PRODUCTION AND CHARACTERIZATION OF BIOETHANOL DERIVED FROM CASHEW APPLE JUICE FOR USE IN INTERNAL COMBUSTION ENGINE

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DECLARATION

I declare that this thesis is my own unaided work and sources that I have used or quoted have been indicated and acknowledged by means of complete references. It is being submitted to the degree of Doctor of Philosophy in Engineering to the University of the Witwatersrand, Johannesburg. This thesis has not been submitted before for any degree or examination to any other University.

(Signature of Candidate)

_____ day of _____ year _____

FACULTY OF ENGINEERING AND THE BUILT ENVIRONMENT.

UNIVERSITY OF THE WITWATERSRAND.

DOCTOR OF PHILOSOPHY IN ENGINEERING (PhD. Eng).

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ABSTRACT

Progressive global dilemmas such as greenhouse gas emissions and depleting crude oil supply have sparked an exponential interest in the use of bioethanol in the motor-fuel industry. To date, bioethanol production from cereal grains and lignocellulose feedstock has been extensively researched but the use of agro-industrial waste products for bioethanol production also has potential. One such product is cashew apples. Cashew apples are regarded as waste after cashew nut harvesting and are an attractive feedstock for bioethanol due to the lack of commercial usage. The present work, therefore aimed to investigate the characteristics of cashew apple juice extracted from cashew apples grown in South Africa and to evaluate the concentration of bioethanol obtainable from cashew apple juice by fermentation using *Saccharomyces cerevisiae* yeast strains NRRL Y2084 and Vin13. Furthermore, this research aimed to show the compatibility of ethanol/petrol blends and determine the change in fuel level of a generator fuelled with ethanol/petrol blends. During the February harvest season, a mixed variety of cashew apples collected from the

KwaNgwanase region revealed the following characteristics: specific gravity of 1.050 and pH of 4.52 by direct measurement; total sugars: 100g/L by HPLC; total minerals: 15.89ppm by AAS; condensed tannins: 55.34mg/L by the Vanillin-HCl assay; proteins: 1.78g/L by the Coomassie Blue assay; Vitamin C: 112mg/100mL by the Iodine Titration. Post characterization, the cashew apple juice was fermented in a BIOSTAT® Bplus fermentor. The fermentation conditions were as follows: pH=4.50, agitation=150rpm, temperature=30ºC (Y2084) and 20ºC (Vin13), oxygen saturation=0% or 50%. During fermentation samples were analysed for sugar consumption, ethanol production and glycerol production by HPLC and yeast viability by plate counts. HPLC revealed that glucose and fructose are the predominant sugars of CAJ and utilized by both strains during fermentation. The maximum ethanol concentration achieved by NRRL Y2084 was 65.00g/L and oxygen saturation affected the fermentation time. The maximum ethanol concentration achieved by Vin13 was 68.00g/L at 50% oxygen, whilst at 0% oxygen; 31.00g/L of ethanol. Both yeast strains produced a higher glycerol concentration at 0% oxygen and yeast viability counts were higher at 50% oxygen. Post fermentation, the product was distilled by simple distillation using a rotary evaporator and 75% of bioethanol was recovered. For the blending experiments, concentrations of E4.4, E5 and E10 ethanol/petrol were prepared using absolute ethanol and SASOL 95 unleaded petrol grade. The blends were analysed by NMR Spectroscopy using the Bruker Ultrashield™ 500 PLUS to determine the ^{1}H and ^{13}C chemical pattern of ethanol/petrol blends. Qualitative analyses revealed ethanol/petrol blends possess structural compounds consistent with neat petrol and ethanol. These blends were used

to fuel a spark ignition generator coupled with a graduated dipstick to monitor the fuel level. From the results, the fuel consumption using neat petrol and the E5 blend were similar. For the E10 blend fuel consumption was slower, whereas when the generator was run using the E4.4 blend, the engine abruptly switched off without complete consumption of the fuel. The fermentation experiments performed under defined conditions in a controlled system showed the suitability of cashew apple juice as a substrate for bioethanol production in conjunction with *S. cerevisiae* yeast strains. Ethanol concentration ranged from 31.00g/L to 68.00g/L, with a higher concentration and shorter fermentation time achieved during aerobic fermentation. From the blending of ethanol with commercial petrol, qualitative analysis confirmed the structure of each individual component is unchanged in a blend and the addition of ethanol to a specific grade of petrol is compatible. Generator analysis revealed the E10 blend decreased the fuel consumption, thus the addition of ethanol to fuel is advantageous.

DEDICATION

My late grandparents **Deenanath Ramnarian, Harriam Jeebodh & Jayanti Jeebodh**, *my late uncle* **Bhimraj Jeebodh** for spiritual wisdom

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MI:	Million litres
GHG:	Greenhouse gas
US:	United States
CA/CAs:	Cashew apple/Cashew apples
CAJ:	Cashew apple juice
ICE/ICEs:	Internal combustion engine/Internal combustion engines
PAHs:	Polynuclear aromatic hydrocarbons
GC/MS:	Gas Chromatography/Mass Spectrometry
MTBE:	Methyl tertiary-butyl ether
MO/MOs:	Microorganism/Microorganisms
S. :	*Saccharomyces*
EMP:	Embden-Meyerhof-Parnas
Z. :	*Zymomonas*
SSF:	Simultaneous saccharification and fermentation
SHF:	Separate hydrolysis and fermentation
GI:	Billion litres
EU:	European Union
SSA:	Sub-Saharan Africa
ACT:	African Conservation Tillage
CNSL:	Cashew nut shell liquid
LAB:	Lactic Acid Bacteria
CAB:	Cashew apple bagasse
RVP:	Reid vapour pressure
CO:	Carbon monoxide
HPLC:	High Performance Liquid Chromatography
RID:	Refractive index detector

AAS:	Atomic Absorption Spectroscopy
BSA:	Bovine Serum Albumin
BPW:	Buffered Peptone Water
YPD:	Yeast Peptone Dextrose
MEA:	Malt Extract Agar
DCU:	Digital control unit
MFC:	Mass Flow Controller
dH_2O:	Distilled water
HCl:	Hydrochloric acid
NaOH:	Sodium hydroxide
O_2:	Oxygen
CO_2:	Carbon dioxide
CFU/mL	Colony forming units per mL
TOC:	Total organic carbon
Ey:	Ethanol yield
Ep:	Ethanol productivity
NMR:	Nuclear Magnetic Resonance
$CDCl_3$:	Deuterated chloroform
TMS:	Tetramethylsilane
^1H:	Proton spectrum
^{13}C:	Carbon spectrum

Chapter 1

Introduction

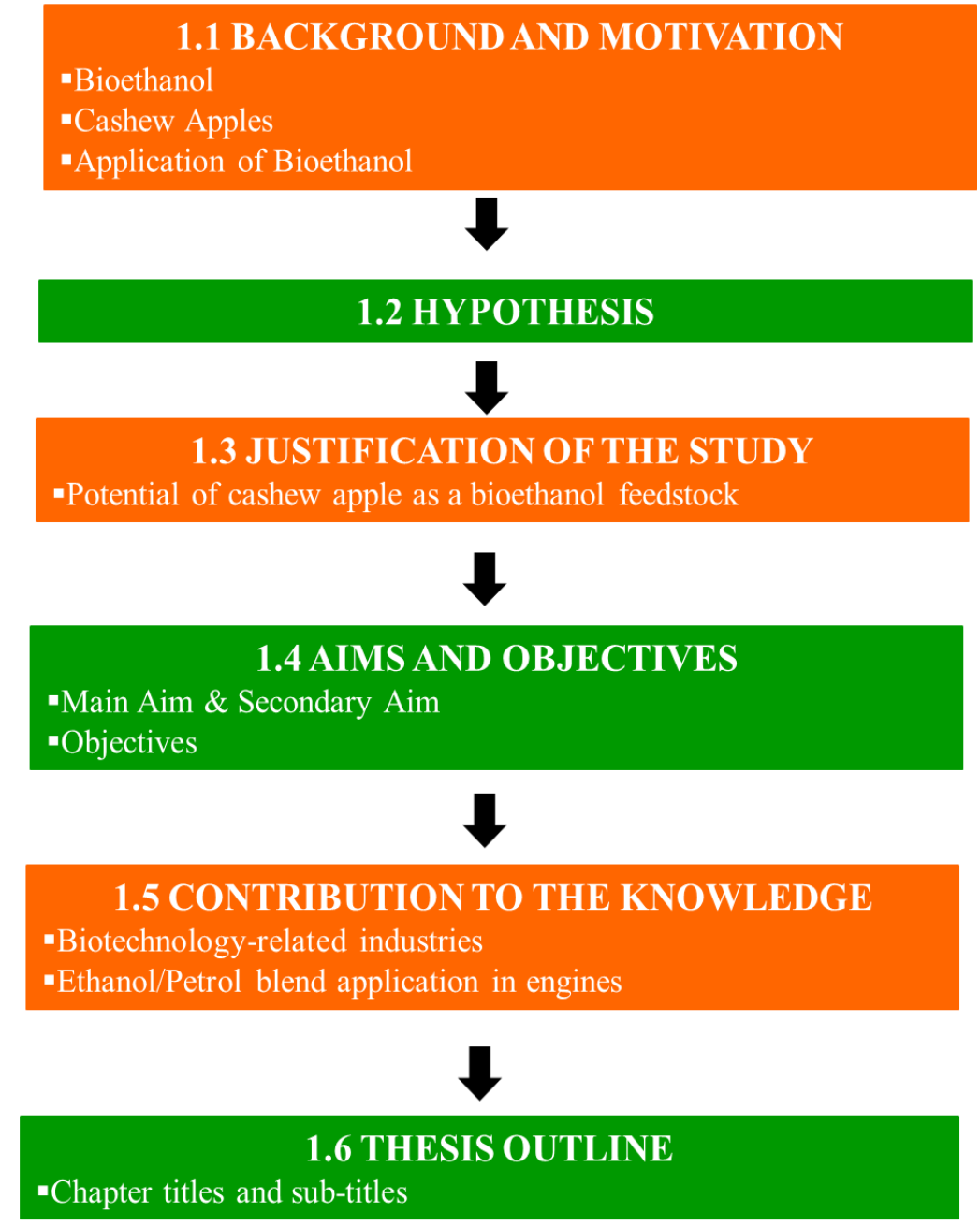


Figure 1.1 A diagram representing the outline of Chapter 1.

1.1 Background and Motivation

It is believed that ethanol production for biofuels began during the 1970s-1980s, however during this period bioethanol production and its usage as a fuel re-entered the industry and product capacity increased. During 1902, in Paris the application of alcohols as fuel to power farm machinery, stoves, heaters and spirit lamps was introduced (Rosillo-Calle and Walter, 2006). In Germany, when the production of stoves and spirit lamps increased, the production of ethanol increased from 38 million litres (MI) to 98.5MI (Rosillo-Calle and Walter, 2006; Mussatto et al., 2010). This paved the way for the use of ethanol in motor vehicles and in the year 1908, another milestone was reached when Henry Ford built the Quadricycle, also known as the Model-T which was run on ethanol (Rosillo-Calle and Walter, 2006; Mussatto et al., 2010). Henry Ford also stated that ethanol would be "the fuel of the future" (Chandel et al., 2007) and "the fuel of the future is going to come from apples, weeds, sawdust-almost anything" (Chandel et al., 2007). Since the 1900s and more importantly in present day, this statement is in the limelight towards alternate energy and sustaining the environment. Furthermore, the interest in bioethanol for fuel purposes was provoked by two additional events, the oil crisis during 1973-1974 and the Kyoto Protocol (Jankowski and Sandel, 2003; Alpanda and Peralta-Alva, 2010; Solomon and Krishna, 2011). The oil crisis of the Organization of Petroleum Exporting Countries embargo during the 1970s caused increasing energy prices (Alpanda and Peralta-Alva, 2010) and this called for developing ideas towards energy saving. The Kyoto Protocol was directed towards climate change with an emphasis on strategies for the reduction in carbon dioxide and other greenhouse

gas (GHG) emissions, as a 70% rise in GHG emissions was reported between the years 1970 to 2004 (Jankowski and Sandel, 2003; Koh and Ghazoul, 2008; Solomon and Krishna, 2011). In the United States (US) bioethanol production dates back to the 1980s and as early as 1975 in Brazil (Balat and Balat, 2009). During 2004 Brazil was the leading bioethanol producer and by the year 2006, the US became the dominant producer (Table 1.1). Other countries contributing to bioethanol production is shown in Table 1.1. In Africa, the production of biofuels is regarded as an "unexploited resource" (Amigun et al., 2008) mainly due to: inadequate economic and political management; underdeveloped infrastructure for commercial energy production; and the use of biomass as important food sources (Amigun et al., 2008). Sub-Saharan regions of Africa such as Malawi, Swaziland, Zimbabwe and South Africa have taken the initiate to contribute to world biofuel production by developing small-scale bioethanol plants (Balat et al., 2008). South Africa is ranked as the seventh leading bioethanol producer worldwide, with 102 million gallons produced in 2006 (Table 1.1). In Zimbabwe, the Triangle Ethanol Plant produced approximately 120,000 litres of bioethanol per day. However, the lack of government support resulted in closing down the plant between 1994-1995 (Amigun et al., 2008; Balat et al., 2008). Hence it is clear that the potential of bioethanol was not fully recognized.

Table 1.1 Bioethanol producing countries and amount of bioethanol produced in million gallons (*Source:* Balat, 2009).

Country	2004	2005	2006
Brazil	3,989	4,227	4,491
USA	3,535	4,264	4,855
China	964	1,004	1,017
India	462	449	502
France	219	240	251
Russia	198	198	171
South Africa	110	103	102
UK	106	92	74
Saudi Arabia	79	32	52
Spain	79	93	122
Thailand	74	79	93
Germany	71	114	202
Ukraine	66	65	71
Canada	61	61	153
Poland	53	58	66
Indonesia	44	45	45
Argentina	42	44	45
Italy	40	40	43
Australia	33	33	39
Rest of the World Total	**545**	**909**	**477**
World Total	**10,770**	**12,150**	**13,489**

With recent global dilemmas such as increased GHG emissions, decrease in the availability of crude oil and rising fuel prices (Chapagain et al., 2009; Girard and Fallot, 2006) the need for bioethanol and biodiesel has generated renewed attention to ease the reduction of non-renewable energy resources and to further prevent global warming. The major contributor to the level of pollution is motor vehicles, emitting approximately 70% of carbon monoxide and 19% of carbon dioxide globally (Balat and Balat, 2009). In light of this information, motor-fuel industries can use bioethanol as a fossil fuel substitute or additive in an attempt to reduce harmful emissions (Suresh et al., 1999; Amigun et al., 2008). The use of bioethanol is advantageous as: the reduction of combustion emissions is possible; it is biodegradable; and accessible from renewable resources (Balat et al., 2008; Demirbas, 2008). Agricultural feedstocks, namely wheat; barley; sorghum; rice; corn and sugar cane have been proven successful for the production of bioethanol. The preferred grain of choice for bioethanol production in the US is corn, whilst in Brazil sugar cane is extensively used as a feedstock (Kim and Dale, 2004; Balat et al., 2008; Linde et al., 2008; Balat and Balat, 2009). However, the use of cereal grains as a mainstream feedstock for bioethanol production is limited due to concerns regarding the availability of the grains as it is important for food and feed (Sun and Cheng, 2002; Kim and Dale, 2004). Agricultural wastes or residues such as barley straw, barley husks, corn stover, sugar cane bagasse and switch grass are attractive starting material that can be utilized for bioethanol production (Kim and Dale, 2004; Linde et al., 2006; Linde et al., 2007; Rosgaard et al., 2007; Najafi et al., 2009). In addition to the above mentioned material, cashew apple (CA) as a bioethanol substrate is an alternative agro-industrial waste that can be

used (Rocha et al., 2006; Honorato et al., 2007; Luz et al., 2008; Honorato and Rodrigues, 2010). CA producing regions are Brazil, India, Mozambique, Nigeria, Guinea-Bissau, Indonesia, Tanzania and Vietnam (Honorato et al., 2007; Luz et al., 2008). Brazil is the leading CA producer, with approximately 2 million tons of cashew apples (CAs) available annually (Honorato et al., 2007). However, 90-94% of the apples are regarded as agricultural waste due to decomposition in the soil and lack of utilization to produce relevant by-products which in turn do not contribute to any economic benefits (Santos et al., 2007; Luz et al., 2008; Pinheiro et al., 2008; Honorato and Rodrigues, 2010). The 10% of CA that is used to produce wine or cashew apple juice (CAJ) is consumed locally and exports of the apple products are stagnant (Rakopoulos et al., 2006; Luz et al., 2008). Biotechnological applications of CAJ to date (Akinwale, 2000; Rocha et al., 2006; Trevisan et al., 2006; Chagas et al., 2007; Rabelo et al., 2009; Honorato and Rodrigues, 2010) include: (i) the production of dextransucrase as a preservative in the food industry, (ii) antioxidant ability by characterizing alkyl phenols, (iii) comparison of nutritional quality with consumable fruits and (iv) production of biosurfactant for the use in oil/water systems. Commercial uses of CAJ involve the production of wine, jam, fruit juice and ice-cream (Osho, 2005; Honorato and Rodrigues, 2010). In relation to bioethanol, research has shown that ethanol yields ranging from 19.82-44.40g/L are achievable from CAJ (Pinheiro et al., 2008; Pacheco et al., 2010). In a study by Neelakandan and Usharani. (2009), ethanol yields of 6.91%, 7.64%, 8.53%, 8.80% and 9.35% (v/v) were possible from fermenting CAJ substrate using immobilized yeast technology. The ethanol concentrations were dependent on various experimental parameters such as

substrate concentration, pH, temperature, yeast cell concentration and fermentation time. Hence, cashew apples (CAs) as an agricultural waste can be exploited for the production of bioethanol as it offers great potential, for example: (i) abundance of reducing sugars (50-200g/L) for yeast assimilation, (ii) rich in organic acids, minerals, and amino acids necessary for yeast growth, (iii) cheap source of sugars and (iv) readily available in large quantities as cashew apples are neglected in their cultivation areas after harvesting of the cashew nut (Rocha et al., 2006; Honorato et al., 2007; Luz et al., 2008; Honorato and Rodrigues, 2010; Silveira et al., 2010).

One of the greatest challenges in current times is the decline in resources of fossil fuels, exponential rise in GHG emissions contributing to global warming and the inability to meet the increasing energy demands. A possible solution to these problems is the use of renewable resources in the production of bioethanol. Currently, on a global scale, there are 700 million operational road vehicles and this is expected to increase to 2 billion by the year 2050 (Balat and Balat, 2009). It has also been reported by the year 2050 global oil production will decrease to 5 billion barrels per annum from a current production of 25 billion barrels (Sun and Cheng, 2002). The reserves of coal, crude oil and natural gas might not be able to keep up with the supply and demand capacity. For this reason, there is an escalating interest in bioethanol as an alternative to petroleum as the abundance of crude oil decreases and as an additive to petroleum to reduce harmful environmental pollutants and hence motivation for this investigation. If bioethanol can be produced and commercialized on a larger, recognizable scale in Africa, then it can contribute as a fuel additive and in turn reduce negative environmental

impacts and offer economic benefits with the future potential is replace petrol for the running of motor vehicles. Recently, the attention has been drawn towards the use of CAs for biosurfactant, lactic acid and dextransucrase production (Rocha et al., 2006; Honorato and Rodrigues, 2010; Silveira et al., 2010). Studies regarding the full potential of CAs as a biofuel substrate are important. In addition, the application of CAs will be useful as they are currently regarded are economically unimportant. Another aspect for the commercialization of bioethanol as a transportation fuel is for the application in internal combustion engines (ICEs). Since the need for biofuels has grown, in an attempt to sustain the environment the application of this technology is also important. Spark ignition and compression ignition engines are types of ICEs (Balat, 2005; Barabas et al., 2010) and bioethanol is a "spark ignition engine fuel" (Rakopoulos et al., 2006). The attractive feature of bioethanol as a fuel substitute or additive in ICEs is chemically, it is an "oxygenated enhancer" because bioethanol has a 35% oxygen content, which petrol lacks and thus combustion efficiency is increased with reduced hydrocarbon and carbon monoxide emissions (Balat et al., 2008; Barabas et al., 2010). Other features of bioethanol for ICEs include possible replacement of toxic octane additives such as benzene, toluene and xylene (Balat, 2009) and the high octane number allows for petrol blends with spontaneous ignition in ICEs (Balat et al., 2008). In a study by Poulopoulos et al. (2001), a fuel blend of E10 bioethanol in a 4-cylinder OPEL 1.6L engine showed an increase in vapour pressure and a decrease in carbon monoxide, benzene and toluene emissions. In a related study (Ameri et al., 2008), using the E20 bioethanol/petrol blend, there was an increase in vapour pressure with an increase in flow availability and a

decrease in the amount of carbon monoxide emitted. These studies (Poulopoulos et al., 2001; Ameri et al., 2008) support the relevance of blending bioethanol with petrol in ICEs, as it shows that improved engine performance and reduction of hazardous environmental emissions are possible. In ICEs, bioethanol/petrol blends range from E5-E20; this contains 5%-20% of bioethanol with 95%-80% of petrol. The E5-E20 blend can be used with no modification of the current engines (Demirbas, 2008) and thus suitable for immediate use. The advantage of petrol blends with bioethanol is, it allows for a slower, complete combustion because of the higher octane number and this reduces toxic emissions as the fuel blend is oxygenated (Balat et al., 2008; Demirbas, 2008). Information on possible emission reductions of blends is provided in Table 1.2.

Table 1.2 Emission changes of bioethanol-petrol blends (*Source:* Balat, 2009).

Emission	Low-level blends (eg., E10)	High-level blends (eg., E85)
Carbon monoxide	25-30% decrease	25-30% decrease
Carbon dioxide	10% decrease	Up to 100% decrease
Nitrogen oxides	5% increase/decrease	Up to 20% decrease
Volatile organic compounds	7% decrease No change (In Canada)	30% or more decrease Decrease
Sulphur dioxide and particulate matter	Decrease	Significant decrease
Aldehydes	30-50% decrease (but negligible due to catalytic converter)	Insufficient data
Aromatics	Decrease	More than 50% decrease

In this study, fuelling of an IG2600 petrol generator with ethanol/petrol blends will assist in interpreting the fuel consumption of an engine run using specific ethanol/petrol blend concentrations as very few studies show the potential for the application of blends.

Furthermore, for the purpose of this investigation bioethanol produced from CAJ will be characterized by chemical fingerprinting. Chemical fingerprinting is an analytical method used to determine compounds that are unique to chemical samples (Boehm et al., 1997). By definition, chemical fingerprinting determines hydrocarbon compounds that are unique to chemical samples such as crude oil and petroleum (Boehm et al., 1997). The use of chemical fingerprinting dates back to 1960, where the focus was on organic geochemistry of fossil fuels and later progressed to hydrocarbon chemistry of petrol (Boehm et al., 1997). Chemical fingerprinting is an approach that is extensively applied in oil spill investigations (Boehm et al., 1997; Barakat et al., 2002; Alzaga et al., 2004). The analysis of oil spills based on fingerprinting provides an understanding of: (i) environmental impacts by the release of toxic compounds, (ii) monitoring of changes in the chemical composition and (iii) to distinguish petrol polynuclear aromatic hydrocarbons (PAHs) and combustible PAHs (Boehm et al., 1997; Alzaga et al., 2004). Barakat et al. (2002) reported the use of Gas Chromatography/Mass Spectrometry (GC/MS) to chemically fingerprint hydrocarbons specific to an oil spill in the Gulf of Suez, Egypt. In this study, compounds from the oil spill were identified as *n*-alkanes PAHs and alkyl PAHs, using petrol from the source of spillage (tankers or pipelines) as a reference in the identification (Barakat et al., 2002). Similar findings were reported for the *Exxon*

Valdez oil spill in 1989 (Boehm et al., 1997) and the Galician coast oil spill in Spain (Alzaga et al., 2004). Other applications of chemical fingerprinting, using GC/MS include: characterizing tar or petrol of polluted soil; analysing the difference between evaporated and unevaporated petrol; quality control of medicinal herbs and comparing metabolic alterations of transgenic crops (Havenga and Rohwer, 1999; Noteborn et al., 2000; Sandercock and du Pasquier, 2003;2004; Eide and Zahlsen, 2005). There are very few investigations which report on the fingerprinting of bioethanol/petrol blends. For this study, chemical fingerprinting is relevant to identify a pattern or signature of bioethanol/petrol blends that has the desired properties to run in ICEs.

This study will contribute knowledge to Bio-Industries in Africa and worldwide regarding the use of cashew apples for bioethanol production, based on chemical fingerprinting and the use of blends in an ICE, as a positive indicator that the end-product is satisfactory as a fuel additive.

1.2 Hypothesis

Cashew apple juice is able to provide essential monosaccharide sugars and nutrients for yeast growth and fermentation. These available compounds are sufficient for bioethanol production by fermentation using *Saccharomyces cerevisiae* yeasts under controlled parameters in a BIOSTAT® Bplus system.

1.3 Justification of the Study

To date, studies have been reported on the use of CAJ for bioethanol production and other biotechnological applications (Akinwale, 2000; Rocha et al., 2006; Trevisan et al., 2006; Chagas et al., 2007; Rabelo et al., 2009; Honorato and Rodrigues, 2010). However, the production of bioethanol from CAJ extracted from CAs growing in Southern Africa has not been reported. Additionally, the potential of the BIOSTAT® Bplus fermentor as a small-scale production plant, specific to bioethanol production has not been documented.

Biofuels in general, is a worldwide phenomenon that is considered as a replacement to methyl tertiary-butyl ether (MTBE) and other petrol additives as an oxygenate in ICEs. In Sub-Saharan Africa there are several hurdles with regards to biofuels (Deenanath et al., 2012). From the review by Deenanath et al. (2012) the use of cereal grains is one major hindrance towards sustainable bioethanol production. A resource that can be utilized to avoid this situation is juice extracted from CAs as a fermentation sugar medium. If the fermentation of CAJ sugars produces a satisfactory yield of bioethanol, then the implementation of large-scale production is an option for Bio-Industries. Bio-Industries are involved in environmental and economic benefits by applying microorganisms (MOs) or biocatalysts to convert biomass resources into bioenergy or chemical products (Hall and Crowther, 1998; Otero et al., 2007; Prasad et al., 2007) and the scope of this research will be of interest to this type of industry. The use of CAJ as a reliable feedstock for bioethanol production will bring about future economical boosts for the cashew industry in South Africa and assist in improving farming

lands in cashew growth regions and could even contribute to the net productivity of bioethanol. Furthermore, the characterization of ethanol/petrol blends and the application in a generator-type engine is indicative that bioethanol as a petrol additive is suitable and therefore the information from this research will contribute towards current, available research in the field of biofuel applications.

1.4 Aims and Objectives

The main aim of this research is to investigate bioethanol production from cashew apple juice substrate, using two types of *Saccharomyces (S.) cerevisiae* yeast strains to obtain maximum ethanol by fermentation carried out in a BIOSTAT® Bplus system. The secondary aim is to blend ethanol with conventional petrol to evaluate the compatibility of the blends and apply these blends to a generator and evaluate the effect on the fuel consumption.

The aims will be achieved by the following objectives:

(i) To physically characterize cashew apples collected from the KwaNgwanase region.

(ii) To extract and characterize cashew apple juice for physical and chemical constituents.

(iii) To ferment cashew apple juice with the use of *S. cerevisiae* strain NRRL Y2084 and Vin13 to produce bioethanol and evaluate the ethanol concentration, sugar consumption, glycerol concentration and yeast viability counts using instrumentation analysis.

(iv) To monitor carbon dioxide and oxygen gas signals and *real time* fermentation parameters during the process by instrumentation analysis.

(v) To purify bioethanol by distillation.

(vi) To evaluate the chemical compatibility of ethanol/petrol blends at various blend concentrations.

(vii) To test the blends in a petrol generator for fuel consumption.

1.5 Contribution to the Knowledge

This report will contribute knowledge towards: (1) Biotechnology-related industries in Africa and worldwide with regards to cashew apple juice as a promising substrate and help direct towards building a stronger foundation for sustained bioethanol production and (2) The application of blends in relation to an engine is to further prove that ethanol can be worthwhile as a fuel additive.

1.6 Thesis Outline

CHAPTER 1:

Chapter 1 is a general introduction to the research and is sub-divided into: Background and Motivation, Hypothesis, Justification of the Study, Scope of the Study, Aims and Objectives, Contribution to the Knowledge and Thesis Outline.

CHAPTER 2:

Chapter 2 is entitled: Literature Review. The Literature Review provides background knowledge on bioethanol, the cashew fruit, the cashew industry and applications of bioethanol. The chapter is sub-divided into: Overview of

Bioethanol, Production Methods for Bioethanol, Bioethanol Perspectives, The Cashew and The Application of Bioethanol.

CHAPTER 3:

Chapter 3 is entitled: Methodology. The Methodology provides in-depth details of the experimental procedures. This chapter is sub-divided into: Raw Materials, Cashew Apple Juice Extraction, Characterization and Preparation, Yeast Inoculum Preparation, Fermentation of Cashew Apple Juice, Analytical Methods, Distillation and Ethanol/Petrol Blends.

CHAPTER 4:

Chapter 4 is entitled: Results and Discussion. The Results are represented by pictures, figures and tables. These are then interpreted and the Discussion is made with comparisons to previous research studies. This chapter is sub-divided into: Raw Materials, Cashew Apple Juice Extraction, Characterization and Preparation, Yeast Inoculum Preparation, Fermentation of Cashew Apple Juice, Distillation and Ethanol/Petrol Blends.

CHAPTER 5:

Chapter 5 is entitled: Summary of the Findings, Concluding Remarks and Future Recommendations. This chapter provides a summary of the main findings, overall conclusion drawn from the results and recommendations that can contribute to future work. Additionally, limitations experienced during the research are provided.

Chapter 2

Literature Review

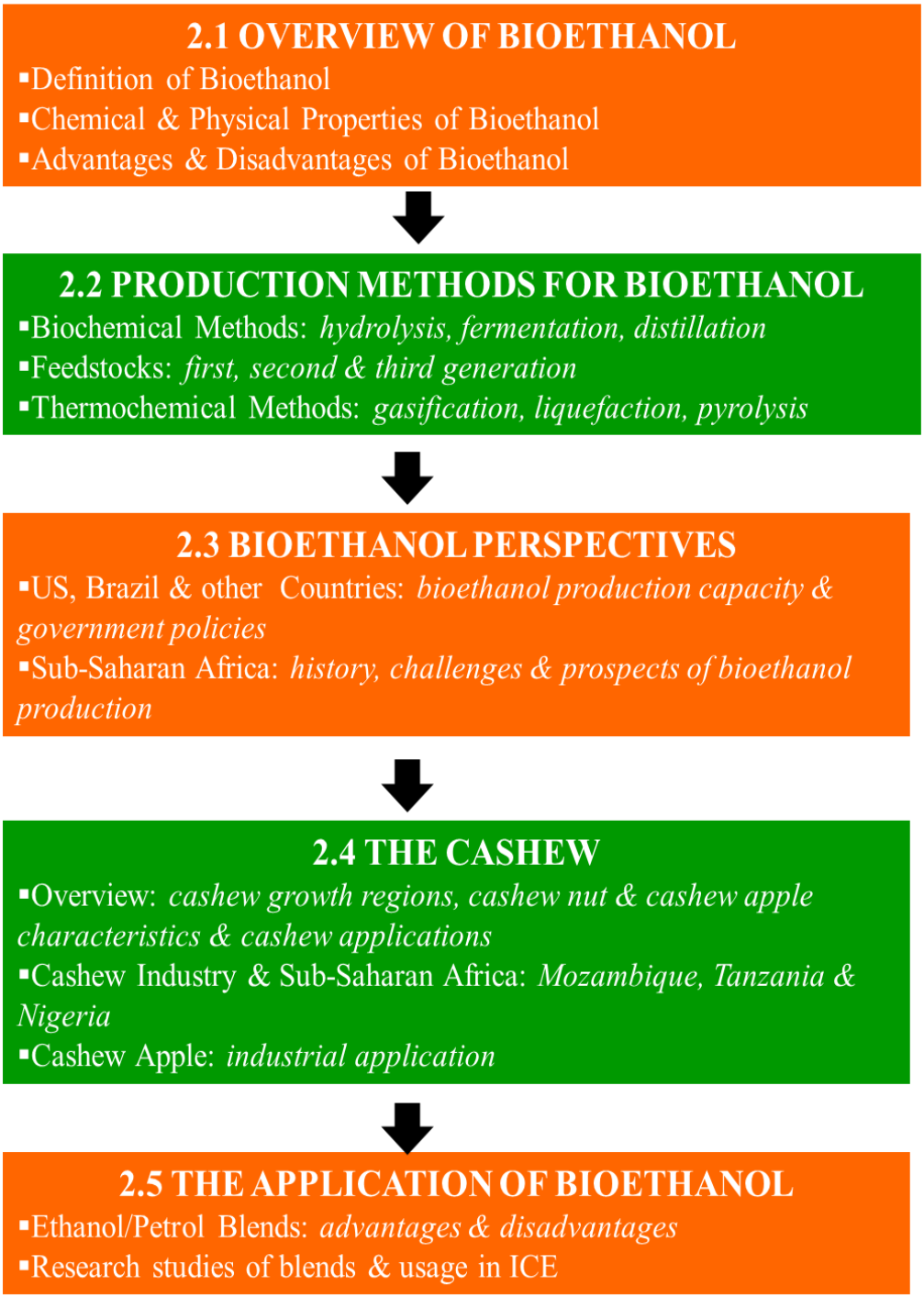


Figure 2.1 A diagram representing the outline of Chapter 2.

The work presented in this chapter is from the article entitled: "The Bioethanol Industry in Sub-Saharan Africa: History, Challenges, and Prospects" published in the journal, *Journal of Biomedicine and Biotechnology*. Information extracted from the published work is cited as the authors and paraphrased. The contributions by this author were concept generation, structure of the article and writing of the article. The copyrights for the use of the published work were acquired (Appendix E).

2.1 Overview of Bioethanol

The term bioethanol is defined as an ethyl alcohol or ethanol (CH_3-CH_2-OH) produced via biological processes (Balat at al., 2008; Demirbas, 2008). Biological processing is the conversion of biomass into "bio"-ethanol by biochemical processes such as hydrolysis and fermentation (Balat et al., 2008; Demirbas, 2008; Walker, 2011). The main application of biologically derived ethanol is in the motor-fuel industry as an additive in conventional petrol or petrol substitute in internal combustion engines (ICEs) (Suresh et al., 1999; Amigun et al., 2008; Demirbas, 2008). As an additive, bioethanol is mixed or blended with conventional petrol at a volume of 5% to 85% (Demirbas, 2008; Walter et al., 2008; Mussatto et al., 2010). The general physical and chemical properties of bioethanol are listed in Table 2.1, followed by Table 2.2 which shows bioethanol properties compared to traditional fuel properties. The properties listed provide insight as to why bioethanol is suitable as a transport fuel.

Table 2.1 Physical and chemical properties of bioethanol (*Source:* Walker, 2011).

Parameter	Characteristic Properties
Molecular Formula	C_2H_5OH
Molecular Mass	46.07g/mol
Appearance	Colourless liquid
Water Solubility	∞ (miscible)
Density	0.789kg/L
Boiling temperature	78.5ºC
Freezing point	-117ºC
Flash point	128ºC
Ignition temperature	425ºC
Explosion limits	Lower 3.5% (v/v) Upper 19% (v/v)
Vapour pressure at 38ºC	50mmHg
Higher heating value (at 20ºC)	29,800kJ/kg
Lower heating value (at 20ºC)	21.090kJ/kg
Specific heat	Kcal/Kg 60ºC
Acidity (pKa)	15.9
Viscosity	1.200mPa.s (20ºC)
Refractive index	1.36 (25ºC)

Table 2.2 Properties of bioethanol as a transport fuel (*Source:* Deenanath et al., 2012).

Desired fuel property	Traditional fuel property	Bioethanol-fuel property	Reference
High octane number	88	107	Balat et al. (2008), Walker. (2011)
High oxygen content	2.7%(w/w)	35% (w/w)	Balat et al. (2008), de Menezes et al. (2006)
Low energy content	31.3MJ/dm3	21.2MJ/dm3	Jegannathan et al. (2009)
High Latent heat of vaporization	0.30MJ/kg	0.91MJ/kg	Balat et al. (2008), Turner et al. (2011)
Low heating value	43.0MJ/kg	26.7MJ/kg	Balat et al.(2008), Turner et al. (2011)

"Bioethanol as a transport fuel (Table 2.2) offer numerous advantages over traditional fuel such as: (i) pre-mature ignition and prevention of cylinder knocking due to the higher octane number and higher heat of vaporization compared to traditional fuel, (ii) reduction in hydrocarbon and carbon monoxide exhaust emission based on the higher oxygen content of bioethanol, (iii) in an internal combustion engine (ICE) the lower energy content of bioethanol allows for direct addition as a bioethanol/petrol blend as the compression ratio is higher and burn time is shorter, (iv) the blending or mixing of bioethanol with traditional fuel is compatible with current engine designs, and (v) bioethanol is chemically miscible in petrol" (Deenanath et al., 2012). "However, there are disadvantages associated with bioethanol. For example, combustion of bioethanol when blended with petrol releases formaldehyde and acetaldehyde, which is toxic compounds

and the lower vapour pressure makes cold starts of an engine difficult. Another major disadvantage is the fuel versus food security issue. The use of agricultural products such as cereal grains will limit food and feed reserves in developing countries, leading to possible food crisis" (Deenanath et al., 2012). "Globally the production of biofuels, which is mainly bioethanol and biodiesel combined, has increased from 4.8 billion gallons to 16.0 billion gallons between the years 2000 to 2007. Currently, Brazil and the United States (US) are the dominating bioethanol producers, contributing approximately 75-80% of the worlds bioethanol production. In Brazil, sugar cane is the preferred feedstock and is estimated to produce 37 billion litres by 2013. In the US, corn grain is the common feedstock used and accounts for 90% of bioethanol production. With 187 commercial bioethanol plants, the US aims to produce 57 billion litres by the year 2012 and 136 billion litres by the year 2022 from the use of additional feedstocks such as maize and sugar cane. During 2011, the European Union (EU) bioethanol production has increased to approximately 2.0 billion gallons. The EU is dependent on wheat and sugar beet as the sole bioethanol feedstocks" (Deenanath et al., 2012).

2.2 Production Methods for Bioethanol

2.2.1 Biochemical Methods

The production of bioethanol is a two stage biochemical procedure of hydrolysis and fermentation, followed by product recovery via distillation (Hahn-Hagerdal, 2006; Balat and Balat, 2009; Jegannathan et al., 2009).

Hydrolysis

Hydrolysis is defined as a chemical reaction that breaks chemical bonds between the starch molecules of polysaccharides into simple sugars or monomers in the presence of water and a catalyst (Thaker and Kastner, 2004; Chandel et al., 2007; Balat and Balat, 2009; Dwivedi et al., 2009, Guo et al., 2012). The catalysts used for hydrolysis are enzymes, metal salts, acids or bases (Mielenz, 2001; Thacker and Kastner, 2004; Guo et al., 2012). Enzymes and acids are the widely used catalysts for hydrolysis in the production of bioethanol from biomass feedstocks (Mielenz, 2001; Taherzadeh and Karimi, 2007). Enzymatic or acidic hydrolysis is via the external addition of commercial enzymes or acids or by native enzymes and acids present in the feedstock (Mielenz, 2001). The advantages of enzyme hydrolysis are that the method conditions are mild and higher substrate yields are possible. The disadvantages are commercial enzymes are expensive, method time is longer and inhibitory molecules can be formed (Taherzadeh and Karimi, 2007). The advantages of acid hydrolysis are the method time is shorter, inhibitory molecules are not produced and commercial acid reagents are cheaper and the disadvantages are substrate yields are lower and method conditions require intense high temperatures (Taherzadeh and Karimi, 2007). The choice of hydrolysis

method is dependent on the type of feedstock used for bioethanol production (described under the heading*: Feedstocks and Methods for Production*).

Fermentation

In ethanol production, the method of fermentation to convert sugars by microorganisms (MOs) is the oldest and frequently used industrial process (Caylak and Vardar Sukan, 1998; Paul Ross et al., 2002; Malherbe et al., 2007; Balat, 2009). As early as 1750-4000BC the Egyptians and Sumerians produced dough and alcoholic beverages such as wine and beer by fermentation (Paul Ross et al., 2002). The fermentation of milk, meat, cereals and vegetables for food products and food preservation dates back to 6000BC in the Middle East (Paul Ross et al., 2002; Blandino et al., 2003). During 6000BC, in Iraq it is believed that cheese was the first fermented product (Paul Ross et al., 2002, Blandino et al., 2003). However, the role of MOs in the fermentation process was unknown (Paul Ross et al., 2002; Blandino et al., 2003). Louis Pasteur in 1861AD developed pasteurization which paved the road to the knowledge of MOs and its link to fermentation gradually progressed (Paul Ross et al., 2002). Although the discovery of fermentation and MOs were milestones, the process or definition of fermentation was not fully understood. A better understanding evolved from three theories, namely Liebigs Chemical Theory; Pasteurs Vitalist Theory; and Berthelots Modified Chemical Theory (Temple, 1986). Liebigs Chemical Theory states: "fermentation is a chemical decomposition caused by putrefaction of animal or vegetable material" (Temple, 1986). Pasteurs Vitalist Theory states: "fermentations are caused by the life activity of specific yeasts" (Temple, 1986). Berthelots Modified Chemical Theory states: "the yeast secretes a chemical

substance which acts on and transforms the sugar, a substance which is very similar to those secretions to be found in parts of an animal body" (Temple, 1986). From these theories, the present day definition of fermentation is simply a chemical process that converts sugar into ethanol and carbon dioxide by the action of yeast enzymatic reactions (Balat, 2009; Walker, 2011).

