biofuel industry, and many other industries requiring the fermentation of sugars to ethanol, *Saccharomyces cerevisiae* is the preferred yeast to be used in the fermentation process (Bai *et al*, 2008). Yeasts are single celled organisms ranging in size and shape, *S. cerevisiae* occurs as globose to

ellipsoidal in shape. The size and shape often varies within a culture (Pelczar *et al*, 1965). The main route of reproduction is through bud formation once a cell has reached a critical size. The bud separates from the mother cell to form daughter cells (Hartwell, 1974). This is an asexual process; however there is also a sexual aspect to *S. cerevisiae* reproduction by the forming of ascospores (Pelczar *et al*, 1965).

The cell wall consists of glucan and mannan, two polysaccharides, together with protein and lipid portions. The cell membrane has a thickness of approximately 80 Å (Pelczar *et al*, 1965), the osmotolerance of which will determine the capacity of the cell to survive in higher concentrations of produced ethanol. Ethanol, although the desired metabolic product of *S. cerevisiae*, can have negative effects on cell viability, growth and fermentation potential, when concentrations are too high (Wang *et al*, 2007). Generally, an ethanol concentration of 10 % v/v is sufficient to halt cell growth and viability (Arai *et al*, 2009). As an attempt to maximise productivity, a strain with a high ethanol tolerance is desired (Hirasawa *et al*, 2007).

1.3.8 - Suitable substrates for fermentation:

As previously mentioned, most starting materials used for bioethanol production are food crops such as sugar cane and corn sugars (Kim *et al*, 2004; Charles *et al*, 2007; Demirbas *et al*, 2007), but these have ethical considerations attached to them. Previously, the focus for ethanol fermentation was on the sugars and starch portions of the crops, resulting in large amounts of plant matter wastes. Also, a vast amount of agricultural wastes are produced globally each year (Chen *et al*, 2007). This plant matter consists, mostly, of lignocellulosic

materials, a target for cellulase biotechnology. This has led to research and development focussing on using the baggase from sugar mills and other agricultural industries, wastes and other sources for lignocellulosic hydrolysis and biofuel production (Chet *et al*, 2007).

Countries in the tropics supporting biofuel programs focus more on sugar cane as the starting material due to its very high energy content and ease of growth in tropical regions (Quintero *et al*., 2008), whereas programs in more temperate regions focus on maize crops (Quintero *et al*, 2008). Maize crops, although easier to grow in temperate regions, have a lower energy output to energy input ratio as compared with sugar cane, and as such require larger crop land to produce the equivalent volume of bioethanol.

A high source of cellulosic material that is often overlooked in the production of biofuel is waste paper. Paper primarily consists of cellulose, which gives it its rigidity and strength (van Wyk *et al*, 1999). Although there are presently many paper recycling programs worldwide, based a review of available literature, there is a very limited biofuel industry associated with waste paper. Paper accounts for approximately 20% of all household wastes in the UK, and amounts to approximately 4 kg of paper waste per household per week (Waste Online, 2004). South Africa has a similar waste per capita figure to the UK (SAWiC, 2006), and as such, similar paper disposal habits are likely to be present. Conventional paper recycling is based around the notion of re-use (Pèlach *et al*, 2003). Interestingly enough, cellulase enzymes have been researched with regards to the de-inking of waste paper (Pèlach *et al*, 2003; Lee *et al*, 2007). The suggested mode of action is that the cellulases cause ink detachment, however

there are several contradictory explanations (Pèlach *et al*, 2003).

A life cycle analysis study performed by Finnveden *et al*, (1998), suggests that, contrary to popular belief, incinerating waste paper is potentially less environmentally detrimental than recycling it, provided that no fossil fuels are used in the incineration process. Also, the quality of recycled paper versus the economic and environmental costs is less than ideal, mostly finding use in art products, packaging and toilet paper (Ikeda *et al*, 2006). The continued recycling of paper results in a shortening of the fibre lengths, ultimately resulting in unusable paper products (Ikeda *et al*, 2006).

This combination between high cellulose content and gaps in the current recycling industry, not to mention the lessened ethical considerations as compared to food crops, suggest enormous potential for the recycling of waste papers into biofuels. Previous enzymatic studies suggest that paper has a very high yield of glucose when the cellulose contained within is hydrolysed into glucose (Spano *et al*, 1976; van Wyk *et al*, 1999).

1.4 - Possible toxicity issues and effects of ink

The majority of paper products discarded as waste have been in contact with inks through the process of printing. These could have potential harmful effects, both for the fermentation organisms as well as humans and the environment by way of toxic industrial effluent. Inks generally consist of some sort of pigment together with other excipients such as surfactants, solvents etc. One of the most common pigments found in black inks is carbon black [CAS #1333-86-4] (Jin *et al*, 1987; Lin *et al*, 2002), which has been used for centuries (Tsai *et al*, 2002).

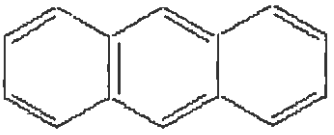
Carbon black is a very fine, black powder, consisting of 90 – 95% elemental carbon (Jin *et al*, 1987; Tsai *et al*, 2002) and is produced by the partial thermal degradation of hydrocarbons (Tsai *et al*, 2002). Due to the nature of manufacture, there are several types of compounds associated with the carbon black itself, such as trace amounts of Polycyclic Aromatic Hydrocarbons (PAH; Tsai *et al*, 2002) and Nitroarenes (Jin *et al*, 1987), both of which are known human carcinogens and potentially mutagenic (Jin *et al*, 1987; Tsai *et al*, 2002). Due to the mutagenic activity of these compounds there is a possibility of fungal mutation or loss of enzymatic activity. There is also the human health issue, especially for people that would be living near or working at an industrial plant dealing with inks.

PAHs are hydrocarbons consisting of at least two ring structures and can contain several rings. Several PAHs are known for producing diol epoxides which form covalent bonds with DNA, possibly causing cancer. The most likely sites to be affected by the PAHs are in areas with rapidly proliferating cells (DHHS/ATSDR, 1995). Reports suggest that workers exposed to PAHs most frequently develop cancer in lung, kidney, urinary tract, and gastrointestinal tissues (National Academy of Sciences, 2003). The aerobic and anaerobic degradation of PAHs have been examined in the context of detoxifying wastes (Chang *et al*, 2003).

PAHs are detected using Gas-Chromatography Mass-Spectrophotometry, and are commonly assessed for environmental and human health issues (Gerhartz, 1995). Some examples of common environmental PAH pollutants can be found in figure 1.5. Due to the nature of PAHs, it is possible for there to be some

influence on the activities of certain enzymes, including the cellulases.

a)



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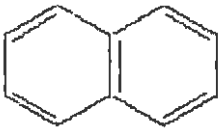

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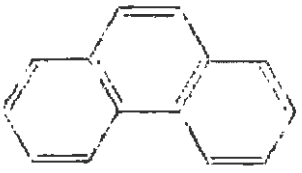


Figure 1.5: PAHs studied. a) Anthracene, b) Naphthalene, c) Phenanthrene

1.5 - Conclusion and aim for future research

Bioethanol is likely to become a major source of energy in the future. There is an abundance of utilizable starting material, both specifically grown for bioethanol production, and wastes. The enzymatic hydrolysis of the cellulose in paper, a major constituent of waste, and subsequent fermentation of the resultant sugars into ethanol could potentially supplement the world's future bioethanol requirements. By utilizing wastes for the production of ethanol, the current high costs of production could be lowered. The development of a low cost laboratory procedure focussing on the production of bioethanol could potentially strengthen biotechnology development in the African context. The proposed areas of study

addressed in this thesis will be the production of cellulase enzymes, the subsequent sugar production, the impact inks could have on the production of sugars from waste paper, the production of bioethanol and the environmental effects to be expected if scale-up is to be considered.

Chapter 2: Enzyme production

2.1 - Introduction

In order to produce fermentable sugars from waste paper, cellulose, which is the main component of its structure, must be hydrolysed (see section 1.3.3). As discussed in section 1.3.1 (Bioethanol production) and 1.3.2 (Cellulose exploitation), this can be achieved through chemical or enzymatic hydrolysis using cellulase enzymes. Enzymatic hydrolysis is potentially more economically favourable due to elimination of costs associated with energy inputs of production and acid proofing of the reaction vessels (see section 1.3.2).

As discussed in section 1.3.3 (Enzyme systems), the enzymatic hydrolysis is carried out by a group of enzymes collectively known as cellulases. These enzymes work synergistically to hydrolyse the complex cellulose molecules into smaller carbohydrates such as cellobiose and glucose, the major product of cellulose hydrolysis (see section 1.3.4). These smaller carbohydrates are readily fermentable into ethanol by several organisms, such as *Saccharomyces cerevisiae*; see section 1.3.6 (Ethanol fermentation).

The cellulase system most commonly used in industrial applications is obtained from *Trichoderma longibrachiatum*, a non pathogenic filamentous fungus (see section 1.3.5). This cellulase system is considered to be one of the most complete cellulase systems found in a single organism, i.e. it has naturally high levels of all the cellulase components in comparison to other organisms capable of cellulose degradation.

Genetically modified strains of *T. longibrachiatum* can produce extremely high levels of extracellular cellulase (see section 1.3.3).

Purchasing purified enzyme extract is one approach to obtain this powerful cellulase cocktail; however, this is extremely expensive, with purified cellulase from *T. longibrachiatum (reesei)* priced at approximately R600 per gram (Sigma, 2008). A more economically sound approach would be to produce the enzymes in-house by the microbiological cultivation of *T. longibrachiatum*, costing approximately R520 for a lyophilised culture (DSMZ, 2009), with the ability to make an infinite supply of enzymes.

In order to produce viable cellulase enzymes, certain factors must be taken into consideration; the rate at which the organism grows, the purity of the culture after manipulation, the rate and extent of cellulase production and the rate and extent of cellobiase production.

2.2 - Materials and Methods

2.2.1 - Materials

The chemicals used in this part of the study are listed in the following text: *Trichoderma longibrachiatum* DSM769 (pure culture, DSMZ, Germany); Potato Dextrose Agar (PDA; Merck Biolab, Gauteng, R.S.A.); office paper (Mondi Rotatrim, Merebank, R.S.A.); newspaper (Grocotts, Grahamstown, R.S.A.); Whatman No. 1 (Whatman, Maidstone, England); GOD (Glucose oxidase) kit (Sigma-Aldrich, Germany); NaOH (98% purity, Merck, Gauteng, R.S.A.); nutrient agar (Merck Biolab, Gauteng, R.S.A.); Bradford reagent (Sigma, Germany); silica gel 60 (particle

size 0.040-0.063mm, Merck, Darmstadt, Germany); NaCl (Merck, Gauteng, R.S.A.); $NaSO_4$ (Merck, Gauteng, R.S.A.); methanol (99.9%, Sigma-Aldrich, Germany); $CHCl_3$ (99.9%, Sigma-Aldrich, Germany); formic acid; n-hexane (Sigma-Aldrich, Germany); bacterial acid methyl esters CP mix (Supelco, U.S.A); FAME mix C4 – C24 (Supelco, U.S.A.); D (+) glucose (Merck, Gauteng, R.S.A.); D (+) cellobiose (Sigma, Germany); bovine serum albumin (Sigma, Germany); sulphuric acid (Merck, Gauteng, R.S.A.); Mandel's medium (appendix A); DNS reagent (appendix B); 0.05 M citrate buffer (appendix C). All chemicals were used as obtained from the manufacturer.

2.2.2 - Methods

2.2.2.1 – Organism

Trichoderma longibrachiatum DSM 769 was obtained from DSMZ. The lyophilised powder was suspended in 1 ml of Mandel's medium and aseptically transferred onto PDA plates. These was incubated at 30 °C for 7 days in a model 3028 incubator (Forma Scientific, Ohio, U.S.A.), thereafter the plates were stored at 4 °C until use.

2.2.2.2 – Seed culture preparation

A 5 mm x 5 mm area of sporulated PDA was aseptically transferred into 100 ml of sterile Mandel's medium containing 10 g/l Whatman No. 1 as the sole carbon source in 250 ml conical flasks sealed with aluminium foil. This was incubated at 28 °C in a Labcon shaking water bath (Labdesign Engineering, Maraisburg, R.S.A.) set at 120 rpm for 5 days. This medium was used to seed experimental cultures.

2.2.2.3 – Growth curve measurements

It is important to assess the rate of growth relative to enzyme production and the rate/extent of product formation in order to maximise the efficiency of cultivation. This is achieved through estimating the biomass in the growth medium. Although the simplest method of cell concentration estimation is the direct measurement of dry weight, this is not always appropriate. In this study, the cellular protein concentration was measured, and correlated to dry weight (Kennedy *et al*, 1992).

A 2 ml aliquot of seed culture prepared in 2.2.2.2 was used to inoculate 100 ml of Mandel's medium with *T. longibrachiatum* DSM 769 in 250 ml shake flasks. Both waste newspaper and office paper respectively were used as the sole carbon sources, in separate flasks. These were placed in a shaking water bath (Labcon, Labdesign Engineering, Maraisburg, R.S.A.) at 28 °C. Each carbon source was examined in triplicate.

An aliquot of 7 ml from the active culture was removed at time = 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 7 days and centrifuged at 1600 x g on a Mixtasel centrifuge (J.P. Selecta, Barcelona, Spain). The supernatant was discarded and 7 ml of physiological saline (0.9% w/v NaCl in deionised water) was added and the pellet re-suspended. This was intended to wash the cells and remove any extracellular protein present in the medium. The samples were centrifuged at 1600 x g again and the supernatants were discarded. Another 7 ml of physiological saline was added and the pellet re-suspended. The samples were centrifuged at 1600 x g again and the supernatant discarded (Tandlich, 2004).

An aliquot of 7 ml of 0.2 M NaOH was added to each sample and the pellet re-suspended. The cells were disrupted using an ultrasonic cell disruptor (Vibracell, Sonics and Materials, Connecticut, U.S.A.) with an amplitude = 60 dB in three 20 second bursts. The samples were boiled for 10 minutes, allowed to cool and centrifuged at 1600 x g on a Mixtasel centrifuge.

The protein concentration was determined using the Bradford method according to the method provided by the supplier of the Bradford reagent (Sigma-Aldrich, 2008). An aliquot of 0.05 ml of supernatant, prepared above, was added to a cuvette. An aliquot of 1.5 ml of lightly mixed, room temperature Bradford reagent was added and the colour was allowed to develop for 10 minutes. The absorbance was measured against a standard curve of bovine serum albumin in 0.2 M NaOH at 595 nm on a UV-1201 spectrophotometer (Shimadzu, Kyoto, Japan). The calibration curve can be seen in Figure 2.1.

The concentration of cellular protein obtained was correlated to direct dry weight determination after 6 days of incubation on office paper, at which point no visible traces of the carbon source were left in the medium. Therefore the interference caused by the paper on the determination was negligible. It was determined experimentally that the glass fibre filters did not hold glucose or cellobiose after the filtration of the respective sugar solutions. Samples of 7 ml were removed and centrifuged on a Mixtasel centrifuge at 1600 x g. The supernatant was discarded and the pellet re-suspended in 7 ml deionised water. This was intended to wash any salts and extracellular protein from the cells. This was centrifuged on a Mixtasel centrifuge at

1600 x g and washed with a further 7 ml of deionised water, centrifuged and re-suspended. This was then filtered through a pre-weighed 0.45 μm pore glass fibre filter on a vacuum filtration apparatus and dried at 108 °C in a Labcon oven (Labdesign Engineering, Maraisburg, R.S.A.) for an hour. After an hour, no change in mass of the biomass was shown indicating all volatile components had evaporated. This was allowed to cool in a dessicator and weighed on an AUW 220 balance (Shimadzu, Tokyo, Japan, precision 0.0001 g). This was repeated in triplicate, and the mean value taken as the correlation factor. This yielded a conversion factor of 16.24.

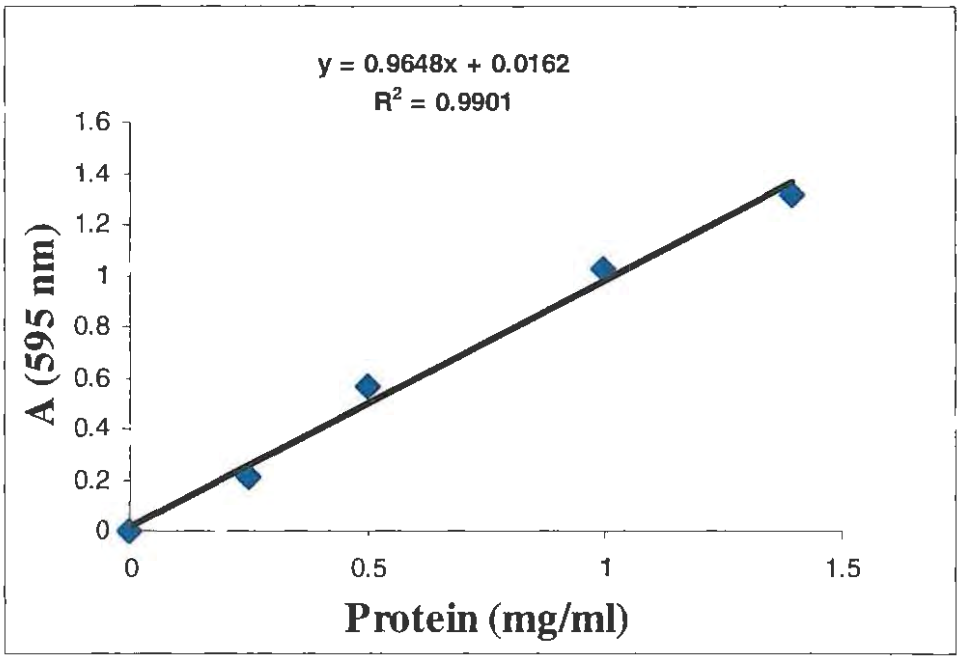


Figure 2.1: Calibration curve for protein determination by the Bradford method, using bovine serum albumin as the standard in 0.2 M NaOH.

Dry weight concentration (g/l) = Protein concentration (g/l) x 16.24 **Eq. 2.1**

Hence, by multiplying the protein concentration obtained by the Bradford method by the correlation factor of 16.24, an estimate of the dry weight equivalent could be determined.

2.2.2.4 – Nutrient agar

Nutrient agar plates are used to confirm the presence of certain, culturable bacterial strains which might be a contaminant in a fungal culture. Incubation for 24 hours is sufficient time for the appearance on bacterial colonies to be visible; however, *T. longibrachiatum* takes considerably more time to grow. Also, *T. longibrachiatum* has a distinctive morphology, as shown in figure 2.2. A 200 µl sample of growth medium was placed on a nutrient agar plate aseptically and spread with a sterilised glass rod. This was placed in a model 3028 incubator at 37 °C for 24 hours and visually inspected for the presence of bacterial colonies.

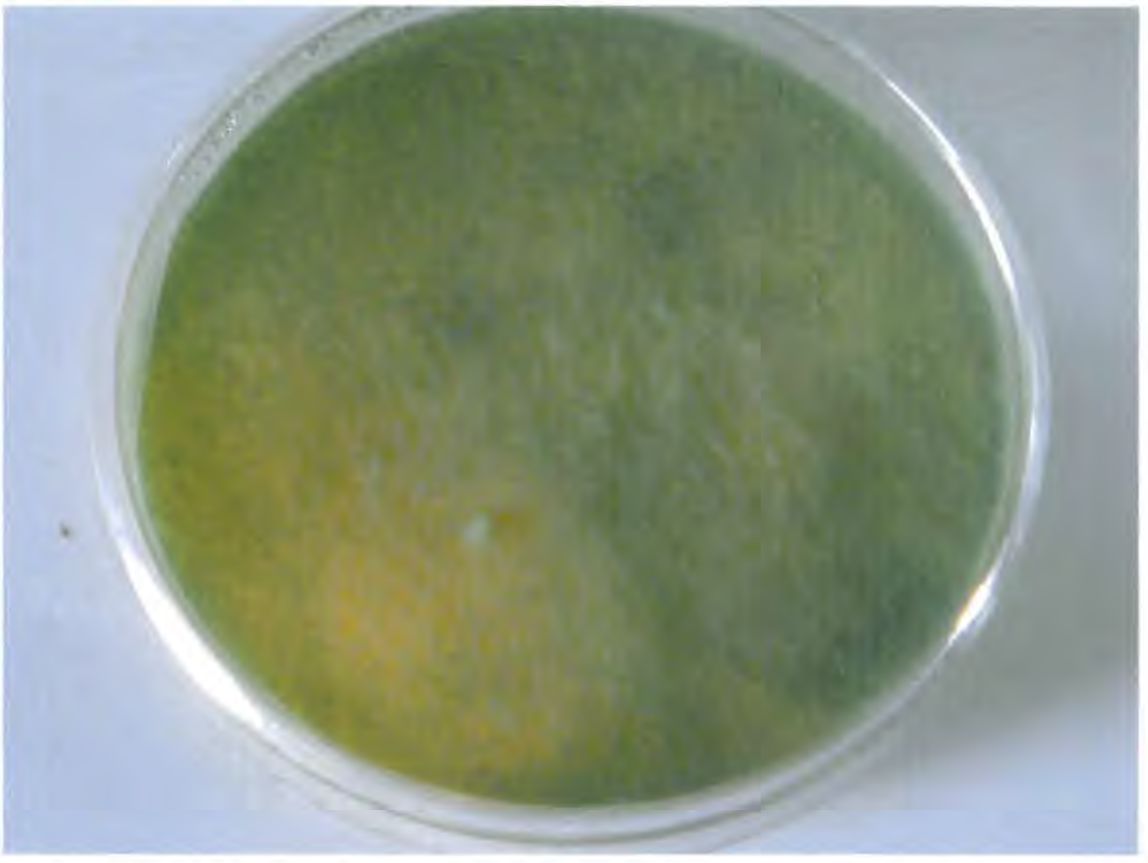


Figure 2.2: *T. longibrachiatum* DSM 769 grown on PDA at 30 °C for 7 days. Note the green conidiophores and white mycelium. This image is for comparative purposes.

2.2.2.5 – FAME analysis

2.2.2.5.1 – Phospholipid extraction

An aliquot of 2 ml of seed culture, prepared in 2.2.2.3, was used to seed 250 ml culture flasks containing 100 ml of Mandel's medium and 10 g/l of newspaper or office paper printed on one side, as carbon sources. The culture flasks were incubated in a Labcon shaking water bath at 28 °C at 120 rpm. Samples of 20 ml were taken after 6 days from each growing culture. These were frozen at -20 °C and freeze-dried using the Freezemobile 6 freeze-drier (Virtis, New York, U.S.A.). A phosphate buffer (pH = 7.4, appendix F):$CHCl_3$: methanol (10:7:3) mixture was prepared and 100 ml

was added to the lyophilised sample and hand shaken every 30 minutes for 2 hours, and thereafter allowed to stand overnight at 4 °C, as described my Melamane (2007).

An aliquot of 10 ml of $CHCl_3$ was added, and the contents shaken for 30 seconds to cause phase separation. The $CHCl_3$ layer was collected for further processing. This process was repeated and all the $CHCl_3$ layers combined. This was concentrated down to approximately 5 – 10 ml on a R-215 rotovapor (Buchi, Switzerland) followed by evaporation to approximately 2 ml under a gentle stream of N_2. This was stored at -20 °C until used for lipid fractionation.

Lipid fractionation was conducted using silica gel column chromatography according to the method of Melamane (2007). The silica gel columns were prepared as follows. Glass wool was used to plug the end of Pasteur pipettes (Merck, Gauteng, R.S.A.). These were filled with silica gel 60, combusted in a muffle furnace at 400 °C overnight and allowed to cool in a dessicator. For lipid fractionation the pipettes were placed in a vacuum manifold (Vac-Elut, Analyticalchem International, California, U.S.A.) and connected to a vacuum pump (Vacuubrand, Germany).

To activate the silica, 2 x 3 ml of methanol was run through the columns, making sure that the silica gel was always below the surface. This was followed by 2 x 3 ml of $CHCl_3$ and 2 x 3 ml of a $CHCl_3$:methanol:formic acid (98.5:1.0:0.5) mixture. The thawed samples were applied to the columns which were then eluted with 8 ml of $CHCl_3$:methanol:formic acid (98.5:1.0:0.5) followed by 2 x 3 ml of acetone. A further 3 ml of $CHCl_3$ was used to restore neutrality. All the effluent at this point, containing the neutral lipids, was discarded and a clean vessel placed in the vacuum chamber for

phospholipid collection. 2 x 3 ml of methanol was run through the column until dry. The phospholipid containing solution was collected and used for methylation.

2.2.2.5.2 – Methylation

In order to increase the volatility of the fatty acids for GC analysis, methyl esters must be formed. The phospholipid containing solutions collected were concentrated to approximately 2 ml under a gentle stream of N_2 and 3 ml of methanol: sulphuric acid (96.8:3.2) was added to each sample in a sealable test tube. These were heated at 80 °C in a Recti-Therm dry block (Pierce, Illinois, U.S.A.) for 2 hours. The solutions were allowed to cool to room temperature and 0.5 ml of a saturated Na_2SO_4 solution and 2 ml of n-hexane was added to each. These were mixed on a vortex mixer for 30 seconds, the layers allowed to separate and the hexane layer was collected. The whole process was repeated one more time, and the n-hexane layers were combined. The resulting organic solution of the FAMEs was pipetted into amber crimp vials (Supelco, Germany), and stored at -20 °C until GC analysis.

