OPTIMIZATION OF ETHANOL YIELD FROM CASSAVA

T.Y Nquma (BSc. Hons)

Dissertation submitted in fulfillment of the requirements for the degree of Master of Science in Chemical Engineering of the North West University (Potchefstroom Campus)

Supervisor : Prof S. Marx

Co-Supervisor : Dr G. Obiero

November 2009

Potchefstroom

ABSTRACT

The energy crisis and worldwide economic depression has highlighted the production of biofuels from agricultural materials as an important national policy. Cassava, a root plant indigenous to Africa, is not cultivated commercially in South Africa because it is not a staple food source and contains some cyanide components in its raw form. Cassava is mostly grown as a food supplement by informal households. Cassava roots are rich in starch (approximately 80%) and are therefore an excellent candidate for the production of bio-ethanol in South Africa. In this research, Cassava roots, which consist mostly of starch, as well as the peels, which consist of cellulose, were converted to bio-ethanol. As a baseline, the Cassava starch and the peels were converted to ethanol separately by using traditional pretreatment methods and *Saccharomyces cerevisiae* as yeast. The hydrolysis process for starch was optimized with respect to substrate concentration, enzyme concentration, enzyme combination, treatment temperature and pH of the different process steps. The best fermentation step was determined through fermentation of the optimized starch hydrolysate using the separated hydrolysis and fermentation process (SHF), the simultaneous saccharification and fermentation process (SSF) as well as a direct fermentation (DF) process from the raw starch using *Schwanniomyces occidentalis* (ATCC 26076). Cassava roots (starch) and peels (cellulose) were then pretreated and fermented simultaneously using different combinations of enzymes. A substrate concentration of 20 wt% biomass gave the highest glucose concentration in the final hydrolysate, while the best enzyme concentration was found to be 0.2% for Termamyl SC, 0.25% for Spirizyme fuel and 0.1% for Celluclast 1.5L. The liquefaction and saccharification treatment temperature that gave the highest ethanol yield were 95°C and 55°C respectively. The best pH for the two hydrolysis steps was found to be 6 and 4.5 for the liquefaction and saccharification steps respectively. The optimum pretreatment conditions with a substrate concentration of 20wt% yielded a final glucose concentration of 141 $g.L^{-1}$ ($Y_{p/s}$ = 0.7 $g.g^{-1}$) for Cassava starch, 109 $g.L^{-1}$ ($Y_{p/s}$ =0.55$g.g^{-1}$) for Cassava cellulose (peels) and for the simultaneous conversion of both the starch and cellulose, a final glucose concentration of 184 $g.L^{-1}$ ($Y_{p/s}$ = 0.9 $g.g^{-1}$) was obtained. It can be concluded from these results that unpeeled Cassava roots (starch and cellulose) yield a higher final glucose concentration in the final hydrolysate than converting the cellulose (peels) and starch (peeled roots) separately.

This means that it is more productive and economical to use unpeeled Cassava roots with the correct combination of starch and cellulose enzymes to produce a glucose rich hydrolysate for ethanol production through fermentation. The direct fermentation (DF) process yielded the lowest final ethanol concentration (0.14%) resulting in a yield coefficient ($Y_{p/s}$) of just 1 %. The SHF process yielded 9.6 % (v/v) ($Y_{p/s}$ = 0.38 $g.g^{-1}$) ethanol for Cassava starch and 10.6 % (v/v) ($Y_{p/s}$ = 0.42 g.g-1) for both roots and peels (starch and cellulose) after 48 hours fermentation. The SSF process resulted in a final ethanol yield of 7 % (v/v) ($Y_{p/s}$ = 0.3g.g-1) for Cassava starch, 4% (v/v) ($Y_{p/s}$ = 0.16 g.g-1) for Cassava peels (cellulose) and 10.6% (v/v) ($Y_{p/s}$ = 0.42 $g.g^{-1}$) for unpeeled Cassava roots (starch and cellulose). These results demonstrate that Cassava waste (peels) can be used as an alternative biomass for bio-ethanol production. However, the SSF process for unpeeled Cassava roots results in a higher ethanol yield than processing the peels and starch separately and then combining the hydrolysates only for the fermentation step. It also became evident that Cassava containing approximately 85% (g/g) starch is a good feedstock for bio-ethanol production in South Africa.

Keywords: Cassava, Liquefaction, Saccharification, Fermentation, Glucose, Ethanol

OPSOMMING

Die energiekrisis en wêreldwye ekonomiese depressie het die produksie van biobrandstof van landboumateriale op die voorgrond geskuif as ‘n belangrike nasionale beleid. Cassava, ‘n wortelplant inheems aan Afrika, word nie in Suid-Afrika kommersieel verbou nie omdat dit nie ‘n stapelvoedsel is nie en sianiedkomponente bevat in sy rou vorm. Cassava word meestal as ‘n voedingsaanvulling geplant deur informele huishoudings. Cassavawortels is ryk aan stysel (ongeveer 80%) en is daarom ‘n uitstekende kandidaat vir die produksie van bio-etanol in Suid-Afrika. In hierdie navorsing word Cassavawortels, wat meestal uit stysel bestaan, sowel as die skille, wat meestal uit sellulose bestaan, omgeskakel in bio-etanol. As ‘n basislyn is die Cassavastysel en die skille apart tot etanol omgeskakel deur tradisionele voorbehandelingsmetodes en *Saccharomyces cerevisiae* as gis te gebruik. Die hidroliseproses vir stysel is geoptimiseer met betrekking tot substraatkonsentrasie, ensiemkonsentrasie, ensiemkombinasie, behandelingstemperatuur en pH van die verskillende prosesseringsstappe. Die beste fermentasiestap is bepaal deur die fermentasie van die geoptimiseerde stysel hidrolisaat deur die gebruik van ‘n afsonderlike hidrolise en fermentasieproses (SHF), die simultane sakkarifikasie en fermentasieproses (SSF) sowel as ‘n direkte fermentasieproses (DF) van die stysel deur die gebruik van *Schwanniomyces occidentalis (ATCC 26076).* Cassavawortels (stysel) en skille (sellulose) is voorbehandel en gefermenteer deur die gebruik van verskillende kombinansies ensieme. ‘n Substraat konsentrasie van 20 wt% biomassa het die hoogste glukosekombinasie in die finale hidrolisaat gelewer, terwyl die beste ensiemkonsentrasie geblyk het te wees 0.2% vir Termamyl SC, 0.25% vir Spirizyme brandstof en 0.1% vir Celluclast 1.5L. Die vervloeiings en sakkarifikasie-behandelingstemperatuur wat die meeste etanol gelewer het was 95°C en 55°C onderskeidelik. Die beste pH vir die twee hidrolisestappe bleik te wees 6 en 4.5 vir die vervloeiing en sakkarifikasiestappe onderskeidelik. Die optimum voorbehandelingskondisies met ‘n substraatkonsentrasie van 20wt% het ‘n finale glukosekonsentraat gelewer van 141 $g.L^{-1}$ ($Y_{p/s}$ = 0.7 $g.g^{-1}$) vir Cassavastysel, 109 $g.L^{-1}$ ($Y_{p/s}$ =0.55$g.g^{-1}$) vir Cassavasellulose (skille) en vir die simultane omskakeling van beide die stysel en sellulose, ‘n finale glukosekonsentraat van 184 $g.L^{-1}$ ($Y_{p/s}$ = 0.9 $g.g^{-1}$). Dit kan uit die resultate afgelei word dat ongeskilde Cassavawortels (stysel en sellulose) ‘n hoër finale glukosekonsentraat in die finale hidrolisaat gelewer het as om

die sellulose (skille) en stysel (geskilde wortels) apart te gebruik. Dit beteken dat dit meer produktief en ekonomies sal wees om ongeskilde Cassavawortels met die korrekte kombinasie van stysel en sellulose-ensieme te gebruik om ‘n glukoseryke hidrolisaat te lewer vir etanolproduksie deur fermentasie. Die direkte fermentasieproses (DF) het die laagste finale etanolkonsentraat gelewer (0.14%) wat uitloop of ‘n leweringskoeffisient ($Y_{p/s}$) van net 1 %. Die SHF proses het 9.6 % (v/v) ($Y_{p/s}$ = 0.38 $g.g^{-1}$) etanol vir Cassavastysel gelewer en 10.6 % (v/v) ($Y_{p/s}$ = 0.42 $g.g^{-}1$) vir beide wortels en skille (stysel en sellulose) na 48 ure van fermentasie. Die SSF proses het uitgeloop op ‘n finale etanollewering van 7 % (v/v) ($Y_{p/s}$ = 0.3$g.g^{-}1$) vir Cassavastysel, 4% (v/v) ($Y_{p/s}$ = 0.16 $g.g^{-}1$) vir Cassavaskille (sellulose) en 10.6% (v/v) ($Y_{p/s}$ = 0.42 $g.g^{-1}$) vir ongeskilde Cassavawortels (stysel en sellulose). Hierdie resultate demonstreer dat Cassava-afval (skille) gebruik kan word as alternatiewe biomassa vir bio-etanol produksie. Die SSF proses vir ongeskilde Cassavawortels lewer egter ‘n hoër etanol hoeveelheid as om die skille en stysel apart te prosesser en dan die hidrolisate slegs vir die fermentasiestap te kombineer. Dit het ook duidelik geword dat Cassava wat omtrent 85% (g/g) stysel bevat ‘n goeie voedingstof vir bio-etanolproduksie in Suid-Afrika is.

Verworde: Cassava, Vervloeiings, Sakkarifikasie, Fermentasie, Glukose, Etanol

Declaration

I Tandokazi Yvonne Nquma hereby declare that I am the sole author of this dissertation

---

Tandokazi Yvonne Nquma.

Acknowledgements

"Give thanks in all Circumstances"

This work has been made possible by a team of inspiration, insights and prayers of distinctive individuals sent by the most sustaining man in this research God Himself. To God I give thanks, honor and glory for the ability He gave me to make it through my research on time. Indeed my God is the greatest engineer.

To my wonderful supervisor, Prof Sanette Marx, I extend my unlimited gratitude for the opportunity you gave me to become a better scientific researcher. You inspired me as a woman scientist and mostly an in sighted one.

To SANERI (CRSES), Thank you so much for your financial support in this work and the opportunity you afforded me to further my career.

To Dr George Obiero, thank you for your advices and recommendations on my work, they have been valuable.

To all my lab mates, fellow Masters students and personnel in the School of Chemical and Minerals Engineering, thank you for your inputs and help in all aspects in this work.

To my mother Mavis, thank you for your support, love and inspiration you gave me to carry out this work. You are my pride.

To my father Jongudumo, thank you for motivating me to better my career, you are a God given gift in my life.

To all my friends, thank you for your support, love and understanding when life became sour and reminding me to pray, laugh and enjoy the simple things in life.

TABLE OF CONTENTS

NOMENCLATURE

Symbol	Description	Units
σ	Standard deviation	-
Y	Yield	$g.g^{-1}$
$\mathbf{Y_{p/s}}$	Yield product per substrate (Cassava)	$g.g^{-1}$
$\mathbf{Y_{g/s}}$	Yield glucose per substrate (Cassava)	$g.g^{-1}$
$\mathbf{Y_{p/g}}$	Yield product (Ethanol) per glucose	$g.g^{-1}$
Z	Z score	-
±	CONFIDENCE	-
n	Number of samples	-
$\bar{x}$	Mean (Average)	-
C	Concentration	$g.^{L-1}$
W	Weight	$g/\ g.^{L-1}$

Acronym	Description
DDGS-	Dried Distiller's Grain with Solubility
SSF	Simultaneous Saccharification and Fermentation
STDEV	Standard deviation
GC	Gas Chromatography
HPLC	High Pressure Liquid Chromatography
C	Celluclast 1.5L
T	Termamyl SC
S	Spirizyme Fuel
ATCC	American Type Culture Collection
S. occidentallis	*Schwanniomyces occidentallis*
SHF	Separate enzymatic Hydrolysis and Fermentation
DF	Direct Fermentation
S. cerevisiae	*Saccharomyces cerevisiae*

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

GENERAL INTRODUCTION

"We must develop knowledge optimization initiatives to leverage our key learnings."

Scott Adams

Overview

This chapter is divided into three sections, starting in Section 1.1 with the background and motivation to this study, Section 1.2 is on the background of bio-ethanol. The hypothesis and research aim are in Sections 1.3 and 1.4 respectively. The research objectives are stated in section 1.5 followed by the detailed scope of investigation in Section 1.6.

1.1 Background and motivation

The worldwide energy crisis and continuous increase in petroleum prices has led to alcohol being considered as an alternative to conventional fuels. Alcohol fuel, which can be produced from Cassava, sugarcane waste and other agricultural products, are considered the most promising fuels for the future.

Any fuel that could be introduced as an alternative to conventional fuel should be evaluated from the aspect of availability, renewability, safety to the environment, energy balance and cost adaptability to the performance in existing engines, economy and finally emission (Nag, 2008). The major concerns of biofuels are related to the energy efficiency of fuel ethanol and its production of a positive energy income (Agu *et al*., 1997).

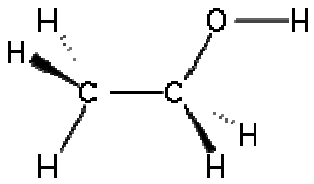


Figure 1.1: *3-d diagram of ethanol*

In the early years bio-ethanol was not used in automobiles due to low energy density, high production cost, and corrosion. However, the current shortage of gasoline has made it necessary to substitute ethanol (see Figure 1.1) as fuel in spark ignition engines.

Ethanol is a renewable fuel that is now widely used in many countries as a power source in vehicles and other internal combustion engines. It is currently produced from starch crops as well as feedstock crops and other biomass materials that can be converted into fermentable sugars (Nzelibe and Okafoagu, 2007). Ethanol is a renewable fuel which is environmentally more friendly than fossil fuel and can contribute to the reduction of the emission of gases such as sulphur dioxide, carbon dioxide and nitrogen oxide that is associated with the utilization of fossil fuels (Cardona and Sanchez, 2007; Dermibas, 2007).

Approximately 7% of Cassava produced all over the world is used by the textile, paper, food and fermentation industries. Another large consumer of Cassava is the animal food industry, using about 33% of the world production (Pandey *et al*., 2000). The comparative properties of ethanol with petrol and diesel are shown in Table 1.1 below.

Table 1.1: *Comparative properties of ethanol with petrol and diesel*

Property	Petrol	Diesel	Ethanol
Specific gravity (15°C)	0.73	0.82	0.79
Boiling point (°C)	30-225	190-280	78.3
Specific heat (MJ/kg)	43.5	43.0	27.0
Heat of vaporization (kJ/kg)	400	600	900
Octane number	91-100	NA	NA
Catani number	Below 15	40-60	Below

Cassava (*Manihot esculent*) from the Euphorbia family (Euphorbiaceae) is a very important source of carbohydrates, which is not only rich in starch but also in cellulose and hemicellulose (Tonukari, 2004) . Cassava is a potential feedstock for ethanol production because of its excellent physical and chemical characteristics of the starch, high attainable ethanol yields and low feedstock costs. Cassava milled flour (also called tapioca flour) is easily and completely hydrolyzed compared to other starchy flours and therefore its use for ethanol production is encouraged. There are few reports (Ramasamy and Paramasamy, 2001) concerning the industrial application of Cassava for ethanol production, possibly because Cassava starch has to be

hydrolyzed into fermentable sugars for bioconversion into ethanol by *Saccharomyces cerevisiae,* making it time consuming (Ayernor *et al*., 2002). Cassava roots also contain coumaric acid, which causes harvested roots to spoil within 24 hours after harvesting. The rapid deterioration of Cassava roots after harvesting is the reason why Cassava is not a major export crop (Bayoumi *et al*., 2008). If Cassava can be used for ethanol production the roots will have be chopped into chips and dried to preserve it long enough for processing into bio-ethanol through fermentation. This additional processing step has thus far limited its use for the production of bio-ethanol.

1.2 Bio-ethanol production from biomass

Biomass can be converted to liquid, solid and gaseous products through different processes. The different stages for the conversion of biomass to these products are shown in Figure 1.2 below.

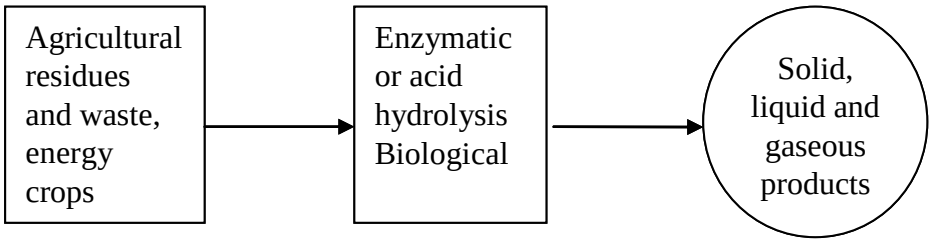


Figure 1.2: *Different products from biomass* (Nag, 2008)

Bio-ethanol liquid can be produced through fermentation of biomass that contains starch and sugar. Starchy tubers such as Cassava that can be used for bio-ethanol production are important staple foods in most of the developing countries in the Tropics. They are widely distributed in these countries and despite their importance, a large proportion of the tubers are lost annually due to inadequate and ineffective storage facilities. In terms of energy utilization and process simplicity, enzymatic conversion of the raw Cassava starch is superior and feasible (Nweke, 2004). Cassava starch has been fermented with amylase, gluco-amylase and ethanol producing organisms, but ethanol yields have been low (Brauman *et al*., 1996).

Methods used to produce bio-ethanol use more energy for ethanol production than the energy in the fuel produced, which is a contradictory energy balance and this becomes costly (Brauman *et al*., 1996). In addition, the concentration of sugars from the

Cassava hydrolysate have a negative impact on the ethanol yield, and therefore methods that lower the negative impact of glucose on ethanol yield and are less time consuming should be found to make the production of ethanol from Cassava economical. The methods and alternative organisms (yeast) that optimize ethanol production conditions, costs and purity are thus the major focus for the prosperity of bio-ethanol from Cassava. The fermentation of Cassava roots prevents the roots from rapid spoilage after harvest. Although bio-ethanol production has greatly improved through the use of better technologies and a wide variety of crops, Cassava has received less attention despite its promising properties and potential in biofuels (Brauman *et al*., 1996).

1.3 Hypothesis

Alternative methods (SSF), additional amylolytic enzymes and yeasts (*Schwanniomyces castellii*) will reduce the impact of glucose concentration on the ethanol yield and increase the ethanol yield obtainable from Cassava.

1.4 Research aim

The main aim of this research was to optimize the ethanol production and yield from the Cassava root fermentation process using alternative methods (SSF) with *Saccharomyces cerevisiae* as well as direct fermentation using *Schwanniomyces castellii/occidentalis*.

1.5 Research objectives

1.5.1 Determine the optimum glucose concentration obtainable from peeled Cassava roots (starch), Cassava peels (cellulose) and unpeeled Cassava roots (starch and cellulose) during liquefaction.

1.5.2 Determine the optimum glucose concentration obtainable from peeled Cassava roots (starch), Cassava peels (cellulose) and unpeeled Cassava roots (starch and cellulose) during saccharification.

1.5.3 Determine the optimum ethanol yield obtainable from Cassava hydrolysate using a separate hydrolysis and fermentation method, a simultaneous

saccharification and fermentation, as well as direct fermentation of Cassava starch.

1.5.4 Determine the cost of ethanol production from Cassava.

1.6 Scope of the investigation

In the process of defining the investigation, three main objectives and aims for this study came to the fore.

1.6.1 Optimization of liquefaction and saccharification steps

The optimum pH, temperature, biomass load, enzyme combination and enzyme concentration for the highest final glucose concentration in the liquefaction and saccharification hydrolysates were determined by varying one parameter at a time while keeping the others constant. The optimum temperature, pH, biomass load and enzyme combination was then used to optimize the fermentation step. Optimization of these parameters was done for the unpeeled Cassava roots (starch and cellulose).

1.6.2 Optimization of ethanol yield

The fermentation of Cassava roots to bio-ethanol was investigated by varying the yeast concentration during fermentation.

Three different processing routes i.e. separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and direct fermentation (DF) were used to determine the best processing route for the optimal ethanol yield from Cassava.

Lastly the SSF process route was used to compare the different Cassava root forms (unpeeled roots, peeled roots and peels only) for optimal ethanol production.

1.7 REFERENCES

AGU, R. C., AMADIFE, A. E., UDE, C. M., ONYIA, A., OGU, E. O., OKAFOR, M. & EZEJIOFOR, E. 1997. Combined heat treatment and acid hydrolysis of Cassava grate waste (CGW) biomass for ethanol production. *Waste Management*, 17(1): 91-96, 25 Sept.

AYERNOR, G.S., HAMMOND, T.K. & GRAFHAM, A. 2002. The combination of rice malt and amyloglucosidase for the production of sugar syrup from Cassava flour. *African Journal of Science Technology*, 3(1): 10-17, June.

BAYOUMI, S.A.L., ROWAN, M.G., BLAGBROUGH, I.S., & BEECHING, J.R. 2008. Biosynthesis of scopoletin and scopolin in Cassava roots during post-harvest physiological deterioration: E-Z-isomerisation stage. *Phytochemistry*, 69(17): 2928-2936, 23 Sept.

BRAUNMAN, A., KELEKE, S., MALONGA, M., MIAMBI, E., & AMPE, F. 1996. Microbiological and Biochemical Characterization of a Traditional Lactic Acid Fermentation for (Cassava Flour) Production. *Applied and environmental microbiology*, 62(8): 2854-2858, 05 Jan.

CARDONA, A. C. & SANCHEZ, O.J. 2007. Fuel ethanol production: Process design trends and integration opportunities. *Bioresource Technology*, 99(12): 2415-57, 01 Mar.

DERMIBAS, A. H. 2007. Combustion of Biomass. *Energy Sources Part A: Recovery, Utilization, and Environmental Effects*, 29(6): 549-56, 06 Jan.

NAG, A. 2008. Biofuels Refining and Performance. United States of America: McGraw-Hill: 5-35, 17 Dec.

NZELIBE, H. C., & OKAFOAGU, C. U. 2007. Optimization of ethanol production from *Garcinia kola* (bitter kola) pulp agro waste. *African Journal of Biotechnology*, **6** (17): 2033-2037, 05 Sept.

PANDEY, C.R., SOCCOL, P., NIGAM, V.T., VANDENBERGHE, L.P.S., & MOHAN, R. 2000. Biotechnological potential of agro-industrial residues. II. Cassava bagasse. *Bioresource Technology*, **74:** 81–87, Aug.

RAMASAMY, A. & PARASAMY, G. 2001. Production of ethanol from liquefied cassava starch using co-immobilized cells of *Zymomonas mobilis* and *Saccharomyces diastaticus*. *Journal of Bioscience and Bioengineering*, 92(6):560-564, 20 Jun.

TONUKARI, N.J. 2004. Cassava and the future of starch. *Electronic Journal of Biotechnology*, 7 (1): 5-8, 15 Apr.

NWEKE, F.I. 2004. "New Challenges in the Cassava Transformation in Nigeria and Ghana." EPTD Discussion Paper No. 118: Environment and Production Technology Division, International Food Policy Research Institute (IFPRI), 18 June. United States of America.

CHAPTER 2

BACKGROUND AND LITERATURE SURVEY

"If we could first learn where we are and where we are going, we could be better able to judge what to do and how to do it."

Abraham Lincoln

Overview

The literature survey is subdivided into various Sections, starting with a brief introduction on bioethanol and Cassava on Section 2.1. Section 2.2 denotes the importance and effect of biofuels followed by a bioethanol review in Section 2.3. Bioethanol production is extensively outlined in Section 2.4 encompassing subsections on bioethanol production methods and organisms. An overview of the Cassava plant, its components and production is outlined in Section 2.5. Energy efficiency of Cassava is reviewed in Section 2.6 followed by the last Section (2.7) on optimization of bioethanol production from Cassava.

2.1 Introduction

Ethanol from biomass has already been introduced in countries such as Brazil, USA and some European countries. In Brazil it is currently produced from sugar and in the USA from starch, both at competitive prices. In recent years, there has been an increase in the efficient utilization of industrial crops such as Cassava for the production of ethanol (Pandey, 1992; 1994). Ethanol is currently produced from sugarcane and starch containing materials, where the conversion of starch to ethanol includes a liquefaction step (to make starch soluble) and a hydrolysis step (to produce glucose). The key parameters that most ethanol research addresses are energy and environmental performance (Ngunyen and Ghweela, 2008). A study by Ngunyen and Gheewala (2008) shows that bioethanol produced from Cassava (E10 fuel), along with its life cycle, reduces certain environmental loads.

Practical investigations have indicated that Cassava as a perennial starch crop proves to be a beneficial raw material for industrial products such as ethanol (Ayernor *et al*., 2002). The Cassava roots specifically produce the most important raw materials, including Cassava flour and Cassava starch, which when hydrolyzed produces fermentable sugars such as glucose for ethanol production (Avancini *et al*., 2007). The roots are rich in energy due to high carbohydrate content. Starch accounts for the highest proportion of the carbohydrate content, making Cassava a potential energy reserving plant. The use of Cassava as a source of ethanol for fuel is already being exploited and is very promising. The work of Roble *et al*., (2003) demonstrates direct ethanol production from Cassava starch by *Aspergillus awamori* and *Saccharomyces cerevisiae* in a circulating loop bioreactor. Optimization of ethanol yield from such crops looks also at the experimental design, its energy consumption and production. Optimal production, bio-processing and chemical processing of bio-ethanol is greatly influenced by proper selection of crops. The optimization of fermentation processes is a crucial tool in bio-ethanol production. The combination of different steps of an integrated bioprocess into one single unit (SSF) is seen as a prospective optimization tool together with the use of amolytic organisms (*S. occidentalis/castellii*) for direct fermentation since no hydrolysis of substrate is required (Cardona and Sanchez, 2007).

2.2 Biofuels

Biofuels form an important emerging business worldwide and the amount of biomass feedstock that can be produced for fuels and other energy purposes are potentially very large (Hoogwijk *et al*., 2003). Fermentation of sugars to alcohol is one of the main routes that have been distinguished to produce biofuels, mainly by conceiving ethanol (see Figure 2.1)

Figure 2.1: *Overview of conversion route from crops to bio-ethanol*

Higher overall energy conversion efficiencies and lower overall costs are the key criteria for selecting biofuels for the longer term, which provides insight into the

possible barriers to implementation that need to be overcome and the technological improvement options that should be stimulated (Hamelinck and Faaij, 2006).

Brazil and USA are the largest producers of bio-ethanol in the world. It is expected that by 2020, 6.9% of the worldwide transportation fuel supply will consist of biofuels, depending on the improvement of technologies. One of the disadvantages of biofuels is the low energy density compared to diesel and petrol. More than a liter of bio-ethanol is necessary to replace a liter of petrol; hence the production cost of bio-ethanol has to be low in order for it to compete with petrol (Bomb *et al*., 2007). The cost of raw material has a significant impact on the cost of bio-ethanol production, and by increasing bio-ethanol production from high ethanol grains and tubers such as Cassava, which are plentiful and less costly, it is anticipated that the future progress in biotechnology will decrease the cost of bio-ethanol production (Bomb *et al*., 2007; Zohreh, 2008).

2.3 Bio-ethanol

Bio-ethanol is a liquid transportation fuel made from renewable resources or plant biomasses such as agricultural wastes, corn, grain, grasses, sugar cane, straw, wood based waste such as newsprint, woodchips, and manufacturing waste materials. Ethanol is a clean burning fuel that lowers overall green house gas emissions (as the biomass absorbs carbon dioxide as it grows), contains a high percentage of oxygen (35%) and therefore produces more complete fuel combustion. Ethanol can be blended with petroleum, integrates into existing fuel delivery systems and provides energy security by reducing our reliance on fossil fuels. The new Flexible Fuel Vehicles currently available operate on E85 ethanol based fuels with a content of 85% ethanol and 15 % petroleum (*Goettemoeller and Goettemoeller, 2007*). Most vehicles on the road use petrol and diesel as their fuel, but fossil fuels are limited. Combustion of petroleum-based fuels increases net emission of carbon dioxide, different toxic and volatile compounds that are responsible for the health hazards and pollutions such as benzene, toluene and xylene (With recent advances in the biotechnology sector, it is plausible to convert biomass into high quality energy carriers such as liquid fuels (Larson *et al*., 1993).