Various fermentation processes such as alcoholic, lactic acid, acetic acid and alkali are used in the production of fermented food and beverages (Paul Ross et al., 2002; Blandino et al., 2003). Alcoholic fermentation is the production of alcohol by *Saccharomyces (S.) cerevisiae* yeasts using cereal grains or fruits as a substrate. The popular commercial products from alcoholic fermentation are wine and beer. Lactic acid fermentation is the use of milk as a substrate to produce cheese, yoghurt and kefir by a variety of MOs such as *Lactococcus lactis, Lactobacillus delbruckii, Lactobacillus bulgaricus* and *Lactobacillus kefir.* Acetic acid fermentation is the production of acetic acid from alcohol by Acetobacter bacteria. Alkali fermentation is the fermentation of a fish substrate by the MOs *Carnobacterium pisciola* or *Carnobacterium divergens* (Paul Ross et al., 2002; Blandino et al., 2003).

Alcoholic fermentation, which is applied in bioethanol production is further classified into three types of systems, namely batch, fed-batch and continuous (Caylak and Vardar Sukan, 1998; Chandel et al., 2007; Sanchez and Cardona, 2008). Batch fermentation is the pitching of MOs; particularly yeast into a highly concentrated substrate and results in a high ethanol production. Yeast is added at the start of fermentation, and post-fermentation the yeast is centrifuged to separate it from the product and re-used in subsequent fermentations (Caylak and Vardar

Sukan, 1998; Chandel et al., 2007; Sanchez and Cardona, 2008). During fed-batch fermentation, yeast is pitched into substrates with a low concentration and the substrate is fed into the fermenting vessel for yeast proliferation, resulting in a gradual increase of the ethanol concentration. The yeast is removed, separated and re-used (Caylak and Vardar Sukan, 1998; Chandel et al., 2007; Sanchez and Cardona, 2008). Continuous fermentation is the addition of yeast at high cell densities in stirred fermentation tanks with intermittent aeration and addition of fresh substrate with the removal of yeast biomass at equal rates of substrate addition and ethanol is produced rapidly (Caylak and Vardar Sukan, 1998; Chandel et al., 2007; Sanchez and Cardona, 2008). Each fermentation system has advantages and disadvantages associated the process, which are listed in Table 2.3. The choice of fermentation system depends on the type of raw material, desired ethanol yield and fermentation time (Caylak and Vardar Sukan, 1998; Chandel et al., 2007; Sanchez and Cardona, 2008).

Table 2.3 Advantages and disadvantages of fermentation systems.

Fermentation System	Advantages	Disadvantages	References
Batch	• Lower operation costs • Easier production control • Contamination is prevented • High ethanol productivity	• Higher labour costs • Lower ethanol productivity • Lengthy and intensive pre-fermentation preparations	Caylak and Vardar Sukan, 1998; Chandel et al., 2007
Fed-Batch	• Yeast viability is not reduced by substrate addition • Low starting concentration of substrate reduces the presence of inhibitory molecules • Higher ethanol productivity	• Additional equipment required • Higher operation costs • Contamination is likely during substrate addition	Caylak and Vardar Sukan, 1998; Chandel et al., 2007; Sanchez and Cardona, 2008
Continuous	• Yeast maintained in exponential phase • Automatic process control systems can be used • Higher ethanol productivity	• Additional equipment required • Higher operation costs • Contamination is likely during substrate addition • Ethanol productivity is affected if process parameters are not stable	Chandel et al., 2007; Sanchez and Cardona, 2008

The widely used microorganism (MO) for ethanol fermentation is the eukaryotic, fungal organism *S. cerevisiae* (Chandel et al., 2007; Asli, 2010; Sicard and Legra, 2011; Bourdichon et al., 2012). *S. cerevisiae* is the preferred MO for the following reasons: (i) easily assimilate and ferment hexose (C6) sugars to ethanol, (ii) tolerate high ethanol concentrations, (iii) proliferate under anaerobic, aerobic and acidic conditions, (iv) not susceptible to bacteriophage contamination, (v) can be easily separated from the fermented product and re-constituted in liquid broth culture for subsequent fermentations, and (vi) able to ferment at a range of temperatures from 15ºC to 30ºC and thermotolerant *S. cerevisiae* strains can ferment at 35ºC or greater (Chandel et al., 2007; van Maris et al., 2007; van Zyl et al., 2007; Edgardo et al., 2008; Sanchez and Cardona, 2008). In addition to *S. cerevisiae*, other Saccharomyces yeasts such as *S. bayanus, S. pastorianus, S. mikatae, S. cariocanus, S. paradoxus, S. kudriavzevii* and *S. arboricolus* are used in food and beverage fermentation (Sicard and Legras, 2011). Other than yeasts, bacteria such as Lactic Acid Bacteria, *Zymomonas mobilis* and *Thermoanaerobacterium* are capable of fermentation (Blandino et al., 2003; Rogers et al., 2007; Walker, 2011). For bioethanol production, *Zymomonas mobilis* is the sort after bacterial MO because like *S. cerevisiae*, this bacteria is not oxygen dependent; it can easily utilize hexose sugars and genetically engineered strains can easily utilize pentose (C5) sugars such as xylose and arabinose (Rogers et al., 2007; Walker, 2011).

During fermentation *S. cerevisiae* produces ethanol by the Embden-Meyerhof-Parnas (EMP) pathway or glycolysis as shown in Figure 2.2.

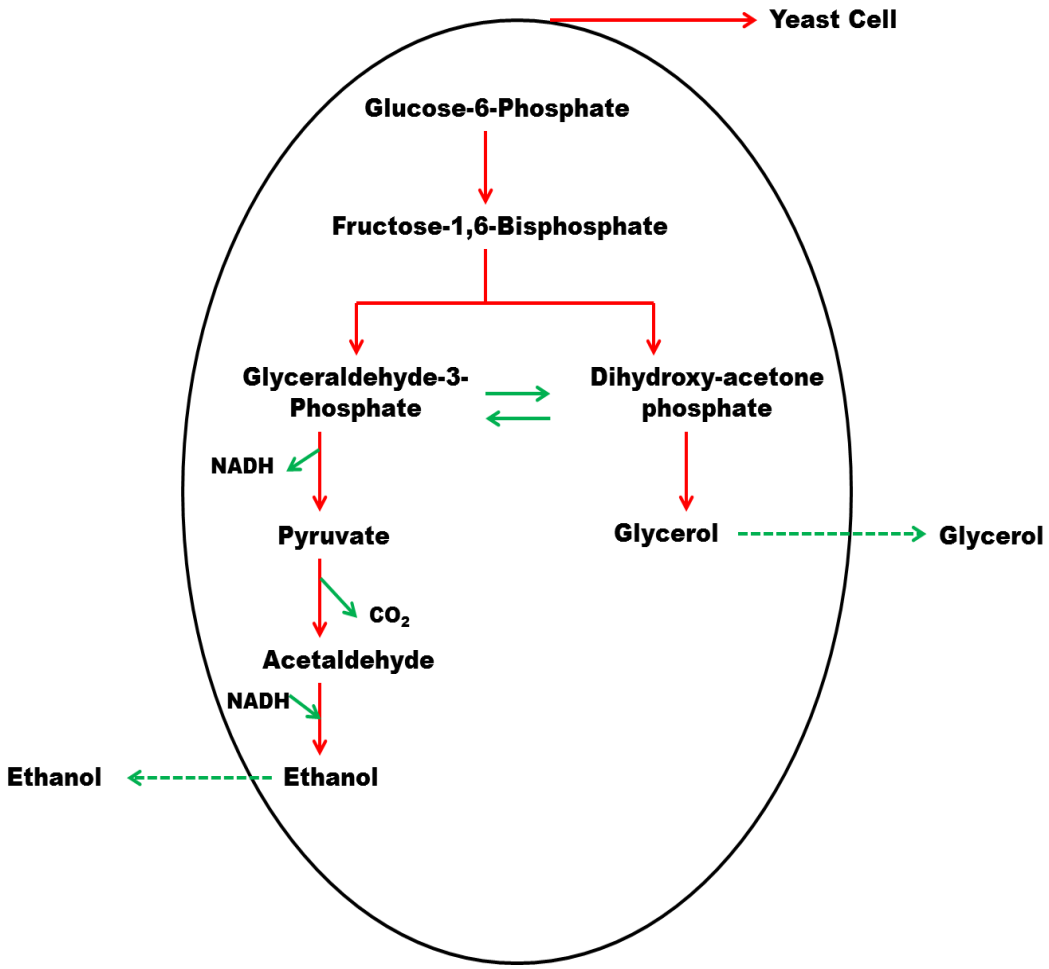


Figure 2.2 Schematic diagram of glycolysis carried out by yeasts during fermentation (*Modified from:* Piskur et al., 2006; Sicard and Legras, 2011).

From Figure 2.2, glucose from the substrate is sequestered by the yeast. Inside the cell (Fig 2.2) glucose is converted to pyruvate and NAD^+ is oxidized to NADH. Pyruvate is then converted to acetaldehyde by the enzyme pyruvate decarboxylase, followed by the conversion of acetaldehyde in to ethanol by alcohol dehydrogenase and NADH is reoxidized to NAD^+ (Fig 2.2) (Piskur et al., 2006; Walker, 2011; Sicard and Legras, 2011).

Distillation

The final step in the production of bioethanol of distillation, which is required to remove substances produced during fermentation in order to obtain a pure ethanol product that can be utilized further. By definition, distillation is a thermo-chemical separation process that concentrates ethanol to 93%-95% purity by the removal of water and other volatiles based on the difference in boiling points between substances when heat is applied (Rose et al., 1951; Ladisch and Dyck, 1979; Balat et al., 2008; Jegannathan et al., 2009). Previously distillation was used to produce fresh water from salt water and to produce wool mat from rosin oil distillation. The first industrial application of distillation to recover ethanol from beverages was between the eleventh to fourteenth centuries and to date is the most widely used industry ethanol recovery method (van Winkle, 1967). In general, distillation requires a still, condenser and collection flask. The heat is applied to the still, cooling water passes through the condenser and the distillate or condensate product drops into the collection flask (Rose et al., 1951). The well-known distillation techniques used are simple, steam or fractional distillation (Rose et al., 1951; Bremner and Keeney, 1965; Barbosa and Perkins, 1978; Barbosa and Doherty, 1988). Simple distillation is used for the separation of non-volatile and organic substances. The liquid is vaporized by heating and condensed by cooling. Distillate is collected and there is a change in the composition of the starting liquid (Rose et al., 1951; Barbosa and Perkins, 1978; Barbosa and Doherty, 1988). Steam distillation is used in the recovery of volatile fatty acids and as the Kjeldahl method for nitrogen content determination (Bremner and Keeney, 1965). For steam distillation, ammonia is first removed by passing steam

through the distillation apparatus. Distilled water is then heated, which supplies the steam required for distillation and the cooling water of the condenser allows the distillate to be collected (Bremner and Keeney, 1965). Fractional distillation is used for the separation of volatiles. In fractional distillation the condensing vapours from the initial liquid sample are separated into fractions of different boiling points. These fractions are then further vaporized to separate and combine fractions of lower boiling points from those of higher boiling points. This type of distillation is mainly used when the composition of the liquid to be separated has similar boiling points and a pure distillate is required (Robinson and Gilliland, 1937; Rose et al., 1951). Furthermore these techniques can be batch or continuous distillation (Rose et al., 1951; van Dongen and Doherty, 1958). Batch distillation is simple and usually applied for small-scale plants or basic laboratory distillation. For batch distillation the liquid is added to the still, heat is applied and the distillate is collected by cooling into the collection flask (Rose et al., 1951; van Dongen and Doherty, 1985). There are two types of condensers used in batch distillation, namely partial or total condensers. For partial condensers the vapours are collected from the top and a second condenser is necessary to form a liquid product. For total condensers the vapours are cooled and the liquid product is collected at the bottom of the condenser (Rose et al., 1951; van Dongen and Doherty, 1985). Continuous distillation is applied in large-scale, industrial plants and is coupled with fractional distillation. For continuous distillation, the liquid is continuously fed into a distillation column and the product is continuously collected from the condenser, either from the top or bottom of the liquid feed site (Rose et al., 1951).

Feedstocks and Methods for Bioethanol Production

The most popular feedstocks for bioethanol production are any material that is rich in carbohydrates. Biomass materials are an abundance of carbohydrates that is renewable and readily available (Balat and Balat, 2009; Balat et al., 2009). Biomass feedstocks are plant materials that accumulate carbon, hydrogen, oxygen, nitrogen and inorganic compounds during photosynthesis (Lal, 2008). There are a variety of biomass feedstocks that can be used for bioethanol production. The feedstocks range from starch and sugar crops to industrial and forestry wastes, even algae. To date, agricultural products contribute 95% of the global bioethanol production which is divided into approximately 60% and 40% from cereal crops and sugar crops, respectively (Reith et al., 2002; Balat et al., 2008; Lal, 2008; Goh and Lee, 2010, Mussatto et al., 2010, Walker, 2011).

Processing of First Generation Feedstocks. First generation feedstocks are starch or sucrose feedstocks. An overview of the methods is outlined in Figure 2.3.

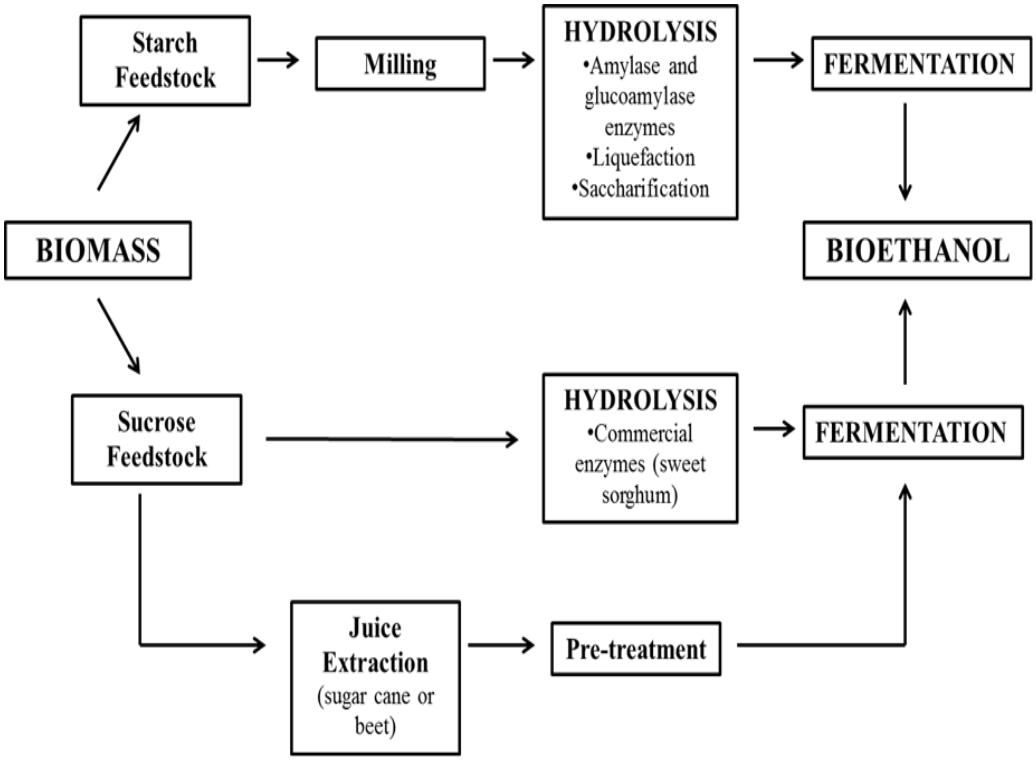


Figure 2.3 A diagram of bioethanol production from starch and sucrose feedstocks (*Source:* Deenanath et al., 2012).

Wheat, corn, maize, barley and cassava are examples of starch feedstocks (Balat et al., 2008; Sanchez and Cardona, 2008; Walker, 2011). These starch feedstocks or cereal grains are solid and subjected to milling, hydrolysis and fermentation (Fig 2.3). The first step is milling (Fig 2.3) which is necessary to crush the grains and expose the starch substrate. Milling can be either dry or wet milling. Dry milling crushes the whole grains into fine particles, whereas in wet milling the grains are steeped in water or dilute acid or alkali solutions. This process separates the starch from other grain components such as gluten and fibre (Taylor et al., 2006; Walker, 2011). The second step is starch hydrolysis (Fig 2.3). Starch is a long chain glucose polymer. This polymer is made up of amylose and amylopectin, the former is a linear glucose polymer and the latter is a branched

glucose polymer. Amylose consists of α-D-(1,4)-linkages, where carbon 1 of one glucose molecule is bonded to carbon 4 of the next glucose molecule. Amylopectin consists of α-D-(1,4)- and α-D-(1,6)-linkages, where carbon 1 and carbon 6 are bonded (Oates, 1997; Mojovic et al., 2006; Balat et al., 2008; Sanchez and Cardona, 2008). Due to this complex structure, a two-step hydrolysis method of liquefaction and saccharification is applied (Fig 2.3). During liquefaction the starch is broken down into sugar oligomers and dextrines by the use of α- and β-amylase enzymes at temperatures ranging between 90ºC to 110ºC (Reith et al., 2002; Mojovic et al., 2006; Sanchez and Cardona, 2008; Walker, 2011). Saccharification is the conversion of oligomers into monosaccharide sugars or monomers by the enzyme glucoamylase at temperatures ranging between 60ºC to 70ºC (Reith et al., 2002; Mojovic et al., 2006; Sanchez and Cardona, 2008; Walker, 2011). Liquefaction breaks down the amylose polymer whilst saccharification targets the amylopectin polymer (Mojovic et al., 2006).

Sugar cane, sugar beet and sweet sorghum are classified as sucrose feedstocks (Balat et al., 2008; Sanchez and Cardina, 2008; Walker, 2011). Sucrose feedstocks require hydrolysis or juice extraction and pre-treatment prior to fermentation (Fig 2.3). The use of sweet sorghum involves hydrolysis by commercial enzymes such as amylase, cellulose and amylopectinases (Fig 2.3) resulting in glucose and fructose monomers (Balat et al., 2008; Sanchez and Cardona, 2008). If sugar cane is used as a feedstock, the sugar cane juice is extracted (Fig 2.3) and conditioned to form crystalline sugar or molasses. This product is then pre-treated and fermented (Balat et al., 2008; Sanchez and Cardona, 2008; Walker, 2011). The pre-treatment of sugar cane juice involves the addition of sulphuric acid followed

by heating at 90ºC (Walker, 2011). For first generation feedstocks, batch or continuous fermentation processes are carried out by *S. cerevisiae*, *Schizosaccharomyces pombe* or *Zymomonas mobilis* (Sanchez and Cardona, 2008).

Processing of Second Generation Feedstocks. Second generation feedstocks are lignocellulosic feedstocks. Wheat straw, barley husks, corn cobs, paper pulp, sugar cane bagasse, wood chips and switch grass are examples of second generation feedstocks (Chandel et al., 2007; Balat et al., 2008; Sanchez and Cardona, 2008; Walker, 2011). Lignocellulosics are complex materials composed of three polymers, namely cellulose; hemicellulose; and lignin (Chandel et al., 2007; Balat et al., 2008; Sanchez and Cardona, 2008; Balat and Balat, 2009; Walker, 2011). Cellulose is a linear molecule of glucose β-(1,4)-glucosidic bonds with a mass of 100,000 Daltons and the empirical formulae $C_6H_{10}O_5$. Hemicellulose is a branched molecule made up of pentose (C5) and hexose (C6) sugars with a mass of 30,000 Daltons and empirical formulae $C_5H_8O_4$. The pentose sugars are xylose and arabinose. The hexose sugars are glucose, mannose and galactose. Lignin is a three dimensional plant cell wall structure made up of methoxylated phenylpropanoid units with the empirical formula $C_6H_{11}O_2$ (Balat, 2009; Balat and Balat, 2009; Walker, 2011). An overview of bioethanol production from these feedstocks is outlined in Figure 2.4.

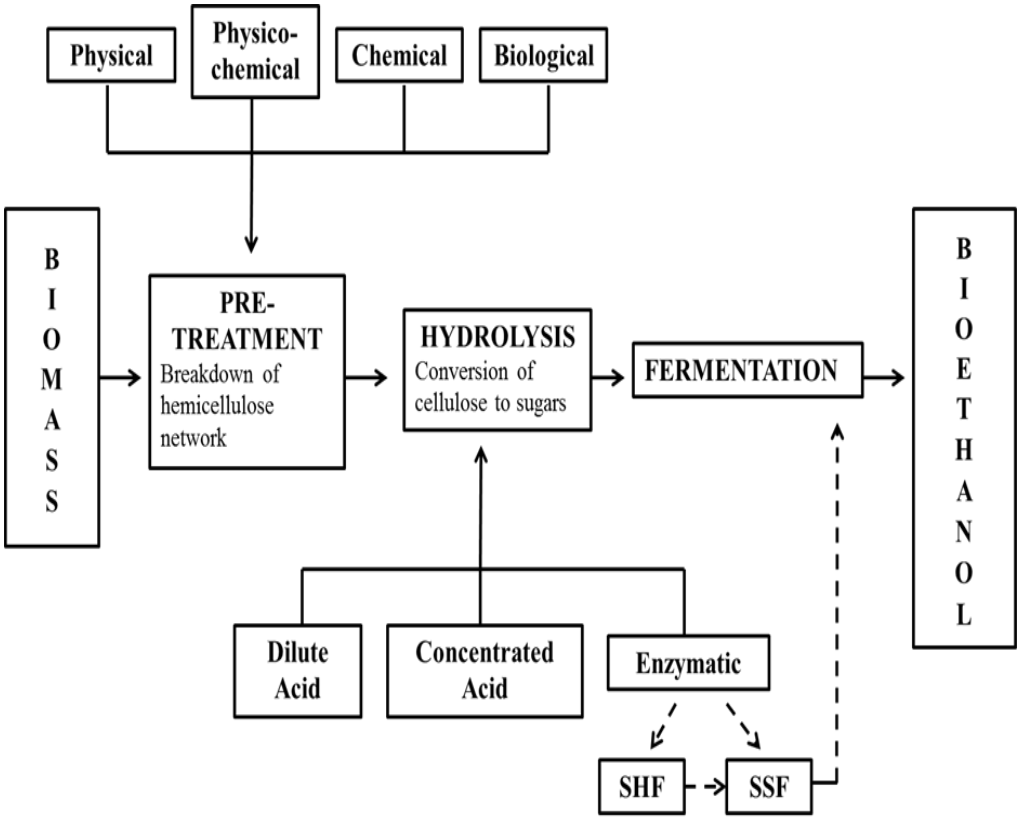


Figure 2.4 A diagram of bioethanol production from lignocellulosic feedstocks (*Source:* Deenanath et al., 2012).

The complicated structure of second generation feedstock requires pre-treatment, hydrolysis and fermentation (Fig 2.4). Pre-treatment methods such as physical, physico-chemical, chemical and biological (Fig 2.4) are necessary to break down the tough lignin exterior, reduce the crystallinity of cellulose, expose the cellulose for hydrolysis and remove hemicelluloses to provide a substrate with increased volume and surface area for hydrolysis (Mielenz, 2001; Hahn-Hagerdahl et al., 2006; Chandel et al., 2007; Balat et al., 2008; Sanchez and Cardona, 2008; Binod et al., 2010). The common pre-treatment methods applied to lignocellulose feedstocks are described in Table 2.4. Following pre-treatment, the cellulose is hydrolysed into monosaccharides by a variety of methods which are summarized in Table 2.5.

Table 2.4 Pre-treatment Methods, Type, Procedures and Effects of Lignocellulosic Feedstocks.

Methods	Type	Procedures	Effects	References
Mechanical Comminution	Physical	Grinding and milling of materials to produce particle sizes of 0.2-2mm and 10-30mm	Breaks down crystallinity of cellulose	Sun and Cheng, 2002; Sanchez and Cardona, 2008; Alvira et al., 2010
Pyrolysis	Physical	Heat treatment at T>300ºC	Cellulose decomposition	Sun and Cheng, 2002; Sanchez and Cardona, 2008
Steam Explosion	Physico-chemical	Steam exposure at 160-260ºC, followed by reduction in pressure from 0.69-4.83MPa to atmospheric pressure for explosive decompression	Disrupts lignin structure and hemicellulose is broken down	Sun and Cheng, 2002; Balat et al., 2008; Sanchez and Cardona, 2008; Alvira et al., 2010
Ammonia Fiber Explosion	Physico-chemical	Material is dosed with liquid ammonium at T=90ºC and MPa=1.36 for 30min, followed by pressure reduction to atmospheric pressure	Solubilizes cellulose	Sun and Cheng, 2002; Balat et al., 2008; Sanchez and Cardona, 2008; Alvira et al., 2010, Binod et al., 2010.
Carbon Dioxide Explosion	Physico-chemical	CO_2 exposure at MPa=5.62 for 24hrs, followed by pressure	Formation of carbonic acid which improves hydrolysis	Sun and Cheng, 2002; Sanchez and Cardona, 2008;

		reduction to atmospheric pressure		Alvira et al., 2010
Ozonolysis	Chemical	Ozone exposure at room temperature and atmospheric pressure	Removes lignin and hemicellulose	Sun and Cheng, 2002; Sanchez and Cardona, 2008; Alvira et al., 2010
Acid	Chemical	5% H_2SO_4 at 140ºC for 15mins, followed by 10mins at 190ºC or 0.75% H_2SO_4 at 121ºC for 1hr	Breaks down cellulose	Sun and Cheng, 2002; Balat et al., 2008; Sanchez and Cardona, 2008; Alvira et al., 2010; Binod et al., 2010
Alkaline	Chemical	Dilute NaOH at 160ºC for 24hrs	Disrupts lignin structure and reduces crystallinity of cellulose	Sun and Cheng, 2002; Balat et al., 2008; Sanchez and Cardona, 2008, Alvira et al., 2010, Binod et al., 2010
Oxidative Delignification	Chemical	Catalyzation by 2% H_2O_2 for 2hrs at 20ºC	Breaks down cellulose and solubilizes lignin and hemicellulose	Sun and Cheng, 2002; Sanchez and Cardona, 2008
Organosolve Process	Chemical	Organic solvent mixture of HCl/H_2SO_4 for 1hr at T=185ºC	Breaks bonds between lignin and hemicellulose	Sun and Cheng, 2002; Sanchez and Cardona, 2008; Alvira et al., 2010, Binod et al., 2010
Fungal	Biological	White-rot fungal cultures produce lignin degrading enzymes	Breaks down lignin and hemicellulose	Sun and Cheng, 2002; Balat et al., 2008; Sanchez and Cardona, 2008

Table 2.5 Hydrolysis Methods, Procedures and Effects of Lignocellulosic Feedstocks.

Methods	Procedures	Effects	References
Dilute Acid Hydrolysis	• Addition of 0.75% H_2SO_4 • Hydrolysis starts at T=50ºC and increased at T=190ºC • Incubation time:2hrs • Addition of 0.4% H_2SO_4 • Hydrolysis at T=170ºC and increased to T=230ºC • Incubation time:3-10mins	• Hemicellulose hydrolysed to xylose • Cellulose hydrolysed to glucose	Chandel et al., 2007; Balat et al., 2008; Sanchez and Cardona, 2008; Walker, 2011
Concentrated Acid Hydrolysis	• Addition of 70% H_2SO_4 at T=40-50ºC • Incubation time:2-4hrs • Water dilution • Soak in 30-40% H_2SO_4 at T=100ºC • Incubation time:50mins	• Hemicellulose hydrolysed to xylose • Cellulose hydrolysed to glucose	Chandel et al., 2007; Balat et al., 2008; Sanchez and Cardona, 2008; Walker, 2011
Enzymatic Hydrolysis	• Commercial enzymes obtained from *Trichoderma reesei* species • T=45-50ºC • pH=4.8	• Endoglucanases reduces the crystalline structure of cellulose • Exoglucanases target the ends of cellulose to degrade cellobiose • B-glucosidase degrades cellobiose	Sun and Cheng, 2002; Hahn-Hagerdahl, 2006; Balat et al., 2008; Sanchez and Cardona, 2008; Walker, 2011

Post hydrolysis, glucose and xylose are the monosaccharides produced (Table 2.5). *S. cerevisiae* is used for fermentation, as it easily assimilates hexose sugars. However, the pentoses cannot be assimilated. Thus, in addition, bacterial species like *Z. mobilis*, *Escherichia coli* and *Klebsiella oxytoca* can be used to obtain efficient yields of bioethanol from lignocellulosic feedstocks (Balat et al., 2008; Walker, 2011). Furthermore, fermentation technologies such as simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) is applied to ferment lignin hydrolyzates (Fig 2.4) (Chandel et al., 2007; Taherzadah and Karimi, 2007). SSF is the combination of cellulase enzymes and preferably genetically modified strains of *S. cerevisiae* in a single fermentor. The modified yeast is designed to release hydrolytic enzymes that convert hexose and pentose sugars to produce ethanol (Chandel et al., 2007; Taherzadah and Karimi, 2007). SHF is focused on optimal factors like: (i) time, (ii) temperature, (iii) pH, (iv) enzyme concentration, and (v) substrate concentration for efficient conversion of cellulose into fermentable glucose (Hahn-Hagerdahl et al., 2006; Chandel et al., 2007; Sanchez and Cardona, 2008). This allows the enzymes to function at enzyme-specific temperatures for cellulases and fermentation to be performed at temperatures suitable for the yeast type (Chandel et al., 2007; Taherzadah and Karimi, 2007).

Processing of Third Generation Feedstocks. Seaweed which is a macroalgae is an example of a third generation feedstock (Goh and Lee, 2010; Mussatto et al., 2010). Compared to first and second generation feedstocks the use of algae in bioethanol production is new. An overview of bioethanol production from a third generation feedstock is outlined in Figure 2.5.

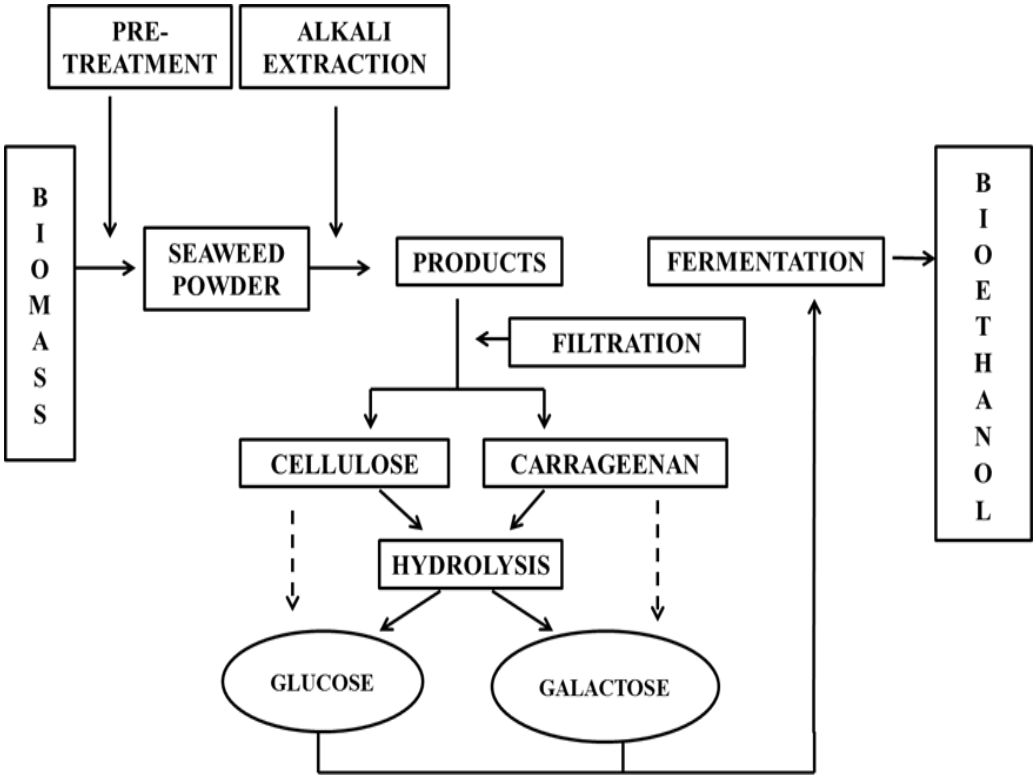


Figure 2.5 A diagram of bioethanol production from seaweed (*Source:* Deenanath et al., 2012).

Bioethanol production from seaweed, which is an example of a third generation feedstock, requires seaweed desalination (pre-treatment), followed by hot water and alkali extraction to produce polysaccharides (Fig 2.5). These polysaccharides are then filtered, centrifuged and precipitated to form cellulose, carrageenan and proteins (Fig 2.5). Carrageenan is a cell wall component, which is linear with alternate residues of α (1-3)-D-galactose-4-sulphate and β-(1-4)-3-6-anhydro-D-galactose (Goh and Lee, 2010). The cellulose and carrageenan is hydrolyzed by acidic or enzymatic hydrolysis techniques. For acid hydrolysis, the polysaccharides are treated with sulphuric acid at 100°C for 3hrs, followed by neutralizing the reaction with barium carbonate (Goh and Lee, 2010). The enzyme hydrolysis technique is unavailable-a possible method is to use the endogenous

amylase of carrageenan (Goh and Lee, 2010; Mussatto et al., 2010). Hydrolysis yields glucose and galactose from cellulose and carrageenan, respectively (Fig 2.5). For fermentation, *S. cerevisiae* is used for glucose fermentation and the bacterial species *Streptococcus lactis* and *Streptococcus cremoris* is used for galactose fermentation (Goh and Lee, 2010).

2.2.2 Thermochemical Methods

Thermochemical methods used to produce biofuels are the application of high temperatures and air within a reactor. Second and third generation feedstocks are used for thermochemical biofuel production. There are three types of thermochemical methods, namely gasification; liquefaction; and pyrolysis (Demirbas, 2009; Dwivedi et al., 2009; Zhang, 2010; Anastasakis and Ross, 2011; Nigam and Singh, 2011; Kwon et al., 2012).

Gasification is the combustion of organic material to produce syngas, which is then conditioned to produce ethanol. Prior to gasification, the feedstock is cleaned to remove foreign particles, chipped to obtain a particle size of <1mm and dried to reduce the moisture to 5-20 weight % (Dwivedi et al., 2009; van der Heijden and Ptasinski, 2012). Gasification can be direct or indirect. For direct gasification, the prepared material is fed into the gasifier at 550ºC which decomposes on contact to form syngas. During this process the addition of air or oxygen is controlled to increase the temperature to approximately 800ºC-900ºC. The syngas is carbon monoxide, hydrogen and nitrogen free. For indirect gasification there is an external supply of steam into the gasifier and the syngas contains a high concentration of tar (Girard and Fallot, 2006; Dwivedi et al., 2009; van der Heijden and Ptasinki, 2012). For ethanol production the syngas product is cleaned

to remove impurities such as tar, chlorides, ash and sulphur. Examples of syngas cleaning methods techniques are cyclones, wet scrubbing, steam reforming or partial oxidation (Girard and Fallot, 2006; Dwivedi et al., 2009; van der Heijden and Ptasinki, 2012). Post cleaning the syngas can be converted into ethanol by fermentation or catalysis. During fermentation, the syngas is introduced into a chamber and converted into ethanol and acetic acid by MOs such as *Clostridium carboxidivorans*, *Clostridium ljungdahlii* or *Clostridium autoethanogenum* (Dwivedi et al., 2009). For catalysis, syngas is heated to 300ºC and fed into a conversion chamber at pressures between 50-105 bar. The gas is mixed with water and methanol and passed through a catalyst to form various products such as methanol; ethanol; pentanol; propanol; butanol; and water. The catalyst is a synthetic catalyst such as molybdenum-disulfide or Rhodium. The products are then cooled to approximately 43ºC and ethanol is purified by distillation (Dwivedi et al., 2009; van der Heijden and Ptasinki, 2012).

Liquefaction is used for the production of biocrude oil from macroalgae or sewage (Anastasakis and Ross, 2011). During liquefaction the feedstock is subjected to high temperatures (473-623K) and pressures (4-10MPa) to decompose the original feedstock material into products such as biocrude, char, water and gases (Demirbas, 2009; Anastasakis and Ross, 2011).

Pyrolysis, similar to liquefaction can be used to produce biocrude oil. During pyrolysis the feedstock is decomposed, without the addition of oxygen at higher temperatures (673-973K) to produce biocrude oil, char and gases (Demirbas, 2009; Anastasakis and Ross, 2011; Iribarren et al., 2012; Kwon et al., 2012). The biocrude oil which is the product of interest as a biofuel is rich in carbon,

hydrogen and oxygen. This product can be used directly as a fuel, blended with diesel or gasified to form ethanol (Ioannidou et al., 2009; Anastaskis and Ross, 2011; Iribarren et al., 2012).

2.3 Bioethanol Perspectives

2.3.1 Bioethanol in the US, Brazil and Other Countries

The front runners in bioethanol production are Brazil and the US (Walter et al., 2008; Harvey and Pilgrim, 2011). From Figure 2.6 it is noted during the early bioethanol production stages, Brazil was the dominant producer and as time progressed the US excelled (Fig 2.6).

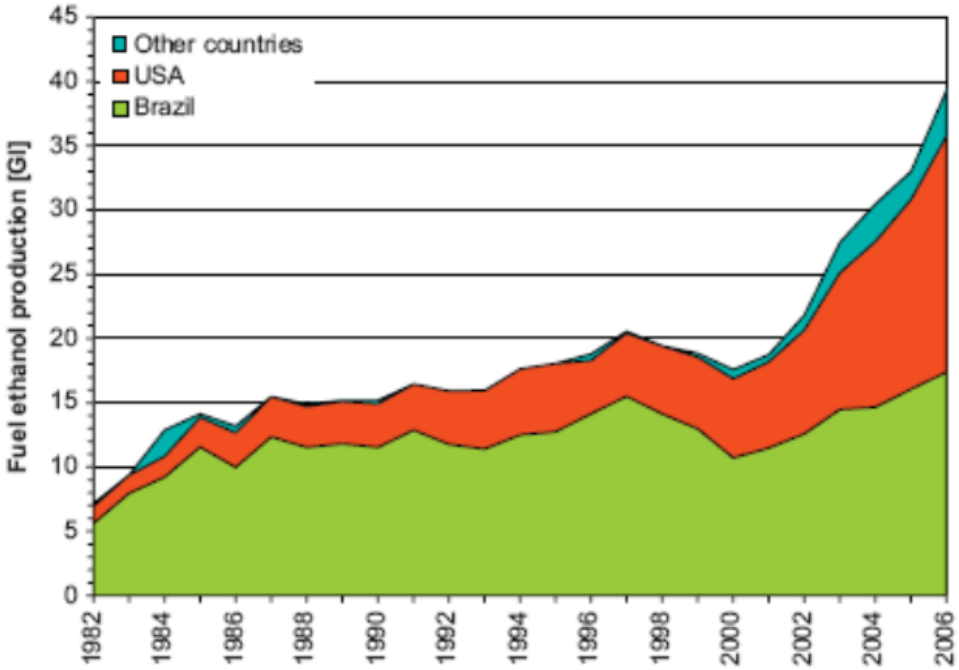


Figure 2.6 Bioethanol production in the US, Brazil and the rest of the world from 1982 to 2006 (*Source:* Walter et al., 2008).

Since the inception of bioethanol in the US, the US government has developed numerous policies and framework to support and sustain the technology. These policies are: (i) in 2000, the Biomass Research and Development Act, (ii) in 2005, the Energy Policy Act and (iii) in 2007, the Energy Independence and Security Act (Walter et al., 2008; Harvey and Pilgrim, 2011). Collectively there policies aimed to reduce the usage of petroleum by 20%, substitute biofuels by 30%, increase biofuel production from 28.4 billion litres (GI) to 132 GI by 2022 and ensure that the majority of biofuels are produced from second generation feedstocks (Walter et al., 2008; Harvey and Pilgrim, 2011). Furthermore, funding from government and corporate investments were used for programmes such as The Genomics Systems Biology Program and The Energy Bioscience Institute. These programmes were directed towards biological science and technological development by incorporating universities for research purposes (Harvey and Pilgrim, 2011). Presently in the US, bioethanol is applied for two purposes: (i) as an octane additive instead of MTBE, and (ii) blending with conventional petrol at 10% (v/v) or 85% (v/v) (Rosillo-Calle and Walter, 2006; Walter et al., 2008; Mussatto et al., 2010).

In Brazil, the Brazilian Alcohol Program (ProAlcool) was implemented in 1975 for bioethanol production and its usage has since provided "energy independence" (Harvey and Pilgrim, 2011). Additionally the country has gained international market expansion, job creation, sustained biofuel production, supporting rural communities through dependence of feedstocks and introducing Flex-Fuel Vehicles which can be utilized with 100% bioethanol (Mabee, 2007; Walter et al., 2008; Harvey and Pilgrim, 2011). Other than the ProAlcool programme, the

FUPA and FUP programmes were directed towards financing the use of bioethanol (Mabee, 2007). These programmes guaranteed the price of 0.12-0.15 US dollars per litre for 20% and 100% bioethanol/petrol blends, respectively (Mabee, 2007). During 1986 there was a decrease in bioethanol production due to the decrease in international oil prices and rise in petrol production. This resulted in the Brazilian government limiting support for sustained bioethanol production and eventually all production was stopped by the late 1990s (Walter et al., 2008). By the year 2001, bioethanol production started again when international oil prices rapidly increased (Walter et al., 2008). Presently, there are 322 sugar and ethanol distilleries in Brazil and 80 plants are under construction. The current usage of bioethanol is as: (i) 100% ethanol in Flex-Fuel Vehicles, and (ii) 20-25% (v/v) ethanol blends of with petrol (Rosillo-Calle and Walter, 2006; Walter et al., 2008; Mussatto et al., 2010).