2.2.2.5.3 – GC FID analysis

A 1 µl sample of the FAME samples was injected onto an HP 6890 Gas Chromatography unit (Hewlett-Packard, Germany) containing a SPB -1, 30 m x 0.25 mm ID, 0.25 µm film column (Supelco, Germany). The oven was set to 150 °C and held for 4 minutes, thereafter the temperature was ramped at 4 °C/minute to 250 °C, where it was held for 5 minutes. The FI detector was set at 280 °C. The resulting chromatograms were compared to a standardised bacterial FAME mixture as well as a standard FAME mixture, ranging from 4 carbon FAMES to 24 carbon FAMES, injected under the same conditions.

2.2.2.6 – Cellulase determination

The cellulase enzymes are the desired product of *T. longibrachiatum* cultivation. These are responsible for the hydrolysis of cellulosic materials, which is an important first step in bioethanol production. Determining how fast these are made and after how long their production stabilises is valuable information for improving process efficiency. Also, the influence of different carbon sources will play a role in their production (Esterbauer *et al*, 1991).

A convenient unit of total cellulase activity is the Filter Paperase Unit (FPU), where 1 FPU = 1 μmol of reducing sugars (as glucose) produced per minute (Ghose, 1987). The FPU measures the total cellulase activity, i.e. it takes all the components of the cellulase system into account.

An aliquot of 2 ml of seed culture, prepared in 2.2.2.2, was used to seed 250 ml culture flasks containing 100 ml of Mandel's medium and 10 g/l of paper as carbon source. Carbon sources examined included, newspaper, office paper printed on one side and Whatman No. 1. The culture flasks were incubated in a Labcon shaking water bath (Labdesign Engineering, Maraisburg, R.S.A.) at 28 °C at 120 rpm. Samples of 10 ml were removed aseptically from each culture every 24 hours. The samples were centrifuged on a Mixtasel centrifuge at 1600 x g.

FPU was determined according to Ghose (1987). A 0.5 ml sample of supernatant was removed from the centrifuged sample and 1 ml of 0.05 M citrate buffer (pH= 4.8) was added. The enzyme/buffer solution was allowed to temperate to 50 °C and a strip of Whatman No. 1 filter paper (1 cm x 6 cm dimension) was added. This was mixed on

an MT19 vortex mixer (Chiltern Scientific, U.K.). It was ensured that the filter paper was completely submerged. The mixture was incubated statically at 50 °C for 60 minutes in a model 132 water bath (Scientific Engineering, R.S.A.).

After 60 minutes, 3 ml of DNS solution was added and the samples placed in vigorously boiling water for 10 minutes to terminate the cellulase reaction and develop the colour of the DNS reaction. The samples were diluted with 20 ml of deionised water in order to fit within the linear range of the DNS protocol used, and the vessel completely inverted to ensure adequate mixing. The samples were left to stand for 20 minutes in order to allow any pulp matter to settle, after which the absorbance of samples was measured on a UV-1201 spectrophotometer at 540 nm and compared to a standard curve using glucose as the standard (appendix B). Each sample was repeated in triplicate.

By comparing the absolute amount of glucose present in the growth medium, in milligrams, i.e. without the strip of Whatman No. 1, to the absolute amount of glucose, produced from the Whatman No. 1 by the cellulase enzymes, the FPU can be determined. At low levels of activities, i.e. in crude enzyme broths, this is done by multiplying the difference in sugar amounts obtained above by a conversion factor of 0.185, as described by Ghose (1987).

FPU = (Glucose blank (mg) – Glucose reaction (mg)) x 0.185 **Eq. 2.2**

2.2.2.7 – Cellobiase determination

An aliquot of 2 ml of seed culture, prepared in 2.2.2.2, was used to seed 250 ml culture flasks containing 100 ml of Mandel's medium and 10 g/l of newspaper and office paper printed on one side as the sole carbon and energy sources, with *T. longibrachiatum* DMS 769. The culture flasks were incubated in a Labcon shaking water bath at 28 °C at 120 rpm. 10 ml samples were taken aseptically from each culture every 24 hours for 8 days. The samples were centrifuged at 1600 x g on a Mixtasel centrifuge. The cellobiase unit was determined according to Ghose (1987).

1 ml of supernatant was removed from the centrifuged sample and mixed with an MT19 vortex mixer with 1 ml of 20 mM cellobiose in 0.05 M citrate buffer (pH 4.8). This was incubated at 50 °C for 30 minutes. The samples where then placed in vigorously boiling water for 5 minutes. The glucose content was then measured using the Glucose Oxidase (GOD) reaction.

The solution was diluted to an appropriate concentration and 0.5 ml of this solution was mixed with 1.0 ml of GOD reagent in a test tube. The contents were mixed on an MT-19 vortex mixer and incubated in a Labcon water bath at 37 °C for 30 minutes. A 1 ml aliquot of 12 N sulphuric acid was added to stop the reaction and stabilise the colour produced. The absorbance was measured on a UV-1201 spectrophotometer at 540 nm against a glucose standard curve (figure 2.3). The glucose standard solutions were prepared by serial dilutions of D (+) glucose in deionised water.

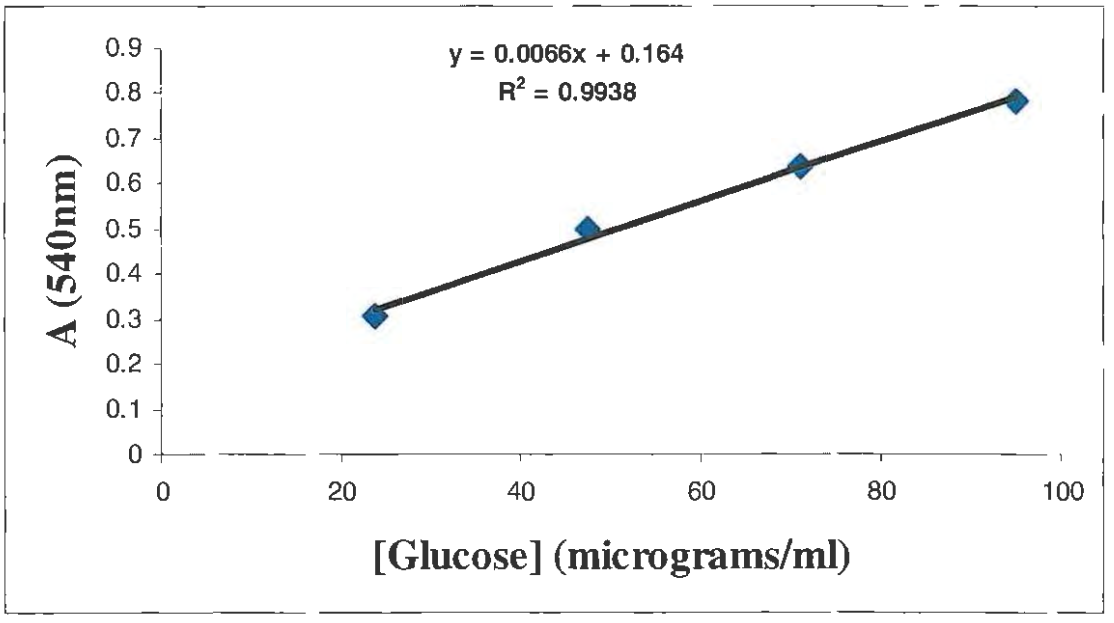


Figure 2.3: Standard curve of D (+) glucose by GOD determination

The absolute amount of glucose produced, in milligrams, can be used to quantify the cellobiase activity. By taking the difference between the glucose produced in the sample containing cellobiose and the sample without (the blank) and multiplying this value by the conversion factor of 0.0926, the cellobiase international unit is determined, where 1 unit = 1 µmol glucose produced per minute at 50 °C (Ghose, 1987)

Cellobiase Unit = (glucose blank (mg) - glucose produced (mg)) x 0.0926 **Eq. 2.3**

2.2.2.8 – Statistical analysis

Data was analysed by means of a one-way ANOVA, using STATISTICA 8.0 (Statsoft Incorporated, Oklahoma, U.S.A.). All one-tailed Student *t*-tests were performed using Excel 2003 (Service pack 3, Microsoft Corporation, California, U.S.A.). All statistics were analysed at a 5% level of significance.

2.3 Results and Discussion

2.3.1 –Growth curves

Measuring the rate of growth of microbial cultures is an important exercise, especially when scale-up of any process under consideration. The rate of growth is determined by estimating the cell concentration over time and is important in designing process control strategies, environment monitoring and control, calculating specific activities and to time inoculations. The most common and simplest method to estimate cell concentration is by a direct dry weight measurement (Kennedy et al, 1992); however, this is not always practical.

The carbon sources used to grow *Trichoderma longibrachiatum* DSM 769 in this study are various sources of paper. Paper is an insoluble substrate, and as such the direct measurement of dry weight biomass is not feasible to quantify the rate of growth of *T. longibrachiatum* DSM 769, as a dry weight determination would overestimate the cell concentration by incorporating the mass of the insoluble substrate, i.e. the un-hydrolysed paper particles.

In order to estimate the biomass, a growth indicator that would not be present in the substrate must be measured. A convenient growth indicator to measure is the cellular protein content (Kennedy *et al*, 1992). After thorough washing of the solid extracts from the medium and extraction of cellular proteins by sonication and boiling in NaOH, the absorbance of the released protein is measured against known standards. This is used to estimate the total concentration of the protein in the growth medium over time. It is assumed that the cellular protein content remains proportional to dry weight at the same ratio throughout the growth of *Trichoderma longibrachiatum*

DSM 769. The conversion factor is calculated from the determination of the dry weight of the fungal biomass in a particular flask, when all visible traces of the paper in question have been eliminated. This was thoroughly washed, and it was determined that no sugars were retained on the filter. By multiplying the protein concentration by the conversion factor of 16.24, obtained in section 2.2.2.4 (equation 2.1), the relative dry weight can be estimated. The results are represented in the growth curve in figure 2.4.

Dry weight concentration (g/l) = Protein concentration (g/l) x 16.24 **Eq. 2.1**

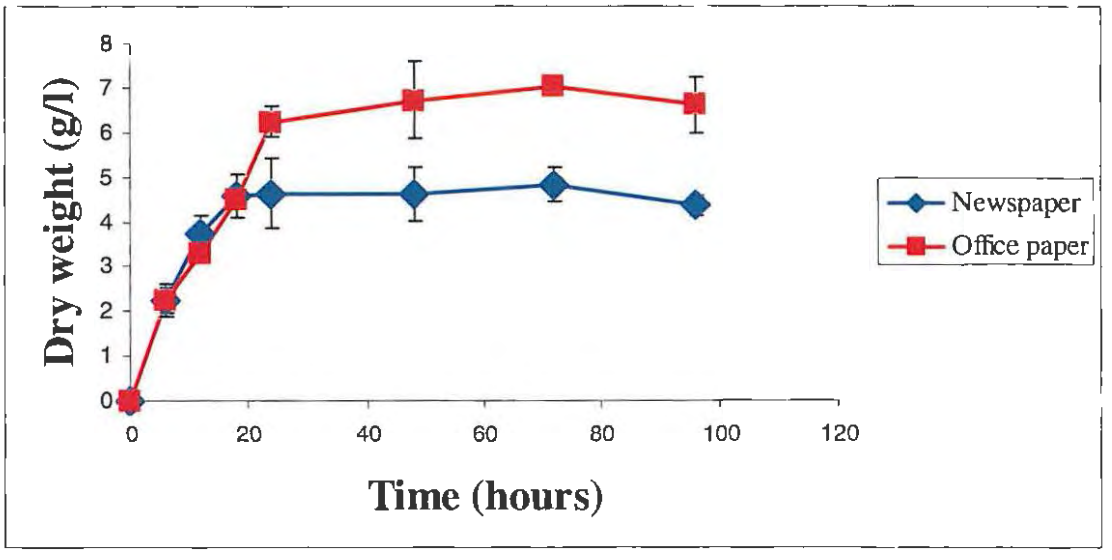


Figure 2.4: Growth curve *T. longibrachiatum* DSM 769 on office paper and newspaper as the sole carbon and energy sources.

After inoculation, there is no lag phase, due to the high concentration of the seed culture's inoculum. The exponential phase continues for 18 hours for *T. longibrachiatum* DSM 769 on newspaper, reaching a maximum dry weight of 4.6 ± 0.49 g/l, and for 24 hours on office paper, reaching a maximum dry weight of 6.2 ±

0.33 g/l. A Student's *t*-test between the dry weights obtained on office paper and newspaper returns a *p*-value > 0.05 for the first 18 hours; hence the rate and extent of growth of *T. longibrachiatum* DSM 769 is equal on each substrate for the first 18 hours. After 24 hours onwards, the *p*-value < 0.05, thus the total dry weight obtained on office paper is higher than that on newspaper.

A one-way ANOVA test of the biomass data obtained from 18 hours to 48 hours on office paper returns a *p*-value of 0.001, thus there is a change between the biomass between these time points. A one-way ANOVA on biomass produced on office paper from 24 hours to 96 hours returns a *p*-value of 0.256, showing that there is no change in biomass and hence the stationary phase has been reached.

The ANOVA test performed on the biomass grown on newspaper returns a *p*-value of 0.202 from 12 hours to 96 hours showing that there is no significant change in biomass in this time. A *p*-value from 6 hours to 24 hours of 0.001 is observed, indicating that there is a significant change in biomass at this time. Therefore it can be stated that the stationary phase of growth of *T. longibrachiatum* DSM 769 is reached after 12 hours when grown on newspaper, and after 24 hours when grown on office paper. These results are likely due to the nutritional values of each source of paper, with office paper having more utilizable material for fungal growth, i.e. more accessible carbohydrates, released through the enzymatic hydrolysis of the cellulose in the paper.

The stationary phase is characterised by a constant dry weight, and follows immediately after the exponential phase. The stationary phase for *T. longibrachiatum*

DSM 769 grown on newspaper and office paper corresponds to 4.6 ± 0.49 g/l and 6.2 ± 0.33 g/l of dry, fungal biomass respectively.

2.3.2 – Contamination control

2.3.2.1 – Test for culturable organisms

After incubation for 24 hours at 37 °C, the nutrient agar plates were visually assessed for the presence of bacterial colonies. These assessments revealed that no culturable bacterial contamination was present in the samples tested, as observed by the lack of visible colonies on the plated media in figures 2.5 through 2.7.

The presence of a bacterial contaminant could impede the cellulase activity, or aid in the degradation of the desired metabolic by products. These contaminating organisms could present problems further on and persist to the ethanol fermentation stage. As such, care must be taken to prevent and monitor contamination by organisms other than those desired.

Figure 2.5: Nutrient agar plate of medium of *T. longibrachiatum* DSM 769 grown on newspaper after incubation. Note the absence of visible colonies.

Figure 2.6: Nutrient agar plate of medium of *T. longibrachiatum* DSM 769 grown on office paper after incubation. Note the absence of visible colonies.

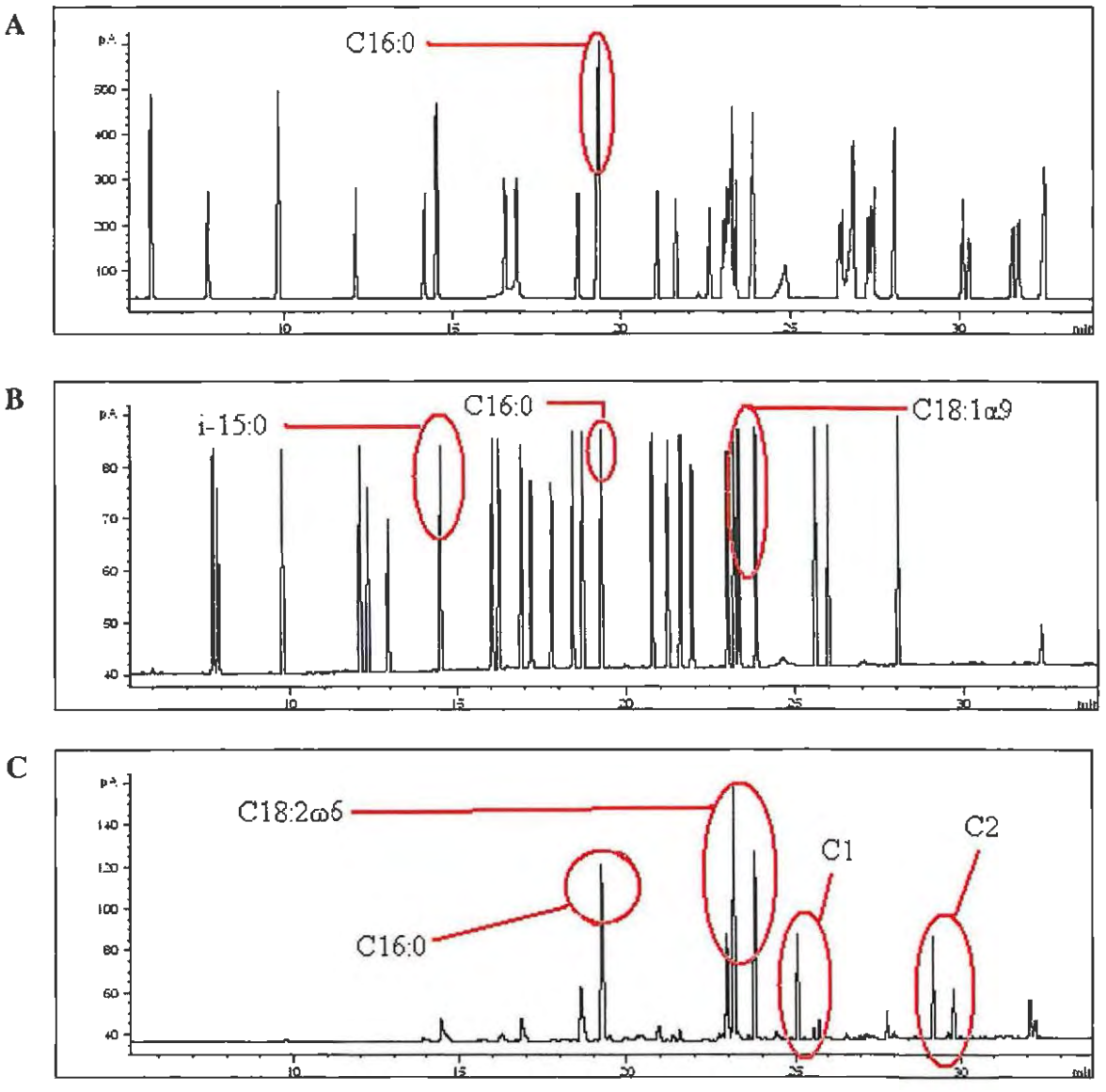
Figure 2.7: Nutrient agar plate of medium of *T. longibrachiatum* DSM 769grown on Whatman filter paper after incubation. Note the absence of visible colonies.

Although nutrient agar is a useful tool to check for the presence of bacterial contamination, there are certain organisms that cannot be cultured (Roszak *et al*, 1987). Further analysis by FAME comparisons would be needed for definitive results.

2.3.2.2 – Test for non culturable organisms

The analysis of PLFAs as FAMEs to identify possible contaminating organisms works in conjunction with nutrient agar plates. As certain organisms are non-culturable, other methods for detecting their presence are necessary since culturable organisms generally only constitute 1 to 10% of the total microbial biomass (Roszak *et al*, 1987). FAMEs have been used to identify certain microbial groups by signature

profiles, and to monitor shifts in biodiversity (Fernandes, 2006; Tunlid *et al*, 1992). Therefore, the comparison of the PLFA FAME profiles of *T. longibrachiatum* DSM 769 at different stages in the cultivation process versus known bacterial standards (figure 2.8) could reveal contamination previously undetected on nutrient agar plates.



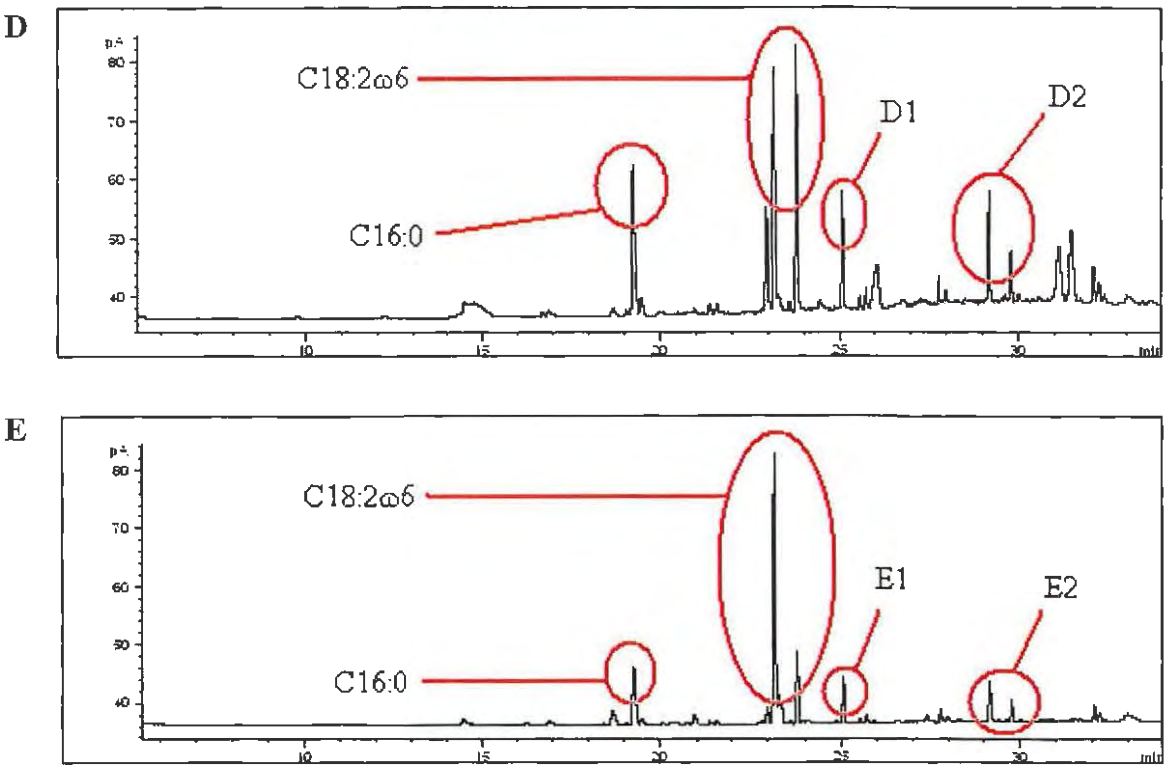


Figure 2.8: Chromatograms of various FAMEs:

A – c4 – c 24 standard FAME mixture

B – Bacterial standards mixture

C – Seed culture PLFA FAME profile

D – Newspaper culture PLFA FAME profile

E – Office paper culture PLFA FAME profile

C15:0 and C17:0 fatty acids are often used to identify the presence of bacteria (Vestal *et al,* 1989; Tunlid *et al*, 1992), i-15:0 is indicative of Gram negative bacteria (Kaur *et al,* 2005). A comparison of the culture PLFA profiles with that of the bacterial standards show's that C15:0, i-C15:0, and C17:0 are not present in the samples from *T. longibrachiatum* DSM 769 medium.

Although C18:1α9 is a biomarker for various bacteria, C18:2ω6 is a biomarker for fungi and eukaryotic organisms (Kaur *et al,* 2005; Fernandez, 2006). The method used for separation will likely only present miniscule separations of these compounds, making their distinction in this case difficult. The moieties present with retention times of 23 and 24 minutes in the samples are likely to be the C18 fungal biomarkers.

The PLFAs marked C1, D1 and E1 (as shown in figure 2.8) are the same acids, as they elute at the same retention time in all the *T. longibrachiatum* DSM 769 cultures. They do not correspond to the bacterial PLFAs, or any of the C4 – C24 standard peaks, and therefore are likely to have originated from *T. longibrachiatum* DSM 769. The same can be said about the PLFAs marked C2, D2 and E2 (figure 2.8).

Based on the PLFA profiles, it is unlikely that bacterial contamination of culture of *T. longibrachiatum* DSM 769 occurred, and therefore there is unlikely to be any interference further along in the production of bioethanol.

2.3.3 – Cellulase activity

The Filter Paperase Unit (FPU) is a good representation of the total cellulase activity as it does not measure individual enzyme activities, but rather their combined effect in degrading Whatman No. 1 filter paper. For a process biotechnologist, this is a useful tool in estimating the optimum time to harvest the enzymes, or other desired products. Enzyme extraction and purification is an expensive step in enzyme production, and the conditions have to be optimized before industrial application (Ahamed *et al*, 2008). In the case of *T. longibrachiatum*, the cellulase enzyme cocktail is excreted into the extracellular growth medium (see section 1.3.5). If the cellulase activity is at

an appreciable level, the growth medium itself may provide sufficient hydrolysing power, and the purification of enzyme unnecessary.

Of principle importance in producing cellulases in the laboratory is the carbon source (Esterbauer *et al*, 1991). One would therefore expect a variation in cellulase activity between the different substrates used in this study, i.e. the different types of paper as each paper has differing degrees of processing and different sources for production associated with them. This will affect the cellulose content of each type of paper, and hence the degree of cellulase induction.