2.4 Bio-ethanol production

Ethanol can be manufactured using a dry mill or wet mill process, where dry milling entails the fermentation of starch into sugars, after which it is distilled into alcohol. The distinct difference between the two processes is the initial treatment of the biomass. Srinorakutara *et al*., (2004) reported that in the 21st century the production of ethanol from abundant, low cost agricultural products is growing due to the certainty that it reduces the cost of ethanol production and has global environmental benefit. Bio-ethanol production had increased sharply from less than 1 GL in 1975 to 49.8 GL in 2006 (Nag, 2008).

Sugar crops constituted 60% of bio-ethanol production in 2000, but its share decreased to 47% in 2006 in Brazil, while the use of starch crops for the production of bio-ethanol increased from 39% to 53% in 2006. The increase in the use of starch crops for the production of bio-ethanol is predicted to increase towards 2015 (Nag, 2008).

2.4.1 Bio-ethanol production from starch-rich biomass

Ethanol is produced largely through biochemical and thermo-chemical processes (see Figure 2.2). Biomass is generally pretreated by mechanically cleaning and sizing the biomass, and then destroying its cell structure to make it more accessible to further chemical and biological treatment. The starch part of biomass is converted by hydrolysis to monomeric sugars such as glucose. The monomeric sugars are fermented to ethanol, which is further purified and dehydrated for industrial use (Hamelinck and Faaij, 2006). Several agricultural products can be used as the raw material for ethanol production, such as sugarcane, rice and Cassava. Among these commercial crops, Cassava, simply transformed to dried chips, is recommended as the most suitable raw material for ethanol production (Dermibas, 2007).

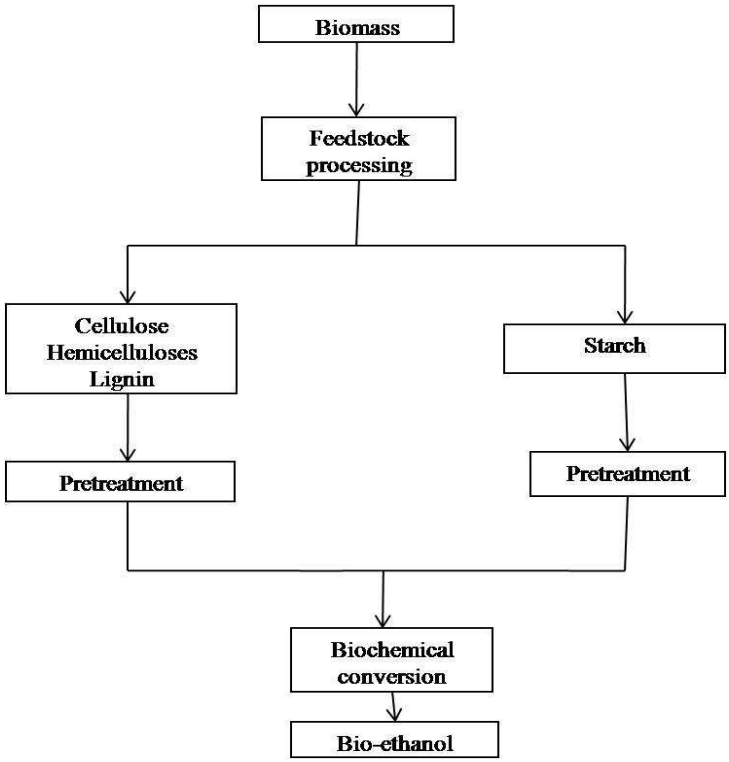


Figure 2.2: *Biomass conversion process* (Dermibas, 2007)

2.4.2 Enzymatic hydrolysis

The thermostable α-amylases (endo- α-1, 4-glucanase) are used in starch hydrolysis to break the starch bonds and thus release the starch for hydrolysis to simple sugars by β-glucosidase. The major end products from the action of α-amylase on starch are dextrins. The glucoamylases and β-amylases (exo- α-1, 4-glucanase) are used in saccharification of liquefied starch producing glucose, maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose, and they are produced by *Aspergillus niger*. The pullulanases and isoamylases (endo- α-1, 6-glucanase) are used for debranching starch into complete fermentable sugars such as glucose (Hema *et al.*, 2006). Fermentation of the simple sugars to ethanol through yeast takes approximately 72 hours. The pH should not drop below 4.5 because α-amylase denatures at low pH-values (Aktinson and Mavituna, 1991). Higher ethanol yields and low concentrations of by-products are obtained at mild process conditions.

2.4.3 Fermentation with yeasts

An increase in the utilization and production of ethanol through the fermentation of starchy materials has aggravated the intensive research into improving the

conventional fermentation procedures through optimization. Non-renewable, commercial bacterial and fungal amylases are used for the common liquefaction and saccharification of starch substrates. However, there are a considerable number of cost effective amylolytic yeasts that produce their own amylases for starch conversion and are also capable of fermentation (Wilson and Ingledew, 1982). Yeasts have been the most commonly used micro-organisms for ethanol production, mainly because they are a species which can produce ethanol as the main fermentation product (Lin and Tanaka, 2008).

2.4.3.1 *Saccharomyces cerevisiae*

S. cerevisiae is the most well-known and widely used yeast on hexose sugars such as glucose and the disaccharide sucrose because it has the ability to grow on these sugars. This yeast is tolerant to ethanol up to 15% of its concentration in the fermentation broth, but it cannot ferment pentoses sugars such as xylose (Saha, 2003). It offers advantages over other yeasts in the bioconversion of sugars. Under excess carbon conditions, its metabolic flux to ethanol is hardly affected by the presence of oxygen (Lagunas, 1979) and it is able to grow under highly anaerobic conditions (Visser, 1995). The main restriction of *S. cerevisiae* is its inability to convert relatively inexpensive polysaccharide-rich substrates, such as starchy materials to fermentable sugars (Hahn-Hagerdal *et al*., 1994). The fermentation step is usually performed in an open vessel that is mechanically agitated and coil refrigerated. *S. cerevisiae* is added and it immediately consumes the glucose in the hydrolysate. The product is continually fed to a distillation column to recover ethanol as an ethanol rich mixture with water (Nag, 2008).

2.4.3.2 *Schwanniomyces castellii*/*occidentalis*

Schwanniomyces castellii also known as *Schwanniomyces occidentalis* has the ability to synthesize alpha amylase and two gluco-amylase enzymes, that is, it can both hydrolyze and ferment Cassava starch (Wilson and Ingledew, 1982; Poonam and Dalel, 1995). The three enzymes produced by *S. castellii* have a common optimum pH of 6 and optimum temperature of 60°C. The two gluco-amylase enzymes of *S. castellii/occidentalis* differ only with their molecular weights and their rates of hydrolysis of the carbon substrates such as maltose, glucose, glycogen and dextrin. The excretion and biosynthesis of the amylases from the yeast depends and is

influenced by the composition of the culture media. It is useful in the direct fermentation of starch to as much as 4 % (w/v) ethanol (Wilson and Ingledew, 1982). *S. castellii* is used as a solid culture and the ethanol produced by the organism is continuously recuperated in a cold trapper tank.

2.4.4 Dry milling process

In the dry milling process (see Figure 2.3) the grain/tuber is ground into fine pulp or flour. The grain is processed without separation of the starch from the fiber components (Avancini *et al*., 2007). The flour or meal is slurred with water to form a mash. The slurry is directly further processed by liquefaction and saccharification and the starch in the mixture is converted to sugars through the use of water and enzymes at high temperatures (55-95°C). The mash is processed in a high-temperature cooker to reduce bacteria levels ahead of fermentation. The mash is cooled and transferred to fermentors where yeast is added and the conversion of sugar to ethanol and carbon dioxide (CO_2) begins (Wyman, 1996).

During the fermentation process, the mash is agitated and kept cool to facilitate the activity of the yeast. After fermentation, the resulting broth is transferred to distillation columns where the ethanol is separated from the remaining silage. The ethanol is concentrated using conventional distillation and then dehydrated in a molecular sieve system (Wayman, 1996). The alcohol product at this stage is called anhydrous ethanol (pure, without water) and is approximately 200 proof. Ethanol that will be used for fuel is denatured, or made unfit for human consumption, with a small amount of petrol (2-5%) added at the ethanol plant. The silage is sent through a centrifuge that separates the coarse grain from the solubles. The solubles are then concentrated to about 30% solids by evaporation, resulting in Condensed Distillers Solubles (CDS). The coarse grain and the syrup are then dried together to produce dried distiller grains with solubles (DDGS), a high quality and nutritious livestock feed. Carbon dioxide is given off in great quantities during fermentation and many ethanol plants collect, compress, and sell it for use in other industries. In addition, the fuel cell industry has developed re-formulators that use ethanol as a source of hydrogen. The CO_2 is also captured and sold for use in carbonating soft drinks and beverages and the manufacture of dry ice (Wyman, 1996).

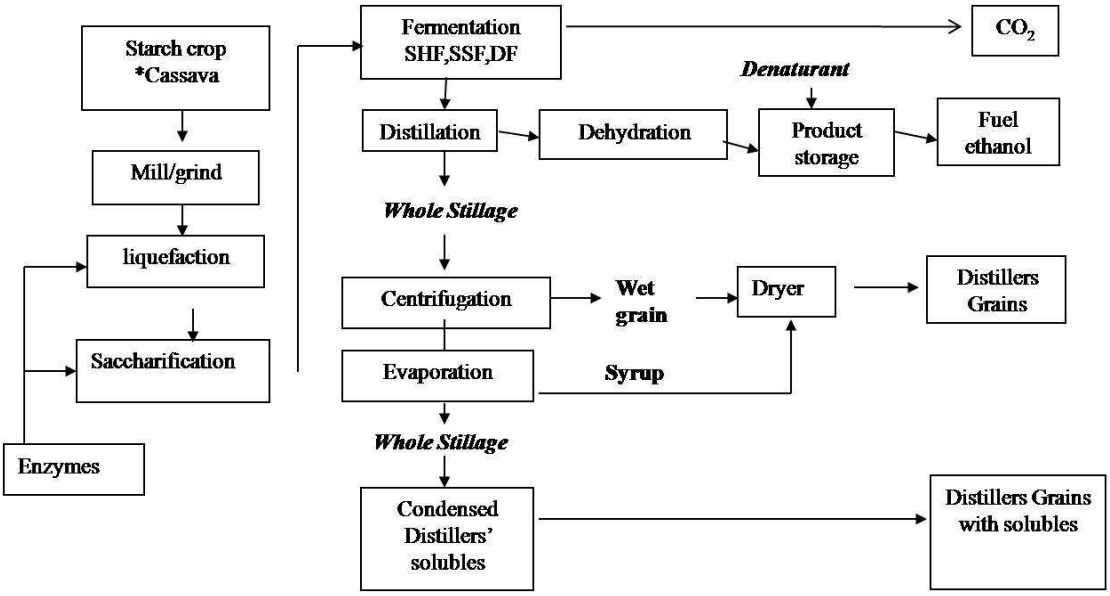


Figure 2.3: *Process flow diagram of dry milling* (Wyman, 1996).

2.4.5 Wet milling process

During the wet milling process the grain is separated into starch and fiber components by soaking or steeping the grain in water and acid for 24 to 48 hours. The slurry produced is processed through degermers to separate the grain germ. The oil from the germ is extracted while the remaining fiber, gluten and starch components are further segregated by centrifugation, screening and hydroclonic separators. The steeping liquid is concentrated in an evaporator. This concentrated product, heavy steep water, is co-dried with the fiber component and is then sold as gluten feed to the livestock industry. The starch and any remaining water from the mash can then be processed in one of three ways: fermented into ethanol, dried and sold as dried starch or processed into syrup. The fermentation process for ethanol is very similar to the dry mill process (Wyman, 1996). The wet milling process is described in Figure 2.4

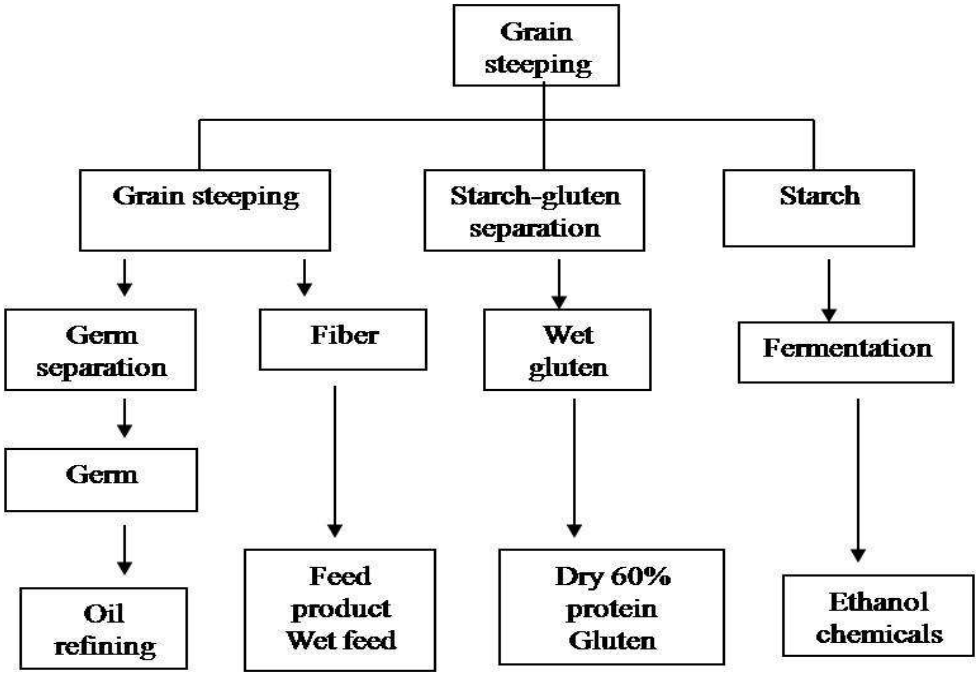


Figure 2.4: *Block diagram of the wet milling process* (Wyman, 1996).

The conversion of starchy biomass to ethanol has been an important focus during recent years. There are two techniques available worldwide for the conversion of the sugar content of starch to ethanol. These are the enzymatic hydrolysis and acid hydrolysis processes.

2.4.6 Separate enzymatic hydrolysis and fermentation (SHF)

Enzymatic hydrolysis of starch to dextrins is the first step (called liquefaction) in this method, where the resulting dextrins are then converted to glucose in a second hydrolysis step (called saccharification). Saccharification is done at an optimum temperature between 50°C and 65°C. The major disadvantage of SHF is that the sugar released from hydrolysis inhibits enzyme activity because glucose is a strong inhibitor for β-glucosidase. The activity of β-glucosidase reduces by 75% at a level of $3g.L^{-1}$ of glucose. Contamination is another problematic factor for SHF because hydrolysis is a lengthy process and a dilute solution of sugar has a risk of contamination by competing external micro-organisms, even at optimum temperatures of 45-50°C (Philippidis and Smith, 1995).

2.4.7 Simultaneous saccharification and Fermentation (SSF)

Simultaneous saccharification and fermentation is an ethanol production method that has been reported to give high ethanol yields and requires minimum amounts of enzyme because end-product inhibition from glucose and cellobiose sugars formed during enzymatic hydrolysis is relieved by yeast fermentation (McMillan, 1999). This is an advantage related to the process of ethanol production from different feedstocks that was first described by Takagi *et al*., (1997). During the simultaneous saccharification and fermentation process, the enzymatic degradation of starch is combined with the fermentation of glucose from hydrolysis of starch by yeasts into ethanol (see Figure 2.5). This method does not need sequential processes, because the glucose is converted to ethanol as soon as it is formed from dextrin in the hydrolysate. The main advantage of this method is its lower energy consumption and a lower content of non-glucosidic impurities, resulting in better ethanol production (Mojovic *et al*., 2006).

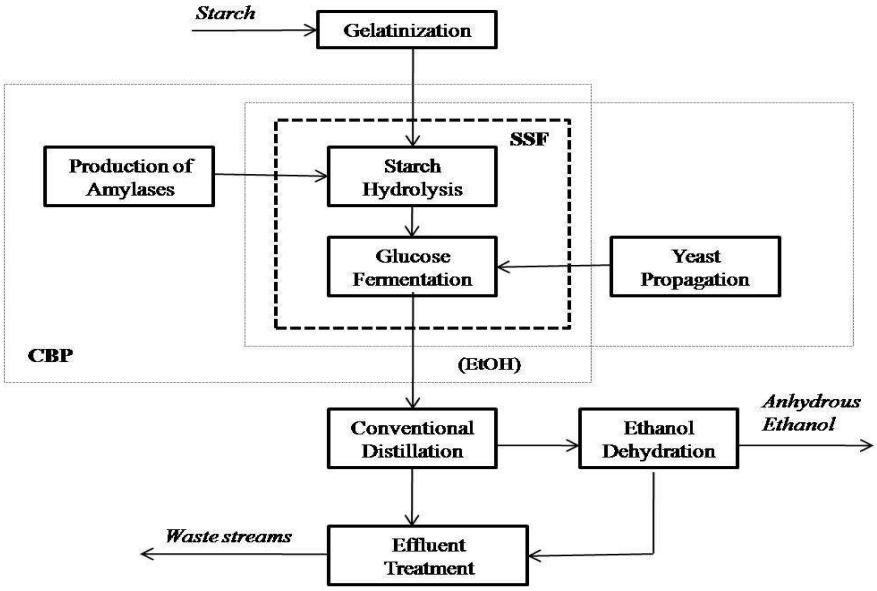


Figure 2.5: *Process flow diagram of ethanol production from starchy material via SSF* (Cardona and Sanchez, 2007).

2.4.8 Direct Fermentation (DF)

Bio-ethanol production has been greatly improved through the use of better technologies and a wide variety of crops. Cassava has received less attention despite its promising properties and potential in biofuels. Production costs tend to be high for

ethanol production since the enzymes have to be utilized throughout. However, direct fermentation through the use of *Schwanniomyces castellii/occidentalis* at a temperature of 30°C is a method that does not require hydrolysis of the Cassava starch (Wilson and Ingledew, 1982).

2.5 Overview of Cassava

The main non-food uses of Cassava are animal feed and starch. Approximately 5.5-6% of world production goes into starch for industrial processes such as alcohol and 10% and more of the total production is classified as lost as waste (see Table 2.1).

Table 2.1: *World utilization of Cassava (Data presented as a percentage of total production)* (Cock, 1985).

Area	Human consumption	Animal feed	Industrial use and starch	Export	Waste
Africa	50.8	1.4	a	a	9.5
World	33.8	11.5	5.5	7.0	10.0

[a] Less than 1%

In the years between the two World Wars, alcohol was produced from Cassava in Brazil and Australia, but that declined because of the availability of cheap supplies of petroleum products. There has been renewed interest in producing alcohol (ethanol) from this neglected crop because of its potential for bio-ethanol prosperity (Cock, 1985). It is a particularly attractive source for alcohol production since it can be grown on marginal land (less favored agricultural areas) and need not compete for land used for food crops. Therefore development of improved technology for conversion of Cassava starch to ethanol should also enhance the net energy ratio as production yields of Cassava rises (Cock, 1985).

2.5.1 Cassava production

In South Africa Cassava is considered a minor crop. It is mainly used as a subsistence crop by resource poor farmers. Plant breeders, agronomists and molecular biologists have made substantial improvements in Cassava yields during the past years and continue to do so. Genetic characterization and mapping has revealed some insights in the molecular nature of Cassava (Fregene *et al*., 2003). The growth of roots and tubers accounts for nearly 122 million metric tons with most of the increase being

Cassava, 80 million metric tons (66%) of the total. Total cassava production is projected to reach 168 million tons by 2020 based on the current production rate (see Table 2.2). Moreover, with the increasing demand and establishment of starch-utilizing industries in developing countries, the production of Cassava will increase beyond expectation (Scott *et al*., 2000), thus making Cassava a prospective crop because of its high yields and low costs.

Table 2.2: *Cassava production and use in 1993 and projected to* 2020 (Scott *et al*., 2000)

Country/region	**Area (million ha)**		**Yield (mt/ha)**		**Production (million mt)**		**Total use (million mt)**	
	1993	**2020**	**1993**	**2020**	**1993**	**2020**	**1993**	**2020**
Sub-Saharan Africa	11.9	15.9	7.4	10.6	87.8	168.6	87.7	168.1
Latin America	2.7	2.7	11.3	15.6	30.3	41.7	30.3	42.9
South-east Asia	3.5	3.5	12.1	13.7	42.0	48.2	18.9	24.4
India	0.2	0.2	23.6	28.4	5.8	7.0	5.7	7.3
Other South Asia	0.1	0.1	9.4	13.5	0.8	1.3	0.9	1.4
China	0.3	0.3	15.1	20.2	4.8	6.5	5.1	6.4
Other East Asia	na	na	na	na	na	na	1.8	1.9
Developing	18.8	22.9	9.2	12.0	172.0	274.7	152.0	254.6
Developed	….	….	12.1	14.7	0.4	0.4	20.7	20.5
World	18.8	22.9	9.2	12.0	172.7	275.1	172.7	275.1

2.5.2 The Cassava plant

Cassava was earlier classified as two species, *M ultissima* Phol and *M. aipi* Phol. These two species with varying cyanogenic glucoside concentrations have recently been classified as being the same species, *M. esculenta* Crantz. It is the only species of 98 species in the Euphorbiaceae family that is widely cultivated for food and industrial benefit. Cassava is a cultigen that originated in Brazil and is grown as an annual worldwide. It is a source of low cost carbohydrates with Brazil being the largest producer, followed by Thailand and African countries such as Nigeria and South Africa, and in Africa its production continues to increase. It is a starch crop that can grow and produce high yields in areas where most crops such as maize cannot grow or produce well. It can tolerate drought and can be grown on soils of pH 4.0 to 8.0 with low nutrient capacity and still respond well to irrigation, use of fertilizers and high rainfall, but not flooding (Ngo *et al*., 2005). This crop is grown for its enlarged starch-filled roots with the leaves containing a high level of protein

and stems rich in potassium. The main products of Cassava are Cassava chips for human consumption, starch for ethanol production and pellets normally used for animal feed.

Cassava is propagated vegetatively as clones that are different cultivars with institutional code names. Classification of cultivars is mostly based on pigmentation and shape of the leaves, stems and roots. Cultivars commonly vary in yield, root diameter and length, harvest time and temperature adaptation. All kinds of Cassava cultivars have potential for ethanol production due to the fact that industrial uses of Cassava include manufacturing of products such as alcohol from its starch (Cock, 1985). The principal parts of the mature Cassava plant are leaves, stem, and roots (see Figure 2.6) and their composition is expressed as a percentage of the whole plant (see Table 2.3).

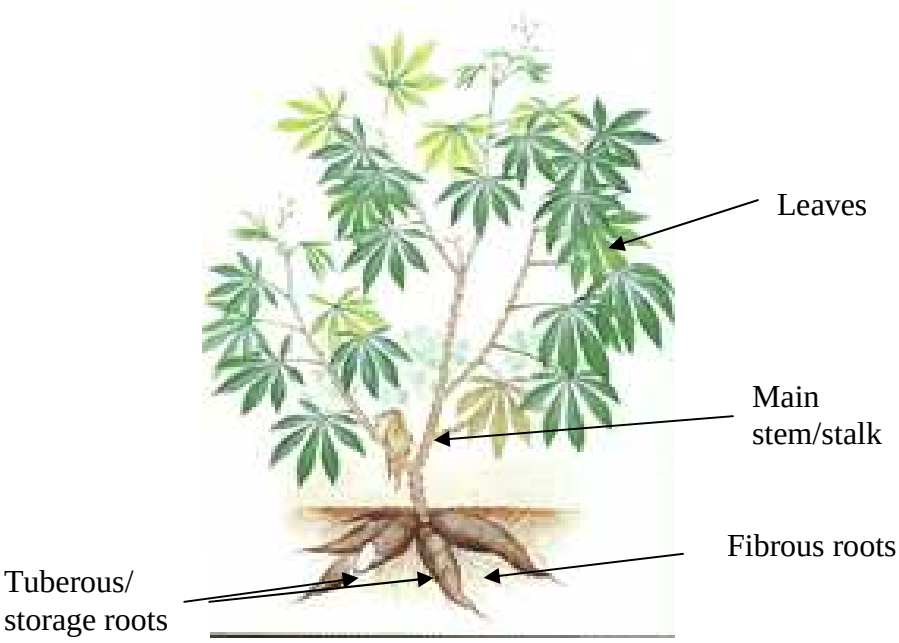


Figure 2.6: *Principal parts of the cassava plant*

Table 2.3: *Percentage composition of Cassava plant* (Ngo *et al*., 2005)

Plant part	Composition
Leaves	6%
Stem	44%
Roots	50%

2.5.3 Cassava stem and leaves

Cassava leaves contain high levels of protein (average 30.5%), vitamins and micronutrients, but low amounts of carbohydrates with starch as the major proportion of the leaf carbohydrate. The amylose content of the leaf starch has been reported to range from 19-24%, with the crude fiber low in contrast to the stem that has higher levels of the fiber (Tewe *et al*., 1976). The leaves are mainly used as a potherb, sometimes for animal feed and for compost (Viljoen and Laurie, 2006).

2.5.4 Cassava roots and peels

The tuberous roots covered with a brown outer bark (peels), grow in clusters with a cream white interior containing high starch content. The root is usually from 1-4 inches in diameter and 8-15 inches long. The peels contain toxic hydrocyanic acid, which is eliminated during cooking (gelatinization). Figure 2.7 shows a typical cassava starch root (a) and the peels (b) that also contain starch, making Cassava a potential producer of ethanol for energy and environmental performance.

a) Cassava storage root

b) Cassava peels

Figure 2.7: *a) Typical Cassava storage root; b) Cassava peels*

Cassava roots mature to harvest within 8 to 24 months of planting, depending on cultivar and climate. The root is circular in cross section (see Figure 2.8), consisting of three principal parts transversely (Ngo *et al*., 2005).

- The periderm: It is the outer most layer of the root, composed mainly of dead cork cells that seal the surface of the root.
- The cortex: A layer beneath the periderm
- The starchy flesh: The central surrounding portion of the root packed with starch grains

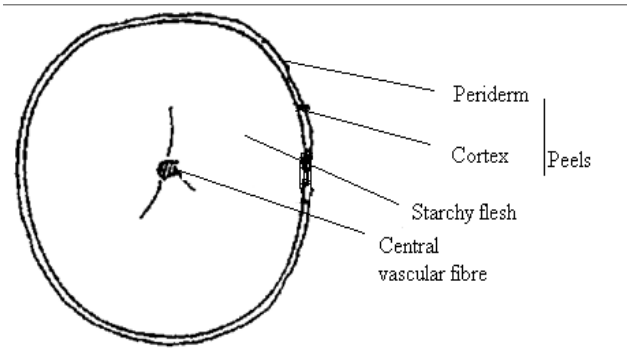


Figure 2.8: *Cross-section of Cassava storage root* (Obadina *et al*., 2006).

Cassava wastes such as the peels constitute 20-35% of the weight of the tuber and have approximately 61 % (w/w) starch content. Consequently, a large amount of Cassava peel waste is generated annually (Obadina *et al*., 2006). However, the possibility of using Cassava peels for ethanol production has not been given much consideration, but a study by Adesanya *et al*., (2008) on milled Cassava peels as substrate for ethanol production revealed that Cassava peel starch can be readily degraded either by amylolytic organisms up to 0.88 $mg.ml^{-1}$ reducing sugar and more with enzymes approximately ($57g.L^{-1}$). The starchy flesh comprises up to 80% to 90% of the root, and that includes all the components in Table 2.2 below. The composition of Cassava roots is shown in Table 2.2 below, amylose and amylopectin are between 24-35% on a dry weight basis, making Cassava a predominantly starchy food with starch having the highest percentage of approximately 64-78% of the carbohydrates (Ngo *et al*., 2005).