Other countries where bioethanol is produced and used in the transport sector are Europe, China, India, Thailand and Canada (Prakash et al., 2005; Rosillo-Calle and Walter, 2006; Mabee, 2007; Walter et al., 2008; Mussatto et al., 2010; Harvey and Pilgrim, 2011).

In Europe, biodiesel (~70 GI/year) is the choice of biofuel over bioethanol. The reason being, approximately 80% of vehicles in the EU have diesel engines and between the years 1990 to 2005, diesel consumption increased from 43% to 65% (Harvey and Pilgrim, 2011). The EU Directive formed in 2001, stipulated that 2% of transport fuel should be biofuels with a progressive increase to 5.75% from 2001 to 2005. However, this target was not met and the lack of biofuel development prompted the EU Directive to separate the member states and it is

required that the states initiate their own policies (Mabee, 2007; Walter et al., 2008; Harvey and Pilgrim, 2011). The EU contributes 6% of worldwide bioethanol production, with the highest production of 2.7 GI in 2005 (Walter et al., 2008). With respect to bioethanol, the majority is produced in Germany, Spain, France, Poland and Sweden with a combined total of 1592 million litres (MI) (Mabee, 2007; Walter et al., 2008). By 2007, this increased to 3300 MI, with France and Germany as the major EU bioethanol contributors of 1150 MI and 706 MI, respectively (Walter et al., 2008). Recent bioethanol policies such as, the Fuel Quality Directive implemented the usage of 5% (v/v) with a potential increase to 10% (v/v) of bioethanol/petrol blends (Harvey and Pilgrim, 2011). In Sweden, Spain and Germany bioethanol usage is at 2.23%, 1.49% and 3.75%, respectively (Mabee, 2007; Walter et al., 2008). Feedstocks used in Europe are sugar beet and wheat (Walter et al., 2008). However, as the demand for bioethanol usage grows the EU, like the US is moving towards policies for the use of second generation feedstocks (Walter et al., 2008; Gnansounou, 2010).

In China, the current estimated production of bioethanol is 3.8GI and this is expected to reach 12.6GI by the year 2020. The feedstocks used are maize, rice, sugar cane and cassava (Walter et al., 2008; Mussatto et al., 2010; Qui et al., 2010). During 2008, bioethanol production from cassava resulted in approximately 0.2 million tons (Mussatto et al., 2010; Qui et al., 2010). There are four operational bioethanol plants which produces approximately 1.3GI per year from maize and three more plants are under construction, which is expected to contribute approximately 1.5GI per year (Walter et al., 2008; Mussatto et al., 2010; Qui et al., 2010). Additionally there are two pilot plants directed towards

the use of second generation feedstocks such as sawdust or rice straw (Walter et al., 2008). Chinese government policies for biofuel development began in the late 1990s and presently there are twenty-seven cities across five China provinces that use the 10% (v/v) blend of bioethanol and petrol (Qui et al., 2010). The earlier policies were aimed towards investment in research and development and by the year 2001 policies such as: (i) The Special Development Plan for Denatured Fuel Ethanol, and (ii) Bioethanol Gasoline for Automobiles in the 10^{th} Five-Year Plan were implemented. These policies were aimed towards the marketing of bioethanol/petrol blends and sustainability support for bioethanol (Qui et al., 2010). In 2002, two new polices were documented, namely The Pilot Testing Program of Bioethanol Gasoline for Automobile and Detailed Regulations for Implementing the Pilot Testing Program (Qui et al., 2010). These policies involved tax exemption if 10% (v/v) ethanol/petrol blends were used; refund of value-added tax; and subsidy and profits for grain feedstocks. Additionally, 3.32 million hectares of unused land can be used for feedstock harvesting and in turn be used to produce at least 12 million tons of bioethanol (Qui et al., 2010).

In India, bioethanol production capacity is estimated at 3.2GI per year from the largely available sugar cane feedstock (Prakash et al., 2005; Rosillo-Calle and Walter, 2006). Currently, the 5% (v/v) blend is under usage at 300 petrol stations and as the demand increases, the Planning Commission of the Indian Government is looking to the use of rice husks, wheat straw or sugar cane bagasse for continued production (Rosillo-Calle and Walter, 2006; Mussatto et al., 2010).

In Thailand, there are twenty-four bioethanol plants and approximately 150MI per year is produced from molasses, cassava and sugar cane (Rosillo-Calle and Walter, 2006; Walter et al., 2008; Mussatto et al., 2010).

In Canada, there are nineteen bioethanol plants producing approximately 1400MI per year from corn or wheat. Additionally there are two plants producing approximately 2MI and 5MI of bioethanol per year from wheat straw and residual wood, respectively (Mabee, 2007; Mabee and Saddler, 2010).

2.3.2 Bioethanol in Sub-Saharan Africa

By the year 2020, the African population will increase from 642 million to 1.3 billion with an expected 17% of this population in Sub-Saharan Africa (SSA) (Amigun et al., 2008; Chakauya et al., 2009). With population growth there are growing demands, especially in the energy sector. However in Africa energy growth is minimal, for example from 1973 to 2006 the population growth rate was between 3% to 4.8% whilst the energy growth rate was 1.7% (Amigun et al., 2008; Chakauya et al., 2009). From Figure 2.7a-b, the majority of energy (80%) in SSA is biomass (Fig 2.7a) and a large percentage of the population is dependent on this biomass for energy resources (Fig 2.7b) such as heat and electricity (Amigun et al., 2008; Chakauya et al., 2009; Jumbe et al., 2009).

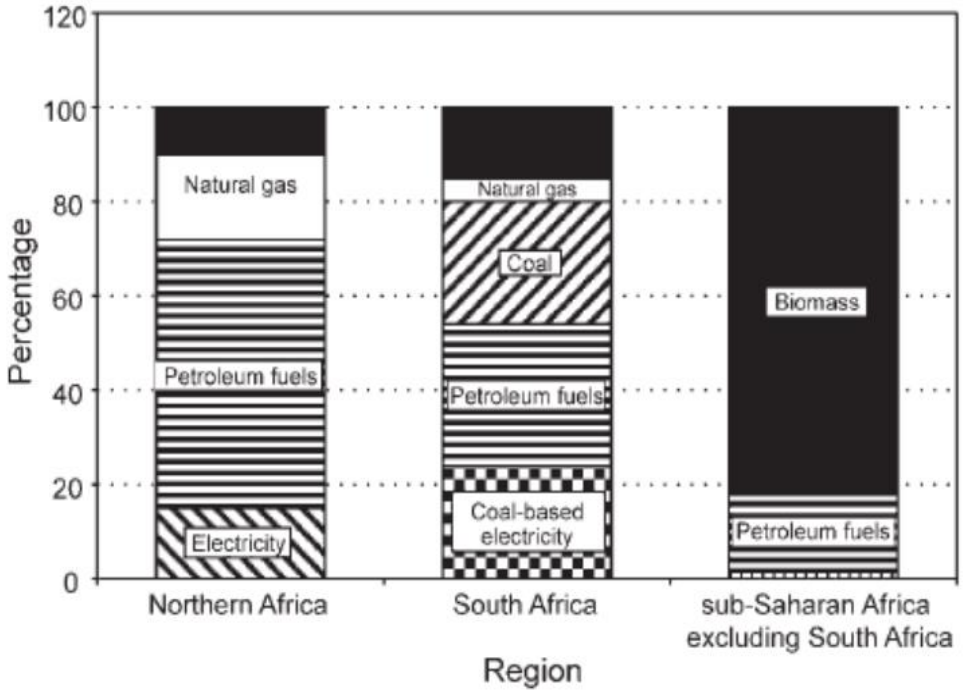


Figure 2.7a Energy demand (%) in Africa (*Source:* Chakauya et al., 2009).

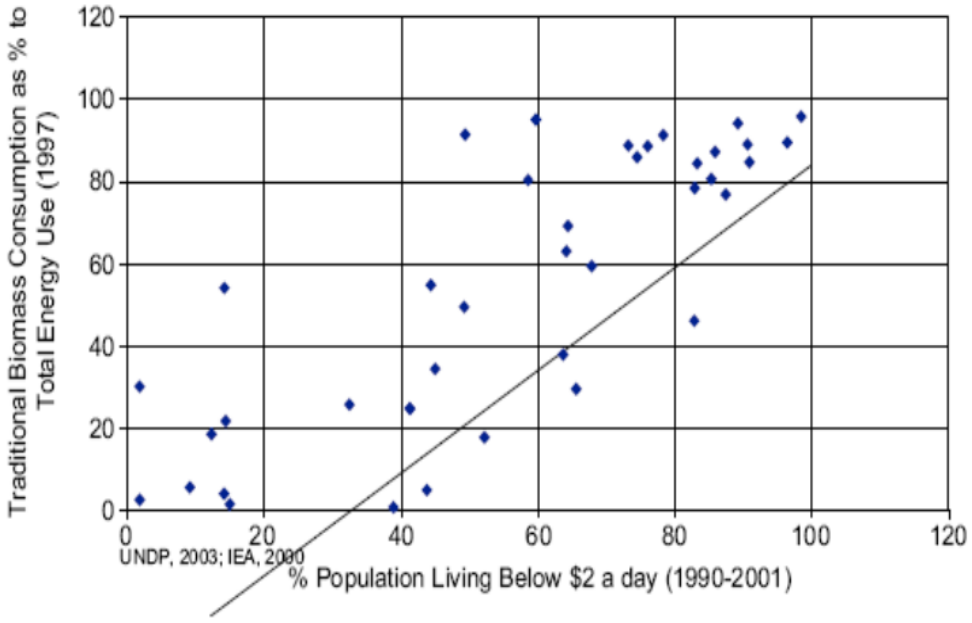


Figure 2.7b The percentage correlation between biomass energy and the Sub-Saharan populations between 1990-2001 (*Source:* Amigun et al., 2008).

Of the fifty poorest worldwide countries, thirty-four of these countries reside in SSA. The reason being that SSA is affected by marginal income, marginal production, inadequate people skills and government management, and high poverty rates (Amigun et al., 2008; Mangoyana, 2009). From there reasons, it is questionable if bioethanol commercialization will be promising for SSA? This question will be addressed as ***History, Challenges,*** and ***Prospects of bioethanol production*** in SSA.

History of bioethanol production

"Bioethanol production in SSA dates back to 1980, with the most successful plants developed in Zimbabwe, Malawi and Kenya. The Zimbabwean Triangle Ethanol Plant began production in 1980, producing approximately 120,000 litres of bioethanol per day and 40MI per annum for 12 years. Sugar cane molasses was the main feedstock used, with the sugar cane being cultivated locally. The Triangle Ethanol Plant was the first in SSA to blend 12% (v/v) to 15% (v/v) of bioethanol with petrol. However, in 1992 drought conditions caused a severe reduction in the availability of the sugar cane feedstock. This resulted in shutting down the plant due to the lack of government support in reviving the land and there was an increased demand for exporting ethanol to Europe as potable ethanol for beverages, instead of using the ethanol as a transport fuel. In Malawi, bioethanol production began in 1982 at the Dwangwa Estate Plant-producing 15-20MI per annum from sugar cane molasses. Currently, this plant is still in existence and in 2004 a second plant, the Nchalo plant was opened with productivity estimated at 12 million litres per annum. Blending of 10% (v/v) bioethanol with petrol is carried out at these plants. The sustainability of

bioethanol production in Malawi is contributed by government policies that: (i) allows for in minimizing dependence of fossil fuel imports, (ii) large quantities of feedstocks from small holder farmers, (iii) consistent water supply from Lake Malawi, and (iv) budgeting and monitoring plant production costs at 8 million US dollars. In Kenya, the Muhoroni Plant produced 45,000 litres of bioethanol per day from sugar cane molasses and like Malawi, 10% (v/v) of ethanol was blended with petrol. However, production was abandoned in 1993 because of expenditure and lack of government management or support from oil companies" (Deenanath et al., 2012).

Challenges of bioethanol production

"***Feedstocks for bioethanol production.*** The availability of crops as feedstocks for bioethanol production exacerbates the debate of food versus fuel. In SSA, the human population is dependent on crops as a major source of food. For example, in Zambia, 95% of maize that is cultivated annually is consumed as the staple diet and the amount produced is equivalent to the amount consumed. If maize is to be used as a fuel providing crop, as in the US, this will in turn cause inflation in food prices because of the strain for the dual use of the crop. In Mauritius, sugar cane and the bagasse are utilized for the generation of electricity and heat and 25% of the electricity is supplied by the sugar cane industry" (Deenanath et al., 2012). Compared to other world regions such as Brazil and India, sugar cane production is on a much smaller scale, as shown in Table 2.6.

Table 2.6 Sugar cane production in Sub-Saharan Africa and other world regions (*Modified from*: Johnson and Matsika, 2006).

Regions	Area Harvested 1000ha	Total Production 1000 tonnes cane (tc)	Average Yield tc/ha
Angola	10	360	38
DR Congo	43	1786	42
Madagascar	69	2460	36
Malawi	20	2100	105
Mauritius	72	5199	73
Mozambique	30	400	13
South Africa	326	20419	63
Swaziland	48	4500	93
Tanzania	17	2000	118
Zambia	17	1800	106
Zimbabwe	45	4533	101
SADC Total	**696**	**45557**	**65**
Australia	448	36995	83
Brazil	5371	396012	74
India	4608	281600	61
Thailand	1139	74259	65
World Total	**20822**	**1359120**	**65**

In SSA, South Africa and Mauritius are the leading sugar cane producers (Table 2.6). Since the majority of sugar cane is harvested for food and electricity, when the average yield (Table 2.6) cannot meet these demands, sugar cane is usually

imported (Johnson and Matsika, 2006). “Hence, in addition to the food supply being affected, if sugar cane is utilized for bioethanol then the electricity supply will also be threatened. Furthermore, the demand for food crops in SSA for basic domestic needs is so great; in the year 2000 33% of crops had to be imported” (Deenanath et al., 2012).

“***Land Availability and development.*** Similar to the food versus fuel debate, the dilemma of land availability is whether or not to use the majority of the land for food crop cultivation or bioethanol crop cultivation. Agricultural land in Tanzania, for example, is owned by smallholder farmers in which farming is their way of life. If their land is converted for bioethanol crop production, will these farmers benefit from this technology? In Burkina Faso, marginal crop lands are governed by women for household needs and medicinal uses. Large-scale bioethanol production requires large amounts of land for on-going production and up-keep of crops. This could result in the use of these marginal crop lands, which negatively affects the amount of land available to African women for obtaining their resources. In Cameroon, 75% of agricultural work is carried out by women, however only 10% of the land is owned by these women. The development of this land purely for bioethanol crops will seriously disadvantage the women owners, as bank credits cannot be received. Furthermore, land development would result in severe deforestation, loss of biodiversity and erosion of the organic soil matter and in order to use the land for successive harvesting, rehabilitation is necessary which will also increase maintenance costs” (Deenanath et al., 2012).

"***Government policies.*** SSA is largely rural with poor income and production markets; severe poverty; high child mortality rates; high HIV infection rates; women marginalization and extreme dependence on agriculture for food and livelihood. The vulnerability of these situations overshadows government policies and full support towards sustainable bioethanol development. To date, bioethanol policies have been proposed in Sub-Saharan regions such as Mozambique, Nigeria, Uganda, South Africa, Malawi and Ghana. The Mozambican government has adopted policies of blending 5% (v/v) to 10% (v/v) of bioethanol with petrol, however this is tentative and solid implementation is unheard of. Whereas in South Africa, the government policies suggest 400MI of biofuels be produced by the year 2013. However, these policies resemble vague phrases and there is a lack of structure for financial obligations and coordinating research, educational training and acquiring skills. Furthermore, these policies do not allow for international trade and investments, thus bioethanol production in SSA will be unrecognizable on the global scale. In Tanzania, the government was forced to render bioethanol production that caused any threat to food and land security as farmers and environmental groups caused unrest. In order for bioethanol policies to be acceptable, the rural lifestyles need to be taken into consideration. Hence, Sub-Saharan governments would prefer to financially support current policies rather than implement fuel development policies at the risk of extreme expenditure and social downfalls" (Deenanath et al., 2012).

Prospects for bioethanol production. It is clear from the aspects reviewed that the challenges facing bioethanol commercialization in SSA is vast and overshadows the advantages but there are beneficial prospects that need to be considered.

“Recently, bioethanol technology is moving towards the use of second generation feedstocks for bioethanol. In SSA, bioethanol development is not at the level of other countries and thus first generation feedstocks need to be used, but it is foreseen that by the year 2050 the potential for bio-energy in SSA will increase from a current production of 347 exajoule to approximately 1548 exajoule. To avoid food security issues, sugar cane and sweet sorghum are first generation feedstocks that have the most potential. Since the starting production of bioethanol in SSA, sugar cane has been extensively exploited and can thus be re-introduced as an industrial crop. Sugar cane cultivation is dominant in Zimbabwe, South Africa and Mauritius as climatic conditions are favourable for growth. Total production in different countries, to date, ranges from 4,533 tonnes in Zimbabwe to 5,199 tonnes in Mauritius and 20,419 tonnes in South Africa (Table 2.6). The production values (Table 2.6) can be increased and in this way crops can be used for current applications and in the near future, for bioethanol. The use of sweet sorghum is also promising, as like sugar cane, growth conditions for sweet sorghum are favourable and the sorghum plant is able to survive drought conditions that are common in SSA. It has been suggested that sorghum processing can take place in sugar cane plants and thus additional costs for ethanol plant developments is avoided” (Deenanath et al., 2012). Other feedstocks and their potential for bioethanol usage are summarized in Table 2.7 and the amount of available land for feedstock cultivation is shown in Table 2.8.

Table 2.7 Available feedstocks for bioethanol production in Sub-Saharan Africa (*Modified from*: Chakauya et al., 2009).

Feedstock	Fit with agroecosystems	Socioeconomic Potential
Sugar Cane	• Minimum rainfall of 600mm required • Widely grown in the region already, possibilities for expansion	• Technology for production established • Ethanol blending already in place • Bagasse usable for fuel
Sweet Sorghum	• Low water requirement • Drought tolerant • Better adapted to marginal growing conditions	• Fits with sugar cane ethanol production • Some varieties have extremely high sugar content
Maize Grain	• Well adapted to region • Grown in commercial and subsistence sectors • Susceptible to drought	• Technology for maize ethanol is well established in other parts of the world
Cassava	• Already grown in the region, potential for expansion • Tolerant to drought, acidity and salinity	• Potentially high yielding
Sugar Beet	• Grows in dry areas • Low water requirement • Fast growing	• Similar sugar output to sugar cane
Sweet Potato	• Already grown in the region • Well adapted to growth region	• 40%-50% more starch than maize • Productivity 3-4X higher than maize

Table 2.8 Land availability (million hectares-Mha) and utilization in Sub-Saharan Africa (*Modified from*: Johnson and Matsika, 2006).

Region	Forest area (Mha)	Agricultural area (Mha)	Cultivated area (Mha)
Angola	69.8	57.6	3.6
Botswana	12.4	26.0	0.4
DR Congo	135.2	22.8	7.8
Lesotho	-	2.3	0.3
Madagascar	11.7	27.6	3.6
Malawi	2.6	4.4	2.6
Mauritius	-	0.1	0.1
Mozambique	30.6	48.6	4.6
Namibia	8.0	38.8	0.8
South Africa	8.9	99.6	15.7
Swaziland	-	1.4	0.2
Tanzania	38.8	48.1	5.1
Zambia	31.2	35.3	5.3
Zimbabwe	19.0	20.6	3.4

From the challenges reviewed the amount of available land was a concern; however in SSA there is a great deal of forest area that can be exploited (Table 2.8). If the forest area is developed and used for harvesting of bioethanol crops then the current agricultural land (Table 2.8) is unharmed and food crop growth continues as normal. Furthermore, to keep crop productivity on the increase for sustained bioethanol production, land maintenance is also important. On-going harvesting and lack of soil restoration causes land deterioration and decrease in

crop production (Amigun et al., 2011a). The undeveloped forest areas and even cultivated areas can possibly be supported by the African Conservation Tillage (ACT) network. The ACT is a non-profit organization formed in 1998 and involved in sustaining agricultural land and water in Africa (Amigun et al., 2011a). For the development and maintenance of land, policies that allow collaboration between the ACT network and Biofuel Associations might be beneficial. In Botswana, Burkina Faso, Kenya, Mali, Senegal, South Africa, Tanzania and Zambia there are high annual yields of cassava ranging from 2.3 to 8.9 tons per hectare as the growth conditions are favourable (Wicke et al., 2011). If cassava is to be exploited for bioethanol in these regions, then marginal lands can be utilized and local women can be involved in harvesting and benefit from profits (Wicke et al., 2011). "South African government has proposed that 20-50% of biofuel renewable energy will be implemented by 2013, using sugar cane and sugar beet as bioethanol feedstocks. Furthermore, in South Africa agricultural and forestry residues can be utilized for bioethanol production. Agricultural and forestry residues are lignocellulosic feedstock such as maize stover, sugar cane bagasse, wheat straw, saw mill residue and paper mill sludge. The capacity of these types of biomass that is indigenous and available in South Africa for bioethanol exploitation was reviewed by Lynd et al. (2003). Agricultural residues accounts for 12.3Mt/a and forestry residues accounts for 5.0Mt/a" (Deenanath et al., 2012). In the Western Cape, South Africa the wheat/rye hybrid known as triticale is being considered for bioethanol production (Amigun et al., 2011b). Triticale is promising as it contains 53% to 64% of starch and can easily adapt to grow in marginal lands where nutrients are limited (Amigun et al., 2011b). There

is approximately 200,000tpa of marginal land available in the Western Cape that can be used to harvest triticale. Furthermore, research into extracting the fibre from triticale is under investigation and this fibre can be converted into bioethanol using procedures similar to second generation feedstock processing (Amigun et al., 2011b). "In 2009, the Mozambican government proposed a National Biofuels Policy and Strategy, "The Resolution". The policy aims to: (i) allocate specific land for bioethanol crops, (ii) choose crops such as sugar cane and sweet sorghum specifically for bioethanol, (iii) blend bioethanol at 5-10% (v/v) with petrol, (iv) promote bioethanol markets by exports and to generate foreign currency, and (v) tariffs for biomass electricity where bioethanol processing will generate electricity as a by-product" (Deenanath et al., 2012). Furthermore this policy showed expansion into various programmes such as: (i) National Programme for Biofuel Development for financial contributions; (ii) National Commission for Biofuels which is a policy supervisory committee; (iii) Biofuels Commercialization Programme to assist with marketing of biofuels; and (iv) Working Group to promote sustainability of biofuels and prevent negative impacts on the agricultural land and the country (Di Lucia, 2010). Thus far the most successful biofuel prospects in SSA lie in Mozambique based purely on active government participation. Additionally, Mozambique has favourable amounts of biomass and land and the weather conditions are suitable for agricultural growth (Di Lucia, 2010; Schut et al., 2010). The potential of Mozambique based on land availability and biomass production is shown in Table 2.9.

Table 2.9 Land availability and estimated biomass production potential of various provinces in Mozambique (*Modified from:* Schut et al., 2010).

Province	Total Available Land (ha)	Annual Biomass Production Potential
Zambezia	1,365,300	883
Niassa	1,220,400	1,176
Inhambane	1.071,660	113
Gaza	866,780	234
Nampula	709,160	1,144
Tete	661,730	576
Sofala	408,650	545
Manica	381,950	642
Cabo Delgado	269,400	1,286
Maputo	11,000	71
Total	**6,966,030**	**6,670**

From the data approximately 7 million ha of land is available (Table 2.9) for biofuel crops without interrupting land used for food crops and 6,670PJ of biomass production is possible by the year 2015 which can contribute to approximately 2.5GI of bioethanol (Di Lucia, 2010; Schut et al., 2010). A total of seventeen biofuel investment projects were submitted to the Mozambican government between the years 2006-2008. Five of these projects were for the production of bioethanol from sugar cane and sweet sorghum with an estimated 8,925 to 11,956 employment opportunities (Schut et al., 2010). The main bioethanol projects that were approved are shown in Figure 2.8.

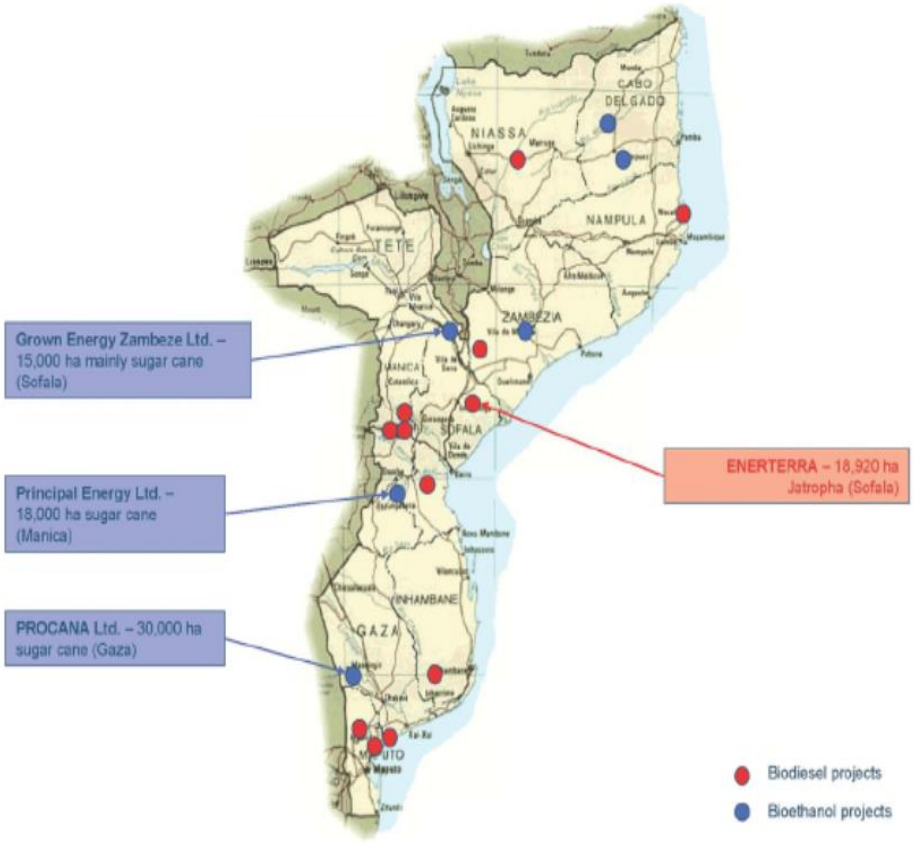


Figure 2.8 National biofuel projects in Mozambique (*Source:* Schut et al., 2010).

From Figure 2.8, three bioethanol projects were based on the cultivation of sugar cane in the Sutala, Manica and Gaza provinces in Mozambique. The Procana Company started the bioethanol project in October 2007 together with Bioenergy Africa Ltd. to produce 30,000 ha of sugar cane (Schut et al., 2010). In 2008, Principle Energy Ltd. was initiated to produce 18,000 ha of sugar cane and Procana and Principle Energy aim to develop biorefineries for sugar cane to bioethanol processing (Schut et al., 2010). In 2009, Grown Zambeze cultivated 15,000 ha of sugar cane and sweet sorghum and is looking towards an ethanol

distillery and cultivation of alternate feedstocks such as beans and soya (Schut et al., 2010). “Another major prospect in SSA is the growing association with the European Union (EU). In SSA, land that is under-utilized is being acquired by the EU to grow bioethanol crops which will then be exported to the EU. This strategy showed promise and resulted in a declaration being signed in September 2008 in Addis Ababa and the “EU-Africa Energy Partnership” was formed. The agreement benefits both parties, where the EU can reduce the use of fossil fuels by 20% by the year 2020 and SSA will gain international investment. This agreement can be improved by involving SSA in the bioethanol production process to achieve bioethanol sustainability and certain percentage of the biofuel crops grown in SSA should be solely used in SSA” (Deenanath et al., 2012).

2.4 The Cashew

2.4.1 Overview of the Cashew

The cashew is a tropical and sub-tropical tree which belongs to the family *Anacardiaceae*, the genus *Anacardium* Linn, the class Dicotyledonae and the species *Anacardium occidentale* Linn var. *nannum* (Ramiakajoto, 2001; Trevisan et al. 2006; Santos et al., 2007; Asogwa et al., 2008; Orwa et al., 2009). Other trees belonging to the *Anacardiaceae* family include mango, marula, poison ivy, karee and poison sumac (Ramiakajoto, 2001; Poulton, 2006; Michodjehoun-Mestres et al., 2009; Orwa et al., 2009). The cashew tree is a branched, evergreen tree with a height range between 6-12m and diameter range of 4-12m (Orwa et al., 2009). Cashew trees are able to grow in regions with the following characteristics: altitude of 0-1000m; average annual temperature of 17ºC to 38ºC; average annual

rainfall of 500-3,500mm; humidity between 65%-80%; wind of 2-25km/hr and sandy soils (Ramiakajoto, 2001; Orwa et al., 2009). The worldwide growth of cashew is shown in Figure 2.9.

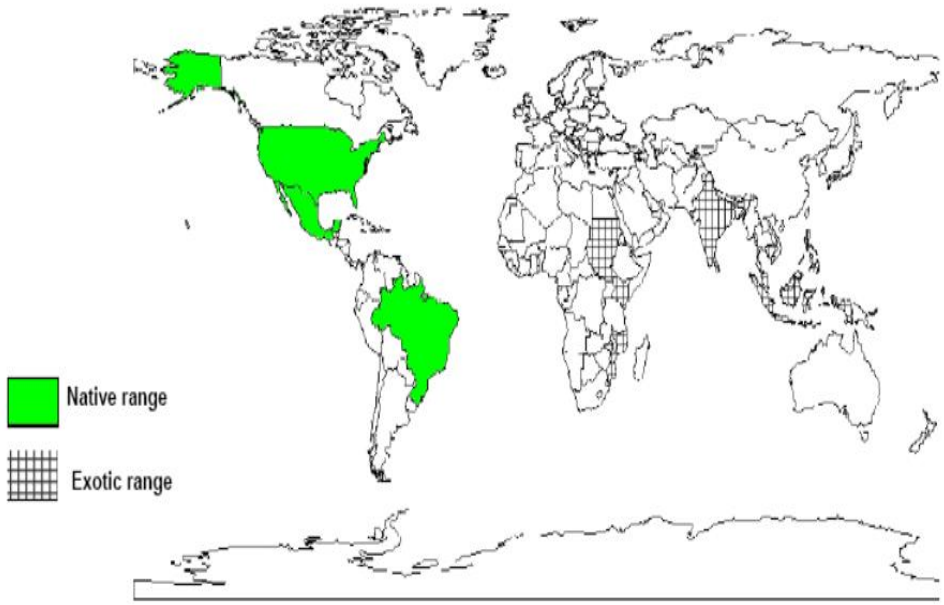


Figure 2.9 Map of native and exotic cashew growth regions in the world (*Source:* Orwa et al., 2009).

The cashew is a native tree of North and South America and is believed to have originated in the *cerrados* of central Brazil and the *restinga* of North-eastern Brazil and has since been planted in a number of regions such as Cambodia, Gambia, India, Indonesia, Kenya, Malaysia, Mozambique, Myanmar, Philippines, Sri Lanka, Sudan, Tanzania, Thailand, Uganda and Vietnam (Fig 2.9) during the fifteenth and sixteenth century by Portuguese and Spanish explorers (Martin et al., 1997; Ramiakajoto, 2001; Asogwa et al., 2008; Hammed et al., 2008; Orwa et al.,

2009). The cashew nut and cashew apple (CA) are the two morphological parts of interest from the cashew tree. The cashew nut and CA are the fruits of the cashew tree, with the nut referred to as the true fruit and the apple or peduncle referred to as the false fruit (Ramiakajoto, 2001). These fruits are attached structures as shown in Figure 2.10 and as the cashew nut grows, the CA grows.

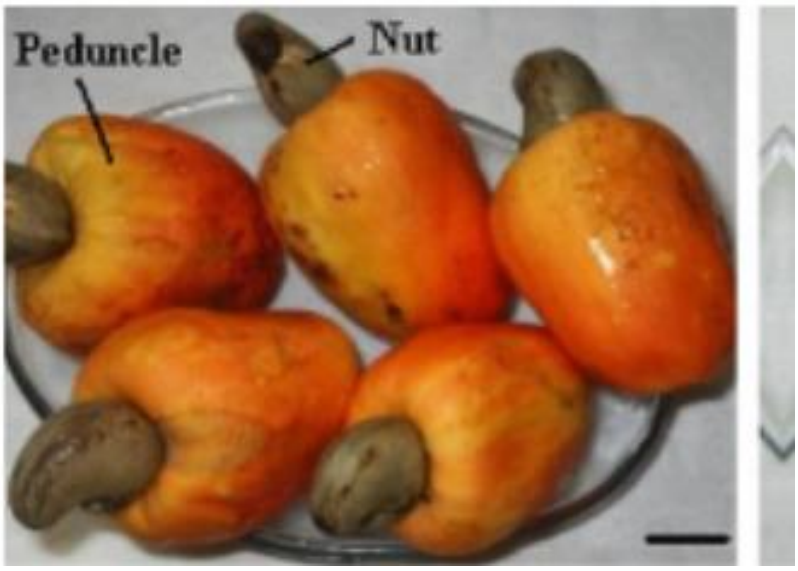
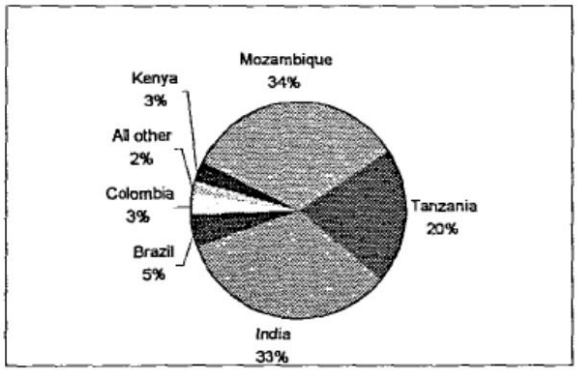


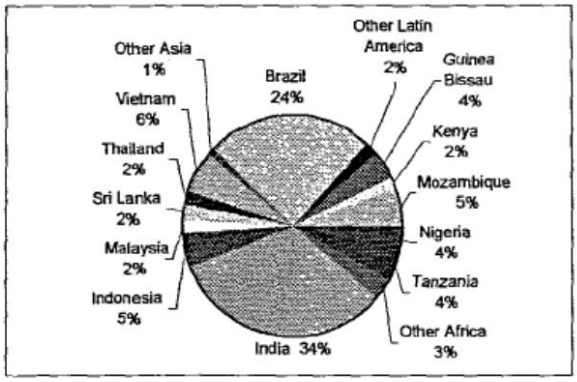
Figure 2.10 The cashew fruits: cashew nut and cashew apple (*Source*: Santos et al., 2007).

The cashew nut is kidney-shaped (Fig 2.10) with an exterior, hard shell and interior white kernel (Fig 2.10). The average weight of the nut is between 2.3-30g, the average length is between 2.5-4.0cm and the average width is between 2-3cm. Roasted cashew nuts are a popular worldwide food product and is rich in phosphorus, magnesium, iron, vitamins A, D, K and E, proteins and linoleic acids (Ramiakajoto, 2001). The CA (Fig 2.10) is a hard, pear-shaped, green fruit that

turns red, yellow or orange during maturation (MacLeod and Gonzalez de Troconis, 1982; Assuncao and Mercadante, 2003; Rocha et al., 2006; Pacheco et al., 2010). It is a sour, astringent fruit during early development and as it matures the CA becomes juicy and fibrous. The CA is rich in sugar, riboflavin, vitamin C, iron, tannins, minerals and organic acids (Ramiakajoto, 2001; Trevisan et al., 2006; Santos et al., 2007; Karuppaiya et al., 2010). The average weight of the apple is 5 to 10 times larger than the nut (Akinwale 1999; 2000; Oduwole et al., 2001). During initial growth stages, growth of the CA is slow and swells into a flesh-like fruit at the base of the nut during the final growth stages (Ramiakajoto, 2001; Orwa et al., 2009; Kuila et al., 2011). Economically, the cashew nut is the main commercial product and is the worlds second largest trade nut after almonds, whilst a small amount of products are derived from the CA (Poulton, 2006; Santos et al., 2007; Michodjehoun-Mestres et al., 2009). The majority of cashew nuts are produced in India, Brazil, SSA and Vietnam (Ramiakajoto, 2001; Poulton, 2006). Data on the cashew nut percentage production capacity of various world regions dates back to 1969 (Ramiakajoto, 2001).



(a)



(b)

Figure 2.11a-b Worldwide cashew nut producing regions between 1969-1971 (a) and 1989-1991 (b) (*Source:* Ramiakajoto, 2001).

As shown in Figure 2.11a-b, during the years 1969 to 1971, Mozambique, India and Tanzania were the leading cashew nut producers (Fig 2.11a). During the years 1989 to 1991, India and Brazil were the leading producers followed by Vietnam and there was a rapid drop in production in SSA (Fig 2.11b). In addition to the cashew nut, oil or liquid extracted from the cashew nut shell is a useful product (Poulton, 2006; Santos et al., 2007; Michodjehoun-Mestres et al., 2009). The cashew nut shell liquid (CNSL) is an export product and examples of CNSL

derived products are resins, friction powders and brake fluid for motor vehicles, paints and cement (Poulton, 2006; Trevisan et al., 2006; Santos et al., 2007). CNSL also has attractive biological activities such as antimicrobial activity, molluscicidal activity and inhibition of α-glucosidase, invertase and aldose reductase (Trevisan et al., 2006). The CA is usually a neglected product and if utilized, CA-derived products are fruit juices, vinegar, jam, syrups, ice-cream and cashew wine (MacLeod and de Troconis, 1982; Assuncao and Mercadante, 2003; Trevisan et al., 2006; Santos et al., 2007; Karuppaiya et al., 2010; Thiripurasundari and Usharani, 2011). In 2006 the estimated worldwide CA production was 30 million metric tons. The leading CA producing regions are Vietnam: 8.4 milliom tons, Nigeria: 5 million tons, India: 4 million tons, Brazil: 1.6 million tons and Indonesia: 1 million tons (Santos et al., 2007; Michodjehoun-Mestres et al., 2009; Karuppaiya et al., 2010). In addition to food and industrial products, the cashew also contributes to fodder; fibre; timber; inks; insect repellent and medicine (Orwa et al., 2009). After cashew nut removal the shell is used as animal fodder. As a fibre, the tree pulp is used to make cardboard boxes. The tree wood is used to make boats and furniture in the timber industry. The bark sap and bark gum is used to produce ink and insect repellent or adhesives, respectively (Ramiakajoto, 2001; Orwa et al., 2009). Medicinal applications are vast such as: (a) treatment of ulcers from cashew tree bark, (b) flower buds and young leaves for skin diseases, bronchitis and diabetes, (c) relief of coughs from cashew apple juice syrup, (d) CNSL for cholera and kidney treatments, (e) fatty acids from the cashew nut can lower cholesterol, (f) anarcardic acid and anacardol of the CNSL acts against Walker carinosarcoma, (g) the tree root is rich in

calcium and can be used for calcium deficiency, and (h) tannins from the CA assists in chronic dysentery (Ramiakajoto, 2001; Lowor and Aygente-Badu, 2009; Orwa et al., 2009; Kuila et al., 2011).

2.4.2 Cashew Industry and Sub-Saharan Africa

Mozambique and Tanzania (Fig 2.11a) were the leading cashew producers and exporters of the cashew nuts in SSA. However, over the years the cashew market in SSA has declined but not ceased. Cashew nut export from Mozambique and Tanzania is present on a small scale and heavily supported by trades with India. This on-going trade partnership with India has kept the cashew industry alive in Africa, together with the involvement of Guinea-Bissau, Benin, Ivory Coast, Nigeria and Ghana (Poulton, 2006). In Mozambique, cashew harvesting began during the 1930s but it was during the 1960s to 1970s when the Mozambican cashew industry became a major exporter of cashew nuts. Approximately 150,000 tons of cashew nuts were produced in factories owned by Portuguese and Asian settlers and 149,800 tons of these nuts were exported (Cramer, 1999; Poulton, 2006). When Mozambique gained independence during 1975, the Portuguese and Asian traders left and the "Caju de Mozambique" (Cramer, 1999; Poulton, 2006); which was a state organization took control of the cashew industry and ceased cashew nut exports and this caused a downfall in the Mozambique cashew industry and international trade industry (Cramer, 1999; Poulton, 2006). In the early 1990s, the Economic Recovery Programme and ENACOMO assisted in reinventing the cashew industry in Mozambique and has since privatised factories and removed the ban on cashew nut exports (Cramer, 1999; Poulton, 2006). In

Tanzania, cashew nut exports began in the year 1938 with 210 tons exported to India. By the year 1973, the production capacity of cashew nuts reached 145,000 tons and like Mozambique, Tanzania became one of the leading cashew countries in SSA (Fig 2.11a) (Martin et al., 1997; Poulton, 2006). The decline in cashew nut production from 145,000 tons to 16,500 tons was during the 1980s when the "Ujamaa" (Martin et al., 1997; Poulton, 2006) government policy moved local, smallholder farmers into new living areas and this resulted in the neglect of many cashew plantations and the fungal disease, powdery mildew attacked and destroyed the cashew trees (Martin et al., 1997; Poulton, 2006). In addition, the decline in production was contributed by investment in new factories but no production was noted in these factories and the production price decreased from 70% to 24% (Martin et al., 1997; Ngatunga et al., 2003; Poulton, 2006). The recovery of the cashew industry in Tanzania was directed by the United Kingdom and Italian governments, where attempts were made to treat the fungal disease by sulphur spraying and identifying cashew tress that were resistant to the disease. The sulphur treatment was applied when the cashew trees were flowering to prevent attack of young leaves, shoots and buds and to prevent poor quality cashew nuts (Martin et al., 1997; Ngatunga et al., 2003, Poulton, 2006). The attempts were purely research based from international involvement and the Tanzanian government provided funds and together with an increase in the price of cashew nuts, the Tanzanian cashew industry improved (Martin et al., 1997).