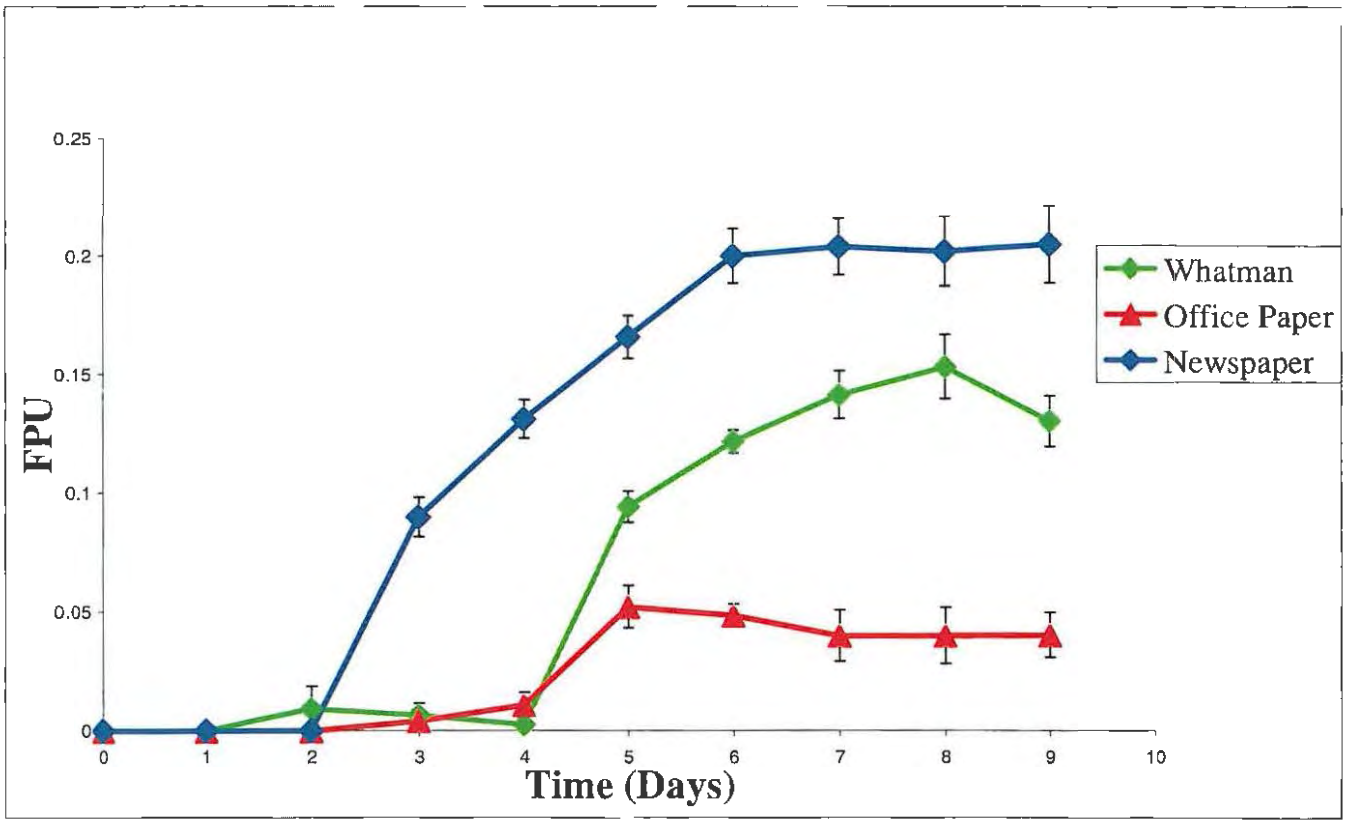


Figure 2.9: Cellulase activity, as FPU per ml, produced extracellularly by *T. longibrachiatum* DSM769, grown on various paper sources over time

As shown in figure 2.9, the cellulase activity is a function of time during the growth of *T. longibrachiatum* DSM 769. The extracellular cellulase activity remains zero for the first few days, increasing on day 3 for newspaper and office paper. This is shown by a one-way ANOVA test from time = 0 days to time = 3 days (p-value < 0.0001). This indicated that on day 3, the cellulase activity is significantly different from the days before.

A one-way ANOVA on the results of cellulase activity produced on Whatman No. 1 filter paper from time = 0 to time = 4 yielded a p-value = 0.138. Therefore there is no enzyme production in the first 4 days of growth of *T. longibrachiatum* DSM 769 on Whatman No.1. The ANOVA p-value between days 3 and 5 was less than 0.0001, indicating that from day 5 the cellulase activity increases significantly. There is no stabilization of enzyme activity in the time period examined and a maximum cellulase activity of 0.142 ± 0.011 FPU.ml^{-1} is achieved.

The maximum cellulase activity obtained on the newspaper substrate is 0.203 ± 0.008 FPU.ml^{-1}. The ANOVA p- value obtained on this substrate, between day 5 and day 7, yields a p-value = 0.01. This shows that the cellulase is still increasing at day 5. From day 6 to day 9 the p-value = 0.407. One can therefore assume that the cellulase activity produced on newspaper stabilises at day 6

Between day 2 and 4 there is a slight increase in cellulase activity from the office paper substrate (ANOVA p-value = 0.037 from day 3 to day 5, the p-value < 0.0001, indicating a large increase in cellulase activity. The cellulase activity reaches $0.052 \pm$

0.009 FPU.ml^{-1} at day 5. The *p*-value obtained from the one way ANOVA test = 0.400 for days 5 to 9, showing that the cellulase activity has stabilised from day 5.

A Student's *t*-test reveals that newspaper achieves the maximum cellulase activity at 0.21 ± 0.016 FPU.ml^{-1}, as compared to office paper and Whatman No. 1 (*p*-values < 0.05).

From day 5, the *p*-value obtained from the Student's *t*-test is < 0.05. After day 5, the cellulase activity is significantly higher on the Whatman No. 1 substrate versus that produced on office paper. Therefore, the cellulase produced on office paper stabilises at a significantly lower activity than that produced on either Whatman No.1 or newspaper.

The difference observed in the cellulase activities produced on different sources of cellulosic materials, i.e. the different paper sources, is due to the susceptibility of the various sources of the cellulose to the cellulases produced by *T. longibrachiatum* DMS 769. Newspaper is highly processed, and the paper obtained for this study contains over 40% recycled material, according to the supplier. This processing has decreased the nutritional content of the substrate, the result of which is more of the cellulase enzymes being produced by *T. longibrachiatum* DSM 769 in an attempt to satisfy its nutritional requirements. The materials used in the office paper are virgin, i.e. have not been recycled and have been subjected to a lesser degree of processing, according to the manufacturer. As such, the nutritional content of office paper is significantly higher than that found in newspaper; hence *T. longibrachiatum* DSM 769 need not invest as much energy into cellulase production. Similarly, the Whatman

No. 1 presented more of a digestive problem than office paper, but is still significantly lower than newspaper.

It can therefore be stated that newspaper is the better cellulosic substrate, of those examined, for the production of cellulase by *T. longibrachiatum* DSM 769 in this system. The enzyme activity stabilizes at day 6, therefore this would be the most economical time to harvest the enzymes produced.

2.3.4 – Cellobiase activity

The determination of cellobiase in the cellulase medium is very important as the accumulation of cellobiose can impair enzyme activity and is itself a glucose dimer, therefore it is essential in maximising cellulose hydrolysing efficiency to hydrolyse this minor product of cellulose hydrolysis (see section 1.3.4). By ensuring that there is an appreciable cellobiase activity present, the total cellulase activity and glucose production can be increased. As cellobiose is a minor product of the hydrolysis, the cellobiase activity is not as high as the total cellulase activity.

The cellobiase activity produced by *T. longibrachiatum* DSM 769 grown on newspaper and office paper was analysed overtime, and is illustrated in figure 2.10.

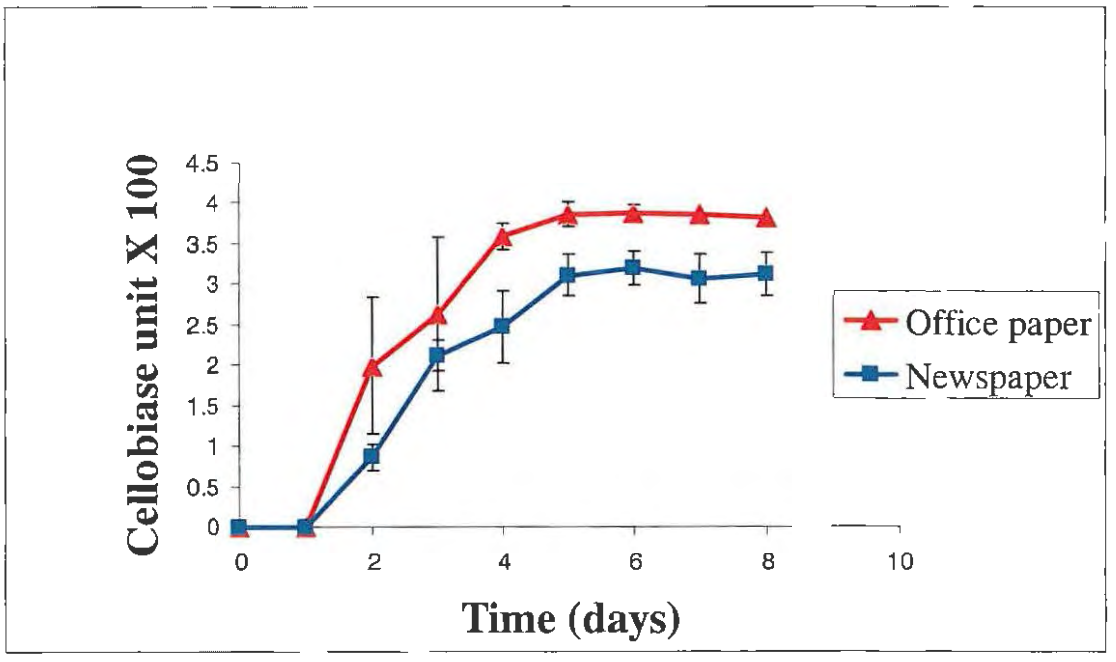


Figure 2.10: Cellobiase activity produced extracellularly by *T. longibrachiatum* DSM 769 grown on newspaper and office paper over time.

There is no measurable cellobiase produced for the first 24 hours on either newspaper or office paper; however on office paper the activity increases from 0 to 0.039 ± 0.009 cellobiase units.ml^{-1} between day 1 and day 5; analysis by a one way ANOVA shows that this is a significant change (p-value < 0.0001). The p-value obtained from day 5 to day 8 = 0.981. This shows that the cellobiase activity remains unchanged, i.e. the cellobiase activity stabilises from day 5 at 0.039 ± 0.009 cellobiase units.ml^{-1}.

The cellobiase activity produced on newspaper increase from 0 to 0.031 ± 0.003 cellobiase units.ml^{-1} between day 1 and day 5 and follows a similar trend to that found on office paper. The p-value obtained from a one-way ANOVA between day 1 and day 4 < 0.0001. This shows that the cellobiase activity is increasing between those times. A one-way ANOVA analysis from day 5 to day 8 returns a p-value = 0.945,

showing that the cellobiase activity remains stabiles after 5 days at a value of 0.031 ± 0.003 cellobiase units.ml^{-1}.

Thus it can be stated that cellobiase production by *T. longibrachiatum* DSM 769 stabilises after 5 days of growth on either newspaper or office paper. This correlates with an absence of free substrate in the office paper medium, on visual inspection.

A Student's *t*-test gives a *p*-value > 0.05 for the first 3 days of growth between the cellobiase activities produced on office paper versus newspaper. Therefore it can be stated that there is no significant difference between the cellobiase activities produced from each substrate in the first 3 days of growth. However, from day 4 the *p*-value < 0.05, i.e. the means are significantly different. Hence, after 4 days, the cellobiase activity produced by *T. longibrachiatum* DSM 769 grown on office paper is significantly higher than that produced on newspaper.

Cellobiase is produced in response to the presence of cellobiose in the growth medium, i.e. cellobiose is an inducer of cellobiase (Sternberg *et al*, 1982). As described previously, these differences in enzyme activities are related to the nutritional contents of the different papers used. As there is a smaller degree of processing involved with the production of office paper, the cellulose chains are longer and more intact. Therefore, upon hydrolysis by the cellulase enzymes produced by *T. longibrachiatum* DSM 769 there is a higher amount of cellobiose produced as compared to the amount of cellobiose produced on the highly processed, less nutritional newspaper and thus, cellobiase production is induced.

2.4 – Conclusion

After growing a culture of *T. longibrachiatum* DSM 769 for 6 days on waste newspaper at 28 °C with shaking agitation at 120 rpm, a maximum cellulase activity is reached of 0.203 ± 0.009 FPU.ml^{-1}. After 5 days, cellobiase activity has reached a maximum. Although the cellobiase produced on waste office paper is higher than that on newspaper, the FPU is much lower. As the FPU is a representation of the total cellulase activity, it is a more important deciding factor on a suitable substrate for cellulase production. As the cellobiase produced on newspaper is still present in an appreciable amount, this is the best substrate, of the wastes examined, to produce the most active cellulase broth.

Upon statistical analysis, the optimum time for enzyme harvest is at day 6, as this is when the cellulase activity produced on newspaper is at its highest. Although the cellobiase activity produced on newspaper reaches a maximum value at day 5, it remains stable through to day 6 and beyond. Both the cellulase and cellobiase activities correspond with the stationary phase of *T. longibrachiatum* DSM 769.

It must be determined whether sugars are produced in sufficient amounts in the growth medium for economical ethanol fermentation, or whether they must be produced secondarily.

Chapter 3: Sugar production

3.1 – Introduction

In order to produce bioethanol, fermentable carbohydrates are needed as a substrate (see section 1.3.1). The most common carbohydrate used in the fermentation of ethanol is glucose. However, certain fermenting organisms can utilize other sugars such as xylose (Agbogbo *et al*, 2006). Glucose is readily used in metabolic processes by yeasts such as *Saccharomyces cerevisiae*, the product of which is ethanol (see section 1.3.6), the ultimate end goal of this study.

The cellulase system produced by *Trichoderma longibrachiatum* DSM 769 is able to hydrolyse the cellulose macromolecule into smaller carbohydrates, the main product being glucose (see section 1.3.4). Smaller quantities of cellobiose may be present, however, this is readily hydrolysed to glucose in the presence of cellobiase, part of the cellulase system of *T. longibrachiatum* DSM 769 (see sections 1.3.4 and 2.6). Paper has high cellulose content and it is this cellulose which gives paper its rigidity (see section 1.3.8). Paper is used in high quantities throughout the world. Although there are several waste paper recycling programs around the world, there is much left to be desired. The high cellulose content and high turnover worldwide makes waste paper a potential target for biotechnological hydrolysis and production of bioethanol (see section 1.3.8). This hypothesis must be tested by analysis of sugar production rates and cellulase activities.

Determining whether the desired product is produced extra- or intracellularly has vast implications in product harvesting, and has economic implications for any industrial

applications and as such must be assessed in detail. Considering that the ultimate aim of bioethanol production is to be as cost effective as possible, the direct production of glucose/fermentable sugars should be tested for viability. The determination of the cellulase activities in harvested cellulase broth must be measured when hydrolysing newspaper and office paper over time to ensure the viability of the enzymes and monitor for their degradation. This monitoring will improve the economy of the process.

3.2 Materials and Methods

3.2.1 – Materials

The following chemicals and reagents were used for this part of the study: acetonitrile (Sigma-Aldrich, Germany); office paper (Mondi Rotatrim, Merebank, R.S.A.); newspaper (Grocotts, Grahamstown, R.S.A.); Whatman No. 1 (Whatman, Maidstone, England); PDA (Merck biolab, Gauteng, R.S.A.); Mandel's medium (appendix A); DNS reagent (appendix B); 0.05 M citrate buffer (appendix C); D (+) glucose (Merck, Gauteng, R.S.A.). These materials were used as supplied.

3.2.2 – Methods

3.2.2.1 – Organism

T. longibrachiatum DSM 769 was maintained on PDA at 4 °C. This was used to inoculate the seed culture, as per section 2.2.2.2.

3.2.2.2 – Sugar production by direct fermentation

3.2.2.2.1 - Extracellular glucose

An aliquot of 2 ml *Trichoderma longibrachiatum* DSM 769 seed culture (see section 2.1.2) was used to inoculate 100 ml of Mandel's medium in 250 ml conical flasks containing 10 g/l waste newspaper. This was incubated at 28 °C in a Labcon shaking water bath set at 120 rpm. A 5 ml sample was taken daily. The sample was centrifuged on a Mixtasel centrifuge at 1600 x g and filtered through a sterile 0.45 µm cellulose acetate membrane Swinney filter (Millipore, Ireland). The solutions were stored in 2 ml amber crimp vials and frozen until analysis.

3.2.2.2.2– Combined Intra- and extracellular glucose

An aliquot of 2 ml of *Trichoderma longibrachiatum* DSM 769 seed culture (see section 2.1.2) was used to inoculate 100 ml of Mandel's medium in 250 ml conical flasks containing 10 g/l waste newspaper. This was incubated at 28 °C in a shaking water bath set at 120 rpm. A 5 ml sample was taken daily and the cells were ruptured using a Vibracell cell disruptor (Vibracell, Sonics and Materials, Connecticut, U.S.A.) for 3 bursts of 20 seconds each at 60 dB. The samples were centrifuged at 1600 x g on a Mixtasel centrifuge and filtered through a sterile 0.45 µm MF cellulose acetate membrane Swinney filter. The solutions were stored in 2 ml amber crimp vials and frozen until use.

3.2.2.2.3 – HPLC analysis

The samples were allowed to warm to room temperature and analysed by HPLC in triplicate, according to appendix D.

3.2.2.3 – Cellulase broth harvest

An aliquot of 2 ml of *Trichoderma longibrachiatum* DSM 769 seed culture prepared in section 2.2.2.2 was used to inoculate 100 ml of sterile Mandel's medium in 250 ml conical flasks with newspaper as the sole carbon source. No office paper was used in the preparation of the cellulase enzyme broth. These were incubated at 28 °C (as per the recommended temperature by Mandel, 1974) in a Labcon shaking water bath set at 120 rpm. After 6 days the contents of the flasks were centrifuged at 1600 x g on a Mixtasel centrifuge. The resulting broth was used to examine the temperature stability of the cellulase enzyme complex and the rate and extent of the sugar production on office waste paper and newspaper. For this, an aliquot of 100 ml of cellulase broth was transferred into a sterile 250 ml flask containing 1 g of sterile waste paper, either newspaper or office paper. Only sections of newspaper printed with black ink were used in this study. The office paper was printed on only one side with Hewlett Packard ink jet ink (Hewlett Packard, Germany). The flasks were sealed with sterile aluminium foil and placed on a SS70 orbital shaker (Chiltern scientific, U.K.) set at 120 rpm in a Model 3028 incubator. Three temperatures were examined; 30 °C, 40 °C, and 50 °C. Samples of 5 ml were taken at t = 0, 6, 12, 18, 24, 48 and 72 hours. These were centrifuged at 1600 x g on a Mixtasel centrifuge and the supernatant was used to test FPU and glucose levels. All manipulations were carried out aseptically and in triplicate.

3.2.2.4 – Cellulase activity (as FPU)

The cellulase activity was calculated according to Ghose (1987). A sample of 0.5 ml of supernatant (3.2.2.3) was placed in a boiling tube together with 1.0 ml of 0.05 M citrate buffer (pH 4.8) and a 1 cm x 6 cm strip of Whatman No. 1 filter paper. This

was mixed on a MT19 vortex mixer and it was ensured that all the paper was submerged. This was placed in a model 132 water bath at 50 °C for 60 minutes. A 3.0 ml aliquot of DNS reagent was added and the tube placed in a water bath at 100 °C for 10 minutes. This was diluted with 20 ml of deionised water and the absorbance of the contents measured at 540 nm on a UV-1201 spectrophotometer against a standard glucose curve (appendix B). This was repeated in triplicate and blanks were prepared as above without the strip of Whatman No. 1, in order to compare produced glucose versus glucose present in the broth.

3.2.2.5 – Reducing sugar levels

Glucose concentrations were determined by the DNS reaction. A 0.5 ml sample of supernatant previously prepared (3.2.2.4) was placed into a boiling tube together with 1.0 ml of 0.05 M citrate buffer (pH 4.8) and 3.0 ml of DNS reagent. This was placed in boiling water for 10 minutes and thereafter diluted with 20 ml of deionised water and the absorbance measured at 540 nm on a UV-1201 spectrophotometer and compared to a standard glucose curve (appendix B). This was performed in triplicate.

3.2.2.6 – Product identification

As the sugar levels have been determined by the DNS method, which is non specific, the identity of the resultant reducing sugars needs to be confirmed. This was achieved through HPLC analysis (see Appendix D). Only the system producing the highest level of reducing sugars was examined.

3.2.2.7 – Statistical analysis

Data was analysed by means of a one-way ANOVA using STATISTICA 8.0 (Statsoft Incorporated, Oklahoma, U.S.A.). All one-tailed Student's *t*-tests were performed by Excel 2003 (Service pack 3, Microsoft Corporation, California, U.S.A.). All statistical analysis was conducted at a 5 % level of significance.

3.3 - Results and Discussion

3.3.1 – Direct substrate fermentation

The levels of glucose and cellobiose in both the extracellular and intracellular medium were found to be below the detection limits of 0.29 and 0.28 mM respectively (see appendix D). This implies that the organism is taking up and utilizing cellobiose and glucose as fast as these are being produced. Considering that no cellobiose was detected in the intra- and extracellular environments, the extracellular cellobiase activity produced by *T. longibrachiatum* DSM 769 is sufficient to hydrolyse all noticeable cellobiose to glucose immediately after its production. This glucose is subsequently taken up and utilized by the cells of *T. longibrachiatum* DMZ 769 for its nutritional requirements.

As the cellulase complex is released into the extracellular environment by *T. longibrachiatum* DSM 769 (see section 1.3.5), glucose levels in the surrounding medium were expected to be of a significant level. However, the organism's glucose transport system appears to be working as fast as glucose is produced from the hydrolysis of the cellulose in the waste paper by the cellulase system.

The intracellular glucose levels were below 0.29 mM, which indicates that once the glucose crosses the fungal cytoplasmic membrane it is rapidly metabolized in order to meet the organism's nutritional requirements. As the glucose levels observed are below the detection limit (see appendix D), it appears as though glucose is not stored intracellularly. Based on these results, the most viable approach for the production of sugars for bioethanol fermentation is the growth of the fungus in the presence of newspaper until maximum cellulase activity is obtained in the extracellular medium. Separation of the fungus is then performed and the enzymes produced are exposed to a new substrate to produce glucose/fermentable sugars and prevent their consumption before the conversion into bioethanol. This approach will be adopted for the remainder of the study.

3.3.2 – Sugars obtained from enzyme broth

Of primary concern at this stage of the study is the rate and the concentrations of fermentable sugars produced, i.e. the optimization of the fermentable sugars available for the production of bioethanol in the subsequent steps of the production. The amount of raw materials/fermentable sugars produced is mainly influenced by temperature and the nature/rigidity of the cellulose source, often altered through pre-treatment (Bommarius *et al*, 2008). Previous literature states that the optimum hydrolysis temperature is 50 °C (Mandel *et al,* 1974), however, many cellulase enzymes are unstable at temperatures exceeding 40 °C (Paljevac *et al*, 2007). Finding the optimum temperature for cellulose hydrolysis by the cellulases produced by *T. longibrachiatum* DSM 769 is important for economic considerations and for the optimization of the stability of the cellulase enzymes produced from *T. longibrachiatum* DMZ 769. This would ultimately lead to higher sugar production.

Finding the balance between production rate and energy required is of paramount importance.

As the different paper sources have different compositions, there are differences in the ease of their respective hydrolysis and sugar ratios (see section 2.3.3). The various extents of different sugars produced can also affect the cellulase activity; most notable is cellobiose, which inhibits cellulase activity (see section 1.3.4).

3.3.2.1 – Enzyme activities

As the production and purification of cellulase enzymes are notoriously expensive with commercial cellulase retailing at approximately R600 per gram (Sigma, 2008), the ability to reuse the enzyme broth would be desirable. As previously discussed, there are several factors which could influence the stability of the enzyme, the most influential being temperature and the maximum cellobiose concentrations reached during enzyme broth sugar production. The monitoring of the cellulase activities are therefore extremely important and are illustrated in figures 3.1 to 3.3.