The Cassava root system is distinguished by various adventitious root types such as fibrous roots (FRs) (see Figure 2.6 above) that absorb water and mineral salts providing support function and storage roots (SRs), which accumulate starch as a reserve compound. Cassava roots also contain sucrose, maltose, glucose and fructose in limited amounts. The storage roots are rich in calcium, phosphorus and vitamin C, but low in protein and minerals, except for the peels that contain more protein and vitamins than is found in the root flesh. The mineral content of the dry bark (periderm) is higher than that of the cortex.

Table 2.4*: A typical composition of Cassava storage root* (Ngo *et al*., 2005; Tonukari, 2004)

Constituent	Composition
Moisture	60-70% (wet basis)
Carbohydrates	24-35%
Fiber (cellulose)	1-2%
Protein	1-2%
Other	3%

2.5.5 Cassava starch

Cassava contains 64-84% of the purest renewable natural polymer (starch) on a dry basis, with a low quantity of other constituents such as proteins and fiber, thus making it useful for a wide range of applications, including biofuels (Lasztity, 1999). Fresh Cassava roots were found to contain 38.7% dry matter and of the 38.7%, 31 % consisted of starch and soluble carbohydrates (Ewell and Wiley, 1893). Further experiments done by Ewell and Wiley (1893) to determine the yield of air dry starch that can be obtained from Cassava roots revealed that 26% of the carbohydrates were pure starch with trace amounts of nitrogenous material. A study on the viscosity of Cassava starch done by Lasztity (1999) indicated that Cassava starch contains a high concentration of amylopectin compared to amylose (80:20), which gives it a high viscosity but low potential for retro-gradation, making resulting in good freeze-thaw stability. Cassava is a low priced carbohydrate feedstock for ethanol fermentation with good susceptibility to acid and/or enzymatic hydrolysis even at low temperatures as shown in Table 2.5 below, which gives Cassava starch properties. Cassava is a relatively cheap source of raw material containing a high concentration of starch (dry-matter basis) that can equal the properties offered by other starch crops, general properties of Cassava are given in Table 2.5.

Table 2.5: *General properties of Cassava starch* (Lasztity 1999).

PROPERTIES	VALUE
Chemical composition (%)	
- Protein	0.15-0.30
- Fat	0.0-0.1
- Ash	0.10-0.15
Granule size (µm by SEM image analysis)	3-34
Amylose content (% by HPSEC)	17-23
Swelling power 85°C (0.1g in 15ml distilled water)	40-62%
Degree of hydrolysis (%, using 1% each of α amylase and gluco-amylase at 37°C, 48 hrs)	25-60

2.6 Potential of Cassava for bio-ethanol production

The use of Cassava for ethanol production has recently attracted attention because it can be cultivated on marginal land where other crops cannot be grown successfully and because it is not considered to be a staple food such as maize, wheat and rice. Cassava roots are rich in energy due to the high carbohydrate content of the roots, which makes Cassava a suitable biomass source for bio-ethanol production (Agu *et al*., 1997)

2.6.1 Energy efficiency of Cassava

In most studies, including an energy analysis by Hodge, (2002), it is concluded that the net energy value of Cassava bio-ethanol production is greater than that of maize bio-ethanol production. One reason for the net gain of energy during the production of bio-ethanol from Cassava roots is the higher yield of Cassava per hectare (12 ton/ha), which is much higher than that of maize. In addition, Cassava cultivation requires less fertilizer (100 $kg.h^{-1}$) than maize (144 $kg.ha^{-1}$). A study conducted in China by Hu *et al*., (2004) concluded that bio-ethanol produced from Cassava is both energy and renewable energy efficient in converting a solid energy source into a liquid energy source, since the solar energy trapped in Cassava is greater than the energy used in the industrial processing of Cassava (Zhang *et al*., 2003).

2.6.2 Cassava starch fermentation to ethanol

Bio-ethanol has already been introduced as a transportation fuel in countries like Brazil, the United States and some European countries. In Brazil it is produced from cane sugar and in the United States from maize starch. Bio-ethanol can be produced from either lignocellulose or starch. Commercial bio-ethanol production from lignocellulose material is hindered due to techno-economic considerations, while bio-ethanol production from starch is widely used in most bio-ethanol producing countries. Furthermore, high ethanol yields from lignocellulosic material require complete hydrolysis of both the cellulose and hemicellulose materials, followed by efficient fermentation of all sugars in the biomass. The bioconversion of Cassava starch into ethanol can be done in three ways. The first is fermentation through hydrolysis of starch by the method of separate enzymatic hydrolysis and fermentation (SHF), while the second method is simultaneous saccharification and fermentation (SSF) where the sugars is utilized as soon as it is formed (John *et al*., 2006). The third method is the use of amylolytic yeast for the direct fermentation of starch that eliminates hydrolysis through solid state fermentation. The amylolytic yeast *Schwanniomyces castellii* for direct fermentation of starch is considered to be suitable for the direct fermentation of starch (Ma *et al*., 2000; Oner *et al*., 2005).

2.7 Optimization of ethanol yield from Cassava

Optimization of ethanol yield is concerned with the energy consumption during production. A combination of different steps in the production process of bio-ethanol into one single unit is seen as a prospective optimization tool, while the immediate continuous removal of ethanol from the biotransformation process is another opportunity to increase product yield, while also reducing product costs (Cardona and Sanchez, 2007). In the earlier works of Mullis and NeSmith, (1984) the economic evaluation of starchy materials as feedstock for bio-ethanol production on a small scale was done to exasperate on the cost affectivity of bio-ethanol.

2.8 REFERENCES

ADESANYA, O. A., OLUYEMO, K.A., JOSIAH, S.J., AESANYA, R.A., SHITU, L.A.J., OFUSORI, D.A., BANKOLE, M.A., & BABALOLA, G.B. 2008. Ethanol Production by Saccharomyces Cerevisiae from Cassava Peel Hydrolysate. *The Internet Journal of Microbiology*, 5(1), Feb

AGU, R. C., AMADIFE, A. E., UDE, C. M., ONYIA, A., OGU, E. O., OKAFOR, M. & EZEJIOFOR, E. 1997. Combined heat treatment and acid hydrolysis of Cassava grate waste (CGW) biomass for ethanol production. *Waste Management*, 17(1): 91-96, 25 Sept.

AKTINSON, B. & MAVITUNA, F. 1991. Upstream processing. *Biochemical Journal of Engineering and Biotechnology*: 834–835, Apr.

AVANCINI, S.R.P., FACCIN, G.L., VIEIRA, M.A., ROVARIS, A.A., PODESTA, R., TRAMONTE, R. & De SOUZA, N.M.A. 2007. Cassava starch fermentation wastewater: Characterization and preliminary toxicological studies. *Food and Chemical Toxicology*, 45:2273-2278, 01 Dec.

AYERNOR, G.S., HAMMOND, T.K. & GRAFFHAM, A. 2002. The combination of rice malt and amyloglucosidase for the production of sugar syrup from cassava flour. *African Journal of Science and Technology*, 3(1): 10-17, 01 Jan.

BOMB, C., McCORMICK, K., DERWAARDER, E., & KÅBERGER, T. 2007. Biofuels for transport in Europe: lessons from Germany and the UK. *Energy Policy*, 35:2256–2267, Apr.

CARDONA, A. C. & SANCHEZ, O.J. 2007. Fuel ethanol production: Process design trends and integration opportunities. *Bioresource Technology*, 99(12): 2415-57, 01 Mar.

COCK, J.H. 1985. Cassava: a basic energy source in the tropics. *Science*, 218(4574): 755-762, 19 Nov.

DERMIBAS, A. H. 2007. Combustion of Biomass. *Energy Sources Part A: Recovery, Utilization, and Environmental Effects*, 29(6): 549-56, 06 Jan.

EWELL, E. E. & WILEY. H. W. 1893. Some products of cassava. *Journal of the American Chemistry Society,* 15:78–82.

FREGRENE, M.A., SUAREZ, M., MKUMBIRA, J., KULEMBEKA, H., NDEDYA, E., KULAYA, A., MITCHELL, S., GULLBERG, U., ROSLING, H., DIXON, A.G., DEAND, R., & KRESOVICH, S. 2003. Simple sequence repeat marker diversity in cassava landraces: genetic diversity and differentiation in an asexually propagated crop. *Theoretical and Applied Genetics,* 107(6): 1083-1093.

GOETTEMOELER, A. & GOETTEMOELER, J. 2007. Sustainable Ethanol. 1st ed. Prairie Oak.

HAHN-HADERDAL, B., JEPPSON, H., SKOOG, K. & PRIOR, B.A. 1994. Biochemistry and physiology of xylose fermentation by yeasts. *Enzyme Microbiology Technology,* 16: 933-943,

HAMELINCK, C.N., & FAAIJ, A.P.C. 2006. Outlook for advanced biofuels. Energy Policy, 34:3268–3283.

HEMA, A., TRIVEDI, U.B. & PATEL, K.C. 2006. Glucoamylase production by solid-state fermentation using rice flake manufacturing waste products as substrate. *Bioresource Technology,* 97: 1161–1166, Jul.

HODGE, C. 2002. Ethanol use in US gasoline should be banned, not expanded. *Oil Gas Journal,* 100: 20-28, Sept.

HOOGWIJK, M., FAAIJ, A., Van Den BROEK, R., BERNDES, G., GIELEN, D. & TURKENBURG, W. 2003. Exploration of the ranges of the global potential for biomass for energy. *Biomass Bioenergy,* 25: 119–133, Aug.

HU, Q., SOMMERFELD, M., JARVIS, E., GHIRARDI, M., POSEWITZ, M., SEIBERT, M. & DARZINS, A. 2004. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant Journal,* 54: 621–639, May.

JOHN, R.P., NAMPOOTHIRI, K.M. & PANDEY, A. 2006. Simultaneous saccharification and fermentation of cassava bagasse for l (+) lactic acid production. *Applied Biochemistry Biotechnology*, **134:** 263–272, Dec.

LAGUNAS, R. 1979. Energetic irrelevance of aerobiosis for *S.cerevisiae* growing on sugars. *Molecular Cell Biochemistry*, 27(3): 139-146, Mar.

LARSON, E.D. & WLLIAMS, R.H. 1993. Biomass plantation energy systems and sustainable development. (In: J. Goldemberg and T.B. Johansson, Editors, *Energy as an Instrument for Socio-Economic Development*). New York: United Nations Development Programme: 91–106, Nov

LASZTITY, R. 1999. Cereal chemistry. Hungary: Akademiai Kiado: 11-51.

LIN, Y. & TANAKA, S. 2008. Ethanol fermentation from biomass resources: current state and prospects. *Applied Microbiology Biotechnology*, 69: 627–642, Apr.

MA, Y. J., Lin, L. L., CHIEN, H. R. & HSU, W. H. 2000. Efficient utilization of starch by a recombinant strain of *Saccharomyces cerevisiae* producing glucoamylase and isoamylase. *Biotechnology Applied Biochemistry*, 31:55-59, 31 Feb.

McMILLAN, J.D. NEWMAN, M.M. TEMPLETON D.W. & MOHAGHEGHI, A. 1999. Simultaneous saccharification and co-fermentation of dilute-acid pretreated yellow poplar hardwood to ethanol using xylose-fermenting *Zymomonas mobilis*. *Applied Biochemistry and Biotechnology*: **77–79, Mar.**

MOJOVIC, L., NIKOLIC, S., RAKIN, M. & VUKASINOVIC, M. 2006. Production of bioethanol from corn meal hydrolyzates. *Fue*l, 85 (12-13): 1750-1755, Oct.

MULLIS, J.T. & NeSMITH, C. 1984. Integrated ethanol production and utilization system for small farms. *Biomass Journal*, **6:** 155–166, 08 Jul.

NAG, A. 2008. Biofuels Refining and Performance. United States of America: McGraw-Hill: 5-35, 17 Dec.

NGO, T. D., INGER, L. & NGUNYEN, T. M. 2005. Intercropping cassava (Manihot esculenta Crantz) with Flemingia (Flemingia macrophylla); effect on biomass yield and soil fertility. *Livestock Research for Rural Development*. 17(6), Feb.

NGUNYEN, T.L.T. & GHWEEWALA , S.H. 2008. Life cycle assessment of fuel ethanol from cassava in Thailand. *International Journal of LCA*, 13(2): 147-154, Jun.

OBADINA, A.O., OYEWOLE, O.B., SANNI, .L. O. & ABIOLA, S. S. 2006. Fungal enrichment of cassava peels proteins. *African Journal of Biotechnology*, 5 (3): 302-304, May.

ONER, E.T. STEOHEN, G. O. & KYRDAR, B. 2005. Production of Ethanol from Starch by Respiration-Deficient Recombinant *Saccharomyces cerevisiae. Applied and environmental microbiology,* 71 (10): 6443–6445, 25 Nov.

PANDEY, A. 1994. Solid State Fermentation; An overview in solid state fermentation. New Delhi, India: Wiley. 3-10

PANDEY, A. 1992. Recent developments in solid state fermentation. *Process Biochemistry,* 27: 109-117, Jul.

PHILIPPIDIS, G.P. & SMITH, T.K. 1995. Limiting factors in the simultaneous saccharification and fermentation process for conversion of cellulosic biomass to fuel ethanol. *Applied and Biochemical Biotechnology,* 51/**52**:117–124, Sept.

POONAM, N. & DALEL, S. 1995. Enzymes and microbial systems involved in starch processing. *Enzyme and Microbial technology,* 17: 770-777, 17 Feb.

ROBLE, N. 2003. Development of production systems for raw cassava starch bioconversion in novel bioreactors with cells immobilized in loofa (Luffa cylindrica) sponge. Japan: University of Tsukuba (Thesis-PhD).

SAHA, B.C. 2003. Hemicellulose conversion. *Journal of Industrial Microbiology and Biotechnology,* **30:**279–291.

SCOTT, G, J., ROSEGRANT, M. W. & RINGLER, C. 2000. Roots and Tubers for the 21st Century: Trends, Projections, and Policy Options. United States of America: 1-71.

SRINORAKUTARA, T., SUESAT, C., PITIYONT, B., KITPREECHAVANIT, W. & CATTITHAMMANIT, S. 2004. Utilization of Waste from Cassava Starch Plant for Ethanol Production. (Joint International Conference on "Sustainable Energy and Environment (SEE), Hua Hin. Thailand. p. 773-76).

TAKAGI, M., ABE, S., SUZUKI, S., EMERT, G.H. & YATA, N. 1977. A method for production of ethanol directly from cellulose using cellulase and yeast. (Proceedings of Bioconversion Symposium. Sweden:Delhi, p 551–71).

TEWE, O.O., JOB, T.A., LOOSLI, J.K. & OYENUGA, V.A. 1976. Composition of two local cassava varieties and the effect of processing on their hydrocyanic acid content and nutrient utilization by the rat. *Nigerian Journal of Animal Production,* 3 (2): 60-66, Jun.

TONUKARI, N.J. 2004. Cassava and the future of starch. *Electronic Journal of Biotechnology,* 7 (1): 5-8, 15 Apr.

VILJOEN, J.C. & LAURIE S.M. 2006. Results obtained from a baseline study on cassava in South Africa. Agricultural Research Council-Roodeplaat and Ornamental Plant Institute. Technical report. Pretoria. 1-50.

VISSER, W. 1995. Oxygen requirements of fermentative yeast. Netherlands: Delft university of Technology. (Thesis-PhD).

WILSON, J.J. & INGLEDEW, W.M. 1982. Isolation and characterization of *Schwanniomyces alluvius* amylolytic enzymes. *Applied and Environmental Microbiology,* **44:** 301–307, Aug.

WYMAN, C.E. 1996. Ethanol production from lignocellulosic biomass: Handbook on Bioethanol Production and Utilization: 1-18.

ZHANG, C. HAN, W.J., PU, G.Q. & WANG, C.T. 2003. Life cycle economic analysis of fuel ethanol derived from cassava in Southwest China. *Renewable and Sustainable Energy Review,* 7: 353-66, 18 Mar.

ZOHREH, A. 2008. Ethanol and glucose tolerance of *M.indicus* in aerobic and anaerobic conditions. Sweden: University College of Boras. (Thesis-M.Sc.) 5-40.

CHAPTER 3

EXPERIMENTAL

"Normal people ... believe that if it isn't broken, don't fix it. Engineers believe that if it isn't broken, it doesn't have enough features yet."

Scott Adams

Overview

In this chapter the experimental work done and experimental procedures followed in this study is discussed in detail. The chapter is subdivided into three sections. The first Section (3.1) gives an overview of the materials used and raw material preparation. The equipment used in this investigation is discussed in Section 3.2, showing the experimental setup. The experimental error and starch degradation process are shown in Section 3.3 and 3.4 respectively and the experimental procedure follows in Section 3.5. Analytical procedures are detailed in Section 3.6 together with the compositional analysis of Cassava cultivar used in this study. Optimization of the enzymatic hydrolysis of cassava roots is discussed in detail in Section 3.7 followed by the optimization of fermentation steps in Section 3.8.

3.1 MATERIALS AND CHEMICALS

All materials, chemicals, enzymes and microorganisms used in this study are listed in Table 3.1. All chemicals and enzymes were used without any prior purification.

Table 3.1: *Material and chemicals used in this study*

Chemicals	Description	Supplier	Purity
Glucose	Glucose calibration curve	Saarchem (Merck)	99.0%
Fructose	Fructose calibration curve	Sigma Aldrich	99.0%
Sucrose	Sucrose calibration curve	Sigma Aldrich	99.0%
Maltose	Maltose calibration curve	Sigma Aldrich	95.0%
Maltotriose	Maltotriose calibration curve	Sigma Aldrich	95.0%
Ethanol	Ethanol calibration curve	Rochelle chemicals	99.9%
Calcium Hydroxide	pH operation	Saarchem (Merck)	95.0%
Sulphuric acid	pH operation	Labchem	98.0%
Iodine	Starch test	Sigma Aldrich	-
Yeast extract	Yeast growth media	Sigma Aldrich	-
Malt extract	Yeast growth media	Sigma Aldrich	-
Peptone	Yeast growth media	Sigma Aldrich	-
Glycerol	Yeast storage	Sigma Aldrich	-
Magnesium Sulfate Heptahydrate	Yeast growth media	Sigma Aldrich	99.0%
Potassium phosphate monobasic	Yeast growth	Sigma Aldrich	99.0%
Urea	Yeast growth	Sigma Aldrich	98.0%
Enzymes			
Termamyl SC	α-Amylase enzyme mixture	Novozymes, South Africa	-
Spirizyme Fuel	Gluco-amylase enzyme mixture	Novozymes, South Africa	-
Celluclast 1.5L	cellulase enzyme mixture	Novozymes, South Africa	-
Micro-organisms			
S. cerevisiae	Bakers' yeast for fermentation	Anchor Yeast, South Africa	-
S. castellii/occidentallis	Amylolytic yeast for fermentation	ATCC, USA	-
Materials			
Cassava roots	Raw feedstock for ethanol production	Agricultural Research Council	-

3.1.1 Preparation of yeasts

3.1.1.1 *Saccharomyces cerevisiae*

S. cerevisiae was revived from the dormant state by using the fermentation broth (medium for fermentation of hydrolysate) as growth medium for ten minutes before use in the batch fermentation.

3.1.1.2 *Schwanniomyces occidentalis/castellii*

Freeze dried organisms were rehydrated with sterile water (water) and plated on malt extract agar and growth was observed after two days. Stock cultures were made in 15% glycerol for long term storage.

Schwanniomyces occidentalis/castellii ATCC 26706 was preserved and stored on glycerol stocks at 4°C; it was subcultured on malt extract agar plates for 72hrs at 32°C, from which an inoculum was prepared. The yeast was grown at 45°C, 150rpm in malt extract broth (YM broth) containing 0.5 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.5 $g.L^{-1}$ $(NH_4)_2HPO_4$, 1.5 $g.L^{-1}$ yeast extract, 5 $g.L^{-1}$ glucose, 1.5 $g.L^{-1}$ malt extract and 2.5 $g.L^{-1}$ peptone pH 5.5 (Srinorakutara *et al*., 2004). The concentration of *S. occidentalis/castellii* used was 10% (v/v) of fermentation sample (Saelim *et al.*, 2008).

3.1.2 Preparation of raw Cassava

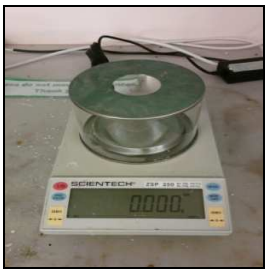
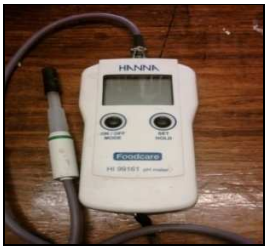
Three forms of Cassava were used in this project, i.e. unpeeled roots (starch and cellulose), peels (cellulose) and peeled roots (starch). The roots were washed thoroughly by hand to remove any soil residues. After washing, the roots were peeled using a knife and chipped/sliced with knives. Some roots were not peeled; they were only washed and sliced. Some Cassava chips were oven dried at 60°C for 24 hours and some were dried in the sun for 3 days. All the Cassava chips were milled into fine flour and sieved with a +1.5 mm screen.

3.2 Equipment

All equipment used in this study is listed in Table 3.2.

Table 3.2: *Equipment used in this study*

Equipment	Description	Model number	Supplier
	Hammer mill for milling of Cassava chips	model TRF-70	Trapp
	HPLC for analysis of hydrolysate and fermentation broth	model 1200 Series	Agilent Technologies
	Oven used to heat distilled water for liquefaction as well as to keep the liquefaction mixture 95°C.	model 276	Scientific
	Shaker incubator used to keep the saccharification temperature at 55°C and fermentation broth at 30°C while agitating constantly.	model FSIE-SPO 8-35	Labcon
	Centrifuge was used for separating the fermentation samples to liquid and solids	Rotilabo-mini-centrifuge	Carl Roth

	Mass balance was used for weighing the material used in this study.	Model ZSP-250	Scientech
	pH meter used to measure pH during all the experiments	Model HI 99161	Hanna Instruments
	Moisture analyzer used for moisture analysis of the materials	Model HR 83	Mettler-Toledo
	Spectrophotometer used to measure absorbance of unhydrolyzed and hydrolyzed samples	Model 20-4001/UV-VIS	Thermo Fischer Scientific
	Glassware used for liquefaction, saccharification and fermentation experiments	1000mL with DIN thread GL 45	Duran group

The analysis equipment used in this study are:

1. HPLC
2. Moisture Analyzer
3. Spectrophotometer

3.3 Experimental error

The experimental error associated was determined for each separate hydrolysis and fermentation step. The details of the calculations can be found in Appendix D. The experimental error associated with each of the process steps utilized in this investigation is listed in Table 3.3.

Table 3.3: *Error percent and product values for SHF*

Process step	Error %
Liquefaction	2
Saccharification	5
Fermentation	5

3.4 Determination of complete starch degradation

The ability of iodine to bind amylose has been used to understand a variety of structural and functional aspects of starch in food systems. In starch granules, the linear amylose polymer binds a significantly higher proportion of iodine than does the branched amylopectin molecule. After molecular dispersion, the amylose-iodine binding ability is commonly used to quantify the amylose content of starches from various botanical sources ((Morrison and Laignelet, 1983).

The presence of starch in this study was determined by a 0.05 mol I_2 (12.69g I_2 + 20g KI) solution. A drop of the iodine solution was inoculated in the slurry before and after hydrolysis. A deep purple/blue color would be an indication of the presence of starch, dark red indicates dextrins and light brown to clear color indicating complete hydrolysis of the starch (see Figure 3.1). The iodine solution was used to determine if all the starch in the Cassava sample used for liquefaction and saccharification has been reduced to glucose.

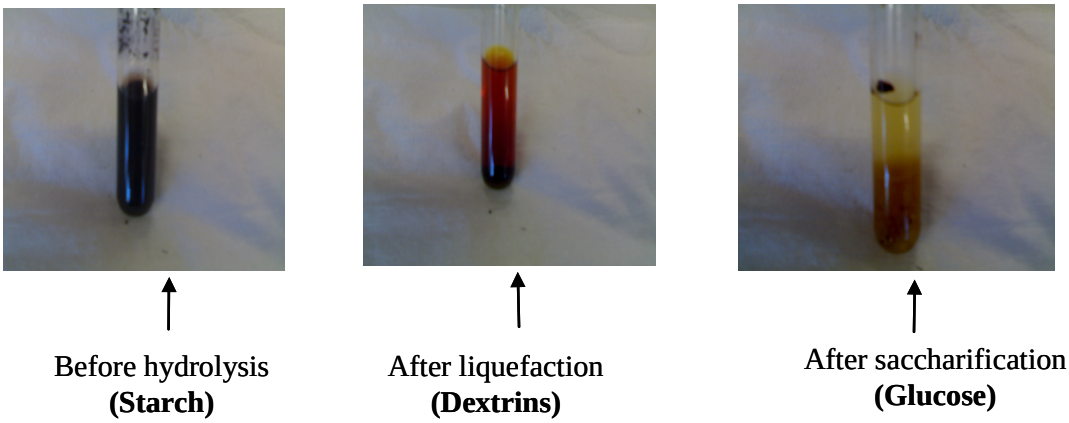


Figure 3.1*: The presence and degradation of starch during liquefaction*

The degradation of starch to reducing sugars was followed using a colorimetric method (Morrison and Laignelet, 1983). An UV-VIS spectrophotometer (see Table 3.2) was used at a wavelength of 620nm to determine the absorbance of iodated hydrolysate samples. A blank solution consisting of water and iodine solution was used to standardize the absorbance measurements (Saibaene and Seetharaman, 2008). Absorbance measured is proportional to the starch concentration of the sample tested. The absorbance of hydrolyzed starch will thus decrease from the original non-hydrolyzed starch solution.

Table 3.4: *Starch content of hydrolysates degradation during starch hydrolysis to glucose*

Time(hrs)	Hydrolysis process	Control	Hydrolysate
0	-	0.9	3.9
1.5	Liquefaction	0.4	2.4
48	Saccharification	2.3	0.6

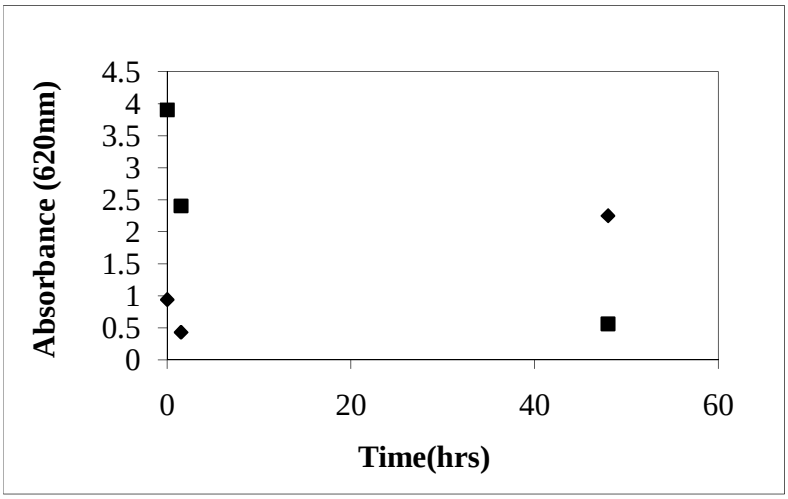


Figure 3.2*: Starch degradation during enzymatic hydrolysis*

(♦ - Control, ■ - Hydrolysate)

The results from Table 3.4 and Figure 3.1 confirm the presence of starch in the hydrolysis process. The results also indicate that starch was degraded to glucose in this study, because the absorbance decreases with time and a definite change in colour of the hydrolysate was observed during this period. the degradation of the starch by the enzymes used, by observing the physical properties of starch determination and the values of the absorbance it clearly shows a decrease in the starch present as hydrolysis succeeds. The control shows an increase in the starch concentration, which is probably reason for the absence of enzymes to degrade the starch. One factor in the increase of starch concentration in the control is that the Cassava granules in the slurry were swelling from exposing more starch. On the contrary, the hydrolysate was immediately hydrolyzed by the enzymes as the starch was exposed more and more.