In addition to Mozambique and Tanzania, the cashew market in Nigeria is on the rise with an estimated contribution of 14% to the total world production (Ngatunga et al., 2003; Hammed et al., 2008). Early on in Nigeria, the cashew tree

was planted for erosion and afforestation purposes. By the year 1953, planting of the cashew tree was on a larger scale to include cashew nut production and in the year 2000 cashew nut production increased from 25,000 tons to 176,000 tons. This is greater than the production capacity of Mozambique and Tanzania as shown in Table 2.10 (Asogwa et al., 2008; Hammed et al., 2008).

Table 2.10 Cashew nut production in various world regions between 1970-2000 (*Source*: Hammed et al., 2008).

Continent/Country	2000	1998	1995	1990	1985	1980	1975	1970
World	1,217,210	1,070,774	944,070	606,681	520,973	464,215	563,795	511,939
Africa	432,955	405,271	274,971	125,745	114,795	162,502	358,035	345,772
Asia	602,237	614,037	494,378	374,427	283,004	218,920	179,016	137,039
South and Central America	176,018	45,466	168,721	102,913	119,226	78,394	23,002	22,813
Nigeria	176,000	152,000	95,000	30,000	25,000	25,000	25,000	25,000
Tanzania	106,500	93,200	63,400	17,060	32,750	41,416	115,840	107,445
Mozambique	35,000	51,716	33,423	22,524	25,000	71,100	188,000	184,000
India	440,000	440,000	321,640	285,500	221,330	180,266	144,254	123,319
Indonesia	69,027	69,027	74,995	29,907	21,114	9,074	9,122	-
Vietnam	41,200	54,000	52,800	26,000	9,000	5,600	3,500	2,100
Brazil	167,123	39,836	164,156	99,367	115,000	75,000	20,490	20,309
El-Salvador	4,000	4,000	3,000	2,098	2,162	2,208	1,702	1,700

The cashew industry in Nigeria continues to thrive and the success is due to: (i) the involvement of entrepreneurs, (ii) the government, (iii) cooperative societies and farmers, (iv) the cultivation of cashew trees in twenty-seven Nigerian states, (v) increased land availability from 40,000ha to 320,000ha for cashew harvesting, (vi) introduction of the Brazilian cashew biotype which produces cashew nuts of an average weight of 16g, and (vii) export of cashew nuts to India, Vietnam and Brazil (Asogwa et al., 2008). In South Africa, there is a cashew nut plantation in the Maputaland area in the North-East region of Kwa-Zulu Natal province. Collaboration between CSIR, The Maputaland Development and Information Centre, DST and The Kellogg Foundation are involved in the production of dried

fruit; jam; fruit juices; spirit coolers and liquors from cashew apples (CAs) Additionally, improvement of the Maputaland Cashew Plantation is under implementation to provide equipment and create jobs for the local community (Lalloo and Thema, 2007).

2.4.3 Cashew Apple and Industrial Applications

As reviewed, the main product of the cashew tree is cashew nuts as a food source. Other than the use of the cashew nut in the food industry, the CA and cashew apple juice (CAJ) has numerous industrial applications. Examples of these applications are: (a) sugar separation from CAJ, (b) beverage production, (c) evaluation of yeast strains for ethanol and sugar tolerance, (d) detection of antioxidant properties, (e) improving nutritional quality of other fruit juices, (f) vinegar production, (g) lactic acid production, (h) biosurfactant production, (i) dextransucrase production, (j) oligosaccharide production, and (k) bioethanol production (Akinwale 1999;2000; Osho, 2005; Rocha et al., 2006; Trevisan et al., 2006; Chagas et al., 2007; Honorato et al., 2007; Luz et al., 2008; Pinheiro et al., 2008; Attri, 2009; Neelakandan and Usharani, 2009; Rabelo et al., 2009; Honorato and Rodrigues, 2010; Pacheco et al., 2010; Silveira et al., 2010; Vergara et al., 2010; Araujo et al., 2011; Makkar et al., 2011; Shenoy et al., 2011; Thiripurasundari and Usharani, 2011).

For sugar separation, pure glucose and fructose can be extracted from CAJ by adsorptive chromatography and used for the production of syrups. This type of syrup is an alternative to the present syrup supply from corn hydrolysis (Luz et al., 2008). Cashew wine, an alcoholic beverage is produced from the fermentation of

CAJ and in Goa, India it is referred to as *fenni* (Attri, 2009). For cashew wine production, the CAJ is fermented using *S. cerevisiae* yeasts at temperatures between 28ºC to 30ºC and pH of 4.0. Alcohol content between 6% (v/v) to 10.6% (v/v) is possible (Akinwale, 1999; Araujo et al., 2011). Yeast tolerance of yeast species isolated from fermenting CAJ was determined in a study by Osho (2005). Seventeen Saccharomyces strains were isolated and four of these strains, namely *S. cerevisiae* strains 0271, 0269, 0260 and *S. uvarum* 0275 show alcohol tolerance ranging from 9% (v/v) to 12% (v/v). These yeasts are also able to grow in various glucose concentrations from 10% (w/v) to 25% (w/v) and respond well to osmotic stress (Osho, 2005). Anacardic acids, which are an alkyl phenol of the CA, possess antioxidant activity by inhibiting superoxide anion and xanthine oxidase (Trevisan et al., 2006). This acid has been shown to have a greater antioxidant capacity then trolox, salicyclic acid and hydroxytyrosol (Trevisan et al., 2006). Additionally, the phytyl side chain of anacardic acid can be used as a Vitamin E substitute in body products (Trevisan et al., 2006). The main nutritional component of CA is vitamin C. It was found that CA has a greater concentration of Vitamin C than other fruits such as oranges, grapes, mangoes, lemons and pineapples (Akinwale, 2000). The CAJ was blended with orange, pineapple, grape and mango fruit juices to increase the Vitamin C concentration of these commercial fruit juices (Akinwale, 2000). The flavour properties of the alternate juices were accepted by consumers and thus CAJ can be mixed with other fruit juices to improve the Vitamin C quality (Akinwale, 2000). Vinegar, as a fermentation product from CAJ is useful in the food industry. The vinegar product as a result of fermentation is a natural product rather than a synthetic product.

This natural vinegar is a rich source of amino acids acquired from the fruit juice (Thiripurasundari and Usharani, 2011). The process of vinegar production from CAJ involves the action of *S. cerevisiae* yeasts on the sugary juice substrate to ethanol, followed by the conversion of the ethanol by acetic acid bacteria to produce acetic acid or vinegar (Thiripurasundari and Usharani, 2011). The production of lactic acid from fermentation of CAJ is of interest, as lactic acid can be used in the preservation of food products, especially dairy products and in the manufacture of polylactic acid biopolymers for food packaging (Silveira et al., 2010). For lactic acid fermentation, the CAJ is fermented by the action of lactic acid bacteria (LAB) such as *Lactobacillus bulgaricus, Lactobacillus leichmanii, Lactobacillus delbrueckii, Lactobacillus amylophilus,* and *Lactobacillus plantarum*. A lactic acid yield of 95% (2.3g/L/h) is possible under fermentation conditions of temperature=37ºC, pH=6.5 and sugar concentration=50g/L (Silveira et al., 2010). Another application of CAJ fermentation is for the production of biosurfactants (Rocha et al., 2006; Makkar et al., 2011). Surfactants are used in household detergents and are derived from petrol. Biosurfactants, on the other hand are alternative products that are derived from plant biomass or vegetable oils by the use of MOs (Rocha et al., 2006; Makkar et al., 2011). Structurally, biosurfactants are made up of hydrophilic and hydrophobic components. The hydrophilic component consists of amino acids and polysaccharides and the hydrophobic component consists of lipids (Makkar et al., 2011). The structure develops from the type of material and processing conditions. This structure enables the biosurfactant molecule to function as emulsifying, foaming and detergent agents (Rocha et al., 2006; Makkar et al., 2011). There is a market for

biosurfactants as they are less toxic products, are environmentally safe and degradable; however due to high costs there is minimal production and usage of biosurfactants (Rocha et al., 2006; Makkar et al., 2011). For this reason, Rocha et al. (2006) demonstrated the use of CAJ as a less expensive substrate to provide a biosurfactant. CAJ was fermented at a temperature of 30ºC and pH of 7.0, with the action of *Acinetobacter calcoacetius* to produce an emulsan biosurfactant. The study revealed the microorganism (MO) produced the biosurfactant as a by-product during the stationary growth phase, when the sugar concentration decreased. The emulsifying activity was found to be 58.8% (Rocha et al., 2006). Other than biosurfactants, CAJ as a fermentation medium can be used to produce dextransucrase and oligosaccharides Dextransucrase can be used as a food preservative and oligosaccharides can be used as a prebiotic (Chagas et al., 2007; Honorato et al., 2007; Rabelo et al., 2009; Honorato and Rodrigues, 2010; Vergara et al., 2010). Dextransucrase is an enzyme produced by the LAB, *Leuconostoc mesenteroides*. This enzyme is a glycosyltransferase that catalyses reactions of glycosyl residues between donor and acceptor molecules to produce fructose, mannitol, dextran, oligosaccharides and polymers (Chagas et al., 2007; Honorato et al., 2007; Rabelo et al., 2009; Honorato and Rodrigues, 2010; Vergara et al., 2010). For dextransucrase production, the CAJ medium was supplemented with sucrose, yeast extract and potassium phosphose followed by fermentation at a temperature of 30ºC, pH of 6.5 and LAB strain *L. mesenteroides* B-512F (Chagas et al., 2007). The study showed that high dextransucrase activity and microbial growth was possible and hence CAJ is a useful substrate for the production of dextransucrase and the supplementation of the CAJ with other

nutrients initiates the enzyme production (Chagas et al., 2007). Additionally, LAB such as *L. citreum* B-742 and *L. mesenteroides* B-742 can be used for dextransucarse production and fructose which is released can be further reduced to mannitol. Mannitol can be used as a sweetener or "texting agent" (Honorato et al., 2007) in food products (Honorato et al., 2007; Rabelo et al., 2009; Vergara et al., 2010). For the production of oligosaccharides, sucrose is also added to the CAJ and the native sugars of CAJ, which are glucose and fructose, serve as acceptor molecules during enzyme reactions to produce oligosaccharides (Honorato et al., 2007; Rabelo et al., 2009; Honorato and Rodrigues, 2010). CAJ fermentation conditions to produce oligosaccharides were temperature=32ºC and pH=6.7. The concentration range of oligosaccharides were 1.80 g/L to 9.30g/L and additionally mannitol was produced with a concentration range of 5.60g/L to 17.44g/L (Honorato et al., 2007). For bioethanol production, CAs and cashew apple bagasse (CAB) has been investigated (Pinheiro et al., 2008; Neelakandan and Usharani, 2009; Pacheco et al., 2010; Shenoy et al., 2011). The use of CAs in bioethanol technology is relatively new and simple procedures are employed to obtain ethanol. In general, CAJ is extracted from CAs and the monosaccharide sugars, namely glucose and fructose are fermented by yeasts. First, CAJ is pre-treated by adding gelatine powder for the precipitation of tannins and juice solids at 4ºC. The CAJ is then filtered or centrifuged and sterilized for fermentation (Pinheiro et al., 2008; Neelakandan and Usharani, 2009). Pinheiro et al. (2008) showed that 44.40g/L of bioethanol can be produced and Neelakandan and Usharani. (2009) showed that 9.35%(v/v) of bioethanol was possible using immobilized yeast cells. The application of cashew apple bagasse (CAB) for bioethanol production has

also been investigated. CAB is the waste product or residue of the CA after CAJ is extracted (Pacheco et al., 2010). Pacheco et al. (2010) described the use of CAB as the support network for yeast cell immobilization, using CAJ as the fermentation substrate. From the methodology used in the study by Pacheco et al. (2010), the general preparation of CAB prior to immobilization was as follows: (1) washing of CAB and drying at 50ºC, (2) acidic treatment at 60ºC for 2.5hrs with 3% HCl, (3) washing and drying to remove acid, (4) alkali treatment for 24hrs with 2% NaOH, (5) sterilization of treated CAB at 110ºC for 10min, (6) addition of synthetic medium (glucose, yeast extract, nutrients) to CAB, (7) inoculation of CAB-synthetic medium with yeast cells, (8) fermentation at 30ºC for 24hrs, (9) centrifugation of fermentation product and (10) the supernatant with CAB-immobilized cells are washed and used for subsequent fermentations (Pacheco et al., 2010). For fermentation, 12.5g of CAB-immobilized cells were added to CAJ and fermented at: temperature of 30ºC; pH of 4.50 and agitation of 150rpm. The bioethanol concentration obtained was 36.91g/L and the immobilized cells were stable for up to ten continuous fermentations (Pacheco et al., 2010). Other than yeast immobilization, CAB can be fermented to produce bioethanol. For CAB fermentation, the CAB is pre-treated by the addition of 2% H_2SO_4. This acidic mixture is kept at 120ºC for 10min and then at 90ºC for 1.5hrs. Following the acidic treatment, the mixture is filtered and titrated with calcium hydroxide until the pH of the mixture is 6.0. The available sugar from the CAB is then fermented at a temperature of 30ºC, pH of 6.0 and agitation of 120rpm, to yield 0.5g of bioethanol per gram of sugar (Shenoy et al., 2011).

2.5 The Application of Bioethanol

As reviewed in Section 2.1, bioethanol as a transport fuel has great potential. The application of bioethanol, specific to the motor-fuel industry is directed towards its usage in a blend or mixture with conventional petrol in order to power ICEs. The usage of fuel ethanol was initiated as early as 1930, but it was during 1970 to 1980 when production of bioethanol from starch increased and the addition of this product to petrol as an oxygenate instead of methyl tertiary-butyl ether (MTBE) was implemented (Renzoni et al., 1985; Lonnon and Hook, 2003; Niven, 2005; da Silva et al., 2005; Barabas et al., 2010). Blending ratios range from E5 to E85, where the ethanol portion is 5% (v/v) and the petrol portion is 95% (v/v) or 85% (v/v) ethanol and 15% (v/v) petrol. Presently, the US, EU and Brazil insist of the utilization of blends in motor vehicles. In the US, blends of 10% (v/v) to 15% (v/v) are used. In the EU, 5% (v/v) of ethanol is used with a possible increase to 10% (v/v). In Brazil, the ethanol volume expected is between 15% (v/v) to 25% (v/v) (da Silva et al., 2005; Muzikova et al., 2009; Paasi et al., 2009; Walker, 2011). The advantages of bioethanol/petrol blends are: (i) octane enhancer when used in ICEs to reduce cylinder knocking, (ii) alternate oxygenate to MTBE and less toxic to the environment, (iii) E10 blends are known to decrease carbon monoxide, benzene, toluene and xylene exhaust emissions by 10-12.5%, (iv) lower energy efficiency and lower volatility during storage and fuelling at petrol stations, (v) reduction in “unburned hydrocarbons” (da Silva et al., 2005), and (v) blends of 5% -24% (v/v) can be used in current engines (Rajan and Saniee, 1983; Jankowski and Sandel, 2003; Niven, 2005; da Silva et al., 2005; Paasi et al., 2009; Barabas et al., 2010). The disadvantages of ethanol/petrol blends are: (i) ethanol

solubility in water is greater than in petrol which leads to ethanol/petrol phase separation and water/ethanol complexes, (ii) difficulty in preventing moisture entering storage tanks of blends, (iii) E20 blends and higher can increase carbon monoxide and nitrogen oxide emissions, (iv) formation of water/ethanol complexes can corrode the storage tanks and damage the engine, (v) sustainability of blends is difficult as in order to add the recommended bioethanol volumes to petrol, bioethanol sustainability is necessary and (vi) the bioethanol product requires distillation and further intensive processing to decrease its water content (Barton and Tjandra, 1989; Poulopoulos, et al., 2001; Jankowski and Sandel, 2003; Niven, 2005; Muzikova et al., 2009). The majority on ethanol/petrol blend research includes the chargeability and conductivity; volatility and phase stability; and the effect of a cosolvent in the blend (Barton and Tjandra, 1989; Muzikova et al., 2009; Paasi et al., 2009). The research by Paasi et al. (2009) investigated the conductivity and chargeability of neat (unblended) petrol versus bioethanol/petrol blends during fuelling of a motor vehicle petrol tank. To measure the conductivity and chargeability, direct current with the use of electrodes and an electrostatic voltmeter was used, respectively (Paasi et al., 2009). Neat petrol, E10 and E85 blends were tested. The data showed, conductivity increases when ethanol is added to petrol from 550pS/m (neat petrol) to 15,000pS/m (E10) and 23,000 000pS/m (E85). The chargeability decreased from -60V (neat petrol) to -10V (E10) and 0V (E85) (Paasi et al., 2009). This indicates that fuelling motor vehicles with blends is safe and a higher blend concentration exhibits no charge due to its high conductivity (Paasi et al., 2009). Muzikova et al. (2009) investigated the volatility of the blends by characterizing the Reid vapour pressure (RVP) and the

stability of the blends in respect to the water content. The RVP was detected according to ASTMD6378 and water content by Ince and Kirlsbar method (Muzikova et al., 2009). The RVP increased from 16.6kPa (neat petrol) to 86.4kPa (E5) (Muzikova et al., 2005). In general, the highest RVP of petrol was reported as 47kPa, dependent on the hydrocarbon composition (da Silva et al., 2005). Thus, the increase in RVP for the blend is advantageous for the antiknock property of the engine but can cause higher exhaust emissions and greater volatility (da Silva et al., 2005). Blending of ethanol with petrol at E5 caused an increase in water solubility but at a temperature of 0ºC a decrease in solubility was noticed and hence water solubility can be influenced by temperature (Muzikova et al., 2009). The effect of water on phase stability of blends can be avoided by the addition of a cosolvent. As investigated by Barton and Tjandra. (1989), the use of 1,8-cineole eucalyptus oil in a E20 blend showed phase stability at a temperature range of -30ºC to 40ºC (Barton and Tjandra, 1989).

Previous research regarding the use of blends in ICEs is directed towards the effect of blends on the engine performance and exhaust emissions (Rajan and Saniee, 1983; Poulopoulus et al., 2001; Ameri et al., 2008; Tangka et al., 2011). Tangka et al. (2011) investigated the effect of bioethanol/petrol blends from E0 to E100 on engine performance using an engine-dynamometer combination. Various aspects such as flash point, specific gravity, octane number, energy density, and vapour pressure were detected (Tangka et al., 2011). With an increase in ethanol from E10 to E100, the energy density decreases from 34.2MJ/L (E10) to 23.5 MJ/L, which can cause an increase in the consumption of fuel. Specific gravity increased from 0.7474 (E0) to 0.7890 (E100) because ethanol has a greater

density than petrol and the increasing gravity will contribute to phase separation of the blend. Flash point increased from 65ºC to 12.5ºC. The flash point temperature is indicative of the flame potential of the blend and the higher the flash point, the lower is the ignition capability and starting of the engine could be almost impossible at E100. The octane number increased from 91 (E0) to 129 (E100) and this is advantageous as premature ignition is prevented and there is steady combustion. The vapour pressure like energy density decreased from 36 (E0) to 9 (E100) which could prevent start-up of engines (Tangka et al., 2011). Overall, the study showed that blends do possess negative attributes and Tangka et al. (2011) suggests to benefit from the use of blends, engine modifications such as changing the carburettor size and piston head are necessary. Rajan and Saniee. (1983) showed that a bioethanol/petrol blend with a water content of 6% (v/v) used to fuel a four cylinder spark ignition engine can be successfully utilized, similar to using neat petrol and nitrogen oxide emissions are reduced. The initial power of the engine for the bioethanol/petrol/water blend was lower (17kW) compared to neat petrol (23kW) and as the engine speed increased, so did the power. For exhaust emissions, there was a reduction from 5,000ppm (neat petrol) to 1,000ppm (blend) of nitrogen oxide. It was suggested that the reduced emissions is due to the blend having a lower energy density and although the blends consisted of water, engine start-up was not affected (Rajan and Saniee, 1983). However, since this study was carried out in the year 1983, knowledge regarding the effect of water on engines and phase stability of blends were scarce. Even though the powering of the engine was possible, long-term usage of bioethanol/petrol/water blends can have a harmful effect on the engine. Ameri et

al. (2008) investigated the temperature and pressure of an engine using E5 to E20 blends and the carbon monoxide (CO) emissions. A four-cylinder spark ignition engine coupled with a dynamometer to measure temperature and pressure and an exhaust gas analyst to measure emission were used (Ameri et al., 2008). The data showed a reduction in CO emissions and increase in temperature and pressure. CO emissions decreased from 3% (v/v) (E5) to <2% (v/v) (E20). The temperature increased from 2150 (E5) to 2220 (E20) and pressure from 40bar (E5) to 50bar (E20). This increase will cause the heating value of the blends to increase and can improve the fuel consumption efficiency (Ameri et al., 2008). Poulopoulos et al. (2001) studied the effect of E3 and E10 blends on exhaust emissions of a four-cylinder OPEL 1.6L engine. Regulated emissions (hydrocarbons and CO) and unregulated emissions (methane, n-hexane, acetone, acetaldehyde, acetic acid, benzene, toluene, 1,3-butadiene and ethanol) were evaluated. The measurements were representative of pre-catalytic and post-catalytic treatment which was emissions at the engine and exhaust, respectively (Poulopoulos et al., 2001). The regulated emissions were decreased, more so for the E10 blend. For the unregulated emissions, acetaldehyde emissions were increased at the engine and decreased at the exhaust for the E10 blend, whereas the E3 blend showed no change. Benzene, toluene, acetic acid, acetone and 1,3-butadiene were reduced for both blends. Ethanol was emitted as an evaporated component of the blends. Hexane and methane emissions were not reduced (Poulopoulos et al., 2011).

Chapter 3

Methodology

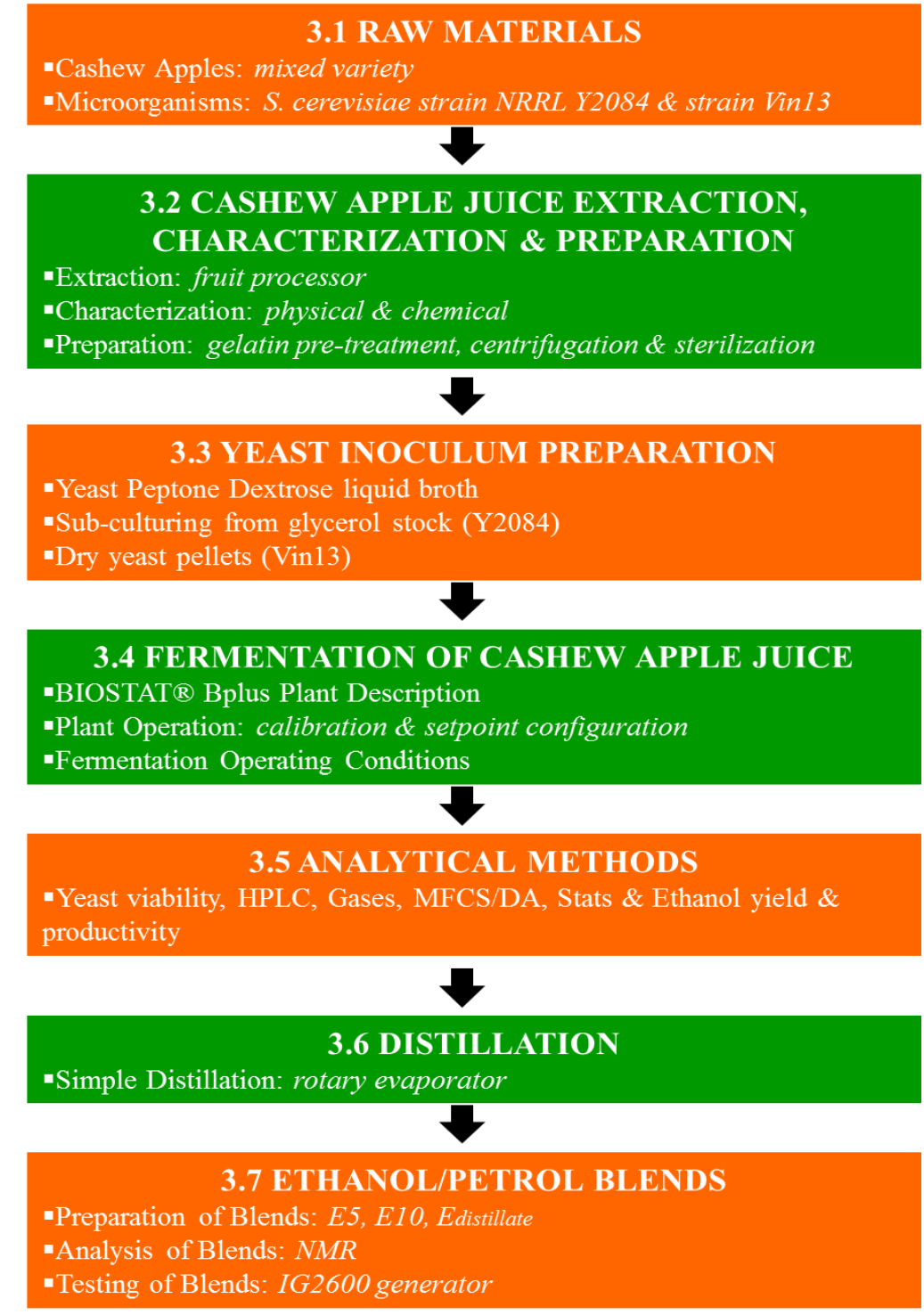


Figure 3.1 A diagram representing the outline of Chapter 3.

The methodology described was applicable for data collection during the research. The methods followed were guided by published research. Certain published methods were modified for the purpose of this research. When equipment training was undertaken for the research, the methods were described as per manufactures instructions and by the researchers own interpretation. The work presented is from the article entitled: "The Production of Bioethanol from Cashew Apple Juice by Batch Fermentation Using *Saccharomyces cerevisiae* Y2084 and Vin13" published in the journal *ISRN Renewable Energy*. Information extracted from the published work is cited as the authors and paraphrased. The contributions of this author were experimental design, execution of all experimental procedures and writing of the article. The copyrights for the use of the published work were acquired (Appendix E).

3.1 Raw Materials

3.1.1 Cashew Apples

"The cashew apples (CAs) used in this study was obtained from the Coastal Cashews plantation during the February 2011 harvest season. The plantation is located along the Coastal Forest Reserve in the rural Manguzi or KwaNgwanase region in Northern Kwa-Zulu Natal, South Africa" (Deenanath et al., 2013) and situated 18km from the Mozambican Farazele border post. "A mixed variety of CAs that did not show cashew nut attachment was selected, by the farm manager (Mr W. Mthembu) from the Coastal Cashews plantation area" (Deenanath et al., 2013). The CAs were collected and kept in a plastic container and the total weight was determined by the farm manager at the Cashew Nut Factory on the plantation.

3.1.2 Microorganisms

"Two strains of *Saccharomyces (S.) cerevisiae* yeasts were the microorganisms used in this study for fermentation. The strain *S. cerevisiae* NRRL Y2084 is a brewers yeast, and the strain *S. cerevisiae* Vin13 is wine yeast. *S. cerevisiae* NRRL Y2084 was previously isolated from dry brewers yeast, purchased from National Food Products (Emmarentia, Johannesburg, South Africa), characterized and maintained at -70°C in 50% glycerol" (Deenanath et al., 2013). Brief details on the isolation and identification procedure is outlined in Appendix D. "*S. cerevisiae* Vin13 is a product of the Wine and Biotechnology Institute, Stellenbosch and was kindly donated by Anchor Wine Yeast (Cape Town, South Africa)" (Deenanath et al., 2013). The wine yeast was supplied as dry yeast pellets. Prior to subsequent culturing for fermentation the glycerol stock (Y2084) was cultured, diluted and plated to determine if the stock was viable. The same procedure was carried out for the dry yeast pellets (Vin13) to ensure that the strain provided by Anchor Wine Yeast was not contaminated. In this study the yeasts were simply referred to as Y2084 and Vin13.

3.2 Cashew Apple Juice Extraction, Characterization and Preparation

3.2.1 Extraction

"Cashew apple juice (CAJ) extraction was done at the Cashew Nut factory on the Coastal Cashew plantation. The whole CAs was washed, to remove external contaminants and cut in half" (Deenanath et al., 2013). The apple pieces were then compressed using a fruit processor (KENWOOD) shown in Figure 3.2.

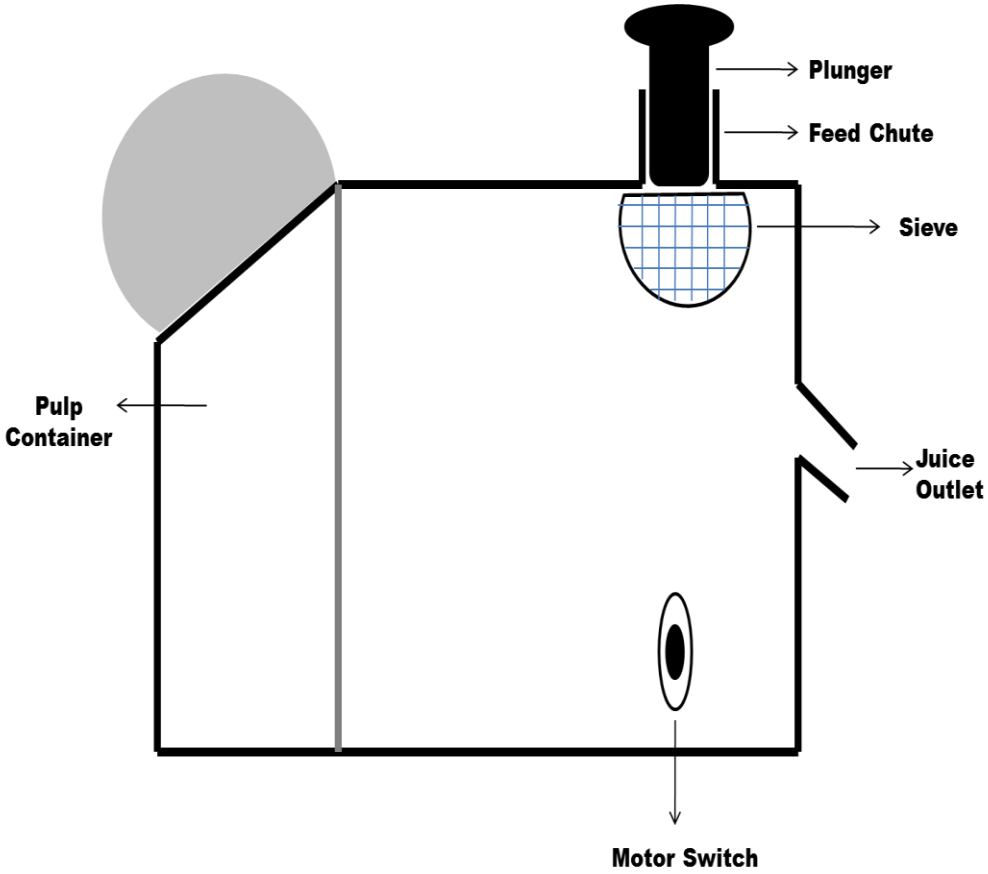


Figure 3.2 KENWOOD fruit processor.

Using the schematic diagram shown in Figure 3.2 for a visual identification, the juice extraction procedure was as follows: (1) the motor was switched ON and a collection jug was placed at the juice outlet, (2) sliced CAs were added to the feed chute, until full, (3) the plunger was used to push the CAs down the feed chute, (4) the extracted juice passed through the stainless steel sieve and flowed through the juice outlet into the collection jug, whilst the pulp accumulated in the pulp container, (5) as the extraction continued the pulp container was emptied when necessary, (6) when the collection jug was full the CAJ was transferred into re-capable plastic bottles and kept cold on ice then frozen. "During transportation to

the University of the Witwatersrand, Johannesburg, the CAJ was maintained frozen in thermal bags and embedded in ice. Upon arrival, the CAJ was stored at -20°C in a freezer available in the School of Molecular and Cell Biology, for subsequent use" (Deenanath et al., 2013). The effectiveness of the fruit processor was determined by calculating: (i) the percentage yield of juice/kg of apples, (ii) extraction capacity, and (iii) extraction efficiency percentage (Akinwale et al., 2001). The equations used were:

$$\text{(1) Percentage yield of juice/kg of apples} = \frac{\text{litres of juice}}{\text{kg of apples}} \times 100 \qquad (3.1)$$

$$\text{(2) Extraction capacity} = \frac{\text{litres of juice}}{\text{time in hours}} \qquad (3.2)$$

$$\text{(3) Extraction efficiency percentage} = \frac{\text{extraction capacity}}{\text{maximum litres extracted}} \times 100 \qquad (3.3)$$

3.2.2 Characterization

Characterization was according to the physical characteristics of the CAs and the physical and chemical characteristics of the CAJ. The physical characteristics of the CAs were based on the visual appearance of shape and colour and the physical measurement to determine the size of the apples. For the size measurements, a total number of ten apples from the total CAs collected were randomly selected and the length and width were measured in millimetres and the average values were reported. The CAJ physical and chemical characteristics were according to the following constituents: specific gravity; pH; sugar composition; condensed tannins; minerals; vitamin C and proteins. Prior to characterization the CAJ was centrifuged (BECKMAN MODEL TJ-6) at 3,500 rpm for 10min to remove

residual pulp that may have passed through the sieve during extraction. A clarified, free-flowing liquid which was cream in colour was used for characteristic analysis.

Specific Gravity

Specific gravity was a direct measurement using a hydrometer (BREWMAKER Limited). Triplicate readings were recorded.

pH

The pH was determined by direct measurement using the Hamilton pH sensor (EF-set 12/200/2K8), which is the pH probe of the BIOSTAT® Bplus fermenter (described in section 3.4.1). Triplicate readings were recorded.

Sugar Composition

Total sugar composition was determined by High Performance Liquid Chromatography (HPLC-Agilent Technologies). "The HPLC system was the LC 1100 series equipped with a solvent delivery system (quaternary pumps); autosampler; refractive index detector; wavelength detector; thermostated column compartment and ChemStation software programme. For the sugar analysis the refractive index detector (RID) and the Aminex Fermentation Monitor column (BIORAD) was used. HPLC conditions were as follows: (i) mobile phase=0.001M sulphuric acid, (ii) flow rate=0.8mL/min, (iii) column temperature=60°C, (iv) RID temperature=40°C, (v) injection volume=20uL, (vi) pressure=60 bar. The ChemStation Software programme was used to monitor the data and concentration of the CAJ was determined by constructing a calibration curve (ChemStation off-line data analysis)" (Deenanath et al., 2013) using

glucose, fructose and maltose sugar standards at a concentration of 2% (w/v). Sugar readings were recorded as six replicates.

Condensed Tannins

Condensed tannins of the CAJ were characterized by the Vanillin-HCl method (Makkar and Becker, 1993; Sun et al., 1998; Lowor and Agyente-Badu, 2009). The composition of condensed tannins was determined for the original CAJ and pre-treated CAJ (pre-treatment described in section 3.2.3). To determine the unknown tannin concentration of the CAJ sample, standard catechin solutions of known concentrations were prepared and a standard curve was constructed. The concentrations of the standard solution were: 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg/L. To achieve these concentrations a catechin stock solution of 120mg/L was prepared (Sun et al., 1998). From this stock solution the various standard solutions were prepared (Table 3.1) by dilutions using the following equation (Reed et al., 2003):

$$C_1V_1 = C_2V_2 \qquad (3.4)$$

C1: Initial Concentration (of stock solution)

V1: Initial Volume (of stock solution required)

C2: Final Concentration (of standard solution)

V2: Final Volume (of standard solution)

Table 3.1 Dilutions of standards for the catechin standard curve.

Standard Solutions (mg/L)	mL of 120mg/L catechin stock solution	mL of dH_2O (V2=10mL)
0	0	10
10	0.83	9.17
20	1.67	8.33
30	2.50	7.50
40	3.33	6.67
50	4.17	5.83
60	5	5
70	5.83	4.17
80	6.67	3.33
90	7.50	2.50
100	8.33	1.67

Once the standard solutions were prepared, a vanillin solution was prepared. The vanillin solution was 4% (w/v) solution where vanillin was dissolved in methanol (Makkar and Becker, 1993; Sun et al., 1998; Lowor and Agyente-Badu, 2009). For the CAJ samples (un-treated and pre-treated) and the standards, 10mL of the CAJ was mixed with a 50% methanol solution. This solution was stirred and incubated at room temperature for 10min (Makkar and Becker, 1993; Lowor and Agyente-Badu, 2009). For the Vanillin-HCl assay, 0.25mL of each standard (0-100mg/L) and CAJ samples were aliquot into pyrex tubes, followed by the addition of 1.5mL vanillin solution. This mixture was gently vortexed and 0.75mL of 37% HCl was added and the mixture vortexed, once again. The reaction

mixture was incubated for 20min at room temperature (Makkar and Becker, 1993; Lowor and Agyente-Badu, 2009). Post-incubation, 1mL of the reaction mixtures were dispensed into cuvettes and the absorbance was measured at 500nm (A_{500nm}) using a spectrophotometer (Spectroquant Pharo 100-Merck) (Makkar and Becker, 1993; Lowor and Agyente-Badu, 2009). Experiments were performed in triplicate for each of the standards and the samples and the measurements were noted accordingly. The A_{500nm} readings for the standards were used to construct a standard curve of A_{500nm} versus catechin concentration (mg/L). The absorbance readings of the CAJ samples were used to determine the concentration of tannins from the standard curve and expressed as mg/L of catechin equivalents from the standard curve.

Minerals

For the analysis of minerals, CAJ was digested according to the method described by Lowor and Agyente-Badu. (2009). The digestion procedure of the CAJ was as follows: (i) 5mL of 65% (w/v) nitric acid was added to 15mL of CAJ, (ii) nitric acid-CAJ mixture was incubated at 130ºC for 3hrs on a heating block (Spectroquant TR320-Merck), (iii) post-incubation the tubes were cooled and the remaining volume was transferred to a 25mL volumetric flask and made up to the final volume with dH_2O (Lowor and Agyente-Badu, 2009). Post-digestion, the minerals present in the CAJ sample were quantified by Atomic Absorption Spectroscopy (AAS). The spectrometer (Spectra AA 55B, Varian Australia Pty Ltd 1996-2000) used for the analysis was equipped with: (i) a flame ionization detector (red cone:1mm), (ii) mineral specific lamps and (iii) air/acetylene gases. Zinc, copper, manganese, magnesium, iron and sodium were the minerals

quantified. Standards of each the above mentioned minerals were prepared using 1000ppm ICP/AAS Standards (SMM Instruments). Specific concentrations of each mineral standard were prepared in 200mL volumetric flasks. Measurements were determined by using wavelength specific lamps (Table 3.2) for each type of mineral standard at a range of concentrations and an online standard curve was constructed. After measuring the absorbance of the standards, the reading of the pre-digested CAJ sample was measured and the concentration calculated in *real time* from the online standard curve. Prior to each measurement the lamps were calibrated using distilled water. Information on the concentration range and wavelength for each mineral was based on the manufacturers recommendation (Analytical Methods Handbook, 1989) and is shown in Table 3.2. The specific concentrations of the standards used in this study are shown in Table 3.3. Experiments for the digestion were performed in triplicate and CAJ readings were recorded in triplicate. The results were expressed as ppm.

Table 3.2 Mineral standards and their concentration range at each respective wavelength.

Mineral Standard	Concentration Range (ug/mL)	Wavelength (nm)
Zinc	0.01-2	213.9
Copper	0.03-10	324.8
Manganese	0.02-5	279.5
Magnesium	0.003-1	285.2
Iron	0.06-15	248.3
Sodium	0.002-1	589.0

Table 3.3 Mineral standards and concentrations used for measurements.

Mineral Standards	Concentrations (ppm)
Zinc	0.50
	1.00
	1.50
	2.00
Copper	2
	4
	6
	8
Manganese	0.5
	1.00
	1.50
	2.00
Magnesium	0.25
	0.50
	0.75
	1.00
Iron	2
	4
	6
	8
Sodium	0.25
	0.50
	0.75
	1.00

Vitamin C

Vitamin C of the CAJ was determined by iodine titration as described by Lowor and Agyente-Badu. (2009), with modifications. The iodine titration method required preparation of the following solutions: (1) 1% starch indicator solution, (2) 3M sulphuric acid solution, (3) iodine solution and (4) vitamin C standards. To

determine the unknown vitamin C concentration of the CAJ sample, standard ascorbic acid solutions of known concentrations were prepared and a standard curve was constructed. The concentrations of the standard solutions were 0.5, 1, 1.5 and 2g/L of ascorbic acid. To achieve these concentrations a stock solution of 5g/L was prepared. From this stock solution the various standard solutions were prepared (Table 3.4) using the equation: C1V1=C2V2.

