BIOETHANOL FERMENTATION OF CORN COB USING IMMOBILISED YEAST CELLS

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0312352M

A dissertation submitted to the Faculty of Engineering and the Built Environment, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Engineering.

Johannesburg, 2011

DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science in Engineering to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any other degree or examination in any other University.

Tessa-Marie Samuel

8th day of July 2011

ABSTRACT

Bioethanol fermentation from non-edible lignocellulosic waste material, such as corn cobs, using immobilised yeast cells will greatly reduce waste, environmental pollution and the world's reliance on crude oil and natural gas. Previous studies have shown that immobilised yeast cells are efficient biocatalysts for repeated batch fermentations and the continuous fermentation of beer and wine. Studies have also shown that immobilisation increased fermentation rates. Corn cob is an attractive feedstock and support for immobilisation because it is cheaply available and in abundance throughout the world. The Ammonia treatment process produced 47.7 % more sugars than the Concentrated Sulphuric Acid treatment process and was therefore used in free and immobilised yeast cell fermentation systems using alcohol tolerant yeast strains, *Saccharomyces cerevisiae* and *Pichia stipitis*. Immobilisations on solid delignified and untreated corn cob supports were carried out. Adsorption of yeast cells on delignified corn cob, suspended for 24 hours, in Yeast Extract Peptone Dextrose (YPD) media resulted in the best immobilisation since it adsorbed approximately 11.9×10^{10} yeast cells and was used in subsequent batch fermentations. The concentration of bioethanol produced from immobilised cell fermentation was 20 % higher than that produced from free cell fermentation. One ton of corn cob would produce 68.4 L of ethanol using this method after purification. Immobilised biocatalysts used in this study are efficient in the fermentation of bioethanol from corn cob and are worthy of further research in repeated batch and continuous fermentation processes.

PUBLICATIONS AND PRESENTATIONS

Publications

Yah, S.C., Iyuke, S.E., Unuabonah E. I., Pillay, O., Chetty, V., **Samuel, T.**, 2010. Temperature Optimization for Bioethanol Production from Corn Cobs Using Mixed Yeast Strains. *Online Journal of Biological Sciences* 10 (2): 103-108.

Conference poster presentations

Samuel, T., Omar, A., Hu, Z, Yah, C. S., Rumbold, K., Iyuke, S. E., 2009. The Efficiency of Pretreatment and Hydrolysis of Corn Cobs for the Production of Bioethanol.*Bio2Biz Conference*, Kwazulu Natal, South Africa.

Samuel, T., Omar, A., Hu, Z, Yah, C. S., Rumbold, K., Iyuke, S. E., 2009. The Efficiency of Pretreatment and Hydrolysis of Corn Cobs for the Production of Bioethanol.*University of the Witwatersrand Postgraduate Symposium*, Johannesburg.

Samuel, T., Iyuke, S. E., Yah, C. S., Rumbold, K., 2010.Bioethanol Production From Corn Cob Using Immobilised Yeast Cells. *14th International Biotechnology Symposium and Exhibition*, Rimini, Italy.

ACKNOWLEDGEMENTS

I first acknowledge GOD, my creator, without Whom none of this would have been possible. **I can do all things through Christ who strengths me. Philippians 4:13**

Tyrone Shaun Grobbelaar, my fiancé, thank you for your unwavering love, care, trust, support and understanding. You have helped me through very difficult times this year and I pray that you are blessed immensely. I LOVE YOU!!!

My family members Charmaine Carmel Samuel, Darren Michael Samuel, and Vicky Du Preez have been my pillars of strength during very difficult times, I LOVE YOU. I would like to thank my extended family members (Michael Edmond & Dolly Paul, Cecil Paul and family, Carleen Bissessar and family, Tanya Grobbelaar and family) and friend Quraisha Moosa for their loving care and support. Vicky, thank you for your unwavering friendship, love and continued support. I thank God that I know you all.

I acknowledge Prof Iyuke as my supervisor. I thank Dr Rumbold and Dr Yah who have assisted, guided and supported me continuously. A special thanks to Miss A. Omar and Mr Z. Hu whom I have worked with to conduct experiments and who have provided me with wheat bran fermentation data.

The Institute of Brewing and Distilling (IBD) and the National Research Foundation (NRF) has provided me with the funding to study toward this Masters Degree. I would not have been able to study without this funding. Thank you!!!

CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE: INTRODUCTION

1.1 Background and motivation

Due to the advancement in technology and the subsequent high demand in energy and fuel, earth's natural resources are diminishing while environmental pollution is on the increase. Therefore a fuel which is secure, modern, sustainable, accessible and environmentally friendly is required. According to Otero et al (2007), bio-based ethanol as an alternative biofuel has emerged as the single largest biotechnology commodity, with close to 46 billion litres produced worldwide in 2005. One way of reducing both the consumption of crude oil and environmental pollution is by producing an oxygenated fuel source, biofuel, as stated by Balat et al (2008), from biomass (recently dead biological and living material).

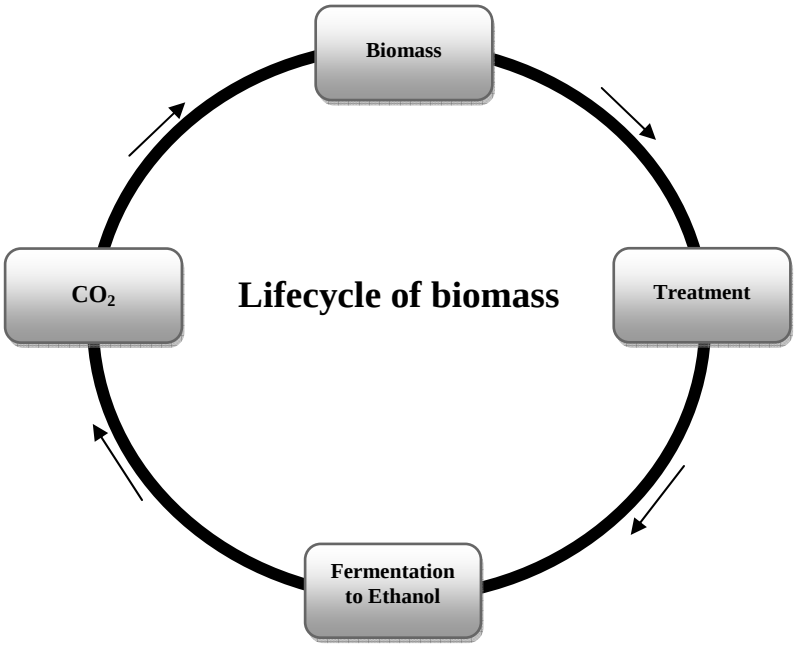

Figure 1.1: Basic process used in the production of bioethanol

One of the major advantages of ethanol fermentation, as illustrated in Figure 1.1, is that carbon dioxide contained in fuel emissions and released into the environment will be reduced since fuel crops absorb this waste by-product for photosynthesis (Sheehan 2001). Other advantages of this biological process include its non-toxicity (Galbe & Zacchi 2007) – improved oxidation of hydrocarbons and a decrease in toxic carbon monoxide and hydrocarbon emissions (Sanchez & Cardona 2008), its suitability for use as a blending fuel when refined further or as a pure ethanol fuel in transportation vehicles and forklifts. Due to this higher octane rating, of approximately 113,less knocking (the pre-ignition of fuel that damages engines) is likely to occur. The US-based National Renewable Energy Laboratory (NREL) states that a 10% ethanol additive in fuel would reduce overall vehicle pollution by 54%.

Amigan et al (2008) reported that higher percentages of quality biofuel energy can be obtained from locally produced agricultural products. Bioethanol has been produced from feedstocks such as corn, sugarcane, wheat, rice, oats, barley and sorghum as stated by Kim and Dale (2003). The cellulose and hemicellulose present in these feedstocks can be broken down into sugars which can then be converted to ethanol by fermentation using yeast cells.

According to Kim and Dale (2003), to prevent competition between human consumption and industrial use of crops as well as use agricultural land more efficiently, it is of utmost importance that non-edible lignocellulosic materials and crops lost in distribution (the agricultural residues such as corn stover) be considered as feedstocks in bioethanol production (Galbe & Zacchi 2007). Corn stover is the world's most abundant agricultural residue as stated by Kadam and McMillan (2003). Other examples of lignocellulosic material include forest residues such as sawdust and dedicated crops such as switch grass (Galbe & Zacchi 2007). Potential bioethanol production of 491 GL per year from crop residues and wasted crops can be expected (Kim & Dale 2003). This is 16 times higher than the current world ethanol production. Corn stover is a mid-term energy supply which would drop fossil fuel

energy input by 86% relative to gasoline and this would result in a decline of petroleum by 69% (Sheehan 2001). The fossil fuel energy benefits translate directly into greenhouse gas reductions (Sheehan 2001). Cao et al (1996) have reported ethanol concentrations of between 45-47 g/Land ethanol yields of 84% and 86% based on the theoretical yield and dry cellulose, from corn cobs, respectively.

However bioethanol fermentation from lignocellulosic material can only be achieved by adequately pre-treating the lignocellulosic material (Patel 2006). Lignocellulosic material contains cellulose which is surrounded by a matrix of hemicellulose and lignin (Galbe & Zacchi 2007). Pre-treatment would remove the lignin and make the cellulose and hemicellulose accessible for conversion to sugars. According to Sun and Cheng (2002) and Galbe and Zacchi (2007), effective pre-treatment methods such as physical, physico-chemical, chemical or biological pre-treatment are crucial in obtaining a high fermentable sugar yield. Pre-treatments such as ammonia steeping and concentrated acid hydrolysis, from previous studies carried out by Cao et al (1996);Chinedu et al (2008) and Sun and Cheng (2002), have been most effective for increasing the sugar yield and decreasing bioethanol production costs (Nwodo et al 2008).

Fermentation can be carried out with alcohol tolerant yeast strains such as *Saccharomyces cerevisiae* and *Pichia stipitis* that converts monosaccharide's, at relatively high conversion rates (Grootjen et al 1990), to bioethanol. Since hydrolysis of corn cobs results in significant amounts of monosaccharide's (Cao et al 1996; Zych 2008), it is necessary in this study to introduce immobilisation of *S. cerevisiae* and *P. stipitis* (Grootjen et al 1990) in order to optimise fermentation and increase bioethanol production.

Ethanol productivity has been enhanced and inhibition caused by high concentration of substrate and products has been eliminated with the inclusion of yeast cell immobilisation. Calcium alginate beads used in continuous fermentation (Nikolic et

al 2009), delignified cellulosic material (Bardi & Koutinas 1994), orange peel (Plessas et al 2007), apple pieces (Kourkoutas et al 2001, 2006) and delignified brewer's spent grains (Kopsahelis et al 2006) used in batch fermentations have been used as supports for immobilisation with increased fermentation rates, which is approximately three fold as compared to free cell fermentation (Bardi & Koutinas 1994).

A study done on the alcohol tolerant yeast strain, *S. cerevisiae*, immobilised on delignified brewer's spent grains for alcohol fermentation at very low temperatures of between 0 and 15ºC have shown good operational stability and increased beer and alcohol productivity during repeated batch fermentations (Kopsahelis et al 2007). Using delignified corn cobs as a support is an attractive option since it is a non-edible agricultural residue that would be cheaply available and in abundance (Kadam & McMillan 2003).

This study therefore seeks to compare the sugar production obtained from corn cobs using two different pre-treatment methods: ammonia steeping and concentrated acid treatment; investigate the effect of media on the immobilisation characteristics of *S. cerevisiae* and *P. stipitis* on corn cobs; and to investigate the effect of these conditions on the fermentation of bioethanol.

1.2 Research Problem

While bioethanol fermentation from edible, cellulosic feedstocks using yeast cells has been carried out with success, very little research has been done on fermenting bioethanol from non-edible, lignocellulosic material using yeast cells immobilised on corn cobs. It has been theorised, however, that the fermentation of bioethanol from lignocellulosic material can be accomplished with adequate pre-treatment methods and that immobilisation increases the rate of fermentation compared to a free yeast cell system. Therefore factors such as concentration of fermentation products

produced and fermentation rate from free and immobilised cell fermentation was investigated in this study.

1.3 Main research aim

The aim of this study is to investigate the impact of using an immobilised yeast cell system for fermentation compared to a free yeast cell system.

1.4 Research objectives

- Investigate the production of fermentable sugars from corn cobs using the two pre-treatment methods: ammonia steeping and concentrated acid hydrolysis.
- Culture and grow yeast cells: *S. cerevisiae* and *P. stipitis*.
- Investigate the immobilisation characteristics of *S. cerevisiae* and *P. stipitis* on corn cobs.
- Fermentation using free yeast cells as compared to an immobilised yeast cell system.

1.5 Dissertation organization

The layout of the dissertation followed an order of Introduction, Literature review, Materials and methods, Results and discussion, Conclusions and recommendations.

CHAPTER TWO: LITERATURE REVIEW

2.1 Non-edible feedstock

The characteristics of biomass which are considered to be desirable for energy production are high cellulose and hemicellulose content; energy density; moisture content; chemical composition; particle size and production rate. The feedstock production must also be sustainable. In order to produce fuel with a high ethanol concentration, the non-edible material must have a significant cellulose and hemicellulose content. After removal of lignin from lignocellulosic material, the cellulose and lignocellulose can be converted to monomer sugars (Galbe & Zacchi 2007). These sugars can then be fermented to ethanol using glucose and xylose fermenting yeast cells.

Feedstocks for bioethanol production include dedicated energy crops, agricultural wastes, crop residues, forest residues, aquatic vegetation and municipal wastes. These materials are cheaply available and in abundance. The chemical compositions (cellulose, hemicellulose and lignin content) of various feedstocks for bioethanol production in Table2.1show that, on average, agricultural wastes with the highest cellulose content are corn cob, softwood stems, cotton seed hairs and saw dust. Forest residues comprise about 80% of the world's biomass (Demirbas 2005) and in the United States alone 33.5-44.6 million metric tons of corn cob are available for harvest each year (Zych 2008).

Table 2.1: Percentage chemical composition of non-edible materials.

	Percentage Composition				
Components	Olive stones (Rodriguez et al 2008)	Corn stover (E Silva et al 2008)	Corn cob (Chen et al 2007)	Wheat straw (Kerstetter et al 2001)	Saw dust (Kerstetter et al 2001)
Cellulose	31.9	40	59.4	38	55
Hemicellulose	21.9	22.5	6.5	22	20
Lignin	26.5	20	22.2	15	20
Other	19.7	17.5	11.9	15	5

Another example of a lignocellulosic material is wheat bran, with industrial bran making up between 14 and 19% of the wheat grain. Wheat bran can be defined as the outer layer, aleuronic layer and all that remains after the starchy endosperm is removed (Maes & Delcour 2001). Typically wheat bran contains starch, arabinoxylans, cellulose, β-glucan, lignin and protein, however, de-starched bran is mainly used for ethanol production (Maes & Delcour 2001).

On average, cellulose makes up about 36-61% of the total dry matter of lignocellulose while hemicellulose makes up to 13-39% both of which can be used in the synthesis of ethanol (Olsson& Hahn-Häigerdal 1996). However, it must be mentioned that this ratio could differ from plant to plant. Cellulose is similar to starch since it too is made up of long chains of glucose molecules; however, there is a difference in their structures as a whole. The structure of hemicellulose follows that of cellulose with the only difference being the inclusion of pentose sugars.

2.1.1 Corn cob as a substrate

Crop residues have been used as a top cover for agricultural land which contributes to soil organic matter and reduction in nutrient depletion (Zych 2008). Residues shield soil from falling rain, wind shear that lead to soil erosion, sun radiation, heat flux and moisture loss(Wilhelm et al 2004).Soil organic matter affects soil water infiltration,

water holding capacity, and aeration (Wilhelm et al 2004) and is associated with continued crop production (Reicosky & Forcella 1988). All of these factors need to be taken into consideration and assessed when considering its removal and use as an energy feedstock.

Corn cob however, has also been used in the past as a fuel in direct combustion in an oxygen-rich environment for cooking and heating (Zych 2008).Corn is a staple food in South Africa with an annual production of 8 million tons. Corn cob can be used as a coal substitute or as mixture of coal and corn cob that would serve to decrease pollution due to the reduction in harmful emissions. When considering the large scale use of corn cob in the bio energy industry the following issues need to be addressed: the production based on harvesting, handling and storage methods; the effect of corn cob removal on soil composition and continued crop production potential, and optimisation of energy conversion methods.

The energy content of lignocellulosic materials needs to be substantial in order to consider its use as an energy feedstock. Energy content is measured in energy per unit volume or weight. These measurements are necessary when considering the volume of feedstock that needs to be harvested, transported, stored, and utilised in an energy production process. The higher the energy density the less volume of feedstock needed for energy production (Zych 2008). A comparison of energy feedstocks, on a dry basis, is given in Table 2.2. Although the energy content of corn cob is less than fossil fuels and coal, it is similar to that of other biomass feedstocks.

Table 2.2: Energy content of biomass feedstocks versus fossil fuels (Zych 2008).

	Corn cob	Corn stover	Switchgrass	Wood pellets	Bituminous coal	Fuel oil
Energy content (MJ/kg)	18.25 -19.18	17	18	19	25.5	43.5

The energy content of all forms of biomass including corn cob should be adjusted to compensate for the moisture content. Corn cobs are not harvested, stored or utilised in a moisture free condition (Zych 2008). Wood pellets have higher energy content due to palletisation. Even though this process increases the density of the product it requires additional energy and equipment which increases cost of production and reduces the products net energy. Corn cobs are sufficiently dense and therefore do not require densification (Zych 2008).

The chemical and physical properties of corn cob make it an ideal second generation energy source. The chemical composition obtained from an African Journal (Akinfemi 2010) is graphically represented in Figure 2.1. Polysaccharides cellulose, the main structural component of the cell walls, and hemicellulose can be broken down to monomer sugars, glucose and xylose. Lignin, a non-carbohydrate, is a complex network that binds cellulose and hemicellulose together. Lignin removal is essential for adequate hydrolysis of the polysaccharides.

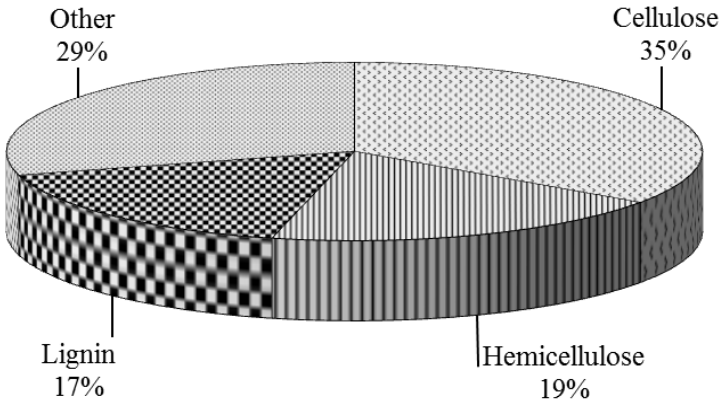

Figure 2.1: Chemical composition of corn cob.

The moisture content of corn cob can affect energy conversion. The weather and time of harvest is of greatest concern. Corn cob with a moisture content of 10-30%, are ideal for energy conversion (Zych 2008). It was found in a study done by Smith et al 1985 that stock piling and ventilating corn cob, with ambient air, for eight to nine months showed a great decrease in moisture. Unventilated cobs showed loss in dry matter due to microbial activity, crop deterioration and spoilage.

Corn cob is the identical alternative to corn grain (Zych 2008) and therefore would eliminate the dependence on a food source, corn, as a feedstock for biofuel. Cao et al (1996) and Chen et al (2007) have recorded percentage yields of ethanol, using corn cob, of 84 and 94%, respectively, thereby making ethanol production from corn cob an attractive option.

2.2 Pre-treatment and hydrolysis methods

Plant cell walls are the source of lignocellulosic materials, also known as biomass. This structure is chiefly represented by the physico-chemical interaction of cellulose, a linear glucose polymer, with hemicellulose, a highly branched heteropolymer, and lignin, a very high molecular weight and cross-linked aromatic macromolecule (Himmel et al 2007; Howard et al 2003; Joseleau et al 1992; Meshitsuka&Isogai 1996; Sakakibara 1991 cited in Bon & Ferrara 2007).

Pre-treatment is the single most crucial step used in bioethanol production, from lignocellulosic material, since it determines the efficiency of the steps that follow. The purpose of pre-treatment is to remove lignin, increase the surface area of cellulose and hemicellulose, and increase porosity of the substrate. Native cellulose is well protected by a matrix of hemicellulose and lignin, as seen in Figure 2.2, which needs to be broken down to expose cellulose and hemicellulose for degradation.

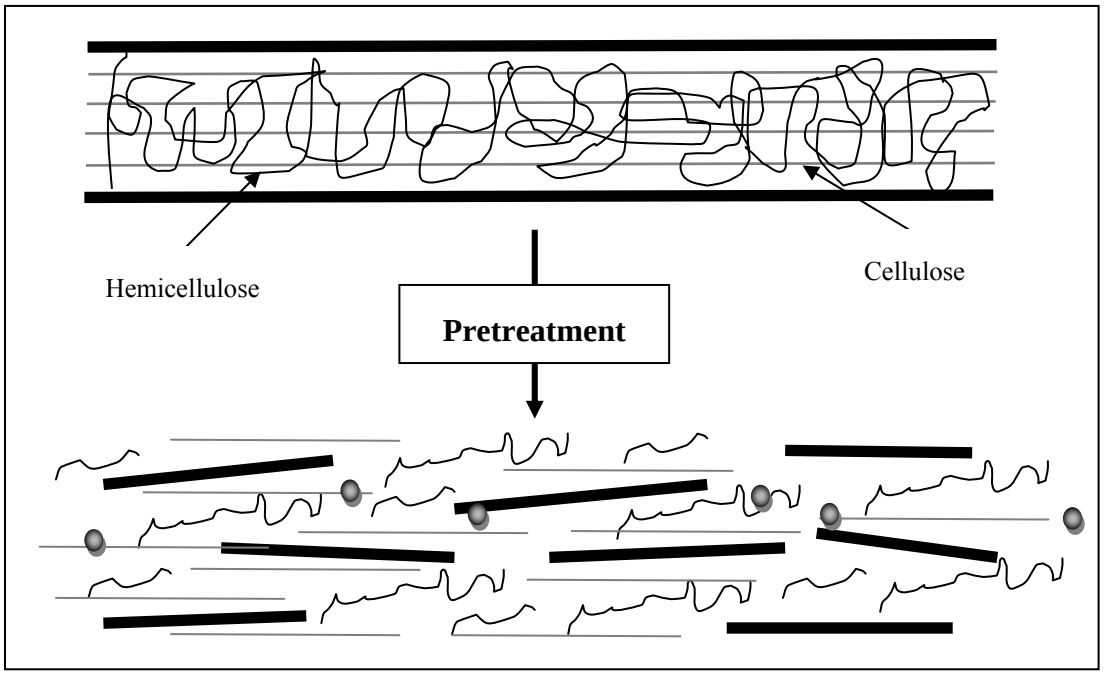

Figure 2.2: Removal of lignin in lignocellulosic substrate exposes cellulose and hemicellulose.

Pre-treatment and hydrolysis of hemicellulose to xylose and cellulose to glucose can be carried out by various processes such as physical, physico-chemical, and biological processes (Sun & Cheng 2002) or a combination of these. According to Sun and Cheng, 2002 for the efficient conversion of hemicellulose and cellulose it is essential that adequate pre-treatment be carried out and must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of by-products inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective.

Methods using low, medium and high pH have been used, which results in high sugar yields of above 90% theoretically, especially for corn stover. A rough classification of treatment methods can also be made according to these (Galbe & Zacchi 2007):

- Acid-based methods, i.e. pre-treatment at low pH, result in hydrolysis of the hemicellulose to monomer sugars and minimize the need for hemicellulases.
- Methods working close to neutral conditions, e.g. steam pre-treatment and hydrothermolysis, solubilise most of the hemicellulose due to the acids released from the hemicellulose, e.g. acetic acid, but do not usually result in total conversion to monomer sugars. Thus this requires hemicellulases acting on soluble oligomer fractions of the hemicellulose.
- Alkaline methods leave a part of the hemicellulose, or in the case of ammonia fibre explosion (AFEX), almost all hemicellulose in the solid fraction. This then requires hemicellulases acting both on solid and on dissolved hemicellulose. An alternative is to perform an acid hydrolysis of this fraction which affects overall costs.

Various studies have been conducted with these treatment methods, on corn stover, which yield high concentrations of fermentable sugars (Table 2.3).

Table 2.3: Summary of treatment studies conducted on corn stover (Galbe & Zacchi 2007).