3.5 Experimental procedure

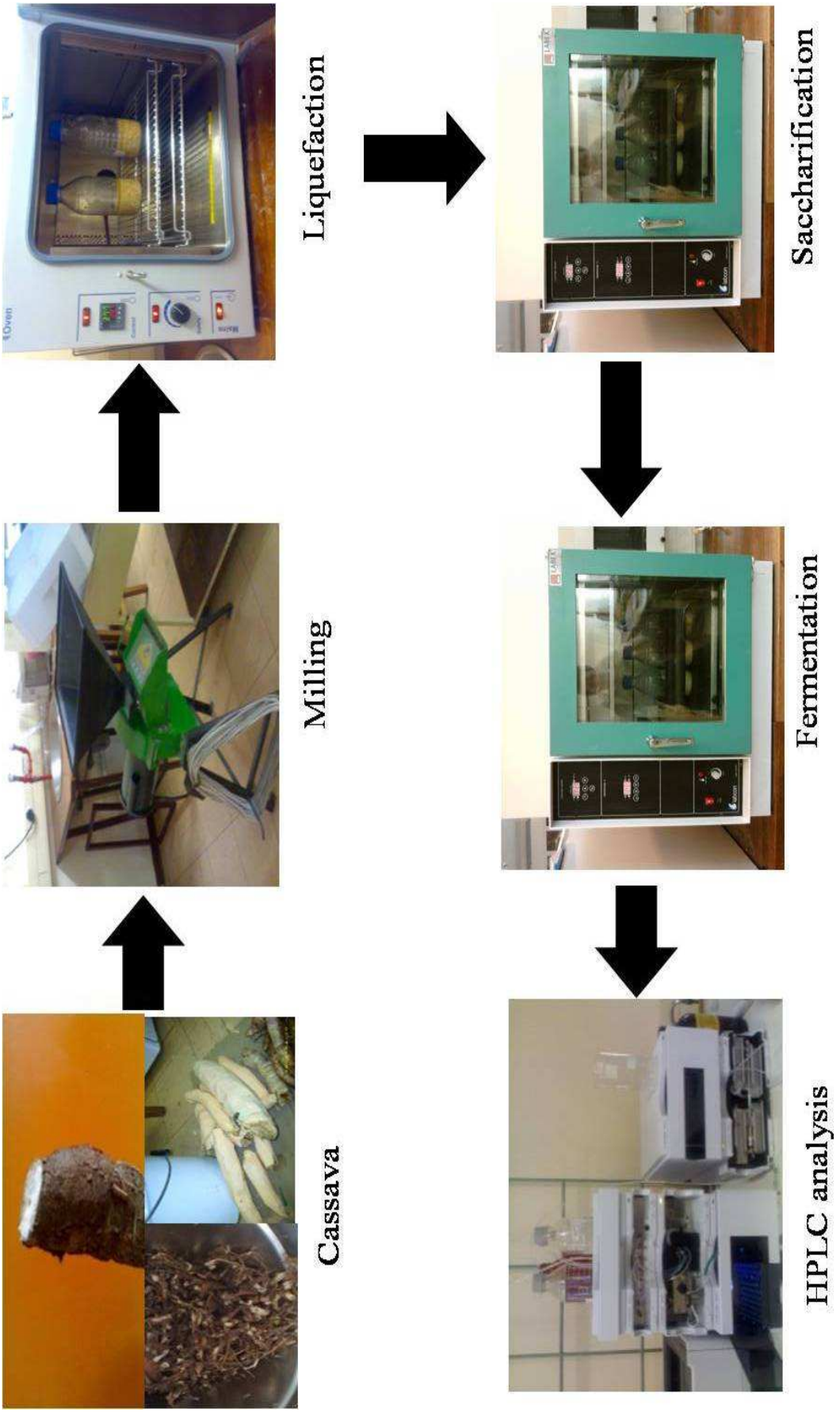


Figure 3.3: *Flow diagram of experimental procedure followed for Cassava hydrolysis and fermentation*

The experimental procedure is also shown schematically in Figure 3.4 and 3.5. In Figure 3.4 the SSF and SHF by *S. cerevisiae* are presented and Figure 3.5 shows the procedure for direct fermentation by *S. occidentalis*.

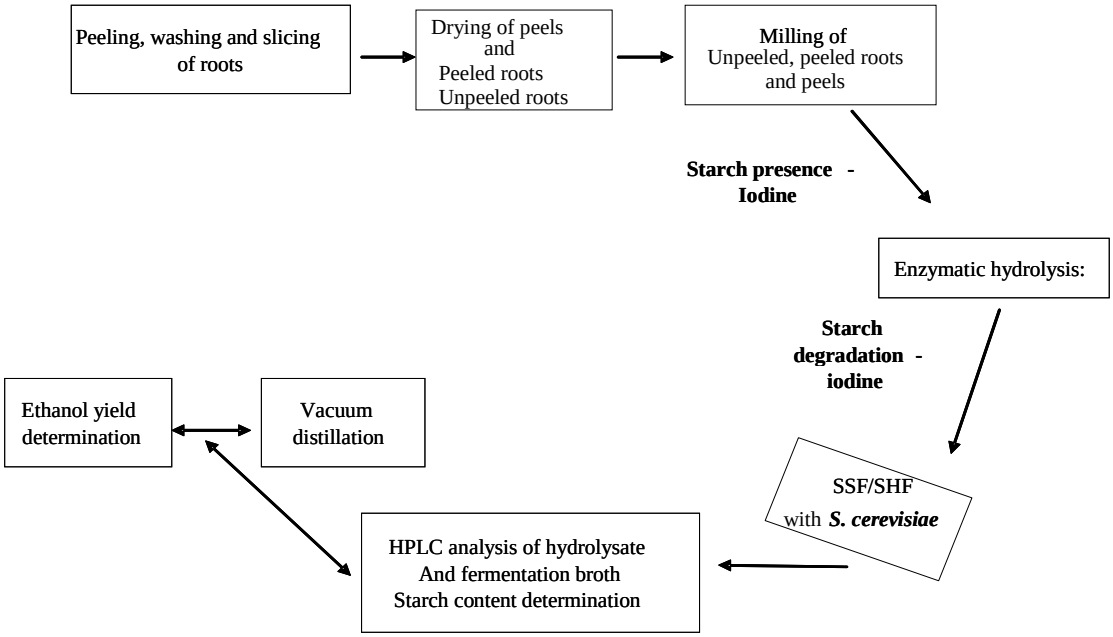


Figure 3.4: *Flow diagram of experimental procedure followed for the SHF and SSF processes with S. cerevisiae*

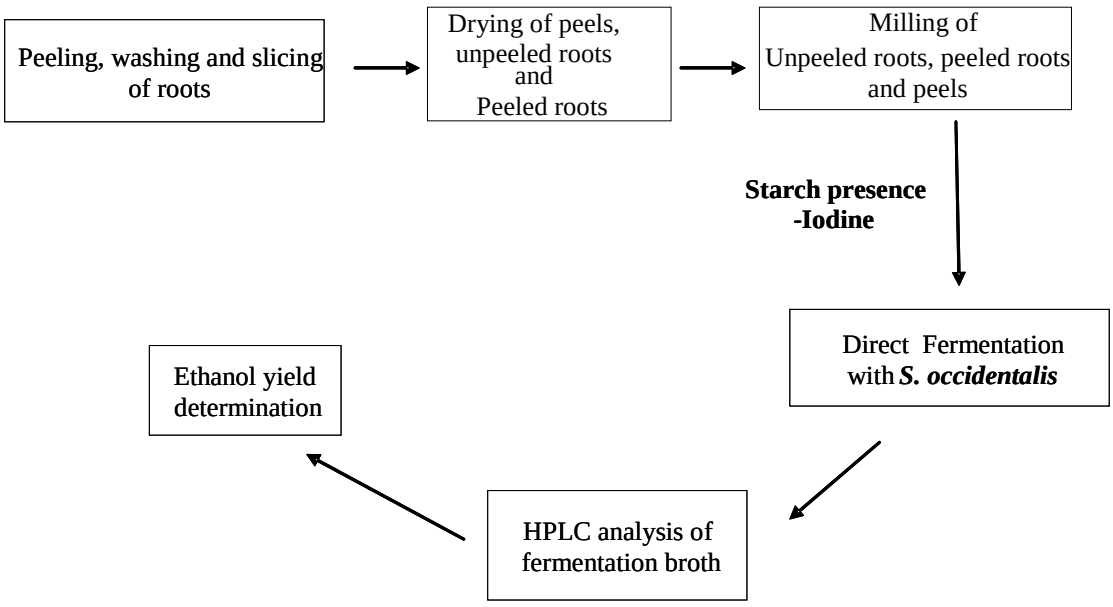


Figure 3.5: *Flow diagram of experimental procedure followed for the DF process with S. occidentalis*

3.6 Analytical procedures

3.6.1 Compositional analysis of Cassava

A complete compositional analysis of the Cassava cultivar used in this study was done by the South African Grain Laboratory for the Cassava peeled roots (starch), Cassava peels (cellulose) and unpeeled Cassava roots (starch and cellulose). The results of the composition analysis done by the SAGL according to the AACC standard methods are presented in Table 3.5.

Table 3.5: *Composition of Cassava root peels, unpeeled Cassava root and peeled root*

Component	Composition (wt %)		
	Unpeeled Cassava root	**Cassava peeled root**	**Cassava peels**
Moisture	9.5	10	9.2
Protein	2.5	2.7	5.1
Starch	81	82	67
Fat	0.6	0.8	1.1
Ash	2.5	2.5	7.0
Crude Fibre	3.9	2.0	11
Total	100	100	100

The main components were found to be starch, protein, fat, ash, crude fiber and moisture. The main component of all the samples submitted for analysis was found to be starch in a range of 67 to 83 wt%. These values correspond to the findings of Aryee *et al.*, (2006). The moisture and starch content are influencing factors in hydrolysis and fermentation for ethanol production. The moisture content lowers the rate of reaction because the higher the moisture content, the higher the viscosity of the feedstock and the more difficult it is for the enzymes to reach the starch particles. The starch content determines how much fermentable sugars (glucose) can be expected as well as the expected ethanol yield. The higher the starch content, the higher the glucose and ethanol yield will be.

3.6.2 Determination of moisture content of Cassava

The initial moisture content (%) of raw Cassava roots as well as the moisture content of the dried and milled Cassava flour was determined for this study. The initial moisture content of the raw Cassava roots were determined by drying a wet slice of unpeeled Cassava root with a known initial mass in an oven at 60°C for 24 hours.

The percentage weight loss during this time was calculated as the initial moisture content of the root. Two methods were used for unpeeled Cassava roots as a comparison to other work done by other researchers on specifically unpeeled Cassava roots moisture content. One single method was the used for the three forms in this study for comparison of the three feedstock forms specific in this study. The moisture content of dried and milled Cassava starch, Cassava peel and unpeeled Cassava roots were determined by the AACC 44-15A standard method, using a Mettler-Toledo HR 83 Halogen moisture analyzer. Each sample (2g) was heated at 130°C ± 1°C for one hour and the recorded weight loss was calculated as the initial moisture content of the samples. The result of the moisture analyses is given in Table 3.6. However the glucose yields were not calculated by the moisture content percentage but rather glucose yield were influenced by the moisture content.

Table 3.6: *Composition of Cassava root peels, unpeeled Cassava root and peeled root*

Sample	Method	Moisture content (w/w)
Raw unpeeled Cassava roots	Oven dried	55-62%
Dried, milled Cassava starch	AACC 44-15A	10%
Dried, milled Cassava peels	AACC 44-15A	9.2%
Dried, milled Cassava roots	AACC 44-15A	9.5%

Moisture content of a Cassava sample is a function of the cultivar, the planting season as well as the soil type and fertilizer used. The maximum reported moisture content in a Cassava sample was 72wt% (Ngo *et al*., 2005) on a wet basis. This result is similar to that of (Ngo *et al*., 2005 and Tonukari, 2004) in their respective work on Cassava intercropping and the future of Cassava starch. Tonukari (2004) stated a moisture content of 70% and Ngo *et al.*, (2005) approximately the same amount.

3.6.3 Determination of starch content of Cassava

The starch content in starch rich biomass can be determined by hydrolysis of the starch to glucose and indirectly determining the starch content with Equation 3.1 by determining the final glucose concentration in the hydrolysate before fermentation (Brunt *et al*., 1998). C is the concentration of glucose (sample solution), 0.93 is the conversion factor taken from Brunt *et al*., (1998) for glucose to starch and W is the

weight of sample to be used for fermentation in ($g.L^{-1}$). The amount of starch calculated with Equation 3.1 is the equivalent amount of starch in the aliquot used.

$$\%\,\text{Starch (g/100g)} = \frac{C \times 0.93}{W} \times 100 \qquad \textbf{[3.1]}$$

The starch content of the Cassava sample used in this study calculated with Equation 3.1 by hydrolysis was determined to be between 69% and 87 % (w/w) of the aliquot used. The starch content determined through hydrolysis with Equation 3.1 of the Cassava cultivar used in this study is the same order of magnitude as the starch content of 31 Cassava varieties determined by Aryee *et al*., (2006), who reported general starch content between 67% and 88% (w/w). The results in this study are also similar to the results reported by Srinorakutara *et al*., (2004) who reported a starch content of between 61.84-69.90% (w/w). The sugar content from HPLC was used to determine the starch content by calculation. The colorimetric measurement was to determine that all the starch had been converted.

3.6.4 HPLC analysis

After liquefaction, saccharification and fermentation, samples were filtered through a 0.2μm syringe filter and analyzed using the High Performance Liquid Chromatography (see Table 3.2). Before analysis of the hydrolysate and fermentation broth calibration curves were prepared from the quantitative determination of the available sugars in Cassava using the HPLC. A calibration curve for the quantitative determination of the weight percentage ethanol in the fermentation broth was prepared from known samples of ethanol concentration using HPLC. The method used for analyzing and processing the standards and the experimental data on the HPLC as well as the calibration are curves discussed in detail in Appendix A. The areas of the peaks, resulting from the analysis of the concentration injected on the HPLC, were used to determine the composition of the sugars and ethanol by converting the measured areas mass/volume using the gradient of the calibration curves.

Reproducibility of the analysis of the samples using the HPLC was confirmed by injection of each sample three times. The peaks were distinct and no significant

overlapping of the peaks occurred. The analysis conditions for the two columns used in the HPLC are given in Table 3.7.

Table 3.7: *Analysis parameters on the HPLC for Cassava hydrolysate and fermentation broth*

Parameters	Shodex	Zorbax
Mobile phase	100% HPLC grade water	75% Acetonitrile: 25% water
Column temperature	80°C	60°C
Detector temperature	55°C	50°C
Flow rate	1.00ml/min	1.00ml/min
Inj. Volume	10µl	10µl
Run time	20 min	10 min

3.7 Optimization of liquefaction and saccharification steps

Enzymatic hydrolysis was done employing the methods used by Ayernor *et al*., (2002) and Mojovic *et al*., (2006) with modifications.

3.7.1 Effect of substrate form on glucose yield

The effect of substrate form on the amount of glucose that can be produced by liquefaction and saccharification was investigated by using three different forms of the Cassava root. Cassava peels, unpeeled roots and peeled roots were investigated for glucose production capability before and during SSF. The peels, peeled root and unpeeled roots were thoroughly washed, sun dried and milled to a +1.5mm sieve size. The substrate concentration was 20wt% (200g milled Cassava + 800mL water) for all the forms of Cassava. Liquefaction was carried out using 7 $\mu L.g^{-1}$ of Termamyl SC at a pH of 6, a temperature of 95°C one hour. The glucose yield for each of the Cassava forms was recorded at various time intervals and after completion of the enzyme hydrolysis. The results of this investigation are shown in Section 4.1.1.

3.7.2 Effect of pH on glucose yield

The effect of pH on glucose concentration was investigated for unpeeled Cassava roots using a substrate concentration of 17% and varying the pH between 5.5, 6 and

6.5 during the liquefaction step. The liquefaction time was 90 minutes at a temperature of 95°C using an oven and a Termamyl SC dosage of 7 $\mu l.g^{-1}$. The liquefaction step was followed by saccharification with 7.5 $\mu l.g^{-1}$ Spirizyme fuel at a temperature of 55°C for 48 hours in a shaker incubator at 150rpm. The effect of varying the pH on the final glucose concentration was investigated by varying the pH for the saccharification step between 4 and 5.5. Control samples (no enzyme) were used for both processes. The results of this investigation can be found in Section 4.1.2.

3.7.3 Effect of temperature on glucose yield

The effect of temperature on the glucose yield during the liquefaction step was investigated on Cassava unpeeled roots by carrying out the liquefaction in an oven at a pH of 6 for 90 minutes using Termamyl SC (7 $\mu l.g^{-1}$) and varying the liquefaction temperature between 85°C and 95°C. The effect of temperature on the glucose yield during saccharification was investigated by carrying out the saccharification step at a pH of 4.5 in an incubator shaker (150 rpm) using Spirizyme fuel (7.5 $\mu l.g^{-1}$) and varying the temperature between 55°C and 65°C. A control sample (no enzyme) was used for each set of experiments. The results of this investigation can be found in Section 4.1.3.

3.7.4 Effect of biomass load on glucose yield

The effect of biomass load on the glucose concentration during liquefaction and saccharification was investigated by using a 10 wt%, and a 20 wt% substrate concentration of milled unpeeled Cassava roots. The same concentration of enzyme was used in the liquefaction and saccharification steps for all substrate concentrations. Liquefaction was performed at a temperature of 95°C with a pH of 6 using Termamyl SC (7 $\mu l.g^{-1}$) for one hour and the saccharification step was performed at a temperature of 55°C and a pH of 4.5 with Spirizyme fuel (7.5 $\mu l.g^{-1}$) and Celluclast 1.5L (4 $\mu l.g^{-1}$) for four hours. The hydrolysis was performed in Duran bottles in a shaker incubator at 170rpm. The results of this investigation are presented in Section 4.1.4.

3.7.5 Effect of enzyme combination on glucose concentration

The effect of different enzyme combinations during liquefaction and saccharification was investigated by using different combinations of Termamyl SC, Celluclast 1.5 L and Spirizyme fuel during the hydrolysis steps. Milled unpeeled Cassava roots (100g) were mixed with distilled water up to 500ml total volume in Duran bottles. In all instances, liquefaction was performed at a temperature of 95°C with a pH of 6 using Termamyl SC (7 $\mu l.g^{-1}$) for one hour. The saccharification step was performed at a temperature of 55°C with a pH of 4.5 for 4 hours, using either only Spirizyme Fuel (7.5 $\mu l.g^{-1}$) or both Spirizyme fuel (7.5 $\mu l.g^{-1}$) and Celluclast 1.5L (4 $\mu l.g^{-1}$). The results of this investigation are presented in Section 4.1.5.

3.7.6 Effect of enzyme load on glucose yield

Unpeeled milled Cassava roots (100g) was mixed with 400ml of distilled water (20wt %). The mixture was hydrolyzed with various concentrations of Termamyl Sc per gram Cassava (7 $\mu l.g^{-1}$, 5$\mu l.g^{-1}$, 2$\mu l.g^{-1}$) for liquefaction at a pH of 6 and at a temperature of 95°C for 1hour, Spirizyme fuel per gram Cassava (7.5$\mu l.g^{-1}$, 5.5$\mu l.g^{-1}$, 2.5$\mu l.g^{-1}$) and Celluclast 1.5L per gram Cassava (4$\mu l.g^{-1}$, 2$\mu l.g^{-1}$, 1$\mu l.g^{-1}$) for saccharification at a pH of 4.5 and a temperature of 55°C for four hours. The hydrolysates were filtered for analysis. The results of this investigation are presented in Section 4.1.6.

3.8 Optimization of fermentation step

3.8.1 Separate hydrolysis and fermentation with *S. cerevisiae*

Termamyl SC, Spirizyme fuel and Celluclast 1.5L (7, 7.5 and $\mu L.g^{-1}$ Cassava) were used in the liquefaction and saccharification steps respectively. Four grams of bakers' yeast was used for fermentation because literature suggests 150g in 20L hydrolysate for bakers' yeast to effect the fermentation. The yeast was first subjected to the hydrolysate to be used for fermentation for ten minutes so as to revive it from the dormant state (see Section 3.1.1)

All samples were liquefied (production of dextrin) with 7µl termamyl per gram Cassava in Duran bottles at a temperature of 95°C, pH 6 for 60 minutes in an oven.

Samples were collected, filtered and analyzed for glucose concentration with the HPLC. All samples were subjected to saccharification (production of glucose) with a mixture of 7µl Spirizyme fuel per gram Cassava and 4µl Celluclast per gram Cassava in Duran bottles at a temperature of 55°C, pH 4.5 for 48 hours in a shaker at 150 rpm. Samples were collected, filtered and analyzed for glucose concentration with the HPLC.

Cassava hydrolysates from all samples obtained from the two-step hydrolysis of the Cassava unpeeled roots were subjected to ethanol fermentation by *Saccharomyces cerevisiae* under anaerobic conditions. Four grams of anchor baker's yeast was used for fermentation in Duran bottles at a temperature of 30°C and pH 4 for 120 hours in a shaker at 70 rpm. Samples of the fermented broth were collected on an hourly basis for three hours, and during the first 24hours of fermentation and thereafter every 24 hours. The results of the SHF process for ethanol production are presented in Section 4.2.1.

3.8.1.1 Effect of yeast concentration on ethanol yield

The effect of yeast concentration on the final ethanol yield was investigated using unpeeled Cassava roots with a 20wt % biomass load and using different concentrations of *S. cerevisiae* for the fermentation ($8g.L^{-1}$, $5g.L^{-1}$ and $3g.L^{-1}$). The fermentation broths were filtered for ethanol analysis. The results of this investigation are presented in Section 4.2.2.1.

3.8.2 Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation was performed using three forms of Cassava i.e. peeled Cassava roots, Cassava root peels and unpeeled Cassava roots. A substrate concentration of 20wt% Cassava was used for all experiments.

SSF was performed the same way for all three Cassava forms. The process was carried out in 1L duran bottles. Pretreatment of the three Cassava slurries with Termamyl SC at a pH of 6 and a temperature of 95°C was performed for one hour prior to SSF. A starting batch size of 20wt% (100g Cassava + 400g distilled water) of the pretreated slurry was used for ethanol fermentation and was suitable for the 1L

duran bottle. The yeast concentration was set at 8g.L^{-1} of the total sample (batch sizes), resulting in the amount of yeast added being 4g. The yeast and the enzymes (Spirizyme fuel 7µl.g^{-1} and Celluclast 1.5L 4 µl.g^{-1}) were added simultaneously, but not until the pH had been adjusted to 4.5 by 0.1 M H_2SO_4 and the temperature had reached 30°C. Samples were taken at time intervals for 72 hrs under careful monitoring of the process. Samples were filtered through micro filters and later analyzed with HPLC (Norgard, 2004). The results of the SSF process can be found in Section 4.2.2.

3.8.3 Direct fermentation with *S. occidentalis*

Direct fermentation of Cassava roots was done with 20wt% substrate concentration. The slurry was inoculated with 1% peptone and was autoclaved at a temperature of 121°C for 15min.

The peels were prepared the same way. The mixture was inoculated with 1% peptone. After the heat pretreatment, the slurry was inoculated with 25 ml of the 24 hour old inoculum at a pH of 4.5 and was processed in a shaker at 150rpm, at a temperature of 37°C. Samples were taken for 7 days and were centrifuge, filtered and HPLC analyzed. The method used for the direct fermentation with *S. occidentalis* was adapted from Rojan *et al*., (2007).

3.9 REFERENCES

ARYEE, F.N.A., ODURO, I., ELLIS, W.O. & AFUAKWA, J.J. 2006. The physicochemical properties of flour samples from the roots of 31 varieties of cassava. *Food Control*, ***17***: 916-922.

ASSOCIATION FOR CLINICALCHEMISTRY (AACC). 1999. "South African Grain Laboratory, S.A.

AYERNOR, G.S., HAMMOND, T.K. & GRAFFHAM, A. 2002. The combination of rice malt and amyloglucosidase for the production of sugar syrup from cassava flour. *African Journal of Science and Technology*, 3(1): 10-17, 01 Jan.

BRUNT, K., SANDERS, P. & ROZAMA, T. 1998. The enzymatic determination of starch in food, feed and raw materials of the starch industry. *Starch*, **50**: 413–419.

MOJOVIC, L., NIKOLIC, S., RAKIN, M. & Vukasinovic, M. 2006. Production of bioethanol from corn meal hydrolyzates. *Fue*l, **85** (12-13): 1750-1755, Oct.

MORRISOM, W. & LAIGNELET, B. 1983. An improved colorimetric procedure for determining apparent and total amylose in cereal and other starches. *Journal of Cereal Science*, **1**:9–20.

NGO, T. D., INGER, L. & NGUNYEN, T. M. 2005. Intercropping cassava (Manihot esculenta Crantz) with Flemingia (Flemingia macrophylla); effect on biomass yield and soil fertility. *Livestock Research for Rural Development*. 17(6), Feb.

NORGAD, J. 2004. Ethanol Production from Biomass - Optimization of Simultaneous Saccharification and Fermentation with Respect to Stirring and Heating, Department of Chemical Engineering, Lund Institute of Technology.

ROJAN, P.J, RAJEEV, K., SUKUMARAN, K., NAMPOOTHIRI, M. & Pandey, A. 2007. Statistical optimization of simultaneous saccharification and l (+)-lactic acid

fermentation from cassava bagasse using mixed culture of lactobacilli by response surface methodology. *Biochemical Engineering Journal*. **36** (3): 262-267.

SAELIM, K., YAOWALUK, D. & ARAN, H. 2008. Saccharification of cassava starch by *Saccharomycopsis fibuligera* isolated from Loog-Pang (rice cake starter). *Journal of Science and Technology*. **30** (1): 65-71.

SAIBENE, D. & SEETHARAMAN, K. 2008. Use of iodine as a tool to understand wheat starch pasting properties. *Starch-starke*, **60**(1): 1-7.

SRINORAKUTARA, T., SUESAT, C., PITIYONT, B., KITPREECHAVANIT, W. & CATTITHAMMANIT, S. 2004. Utilization of Waste from Cassava Starch Plant for Ethanol Production. (Joint International Conference on "Sustainable Energy and Environment (SEE), Hua Hin. Thailand. p. 773-76).

TONUKARI, N.J. 2004. Cassava and the future of starch. *Electronic Journal of Biotechnology*, 7 (1): 5-8, 15 Apr.