Table 3.4 Dilutions of standards for the ascorbic acid standard curve.

Standard Solution g/L	mL of 5g/L Ascorbic Acid Stock Solution	mL of dH_2O (V2=50mL)
0	0	50
0.5	5	45
1	10	40
1.5	15	35
2	20	30

The titration assay was carried out for the Ascorbic Acid standards (0-2g/L) and CAJ sample. For the assay, 25mL of each standard and sample was aliquot into a 150mL Erlenmeyer flask and a few drops of starch indicator solution were added. This mixture was placed onto a stirrer and titrated with the iodine solution, using a graduated burette until a persistent blue colour was noticed and the volume of titrant was recorded (Silva et al., 1999; Lowor and Agyente-Badu, 2009). Experiments were performed in triplicate for the standards and sample and volume measurements were noted accordingly. The volume recorded for the standards were used to construct a standard curve of volume of titrant (mL) versus ascorbic acid concentration (g/L). The volume of titrant recorded for the CAJ

sample was used to determine the amount of ascorbic acid from the standard curve and the concentration was expressed as mg/100mL.

Proteins

Protein composition was determined by the Bradford Method, also known as the Coomassie Blue assay (Boyes et al., 1997, Reed et al., 2003). The Coomassie Blue assay required the preparation of a dye and standard solutions of bovine serum albumin (BSA) to determine the unknown protein content of the CAJ. The concentrations of the standard solutions were: 5, 10, 15, 20, 25 and 30mg/L of BSA. To achieve these concentrations a stock solution of 250mg/L of BSA was prepared (Boyes et al., 1997). From this stock solution the various standard solutions (Table 3.5) were prepared using the equation: C1V1=C2V2.

Table 3.5 Dilutions of standards for the bovine serum albumin standard curve.

Standard Solutions (mg/L)	mL of 250mg/L Stock Solution	mL of dH_2O V2=5mL
0	0	5
5	0.1	4.9
10	0.2	4.8
15	0.3	4.7
20	0.4	4.6
25	0.5	4.5
30	0.6	4.4

The Coomassie Blue assay was carried out for the BSA standards (0-30mg/L) and the CAJ sample. For the assay, 3mL of the Coomassie Blue dye was added to

100uL of the BSA standards and CAJ sample and mixed by gentle vortexing. The reaction mixture was incubated for 30mins at room temperature. Post-incubation, 1mL of the reaction mixtures were dispensed into cuvettes and absorbance was measured at 595nm (A_{595nm}) using a spectrophotometer (Boyes et al., 1997). Experiments were carried out in triplicate for the standards and sample and measurements were noted accordingly. The A_{595nm} readings for the standards were used to construct a standard curve of A_{595nm} versus BSA concentration (mg/L). The absorbance readings of the CAJ sample were used to determine the amount of proteins from the standard curve. When the absorbance readings of the CAJ sample were >1.000, serial dilutions of the sample was carried out. Protein composition was expressed as g/L.

3.2.3 Preparation

"CAJ was prepared, prior to each fermentation by pre-treatment, centrifugation and sterilization. Pre-treatment involved the addition of 1% (w/v) gelatine powder to the raw CAJ and maintained at 4°C for 24hrs. This was followed by centrifugation at 3,500rpm for 20min, addition of 2.5g/L of ammonium sulphate and sterilization at 121°C for 15min. The CAJ was sterilized in conjunction with the fermenting vessel" (Deenanath et al., 2013).

3.3 Yeast Inoculum Preparation

“Inoculum of the yeast species (Y2084 and Vin13) were prepared in sterilized yeast peptone dextrose (YPD) liquid broth as shown in Figure 3.3. The composition of YPD in g/L was: 10g yeast extract powder, 20g peptone powder and 20g D-glucose” (Deenanath et al., 2013).

Figure 3.3 Yeast Peptone Dextrose liquid broth.

“The culture conditions (Labcon bench top incubator) were as follows: (i) volume=200mL, (ii) temperature=30°C, (iii) agitation=150rpm, (iv) duration=18hrs. The yeast strain Y2084 was activated by extracting the glycerol from the glycerol stock (Fig 3.4a) and re-suspending the pellet in 1mL of YPD broth. This suspension was added to 50mL of YPD liquid. The cell count was increased by sub-culturing at 10% every 18hrs from 50mL to 200mL. For the yeast strain Vin 13, 1g of dry yeast pellets (Fig 3.4b) was added to 200mL of YPD liquid broth. After cultivation, the cell viability was determined (described in

section 3.5.1) and 10% of the initial fermentation volume was used as a starter culture" (Deenanath et al., 2013).

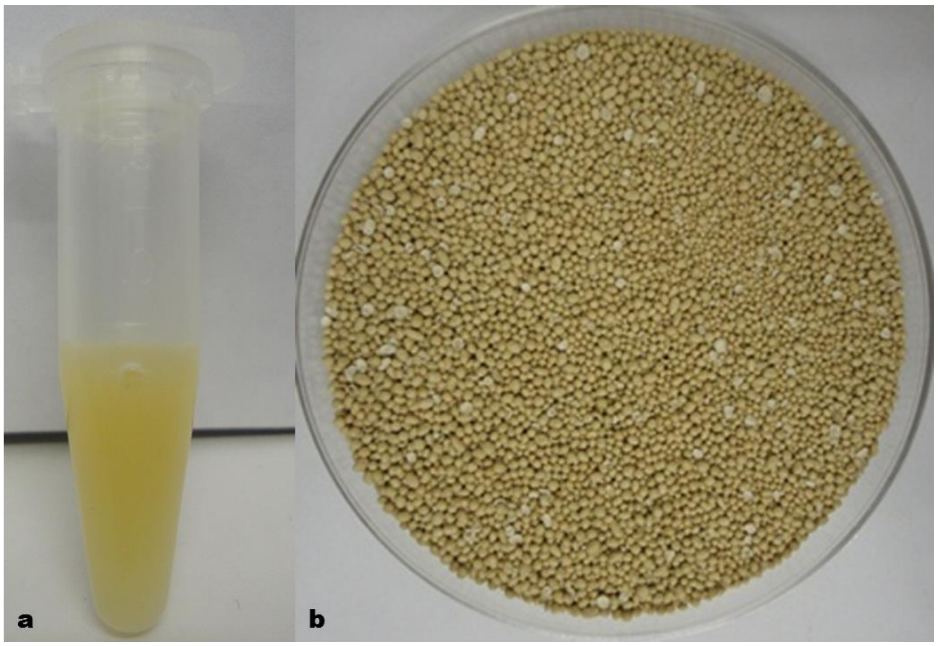


Figure 3.4a-b Glycerol stock of Y2084 (a) and dry yeast pellets of Vin13 (b).

3.4 Fermentation of Cashew Apple Juice

3.4.1 Lab-Scale Plant Description

"A BIOSTAT® Bplus 2L MO-O_2 fermentor (Sartorius Stedim Systems GmBH, Germany) was used for the fermentation of cashew apple juice to produce bioethanol" (Deenanath et al., 2013). The lab-scale set-up of the plant is shown in Figure 3.5.

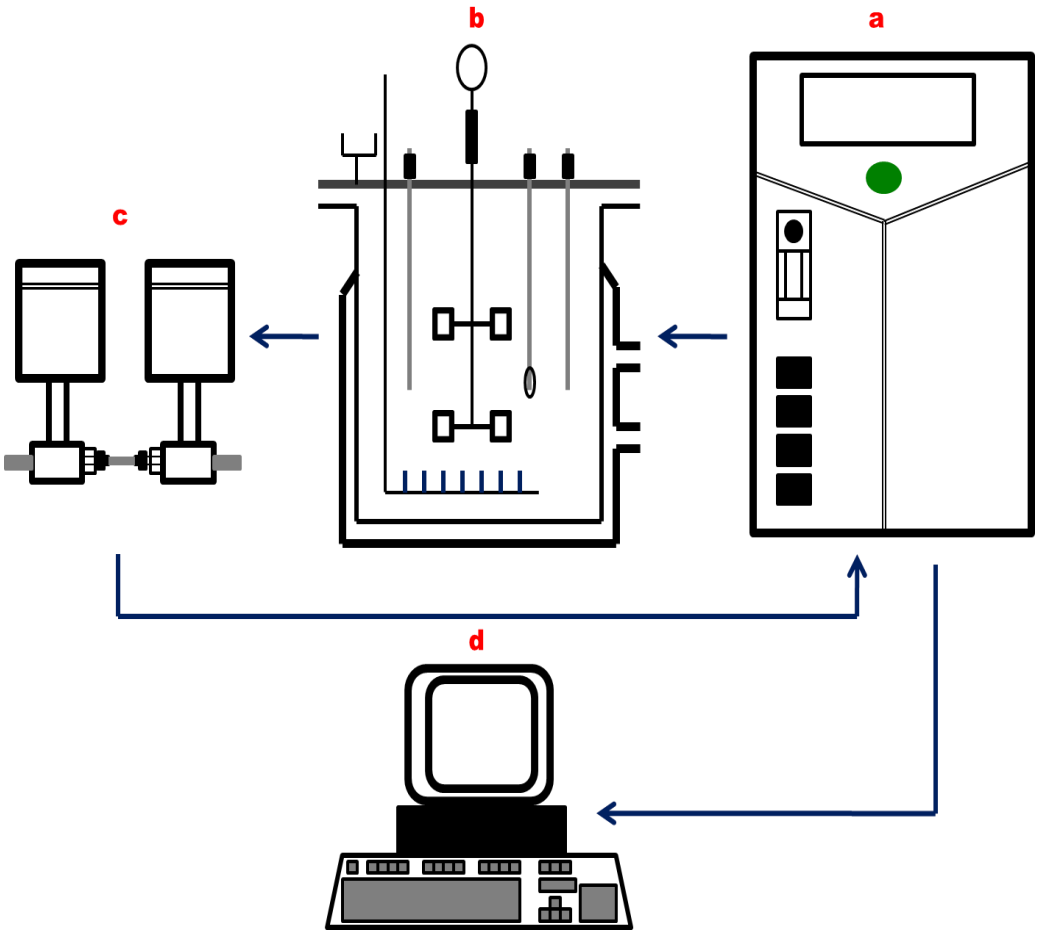


Figure 3.5 Fermentor Plant housed at the University of Witwatersrand, Johannesburg and supplied by LABOTEC, South Africa. a: Digital control unit, b: 2L Univessel, c: Gas sensors, d: Host computer.

The lab-scale plant used to collect the fermentation experimental data is a four-part system consisting of the digital control unit (DCU), univessel, gas sensors and host computer installed with a software programme (Fig 3.5). Details on each part of the system are represented by figures and described as separate entities.

The digital control unit (Fig 3.5) is shown in detail in Figure 3.6a-c.

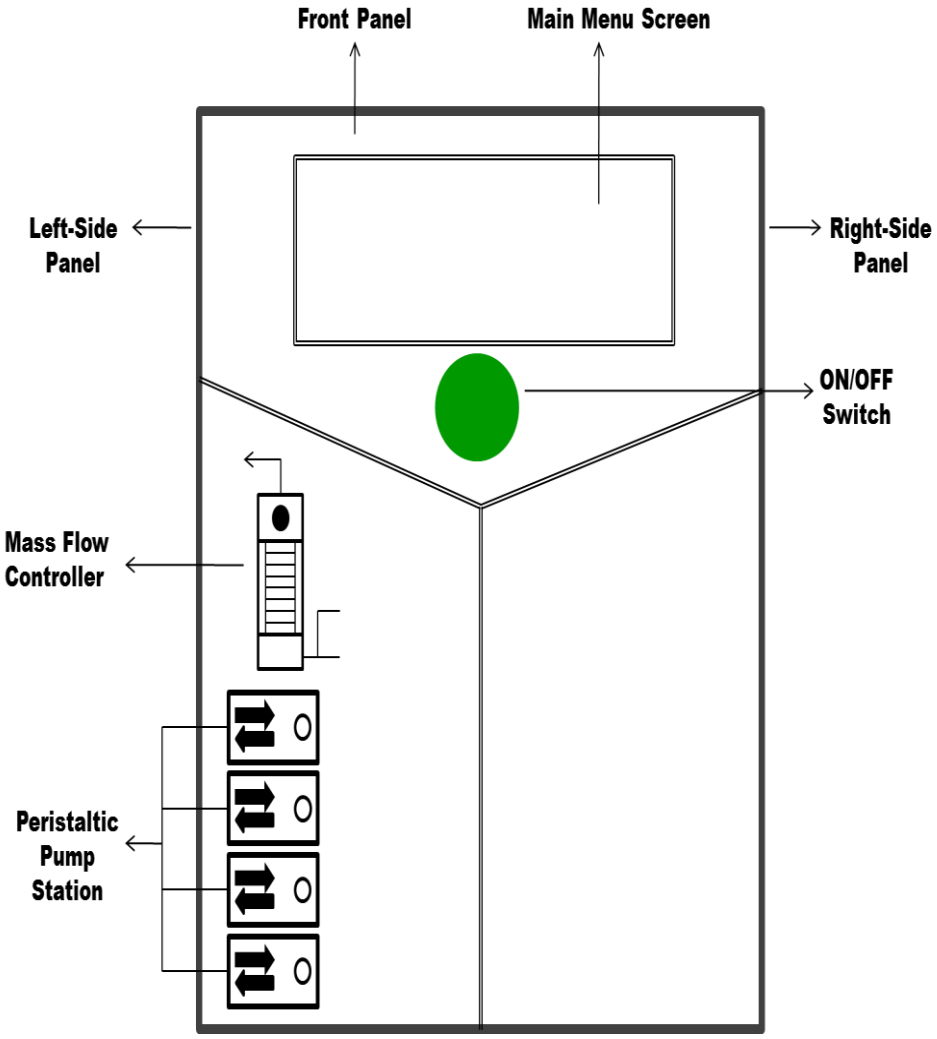


Figure 3.6a Digital control unit of the BIOSTAT® Bplus System.

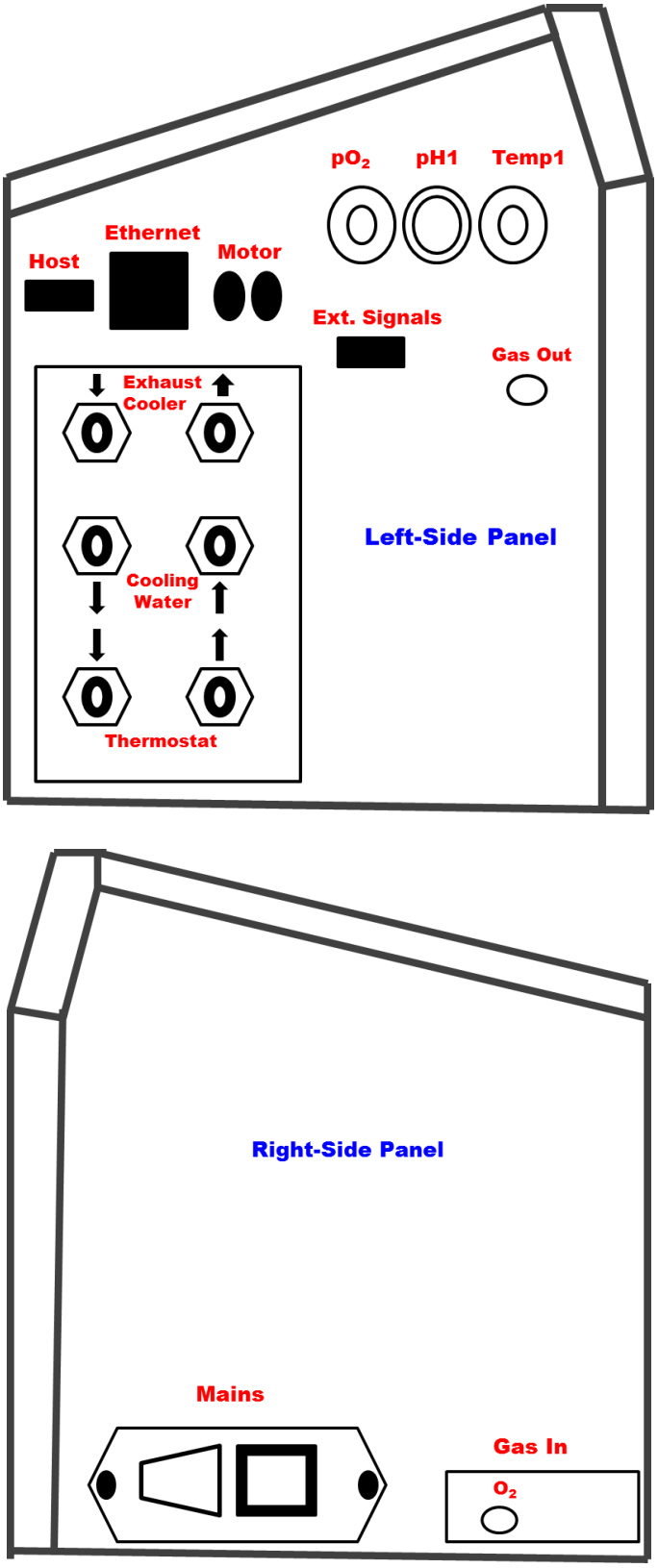


Figure 3.6b Side panels of the Digital Control Unit.

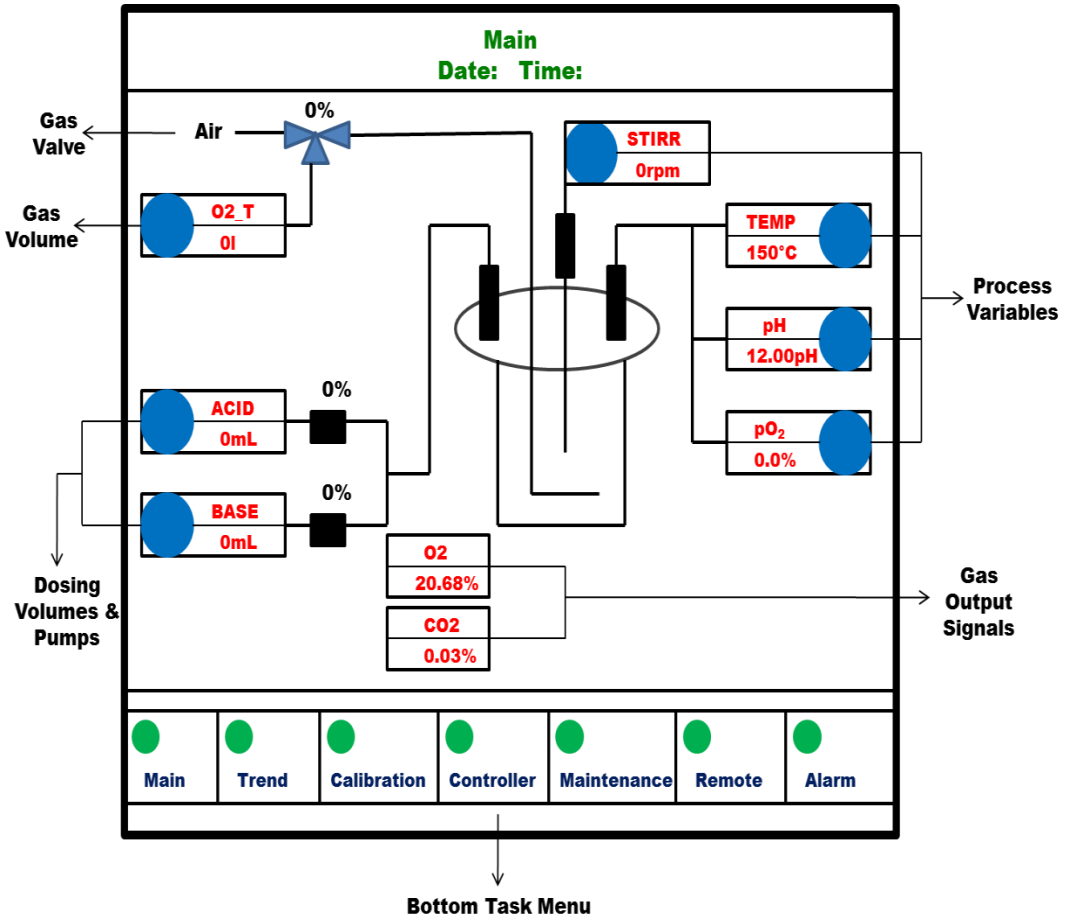


Figure 3.6c Touch-screen graphical interface of the digital control unit where the main menu is displayed.

The DCU is a micro-DCU touch screen graphical interface which is the measurement and control system of the BIOSTAT® system. It consists of a front panel (Fig 3.6a), left-side panel (Fig 3.6a-b) and right-side panel (Fig 3.6a-b) which is connected to the main power supply. The front panel has the ON/OFF switch for the digital controller, peristaltic pump station and the mass flow controller (MFC) (Fig 3.6a). The right-side panel has the connectors for the oxygen gassing system (gas input) and main power supply (Fig 3.6b). A hose is connected from the gas tank to the gas input connector on the right-side panel. The left-side panel has the connectors for the temperature sensor, pH probe, pO_2

probe, stirrer motor, cooling water system, thermostat system, Ethernet connection to the host computer and gas output tubing (Fig 3.6b). The graphical interface (Fig 3.6c) shows the main menu which displays an overview of all possible configurations. From the main menu the submenus for the process variables, acid/base volume readings, peristaltic pump configurations, oxygen levels, solenoid valve configuration and gas output signals are accessed (Fig 3.6c). Through these submenus the input values are entered. At the bottom of the screen (Fig 3.6c) are the TREND, CALIBRATION, CONTROLLER, MAINTENANCE, REMOTE and ALARM configuration menus. The TREND menu is a visual data monitoring function, where changes in the selected process variables are recorded in *real time* over a specified time period and displayed in the TREND function window. However, data storage is not possible through this function and the data is lost when the main system is switched off. The SCADA software programme, installed on the host computer (Fig 3.5) is used for data acquisition. The CALIBRATION menu is used for optimizing the pH and pO_2 probes and peristaltic pumps. The CONTROLLER menu is for controlling pumps, valves and input values. The MAINTENANCE menu provides information on the system settings and is activated by the suppliers of the system (LABOTEC-South Africa) by the use of a password for service and repair purposes. The REMOTE menu is used to able/disable the host computer software. When the "able" function is selected, the touch screen interface is activated and when the "disable" function is selected, the touch screen is inactivated and input values are entered and measured through the SCADA software programme on the host computer. The ALARM menu is signalled when there is a disturbance in the system.

The fermentor of the BIOSTAT® Bplus system is a 2L glass univessel (Figs 3.5; 3.7a) which is double-walled or jacketed for temperature control (Fig 3.7a) and closed by a stainless steel lid (Fig 3.7b). The univessel together with lid is supported by a stainless steel structure.

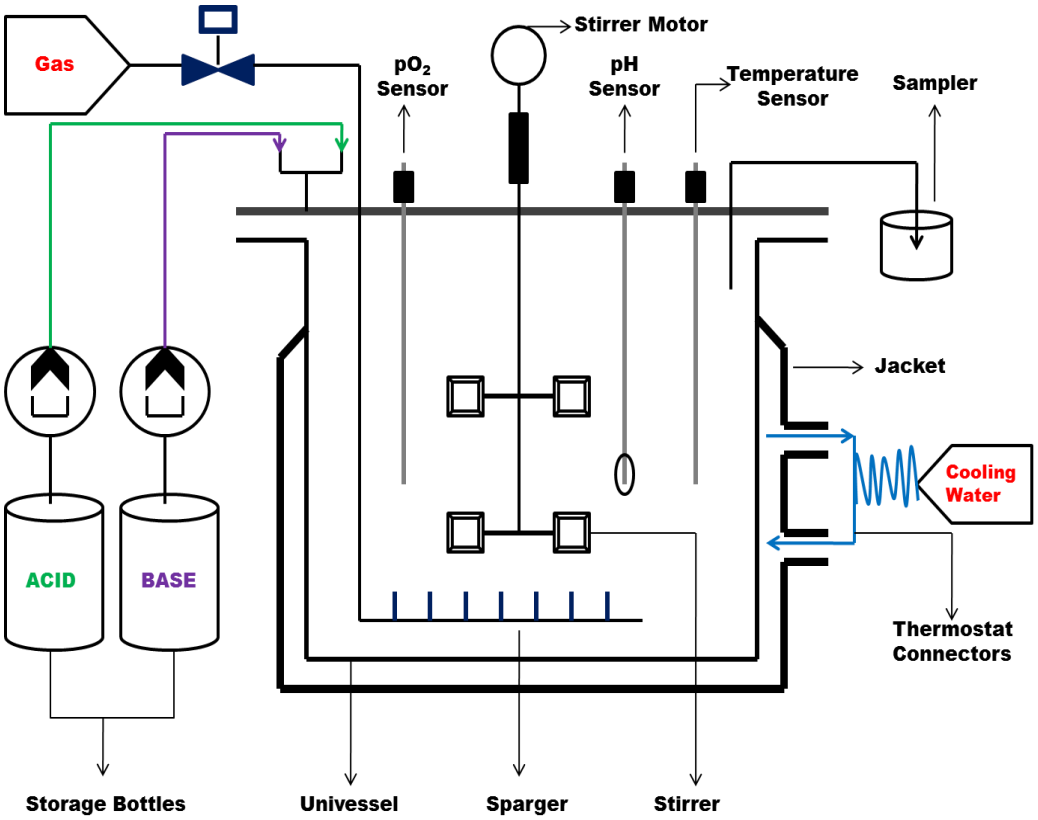


Figure 3.7a 2L glass univessel of the BIOSTAT® Bplus System.

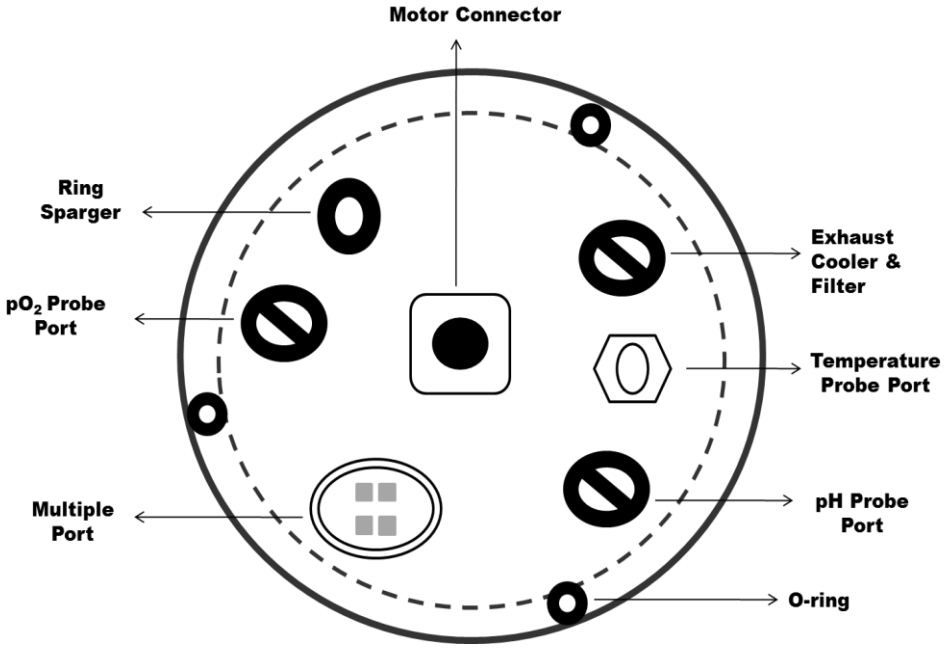


Figure 3.7b Stainless steel lid of the univessel.

The jacket of the univessel is equipped with water inlet/outlet valves (Fig 3.7a) for temperature control through the exhaust cooler and thermostat system. Hoses are connected from the water supply (laboratory tap) to the water-in on the left-side panel of the DCU (Fig 3.6b) and from the water-out to the drain in the laboratory. The thermostat connections are from the water-in and water-out of the univessel (Fig 3.7a) to the left-side panel of the DCU (Fig 3.6b). In addition, there are connections from the DCU to the exhaust filters. The storage bottles are 250mL in volume and connected to the front of the univessel (Fig 3.7a). These are used for the storage of acid and base solutions for pH control. The storage bottles are equipped with screw cap lids installed with a stainless steel covering with two ports. One port is for the attachment of a filter and the second port is for the connection of transfer tubing which is used for the addition of acid/base solutions into the univessel via the multiple ports on the univessel lid (Fig 3.7b). The tubing

is connected from the port on the storage bottle lid to the respective peristaltic pump for acid/base on the DCU (Fig 3.6b) and to one of the multiple ports on the univessel lid (Fig 3.7b). The 12mL manual sampler is attached to the side of the univessel (Fig 3.7a) and equipped with a harvest pipe and filter for sample collection. The connecting or transfer tubing of the system used in this study were transparent Silicon Schlauch Tubing (6.4mm) and filters are 0.20 micron PTFE Midisart® 2000. The stainless steel lid is secured onto the univessel by O-rings (Fig 3.7b) and equipped with single ports for the temperature sensor, pH and pO_2 probes (Fig 3.7b) and a multiple port (Fig 3.7b) for the tubing connection from the storage bottles (Fig 3.7a). The agitator or stirrer shaft is a double impeller, each with six blades and secured onto the lid. The agitator is operated by a 200W motor (Fig 3.7a), connected to the left-side panel of the digital controller (Fig 3.6b). For oxygen enrichment, the univessel is equipped with a ring sparger, with the gas addition port situated on the lid (Fig 3.7b). Gas addition into the univessel is via a hose, connected from the oxygen tank (laboratory supply) to the right-side panel of the DCU (Fig 3.6b) and the gas outlet connection is silicon tubing from the left-side panel of the DCU into the ring sparger port of the univessel (Fig 3.7b). Oxygen supply is controlled at 1.5 bar by a 3/2-way solenoid valve (Fig 3.6c) and the mass flow controller (MFC) (Fig 3.6a).

The BCP-O_2 and BCP-CO_2 gas sensors (BlueSens GmBH, Germany) are connected as shown in Figure 3.8.

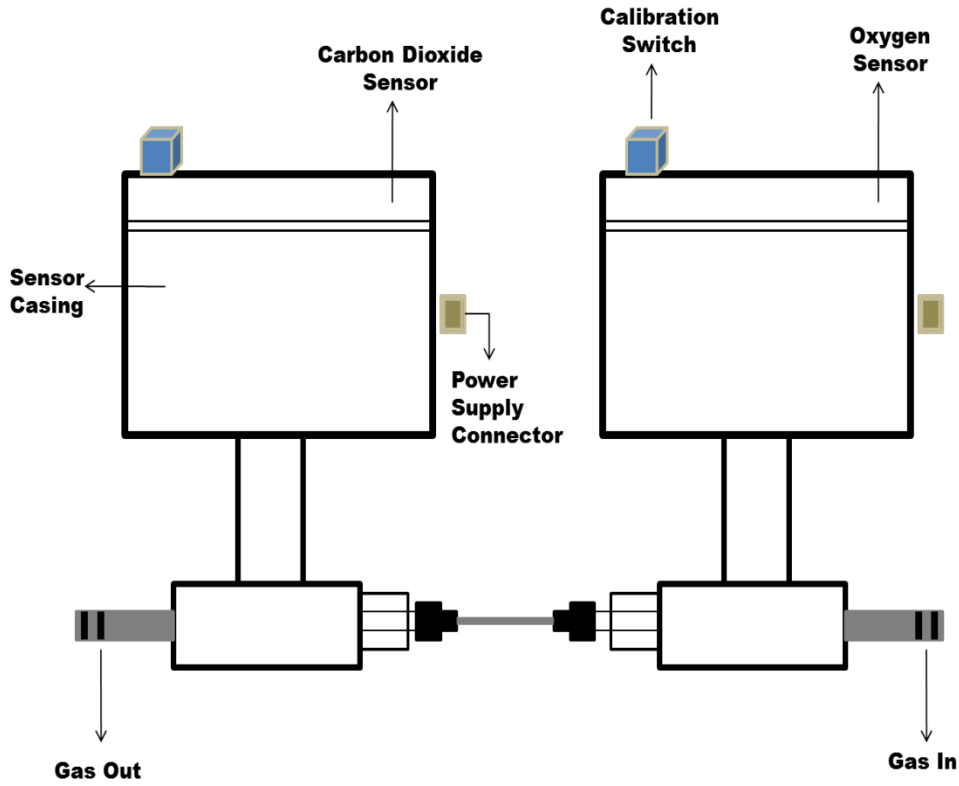


Figure 3.8 BlueSens gas sensors.

The gas sensors are connected in the off-gas line. Tubing from the exhaust filter of the univessel (Fig 3.7b), equipped with a filter is connected directly to the BCP-O_2 sensor (Fig 3.8), followed by the BCP-CO_2 sensor (Fig 3.8). The sensors are housed within an aluminum casing (IP65) with a measuring range of 0-10% volume for BCP-CO_2 and 0-25% volume for BCP-O_2. The operating temperature and pressure range of the sensors are 25°C and 0.8-1.3 bar, respectively. The signals from the sensors are recorded on the main menu DCU screen (Fig 3.6c).

The final section of the plant is the host computer (Fig 3.5), installed with the BioPAT® MFCS/DA software programme (Sartorius Stedim, Germany, Serial No. 41562, 2010) and identified by the operator service icon on the desktop. The

schematic of the software, shown in Figure 3.9 is designed to handle multiple input/output process variables of the fermenter control system. Data is continuously transferred and stored to the software. From the stored data, tracking the process changes of each batch is possible.

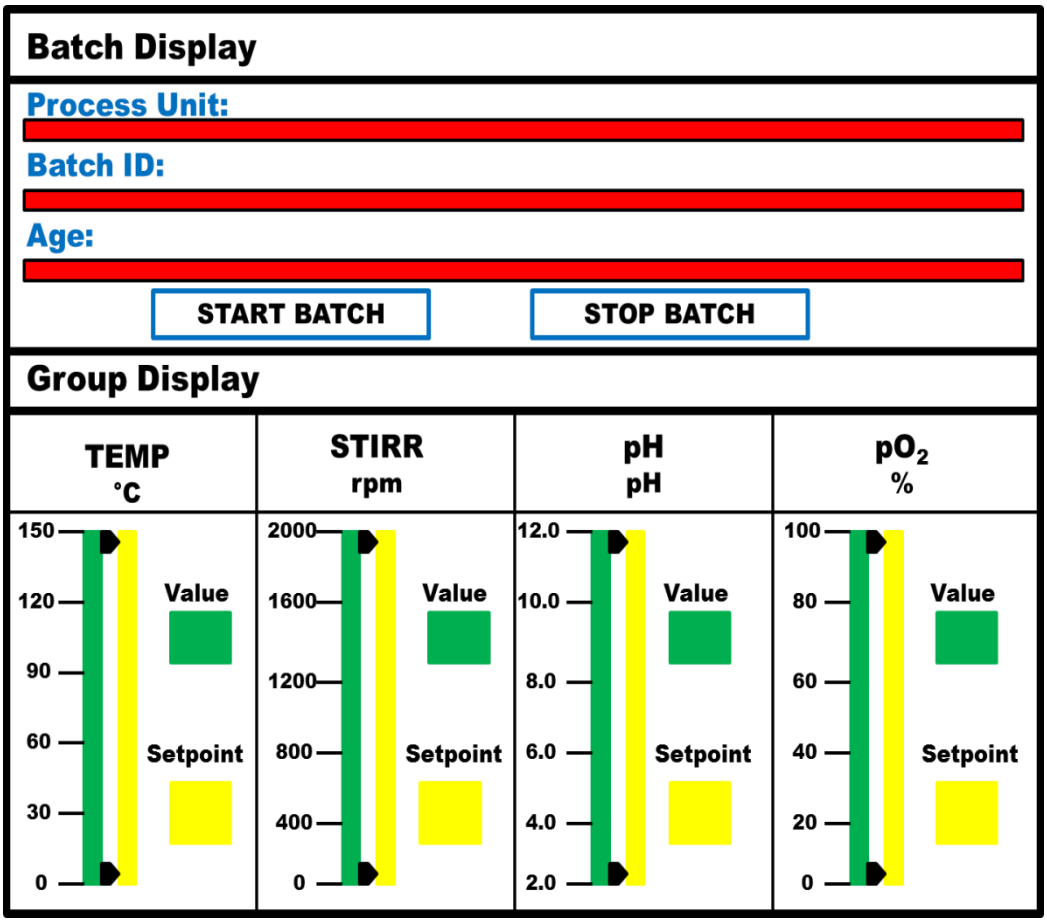


Figure 3.9 MFCS/DA software programme configuration menu.

The GROUP DISPLAY shows the process variables (Fig 3.9). The "setpoint" is the value configured according to fermentation conditions/parameters. The "value" is the *real time* reading of the system (Fig 3.9). The BATCH DISPLAY (Fig 3.9) shows: (i) Process Unit; which is the univessel (single fermenter), (ii) Batch ID; is the display of the start date of the fermentation run, (iii) Age; is the

time of the fermentation (hrs:min), and (iv) START/STOP; is to record and store the data (Fig 3.9). In addition to the variables shown (Fig 3.9), acid/base volumes, oxygen volume, opening/closing of the solenoid valve, jacket temperature and flow rate of the gas was recorded by the software.

3.4.2 BIOSTAT® Bplus Operation and Fermentation

For fermentation, 1L of CAJ was added to the 2L univessel through the single addition port on the lid (Fig 3.7b). The jacket was filled mid-way with water by activating the THERM FILL on the CONTROLLER function (Fig 3.6c). The peristaltic pumps and pH electrode (Hamilton-EASYFERM PLUS K8200) were calibrated as per manufacturers instructions. Pump calibration was as follows: (1) tubing was placed into a beaker filled with dH_2O, (2) this tubing was connected through the pump head and into a measuring cylinder, (3) on the main menu of the DCU the respective acid/base pump was switched ON using the pump configuration menu, (4) the tubing was filled with dH_2O until the tubing end and the pump was switched to AUTO, (5) the calibration menu was selected and the submenu ACID/BASE totalizer calibration was used for the respective pump, (6) START calibration function was selected and dH_2O from the tubing was collected drop-wise into the measuring cylinder, (7) after 2min the calibration was stopped, (8) the volume (mL) of water collected in the measuring cylinder was entered into the QUANTUM window. Each pump from the pump station was calibrated individually. From the calibration, the flow rate of the pumps was calculated in *real time* from the volume recorded by the time taken to accumulate this volume. The flow rate was displayed in the FLOW window. A flow rate of 10mL/min was

noted for the pumps. Once calibration was complete the main menu was accessed and the pump was switched from AUTO to ON to drain the remaining dH_2O from the tubing and the pump was switched OFF for sterilization. Calibration of the pH electrode was as follows: (1) the pH electrode was connected to the pH socket connector on the left-side panel of the DCU, (2) beakers were filled with 20mL of pH 7.00 and pH 4.00 buffer solutions (Hamilton Durcal Solutions), (3) the CALIBRATION menu was selected, followed by the selection of the pH sensor submenu, (4) the pH electrode was placed into the beaker filled pH 7.00 buffer solution and the MODE menu was activated, (5) from the MODE menu the ZBuffer window appears and the value 7.00 was entered and calibration started, (6) pH 7.00 calibration stopped automatically when the pH measurement was stable. When calibration of the pH 7.00 buffer was complete the ZBuffer window re-appeared, (7) the pH electrode was removed from the pH 7.00 solution, rinsed with dH_2O and placed into the beaker filled with pH 4.00 buffer solution, (8) the value 4.00 was entered into the ZBuffer window and calibration started, (9) when the pH4.00 calibration was complete, the main calibration menu was closed. The electrode was then inserted into the univessel via the pH port on the lid (Fig 3.7b) for sterilization. For the pH calibration, the pH 7.00 buffer calibration was the zero reference and the pH 4.00 buffer was selected as this was the measuring range for the fermentation (Table 3.6). Post-calibration, the 200W motor; pump tubing; sensor cables; probes cables and thermostat cables were disconnected, openings were closed-off and the univessel was placed into an autoclave and sterilized at 121°C for 15min with the fermentation medium (CAJ). Post-sterilization, the equipment was cooled and the cables re-connected to their

respective side-panel connectors (Fig 3.6b). The jacket was filled to the maximum with the water continuously running for temperature control. The 250mL storage bottles were filled with pre-prepared 1M HCl and 1M NaOH and the pump tubings were re-connected from the storage bottles to the respective ACID/BASE pump of the peristaltic pump station and to the multiport of on the univessel (Figs 3.6a; 3.7b). When the pO_2 electrode (Hamilton-OXYFERM™ O_2 sensor) was used for oxygen enrichment, sterilization was performed 24hrs prior to the start of fermentation as the pO_2 electrode required polarization by charging the electrode overnight in the fermentation medium. For the fermentation process, the DCU was used to set the fermentation operating conditions (Table 3.6) by selecting each variable and entering a setpoint value. The temperature, pH, pO_2 and stirrer speed were the process variables set on the DCU. For each variable the TEMP; pH; pO_2 and STIRR submenus were selected from the main menu (Fig 3.6c). The submenus allowed access to a window where the setpoint value and the mode operation was entered. First the setpoint value, which is the numerical input value, was entered for each variable and then the mode was set on AUTO to activate the controller functions for automatic control of the setpoint value. Setpoint values were displayed on the main menu and these were the measured values for the fermentation. In addition, the ACID and BASE pumps and the 3/2-way solenoid valve were set to AUTO through the pump configuration and valve configuration, respectively, for the control of the setpoint value. The controller functions are enabled when AUTO was selected for the process variables and this triggers the control system or control loops to maintain the setpoint values. Temperature controller is a PID controller through the jacket temperature (JTEMP) controller.