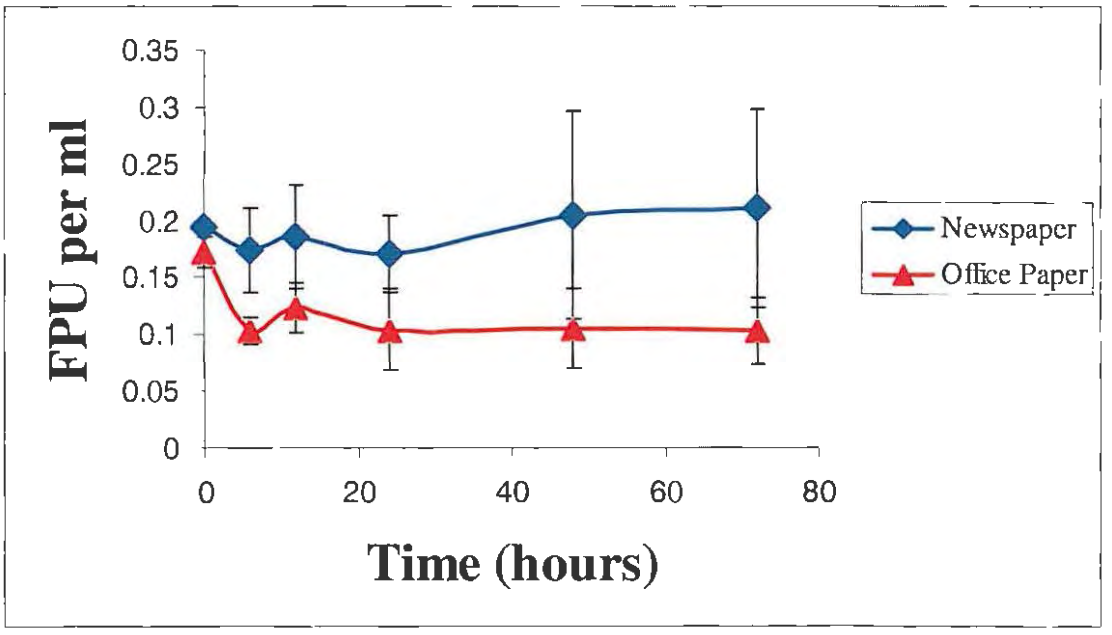


Figure 3.1: Cellulase activity as FPU per ml over time in the enzyme broths at 30 °C.

At t = 0 at 30 °C the initial cellulase activities in the newspaper and office paper broths were equal to 0.194 ± 0.005 $FPU.ml^{-1}$ and 0.178 ± 0.013 $FPU.ml^{-1}$ respectively. Results of the Student's *t*-test shows that these values are not significantly different (*p*-value = 0.83). Using the one-way ANOVA the cellulase activity in the newspaper medium was independent of time (*p*-value = 0.944). The one-way ANOVA for cellulase activity values in the office paper broth shows that the cellulase activity decreases during the initial 12 hours of incubation (*p*-value = 0.047) for the data after 0, 6 and 12 hours of incubation. The cellulase activity remains constant after 12 hours of incubation (*p*-value = 0.894), with an average value of 0.108 ± 0.013 $FPU.ml^{-1}$.

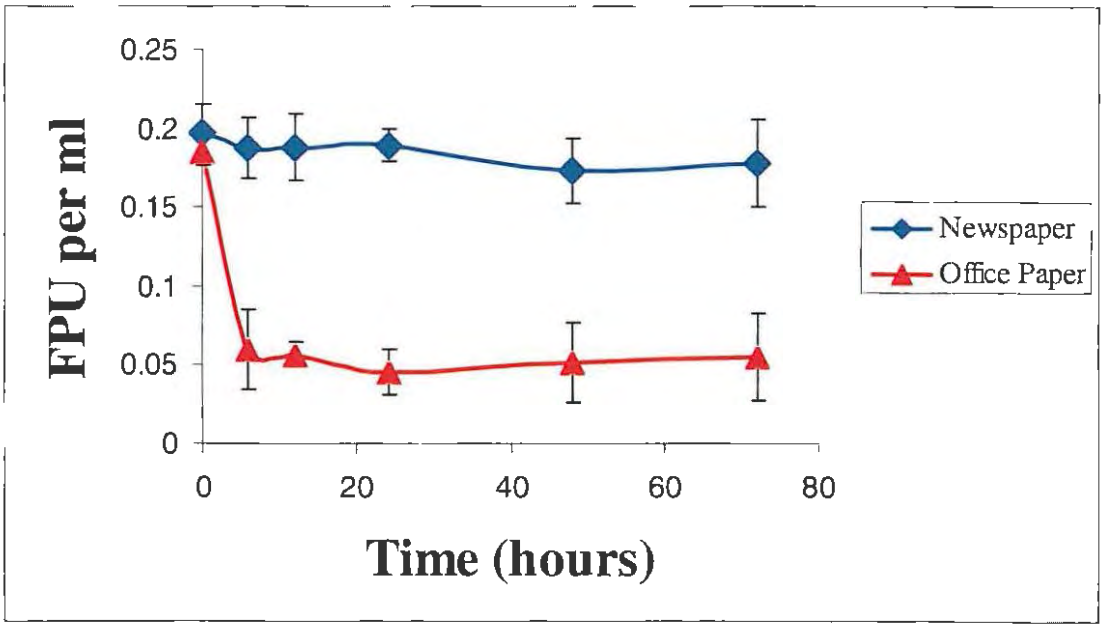


Figure 3.2: Cellulase activity as FPU per ml over time in the enzyme broths at 40 °C.

For the incubation at 40 °C, the initial cellulase activity in the newspaper and office paper medium is 0.196 ± 0.018 $FPU.ml^{-1}$ and 0.185 ± 0.009 $FPU.ml^{-1}$ respectively. Results of a Student's *t*-test shows that the means were statistically similar (*p*-value = 0.093). After 6 hours of fermentation, the cellulase activity in the office paper broth decreases rapidly to 0.060 ± 0.025 $FPU.ml^{-1}$, while the cellulase activity observed in the newspaper broth is equal to 0.188 ± 0.020 $FPU.ml^{-1}$. The value of the activity obtained on newspaper was statistically different to that obtained on office paper at the 5 % level of significance (*p*-value = 0.019). In fact the cellulase activity in the newspaper broth did not change throughout the incubation period (one-way ANOVA *p*-value = 0.743). However, the cellulase activity in the office paper broth decreases in the first 12 hours, illustrated with a one-way ANOVA *p*-value less than 0.0001. After this decrease in cellulase activity, it stabilises at 0.052 ± 0.005 $FPU.ml^{-1}$ (*p*-value = 0.934). It can therefore be deduced that the decrease in cellulase activity observed is

not caused by high temperatures, as it remains constant in the newspaper medium, but is rather the result of enzyme inhibition.

As shown in section 2.3.4, *T. longibrachiatum* DSM 769 grown on office paper produces higher cellobiase activities versus the newspaper-grown fungus. Since the enzymes used during the incubations in this section were produced on newspaper, it is likely that the cellobiase activity present in the enzyme broth at the beginning of the incubation was relatively low. The structure of the office paper probably leads to a faster rate of cellulose hydrolysis and thus to a faster build-up of cellobiose in the incubation medium. The low cellobiase activity led to a build-up of cellobiose which likely led to the faster onset of the inhibitory effect of cellobiose in the office paper broth versus the newspaper broth.

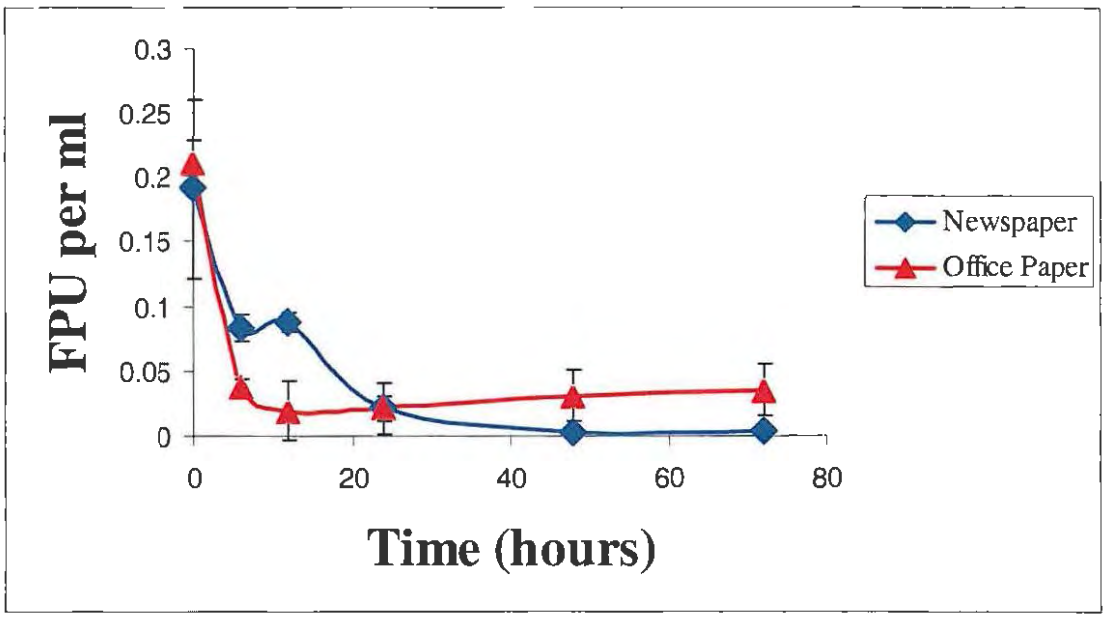


Figure 3.3: Cellulase activity as FPU per ml over time in the enzyme broths at 50 °C.

At time = 0 at 50 °C the cellulase activity in the newspaper and office paper broths were 0.191 ± 0.070 FPU.ml^{-1} and 0.211 ± 0.017 FPU.ml^{-1} respectively. Results of the Student's *t*-test shows that these values are equal (*p*-value = 0.830). The cellulase activities in both the newspaper and office paper broths decreased between 0 and 12 hours of incubation (one-way ANOVA *p*-value < 0.001). After 6 and 12 hours the Student's *t*-test shows that the cellulase activity in the newspaper broth is higher than in the office paper broth (*p*-values = 0.016 for 6 hours and *p*-value = 0.017 after 12 hours of incubation). Results of the Student's *t*-test indicate that the cellulase activities decrease at different rates in the newspaper and office paper broths during the initial 24 hours of fermentation. After 24 hours there were no statistically significant differences between the cellulase activity measured in the newspaper medium and the office paper medium (Student's *t*-test *p*-values > 0.05 paired time points, and one-way ANOVA *p*-value = 0.117 and 0.613 for newspaper and office paper broths respectively). The residual cellulase activities are equal regardless of the substrates used in this study and the average value is equal to approximately 0.020 ± 0.002 FPU.ml^{-1}after 24 hours of incubation, 10 times less than the starting activity.

The differing rates of cellulase activity decrease during the initial 24 hours of fermentation could be explained as follows; different rates of cellobiose production could have been observed and hence a higher cellobiose level in the office paper broth which resulted in the onset of the inhibitory effect of cellobiose towards certain enzymes in the cellulase complex. However, the total decrease in activity is a combination of temperature and the cellobiose inhibitory effect. In the newspaper medium the decrease in cellulase activity is caused almost exclusively by the

temperature, causing the enzymes to denature. This point is illustrated further by examining the results obtained at 30 and 40 °C (figures 3.1 and 3.2), where there was no decrease in activity observed.

A one-tailed Student's *t*-test shows that once the cellulases had stabilised, the activity in the 30 °C broth (on both the newspaper and office paper substrates) was the highest (*p*-values < 0.05). However, this may not necessarily yield the maximum sugar concentrations due to enzyme kinetics.

3.3.2.2 – Sugar production

Of primary concern at this stage of the study is the production of fermentable sugars. As the cellulase system hydrolyses the cellulose in the various paper sources, sugars are produced. For the development of an economically viable process for bioethanol production, the effects of temperature and starting material on the rate and extent of sugar production must be examined. A quick and simple method to analyse the concentration of these sugars in the broth is by DNS determination, which measures reducing sugars. Data with incubations at various temperatures are shown in figures 3.4-3.6.

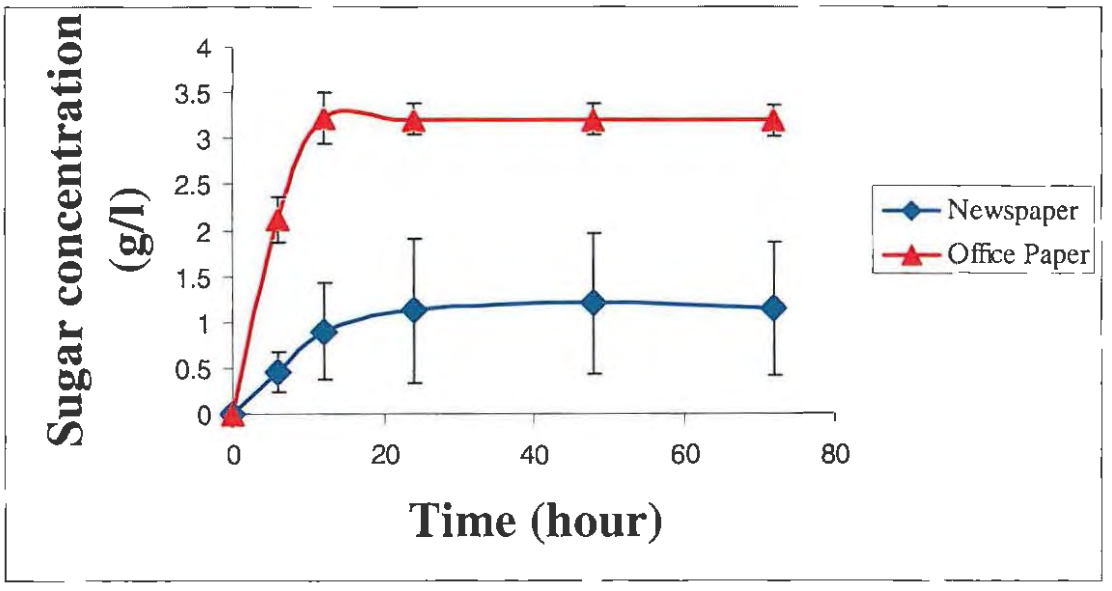


Figure 3.4: Reducing sugar production at 30°C.

At 30 °C, the one-way ANOVA shows that the concentrations of reducing sugars in the newspaper and the office paper media increases during the initial 12 hours of fermentation (p-values = 0.001 for office paper and p-values = 0.043 for newspaper, respectively). The maximum concentrations of reducing sugars reached 3.23 ± 0.30 g/l and 1.20 ± 0.80 g/l in the office paper and newspaper broths respectively. The reducing sugar concentrations were shown to be higher in the office paper broth than in the newspaper broth for all time points (based on the Student's t-test p-values < 0.05). No changes in the concentrations of reducing sugars were detected in either of the two media after 12 hours of incubation (based on the one-way ANOVA with both p-values > 0.9). As 1 g/l of waste paper was used, it can be concluded that 32.5% of the office paper and 12% of the newspaper were hydrolysed into sugars.

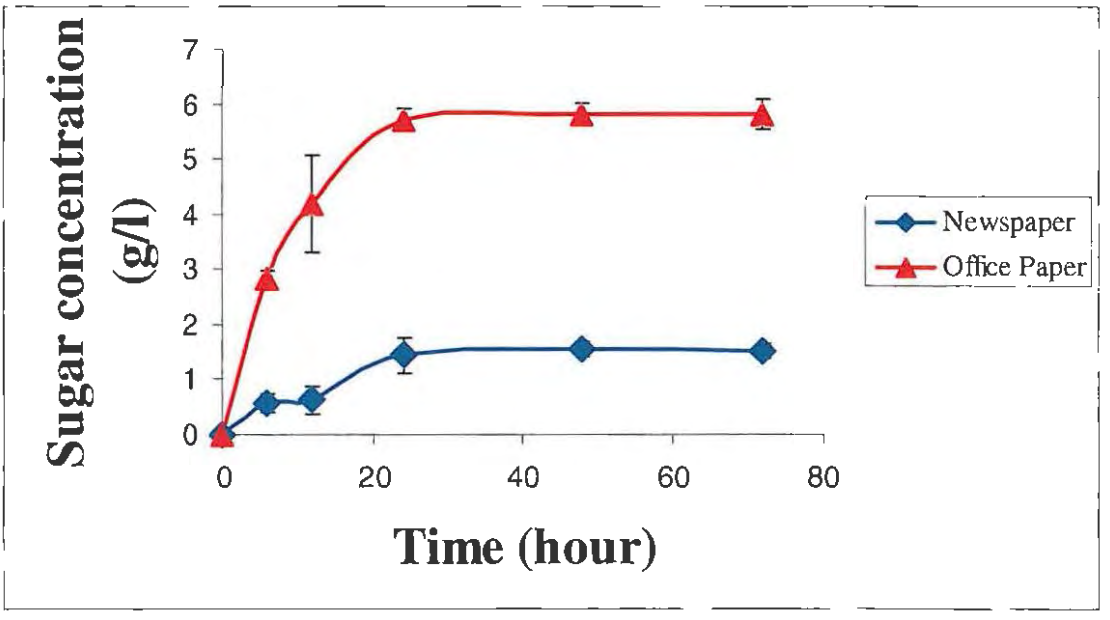


Figure 3.5: Reducing sugar production at 40°C

The reducing sugar concentrations, produced at 40 °C increased with time during the initial 24 hours in both the office paper broth and the newspaper medium (based on the one-way ANOVA *p*-values = 0.0001 and 0.009 respectively). The maximum concentrations of reducing sugars achieved were 5.80 ± 0.19 g/l in the office paper broth and 1.50 ± 0.12 g/l in the newspaper broth. A statistically higher maximum concentration of reducing sugars was measured in the office paper medium than in the newspaper medium (based on the Student's *t*-test *p*-value = 0.0016). The concentration of reducing sugars remains constant in both hydrolysis broths after 24 hours of incubation (one-way ANOVA *p*-values = 0.806 and 0.843 in office paper and newspaper broths respectively).

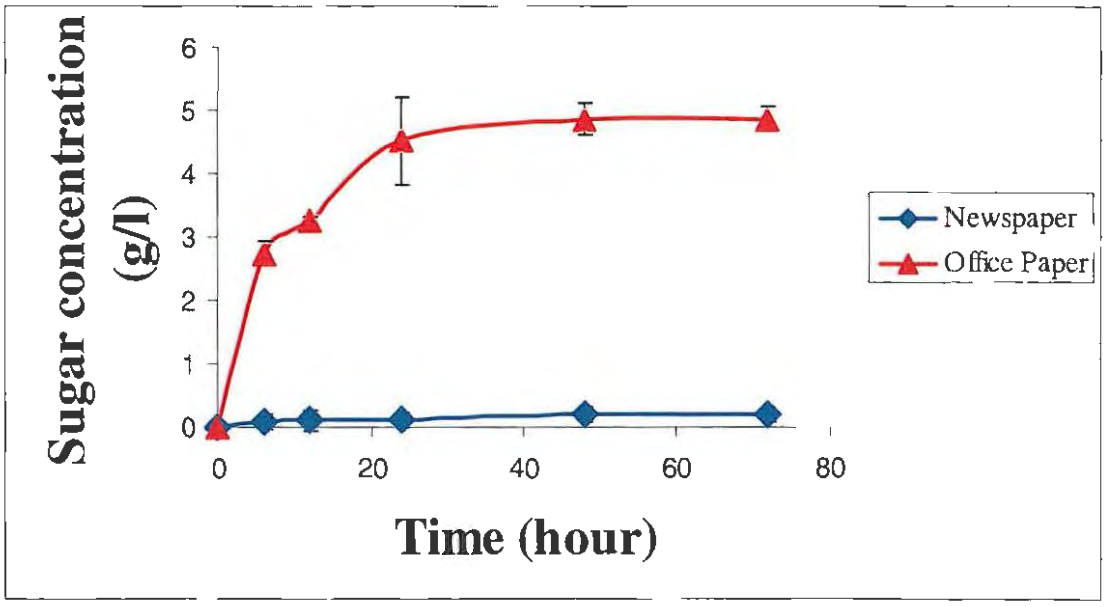


Figure 3.6: Reducing sugar production at 50°C

The reducing sugar yield produced at 50 °C increases for the first 24 hours in the office paper broth, illustrated with a one-way ANOVA test (p-value = 0.0001). The maximum concentration achieved was 4.75 ± 0.20 g/l, corresponding to a 47.5% conversion rate. The reducing sugar production in the newspaper broth is statistically insignificant (p-value = 0.298 for all times points from t = 0 to t = 72). The reducing sugar concentrations produced in the office paper and newspaper broths are statistically different throughout the incubation period, illustrated with a Student's t-test (p-values < 0.004).

The differences in sugar levels produced by these different paper sources have been discussed in various other sections as the relative amounts of resultant sugars affect other processes involved with the production and inhibition of the cellulase system, see sections 2.3.3, 2.3.4 and 3.3.2.1. These differences are caused by the differences

in paper structure, and ease with which the cellulose can be accessed and hydrolysed by the cellulase enzymes. The effects of the various inks could potentially alter the glucose production, and is discussed in the following chapter.

Incubation of office paper at 40 °C in the cellulase medium results in the highest level of sugar production (Student's *t*-test *p*-value <0.05 when compared to the sugar concentrations obtain at other temperatures), stabilizing at 5.80 ± 0.06 g/l of reducing sugars, such as glucose, which accounts for 58% of the starting mass of paper being hydrolysed into sugars.

3.3.3 – HPLC analysis

As the DNS method of sugar determination is non specific, HPLC analysis of the resultant sugar solution produced from office paper at 40 °C is necessary to determine how effective the cellulase system is at producing glucose, as glucose is the desired product at this stage of ethanol production.

As shown in figure 3.7, glucose is the major product of hydrolysis in the office paper broth at 40 °C, yielding a value of 4.35 ± 0.12 g/l. However cellobiose is also produced, with a concentration of 1.97 ± 0.71 g/l. The presence of cellobiose in this broth supports the hypothesis in sections 3.3.2.1 that cellobiose is, in part, responsible for the enzyme inhibitions observed in the office paper broths at 40 and 50 °C. The various sugars were identified by retention time comparisons to known standards (appendix E).

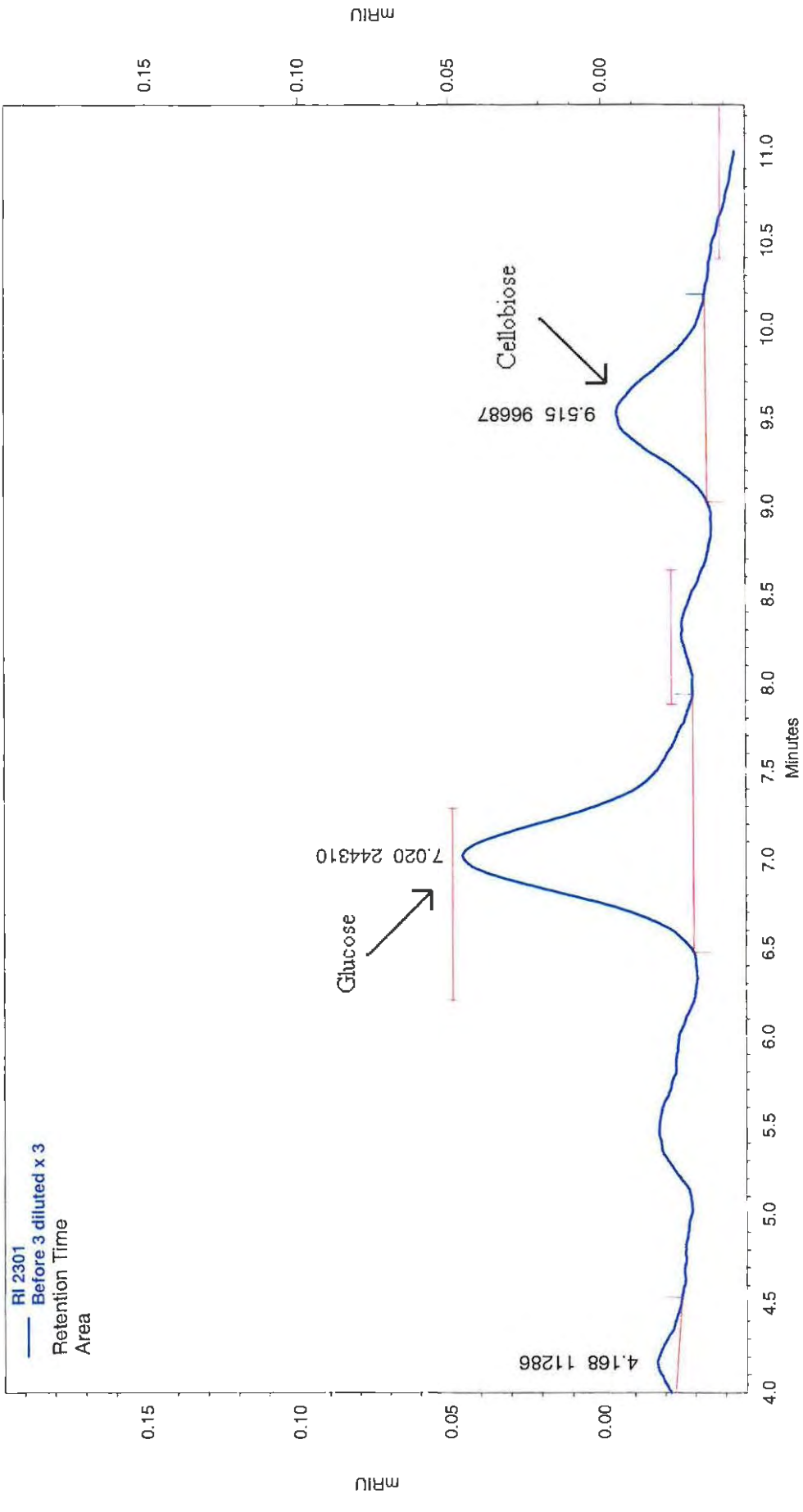


Figure 3.7: Chromatogram of the resultant reducing sugars produced at 40 °C in the office paper broth.