CHAPTER 4

RESULTS AND DISCUSSION

"Creativity is allowing yourself to make mistakes. Art is knowing which ones to keep"
Unknown

Overview

All the hydrolysis and fermentation experiments were done according to the procedures described in chapter 3. In this chapter, the results of the optimization of the enzymatic hydrolysis of Cassava are presented and discussed. The chemical composition of Cassava is given in Section 4.1. Section 4.2 focuses on the results of the enzymatic hydrolysis experiments, after which the results of the fermentation are documented and discussed extensively in Section 4.3. Summary of results for this work are tabled in Section 4.4

4.1 Optimization of liquefaction and saccharification steps

4.1.1 Effect of substrate form on glucose yield

The effect that substrate form has on the final glucose yield and rate of glucose production was investigated by liquefying three different forms of Cassava (Cassava starch, Cassava peels and unpeeled Cassava roots) according to the method described in Section 3.7.1. Figure 4.1 and Table 4.1 illustrate that Cassava peels can be readily degraded by enzymes to glucose, but that a lower glucose yield is obtainable than for the Cassava starch and unpeeled Cassava roots. Cassava peels have almost the same glucose yield as the Cassava starch. This is due to the fact that Cassava peels consist of two layers (periderm and cortex), which results in a high starch and cellulose content (77wt %). Furthermore, Sulphuric acid was used to operate the pH during liquefaction, the acid may have hydrolyzed the cellulose in the peels since cellulose can be broken down through acid hydrolysis by sulphuric acid at high temperatures of 90ºC which were used during liquefaction and above. The combined starch and cellulose content of the peels is close to the starch content of Cassava starch (82wt %). Moreover during the liquefactions step, only the starch was liquefied though not

completely liquefied, most possibly the starch attached to the peels were more accessible for liquefaction by the enzymes than just the peels and the swelling of the starch in the presence of water can hinder the enzymes from getting the starch particles.

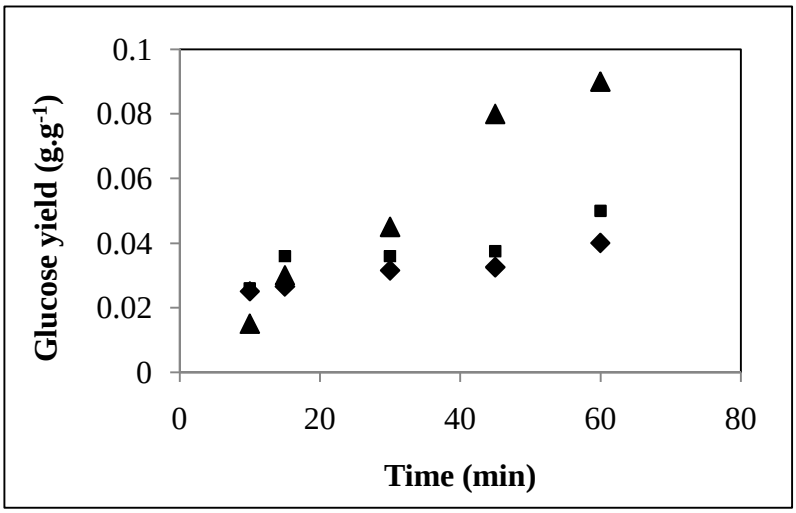


Figure 4.1: *Influence of Cassava substrate form on the glucose yield during liquefaction*
(♦ - Peels, ■ - peeled Cassava roots, ▲ -unpeeled Cassava roots)

The glucose yield increases with time for all Cassava forms as the starch is converted to dextrin and glucose by the added enzymes. The final glucose yield for the three different substrate forms are summarized in Table 4.1

Table 4.1: *Final glucose yield for different Cassava forms during liquefaction*

Substrate form	Final glucose yield ($g.g^{-1}$)
Cassava starch	0.05
Cassava peels	0.04
Unpeeled Cassava roots	0.09

The unpeeled Cassava roots yielded a much higher glucose yield than the peels or the starch samples even though the starch content for all the three samples are in the same order of magnitude. The unpeeled roots contain both the peels with the two epidermal layers (Cellulose) with higher fiber content (3.47%) than the peeled roots (2.01%) and the starch. The acid (Sulphuric acid) was also used for pH operation for unpeeled Cassava roots, the acid hydrolysis of cellulose was likely to take place hence a glucose yield similar to the sum of the glucose yield for peels and Cassava starch was obtained. The difference in glucose yield for the unpeeled Cassava roots and the sum of the peels

and starch glucose yield is higher than the experimental error (see Section 3.3). The yield per mass of biomass for the unpeeled Cassava roots was obtained with the same amounts of enzymes per gram of biomass as for the peels and the Cassava starch. This means that the mixture of peels and starch in the unpeeled roots were better utilized/converted to produce glucose than for separate hydrolysis of the two forms of biomass. This is evident when the glucose production rates for the liquefaction of the different substrate forms are compared. The best substrate form for glucose production from Cassava is thus unpeeled Cassava roots.

The initial rate of glucose production for the three different substrate forms were calculated using Equation 4.1 and the values are presented in Table 4.2

$$r_{glucose} = \frac{dC_{glucose}}{dt} \qquad \textbf{[4.1]}$$

Table 4.2: *Initial glucose production rate (15 min) for different substrate forms*

Substrate form	**Production rate (r) ($g.g^{-1}.min^{-1}$)**
Unpeeled roots	0.0015
peeled roots	0.0007
peels	0.0002

The initial rate (0.0015 $g.g^{-1}.min^{-1}$) of glucose production for the unpeeled roots is much faster than the other two substrate forms which have rates of 0.0007 $g.g^{-1}.min^{-1}$ and 0.0002 $g.g^{-1}.min^{-1}$ for unpeeled roots and peels respectively. The starch is bound by the peels which have a higher cellulose component and thus not as accessible to enzyme attack as pure starch granules, resulting in a low glucose production rate when comparing pure starch and the unpeeled root mixture of starch and cellulose. The density of the latter is lower due to the presence of the cellulose pieces in the starch. A lower density facilitates the easy access of enzymes to both the cellulose and starch components, resulting in a very high glucose production rate. Starch swells in the presence of water, forming agglomerates that are not easily accessible for conversion to glucose, (Komolprasert and Ofoli, 2007) by the enzymes and thus a lower glucose production rate is obtained for the pure starch substrate than for the unpeeled roots.

4.1.2 Effect of pH on glucose yield

The effect of pH on the production of glucose during the liquefaction and saccharification was investigated according to the methods discussed in Section 3.7.2. The change in glucose concentration with a change in pH during the liquefaction step is presented in Figure 4.2.

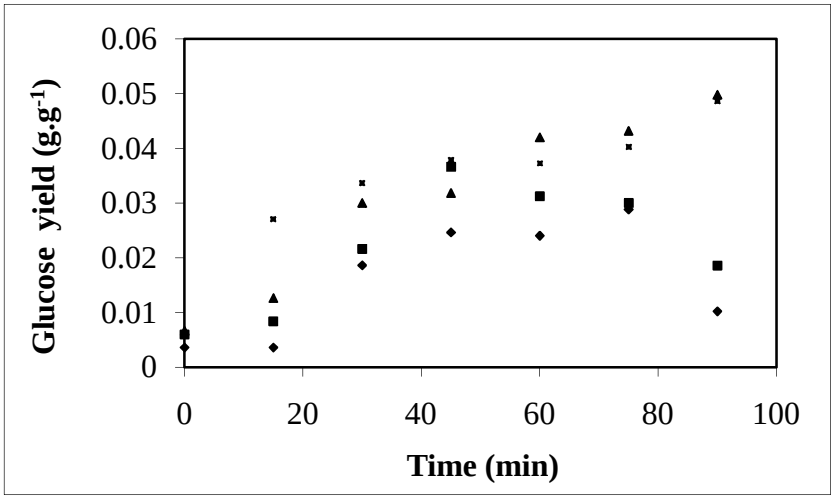


Figure 4.2: *Effect of pH on glucose yield during liquefaction*
(♦ - control, ▲ - pH 6, ■ - pH 6.5, ■ - pH 5.5)

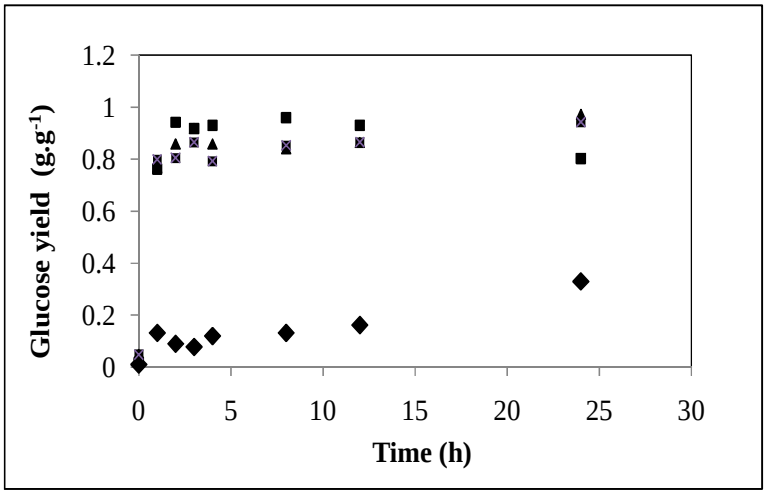


Figure 4.3: *Effect of pH on glucose yield during saccharification*
(♦ - control, ▲ - pH 4.5, ■ - pH 4, ■ - pH 5)

The effect of pH on the enzyme activity and glucose concentration (Figure 4.2 and 4.3) indicates that the α-amylase (Termamyl) is active at pH 6-6.5 and its activity declines at pH 5.5. The optimal pH for liquefaction was found to be 6. The glucose

yield initially increases with time for all samples. According to the manufacturer's specification sheet, the optimum treatment time for starch with Termamyl SC is 60 minutes. From Figure 4.2 it can be seen that after 60 minutes, the control samples have the lowest glucose yield and the sample treated at a pH of 6 has the highest glucose yield. The increase in glucose yield for the control sample is most probably due to the acid hydrolysis of starch caused by Sulphuric acid during pH operation. The fact that all samples treated with Termamyl SC have a higher glucose yield than the control sample after 60 minutes indicates that the addition of Termamyl SC to the samples had a positive effect on the glucose yield and the activity of the enzyme is thus confirmed. The final glucose for samples treated at a pH of 6 and 6.5 increased after 60 minutes and was approximately the same at the end of the liquefaction process (90 minutes) while the glucose yield for the sample treated at a pH of 5 as well as the glucose yield of the control decreased slightly after 60 minutes. This suggests that the best pH to use during liquefaction is thus a pH of 6.

The effect of pH on the glucose yield during saccharification is presented in Figure 4.3. It can be seen that the glucose yield of the control sample remained constant, while the glucose yield of all the samples treated with Spirizyme fuel increased with time, thus confirming the activity of the enzyme to convert dextrin into glucose. At the end of the saccharification process, all samples treated at different pH values yielded approximately the same glucose yield. This means that the pH had no significant effect on the glucose yield during saccharification for the pH range investigated in this study.

This result is in accordance with the work of Oboh (2008). The optimum pH for saccharification was found to be 4.5 (Figure 4.6). This result is in agreement with the work of Srinorakutara *et al.,* (2004) who used the same pH for saccharification and obtained 75% glucose concentration.

4.1.3 Effect of temperature on glucose yield

The effect of temperature on the glucose yield during liquefaction and saccharification was investigated according to the method discussed in Section 3.7.3. The influence of temperature on glucose yield during liquefaction and saccharification is shown in Figure 4.4 and 4.5 respectively.

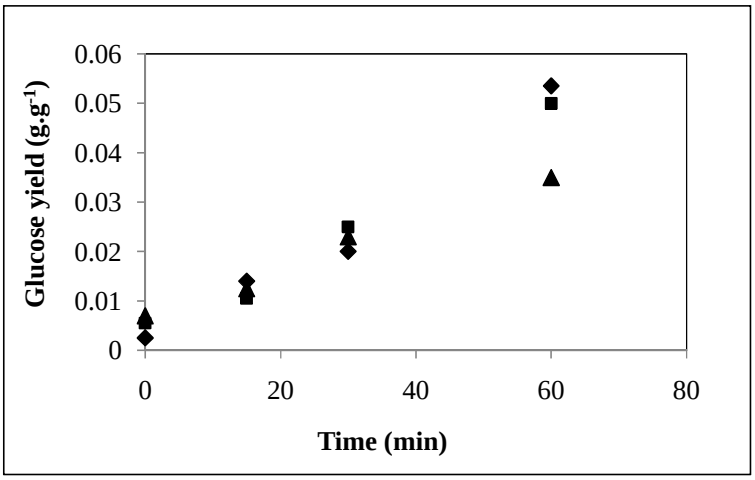


Figure 4.4: *Effect of temperature on glucose yield during liquefaction*
(♦ - 95°C, ■ - 90°C, ▲ - 85°C)

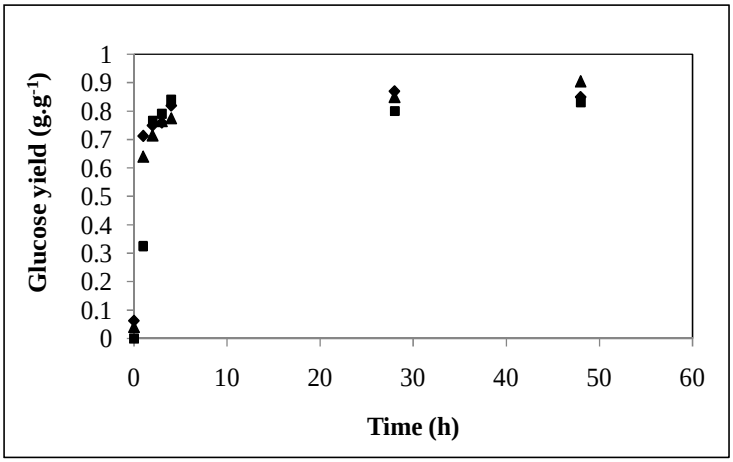


Figure 4.5*: Effect of temperature on glucose yield during saccharification*
(♦ - 65°C, ■ - 60°C, ▲ - 55°C)

From Figure 4.4 it can be seen that all samples treated at different temperatures initially has the same production rate up to approximately 35 minutes. After 35 minutes the production of glucose increases faster for samples treated at 95°C than at the other temperatures. Glucose production drastically decreases from 0.05g.g^{-1} to 0 after 60 minutes for samples treated at 85°C while the glucose yield only levels off at approximately 75 minutes for samples treated at 95°C. The extra 25 minutes of increased glucose production for samples treated at 95°C results in a much higher

final glucose concentration than for samples treated at 85°C and 90°C. This result corresponds to the manufacturer's specification sheet for Termamyl SC.

From Figure 4.5 it can be seen that all treatment temperatures initially have high production rates and that the saccharification reaction is almost complete after 4 hours. The highest final glucose yield was obtained for a treatment temperature of 55°C. According the error analysis in appendix D.2 the difference between the temperatures was insignificant and therefore the lowest temperature was chosen for energy conservation. The best temperature to use during saccharification of Cassava roots with Spirizyme Fuel is thus 55°C.

4.1.4 Effect of biomass load on glucose yield

The effect of biomass load on the glucose yield was investigated according to the methods described in Section 3.7.4. The effect of biomass load on glucose yield can be seen in Figure 4.6.

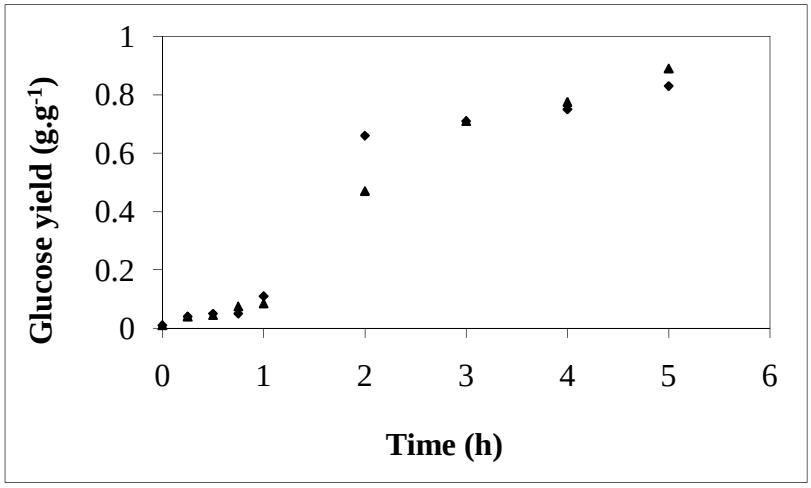


Figure 4.6: *Influence of biomass load on glucose yield during liquefaction and saccharification*
(♦ - 10wt% substrate, ▲ - 20wt% substrate)

From Figure 4.6 it can be seen that during liquefaction (up to one hour) the glucose yield for a biomass load of 10wt% is higher than for a biomass load of 20wt%. With more biomass present, the viscosity of the mixture is higher and thus the biomass is not that easily accessible to the enzymes. The lower viscosity for a biomass load of 10wt% also results in a faster conversion of dextrins to glucose during the saccharification step, as can been seen at a time of two hours in Future 4.6. After approximately four hours, enough biomass/dextrins has been converted to glucose so

that the effect of viscosity on glucose production is starting to become limited and thus the enzymes can now access all of the available dextrins/biomass for conversion to glucose. At the end of the hydrolysis process it is clear that a 20wt% biomass load gives a higher final glucose yield. The result is in agreement to work done by Aggarwal *et al*., (2001) that reported an optimum biomass load of 25wt% of Cassava in their study.

4.1.5 Effect of enzyme combination on glucose yield

The effect of enzyme combination on the glucose yield was studied according to the procedure described in Section 3.7.5. The purpose of this investigation is to verify whether the addition of Celluclast 1.5L to the hydrolysis step would have a significant influence on the final glucose yield. The effect of adding Celluclast 1.5L to the hydrolysis step is summarized in Table 4.3

The glucose yield from enzymatic hydrolysis (5 hours) of 20 wt% and 10 wt% Cassava flour was 178 $g.L^{-1}$ (0.9 $g.g^{-1}$), and 91$g.L^{-1}$ (0.91 $g.g^{-1}$) respectively with three enzymes (Termamyl SC, Spirizyme fuel and Celluclast 1.5L) compared to 156 $g.L^{-1}$ (0.78 $g.g^{-1}$) and 83 $g.L^{-1}$ (0.83$g.g^{-1}$) respectively (see Figure 4.7 and 4.8), for two enzymes (Termamyl SC and Spirizyme fuel) under the same hydrolysis conditions of pH and Temperature. The results are in agreement with the results reported by Sriroth *et al.*, (2000). The use of additional enzymes also helped to reduce the viscosity of the sample solution and improved starch hydrolysis.

Table 4.3: *Effect of enzyme treatment on glucose yield*

Cassava roots and peels (wt %)	**Glucose yield ($g.g^{-1}$)**	
	Termamyl SC Spirizyme fuel	Termamyl SC Spirizyme fuel Celluclast 1.5L
10	0.83	0.91
20	0.78	0.90

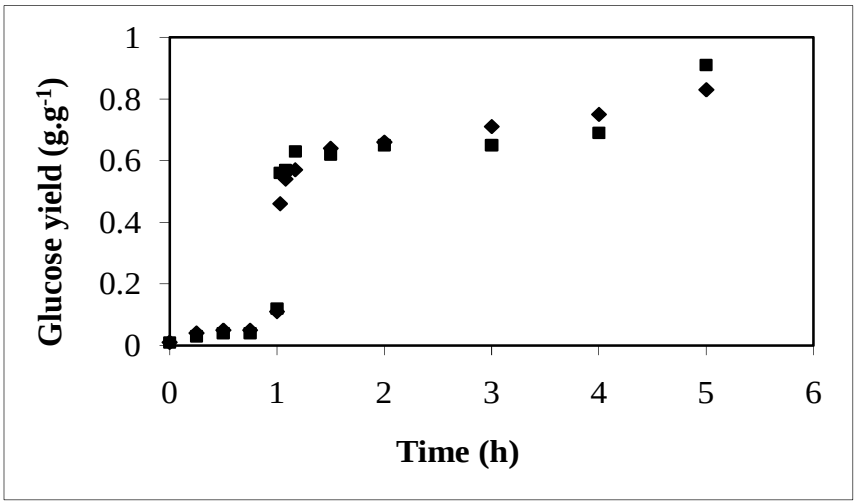


Figure 4.7: *The effect of enzyme treatment on glucose yield with 10wt% substrate concentration*

(♦ - Without Celluclast, ■ - With Celluclast)

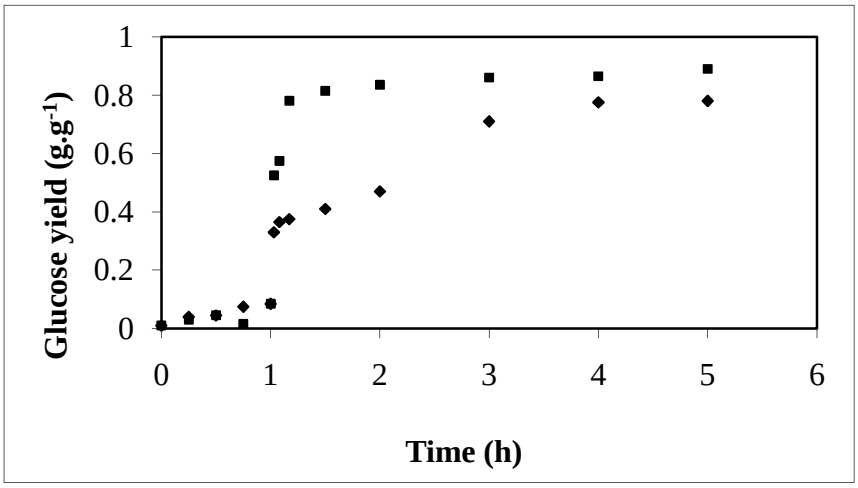


Figure 4.8: *The effect of enzyme treatment on glucose yield with 20wt% substrate concentration.*

(♦ - Without Celluclast, ■ - With Celluclast)

4.1.6 Effect of enzyme loading on glucose yield

The effect of enzyme loading on the final glucose yield after hydrolysis was investigated with the methods described in Section 3.7.6. The concentration of enzymes used is summarized in Table 4.4

Table 4.4: *Enzyme loadings in different treatment combinations*

Loading	Termamyl SC ($\mu L.g^{-1}$)	Spirizyme fuel ($\mu L.g^{-1}$)	Celluclast ($\mu L.g^{-1}$)
Loading 1	7	5	2
Loading 2	7.5	5.5	2.5
Loading 3	4	2	1

The results of hydrolysis of a 20wt% biomass load slurry with the different enzyme loadings is presented in Figure 4.8.

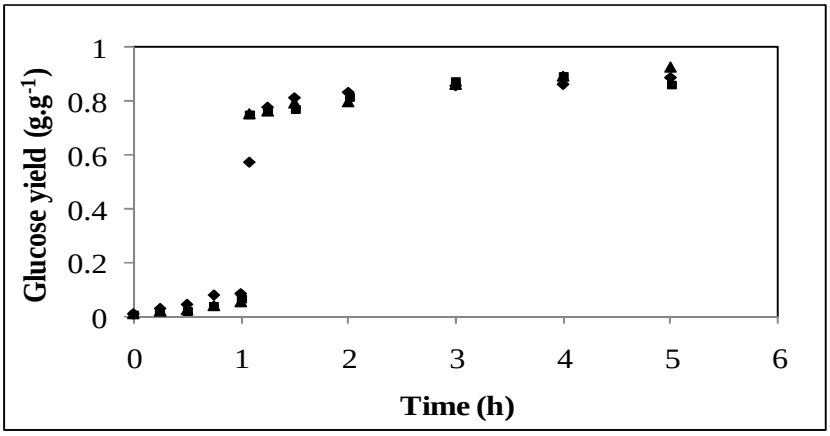


Figure 4.9: *Influence of enzyme concentration on glucose concentration* (▲ - Loading 1, ■ - Loading 2, ♦ - Loading 3)

Enzyme combination 1 gave a better glucose yield (0.925 $g.g^{-1}$) followed by loading 3 and loading 2 though the difference was insignificant, given a 5% error. The lower glucose yield at higher enzyme loading can be attributed to the fermentation of by-products when more enzymes are present, resulting in less glucose being produced. This corresponds with was reported by Ku Ismail *et al.*, (2008).

4.2 Optimization of fermentation step

For the study on glucose consumption by *Saccharomyces cerevisiae*, 20wt% substrate concentration was used, and the concentration was hydrolyzed with 0.7% T, 0.75% S & 0.4% C.

4.2.1 Separate hydrolysis and fermentation with *S. cerevisiae*

Separate hydrolysis and fermentation was carried out with a 20 wt % biomass loading according to the method described in Section 3.8.1. Conditions used are summarized in Table 4.5.

Table 4.5: *Hydrolysis conditions used during separate hydrolysis and fermentation (SHF) process*

Process	pH	T (°C)	Termamyl SC ($\mu L.g^{-1}$)	Spirizyme fuel ($\mu L.g^{-1}$)	Celluclast ($\mu L.g^{-1}$)
Liquefaction	6	95	7	-	-
Saccharification	4.5	55	-	7.5	4

The increase in glucose yield during liquefaction and saccharification is shown in Figure s 4.10 and 4.11 respectively.

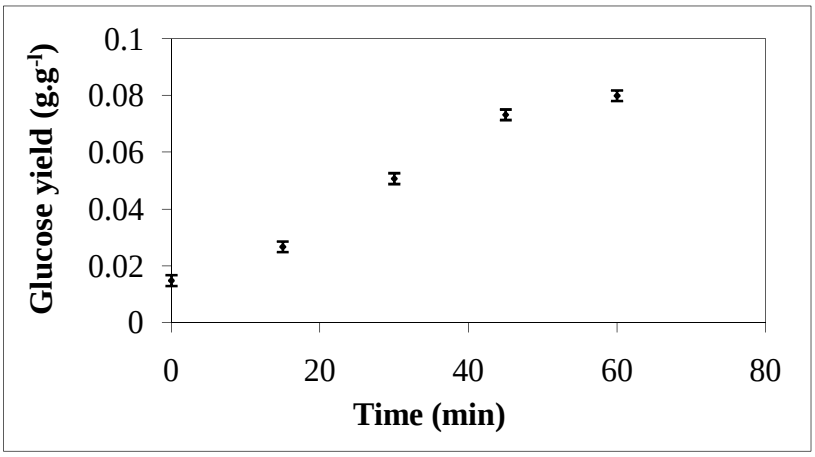


Figure 4.10*: Increase in glucose yield during liquefaction in the SHF process*

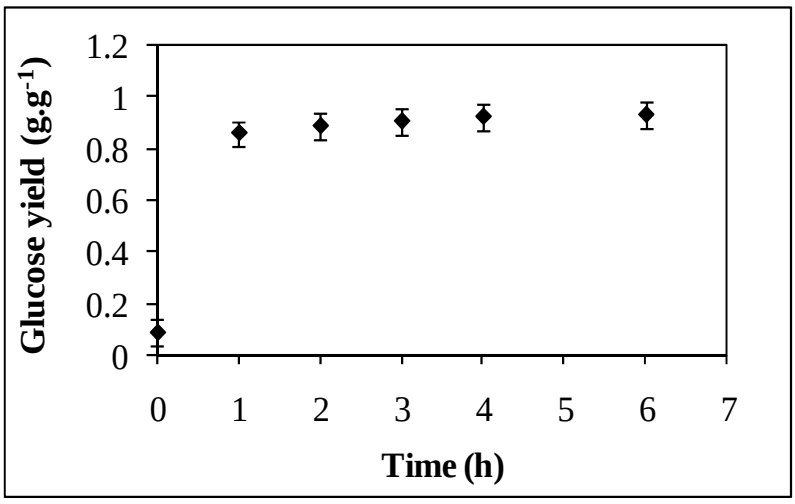


Figure 4.11: *Increase in glucose yield during saccharification in the SHF process*

The glucose rich hydrolysate from hydrolysis was inoculated with *S cerevisiae* for ethanol production. Figure 4.12 shows the ethanol yield during fermentation and the ethanol yield in comparison to the glucose uptake during fermentation are shown in Figure 4.13. The initial ethanol concentration (v/v) in the broth was 9.6%.