Treatment method	**Catalyst**	**Time(min); Temp(˚C)**	**Enzymatic hydrolysis conditions**	**Glucose yield (%)**	**Xylose yield(%)**
AFEX	Conc. NH_3	5; 90	1% glucan, washed, 50˚C, 15FPU/g cellulose	96	77.7
Ammonia recycle percolation	NH_3	10; 17	1% glucan, washed, 50˚C, 15FPU/g cellulose	90	41.1
Alkali	$Ca(OH)_2$	4 weeks;55	1% glucan, washed, 50˚C, 15FPU/g cellulose	92	52.8
Dilute acid hydrolysis-1	0.49% H_2SO_4	20; 16	1% glucan, washed, 50˚C, 15FPU/g cellulose	91.6	91.2
Dilute acid hydrolysis-2	5% H_2SO_4	90; 12	3% solids, 50˚C, 15FPU/g solids, 72h	54.6	100
Steam-1	H_2SO_4	5; 19	5% solids, washed, 50˚C, 25FPU/g solids, 48h	73.6	61
Steam-2	SO_2	5; 19	2% solids, washed, 40˚C, 15FPU/g solids, 96h	90	84
Liquid hot water	Water	15; 19	1% glucan, washed, 50˚C, 15FPU/g cellulose	85.2	26.3
Wet Oxidation	O_2, Na_2CO_3	15; 20	2% solids, washed, 50˚C, 25FPU/g solids, 24h	74	53.7

In Table 2.3 it can be seen that ammonia and dilute acid treatment yield the highest xylose and glucose concentrations. Another effective pre-treatment method proposed by Cao et al in 1996 requires the steeping of lignocellulosic biomass in dilute NH_4OH. This process differed from the AFEX process both in concept and technique. Unlike the AFEX process all the hemicellulose was kept intact during the lignin removal and not subjected to degradation. The primary objective of this process was to separate the major components which are the lignin, hemicellulose and cellulose.

According to Cao et al (1996), by steeping the lignocellulosic material in a 2.9M ammonia solution at 26˚C about 80-90% of the lignin and almost all the acetate and alkali-soluble extractives were removed. This was a significant step in the process as these were identified as inhibitors during the fermentation of sugars by yeast. Studies with the above process have shown that the solubility of lignin in dilute ammonia was much higher than reported elsewhere (Cao et al 1996). In order to carry out an effective pre-treatment, different steeping conditions are required for materials with different lignin contents. Ammonia can be recovered under a vacuum at below 60˚C with a recovery of 98%. A pure, high quality lignin can be isolated from the steeped extract and allows its use in synthesis of polymers and chemicals.

Cao et al (1996) went on to say that a combination of ammonia steeping followed by dilute acid hydrolysis gave the highest glucose yield of 92% based on dry cellulose. A cellulose rich residue was obtained after the dilute acid hydrolysis step. By removing all the lignin, hemicellulose, acetate and alkali extractives, a lower enzyme dosage was required for effective hydrolysis of the cellulose to glucose. The glucose-rich solution obtained did not require further treatment and can be used as a substrate for ethanol production. The hemicellulose fraction can be hydrolysed readily by dilute acid hydrolysis and separates from the cellulosic fraction. The hemicellulose hydrolysate which is rich in xylose has no acetate and alkali extractives that can be used as a substrate for xylose fermenting yeast.

It is well known that more severe conditions during pre-treatment will cause greater degradation of hemicellulose sugars and enhance the enzymatic digestibility of cellulose, however, both is not achieved at the same severity (Galbe & Zacchi 2007). The severity correlation describes the severity (Ro) of the pre-treatment as a function of treatment time (minutes) and temperature (˚C), $T_{ref} = 100°C$.

$$\log(Ro) = \log\left(t \times \exp\frac{\left(T - T_{ref}\right)}{14.75} \right) \quad (1)$$

When pre-treatment is performed under acidic conditions, the effect of pH needs to be taken into consideration by the combined severity:

$$\text{Combined Severity (CS)} = \log(Ro) - pH \quad (2)$$

Equations 1 and/or 2 can be used to assess and compare various pre-treatment methods.

2.2.1 Delignification

Lignin is a branched polymer of aromatic compounds (Figure 2.3).In contrast to hemicelluloses, and according to Bon and Ferrara (2007), it is a three-dimensional polyphenolic network built up of dimethoxylated (syringyl), monomethoxylated (guaiacyl) and non-methoxylated (p-hydroxyphenil) phenylpropanoid units, derived from the corresponding p-hydroxycinnamyl alcohols. These give rise to a variety of sub-units including different ether and C-C bonds. Lignin is highly resistant to chemical and biological degradation. This branched polymer is hydrophobic and acts as cement between the plant cells in the middle lamella, and in the layers of the cell wall, which forms, together with hemicellulose, an amorphous matrix in which the cellulose fibrils are embedded and protected against biodegradation. Lignin content and composition vary among different plant groups and between the different wood tissues and cell wall layers.

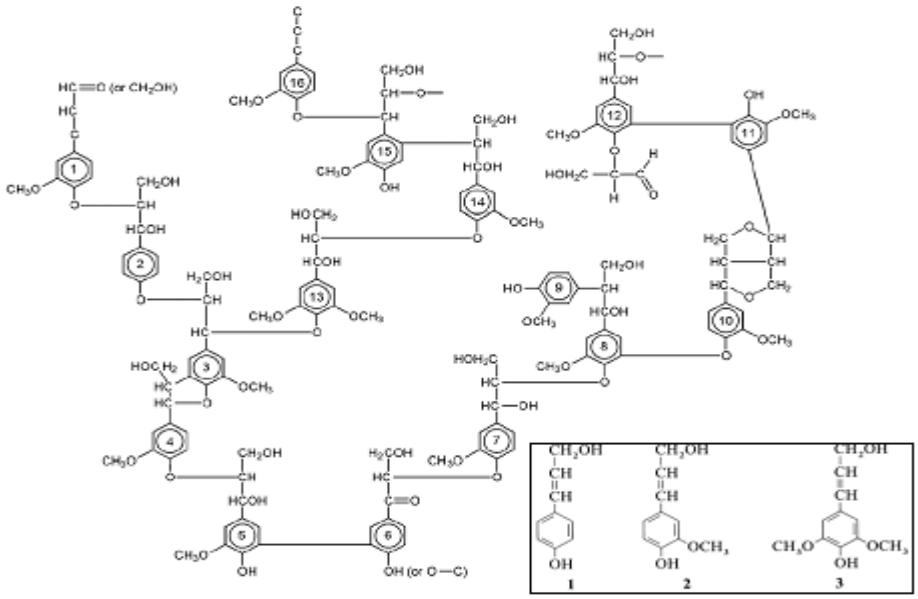

Figure 2.2: Representation of spruce lignin.

Lignin interferes with hydrolysis by blocking access of cellulases to cellulose and by irreversibly binding hydrolytic enzymes. Therefore, removal of lignin can dramatically increase the hydrolysis rate (McMillan 1994). Once the lignin is removed it can be used to produce hydrogen through the thermochemical process and for synthesis of polymers and chemicals (Cao et al 1996). The energy from all fractions of the biomass is therefore utilized and creates the highest net conversion efficiency of any known cellulosic ethanol process.

It was observed by Iconomou et al in 1994 that delignified cellulosic material, which is suitable for cell immobilisation (Bardi & Koutinas 1994), affects the fermentation rate of S. *cerevisiae*. Iconomou et al (1994) reported delignification of 95-100% of the starting material.The presence of delignified cellulosic material speeded up the rate of fermentation and resulted in a 120% increase in ethanol productivity. This can be attributed to the increase in surface area (holes and pores) of the substrate formed after delignification.

2.2.2 Dilute and concentrated acid hydrolysis

Hemicellulose is a copolymer of different C5 and C6 sugars including e.g. xylose, mannose and glucose, depending on the type of biomass. Hemicelluloses (polyoses) are the linking material between cellulose and lignin. Wood hemicelluloses are short(degree of polymerisation within 100 to 200), highly branched heteropolymers of the predominant xylose, plus glucose, mannose, galactose and arabinose, as well as different sorts of uronic acids. Depending on the three predominant sugar types, the hemicelluloses are referred to as mannans, xylans or galactans. The C5 and C6 sugars, linked through 1,3, 1,6 and 1,4 glycosidic bonds and often acetylated, form a loose, very hydrophilic structure that acts as glue between cellulose and lignin (Bon & Ferrara 2007).

According to Cao et al (1996), which was used as the basis of this research, the hemicellulose fraction can be hydrolysed readily by dilute hydrochloric acid hydrolysis, the oldest technology for converting biomass to ethanol. It is then separated from the cellulosic fraction. The hemicellulose hydrolysate (or dilute acid hydrolysate), which is rich in xylose, has no acetate and alkali extractives that can be used as a substrate for xylose fermenting yeast. Treating the hemicellulose hydrolysate with a weak based anion exchange resin removes the salt before fermentation. This shortens the time needed for fermentation and increases the ethanol yield by approximately 50%.

Dilute acid hydrolysis, using H_2SO_4 and HCl, has been reported by Sun and Cheng (2002) to treat lignocellulosic material efficiently. Esteghlalian et al noted earlier in 1997 that dilute sulphuric acid treatment achieved high reaction rates and significantly improved cellulose hydrolysis. High temperature (below 180°C) and acid concentrations below 1% are favourable for cellulose hydrolysis as compared to moderate temperature direct saccharification that yields low sugar concentrations due to sugar decomposition. There are strictly speaking, two types of dilute acid hydrolysis: high temperature (above 160˚C) used for continuous flow processes

which have a low solids loading (Brennan et al 1986; Converse et al 1989 as cited in Sun & Cheng 2002), and low temperature (below 160˚C) which is used for batch processes with high solid loading (Cahela et al 1983; Esteghlalian et al 1997). Even though dilute acid treatment costs are more than physico-chemical processes such as steam explosion and AFEX, it significantly improves cellulose hydrolysis and has a short reaction time. However, a pH adjustment of the resulting acid hydrolysate is necessary when considering further treatment by enzymatic hydrolysis and fermentation.

Strong acids such as HCl and H_2SO_4 have been powerful agents in treatment of lignocellulosic material. However they are toxic, corrosive, and hazardous and require corrosion resistant reactors according to Sun and Cheng (2002). In order to make the process economically feasible, the concentrated acid must be recovered after hydrolysis (Sivers & Zacchi (1995) as cited in Sun & Cheng (2002)). Conventional hydrolysis methods use high temperatures and or high acid concentrations which lead to degradation of hemicellulose sugars to fermentation inhibitors such as furfural and 5-hydroxymethylfurfural.

2.2.3 Enzyme technology and hydrolysis

Cellulose (a polymer of glucose),which is the most abundant polysaccharide on earth (Bon & Ferrara 2007) and provides much of the strength in plant cell walls, makes its decomposition into fermentable monomer glucose molecules one of the most important natural degrading processes (White & Brown 1981) in biotechnology. Therefore this reaction is of significant value when considering cellulose as a renewable energy substrate which can be converted to bioethanol.

Cellulosic materials present crystalline domains separated by less ordered, amorphous, regions that are potential points for chemical and biochemical attacks. The decomposition of cellulose into glucose takes place by synthesis of highly specific cellulose-degrading enzymes, cellulases. Cellulase, in effect, actually refers

to a system of three different enzymes that work together synergistically to efficiently degrade cellulose according to White and Brown (1981). The endo-1,4-β-D-glucanase (endoglucanase) cuts through internal glucosidic bonds within an unbroken glucan chain. These non-reductive chain ends are then acted upon by 1,4-β-D-glucan cellobiohydrolase (cellobiohydrolase) which separates and removes the cellobiose dimers from the glucan chain, into solution. The hydrolysis reaction of cellulose into glucose is then completed by β-glucosidase which splits cellobiose into glucose monomers (White & Brown 1981). Ancillary enzymes, in addition to the three major groups of cellulase enzymes, such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase attack hemicellulose (Sun & Cheng 2002).

Bacteria and fungi can be used to produce cellulase enzymes responsible for degradation of lignocellulosic material (Sun & Cheng 2002). Cellulolytic anaerobic bacteria such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* produce cellulases with high specific activity but low enzyme titres and low growth rate in anaerobic environments (Sun & Cheng 2002).Therefore, cellulases currently being utilised by cellulosic ethanol producers are derived from fungi such as, *Trichoderma reesei*. This discovery was actually made during the Second World War when it was found digesting tents and clothing of US soldiers. New ways to improve the cellulose to glucose process has been researched and new strains of *T. reesei* that can produce cellulases at enhanced rates have been discovered. Other fungi that produce cellulases include *Sclerotium rolfsii*, *P. chrysosporium* and species of *Aspergillus*, *Schizophyllum* and *Penicillium* (Sternberg 1976; Fan et al 1987; Duff & Murray 1996).

Cellulase from *Trichoderma viride*, an enzyme complex derived from the fermentation of a selected strain, hydrolyses or degrades cellulosic materials from a wide variety of sources depending on enzyme dosage, reaction conditions and the type of material being treated. Besides a high cellulase activity, the preparation

exhibits hemicellulase and amylase activity. Another feature is its macerase activity which is capable of cell wall decomposition. *T. viride* functions optimally at a pH of between 4-5 and temperature of between 50-60˚C (1988). Due to the mild conditions of enzymatic hydrolysis as compared to acid and alkaline hydrolysis, the utility costs are low (Sun and Cheng 2002).According to Sun and Cheng (2002) enzymatic hydrolysis of cellulose is affected by three factors: substrate, cellulase activity, and reaction conditions (temperature, pH and other parameters).

The yield and initial rate of enzymatic hydrolysis is mainly affected by substrate concentration. An increase in substrate concentration, at low substrate levels, causes an increase in yield and reaction rate of hydrolysis (Cheung & Anderson 1997 cited in Sun & Cheng 2002). In contrast, a high substrate concentration causes substrate inhibition which lowers the rate of hydrolysis. The extent of substrate inhibition is dependent on the total substrate to total enzyme ratio. According to Huang and Penner (1991) substrate inhibition occurred when the ratio of microcrystalline substrate Avicel pH 101 to the cellulase from T. reesei was greater than 5. It was discovered later on by Penner and Liaw (1994) that the optimum ratio of microcrystalline substrate Avicel pH 105 to cellulase from *T. reesei* was 1.25. The affinity of cellulases to substrate is dependent upon the substrates structural features including cellulose crystallinity, degree of cellulose polymerisation, surface area and lignin content. Lignin blocks access of cellulases to cellulose and irreversibly binds hydrolytic enzymes. It is therefore imperative that lignin be removed to increase the hydrolysis rate (McMillan 1994).

The yield and rate of hydrolysis can be increased, to a certain extent, by increasing the cellulase dosage in the process which results in a significant increase in process costs (Sun & Cheng 2002). In order to conduct laboratory studies and obtain high glucose yields in reasonable time (48-72 hours) and cost a cellulase dosage of10 FPU/g is often used (Gregg &Saddler 1996 cited in Sun & Cheng 2002). Depending on the type and concentration of substrates, enzyme loadings vary from 7-33 FPU/g

substrates (Sun & Chen 2002). The three steps involved in enzymatic hydrolysis of cellulose are adsorption of cellulase onto the surface of the cellulose, biodegradation of cellulose to fermentable sugars and desorption of cellulase. Cellulase activity decreases during hydrolysis, which is, partially due to the irreversible adsorption of cellulase on cellulose (Converse et al 1988). However, this can be minimized by the addition of surfactants. Non-ionic surfactants are believed to be most suited to enhance cellulose hydrolysis such as Tween 20 and 80, and Pluronic F68 and F88 as seen in Table 2.4 where an enzyme loading of 2 g/L was used with a solid substrate concentration of 10 %. Hydrolysis can be enhanced further by the addition of a mixture of hemicellulases or pectinases with cellulases.

Table 2.4: Surfactants used to enhance enzymatic hydrolysis (Wu &Ju 1998 cited in Sun & Cheng 2002).

Surfactants		**Percentage cellulose conversion**			
Type	Concentration (%)	10 hours	15 hours	44.5 hours	123.5 hours
Control	0	11.9	17.5	20.7	27.5
Tween 20	0.5	14.1	21.6	27.2	43.6
	2.0	16.0	24.7	32.1	46.8
Tween 80	0.5	14.5	22.0	28.0	43.1
	2.0	14.2	24.7	29.6	43.6
F68	0.5	17.3	26.7	34.4	51.0
	2.0	16.6	27.5	34.0	56.5
F88	0.5	15.4	24.7	32.8	47.8
	2.0	14.5	24.6	33.9	51.2

Cellulase can be recovered from the liquid supernatant or the solid residue to lower enzyme costs, however, the efficiency of cellulose hydrolysis decreases gradually with each recycling step (Sun & Cheng 2002).

Inhibition of cellulase activity is usually due to cellobiose, glucose, and ethanol when considering cellulase activity in the Simultaneous Saccharification and Fermentation (SSF) process. In this process, product inhibition to the hydrolysis is greatly reduced due to reducing sugars produced in saccharification being simultaneously fermented to ethanol. The optimal temperature for SSF is around 38˚C which is a compromise between the optimal temperature for hydrolysis (45-50˚C) and fermentation (30˚C) (Philippidis 1996 cited in Sun & Cheng 2002). Hydrolysis is usually the rate limiting step in SSF (Philippidis & Smith 1995). Compared to a two stage hydrolysis fermentation, SSF has the following advantages: increase in rate of hydrolysis by conversion of sugars that inhibit cellulase activity; lower enzyme titre; higher product yields; lower requirements for sterile conditions since glucose produced is immediately removed in ethanol production; shorter process time reduced reactor volume because a single reactor is used (Sun & Cheng 2002). However, SSF does have disadvantages such as incompatible temperature of hydrolysis and fermentation, low ethanol tolerance of microbes and inhibition of enzymes caused by ethanol (Sun & Cheng 2002).

2.3 Yeast technology

Eukaryotic microorganisms, yeast cells, are from the fungi kingdom. They mostly reproduce asexually by budding although some do reproduce by binary fission. Yeasts are unicellular and can vary in size depending on species. Typical sizes of yeasts are 3-4 μm in diameter. The yeast species *S. cerevisiae* has been used in baking, in fermentation of alcoholic beverages for thousands of years, in the generation of electricity in microbial fuel cells and most recently in the biofuel industry to produce ethanol.

Yeasts are chemoorganotrophs, as they do not require sunlight to grow but rather thrive on organic compounds. This carbon energy source is mostly obtained from hexose sugars although some do utilise pentose sugars. *S. cerevisiae* rapidly converts hexoses and *P. stipitis* converts both hexoses and pentoses, at relatively high conversion rates according to Grootjen et al (1990) and Boynton & McMillan (1994), to bioethanol. According to Grootjen et al (1990), with co-immobilisation of both these yeasts it is possible to convert glucose and xylose simultaneously. Fermentation can therefore be carried out with alcohol tolerant yeast strains such as *S. cerevisiae and P. stipitis*.

Hydrolysis of corn cob results in significant amounts of xylose and glucose. Therefore, it is of valuable research to immobilise yeast cells *S. cerevisiae* and *P. stipitis* (Grootjen et al 1990) in order to optimise fermentation and increase production of bioethanol whilst keeping in mind that yeast cells can undergo ethanol inhibition when concentration of ethanol exceeds 1-2% (w/v) and at 10% (w/v) microbe growth rate is nearly halted.

2.3.1 Yeast metabolism

All yeast cells metabolise nutrients, by chemical reactions, to obtain energy needed for respiration, growth, maintenance of their structure and to respond to the given environment. A small concentration of oxygen must be provided to the fermenting

yeast, since it is a necessary component in the biosynthesis of polyunsaturated fats and lipids. Typical amounts of O_2 maintained in the broth are 0.05 – 0.10 mm Hg oxygen tension. The relative requirements for nutrients not utilized in ethanol synthesis are in proportion to the major components of the yeast cell. These include carbon, oxygen, nitrogen and hydrogen. To lesser extent quantities of phosphorus, sulphur, potassium, and magnesium must also be provided for the synthesis of minor components. Minerals (i.e. Mn, Co, Cu, Zn) and organic factors (amino acids, nucleic acids, and vitamins) are required in trace amounts.

Cellular respiration is the set of metabolic reactions and processes that take place in the cells of organisms to convert biochemical energy from nutrients into adenosine-5-triphosphate (ATP), and then release waste products. The reactions involved in respiration are catabolic and involve a series of redox reactions (which is the oxidation of one molecule and the reduction of another). Respiration is one of the key ways a cell gains useful energy to fuel cellular reformations. The energy released in respiration is used to synthesize ATP to store this energy. The energy stored in ATP can then be used to drive processes requiring energy, including biosynthesis, locomotion or transportation of molecules across cell membranes.

Figure 2.4 and equations 3, 4 and 5detailthe metabolic pathway of glucose. According to Kompala (1996) the metabolism of yeast cells, *S. cerevisiae*, follows three major pathways. Balanced equations can be found in Appendix B.5. One glucose molecule is converted into two ethanol molecules and two carbon dioxide molecules as in Equation 6. The yield attained in practical fermentations, however, does not usually exceed 90 – 95% of the theoretical. The process begins with a molecule of glucose being broken down by the process of glycolysis or the Embden-Meyerhof-Parnas (EMP), pathway to pyruvate:

$$C_6H_{12}O_6 \rightarrow 2\ CH_3COCOO^- + 2H^+ \qquad 3$$

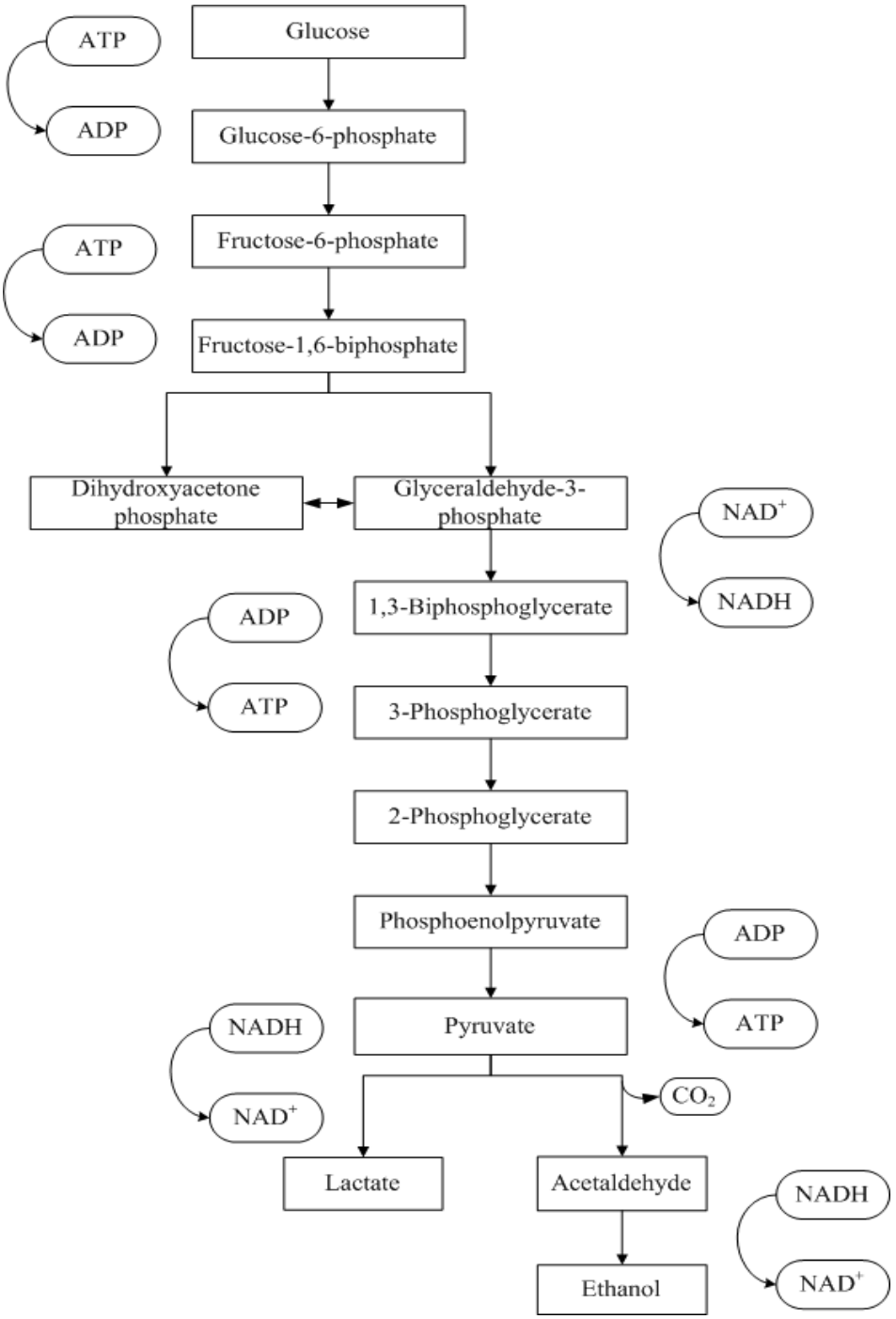

Figure 2.4: Metabolic pathway of glucose to ethanol. (Wolf & Heinrich 2000)

This reaction is accompanied by the reduction of two molecules of nicotinamide adenine dinucleotide (NAD^+) to NADH and a net of two adenosine diphosphate (ADP) molecules converted to two ATP plus the two water molecules. NAD^+ is a coenzyme found in all living cells that is involved in redox reactions in metabolism. ATP is a multifunctional nucleotide used in cells as a co-enzyme. It transports chemical energy within cells for metabolism. Pyruvate is then converted to acetaldehyde and carbon dioxide by an enzyme called pyruvate decarboxylase and requiring thiamine diphosphate as a cofactor (equation 4). In Equation 5 the acetaldehyde is subsequently reduced to ethanol by the NADH from the previous glycolysis, which is returned to NAD^+. One glucose molecule is converted into two ethanol molecules and two carbon dioxide molecules in the overall reaction as in Equation 6a.

$$CH_3COCOO^- + H^+ \rightarrow CH_3CHO + CO_2 \quad (4)$$

$$CH_3CHO + NADH \rightarrow C_2H_5OH + NAD^+ \quad (5)$$

Fermentation of glucose occurs when the glucose concentration is high or when oxygen is absent. The maximum specific growth rate attainable by the cells is approximately 0.45 hr^{-1} with a low biomass yield of 0.15 g dry mass per gram glucose consumed. A high respiratory quotient, the ratio of CO_2 production rate to the O_2 consumption rate, and a low energy yield of only about 2 ATP's (adenosine triphosphates) per mole of glucose metabolised is also reached. Stoichiometric representation of this reaction and xylose is in Equations 6a and 6b, respectively:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + \varepsilon \quad (6a)$$

$$C_5H_{10}O_5 \rightarrow 1.67\ C_2H_5OH + 1.66\ CO_2 + \varepsilon \quad (6b)$$

where ε represents chemical energy utilized in the growth processes.