When the TEMP setpoint was entered and the mode switched to AUTO, the JTEMP controller was triggered. The temperature sensor monitored the temperature of the fermentation medium and deviations from the setpoint value enabled JTEMP controller to supply hot or cold water from the thermostat system through the jacket of the univessel. The stirrer controller is a setpoint controller and acts on the motor for continuous stirring at the specified setpoint value. The pH controller is a PID controller. The pH electrode measures the pH value and sensors deviations from the setpoint value which triggers the controller to supply acid or base via the peristaltic pumps. Addition of acid/base from the storage bottles were drop-wise. Once all conditions were stabilized according to the setpoints, the CAJ medium was inoculated with ~8.00 Log CFU/mL of active yeast cells (Table 3.6) at 10% of the initial fermentation volume. In addition, the MFCS/DA software on the host computer was enabled and the START BATCH function (Fig 3.9) was selected for recording of the input/output variables and data acquisition. Furthermore, gas measurements were recorded using the BluSens BCP gas sensors (Fig 3.8). For gas measurements, tubing was connected from the exhaust filter to the sensors to continuously measure the volume fraction in percentage of oxygen (O_2) and carbon dioxide (CO_2) during the course of fermentation. The BCP sensors were calibrated by a 1-point calibration. The sensors were switched on and allowed to warm up to 1hr, after which the calibration switch (Fig 3.8) was pushed down for 5secs. Once calibrated, the O_2 and CO_2 readings on the main menu displayed as 20.68% and 0.03% for O_2 and CO_2, respectively. The operating conditions of the batch fermentations are summarized in Table 3.6. Fermentations were performed in duplicate and data

analysis was carried out in triplicate for the fermentations with a resulting data set of six replicates. Prior to experimental fermentations for the research, the equipment was tested with water to ensure stable functioning of the sensors and electrodes after calibration.

Table 3.6 Operating conditions of batch fermentations (*Source:* Deenanath et al., 2013).

Conditions	Run 1	Run 2	Run 3	Run 4
Working Volume	1L	1L	1L	1L
Yeast Strain	Y2084	Y2084	Vin 13	Vin 13
Inoculum Concentration(Log CFU/mL)	8.62	8.58	8.64	8.69
Temperature	30°C	30°C	20°C	20°C
pH	4.5	4.5	4.5	4.5
pO_2	0%	50%	0%	50%
Stirrer Speed	150rpm	150rpm	150rpm	150rpm

For run 2 and run 4 (Table 3.6), the polarized pO_2 electrode was calibrated for oxygen as per manufacturers instructions. Calibration of the pO_2 electrode was as follows: (1) the pO_2 electrode was polarized overnight in the uinvessel and then disconnected for 5min, which was the zero reference, (2) the gasflow (GASFL) function from the CONTROLLER menu and the solenoid valve were set on AUTO, (3) fermentation medium was supplemented with oxygen (from the laboratory gas supply) at 50% saturation by using the pO_2 submenu, (4) the pO_2 electrode was then re-connected and the stirrer was switched ON and set at 150rpm, at this stage the pO_2 value on the main menu was displayed as 0.0%, (5)

from the CALIBRATION menu, the pO_2 sensor calibration was selected and in the mode display window the CALIB AIR was selected and the value 100% was entered and calibration started, (6) calibration was complete when the pO_2 value on the main menu was displayed as 100.00%. For the pO_2 calibration, the pO_2 and stirrer setpoint values used were the same as the measuring range for the fermentation (Table 3.6) to obtain stability of the pO_2 electrode. Post-calibration, the pO_2 value on the main menu was monitored until the 50% setpoint was reached. When it was reached, the other variables such as temperature and pH were set and allowed to stabilize. The yeast was then added and fermentation continued. In addition, for the oxygen enrichment assays the regulator on the gas tank was set at 1.5 bar and the MFC on the digital controller was opened (Fig 3.6a). The pO_2 controller is a PID controller and triggers the GASFL and MFC controllers. Through the pO_2 probe the setpoint value is monitored and deviations trigger the addition of oxygen through the GASFL controller and oxygen is fed via the gas supply line, through the valve and ring sparger into the univessel. Simultaneously the flow of the gas is controlled by the MFC, integrated at the gas outlet on the DCU (Fig 3.6a-b). The addition of the gas is by pulses through the ring sparger and the flow rate of the gas was adjusted, from the calibration as 1l/min.

3.5 Analytical Methods

"During fermentation, samples were extracted using the manual sampler every 24hrs and analysed for yeast viability, sugar consumption, ethanol production and glycerol production. Additionally, gas output signals were recorded and

MFCS/DA plots were constructed. Furthermore, CAJ and the final fermented product were evaluated for total organic carbon. Analysis was performed in triplicate for the batch fermentations. Samples were collected in three separate aliquots and analysed accordingly. Statistical analysis was performed for the replicates" (Deenanath et al., 2013).

3.5.1 Yeast Viability

"Yeast cell viability of the starter culture (section 3.3) and during fermentation was determined by serial dilutions and standard plate counts. From the initial sample (10^0), 1mL of the sample was aliquot into 9mL of buffered peptone water (BPW) and tenfold serial dilution was performed as shown in Figure 3.10. From the dilutions 100uL of the diluted suspension was spread plate onto malt extract agar (MEA) (Fig 3.10). The agar plates were incubated at 30°C, in a temperature controlled incubator for 24-48hrs and examined for the growth of colonies. Plates showing between 30-300 colonies were selected and counted" (Deenanath et al., 2013). When resulting colonies from the dilutions were >300 colonies, further dilutions were carried out and plated accordingly. Viability was expressed by calculating the colony forming units per mL (CFU/mL) using the following equation: (Pattison et al., 1998; Reed et al., 2003).

$$\text{CFU/mL} = \frac{\text{number of colonies X dilution factor}}{\text{volume (100uL)}} \qquad (3.5)$$

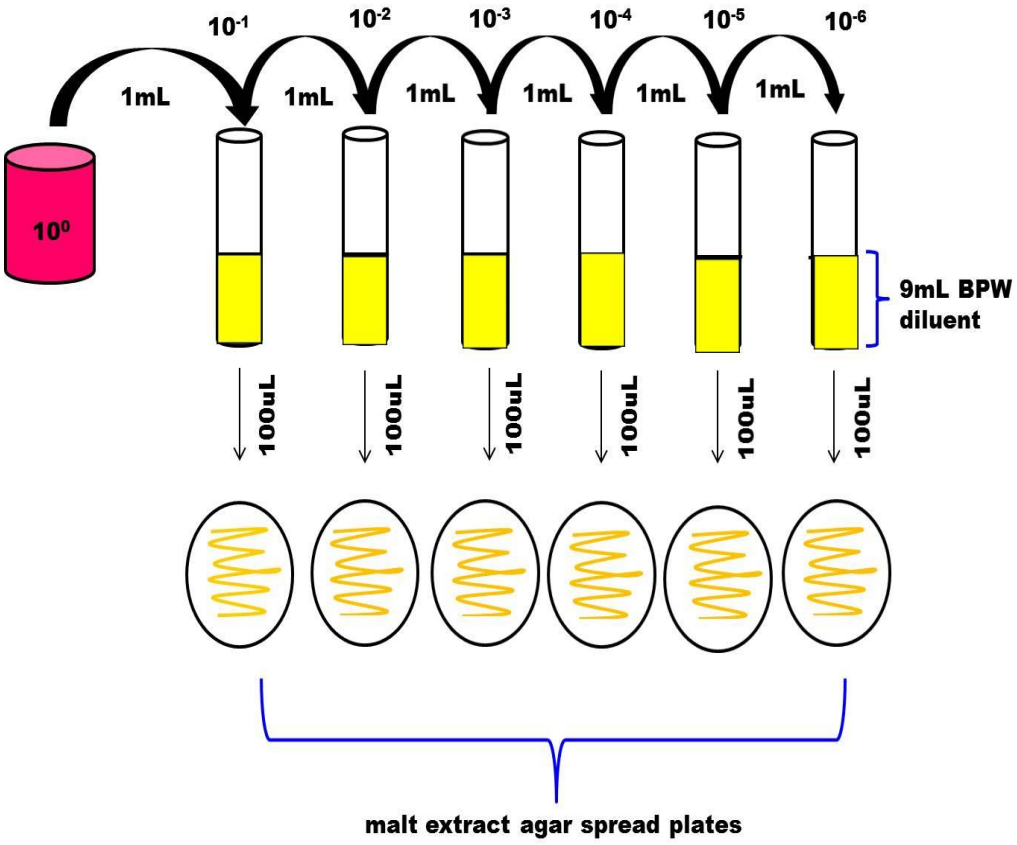


Figure 3.10 Illustration showing tenfold serial dilutions and spread plates.

3.5.2 High Performance Liquid Chromatography Analysis

HPLC was performed as described in section 3.2.2. In addition to the sugar standards, ethanol and glycerol standards were prepared for calibration at concentrations of 12% (w/v) and 1% (w/v), respectively. "Prior to HPLC analysis, the fermented samples were centrifuged at 5,000rpm for 60secs using a microcentrifuge (Eppendorf Minispin Plus-Merck). The supernatant was filtered using a 0.45um syringe filter tip (Minisart cellulose acetate-Sigma) and a volume of 1mL was aliquot into HPLC vials" (Deenanath et al., 2013).

3.5.3 Gas Analysis

Carbon dioxide and residual oxygen was measured by the use of BCP-sensors (section 3.4.1/3.4.2). The output signals were recorded in *real time* and displayed on the main menu of the DCU (section 3.4.1, Fig 3.6b). The output signals are the volume fractions of the gases. To determine the volume of each gas, detected by the sensors, it is assumed that the gas in=gas out from the exhaust filter. Since the calibration for the sensor was at 100% of oxygen at 1l/min (gas in), thus 100% of oxygen at 1l/min is also at the gas out. By using the following equations the volume percentage of each gas was calculated:

$$\mathbf{CO_2\ Output\ Signal\ (\%)\ X\ \frac{gas\ conversion\ factor\ for\ carbon\ dioxide}{gas\ conversion\ factor\ for\ oxygen}} \quad (3.6)$$

$$\mathbf{O_2\ Output\ Signal\ (\%)\ X\ \frac{gas\ conversion\ factor\ for\ oxygen}{gas\ conversion\ factor\ for\ oxygen}} \quad (3.7)$$

3.5.4 MCFS/DA

"Data monitoring of the fermentations were made possible by the MCFS/DA BioPat® software programmer" (Deenanath et al., 2013). As described in section 3.4.1, the START BATCH function (Fig 3.9) was selected to automatically record the input and output process variables that were set for the fermentations (Table 3.6). Every 24hrs, the STOP BATCH function (Fig 3.9) was selected and graphs were constructed. The graphs were constructed by the PLOT function on the taskbar. In the PLOT display window, the batch run date was selected and the

measured process variables were individually added to the PLOT attributes and the graphs were automatically constructed.

3.5.5 Total Organic Carbon Analysis

"The total organic carbon (TOC) of CAJ and the final day fermentation product was determined as described by LaPara et al. (2000), with minor modifications" (Deenanath et al., 2013). Prior to the TOC assay, a digestion solution (2.6g potassium dichromate; 8.33g mercuric sulphate; 42mL sulphuric acid), a catalyst solution (500mL sulphuric acid; 5.06g silver sulphate) (LaPara et al., 2000) and serial dilutions (50%-3.13%) of the samples were prepared. The dilutions of the initial pre-treated CAJ and final fermented product were: 50%, 25%, 12.5%, 6.25% and 3.13%. The prepared dilutions are shown in Figure 3.11.

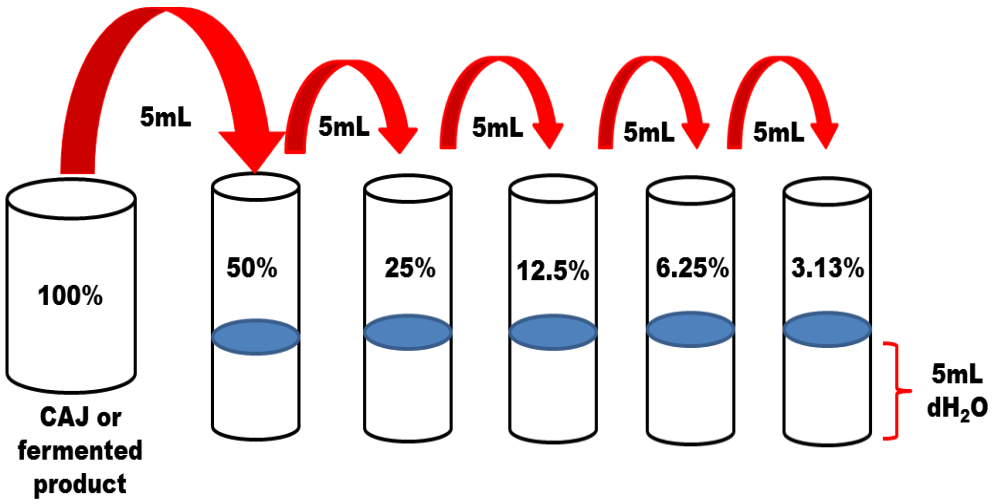


Figure 3.11 Illustration showing dilutions for the total organic carbon assay.

For the fermented product, samples were measured for the insoluble and soluble TOC. The former was an uncentrifuged sample comprised of yeast cells and the latter was a centrifuged sample from which the supernatant was used for the

assay. The TOC assay was as follows: (1) 2mL of each diluted sample was aliquot into pyrex tubes consisting of 1.5mL of digestion solution and 3.5mL of catalyst solution, (2) this mixture was digested for 2hrs at 150°C (LaPara et al., 2000), using a heating block (Spectroquant TR320-Merck), (3) following digestion, the tubes were cooled and the concentration readings at 25-1500mg/L and 1500-5000mg/L were recorded using a spectrophotometer (Spectroquant Pharo 100-Merck). The dilutions and assays were performed in triplicate for the fermentations. The TOC data set collected was a total of six replicates. The carbon percentage of the yeast was determined by subtracting the total concentration of the insoluble TOC from the total concentration of the soluble TOC. The concentration reading at a specific dilution was expressed as a 100%, which is the concentration in the original sample and the total carbon concentration was expressed as g/L.

3.5.6 Statistical Analysis

Statistical analysis was carried out using the software programme STATA 21.1 (StataCorp LP, College Station, Texas 77845 USA) and Microsoft Excel 2010 for the following data: HPLC; yeast viability counts; TOC; tannin; mineral and protein characteristic results. HPLC and TOC data was statically analysed for inferential statistics. Yeast cell counts and characteristic data was analysed for descriptive statistics. “For the inferential statistics, analysis of variance (ANOVA) and significant differences among the means were tested by one-way ANOVA at the 95% confidence interval. Standard deviation was also reported. The results

were declared significant when p≤0.05. For the descriptive statistics, the mean and standard deviations were tested" (Deenanath et al., 2013).

3.5.7 Ethanol Yield and Productivity

At the end of fermentation the ethanol yield (E_y) and ethanol productivity (E_p) were calculated using the following equations (Pacheco et al., 2010):

$$\text{Ethanol yield} = \frac{P_f}{S_o - S_f} \qquad (3.8)$$

$$\text{Ethanol productivity} = \frac{P_f}{t} \qquad (3.9)$$

Where: P_f= maximum ethanol concentration

S_o= initial sugar concentration

S_f= final sugar concentration

t= time (hours)

3.6 Distillation

Post-fermentation the remaining product was collected into re-capable plastic bottles from the fermenter via a single addition port with the use of tubing. The recovered product was centrifuged at 3,500rpm for 10min to remove residual yeast and stored at -20ºC, until distilled. For distillation, simple batch distillation was carried out using the rotary evaporator (Heidolph Instruments-United Scientific) shown in Figure 3.12. Prior to distillation of the product, the equipment was tested with absolute ethanol.

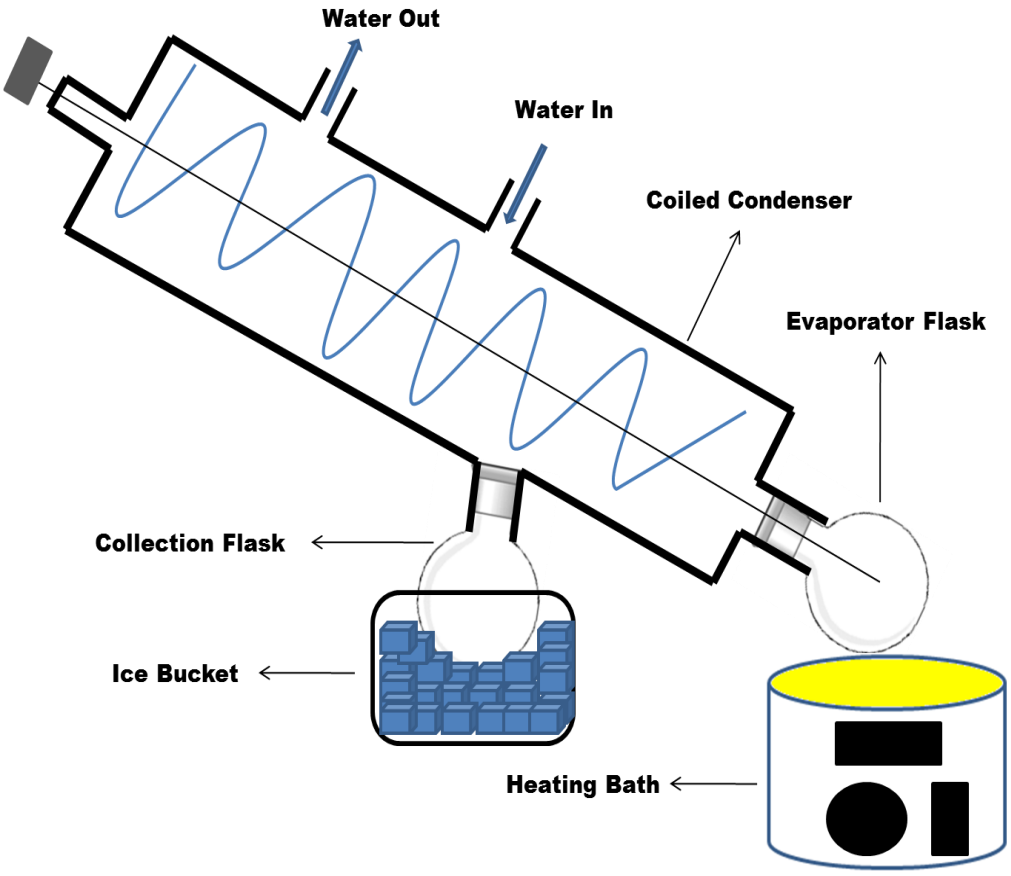


Figure 3.12 Rotary evaporator housed at the University of the Witwatersrand, Johannesburg and supplied by United Scientific, South Africa.

The rotary evaporator unit (Fig 3.12) consisted of: a 1L evaporator flask; 1L collection flask; coiled condenser with water inlet and outlet points; heating bath (Heidolph LABOROTO-4000eco) and power connections (Fig 3.12). The procedure was as follows: (1) the heating bath was filled with Silicon Fluid Oil (Silicon Oil 350cst-United Scientific), (2) the temperature of the heating bath was set at 90ºC using the temperature control settings, (3) the evaporator flask was filled with 500mL of the fermented sample, (4) when the setpoint temperature was reached, the evaporator flask was lowered into the heating bath, (5) the flask was

rotated at 60rpm by using the rotation control on the heating bath, (6) the tap (laboratory water supply) was opened for cooling via the condenser and the distillate was collected drop-wise into the collection flask, which was embedded on ice, (7) distillation continued until condensation was no longer visible. The product from the duplicate fermentations was distilled separately. During the course of distillation, when a sufficient or extractable volume of distillate was present, the process was stopped and the distillate was collected and aliquot into graduated tubes. The volume of distillate and the collection time was noted. Collection time for the first fermentation run product was used as a reference time for the duplicate fermentation run product. The distilled product was then analysed for water content and concentration. Water content was detected by drying aliquots of the distillate in a temperature-controlled oven (Labcon) set at 100ºC for 1hr. Post-drying; the aliquots were collectively transferred to a graduated measuring tube. The amount of water was calculated by subtracting the initial volume from the final volume and the water content expressed as a percentage. Concentration of the remaining volume was detected by HPLC as described in section 3.2.2. For HPLC analysis of the distilled product, an ethanol standard was used and six replicates of the remaining sample was analysed.

3.7 Ethanol/Petrol Blends

3.7.1 Preparation of Blends

Ethanol/petrol blends were prepared using absolute ethanol with commercial petrol (95 unleaded petrol SASOL). Three blends were prepared at the following

concentrations: E5, E10 and $E_{distillate}$. The E is the denotation for the ethanol and the numerical value is the percentage of ethanol. For the blends, ethanol volumes were aliquot into 50mL NUNC tubes and made-up to the final volume with petrol (Leeper and Wankat, 1982; Renzoni et al., 1985; Tangka et al., 2011). The volume compositions of the blends are represented in Table 3.7.

Table 3.7 Volume compositions of ethanol and petrol for blends.

Blend	Volume of Ethanol (mL)	Volume of petrol (mL) Vf=25mL
E5	1.25	23.75
E10	2.5	22.50
$E_{distillate}$	1.5	23.50

Once prepared the blends were mixed at room temperature using a sonicator (Ultrsonicator Processor UP200s-United Scientific), shown in Figure 3.13. The $E_{distillate}$ blend was prepared according to the water content and concentration of the distillate obtained from the distillation of the fermented CAJ product (section 3.6).

For the mixing the sonicator settings were amplitude: 50% and cycle: 1 (Fig 3.13). The samples were placed under the sonicator probe and the motor was switched on (Fig 3.13). Samples were sonicated for 60secs and cooled down on a bed of ice for 60secs (Reed et al., 2003). This procedure was continued for 15mins.

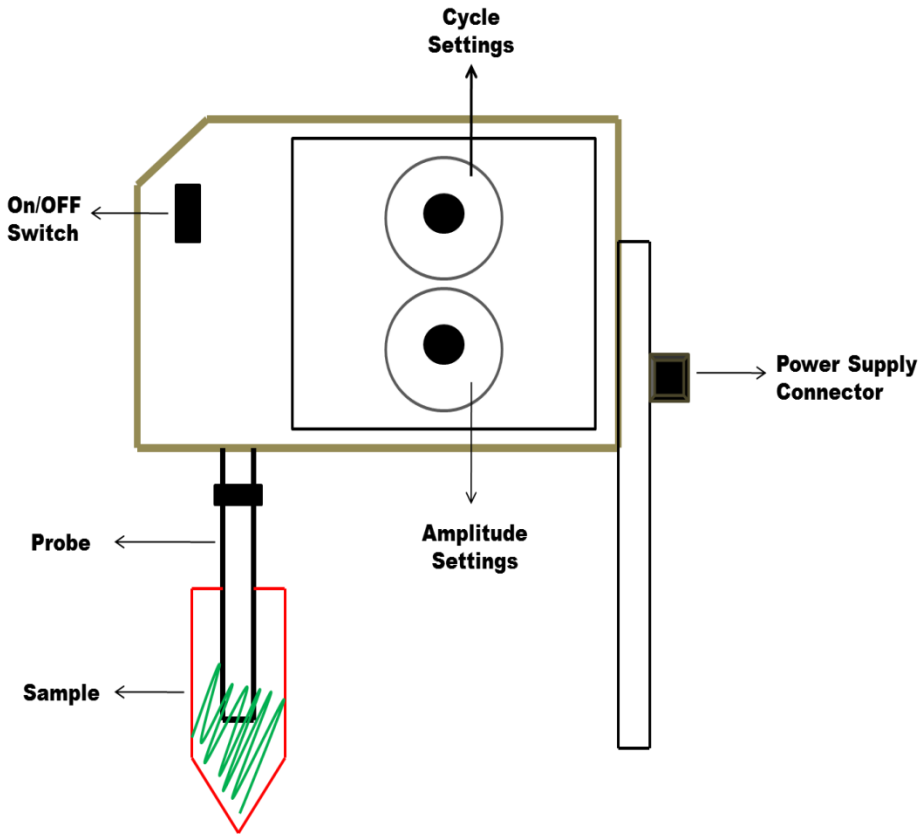


Figure 3.13 ULTRASONIC PROCESSOR UP200s sonicator.

3.7.2 Analysis of Blends

The prepared blends were analysed by Nuclear Magnetic Resonance (NMR) Spectroscopy. Samples for the NMR were prepared in quartz NMR tubes (volume:1mL; width:5mm) by dissolving 0.1mL aliquots of 95 unleaded petrol 100% ethanol or blended samples (E5, E10, $E_{distillate}$) into 0.6mL of deuterated chloroform ($CDCl_3$). The petrol and 100% ethanol were used as reference lines for the analysis of blends. Additionally, the solvent ($CDCl_3$) was also used as a reference line and the chemical shifts were referenced by tetramethylsilane

(TMS), which is an internal standard of $CDCl_3$ (Renzoni et al., 1985; Sarpal et al., 1997; Bansal et al., 2008). Hydrogen (^1H) and carbon (^{13}C) spectra of the samples were acquired using the Bruker Ultrashield™ 500PLUS (School of Chemistry, University of the Witwatersrand). The spectral parameters were as follows: frequency: 500MHz; temperature: 32K; line width: 0.75Hz; and min J-value: 0.3Hz. Sample preparation and spectral parameters were as suggested by the NMR specialist, Dr. RM. Mampa (School of Chemistry, University of the Witwatersrand). Data processing of the chemical shifts and spectra was by the use of the MestReNova Software, 2008, Version 5.2.5-4119 (School of Chemistry, University of the Witwatersrand).

3.7.3 Testing of blends

Ethanol/petrol blends of E5; E10; and $E_{distillate}$ were tested in an IG2600 petrol-fuelled generator shown in Figure 3.14. The generator (Fig 3.14) is a single cylinder, 4-stroke, air-cooled, 95 unleaded petrol generator with a fuel tank capacity of 4.6L and operated as a spark ignition engine. Specifications of the engine, provided by the manufacturers are summarized in Table 3.8.

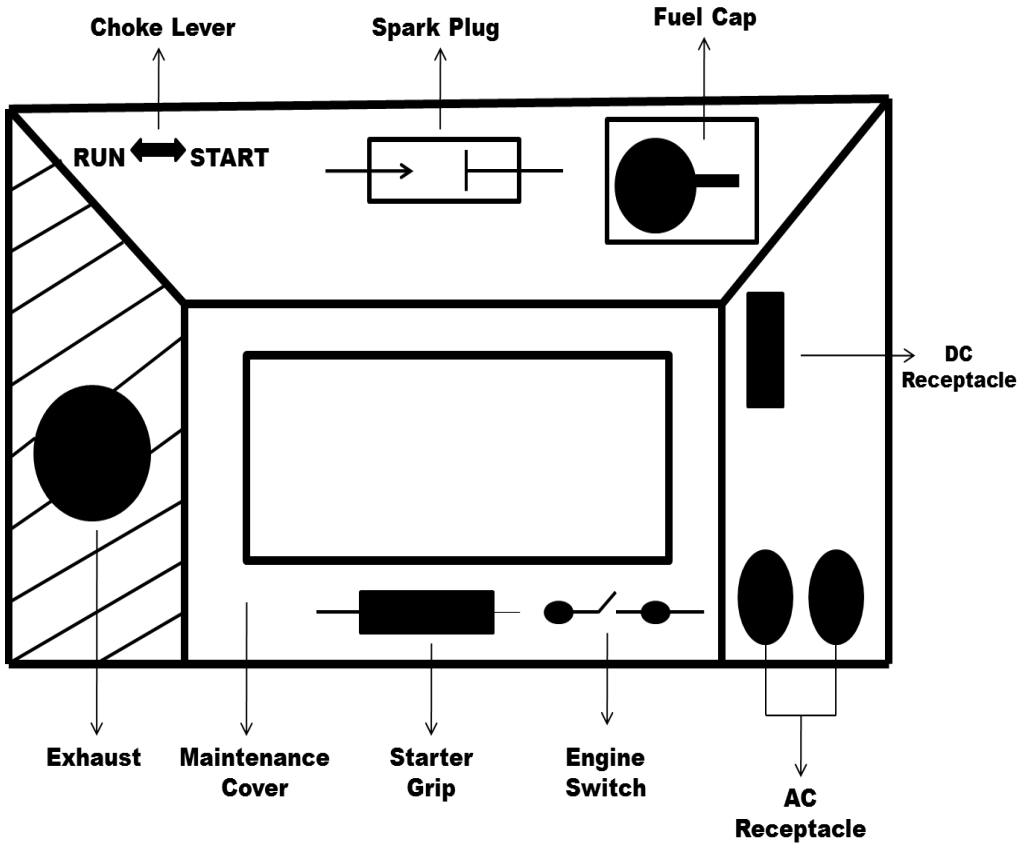


Figure 3.14 BUNDU POWER generator operated at the University of the Witwatersrand, Johannesburg and supplied by Absolute Power Africa, South Africa.

Table 3.8 Engine specifications of the BUNDU POWER generator.

Model Type	Specifications
Displacement (BorexStroke)	171cc (66X50mm)
Compression Ratio	8.5:1
Rated Power [kW/(r/min)]	3.3/3600
Spark Plug	WRTDC
Lube Oil	CD grade or SAE 10W-30, 15W-40

For the generator experiments, the blends were prepared using absolute ethanol and 95 unleaded petrol. The E5 and E10 blends were as described in section 3.7.1 and made up to a final volume of 1L. The $E_{distillate}$ blend was prepared by using the water content (section 3.6) of the distilled product as a guideline. Prior to running the blends, neat or un-blended petrol was tested and used as a reference sample.

Using the schematic provided (Fig 3.14) for a visual identification, the operation of the generator (as per manufacturers instructions) for the testing of blends and neat petrol was as follows: (1) 15W-40 commercially available engine oil (ENGEN) was added until the upper limit of the oil filter within the maintenance cover, (2) the fuel tank was filled with 1L of neat petrol or blends through the fuel cap, (3) the fuel cap lever was turned to the ON position, (4) the engine switch was then turned ON and the choke lever was moved to the START position, (5) the starter grip was pumped by pulling the grip outward until the generator started and the output indicator light was green, (6) once the generator was started, the choke lever was moved to the RUN position, (7) the generator was run for approximately 1hr.

During the generator run time of the neat petrol and blended samples, the level of fuel was recorded at time intervals of: 0, 15, 30, 45 and 60min. To detect the level of fuel, the fuel cap was opened and a graduated dipstick was inserted into the fuel tank of the generator. The dipstick ranged from 0-42cm and at the specified time intervals, the level of fuel was recorded (cm) directly from the dipstick. Triplicate readings, from the dipstick, were recorded at each time interval and generator experiments of the neat petrol and each blended sample were performed in triplicate.

Chapter 4

Results and Discussion

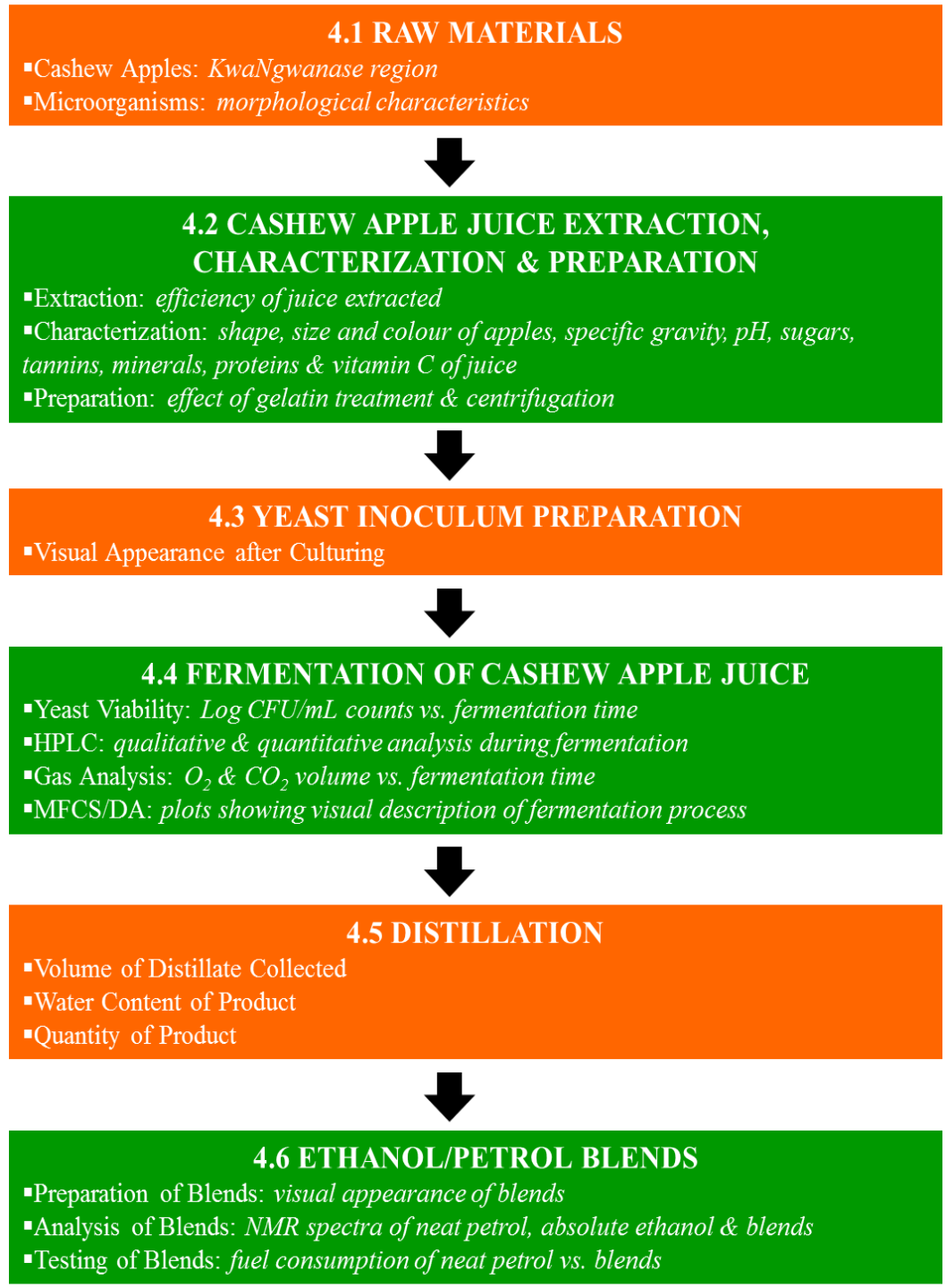


Figure 4.1 A diagram representing the outline of Chapter 4.

The work presented in this chapter is from the publication entitled: "The Production of Bioethanol from Cashew Apple Juice by Batch Fermentation Using *Saccharomyces cerevisiae* Y2084 and Vin13" published in the journal *ISRN Renewable Energy*. Information extracted from the published work is cited as the authors and paraphrased. The contributions of this author were data collection, data analysis and interpretation and writing of the article. The copyrights for the use of the published work were acquired (Appendix E).

4.1 Raw Materials

Cashew apples and *Saccharomyces cerevisiae* yeasts are the raw materials of this research for bioethanol production. The purpose of this section is to pictorially introduce the raw materials. The cashew apples growing in a plantation is presented together with the location of the growth region. Two types of yeast microbes, namely *Saccharomyces cerevisiae* strain Y2084 and Vin13 is shown by means of their specific growth on agar plates.

4.1.1 Cashew Apples

"For this research cashew apples (CAs) were obtained from Coastal Cashews located in the KwaNgwanase region in Northern Kwa-Zulu Natal, South Africa" (Deenanath et al., 2013), as depicted in the map (Fig 4.2a-map courtesy of Mr W. Mthembu). This area is also known as Maputaland, Kosi Bay or rural Manguzi. The Coastal Forest Reserve (Fig 4.2a) is the main region on the east coast of Sub-Saharan Africa (SSA) where cashew trees grow and the reserve stretches for approximately 18km along the north-east Kwa-Zulu Natal coastline in South Africa to Mozambique (Fig 4.2a). The Coastal Cashews plantation (Fig 4.2b) is

1000 hectares (ha) in size and is the only cashew tree plantation in South Africa*. The in-land plantation (Figs 4.2a-b), is comprised of 650ha of cashew trees, 42.5ha of cassava trees, 8ha of mangoes trees and 5ha of pineapple trees*. The main cashew apple harvest season in South Africa is between January to April, when the average rainfall increases to 96% and approximately 380 tons of cashew apples are produced*.

*Information based on verbal communication with Mr W. Mthembu, Farm Manager at Coastal Cashews.

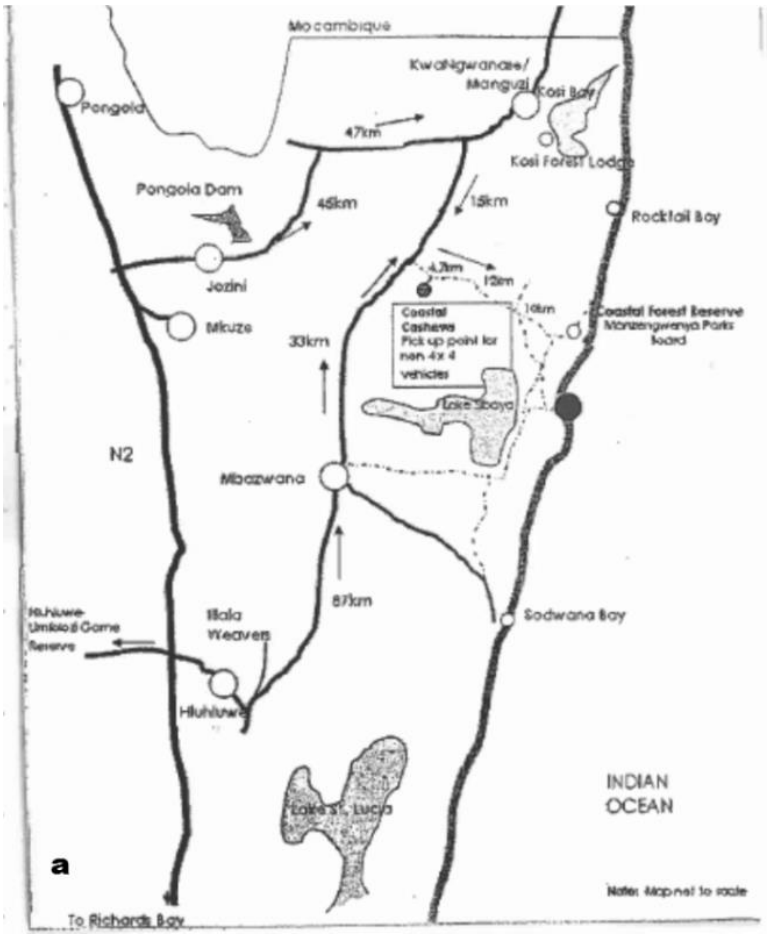


Figure 4.2a-b The location of the cashew apple growth region in Southern Africa (a) and the Coastal Cashews plantation (b).

"During the February 2011 harvest season, CAs was collected (Fig 4.3a-b) from the cashew trees at the Coastal Cashew plantation (Fig 4.2b)" (Deenanath et al., 2013). The CAs that spontaneously fell from the trees or did not show cashew nut attachment was collected (Fig 4.3a-b) by the farm manager (Mr. W Mthembu). "Figure 4.3a shows a single CA which is partial red/yellow to orange in colour. Figure 4.3b shows a mixed variety of CAs such as red apples, green apples, and partial red/yellow to orange apples and even damaged (reddish brown) apples due to natural falling from the trees. In total, thirty-three kilograms (33kg) of CAs were obtained" (Deenanath et al., 2013).

Figure 4.3a-b The cashew apples from the Coastal Cashews plantation in KwaNgwanase, South Africa (*Source:* Deenanath et al).

4.1.2 Microorganisms

Macroscopic morphological characteristics of spread plates revealed colony growth of Y2084 and Vin13 is circular, cream colonies as shown in Figure 4.4a-b.

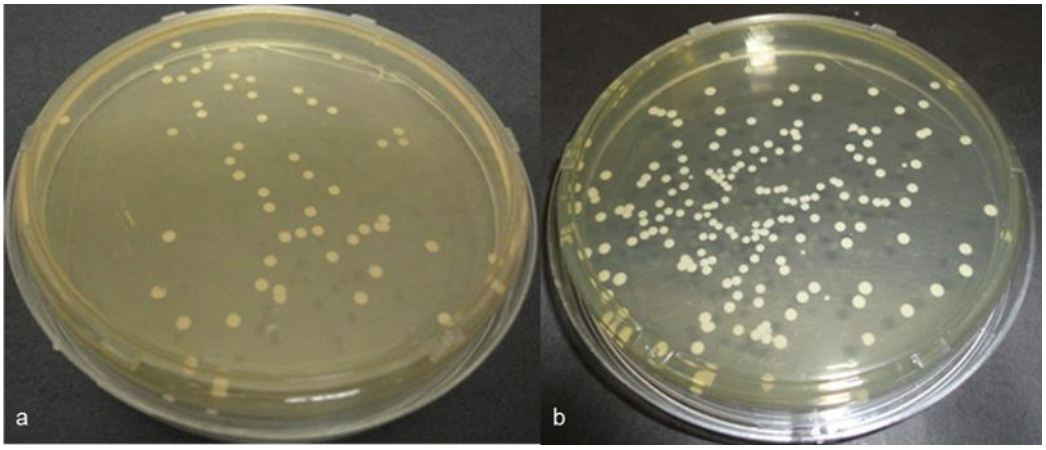


Figure 4.4a-b Malt extract agar spread plates showing colony growth of *Saccharomyces cerevisiae* yeast strains Y2084 (a) and Vin 13 (b).