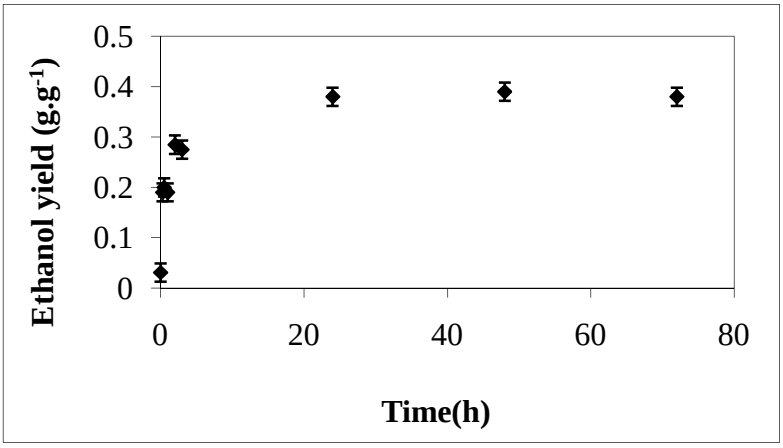


Figure 4.12: *Ethanol production in shake flasks by Saccharomyces cerevisiae using the SHF process*

From Figure 4.12 it can be seen that a final average ethanol concentration of 75g.L^{-1}, corresponding to a yield coefficient ($Y_{p/s}$) of 0.38g.g^{-1} (gram of ethanol per gram biomass) was reached after only 24 hours. The initial glucose yield in the 20wt% biomass load hydrolysate was 0.92g.g^{-1}. The ethanol yield per gram of glucose ($Y_{ap/g}$) was 0.4g.g^{-1}.

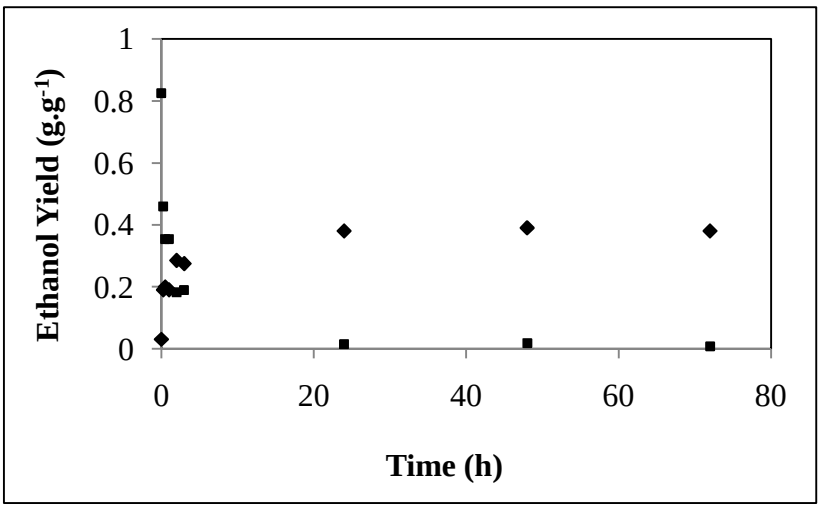


Figure 4.13: *Ethanol production in comparison to glucose uptake in shake flasks by Saccharomyces cerevisiae using SHF*
(♦ - Ethanol, ■ - Glucose)

From Figure 4.13 it can be seen that the final glucose yield in the fermentation broth was 0.006g.g^{-1}. Equivalent amounts of ethanol and carbon dioxide (CO_2) are formed

during the fermentation reaction. Theoretically 10 kg of sugar will produce 5.1 kg of ethanol and 4.9 kg of carbon dioxide (Salle, 1993). Therefore if 40 % of the available glucose in the initial hydrolysate was utilized for the production of ethanol as the fermentation results above show, theoretically, approximately 38% must have been utilized for the production of CO_2. From a mass balance on glucose it can then be said that approximately 21.4% of the glucose was utilized for yeast production. The same analogy was used by Yusaku et al., (2004) on a study focusing on ethanol fermentation of raw cassava starch with *Rhizopus koji* in a gas circulation type fermentor. These results are in agreement with the results reported by Srinorakutara *et al.*, (2004) for the production of ethanol from Cassava in a fermentor. In other studies (Thailand Institute of Scientific and Technological Research, 2002) a maximum ethanol concentration of only 5% after 69 hours of fermentation was reported. It can be thus be concluded that optimization of the hydrolysis steps had a positive effect on the final ethanol yield in this study.

4.2.2 Simultaneous saccharification and fermentation with *S. cerevisiae*

4.2.2.1 Influence of yeast concentration on ethanol yield

The influence of yeast concentration on ethanol yield during the SSF was investigated on a 20wt% biomass load of unpeeled Cassava roots according to the methods presented in Section 3.8.1.1. The process conditions used to investigate the influence of yeast concentration on the ethanol yield during SSF is given in Table 4.6.

Table 4.6: *Influence of yeast concentration on ethanol yield*

Process	T (°C)	pH	Termamyl ($\mu L.g^{-1}$)	Spirizyme fuel ($\mu L.g^{-1}$)	Celluclast ($\mu L.g^{-1}$)	Yeast ($g.L^{-1}$)
Liquefaction	95	6	7	-	-	-
Saccharification and Fermentation	30	4.5	-	7.5	4	8,5,3

The ethanol yield and glucose yield during fermentation using SSF process for a 20wt% biomass load of unpeeled Cassava and different yeast concentrations are presented in Figures 4.14 and 4.15 respectively

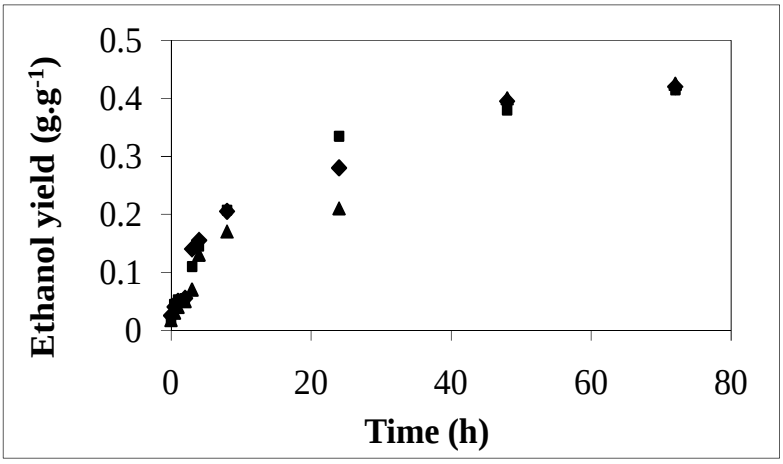


Figure 4.14: *Effect of yeast concentration on ethanol yield after 72 hours, using the SSF process*
(♦ - 8 g.L^{-1}, ■ - 5g.L^{-1}, ▲ - 3g.L^{-1})

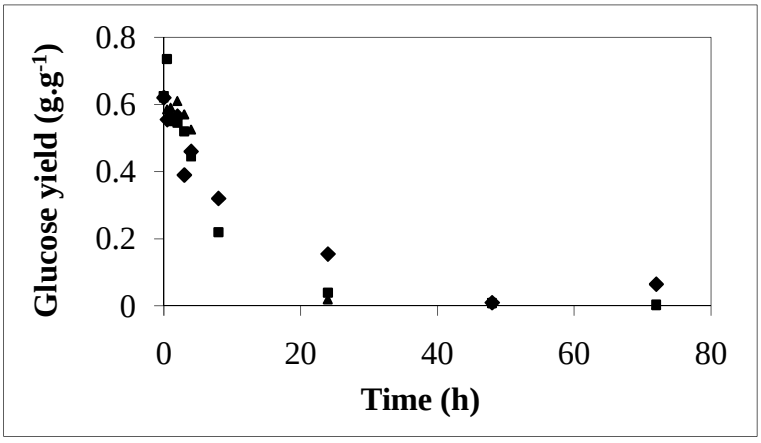


Figure 4.15: *Glucose uptake during fermentation by Saccharomyces cerevisiae in the SSF process*
(♦ - 8 g.L^{-1}, ■ - 5g.L^{-1}, ▲ - 3g.L^{-1})

From Figures 4.14 and 4.15 it can be seen that all the yeast concentrations used resulted in approximately the same ethanol yield. The initial ethanol production rates for the different yeast concentrations used are given in Table 4.7

Table 4.7: *Initial ethanol production rate for different yeast concentrations*

Yeast Concentration (g.L^{-1})	Initial ethanol production rate
3	0.025
5	0.029
8	0.035

From Table 4.7 it can be seen that the initial ethanol production rate is faster at the higher yeast concentration. This is expected since more yeast organisms will convert the glucose faster, but in the end, given enough time the same ethanol yield should be

reached. In this study all three chosen yeast concentrations resulted in almost complete utilization of glucose, implying that 3g.L^{-1} yeast is sufficient to convert all available glucose formed in the SSF process to bio-ethanol within 72 hours.

4.2.2.2 Influence of substrate form on ethanol yield

Simultaneous saccharification and fermentation was carried out with a 20wt% biomass loading according to the methods presented in Section 3.8.2. The hydrolysis and fermentation process conditions for all three Cassava substrate forms (Cassava starch, Cassava peels and unpeeled Cassava roots) are given in Table 4.8.

Table 4.8: *Hydrolysis and fermentation process conditions for all three Cassava substrate forms*

Process	T (°C)	pH	Termamyl (µL.g^{-1})	Spirizyme fuel (µL.g^{-1})	Celluclast (µL.g^{-1})	Yeast (g.L^{-1})
Liquefaction	95	6	7	-	-	-
Saccharification and Fermentation	30	4.5	-	7.5	4	8

The increase in the glucose concentration during liquefaction in the SSF process is presented in Figure 4.16

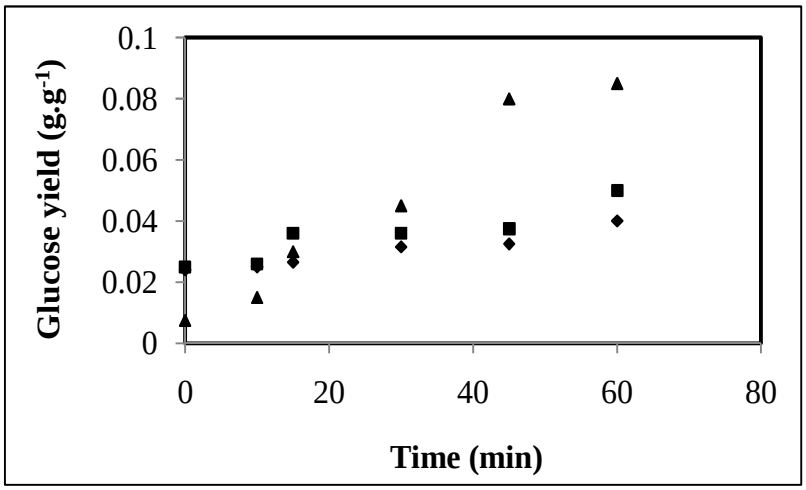


Figure 4.16: *Increase in glucose concentration during liquefaction step of the SSF process*
(♦ - Peels, ■ - peeled Cassava roots, ▲ -unpeeled Cassava roots)

From Figure 4.17 it can be seen that the unpeeled Cassava roots gave the highest final ethanol yield. The higher ethanol yield for unpeeled Cassava roots compared to Cassava starch and Cassava peels is due to the higher glucose production during liquefaction (see Table 4.8).

The hydrolysate for liquefaction was inoculated with *S. cerevisiae* as well as saccharification enzymes. The influence of substrate form on the final ethanol yield and glucose uptake during SSF at a yeast concentration of 8g.L^{-1} is presented in Figures 4.17 and 4.18 respectively.

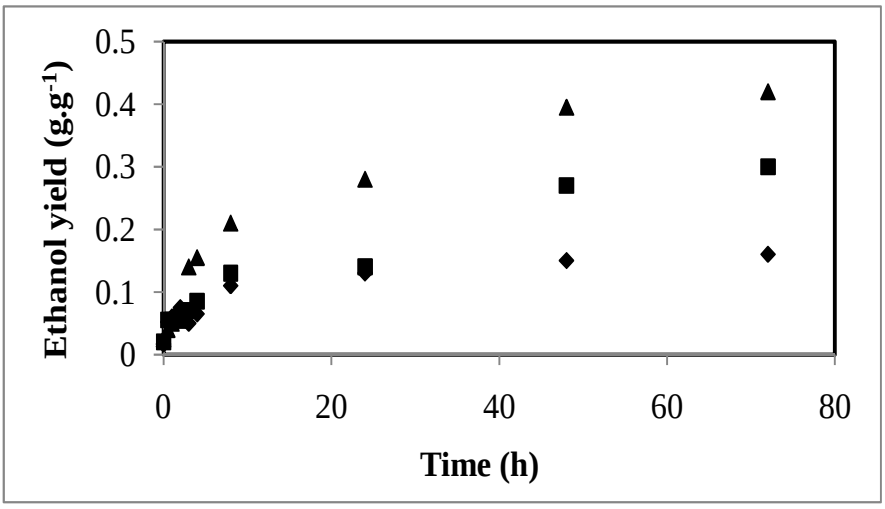


Figure 4.17: *Influence of substrate form on ethanol yield*
(♦ - Peels, ■ - peeled Cassava roots, ▲ -unpeeled Cassava roots)

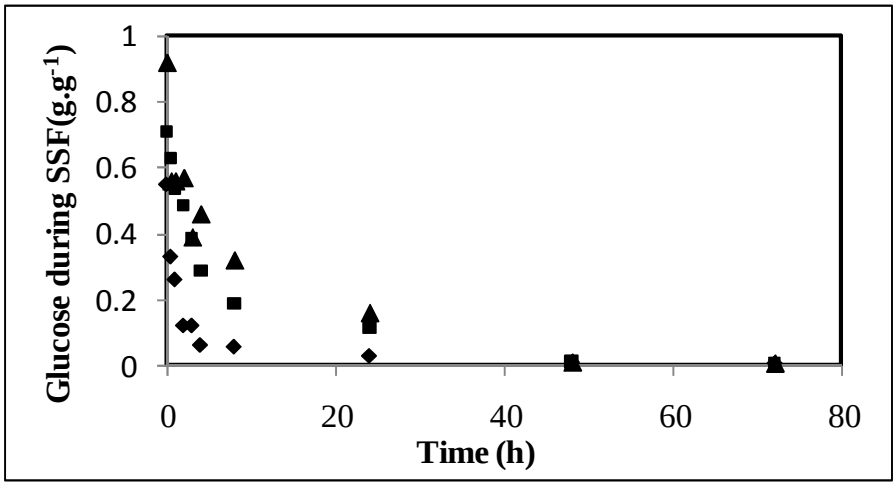


Figure 4.18: *Glucose uptake during fermentation of peels, peeled roots and unpeeled roots*
(♦ - Peels, ■ - peeled Cassava roots, ▲ -unpeeled Cassava roots)

The results graphically presented in Figure 4.17 are summarized together with yield coefficients for the SSF process in Table 4.9.

Table 4.9: *Ethanol and glucose yield coefficients for the SSF process*

Substrate form	Glucose [a]	Ethanol			
	$Y_{p/s}$ ($g.g^{-1}$)	$Y_{p/s}$ [b] ($g.g^{-1}$)	$Y_{p/g}$ [c] ($g.g^{-1}$)	$Y_{p/s}$ [d] ($ml.g^{-1}$)	$Y_{p/g}$ [e] ($ml.g^{1}$)
Unpeeled roots	0.90	0.42	0.45	0.53	0.58
Peeled roots (starch)	0.70	0.30	0.40	0.40	0.50
Peels (cellulose)	0.55	0.16	0.30	0.20	0.37

[a] gram glucose per gram Cassava substrate;
[b] gram ethanol per gram glucose hydrolysate
[c] gram ethanol per gram Cassava substrate
[d] mL ethanol per gram glucose hydrolysate
[e] mL ethanol per gram Cassava substrate

The conversion efficiencies for the SSF process are given in Table 4.10.

Table 4.10: *Conversion efficiencies for SSF process in wt%*

Substrate form	Substrate utilized for glucose production	Glucose utilized for ethanol production	Glucose utilized for CO_2 production	Glucose utilized for cell growth and other products	Total glucose conversion
Unpeeled roots	90%	45%	43%	11.4%	0.994
Peeled roots	70%	40%	38%	20.8%	0.988
Peels	55%	30%	29%	40.5%	0.995

From Table 4.10 it can be seen that the unpeeled Cassava roots performed better than the other substrate forms in terms of conversion efficiency for substrate to glucose, as well as glucose conversion to ethanol. The total final glucose to products is approximately the same for all the substrate forms. This implies that more by-products were formed during the fermentation of pure Cassava starch and Cassava peels than for the unpeeled Cassava roots. The identification of the formed by-products fell outside the scope of this investigation. In conclusion it can be said that unpeeled Cassava roots are the best substrate form to use for the production of bio-ethanol and that a 45% conversion of substrate to bio-ethanol can be achieved.

4.2.3 Direct fermentation with *S. occidentalis*

Direct fermentation with unpeeled Cassava roots with *S. occidentallis* was done with 20wt% biomass loading according to the procedure described in Section 3.8.3. The final ethanol yield obtained was only $0.0025 g.g^{-1}$. The final yield was too low to be

economically feasible; therefore this production was not investigated further. From this it could be concluded that direct fermentation is not an ideal method for bio-ethanol production from starch crop that require hydrolysis.

4.3 Comparison of bio-ethanol production processes

The different production processes (SHF, SSF and DF) for the production of bio-ethanol from Cassava roots were quantitatively compared to select the best process to use. The ethanol yield for the three different processes is compared in Figure 4.19. The ethanol yield obtained from DF process was too low to show on Figure 4.19.

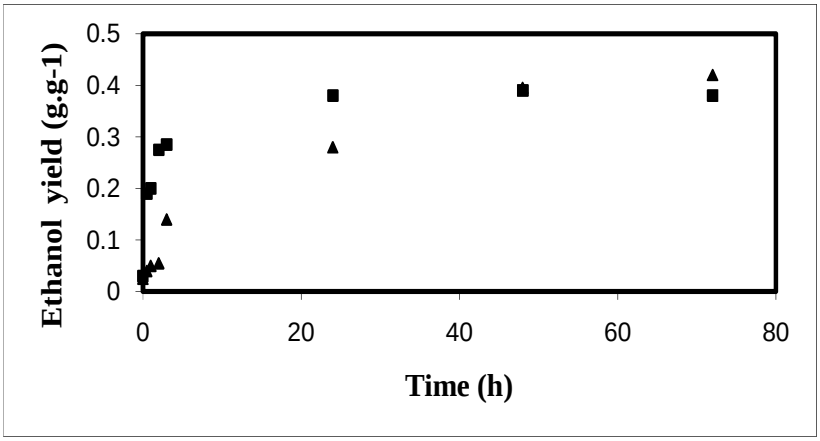


Figure 4.19: *Comparison of ethanol production between **SSF,** and **SHF** in shake flasks*
(▲- SSF, ■ - SHF)

From Figure 4.19 it can be seen that although the SHF process initially produces more ethanol faster, the final ethanol yield for the SSF process is higher. The final ethanol yields and conversion efficiencies for the three processes investigated is presented in Table 4.11.

Table 4.11: *Comparison of glucose and ethanol yields of different bio-ethanol production processes*

Process	$Y_{p/g}$ ($g.g^{-1}$)	$Y_{p/s}$ ($g.g^{-1}$)
SHF	0.92	0.38
SSF	0.90	0.43
DF	-	0.03

From Table 4.11 it can be seen that although the SHF process produced more glucose from the same amount of biomass, more of the glucose was converted to ethanol in the SSF process, making the latter economically more attractive and promising. The

better conversion of glucose to ethanol in SSF process is because the end-product inhibition from glucose formed during enzymatic hydrolysis is relieved by yeast fermentation.

4.4 Summary of hydrolysis and fermentation results

A comparative summary of all results obtained in this study is presented in Table 4.12. The Table shows that this work has been validated through other sources of similar work. The different forms of Cassava have been used for ethanol production, but peels has not been done extensively hence this study shared a focus on that as well. Therefore this table strives to reveal how much work has been done on Cassava ethanol production. This study adds value to the present work as well as it might be seen as an expansion of it.

Table 4.12: *Conclusion and validation of results against literature*

	Theoretical/ Literature	**Nquma *et al.*, 2009 (this study)**
Moisture content	Unpeeled root- 60-72% (wet basis) (Ngo *et al*. 2005 and Tonukari, 2004) peels – 8.50wt% (db) peeled root--------	61% (wet basis)- unpeeled root 9.2% (db)- unpeeled root 9.2-9.7wt% (db)- peels 10.2wt% (db)- peeled root
Starch content (db) **(unpeeled roots)** **(peels)** **(peeled roots)**	70-88% (Aryee *et al*., 2006 and (Lasztity,1999) 61% (Obadina *et al*., 2006) ---	83% - unpeeled root 51-67% - peels 70-82% -peeled root
Glucose Yield conversion (Y*p/s*) **(unpeeled roots)** **Peeled roots** **(peels)**	60-93% (Ejiofor *et al*. 1996) (Krzysztof *et al*., 2007) ------	60-92%, (0.9;0.6 $g.g^{-1}$) – unpeeled roots 70% (0.7 $g.\ g^{-1}$)- peeled roots 55% (0.55$g.\ g^{-1}$)- peels
Ethanol Yield coefficient (Y*p/s*) **(peeled and unpeeled roots)** **(peels)**	0.45 $ml.g^{-1}$ (Drapcho *et al*., 2008) 0.41 $g.g^{-1}$ ---	0.53ml. g^{-1} (SSF); 0.4 ml. g^{-1} 0. 42 $g.g^{-1}$ – unpeeled roots 0.3 $g.g^{-1}$ – peeled roots 0.16 $g.g^{-1}$ –peels
Ethanol Yield coefficient (Y*p/g*) **(peeled and unpeeled roots)** **(peels)**	0.51 g. g^{-1} (Ejiofor *et al*., 1996) --------	0.41 $g.g^{-1}$ (SHF); 0.45 g. g^{-1} (SSF); - unpeeled roots 0.4 g. g^{-1} – peeled roots 0.3 $g.g^{-1}$ –peels
Ethanol percent **(peeled and unpeeled roots)** **(peels)**	Roots-8-12% (Atthasampunna *et al.*, 1987) Peels-1.05% (Adesanya *et al.,* 2008)	9.8% (SHF); 10.6% (SSF); - unpeeled roots 7%- peeled roots 4% -peels
Substrate concentration	25-30% (Aggarwal *et al*., 2001); (Ku Ismail *et al*., 2008)	20%
Enzyme concentration	0.3% Term, 0.2 % Spir (Ku Ismail *et al*., 2008)	0.2% Term, 0.25 % Spir and 0.1% Cell
Yeast concentration	$10g.L^{-1}$ -5 $g.L^{-1}$ (Norgard, 2008)	3 $g.L^{-1}$
Optimum pH	6 (liq), (Oboh, 2008). 4.5 (Sacc) (Srinorakutara *et al.*, 2004)	6 (liq), 4.5 (Sac)
Optimum Temperature (°C)	100-85 (liq), 55-65 (Sac) (Ejiofor *et al*. 1996 and Srinorakutara *et al.* 2004)	95 (liq), 55 (Sac)
Efficient enzyme combination	Termamyl, Spirizyme and Cellulase (Sriroth *et al*,. 2000)	Termamyl SC, Spirizyme Fuel and Celluclast 1.5L

4.5 REFERENCES

ADESANYA, O. A., OLUYEMO, K.A., JOSIAH, S.J., AESANYA, R.A., SHITU, L.A.J., OFUSORI, D.A., BANKOLE, M.A., & BABALOLA, G.B. 2008. Ethanol Production by Saccharomyces Cerevisiae from Cassava Peel Hydrolysate. *The Internet Journal of Microbiology,* 5(1), Feb

AGGARWAL, N.K., NIGAM, P., SINGH, D. & YADAV, B.S. 2001. Process optimization for the production of sugar for bioethanol industry from tapioca, a non-conventional source of starch. *World Journal of Microbiology and Biotechnology,* **17**: 783-787.

ARYEE, F.N.A., ODURO, I., ELLIS, W.O. & AFUAKWA, J.J. 2006. The physicochemical properties of flour samples from the roots of 31 varieties of cassava. *Food Control, **17***: 916-922.

ASSOCIATION FOR CLINICALCHEMISTRY (AACC). 1999. "South African Grain Laboratory, S.A.

ATTHASAMPUNNA, P., SOMCHAI, P., EU-AREEU, A. & ARTJARIYASRIPONG, P. 1987. Production of fuel ethanol from cassava. *MIRCEN Journal,* **3**: 135-142.

CARTA, F.S., SOCCOL, C.R., VANDENBERGHE, L.P.S. & Lebault, J.M. 1997. Enzymatic hydrolysis of cassava bagasse for production of metabolites obtained by fermentation. (Paper presented in Enzytec- 97, Brazil: Rio de Janeiro).

DRAPCHO, C. M., NHUAN, N.P. & WALKER, T.H. 2008. *Biofuels engineering process technology,* McGraw-Hill.

Ejiofor, A.O., Yusuf, C., and Moo-Young M. (1996). Culture of *Saccharomyces cerevisiae* on hydrolyzed waste cassava starch for production of baking-quality yeast, *Enzyme and Microbial Technology.* **18**, 519-525.

Komolprasert, V., and Ofoli, R.Y., Starch hydrolysis kinetics of Bacillus licheniforms alpha-amylase. *Journal of Chemical Technology & Biotechnology*. **51**(2):209-223

Krzysztof, N., Waliszweski., Miguel Darcia Avarado Javier De La Cruz M. (2007) Kinetics of enzymic hydrolysis of cassava flour starch — optimization and modeling, *International Journal of Food Science & Technology*. **27**(4):465-472.

Ku ISMAIL, K. S., AHMAD A.A., INBA, K.F. & DAUD M.Z.M. 2008. Thermo-enzymatic Hydrolysis of Cassava Starch by α-amylase and amyloglucosidase. (Proceedings of MUCET-2008, Malaysia:Perlis).

LASZTITY, R. 1999. Cereal chemistry. Hungary: Akademiai Kiado: 11-51.

McMILLAN, J.D. NEWMAN, M.M. TEMPLETON D.W. & MOHAGHEGHI, A. 1999. Simultaneous saccharification and co-fermentation of dilute-acid pretreated yellow poplar hardwood to ethanol using xylose-fermenting *Zymomonas mobilis*. *Applied Biochemistry and Biotechnology*: **77–79, Mar.**

MORRISOM, W. & LAIGNELET, B. 1983. An improved colorimetric procedure for determining apparent and total amylose in cereal and other starches. *Journal of Cereal Science,* **1**:9–20.

NGO, T. D., INGER, L. & NGUNYEN, T. M. 2005. Intercropping cassava (Manihot esculenta Crantz) with Flemingia (Flemingia macrophylla); effect on biomass yield and soil fertility. *Livestock Research for Rural Development*. 17(6), Feb.

NORGAD, J. 2004. Ethanol Production from Biomass - Optimization of Simultaneous Saccharification and Fermentation with Respect to Stirring and Heating, Department of Chemical Engineering, Lund Institute of Technology.

OBADINA, A.O., OYEWOLE, O.B., SANNI, .L. O. & ABIOLA, S. S. 2006. Fungal enrichment of cassava peels proteins. *African Journal of Biotechnology*, 5 (3): 302-304, May.

OBOH, G. 2005. Isolation and characterization of amylase from fermented cassava (*Manihot esculenta* Crantz) wastewater. *African Journal of Biotechnology*, **4**(10), 1117-1123.

OCLOO, F. C. K. & AYENOR, G. S. 2008. Physical, chemical and microbiological changes in alcoholic fermentation of sugar syrup from cassava flour. *African Journal of Biotechnology*, **7** (2): 164-168.

OMEMU, A.M., AKPAN, I., BANKOLE, M.O. & TENIOLA, O.D. 2005. Hydrolysis of raw tuber starches by amylase of *Aspergillus niger* AM07 isolated from the soil. *African Journal of Biotechnology*, **4**: 19-25.