Oxidation of glucose predominates at glucose concentrations below 50 mg/L in aerobic cultures. The cells attain a maximum specific growth rate of approximately 0.25 hr^{-1} with a biomass yield of about 0.5 g dry mass per gram glucose consumed, a

respiratory quotient of about 1, and a high energy yield of 16-28 ATP per mole of glucose metabolised (Equation 7).

The stoichiometry of the reaction:

$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + \varepsilon$ 7

Oxidation of ethanol predominates when fermentative substrates are not available or in very limited supply. The cells attain a maximum specific growth rate of about 0.2 hr^{-1} with a high biomass yield of about 0.6-0.7 g dry mass per gram ethanol consumed, a low respiratory quotient of about 0.7, and an energy yield of about 6-11 ATP per mole of ethanol metabolized. The stoichiometry of this reaction is given in Equation 8:

$C_2H_5OH + 3O_2 \rightarrow 2CO_2 + 3H_2O + \varepsilon$ 8

It is desirable to ferment D-xylose to ethanol. However micro-organisms that can do this naturally have some disadvantages. One such organism that can convert D-xylose to ethanol naturally is *P. stipitis*: however it is not as tolerant to ethanol as the traditional ethanol producing yeast, *S. cerevisiae*. The catabolism of D-xylose by eukaryotic micro-organisms, yeasts, takes place via the oxi-reductive pathway. This pathway is called the Xylose Reductase-Xylitol Dehydrogenase (XR-XDH) pathway which is depicted in Figure 2.5.

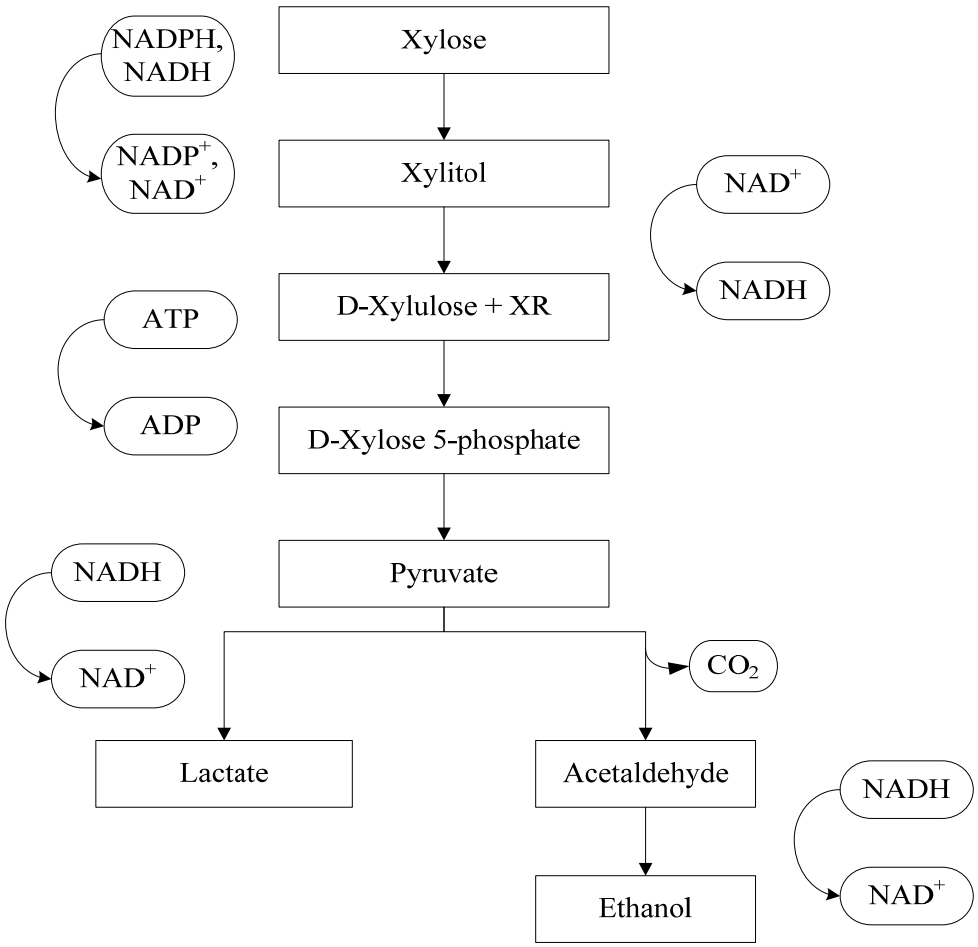

Figure 2.5: Metabolic pathway of xylose to ethanol.

The first two enzymes in this pathway are XR and XDH. XR reduces D-xylose to xylitol using NADH or NADPH to NAD^+ and $NADP^+$ (nicotinamide adenine dinucleotide phosphate), respectively. Xylitol is then oxidised to D-xylulose by XDH, using the co-factor NAD. In the last step D-xylulose is phosphorylated by an ATP utilising xylulokinase (XK) to result in D-xyulose-5-phosphate which is an intermediate of the pentose phosphate pathway. Because of the varying cofactors needed in this pathway and the degree which they are available for usage, an imbalance can result in an overproduction of xylitol by-product. D-xylulose 5-phosphate is then converted to pyruvate through both the pentose phosphate pathway (PPP) and the Embden-Myerhof-Parnas (EMP) pathway (Chiang et al 1981).

Pyruvate is then converted to acetaldehyde and carbon dioxide by an enzyme called pyruvate decarboxylase and requiring thiamine diphosphate as a cofactor as seen in equation 7). In equation 8 the acetaldehyde is subsequently reduced to ethanol by the NADH from the previous glycolysis, which is returned to NAD^+. The net of one ADP is converted to one ATP, balanced equations can be found in Appendix B.5. When considering the metabolic pathways of glucose and xylose it can be deduced that fermentation is less efficient at using the energy from xylose since 1 ATP is produced per xylose, compared to the 2 ATP produced per glucose metabolised by aerobic respiration.

2.3.2 Immobilisation of yeast cells

2.3.2.1 Background

Increasing interest in cell immobilisation technology, in beer brewing and potable alcohol production, has been brought about by the numerous advantages that immobilisation offers. Advantages include enhanced fermentation productivity, feasibility of continuous processing, cell stability and lower costs of recovery and recycling and downstream processing (Margaritis & Merchant 1984; Stewart & Russel 1986 cited in Kourkoutas et al 2004). Industrial use of this technology has been limited and will only increase in popularity once processes have been successfully developed and efficient scale-up carried out (Kourkoutas et al 2004).

Whole cell immobilisation has been defined as ‘‘the physical confinement or localization of intact cells to a certain region of space with preservation of some desired catalytic activity’’ (Karel et al 1985). This often occurs naturally with many microorganisms in nature. As described by Kourkoutas et al (2004) the four categories of techniques employed in immobilisation include (Figure 2.6): (A) attachment or adsorption on solid carrier surfaces, (B) entrapment within a porous matrix, (C) self-aggregation by flocculation (natural) or with cross-linking agents (artificially induced), and (D) cell containment behind barriers (Kourkoutas et al 2004).

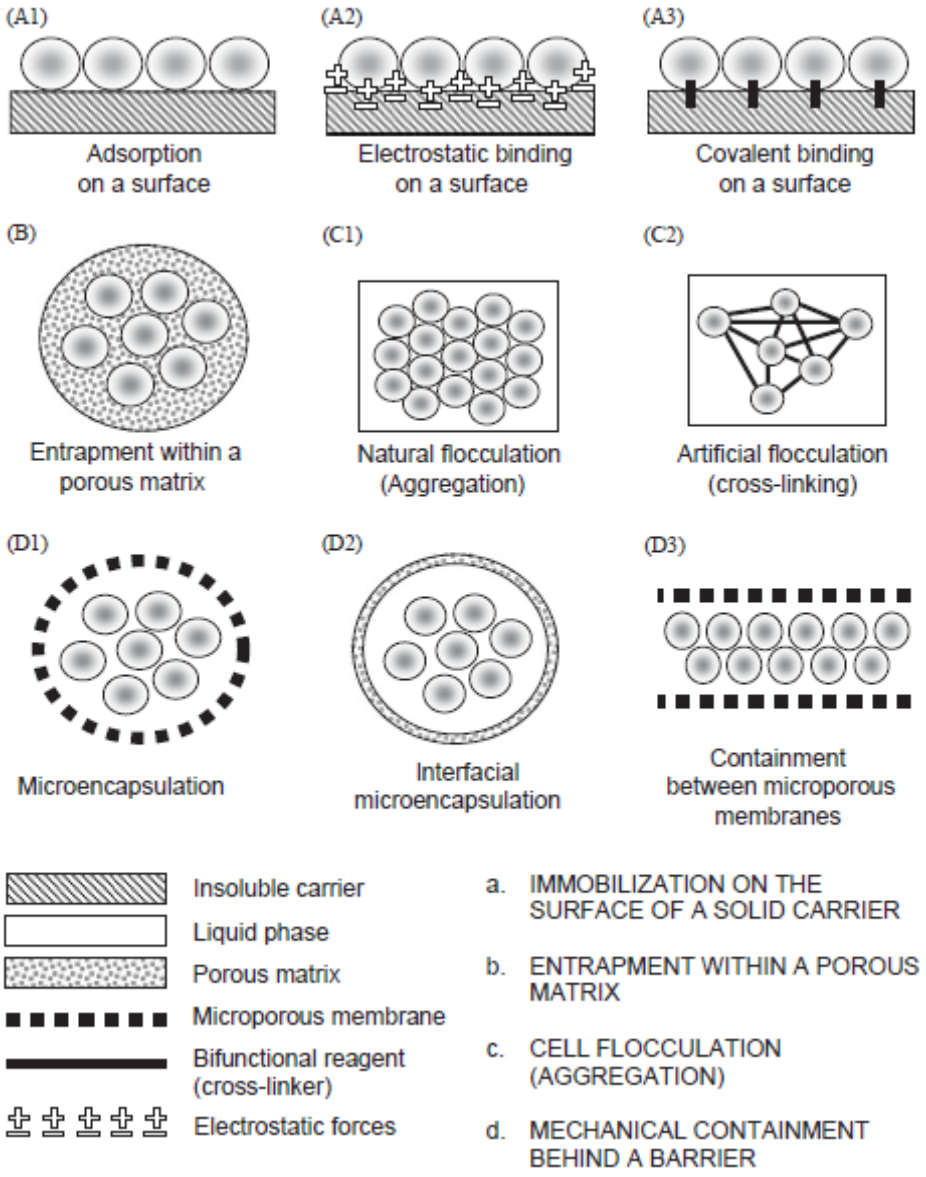

Figure 2.6: Immobilisation techniques (Kourkoutas et al 2004).

2.3.2.2 Immobilisation on the surface of a solid substrate

Immobilisation of yeast cells on a solid surface is carried out by physical adsorption due to electrostatic forces or covalent bonding between the cell membrane and the surface of the substrate (Kourkoutas et al 2004). The thickness of the biofilm that forms on this surface can range from one layer of cells to 1 mm or more of cells. This

type of immobilisation technique is quite easy to perform and is therefore very popular. The strength of cell attachment cannot be easily determined. Due to the absence of a barrier between the fermentation medium and cells, detachment and relocation of cells takes place with the potential establishment of equilibrium between adsorbed and free suspended cells. Cellulosic and lignocellulosic materials such as apple pieces (Kourkoutas et al 2006), orange peel (Plessas et al 2007),spent grains and delignified spent grains (Kopsahelis et al 2007), wood, sawdust, and delignified sawdust have been used in this type of application (Kourkoutas et al 2004). The adsorption ability of cellulose and solid materials, like glass, can be enhanced by treating it with polycations or chitosan (Norton & D'Amore 1994; Navarro &Durand 1977). Supports used in this type of immobilisation are classified as organic, inorganic, natural supports and membrane systems.

2.3.2.3 Entrapment within a porous matrix

This type of technique refers to cells that penetrate a porous matrix until mobility is halted by the presence of other cells or when the porous material is formed in situ into a culture of cells (Kourkoutas et al 2004). This type of network still allows mass transfer of nutrients and metabolites. Entrapment into polysaccharide gels such as alginates, k-carrageenan, agar, chitosan and polygalacturonic acid or other polymeric matrices like gelatin, collagen and polyvinyl alcohol (Norton & D'Amore 1994; Park & Chang 2000) are characteristic examples of this type of immobilisation. The disadvantage of this type of cell entrapment is the ability of cells located on the outer surface of the beads to multiply and be released from the inclusion bead, which results in a system comprising of immobilised and free cells.

2.3.2.4 Cell flocculation

As stated by Jin & Speers in 1998, flocculation is the property of cells in suspension to adhere in clumps and sediment rapidly. Due to the large size of the resulting clumps, its use in certain reactors (packed-bed, fluidized-bed and continuously stirred-tank reactors) is justified. Cross-linkers and artificial flocculating agents can

be used to enhance coalescence of cells that do not naturally flocculate. Many factors such as cell wall composition, pH, dissolved oxygen and medium composition affect flocculation and yeast recovery and removal in beer brewing.

2.3.2.5 Containment of cells behind a barrier

This type of immobilisation can be attained in three ways:

1. Use of microporous membrane filters.
2. Entrapment of cells in a microcapsule.
3. Cell immobilisation onto an interactive surface layer of two immiscible liquids.

According to Park and Chang (2000), this type of immobilisation technique can be used when the product must be free of cells and when minimum transfer of compounds is required. The major disadvantages of cells immobilisation between microporous membranes are mass transfer limitations (Lebeau et al 1998) and possible membrane biofouling caused by cell growth (Gryta 2002).

2.3.2.6 Effects of immobilisation

Immobilisation of yeast cells using these various techniques results in alterations of cell growth, physiology and metabolic activity. The magnitude and type of these is, however, very difficult to predict. Parameters responsible for these alterations include mass transfer limitations by diffusion (Webb et al 1986), disturbances in the growth pattern (Doran & Bailey 1986) and surface tension and osmotic pressure effects (Vijayalakshmi et al 1979 cited in Kourkoutas et al 2004). Reduced water activity (Mattiasson et al 1984 cited in Kourkoutas et al 2004), cell-to-cell communication (Shuler 1985), changes in the cell morphology (Shirai et al 1988), altered membrane permeability and media component availability (Chen et al 1990) also contribute to these alterations.

A study was conducted by Norton & D'Amore in 1994 to compare immobilised and free cell effects on activation of yeast energetic metabolism, which showed an increase in storage polysaccharides, altered growth rates, increased substrate uptake and product yield, lower yield of fermentation by-products, higher intracellular pH values, increased tolerance against toxic and inhibitory compounds, and increased invertase activity.

2.3.2.7 Advantages of immobilisation according to Kourkoutas et al (2004)

Advantages offered by immobilised cell fermentation are vast as compared to free cell fermentation:

- The biocatalyst exhibits prolonged stability and activity. This can be due to the immobilisation support acting as a protective agent against physicochemical effects of pH, temperature, solvents or even heavy metals. This allows for regeneration and reuse of the biocatalyst for extended periods in batch operations, without removing it from the bioreactor.
- Immobilisation allows for high yeast cell densities to be accommodated in the bioreactor. This leads to shorter fermentation times, high productivity, elimination of non-productive cell growth phases and shorter maturation times for certain products.
- Greater substrate utilisation, thereby improving the yield, whilst displaying an increased tolerance to high substrate concentration and reduced end product inhibition.
- Continuous processing is made feasible with immobilised cells.
- Product quality is improved by low temperature fermentation which can now be carried out successfully using immobilised cells.
- The reduction of separation and filtration requirements reduces the cost for equipment and energy demands, and makes product recovery easier.
- The risk of microbial contamination is reduced due to high cell densities and fermentation activity.

- Capital costs can be reduced by using smaller bioreactors with simplified process designs.

2.4 Ethanol Fermentation

73% of the ethanol produced globally is used as fuel, 17% is used in beverages and 10% is utilised industrially (Fukuda et al2009).Conventionally fuel ethanol has been produced from fossil fuels. The last two decades has seen the technology for bio-ethanol production from non-food biomass advance drastically and large-scale production of fuel ethanol will materialize in the not too distant future (Yan & Tanaka 2006). In 1995 it was reported that 93% of global ethanol production was as a result of fermentation, with a mere 7% from the synthetic method (Badger 2002).

The fermentation of ethanol is the biological process that converts fermentable sugars such as glucose and xylose to cellular energy with microorganisms which produce waste by-products ethanol and carbon dioxide anaerobically by the metabolic pathways of sugars. Many species of yeast will favour respiration, production of carbon dioxide and water instead of fermentation in the presence of oxygen. This is unlike yeast cells *S. cerevisiae* (baker's yeast) and *Schizosaccharomycespombe* that prefer fermentation even in the presence of oxygen and will produce ethanol given a suitable source of nutrition. *S. cerevisiae* is yeast that is most widely used in the production of ethanol from hexoses but cannot utilise pentoses. *P. stipitis* converts both hexoses and pentoses into ethanol at relatively high conversion rates according to Grootjen et al (1990).

According to recent studies, careful control of aeration and pH are necessary to attain maximum ethanol yield, making shake-flask fermentation a suitable method (Boynton & McMillan 1994) for this research. Fermentation can be carried out using Simultaneous Saccharification and Fermentation (SSF), Simultaneous Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Co-fermentation (SSCF) and continuous fermentation. The SSF process has been identified as economically viable for the conversion of lignocellulosic substrates to fermentation products (Cao et al 1996).

2.5 Kinetics in fermentation technology

In order to operate the fermentation process in the most efficient and economical manner, reliable numerical data is necessary. This information will allow for the improvement, optimisation and trouble shooting of the fermentation process. One, initially, also needs to decide which substrate and biomass will enable for the most efficient fermentation process in terms of yield and productivity.

It is necessary to begin with a material balance of the microbial biomass (X) around the chemostat yields in Equation 9a (Shuler & Kargi 2002). This equation reduces to Equation 9b when considering a batch reactor, as in this study with no inlet and outlet flows.

$$FX_0 - FX + V_R \mu_g X = V_R \frac{dX}{dt} \qquad 9a$$

$$\frac{dX}{dt} = \left(\mu_g - k_d\right)X \qquad 9b$$

Where F is the flowrate, X is the quantity of microbes, V_R is the volume of the reactor, μ_g and k_d are the growth and death, first order, rate constants, respectively, $\frac{dX}{dt}$ is the rate of microbial growth. $k_d = 0$ when endogeneous metabolism is unimportant (Shuler & Kargi 2002).

The Monod equation (Bouville 2008), Equation 10, is used in modelling the resource-limited growth of microbial yeast cells.

$$\mu_g = \mu_m \left(\frac{S}{S + K_s}\right) \qquad 10a$$

where μ_m is the maximum specific growth rate, S is the substrate concentration and K_s is the Monod constant.

By combining Equations 9b and 10a we get equation 10b (Shuler & Kargi 2002):

$$\frac{dX}{dt} = \mu_m \left(\frac{S}{S + K_s} \right) X \qquad 10b$$

Linearisation (enables determination of constants) of Equation 10b gives:

$$\frac{SX}{\left(\frac{dX}{dt} \right)} = \frac{1}{\mu_m} S + \frac{K_S}{\mu_m} \qquad 10c$$

The substrate as well as biomass dynamics can be modelled when the yield of organisms per unit substrate consumed is quantified. This can be accomplished using the yield coefficient, $Y_{X/S}$, in Equation 11.

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \frac{dX}{dt} - mX \qquad 11a$$

$$X - X_0 = Y_{X/S} (S_0 - S) \qquad 11b$$

Y is the yield coefficient, and m is the maintenance coefficient. This equation shows that the quantity of microorganisms is directly affected by the concentration of substrate over time. A lot of valuable information can be extrapolated once the values of the constants are determined. By substituting for S in Equation 10b with Equation 11b the result is given by Equation 12a:

$$\frac{dX}{dt} = \left(\frac{\mu_m (Y_{X/S} S_0 + X_0 - X)}{K_s Y_{X/S} + (Y_{X/S} S_0 + X_0 - X)} \right) X \qquad 12a$$

$$\frac{dX}{dt} = \left(\frac{\mu_m (Y_{X/S} S_0 + X_0 - X)}{K_s Y_{X/S} + (Y_{X/S} S_0 + X_0 - X)} \right) X - k_d X \qquad 12b$$

where k_d is the first order death rate constant. Cell death is due to harsh environments, shear mixing forces, local depletion of nutrients and production of toxic substances (Fogler 2006). Representative values of k_d are from 0.1 to lower than

0.0005 h^{-1} (Fogler 2006). Equation 12 can be affected by the diversity of varying microbial reactions. When integrated, this equation can be used to model experimental microbial growth patterns.

CHAPTER THREE: MATERIALS & METHODS

This chapter details the experimental procedure used to ferment bioethanol in this study.

3.1 Raw materials

3.1.1 Corn cob

3.1.1.1 Raw material preparation

Maize, having its origin in Limpopo, South Africa, was purchased from local vendors in Gauteng, Midrand, South Africa. The maize was then decorned/pitted and the corn cobs were dried in an oven at low to moderate temperature to a constant mass. The corn cob was then crushed to fine grist before being used in subsequent experiments.

3.1.1.2 Corn cob composition

The cellulose, hemicellulose and lignin content from Brazilian and African sources are presented in Table 3.1. The chemical composition data only highlights greater cellulose content when compared to hemicellulose. This data was not characterized in this study because it cannot predict the efficiency of hydrolysis treatments and because these complex polysaccharides are not stoichiometrically related to the amount of fermentable sugars that can be produced. The variable temperatures and precipitation affects the yield alone and not the chemical composition of corn cob, including the corn cob (Limpopo) used in this study (Akpalu et al 2009) as was supported with personal communication by Dr Dries Fourie (2011) in the Agricultural Research Council (ARC).

These chemicals (cellulose and hemicellulose) are converted to sugars by hydrolysis and the sugars are then fermented to ethanol using yeast cells.

Table 3.1: Corn cob composition.

Component	Percentage composition	
	E Silva et al (2008)	**Akinfemi (2010)**
Cellulose	40	34.7
Hemicellulose	22.5	19.1
Lignin	20	16.9
Other	17.5	29.3

3.1.2 Chemicals and reagents

All chemicals and reagents used in experiments were purchased from Merck Chemicals Pty (Ltd), Laboratory Supplies Division, South Africa and were all of laboratory grade.