3.4 – Conclusion

The direct fermentation of waste papers into fermentable sugars by *T. longibrachiatum* DSM 769 is an infeasible approach to produce fermentable sugars as the fungus readily consumes the sugars as they are released from the cellulose hydrolysis. The maximum cellulase activities were produced extracellularly during the growth of the fungus of newspaper as the sole carbon and energy source (chapter 2). After biomass separation by centrifugation, the resulting culture broth containing the extracellularly produced cellulase complex was used for the production of reducing sugars. The maximum fermentable sugar yield was obtained with office paper as the substrate for cellulase-catalyzed hydrolysis. The optimum conditions of the cellulose hydrolysis were 40 °C for 24 hours, and the maximum reducing sugar concentration reached was equal to 5.8 g/l, which corresponds to a 58% conversion. Under these conditions, however, cellobiose is produced, which could impede the re-use of the enzyme broth due to its inhibitory effect on cellulase enzymes. This could be separated and sold as a separate product in addition to the bioethanol. Alternatively, methods to improve the hydrolysis of cellobiose into glucose could be explored.

Chapter 4: Printing inks and their possible interference

4.1 – Introduction

As discussed in section 1.4, most waste paper sources have been exposed to inks, as most commercial paper products have been printed on using various inks. These inks contain different pigments, solvents and surfactants, each of which could affect certain aspects of the cellulosic degradation process. In this study, only paper printed with black ink is used, as the analysis of colour inks is a daunting process due to the sheer number of pigments found in such inks (Survey on new printing inks on the US market, 2002). The major pigment found in black inks is carbon black. Carbon black is produced by the partial thermal degradation of hydrocarbons, which leads to the production of finite amounts of PAHs, which are known to have biological effects (see section 1.4). Due to this, there are likely trace amounts of PAHs found in black printing ink. Carbon black itself has sorbent properties (Crescenzi *et al*, 1996), which could cause the appearance of a low sugar yield by sorbing sugars to its surface. The effects that these various compound have on the cellulase activities and production of glucose to be used for ethanol production must be examined.

4.2 – Materials and methods

4.2.1 – Materials

The following chemicals and reagents were used in this section of the study; cellulase from *Trichoderma reesei* (Fluka, Switzerland); 1-octanol (>99%, Merck, Hohenbrunn, Germany); phenanthrene (>96%, Sigma, Germany); naphthalene (99%, Aldrich, Germany); anthracene (>99%, Fluka, U.S.A.); D (+) glucose (Merck, Gauteng, R.S.A.); methanol (99.9%, Sigma-Aldrich, Germany); sodium azide (99%,

BDH laboratory supplies, England); DNS reagent (appendix B); Mandel's medium (appendix A); 0.05M citrate buffer pH = 4.8 (appendix C). The chemicals and reagents were used as supplied.

4.2.2 –Methods

4.2.2.1 – Inhibition of cellulase by PAHs

A cellulase solution of approximately 1 mg/ml was prepared in 0.05 M citrate buffer (pH = 4.8). Solutions of the PAHs anthracene, naphthalene and phenanthrene were prepared in methanol with concentrations ranging from approximately 0 mg/ml to 90 mg/ml. The effects of the various PAHs were assessed by injecting 10 µl of the relative PAH/methanol solution into a 2 ml amber crimp vial for cellulase determination. Care was taken that no headspace was present in the assay vial in question.

The cellulase activity was determined according to Ghose (1987), with slight modifications. A strip of Whatman No. 1 filter paper with dimensions 1 cm x 6 cm was rolled up and placed inside a 2 ml amber crimp vial. An aliquot of 1 ml of 0.05 M citrate buffer (pH = 4.8) was added. An aliquot of 0.5 ml of the cellulase solution was introduced to the crimp vial. 10 µl of the relative PAH solutions were injected under the liquid surface, resulting in reaction concentrations of relative PAHs ranging from 0 µg/l to approximately 600 µg/l. The vials were sealed and place upright in a Labcon water bath at 50 °C for 60 minutes. The vials were then placed in boiling water for 10 minutes.

Glucose levels were determined by extracting the contents of the vials, including all paper fragments, into boiling tubes. An aliquot of 3 ml of DNS reagent was added to the boiling tubes and placed in boiling water for 10 minutes. The samples were diluted with 20 ml of deionised water and the absorbance at 540 nm measured on a UV-1201 spectrophotometer. All samples were processed in triplicate. Blanks were prepared as above for each concentration of PAH in the absence of the filter paper strip. The cellulase activity was expressed as FPU per ml by multiplying the absolute amounts of glucose by the conversion factor of 0.185, according to Ghose (1987), as in section 2.2.2.8.

4.2.2.2 – Sorption onto ink pigments

A solution of 2 g of newsprint ink was made in 70 ml of 1-octanol. A test solution of 200 ml Mandel's medium, with 6 g/l glucose and 0.05% m/v sodium azide to prevent biodegradation of the reducing sugars present, (pH = 6.8) was placed in a 250 ml Schott bottle with a magnetic stirring bar inside. An aliquot of 20 ml of the ink/1-octanol solution was carefully surfaced over the medium to prevent formation of 1-octanol emulsions in the aqueous phase (Brook *et al*, 1990). The Schott bottle was placed on a magnetic stirrer set at 45 rpm and covered with aluminium foil to protect from light. This was allowed to stand at room temperature and was sampled at t = 0, 2, 8, 24, 48 hours by the removal of 20 ml of the aqueous phase from the bottom of the reaction vessel. This was centrifuged at 1600 x g on a Mixtasel centrifuge and the concentration of reducing sugars was measured as glucose equivalents by the DNS method (appendix D).

A 0.5 ml aliquot of the centrifuged sample was added to a boiling tube followed by the addition of 1.0 ml of 0.05 M citrate buffer with pH = 4.6. An aliquot of 3.0 ml DNS reagent was added and the tubes placed in boiling water for 10 minutes. This was diluted with 20ml of deionised water. The resultant solution's absorbance was determined on a UV-1201 spectrophotometer versus the DNS blank.

4.2.2.3 – Statistical analysis

One-way ANOVA tests were performed by Statistica 8.0 (Statsoft Incorporated, Oklahoma, U.S.A.) at a 5% level of significance.

4.3 – Results and Discussion

4.3.1 – PAH inhibitory effects

Due to the production of carbon black, a common black pigment used in printing inks, PAHs are formed, which could have certain biological activities (see section 1.4). The presence of these PAHs could affect the cellulase enzyme activities, by potentially altering the active or binding site of the cellulase enzymes and thus create lower than optimal produced glucose levels. In this study, 3 commonly found PAHs were examined for their ability to inhibit cellulase activity. The results are displayed in figures 4.1 to 4.3.

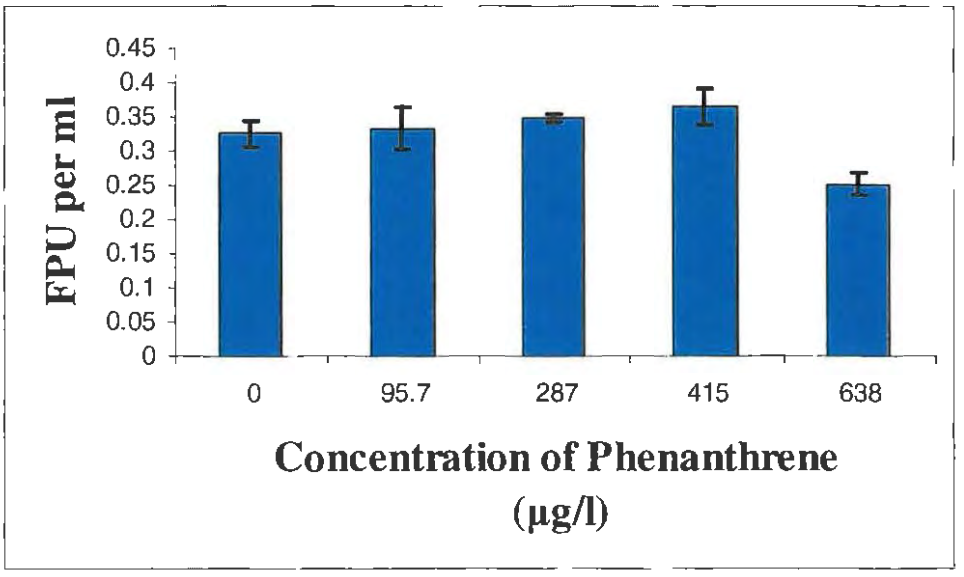


Figure 4.1: The effect of various concentrations of phenanthrene on the cellulase activity.

A one-way ANOVA analysis of the concentrations of phenanthrene from 0 to 415 µg/l yields a *p*-value of 0.438. This indicates that there is no significant change in cellulase activity at these concentrations of phenanthrene, i.e. the cellulase activity remains constant at 0.342 ± 0.017 $FPU.ml^{-1}$. However, the *p*-value obtained incorporating the phenanthrene at a concentration of 638 µg/l was = 0.02, showing that there is a decrease in cellulase activity at these high concentrations of phenanthrene.

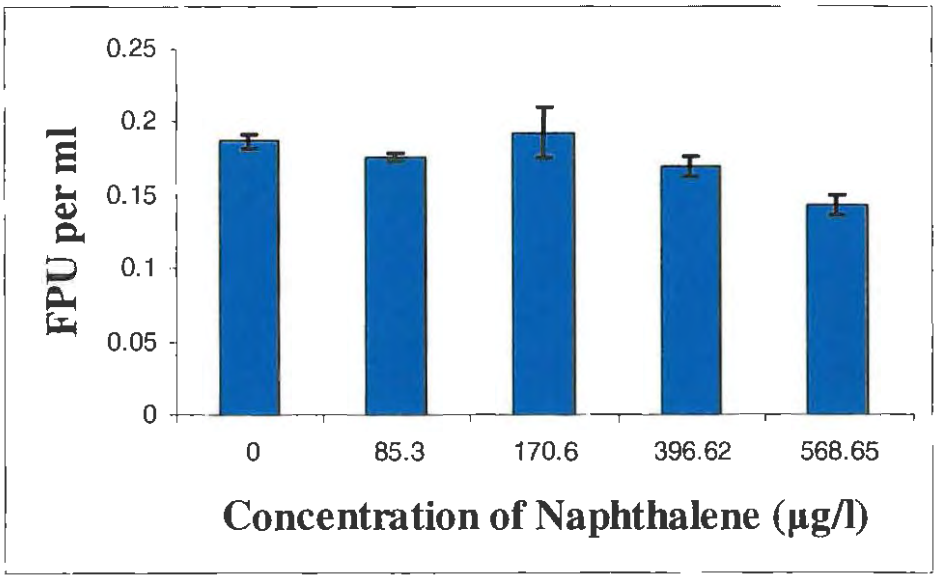


Figure 4.2: The effect of various concentrations of naphthalene on the cellulase activity.

One-way ANOVA analyses of the concentrations of naphthalene from 0 to 396.62 µg/l yields a *p*-value of 0.066. This shows that there is no significant change in cellulase activity at these concentrations of naphthalene, i.e. the cellulase activity remains constant with a mean of 0.181 ± 0.011 $FPU.ml^{-1}$. If the naphthalene concentration of 568.65 µg/l is incorporated in the data for analysis then a *p*-value < 0.0001 is obtained showing that there is a decrease in cellulase activity at these high concentrations of naphthalene.

As PAHs are electron rich molecules due their aromatic character, they can be involved in $\pi - \pi$ donor –acceptor interactions with different biological molecules (He *et al*, 2009). It has been shown that certain PAHs cause protonation of certain ring structures (He *et al*, 2009), which could account for the decrease in enzyme activity by altering the binding or active sites of the cellulase enzymes.

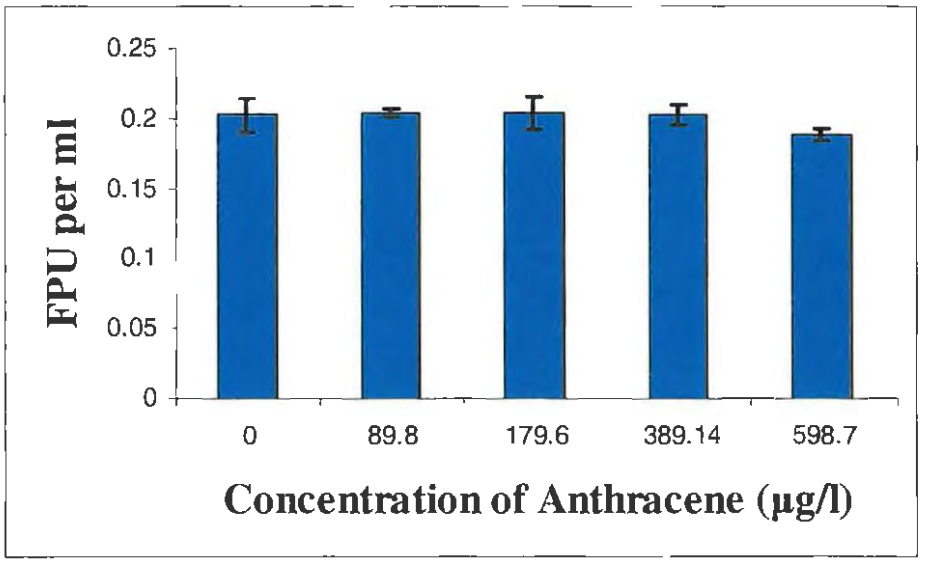


Figure 4.3: The effect of various concentrations of anthracene on the cellulase activity.

The one-way ANOVA analysis of cellulase activity in the presence of anthracene yields a *p*-value = 0.201. This shows that anthracene has no inhibitory effect at these levels and the cellulase activity remains constant with a mean of

0.20 ± 0.01 FPU.ml^{-1}.

4.3.2 – Sorption study

Carbon black has been shown to provide sorption surfaces for a wide variety of organic compounds (Crescenzi *et al*, 1996). Therefore, sorption of reducing sugars could occur on the carbon black particles associated with waste paper, thus leading to a decrease in the concentration of the reducing sugars available for bioethanol production. The extent of the sorption of organic compounds onto particulate matter is generally assessed using sorption isotherm measurements (Tandlich, 2004). A

suspension of the particulate matter, i.e. carbon black, is prepared in the aqueous phase in question and the suspension is exposed to the organic compound studied (Tandlich, 2004). After a given equilibration period the residual concentration of the organic compound in the aqueous phase is measured and the extent of sorption is assessed using mass balance of the organic compound (Tandlich, 2004).

Separation problems were observed during preliminary sorption experiments with the newsprint ink used in the printing of the newspaper used in the study. Therefore it was impossible to assess the extent of the reducing sugar sorption onto the carbon black particles using the sorption isotherm method. A solution of the newsprint ink in 1-octanol was used as a substitute matrix, given the hydrophobic nature of 1-octanol and the solution of the carbon black from the newsprint ink in it. If the carbon black functions as a sorption sink for the reducing sugar molecules, then a concentration gradient will be formed between the 1-octanol/ newsprint mixture and the cultivation medium. If the cultivation medium is exposed to a 1-octanol solution of the carbon black, then a decrease in the reducing sugar concentration in the cultivation medium will be observed if the sorption onto carbon black is significant.

The glucose molecule is unlikely to dissolve in the 1-octanol phase due to the value of its 1-octanol/water partition coefficient of log K_{OW} of -3.24 (Sangster, 1994). However, the possible effects were assessed in a control experiment, using 1-octanol with carbon black omitted from the solution.

D (+) glucose was used as a model reducing sugar. The control results are found in figure 4.4.

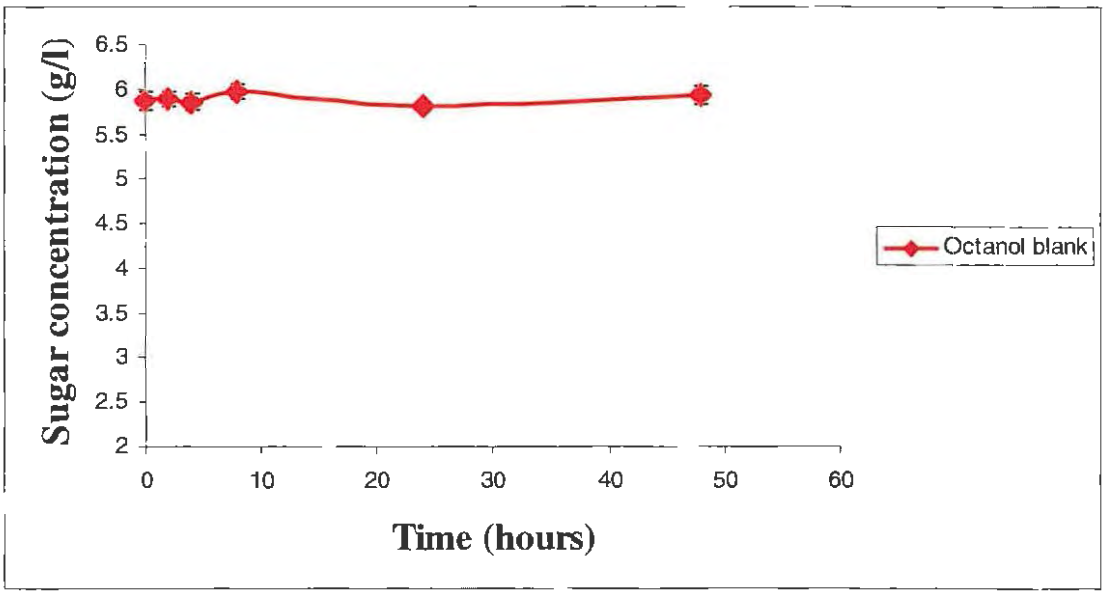


Figure 4.4: Sugar concentration (g/l), as determined by DNS, over time in the 1-octanol blank.

The sugar concentration remains constant throughout the period of the study at 5.89 ± 0.06 g/l. This is illustrated by a one-way ANOVA *p*-value of 0.839. Thus the glucose concentration does not change over time and it can be stated that there is no sorption, or dissolution, of glucose into 1-octanol; hence study into the effect of the ink can be carried out and is shown in figure 4.5.

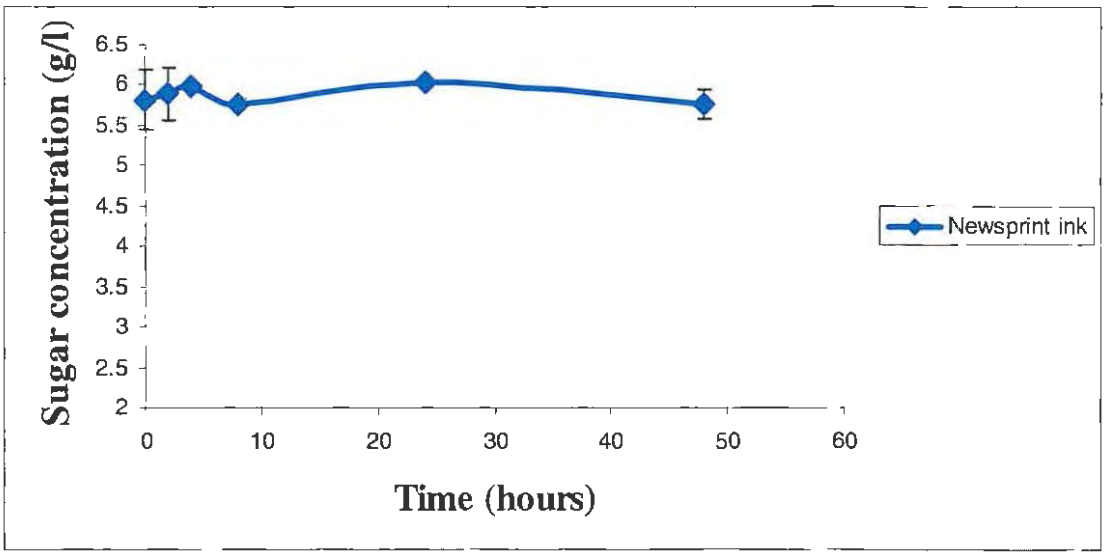


Figure 4.5: Sugar concentration (g/l), as determined by DNS, over time in the presence of newsprint ink.

The sugar concentration remains constant throughout the period of study at 5.88 ± 0.11 g/l. This is shown with a one-way ANOVA, conducted from t = 0 to t = 48 hours (p-value = 0.956). Thus there is no change in the sugar concentration over the time period examined; hence the newsprint ink does not sorb glucose in Mandel's medium in the presence of sodium azide as a preservative.

4.4 – Conclusion

Phenanthrene and naphthalene cause inhibition of cellulase activity at relatively high concentrations. The concentrations found to cause cellulase activity are unlikely to be present in waste paper, as PAHs associated with carbon black are only found in trace amounts (see section 1.4); however, this remains to be confirmed. Anthracene does not cause cellulase inhibition at the concentrations examined. From this study, it has been shown that the presence of newsprint ink does not adsorb D (+) glucose in solution and as such would not affect the glucose yield from the hydrolysis of waste

paper exposed to newsprint ink and is therefore not a concern for industrial scale-up. Therefore, the presence of inks on the waste papers used for cellulase production (chapter 2) and in waste papers as cellulase substrates (chapter 3) have not shown an influence the extent of sugar production and therefore will not influence the production of bioethanol.

Chapter 5: Ethanol production

5.1 – Introduction

Bioethanol is becoming an increasingly desirable alternative fuel for several reasons, the most notable begin its renewability and carbon neutral character (see sections 1.1 and 1.3.2). It is produced by the fermentation of carbohydrates, such as glucose, by a diverse range of organisms, such as yeast (see section 1.3.6) and bacteria, for example *Zymomomas mobilis* (Bai *et al*, 2008). In the alcoholic brewing industry, the most commonly used yeast is *Saccharomyces cerevisiae* (see section 1.3.1). Ethanol is produced by *S. cerevisiae* as part of the anaerobic conversion of glucose into ATP and CO_2 (see section 1.3.7) and is therefore associated with a decrease in environmental glucose levels and an increase in biomass. In order to monitor bioethanol production, the rate of cell proliferation and sugar consumption are important variables to determine. As the substrate utilized in this study results from the enzymatic degradation of the cellulose found in waste office paper certain other carbohydrates aside from glucose, such as cellobiose, could be present. Therefore, the analysis of which carbohydrates are utilized is an important consideration for future process enhancement.

5.2 – Materials and Methods

5.2.1 – Materials

The following chemicals an reagents were used for this part of the study: KH_2PO_4 (98-100.5%, Merck, Gauteng, R.S.A.); NH_4Cl (99%, Merck, Gauteng, R.S.A.); $MgSO_4 \cdot 7H_2O$ (Minimum 99% purity, Saarchem, Krugersdorp, R.S.A.); $MgSO_4$ (anhydrous, Merck, Gauteng, R.S.A.); KCl (99%, Merck, Gauteng, R.S.A.); YPE

(Yeast Peptone Extract) agar (Fluka, Switzerland); yeast extract (Fluka, Spain); HCl 32 % v/v (Merck, Gauteng, R.S.A.); acetonitrile (99.9%, Sigma-Aldrich, Germany); CDCl3 (99.8%, Merck, Germany); ethanol (absolute, distilled solvent from Rhodes University chemistry stores, Grahamstown, R.S.A.); DNS reagent (Appendix B); 0.05 M citrate buffer (pH = 4.8, appendix C). The chemicals were used as provided by the suppliers.

5.2.2 – Methods

5.2.2.1 – Preparation of the stock culture

The following medium was used for reconstitution of lyophilised culture and for the growth of the seed culture: 6 g/l yeast extract; 5 g/l KH_2PO_4; 1.5 g/l NH_4Cl; 1 g/l $MgSO_4$; 1 g/l KCl; 6 g/l D (+) glucose; deionised water to volume. This was then sterilised at 121 °C in a HA-3D autoclave for 15 minutes.

Saccharomyces cerevisiae DSM 1333 was purchased from DSMZ (Germany) as a lyophilised culture which was reconstituted with the preparation medium, prepared above. This was plated on YPE agar and incubated at 30 °C in a model 3028 incubator for 5 days and thereafter maintained on the YPE agar at 4 °C until use.

A 5 mm x 5 mm section of the incubated YPE agar was aseptically inoculated into 200ml of preparation medium in an air tight vessel with a gas trap to make the seed culture. This was agitated with a magnetic stirrer at 50 rpm at 32 °C for 2 days and a 2.5 ml aliquot was used to inoculate the experimental cultures.

5.2.2.2 – Experimental medium preparation

The crude sugar solution produced as described in chapter 3 was centrifuged on a Mixtasel centrifuge at 1600 x g for 10 minutes. The supernatant was removed and the remaining pulp discarded. Yeast extract was added to produce a concentration of 5 g/l and the pH was adjusted to 5.0 (Andrietta *et al*, 2007) by the gradual addition of 3.2% v/v HCl (diluted from 32% v/v HCl). The experimental vessel had 250 ml of this medium introduced to it, and was sterilised at 121 °C in a HA-3D autoclave for 15 minutes. This was inoculated with 2.5 ml of seed medium (prepared in section 5.2.2.1) and incubated at 32 °C in a model 3028 incubator and agitated at 50 rpm by a magnetic stirrer.

