Optimization of maize starch fermentation by *Saccharomyces cerevisiae* using pervaporation

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DECLARATION

I, Sinethemba Aubrey Nongauza, the undersigned, hereby declare that this dissertation titled, **Optimization of maize starch fermentation by *Saccharomyces cerevisiae* using pervaporation,** is my own work.

_____

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Potchefstroom

May 2010

ABSTRACT

Due to the depletion of petroleum reserves and environmental concerns, bioethanol has been identified as an alternative fuel to petrol. Bioethanol is a fuel of bio-origin derived from renewable biomass. Starch and sugar containing materials are the primary sources of carbon for bioethanol production. Starch is firstly hydrolysed into simple sugars which are later fermented to bioethanol using *Saccharomyces cerevisiae* (*S. cerevisiae*). The fermentation of sugars to bioethanol is however limited by inhibition of *S. cerevisiae* by the major product of the process, bioethanol. The challenge is thus in keeping the bioethanol concentration at levels which are not harmful to the fermenting organism. Keeping bioethanol concentration low in the broth will provide a suitable environment for yeast to grow and thus increase the overall production. Currently bioethanol producers use high water dilution rates to keep the bioethanol concentrations in the broth low enough so that yeast is not harmed. This excess water has to be removed in the downstream process, which is expensive. The use of excessive amounts of water in the fermentation can be avoided by continual removal of bioethanol from the broth.

During this investigation the experimental conditions for the hydrolysis process were determined. A pH of 5.5 was determined as the best pH for Termamyl SC at 95°C with a pH of 5.0 for Spirizyme Fuel at 55°C during the liquefaction and the saccharification step, respectively. During the fermentation process the influence of yeast concentration on bioethanol production was investigated by varying the yeast concentration between 2 $g.L^{-1}$ and 7 $g.L^{-1}$. A yeast concentration of 5 $g.L^{-1}$ produced the highest bioethanol yield of 0.48 $g.g^{-1}$ after 48 hours of fermentation using *S. cerevisiae*. Later during the investigation a coupled fermentation/pervaporation system was employed in a batch system for continual removal of bioethanol in the fermentation broth in a process called simultaneous fermentation and separation (SFS). Through the continuous removal of bioethanol from the fermentation broth, the bioethanol concentration in the broth was kept low enough so that it was not harmful to the fermenting organism but the overall fermentation yield was not improved. Pervaporation is a membrane separation process used to separate azeotropic mixtures such as bioethanol and water. It is highly efficient, cost effective and uses less energy than distillation. During the SFS process a bioethanol yield of 0.22 $g.g^{-1}$ was obtained. The SFS

process yield for bioethanol was low compared to 0.45 $g.g^{-1}$ of the traditional batch fermentation process. The lower overall bioethanol yield obtained in the SFS process could be attributed to only the supernatant being used in the SFS process and not the entire fermentation broth as in the traditional process. The results from this study proved that the SFS process was less efficient compared to the traditional batch fermentation process with respect to the bioethanol yield, but that the fermentation could be carried out without the necessity for additional process water.

Keywords: Hydrolysis, Fermentation, Bioethanol, Inhibition, Simultaneous fermentation and separation.

OPSOMMING

As gevolg van die uitputting van petroleumreserwes en omgewingsbesorgdhede is bio-etanol geïdentifiseer as 'n alternatiewe brandstof vir petrol. Bio-etanol is ‘n brandstof van bio-oorsprong, verkry vanaf hernubare biomassa. Stysel- en suikerbevattende materiale is die primêre bronne van koolstof vir bio-etanolproduksie. Stysel word eers gehidroliseer tot eenvoudige suikers wat later gefermenteer word tot bio-etanol deur gebruik te maak van *Saccharomyces cerevisiae* (*S. cerevisiae*). Die fermentering van suikers tot bio-etanol word egter beperk deur die inhibering van *S. cerevisiae* deur die hoofproduk van die proses, bio-etanol. Die uitdaging is dus om die bio-etanolkonsentrasie op vlakke te hou wat nie skadelik is vir die fermenteringsorganisme nie. Deur die bio-etanolkonsentrasie in die reaksiemengsel laag te hou, word ’n geskikte omgewing geskep vir gis om te groei en sodoende word die totale produksie verhoog. Tans gebruik bio-etanolproduseerders hoë waterverdunningstempo’s om die bio-etanolkonsentrasies in die reaksiemengsel laag genoeg te hou sodat die gis nie benadeel word nie. Die oormaat water moet in die afstroomproses verwyder word, wat duur is. Die gebruik van oormatige hoeveelhede water in die fermentering kan vermy word deur die voortdurende verwydering van bio-etanol uit die reaksiemengsel.

In hierdie ondersoek is die eksperimentele kondisies vir die hidroliseproses vasgestel. ‘n pH van 5.5 is bepaal as die beste pH vir Termamyl SC by 95°C en ‘n pH van 5.0 vir Spirizyme Fuel by 55°C gedurende die vervloeiing- en die versuikeringstappe, respektiewelik. Gedurende die fermenteringsproses is die invloed van giskonsentrasie op bio-etanolproduksie ondersoek deur die giskonsentrasie te wissel tussen 2 $g.L^{-1}$ en 7 $g.L^{-1}$. ‘n Giskonsentrasie van 5 $g.L^{-1}$ het die hoogste bio-etanol opbrengs van 0.48 $g.g^{-1}$ gelewer na 48 uur fermentering deur gebruik te maak van *S. cerevisiae*. Later gedurende die ondersoek is ’n gekoppelde gisting-/pervaporasiesisteem gebruik in ‘n lotsisteem vir kontinue verwydering van bio-etanol uit die fermenteringsreaksiemengsel in ‘n proses bekend as gelyktydige fermentering en skeiding (GFS). Deur die kontinue verwydering van bio-etanol uit die fermenteringsreaksiemengsel is die bio-etanolkonsentrasie in die reaksiemengsel laag genoeg gehou sodat dit nie skadelik vir die fermenteringsorganisme was nie, maar die totale fermenteringsopbrengs het nie verbeter nie. Pervaporasie is ‘n membraan-skeidingsproses

wat gebruik word om azeotroop-mengsels soos bio-ethanol en water te skei. Dit is hoogs doeltreffend, koste-effektief en gebruik minder energie as distillasie. Met die GFS-proses is ’n bio-etanolopbrengs van 0.22 $g.g^{-1}$ verkry. Die GFS-proses-opbrengs vir bio-etanol was laag in vergelyking met die 0.45 $g.g^{-1}$ van die tradisionele lot-fermenteringsproses. Die laer bio-etanol opbrengs met die GFS proses kan toe geskryf word aan die feit dat slegs die filtraat van die fermentasie mengsel gebruik is in die GFS proses en nie die hele mengsel soos in die geval van die tradisionele proses nie. Die resultate van hierdie studie bewys dat die GFS-proses minder doeltreffend was in vergelyking met die tradisionele lot-fermenteringsproses met betrekking tot die bio-etanol-opbrengs, maar dat die proses uitgevoer kan word sonder die toevoeging van addisionele proses water.

Sleutelwoorde: Hidrolise, Fermentering, Bio-etanol, Inhibering, Gelyktydige fermentering en skeiding.

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NOMENCLATURE

C_c	Cell concentration	$(g.L^{-1})$
C_f	Solute concentration in the feed	$(g.L^{-1})$
C_g	Glucose concentration	$(g.L^{-1})$
C_p	Solute concentration in the permeate	$(g.L^{-1})$
K_p	Specific rate constant with respect to product	$(L.g^{-1}.s^{-1})$
K_{sn}	Monod constant	$(g.L^{-1})$
r_p	Ethanol production rate	$(g.L^{-1}.s^{-1})$
W	Weight of starting material	(g)
X	Bioethanol mass fraction	(wt%)
X_A	Concentration of component A in feed	$(g.L^{-1})$
X_B	Concentration of component B in feed	$(g.L^{-1})$
Y_A	Concentration of component A in permeate	$(g.L^{-1})$
Y_B	Concentration of component B in permeate	$(g.L^{-1})$
$\gamma_{cell/glucose}$	Mass of new cells per mass of substrate consumed	$(g.g^{-1})$
$\gamma_{CO2/glucose}$	CO_2 yield per gram of substrate	$(g.g^{-1})$
$\gamma_{EtOH/glucose}$	Bioethanol yield per substrate	$(g.g^{-1})$
$\gamma_{Glucose/Starch}$	Glucose yield per substrate	$(g.g^{-1})$

Subscripts

D	Dioxane
E	Ethanol

Abbreviations

ADP	Adenosine Diphosphate
AGU	Amyloglucosidase Unit
Al^{3+}	Aluminium Ion
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
C_2H_2OH	Ethanol
$C_6H_{12}O_6$	Glucose
Ca^{2+}	Calcium ion
CO_2	Carbon Dioxide
Cr^{3+}	Chromium (III) Ion
CS	Cellulose Sulphate
CVDP	Chemical Vapour Deposition and Polymerization
DE	Dextrose Equivalent
DMC	Dimethyl Carbonate
EFB	Empty Fruit Batch
GFT	Gesellschaft Für Trenntechnik
H_2O	Water

KNU	Kilo Novo Unit
Mg^{2+}	Magnesium Ion
MTBE	Methyl *tert*-Butyl Ether
NAD	Nicotinamide Adenine Dinucleotide
NH_2	Amide Group
NH_3^+	Ammonium Ion
NO	Nitrogen Monoxides
O_2	Oxygen
PAA	Poly(acrylic acid)
PAN	Polyacrylonite
PB	Polybutadiene
PDMS	Polydimethyl Siloxane
PEBA	Polyether-Block-Polymide
PEI	Polyethyleneimine
PTFE	Polytetraflouro-ethylene
PTMSP	Poly [1-(trimethylsilyl)-1-propyne]
PVA	Polyvinyl Alcohol
R	Retention
SEM	Scanning Electron Microscopy
SFS	Simultaneous Fermentation and Separation
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation

STDEV	Standard Deviation
USA	United States of America
VOC	Volatile Organic Compound
Zn^{2+}	Zinc Ion

CHAPTER 1

GENERAL INTRODUCTION

"The journey of a thousand miles begins with a single step"

Lao Tzu

"The most important thing about goals is having one"

Geoffrey F. Abert

OVERVIEW

This chapter gives a brief introduction of the study. The background information concerning the study and the motivating factors for conducting the investigation are given in Section 1.1. The objective of the study is described in Section 1.2. The scope of the study is presented in Section 1.3, followed by the scope of the dissertation in Section 1.4.

1.1 Background and Motivation

For centuries people from all over the world have been using fossil fuels to produce energy. The use of fossil fuels has however been associated with pollution (Shafaghat *et al*., 2009). During energy extraction from fossil fuels, a huge amount of harmful gases is released into the atmosphere. Such gases include carbon dioxide, carbon monoxide, nitrogen oxide and sulphur dioxide (Barnwal and Sharma, 2005; Hahn-Hägerdal *et al.*, 2006; Demirbas, 2007; Kaminski *et al.*, 2008). In addition, it has also been reported that fossil fuel reserves are depleting (Mielenz, 2001; Najafpour *et al.*, 2005; Shen *et al.*, 2008; Bai *et al.*, 2008). The depletion of fossil fuel reserves and their negative effect on the environment have led to the search for alternative fuels (Mielenz, 2001; Zaldivar *et al.*, 2005; Demirbas, 2005; Hahn-Hägerdal *et al.*, 2006; Shafaghat *et al*., 2009). Renewable energies were identified as potential alternatives to fossil fuels for energy generation.

Fuels of bio-origin, produced from renewable biomass have been identified as possible alternatives to fossil fuels for both domestic and transportation requirements. Examples of such fuels include alcohols, vegetable oils and biogas. In the transportation sector bioethanol which is an alcohol and biodiesel made from vegetable oils were identified as possible alternatives to petrol and diesel respectively (Barnwal and Sharma, 2005; Hahn-Hägerdal *et al.*, 2006; Öhgren *et al.*, 2006; Bai *et al.*, 2008). The production of bioethanol was investigated in this study.

Bioethanol can be produced from a variety of biomass that contain cellulose, starch and sugar (Demirbas, 2005; Dawson and Boopathy, 2008). Maize, cassava, potatoes and wheat are examples of starch biomass (Moore *et al.*, 2005; Jamai *et al.*, 2007; Mohammad and Keikhosro, 2008, Ocloo and Ayenor, 2008). Recently, starch has gained recognition in the fuel industry as a raw material for bioethanol production (Öhgren *et al.*, 2006). Starch is considered to be a clean, non-toxic source of carbon for bioethanol production (Moore *et al.*, 2005; Kunamneni and Singh, 2005; Chen *et al*., 2008).

From the different sources of starch, maize starch, the primary source for bioethanol production in the United States of America (USA) (Mielenz, 2001; Torney *et al.*, 2007), occurs as either amylose (see Figure 1.1) or amylopectin (see Figure 1.2) (Mojović *et al.*, 2006; Torney *et al.*, 2007).

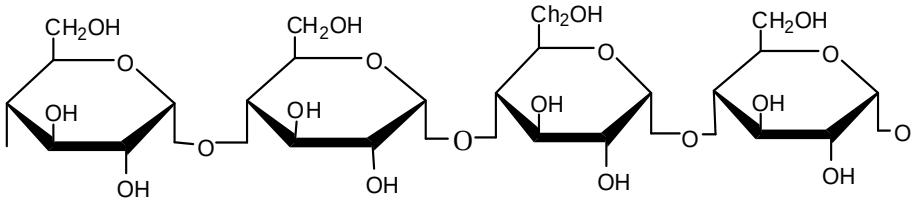

Figure 1.1: Linear form of starch (amylose)

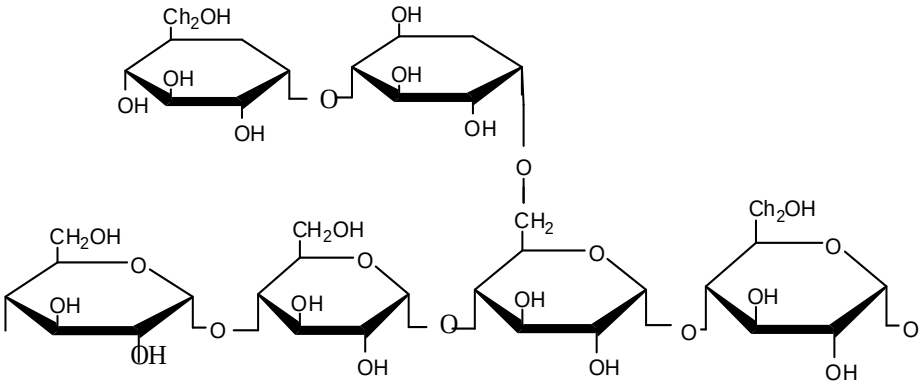

Figure 1.2: Complex form of starch (amylopectin)

Amylose is the linear form of starch, formed by glucose molecules joined together by α-D-(1-4) glycosidic linkages. Amylopectin, a major form of starch, is composed of α-D-(1-6) glycosidic linkages in addition to the α-D-(1-4) glycosidic linkages, occurring in every 20–30 units of glucose molecules of amylose (Mathews *et al.*, 2000; Torney *et al.*, 2007).

There are two feasible methods of producing bioethanol from maize, viz. through the dry milling or the wet milling process (Sanchez and Cardona, 2008; Gnansounou, 2009). The dry milling process is the most commonly employed technique in the United States, accounting for almost 80% of the production (Kim *et al.*, 2008a; Kim *et al.*, 2008b; Murthy *et al.*, 2009; Gnansounou, 2009). A simple schematic flow diagram of the unit processes involved in bioethanol production through the dry milling process is shown in Figure 1.3.

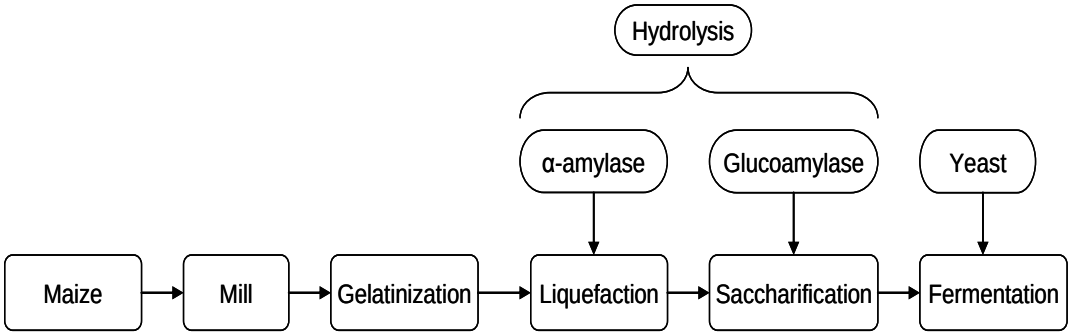

Figure 1.3: Bioprocessing steps of starch/maize to bioethanol through the dry milling process (Modified from Mohammad and Keikhosro, 2008)

The smallest particle size of the maize meal is recommended for the optimum penetration of water into the starch granules in preparation of starch for the hydrolysis process. As shown in Figure 1.3 the maize meal formed from the milling step is gelatinized, followed by hydrolysis and subsequently fermentation. Gelatinization is a process for dissolving starch into water, as well as the reduction of bacterial contamination/infection (Torney *et al.*, 2007; Mojović *et al.*, 2006; Franceschin *et al.*, 2008). The degradation of the two forms of starch is performed immediately after gelatinization during the hydrolysis process which is considered to be the most important step in bioethanol production. The hydrolysis process involves the breaking down of amylose and amylopectin into glucose. The glucose molecules produced during the hydrolysis process are further converted to bioethanol in the presence of the fermenting organism through a process called fermentation (Mojović *et al.*, 2006). Fermentation is the most commonly used method of producing bioethanol from sugars such as sucrose, glucose and fructose using *Saccharomyces cerevisiae* (*S. cerevisiae*) (Khaw *et al.*, 2007). According to Mojović *et al.* (2006) fermentation is responsible for 60% of the world's bioethanol production.

The major challenge of the fermentation process is the inhibition of *S. cerevisiae* by the major product of the fermentation process, namely bioethanol. The inhibition of *S. cerevisiae* affects the overall productivity of the fermentation process (Dombek and Ingram, 1986). As reported by Bai *et al.* (2008), the effect of bioethanol on *S. cerevisiae* is prominent at 9-12 wt% bioethanol. Another challenge is the reduction of nutrients in the fermentation broth as the fermentation process progresses. The nutrients are known to keep the enzymes active and

stable for a prolonged period. High temperatures, on the other hand, denature the structure of *S. cerevisiae*, thus resulting in a decrease in activity which eventually affects the overall productivity.

It has been reported that supplementing nutrients into the fermentation broth during the fermentation process has the potential of keeping *S. cerevisiae* stable and active for an extended time. However, the addition of such nutrients does not improve the overall output of the traditional batch fermentation process (Dombek and Ingram, 1987). Developing *S. cerevisiae* strains with high bioethanol tolerance can improve the output of the fermentation process to some extent. A high bioethanol tolerant yeast strain will have the potential of utilizing all the available sugars in the broth (Öner *et al.,* 2005). The high water dilution rate of the fermentation broth can prevent the inhibition of *S. cerevisiae*. However, the removal of the added water will require more energy, thus increasing the cost of the fermentation process. On the other hand, separating bioethanol from the fermentation broth during fermentation has the potential of minimizing the inhibitory reaction conditions for *S. cerevisiae* (O'Brien *et al.,* 2004). The separation of bioethanol from the broth and the adjustment of temperature and pH to the right conditions have the potential of improving the productivity of the fermentation process.

The approach of separating bioethanol from the fermentation broth during fermentation and setting the right experimental conditions (such as pH and temperature) was adopted in this study. An integrated system of fermentation and pervaporation was developed to carry out the continuous separation and fermentation of bioethanol. The fermentation-pervaporation system allows continual removal of bioethanol from the broth during fermentation, thus keeping the bioethanol concentration at levels which are less harmful to *S. cerevisiae*. This process of separating and fermenting bioethanol was termed the simultaneous fermentation and separation (SFS) process.

The SFS process utilizes pervaporation for the separation of bioethanol by circulating the fermentation broth across the active membrane layer inside the pervaporation module. The pervaporation cell is a two-phased system which allows easy separation of components of the mixture through a selective membrane. The selective membrane acts as the barrier between the two phases of pervaporation (Van der Gryp, 2003; Shao and Huang, 2007). Pervaporation

is described as an energy saving process that is highly efficient and easy to operate (Van der Gryp, 2003; Mzinyane, 2005; Qi *et al.*, 2006).

1.2 Objective of the study

The main objective of this study was to minimize the inhibitory effect of bioethanol on *S. cerevisiae* through the SFS process. The hydrolysis and the fermentation process experimental conditions were optimized prior to performing the SFS process experiments. Commercially available composite membranes were assessed for their efficiency towards separating ethanol from an ethanol/water mixture. The membrane showing the best performance (with regard to selectivity, stability and flux) was used throughout the study to perform the SFS process experiments.

1.3 Scope of the study

The main objective of the study was guided and limited by the information given in Figure 1.4.

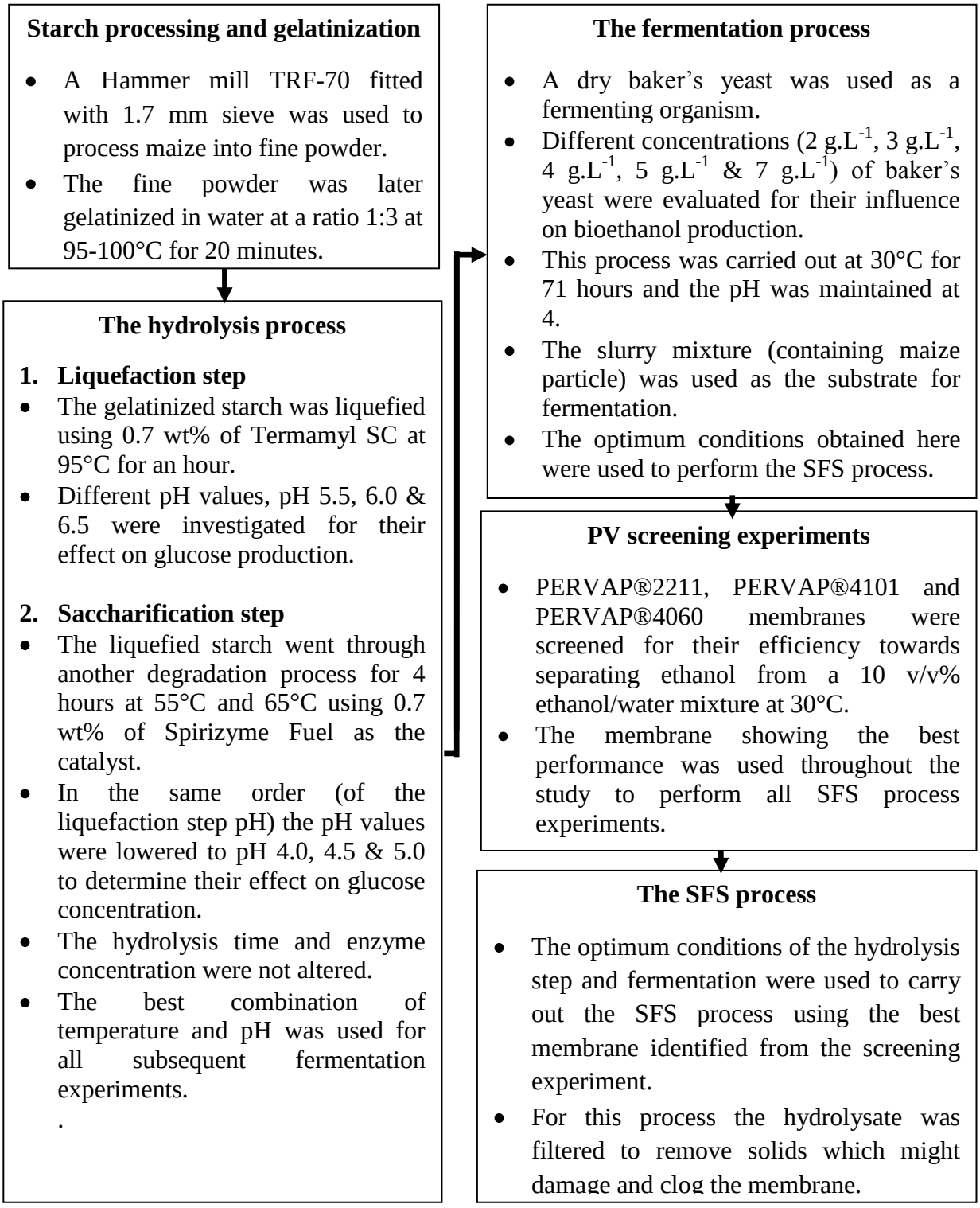

Figure 1.4: Scope of the study

1.4 The Scope of the dissertation

The dissertation is divided into five chapters as shown in Figure 1.5.