3.1.3 Yeast Cells

Plated yeast strain *S. cerevisiae* (NRRLY2084), dry brewer's yeast, was provided by the School of Molecular and Cell Biology, University of the Witwatersrand, South Africa and it will be referred to as *S. cerevisiae*. *P. stipitis* was obtained from the DSMZ culture collection in Germany (DSM3651), will be referred to as *P. stipitis*. Both cultures (Figure 3.1) were maintained on agar plates at 4°C.

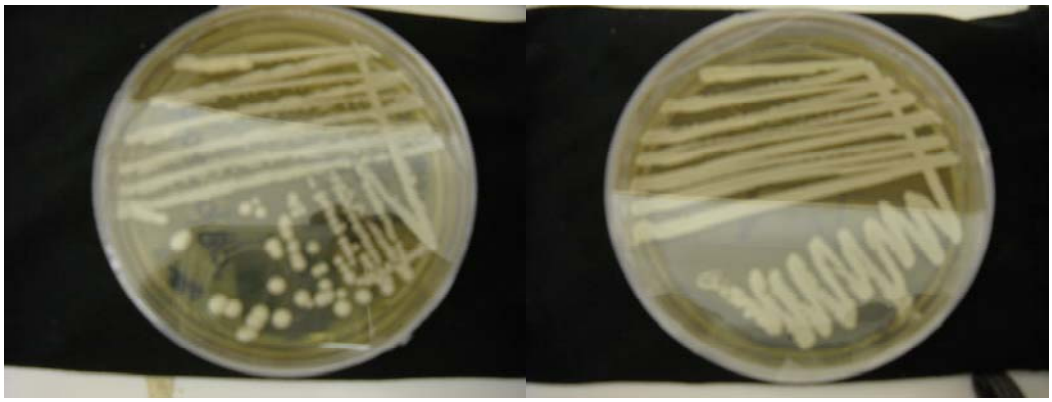

Figure 3.1: Plated yeast cells *S. cerevisiae* and *P. stipitis*, respectively.

3.1.4 Cellulase enzymes

Cellulysin Cellulase Trichoderma Viride, CAS number [9012-54-8], was purchased from Merck Chemicals Pty (Ltd), Laboratory Supplies Division, South Africa.

3.2 Methodology

Hydrolysis, Immobilisation and Fermentation experiments were carried out.

Figure 3.2 shows two alternative methods of treatment conducted in parallel. These parallel processes thereafter followed the same experimental procedure.

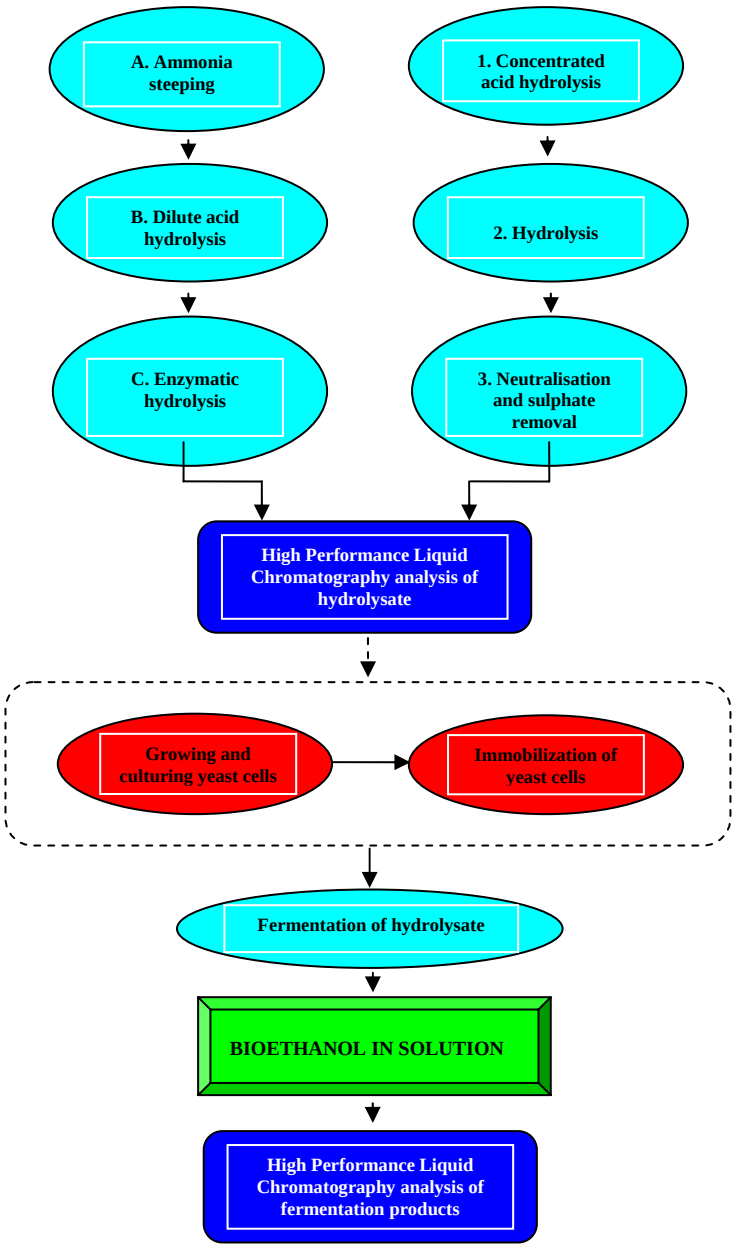

Figure 3.2: Experimental procedure for bioethanol fermentation.

3.3 Methods of analysis

3.3.1 High Performance Liquid Chromatography (HPLC)

HPLC is a powerful tool in analysis of carbohydrates. See Appendix B.3 for a sample chromatogram showing the presence of glucose, xylose and ethanol. There is a stationary phase (solid, or a liquid supported on a solid) and a mobile phase (liquid or gas). The mobile phase, together with the components of the mixture is forced through the columns stationary phase under high pressures. Different components travel at different rates.

HPLC (Agilent Technologies-1200 Series) was used to analyse the presence and concentration of sugars in the hydrolysate, to measure the ethanol produced and monitor the periodic utilisation of fermentable sugars, and finally to measure the concentration of the fermentation product and distillate. The HPLC parameters and specifications are presented in Table 3.2.

Table 3.2: HPLC column specifications.

Column	
BIORAD column	Aminex® Fermentation Monitor
Dimensions	150×7.8mm
Part number	1250115
Conditions	
Mobile Phase	0.001M H_2SO_4
Flow	0.8mL/min
Temperature	60˚C
Detector	Refractive Index @ 40˚C
Injection volume	10µL

3.3.2 Haemocytometer

The haemocytometer was used to count the number of yeast cells present in the yeast broth culture. The number of immobilised yeast cells were also determined using this method of analysis. The number of cells in the yeast broth culture was counted before and after immobilisation. The difference was the number of immobilised yeast cells.

The haemocytometer, which was invented by Louis-Charles Malassez, was first used to count blood cells. Now it is used to count many types of cells as well as microscopic particles. It contains a thick glass microscope slide with a rectangular chamber that is laser-etched with a grid of perpendicular lines so that the area bounded by the lines and the depth of the chamber is also known as seen in Figure 3.3. This construction makes it possible to count the number of cells in a specific volume of fluid and hence the concentration of cells in a fluid using a microscope.

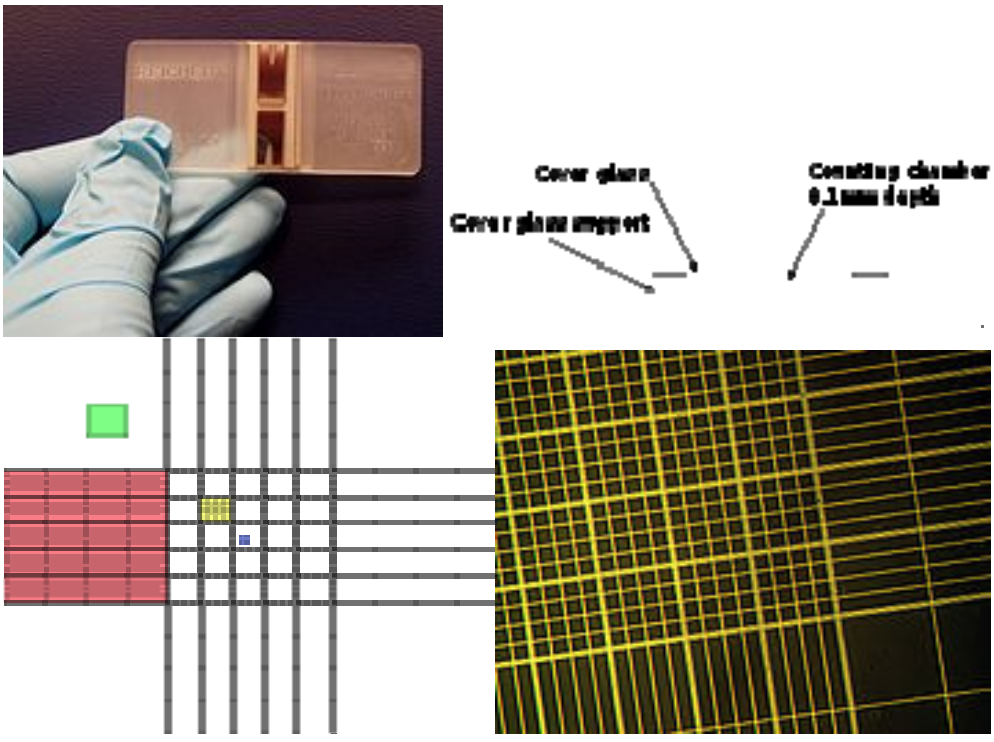

Figure 3.3: Top and cross-sectional view of a haemocytometer (top).

Perpendicular grid lines viewed using a microscope (bottom).

3.3.3 Spectrophotometer

Optical density (OD) was determined by measuring absorbance of 1mL of culture mixture at 600 nm using a Unico UV-visible spectrophotometer. 1mL of sterile YPD media was used to blank the spectrophotometer and set the reference point. OD measurements were converted to cell concentrations using correlation factors developed from final OD measurements (under experimental conditions at 600 nm 1.0 = 0.24 g/L cells). Growth rate of biomass was determined using the gradient or slope of the growth curve (g/(L·h)).

3.3.4 Scanning Electron Microscope

Surfaces of objects can be seen in their natural state without staining using the JSM-840scanning electron microscope (SEM). The specimen is first covered with a thin gold coating to increase electrical conductivity, hence forming a less blurred image. After positioning the specimen in the vacuum chamber the electron beam sweeps across the object forming an image line by line as in a television camera. Whilst striking the object the electrons knock loose showers of electrons which are captured by a detector to form the image. Magnifications with this microscopy are limited to about 75,000-100,000 diameters.

3.4 Ammonia treatment

3.4.1 Ammonia steeping - Delignification

The corn cob was steeped in dilute ammonia at ambient temperature to remove the lignin, acetate and extractives present and to cause the cellulosic fraction to swell and enhance the acid hydrolysis step according to Cao et al (1996). 100 g of corn cob was mixed with 500 mL 2.9 M NH_4OH in a 1000 mL Schott bottle. The mixture was incubated at 50 rpm and 30˚C for 24 hours in an incubator shaker. After 24 hours the mixture was washed and filtered four times with distilled water in a Büchner funnel. The resulting solid residue was then dried in an oven at 40˚C to a constant mass.

3.4.2 Dilute acid hydrolysis

The hemicellulose fraction of the delignified corn cob was hydrolysed to mainly xylose with dilute hydrochloric acid at high temperatures. This treatment subsequently increases the surface area of cellulose for enzymatic hydrolysis, according to the modification of Cao et al (1996). Delignified corn cob was treated with 0.3 M HCl in an autoclave at 121 ˚C and 15 psi (103.4 kPa) for1 hour with a ratio of 1:10 w/v (dry basis). The acidic hemicellulose hydrolysate was filtered from this mixture using a Büchner funnel and neutralised with sodium hydroxide to a pH of 5.5-6.5. This supernatant was then filtered and analysed by HPLC using the method prescribed by Duke & Henson (2008). The hydrolysate was stored at -20 ˚C for six months or until use. The residual cellulosic residue was then washed twice with distilled water to remove residual acid and used in enzymatic hydrolysis experiments.

3.4.3 Enzymatic hydrolysis

The cellulosic fraction was enzymatically hydrolysed to glucose, according to the modification of Cao et al (1996).

3.3.3.1 Dilution of enzymes

1.145 mL of acetic acid was added to 198.86 mL of distilled water to make 200 mL of the acetate buffer solution. The pH of the 0.1 M acetic acid was increased with the addition of sodium hydroxide pellets to pH 4.0 according to Okada (1988). 0.5 g cellulose enzymes were added to 100 mL of the acetate buffer solution. 200 µL of the enzyme solution, which had an activity of 10 U/g, was aliquoted into Falcon tubes and stored at -20 ˚C until use.

3.3.3.2 Experimental method

200 µL cellulose enzyme and 50 mL distilled water were added to the cellulosic residue obtained from 20 g corn cob in a 250 mL Erlenmeyer flask. This enzymatic hydrolysis was carried out at 50 ˚C for 48 hours in a water bath. The enzymes were then denatured by heating the resultant hydrolysate to 90˚C and keeping it at that constant temperature for 30 minutes. The supernatant was removed, filtered and analysed by HPLC using the method prescribed by Duke & Henson (2008). The hydrolysate was stored at -20 ˚C for six months or until use.

3.5 Concentrated sulphuric acid hydrolysis

3.5.1 Treatment of biomass

At ambient temperature 200 g corn cob and 336 mL of 72 % sulphuric acid were added to a 2 L Schott bottle (sulphuric acid: pulp = 2:1 [w/w]). This mixture was then treated in an incubator shaker at 30 ˚C and 50 rpm for 24 hours.

3.5.2 Hydrolysis

The pre-treated corn cob was then poured into a 5 L Schott bottle. The remaining residue was washed with 2.4 L distilled water and thereafter transferred to the 5 L Schott bottle. The 5 L Schott bottle was then partially covered with aluminium foil and placed in an oven at 95 ˚C for 3 hours. This allowed the gases that formed to escape.

3.5.3 Neutralisation and sulphate removal

Calcium hydroxide was thereafter added to this suspension and stirred using a mechanical or magnetic stirrer, for approximately 20 minutes, until a pH of 5.5-6.5 was reached. The fluid thickened and resembled cement-like slurry. The slurry was then centrifuged at 6000 rpm for 20 minutes. The supernatant was removed, filtered and analysed by HPLC using the method prescribed by Duke & Henson (2008). The hydrolysate was stored at -20 ˚C for six months or until use.

3.6 Yeast growth and immobilisation

3.6.1 Culturing and growing of yeast cells

The yeast cells *Saccharomyces cerevisiae* and *Pichia stipitis* were cultured and grown in a yeast culture medium, the YPD media, which consisted of 1 % yeast extract, 2 % peptone and 2 % glucose. It was prepared by adding 10 g yeast extract, 20 g peptone, 20 g glucose and 1 L of distilled water to an Erlenmeyer flask. The flask was covered with aluminium foil in order to prevent contamination and autoclaved at 121 ˚C for 20 minutes. This broth was then cooled to room temperature. 50 ml of YPD broth culture and 20 % of a packet of yeast were added to a 100 mL Erlenmeyer flask and incubated at 25-30˚C and 220-250 rpm for 16-18 hours or 110 rpm for 24 hours. Milky broth culture is an indication of growth. 10 % sub-culture of the final volume was prepared by adding 50 mL of yeast broth culture to 200 mL of fresh YPD media. The total volume of 250 mL was then added to a 500 mL Erlenmeyer flask which was incubated at 25-30 ˚C and 220-250 rpm for 16-18 hours (or 110 rpm for 24 hours). Both monocultures were then added to the hydrolysate, for fermentation, or delignified corn cob for immobilisation.

3.6.2 Plating and storage of yeast cells

20 % of a petri dish was filled with yeast agar and allowed to harden at room temperature; this was streaked with a drop of cultured yeast cell broth using the round end of a sterilized pin, needle or lube. The five steps used to streak the plate are illustrated in Figure 3.4making sure to use a new lube in each step.

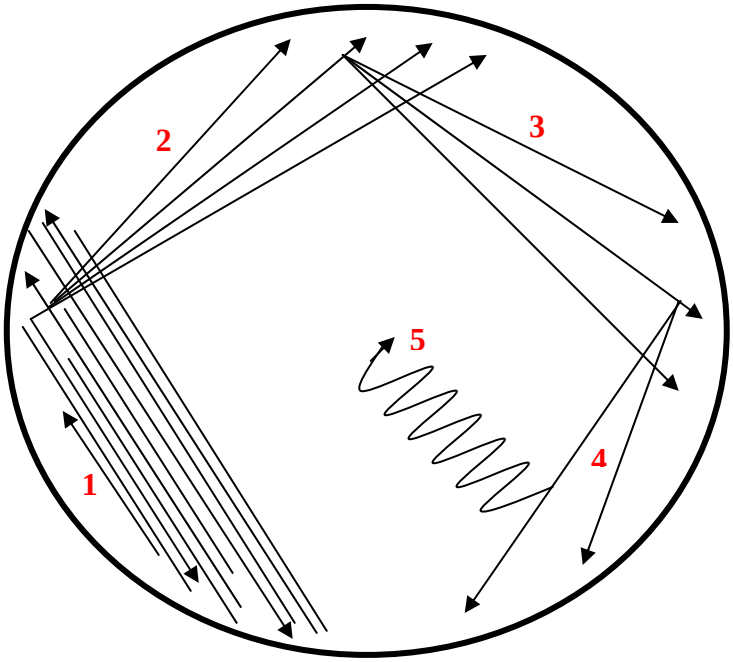

Figure 3.4: Technique used to culture yeast cells, streak plating.

The petri dishes were closed and left inverted for 48 hours at 30 ˚C. Thereafter it was wrapped with electricians tape, kept airtight and stored in the fridge at 4 ˚C for up to six months or until use. When storing or incubating plates always seal and keep inverted.

3.6.3 Type 1 immobilisation

The synthetic media was prepared by adding 60 g glucose, 2 g yeast extract, 0.5 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4 and 5 g $MgSO_4$ to 500 mL distilled water in an Erlenmeyer flaskaccording to Kourkoutas et al (2006) and Plessas et al (2007). The immobilisation medium was sterilised in an autoclave at 121 ˚C for 20 minutes. Adjust pH to 4.8-5.6 with sulphuric acid. 5 g of crushed delignified corn cob of size fraction +500-2000 μm and 2 yeast cell colonies were added to 100 mL of this culture medium in a 250 mL Erlenmeyer flask. This was fermented for 6-24 hours at 180 rpm in an incubator shaker. The liquid was then decanted and the biocatalyst was washed with fresh culture media before use in subsequent fermentation experiments.

In order to compare ethanol production of free cells with that of immobilised cells, an estimation of the number of yeast cells is required. The amount of yeast cells immobilised was determined by comparison of the synthetic culture media in the presence and absence of corn cobs, using a haemocytometer. The difference in yeast cell concentration is the amount of cells immobilised on the corn cob.

The adhesion of cells on the surface of the corn cob was observed by drying the biocatalyst in an oven at 30 ˚C overnight. The biocatalyst was then mounted to a stub, gold and platinum coated and then viewed with an SEM. The larger the size of corn cob the larger the yeast cell loading, this large size however cannot be mounted on a stub to view the immobilised cells on the surface of the support by SEM. Another method to view yeast cells will therefore be required.

3.6.4 Type 2a and 2b immobilisation

Immobilisation was carried out in a two-step process. The first step involved culturing the yeast cells in an incubator shaker, for 16-18 hours at 220 rpm, by adding 2 colonies of yeast cells to 50 mL of complex YPD culture media in a 250 mL Erlenmeyer flask under a laminar flow flame. The second step involved the addition of 1 g of sterilized delignified (2a) and untreated (2b) corn cob to this yeast broth culture and fermentation for 6-24 hours in an incubator shaker at 180 rpm to facilitate immobilisation of yeast cells onto the corn cob. The liquid was then decanted and the biocatalyst was washed with fresh culture media. The amount of yeast cells immobilised was then analysed using a haemocytometer as before and immobilisation was viewed using an SEM. The biocatalyst was used in subsequent fermentation experiments.

3.7 Fermentation

3.7.1 Free cell fermentation

2.5 mL *S. cerevisiae*, 2.5mL *P. stipitis* and 5 mL fresh YPD media were added to 50 mL of hydrolysate in a 250 mL Erlenmeyer flask. The flask was sterilised with alcohol and covered with foil. Fermentations were carried out at 30-33 °C and 180 rpm for three days. Rapid sampling was carried out periodically to measure fermentable sugar utilisation and ethanol production with time.

3.7.2 Immobilised cell fermentation

0.1 g *S. cerevisiae* biocatalyst, 0.1 g *P. stipitis* biocatalyst and 5 mL fresh YPD media was added to 50 mL hydrolysate in an Erlenmeyer flask. The flask was sterilised before being covered with foil and placed in an incubator shaker at 30-33 °C and 180 rpm for three days. This was allowed to ferment until complete utilisation of fermentable sugars. Rapid sampling was carried out periodically to monitor sugar utilisation and ethanol production with time.

CHAPTER FOUR: RESULTS & DISCUSSION

4.1 Ammonia treatment

4.1.1 Dilute acid hydrolysate

Dilute acid hydrolysis followed delignification of corn cob, at ambient temperature, by ammonia steeping. According to Cao et al (1996), ammonia steeping in dilute NH_4OH enables the removal of 80-90 % lignin, alkali-soluble extractives and acetates, which have been known to inhibit fermentation by yeast cells.

All five samples of hydrolysate from five experiments show higher xylose yields as compared to glucose and are presented in Figure 4.1. The standard deviation between values was 0.3 and 3.9 for glucose and xylose, respectively.

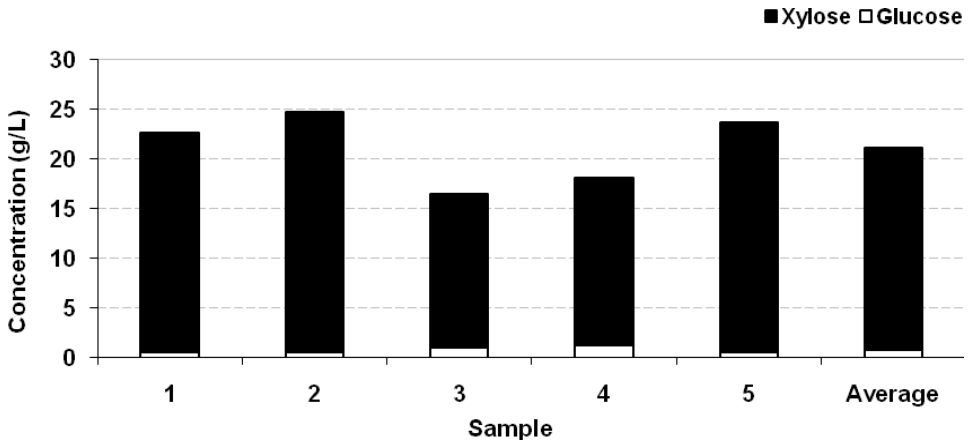

Figure 4.1: Glucose and xylose concentration of acid hydrolysate.

The average xylose and glucose concentrations obtained from the ammonia treatment are 20.3 g/L and 0.8 g/L, respectively (Table A1, Appendix A). D-xylose is the major product of the hydrolysis of hemicellulose which is a common occurrence when hydrolyzing various plant materials including corn cob. It often comprises more than 60 % of the recoverable sugars derived from hemicelluloses (Lin-Cheng et al 1981).

High extraction of xylose, as compared to glucose, by dilute acid hydrolysis at 121 °C for one hour was confirmed in this experiment. According to Cao et al (1996) dilute acid hydrolysis allows for treatment of the hemicellulose fraction to pentose sugars with xylose being of the highest concentration. The xylan to xylose conversion ratio is extremely high.

The concentrations achieved, however, were not as high as reported by Cao et al (1996) which were 51 g/L xylose and 4 g\L glucose. The extreme high pressure and temperature reached in the autoclave resulted in the loss of part of the pentose fraction as was discovered by Dale and Moreira (1982) cited in Cao et al (1996) or to inefficient lignin removal as suspected by Yah et al (2010). The presence of lignin in the substrate would have prevented the adequate extraction of sugars.

4.1.2 Enzymatic hydrolysate

Enzymatic hydrolysis was satisfactory. Five experiments reported in Figure 4.2 indicate the presence of a low glucose concentration and the absence of xylose with a standard deviation of 0.1 between values.