SAIBENE, D. & SEETHARAMAN, K. 2008. Use of iodine as a tool to understand wheat starch pasting properties. *Starch-starke*, **60**(1): 1-7.

SALLE, A. J. 1993. The Permaculture Book of Ferment and Human Nutrition Tyalgum: Tagari.

SCHENCK, F.W. 2002. Starch hydrolysates - An overview. *Intenational Sugar Journal*, **104**, 82-89.

SRIROTH, K., CHOLLAKUO, R., CHOTNEERANAT, S., PIYACHOMKWAM, K. & OATES, C.G. 2000. Processing of Cassava waste for improved biomass utilization. *Bioresource Technology*, **71**: 63-69.

SRINORAKUTARA, T., SUESAT, C., PITIYONT, B., KITPREECHAVANIT, W. & CATTITHAMMANIT, S. 2004. Utilization of Waste from Cassava Starch Plant for Ethanol Production. (Joint International Conference on "Sustainable Energy and Environment (SEE), Hua Hin. Thailand. p. 773-76).

THAILAND INSTITUTE OF SCIENTIFIC AND TECHNOLOGICAL RESEARCH (TISTR). 2002. The improvement on ethanol quality: the Royal Chitralada Project. Ministry of Science and Technology, Thailand :Bangkok.

TONUKARI, N.J. 2004. Cassava and the future of starch. *Electronic Journal of Biotechnology*, 7 (1): 5-8, 15 Apr.

WEBB, F.C. 1964. Biochemical Engineering, D.Van Nostrand Ltd. London.

YUSAKU, F., MASAFUMI, O., and SEINOSUKE, U. 2004. Ethanol fermentation of raw cassava starch with Rhizopus koji in a gas circulation type fermentor. *Biotechnology and Bioengineering*, 27 (8): 1270-1273, 18 Feb.

CHAPTER 5

CONCLUSIONS

5.1 Conclusions

This study was undertaken to investigate the optimization of ethanol production from enzymatic pretreatment of the Cassava roots. From this study some conclusions and recommendations can be made.

- In this dissertation it was shown that Cassava roots have a high starch content that can be enzymatically utilized for the production of glucose that can be converted to bio-ethanol.

- All Cassava root material used in this study had a moisture content of between 9 and 10 wt% (dry basis).

- The starch content of all Cassava roots used in this study was determined to be between 69 wt% and 87wt%.

- Iodine solution was used successfully in this study to determine complete conversion of starch to dextrins during liquefaction.

- It was shown conclusively in this study that substrate form has a significant influence on the glucose produced during liquefaction, and that unpeeled Cassava roots are the best substrate form to use.

- It was found that pH significantly influences the liquefaction step, but not the saccharification.

- Liquefaction of Cassava unpeeled roots should be done at a pH of 6 for optimum glucose production.

- It was found that temperature significantly influenced the amount of glucose produced during hydrolysis, and that a temperature of 95°C during liquefaction and a temperature of 55°C during saccharification will produce the highest glucose yield.

- A 20 wt% biomass load resulted in a higher glucose yield during liquefaction and saccharification.

- Enzyme loading had a significant influence on the final glucose yield during hydrolysis and it was found that an enzyme loading of 2 $\mu L.g^{-1}$ Termamyl SC, 2.5 $\mu L.g^{-1}$ Spirizyme fuel and 1 $\mu L.g^{-1}$ Celluclast 1.5L resulted in the highest glucose yield.

- It was shown in this study that the addition of Celluclast 1.5L to the hydrolysis step had a positive effect on the glucose yield obtained.

- Direct fermentation of unpeeled Cassava roots produced a very small amount of ethanol.

- The SHF process showed a better conversion of biomass to glucose, while the SSF process showed better conversion of glucose to ethanol.

- It was shown that a yeast concentration of only $3g.L^{-1}$ is sufficient to convert the available glucose to ethanol in 72 hours.

- An ethanol yield of 45wt% (g ethanol per g substrate) was achieved with the SSF process, resulting in a production potential of 580L of ethanol per ton of unpeeled Cassava roots.

APPENDIX A

CALIBRATION CURVES

Overview

In this Appendix the preparation of calibration curves for the determination of sugar concentration and ethanol concentration in a Cassava fermentation broth using high performance liquid chromatography (HPLC) is presented. The Appendix is subdivided into two sections. The results for the reducing sugar calibration curves are given in Section A.1. The tabulated results and calibration curve for ethanol is given in Section A.2. The Chromatograms for the sugars and ethanol are shown in Section A.3.

In order to use high performance liquid chromatography as an analytical tool, a calibration curve is required. Standard mixtures of different glucose concentration are analyzed with HPLC and the response area of the peak for each concentration used is recorded. The areas of the peaks are then plotted against the concentration used to obtain each of the peaks and a straight line is fitted to the data to obtain an equation that can be used to determine the glucose concentration in any Cassava fermentation broth.

$$y = mx + b$$

m: gradient
x: Amount (concentration)
y: Height (area)

Where m is the gradient, for every sample, y is known and thus x can be calculated. The same principle holds for all the calibration curves used in this study.

A.1 Sugar calibration curves

The fermentable sugars available in Cassava that were prepared as standards are; glucose, sucrose, fructose, maltose and maltotriose. The calibration curves (slope) were used to determine the amount/yield of sugars present in a specific hydrolysate

and fermentation broth, reported in chapter 4. The concentration prepared for standards are concentrations that were expected to be obtained during and after hydrolysis. The amount of water and sugar used to prepare the standard sugar mixtures for the calibration curves are given in Table A.1.

Table A.1: *Preparation (dilutions) of sugar standards*

Concentration ($g.L^{-1}$)	**Volume of sugar (ml)**	**Volume of water (ml)**	**Final Volume (ml)**
200	2	0	2
100	1	1	2
50	1	1	2
10	0.4	1.6	2
5	1	1	2
1	0.4	1.6	2

Table A.2: *Peak areas obtained for each sugar concentration using HPLC*

Concentration ($g.L^{-1}$)	**Glucose**	**Fructose**	**Maltose**	**Sucrose**	**Maltotriose**
1	8.09E+04	7.89E+04	1.06E+05	7.23E+04	9.04E+04
5	3.85E+05	2.66E+05	4.33E+05	3.71E+05	4.06E+05
10	6.76E+05	4.73E+05	8.28E+05	7.96E+05	7.99E+05
50	3.79E+06	2.90E+06	3.95E+06	3.90E+06	3.96E+06
100	7.76E+06	5.82E+06	7.83E+06	7.78E+06	7.98E+06
200	1.54E+07	1.30E+07	1.47E+07	1.53E+07	1.56E+07

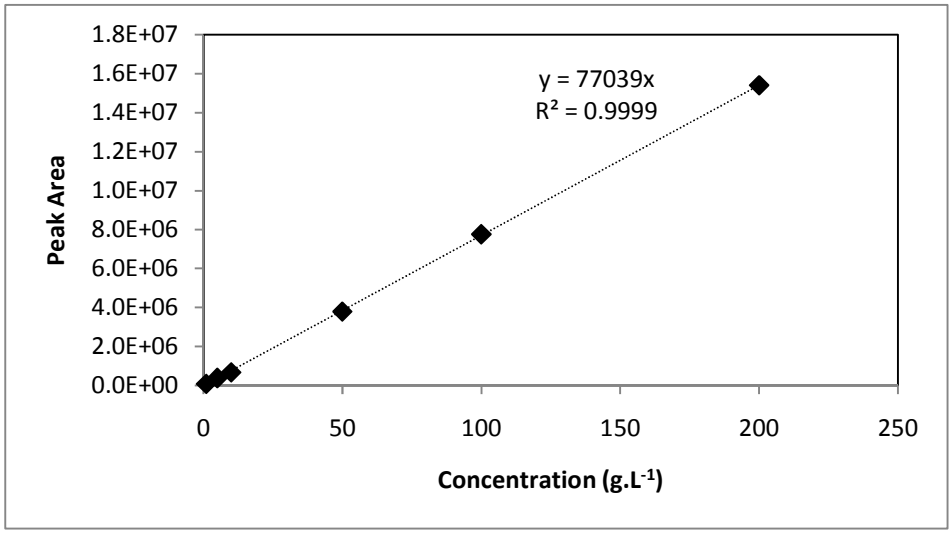


Figure A.1: *Glucose calibration curve*

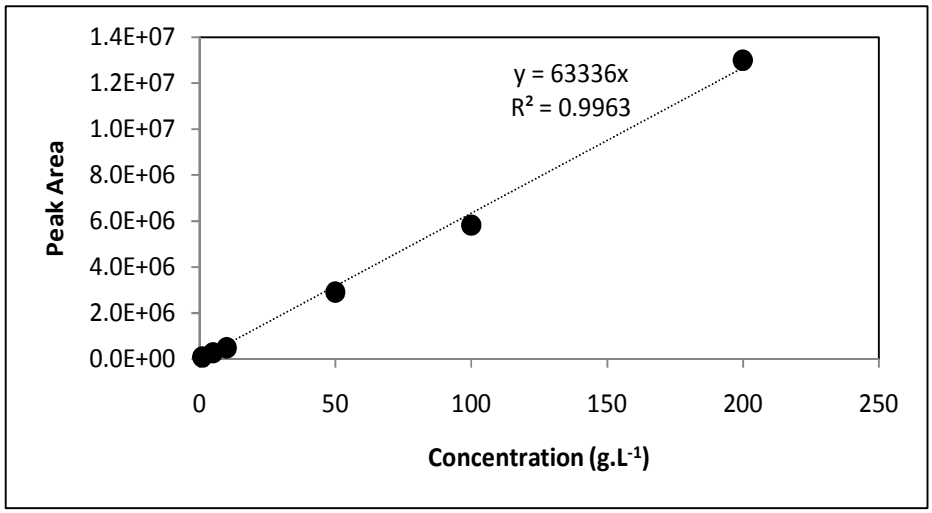


Figure A.2: *Fructose calibration curve*

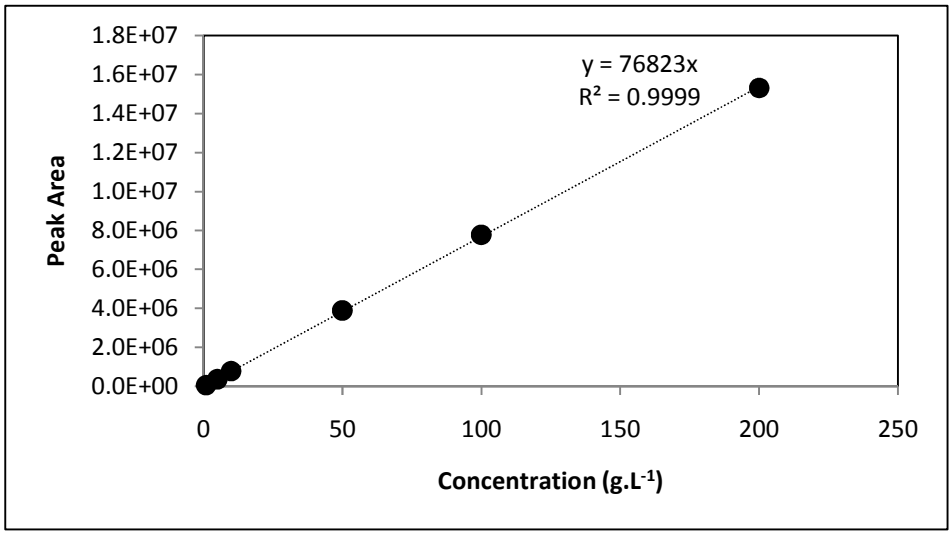


Figure A.3: *Sucrose calibration curve*

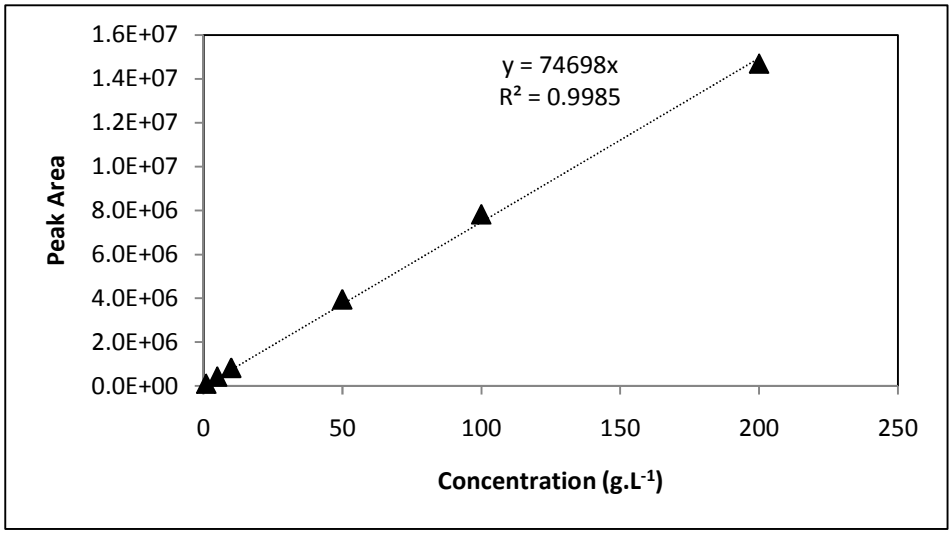


Figure A.4: *Maltose calibration curve*

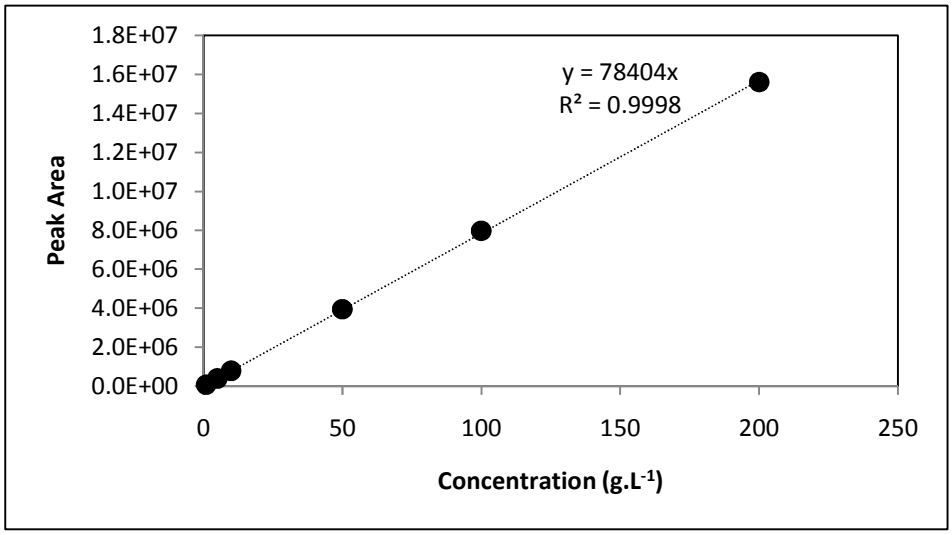


Figure A.5: *Maltotriose calibration curve*

A.2 Ethanol calibration curve

The ethanol concentrations in each of the standard solutions used were determined with HPLC. The concentration prepared for standards are concentrations that were expected to be obtained after fermentation of the sugars. The amount of water and ethanol used to prepare each of the standard solutions is given in Table A.3.

Table A.3: *Preparation of ethanol standard solutions*

Concentration ($g.L^{-1}$)	Volume of ethanol (ml)	Volume of water (ml)	Final Volume (ml)
150	2	0	2
100	0.7	1.3	2
50	1	1	2
25	1	1	2
15	0.8	1.2	2
7.5	1	1	2
3.75	1	1	2

Table A.4: *Ethanol peak areas obtained for each standard solution*

Concentration ($g.L^{-1}$)	Area
15	5.70E+05
25	9.30E+05
50	2.00E+06
100	3.66E+06
150	5.56E+06

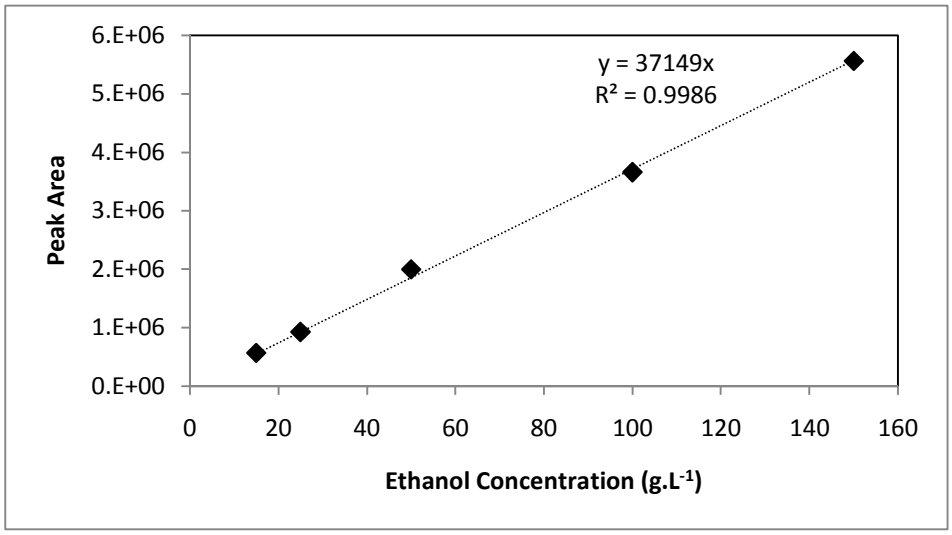


Figure A.6: *Ethanol calibration curve*

A.3 Sugar and ethanol chromatograms

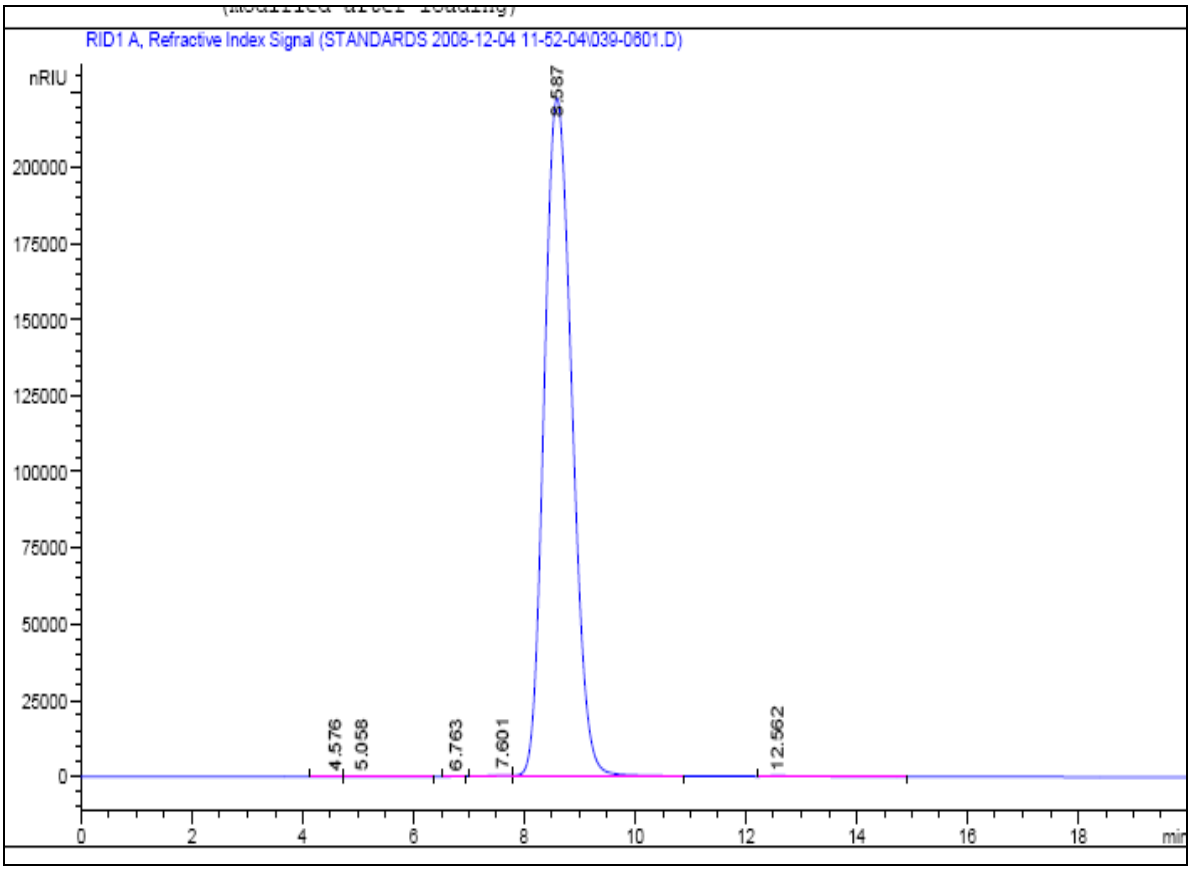


Figure A.7: *Typical glucose chromatogram obtained from HPLC analysis*

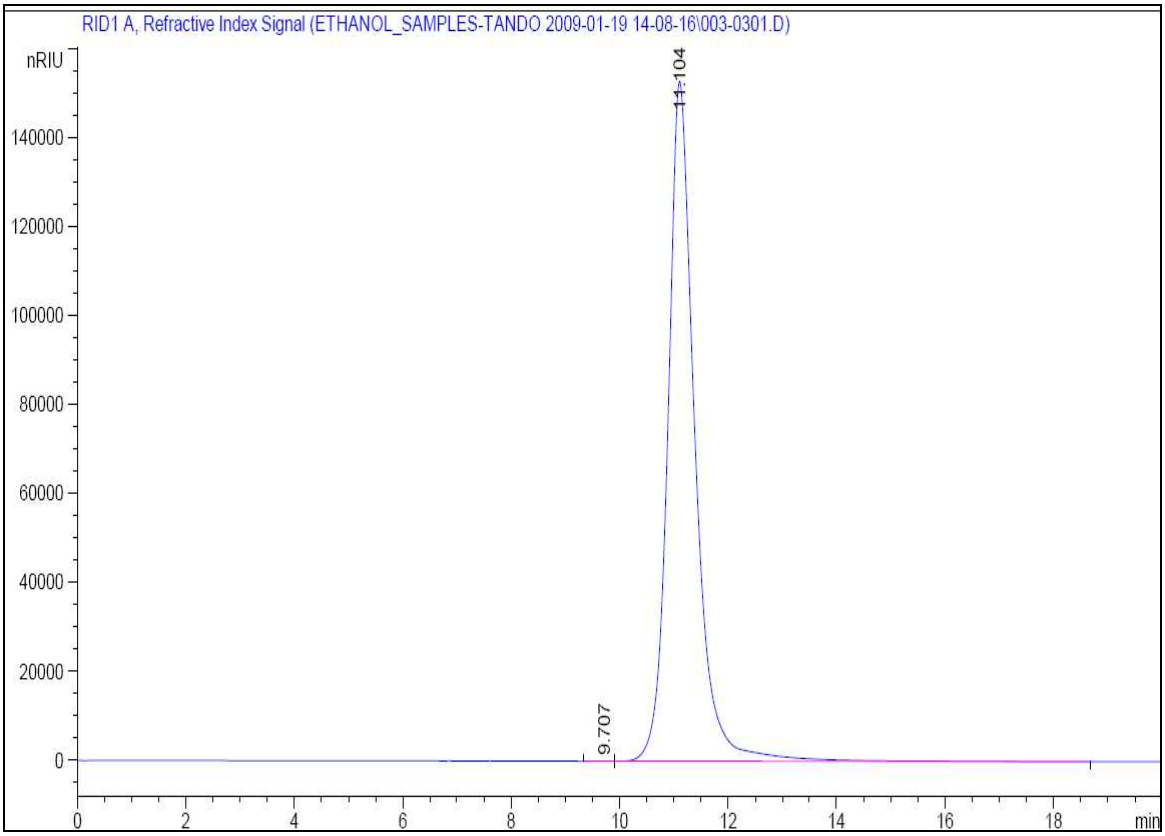


Figure A.8: *Typical ethanol chromatogram obtained from HPLC analysis*

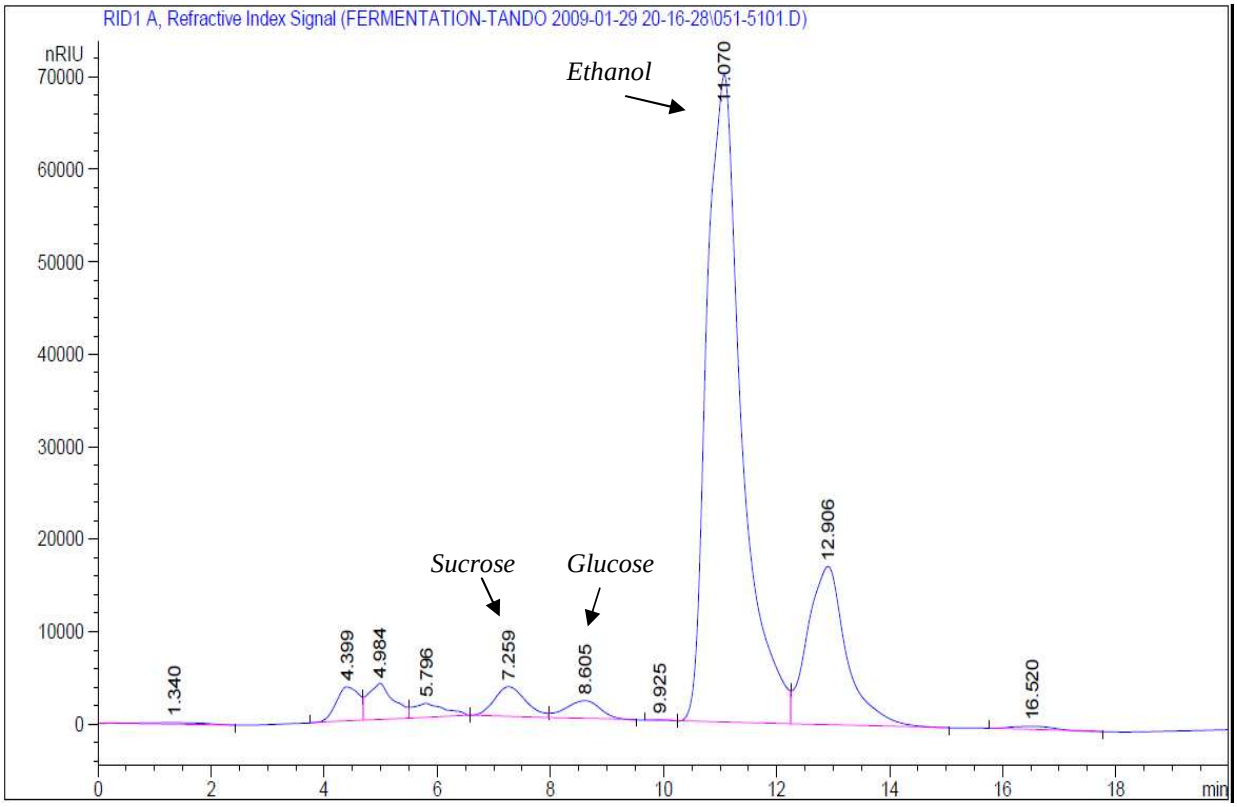


Figure A.9: *Typical HPLC chromatogram of a fermentation broth sample*

APPENDIX B

ENZYMATIC HYDROLYSIS

Overview

In this Appendix the glucose yields obtained during enzymatic hydrolysis of Cassava for different values of the manipulated variables are listed. The results given in this Appendix is graphically presented and discussed in chapter 4 of this dissertation.

B.1 Optimization of liquefaction and saccharification steps

The glucose yield is recorded as $Y_{g/s}$ ($g.g^{-1}$) (gram glucose per gram Cassava).