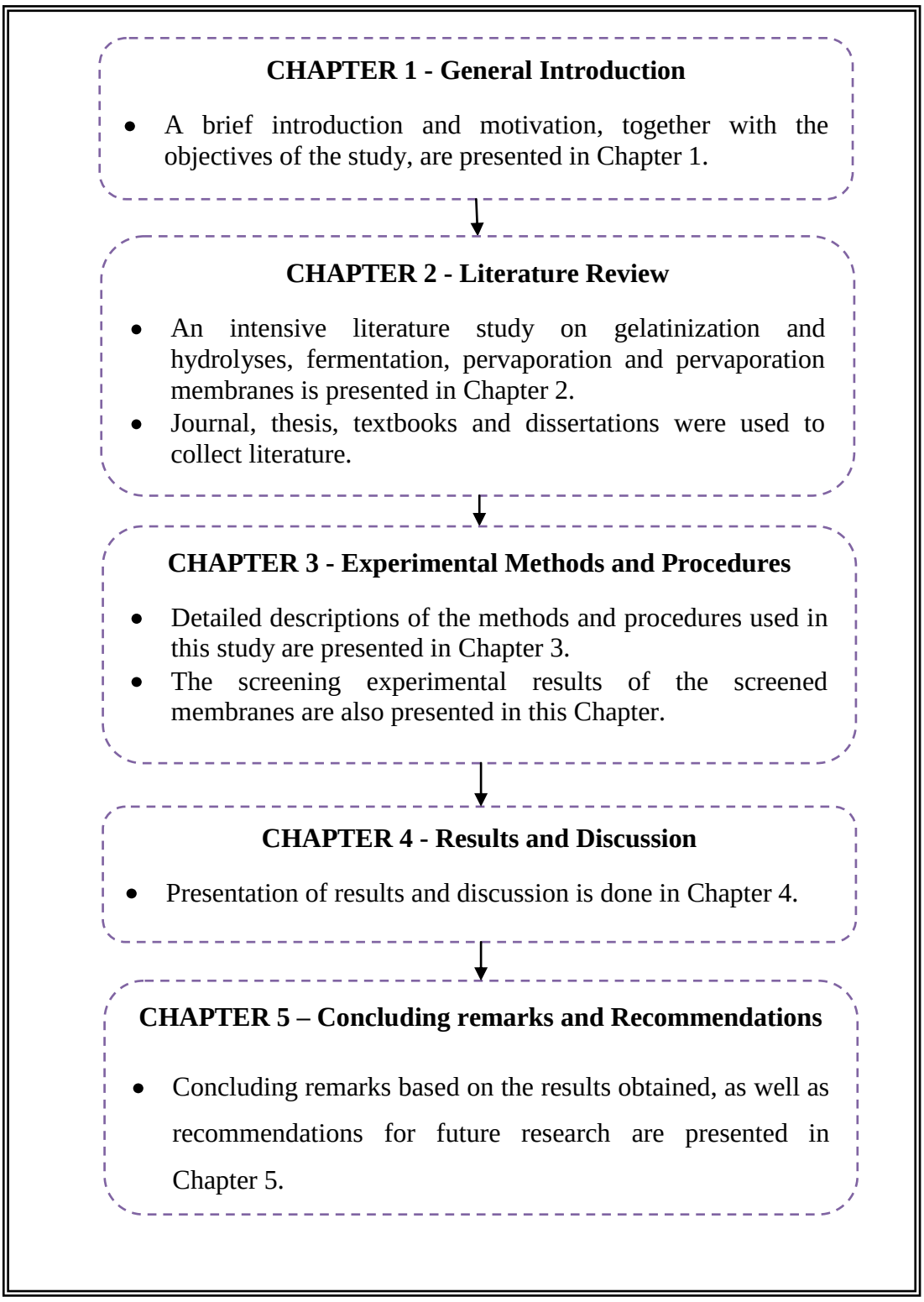

Figure 1.5: The Scope of the dissertation

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CHAPTER 2

LITERATURE SURVEY

"Time ripens all things; no man is born wise".

Miguel de Cervantes

OVERVIEW

In this chapter a detailed discussion of the literature encompassing the subjects of fermentation and pervaporation are presented. This chapter is divided into different sections and sub-sections. The fermentation process for the production of bioethanol is introduced and discussed in Section 2.1. The pervaporation process together with the coupled fermentation and pervaporation system is presented and discussed in Section 2.2. The concluding remarks are presented in Section 2.3

2.1 The fermentation process

2.1.1 Introduction

The fermentation process is the oldest and yet the most commonly employed technique of producing bioethanol from renewable biomass containing starch and/or sugars through the use of *S. cerevisiae* (Khaw *et al.*, 2007). According to Demirbas (2005) 91% of the world's total bioethanol production is through the fermentation process.

In a glucose ($C_6H_{12}O_6$) rich mixture, *S. cerevisiae* can directly utilize and convert glucose into ethanol and carbon dioxide (CO_2) as shown in equation 2.1 (Cheng *et al.*, 2007, Russel, 2003).

$$C_6H_{12}O_6 + \text{yeast} \rightarrow 2C_2H_5OH + 2CO_2 + 2ATP + 2H_2O \quad (2.1)$$

The major pathway of converting glucose to bioethanol is through the glycolysis process (Bai *et al.*, 2008). Through the glycolysis process a molecule of glucose is broken down into two molecules of pyruvate which can subsequently be converted to bioethanol under different step reactions as shown in Figure 2.1.

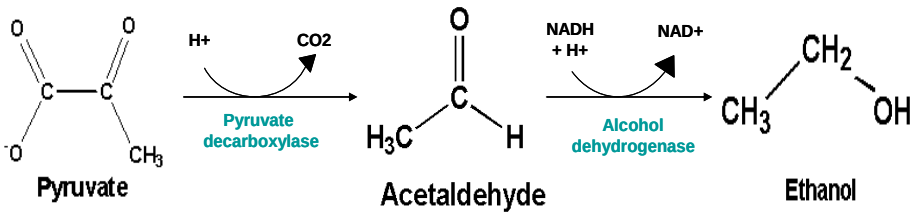

Figure 2.1: The reduction of pyruvate to bioethanol (Modified from Mathews *et al.*, 2000)

The first reaction is the decarboxylation reaction of pyruvate to acetaldehyde in the presence of pyruvate decarboxylase. In the second reaction, acetaldehyde formed from the decarboxylation reaction is further reduced into bioethanol by alcohol dehydrogenase (Stryser, 1995; Mathews *et al.*, 2000; Najafpour *et al.*, 2004; Bai *et al.*, 2008). The

decarboxylated CO_2 can be trapped during the fermentation process and be sold for other applications (Kheshgi and Prince, 2005; Gnansounou, 2009).

In a mixture containing sucrose (which is a disaccharide), invertase, an enzyme secreted by *S. cerevisiae*, is able to degrade sucrose into glucose and fructose. The produced glucose and fructose are further converted to bioethanol by Zymase, another enzyme secreted by *S. cerevisiae*, through the glycolytic pathway (Sánchez and Cardona, 2008; Demirbas, 2009; Gnansounou, 2009). The *S. cerevisiae* activity is limited to sugars, unable to directly utilize starch. Therefore, hydrolysis is important for converting starch into simple sugars (such as sucrose, fructose and glucose) which can be utilized by *S. cerevisiae* to produce bioethanol. The conversion of starch to bioethanol involves a series of processes which include gelatinization and hydrolysis, fermentation and product recovery.

2.1.2 The bioconversion of starch to bioethanol

Starch is the major source of carbon for bioethanol production and can be obtained from a variety of sources which include maize (Moore *et al.*, 2005; Kunamneni and Singh, 2005; Bai *et al.*, 2008). Maize consists of 20-30% amylose and 70-80% amylopectin (Torney *et al.*, 2007). Amylose is more soluble in water than amylopectin and both forms need to be dissolved completely before it can be hydrolysed. Dissolving maize starch in water prepares the starch granules for hydrolysis and fermentation.

The two forms of starch (especially amylopectin) are dissolved in water through the gelatinization step. The gelatinization step is also important for reducing bacterial infection or contamination which may affect the performance of the hydrolysis enzymes (Torney *et al.*, 2007; Mojović *et al.*, 2006; Franceschin *et al.*, 2008). The gelatinization process involves the mixing of water and starch (at a specified ratio) at high temperatures, thus producing a highly viscous mixture (Janušić *et al.*, 2007). At high temperatures the starch granules absorb water and swell (Patel and Seetharaman, 2006). The swelling makes the starch granules easily accessible by the hydrolysis enzymes (Göksungur and Güvenc, 1994).

The gelatinization step is followed by the liquefaction step in which α-amylase enzyme is added to hydrolyse the swelled starch. Liquefaction is the first step of hydrolysis. The α-amylase enzyme mixture is responsible for a random cleavage of α-(1-4) glucosidic linkages

of the two polymers of starch. The product of the liquefaction step is a slurry mixture containing a solubilised starch and different sized chains of saccharides including glucose (Mojović *et al.*, 2006; Sánchez and Cardona, 2008; Nair *et al.*, 2009). The α-amylase enzyme mixture is a thermostable enzyme, therefore, the liquefaction step is performed between 85°C and 100°C usually for an hour (Mojović *et al.*, 2006; Sánchez and Cardona, 2008; Nikolić *et al.*, 2009). The second step of hydrolysis is called the saccharification step. The saccharification step is initiated by adding glucoamylase enzymes into the liquefied mash. The glucoamylase enzyme mixture cleaves both the α-(1-4) and α-(1-6) glucosidic linkages from the non-reducing ends, thus releasing glucose as the main product (Mojović *et al.*, 2006; Nair *et al.*, 2009). Unlike α-amylase, the glucoamylase performs optimally between 55°C and 65°C (Kunamneni and Singh, 2005; Mojović *et al.*, 2006; Sánchez and Cardona, 2008). In reported cases by Kunamneni and Singh (2005) and Mojović *et al.* (2006) the saccharification step was carried out for 4 hours. In industry, the saccharification step and the fermentation step are carried out simultaneously through a process called the simultaneous saccharification and fermentation (SSF) process. When this is the case, the simultaneous process is continued for 45 to 72 hours at temperatures between 30°C and 35°C (Kunamneni and Singh, 2005; Nikolić *et al.*, 2009). Under these conditions, the optimum temperature of glucoamylase is neglected (Öhgren *et al.*, 2006). A successful hydrolysis process is dependent on the performance of the hydrolysis enzymes, which rely on the experimental conditions. Under optimal experimental conditions, the enzymes can produce high quantities of sugars during hydrolysis. For example, enzymes are sensitive to pH and temperature, thus neglecting either of the two or both will greatly affect the performance of the enzyme(s) and the productivity of the hydrolysis process. The optimum pH range of α-amylase as reported by Mojović *et al.* (2006) is between 5.5 and 6.2, and for glucoamylase is between 4.0 and 5.0 (Kunamneni and Singh, 2005).

The hydrolysed starch is subsequently fermented into bioethanol by *S. cerevisiae* through a traditional batch fermentation process. However, the efficiency of the fermentation process is influenced by a number of factors which include temperature, pH, inhibition, growth rate, productivity, osmotic tolerance and the condition of the medium (Alterthum *et al.*, 1989; Demirbas, 2005). *S. cerevisiae* is the most commonly used organism in bioethanol producing industries. The popularity of yeast in industry is due to its ability to ferment different sugars and utilise cheap raw material for growth and production (Shafaghat *et al*, 2009). The *S.*

cerevisiae functions optimally at temperatures between 27°C and 34°C within the pH range of 4-5. Under optimum conditions *S. cerevisiae* can readily convert sugars to bioethanol at a fast rate. However, the activity of *S. cerevisiae* is limited due to inhibition by the accumulating bioethanol in the fermentation broth (Dombek and Ingram, 1987). A bioethanol concentration around 9-12 wt% is sufficient enough to cause inhibition of *S. cerevisiae* (Bai *et al.*, 2008). Inhibition lowers the productivity and efficiency of the fermentation process.

Dombek and Ingram (1986b) conducted an investigation to determine the effect of an intracellular bioethanol on *S. cerevisiae* during the fermentation process. The study was performed in an effort to clarify the controversy concerning *S. cerevisiae* inhibition by bioethanol. During the study the effect of added bioethanol on the fermentative activity was assessed by adding bioethanol in a cell medium containing yeast. Findings showed that the growth and fermentative activity were less sensitive to inhibition by the added bioethanol. Later findings by Dombek and Ingram (1987) showed that both the added and the accumulated bioethanol had the same effect on the growth and the fermentative activity. According to Dombek and Ingram (1987), the availability of bioethanol in the fermentation broth causes physiological changes to the plasma membrane of the yeast responsible for the uptake of nutrients and other components in and out of the cell. The physiological change in the plasma membrane greatly affects the supply of nutrients such as glucose and other components necessary for the fermentation activity inside the cell. The requirement of the fermentation process as reported by Dombek and Ingram (1987) includes glucose, functional enzymes, coenzymes (such as NAD, thiamine pyrophosphate, ADP, ATP), cofactors (such as Mg^{2+}, Zn^{2+}), appropriate pH, a functional membrane to maintain the concentration of reactants and enzymes and a glucose uptake system. A functional membrane plays a vital role in regulating the uptake of some of the nutrients required for fermentation. When the membrane is altered and disrupted, the supply of these nutrients into the cell is greatly affected.

Alterthum *et al.* (1989) also highlighted that the accumulation of adenosine monophosphate (AMP) in the fermentation broth affects the performance of fermentation. According to Alterthum *et al.* (1989) AMP inhibits the major glycolytic enzyme, hexokinase, responsible for converting glucose to glucose-6-phosphate. Dombek and Ingram (1986a) and Alterthum *et al*. (1989) identified magnesium as another limiting factor of fermentation. Magnesium is

an important cofactor of glycolytic enzymes. The supply of this cofactor into the fermentation broth can extend the exponential growth, increasing the yeast cell mass and thus improving the fermentative activity (Dombek and Ingram, 1986a).

Nikolić *et al.* (2009) added that the SFS process avoids inhibition since the sugars are immediately consumed by the yeast as they are produced. The SFS process has high yields and productivity, better glucose utilization and low energy consumption compared to SHF (Nikolić *et al.*, 2009). The SFS process is usually carried out at temperatures between 30°C and 35°C. Even though the temperature of the saccharification enzymes is neglected at these temperatures it does not affect the overall performance of the process.

Nikolić *et al.* (2009) highlighted that a constant supply of nutrients in the fermentation broth can maintain fermentation and productivity for longer hours. However, Nikolić *et al.* (2009) also highlighted that the addition of nutrients is determined by the substrate in use; some substrates have poor chemical compositions, which require addition of other nutrients in the reaction to activate and stabilizes the catalyst, yeast. The nutrients as reported by Nikolić *et al.* (2009) have a protective effect in growth or fermentation and viability, thus resulting in an increased rate of production. The results show that adding yeast activators results in an improved bioethanol concentration.

Studies have been conducted in many laboratories in an attempt to develop methods of improving the process technologies involved in the production of bioethanol with regard to productivity and efficiency. Starch is the major source of carbon for bioethanol production in the USA and its conversion method is well-established, but the limiting factor in the process is the inability of yeast to directly utilize the starch. Therefore, starch must first be hydrolysed to simple sugars (such as glucose, fructose and sucrose) for yeast to produce bioethanol. Improving the process technologies involved in the conversion of starch (and other polymers) to sugars can improve the efficiency of the fermentation. Cheng *et al.* (2007) proved that the sugar concentration in the broth determines the yield. In a high glucose medium, a high bioethanol yield is expected (Cheng *et al*., 2007; Shafaghat *et al*., 2009).

Mojović *et al.* (2006) performed a two-step hydrolysis of maize starch using the commercially available α-amylase (Termamyl SC) and glucoamylase (Supersan 240L) to determine the best starch to water ratio and enzyme concentration. Maize meal prepared by

the dry milling process with an average particle size of 0.5mm was used as the starch source. Different starch-to-water ratios (1:1.25, 1:3, 1:4, and 1:5) were assessed. The liquefaction step was performed at 85°C with different enzyme concentrations at a pH of 6.0 for an hour. The saccharification step was later performed on the liquefied mash at 55°C with different enzyme concentrations at pH 5.0 for 4 hours. High Termamyl 120 L concentrations produced a high dextrose equivalent (DE) in a short time. It was, however, mentioned that low concentrations of Termamyl 120L could produce the same amount of DE in a longer period. The use of low Termamyl SC concentration was rejected to minimize energy consumption since the process was carried out at high temperatures and it would not be economically feasible to extend the running time. The same conclusions were made with regard to enzyme concentration for saccharification. Mojović *et al.* (2006) therefore, reported that the best concentrations of enzyme to use for liquefaction and saccharification were 12 KNU Termamyl SC and 48 AGU Supersan 240L, respectively. The lowest starch-to-water ratio (1:5) produced the highest glucose yield. The 1:3 starch-to-water ratio produced the highest bioethanol concentration and was thus suggested to be the more economic ratio to use. Attaining high bioethanol concentrations decreases the cost of the downstream process for product recovery, because the energy required to separate high bioethanol concentration would be lower compared to low bioethanol concentration in the broth. Mojović *et al.* (2006) also assessed the effect of different yeast inoculum concentrations on the final bioethanol concentration and reported that the different concentrations did not have a significant effect on the final bioethanol concentration. The only effect observed was that the duration of the fermentation process decreased with an increase in inoculum concentration.

Öner *et al.* (2005) on the other hand performed a study on bioethanol production using a 100% respiration deficient nuclear peptide amylolytic *S. cerevisiae* NPB-G for direct fermentation of starch to bioethanol. A comparison experiment was conducted using a 100% respiration sufficient nuclear peptide amylolytic WTPB-G strain. From the results NPB-G produced the best performance, with an increase of 48% in both yield and productivity compared to the respiration-sufficient yeast. According to Öner *et al.* (2005) the amylolytic yeast strain has a potential of substituting dried baker's yeast due to its high bioethanol productivity and yield. However, the major challenge with engineered yeast strains is their low bioethanol tolerance; over 150 amylolytic yeast strains with the same problem have been developed (Öner *et al*., 2005). This limits their application in industries. Developing strains

with high bioethanol tolerance can reduce the cost involved in the production of bioethanol from starch.

2.1.3 Applications and advantages of bioethanol

Bioethanol is the end product of the fermentation process and has been used in the fuel industry for different applications. Bioethanol has been used as an octane enhancer and as an oxygenate in petrol for many years (Shafaghat *et al*., 2009). Bioethanol has a high octane number and contains 35% oxygen. As an oxygenate, bioethanol has the potential of reducing NO_x emission from combustion. The use of bioethanol as fuel has a great potential of reducing air pollution (Demirbas, 2005; Shafaghat *et al*., 2009). Bioethanol leads to zero net carbon dioxide when burned and can be considered as an environmental friendly fuel (see Figure 2.2).

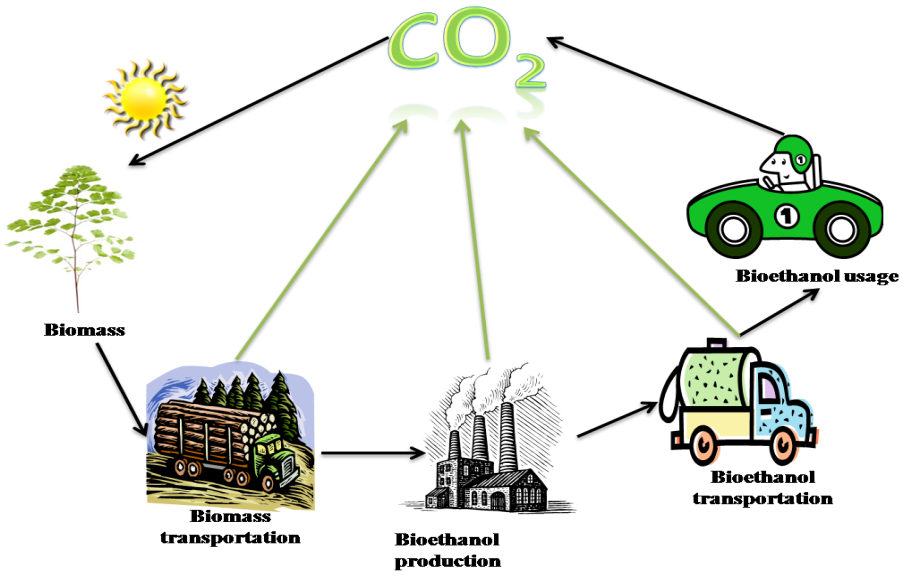

Figure 2.2: Carbon dioxide cycle

The CO_2 that is released during the bioethanol production, including transportation of biomass and product, is captured through photosynthesis by the biomass used in the production and will be captured upon release (Mathews *et al.*, 2000; Reijnders and Huijbregts, 2007; Kheshgi and Prince, 2005; Saxena *et al.*, 2007). During photosynthesis

plants take in CO_2 and water from the soil in the presence of light to produce glucose. Excess glucose is stored in the form of starch and the same plant can be used as the source of carbon for bioethanol production (Mathews, 2000; Saxena *et al.*, 2007). A typical photosynthesis reaction is shown in equation 2.2.

$$6CO_2 + 12H_2O + \text{Light} \rightarrow C_6H_{12}O_6 + 6O + 6H_2O \qquad (2.2)$$

Theoretically, in batch fermentation, 1g of glucose yields 0.511 g of bioethanol and 0.489 g of CO_2. In practice the bioethanol yield is always below the theoretical value since some of the glucose is used up for cell growth and for the maintenance of the process (Demirbas, 2005; Bai *et al.*, 2008).

Renewable biomass as reported by Kim and Dale (2004) and Saxena *et al.* (2007) is rated as the fourth largest source of energy, contributing 9-14% of the world's total energy consumption.

2.2 The pervaporation process

2.2.1 Introduction

The pervaporation process is a membrane process technology commonly used to separate mixtures with similar physical and chemical characteristics. Separation of azeotropic, as well as heat sensitive mixtures and mixtures with close boiling points, are some of the common applications of pervaporation. Moreover pervaporation can be used to purify water contaminated with organics (Jou *et al.*, 1999; Kujawski, 2000; Smitha *et al.*, 2004; Qi *et al.*, 2006). Amongst other things the importance of separation is to concentrate solvents and purify components (Mulder, 1998).

The separation of components by pervaporation involves a phase change, i.e. from liquid to vapour due to the applied vacuum downstream. Distillation and adsorption are some of the examples of phase change processes (Shao and Huang, 2007). Distillation is the most commonly used process in bioethanol production industries for the separation of bioethanol from water (Öhgren *et al.*, 2006). The major challenge with distillation is that it is energy

intensive and it cannot purify the bioethanol beyond the azeotrope. Its separation is based on the difference in volatility of compounds present in the mixture (Qi *et al.*, 2006; Zhu, 2006). Pervaporation was subsequently introduced as an energy saving process technology which could be implemented for the same applications as distillation, but with higher efficiencies (Van der Gryp, 2003; Smitha *et al.*, 2004; Qi *et al.*, 2006; Shao and Huang, 2007). Pervaporation separates components of the mixture based on their relative affinity and/or diffusivity and solubility toward the membrane at low temperatures (Feng and Huang, 1997; Shao and Huang, 2007). Pervaporation is economically viable, safe and environmental friendly (Smitha *et al.*, 2004). Recently (O'Brien *et al*., 1996) pervaporation has been assessed for its ability to separate and concentrate bioethanol from the fermentation broth. It has also been coupled with fermentation in an attempt to limit the inhibitory reaction conditions for *S. cerevisiae* by bioethanol during the fermentation process (O'Brien *et al*., 2004).

2.2.2 The pervaporation process

A typical pervaporation system consists of a membrane module, vacuum pump and a condenser as shown in Figure 2.3.

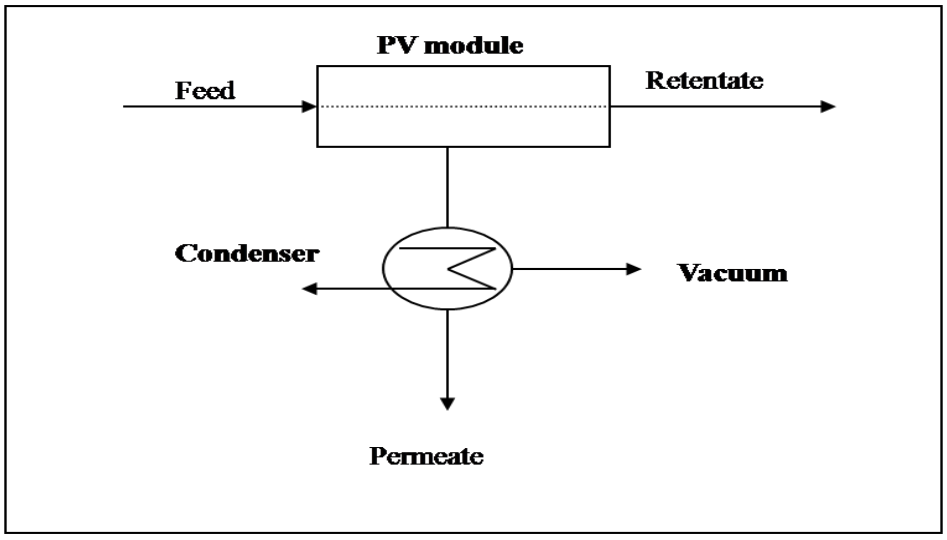

Figure 2.3: Schematic representation of a typical pervaporation system

The membrane for separation is located inside the membrane module. When the liquid feed enters the membrane module, it is split into the retentate and the permeate stream. The retentate represents a component of the mixture which does not diffuse through the membrane, and the permeate represents a component of the mixture which diffused through the membrane (Zhu, 2006). The diffused component of the mixture is later condensed downstream to liquid in a cold trap immersed in liquid nitrogen. Separation of components in pervaporation is achieved when one component of the mixture is continuously evaporated through a selective membrane under a driving force at low temperature (see Figure 2.4) (Mulder, 1998; Van der Gryp, 2003; Shao and Huang, 2007).

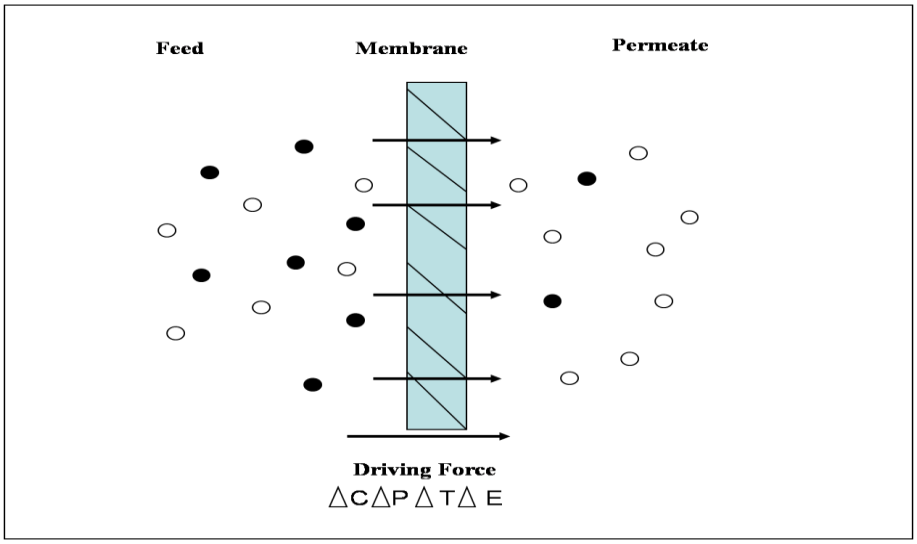

Figure 2.4: The schematic representation of the two-phase system of pervaporation (Modified from Mulder, 1998)

Mass transport of permeate across the membrane is induced by creating a driving force across the membrane by the application of a vacuum on the permeate side. Depending on the purpose of separation and the composition of the mixture, together with the membrane used, the permeate or retentate streams, or both, can be regarded as the product (Mulder, 1998).