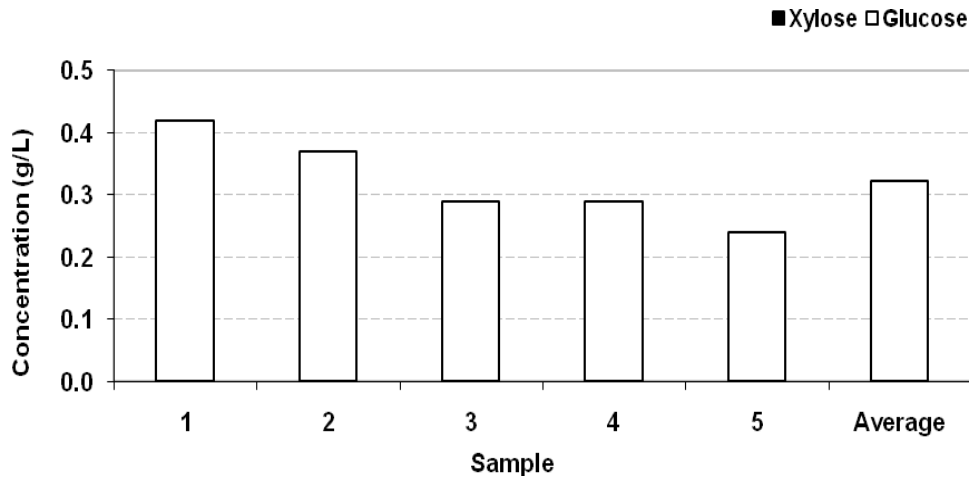

Figure 4.2: Glucose and xylose concentration of enzymatic hydrolysate.

Ammonia steeping and dilute acid hydrolysis followed by enzymatic hydrolysis has been theorized to produce high concentrations of glucose (Cao et al 1996). The low average concentration of glucose, 0.32 g/L (Table A1, Appendix A), extracted from corn cob in this study can be attributed to three factors: substrate, cellulase activity, and reaction conditions (temperature, pH and other parameters) according to Sun and Cheng (2002).

The pH and temperature are not attributable factors to the low concentration of glucose as this was monitored and remained within functional specifications, of 50-60 °C and pH of 4-5, provided by the supplier, Merck Pty (Ltd). It was suspected, however, that the extremely high temperature (121 °C) and pressure (103.4 kPa) during the hour of dilute acid hydrolysis in the autoclave may have caused high losses of the cellulose fraction.

The cellulase activity during hydrolysis may have decreased which was partially due to the irreversible adsorption of cellulase on cellulose (Converse et al 1988). This, however, could have been minimized by the addition of surfactants. Another reason for a low glucose concentration could be attributed to inefficient lignin removal. Lignin blocks access of cellulases to cellulose and irreversibly binds hydrolytic enzymes (McCarter et al 2002). Therefore, it is imperative that lignin be removed to increase the hydrolysis rate (McMillan 1994).

4.1.3 Comparison of acid and enzymatic hydrolysate

It is necessary to compare the concentrations of fermentable sugars extracted, as indicated in Figure 4.3, in order to determine the feasibility of conducting both treatments. According to Cao et al (1996) a mixture of equal quantities of both hydrolysates should provide a solution with concentrations of approximately 56 g/L xylose and 54 g/L glucose.

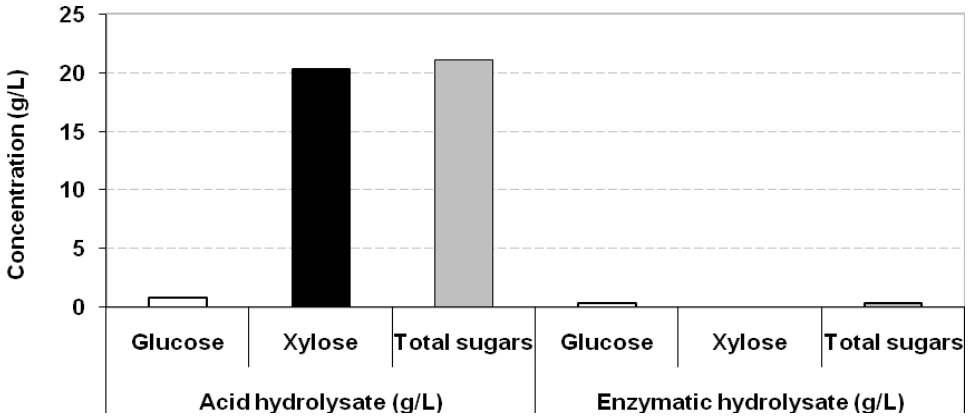

Figure 4.3: Total sugar concentration of acid and enzymatic hydrolysate.

Overall, by using Cao et al (1996) findings as a comparison it can be concluded that both acid and enzymatic hydrolysis was only partially successful. The fermentable sugar concentrations extracted was also lower than that reported by Yah et al (2010). This was due to autoclaving of corn cob that could have been caused loss of cellulose and the suspected inadequate removal of lignin. Hydrolysis, however, can be further enhanced by the addition of a mixture of hemicellulases or pectinases with cellulases; by addition of surfactants; and efficient removal of lignin.

Enzymatic hydrolysis is not a favoured hydrolysis method. Hydrolysis rate by cellulases was very slow. This type of hydrolysis gives rise to product and substrate inhibition. Cellulases are expensive and difficult to reuse due to its specific activity, high production costs and difficulty of kinetic analysis.

When combining equal quantities of the acid and enzymatic hydrolysates the resulting solution had concentrations of 10.17 g/L xylose and 0.56 g/L glucose (Calculated in Appendix A.2). Combining equal quantities of acid and enzymatic hydrolysate would decrease the total concentration of sugars in the mixture. It is

therefore feasible to use acid hydrolysate alone, without enzymatic hydrolysate, in subsequent fermentations.

4.2 Concentrated sulphuric acid treatment

Lignocellulosic materials have been treated in the past with concentrated acids, H_2SO_4 and HCl.

Concentrated sulphuric acid hydrolysis was moderately successful in this study. The average of five experimental results, presented in Figure 4.4, had a standard deviation of 0.9 for both glucose and xylose.

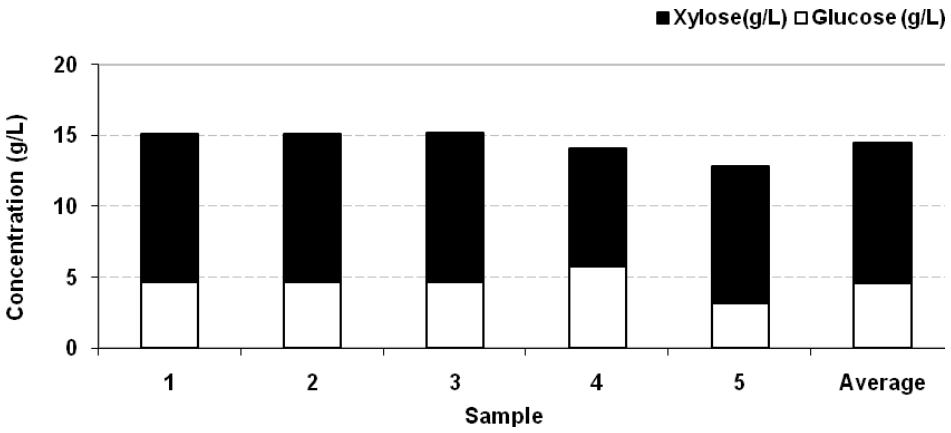

Figure 4.4: Glucose and xylose concentration of concentrated sulphuric acid hydrolysate.

The concentration of fermentable sugars extracted was 9.9 g/L xylose and 4.6 g/L glucose. The total concentration of fermentable sugars was 14.5 g/L. The concentration of xylose extracted was 2.2 times higher than the glucose concentration. Acid treatment has been reported by Cao et al (1996) to readily hydrolyse the hemicellulose fraction to, primarily, pentose sugars, such as xylose. Therefore the finding in this study is justified.

4.3 Comparison of ammonia and concentrated sulphuric acid treatments

The two treatment processes employed in this study differed both in concept and technique. Unlike the concentrated sulphuric acid treatment, during ammonia treatment all the hemicellulose was kept intact during lignin removal and not subjected to degradation. The primary objective of the latter experiment was to separate the major components lignin, hemicellulose and cellulose and treat them separately so as to obtain maximum yield from each component.

Figure 4.5 compares the ammonia treatment process to the concentrated sulphuric acid treatment which produced hydrolysate with a 47.7 % higher total fermentable sugar concentration. The concentration of fermentable sugars obtained by ammonia and concentrated sulphuric acid treatment was 21.5 and 14.5 g/L, respectively. This finding was unlike that of Badger (2002) who stated that concentrated sulphuric acid processing has a high sugar recovery when compared to dilute acid hydrolysis. This was due to the initial steeping of corn cob in ammonia to remove lignin and increase the surface area of hemicellulose which enabled a higher sugar recovery

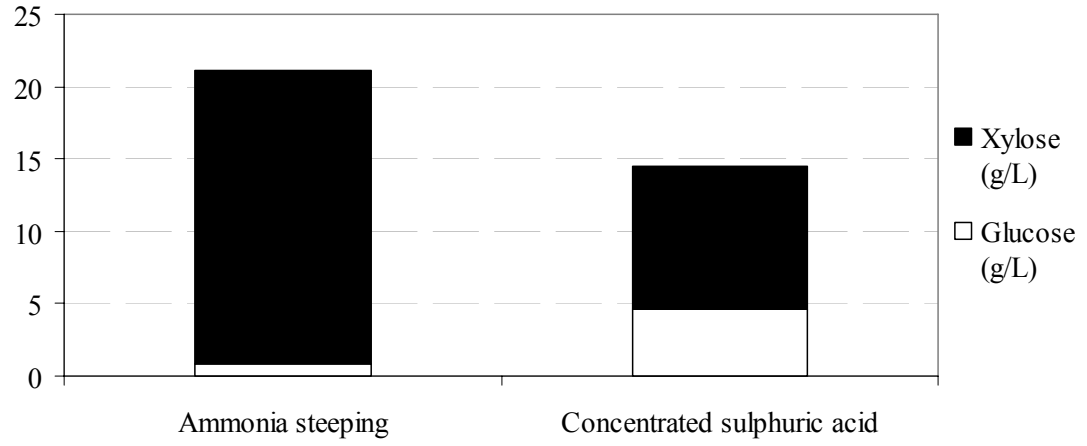

Figure 4.5: Glucose and xylose concentration achieved from ammonia steeping and concentrated sulphuric acid pre-treatment methods.

According to Galbe & Zacchi (2007) the more severe conditions during pre-treatment the greater the degradation of hemicelluloses to pentose sugars and enzymatic digestibility of cellulose to monomer sugars. The severity results of pre-treatment methods, employed in this research, are presented in Table 4.1 Calculations for this data can be found in Appendix A.4.

Table 4.1: Severity correlation.

		Severity	Combined severity
Ammonia treatment process	2.9 M NH_4OH	1.097	1.097
	0.3 M HCl	2.396	2.919
	Enzymatic hydrolysis	1.987	1.987
	Total severity	**5.480**	**6.003**
Concentrated H_2SO_4 process	72 % H_2SO_4	1.097	-0.033
	Hydrolysis	2.108	3.782
	Total severity	**3.205**	**3.748**

The severity correlation shows that the ammonia treatment process is 1.6 times more severe than the concentrated H_2SO_4 process in degradation of hemicelluloses and cellulose to fermentable sugars.

Even though concentrated acids have been powerful agents in treatment of lignocellulosic material they are toxic, expensive, and hazardous, and require corrosion resistant reactors according to Sivers and Zacchi1995 cited in Sun and Cheng 2002. High acid concentrations lead to degradation of hemicellulose sugars to fermentation inhibitors such as furfural and 5-hydroxymethylfurfural.Neutralisation requires the purchasing of large quantities of the neutralizing agent, calcium hydroxide. The cost of calcium hydroxide and the proper disposal of the resultant calcium sulphate residue from neutralisation drastically contribute to the overall cost

of the process. In order to make the process economically feasible the concentrated acid must be recovered after hydrolysis. Studies have also shown that the majority of the concentrated acid processes available have a relatively slow processing time and are more costly (Badger 2002). The ammonia treatment process is therefore the preferred treatment method due to its high fermentable sugar production, high severity, economic feasibility, and ease of application.

4.4 Immobilisation

4.4.1 Type 1 immobilisation

A synthetic immobilisation medium was used to immobilise the yeast cells on corn cob supports.

In contrast to results obtained by Kourkoutas et al (2006) and Plessas et al (2007), the attachment of yeast cells to the corn cob support was sparse as seen in Figure 4.6.

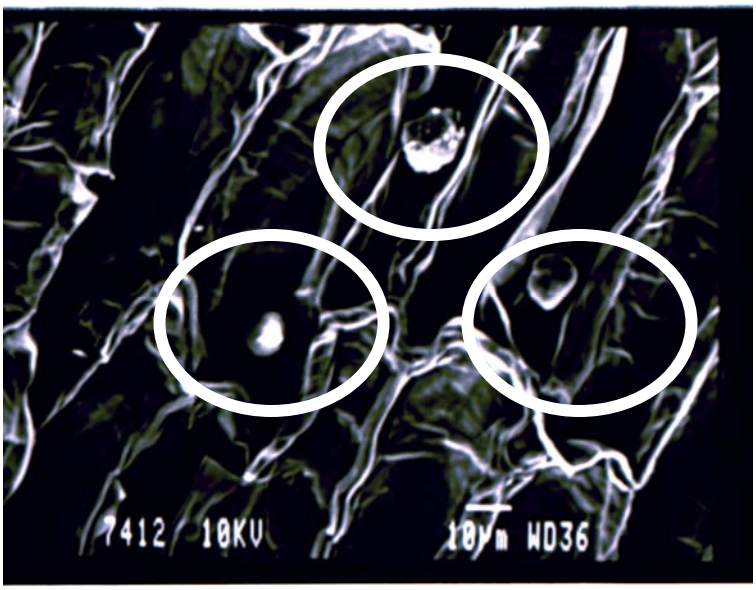

Figure 4.6: Scanning Electron micrograph of immobilised yeast cells on the surface of corn cob.

At a $\times 900$ magnification only three yeast cells were viewed by SEM. The number of yeast cells immobilised per millilitre was 0.5×10^{10}, after 24 hours of incubation and shaking, which was counted using a haemocytometer. The low concentration of yeast cells immobilised can be attributed to the use of the synthetic medium. On sterilisation of the media, precipitation occurred. This media was then filtered to remove precipitate and used in immobilisation experiments. The precipitation could have been caused due to the high concentration of salts which caused the metal ions

to be precipitated out of the solution. This can be avoided by the addition of a chelating agent. However, this will increase costs. This chemically defined synthetic media contained carbon, and nitrogen in trace amounts. In order to grow yeast cells effectively a crude source of nitrogen is needed.

4.4.2 Type 2a immobilisation

Cell immobilisation on untreated corn cob is shown by the electron micrograph in Figure 4.7.

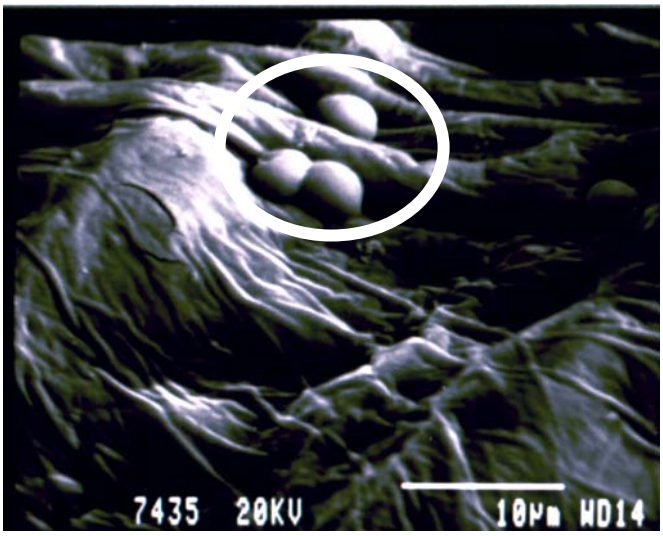

Figure 4.7: Scanning Electron micrograph of immobilised yeast cells on the surface of untreated corn cob.

Yeast cells have adhered very sparsely to the surface of the corn cob support as indicated by the white ring in Figure 4.7. At ×3000 magnification three yeast cells could also be viewed by SEM on untreated corn cob support. The number of yeast cells immobilised per millilitre was 2.7×10^{10}, after 24 hours of incubation and shaking, which was counted using a haemocytometer. The lignin present on the surface of the untreated corn cob covered the cellulose needed for cell adhesion thereby preventing adsorption of yeast cells onto the cellulose surface. The adsorption ability of cellulose and solid materials, like glass, can be enhanced by treating it with

polycations, or chitosan (Norton & D'Amore 1994; Navarro & Durand 1977). This will, however, increase costs drastically and make it economically unfeasible.

4.4.3 Type 2b immobilisation

Cell immobilisation on delignified corn cob is shown by the electron micrograph in Figure 4.8.

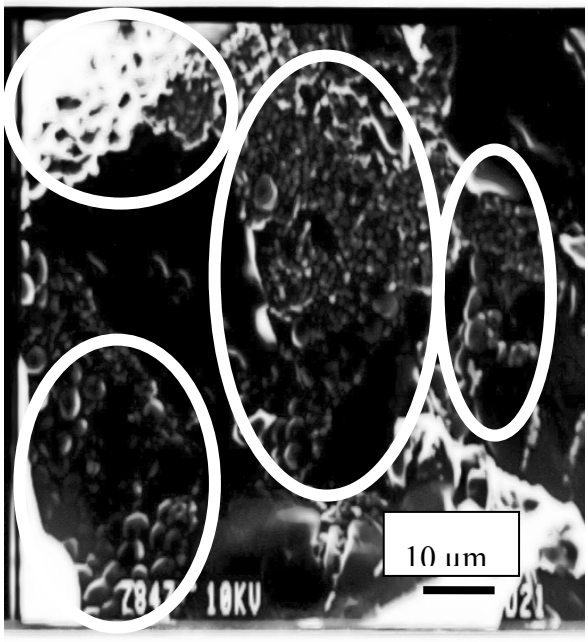

Figure 4.8: SEM micrograph of immobilised yeast cells on the surface of delignified corn cob.

Yeast cells adsorbed densely and homogenously on the surface of the corn cob as indicated by the white rings on Figure 4.8. This yeast cell immobilisation was viewed at a ×1200 magnification by SEM. The number of yeast cells immobilised per millilitre was 11.9×10^{10}, after 24 hours of incubation and shaking, which was counted using a haemocytometer. Yeast cells were able to adsorb the best due to the absence of lignin that exposed cellulose thereby causing adherence of yeast cells to the surface. Other reasons for immobilisation include natural entrapment into the porous cellulosic material of delignified corn cob, or due to physical adsorption by electrostatic forces or covalent binding between the cell membrane and the support as

reported by Plessas et al in 2007 who studied the adhesion of yeast cells on cellulosic orange peel.

4.4.4 Comparison of immobilisation methods

It is imperative that the different immobilisation techniques employed be compared to each other in order to determine the most suitable biocatalyst which was used in fermentation experiments as shown in Figure 4.9.

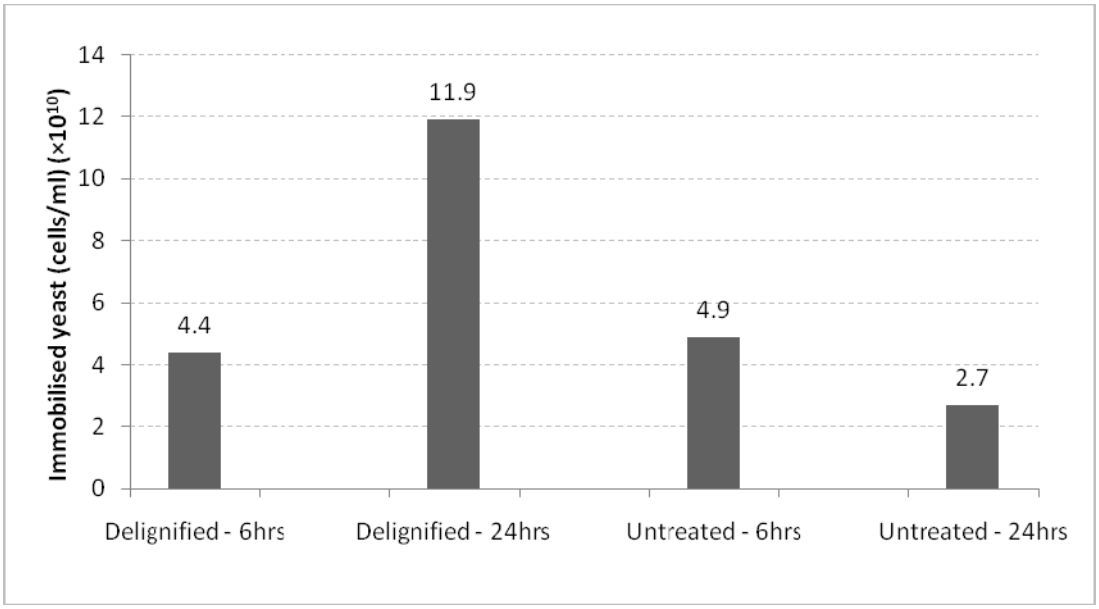

Figure 4.9: Comparison of immobilisation techniques.

The mean values of duplicate tests are displayed in Figure 4.9. Haemocytometer yeast cells counts show that the greatest adsorption of yeast cells was achieved on delignified corn cob supports. An average of 11.9×10^{10} yeast cells adsorbed on delignified corn cob as compared to immobilisation on untreated corn cob which adsorbed 2.7×10^{10} yeast cells after 24 hours. The biocatalyst was allowed to ferment at 30 °C in an incubator shaker for 24 hours. This period of incubation allowed yeast cells to multiply exponentially in the complex YPD media. The YPD media contained peptone which is a crude source of carbon, nitrogen, fats, metals, salts,

vitamins and many other biological compounds unlike the synthetic media. More yeast cells were therefore available for adherence to the surface of the delignified corn cob. The exposed cellulose also enabled better adhesion of yeast cells on corn cob. This biocatalyst was therefore used in subsequent immobilised cell fermentations and was compared to free cell fermentation.

4.5 Fermentation

This section aims at qualifying a biocatalyst in batch fermentation which can be used in further research for the continuous production of ethanol. All fermentations were performed in duplicate, one of which is presented in subsequent sections. Wheat bran fermentation results were taken from previous unpublished studies conducted by Aadilah Omar and Zi Hu at the School of Molecular and Cell Biology at the University of the Witwatersrand.

4.5.1 Free cell fermentation

4.5.1.1 Fermentation of wheat bran hydrolysate by *P. stipitis*

Wheat bran hydrolysate was produced using concentrated H_2SO_4 treatment. Concentrated acid hydrolysis resulted in a higher xylose than glucose concentration which was consistent with findings reported by Maes & Delcour in 2001 where they found that cellulose and lignin combined made up to 25.2 % and hemicellulose had a higher concentration of 18.9 % alone of the dry matter in de-starched wheat bran.

Figure 4.10 shows, not only sugar utilization of wheat bran hydrolysate and ethanol production by a monoculture of *P. stipitis*, but biomass growth as well. All data for Figure 4.10 are available in Appendix B.1.1.

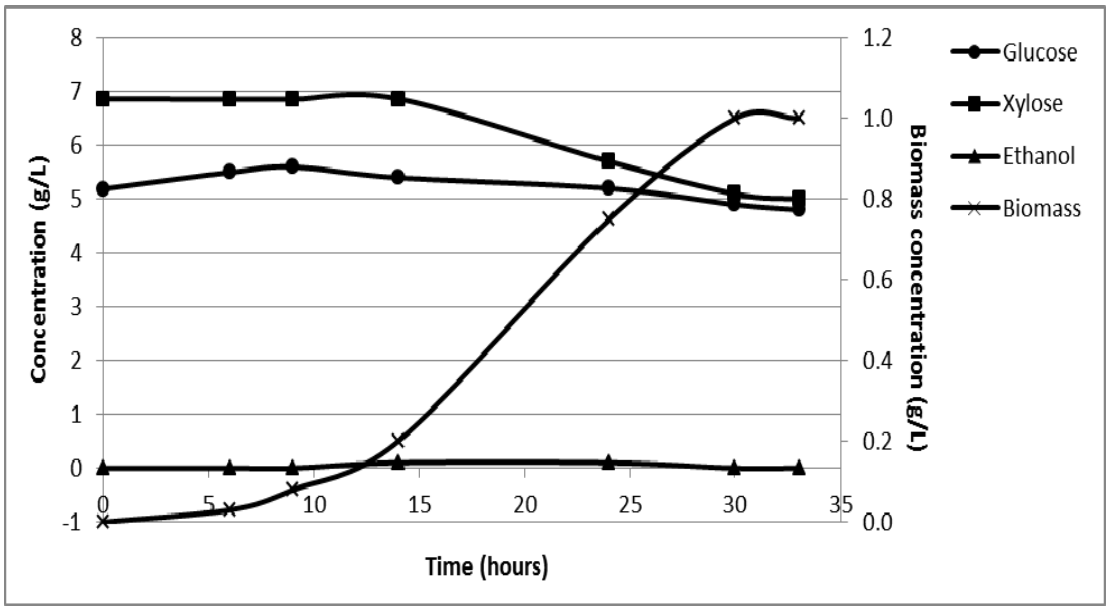

Figure 4.10: Sugar consumption and ethanol production by *P. stipitis* (Omar & Hu 2009).