5.2.2.3 – Growth curve and sugar utilization

The medium prepared in 5.2.2.2 was sampled at t = 0, 1, 4, 8, 10, 12, 24, 36, 48 hours by the aseptic removal of 5 ml from the reaction vessel into pre-weighed centrifuge tubes. These were centrifuged in a Mixtasel centrifuge at 1600 x g for 10 minutes. The supernatant was removed and analysed for reducing sugar. The pellet was re-suspended in 5 ml of physiological saline (0.9 % w/v NaCl in deionised water). This was intended to wash any remnants of extracellular compounds. The samples were centrifuged in a Mixtasel centrifuge at 1600 x g for 10 minutes again, and the supernatant was discarded. The pellet was again re-suspended, this time in 5 ml of deionised water. This was intended to remove any NaCl and other extracellular compounds, since residual salt could compromise the determination of the dry weight of *S. cerevisiae* DSM 1333. This was centrifuged in a Mixtasel centrifuge at 1600 x g for 10 minutes. The washing with deionised water was repeated. After the final

supernatant was discarded, the centrifuge tubes were placed in a Labcon drying oven at 108 °C for 6 hours. The tubes were allowed to cool in a desiccator for 30 minutes and weighed on an AUW 220 balance. The post-drying mass was subtracted from the pre-weighed tube mass, and the dry weight calculated in g/l. This was performed in duplicate, in two separate fermentation vessels.

A 0.5 ml aliquot of the original supernatant was placed in a boiling tube, together with 1.0 ml of 0.05 M citrate buffer (pH = 4.8) and 3.0 ml of DNS reagent (prepared in appendix B). The boiling tube was placed in boiling water for 10 minutes and diluted with 20 ml of deionised water. After cooling for 10 minutes, the absorbance was measured against a glucose standard curve (appendix B) at 540 nm on a UV-1201 spectrophotometer. This was performed in duplicate.

A 5 ml sample was taken from the reaction vessel before inoculation with *S. cerevisiae* DSM 1333 and after 48 hours to analyse the sugars present before and after fermentation by means of HPLC (see Appendix D).

5.2.2.4 – Ethanol production

The experimental medium (section 5.2.2.2) was sampled at t = 0 and 48 hours by the aseptic removal of 5 ml from the reaction vessel into centrifuge tubes. These were centrifuged on a Mixtasel centrifuge at 1600 x g for 10 minutes. Supernatant was transferred in 2 ml amber crimp vials so that no headspace was present. The vials were sealed and stored at 4 °C until analysis by GC.

An amber crimp vial had 50 µl of 32 % HCl added to it together with an aliquot of 450 µl of sample and was immediately sealed. A SPME fibre apparatus containing a 65 µm Carbowax ™ – Divinylbenzene fibre was placed inside the vial for 10 minutes, at a depth of 0.6 cm. This was injected into an HP 6830 Gas Chromotography unit, to a depth of 3 cm, as per the ethanol detection method (Appendix E). The presence of ethanol was confirmed by repeating the aforementioned procedure with the addition of 7 µl of absolute ethanol. This was performed in duplicate.

5.2.2.5 – NMR

A sample of 1.5 ml of the experimental medium was taken after 48 hours of fermentation by *S. cerevisiae* DSM 1333. This had 1 ml of $CDCL_3$ added to it and thoroughly mixed on an MT19 vortex mixer for 30 seconds. The layers were allowed to separate and the $CDCL_3$ layer collected and passed through an anhydrous $MgSO_4$ column. After collection, the liquid was placed in a clear glass vial and stored at 4 °C until ethanol analysis.

The sample was analysed in a 600 MHz NMR (Bruker, Germany). A standard containing 2 µl of ethanol in 1 ml of $CDCL_3$ was similarly analysed.

5.2.2.6 – Statistical analysis

The ANOVA test was performed using Statistica 8.0 (Statsoft Incorporated, Oklahoma, U.S.A.) at a 5% level of significance.

Student's *t*-tests were performed by Excel 2003 (Service pack 3, Microsoft Corporation, California, U.S.A.) at a 5% level of significance.

5.3 – Results and Discussion

5.3.1 – Growth curve

Ethanol production by *S. cerevisiae* is associated with the cell growth as it is a by-product of energy production (Bai *et al*, 2008, see section 1.3.6). As such, the rate of biomass growth is an important parameter to monitor.

As shown in figure 5.1, no lag phase was observed during the growth of *S. cerevisiae* DSM 1333, as illustrated at a 5 % level of significance by a one-way ANOVA *p*-value of 0.031, obtained from analysis of biomass concentrations from 0 to time = 10 hours. The biomass concentration within this time period increased from below the detection limit to 1.02 ± 0.03 g/l. The growth of *S. cerevisiae* DSM 1333 ceases after 10 hours, and stabilises at 1.14 ± 0.10 g/l (*p*-value = 0.822).

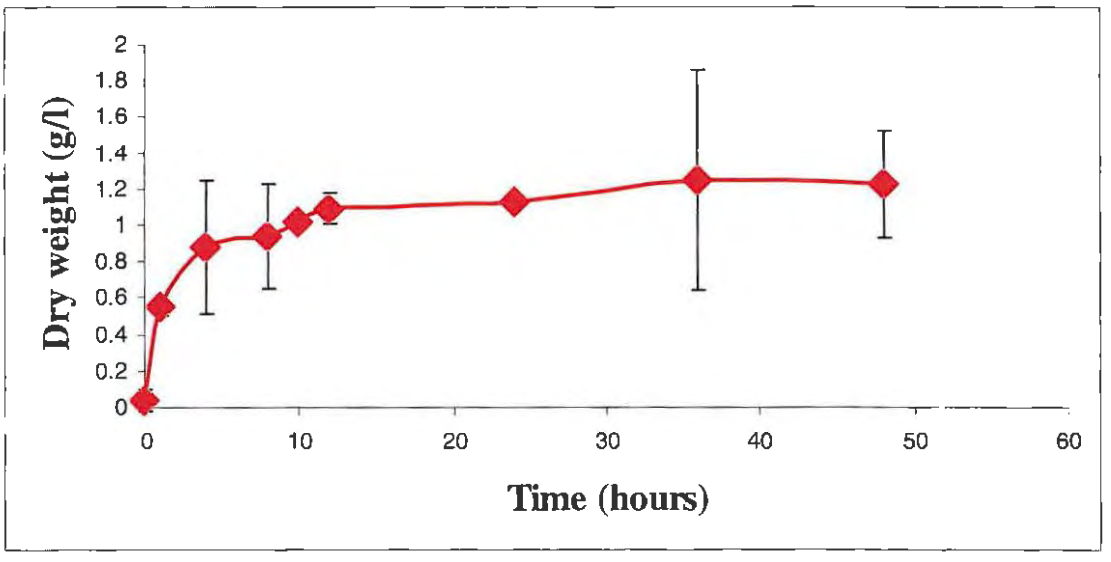


Figure 5.1: Growth curve of *S. cerevisiae* DSM 1333, as dry weight in g/l over time with sugars produced on office paper at 40 °C as per chapter 3 as the substrate.

5.3.2 – Rate of sugar utilization

The ethanol produced by *S. cerevisiae* is due to the anaerobic metabolism of sugars, such as glucose (see section 1.3.7). Due to this, there is likely to be a decrease in sugar levels as the organism grows, and produces ethanol. Due to the nature of the substrate utilized in this study, the presence of cellobiose is expected. Whether or not this carbohydrate is fermentable by *S. cerevisiae* needs to be determined.

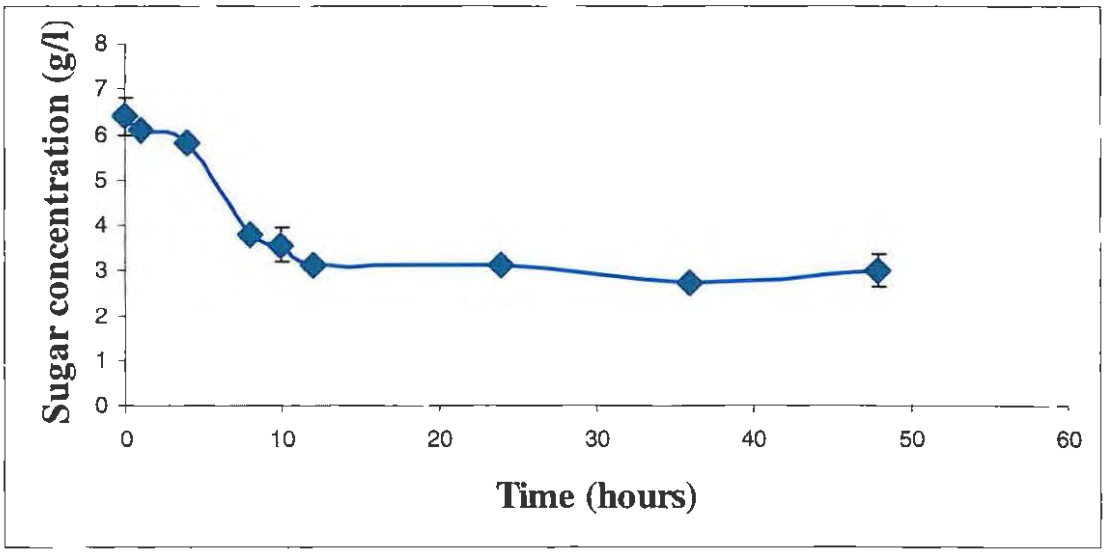


Figure 5.2: Rate of reducing sugar consumption by *S. cerevisiae* DSM 1333 at 32 °C.

There is a delay in the onset of sugar consumption and metabolism by *S. cerevisiae* in the first 4 hours of incubation, with the concentration remaining at 6.12 ± 0.29 g/l (ANOVA *p*-value = 0.194). Between time = 4 and time = 12 hours, the yeast consumes the reducing sugars and the concentration decreases to 3.00 ± 0.19 g/l, (ANOVA *p*-value = 0.0001) where it stabilises for the remainder of the incubation period (ANOVA *p*-value = 0.264). The fact that not all the reducing sugars are consumed suggests that certain reducing sugars are neither utilizable nor fermentable by *S. cervisiae* DSM 1333. As illustrated in section 3.3.3, cellobiose is present in this

fermentation medium. It must be determined whether or not this disaccharide is fermentable by *S. cerevisiae* DSM 1333. According to section 3.3.3, the concentration of cellobiose in the fermentation medium is 1.97 ± 0.71 g/l. As this test indicates that there is 3.00 ± 0.19 g/l remaining in the fermentation medium, it can be speculated that other reducing sugars are present or that glucose is not completely utilized by *S. cerevisiae* DSM 1333.

5.3.3 – Specific sugar utilization

As shown in chapter 3, the sugars produced by the cellulase catalysed hydrolysis of office paper are most notably glucose and cellobiose. Glucose is readily fermented by *S. cerevisiae*, however it remains to be seen whether cellobiose is. HPLC analysis of the sugar content in the medium before and after the fermentation by *S. cerevisiae* DSM 1333 can be seen in figures 5.3 – 5.4.

The glucose concentration decreased from 24.14 ± 0.69 mM (4.35 ± 0.12 g/l; figure 5.3) to below the detection limit of 0.29 mM during the fermentation by *S. cerevisiae* DMS 1333 (figure 5.4, appendix D. The cellobiose concentration before fermentation was 5.75 ± 2.06 mM and after fermentation was 4.46 ± 1.31 mM. A one tailed Student's *t*-test returns a *p*-value of 0.342, thus *S. cerevisiae* DSM 1333 does not consume, nor ferment cellobiose into ethanol. Therefore, not only does cellobiose present cellulose hydrolysis issues (see sections 1.3.4 and 3.3.2.1), but is also resulting in a lower conversion of starting material, i.e. waste office paper, into ethanol.

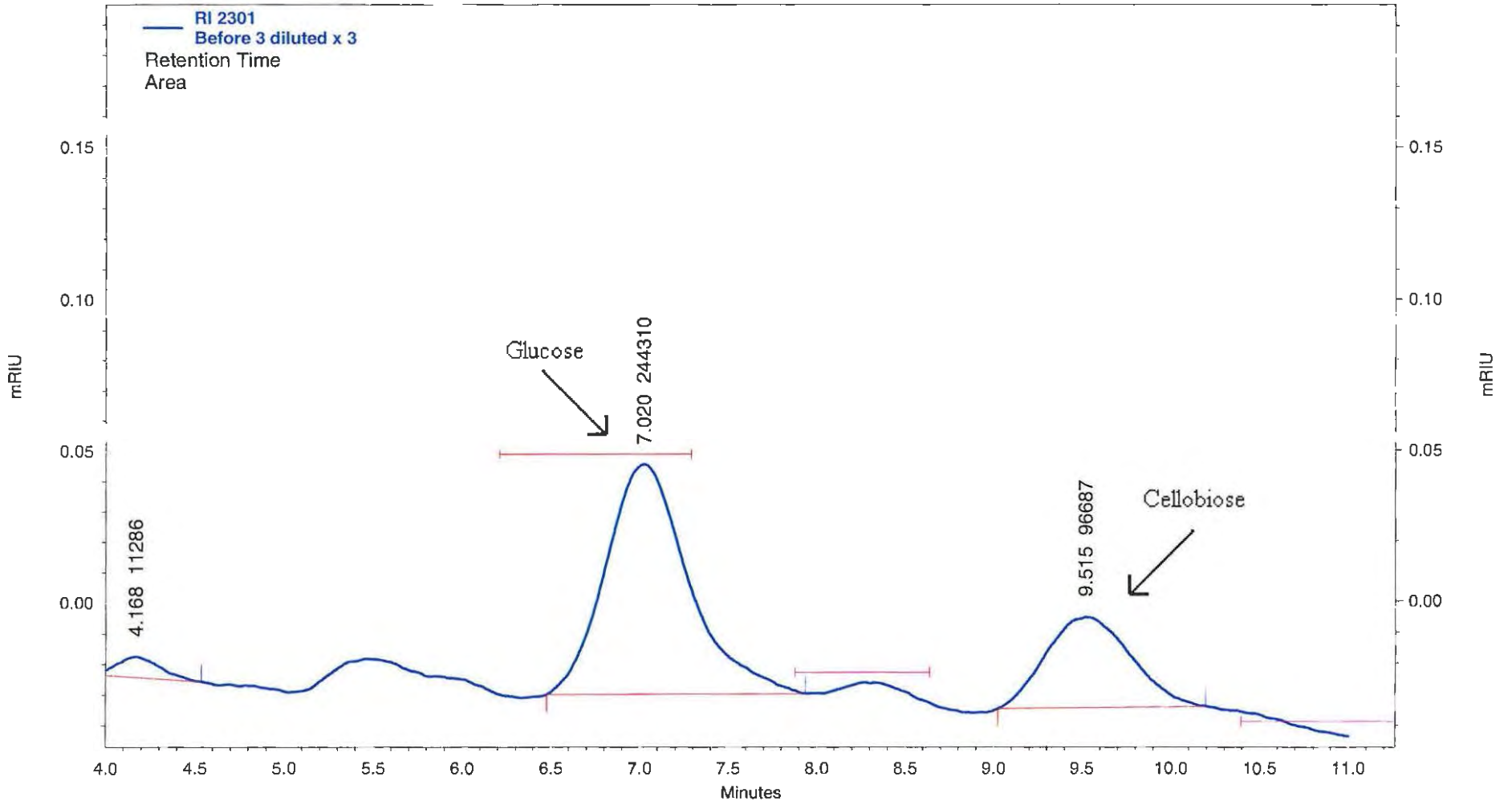


Figure 5.3: Chromatogram of the medium before fermentation by *S. cerevisiae* DSM 1333, diluted by 3.

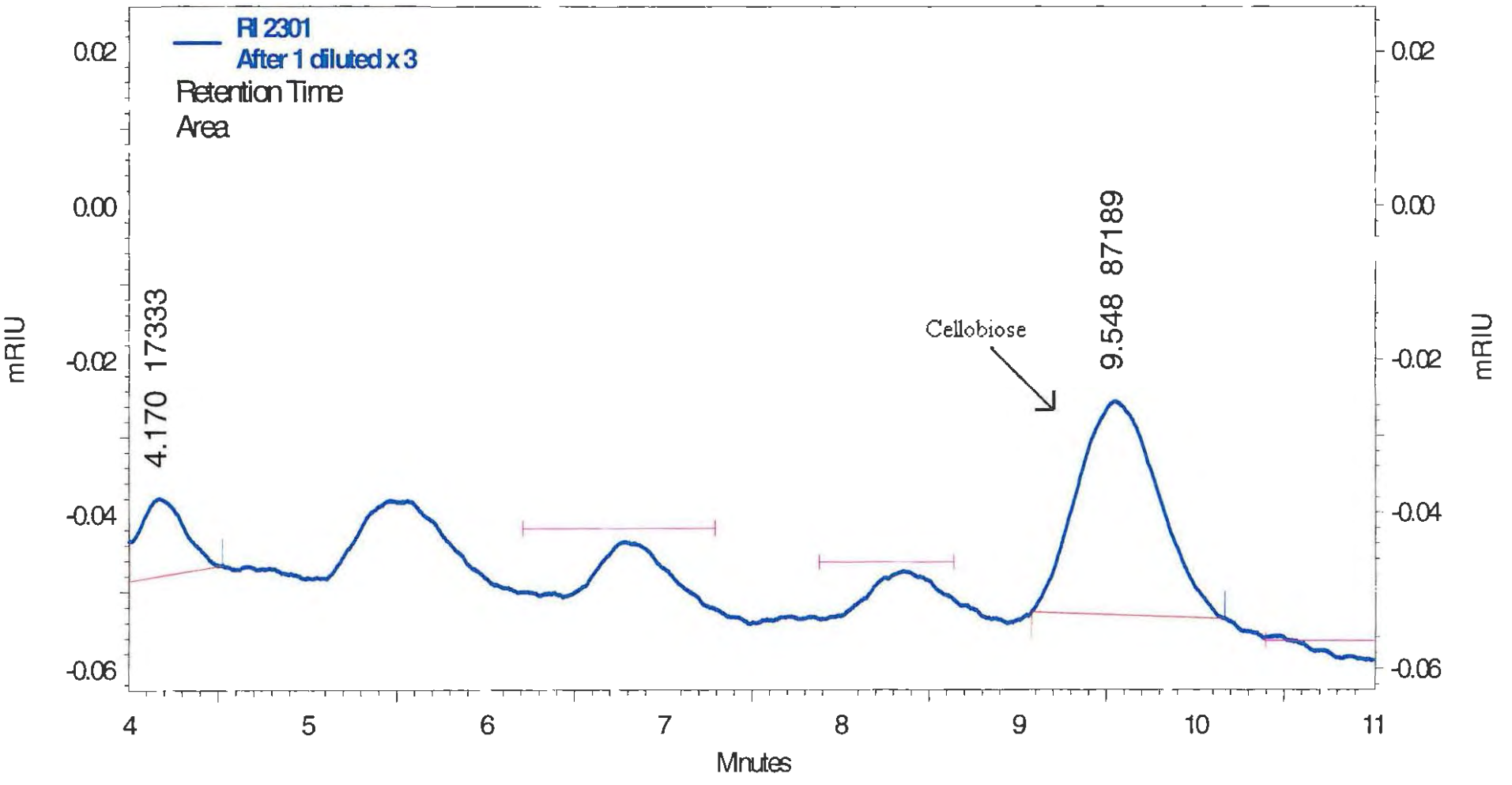


Figure 5.4: Chromatogram of the medium after fermentation by *S. cerevisiae* DSM 1333, diluted by 3.

5.3.4 – Ethanol production

The ethanol produced by *S. cerevisiae,* during the fermentation of glucose, can be detected by headspace absorption and GC-FID separation and analysis. As shown in figure 5.5, after a retention time of 2.5 minutes, a noticeable peak appears after 48 hours of fermentation with *S. cerevisiae* DSM 1333 that is not present before the fermentation

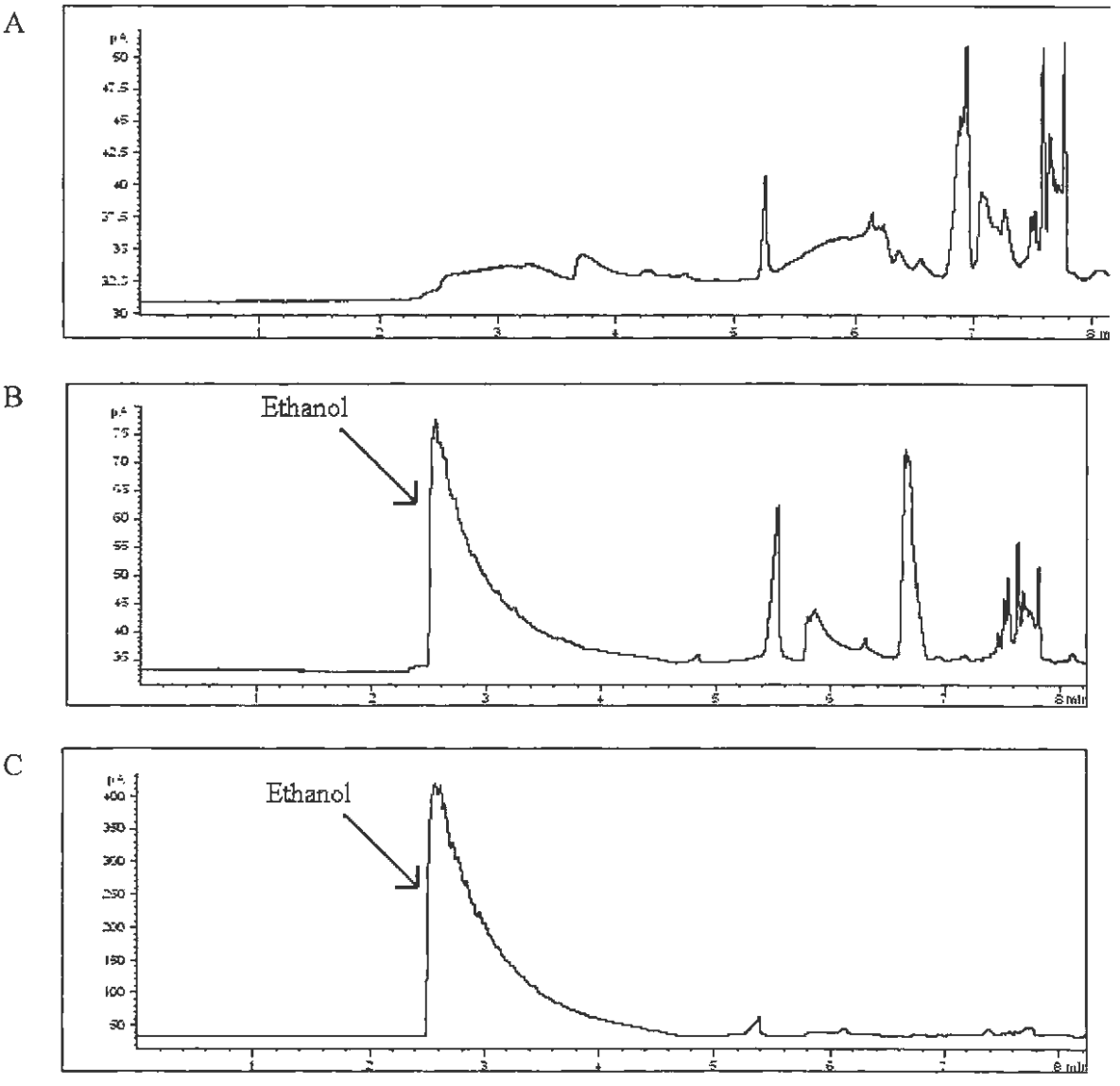


Figure 5.5: Ethanol chromatograms

A – GC-FID chromatogram of the fermentation medium before fermentation with *S. cerevisiae* DSM 1333

B – GC-FID chromatogram of the fermentation medium after fermentation with *S. cerevisiae* DSM 1333, clearly showing an ethanol peak

C – GC-FID chromatograms of the fermentation medium after fermentation with *S. cerevisiae* DSM 1333 and the addition of 7 µl of ethanol as a reference.

After 48 hours of fermentation, the resultant ethanol produced = 1.79 ± 0.26 g/l. As the amount of glucose present = 4.35 ± 0.12 g/l (section 5.3.3) and the theoretical yield of ethanol production = 0.511 x the mass of glucose present (Bai *et al* 2008), this accounts for 80% of the theoretical maximum (2.22 g/l). In order to ensure that the peak observed was indeed ethanol, a 7 µl aliquot of standardised ethanol was added to the head space analysis vessel. As shown in figure 5.5 B and C, the peak at time 2.5 minutes increases from approximately 80 pA to 420 pA with the addition of this aliquot, proving that the observed peak in section B of figure 5.5 is ethanol.

5.3.5 – Ethanol confirmation

To absolutely confirm that ethanol was produced, a crude extraction with $CDCl_3$ was conducted and NMR spectra compared to a standard. As shown in figures 5.6 and 5.7, it is clear that ethanol was produced, with distinct CH_2-0 and CH_3 moieties present.