The efficiency of the pervaporation process can be determined by membrane flux and selectivity (Kujawski, 2000). The performance of the membranes is rated according to stability, permeate flux and selectivity (i.e. the way in which the membranes are able to

distinguish between the component they pass in and the one(s) it reject) (Van der Gryp, 2003). Flux is defined as the volume flowing through the membrane per unit area and time (Mulder, 1998). The extent of separation can be expressed through retention (R) or selectivity (α). Retention is mainly used for determining the selectivity of a membrane towards a heterogeneous mixture of a solute and a solvent. Equation (2.3) can be used to calculate retention, with R varying between 0 and 100% depending on the degree of purification or separation. Complete separation is expressed as 100% and no separation as 0% (Mulder, 1998).

$$R = \frac{C_f - C_p}{C_f} = 1 - \frac{C_p}{C_f} \qquad (2.3)$$

where C_f is solute concentration and C_p is the solute concentration in permeate.

For homogeneous binary mixtures selectivity can be calculated, using equation (2.4) (Mulder, 1998).

$$\alpha_{A/B} = \frac{Y_a / Y_b}{X_a / X_b} \qquad (2.4)$$

where Y_a and Y_b are the concentrations of component A and B in the permeate, X_a and X_b are the concentrations of component A and B in the feed.

2.2.3 The transport mechanism of components across the membrane

Mass transport of permeate across a non-porous membrane can be described by a solution-diffusion model. The efficiency of the non-porous membrane can be determined by diffusion coefficiency and solubility coefficiency (Feng and Huang, 1997; Huang *et al.*, 2001). Following the solution-diffusion model, as depicted in Figure 2.5, according to Van der Gryp (2003), Smitha *et al.* (2004) and Zhu (2006), the separation of components by pervaporation is achieved in three steps, namely

- sorption of permeate by the membrane,
- diffusion of permeate across the membrane,
- desorption of permeate on the downstream side.

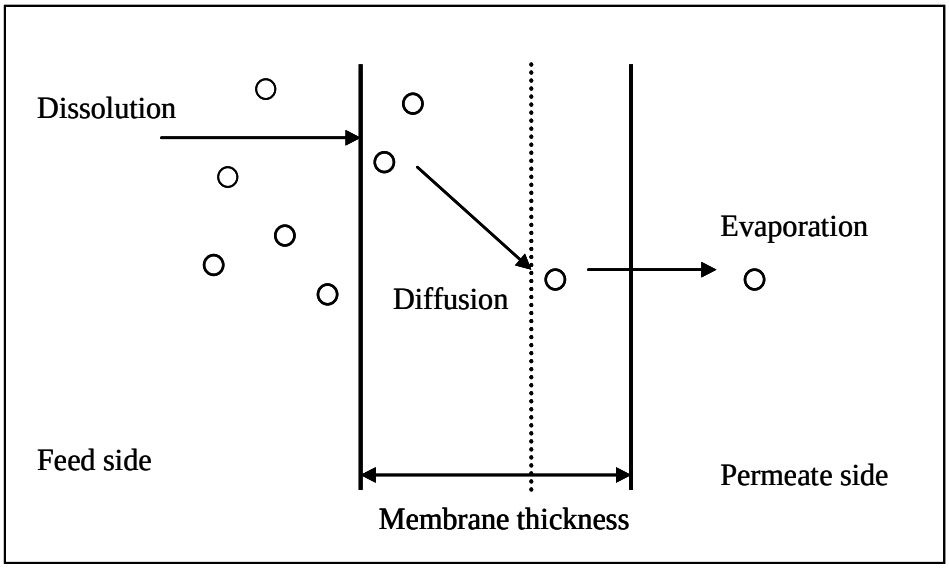

Figure 2.5: A schematic representation of the solution-diffusion model (Van der Gryp, 2003; Zhu, 2006)

The sorption of the permeate occurs in the feed side where the liquid feed comes in contact with the membrane. The components of the liquid feed mixture are absorbed and diffuse through the membrane as permeate at different rates. The diffused permeates are desorbed in the last step of the solution-diffusion model and condensed to liquid. The partial swelling of the membrane is regarded as a unique feature of pervaporation, with the upstream part of the membrane fully swollen and the downstream part almost dry due to the applied vacuum.

2.2.4 Membrane polymers and pervaporation membranes

The different types of polymers for membrane preparation can be classified into three groups, i.e. glassy polymers, rubbery or elastomeric polymers and ionic polymers. These polymers are naturally occurring polymers and they can be used to prepare different pervaporation membranes for different applications (Böddeker, 1990; Feng and Huang, 1997).

Glassy polymers are commonly used for the preparation of solvent dehydration membranes. The membranes made from glassy polymers are water-selective with few exceptions. For example polyacetylene derivatives and poly(1-trimethylsilyl)-1-propyne (PTMSP) have affinity towards organic components instead of water (Feng and Huang, 1997).

Rubbery or elastomeric polymers are mostly used for the preparation of membranes for the separation of organics in water. The most common rubbery polymer used to prepare these membranes is silicone rubber usually coated on poly(dimethylsiloxane). The silicone rubbery membranes are, however, reported to have low selectivity towards low alcohol concentrations in an alcohol-water mixture. This problem can be resolved by filling the membrane with an organophilic adsorbent. The organophilic adsorbent (or adsorbent filters) improves the sorption capacity and/or sorption selectivity of the membrane, thus causing a decline in membrane flux. The decline in membrane flux is due to strong interaction (or adsorption) of the permeating component with the membrane. Therefore, an appropriate adsorbent filter with proper organophilicity or hydrophobicity and pore size characteristics can be used to overcome the adsorption problem on the membrane surface. Glassy and rubbery polymers have both shown selectivity for organics and therefore it is unclear which of the two polymers is most appropriate to use for the preparation of membranes for the separation of organic mixtures (Feng and Huang, 1997).

The third group of polymers is comprised of ionic polymers. The membranes produced from ionic polymers are water-selective (Böddeker, 1990; Feng and Huang, 1997). Ionic polymers contain neutral ionic groups in their structures. This group of polymers can further be divided into cationic and anionic polymers. Ionic polysaccharides have also been reported to have a potential as a polymer for the preparation of dehydration membranes (Feng and Huang, 1997).

The polyvinyl alcohol (PVA) and the poly(acrylic acid) PAA are the most commonly used polymers for the preparations of dehydration membranes and both these polymers have high hydrophilic characteristics. On the other hand, chitosan and aromatic polyimides are under serious investigation as potential polymers for membrane preparation (Feng and Huang, 1997). Membranes made from PVA and PAA polymers have been reported to have high water flux (with poor selectivity) compared to membranes with low hydrophilic characteristics. To improve the selectivity of these membranes they need to be cross-linked and the balance in hydrophilicity and hydrophobicity characteristics should be maintained (Feng and Huang, 1997).

During pervaporation with PVA membranes, strong interactions of water with the hydroxyl group of the PVA polymer occur through hydrogen bonding. These interactions allows for the dehydration of water. PVA is amongst a few polymers with high water-solubility and can be easily modified for an improved performance, either by chemical or thermal cross-linking (Feng and Huang, 1997). According to Feng and Huang (1997), the membranes produced from PVA based polymers are the most studied pervaporation membranes thus far. Up to date, the focus in membrane preparation research is on modifying PVA for an improved selectivity, stability and performance for various prospective applications. The commercially available dehydration membranes produced by GTF Co. are made from PVA polymers that are thermally cross-linked (Feng and Huang, 1997).

PAA is water-selective with carboxyl groups in its structure, making it flexible to perform structural modification through cross-linking and salt formation with alkali metals. Dissociation of PAA polymers in an aqueous solution is a common occurrence but can be prevented by using multicovalent ions such as Al^{3+}, Cr^{3+}, Ca^{2+} and Mg^{2+} which induce ionic cross-linking for an improved resistance. Ionisation of PAA from an acidic form to alkali-metal form increases both the permselectivity and selectivity. However, during pervaporation the membrane looses the alkali-metal ions. This leads to the restoration of the PAA acidic form. This can, however, be prevented by maintaining the appropriate feed pH. Maintaining the pH will immobilise the alkali-metal ions in the membrane, but this method has its shortcomings and its applicability according to Feng and Huang (1997) is limited. Using certain polycations instead of alkali-metals can stabilize the PAA to form a stable polyion

complex. This method has been reported to be more effective than other methods tried (Feng and Huang, 1997).

Chitosan, a product of chitin, is rated as the second most abundant polymer occurring in nature with a linear structure of glucosamine with the reactive amino groups together with the primary and the secondary hydroxyl groups linked to its structure. The amino and hydroxyl groups of the chitosan play an important role during chemical modification of the polymer. The structural behaviour of chitosan is mostly affected by pH. At a neutral pH, the free amine groups of chitosan are insoluble in water, but in acidic pH the free amine group is protonated to form NH_3^+. The protonated amine group (NH_2) can be regenerated by treating the cationic structure (NH_3^+) with an alkaline solvent. The free amine form of chitosan has high water permeability. For stability, chitosan membranes should be prepared by spreading an aqueous solution of an appropriate chitosan followed by neutralization with alkali. Most work has been done on the separation of ethanol-water and acetic acid-water mixture using chitosan membranes (Feng and Huang, 1997).

Aromatic polyimides, according to Feng and Huang (1997), have outstanding thermal stability, mechanical strength and chemical resistance. Aromatic polyimides are produced by polycondensing aromatic dianhydrides and diamines. A soluble poly(amic acid) is formed from the polycondensation process which can be condensed to polyamine. Imide polymers are known for their extreme resistance to solvent dissolution and they can be good as material for preparing membranes for organic-organic separation. However, the strong resistance of the imide group makes it difficult to choose the suitable solvent to use during the conversional solution casting method when preparing polyamides membranes. This problem can, however, be avoided by casting the membrane with poly(amic acid) solution during the membrane preparation stages (Feng and Huang, 1997). Another way of preparing the polyimide membrane is by chemical vapour deposition and polymerization (CVDP). Through this process, aromatic dianhydrates and diamines are effused and deposited on the surface of the membrane substrate simultaneously. The deposited layers are then heated to form polyimide. The CVDP is ideal for producing membranes with high solvent resistance (Feng and Huang, 1997).

According to Smitha *et al*. (2004) and Shao and Feng (2007), pervaporation membranes are classified into two groups, i.e. homogeneous and composite membranes. Composite

membranes provide better flux due to their thin active layer compared to the thick layer of the homogeneous membranes (Smitha *et al*., 2004; Shao and Huang, 2007). The high flux of composite membranes makes them suitable for industrial purposes and these membranes have little resistance to mass transport. High resistance of membrane to mass transport result in a decrease in productivity and selectivity of the membrane (Shao and Huang, 2007). The internal property of the non-porous composite membranes determines the degree of separation (i.e. in terms of selectivity and permeability) (Mulder, 1998). Between 1982 and 1983 the first ever composite membrane made from a thin PVA on a porous PAN support and non-woven support fabric for the dehydration of ethanol was produced by GFT Co. The thin PVA layer was cross-linked to ensure membrane stability against high degree of swelling and high temperatures (Shao and Huang, 2007). The cross-linked thin PVA layer provides the membrane with higher flux and the porous supports are only there to provide the membrane with mechanical strength (Smitha *et al*., 2004). Asymmetric or homogeneous membranes are two layered membranes of the same polymer and/or a dense film. Homogeneous membranes with a dense layer have low permeation flux compared to symmetric membranes (Smitha *et al*., 2004).

2.2.5 Factors affecting pervaporation

There are various factors which affect membrane performance during the pervaporation process. These factors include feed concentration, feed temperature, permeate pressure and membrane fouling. These factors should be taken into consideration when performing the pervaporation process.

2.2.5.1 Permeate pressure

According to Smitha *et al.* (2004) the active gradient of the components of the mixture in the membrane is the main driving force of pervaporation. The permeation rate or flux can be increased by lowering the permeate pressure. A zero permeate pressure will create a maximum gradient of the components. The permeate pressure also determine the activity of the components in the permeate side of the membrane, i.e. it strongly affects the pervaporation characteristics. The rapid evaporation of one component than the other(s) in

the mixture at low temperatures still remains as the distinguishing feature of pervaporation (Feng and Huang, 1997; Smitha *et al.*, 2004).

2.2.5.2 Feed concentration

Feed concentration refers to the concentration of the targeted component in a mixture. As the process progresses, this component gets depleted in the mixture (Böddeker, 1990). According to Van der Gryp (2003) an increase in feed concentration of the targeted component will result in an increase in membrane flux and poor selectivity. According to solution-diffusion model the feed concentration has a direct effect on the sorption of the permeating component (Smitha *et al*., 2004). There are two features which can be used to determine the effect of feed concentration on pervaporation, viz. the driving force and the concentration polarization.

Concentration polymerisation occurs as a result of the less permeable component(s) of the mixture forming a boundary layer near the membrane surface area. The concentration of the faster permeating component in the feed mixture decreases, while the concentration of the slower permeating components increases on the membrane surface. This phenomenon lowers the selectivity of pervaporation and is most likely to occur in membranes with high permselectivity. In membranes with low permeation flux the concentration polymerization becomes insignificant (Feng and Huang, 1997; Smitha *et al.*, 2004).

2.2.5.3 Feed temperature

Both the selectivity and flux are strongly influenced by temperature. An increase in temperature will cause an increase in flux and it is often associated with a decrease in selectivity (Böddeker, 1990; Smitha *et al*., 2004). It can be mentioned that flux is directly proportional to temperature whereas selectivity is not.

2.2.5.4 Membrane fouling

By definition membrane fouling is the deposition of retained particle, colloids, suspension, macromolecules and salts in the membrane. These particles are associated with a decline in

membrane flux. Pore blockage, adsorption and precipitation also contribute to membrane fouling (Mulder, 1998). This phenomenon is mostly observed in microfiltration or ultrafiltration processes and is rarely observed in pervaporation (Mulder, 1998).

2.2.6 Application of pervaporation and pervaporation membranes

Pervaporation has different applications in different sectors and its applications can be classified into three branches of separation, namely for, (Feng and Huang, 1997; Smitha *et al.*, 2004; Shao and Huang, 2007):

- Dehydration of organic solvents (e.g. alcohols, ethers, esters, acids).
- The removal of dilute organic compounds from aqueous streams (e.g. removal of volatile organic compounds (VOC), recovery of aroma and biofuels from the fermentation broth).
- The separation of organic-organic mixtures (e.g. methyl *tert*-butyl ether (MTBE)/methanol, dimethyl carbonate (DMC)/methanol).

Membranes are the basis of separation for any membrane process and therefore, the choice of membrane will determine the effectiveness of separation (Feng and Huang, 1997; Smitha *et al.*, 2004). The different branches of pervaporation separation can be achieved by utilizing one of the membranes in the groups as classified in Figure 2.6.

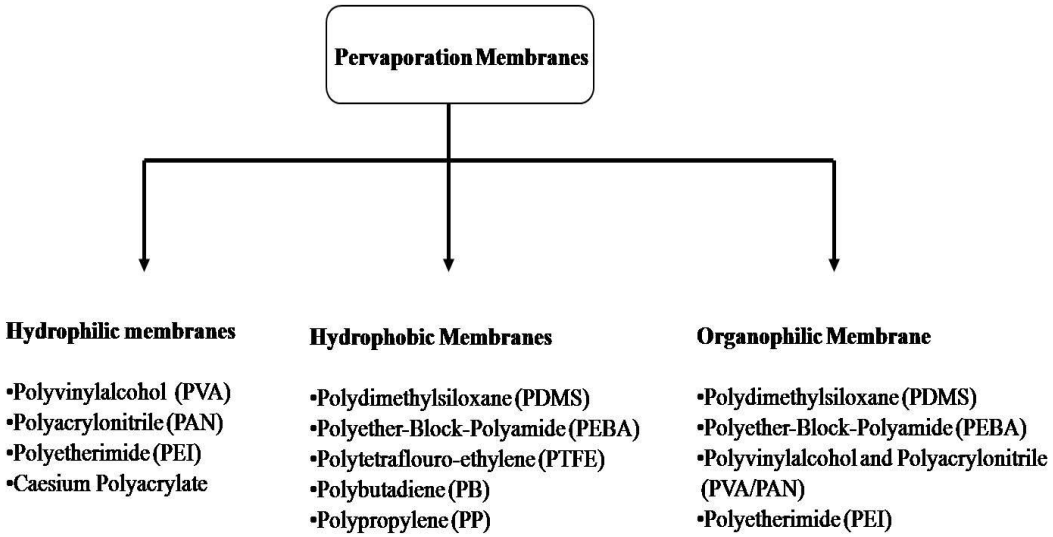

Figure 2.6: The classification of pervaporation membranes for the separation of different mixtures (Van der Gryp, 2003; Thongsukmak and Sirkar, 2007)

The commercial application of pervaporation started with the dehydration of organic solvents employing hydrophilic membranes produced from PVA. The hydrophilic membranes have high affinity for water, and therefore in an ethanol-water mixture the membrane will preferentially diffuse water across the membrane. Up to date, the dehydration of organic solvents is the most developed process and still remains the main application of pervaporation in industry. Pervaporation membranes need to be thermally cross-linked or cross -linked by special agents to ensure stability when exposed to acidic or strong salivating media. Grafting, blending and copolymerization of membranes can also improve the structure and the functionality of the membrane for better performance, hence the performance of the pervaporation process is determined by the performance of the membrane (Smitha *et al.*, 2004). Feng and Huang (1997) also added that copolymerization, and the incorporation of adsorbent material to membranes can improve their performance. These membranes have proven to have high chemical resistance and stability at high temperatures (Marin *et al.*, 1996; Smitha *et al*., 2004).

According to Shao and Huang (2007) the first pervaporation plant with a daily capacity of 15 m^3 was installed in France during 1988. This plant was installed after a breakthrough in membrane production by Gasellschaft Für Trenntechnik (GTF) a membrane technology

company. During the developmental stages of pervaporation the materials used for the dehydration process were naturally occurring polymers such as cellulose and cellulose derivatives. Following the unsuccessful attempts of using synthetic polymers, GTF later succeeded in creating membranes based on cross-linked PVA coated on a PAN substrate. Since the invention of GTF membranes, researchers have also investigated other synthetic polymers such as sodium alginate, chitosan, nylon 6, polyetherimine (PEI), cellulose sulphate (CS) and synthetic zeolite for bioethanol dehydration (Shao and Huang, 2007). Synthetic polymers since then have demonstrated better thermal and mechanical properties over natural polymers (Smitha *et al.*, 2004).

The removal of dilute organic compounds from aqueous streams is commonly performed using hydrophobic membranes. Unlike the hydrophilic membranes, hydrophobic membranes have a low affinity towards water and thus organic compounds are preferentially permeated across the membrane. The organic-organic mixtures can be separated using organophilic membranes. The organophilic membranes and the hydrophobic membranes can both be used for the removal of volatile organic compounds found in drinking water and in the fermentation broth (Thongsukmak and Sirkar, 2007; Ghoreyshi *et al.*, 2008). The separation of organic-organic mixtures is considered as the most difficult form of separation in pervaporation and its applications are of industrial importance (Shao and Huang, 2007).

According to Smitha *et al.* (2004), between 1984 and 1996, a total of 63 pervaporation systems were installed for commercial applications. Thirty two (32) of these systems were for the dehydration of organic solvents (i.e. bioethanol), 16 were for the dehydration of isopropanol and the last 15 were multifunctional systems. The recent global analysis on pervaporation and the technical brochures of Sulzer Chemtech cited by Smitha *et al.* (2004) reports that 90% of the commercialized pervaporation systems worldwide are produced by GTF.

2.2.6.1 The fermentation-pervaporation process

In addition to its other applications, pervaporation has been coupled with the fermentation system for the recovery of bioethanol from the fermentation broth. The coupled fermentation and pervaporation system has potential of reducing the overall cost of fermentation

technology. This system can also be used to minimize the inhibitory effects of yeast by continuous removal of the accumulating bioethanol in the fermentation broth.

O'Brien and Craig (1996) performed a study on bioethanol production using the coupled fermentation and pervaporation system with the aim of improving the productivity of the fermentation process by continuously recovering the produced bioethanol in the fermentation broth. To establish the best operating conditions for the process, 8 wt% ethanol and water mixture was used with a commercially available MPF-50 membrane as a selective barrier between the feed and the permeate side. The results demonstrated that high temperatures, high feed flow rate together with low vacuum were the best conditions. Later O'Brien and Craig (1996) performed a coupled fermentation and pervaporation at 35°C. A flux of 0.79 $L.m^{-2}.hr^{-1}$ from a membrane area of 0.1 m^2 with the selectivity of 6.5 was obtained. During the process the permeate concentration was kept between 20 wt% and 23 wt%, whilst the concentration of bioethanol in the broth was kept between 4 wt% and 6 wt%. The bioethanol yield obtained from a glucose concentration of 619 $g.L^{-1}$ with cell density of 23 $g.L^{-1}$ was 0.51 $g.g^{-1}$with the productivity of 7.8 $g.L^{-1}.hr^{-1}$.

Kaseno *et al.* (1998) confirmed that the removal of the major product of fermentation in the broth can improve the efficiency of the fermentation process. However, Dombek and Ingram (1987) highlighted that the removal of bioethanol in the broth does not immediately restore the fermentative activity of yeast. Kaseno *et al.* (1998) performed three experiments, batch fermentation without pervaporation, fed batch fermentation without pervaporation and fed batch fermentation with pervaporation in an effort to demonstrate that coupled fermentation and pervaporation can improve the overall yield or the efficiency of the fermentation process. A hydrophobic porous membrane made from polypropylene (PP) was used as the separating barrier with a feed contact area of 0.5 m^2. Dry baker's yeast without pre-cultivation was used as the fermenting organism. All experiments were carried out at 30°C in the absence of nutrients and the pH was maintained between 4 and 5. Under these conditions, productivity of the coupled system was two times higher than batch and fed batch fermentation without pervaporation.

O'Brien *et al.* (2004) also affirmed that one way of improving the fermentation efficiency is to continuously remove bioethanol from the broth as it is produced, to minimize the inhibitory reactions for baker's yeast. O'Brien *et al.* (2004) demonstrated that pervaporation

could maintain the concentration of bioethanol at a low stable range without affecting the activity of the fermenting organism.

Fadeev *et al.* (2003) performed a series of experiments to determine the effect of the fermentation broth by-products on the performance of poly[1-(trimethylsilyl)-1-propyne] (PTMS) membrane with regard to membrane flux. A decrease in membrane flux was observed during the fermentation and pervaporation process. This observation led to the assumption that the decline in flux was as a result of high yeast cell deposition on the membrane layer. This assumption was made following the results obtained from using a standard solution of ethanol and water which showed a stable performance of the membrane. A model system containing nutrients, 2 w/v% yeast extract, 1 w/v% bacto peptone, 15 w/v% glucose and 6 wt% ethanol was later assessed for its effect on PTMS membrane. A slight decrease in membrane flux was observed. Another experiment was performed using a standard mixture of 6 wt% ethanol, 0.5 % glycerol and 93.5 % water. The results showed that glycerol was responsible for at least 30% decrease in membrane flux. The low volatile components found in the fermentation broth were also found to be responsible for a decrease in membrane flux. These components occupy the polymer free volume thus making it inaccessible for mass transport of components across the membrane. PTMSP membranes are polymers with nanoporous morphology of about 20 % - 26 % fraction of nonequilibrium free volume. The PTMSP membranes are rare membranes which can produce high selectivity (ca. 12-20) for ethanol-water mixtures. These membranes have not yet found a practical application as pervaporation membranes due to low resistance to physical and chemical harshness (Fadeev *et al.*, 2003).

González-Velasco *et al.* (2002) performed a study on PTMSP membranes to determine the stability of the membrane over time, using a 10 wt% ethanol-water mixture. The findings of this study revealed that the selectivity and permeation rate were negatively influenced by the duration of the process. The experiment was conducted for 450 hours with the feed temperature maintained at 75°C. A slight decrease in the selectivity was observed after 200 hours of operation. However, for runs which lasted less than 100 hours the selectivity was less affected. PTMSP membranes also allow manufacturing of thin membranes which can be used for gas separation, vapour permeation and, of course, pervaporation.

Weyd *et al. (*2008) studied the separation of ethanol from ethanol-water mixtures using a hydrophobic ZSM-5 zeolite membrane under different feed concentration, feed temperatures and permeate pressure with regard to permeate flux and permeate concentration. The hydrophobic ZSM-5 zeolite membrane, like all the pervaporation membranes, separates components of the mixture on the basis of their differences in sorption and diffusion characteristics. An 8 wt% ethanol-water mixture was used to determine the duration of pervaporation under different temperature levels to reach 2 wt%. At 120°C it took approximately 2-4 hours to reach the targeted 3 wt%, whilst at lower temperature it took 8-10 hours. This is an indication that temperature has a direct influence on membrane flux and selectivity. An increase in membrane flux was observed with an increase in temperature. However, this increase in flux is mostly associated with a decrease in membrane selectivity. Inversely to temperature, an increase in permeate pressure resulted in a decrease in membrane flux. About 30% of the permeate flux was reduced with an increase in permeate pressure from 400 Pa to 2500 Pa. Increasing the feed temperature compromises the efficiency of pervaporation with regard to selectivity (Weyd *et al*., 2008).

2.3 Conclusion

Using the right ratio of starch to water and the best yeast concentration is a start in establishing a cost-effective fermentation process with good productivity. For this study choosing the right membrane and establishing the best operating conditions for the pervaporation and the SFS process can influence the separation of bioethanol thus improve the overall fermentation productivity.

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CHAPTER 3

EXPERIMENTAL METHODS AND PROCEDURES

"The lure of the distance and the difficult is deceptive. The great opportunity is where you are" John Burroughs

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OVERVIEW

In this chapter a detailed description of the methods and procedures used for the fermentation process and the SFS process experiments are presented. The chapter is divided into four sections. The chemicals and the membranes used in this study are presented and described in Section 3.1. Section 3.2 describes the steps involved in the processing of maize meal starch to bioethanol. In Section 3.3 the apparatus used and the method description of the SFS process are presented. The analytical techniques are presented in Section 3.4.