The yeast reached exponential growth after about 15 hours, reaching a maximum of 1.2 g/L cells. There is no data between 14 and 24 hours due to experimental constraints as fermentation was conducted overnight. Glucose and xylose were utilized by the yeast but not to the extent that was anticipated with a 25.3 % decrease in xylose and an 11.8 % decrease in glucose after 30 hours of fermentation. It was deduced, therefore, that the sugars utilized from the hydrolysate were used for respiration and growth not fermentation hence there was no ethanol produced during this time. Low ethanol production, by *P. stipitis*, in this research could be attributed to oxidation of ethanol where high levels of aeration led to great cell mass production and consequently a low ethanol yield as was reported in 1994 by Boynton and McMillan. Oxidation usually results when fermentative substrates are not available or in very limited supply. Fully anaerobic conditions should therefore be employed.

With minimal sugar utilization a growth rate of 0.036 g/(L·h) was achieved. The growth rate was determined by calculating the gradient of the yeast cell growth curve from Figure 4.10.

4.5.1.2 Fermentation of wheat bran hydrolysate by *S. cerevisiae*

Figure 4.11 illustrates the characteristic behaviour of *S. cerevisiae* in a single culture. Data is available in Appendix B.1.2.

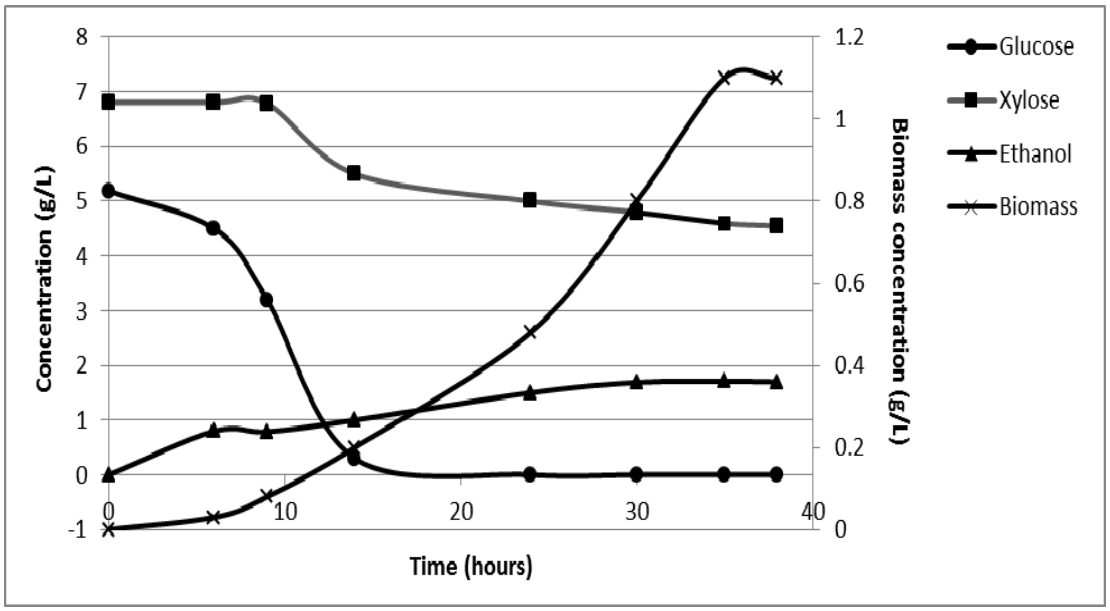

Figure 4.11: Sugar consumption and ethanol production by *S. cerevisiae* (Omar & Hu 2009).

Both xylose and glucose utilization is significantly higher at 30.1 % and 100 %, respectively. The complete depletion of glucose occurred after approximately 14 hours of fermentation. There is no data between 14 and 24 hours due to experimental constraints as fermentation was conducted overnight. This indicated efficient utilization of glucose by *S. cerevisiae* leading to a 23.4 % conversion rate of sugars to ethanol. The maximum concentration of ethanol produced was 1.69 g/L.

A lower growth rate of 0.032 g/(L·h) was observed with a maximum of 1.09 g/L of biomass. The growth of yeast cells was limited due to the lack of glucose present in the hydrolysate. Exponential growth started after 10 hours of fermentation. The rate of growth between 15 and 25 hours was lower than that of *P. stipitis* due to glucose exhaustion. Ethanol production began early in fermentation reaching its plateau after 15 hours which also coincided with glucose depletion. The decline in xylose leveled off after 15 hours.

4.5.1.3 Fermentation of wheat bran hydrolysate by *S. cerevisiae* and *P. stipitis*

The results presented in Figure 4.12 show fascinating trends in the mixed culture of yeast cells. All data for Figure 4.12 are available in Appendix B.1.3.

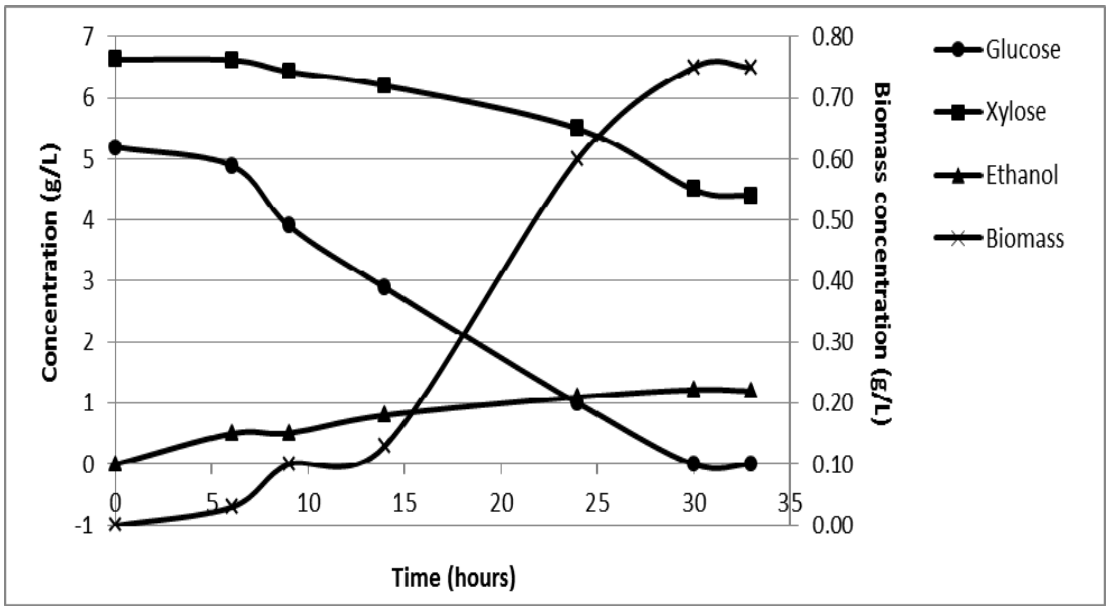

Figure 4.12: Sugar consumption and ethanol production by mixed culture (Omar & Hu 2009).

Xylose utilization was 32.0 % while glucose was 100 % after 30 hours of fermentation. Glucose was depleted after 30 hours unlike the *S. cerevisiae* monoculture that reached 100% utilization after 14 hours of fermentation. There is no

data between 14 and 24 hours due to experimental constraints as fermentation was conducted overnight. The mixed culture biocatalyst takes a longer time to get accustomed to the environment. The rate of xylose utilization to ethanol was lower than that of glucose which is due to their difference in metabolic pathways by yeast cells. The xylose metabolic pathway by yeasts cells produces less ATP in the form of energy than the glucose metabolic pathway for the production of ethanol. The conversion of sugars to ethanol was 17.8 %; this is lower than that of *S. cerevisiae* on its own which indicates that placing the two types of yeasts together did not greatly affect ethanol production.

However, the fact that the xylose consumption had increased slightly gives reason to believe that a mixed culture of the two yeasts does have a positive effect on the pentose utilization.

The concentration of ethanol produced was 1.22 g/L. The growth rate was the lowest at 0.027 g/(L·h)and reached a maximum cell concentration of 0.75 g/L after 30 hours, most probably due to competition between the species for sugar utilization. There is evidence of a lag from 0 to 15 hours, after which growth reached its exponential phase. The increased growth rate between 15 and 25 hours did not seem to have much of an effect on the rate of ethanol production which can be attributed to sugar utilization by yeast cells predominantly for growth not fermentation.

4.5.1.4 Fermentation of corn cob hydrolysate by *S. cerevisiae* and *P. stipitis*

This free cell fermentation of corn cob hydrolysate produced by the ammonia treatment process was used as a basis to compare immobilised cell fermentation. Fermentable sugar consumption and ethanol production is shown in Figure 4.13. All data for Figure 4.13 are available in Appendix B.1.4. There is no data between 9 and 21 hours due to experimental constraints as fermentation was conducted overnight.

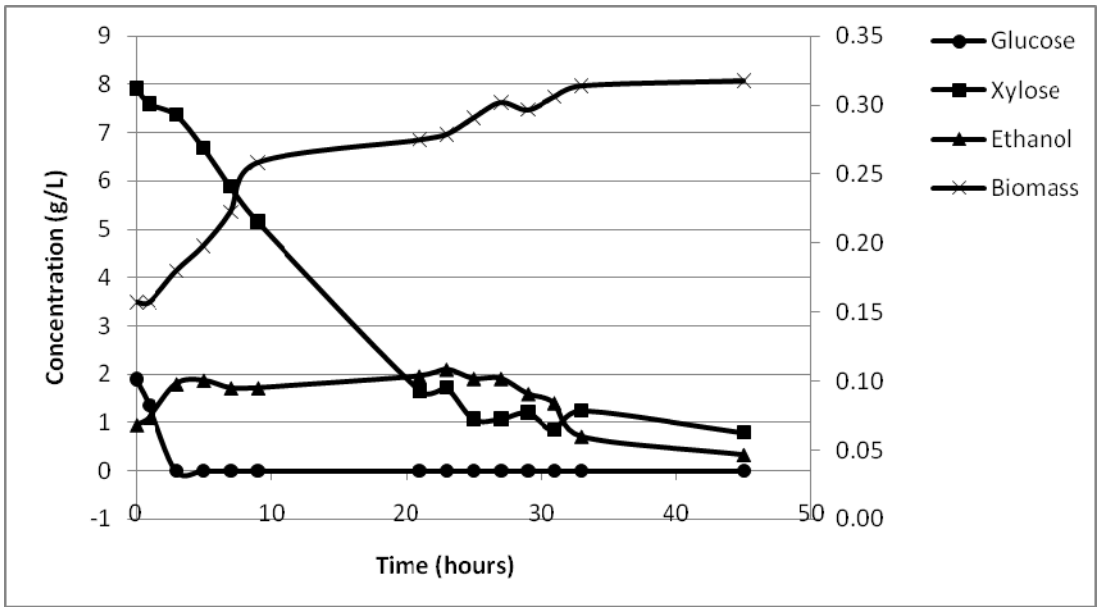

Figure 4.13: Sugar consumption and ethanol production of corn cob hydrolysate by mixed culture.

In batch fermentations hexose sugars are usually metabolized preferentially by *P. stipitis* however since the glucose conversion rate of *S. cerevisiae* is much higher than *P. stipitis* (Grootjen et al 1990), *S. cerevisiae* is responsible for the initial utilization of glucose. *S. cerevisiae* and *P. stipitis* readily fermented the corn cob hydrolysate containing 32.8 g/L xylose and 1.1 g/L glucose to produce 4.5 g/L ethanol within 29 hours. This occurred at a yield of 27.1% (based on the theoretical yield of 10.8 g/L ethanol), see Appendix B.6 for calculations. This was unlike the results reported by Cao et al (1996) who obtained a yield of 80 % which could have

been attributed to his use of a weak-based anion exchange resin to remove salts which inhibits fermentation and a higher initial glucose concentration.

The lowest growth rate of yeast cells, 0.0052 g/(L·h), was obtained as compared to all other free cell fermentations conducted. This was due to the lowest concentration of glucose in the hydrolysate which was utilised for yeast cell growth only and not fermentation. The utilisation of pentose sugars by yeast cells is more difficult when compared to that of glucose. The metabolic pathway of xylose by yeast cells produces only one ATP unlike the glucose metabolic pathway that produces 2 ATP's per molecule of glucose. This energy is generally used for cell division, growth and fermentation which mean that yeast cells would have more energy to produce ethanol using glucose than xylose.

The addition of *P. stipitis* and *S. cerevisiae* to corn cob hydrolysate enhanced the conversion rate of the sugars into bioethanol since 100 % of the glucose was metabolised after only 2 hours and 48 % xylose in 29 hours, when compared to fermentation of wheat bran hydrolysate. The xylose and glucose utilisation by yeast cells of wheat bran hydrolysate was 90.9 and 32 %, respectively after 30 hours of fermentation. According to Jeffries et al (2007), by using a monoculture of *S. cerevisiae*, the glucose is converted after about 12.5 hours, while the xylose conversion to bioethanol and other products takes approximately 48 hours which reiterates the fact that using a mixed culture increases the rate of sugar utilisation of corn cob hydrolysate.

The yeast cell growth rate in wheat bran hydrolysate was five times faster than in corn cob hydrolysate due to the glucose concentration being five times higher. The ethanol production from wheat bran hydrolysate, however, was 3.3 g/L lower when compared to fermentation with corn cob hydrolysate due to the utilisation of xylose by *P. stipitis* for fermentation and growth.

4.5.2 Immobilised cell fermentation

Immobilised cell fermentation was also conducted using corn cob hydrolysate. All data for Figure 4.14 are presented in Appendix B.2. There is no data between 11 and 23 hours due to experimental constraints as fermentation was conducted overnight.

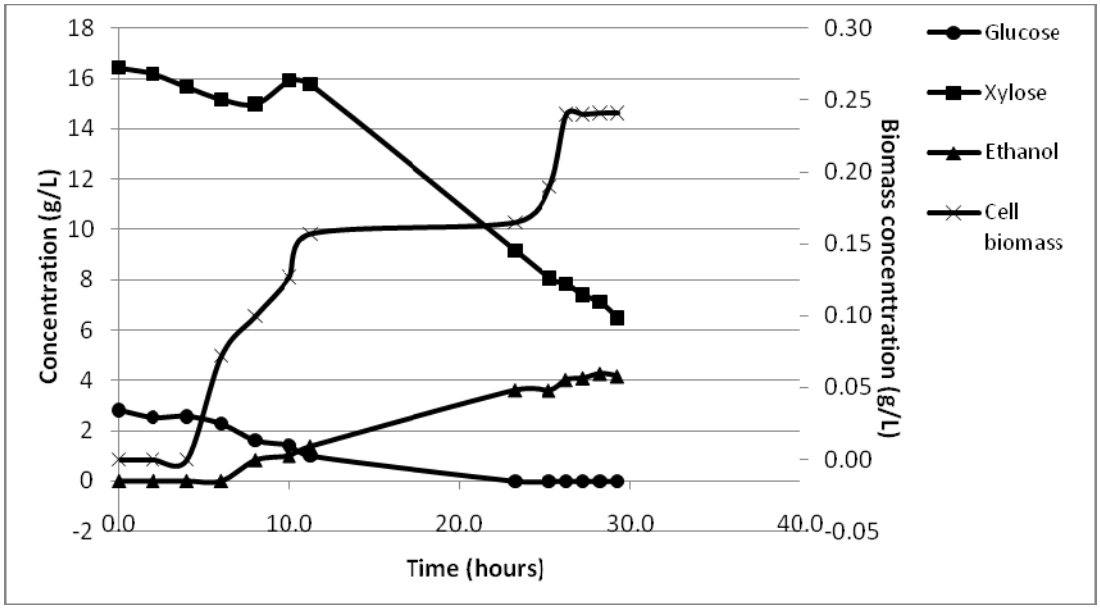

Figure 4.14: Glucose and xylose consumption and ethanol production in immobilised cell fermentation.

Corn cob hydrolysate was partially fermented to 4.2 g/L ethanol after 29 hours (Figure 4.14), giving a yield of 47.3% (Figure 4.15) based on the theoretical value, see Appendix B.6 for calculations. All the glucose was metabolised within 23.3 hours whilst the final concentration of xylose was 6.5 g/L after 29 hours having metabolised 60 % of the xylose.The metabolism of fermentable sugars to ethanol started taking place after only 8 hours and reached its maximum after 29.3 hours as compared to that reported by Jeffries et al (2007) which were 48 hours, thereby indicating an enhancement in the rate of fermentation due to immobilisation. The growth rate obtained from the gradient of the biomass growth curve was 0.0081 g/(L·h). After 26 hours of fermentation xylose continued to decrease even though the cell biomass

concentration started to level out. This is due to the death rate becoming equal to the growth rate which can be due to toxic by-products, the harsh semi-anaerobic environment and/or the depletion of essential nutrients and metabolites (Fogler 2006). It can also be due to the cell utilization of the secondary nutrient, xylose, for maintenance, production of desired product and not growth during this stationary phase after exhausting the primary nutrient glucose (Fogler 2006).

4.5.3 Comparison of fermentation methods

Free cell fermentation and immobilised cell fermentation are compared to each other in Figure 4.15. Detailed calculations for Figure 4.15 are presented in Appendix B.6.

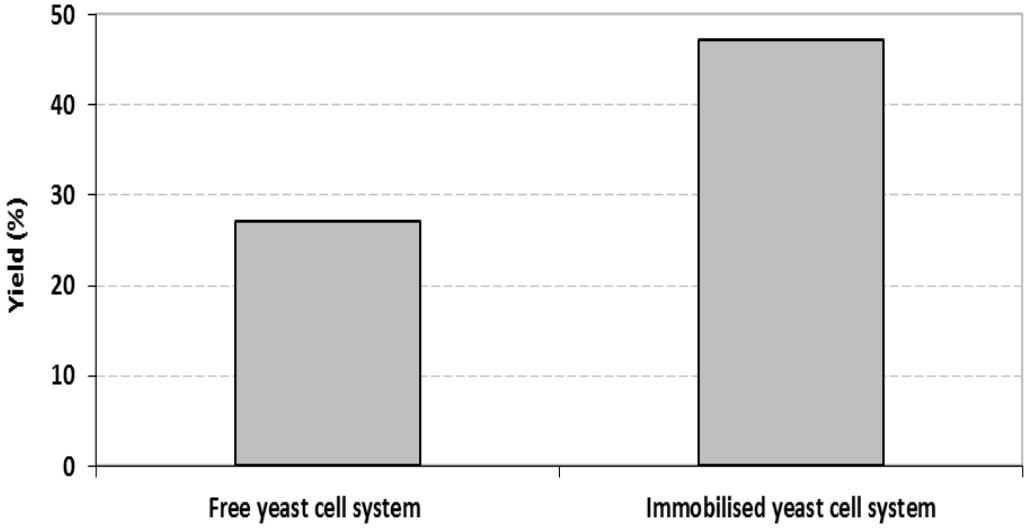

Figure 4.15: Stoichiometric yield of free and immobilised cell fermentation of corn cob hydrolysate.

Immobilised cell fermentation was partially successful in utilising sugars to produce ethanol as was reported in previous studies carried out by Kourkoutas et al (2006). Immobilised yeast cell fermentation was more successful than free cell fermentation. This result was also claimed previously by Plessas et al (2007) and Bardi & Koutinas

(1994). The percentage yield obtained from free cell fermentation and immobilised cell fermentation was 27.1 and 47.3 %, respectively. Therefore, the yield obtained by immobilised cell fermentation was 20.2 % higher than free cell fermentation. This in understandable since the actual yield will always be lower than the theoretical yield due to the utilization of fermentable sugars by yeast cells for respiration and growth as well as fermentation. The overall ethanol yield can be increased by treating the hydrolysate with a weak based anion exchange resin to remove the salt before fermentation (Cao et al 1996).

The growth rate obtained from immobilised cell fermentation was higher at 0.0081 g/(L·h), as compared to free cell fermentation which was 0.0052 g/(L·h). The growth rates were extrapolated from the gradient of the yeast cell growth curve. The increase in rate of fermentation is an important characteristic of immobilised yeast cells, which was also reported by Bardi & Koutinas (1994).

4.5.4 Fermentation modelled by Monod kinetics

4.5.4.1 Free cell fermentation kinetics

Linearisation was performed (see data in Appendix C.2.1) using Equation 10c. Good estimates of the maximum specific growth rate and Monod constant, μ_{max} and K_s, respectively were then calculated from the slope and intercept (Figure 4.16).

$$\frac{SX}{\left(\frac{dX}{dt}\right)} = \frac{1}{\mu_m} S + \frac{K_S}{\mu_m} \qquad 10c$$

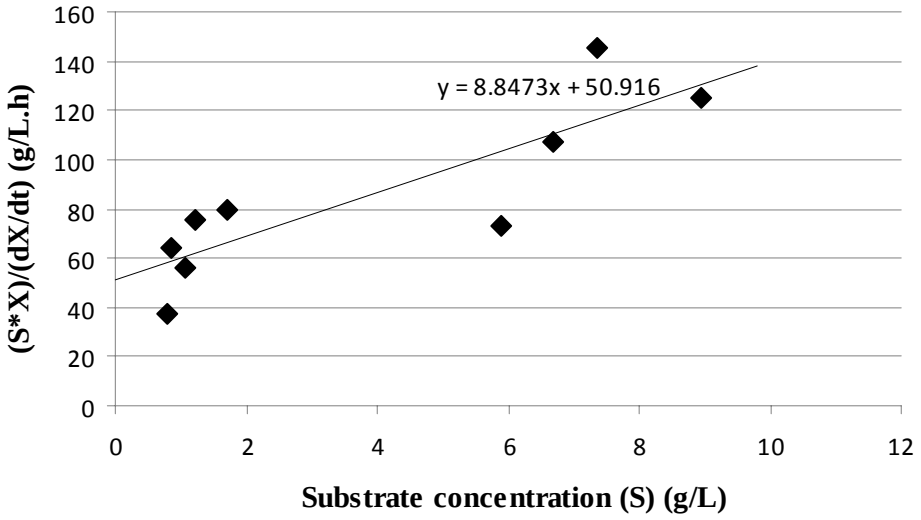

Figure 4.16: Linearisation of free cell fermentation data.

The values obtained for μ_{max} and K_s were 0.113029 h^{-1} and 5.754976 g/L, respectively.

The experimental data of free cell fermentation was modelled with the Monod Equation, equation 12a. The graph showing this model is presented in Figure 4.17 on the following page. Please refer to Table C.2.1 in Appendix C.2.1 for the data.

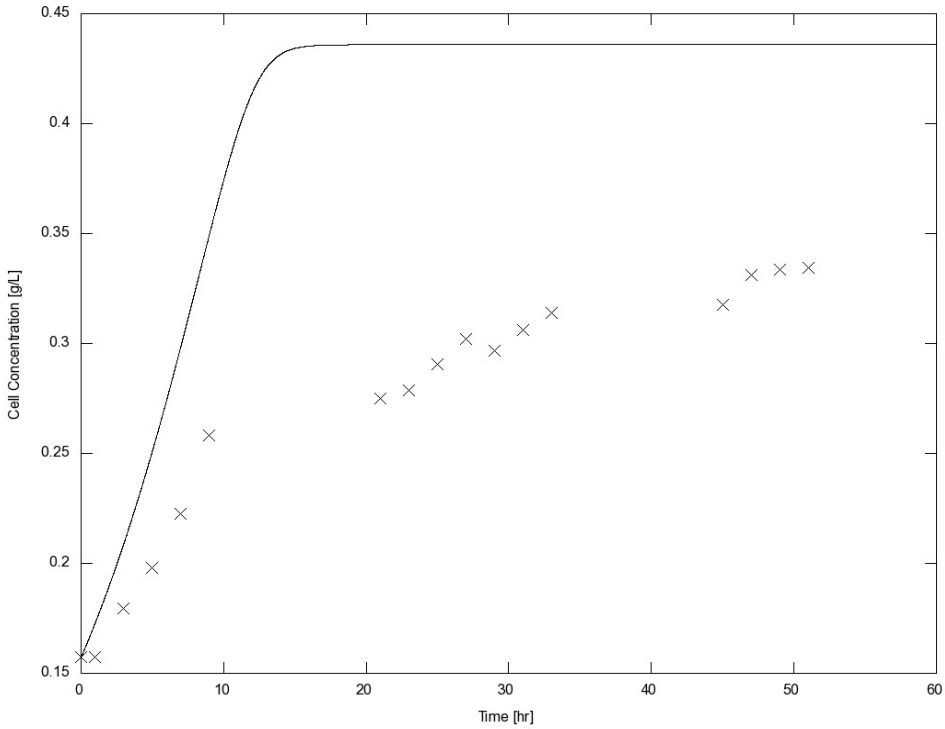

Figure 4.17: Experimental data (×) was fitted to the theoretical Monod equation (──) for Free cell fermentation.