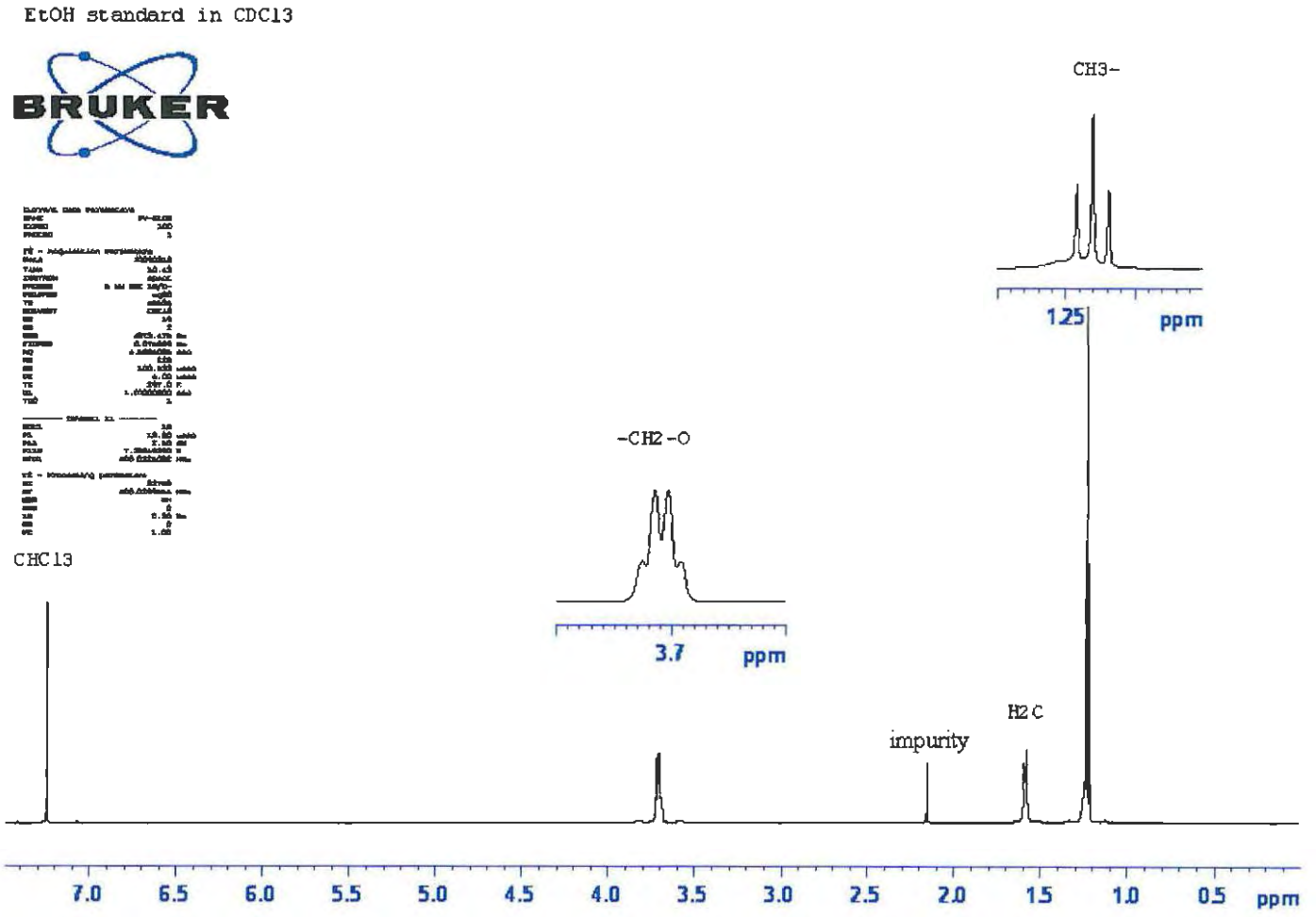


Figure 5.6: NMR spectrum of standard ethanol in $CDCL_3$

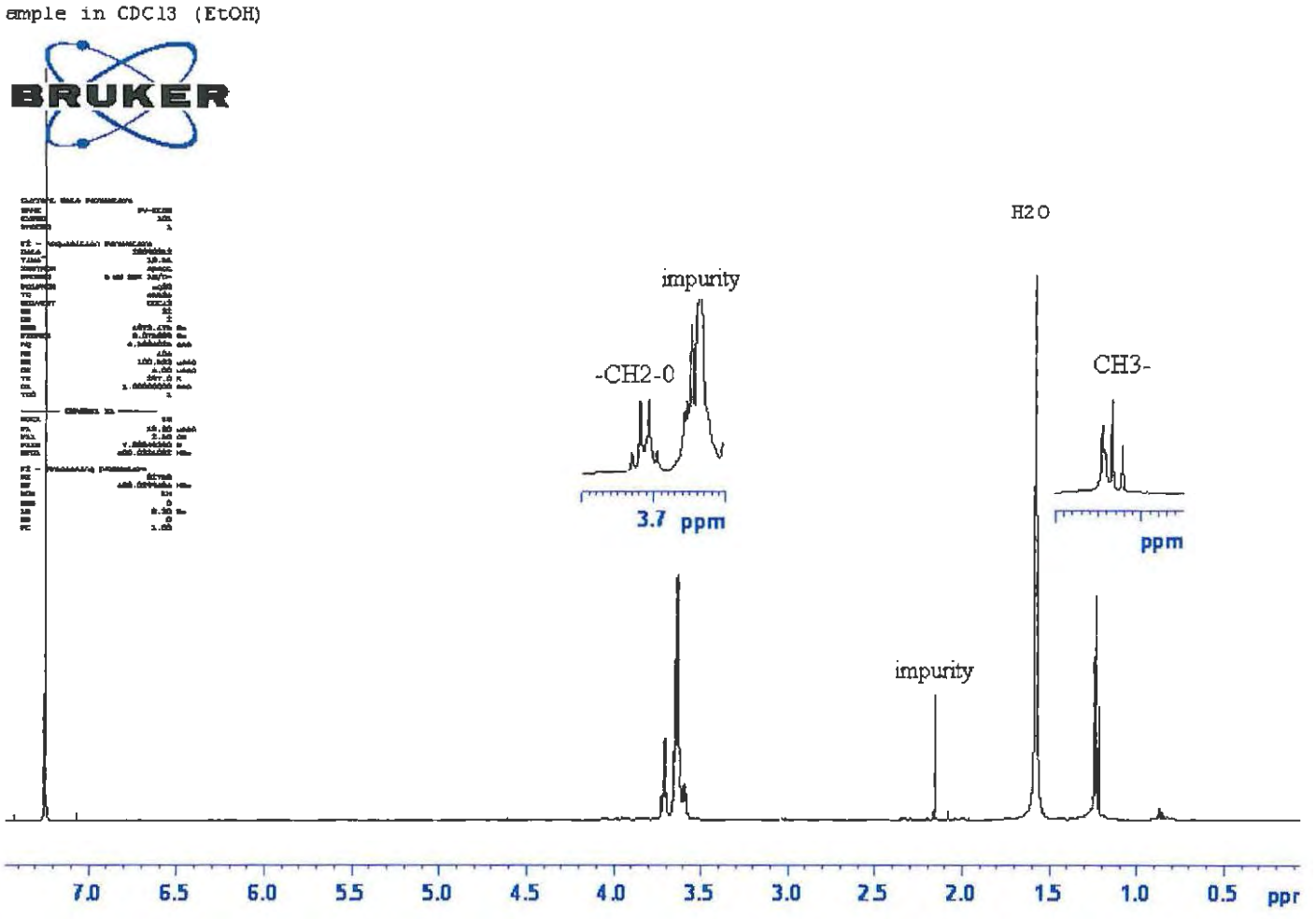


Figure 5.7: NMR spectrum of crude ethanol extract in $CDCL_3$ after 48 hours of fermentation with *S. cerevisiae* DSM 1333

5.4 – Conclusion

Fermenting the crude sugar solution produced by the enzymatic hydrolysis of waste office paper, after adjusting the pH to 5.0 and adding yeast extract to 5 g/l, with *S. cerevisiae* DSM 1333 for 48 hours produces an 80 % yield of ethanol, based on the glucose present. This amounts to 1.79 ± 0.26 g/l of ethanol. As the crude sugar solution contained glucose and cellobiose, and cellobiose is not utilizable by *S. cerevisiae*, not all the reducing sugars were fermented. It can be concluded that cellobiose is not fermentable by *S.* cerevisiae DSM 1333. Increasing the hydrolysis of cellobiose into glucose could potentially increase the ethanol yield. As it stands at present, only 17.9% w/w of the office paper starting material is converted into ethanol.

Chapter 6: Effluent reuse and environmental effects

6.1 – Introduction

Industrial water effluent often has a composition capable of impairing the running of industrial equipment (DWAF 3, 1996), having environmentally damaging effects (DWAF 7, 1996) or potential human health hazards (DWAF 1, 1996). As such, many types of industrial effluent require treatment before reuse (DWAF 3, 1996). Discharge into natural aquatic systems can also lead to harmful effects, and must therefore be analyzed in detail before any discharge takes place (DWAF 7, 1996). Several factors need to be taken into account when handling effluents; pH, dissolved oxygen, conductivity, alkalinity, chemical oxygen demand, nitrogen content (as ammonium), phosphate content (DWAF 1, 1996; DWAF 3 1996; DWAF 7, 1996) and the concentration of effluent in water responsible for 50% lethality of *Daphnia pulex* after 48 hours (LC_{50}) (Slabbert, 2004). By taking these factors into consideration, the most cost effective method and environmentally friendly solution in handling effluents must be researched before implementation of any process at pilot-scale and/or full-scale production. Preliminary assessments of the potential environmental and reuse implications of the effluents produced during the fermentation of paper into bioethanol are presented in this chapter.

6.2 – Materials and Methods

6.2.1 – Materials

The following materials were used to test the water quality: office paper (Mondi Rotatrim, Merebank, R.S.A.) ; Mandel's medium (Appendix A); moderately hard water (prepared as described by Slabbert, 2004, and prepared by the Institute for

Water Research (IWR), Rhodes University); *Daphnia pulex* (less than 24 hours old, IWR, Rhodes University); ammonium kit (1.14752.0001, Merck, Gauteng, R.S.A.); Phosphate kit (1.14848.0001, Merck, Gauteng, R.S.A.); Chemical oxygen demand (COD) kit (500 – 10000mg/l range, Merck, Gauteng, R.S.A.); production effluent (Chapter 2); sugar effluent (Chapter 3); ethanol effluent (Chapter 5). All materials were used as provided by the manufacturer.

6.2.2 – Methods

6.2.2.1 – Sample preparation

Trichoderma longibrachiatum DSM 769 was grown in Mandel's medium with newspaper as the carbon source at 28 °C for 6 days (chapter 2). This was centrifuged on a Mixtasel centrifuge at 1600 x g for 10 minutes to separate the broth medium from the biomass. This was named the production effluent and a sample of 200 ml was set aside for *D. pulex* lethality testing. No chemical characterization of this effluent was undertaken, since this effluent was used in the further production of fermentable sugars for the production of bioethanol. Aliquots of 300 ml were used to hydrolyse office paper at 40 °C for 24 hours (chapter 3). This was named sugar effluent, and centrifuged on a Mixtasel centrifuge at 1600 x g for 10 minutes in order to separate the paper pulp from the sugar solution. The sugar effluent obtained from the office paper broth was used for the *D. pulex* lethality test, and effluent characterization with respect to selected physical and chemical parameters. A 300 ml sample was adjusted to pH = 5.0, yeast extract added to provide a concentration of 5 g/l and inoculated with *Saccharomyces cerevisie* DSM 1333. This was incubated at 32 °C for 48 hours (chapter 5), centrifuged on a Mixtasel centrifuge at 1600 x g to separate the medium from the biomass and named ethanol effluent. This was used in

the *D. pulex* lethality test and to characterise the chemical composition of the medium.

6.2.2.2 – Effluent characterisation

Ammonium ion concentration was determined according to the U.S. EPA 350.1 method (U. S. EPA, 1978), utilizing the colorimetric reaction of ammonium with sodium phenol and sodium hypochlorite. The ammonium kit was used, and the resultant absorbance measured on a SQ 118 spectrophotometer (Merck, Germany). Phosphate was determined according to U.S. EPA 365.2 method (U. S. EPA, 1971), utilizing the colorimetric reaction of low concentrations of phosphorous with ammonium molybdate and antimony potassium tartrate. The resultant absorbance was measured on the SQ 118 spectrophotometer.

The pH was determined using a Eutech 5000 pH meter and the dissolved oxygen on a Eutech DO 1500 (Eutech Instrument, Singapore). For turbidity measurements, the samples were shaken vigorously and the turbidity measured on a Model 966 portable turbidity meter (Orbeco-Hellige, Florida, U.S.A.). Alkalinity was measured using a portable test kit (Blu 52, Midrand, R.S.A.). COD was determined using the COD kit. Conductivity was measured on a 160 conductivity meter (Amel instruments, Milano, Italy).

6.2.2.3 - *Daphnia pulex* lethality test

The *D. pulex* lethality test was carried out according to Slabbert (2004). Solutions of the effluents prepared as in 6.2.2.1 (production effluent, sugar effluent and ethanol effluent) with concentrations ranging from 0% v/v of effluent in moderately hard

water to 50% v/v effluent in moderately hard water were made. Five young *Daphnia pulex* (less than 24 hours old) were carefully pipetted into the solutions with volume = 25 ml, ensuring no air was trapped in their carapace. The temperature was kept constant at 20 ± 2 °C in ambient light. The number of live *D. pulex* present after 48 hours was recorded and used to calculate the lethality, in percentage:

$$\text{Lethality (\%)} = (N_{t0} - N_{t48})/N_{t0} \times 100\% \qquad \textbf{Eq. 6.1}$$

In Eq. (6.1.), N_{t0}: Number of live *D. pulex* at t = 0 h, and N_{t48}: Number of live *D. pulex* at t = 48 h

The lethality versus concentration was used to calculate the LC_{50} (the concentration causing 50% lethality) by extrapolation.

6.3 Results and Discussion

6.3.1 – Effluent chemical characterisation

The chemical character of effluent can have several effects on its disposal and reuse. The results of the characterization are displayed in table 6.1, and each variable is discussed separately. According to DWAF 3 (1996) there are four categories of industrial processes using water, labelled category 1 to category 4. Descriptions and examples of each category follow:

- Category 1 industrial processes require high quality water and as such have strict guidelines. Examples of category 1 processes include evaporative cooling, high pressure boilers, phase separation and washing of high

technology parts. Purifying industrial effluent to use in category 1 processes potentially has high economic implications.

- Category 2 process guidelines are somewhat less stringent than category 1 guidelines, but more so than domestic water guidelines. Examples of category 2 processes include beverage manufacture, solvent cooling, and heat transfer. The economic implications are less than those for purifying effluent for use in category 1, however these are still significant.
- Water for the purposes of category 3 industrial processes has the same quality regulations as domestic water. Generally, a low degree of treatment is needed and the costs are often seen as insignificant to the process in question. Examples include surface washing, food manufacture and low pressure boilers.
- Category 4 processes can use water of practically any quality without causing any significant problems. Examples include fire fighting, rough washing and irrigation.

In general, the vast majority of industrial water is used in category 3 processes, accounting for between 65 and 70% of industrial water usage (DWAF 3, 1996).

The pH represents the balance between H^+ and OH^- ions present. According to the DWAF 3 (1996), the target pH range for water for industrial use is between 7 and 8. A pH outside of this range has the potential to damage machinery, interfere with processes and alter product integrity (DWAF 3, 1996). The pH obtained in the sugar solution was 6.61. According to the DWAF 3 (1996) guidelines, a pH at this level could potentially cause minor machinery corrosion, which could lead to product

impairment in category 1 industrial processes, and provides no functionality changes in category 2 to 4 processes and poses no problems for disposal. The pH of the ethanol effluent is 4.38. This would cause major machinery damage, moderate product impairment and pH adjustment is required before disposal for all categories (1 to 4) of industrial processes. Due to the slightly acidic nature of the processes to produce bioethanol from waste paper, neither of these effluents are expected to interfere with these processes.

Table 6.1 - Chemical characterisation

	Sugar Effluent	Ethanol Effluent
pH	6.61	4.38
Dissolved oxygen concentration (mg/l)	5.80	7.30
Conductivity (dS/m)	5.33	3.38
Turbidity (NTU)	18.2	87.5
Alkalinity	160	40
Chemical Oxygen Demand (mg/l)	3980	3586
Ammonium (mg/l)	< 0.1	< 0.1
Phosphate (mg/l)	5.76	4.95

Atmospheric oxygen is moderately soluble in water and the maintenance of adequate levels is vital to the functioning of aquatic ecosystems (DWAF 7, 1996). The dissolved oxygen content of the sugar solution was 5.80 mg/l, which corresponds to

63.81% of the saturation concentration at 20 °C (DWAF 7, 1996). This level of dissolved oxygen is referred to as sub-toxic, and could exert acute toxic effects if discharged into an aquatic ecosystem, and thus should be adequately oxygenated before being discarded. The dissolved oxygen in the ethanol effluent was 7.30 mg/l, 80.31% of the saturation concentration at 20 °C (DWAF 7, 1996), and thus no oxygenation would be needed for the safe discharge into aquatic ecosystems.

Electric conductivity is the measure of total dissolved solids (TDS) in an effluent, as the higher the level of dissolved ions, the greater the conductivity of the effluent (DWAF 3, 1996). The main issue in industrial processes with effluents with high conductivity is the corrosion to the machinery and subsequent damage (DWAF 3, 1996). The conductivity for the sugar and ethanol effluents was 5.33 and 3.38 respectively. Both of these effluents would therefore cause major damage, process interference and product impairment with category 1, 3 and 4 industrial processes (DWAF 3, 1996). The ethanol effluent would cause minor damage, interference, and impairment in category 2 processes, whereas the sugar solution would cause moderate damage, interference, and impairment, according to DWAF (3, 1996) guidelines.

Turbidity is a measure of the light scattering properties of water and is closely associated with the presence of micro-organisms, many of which are potentially pathogenic to humans. The turbidity is expressed as Nephelometric Turbidity Units (NTU). Ideally, effluents should have as low turbidity as possible (DWAF 1, 1996). The turbidity in the sugar effluent and ethanol effluent was found to be 18.2 NTU and 87.5 NTU respectively. According to the DWAF (1, 1996), turbidity above 10 NTU carries an increased risk of disease. At levels as high as those reported in the sugar

and ethanol effluents, the risk of an epidemic infection rate is present. Due to these high turbidity levels, effluent needs to be handled carefully. In a scaled-up process, great care needs to be taken by personnel when handling and disposing of effluent. In order to reduce the turbidity, the DWAF (1, 1996) recommends the addition of a flocculating agent to the effluent.

Alkalinity is a measure of the acid neutralizing capacity of the effluent in question, as well as a measure of bicarbonate, carbonate and at high pH levels, hydroxide ions and is dependent on all acid and base constituents of the effluent (DWAF 7, 1996). Of added significance, high alkalinity levels coupled with high electric conductivity could result in a high salt precipitation rate (DWAF 3, 1996; DWAF 7, 1996). The alkalinity is expressed in mg $CaCO_3$/l. The alkalinity of the sugar effluent was 160 mg $CaCO_3$/l. This would cause moderate machine damage, moderate process interference and moderate product impairment in category 1 industrial processes. Disposal would cause little problems, apart from desalinization in repeatedly reused effluent. The effluent would cause mild damage, interference and product impairment in category 2 industrial process and is suitable with no treatment for category 3 and 4 industrial processes. The ethanol effluent is suitable for category 1 – 4 industrial processes, in terms of alkalinity. As the alkalinity is relatively low, it is unlikely to contribute significantly to the precipitation of salts.

The definition of COD is a measure of the oxygen equivalent of the organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant (DWAF 3, 1996). As organic matter can be oxidised, the COD gives an estimate of the total organic content of the effluent. Both the sugar and ethanol effluents have

COD levels far exceeding those recommended by the DWAF (3, 1996), and would likely cause severe machinery damage by the development of biofilms and fouling, increased foaming in industrial processes and could severely affect desired end-products, if the effluent is reused. As such, the effluent would have to be treated before use in any industrial application or being discarded. Treatment options include: filtration, oxidising organic material, and flocculation of organic material.

The ammonium concentration in the effluent before fermentation and after fermentation with *S. cerevisiae* DSM 1333 was < 0.1 mg/l. According to the DWAF (7, 1996) guidelines, the target water quality level is 7 mg/l. The level obtained is below this level, and meets the DWAF (7, 1996) limits.

The phosphorous level for the sugar solution is 5.76 mg/l. This just falls into the mesotrophic level, as described by the DWAF (7, 1996). At this concentration, a large biodiversity is observed, with the potential growth of so-called nuisance aquatic organisms, such as blue-green algae (DWAF 7, 1996). The phosphorous level in the ethanol effluent is 4.95 mg/l, which falls into the oligotrophic level (DWAF 7, 1996). At this level, slightly less biodiversity is observed, in comparison to the mesotrophic level, however, nuisance growth are less likely (DWAF 7, 1996). Both the effluents do not contain toxic levels of phosphorous and ammonium, however, the extremely high COD poses problems for aquatic ecosystems. In studying South African waters, it has been found that waters with similar results to these often show evidence of permanent faecal pollution (Palmer *et al*, 1994).

6.3.2 - *Daphnia pulex* lethality test

The lethality test measures the acute toxicity of effluent by determining the concentration of the effluent causing 50 % lethality of *D. pulex* (LC_{50}). The results are shown in table 6.2.

Table 6.2 - LC_{50} of the various stages of effluent production.

Effluent	LC_{50}
Production effluent	7.97% 95% lower confidence limit: 7.13% 95% upper confidence limit: NA*
Sugar effluent	11.27% 95% lower confidence limit: 9.54% 95% upper confidence limit: 13.31%
Ethanol effluent	2.02% 95% lower confidence limit: NA* 95% upper confidence limit: NA*

***NA = Not Applicable**

In order to be classified as non-toxic (according to Slabbert, 2004), the lethality must be less than 10%. This implies that solutions with lethality greater than 10% must be diluted before being discharged into aquatic ecosystems, i.e. all the effluents must be diluted before they can be discharged. In the case of the effluents produced during the course of this study, the production effluent, with a LC_{50} of 7.97%, would have to be diluted to a solution of less than 1.59% v/v in water before being discharged.

Likewise, the sugar (LC_{50} = 11.27 %) and the ethanol (LC_{50} = 2.02 %) effluents would have to be diluted to a minimum of 2.25% v/v and 0.40% v/v in water before being discharged. The dilutions are calculated by dividing the LC_{50} by 5, to find the concentration with a lethality of 10%. These big dilutions could pose large logistical and economic problems when dealing with discarding the effluent, be it after final production or during a spill mid process. Methods of de-toxifying the ethanol effluent (i.e. the end product effluent) should be examined, or the water re-used in other industrial processes, provided that is a suitable, based on the South African Water Quality guidelines for industrial use (DWAF, 1996).

6.4 – Conclusion

Before the reuse of the sugar and ethanol effluents in industrial processes, several forms of processing must be performed in order to minimise machinery damage and product impairment. The costs of performing the necessary processing must be weighed against the costs of simply discarding the effluent and using fresh water in the industrial processes, however; these effluents cannot simply be discarded, and various forms of processing must be performed before being discharged into aquatic ecosystems. In order to develop a cost effective industrial process, the costs of the different processing options need to be examined.

Conclusion

Bioethanol is likely to play a big part in the fuel system of the near future. Although there are several controversies associated with some fermentation substrates, development is likely to continue. Waste paper provides a somewhat untapped resource of cellulosic materials. By producing cellulase enzymes from the fungus *T. longibrachiatum* DSM 769 on waste newspaper, sufficient enzyme activities were produced to hydrolyse the cellulose found in common office paper at 40 °C. A solution of approximately 5.8 g/l of reducing sugars was produced which accounts for approximately 58% dry weight conversion of office paper into reducing sugars. Although glucose is the desired product from the cellulosic hydrolysis, cellobiose was also produced. This contributed to high rates of cellulase deactivation and potentially reduced the end sugar yield due to cellobiose inhibition effects on the cellulase enzymes.

As most waste paper has been exposed to ink, the effects of various constituents of ink were examined. Two of constituents examined (the PAHs phenanthrene and naphthalene) showed a deactivation of cellulase enzymes at high concentrations, at approximately 600 µg/l. Whether or not these concentrations would be found in the fermentation medium remains to be tested. Anthracene did not cause inhibition at the concentrations examined. Newsprint ink particles, i.e. carbon black particles, were shown not to sorb glucose.

The desired end product of this study was ethanol, produced by the fermentation of various carbohydrates produced by the hydrolysis of waste paper. The fermenting

organism used in this study was *S. cerevisiae* DSM 1333, which readily fermented the glucose produced into ethanol over a 48 hour period. However, the cellobiose produced by the cellulose hydrolysis was not fermented by this strain of *S. cerevisiae*. The presence of ethanol was confirmed by GC FID upon the addition of standard ethanol in the sample as well as by NMR spectra at 600 MHz.

As part of the argument for the development of biofuel production is to be environmentally benign, the effluents were examined for toxicity and reusability. The final effluent was found to be severely toxic to aquatic life forms and would cause extensive machinery damage if it were to be reused without sufficient processing. The effluent produced during the production of intermediate products is less toxic than the final effluent, but must still be processed in such a way to make it safe to discharge or reuse.

Summary of the proposed process:

1. Incubate *T. longibrachiatum* DSM 769 on newspaper (10 g/l) in Mandel's medium for 6 days at 28 °C.

2. Remove biomass from cellulase rich liquid medium under aseptic conditions.

3. Incubate office paper (10 g/l) in the cellulase rich liquid medium for 24 hours at 40 °C and remove pulp material from the resultant sugar solution.