3.1 Materials used

The maize used for this investigation was obtained from a local farm in Potchefstroom. The starch content of the maize used in this study was determined (using equation 3.1) (Brunt *et al.*, 1998) to be between 65 wt% and 75 wt%. The starch percentage error observed during the determination of starch content was 3.1% (see Appendix E).

$$\text{Starch}\% = \frac{c * 0.98}{w} * 100\% \qquad (3.1)$$

where c = glucose concentration ($g.L^{-1}$)

w = weight of starting material (g)

0.98 = conversion factor of glucose

Mojović *et al.* (2006) reported 70.8 wt% starch content for the maize used in their study. A starch content of 73.4 wt% was reported by Dale and Tyner (2006) and Nikolić *et al.* (2009) reported 73.8 wt% starch content for the maize they used in their study. The starch content of the maize used in this study is thus comparable to the starch content of maize used in other studies.

The hydrolysis enzymes (i.e. Termamyl SC and Spirizyme Fuel) were supplied by NOVOZYME, South Africa. Termamyl SC activity according to Novozyme standards for the determination of α-amylase was 133 KNU/g (the amount of enzyme which breaks down 5.26 g of starch per hour).

Baker's yeast or *Saccharomyces cerevisiae* manufactured by Anchor yeast (South Africa) was purchased from the local store (Friendly supermarket) in Potchefstroom.

3.1.1 Chemicals used

All chemicals and reagents used in this study are given in Table 3.1, each with its application.

Table 3.1: Chemicals and reagents used

Chemical	Purity	Supplier	Use
NaOH	≥ 98 wt.%	Fluka	pH adjustment
HCL	30 wt.%	Saarchem Merck	pH adjustment
Glucose	≥ 99%	Fluka	Calibration Curve
Fructose	99%	Sigma Aldrich	Calibration Curve
Sucrose	99%	Sigma Aldrich	Calibration Curve
Maltose	95%	Sigma Aldrich	Calibration Curve
Ethanol	99.95%	Rochelle Chemica	Calibration Curve
1,4-Dioxane	99%	Saarchem Merck	GC Internal standard
Acetonitrile	99.9%	Sigma Aldrich	HPLC mobile phase
Buffer solution pH 7	-	HANNA INSTRUMENTS	pH meter calibration
Buffer solution pH 4.0	-	HANNA INSTRUMENTS	pH meter calibration

All reagents were used without any further purification.

3.1.2 Membranes used

Commercially available poly(vinyl alcohol) (PVA) membranes supported on a poly(acrylonitrile) (PAN) support layer coated on a polymer fleece were used in this investigation. PERVAP® 2211, PERVAP®4101 and PERVAP®4060 membranes were purchased from Sulzer Chemtech GmbH and screened for their efficiency towards separating ethanol from an ethanol-water mixture. The manufacture's specification sheet (PERVAP_Datasheets0809) for the three membranes is given in Table 3.2.

Table 3.2: Membrane specification sheet of membranes used in this study (PERVAP-_Datasheets0809)

Conditions	**PERVAP®2211**	**PERVAP®4101**	**PERVAP®4060**
Main application	For removal of organics and their mixture	For volatile organics and their mixtures	Removal of volatile organics from water
Max. temp. long term, ^{o}C	105 for EtOH	103 for ethanol	80
Max, temp. short term, ^{o}C	107	105	85
Max. water content in feed, % b.w	≤ 40	≤ 30	-
Major Limitations			
Aprotic solvents	≤ 0.1%	≤ 0.1%	≤ 0.1%
Aldehydes & derivatives (as acetaldehyde)	≤ 100 ppm	≤ 100 ppm	-
Organic acids (e.g. acetic acid)	≤ 10%	≤ 0.1%	≤ 1%
Formic acid	≤ 0.1%	≤ 0.005%	-
Aromatic HCs, Ketones, Esters, Cyclic Ethers, Halogenate HCs	No limitations	No limitations	No limitations
Aromatic Amines (e.g. Pyridine)	≤ 50%	≤ 0.01%	≤ 0.1%
pH (indictive)	5 – 7	5 – 7	5 – 7

According to PERVAP_Datasheets0809 high concentrations of organics and minerals outside the stipulated pH range, as well as alkali and aliphatic amines can damage the membrane. All membranes used in this study were characterized by scanning electron microscopy (SEM) to

determine the different layers of the membrane. The different layers of the membranes were measured and labelled as shown in Figure 3.1, Figure 3.2 and Figure 3.3.

3.1.2.1 PERVAP®2211 membrane

An SEM image of a PERVAP®2211 membrane showing the three layers of the membrane is shown in Figure 3.1.

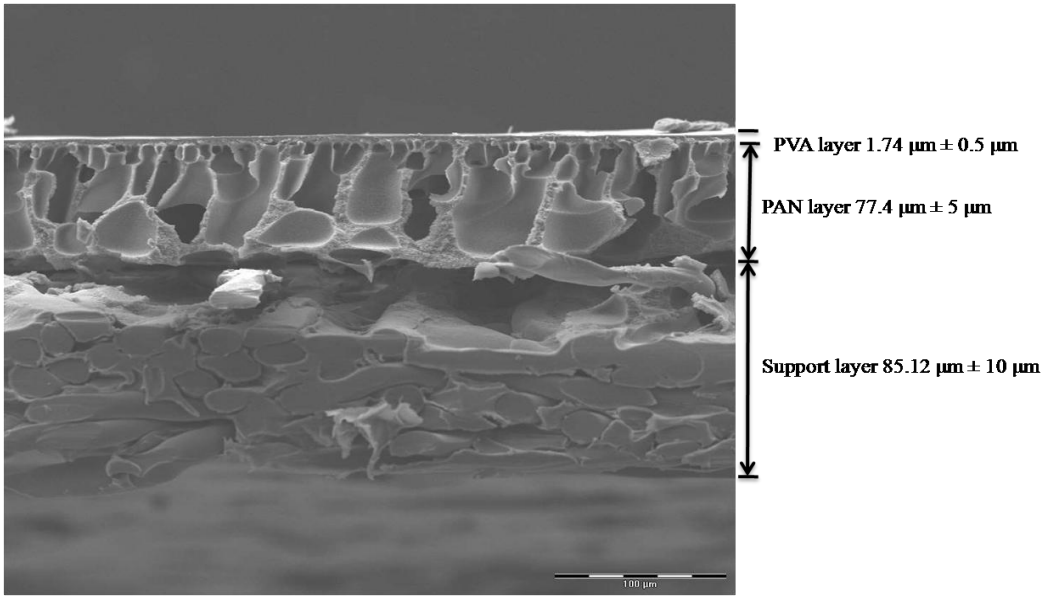

Figure 3.1: SEM image of the PERVAP®2211 membrane (bar = 100 µm)

The PVA layer, PAN layer and the mechanical support layer of the PERVAP®2211 membrane were measured to be 1.74 µm, 77.4 µm and 85.12 µm, respectively. Recent literature has shown no application of PERVAP®2211 membrane and, therefore, its performance was evaluated in this study.

3.1.2.2 PERVAP®4101 membrane

An SEM image of a PEVAP®4101 membrane showing the membrane's three layers is given in Figure 3.2.

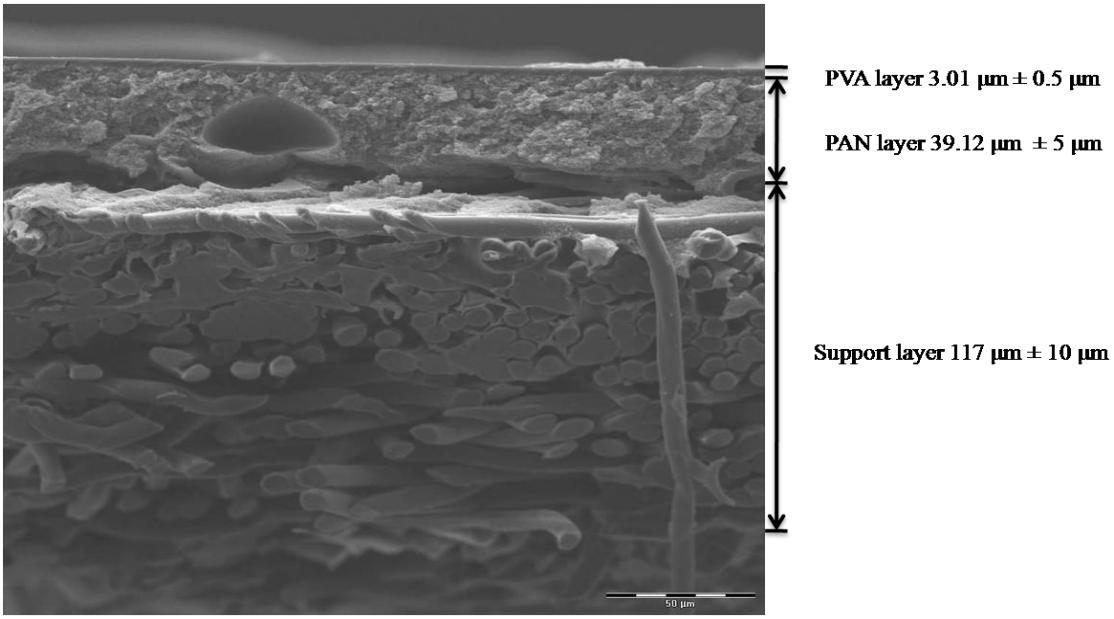

Figure 3.2: SEM image of the PERVAP®4101 membrane (bar = 50 µm)

The PVA, PAN and the mechanical support of the PERVAP®4101 membrane were determined to be 3.01 µm, 39.12 µm and 117 µm, respectively. Recent literature has shown no application of PERVAP®4101 membrane and its performance was evaluated in this study.

3.1.2.3 PERVAP®4060 membrane

An SEM image of a PERVAP®4060 membrane showing the different layers of the membrane is presented in Figure 3.3.

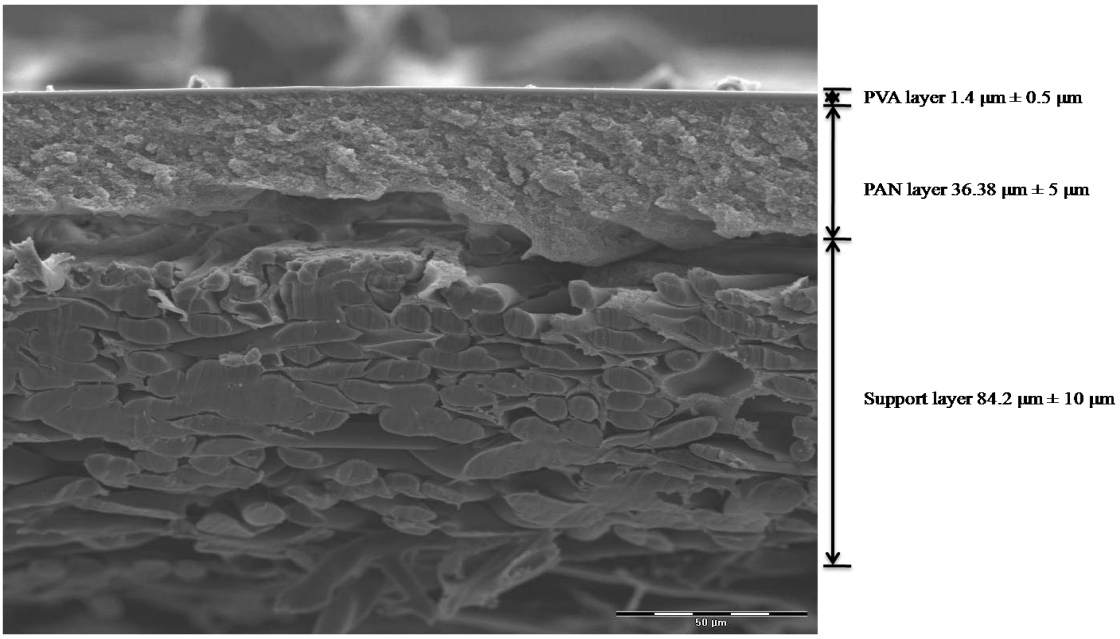

Figure 3.3: SEM image of the PERVAP®4060 membrane (bar = 50 µm)

The thickness of the active PVA layer was measured to be 1.4 µm, the thickness of the PAN layer was 36.38 µm and of the mechanical support 84.20 µm. Khayet *et al.* (2008b) used PERVAP®4060 membrane to determine the extent of separation of ethanol, acetonitrile and acetone from different water solutions. Their (Khayet *et al.* (2008b)) results showed that the membrane was more selective and produced good flux in the order: acetone, acetonitrile and ethanol. Khayet *et al.* (2008a) later performed a study to determine the effect of the operating conditions for PERVAP®4060 membrane with different mixtures of acetonitrile concentrations in water. From the results it was observed that an increase in organic feed concentration resulted in an increase in both the organic permeate and the permeate flux. Amongst the three PVA membranes evaluated in this study, PERVAP®4060 membrane had the smallest active layer thickness.

3.2 The fermentation process

3.2.1 Introduction

Maize meal was used as the source of carbon for bioethanol production in this study. The Termamyl SC and Spirizyme Fuel enzyme mixtures were used to hydrolyse the gelatinized maize meal starch into a glucose rich mixture known as the hydrolysate. The *S. cerevisiae* (baker's yeast) was used in all fermentation experiments to convert glucose to bioethanol. Details of the method and procedures used to perform the fermentation process are given from Section 3.2.2 to Section 3.2.5.

3.2.2 The gelatinization step

Gelatinization is the first key step before hydrolysis, when performing the dry milling process for the production of bioethanol (Torney *et al.*, 2007; Franceschin *et al.*, 2008). The gelatinization step was performed at high temperatures between 90°C and 100°C for approximately 20 minutes. Increasing the gelatinization time was demonstrated by Kunamneni and Singh (2005) to have no significant effect on the overall output of sugars during the hydrolysis step. The ratio of starch to water is another important factor when performing the gelatinization step. Mojović *et al.* (2006) performed a study to determine the effect of using different starch to water ratios on bioethanol production. Their results showed that lower substrate concentration (1:1.25) (Starch:Water) was more appropriate to use than higher substrate concentrations. In this way inhibition could be avoided. However, for economic reasons Mojović *et al.* (2006) highlighted that higher substrate concentrations are required to produce high bioethanol concentrations. High bioethanol concentration in the broth reduces the cost of product recovery. Therefore the ratio of 1:3 was adapted from Mojović *et al.* (2006) after careful consideration of all the factors involved. The steps involved during the gelatinization through the dry milling process are shown in Figure 3.4.

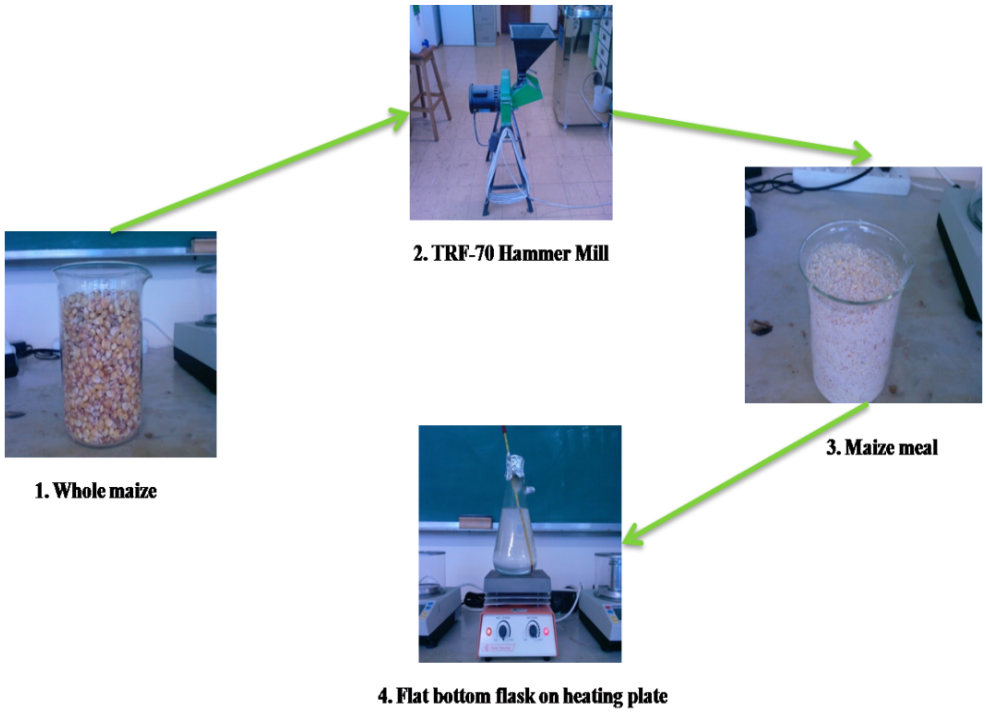

Figure 3.4: The processing of maize and the gelatinization process

The whole maize kernel (1) was milled (2) to +1.7 mm sieve size using a Hammer mill TRF-70 to produce maize meal (3) which was later used as the source of starch for bioethanol production. The gelatinization step was carried out in a flat bottom flask suspended on a heating plate (4) with the thermocouple immersed inside to monitor the temperature of the mixture inside the flask. A jelly-like, highly viscous consistent mixture referred to as mash was produced at the end of gelatinization step.

3.2.3 The liquefaction step

After adjusting the temperature of the mash formed during the gelatinization step to 95°C, 0.7 wt% (based on starch content) of Termamyl SC was added and the pH was adjusted to a required value using 30% hydrochloric acid (HCl) and 2 M solution of sodium hydroxide (NaOH). The content of the mixtures was later incubated in an oven at a fixed temperature for 60 minutes and samples were collected every 15 minutes for analysis using high performance liquid-gas chromatography (HPLC). The effect of pH on glucose production was investigated by varying the pH between 5.5 and 6.5.

3.2.4 The saccharification step

The liquefied mash was further hydrolysed by Spirizyme Fuel during the saccharification step. Prior to the addition of the saccharifying enzyme, the content of the liquefied mixtures was cooled to a desired temperature and the pH was adjusted accordingly. The temperature range of glucoamylase as reported by Kunamneni and Singh (2005), Mojović *et al.* (2006) and Sánchez and Cardona (2008) is between 55°C and 65°C; therefore the saccharification step was performed at 55°C and 65°C. The influence of pH on the final glucose concentration was investigated by varying the pH between pH 4.0 and 5.0. After adjusting the temperature and the pH, the content of the mixtures was later incubated in an oven at a fixed temperature for 4 hours with samples collected every hour. The same amount of enzyme (0.7 wt% Spirizyme Fuel based on starch content) was used to saccharify the liquefied starch.

3.2.5 The fermentation step

The traditional batch fermentation step was performed from a glucose rich mixture obtained through the hydrolysis step of maize meal starch employing baker's yeast as the fermenting organism. Prior to the addition of yeast in the hydrolysate, the temperature of the hydrolysate was cooled to 30°C and the pH was adjusted to pH 4. After adjusting the pH and the temperature a fraction of the hydrolysate was collected into a separate beaker. This was used to activate the dry baker's yeast. This mixture was incubated in an oven at 33°C for approximately 10 minutes to allow the yeast to adapt and grow in the hydrolysate medium. The activated yeast was later added to the hydrolysed maize meal starch to begin fermentation. Different yeast concentrations (between 2 $g.L^{-1}$ and 7 $g.L^{-1}$) were used to determine the influence of yeast concentration on bioethanol production. The fermentation broth was incubated in an oven at a fixed temperature of 30°C for 71 hours and samples were collected at various time intervals. No agitation was performed for any fermentation experiments. The activation step allowed yeast to adapt quickly to the hydrolysate medium. The result of activation is a short or no lag phase, allowing the exponential phase to take place within the first hour of operation.

3.3 The pervaporation process

3.3.1 Introduction

The pervaporation screening experiments of PERVAP®2211, PERVAP®4101 and PERVAP®4060 membranes were performed to assess their efficiency towards separating ethanol from a 10 %(v/v) ethanol-water mixture. The 10 % (v/v) ethanol-water mixture was selected since it falls within the bioethanol concentration range (i.e. 9-13 wt%) expected during the traditional batch fermentation process. A lower ethanol concentration (i.e. 5 wt%) could have been used, however, for a better flux and selectivity, according to Khayet *et al.* (2008a), a higher ethanol concentration would be ideal. All screening experiments were conducted under the same temperature of 30±2°C using a pervaporation unit (see Figure 3.5). The membrane exhibiting high flux, good selectivity and stability was used for all subsequent SFS process experiments.

During the SFS process, the membrane was exposed to the fermentation broth which is believed to affect the performance of membranes as a result of interactions of the membrane with the fermentation broth by-products. Therefore membrane stability was a critical factor when choosing the best membrane for the SFS process experiments. Good selectivity was also crucial since it forms the basis of separation. High flux was also regarded as an imported factor since the aim of the SFS process was to remove as much bioethanol from the broth as possible.

3.3.2 Apparatus and method description

A picture of the pervaporation system used to carry out the screening experiments and the SFS process experiments is shown in Figure 3.5.

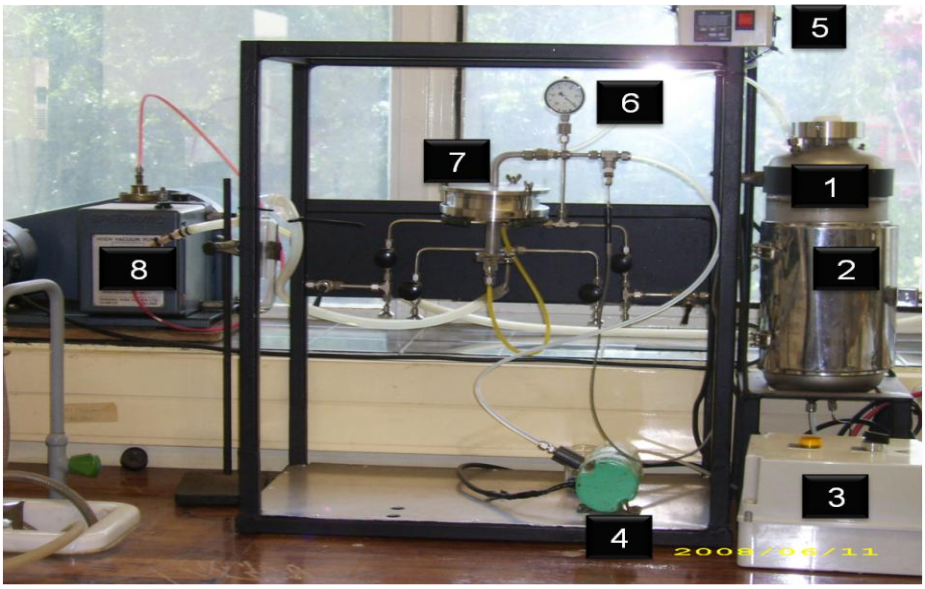

Figure 3.5: Picture of the fermentation-pervaporation system used in this study

Legend: 1)Feed tank 2)Heating jacket 3)Power supply 4)Slurry pump 5)Temperature probe, 6)Pressure gauge 7)Membrane module 8)Vacuum pump

During pervaporation, the feed mixture in the feed tank (1) is circulated across the membrane module (7) and back to the feed tank using the slurry pump (4). As the feed enters the membrane module it comes into contact with the active membrane surface. A vacuum (8), created on the permeate side of the membrane, affects the diffusion of water and ethanol through the membrane, due to the chemical potential difference induced by the vacuum. During the diffusion process, the diffusing component(s) undergo a phase transition to the vapour phase. The (bio)ethanol rich vapour is condensed by liquid nitrogen and then analysed for flux and selectivity calculations. A simplified schematic representation of the pervaporation system is shown in Figure 3.6.

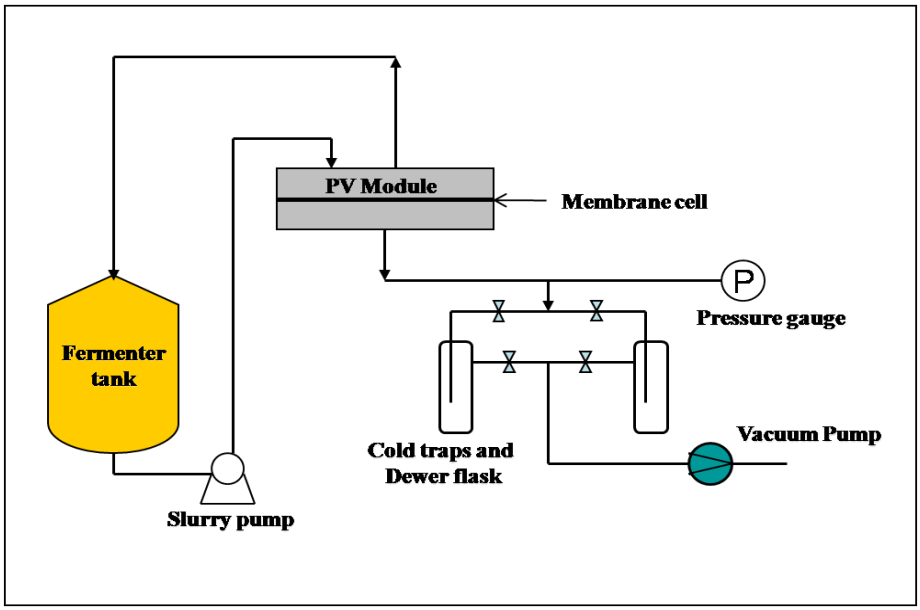

Figure 3.6: The schematic representation of the pervaporation system (Marx, 2002)

The membrane module consists of a membrane cell and flanges that seal the cell with o-rings as shown in Figure 3.7.