The fit of the experimental data to the theoretical Monod equation gives an error of 0.26283. The variance in error was calculated to be 0.0094434 (see Appendix C.2.1. for calculations).

The Monod equation is used to model the substrate-limited growth phase of cells in a steady-state reactor only (Shuler and Kargi 2002) therefore, the experimental data is modelled fairly well up to 10 hours of fermentation, during the growth phase. As seen in Figure 4.17 the error gradually increases after the growth phase. This experiment was conducted in a batch reactor shake flask which could have contributed to the error also.

Equation 12a was then integrated and experimental data was modelled (see program in Appendix C.2.1). The constant yield coefficients calculated from experimental data for $Y_{X/S}$ and $Y_{P/X}$ were 0.0839 g/g and 0.0579 g/g, respectively.

The error could also attributed to the absence of yeast cell death which is going on continuously in the form of endogenous respiration which is a 'sink' in growth models and therefore needs to be taken into account. Death is due to harsh environments, shear mixing forces, local depletion of nutrients and production of toxic substances (Fogler 2006). In order to obtain a better fit of experimental data to theoretical the constants μ_{max}, K_s, and k_d were regressed using Octave, see Figure 4.18 and Appendix C.2.2 for programming using Octave.

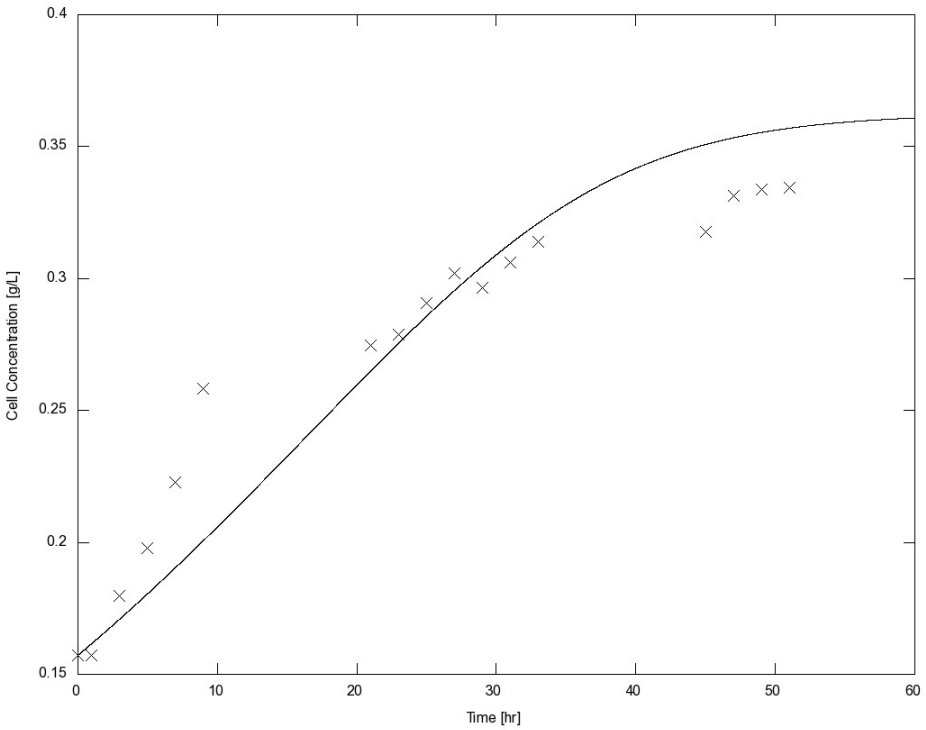

Figure 4.18: Experimental data for free cell fermentation (×) was fitted by a rate expression (—) that includes cell death.

The values obtained for μ_{max}, K_s and k_d were 0.1135 h^{-1}, 5.76 g/L and 0.0684 h^{-1}, respectively. The Monod equation 12b includes the death rate of cells, k_d, which provides a good fit to the experimental data with an error of 0.066877. The variance of error was calculated to be 0.0050693. There is no death of the cells from 0 to 10 hours of fermentation, which is why the experimental data is not modelled well during this time.

4.5.4.2 Immobilised cell fermentation kinetics

Linearisation (see Table C.2.2 and Figure 4.19) of the equation gave values for μ_{max} and K_s were determined from the slope and intercept and were 0.12798 h^{-1} and 29.2379 g/L, respectively.

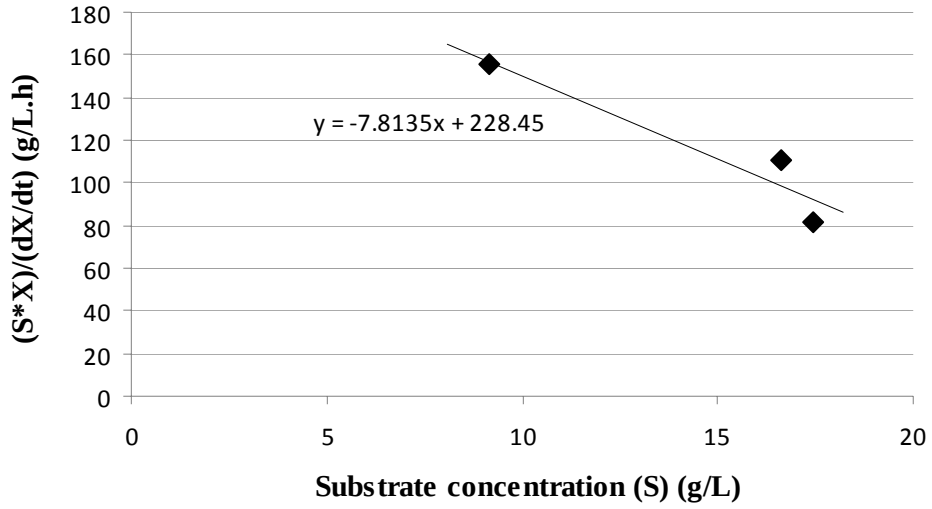

Figure 4.19: Linearisation of immobilised cell fermentation data

The experimental data of immobilised cell fermentation and μ_{max}, K_s values, which were determined from linearisation, were modelled by the Monod kinetic equation 12a and is presented in Figure 4.20. See Table C.2.2 in Appendix C.2.3 for data.

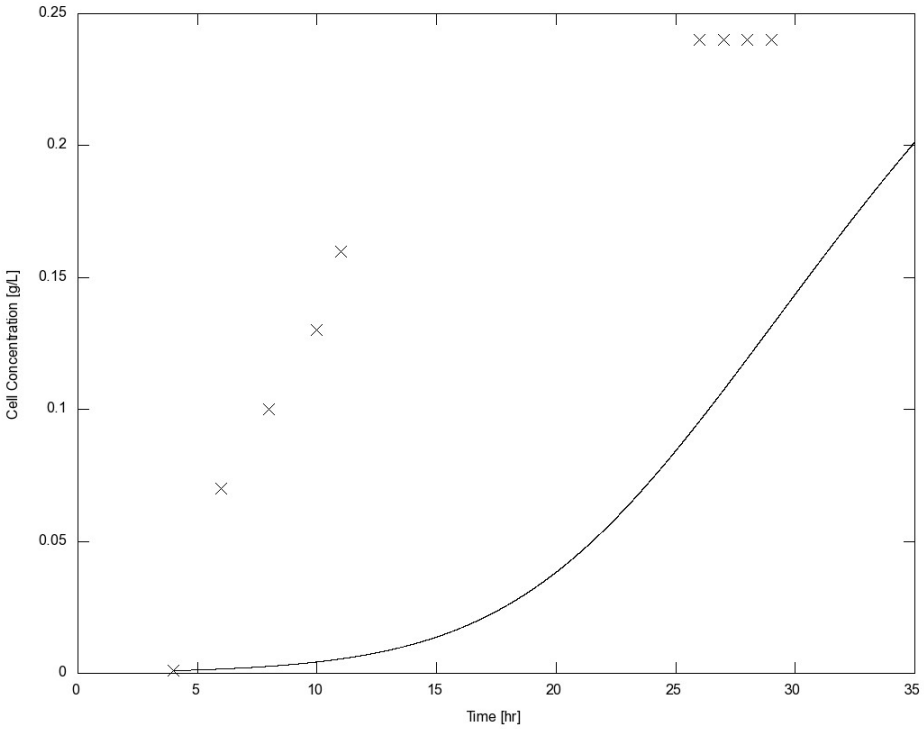

Figure 4.20: Experimental data for immobilised cell fermentation (×) was fitted by a rate expression (—) that does not consider cell death.

Equation 12a used to model free cell fermentation data was the same equation used to model immobilised cell fermentation data. The constant yield coefficients calculated from experimental data for $Y_{X/S}$ and $Y_{P/X}$ were 0.0188 g/g and 0.0572 g/g, respectively. The data was fitted in Octave with an error of 15.608 and a variance of 283.81. Comparing the constants of the free cell model to that of the immobilised model constants revealed that the reaction rate for immobilised yeast cells appear to be influenced by external mass transfer effects because of diffusion of substrate across a boundary layer. The effects of mass transfer control the overall reaction, which can be noticed by a lower cell concentration and final conversion (Fogler

2006). The error can be due to mass transfer effects of immobilisation and the use of the Monod equation for expressing the growth of immobilised cell.

It is necessary again to include the death rate constant, k_d, as it is taking place continuously and contributes to a sink in growth models. As a result of this a better fit is obtained which can be seen in Figure 4.21.

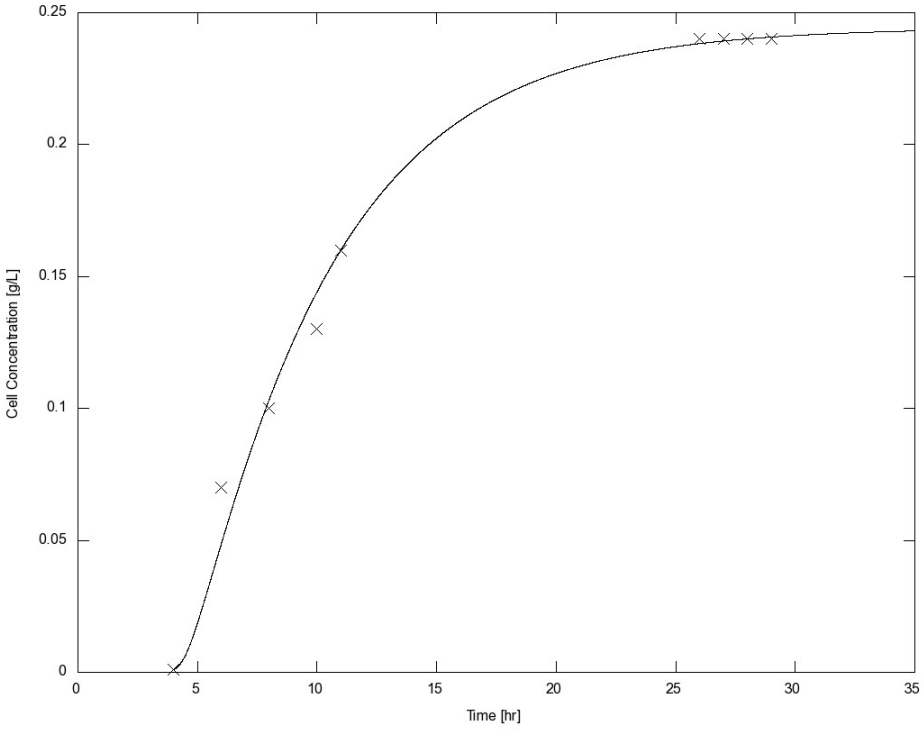

Figure 4.21: Experimental data for free cell fermentation (×) was fitted by a rate expression (—) that includes cell death.

The values obtained for μ_{max}, K_s and k_d were 0.125 h^{-1}, 19.75 g/L and 0.058 h^{-1}, respectively. These constants were regressed using Octave obtaining an error of 0.064409 and a variance of 0.017963, see Appendix C.2.4.

The yield coefficients calculated for free cell fermentation are higher than the immobilised cell fermentation. This can be due to the determination of yeast cells in solution. Since the yeast cells have adsorbed on the surface of the corn cob there are fewer in solution. The cells take time to acclimatise to the environment before using sugars to produce ethanol.

Immobilised cell fermentation has a higher maximum specific growth rate than free cell fermentation. The growth rate is directly related to cell concentration therefore the higher the growth rate the higher is the cell concentration which allows for a higher production of bioethanol. This confirms the results that immobilised cell fermentation produces a higher concentration of bioethanol than free cell fermentation.

CHAPTER FIVE: CONCLUSIONS & RECOMMENDATIONS

5.1 Conclusions

All aims and objectives were met in this study. Corn cob, available cheaply and in abundance, proved to be an effective substrate for the production of fermentable sugars and as a support for the immobilisation of yeast cells. It contained a substantial amount of starches, hemicelluloses and celluloses that were converted to fermentable sugars using the Ammonia treatment and Concentrated Sulphuric Acid treatment methods.

Ammonia treatment was favoured over the Concentrated Sulphuric Acid treatment as it produced 47.7 % more fermentable sugars. Besides enabling swelling of the cellulosic layer, which enhanced dilute acid hydrolysis, Ammonia steeping was also less toxic than concentrated sulphuric acid due to the use of dilute ammonia and hydrochloric acid. Enzymatic hydrolysis is extremely expensive, it has a very slow sugar production rate, its specific activity decreases with time as found in this study, and it gives rise to product and substrate inhibition and was therefore not favoured as a treatment method.

Immobilisation of yeast cells, in YPD media, on delignified corn cob was more successful than immobilisation on untreated supports as discovered in this study. The highest yeast loading of 11.9×10^{10} cells was achieved on this support.

It was found that fermentation of wheat bran hydrolysate with a monoculture of *S. cerevisiae* produced fermentation products with the highest ethanol concentration, 1.69 g/L, when compared to the monoculture of *P. stipitis* and mixed cultures of both species. However, fermentation of corn cob hydrolysate using a mixed culture of

yeast cells was more successful than wheat bran hydrolysate. Corn cob hydrolysate was used in immobilised cell fermentation experiments. Immobilised cell fermentation showed higher fermentation rates and greater ethanol production than free cell fermentation. This was due to higher growth rates of yeast cells in an immobilised system as a result of the ability of immobilised top fermenting yeasts to utilise the entire available sugars not just the top contact portion of substrates as with free cells.

For the same initial fermentable sugar concentration in the hydrolysate, the immobilised yeast cell system produced fermentation products with a higher ethanol concentration. Proportional calculations show that since free cell fermentation will produce 5.3 ml of ethanol from 139 g corn cob, therefore, one ton of corn cob would produce 38.2 L of ethanol. Immobilised yeast cell fermentation would produce 9.3 ml ethanol from 139g corn cob, therefore one ton of corn cob would produce 68.4 L ethanol.

In summary, the Ammonia treatment is most effective treatment in producing hydrolysate with a substantial amount of fermentable sugars that can be converted to ethanol during fermentation. Immobilisation of yeast cells on corn cob supports, with YPD media was successful. Immobilised yeast cells are more effective at bioethanol fermentation than free yeast cells with increases in the specific growth rate and the concentration of ethanol produced.

5.2 Recommendations

With the knowledge gathered from this research the following recommendations for further studies are proposed.

Whilst researching non-edible materials it was found that substrates such as rice straw, sugar cane bagasse as well as other industrial and agricultural wastes were also effective non-edible materials to use for cost-effective bioethanol fermentation in future research and this will in turn solve their problem of disposal.

Immobilisation of yeast cells on cellulose or delignified cellulosic supports are feasible option for a biocatalyst since yeast cells cannot utilise this complex starch cellulose support for respiration and fermentation and will not deplete it. These biocatalysts, therefore, can be used successfully for further research in repeated batch and in continuous fermentation. Previous research has found that adsorption ability can be improved by polycations or chitosan.

Previous studies have also shown that fermentation can be improved by treating the hydrolysate with a weak based anion exchange resin before fermentation to remove the salt present as this is seen as a fermentation inhibitor. The use of LED light by the fermentation laboratory in Chemtex Italia has proven to increase ethanol production by 20 % and should be considered in further research. The hydrolysate produced, by various treatments, can be concentrated or purified, by evaporating water before fermentation, to enhance ethanol production during fermentation. Another improvement that can be made is to conduct experiments at the optimal temperature of 25 ℃, as reported in previous research for a mixed culture of yeast cells *S. cerevisiae* and *P. stipitis*.

In order for ethanol production, by lignocellulosic substrates, to develop into an industrial technology much research still needs to be conducted, specifically in the field of developing robust fermenting micro-organisms such as pentose-fermenting

fungi. These micro-organisms must display certain characteristics including tolerance to inhibitors, ability to efficiently ferment all sugars in concentrated hydrolysate while maintaining a high yield of ethanol in contrast to the yeast cells used in this experiment. One potential approach is to look at other organisms that possess the cellulose and hemicellulose-converting ability, such as certain species of bacteria and genetically engineered yeast cells.

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APPENDICES

Appendix A: Hydrolysis

Appendix A.1: Ammonia treatment process

This process was carried out successfully with the extraction of glucose and xylose. Five experiments were reported detailing the quantities extracted. An average was calculated for the five experiments.

Table A1: Acid and enzymatic hydrolysate.

Sample	Acid hydrolysate (g/L)		Enzymatic hydrolysate (g/L)		Total fermentable sugars (g/L)
	Glucose	Xylose	Glucose	Xylose	
1	0.58	22.12	0.42	0.00	23.12
2	0.55	24.17	0.37	0.00	25.08
3	1.09	15.39	0.29	0.00	16.77
4	1.23	16.91	0.29	0.00	18.42
5	0.56	23.12	0.24	0.00	23.93
Average	**0.80**	**20.34**	**0.32**	**0.00**	**21.46**

Appendix A.2: Calculation of glucose and xylose concentration in the mixture of acid and enzymatic hydrolysate

Glucose from acid hydrolysate:

$$C_{1a}V_{1a} = C_{2a}V_{2a}$$
$$(0.8g/l)(0.5g/l) = C_{2a}(1l)$$
$$C_{2a} = 0.4g/l$$

Glucose from enzymatic hydrolysate:

$$C_{1e}V_{1e} = C_{2e}V_{2e}$$
$$(0.32g/l)(0.5g/l) = C_{2e}(1l)$$
$$C_{2e} = 0.16g/l$$

Total glucose in mixture of acid and enzymatic hydrolysates:

$C_{2a} + C_{2e}$
$0.4g/l + 0.16g/l = 0.56g/l$

Xylose is absent in the enzymatic hydrolysate therefore **total xylose in the mixture** is based only on the xylose available in the acid hydrolysate:

$C_1V_1 = C_2V_2$
$(20.34g/l)(0.5g/l) = C_2(1l)$
$C_2 = 10.17g/l$

Appendix A.3: Concentrated sulphuric acid hydrolysate

The results for five experiments were reported and presented in Table A1. An average was taken for the five experiments. The ammonia treatment process has a 6.96 g/L higher concentration of fermentable sugars and will therefore be used fermentation experiments.

Table A2: Hexose and pentose sugar concentration.

Sample	Glucose (g/L)	Xylose (g/L)	Total fermentable sugars (g/L)
1	4.7	10.4	15.1
2	4.7	10.5	15.2
3	4.7	10.5	15.2
4	5.8	8.3	14.1
5	3.2	9.7	12.9
Average	**4.6**	**9.9**	**14.5**

Appendix A.4: Calculating severity of pre-treatment methods

It is well known that more severe conditions during pre-treatment will cause greater degradation of hemicellulose sugars and enhance the enzymatic digestibility of

cellulose, however, both is not achieved at the same severity (Galbe & Zacchi 2007). The severity correlation describes the severity of the pre-treatment as a function of treatment time (minutes) and temperature (˚C), T_{ref} = 100˚C. The severity can be calculated using Equation 1. When pre-treatment is performed under acidic conditions, the effect of pH needs to be taken into consideration by the combined severity which can be calculated using Equation 1 and 2. Calculating combined severity of 0.3 M HCl:

$$\text{Combined severity} = \log(\text{Ro}) = \log\left(t \times \exp\left(\frac{(T - T_{ref})}{14.75}\right)\right) - \text{pH}$$

$$= \log\left(60 \times \exp\left(\frac{121-100}{14.75}\right)\right) - \left(\log\left[H^+\right]\right)$$

$$= 2.396 - \log[0.3\text{M}]$$

$$= 2.396 - (-0.593)$$

$$= 2.919$$

A similar calculation can be performed for the other pre-treatments employed.

Table 4.1 gives a comparison of pre-treatment methods. The Ammonia treatment process is more severe than the Concentrated H_2SO_4 process.

Appendix B: Fermentation results

Appendix B.1: Free cell fermentation data

Appendix B.1.1 Fermentation of wheat bran hydrolysate by *P. stipitis*

Table B.1.1: Sugar utilisation and ethanol production.

	Concentration (g/L)			
Time (hours)	Biomass	Glucose	Xylose	Ethanol
0	0.0	5.2	6.9	0.0
6	0.0	5.5	6.9	0.0
9	0.1	5.6	6.9	0.0
14	0.2	5.4	6.9	0.1
24	0.8	5.2	5.7	0.1
30	1.0	4.9	5.1	0.0
33	1.0	4.8	5.0	0.0

Appendix B.1.2 Fermentation of wheat bran hydrolysate by *S. cerevisiae*

Table B.1.2: Sugar utilisation and ethanol production.

	Concentration (g/L)			
Time (hours)	Biomass	Glucose	Xylose	Ethanol
0	0	5.19	6.81	0
6	0.03	4.5	6.81	0.8
9	0.08	3.2	6.79	0.79
14	0.2	0.3	5.5	1
24	0.48	0	5	1.5
30	0.8	0	4.8	1.69
35	1.1	0	4.6	1.71
38	1.1	0	4.55	1.70

Appendix B.1.3 Fermentation of wheat bran hydrolysate by *S. cerevisiae* and *P. stipitis*

Table B.1.3: Sugar utilisation and ethanol production.

	Concentration (g/L)			
Time (hours)	**Biomass**	**Glucose**	**Xylose**	**Ethanol**
0	0	5.19	6.63	0
6	0.03	4.89	6.62	0.5
9	0.1	3.9	6.43	0.51
14	0.13	2.89	6.2	0.8
24	0.6	1	5.5	1.1
30	0.75	0	4.5	1.22
33	0.75	0	4.4	1.20

Appendix B.1.4 Free cell fermentation of corn cob hydrolysate by mixed yeast culture

Table B.1.4 and B.1.5 highlights the glucose and xylose consumption and ethanol production during free cell fermentation. The highest ethanol production of 4.5 g/L took place after 29 hours of fermentation with an initial glucose and xylose concentration of 1.0 and 32.2 g/L, respectively.

Table B.1.4: Biomass concentration with time.

	Concentration (g/L)			
Time (hours)	**Biomass**	**Glucose**	**Xylose**	**Ethanol**
0	0.157	1.89	7.92	0.95
1	0.157	1.35	7.60	1.11
3	0.180	0	7.37	1.80
5	0.198	0	6.69	1.87
7	0.223	0	5.88	1.71
9	0.258	0	5.16	1.72
21	0.275	0	1.66	1.96
23	0.279	0	1.72	2.10
25	0.291	0	1.08	1.91
27	0.302	0	1.08	1.91
29	0.297	0	1.21	1.59
31	0.306	0	0.85	1.41
33	0.314	0	1.25	0.71
45	0.318	0	0.80	0.34

Table B.1.5: Sugar consumption and ethanol production.