The brewers yeast Y2084 was previously isolated and identified as described by Deenanath et al., 2010b. The details of the procedure are briefly outlined in

Appendix D. The preserved glycerol stock was cultured and plated, prior to the culturing for fermentation to ensure that the stock was not contaminated. Colony growth on malt extract agar (MEA) plates from the glycerol stock was uniform (Fig 4.4a). To ascertain the wine yeast Vin 13 was a pure culture, the dry yeast was cultured and plated onto MEA plates. Macroscopic colony growth showed distinct colonies of the same size, colour and appearance (Fig 4.4b). This confirmed the dry yeast pellets provided by the Anchor Yeast was pure and preserved within its packaging.

4.2 Cashew Apple Juice Extraction, Characterization and Preparation

This section deals with determining the extraction efficiency of cashew apple juice which is obtained from compressing the apples. Following extraction, the juice is characterized for chemical constituents such as total sugars, tannins, minerals, vitamin C and protein in order to show the available concentrations for fermentation. Additionally, the apples are physically characterized based on colour, size, shape, specific gravity and pH to determine the difference in cashew apples from other growth regions. Lastly, the preparation of the juice for subsequent fermentation is presented.

4.2.1 Extraction

The 33kg of CAs obtained from the cashew trees, were sliced in half (Fig 4.5a) and 8.75L of cashew apple juice (CAJ) was extracted (Fig 4.5b) using a fruit processor (section 3.2.1).

Figure 4.5a-b Cashew apple slices (a) used for the extraction of cashew apple juice (b).

The percentage yield of juice, extraction capacity and percentage extraction efficiency was calculated as described by Akinwale et al. (2001). Values obtained for the percentage yield of juice, extraction capacity and percentage extraction efficiency were 26.52%, 2.9L/hr and 33.14% respectively. The percentage yield of juice would be higher if a larger number of CAs were available for use. On the basis of the extraction efficiency, the result in the current study was lower compared to that of Akinwale et al. (2001). In this investigation a simple fruit processor was used and the capacity and efficiency could be greatly improved if mechanical processing equipment was used, as in the study by Akinwale et al. (2001). For the purpose of this research the use of mechanical press equipment was not possible as the Coastal Cashew Nut Factory was not equipped with extracting machinery as the main commercial product are the cashew nuts. Furthermore, due to long distance traveling it was safer to extract and transport

the juice rather than transporting the apples as CAs are easily perishable (Akinwale et al., 2001). Hence, the manually operated fruit processor used in this study was suitable for on-site juice extraction.

4.2.2 Characterization

The physical and chemical characteristics of the KwaNgwanase cashew apples and the extracted juice are listed in Table 4.1.

Table 4.1 Physical and chemical compositional characteristics of cashew apples and cashew apple juice from the KwaNgwanase region in South Africa.

Characteristics	Composition
Shape of cashew apple	Pear-shaped
Size of cashew apple	Average Length: 58mm Average Width: 44mm
Colour of cashew apples	Mixed variety: green, red, yellow, orange
Total weight of cashew apples	33kg
Specific gravity	1.050
pH	4.52
Total sugars	100.00g/L
Fructose	57.06g/L±0.30
Glucose	40.56g/L±0.32
Maltose	2.18g/L
Condensed tannins (un-treated)	55.34mg/L±0.003
Condensed tannins (pre-treated)	15.34mg/L±0.003
Total minerals	15.89ppm
Zinc	1.39ppm±0.01
Copper	2.18ppm±0.02
Magnesium	4.32ppm±0.01
Iron	1.32ppm±0.03
Sodium	5.44ppm±0.04
Manganese	1.24ppm±0.01
Vitamin C	112mg/100mL±0.58
Total proteins	1.78g/L±0.01

Cashew Apple Physical Characteristics

From the mixed variety of CAs, most of the apples were pear-shaped as shown in Figure 4.3a; other shapes were conical and oblong. For the size, CAs was randomly selected from the 33kg of collected apples and the size measured. The value represented in Table 4.1 is the average length and width of various CAs. On the basis of shape and size (Table 4.1) CAs in the current study is closely related to the Zambian variety of CAs; specifically strain NZ34 of CA characteristics (Unpublished dissertation Ramiakajato et al., 2001). The research by Ramiakajato. (2001) investigated the morphological and phenotypic characteristics of a variety of cashew trees from Brazil and Zambia that were planted in the Coastal Cashews plantation. A total of 130 cashew varieties were studied and the characteristics of the cashew trees, cashew nut, cashew apple and cashew tree flowers and leaves were reported. The colours of the CAs in this study are similar to the typical colours of CAs (MacLeod and Gonzalez de Troconis, 1982; Assuncao and Mercadante, 2003; Rocha et al., 2006; Lowor and Agyente-Badu, 2009, Pacheco et al., 2010). The weight of individual apples is not comparable as in this study the total weight after collection is reported (Table 4.1). Weighing of each apple was not carried out as a lab-scale weigh balance was not available at the Coastal Cashew Nut Factory. Based on the research by Ramiakajato. (2001) it is provided that the weight of the CAs growing from the cashew trees at Coastal Cashews range from 19.92 to 82.85 grams and the average size of CAs from various trees range from length: 51mm to width: 40mm (Unpublished dissertation Ramiakajoto, 2001).

Cashew Apple Juice Physical Characteristics

Specific gravity and pH. The specific gravity value (Table 4.1) was recorded at the point where the liquid intersected the hydrometer. This value is a preliminary prediction of the ethanol level achievable from the CAJ. From the specific gravity value of 1.050 (Table 4.1), ethanol production between 6% (v/v) to 7% (v/v) is possible. This specific gravity value is similar to the value of 1.046 reported by Akinwale et al. (2000;2001) where the characteristics of CAJ extracted from the CAs growing in Nigeria were investigated (Akinwale et al., 2000;2001). Attri. (2009) reported a specific gravity of 1.069 for CAJ from apples growing in Bengal, India which is higher than the value shown in the current study.

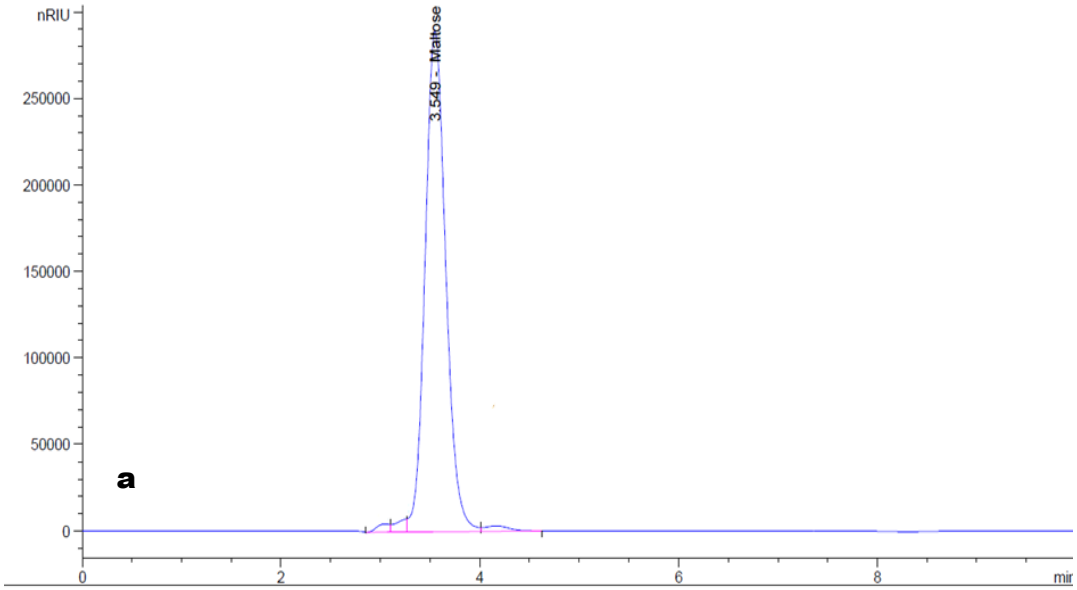
The acidic pH of 4.52 (Table 4.1) is close to the common pH range of 3 to 4 for fruit juices (Boyes et al., 1997). This value is similar to the pH value of 4.15 reported by Akinwale et al. (2000;2001), based on the study in Nigeria. Other CAJ characteristic studies carried out in SSA include Ghana and Benin (Lowor and Agyente-Badu, 2009; Michodjehoun-Mestres et al., 2009). Lowor and Agyente-Badu. (2009) compared CAJ characteristics from CAs growing in three ecological regions of Ghana, namely the Forest Savannah, Northern Savannah and Coastal Savannah. The Forest Savannah CAJ pH values ranged from 4.46 to 4.59, which was dependent on the colour of the apples and the pH value of the KwaNganase CAJ is in accordance with these values. The pH values of 3.85 and 4.02 obtained by Michodjehoun-Mestres et al. (2009) from CAJ in Benin was lower compared to Nigeria, Ghana and KwaNgwanase South Africa. In addition to Africa, CA research is popular in Brazil and India. In Brazil, pH values of CAJ ranged from 3.80 to 6.00, based on the growth region of the CAs (Campos et al.,

2002; Assuncao and Mercadante, 2003; Michodjehoun-Mestres et al., 2009; Vergara et al., 2010). CAJ from Rio de Janeiro, Brazil showed a pH value of 3.91 (Campos et al., 2002) which is lower than the KwaNgwanase CAJ pH. In Ceara State, Brazil the pH of juice extracted from two varieties of CAJ was reported as 4.00 and 4.60 (Michodjehoun-Mestres et al., 2009) and the pH value of KwaNgwanase CAJ reported here is similar. In the study by Assuncao and Mercadante. (2003), a pH value of 4.50 for CAJ from CAs growing in Sao Paulo, Brazil was observed. This is the closest related pH value to the value reported in the current study. The highest pH value of 6.0 for CAJ was shown by Vergara et al. (2010) based on a study using CAs in Fortaleza, Brazil. In India, CAJ characteristic studies were carried out in Bengal and the Ariyalus District (Attri, 2009; Sivagurunathan et al., 2010). CAJ extracted from CAs growing in Bengal was shown to have pH values ranging from 3.90 to 4.14 and the Ariyalus District showed a value of 5.54 (Attri, 2009; Sivagurunathan et al., 2010). These pH values are different from the pH value reported in this investigation but similar to the values reported for CAJ in Brazil. From the collective research data of the physical CAJ characteristics in Africa, Brazil and India, the CAJ pH range is from 3.00 to 6.00 and the specific gravity is from 1.046 to 1.069. By comparison, the CAJ specific gravity and pH values are similar to the values obtained for the juice characteristics from the West Coast of Africa such as Nigeria and Ghana. However, because of limited studies on CAJ characteristics in Sub-Saharan Africa this assumption is speculative. Furthermore, KwaNgwanase CAJ is similar to the pH characteristics of Brazil CAJ and different to India CAJ. This could be attributed to climatic conditions such as rainfall, wind or sunlight and possibly

even land conditions such as soil composition. It can be assumed that conditions experienced in the KwaNgwanase area of Kwa-Zulu Natal, South Africa is similar to the West Coast of Africa and Brazil.

Cashew Apple Juice Chemical Characteristics

Sugar Composition. The sugar composition listed in Table 4.1 shows KwaNganase CAJ composed of fructose, glucose and maltose. The highest sugar composition was fructose: 57.06g/L, followed by glucose: 40.56g/L and maltose: 2.18g/L (Table 4.1). These sugar concentrations were quantitatively determined by High Performance Liquid Chromatography (HPLC). By the use of standards shown in Figure 4.6a-c, the area under each peak is detected at specific retention times and calibration curves shown in Figure 4.7a-c were constructed using the ChemStation software.



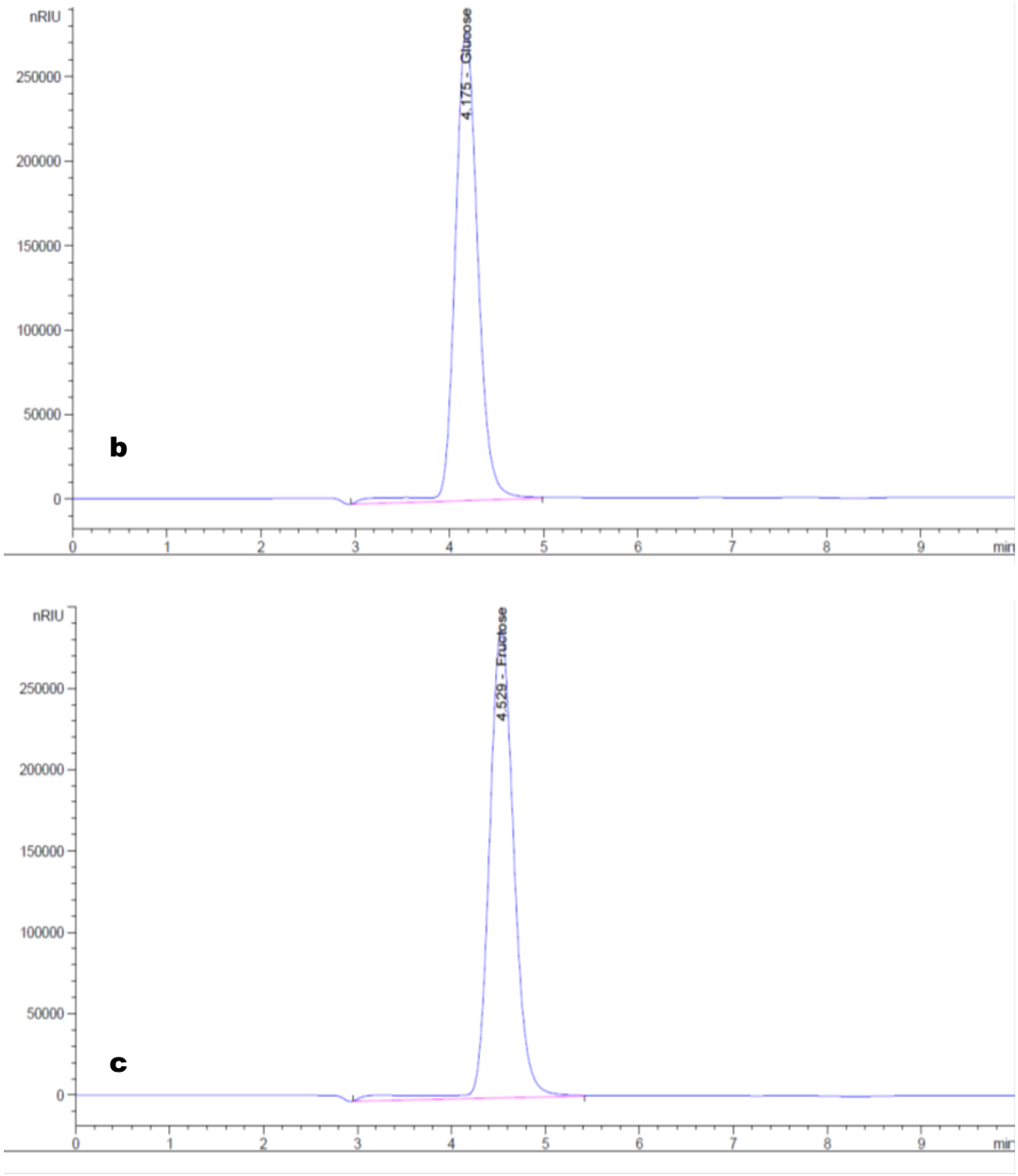


Figure 4.6a-c HPLC chromatogram of three sugar standards. Aminex® Fermentation Monitor Column (150x7.8mm) conditions: column temperature: 60ºC; mobile phase: 0.001M H_2SO_4; RID temperature: 40ºC; flow rate: 0.8mL/min; injection volume: 20uL. The peak identification is indicated on the chromatogram as maltose (a), glucose (b) and fructose (c).

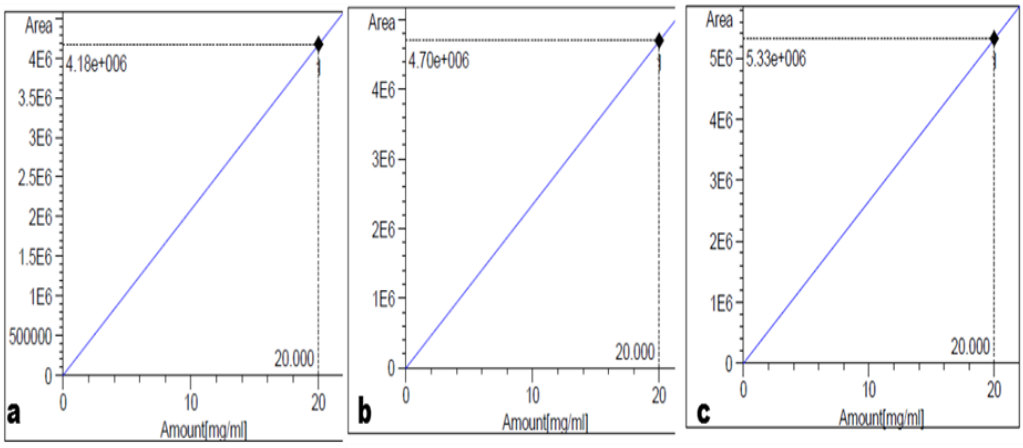


Figure 4.7a-c Calibration curves of maltose (a), glucose (b) and fructose (c) standards as constructed from the ChemStation software.

The chromatogram of the CAJ is shown in Figure 4.8 below. From the HPLC chromatogram (Fig 4.8) two major sugars and one minor sugar is noticeable. The major sugars are glucose and fructose and the minor sugar is maltose. Maltose is the first sugar eluted from the column with the shortest retention time, followed by glucose and fructose (Fig 4.8). Glucose and fructose are coupled peaks as their retention times are almost identical (Fig 4.8). Glucose and fructose are regarded as the major sugars as the peak areas (Fig 4.8) and concentrations (Table 4.1), as determined from the standards (Figs 4.6a-c;4.7a-c) are higher than maltose.

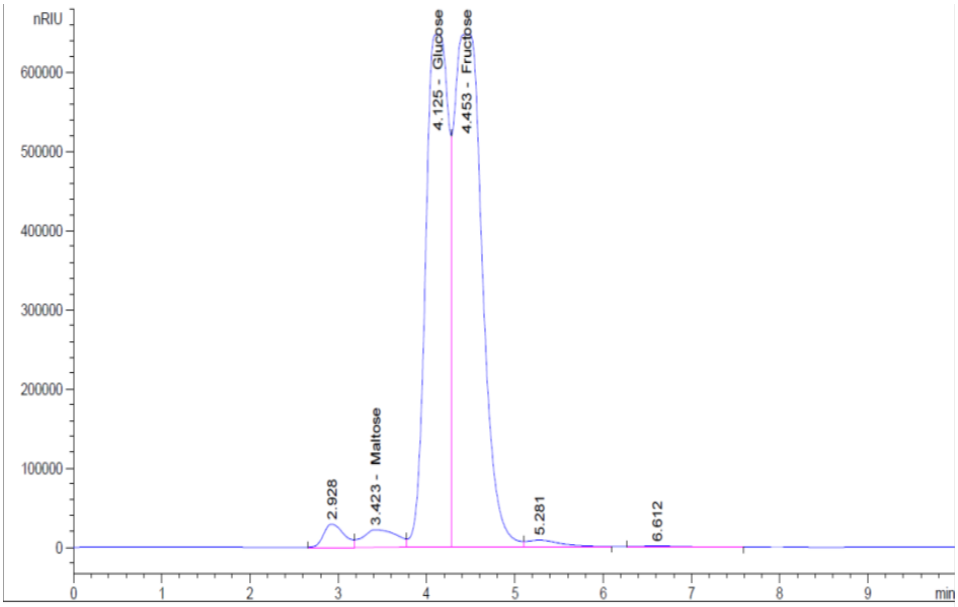


Figure 4.8 HPLC chromatogram showing the cashew apple juice sugar profile. Peak identification based on the refractive index and retention time is indicated on the chromatogram.

HPLC conditions and separation of the sugars are valid as the refractive index and retention times are comparable to the standards. The abundance of glucose and fructose is in agreement with previous findings (Chagas et al., 2007; Luz et al., 2008; Pinheiro et al., 2008; Rabelo et al., 2009; Honorato and Rodrigues, 2010; Pacheco et al., 2010; Araujo et al., 2011). From the various research studies glucose concentrations ranged from 43.28g/L to 46.34g/L and fructose from 37.46g/L to 56.00g/L (Chagas et al., 2007; Luz et al., 2008; Pinheiro et al., 2008; Rabelo et al., 2009; Honorato and Rodrigues, 2010; Pacheco et al., 2010; Araujo et al., 2011). The glucose concentration reported for the KwaNgwanase CAJ was lower and the fructose concentration was higher and similar to the 56.00g/L reported by Rabelo et al. (2009). From the previous studies and the current study, the identification of glucose and fructose is consistent, hence these are native sugars of CAJ and the difference in concentration is probably dependent on the

CA variety. The detection of maltose for the KwaNgwanase CAJ (Table 4.1;Fig 4.8) was unusual. Even though the HPLC analysis showed higher concentrations of glucose and fructose and these will naturally be the main sugars utilized during fermentation, the considerable amount of maltose can provide an additional source of sugar for ethanol production or yeast growth. Furthermore, the use of a mixed variety of CAs and questionable quality of the apples in the present study did not affect the presence or concentration of sugars and is favourable for fermentation.

Condensed Tannins. The tannin composition (Table 4.1) of un-treated CAJ: 55.34mg/L and pre-treated CAJ: 15.34mg/L was determined from the catechin calibration curve shown in Figure 4.9. From the calibration curve, as the catechin concentration increases, the absorbance increases during the Vanillin-HCl assay (Fig 4.9).

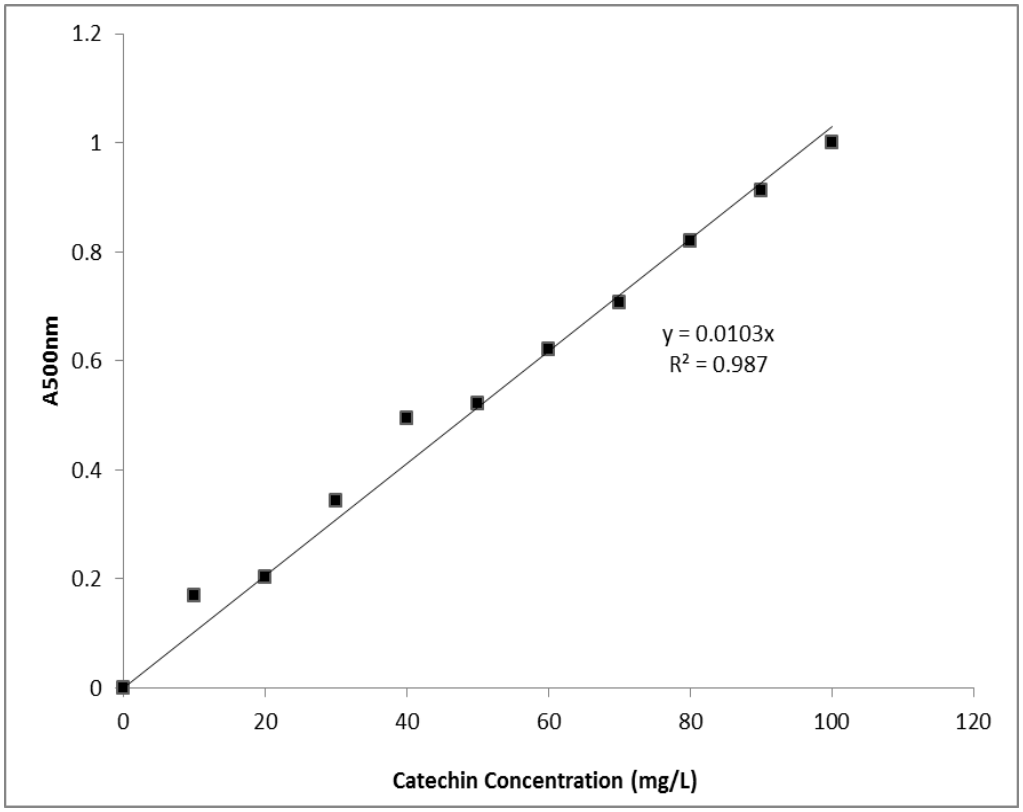


Figure 4.9 Calibration curve of the Catechin standard at different catechin concentrations in 50% methanol for the Vanillin-HCl assay. The values plotted are an average from three replicates.

An average absorbance reading of 0.570 and 0.158 was recorded for the un-treated and pre-treated CAJ, respectively. The values reported (Table 4.1) was determined from the calibration curve (Fig 4.9). For the Vanillin-HCl assay, methanol is used to complex or bind with catechin in the case of the standards and with the tannins in the CAJ sample. The Vanillin-HCl solution then reacts with the catechin-methanol or tannin-methanol complex to produce an intermediate compound that absorbs light at 500nm (Makkar and Becker, 1993; Sun et al., 1998; Nakamura et al., 2003). Hence by using the calibration curve (Fig 4.9) the tannins of the CAJ sample, expressed as catechin equivalents could be determined. By comparison, the tannin value of 55.34mg/L was lower than the

value reported by Lowor and Agyente-Badu. (2009). In the study by Lowor and Agyente-Badu. (2009), the CAJ tannins from CAs growing in Ghana was characterized and the values ranged from 184.7mg/100mL to 412.8mg/100mL. Although other KwaNgwanase CAJ characteristics such as specific gravity and pH are similar to Ghana CAJ the tannin composition is not. Condensed tannins, as suggested by Lowor and Agyente-Badu. (2009) is influenced by climate conditions and drier climates contributes to a higher tannin content. From this information it can be assumed that the KwaNgwanase area is less dry than other CA growth regions. These condensed tannins, also known as proanthocyandins are phenolic compounds made up of oligomers and polymers of flavans which are mainly found in plant materials (Mullins and Lee, 1991; Sun et al., 1998; Nitao et al., 2001). Tannins are known to reduce microbial contamination (Nitao et al., 2001) and for the purpose of this research the initial high tannin composition (un-treated CAJ) was beneficial during transportation to prevent spoilage of the juice.

Total Minerals. Mineral compositions examined were zinc:1.39ppm, copper:2.18ppm, manganese:1.24ppm, magnesium:4.32ppm, iron: 1.32ppm and sodium:5.44ppm (Table 4.1). The highest mineral compositions were recorded for sodium and magnesium and the lowest for iron and manganese (Table 4.1). These mineral compositions of the KwaNganase CAJ were similar to that of Ghana CAJ (Lowor and Agyente-Badu, 2009). In the study by Lowor and Agyente-Badu. (2009) mineral results showed that sodium and magnesium possess the highest composition and iron the lowest. The results in this study are in accordance with the exception of the lowest mineral composition. In the present study, manganese showed the lower composition than iron and this mineral was not previously

investigated for CAJ. Other than iron and manganese, the zinc composition in the present study and previous study (Lowor and Agyente-Badu, 2009) was low which is common for fruit juices (Krejpcio et al., 2005). Similarities in mineral compositions are probably due to similar mineral deposits in the cashew tree cultivation areas in KwaNgwanase area and Ghana. The total minerals (15.89ppm) are beneficial and will be acquired by the yeast during fermentation as a growth source.

Vitamin C. The vitamin C composition of 112mg/100mL (Table 4.1) CAJ was determined from the ascorbic acid calibration curve shown in Figure 4.10. From the calibration curve, as the ascorbic acid concentration increases, the volume of titrant increases during the iodine titration assay (Fig 4.10).

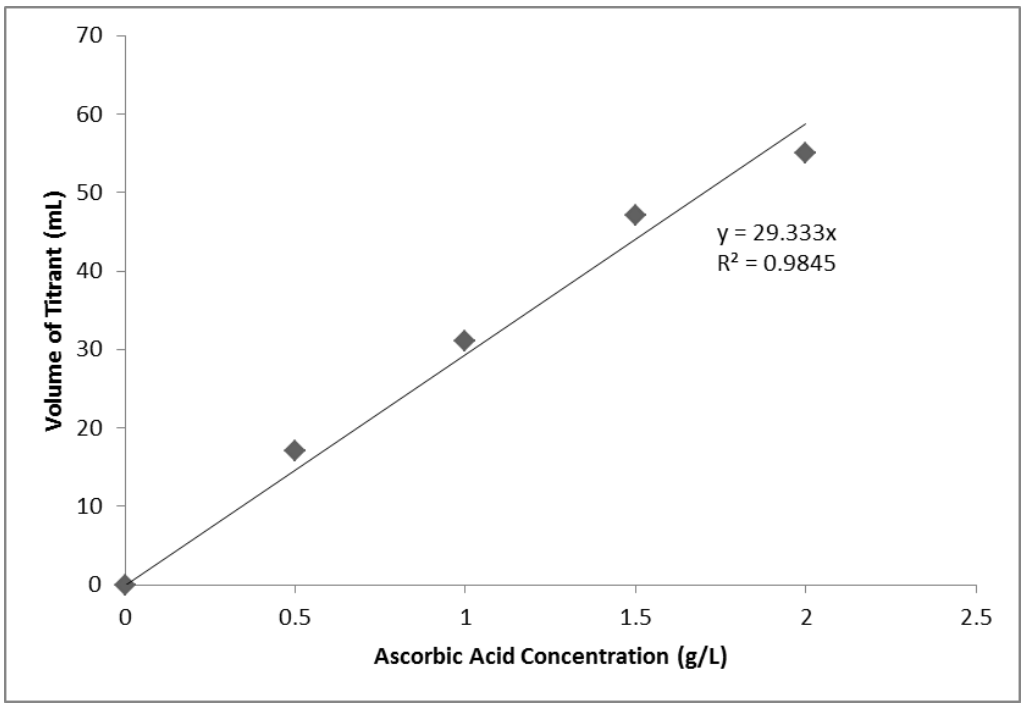


Figure 4.10 Calibration curve of the Ascorbic Acid standard at different ascorbic acid concentrations for the Iodine Titration assay. The values plotted are an average from three replicates.

For the iodine titration assay, an iodine solution is used to complex or bind with ascorbic acid in the case of the standards and with the vitamin C in the CAJ sample. The iodine solution reacts with the ascorbic acid to form a blue-black colour (Lowor and Agyente-Badu, 2009) and the volume of iodine corresponds to the amount of ascorbic acid. An average volume of 33mL of iodine titrant was recorded for CAJ and using the calibration curve (Fig 4.10) the vitamin C of the CAJ sample was determined and expressed in mg/100mL (Table 4.1). The vitamin C composition of 112mg/100mL value in this study (Table 4.1) was lower compared to the vitamin C content of CAJ from Ghana, Nigeria and India (Akinwale et al., 2000;2001; Attri, 2009; Lowor and Agyente-Badu, 2009). In the study by Akinwale et al. (2000;2001) vitamin C content was found to be 203.5mg/100mL of Nigeria CAJ. Lowor and Agyente-Badu. (2009) reported a vitamin C content of 268.60mg/100mL of Ghana CAJ. In India, it was found that CAJ from Bengal CAs have a vitamin C content of 209.76mg/100g (Attri, 2009). The vitamin C value for the KwaNganase CAJ was similar to the value detected by Assuncao and Mercadante. (2003) for the CAJ extracted form CAs growing in Piaui and Sao Paulo in Brazil. It can deduced that vitamin C content is determined by the soil composition, similar to minerals and CAJ characteristics are growth regional dependent. Vitamin C composition is an important requirement if the CAs or the juice is consumed as food or used in the manufacture of food or medicinal products as it provides nutrition and can be used for the treatment of diseases such as scurvy, gastritis and rheumatism (Akinwale, 2000; da Silva, 2000; Campos et al., 2002; Assuncao and Mercadante, 2003; Krejpcio et al., 2005; Attri, 2009; Lowor and Agyente-Badu, 2009; Sivagurunathan et al., 2010).

In the context of this study vitamin C probably contributes to the acidic pH of the juice and can serve as a co-factor for yeast enzymatic reactions during fermentation (Soubeyrand et al., 2005; Malherbe et al., 2007; Walker, 2011). Furthermore, Vitamin C was determined as the characteristics of CAJ from the KwaNgwanase area in South Africa have not been documented.

Total Proteins. The protein composition of KwaNgwanase CAJ was 1.78g/L (Table 4.1) as determined by the Bovine Serum Albumin (BSA) calibration curve shown in Figure 4.11. From the calibration curve, as the BSA concentration increases, the absorbance increases during the Coomassie Blue assay (Fig 4.11).

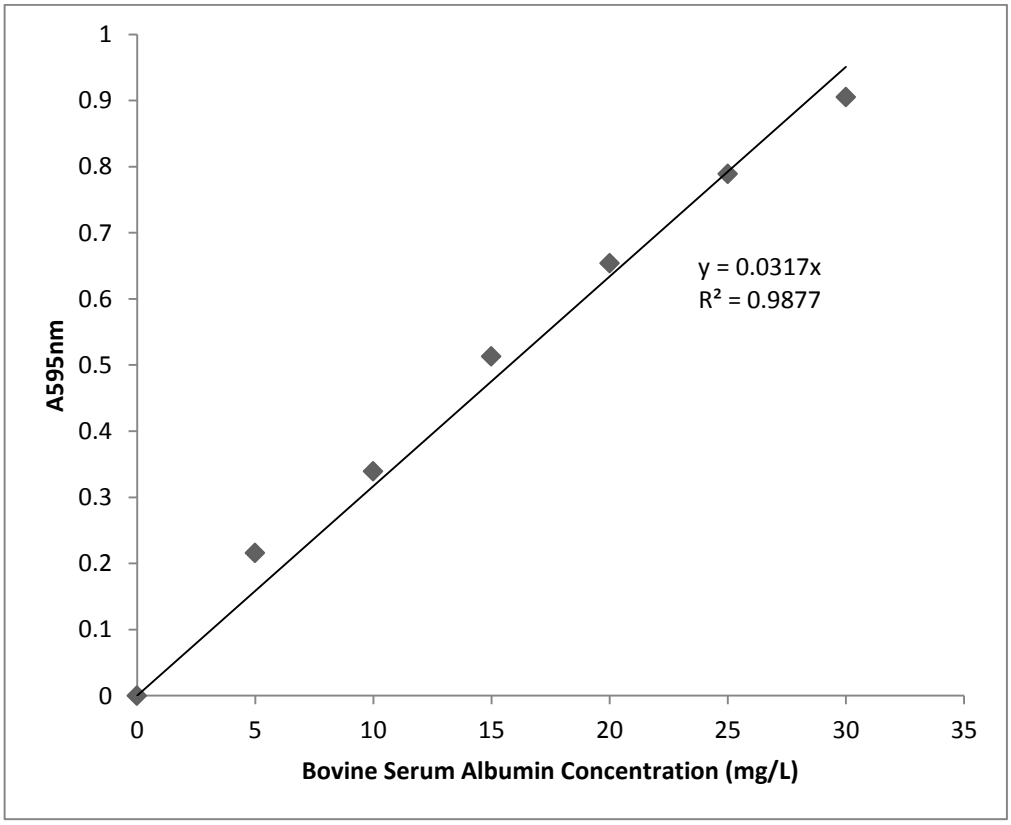


Figure 4.11 Calibration curve of the Bovine Serum Albumin standard protein at different bovine serum albumin concentrations for the Coomassie Blue assay. The values plotted are an average from three replicates.

The Coomassie Blue assay is a direct measurement of the protein content by the use of BSA which is the protein standard. The Coomassie Blue dye binds to this BSA protein to form a compound that absorbs light at 595nm (Bradford, 1976; Boyes et al., 1997). An average absorbance of 0.845 was recorded for the 1.5% dilution and the BSA concentration at this dilution was determined using the calibration curve (Fig 4.11). The initial assay resulted in a reading >1.000, the CAJ sample was then serially diluted to obtain an accurate reading. The value reported (Table 4.1) is indicative of the protein content in the original CAJ (undiluted). The total protein value reported in this study is lower compared to previous studies (Chagas et al., 2007; Honorato et al., 2007; Honorato and Rodrigues, 2010), which reported a total protein content of 2.58g/L. These studies were based on CAJ characterization from CAs grown in Brazil (Chagas et al., 2007; Honorato et al., 2007; Honorato and Rodrigues, 2010). To date, protein characterization values for CAJ in SSA is not documented. The available protein of the juice will be acquired during fermentation by the yeast as a nitrogenous growth source.

4.2.3 Preparation

"Prior to fermentation CAJ was thawed (Fig 4.12a) and refrigerated to settle out suspended solids (Fig 4.12b). The clear, top layer juice (Fig 4.12b) was centrifuged and analysed chemically (as discussed in section 4.2.2) and pre-treated with gelatine powder. Additionally the bottom layer (Fig 4.12b) was centrifuged to extract residual juice.

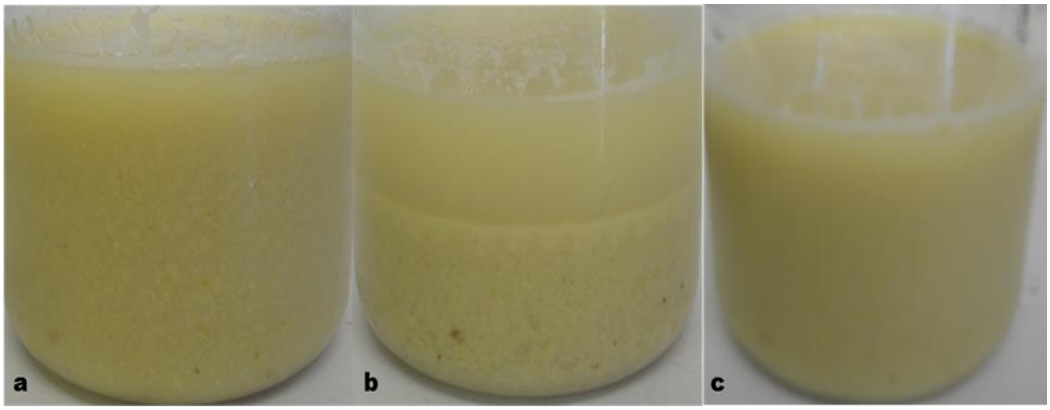


Figure 4.12a-c The preparation phases of cashew apple juice for fermentation. a: un-treated cashew apple juice. b: phase separation of suspended solids from pure juice. c: clarified cashew apple juice.

Overnight pre-treatment resulted in a decrease in tannins (Table 4.1). Following pre-treatment the juice was centrifuged to remove residual gelatine and remaining solids, resulting in a clarified CAJ as shown in Figure 4.12c. The preparation of CAJ was necessary as the clear juice with a lower tannin concentration and minimal to zero suspended solids will allow the yeast to easily assimilate the sugars and other components of the CAJ for growth and the production of bioethanol. Furthermore the reduction in tannins, which complexes with gelatine will reduce the negative effects on enzyme activity during fermentation as condensed tannins usually form insoluble compounds with proteins" (Deenanath et al., 2013).

4.3 Yeast Inoculum Preparation

An inoculum of the yeast strains is prepared prior to fermentation in order to activate the dry yeast pellets and preserved yeast strain. Inoculation of the 200mL

YPD liquid broth with the yeast strains Y2084 or Vin13 resulted in dense growth after the 18hr incubation period. The accumulation of yeast cells in the YPD broth is shown in Figure 4.13a-b. Both yeast strains displayed the same colour after growth. The culture of Y2084 is depicted in Figure 4.13b.

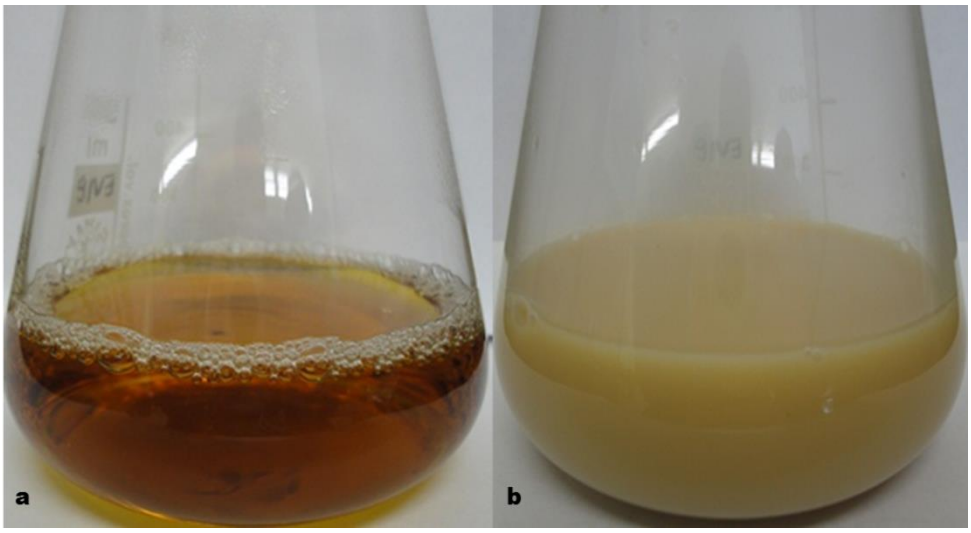


Figure 4.13a-b Yeast Peptone Dextrose liquid broth before inoculation (a) and after yeast growth (b).