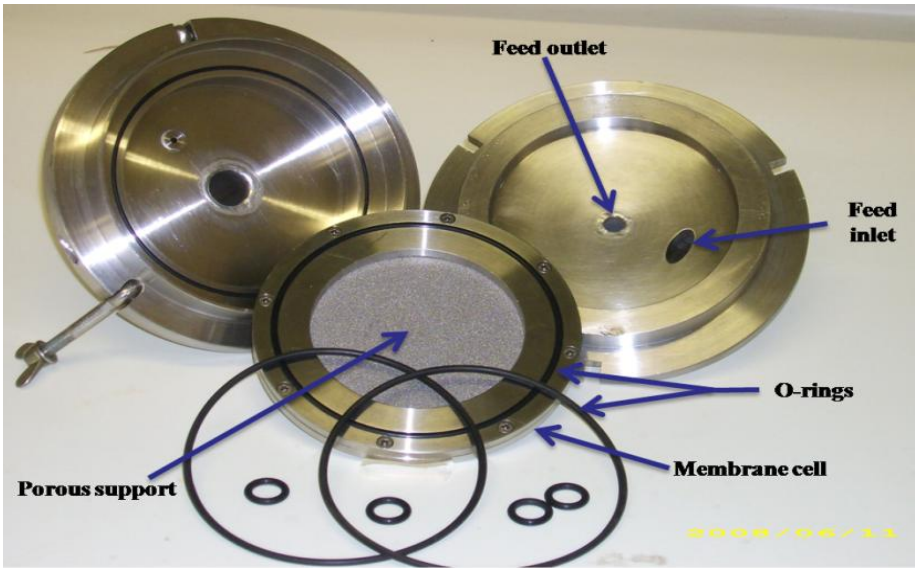

Figure 3.7: Components of the membrane module

The membrane cell consists of the retentate and permeate side flanges that forms the feed side and permeate side chambers on either side of the membrane cell. The membrane cell consists of two plates that hold the membrane on top of a porous support frit between o-rings. The porous support acted to support the membrane during operation**.**

3.3.2.1 The SFS process method description

The optimum operating conditions of the hydrolysis and the fermentation process obtained from this study were used to carry out the SFS process experiments. A flow diagram showing the different steps performed during the SFS process is shown in Figure 3.8.

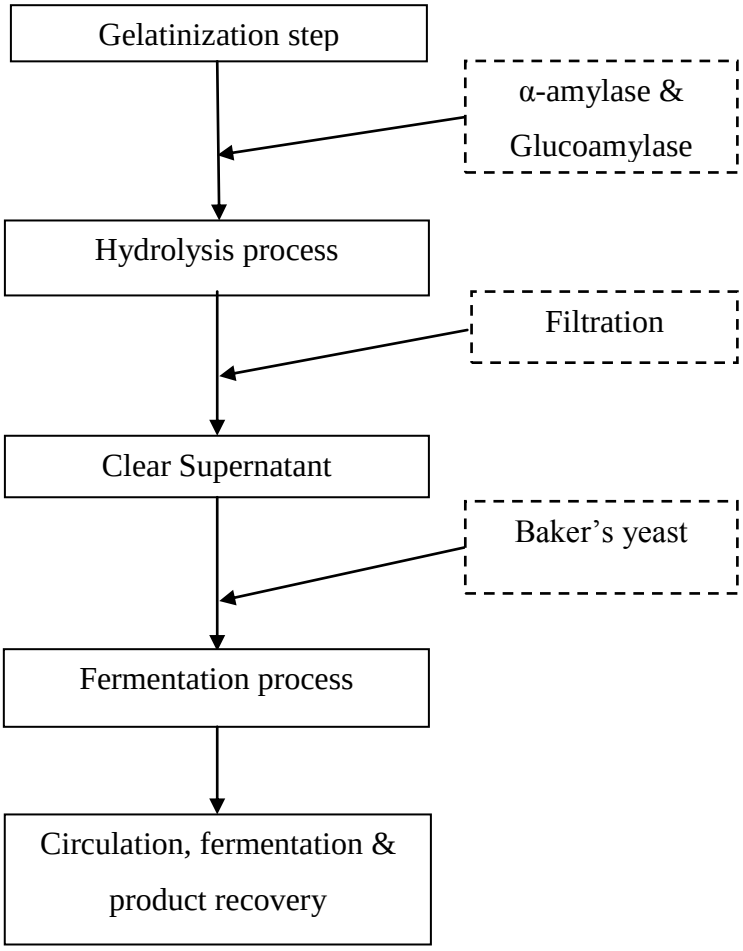

Figure 3.8: Flow diagram of the SFS process

The hydrolysate was prepared according to the procedure described in Section 3.2.2 to Section 3.2.4. Unlike the traditional batch fermentation, the hydrolysate was filtered and used as a substrate for fermentation. As this was the first attempt of performing the SFS process moving from starch to bioethanol through a continuous system, an assumption was made that some of the glucose molecules would be lost during the filtration step. The assumption was later proven to be correct as it is shown in Chapter 4 (page 83). After filtration the pH of the clear supernatant was adjusted to a pH value of 4.0 using the HCL to lower the current pH value. The effect of filtration on the glucose concentration will be discussed in Chapter 4. Yeast activation was performed according to the procedure described in Section 3.2.5. After adding the activated yeast to the clear supernatant it started producing bioethanol and was allowed to do so for 15 hours before the broth was circulated across a polymer membrane (for 4 hours). At the end of the membrane saturation the vacuum pump was applied downstream to start separation and permeates were collected and analysed using gas chromatography (GC). Two samples were collected per sampling time, permeate and the feed. Sampling was necessary to monitor the bioethanol concentration in the broth and to determine the selectivity and flux of the chosen membrane for the separation of bioethanol.

3.3.3 Screening experiment results

The raw and the calculated data of the membrane screening experiments is presented in Appendix B.

3.3.3.1 PERVAP®2211 membrane

The PERVAP®2211 membrane selectivity and flux obtained from an ethanol-water mixture is presented in Figure 3.9.

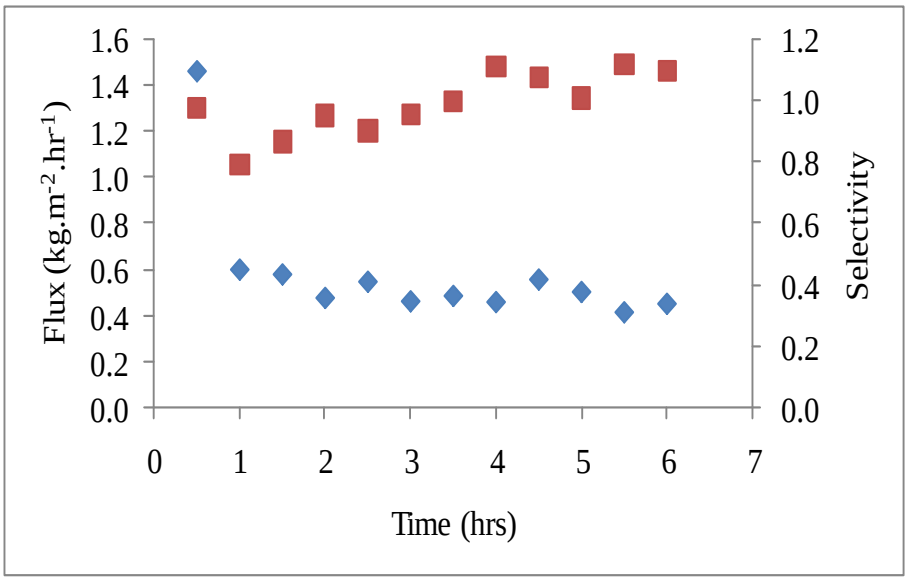

Figure 3.9: PERVAP®2211 membrane flux and selectivity obtained from a 10 (v/v)% ethanol/water mixture at 30°C (♦**:** Flux; ■**:** Selectivity)

A steady state flux of 0.48 $kg.m^{-2}.hr^{-1}$ was obtained after six hours of operation. A poor selectivity of 1.1 was obtained. This observation is common amongst pervaporation processes; most membranes with good flux often have poor selectivity. High flux and poor selectivity is an indication that the membrane was also permeating water with the ethanol.

3.3.3.2 PERVAP® 4101 membrane

The selectivity and flux of PERVAP®4101 membrane obtained from the separation ethanol from an ethanol-water mixture are presented in Figure 3.10.

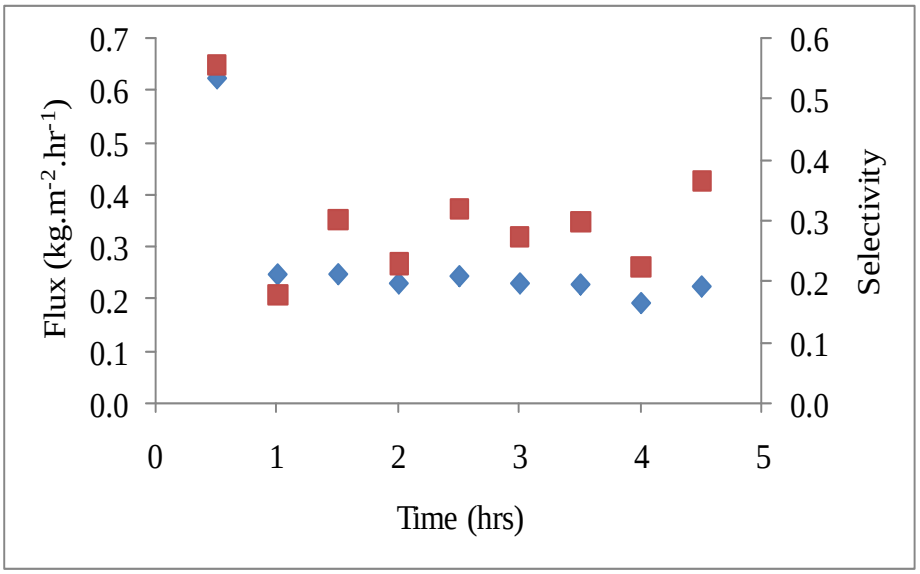

Figure 3.10: PERVAP®4101 membrane flux and selectivity obtained from a 10% (v/v) ethanol/water mixture at 30°C (♦: Flux; ■: Selectivity)

The results shown in Figure 3.10 demonstrate that the PERVAP®4101 membrane was preferential permeating water, hence the low selectivity of 0.29 towards ethanol. With regard to flux, the PERVAP®4101 membrane maintained stability throughout the operation with a flux of 0.23 $kg.m^{-2}.hr^{-1}$.

3.3.3.3 PERVAP®4060 membrane

The membrane flux and selectivity results of the PERVAP®4060 membrane obtained from the separation of ethanol from an ethanol-water mixture are presented in Figure 3.11.

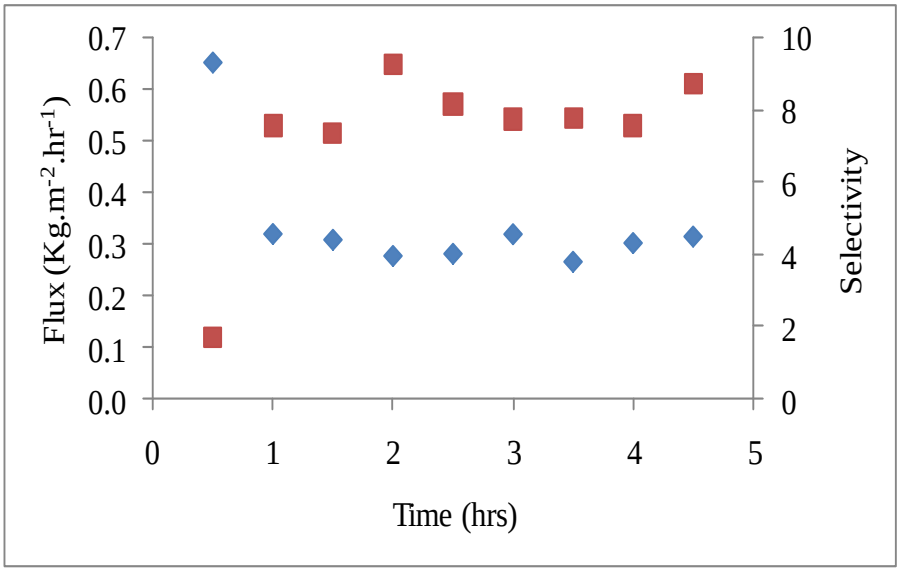

Figure 3.11: PERVAP®4101 membrane flux and selectivity obtained from a 10% (v/v) ethanol/water mixture at 30°C (♦**:** Flux; ■**:** Selectivity)

The PERVAP®4060 membrane performed better than the other membranes screened with respect to selectivity. A high selectivity of 9.3 was obtained with an average selectivity of 8.0, reached after 4.5 hours of operation. However, with respect to flux, the membrane showed a steady state flux of 0.30 $kg.m^{-2}.hr^{-1}$ that was lower than that of the PERVAP®2211 membrane and almost the same as that of the PERVAP®4101 membrane. The PERVAP®4060 membrane showed a stable performance throughout the 4.5 hours of operation. Khayet *et al.* (2008b) reported a selectivity of approximately 7.5 with a flux of 0.18 $kg.m^{-2}.hr^{-1}$ from a 10 wt% ethanol-water mixture at a feed temperature of 40°C.

3.3.3.4 Discussion of screening results

The steady state results of the different membranes assessed for the separation of ethanol from a 10 %(v/v) ethanol-water mixture during the pervaporation experiments are presented in Table 3.3.

Table 3.3: Pervaporation **s**teady state results

Membrane	**Flux ($kg.m^{-2}.hr^{-1}$)**	**Selectivity**
PERVAP®2211	0.48	1.1
PERVAP®4101	0.23	0.29
PERVAP®4060	0.30	8.0

With respect to membrane flux the PERVAP®4101 membrane and the PERVAP®4060 membrane performed almost the same with a flux of 0.23 $kg.m^{-2}.hr^{-1}$ and 0.30 $kg.m^{-2}.hr^{-1}$, respectively. The PERVAP®2211 membrane had the highest flux. Overall the PERVAP®4101 membrane had the worst performance compared to the other two membranes assessed. Despite the low flux, the PERVAP®4060 membrane was selected to carry out all SFS experiments due to its performance with regard to selectivity and stability.

3.4 Analytical techniques

3.4.1 The HPLC

The Agilent technology 1200 series HPLC system fitted with a Zorbex 5 µm carbohydrates column (4.6x250 mm) was used to analyze all samples collected during the starch hydrolysis process. The operating conditions used in the analysis are listed in Table 3.4.

Table 3.4: HPLC operating conditions for the analysis of sugars

Flow rate of mobile phase	1.00 $ml.min^{-1}$
Pressure range	0-400 bars
Solvent (Mobile phase)	75 vol.% Acetonitrile + 25vol.% dH_2O
Run-time	15 min
Injection	Standard injection
Injection volume	10 µl
Column temperature	60°C
RID signal: Optical unit temperature	55°C
Detector	Refractive Index (RI)

3.4.1.1 Sample preparation

All samples collected during the hydrolysis process were centrifuged at high speed using a Carl Roth SD micro centrifuge. The supernatant was then filtered using 0.2 µm and 0.45 µm filters fitted to a syringe, to remove solid particles. These samples were analysed within 24 hours after sampling to prevent bacterial infection or contamination and retrogradation.

The HPLC analysis was important for the identification and quantification of sugars present in the hydrolysate. A 200 $g.L^{-1}$ stock solution of different sugars present in the hydrolysis mixture was prepared. For calibration, the stock solutions were diluted with water to different concentrations as shown in Table A.1 (Appendix A) and the results were plotted as shown in Figure 3.12 to Figure 3.14.

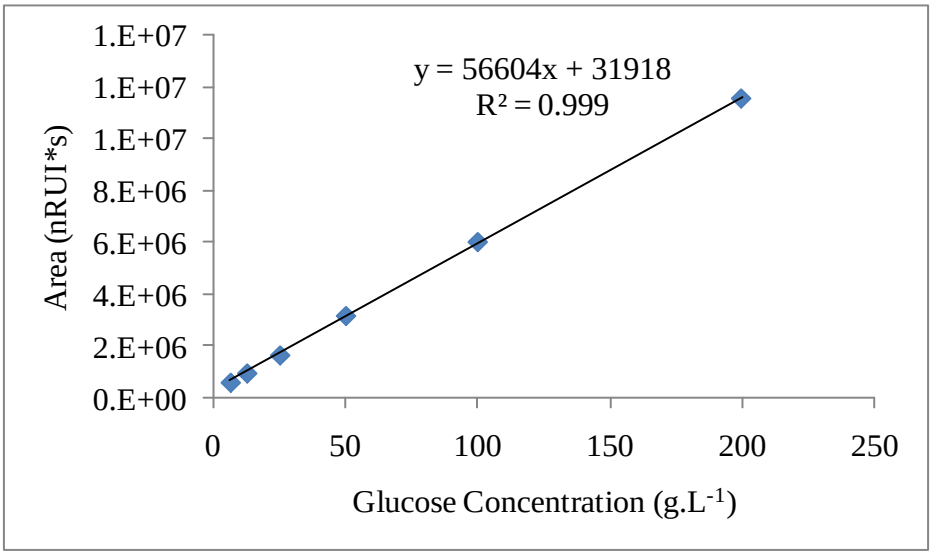

Figure 3.12: Glucose calibration curve

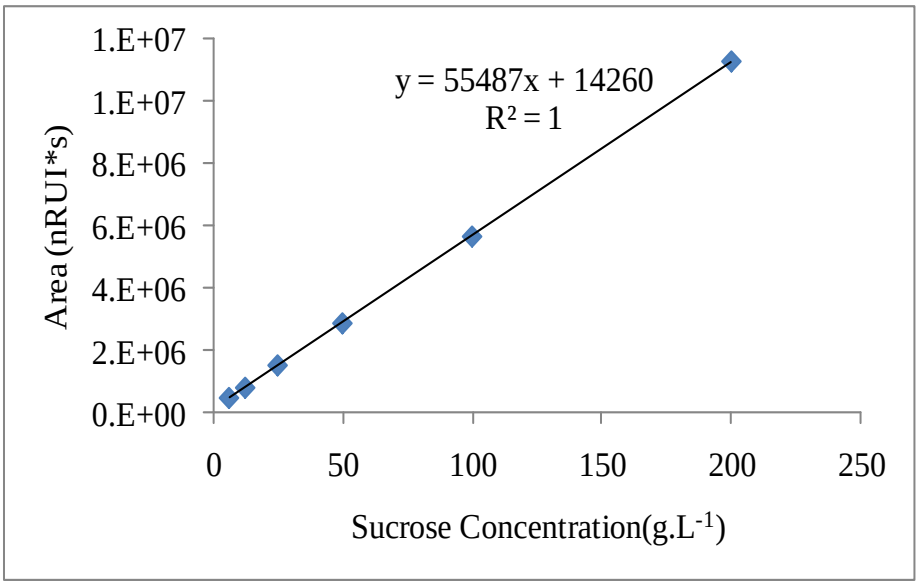

Figure 3.13: Sucrose calibration curve

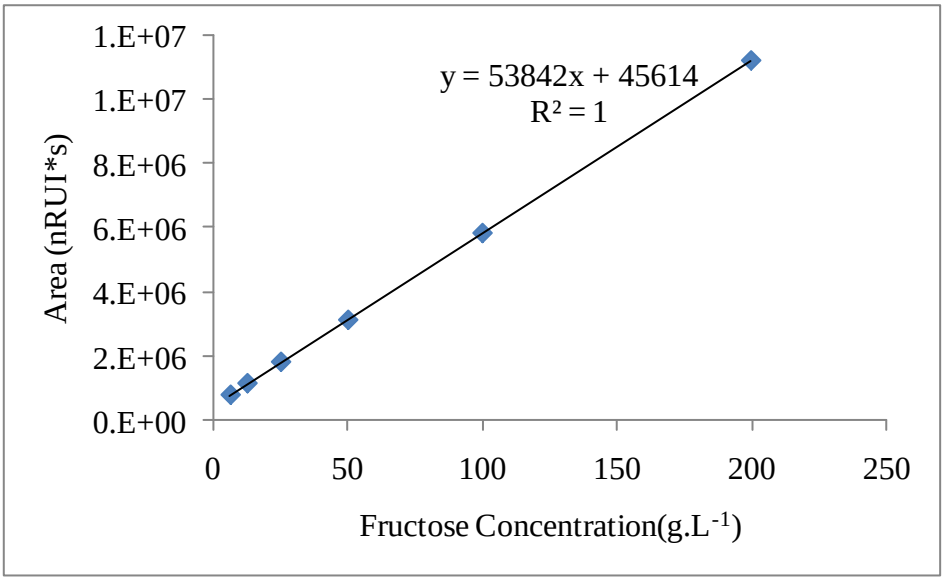

Figure 3.14: Fructose calibration curve

3.4.2 The Gas Chromatography (GC) analysis

The Agilent Technologies 1200 series GC system fitted with HP-5 column and flame ionisation detector (FID) was used to analyse all samples collected during the fermentation and the SFS process experiment for (bio)ethanol content. The HP-5 column is described as a non-polar general column that can be used for a wide range of applications. Adding to its advantage is its high temperature limitation.

The initial temperature of the GC oven was set at 45°C and was ramped by 15°C every 5 minutes until the highest temperature of 240°C was reached. Helium with a flow rate of 10 $ml.min^{-1}$ was used as carrier gas for all ethanol analyses. After each run the column was heated to 300°C for 5 minutes to clear the column of any compounds which might still be left. An injection volume of 0.2 µl was used to inject the samples from the sample vials into the column. The two gases used in the detector were hydrogen (H_2) and air with a flow rate of 40 $ml.min^{-1}$ and 400 $ml.min^{-1}$, respectively. The detector and the injector's temperature were set at 350°C and 250°C respectively.

3.4.2.1 Sample preparation

All samples collected during the fermentation process and the SFS process were centrifuged using a Carl Roth SD micro centrifuge. The supernatant was filtered using three different sized filters, a yeast removal filter, a 0.2 µm and a 0.45 µm filter supplied by PALL. The yeast removal filter was used to filter the yeast in the sample, thus stopping the reaction. The 0.2 µm and 0.45 µm filters were used to remove residual solids from the samples. For GC analysis an internal standard was required since the FID detector can not detect water in the samples. Therefore, 1,4-dioxane with high solubility in many organic solvents and with a retention time close to that of ethanol was chosen as an internal standard. A 10 wt% solution mixture of 1,4-dioxane was prepared and used for all GC samples to be analysed. All samples were diluted two-fold with the internal standard. The ethanol calibration for determination of bioethanol content is shown in Figure 3.15.

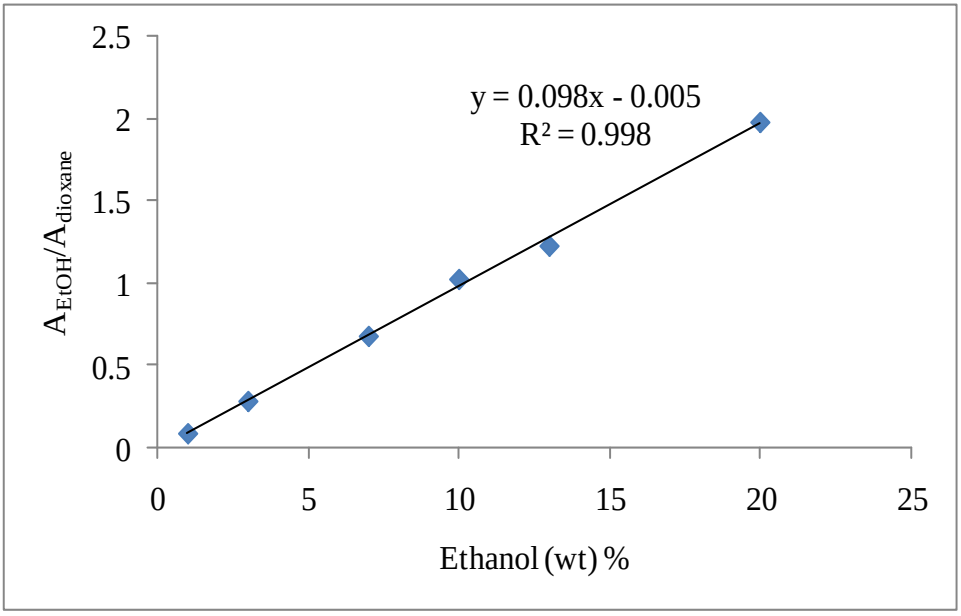

Figure 3.15: Ethanol standard curve for the identification and quantification of ethanol in broth

3.4.3 The pH

A HANNA HI 99161 pH meter was used to monitor the pH during the hydrolysis and the fermentation process. A 2 M solution of NaOH and 30 wt% HCL were used to adjust the pH of the mixtures to the required pH value. The pH meter had a maximum temperature

limitation of 60°C and thus samples were cooled to below 55°C before reading the pH. The pH meter was calibrated continuously with buffer solutions during use.

3.5 References

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CHAPTER 4

RESULTS AND DISCUSSION

"Being an optimist after you've got everything does not count"

Kin Hubbard

"Every great mistake has a halfway moment, a split second when it can be recalled and perhaps remedied" Pearl S. Buck

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OVERVIEW

In this chapter all results obtained in this study are presented and discussed. The results of the traditional batch fermentation experiments can be found in Section 4.1 with the SFS results presented in Section 4.2. Concluding remarks are presented in Section 4.3.

4.1 The fermentation process

4.1.1 The liquefaction step

Starch has to go through a gelatinization step at high temperature to prepare the starch granules for subsequent saccharification (Mojović *et al.*, 2006; Franceschin *et al.*, 2009). The liquefaction of milled whole maize kernels was carried out at 95ºC for an hour for all fermentation experiments in this study, according to the procedure discussed in Section 3.2.3. The results on the influence of pH on the liquefaction of the gelatinized mash at 95ºC are shown in Figure 4.1.