Sample			**Time taken**		**Time elapsed (hours)**		**Glucose (g/L)**			**Xylose (g/L)**			**Ethanol (g/L)**	
			a	b	c	**Average**	a	b	c	**Average**	a	b	c	**Average**
Day 1-1	10.50	0.00	0.748	0.996	1.102	**1.049**	31.972	32.214	32.345	**32.177**	1.614	1.561	1.483	**1.522**
2	11.50	1.00	0.467	0.506	0.447	**0.473**	27.858	28.060	28.116	**28.011**	2.814	2.462	2.452	**2.576**
3	13.50	3.00	0	0	0	**0**	24.777	34.928	28.270	**29.325**	2.035	2.747	2.172	**2.318**
4	14.50	4.00	0	0	0	**0**	27.194	26.016	24.479	**25.896**	3.049	3.179	2.374	**2.867**
5	16.50	6.00	0	0	0	**0**	23.197	27.427	23.371	**23.284**	14.157	2.829	2.890	**2.860**
6	18.00	7.50	0	0	0	**0**	26.577	26.179	26.227	**26.328**	2.780	2.728	2.698	**2.735**
Day2-1	7.50	19.50	0	0	0	**0**	23.275	24.274	24.056	**23.868**	3.462	3.437	3.367	**3.422**
2	9.00	21.00	0	0	0	**0**	36.127	22.399	23.578	**22.989**	10.679	3.175	3.483	**3.329**
3	11.00	23.00	0	0	0	**0**	21.883	23.242	22.915	**22.680**	3.863	3.712	3.686	**3.754**
4	13.00	25.00	0	0	0	**0**	22.286	22.802	21.986	**22.394**	3.540	3.701	3.982	**3.741**
5	15.00	27.00	0	0	0	**0**	16.623	18.887	17.757	**17.756**	4.458	4.163	4.197	**4.180**
6	17.00	29.00	0	0	0	**0**	15.756	17.477		**16.617**	4.583	4.366		**4.475**

Appendix B.2: Immobilised cell fermentation

Table B.2.1 and B.2.2 highlights the glucose and xylose consumption and ethanol production during free cell fermentation. After 29 hours of fermentation 4.2 g ethanol/L was produced having an initial glucose and xylose concentration of 2.8 and 16.4 g/L, respectively (total sugar concentration is 19.2). Ethanol production would have been 7.2 g/L if the total sugar concentration was 33.2 g/L as was the case in free cell fermentation hydrolysate.

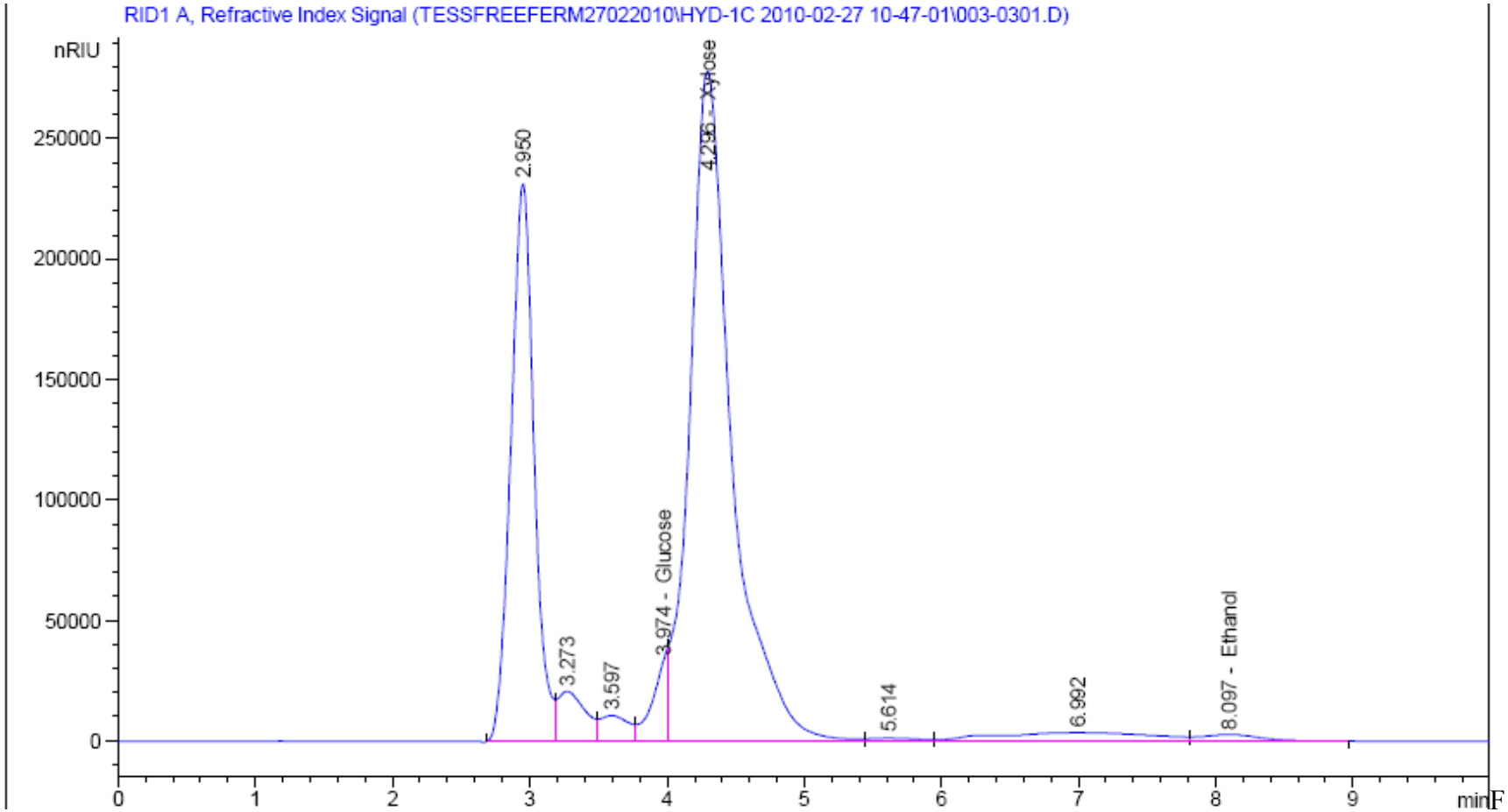
Table B.2.1: Biomass concentration with time.

	Concentration (g/L)			
Time (hours)	Cell biomass	Glucose	Xylose	Ethanol
0	0.001	2.81	16.42	0.00
2	0.001	2.52	16.19	0.00
4	0.001	2.57	15.66	0.00
6	0.070	2.27	15.18	0.00
8	0.100	1.61	14.99	0.83
10	0.130	1.42	15.93	1.01
11	0.160	1.00	15.77	1.38
23	0.170	0.00	9.16	3.62
25	0.190	0.00	8.06	3.61
26	0.240	0.00	7.84	4.03
27	0.240	0.00	7.39	4.10
28	0.240	0.00	7.11	4.27
29	0.240	0.00	6.49	4.18

Table B.2.2: Immobilised cell fermentation ethanol production.

Sample	Time taken	Time elapsed (hours)	Glucose (g/L)				Xylose (g/L)				Ethanol (g/L)			
			a	b	c	**Average**	a	b	c	**Average**	a	b	c	**Average**
Day1-1	7.45	0.00	2.762	2.795	2.861	**2.812**	16.175	16.533	16.559	**16.422**	0	0	0	**0**
2	9.45	2.00	2.758	2.758	2.285	**2.522**	15.922	15.903	16.752	**16.192**	0	0	0	**0**
3	11.45	4.00	2.739	2.532	2.611	**2.572**	15.822	15.646	15.518	**15.662**	0	0	0	**0**
4	13.45	6.00	2.172	2.153	2.476	**2.267**	15.318	14.226	15.983	**15.176**	0	0	0	**0**
5	15.45	8.00	0.758	1.999	2.072	**1.610**	13.602	15.689	15.690	**14.994**	0.970	0.730	0.802	**0.834**
6	17.45	10.00	0.594	1.443	1.397	**1.420**	15.592	16.061	16.126	**15.926**	1.072	0.952	0.995	**1.006**
7	19.00	11.25	0	1.102	0.901	**1.002**	15.380	16.057	15.864	**15.767**	1.765	1.087	1.282	**1.378**
Day2-1	7.00	23.25	0	0	0	**0**	9.439	9.339	8.695	**9.158**	3.688	3.546	3.564	**3.617**
2	9.00	25.25	0	0	0	**0**	8.271	8.089	8.032	**8.061**	3.548	3.487	3.725	**3.606**
3	10.00	26.25	0	0	0	**0**	8.009	8.037	7.477	**7.841**	4.121	4.071	3.897	**4.030**
4	11.00	27.25	0	0	0	**0**	7.610	7.675	7.111	**7.393**	4.099	4.013	3.832	**4.099**
5	12.00	28.25	0	0	0	**0**	7.283	7.301	6.751	**7.112**	4.231	4.318	4.119	**4.275**
6	12.45	29.00	0	0	0	**0**	6.457	6.590	6.413	**6.487**	4.176	4.032	4.025	**4.176**

Appendix B.3: Sample and HPLC column conditions



igure B1: HPLC chromatogram of two carbohydrate standards, glucose and xylose and ethanol.

Fermentation media and hydrolysate samples were filtered using a 0.45 micron filter paper. The chromatogram in Figure B1 shows the peaks for glucose, xylose and ethanol. Prior to analysis of the fermentation medic and hydrolysate by HPLC, the system was purged for five minutes with 0.001 M H_2SO_4 before running standards. The column operations were run as stated in Table 3.

Appendix B.4: Bioethanol production

The ethanol produced using the same initial fermentable sugar concentration was determined using proportions.

B.4.1 Free yeast cell system

$33.2\ \text{g/L fermentable sugars} \rightarrow 4.5\ \text{g/L ethanol}$

$21.1\ \text{g/L fermentable sugars} \rightarrow 2.9\ \text{g/L ethanol}$

Calculating volume of ethanol produced from 136 g corn cob using free cell fermentation:

$2.9\text{g ethanol/L is produced from } 1.435\ \text{L acid hydrolysate}$

$M_{ethanol} = 4.1615\ \text{g}$

$\rho_{ethanol} = 0.789\ \text{g/ml}$

$V_{ethanol} = 5.3\ \text{ml}$

B.4.2 Immobilised yeast cell system

$19.2\ \text{g/L fermentable sugars} \rightarrow 4.6\ \text{g/L ethanol}$

$21.1\ \text{g/L fermentable sugars} \rightarrow 5.1\ \text{g/L ethanol}$

Calculating volume of ethanol produced from 136 g corn cob using immobilised cell fermentation:

$5.1\ \text{g ethanol/L is produced from } 1.435\ \text{L acid hydrolysate}$

$M_{ethanol} = 7.3185\ \text{g}$

$\rho_{ethanol} = 0.789\ \text{g/ml}$

$V_{ethanol} = 9.3\ \text{ml}$

Appendix B.5: Balancing ATP and redox reactions in metabolic pathways of sugars

The metabolic pathway of glucose to ethanol by eukaryotic yeast cells:

(1a) $2\,NAD^+ + 4\,H \rightarrow 2\,NADH + 2\,H^+$

(1b) $NADH + H^+ \rightarrow NAD^+ + 2\,H$

Overallreaction(1):

$NAD^+ + 2\,H \rightarrow NADH + H^+$

Overallreaction(2):

$2\,ADP + 2\,PO_4^{3-} + \text{nutrients} \rightarrow 2\,ATP + 2\,H_2O$

The metabolic pathway of xylose to ethanol by eukaryotic yeast cells:

(1a) $NADPH + H^+ \rightarrow NADP^+ + 2\,H$

(1b) $NADH + H^+ \rightarrow NAD^+ + 2\,H$

(1c) $NAD^+ + 2\,H \rightarrow NADH + H^+$

(1d) $2\,NAD^+ + 4\,H \rightarrow 2\,NADH + 2\,H^+$

(1e) $NADP^+ + 2\,H \rightarrow NADPH + H^+$

(1f) $NADH + H^+ \rightarrow NAD^+ + 2\,H$

Overall reaction (1):

$NAD^+ + 2\,H \rightarrow NADH + H^+$

(2a) $ATP + H_2O \rightarrow ADP + PO_4^{3-} + \text{energy}$

(2b) $2\,ADP + 2\,PO_4^{3-} + \text{nutrients} \rightarrow 2\,ATP + 2\,H_2O$

Overall reaction (2):

$ADP + PO_4^{3-} + \text{nutrients} \rightarrow ATP + H_2O$

The metabolic pathway of glucose is more efficient at utilising the nutrient sugars and producing ATP than the metabolic pathway of xylose.

Appendix B.6: Percentage yield calculated stoichiometrically

The theoretical yield reached by the two hydrolysis methods, Ammonia treatment and concentrated H_2SO_4 were calculated stoichiometrically and compared to each other. This was calculated using Equation 3a and Equation 3b.

Calculating theoretical yield of ethanol using Ammonia treatment hydrolysate:

$$2\times\left(\frac{\mathrm{m_{glucose}}}{\mathrm{M_{glucose}}}\right)\times\mathrm{M_{ethanol}}+1.67\times\left(\frac{\mathrm{m_{xylose}}}{\mathrm{M_{xylose}}}\right)\times\mathrm{M_{ethanol}}$$

$$=2\times\left(\frac{0.8\mathrm{g}}{180\mathrm{g/mol}}\right)\times 46+1.67\times\left(\frac{20.3}{150\mathrm{g/mol}}\right)$$

$$=10.8\,\mathrm{g/L}$$

All the results together with theoretical yield of concentrated H_2SO_4 using Equations 3a and 3b are presented in Table B3.

Table B.6.1: Theoretical yield of ethanol.

	Ammonia treatment	**Concentrated H_2SO_4**
Glucose (g/L)	0.8	4.6
Theoretical yield of ethanol from glucose (g/L)	0.4	2.4
Xylose (g/L)	20.3	9.9
Theoretical yield of ethanol from xylose (g/L)	10.4	5.1
Total sugars (g/L)	**21.1**	**14.5**
Theoretical yield of ethanol from total sugars (g/L)	**10.8**	**7.5**

A comparison of the actual to the theoretical yield was carried out and presented in Table B.6.2. The overall percentage yield of an immobilised yeast cell system is 20.2 % greater than a free yeast cell system. This experiment was therefore successful and an immobilised yeast cell was preferred.

Table B.6.2: Actual versus the theoretical yield calculated from stoichiometry.

	Actual grams ethanol/L	**Theoretical grams ethanol/L**	**% Yield**
Free yeast cell system	2.9	10.8	**27.1**
Immobilised yeast cell system	5.1	10.8	**47.3**

Appendix C: Fermentation Kinetics

Appendix C.1 Experimental yield coefficients

Yields were calculated from experimental results with $Y_{X/S} = -\frac{dX}{dS} = \frac{X_2 - X_1}{S_1 - S_2}$. $Y_{X/S}$ is dependant on biomass and substrate concentration. $Y_{P/X}$ calculated in a similar fashion is dependant on product and biomass concentration and $Y_{P/S}$ is dependant on product and substrate concentration.

Appendix C.2 Experimental data modelled by Monod kinetics

The Monod equation, equation 10a, was modified to include the death rate constant k_d resulted in equation 12b. The average error and variance is calculated by:

$$\text{Average error (AE)} = \frac{1}{N}\sum_{i=1}^{N} E_i$$

$$\text{Variance (V)} = \frac{1}{N}\sum_{i=1}^{N} (E_i - AE)^2$$

where E_i is the error at a point in time.

Appendix C.2.1 Free cell fermentation linearised modelling

The Monod equation was linearised, equation 13, in order to determine an approximate value of μ_{max} and K_s. Octave was used to integrate equation 12a and plot biomass concentration (g/L) versus time (hours). This was helpful fine tuning the other parameters when finding the best fit.

Table C.2.1: Data for linearisation of free cell fermentation data

Time	X	S	dX/dt	Ydata
1	0.1572	8.947167	0.01122	125.356
3	0.17964	7.367667	0.00912	145.1236
5	0.19788	6.691	0.01238	106.9479
7	0.22264	5.882	0.01788	73.24209

23	0.27864	1.722	0.006	79.96968
25	0.29064	1.08	0.00564	55.65447
29	0.29652	1.208333	0.00474	75.58966
31	0.306	0.848	0.00402	64.54925
45	0.31776	0.796	0.00676	37.41671

The values calculated from the slope, $\frac{1}{\mu_{max}}$, and intercept, $\frac{K_s}{\mu_{max}}$, are

μ_{max} = 0.113029 h^{-1} and K_s= 5.754976 g/L. Programming was done using Octave, these values and k_d were then fine tuned and are presented below.

The following mathematical program was used:

```
% Experimental

te = [0 1 3 5 7 9 21 23 25 27 29 31 33 45 47 49 51]';

Se = [33.226 28.484667 29.325 25.89633 22.9885 22.68 22.394 17.755667 16.6165]';

Xe = [0.1572 0.1572 0.17964 0.19788 0.22264 0.2584 0.2748 0.27864 0.29064
0.30192 0.29652 0.306 0.31404 0.31776 0.33128 0.3336 0.33448]';

Pe = [0 1.107 1.796 1.8703 1.7133 1.7153 1.9583 2.096 1.9077 1.9077 1.593 1.4053
0.7135 0.3357]';


%%

Yxs = (-(0.29652-0.1572)./(16.6165-33.226))

Yxp = (-(0.1572-0.27864)./(2.096 - 0))

S0 = Se(1);

P0 = Pe(1);

X0 = Xe(1);
```

```
%% Guess

umax = 0.113029

Ks = 5.754976


%% Integrate

tspan = linspace(0, 60, 10000);

dXdt = @(X, t)(((umax.*(Yxs.*S0 + X0 - X(1))./(Ks.*Yxs + Yxs.*S0 + X0 - X(1))).*X(1));

Xi = lsode(dXdt, X0, tspan);


%% Plot

plot(te, Xe, 'xk', tspan, Xi, 'k');

ylabel('Cell Concentration [g/L]')

 xlabel('Time [hr]')

print 'JustMonod_free_cell.jpg'


% DETERMINE THE ERROR

Xi2 = lsode (dXdt, X0, te);


ARE = (abs(Xi2(:,1) - Xe))./(Xi2(:,1));


disp('The average absolute error is:')

AARE = sum(ARE(2:17))/length(ARE(2:17))

AE=0.26283
```

```
% DETERMINE THE VARIANCE

disp('The variance in absolute error is:')

VARE = (sum((ARE - AARE).^2))./length(ARE(2:17))

V=0.0094434
```

Appendix C.2.2 Free cell fermentation best fit modelling with death rate

The following mathematical program was used:

```
% Experimental

te = [0 1 3 5 7 9 21 23 25 27 29 31 33 45 47 49 51]';

Se = [33.226 28.484667 29.325 25.89633 22.9885 22.68 22.394 17.755667 16.6165]';

Xe = [0.1572 0.1572 0.17964 0.19788 0.22264 0.2584 0.2748 0.27864 0.29064
0.30192 0.29652 0.306 0.31404 0.31776 0.33128 0.3336 0.33448]';

Pe = [0 1.107 1.796 1.8703 1.7133 1.7153 1.9583 2.096 1.9077 1.9077 1.593 1.4053
0.7135 0.3357]';


%%

Yxs = (-(0.29652-0.1572)./(16.6165-33.226))

Yxp = (-(0.1572-0.27864)./(2.096 - 0))

S0 = Se(1);

P0 = Pe(1);

X0 = Xe(1);


%% Guess

umax = 0.1135
```

```
Ks = 5.76

Kd = 0.0684


%% Integrate

tspan = linspace(0, 60, 10000);

dXdt = @(X, t)(((umax.*(Yxs.*S0 + X0 - X(1)))./(Ks.*Yxs + Yxs.*S0 + X0 -
X(1))).*X(1)) - (Kd).*X(1);

Xi = lsode(dXdt, X0, tspan);


%% Plot

plot(te, Xe, 'xk', tspan, Xi, 'k');

ylabel('Cell Concentration [g/L]')

 xlabel('Time [hr]')

print 'Monod_free_cell.jpg'


% DETERMINE THE ERROR

Xi2 = lsode (dXdt, X0, te);

ARE = (abs(Xi2(:,1) - Xe))./(Xi2(:,1));


disp('The average absolute error is:')

AARE = sum(ARE(2:17))/length(ARE(2:17))

AE=0.066877
```

% VARIANCE

disp('The variance in absolute error is:')

VARE = (sum((ARE - AARE).^2))./length(ARE(2:17))

V=0.0050698

Appendix C.2.3 Immobilised cell fermentation modelling using linearisation

Octave was used to integrate equation 13 and plot biomass concentration (g/L) versus time (hours). The Monod equation was linearised in order to determine an approximate value of μ_{max} and K_s. This was helpful when guessing k_d and fine tuning the other parameters when finding the best fit.

Table C.2.2: Data for linearisation of free cell fermentation data

Time	X	S	dX/dt	Ydata
6	0.07	17.44267	0.015	81.39911
8	0.1	16.60333	0.015	110.6889
23	0.17	9.157667	0.01	155.6803

The values calculated from the slope, $\frac{1}{\mu_{max}}$, and intercept, $\frac{K_s}{\mu_{max}}$, are

μ_{max} = -0.12798 h^{-1} and K_s= -29.2379 g/L. Programming was done using Octave and is presented below. The Monod model was modified to accommodate the negative μ_{max} and K_s and subsequently fit the experimental data to the theoretical with an error of 17.559.

The following mathematical program was used:

% Experimental

te = [4 6 8 10 11 26 27 28 29]'; % took out dead time

```
Se = [19.23 17.44 16.60 17.35 16.77 7.84 7.39 7.11 6.49]';

Xe = [0.001 0.070 0.100 0.130 0.160 0.240 0.240 0.240 0.240]';

Pe = [0.00 0.00 0.83 1.01 1.38 4.03 4.10 4.27 4.18]';


%%

Yxs = (-(0.24-0.001)./(6.49-19.23))

Yxp = (-(0.001-0.24)./(4.18 - 0))

S0 = Se(1);

P0 = Pe(1);

X0 = Xe(1);


%% Guess

umax = 0.12798                  %-0.081

Ks = 29.2379            % modified down from -29


%% Integrate

tspan = linspace(4, 35, 10000);

dXdt = @(X, t)(((-umax.*(Yxs.*S0 + X0 - X(1)))./((Yxs.*S0 + X0 - X(1)) -
Ks.*Yxs)).*X(1));

Xi = lsode(dXdt, X0, tspan);


%% Plot

plot(te, Xe, 'xk', tspan, Xi, 'k');

ylabel('Cell Concentration [g/L]')
```

```
 xlabel('Time [hr]')
print 'JustMonod_immobilised.jpg'

% DETERMINE THE ERROR
Xi2 = lsode (dXdt, X0, te);

ARE = (abs(Xi2(:,1) - Xe))./(Xi2(:,1));

disp('The average absolute error is:')
AARE = sum(ARE(1:9))/length(ARE(1:9))
AE=15.608

% VARIANCE
disp('The variance in absolute error is:')
VARE = (sum((ARE - AARE).^2))./length(ARE(1:9)
V=283.81
```

Appendix C.2.4 Immobilised cell fermentation modelling for a best fit with death rate

The following mathematical program was used:

```
% Experimental
te = [4 6 8 10 11 26 27 28 29]'; % took out dead time
Se = [19.23 17.44 16.60 17.35 16.77 7.84 7.39 7.11 6.49]';
```

```
Xe = [0.001 0.070 0.100 0.130 0.160 0.240 0.240 0.240 0.240]';

Pe = [0.00 0.00 0.83 1.01 1.38 4.03 4.10 4.27 4.18]';


%%

Yxs = (-(0.24-0.001)./(6.49-19.23))

Yxp = (-(0.001-0.24)./(4.18 - 0))

S0 = Se(1);

P0 = Pe(1);

X0 = Xe(1);


%% Guess

umax = 0.125            %-0.081

Ks = 19.75              % modified down from -29

Kd = 0.058              %0.028


%% Integrate

tspan = linspace(4, 35, 10000);

dXdt = @(X, t)(((-umax.*(Yxs.*S0 + X0 - X(1)))./((Yxs.*S0 + X0 - X(1)) -
Ks.*Yxs)).*X(1)) - (Kd).*X(1);

Xi = lsode(dXdt, X0, tspan);


%% Plot

plot(te, Xe, 'xk', tspan, Xi, 'k');

ylabel('Cell Concentration [g/L]')
```

```
 xlabel('Time [hr]')
print 'Monod_immobilised.jpg'

% DETERMINE THE ERROR
Xi2 = lsode (dXdt, X0, te);

ARE = (abs(Xi2(:,1) - Xe))./(Xi2(:,1));

disp('The average absolute error is:')
AARE = sum(ARE(1:9))/length(ARE(1:9))
AE=0.064409

% VARIANCE
disp('The variance in absolute error is:')
VARE = (sum((ARE - AARE).^2))./length(ARE(1:9))
V=0.017963
```