4. Add yeast extract to a level of 5g/l in the sugar solution and adjust the pH to 5.0. Sterilise.

5. Incubate *S. cerevisiae* DSM 1333 in this medium for 48 hours at 32 °C.

Recommendations for future research:

Cellobiose was found to be produced in the paper hydrolysis. This carbohydrate inhibited cellulase activity and could have lead to a lower than optimal reducing sugar yield. It was also found that cellobiose was not fermented into ethanol and as such its total effect on this production system is negative, resulting in less ethanol being produced. By developing an advanced process of cellobiose hydrolysis, the potential for increased bioethanol production becomes apparent. As discussed in section 1.3.4, the co-cultivation of *T. longibrachiatum* with *Aspergillus niger* presents a cellulase system with a higher cellobiase portion. Alternatively, a method for cellobiose separation could be developed and the resultant cellobiose used to either induce higher cellobiase from *T. longibrachiatum* or for commercial sale.

Although the effect of ink on the cellulase activities was investigated, the effect on the production of cellulase was not. This is an important consideration for the future.

A time scale for ethanol production should be examined, as in this project the ethanol production was a feasibility study. Optimising the process and incubating for the shortest possible time could improve the economics of the system. The development of an ethanol distilling process needs to be conducted before this proposed technology can be fully implemented and the overall yield of ethanol needs to be maximised.

Appendix A: Mandel's Medium

Materials

$(NH_4)_2SO_4$ (minimum 99% purity, Saarchem, Krugersdorp, R.S.A.); KH_2PO_4 (98-100.5%, Merck, Gauteng, R.S.A.); $(NH_2)_2CO$ (pure, Saarchem, Krugersdorp, R.S.A.); $CaCl_2$ (anhydrous, minimum 95% purity, Associated Chemical Enterprises, Glenvista, R.S.A.); $MgSO_4$ (Minimum 99% purity, Saarchem, Krugersdorp, R.S.A.); tween 80 (Lennon Ltd, Port Elizabeth, R.S.A.); peptone powder (Merck Biolab, Gauteng, R.S.A.); HCl (32% aqueous solution; Merck, Gauteng, R.S.A.); $FeSO_4 \cdot 7H_20$ (minimum 99.5% purity, Merck, Gauteng, R.S.A.); $MnCl_2 \cdot 4\ H_20$ (99% purity, Merck, Gauteng, R.S.A.); $ZnCl_2$ (minimum 97% purity, Merck, Gauteng, R.S.A.); $CoCl_2 \cdot 7H_20$ (99%, Merck, Gauteng, R.S.A.).

Methods

The medium of Mandel (1974) was used for the growth of *T. longibrachiatum* DSM 769, and in all experiments where *T. longrachiatum* DSM 769 is used during the entire course of this study. The composition of the Mandel's medium is as follows: 0.14% m/v $(NH_4)_2SO_4$; 0.20% m/v KH_2PO_4; 0.03% m/v $(NH_2)_2CO$; 0.03% m/v $CaCl_2$; 0.03% m/v $MgSO_4$; 0.20% v/v tween 80; 1g/l Peptone; 1ml/l Trace metal stock solution; Deionised water to volume. The trace element solution contained the following salts: 10ml/l HCl (32%); 9.2g/l $FeSO_4 \cdot 7H_20$; 1.8g/l $MnCl_2 \cdot 4\ H_20$; 1.66g/l $ZnCl_2$; 2g/l $CoCl_2 \cdot 7H_20$; Deionised water to volume. This was sterilized at 121 °C in a HA-3D autoclave (Hirayama Manufacturing Corporation, Tokyo, Japan).

Appendix B: DNS reagent

DNS reagent

The reaction of 3,5 - dinitrosalicylic acid (DNS) with reducing sugars yieds a highly coloured product. This reaction can be used to estimate reducing sugar levels either produced by cellulase enzymes, and hence the cellulase activity, or as a measurement of sugars present in a solution. It is not specific, and reacts with all reducing sugars (Ghose, 1987).

Methods and materials:

Materials:

The following chemicals were used to make the DNS reagent: 3,5 - dinitrosalicylic acid (Sigma, Germany); NaOH (98% purity, Merck, Gauteng, R.S.A.); sodium potassium tartrate (99.5% purity, Sigma-Aldrich, Germany); $Na_2S_2O_5$ (95% purity, GPR, R.S.A.); phenol (Merck, Gauteng, R.S.A.); D (+) glucose (Merck, Gauteng, R.S.A.);

Methods:

The DNS reagent according to Ghose (1987), with a composition of: 10.6 g of 3,5 Dinitro salicylic acid; 19.8 g NaOH; 306 sodium potassium tartrate; 8.28 g phenol; 8.3 g $Na_2S_2O_5$; 1416 ml Deionised water.

Calibration curve

The standard curve (figure B 1) was prepared with serial dilution of D (+) glucose in 0.05 M citrate buffer (pH = 4.8), as prepared in appendix C, creating 6 different

concentrations ranging from 0 g/l to approximately 10 g/l. An aliquot of 3.0 ml of the DNS reagent prepared above was added to 0.5ml of each of the standard dilutions, together with 1.0 ml of 0.05 M citrate buffer (pH = 4.8). These were placed in boiling water for 10 minutes, diluted with 20.00 ml of deionised water, and allowed to cool to room temperature. The absorbances were read on a UV-1201 spectrophotometer at 540 nm.

As the DNS reacts with the absolute amount of glucose to produce the colour change, the calibration curve is expressed as absolute amounts of glucose, i.e. the concentration, in mg/ml, divided by 2 (as 0.5ml was used). This reaction is used to estimate reducing sugars at various stages throughout this thesis.

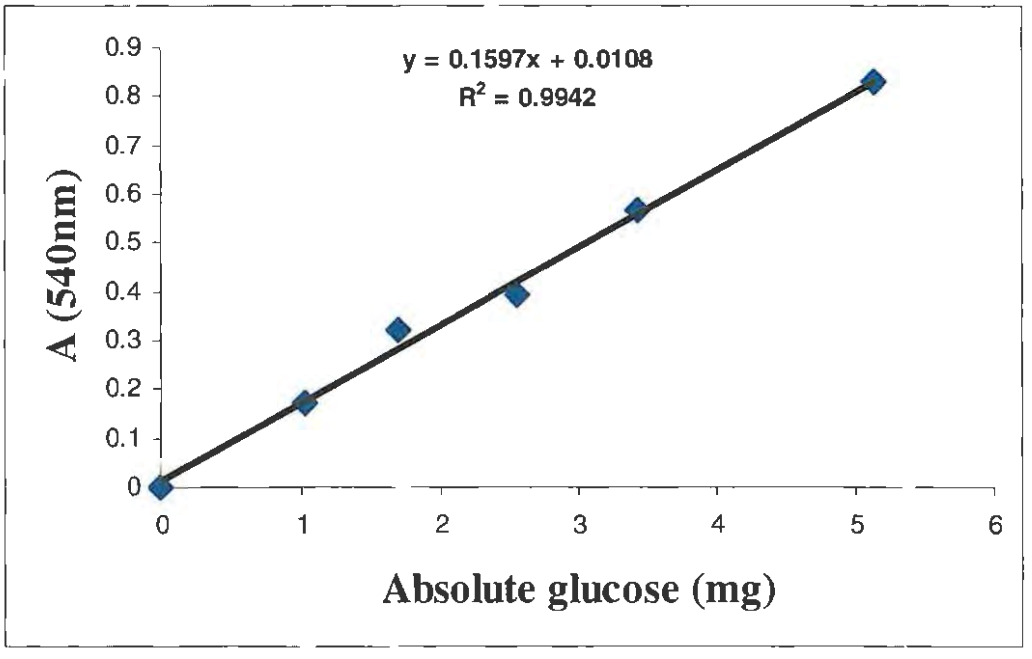


Figure B.1: An example of the standard curve of the DNS reducing sugar determination as D (+) glucose dissolved in 0.05 M citrate buffer, pH = 4.8

Appendix C - 0.05 M Citrate buffer (pH = 4.8)

Materials:

The following chemicals were used to make 0.05 M citrate buffer, with a pH of 4.8: citric acid monohydrate (minimum 99.5% purity, Saarchem, Krugersdorp, R.S.A.); NaOH (Merck, Gauteng, R.S.A.). The chemicals were used as supplied by the respective manufacturer.

Method:

Citrate buffer was used for all enzyme activity assays in this chapter.

1 M citrate buffer with a pH = 4.6 was first prepared; 21 g citric acid monohydrate, 100 ml deionised water, NaOH until pH = 4.6. This buffer was then diluted to 0.05 M. The pH was checked and NaOH added until pH = 4.8.

Appendix D – HPLC validation and analysis

Materials:

The following materials were used to construct calibration curves and to process samples: D (+) glucose (99%, Merck, Gauteng, R.S.A); D (+) cellobiose (96%, Sigma, Germany); acetonitrile (99.9%, Sigma-Aldrich, Germany); Milli Q water (provided by an Academic A10 Milli Q system, Millipore, Ireland).

Mobile phase:

The mobile phase consisted of a 73:27 acetonitrile: Milli Q water solution.

Instrument:

The following instrument components were used to conduct the HPLC analysis; Beckman 110B Solvent Delivery Module (Beckman Incorporated, California, U.S.A.), set at a flow rate of 1 ml/min; Prevail Carbohydrate ES 250 column (Alltech, Kentucky, U.S.A.); K-230I RI detector (Knauer, Berlin, Germany). The run time was 11 minutes.

Calibration curves:

Calibration curves were created using D (+) glucose and D (+) cellobiose standards to formulate standard solutions in Milli Q water. Samples of 50 µl were injected into the HPLC system, with a loop volume of 20 µl, for analysis.

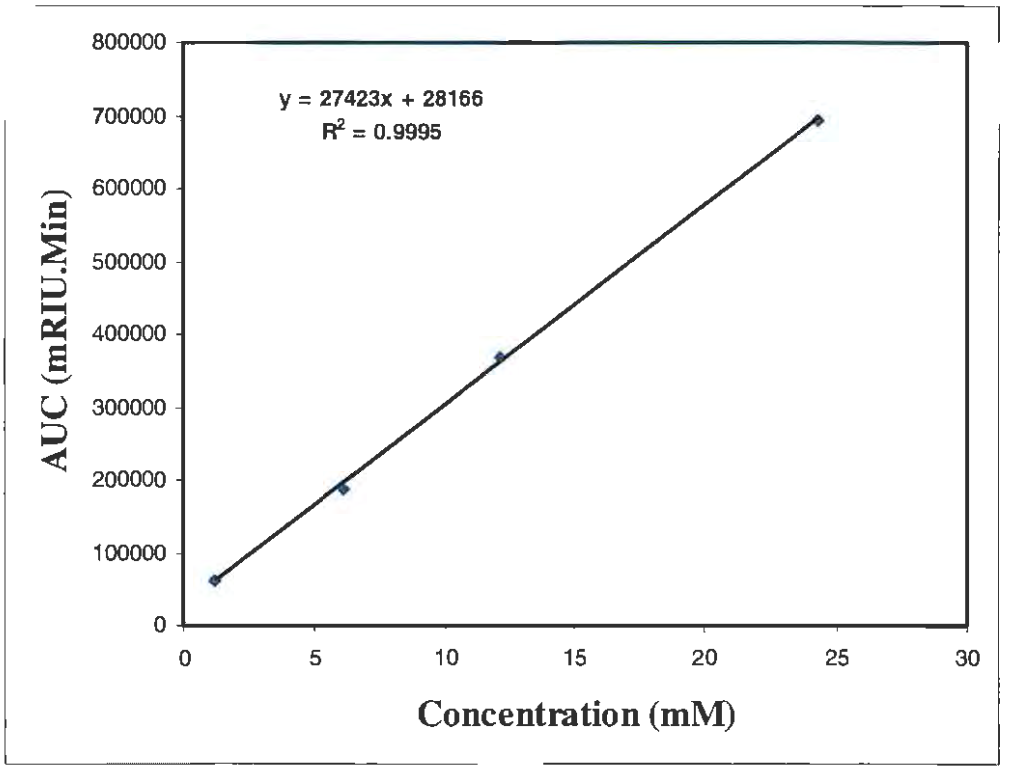


Figure D.1 – Glucose calibration curve.

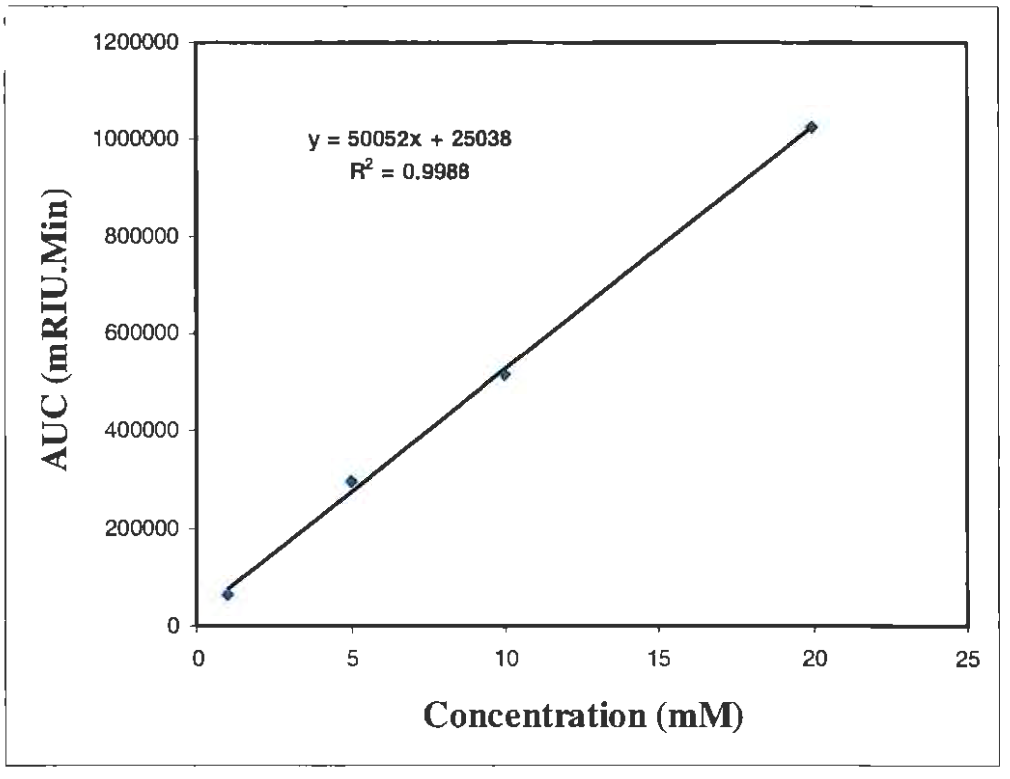


Figure D.2 – Cellobiose calibration curve.

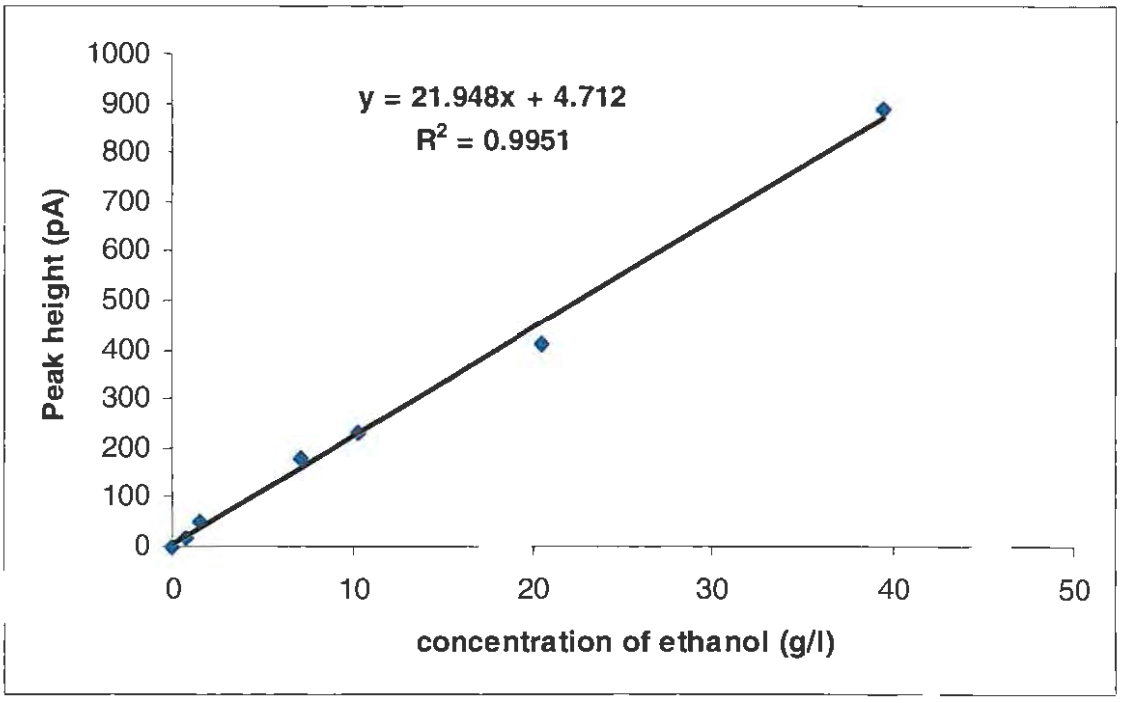


Figure E.1 – calibration curve of ethanol concentration (g/l) versus peak height (pA).

MDL, LOQ, Intra- and Inter day variability:

The following parameters were calculated according to Wisconsin Department of Natural Resources Laboratory Certification Program (1996) where 10 replicates of an ethanol standard is analysed, the means and standard deviation is then used to determine;

Method Detection Limit (MDL)

The MDL is the lowest concentration detectable by the method.

Equation E.1 – MDL calculation

$MDL=t_{(n-1,1-\mu=0.99)}$ (S)

Method Detection Limit (MDL)

The MDL was calculated according to Wisconsin Department of Natural Resources Laboratory Certification Program (1996);

Equation D.1 – MDL calculation

$MDL=t_{(n-1,1-\mu=0.99)}(S)$

Where:

$t_{(n-1,1-\mu=0.99)}$ = t test value at 99 % confidence level, degrees of freedom = 9, read oft test table (Table of Critical values for T, 2009)

S = Standard deviation

The MDL's were 0.29 mM and 0.28 mM for glucose and cellobiose, respectively.

Limit of quantification (LOQ)

In this study, the LOQ was calculated by multiplying MDL by 5,.

Equation D.2 – LOQ calculation

LOQ = MDL x 5

Therefore, the LOQ for glucose = 1.46 mM and cellobiose LOQ = 1.41 mM.

Intra-day variability

For glucose, the intra-day variability was 4.30%. For cellobiose, the intra-day variability was 1.98%.

Inter-day viability

For glucose, the inter-day variability was 5.16%. For cellobiose, the inter day variability was 4.90%.

Sample analysis

All samples were filtered through 0.45 μm cellulose acetate filter (Millipore, Ireland). These were diluted appropriately to fit within the calibration range and 50 μl injected into the HPLC system, with a loop volume of 20 μl

Appendix E – GC FID validation and analysis

Materials:

Absolute ethanol (Distilled solvent, Rhodes University chemistry stores); experimental medium (produced in section 5.2.2.2); 32% HCl (Merck, Gauteng, R.S.A).

Instrument

HP 6890 GC system (Hewlett Packard, Germany) fitted with a SPB -1, 30 m x 0.25 mm ID, 0.25 μm film column (Supelco, Germany). Injector temperature was 250 °C and the detector temperature was 280 °C. Initial oven temperature was held at 35 °C for 4 minutes, thereafter it was ramped at 20 °C per min until it reached 120 °C.

Calibration Curves

Solutions of absolute ethanol were made in the experimental medium, ranging from 0 to 40 g/l. A sample of 450 μl of the standard solution was mixed with 50 μl of 32 % HCl in an amber crimp vial and immediately sealed. A SPME fibre was insert at a depth of 0.6 cm for 10 minutes, after which the fibre was inserted into the HP 6890 GC system injector at a depth of 3 cm. Concentration of ethanol was correlated to peak height (pA).

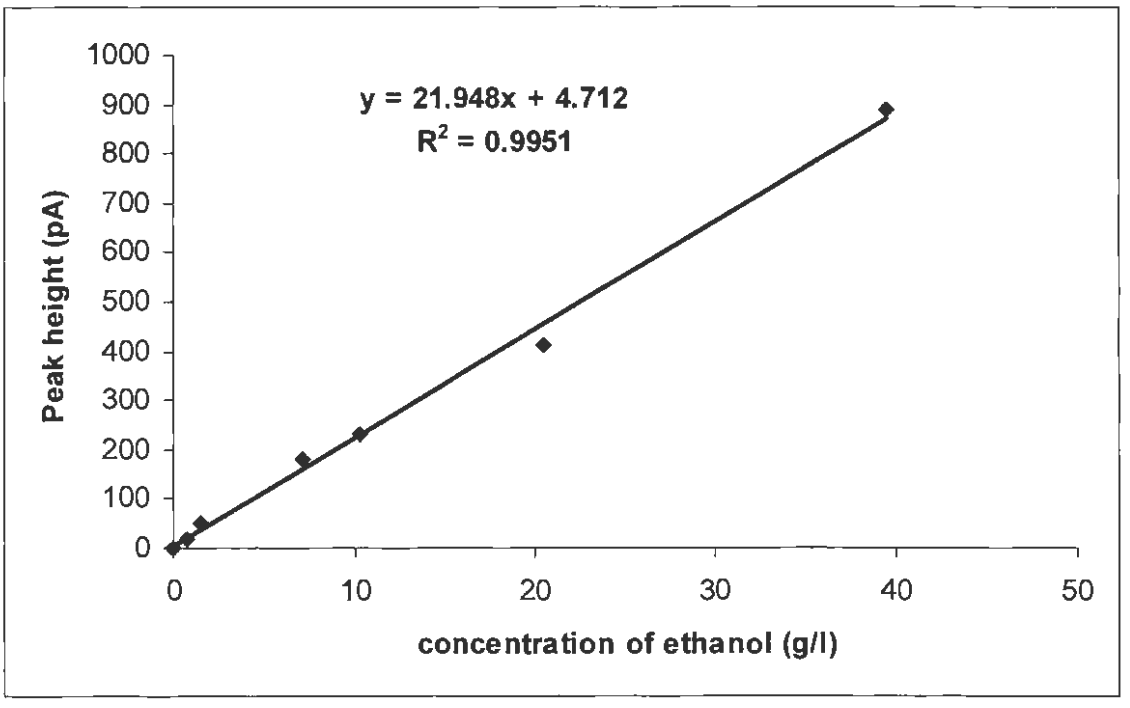


Figure E.1 – calibration curve of ethanol concentration (g/l) versus peak height (pA).

MDL, LOQ, Intra- and Inter day variability:

The following parameters were calculated according to Wisconsin Department of Natural Resources Laboratory Certification Program (1996) where 10 replicates of an ethanol standard is analysed, the means and standard deviation is then used to determine;

Method Detection Limit (MDL)

The MDL is the lowest concentration detectable by the method.

Equation E.1 – MDL calculation

$MDL=t_{(n-1,1-\mu=0.99)}(S)$

Where:

$t_{(n-1,1-\mu=0.99)}$ = t test value at 99 % confidence level, degrees of freedom = 9, read oft test table (Table of Critical values for T, 2009)

S = Standard deviation

The MDL for this method is 0.33 g/l.

Limit of quantification (LOQ)

In this study, the LOQ was calculated by multiplying MDL by 5.

Equation E.2 – LOQ calculation

LOQ = MDL x 5

Therefore, the LOQ for this method is 1.29 g/l.

Intraday variability

The intraday variability is calculated by expressing the standard deviation as a percentage of the mean of 5 replicates of a standard ethanol solution in the experimental medium, analysed on one day. In this case, the mean equals 1.55 g/l and standard deviation = 0.09 g/l. Therefore the intraday variability of this method is 5.73%.

Interday variability

The interday variability is calculated by expression the standard deviation as a percentage of the mean of 10 replicates of a standard ethanol solution in the

experimental medium, analysed over two days, 5 replicates per day. In this method, the mean was = 1.53 g/l and the standard deviation was = 0.08 g/l. The interday variability of this method therefore = 5.18%.

Sample analysis

An aliquot of 450 µl of sample was mixed with 50 µl of 32% HCl in an amber crimp vial. This was sealed immediately. A SPME fibre was placed in the vial at a depth of 0.6 cm for 10 minutes. This was then placed in the HP 6890 GC system injector at a depth of 3 cm, and the method allowed to run.

Appendix F – 0.1 M Phosphate buffer (pH = 7.4)

Materials

Anhydrous Na_2HPO_4 (99%, Saarchem, Krugersdorp, R.S.A); Anhydrous NaH_2PO_4 (99%, Saarchem, Krugersdorp, R.S.A).

Method

A solution containing 13.9 g of NaH_2PO_4 in 500 ml of deionised water and a solution containing 24.4 g of Na_2HPO_4 in 1 l of deionised water was made. An aliquot of 57 ml of the NaH_2PO_4 solution and 243 ml of the Na_2HPO_4 solution were combined. This solution was made up to 600 ml with deionised water

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