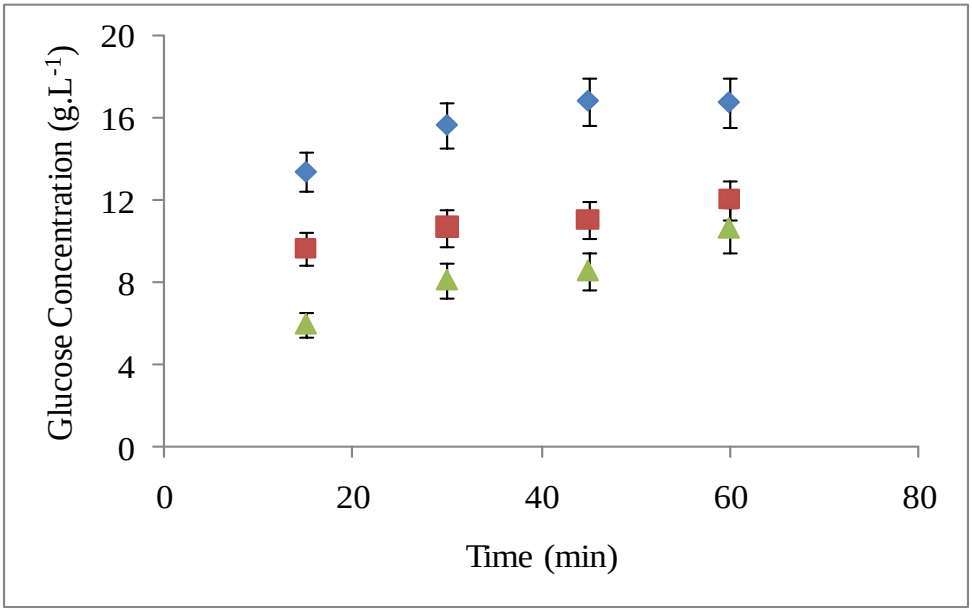

Figure 4.1: The effect of pH on the amount of glucose produced during the liquefaction step of the gelatinized maize mash at 95ºC (♦**:** pH 5.5; ■**:** pH 6.0; ▲**:** pH 6.5)

From Figure 4.1 it can be observed that the highest average glucose concentration of 16.8 $g.L^{-1}$ was obtained at a pH of 5.5, while the lowest average glucose concentration of 10.6 $g.L^{-1}$ was obtained at a pH of 6.5. The pH of the mixture during liquefaction can have a significant influence on the amount of glucose produced, because the pH affects the activity of the liquefaction enzymes. An increase in pH modifies the structure of the enzyme, particularly the active site, in such a way that the substrate can no longer bind to the enzyme.

During this time the enzyme is rather denatured and thus less glucose is obtained. This was observed in the decrease in glucose concentration with an increase in pH during liquefaction from 5.5 to 6.5. Little has been reported in literature on glucose concentration obtained during the liquefaction step. Table 4.1 presents the glucose yield obtained during the liquefaction of the gelatinized mash using Termamyl SC as a catalyst.

Table 4.1: Glucose yield obtained during the liquefaction step

pH	$\gamma_{Glucose/Starch}$ **(g.g^{-1})**
5.5	0.051
6.0	0.036
6.5	0.032

The experimental error observed during the liquefaction step at 95°C was 7.1% (see Appendix E). The data given in Table 4.1 demonstrate that Termamyl SC was greatly influenced by the change in pH, thus the difference in glucose yield. A high glucose yield is an indication that Termamyl SC was more active and productive at a pH of 5.5 than at 6.0 and 6.5. However, researchers such as Apar and Özbek (2005), Mojović *et al.* (2006) and Nikolić *et al.* (2009) reported good findings at pH values different from pH 5.5. Apar and Özbek (2005) investigated the effect of pH on the hydrolysis of rice starch using α-amylase, and found pH 6.5 to be the best pH for Termamyl SC. Mojović *et al.* (2006) chose pH 6.0 to perform the liquefaction step with the aim of optimizing substrate and enzyme concentration, and yeast for an improved bioethanol production using Termamyl 120L. Following the work of Mojović *et al.* (2006), Nikolić *et al.* (2009) performed the liquefaction step at pH 6.0 using Termamyl SC with the aim of improving the productivity of bioethanol production by employing the SSF process. Kunamneni and Singh (2005) reported an increase in glucose production at pH 6.0 using an extracted α-amylase from *B. substilis*. However, in this study it was found that better glucose yields are found at a lower pH value (Table 4.1), using Termamyl SC enzyme mixture.

4.1.2 The saccharification step

The saccharification of the liquefied starch to glucose was performed according to the procedure described in Section 3.2.4. After liquefaction, the pH of the mixture(s) was lowered to the desired pH values for saccharification using a 30% hydrochloric acid (HCl) solution. The influence of pH on the glucose produced during the saccharification process was assessed by varying the pH between 4 and 5. In most published papers (Mojović *et al.*, 2006; Öhgren *et al.*, 2006; Nikolić *et al.*, 2009) the saccharification step is performed simultaneous with the fermentation process through a process called SSF. In this study the saccharification and fermentation steps were performed separately because a glucose rich hydrolysate was required for the pervaporation process. The results of the effect of pH on glucose concentration during saccharification of the liquefied mash performed at 55°C are given in Figure 4.2.

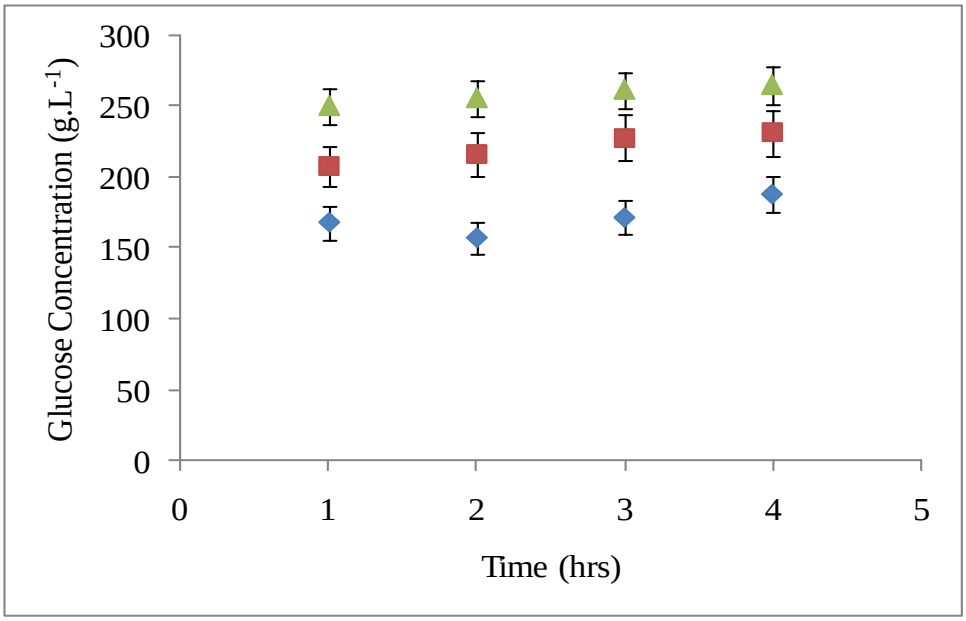

Figure 4.2: Effect of pH on glucose concentration during the saccharification of the liquefied maize starch at 55ºC (♦**:** pH 4.0; ■**:** pH 4.5; ▲**:** pH 5.0)

From Figure 4.2 the highest average glucose concentration of 265.3 $g.L^{-1}$ was obtained at a pH of 5.0 with the lowest glucose concentration of 188.7 $g.L^{-1}$ obtained at a pH value of 4.0.

An average glucose concentration of 231.9 $g.L^{-1}$ was obtained at a pH of 4.5. The second set of results on the saccharification of the liquefied mash was conducted at 65°C and the results are presented in Figure 4.3.

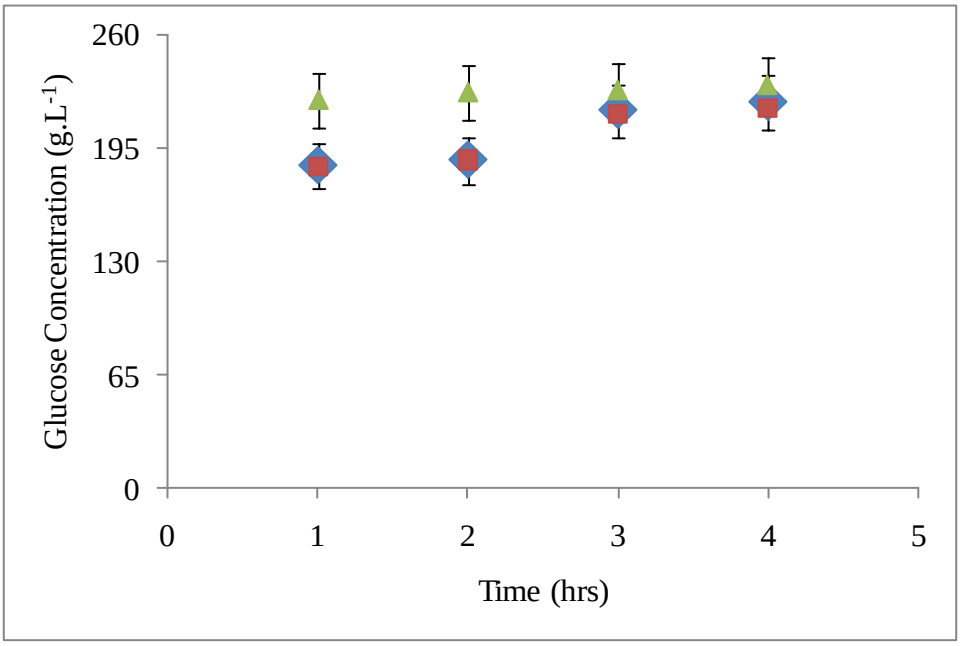

Figure 4.3: Effect of pH on the glucose concentration during the saccharification of liquefied maize starch at 65ºC (♦**:** pH 4.0; ■**:** pH 4.5; ▲**:** pH 5.0)

The same trend in the influence of pH on glucose production was observed at 65ºC (see Figure 4.3) as at 55ºC. The highest average glucose concentration of 230.9 $g.L^{-1}$ was obtained at pH 5.0 with the lowest concentration of 218.2 $g.L^{-1}$ obtained at pH 4.0. Little has been reported on separate hydrolysis and fermentation (SHF), particularly when maize starch is used as the substrate for bioethanol production. Most reported literature is on simultaneous saccharification and fermentation (SSF).

The results in Figure 4.2 and Figure 4.3 clearly indicate that the pH does affect the overall production of glucose (see Table 4.2).

Table 4.2: Glucose yield obtained during the saccharification step

	$\gamma_{Glucose/Starch}$ **(g.g^{-1})**	
pH	**55°C**	**65°C**
4.0	0.56	0.66
4.5	0.70	0.67
5.0	0.80	0.70

The glucose yields ($\gamma_{Glucose/Starch}$) presented in Table 4.1 and Table 4.2 were calculated by first determining the mass of glucose in a batch system using the equation (4.1).

$$c = \frac{m}{V} \qquad (4.1)$$

where c = concentration of glucose in g.L^{-1}

m = mass of glucose in g

V = volume in L

The mass of glucose obtained from equation (4.1) was subsequently divided by the mass of the starting material (i.e. starch) in grams to get the glucose yield in g.g^{-1}.

As is shown in Table 4.1, pH 5.5 resulted in the highest glucose yield, followed by pH 6.0 and lastly pH 6.5 with the lowest glucose yield. During the saccharification step, pH 5.0 resulted in the highest glucose yield followed by pH 4.5 and 4.0 in a decreasing order for both temperatures investigated (see Table 4.2). During the Saccharification step it was also observed that the substrate gets depleted as the process progresses. This was also revealed by the little variation in glucose concentration at different sampling time (Figure 4.2 and Figure 4.3).

In the same observation, temperature has also proven to have a significant effect on the glucose production. During saccharification two temperatures (55°C and 65°C) were investigated for their effect on glucose production at pH 5.0 and the results are presented in Figure 4.4.

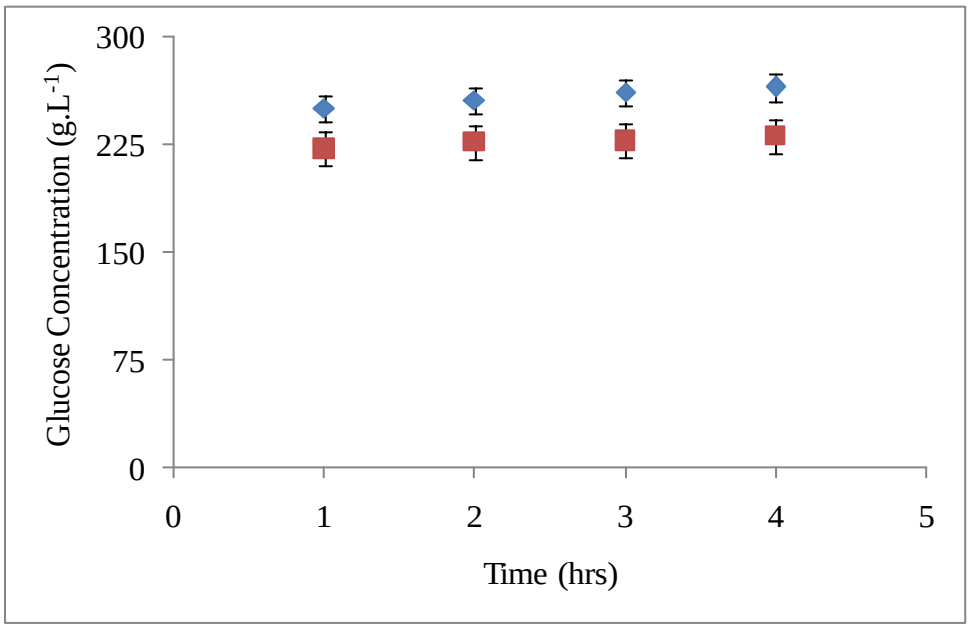

Figure 4.4: Influence of temperature on glucose concentration during saccharification at a pH 5 (♦**:** Temp. 55ºC; ■**:** Temp. 65ºC)

From Figure 4.4 it can be observed that Spirizyme Fuel was more active and productive at 55ºC (6.25 $g.L^{-1}.s^{-1}$) than at 65ºC (3.75 $g.L^{-1}.s^{-1}$), hence the higher glucose concentration at 55°C. The higher rate of production of glucose at low temperature emphasises the influence of temperature on enzyme activity. The experimental error observed during the saccharification step was 3.1% (see Appendix E).

From the foregoing results, the best conditions for liquefaction and saccharification could be identified. The best conditions are summarized in Table 4.3.

Table 4.3: The best experimental conditions for the hydrolysis step

Conditions	**Processes**		
	Gelatinization	**Liquefaction**	**Saccharification**
pH	-	5.5	5.0
Temperature (°C)	90-100	95	55
Time (hrs)	0.33	1	4
Ratio (Maize meal: water)	1:3	-	-
Enzyme concentration (v/w of starch)	-	0.7	0.7
Enzymes	-	Termamyl SC	Spirizyme Fuel

The experimental conditions presented in Table 4.3 were used for all subsequent fermentation experiments as well as the SFS process experiments.

4.1.3 The fermentation step

4.1.3.1 Fermentation of a traditional hydrolysate

One temperature (30ºC) was chosen to perform all fermentation experiments. In most reported cases (Kaseno *et al.*, 1998; Öhgren *et al.*, 2006; Chen *et al.*, 2008) the optimum temperature for the fermentation process is 30ºC. All fermentation experiments were done according to the procedure described in Section 3.2.5.

The influence of yeast concentration on bioethanol production was investigated by varying the yeast concentration between 2 $g.L^{-1}$ and 7 $g.L^{-1}$. The hydrolysate produced through the hydrolysis process was fermented using the different yeast concentrations. The fermentation

error observed in this study was 25.9% (see Appendix E). Figure 4.5 presents the different bioethanol concentrations obtained during the fermentation of the hydrolysate using the different yeast concentrations.

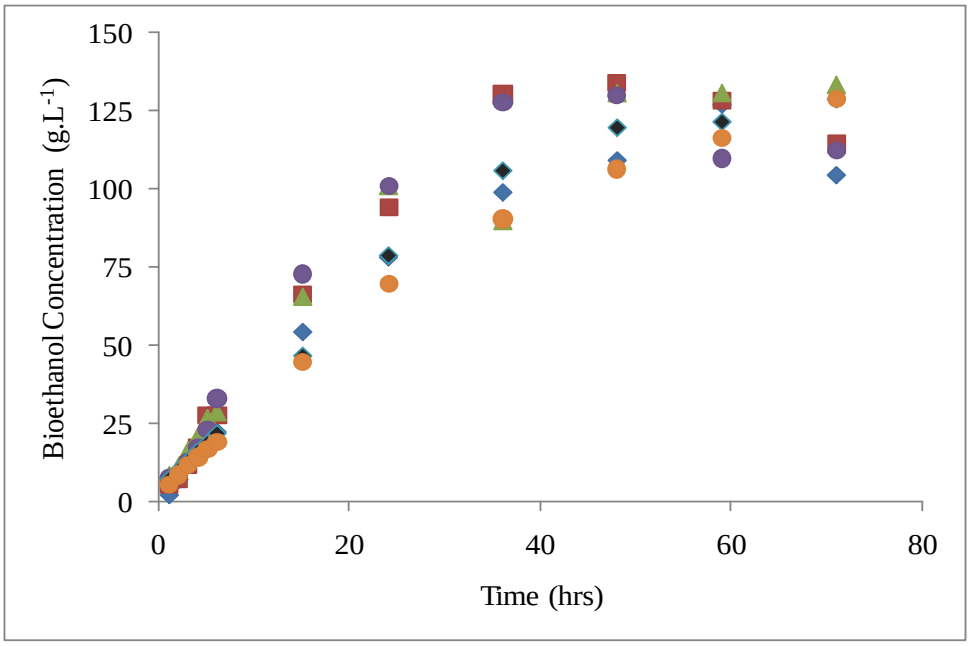

Figure 4.5: Influence of yeast concentration on bioethanol production during the fermentation of the hydrolysate (●**:** 2 g.L^{-1}; ♦**:** 3 g.L^{-1}; ◆**:** 4 g.L^{-1}; ■**:** 5 g.L^{-1}; ▲**:**6 g.L^{-1}, ●**:**7 g.L^{-1})

The highest bioethanol concentration obtained from the fermentation of the hydrolysate at pH 4.0 using the different yeast concentrations was 134 g.L^{-1}. This concentration was produced after 48 hours of fermentation with the yeast concentration of 5 g.L^{-1}. Both the 2 g.L^{-1} and 3 g.L^{-1} yeast concentration produced the same bioethanol concentration of 129 g.L^{-1} after 71 hours of fermentation. The data plotted in Figure 4.5 correlate with the findings by Mojović *et al.* (2006) in that no matter how much yeast you begin with, the concentration of the end product (bioethanol) will almost be the same; however, the time each concentration takes to reach the maximum concentration will differ. Cheng *et al.* (2007) confirmed the findings of Mojović *et al.* (2006) in that the different yeast concentrations only affect the duration of the fermentation process to reach the maximum bioethanol concentration. High yeast

concentrations have been shown to reach the maximum bioethanol concentration in less time than low yeast concentrations. Mojović *et al.* (2006) and Nikolić *et al.* (2009) reported the same bioethanol concentration of 80 $g.L^{-1}$ after 48 hours of fermentation, which was low compared to 134 $g.L^{-1}$ obtained in this study.

The bioethanol yields obtained from using different yeast concentrations are therefore, presented in Figure 4.6.

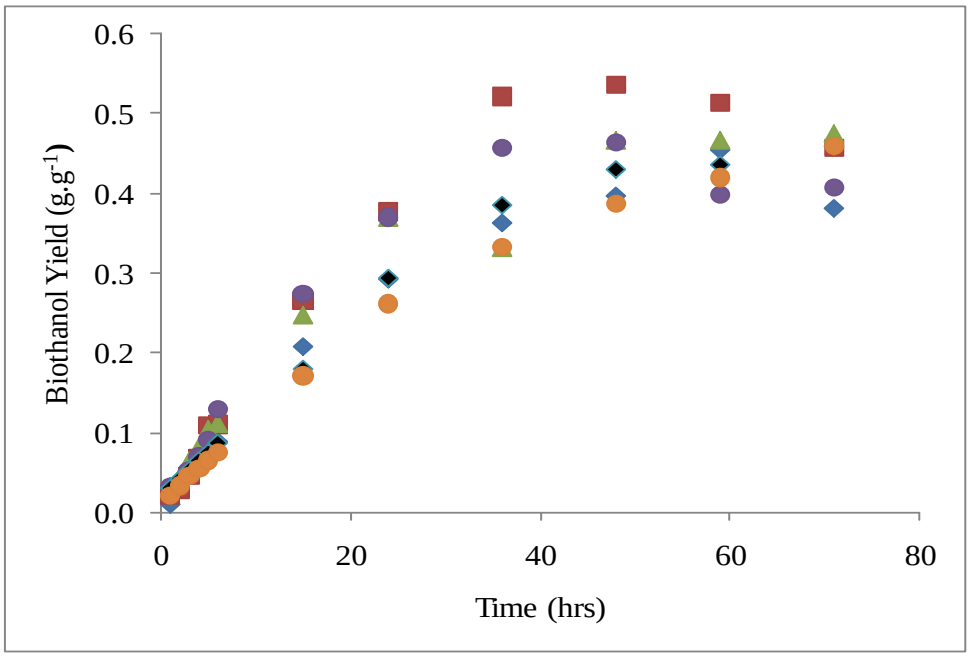

Figure 4.6: The fermentation yield of bioethanol using the different yeast concentration

(●: 2 $g.L^{-1}$; ♦: 3 $g.L^{-1}$; ♦: 4 $g.L^{-1}$; ■: 5 $g.L^{-1}$; ▲: 6 $g.L^{-1}$; ●:7 $g.L^{-1}$)

Bioethanol production, as shown in Figure 4.6, is directly proportional to the amount of yeast used. High yeast concentration gave a high production rate during the initial hours of fermentation. However, as the process progresses the yeast starts experiencing stresses as a result of change in broth composition (Dombek and Ingram, 1987; Alterthum *et al.*, 1989). The change in broth composition is a result of nutrient deficiency, formation of by-products and high bioethanol concentration. The change in broth composition has a direct effect on the yeast activity and the production rate. Alterthum *et al.* (1989) reported that a decline in

bioethanol production rate was due to stress induced on yeast by change in broth. According to Dombek and Ingram (1986) and Alterthum *et al.* (1989), high concentrations of bioethanol in the broth and the accumulation of by-products together with the depletion of nutrients affect the performance of yeast. Amongst the different factors that affect yeast performance, bioethanol is regarded as the major factor**.** Bioethanol inhibits the activity of yeast. During inhibition the enzyme secreted by the yeast is responsible for degrading sucrose to glucose and fructose is inactivated together with the glycolytic enzymes responsible for converting glucose to pyruvate (Dombek and Ingram, 1986; Alterthum *et al.*, 1989; Russel, 2003, Shafaghat *et al.*, 2009).

The production rate of bioethanol was calculated (using equation (4.2)) and the glucose concentration was monitored throughout the fermentation process.

$$\text{Rate of Production} = \frac{dC_p}{dt} = \frac{C_{n+1} - C_n}{T_{n+1} - T_n} \qquad (4.2)$$

Where dC_p = Change in bioethanol concentration ($g.L^{-1}$)

dt = Change in time (hrs)

n = 1

The change in bioethanol concentration over the change in time (dCp/dt) was used to calculate the rate of bioethanol production during fermentation (see Appendix D). The data in Table D.1 to Table D.6 indicate that as the fermentation process progressed the rate at which bioethanol was produced was slowly decreasing due to high bioethanol concentrations and the depletion of substrate in the broth. Alterthum *et al.* (1989) also highlighted that the accumulation of bioethanol in the fermentation broth occurs at the same time as the shift in metabolism from balance to stationary phase.

The mass balance of the fermentation process together with the fermentation efficiency is given in Table 4.4.

Table 4.4: The fermentation process mass balance

Yeast Concentration ($g.L^{-1}$)	**$\gamma_{EtOH/glucose}$ ($g.g^{-1}$)**	**$\gamma_{CO2/glucose}$ ($g.g^{-1}$)**	**$\gamma_{cells/glucose}$ ($g.g^{-1}$)**	**Fermentation Efficiency (%)**
2	0.46	0.44	0.10	90.2
3	0.46	0.44	0.10	90.2
4	0.46	0.44	0.10	90.2
5	0.48	0.42	0.10	94.1
6	0.47	0.43	0.10	92.2
7	0.46	0.44	0.10	90.2

The bioethanol yield in Table 4.4 was calculated by first determining the mass of bioethanol produced using equation (4.3).

$$m_{(EtOH)} = \frac{m_{(Broth)} * x}{1 + 0.957 * x} \qquad (4.3)$$

Where x = bioethanol mass fraction (wt %),

$m_{(broth)}$ = mass of the broth (g)

0.957 = Molar ratio of EtOH over CO_2

The mass of bioethanol obtained from equation (4.3) was later divided by the mass of the starting biomass (i.e. maize) to get the bioethanol yield in $g.g^{-1}$.

In order to determine the yield of CO_2, the mass of CO_2 produced during fermentation had to be established. Theoretically,

$$1 \text{ mol Glucose} \rightarrow 2 \text{ mol Ethanol} + 2 \text{ mol } CO_2 \quad (4.4)$$

Therefore, equation (4.5) was used as a basis to derive an equation to calculate the mass of CO_2.

$$n = \frac{m}{M} \quad (4.5)$$

Where n = the number of moles,

m = mass in grams and,

M = the molar mass

Using the theoretical relationship presented above, equation (4.6) was developed,

$$\frac{n_{(EtOH)}}{n_{(CO2)}} = \frac{m_{(EtOH)}M_{(CO2)}}{M_{(EtOH)}m_{(CO2)}} = 1 \quad (4.6)$$

From equation (4.6), the equation (4.7) was derived,

$$m_{(CO2)} = \frac{m_{(EtOH)}M_{(CO2)}}{M_{(EtOH)}} \quad (4.7)$$

The mass of CO_2 calculated from equation (4.6) was divided by the mass of the starting biomass to determine the yield. The sum of the bioethanol yield and the CO_2 yield adds up to 0.9 $g.g^{-1}$, therefore an assumption was made that the remaining 0.1 $g.g^{-1}$ goes to the maintenance and growth (i.e. γ cells/glucose) of the yeast cells, since it could not be

calculated directly. The fermentation efficiency was calculated by dividing the bioethanol yield obtained in the study by the theoretical yield value (i.e. 0.51 g). The fermentation efficiency reported in this study was slightly higher (90-94%) than 76.8% and 89.2 % reported by Nikolić *et al.* (2009) and Mojović *et al.* (2006), respectively. This efficiency is an indication that the conditions chosen were conducive to fermentation.