B.1.1 Effect of substrate form on glucose yield

Table B.1: *Glucose yield ($g.g^{-1}$) obtained during liquefaction of different substrate forms of Cassava*

Time (minutes)	Substrate form		
	Peels (Cellulose)	Peeled roots (Starch)	Unpeeled roots (Starch and Cellulose)
0	0.0240	0.0250	0.0075
10	0.0250	0.0260	0.0150
15	0.0265	0.0360	0.0300
30	0.0315	0.0360	0.0450
45	0.0325	0.0375	0.0800
60	0.0400	0.0500	0.0900

B.1.1 Effect of pH on glucose yield

Table B.2: *Glucose yield ($g.g^{-1}$) obtained at different pH values during liquefaction of unpeeled Cassava roots*

Time (min)	Control	pH 6.5	pH 6	pH 5.5
0	0.0036	0.0060	0.0066	0.0060
15	0.0036	0.0084	0.0126	0.0270
30	0.0186	0.0216	0.0300	0.0336
45	0.0246	0.0366	0.0318	0.0378
60	0.0240	0.0312	0.042	0.0372
75	0.0288	0.0300	0.0432	0.0402
90	0.0102	0.0186	0.0498	0.0486

Table B.3: *Glucose yield ($g.g^{-1}$) obtained during saccharification of unpeeled Cassava roots at different pH values*

Time(h)	Control	pH 5.5	pH 4.5	pH 4
0	0.0102	0.0186	0.0498	0.0486
1	0.1320	0.7620	0.7680	0.7980
2	0.0900	0.9420	0.8580	0.8040
3	0.078	0.9180	0.9180	0.8640
4	0.1200	0.9300	0.8580	0.7920
8	0.132	0.9600	0.8400	0.8520
12	0.162	0.9300	0.8640	0.8640
24	0.330	0.8020	0.9720	0.9420
28	0.306	0.8580	0.8880	0.8520

B.1.3 Effect of temperature on glucose yield

Table B.4: *Glucose yield ($g.g^{-1}$) obtained during liquefaction of unpeeled Cassava roots at different temperatures*

Time (min)	95°C	90°C	85°C
0	0.0025	0.0055	0.0070
15	0.0140	0.0105	0.0125
30	0.0200	0.0250	0.0230
60	0.0535	0.0350	0.0500

Table B.5: *Glucose yield ($g.g^{-1}$) obtained during saccharification of unpeeled Cassava roots at different temperatures*

Time (h)	65°C	60°C	55°C
0	0.063	0.000	0.0415
1	0.713	0.325	0.6400
2	0.750	0.765	0.7150
3	0.760	0.790	0.7650
4	0.820	0.840	0.7750
28	0.870	0.800	0.8500
48	0.850	0.830	0.9050

B.1.4 Effect of biomass load on glucose yield

Table B.6: *Glucose yield ($g.g^{-1}$) obtained during liquefaction of unpeeled Cassava roots at different biomass loadings*

Time(min)	10wt%	20wt%
0	0.01	0.010
15	0.04	0.040
30	0.05	0.045
45	0.05	0.075
60	0.11	0.085
120	0.66	0.470
180	0.71	0.710
240	0.75	0.775
300	0.83	0.890

B.1.5 Effect of enzyme combination on glucose yield

Table B.7: *Glucose yield ($g.g^{-1}$) obtained during hydrolysis of unpeeled Cassava roots using a 10wt% biomass loading with and without the addition of Celluclast 1.5L to the hydrolysis mixture*

Time(min)	Without Celluclast 1.5 L	With Celluclast 1.5L
0	0.01	0.01
15	0.04	0.03
30	0.05	0.04
45	0.05	0.04
60	0.11	0.12
65	0.46	0.56
73	0.54	0.57
88	0.57	0.63
90	0.64	0.62
120	0.66	0.65
180	0.71	0.65
240	0.75	0.69
300	0.83	0.91

Table B.8: *Glucose yield ($g.g^{-1}$) obtained during hydrolysis of unpeeled Cassava roots using a 20wt% biomass loading with and without the addition of Celluclast 1.5L to the hydrolysis mixture*

Time(min)	Without Celluclast 1.5L	With Celluclast 1.5L
0	0.010	0.010
15	0.040	0.030
30	0.045	0.045
45	0.075	0.016
60	0.085	0.085
65	0.330	0.525
73	0.365	0.575
88	0.375	0.780
90	0.410	0.815
120	0.470	0.835
180	0.710	0.860
240	0.775	0.865
300	0.780	0.890

B.1.6 Effect of enzyme loading on glucose yield

Table B.9: *Enzyme loadings (wt %) used in different combinations in this study*

Combination	Termamyl SC	Spirizyme Fuel	Celluclast 1.5 L
Combo 1	0.2	0.25	0.1
Combo 2	0.5	0.55	0.2
Combo 3	0.7	0.75	0.4

Table B.10: *Glucose yield ($g.g^{-1}$) obtained during liquefaction of unpeeled Cassava roots using different enzyme loadings*

Time(h)	Combo 1	Combo 2	Combo 3
0	0.010	0.005	0.005
0.25	0.030	0.015	0.015
0.5	0.045	0.020	0.020
0.75	0.080	0.040	0.035
1	0.085	0.060	0.050
1.08	0.575	0.750	0.750
1.25	0.780	0.760	0.760
1.5	0.815	0.770	0.790
2	0.835	0.810	0.795
3	0.860	0.870	0.860
4	0.865	0.890	0.890
5	0.890	0.860	0.924

APPENDIX C

FERMENTATION

Overview

In this Appendix the ethanol and glucose yield values (data) during fermentation will be given. The manipulative variable which was the fermentation route for the optimization of ethanol yield are reported in data form with respect to Section C.1, SHF and Section C.2 SSF. The ethanol yield is recorded as $Y_{g/s}$ ($g.g^{-1}$) of cassava.

C.1 Separate Hydrolysis and Fermentation

Table C.1: *Ethanol yield ($g.g^{-1}$) obtained during SHF of Cassava roots by Saccharomyces cerevisiae*

Time (h)	Y ($g.g^{-1}$)
0	0.031
0.25	0.190
0.5	0.200
1	0.190
2	0.285
3	0.275
24	0.380
48	0.390
72	0.380
96	0.395
120	0.375

Table C.2: *Ethanol yield compared to glucose uptake during SHF of Cassava roots by Saccharomyces cerevisiae*

Time (h)	Ethanol ($g.g^{-1}$)	glucose ($g.g^{-1}$)
0	0.031	0.8250
0.25	0.190	0.4600
0.5	0.200	0.3550
1	0.190	0.3550
2	0.285	0.1825
3	0.275	0.1900
24	0.380	0.0150
48	0.390	0.0185
72	0.380	0.0080
96	0.395	0.0065
120	0.375	0.0060

C.2 Simultaneous Saccharification and Fermentation

Table C.3: *Ethanol yield ($g.g^{-1}$) and glucose uptake ($g.g^{-1}$) during SHF of Cassava roots using different yeast concentrations*

	Ethanol ($g.g^{-1}$)			Glucose ($g.g^{-1}$)		
Time (h)	8 $g.L^{-1}$	5 $g.L^{-1}$	3 $g.L^{-1}$	8 $g.L^{-1}$	5 $g.L^{-1}$	3 $g.L^{-1}$
0	0.025	0.0200	0.0175	0.620	0.625	0.6200
0.5	0.040	0.0450	0.0300	0.555	0.735	0.5850
1	0.050	0.0525	0.0400	0.560	0.550	0.5900
2	0.055	0.0550	0.0500	0.565	0.545	0.6100
3	0.140	0.1100	0.0700	0.390	0.520	0.5700
4	0.155	0.1450	0.1300	0.460	0.445	0.5250
8	0.205	0.2075	0.1700	0.320	0.220	0.3250
24	0.280	0.3350	0.2100	0.155	0.040	0.0200
48	0.395	0.3800	0.4000	0.010	0.010	0.0085
72	0.420	0.415	0.4250	0.065	0.005	0.0030

Table C.4: *Ethanol yield ($g.g^{-1}$) obtained during SHF of different forms of Cassava roots with S. cerevisiae*

Time (h)	Peels (Cellulose)	Peeled Cassava (Starch)	Unpeeled Cassava (Starch and Cellulose)
0	0.017	0.020	0.025
0.5	0.052	0.055	0.040
1	0.06	0.055	0.050
2	0.075	0.060	0.055
3	0.050	0.070	0.140
4	0.065	0.085	0.155
8	0.110	0.130	0.210
24	0.130	0.140	0.280
48	0.150	0.270	0.395
72	0.160	0.300	0.420

Table C.5: *Glucose uptake ($g.g^{-1}$) during SHF of different substrate forms of Cassava using S. cerevisiae*

Time (h)	Peels (Cellulose)	Peeled Cassava (Starch)	Unpeeled Cassava (Starch and Cellulose)
0	0.550	0.710	0.9200
0.5	0.330	0.630	0.5600
1	0.260	0.540	0.5600
2	0.120	0.490	0.5700
3	0.120	0.390	0.3900
4	0.060	0.290	0.4600
8	0.055	0.190	0.3200
24	0.027	0.120	0.1600
48	0.008	0.015	0.0100
72	0.005	0.012	0.0065

Table C.6: *Ethanol yield ($g.g^{-1}$) compared to glucose uptake ($g.g^{-1}$) during SHF of Cassava peels using 8 $g.L^{-1}$ S cerevisiae*

Time (h)	Ethanol yield ($g.g^{-1}$)	Glucose uptake ($g.g^{-1}$)
0	0.017	0.550
0.5	0.052	0.330
1	0.060	0.260
2	0.075	0.120
3	0.050	0.120
4	0.065	0.060
8	0.110	0.055
24	0.130	0.027
48	0.150	0.008
72	0.160	0.005

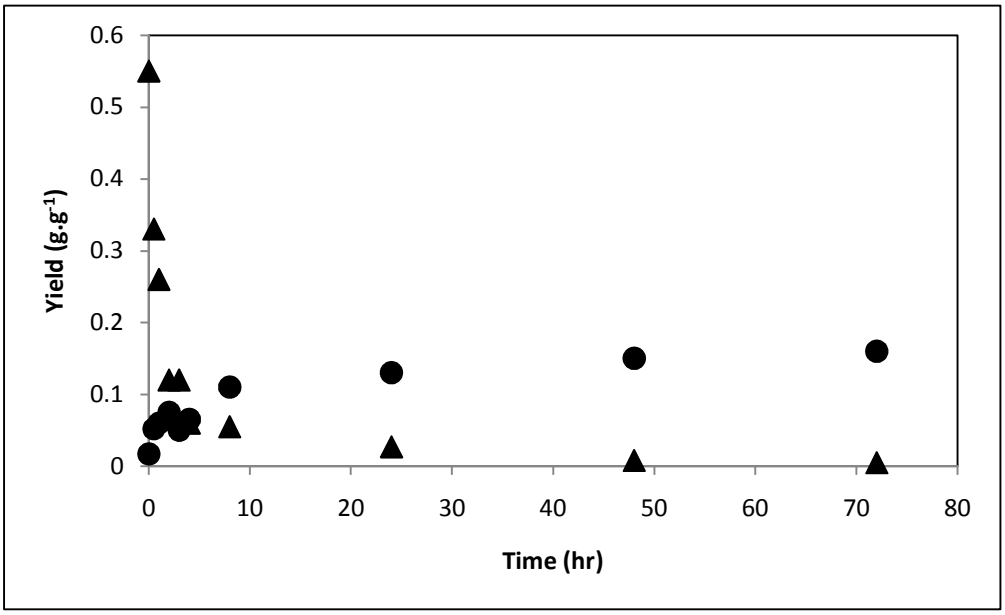


Figure C.1: *Ethanol yield ($g.g^{-1}$) compared to glucose uptake ($g.g^{-1}$) during SHF of Cassava peels using 8 $g.L^{-1}$ S cerevisiae*

Table C.7: *Ethanol yield ($g.g^{-1}$) and glucose uptake ($g.g^{-1}$) during SSF of peeled Cassava roots using 8 $g.L^{-1}$ of S. cerevisiae*

Time (h)	**Ethanol yield ($g.g^{-1}$)**	**Glucose uptake ($g.g^{-1}$)**
0	0.020	0.7050
0.5	0.055	0.6250
1	0.055	0.5400
2	0.060	0.4850
3	0.070	0.3900
4	0.085	0.2900
8	0.125	0.1900
24	0.140	0.1200
48	0.270	0.0150
72	0.30	0.0115

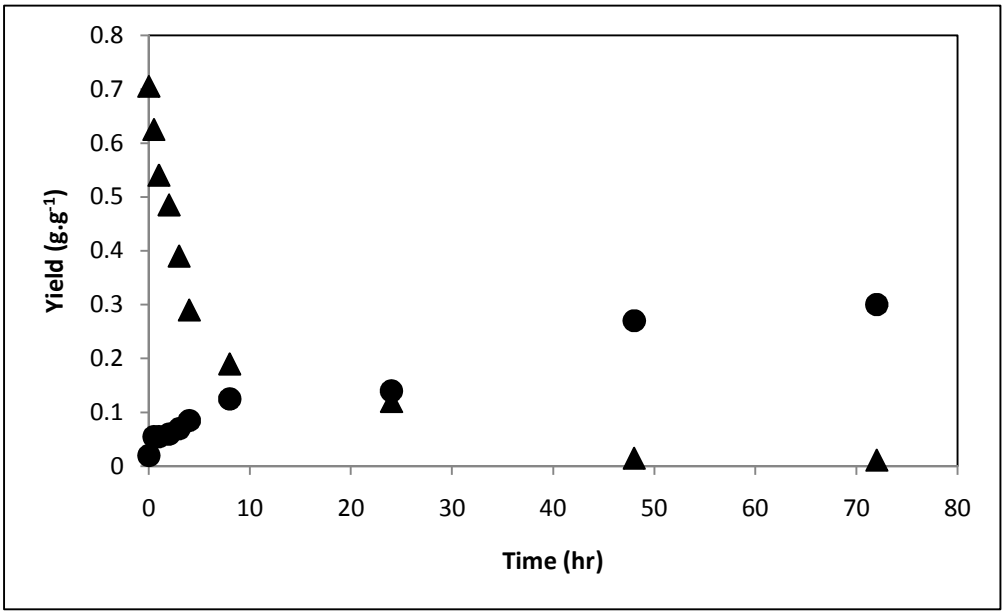


Figure C.2: *Ethanol yield ($g.g^{-1}$) and glucose uptake ($g.g^{-1}$) during SSF of peeled Cassava roots using 8 $g.L^{-1}$ of S. cerevisiae*

Table C.8: *Ethanol yield ($g.g^{-1}$) and glucose uptake ($g.g^{-1}$) during SSF of unpeeled Cassava roots using 8 $g.L^{-1}$ S. cerevisiae*

Time (h)	**Ethanol yield ($g.g^{-1}$)**	**Glucose uptake ($g.g^{-1}$)**
0	0.025	0.9200
0.5	0.040	0.5600
1	0.050	0.5600
2	0.055	0.5700
3	0.140	0.3900
4	0.155	0.4600
8	0.210	0.3200
24	0.280	0.1600
48	0.395	0.0100
72	0.42	0.0065

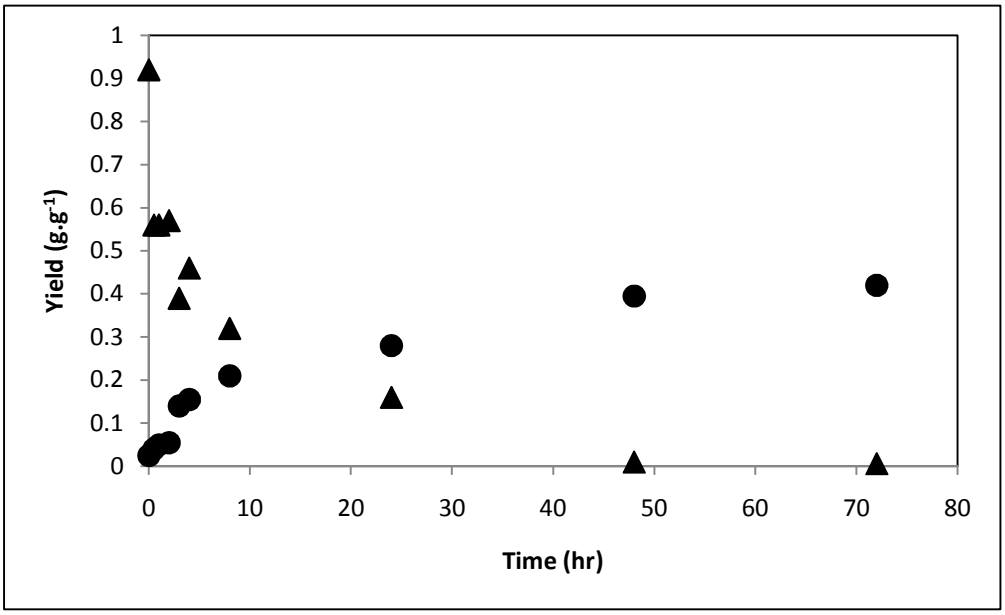


Figure C.3: *Ethanol yield ($g.g^{-1}$) and glucose uptake ($g.g^{-1}$) during SSF of unpeeled Cassava roots using 8 $g.L^{-1}$ S. cerevisiae*

Table C.9: *Comparison of ethanol yield ($g.g^{-1}$) for SHF and SSF*

Time (hr)	Ethanol yield($g.g^{-1}$)	
	SSF	SHF
0	0.025	0.030
0.5	0.040	0.190
1	0.050	0.200
2	0.055	0.275
3	0.140	0.285
24	0.280	0.380
48	0.395	0.390
72	0.420	0.380

APPENDIX D

EXPERIMENTAL ERROR

Overview

In this Appendix the experimental error values (data) during liquefaction, saccharification and fermentation will be given. The Appendix is subdivided into three Sections with respect to Section D.1, liquefaction, Section D.2 saccharification and fermentation in Section D.3.

The experimental error was done according to the following principles to validate this study.

Average ($\bar{x}$) - The arithmetic mean, and is calculated by adding a group of numbers and then dividing by the count of those numbers.

Z score (Z) -The z score for an item, indicates how far and in what direction, that an item deviates from its distribution's mean, expressed in units of its distribution's standard deviation. The equation (2) is used for samples less than 100 (small samples), where TINV returns the inverse of the t-distribution, which is used in the hypothesis testing of small sample data sets.

STDEV (σ) - The standard deviation is the unit of measurement of the z-score. It allows comparison of observations from different normal distributions, which is done frequently in research (see equation 1)

Confidence Limit (±) - Returns a value that you can use to construct a confidence interval for a population mean. The confidence interval is a range of values. Your sample mean, $\bar{x}$, is at the center of this range and the range is ± CONFIDENCE (see equation 3)

Experimental error - The **approximation error** in the data is the discrepancy between an exact value and some approximation to it. (see equation 4)

$$\sigma = \sqrt{\sum \frac{(x-\bar{x})^2}{(n-1)}} \quad (1)$$

$$\bar{Z} = TINV\ (0.05, COUNT\ (n)) \quad (2)$$

$$Confidence\ Limit = 2 \times \bar{Z}\left(\frac{\sigma}{\sqrt{n}}\right) \quad (3)$$

$$Error\ \% = \left(\frac{Confidence}{\bar{x}}\right) \times 100 \quad (4)$$

D.1 Liquefaction

The experimental error for the liquefaction step was determined by repeating the liquefaction of raw Cassava starch at a pH of 6 and a temperature of 95 °C five times. The glucose concentration ($g.g^{-1}$) obtained for each time interval for the five repeated experiments are listed in Table D.1. The statistical parameters used to calculate the experimental error are listed in Table D.2. The glucose concentration determine at each time interval for the five repeated liquefaction experiments is graphically presented in Figure D.1.

Table D.1: *Glucose concentration for repeated liquefaction experiments at pH 6 and 95°C.*

Time (min)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
0	0.0155	0.015	0.0155	0.014	0.014
15	0.025	0.026	0.029	0.0275	0.026
30	0.048	0.0505	0.0505	0.0535	0.051
45	0.07	0.0765	0.074	0.0705	0.075
60	0.079	0.0805	0.0795	0.0795	0.081

Table D.2: *Statistical parameters used to calculate the experimental error for the liquefaction step*

Sample	Final Glucose Concentration
1	0.079
2	0.0805
3	0.0795
4	0.0795
5	0.081
Mean	0.0799
Standard deviation	0.0008
Confidence limit (95%)	0.002
Experimental error	2.36%

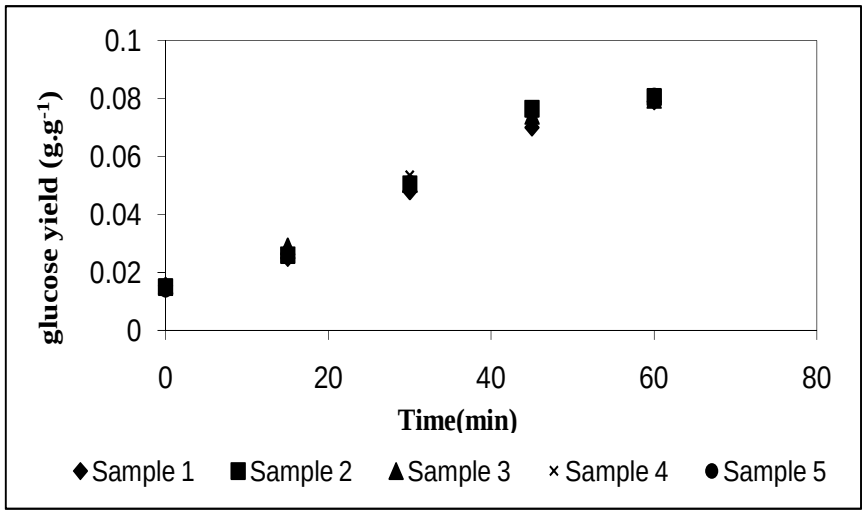


Figure D.1: *Five replicates of increasing glucose yield during liquefaction in the SHF process*

D.2 Saccharification

The experimental error for the saccharification step was determined by repeating the saccharification of a liquefied Cassava hydrolysate at a pH of 4.5and a temperature of 55°C five times. The glucose concentration ($g.g^{-1}$) obtained for each time interval for the five repeated experiments are listed in Table D.3. The statistical parameters used to calculate the experimental error are listed in Table D.4. The glucose concentration determine at each time interval for the five repeated saccharification experiments is graphically presented in Figure D.2.

Table D.3: *Glucose concentration for repeated saccharification experiments at pH 4.5 and 55°C. Five replicates of samples*

Time(h)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
0	0.079	0.0805	0.0795	0.0795	0.081
0.3	0.875	0.905	0.915	0.905	0.905
0.7	0.91	0.915	0.915	0.895	0.89
1	0.85	0.85	0.865	0.855	0.865
2	0.865	0.885	0.89	0.89	0.89
3	0.885	0.905	0.905	0.905	0.91
4	0.905	0.92	0.93	0.915	0.93
6	0.915	0.93	0.93	0.935	0.925
8	0.93	0.94	0.93	0.93	0.935
24	0.925	0.935	0.93	0.93	0.935
28	0.925	0.935	0.93	0.905	0.93
48	0.935	0.89	0.9	0.935	0.93

Table D.4: *Statistical parameters used to calculate the experimental error for the saccharification step*

Sample	Final Glucose Concentration
1	0.935
2	0.89
3	0.9
4	0.935
5	0.93
Mean	0.918
Standard deviation	0.021
Confidence limit (95%)	0.049
Experimental error	5.36 %

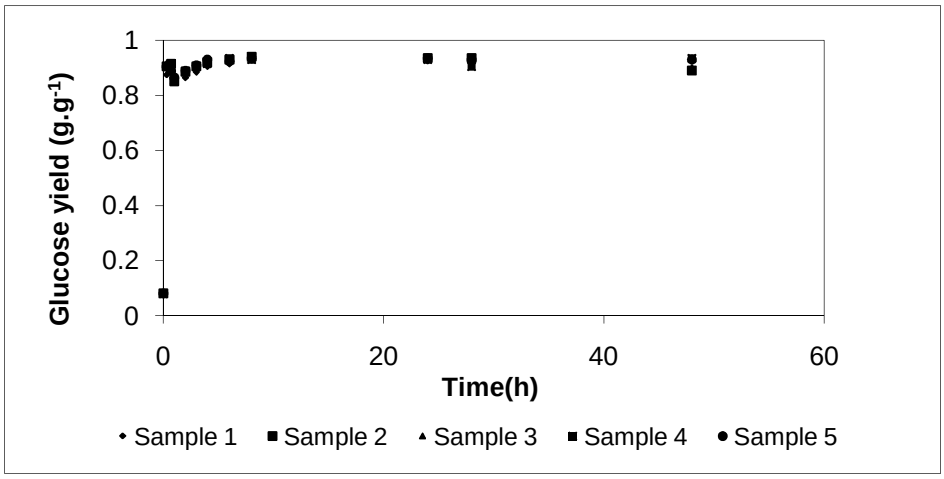


Figure D.2: *Five replicates of increasing glucose yield during saccharification in the SHF process*

D.3 Separate Hydrolysis and Fermentation

The experimental error for the fermentation step was determined by repeating the fermentation step using a yeast concentration of $8g.L^{-1}$ five times. The ethanol yields ($g.g^{-1}$) for each experiment at different time intervals are listed in Table D.5. The statistical parameters used to calculate the experimental error is listed in Table D.6. The ethanol yield for each experiment at each time interval for the five repeated fermentation experiments are graphically presented in Figure D.3.

Table D.5: *Ethanol yield ($g.g^{-1}$) at different time intervals for five repeated fermentation experiments*

Time (min)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
0	0.031	0.031	0.031	0.031	0.031
0.25	0.245	0.1765	0.1775	0.2	0.17
0.5	0.23	0.195	0.195	0.2	0.175
1	0.19	0.19	0.19	0.18	0.19
2	0.215	0.35	0.255	0.295	0.32
3	0.275	0.3	0.27	0.26	0.275
24	0.365	0.38	0.385	0.385	0.375
48	0.38	0.395	0.39	0.39	0.39
72	0.375	0.39	0.395	0.395	0.375
96	0.38	0.395	0.405	0.4	0.395
120	0.385	0.375	0.37	0.365	0.38

Table D.6: *Statistical parameters used to calculate the experimental error for the liquefaction step*

Sample	Final ethanol yield
1	0.385
2	0.375
3	0.37
4	0.365
5	0.38
Mean	0.375
Standard deviation	0.008
Confidence limit (95%)	0.018
Experimental error	4.85 %

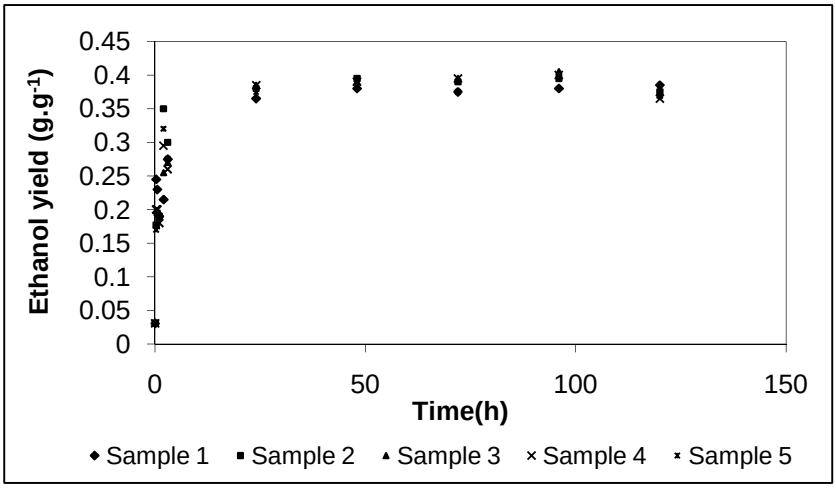


Figure D.3: *Five replicates of ethanol production in shake flasks by Saccharomyces cerevisiae using the SHF process graph of fermentation samples*