The data in Table 4.4 emphasize the point that, irrespective of the yeast concentration used to ferment the substrate, the product yield will always be the same, but the time each yeast concentration takes to reach the maximum yield will differ.

4.1.3.2 Determination of fermentation kinetics

During the fermentation process, the yeast undergoes at least four different phases, namely, lag, exponential, stationary and death phases (Diaz *et al*., 1999). It is generally believed that bioethanol production takes place during the stationary phase of the yeast's life cycle (Russel, 2003). No growth of yeast takes place during the stationary phase (Russel, 2003) and thus the assumption can be made that most of the substrate consumed during this phase can be directly related to the production of bioethanol with only a small portion allocated to the maintenance of cells or to increase the cell mass (Russel, 2003). Bioethanol production by *S. cerevisiae* is product-limiting (Kaseno *et al.*, 1998; Bai *et al*., 2008). This means that the cells will start to enter the death phase when the bioethanol concentration in the broth becomes high enough to be poisonous to the yeast (Dombek and Ingram, 1986; Alterthum *et al*., 1989). Although there are a number of equations that can be used to account for the growth of cells during the exponential growth phase and the rate of bioethanol production during the stationary phase, the most commonly used equation for product formation during the non-growth stationary phase is the Monod equation (see equation (4.8)).

$$r_p = \frac{K_p C_g C_c}{K_{sn} + C_g} \quad (4.8)$$

Where r_p = Ethanol production rate ($g.L^{-1}.s^{-1}$)

k_p = Specific rate constant with respect to product ($L.g^{-1}.s^{-1}$)

K_{sn} = Monod constant ($g.L^{-1}$)

C_g = Glucose concentration ($g.L^{-1}$)

C_c = Cell concentration ($g.L^{-1}$)

Equation (4.8) can be linearised to yield equation (4.9)

$$\frac{C_c}{r_p} = \frac{K_{sn}}{K_p} + \frac{C_g}{K_p C_c} \quad (4.9)$$

The K_p and the K_{sn} constants were determined by regression analysis. The statistic version of Excel 2007 with standard least squares method was used to regress experimental data to obtain k_p and K_{sn}. The results of the regression are presented in Figure D.1 to D.6 and Table 4.5.

Table 4.5: Kinetic parameters obtained by linear regression of fermentation data

Yeast Concentration **($g.L^{-1}$)**	**Specific rate constant** **($L.g^{-1}.s^{-1}$)**	**Monod constant** **($g.L^{-1}$)**
2	0.80	5.6
3	1.01	6.5
4	2.20	24.8
5	1.10	20.4
6	2.05	94.2
7	1.60	27.1

It can be seen from Table 4.5 that the yeast concentration significantly influences the Monod constant, while having less effect on the specific reaction rate. As shown in Table 4.5 there is no indication as to how the yeast concentration affects the specific reaction rate. According to Cooney and McDonald (1993) the Monod constant is greatly influenced by the composition of the medium and the exposure of organism to the medium. The Monod constant also acts as the representative to interdependent reactions from nutrient uptake into the cell to cell metabolism reactions. The data in Table 4.5 indicates that the Monod constant was also influenced by the cell concentration. However, it is not clear as to why the Monod constant increases with an increase in cell concentration. There is no clear explanation as to how the yeast concentration affects Monod constant during the synthesis of bioethanol. Liu (2006) reported that the variation in the Monod constant value was due to mass transfer and that the alteration of any factor influencing the yeast and substrate interaction would greatly affect the determination of the Monod constant. On the other hand, the substrate uptake influences the specific growth rate which can be used to determine maximum specific rate (Liu, 2006). The Monod constant can therefore be thought of as a fitting parameter.

4.1.3.3 Fermentation of a filtered hydrolysate

Unconverted starch granules will clog and damage the membrane in the SFS process. The starch granules thus needed to be filtered from the hydrolysate. The influence of using a filtered hydrolysate on the final bioethanol concentration was investigated by filtering the hydrolysate after saccharification and adding the yeast to the supernatant mixture. The bioethanol yield from the supernatant mixture was compared to the bioethanol yield obtained from an unfiltered hydrolysate. The results are shown in Figure 4.7.

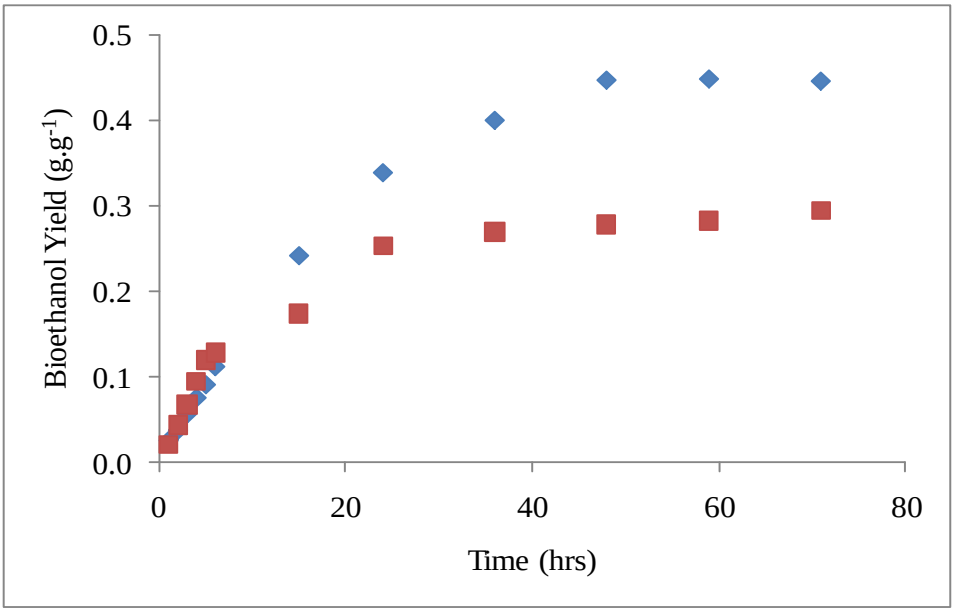

Figure 4.7: Bioethanol yield ($g.g^{-1}$) obtained from fermentation of a traditional broth (slurry) and a filtered broth (supernatant) using 5 $g.L^{-1}$ yeast concentration (■: Supernatant; ♦: Slurry)

As anticipated, a lower bioethanol yield was produced with the supernatant. This was due to the fact that 15 wt% of glucose was lost in the filtration step. The supernatant produced a maximum yield of 0.29 $g.g^{-1}$ after 71 hours of fermentation compared to 0.45 $g.g^{-1}$ of traditional hydrolysate (slurry). As a result of removing the solids present in the slurry, a higher initial yield was observed with the supernatant. The removal of solids from the hydrolysate presented the yeast with better access to the glucose molecules.

4.2 The simultaneous fermentation and separation (SFS) process

The final objective of this study was to minimize the inhibition of *S. cerevisiae* by bioethanol through the SFS process. The SFS process creates a suitable environment for the yeast cells to continue producing bioethanol in a batch system until all sugars are consumed (O'Brien and Craig, 1996; O'Brien *et al.*, 2004).

The SFS process was performed according to the procedure described in Section 3.3.2.1. The hydrolysate obtained through the hydrolysis of maize meal was subsequently fermented using 5 $g.L^{-1}$ baker's yeast during the SFS process. The activated baker's yeast was added to the hydrolysate in the fermenter tank and left to ferment for 15 hours to allow the production of bioethanol up to 6 wt%. The membrane was subsequently saturated by circulating the fermentation broth across a polymer membrane for 4 hours. After 4 hours of membrane saturation the first permeate was collected. During this time the concentration of bioethanol in the broth was 64.5 $g.L^{-1}$ and remained at the same concentration throughout the experiment. At this concentration the inhibitory conditions of yeast as reported by O'Brien and Craig (1996) are minimal. O'Brien and Craig (1996) maintained the bioethanol concentration in the broth between 40 $g.L^{-1}$ and 60 $g.L^{-1}$ in a batch system. O'Brien *et al.* (2004) maintained the bioethanol concentration in the broth at 25 $g.L^{-1}$ throughout pervaporation in a fed batch system. Kaseno *et al.* (1998) kept the bioethanol concentration in the broth at 50 $g.L^{-1}$. Therefore, 64.5 $g.L^{-1}$ was in line with other researchers' methods.

All permeates were collected hourly with 2 samples taken per sampling (in the broth and permeate). The results showing the membrane flux and selectivity of the membrane towards bioethanol from the broth are presented in Figure 4.8.

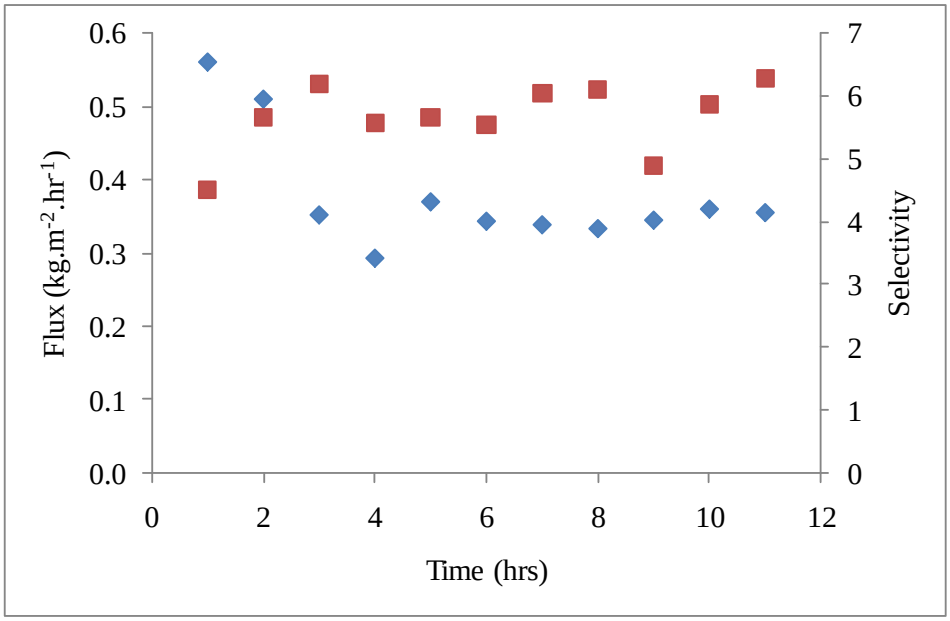

Figure 4.8: Pervaporation results of the fermentation broth showing membrane flux and selectivity **(♦:** Flux; **■:** Selectivity)

A selectivity of 5.7 was obtained with a steady state flux of 0.35 $kg.m^{-2}.hr^{-1}$ after 11 hours of operation. O'Brien and Craig (1996) obtained a higher flux of 0.74 $L.m^{-2}.hr^{-1}$ and a poor selectivity of 2.6 using a polydimethyl-siloxane (MPF-50) membrane at 35°C after 26.1 hours of pervaporation. O'Brien *et al.* (2004) obtained a low flux of 0.135 $kg.m^{-2}.hr^{-1}$ and a good selectively of 7.7. Kaseno *et al.* (1998) reported a high flux of 1.4 $kg.m^{-2}.hr^{-1}$ and a selectivity of 2.3. The observations presented above indicate that high flux is associated with low selectivity. This is, however, not always the case, there are exceptions but generally flux is inversely proportional to selectivity.

On the other hand, the membrane used in this study showed great stability throughout the SFS process. Fadeev *et al.* (2003) observed a decline in flux and selectivity after exposing the membrane to the fermentation broth. The decline in the performance (i.e. in flux and selectivity) of the membrane as reported by Fadeev *et al.* (2003) is a result of interaction of the membrane with the fermentation process by-products such as the acetic acid, glycerol, acetate, lactate (Fadeev *et al.*, 2003). A strong interaction of acetic acid with the membrane according to Fadeev *et al.* (2003) may lead to structural alteration of the membrane characteristic from its hydrophobic state to a hydrophilic state, thus lowering the flux and

selectivity in the process. The glycerol (a major by-product of fermentation) and low volatile components of fermentation are also responsible for a decline in membrane flux (Fadeev *et al.*, 2003). Contrary the findings of Fadeev *et al.* (2003), no significant decline in membrane performance (flux and selectivity) was observed in this study when the membrane was exposed to the fermentation broth. The membrane was proven to be stable throughout the experiment. The bioethanol mass fraction in the broth and permeate as well as the membrane flux with respect to time are presented in Figure 4.9.

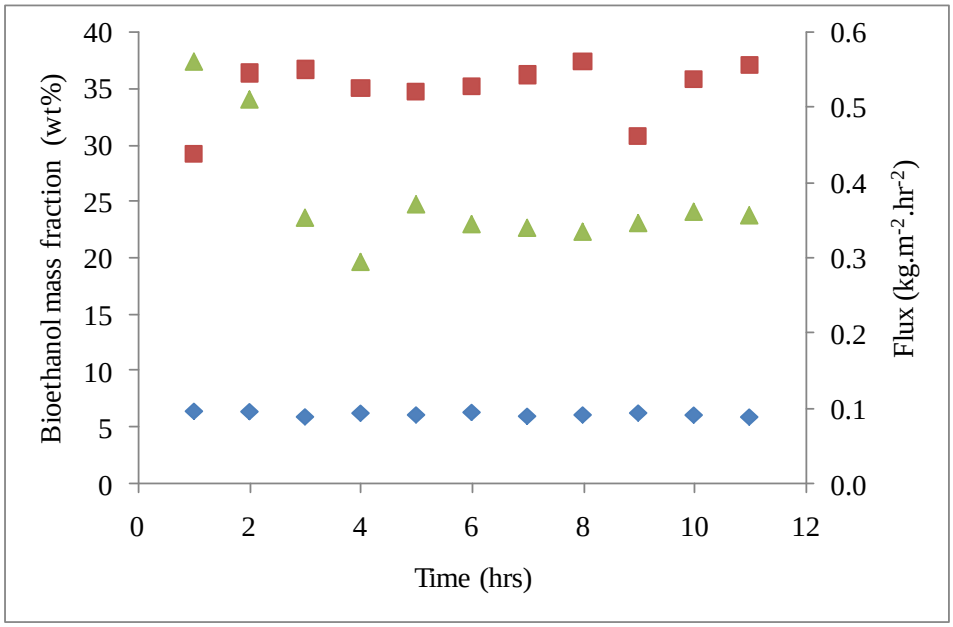

Figure 4.9: Bioethanol weight fraction in the broth (♦) and the permeate (■) during the SFS process, Flux (▲)

The bioethanol mass fraction in the broth was kept between 5.9 wt% and 6.5 wt% as shown in Figure 4.9. This data demonstrate that as the bioethanol was being separated (as permeate) from the broth, some was produced, hence the stable bioethanol fraction in the broth. The low bioethanol fraction in the broth kept the yeast active throughout the SFS process; however, the overall yield was not improved. An average bioethanol fraction of 35.5 wt% in the permeate was obtained in this study, which was higher than 23 wt% and 33.5 wt% reported

by Kaseno *et al.* (1998) and O'Brien and Craig (1996). The mass of bioethanol recovered in the permeate at the end of 11 hours of pervaporation was 0.02 kg.

The bioethanol yields obtained through the traditional batch fermentation and the SFS process were compared and the results are plotted in Figure 4.10.

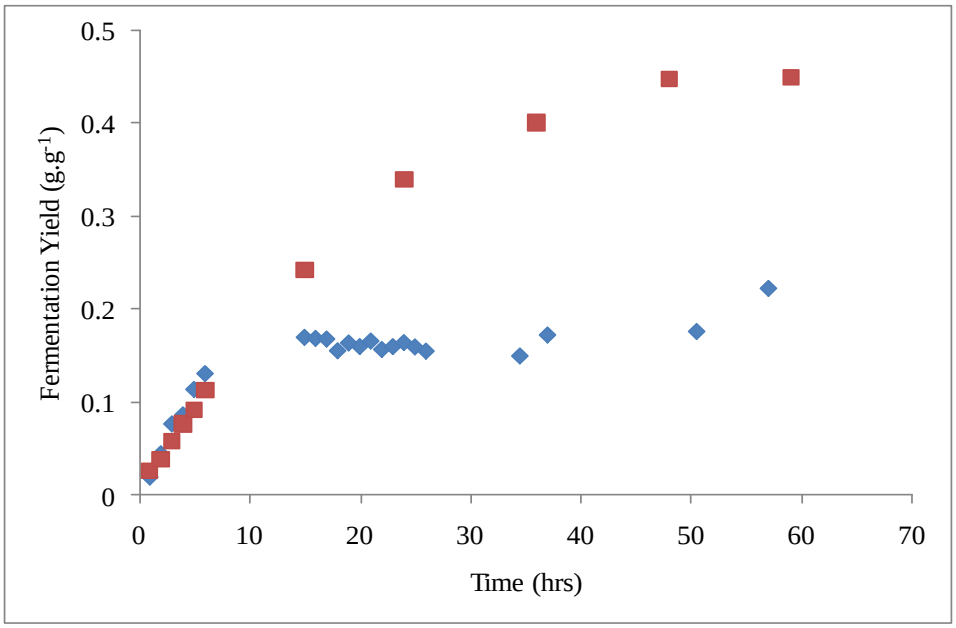

Figure 4.10: Traditional batch fermentation versus the SFS process with regard to bioethanol yield (♦: SFS process, ■: Traditional batch fermentation)

The bioethanol yield obtained during the SFS process was less than half of the bioethanol yield obtained from the traditional batch fermentation process. The traditional batch fermentation process produced a high bioethanol yield of 0.45 $g.g^{-1}$ compared to 0.22 $g.g^{-1}$ obtained through the SFS process. The yield (0.22 $g.g^{-1}$) obtained through the SFS process did not vary much compared to that obtained by the batch fermentation (0.29 $g.g^{-1}$) with the supernatant as substrate. The filtration of the hydrolysate thus had a significant negative effect on the final bioethanol yield during the SFS process. During the filtration step a large amount of glucose, as mentioned in Section 4.1.3.3, was lost and this resulted in a decrease in the overall amount of glucose in the clear hydrolysate, which eventually affected the overall bioethanol yield. The results of the SFS process clearly demonstrated that glucose is indeed a

major nutrient or source of carbon for yeast fermentation; and, deficiency of glucose in the broth will result in low bioethanol yields. Similar observations were reported by Cheng *et al.* (2007) after performing a study on the effect of substrate concentration on the bioethanol yield. Cheng *et al.* (2007) reported that a high glucose concentration medium produced the highest bioethanol yield compared to a lower glucose medium concentration. It can also be mentioned that in this study glucose was the limiting factor during the SFS process. Despite the low bioethanol yield, the SFS process demonstrated its ability of keeping the bioethanol concentration below the inhibitory concentrations without any addition of water. Current bioethanol producers use high water dilutions to keep the bioethanol concentration in the broth less harmful to the yeast. This addition of water in the broth increases the cost of product recovery in the downstream process at the end of fermentation. Low bioethanol concentrations in the fermentation broth require more energy to recover the product than a highly concentrated medium. A highly concentrated permeate of 37.3 wt% bioethanol concentration obtained in this study will require less energy to further concentrate it to +99.99 wt% bioethanol, than the bioethanol concentration obtained from traditional batch fermentation, which is usually between 12 wt% and 13.5 wt%. Using pervaporation to recover bioethanol from the fermentation broth has a great potential of reducing the overall cost of the downstream process. All the bioethanol produced during the fermentation process can be evaporated through pervaporation to form a highly concentrated permeate of over 30 wt%, making it easy to further concentrate for later application.

4.3 Conclusion

The best experimental conditions for starch hydrolysis were determined (see Table 4.3). The best pH for liquefaction and saccharification were determined to be pH 5.5 and pH 5.0 respectively (see Table 4.1 and Table 4.2). The traditional batch fermentation process was performed successfully and the results were comparable with literature. The average bioethanol concentration obtained in this study was above 12 wt% for most of the traditional batch fermentation experiments except when the supernatant was used as a substrate. The highest concentration produced from traditional batch fermentation using the supernatant as a substrate was 11.2 wt% (0.29 $g.g^{-1}$). Part of the main objective of the study was achieved, i.e. minimizing the inhibitory reactions for yeast, but the overall yield was not improved when the batch SFS process was used. Kinetics has shown that at the end of the traditional batch

fermentation process the glucose concentration was almost depleted, which explains why the overall yield of the SFS process was not improved. In addition to the data given by the kinetics, the filtration step was also responsible for 15 wt% glucose loss, which also contributed to decrease in the overall yield of the SFS process.

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CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

"*Life does not require us to make good; it asks only that we give our best at each level of experience"* Harold Ruopp

"Satisfaction lies in the effort, not in the attainment, full effort is full victory"
Mahatma Ghandhi

OVERVIEW

In this chapter the conclusions drawn from this study and the recommendations for further investigation are presented. This chapter is divided into two sections. The conclusions based on the outcome of the study are presented in Section 5.1 and recommendations for future experimental work are suggested in Section 5.2.

5.1 Conclusions

The study was undertaken in an attempt to improve the efficiency of the fermentation process by reducing the inhibitory effects of bioethanol on the fermenting organism (*S. cerevisiae*) in a batch fermentation system coupled with pervaporation. The following conclusions were made, based on the objectives and the results obtained in the study

- The pH 5.5 and 5.0 were determined as the optimum pH for Termamyl SC and Spirizyme Fuel enzyme mixture, respectively. The effect of temperature on glucose concentration during the saccharification step was also investigated. The results showed that 55ºC was the optimum temperature for Spirizyme Fuel enzyme mixture at a pH of 5.0.
- The effect of yeast concentration on bioethanol yield was investigated using 6 different yeast concentrations. Amongst the different yeast concentrations investigated, 5 $g.L^{-1}$ produced the highest bioethanol yield after 48 hours of fermentation. The 5 $g.L^{-1}$ was therefore considered as the best yeast concentration to carry out all subsequent fermentation experiments.
- It was also demonstrated that yeast concentration does not have an effect on the final bioethanol concentration in a batch system but does affect the duration of the fermentation process. With low yeast concentrations it took longer to reach the maximum bioethanol yield.
- The highest bioethanol yield obtained with a supernatant was 0.29 $g.g^{-1}$ compared to 0.45 $g.g^{-1}$ with the traditional slurry. The bioethanol yield of the supernatant was greatly affected by the loss of glucose molecules during the filtration step. Approximately 15 wt% of glucose was lost during the filtration step of the hydrolysate.
- Membrane screening experiments were conducted to determine the best membrane for the separation of bioethanol in an ethanol-water mixture. PERVAP®4060 membrane performed well, compared to the other membranes investigated. The PERVAP®4060 membrane produced the highest average selectivity of 8 with the average flux of 0.3 $kg.m^{-2}.hr^{-1}$. The high selectivity was a clear indication that the membrane was preferentially permeating bioethanol, with the highest permeate concentration obtained being 37.3 wt%.

- Bioethanol was produced and maintained between 59 $g.L^{-1}$ and 65 $g.L^{-1}$ throughout the SFS process. The SFS process produced a bioethanol yield of 0.22 $g.g^{-1}$.
- The SFS process did not produce more bioethanol than the traditional batch process but did demonstrate its ability to keep the concentration of bioethanol in the broth below harmful levels and to produce highly concentrated permeates.

5.2 Recommendations

As it has been demonstrated, the SFS process was less efficient compared to the traditional batch fermentation using maize meal starch as a source of carbon for bioethanol production. The following recommendations are suggested for future work:

- The substrate (i.e. maize meal starch) used in this study had a huge effect on the yield of the SFS process. Due to the filtration step approximately 15 wt% of glucose molecules were lost which resulted in lower sugar content in the supernatant to be fermented. Using a different substrate which does not require hydrolysis and filtration will improve the overall yield of the SFS process.

- The fed batch system has the potential of improving the yield and reducing the cost of fermentation. During the SFS process the yeast demonstrated the ability to retain its activity after long hours of pervaporation. Therefore, during the fed batch system the same yeast can be used repeatedly throughout the process without the addition of fresh yeast but only the substrate.

APPENDIX A

PREPARATION OF STANDARDS

A.1 Preparation of standard solutions

Stock solutions containing 200 $g.L^{-1}$ of fructose, glucose, and sucrose were prepared. The stock solutions were diluted to different concentrations as shown in Table 1.A for the calibration curves.

Table A.1: Dilution of stock solution to different concentrations for calibration curves

Concentration ($g.L^{-1}$)	**Volume Stock (mL)**	**Volume Water (mL)**
200	5	0
100	2.5 ml of 200 $g.L^{-1}$	2.5
50	2.5 ml of 100 $g.L^{-1}$	2.5
25	2.5 ml of 50 $g.L^{-1}$	2.5
12.5	2.5 ml of 25 $g.L^{-1}$	2.5
6.25	2.5 ml of 12.5 $g.L^{-1}$	2.5

The prepared sugar solutions in Table A.1 were analysed using the HPLC with each concentration injected three times. The average areas against concentration were plotted as shown in Figure 3.12 to Figure 3.14. Table A.2 presents the average areas of the sugar solutions analysed using the HPLC.

Table A.2: The average area of the standard sugar solutions

Concentration ($g.L^{-1}$)	**Avg. Area Glucose (nRUI*s)**	**Avg. Area Sucrose (nRUI*s)**	**Avg. Area fructose (nRUI*s)**
6.25	$3.74e10^5$	$5.08x10^5$	$7.85x10^5$
12.5	$8.14e10^5$	$8.34x10^5$	$1.15x10^6$
25	$1.74e10^6$	$1.55x10^6$	$1.81x10^6$
50	3.42e106	$2.89x10^6$	$3.13x10^6$
100	$7.16e10^6$	$5.66x10^6$	$5.84x10^6$
200	$1.46e10^7$	$1.13x10^7$	$1.12x10^7$

A.2 Preparation of ethanol standard solution

A commercially available ethanol (99.95 wt%) was used to prepare the different concentrations of ethanol standards for the calibration curve which was later used to identify and quantify the bioethanol produced during the fermentation process. The standard ethanol solutions were prepared on a weight per weight basis as shown in Table A.3.

Table A.3: Preparation of ethanol standard solution

Ethanol mass fraction (wt%)	**Mass of Ethanol (g)**	**Mass of Water (g)**
20	2.0	8.0
13	1.3	8.7
10	1.0	9.0

0.7	0.7	9.3
0.5	0.5	9.5
0.3	0.3	9.7
0.1	0.1	9.9

A 10 wt% solution mixture of 1,4-dioxane was prepared and used as an internal standard. All samples were diluted twofold with this solution. Each concentration was injected three times in the GC and the ratio(s) of the two solvents (ethanol and 1,4-dioxane) were plotted against ethanol concentration. Average ratios in Table A.4 were plotted as shown in Figure 3.15.

Table A.4: GC analysis results of the ethanol standard solutions

Ethanol Concentration (wt %)	**Ratio (A_E/A_D)**
1	0.092
3	0.287
5	0.491
7	0.682
10	1.028
13	1.201
20	1.980

E= ethanol & D= 1,4-dioxane

APPENDIX B

PERVAPORATION MEMBRANE SCREENING EXPERIMENTS

The separation efficiency of ethanol from an ethanol-water mixture using PERVAP®2211, PERVAP®4101 and PERVAP®4060 membrane was investigated. The ethanol-water solution mixture was circulated across a membrane area of 0.0048 m^2 to saturate the membrane. Full data on the results obtained during the screening experiments is presented in Table B1 to B3.

Table B.1: Screening results of PERVAP®2211 membrane

Time (hrs)	**c Feed (wt %)**	**c Perm (wt %)**	**m Perm (kg)**	**Flux ($kg.m^{-2}.hr^{-1}$)**	**Selectivity**
0.5	9.8	9.6	0.00349	1.461	0.98
1	10	8.1	0.00143	0.600	0.80
1.5	10	8.8	0.00138	0.579	0.87
2.0	9.6	9.2	0.00114	0.477	0.95
2.5	10.2	9.3	0.00131	0.547	0.90
3.0	10.2	9.8	0.00110	0.462	0.96
3.5	10.3	10.3	0.00116	0.485	1.00
4.0	9.9	10.9	0.00110	0.459	1.11
4.5	9.8	10.5	0.00133	0.556	1.08
5.0	10.4	10.5	0.00120	0.503	1.01
5.5	10.2	11.3	0.0099	0.414	1.12
6.0	10.3	11.2	0.00108	0.451	1.10

Table B.2: Screening results of PERVAP®4101 membrane

Time (hrs)	c Feed (wt %)	c Perm (wt %)	m Perm (kg)	Flux ($kg.m^{-2}.hr^{-1}$)	Selectivity
0.5	9.8	5.7	0.00149	0.623	0.56
1	10.3	2	0.00059	0.247	0.18
1.5	9.3	3	0.00059	0.248	0.30
2.0	10.1	2.5	0.00055	0.230	0.23
2.5	10.2	3.5	0.00058	0.244	0.32
3.0	10.2	3	0.00055	0230	0.27
3.5	10.3	3.3	0.00055	0.228	0.3
4.0	9.9	2.4	0.00046	0.192	0.22
4.5	10	3.9	0.00054	0.224	0.37

Table B.3: Screening results of PERVAP®4060 membrane

Time (hrs)	c Feed (wt %)	c Perm (wt %)	m Perm (kg)	Flux ($kg.m^{-2}.hr^{-1}$)	Selectivity
0.5	9.75	15.84	0.00156	0.654	1.7
1	9.75	45.08	0.00077	0.321	7.6
1.5	9.85	44.68	0.00074	0.309	7.4
2.0	9.85	50.36	0.00066	0.278	9.3
2.5	9.95	47.52	0.00067	0.282	8.2
3.0	9.85	45.92	0.00077	0.320	7.8
3.5	9.65	45.48	0.00064	0.267	7.8
4.0	9.75	45.08	0.00072	0.303	7.6
4.5	9.65	48.32	0.00075	0.315	8.8

APPENDIX C

THE SFS EXPERIMENTAL RESULTS

The SFS process experiment was conducted according to the procedure described in Section 3.3.2.1. The raw data of the results is presented in Table C.1.

Table C.1: Experimental results of the SFS process

Time (hrs)	**c Feed (wt %)**	**c Perm (wt %)**	**m Perm (kg)**	**Flux ($kg.m^{-2}.hr^{-1}$)**	**Selectivity**
1	6.5	29.2	0.002678	0.56	4.5
2	6.4	36.3	0.002437	0.51	5.7
3	5.9	36.6	0.001685	0.35	6.2
4	6.3	35.0	0.001404	0.29	5.6
5	6.1	34.6	0.00177	0.37	5.7
6	6.3	35.1	0.001644	0.34	5.5
7	6.0	36.1	0.001621	0.34	6.0
8	6.1	37.3	0.001596	0.33	6.1
9	6.3	30.8	0.001651	0.35	4.9
10	6.1	35.7	0.001723	0.36	5.9
11	5.9	37.1	0.0017	0.36	6.3

The amount of bioethanol recovered per sampling during pervaporation is given in Table C.2.

Table C.2: Bioethanol recovered during the SFS process experiment

Permeate	**m Perm (g)**	**c Perm (wt)%**	**m Ethanol/ permeate (g)**
1	2.678	29.1	0.779
2	2.437	36.3	0.885
3	1.685	36.6	0.617
4	1.404	34.9	0.490
5	1.77	34.6	0.612
6	1.644	35.1	0.577
7	1.621	36.1	0.585
8	1.596	37.2	0.594
9	1.651	30.7	0.507
10	1.723	35.7	0.615
11	1.7	37.0	0.629

APPENDIX D

FERMENTATION RESULTS

Table D.1 to Table D.7 present the raw data, as well as the calculated data from the batch fermentation experiments, followed by the regression results in Figure D.1 to Figure D.6.

Table D.1: Fermentation experiment using baker's yeast concentration of 2 $g.L^{-1}$

Time (hrs)	**Bioethanol (wt%)**	**Bioethanol ($g.L^{-1}$)**	**m (EtOH) (g)**	**Bioethanol yield ($g.g^{-1}$)**	**Glucose Concentration ($g.L^{-1}$)**	**dC_p/dt**	**C_g/r_p**	**C_g/C_c**
1	0.5	5.3	1.8	0.02	257.83	0.09	49.58	128.91
2	0.8	8.3	2.8	0.03	241.35	0.05	74.91	120.67
3	1.2	11.7	3.8	0.05	217.82	0.06	60.45	108.91
4	1.4	14.0	4.6	0.06	189.35	0.04	74.99	94.68
5	1.6	16.5	5.4	0.07	155.69	0.04	58.66	77.85
6	1.9	19.1	6.2	0.08	116.65	0.05	42.40	58.32
15	4.5	44.5	14.2	0.17	23.98	0.05	7.87	11.99
24	7.0	69.6	21.7	0.26	0.00	0.05	0.00	0.00

36	9.0	90.3	27.7	0.33	0.00	0.03	0.00	0.00
48	10.6	106.2	32.1	0.39	0.00	0.02	0.00	0.00
59	11.6	116.3	34.9	0.42	0.00	0.02	0.00	0.00
71	12.9	128.6	38.1	0.46	0.00	0.02	0.00	0.00

Table D.2: Fermentation experiment using baker's yeast concentration of 3 $g.L^{-1}$

Time (hrs)	**Bioethanol (wt%)**	**Bioethanol ($g.L^{-1}$)**	**m (EtOH) (g)**	**Bioethanol yield ($g.g^{-1}$)**	**Glucose Concentration ($g.L^{-1}$)**	**dC_p/dt**	**C_g/r_p**	**C_g/C_c**
1	0.7	7.4	2.4	0.03	253.598	0.12	34.45	84.53
2	1.0	10.2	3.4	0.04	233.142	0.05	75.36	77.71
3	1.3	13.1	4.3	0.05	206.685	0.05	67.38	68.90
4	1.6	16.2	5.3	0.06	173.708	0.06	52.13	57.90
5	1.9	18.8	6.1	0.07	135.249	0.05	48.26	45.08
6	2.2	21.9	7.1	0.09	90.246	0.06	26.98	30.08
15	4.7	46.6	14.9	0.18	0.000	0.05	0.00	0.00
24	7.9	78.7	24.4	0.29	0.000	0.06	0.00	0.00
36	10.6	105.7	32.0	0.39	0.000	0.04	0.00	0.00
48	11.9	119.5	35.7	0.43	0.000	0.02	0.00	0.00
59	12.1	121.3	36.2	0.44	0.000	0.00	0.00	0.00
71	12.9	128.6	38.1	0.46	0.000	0.01	0.00	0.00

Table D.3: Fermentation experiment using baker's yeast concentration of 4 $g.L^{-1}$

Time (hrs)	Bioethanol (wt%)	Bioethanol ($g.L^{-1}$)	m (EtOH) (g)	Bioethanol yield ($g.g^{-1}$)	Glucose Concentration ($g.L^{-1}$)	dC_p/dt	C_g/r_p	C_g/C_c
1	0.23	2.26	0.75	0.01	244.84	2.259	108.39	61.21
2	0.72	7.22	2.39	0.03	230.43	4.966	46.40	57.61
3	1.21	12.07	3.97	0.05	220.12	4.845	45.43	55.03
4	1.86	18.59	6.08	0.07	210.61	6.521	32.30	52.65
5	2.07	20.71	6.76	0.08	194.86	2.123	91.78	48.71
6	2.24	22.42	7.31	0.09	186.58	1.708	109.24	46.64
15	5.44	54.42	17.2	0.21	101.08	3.555	28.43	25.27
24	7.83	78.34	24.3	0.29	52.98	2.658	19.93	13.24
36	9.91	99.09	30.14	0.36	3.67	1.729	2.12	0.92
48	10.93	109.33	32.96	0.40	0.96	0.853	1.12	0.24
59	12.72	127.20	37.76	0.45	4.90	1.625	3.01	1.22
71	10.46	104.61	31.66	0.38	0.07	-1.883	-0.04	0.02

Table D.4: Fermentation experiment using baker's yeast concentration of 5 $g.L^{-1}$

Time (hrs)	**Bioethanol (wt%)**	**Bioethanol ($g.L^{-1}$)**	**m (EtOH) (g)**	**Bioethanol yield ($g.g^{-1}$)**	**Glucose Concentration ($g.L^{-1}$)**	**dC_p/dt**	**C_g/r_p**	**C_g/C_c**
1	0.53	5.34	1.78	0.02	236.24	5.344	44.21	47.25
2	0.73	7.28	2.42	0.03	232.80	1.934	120.38	46.56
3	1.16	11.62	3.87	0.05	216.79	4.343	49.92	43.36
4	1.70	16.95	5.65	0.07	208.51	5.334	39.09	41.70
5	2.74	27.38	9.12	0.11	195.29	10.425	18.73	39.06
6	2.76	27.57	9.18	0.11	177.94	0.188	948.53	35.59
15	6.61	66.15	22.03	0.27	83.38	4.287	19.45	16.68
24	9.41	94.11	31.34	0.38	29.07	3.107	9.36	5.81
36	13.02	130.16	43.34	0.52	2.66	3.004	0.89	0.53
48	13.38	133.81	44.56	0.54	1.24	0.305	4.06	0.25
59	12.81	128.14	42.67	0.51	0.64	-0.516	-1.23	0.13
71	11.42	114.25	38.04	0.46	0.57	-1.158	-0.49	0.11

Table D.5: Fermentation experiment using baker's yeast concentration of 6 $g.L^{-1}$

Time (hrs)	Bioethanol (wt%)	Bioethanol ($g.L^{-1}$)	m (EtOH) (g)	Bioethanol yield ($g.g^{-1}$)	Glucose Concentration ($g.L^{-1}$)	dC_p/dt	C_g/r_p	C_g/C_c
1	0.82	8.21	2.71	0.03	230.44	8.213	28.06	38.41
2	1.12	11.23	3.70	0.04	211.92	3.022	70.13	35.32
3	1.63	16.33	5.36	0.06	195.91	5.100	38.42	32.65
4	2.07	20.67	6.75	0.08	192.92	4.337	44.48	32.15
5	2.66	26.57	8.63	0.10	176.77	5.902	29.95	29.46
6	2.85	28.46	9.23	0.11	169.32	1.888	89.70	28.22
15	6.54	65.39	20.49	0.25	81.28	4.103	19.81	13.55
24	10.08	100.84	30.63	0.37	35.47	3.939	9.00	5.91
36	8.96	89.64	27.49	0.33	5.23	-0.933	-5.60	0.87
48	13.06	130.56	38.65	0.47	2.52	3.410	0.74	0.42
59	13.06	130.62	38.66	0.47	2.01	0.005	376.94	0.33
71	13.32	133.22	39.35	0.47	1.75	0.217	8.08	0.29

Table D.6: Fermentation experiment using baker's yeast concentration of 7 $g.L^{-1}$

Time (hrs)	**Bioethanol (wt%)**	**Bioethanol ($g.L^{-1}$)**	**m (EtOH) (g)**	**Bioethanol yield ($g.g^{-1}$)**	**Glucose Concentration ($g.L^{-1}$)**	**dC_p/dt**	**C_g/r_p**	**C_g/C_c**
1	0.80	8.00	2.64	0.03	175.48	8.00	21.94	25.07
2	0.84	8.36	2.76	0.03	210.27	0.36	587.76	30.04
3	1.32	13.17	4.33	0.05	212.52	4.81	44.21	30.36
4	1.74	17.44	5.71	0.07	203.24	4.28	47.50	29.03
5	2.31	23.09	7.52	0.09	186.75	5.64	33.09	26.68
6	3.33	33.30	10.74	0.13	161.02	10.21	15.77	23.00
15	7.30	73.00	22.72	0.27	64.75	4.41	14.68	9.25
24	10.12	101.16	30.71	0.37	21.31	3.13	6.81	3.04
36	12.78	127.79	37.92	0.46	3.10	2.22	1.40	0.44
48	13.01	130.06	38.51	0.46	1.73	0.19	9.18	0.25
59	10.99	109.88	33.11	0.40	1.21	-1.83	-0.66	0.17
71	11.27	112.71	33.88	0.41	1.16	0.24	4.94	0.17

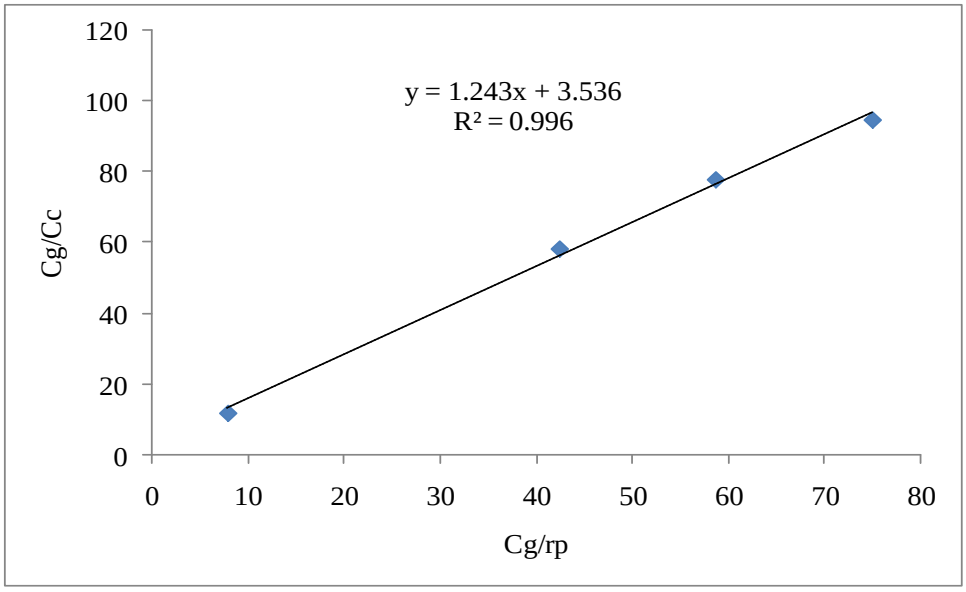

Figure D.1: Regression of experimental fermentation data at a yeast concentration of 2 $g.L^{-1}$

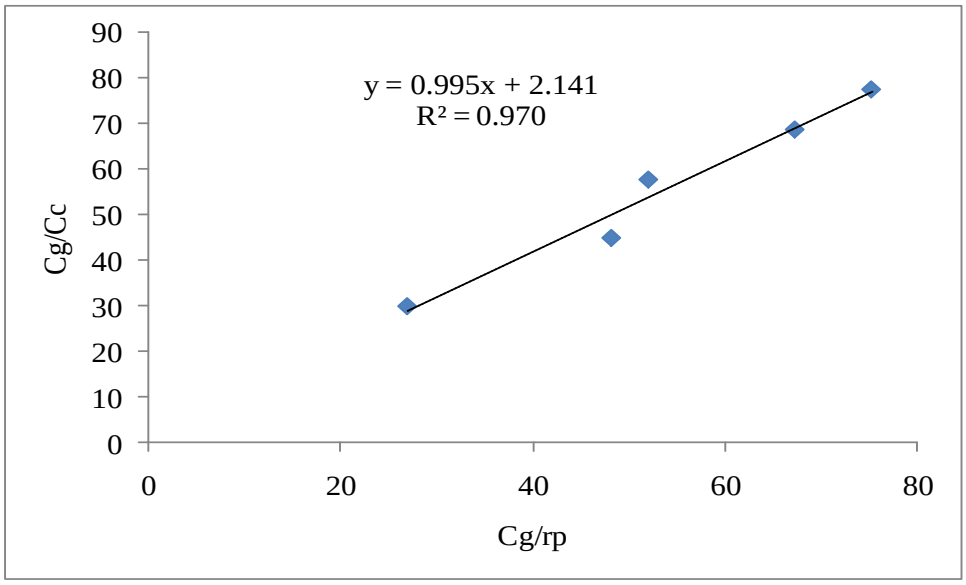

Figure D.2: Regression of experimental fermentation data at a yeast concentration of 3 $g.L^{-1}$

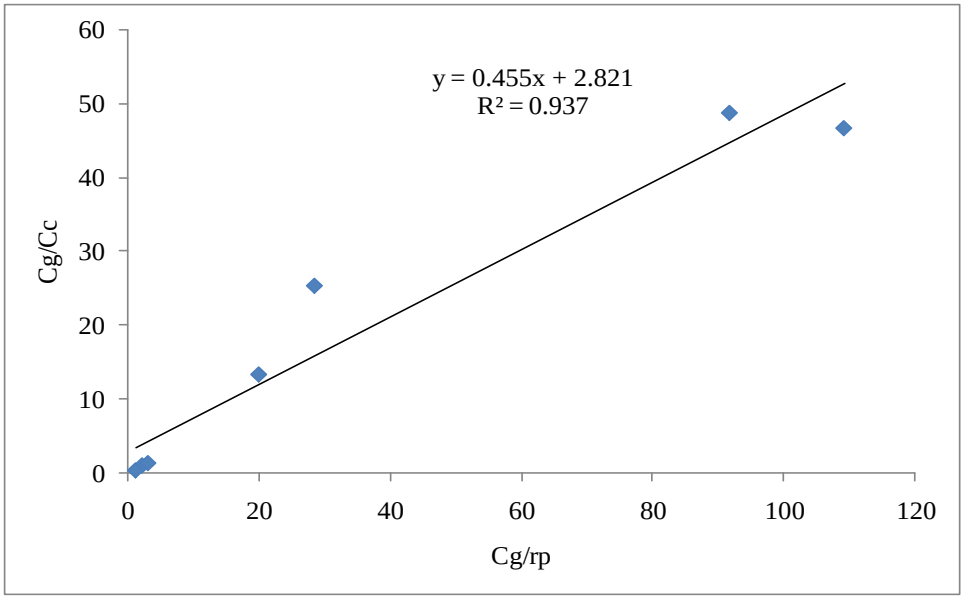

Figure D.3: Regression of experimental fermentation data at a yeast concentration of 4 $g.L^{-1}$

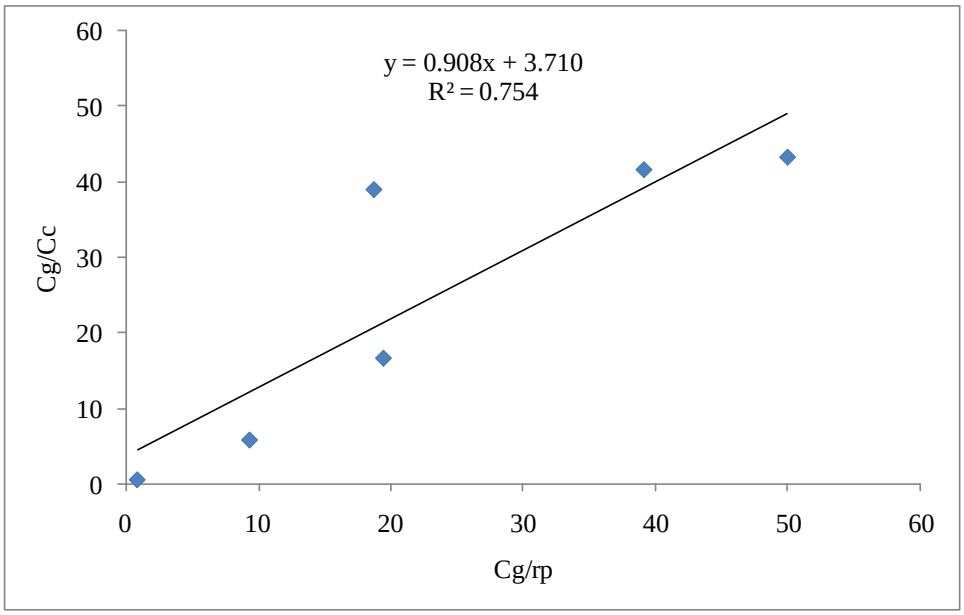

Figure D.4: Regression of experimental fermentation data at a yeast concentration of 5 $g.L^{-1}$

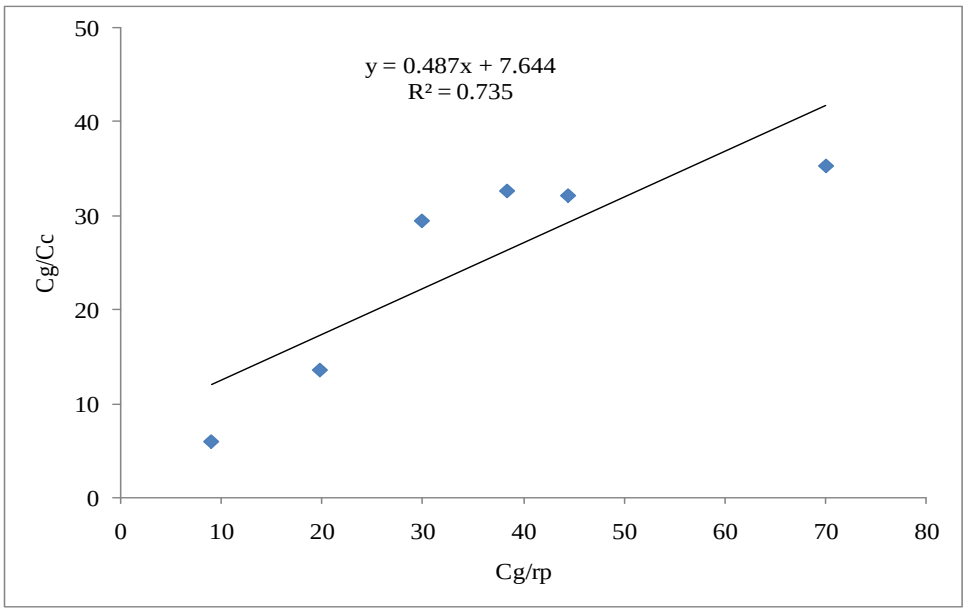

Figure D.5: Regression of experimental fermentation data at a yeast concentration of 6 $g.L^{-1}$

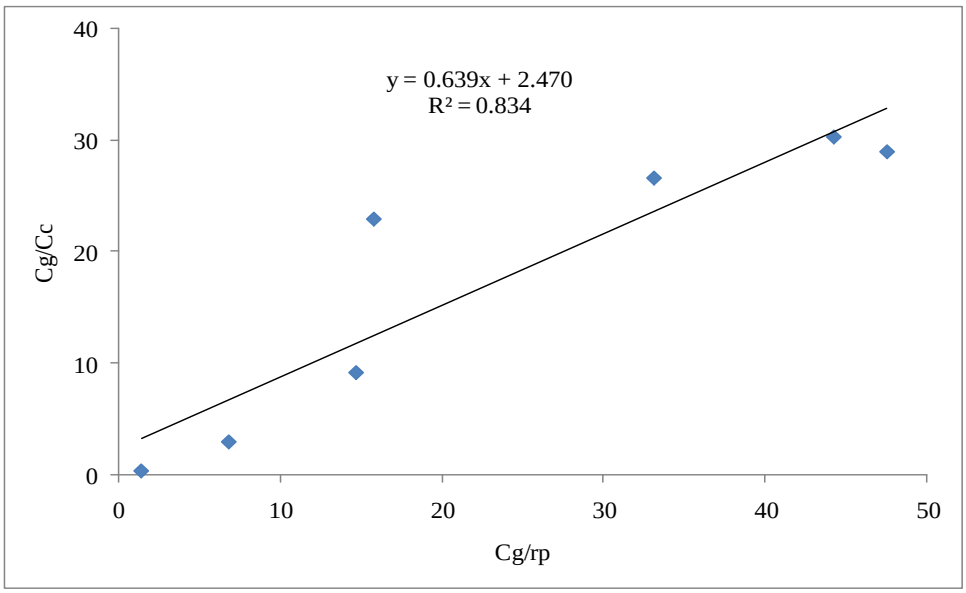

Figure D.6: Regression of experimental fermentation data at a yeast concentration of 7 $g.L^{-1}$

APPENDIX E

EXPERIMENTAL ERROR

E.1 Hydrolysis experimental error

The experimental errors for the hydrolysis and fermentation steps were calculated as shown in Table E.1.

Table E.1: Calculations of the experimental error during hydrolysis and fermentation

	Hydrolysis			
	Starch content	**Liquefaction**	**Saccharification**	**Fermentation**
Average	231.9	10.9	221.2	60.5
STDEV	6.3	1.4	12.3	48
95% conf.	7.2	0.8	7.0	15.7
Error %	3.1	7.1	3.1	25.9

Each experiment was repeated three times in order to determine the average.