The nutritional composition of the growth medium and the provided growth conditions of: temperature: 30ºC; agitation: 150rpm and time: 18hrs was suitable for the culturing of the Y2084 and Vin13 yeast strains.

4.4 Fermentation of Cashew Apple Juice

This section studies the fermentation of cashew apple juice in a BIOSTAT® Bplus fermentor with the use of Saccharomyces yeast strains Y2084 and Vin13 to produce bioethanol. The fermentation potential of the substrate and these yeasts is shown by evaluating the yeast viability, sugar consumption, ethanol production and glycerol concentration by instrumentation analysis. Additionally this section explores gas analysis which shows carbon dioxide and oxygen gas signals in conjunction with bioethanol production by yeast activity; on-line fermentation parameters for monitoring of the process; total organic carbon for determining the carbon input versus output; and statistical analysis for validity of results.

4.4.1 Yeast Viability

Viability counts of the strains Y2084 and Vin13 after culturing (section 4.3) and during the course of fermentation is listed in Table 4.2a-d.

Table 4.2a-d Viability counts (Log CFU/mL±SD) of Y2084 and Vin13. 4.2a: run 1 Y2084, 4.2b: run 2 Y2084, 4.2c: run 3 Vin13, 4.2d: run 4 Vin13.

4.2a:

Fermentation Time (Days)	Viable Cell Counts (Log CFU/mL)
0	8.62±0.30
1	7.37±0.30
2	7.46±0.42
3	8.62±0.35
4	8.60±0.35
5	8.65±0.40
6	8.57±0.22
7	8.52±0.33
8	8.47±0.34
9	7.50±0.40
10	7.42±0.33
11	7.29±0.37
12	7.27±0.48
13	6.21±0.55
14	6.13±0.66

4.2b:

Fermentation Time (Days)	Viable Cell Counts (Log CFU/mL)
0	8.58±0.26
1	7.50±0.29
2	8.51±0.24
3	9.49±0.47
4	9.58±0.37
5	9.67±0.40
6	9.47±0.63
7	8.49±0.45
8	8.39±0.54

4.2c:

Fermentation Time (Days)	Viable Cell Count (Log CFU/mL)
0	8.64±0.24
1	8.48±0.47
2	7.41±0.53
3	6.52±0.61
4	6.51±0.67
5	6.71±0.10

4.2d:

Fermentation Time (Days)	Viable Cell Count (Log CFU/mL)
0	8.67±0.29
1	9.71±0.51
2	9.53±0.40
3	9.59±0.64
4	8.33±0.62
5	8.23±0.57

The initial cell count at day 0 achieved for both yeast strains (Table 4.2a-d) is in accordance with viable counts of yeast cells in previous studies (Thuesombat et al., 2007; Deenanath et al., 2010a;b) and the expected viable count of pure *S. cerevisiae* cultures (Ciani et al., 2006). Viability of approximately 8.00 Log CFU/mL (Table 4.2a-d) is higher than previous studies and the initial concentration of approximately 6.00 to 7.00 Log CFU/mL which is required for fermentation (Lafon-Lafourcabe et al., 1984; Malherbe et al., 2007; Mannazzu et al., 2008; Torrea et al., 2011). For the yeast strain Y2084, subculturing was useful in obtaining a high cell count (Table 4.2a-b). Between day 0 and day 1 there was a decrease in cell count of runs 1, 2 and 3 (Table 4.2a-b,c). This is expected as the yeast cells were inoculated into the environment of the CAJ and there was an adjustment period to the new environment. When the cells began to utilize the available nutrients of the CAJ, cell viability increases. The increase is observed at day 3 for run 1 and at day 2 for run 2 (Table 4.2a-b). For run 3, no increase is noticed instead there is a decline in viability (Table 4.2c). This is the opposite of

run 4, where the viability does not decline but increases from day 0 to day 3 (Table 4.2d). At these time points the cells are in the exponential growth phase and there is sugar consumption and sugar metabolism (Bellissimi and Ingledew, 2005) Moreover, the high viability counts could be stimulated by the presence of minerals, vitamin C and proteins in the CAJ (Table 4.1) and supplementation of the CAJ with ammonium sulphate (section 3.2.3). In comparison to previous research studies, viable cell counts between 5.00 to 7.00 Log CFU/mL were obtained (Lafon-Lafourcabe et al., 1984; Mannazzu et al., 2003; Torrea et al., 2011). A viable cell count of 7.00 Log CFU/mL was reported by Lafon-Lafourcabe et al. (1984). The cell count was obtained by culturing the yeast, *S. cerevisiae* (Rapidase) in grape juice, which was also the fermentation medium (Lafon-Lafourcabe et al., 1984). Mannazzu et al. (2003) reported a value of 5.00 Log CFU/mL of the *S. cerevisiae* strains: BY4743; L2056; and M25 by culturing the yeasts in YPD liquid broth. In another study, a value of 6.00 Log CFU/mL was obtained by culturing the yeast *S. cerevisiae* AWRI 796 in MYPG liquid broth and incubated at 27ºC with agitation at 180rpm (Torrea et al., 2011). The MYPG broth consisted of malt extract, yeast extract, peptone and glucose (Torrea et al., 2011) which are similar to the broth used in this study (section 3.3). From the collective research data, the initial viable cell count in the present study is higher but similar to the cell count reported by Lafon-Lafourcabe et al. (1984). Thus, yeast can be pre-cultured in the fermentation medium to obtain a high viability. However, in the present study the limited volume of CAJ could not permit the dual usage of the juice as a pre-culture broth. In the study by Mannazzu et al. (2003), a much lower cell count was obtained even though the same broth

was used for culturing the yeasts. Since different yeast strains were used, it is assumed that various strains of *S. cerevisiae* yeasts show different growth in identical nutritional compositions. From the results of Torrea et al. (2011), the liquid broth was similar to the present study but the growth conditions were different. Thus, in addition to the choice of liquid broth and the growth conditions selected for culturing can also influence the viability outcome. The liquid growth medium and culture conditions selected in this study were ideal to obtain a high initial cell count of both yeast strains and the CAJ components together with the fermentation conditions supported the yeast growth during the course of fermentation.

4.4.2 High Performance Liquid Chromatography Analysis

The HPLC system used was the LC1100 series (Agilent Technologies) equipped with a solvent delivery system (quaternary pumps); autosampler; refractive index and wavelength detectors; thermo stated column compartment and ChemStation software programme. For the HPLC analysis, samples are eluted from the column with 0.001M H_2SO_4 (mobile phase prepared using Millipore filtered water) at a flow rate of 0.8mL/min. Peaks are detected by the refractive index detector (RID) and quantified by comparison to the retention times (RT) of the standards. HPLC was used in the identification of sugar, ethanol and glycerol profiles during the course of fermentation. The ChemStation software programme generated qualitative and quantitative data. Qualitative data will be represented by HPLC chromatograms and quantitative data will be represented by graphs depicting the concentrations obtained by the calibration of standards. The sugar concentrations

were determined by sugar standards and calibration curves are shown in Figures 4.6a-c and 4.7a-c (section 4.2.2). The ethanol and glycerol concentrations were determined by the standards and calibration curves depicted in Figures 4.14a-b and 4.15a-b.

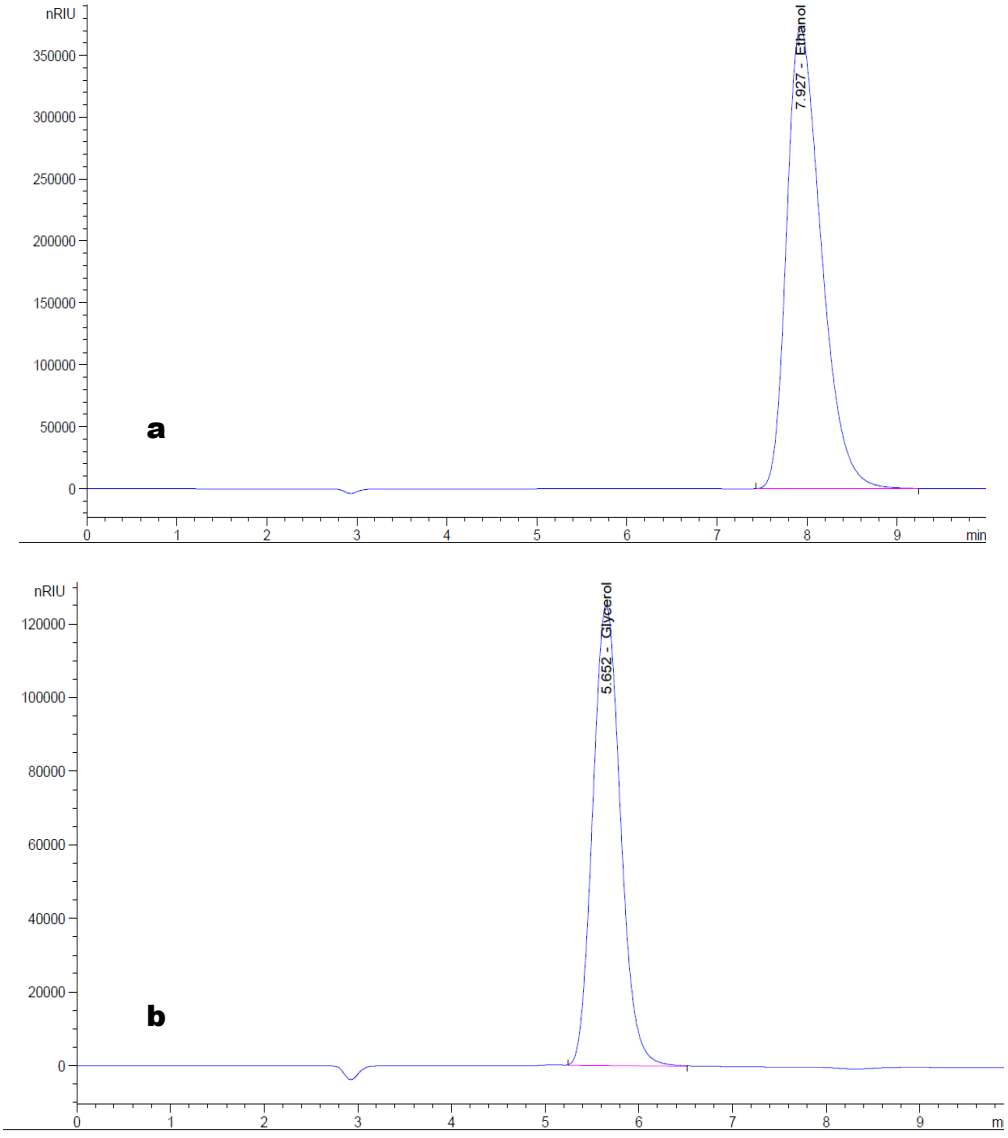


Figure 4.14a-b HPLC chromatograms of ethanol (a) and glycerol (b) standards. Aminex® Fermentation Monitor Column (150x7.8mm) conditions: column temperature: 60°C; mobile phase: 0.001M H_2SO_4; RID temperature: 40°C; flow rate: 0.8mL/min; injection volume: 20uL. Peak identification is indicated on the chromatogram as ethanol (a) and glycerol (b).

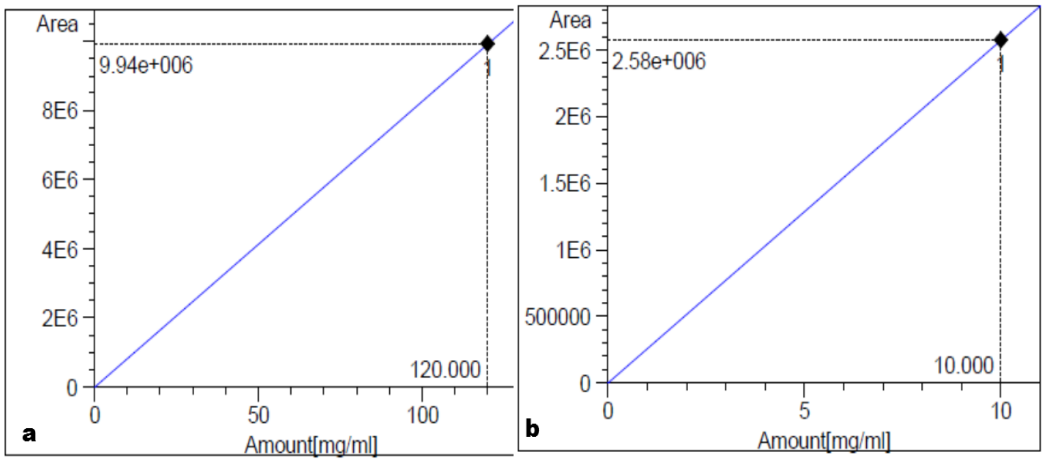
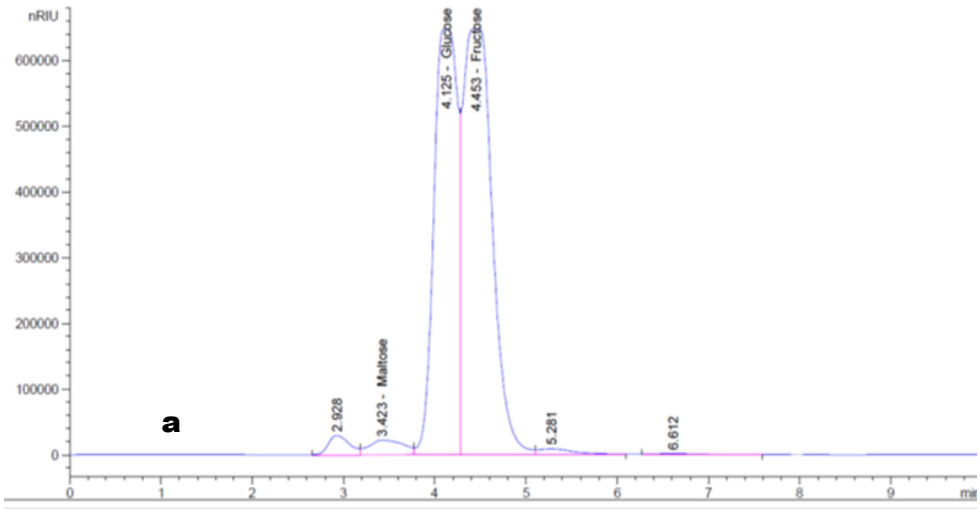
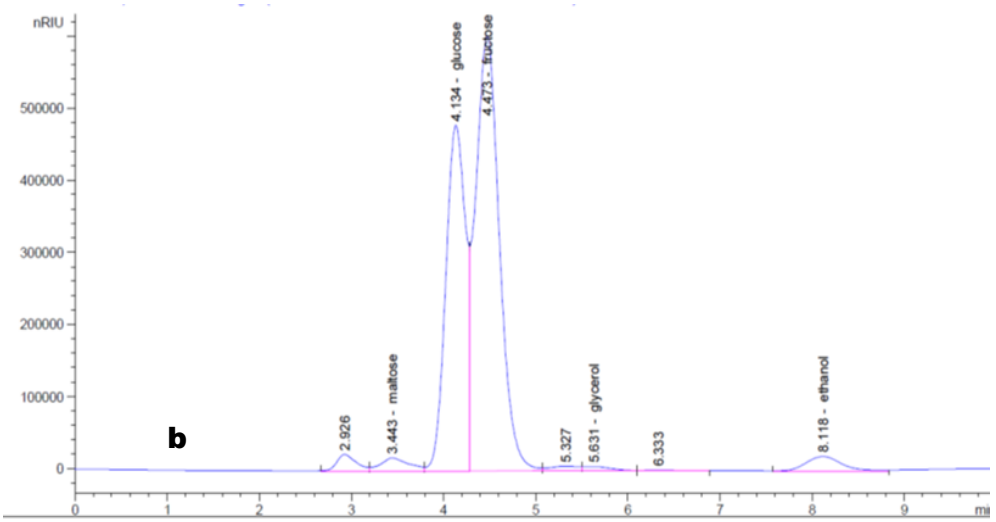


Figure 4.15a-b Calibration curves of ethanol (a) and glycerol (b) standards as constructed from the ChemStation software.

Fermentation Run 1

The qualitative data for fermentation run 1 is depicted in Figure 4.16a-l.

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4.134 - glucose
4.473 - fructose
5.327
5.631 - glycerol
6.333
8.118 - ethanol
0
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2
3
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5
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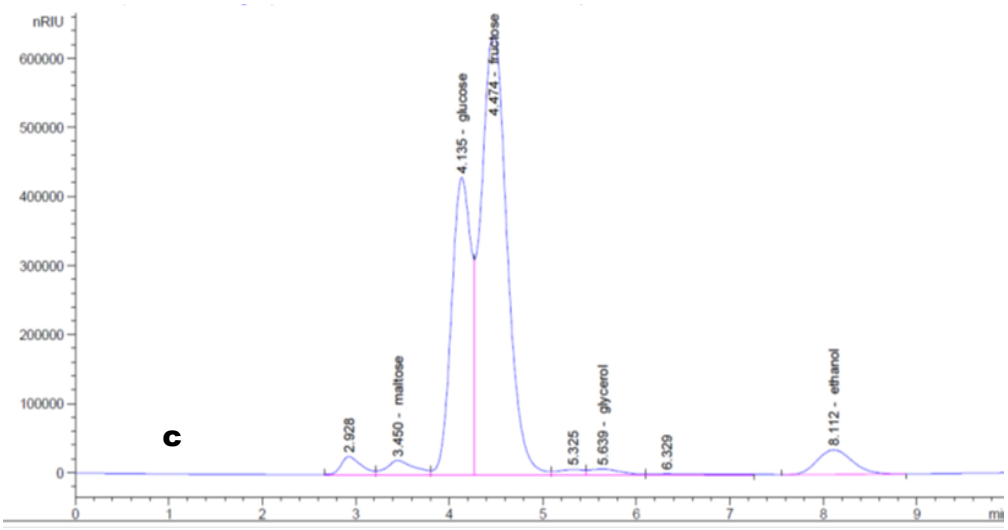
9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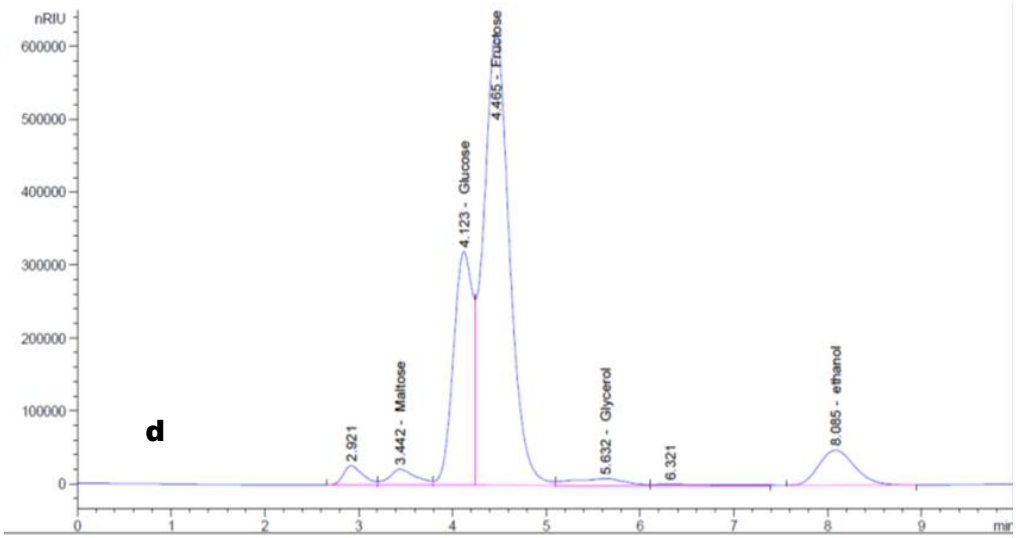
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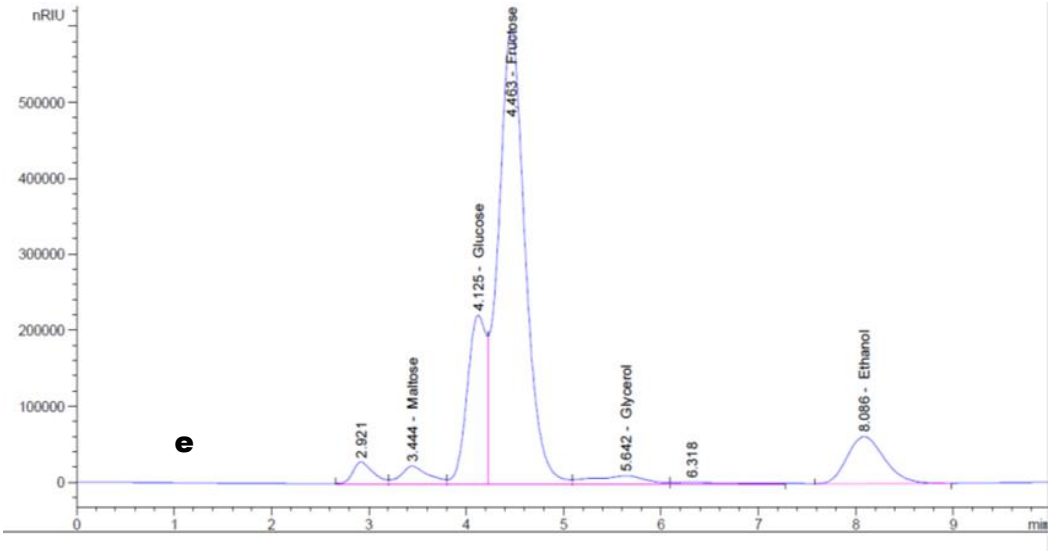
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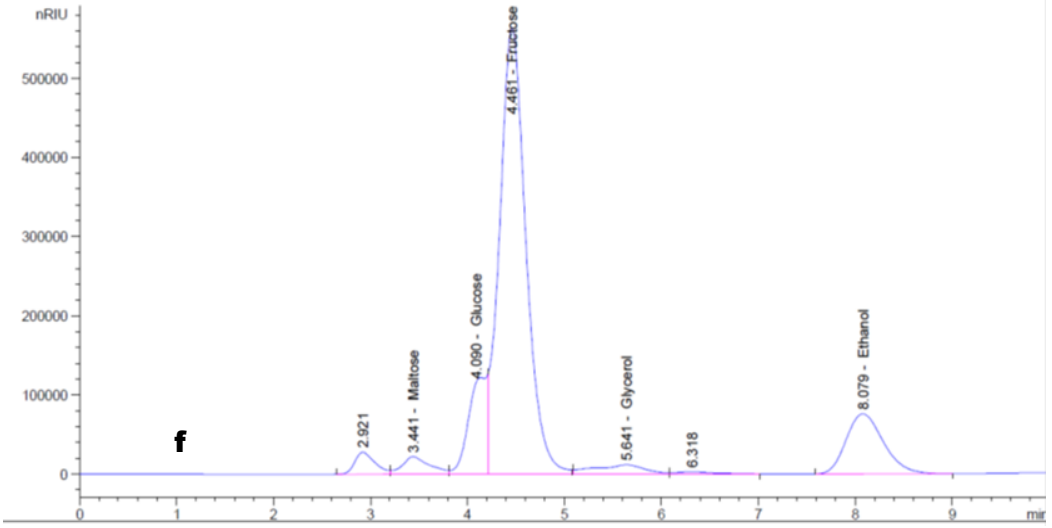
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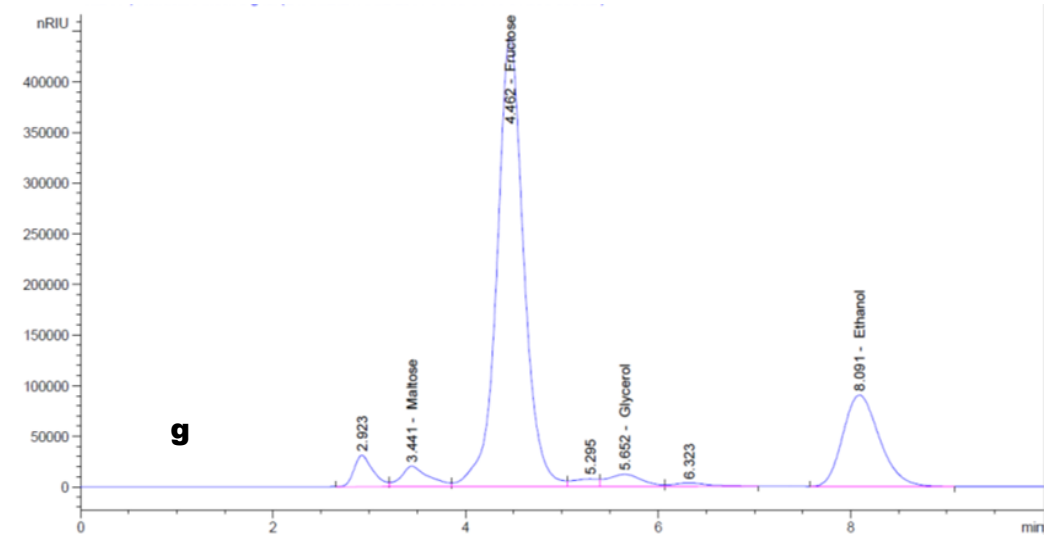
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6.323
8.091 - Ethanol
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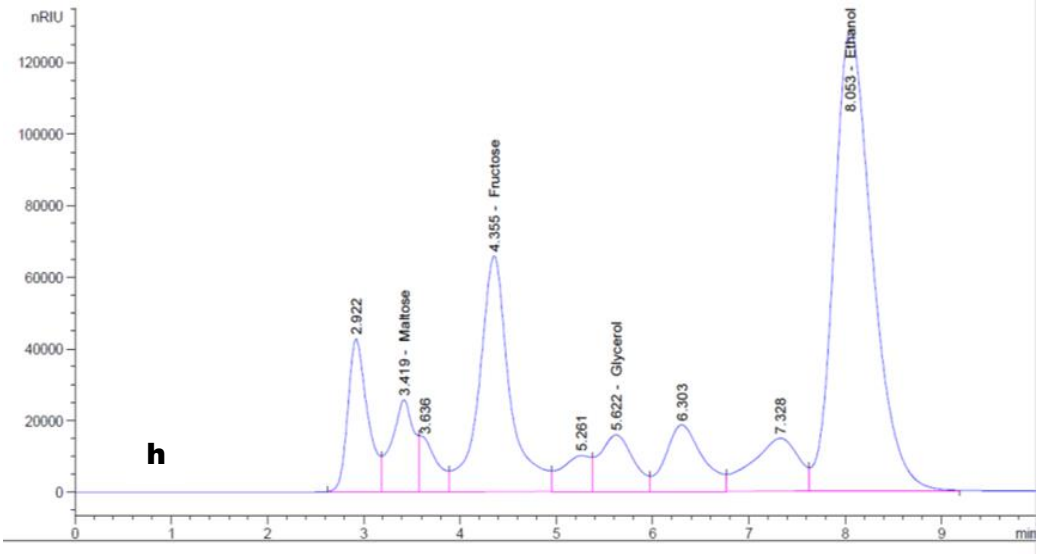
6
8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5.622 - Glycerol
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7.328
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3
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9
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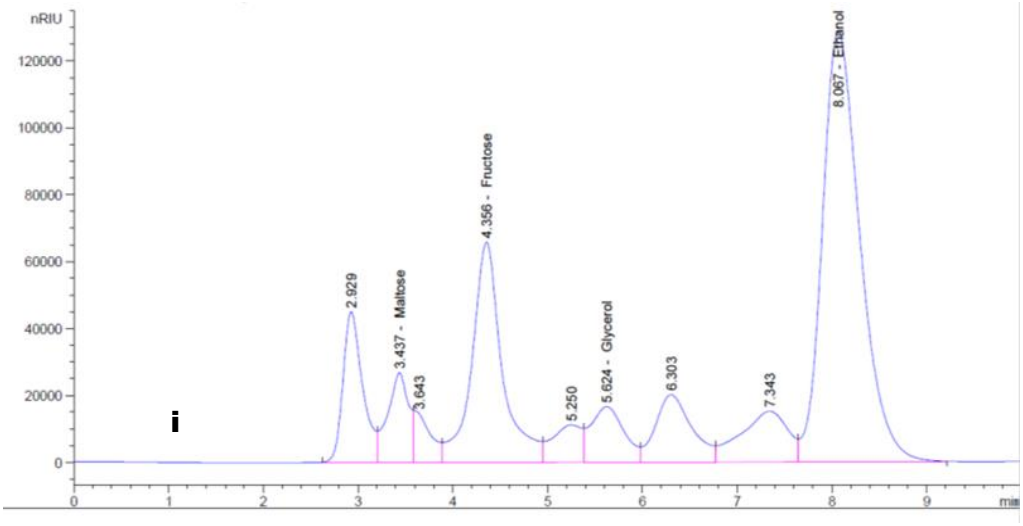
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5.624 - Glycerol
6.303
7.343
8.067 - Ethanol
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3
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9
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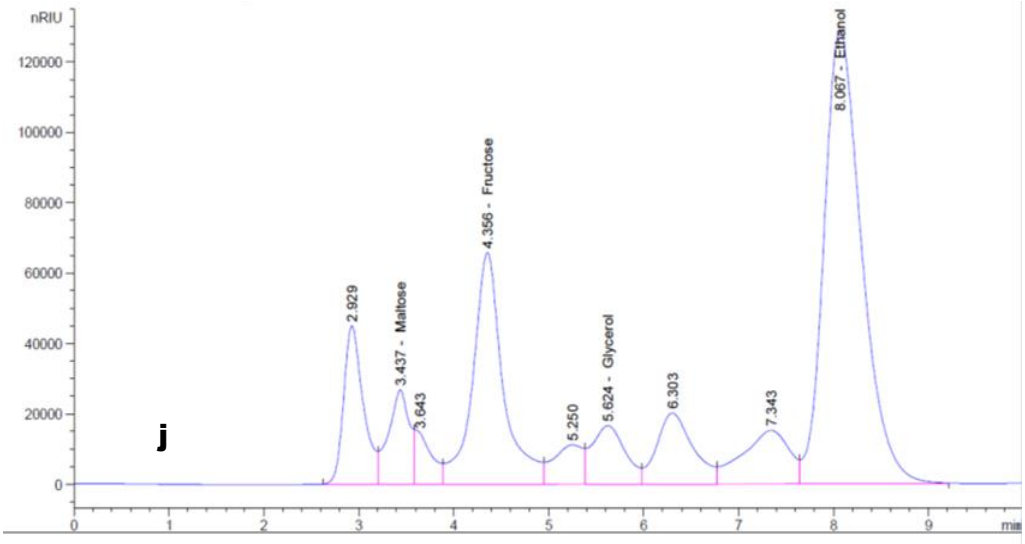
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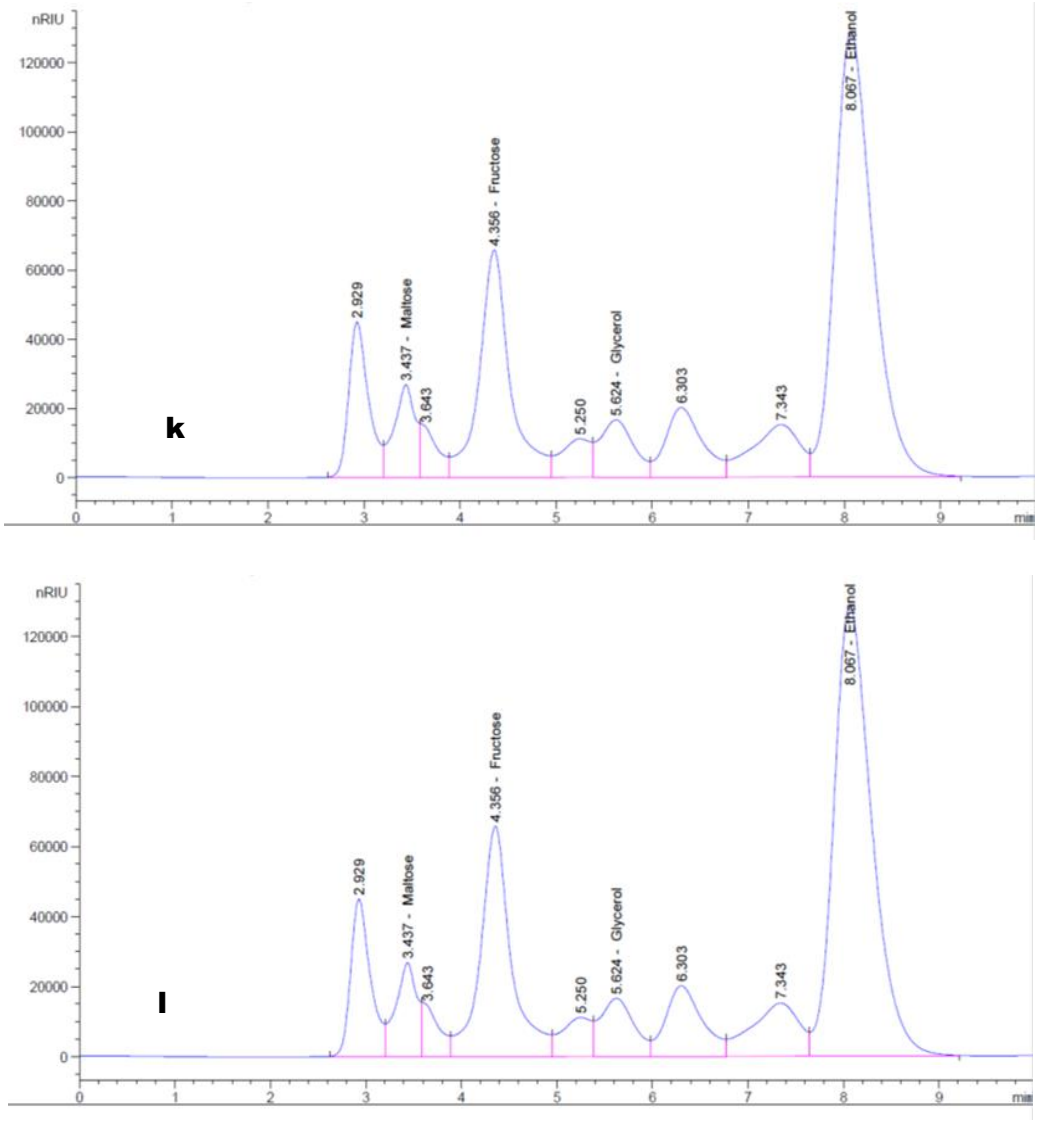


Figure 4.16a-l HPLC chromatograms of sugars, ethanol and glycerol from day 0 to day 11 of run 1. Peak identification based on the refractive index and retention time is indicated on the chromatograms.

The HPLC chromatograms revealed maltose has the shortest retention time followed by glucose, fructose, glycerol and ethanol (Fig 4.16a-l). Highest sugar concentrations are glucose and fructose and the lowest is maltose. This is visible from the height of the peaks (Fig 4.16a). At first glance, the glucose and fructose peaks appear as a single peak. There is co-elution of these sugars from the column as they have similar retention times. During run 1, glucose is utilised first then

fructose. Maltose is not utilized by the yeast strain Y2084. As the sugar peaks decrease in height, the ethanol and glycerol peaks increase (Fig 4.16b-l). Ethanol peaks are visible from day 1 to day 11 (Fig 4.16b-l) and glycerol peaks are visible from day 6 to day 11 (Fig 4.16f-l).

The fermentation profile of run 1 is presented in Figure 4.17 from the quantitative HPLC data. Additionally, the viable yeast cell counts are shown in Figure 4.17 to correlate the yeast viability (section 4.3) and HPLC data.

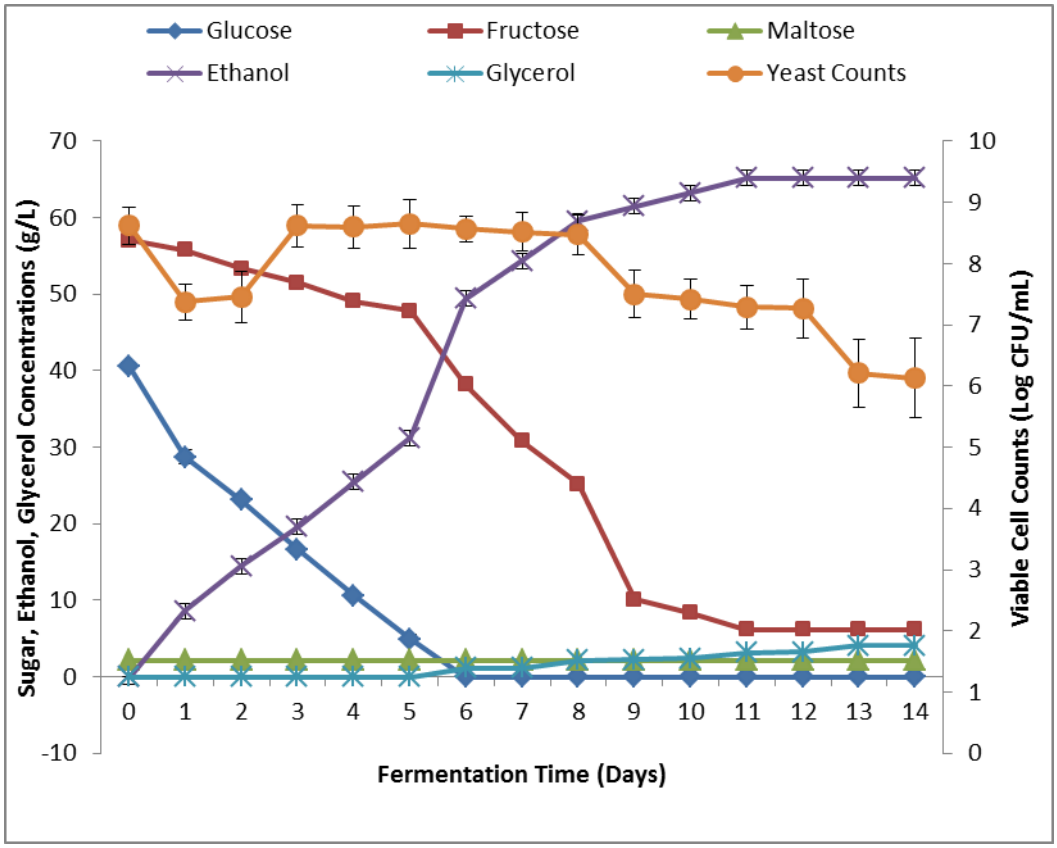


Figure 4.17 Fermentation profile of Y2084 at temperature: 30ºC; pH: 4.50; oxygen saturation: 0%; stirrer speed: 150rpm; yeast inoculum concentration: 8.62 Log CFU/mL. The values plotted are an average from six replicates. Error bars represent ±SD, where not seen, they lie within the plot symbol.

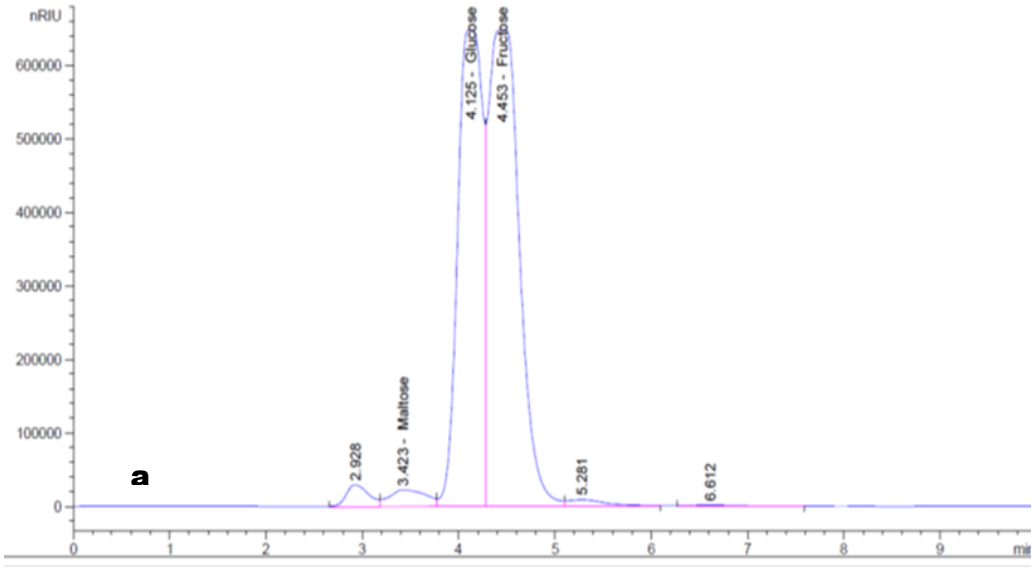
From day 0 to day 1 there is a change in the initial starting concentrations of glucose, fructose and ethanol (Fig 4.17). Glucose is almost halved from 40.56g/L to 28.73g/L with a minimal decrease in fructose from 57.06g/L to 55.74g/L. The decrease in sugar resulted in an increase in ethanol from 0g/L to 8.59g/L (Fig 4.17). A drop in viable cells from 8.62 to 7.38 Log CFU/mL is noticed at day 1 (Table 4.2;Fig 4.17) due to a change in nutritional environment from the YPD liquid culture broth to the CAJ substrate. At this point the yeast cells enter the lag phase. At day 2 the glucose and fructose concentrations continue to decrease, the ethanol concentration increases and the yeast cells remain in the lag phase (Fig 4.17). Between day 2 and day 3 a high concentration of glucose is consumed (Fig 4.17) and this coincides with the increase in viable cells (Table 4.2; Fig 4.17). The yeasts enter the exponential phase by day 3 and ethanol concentration increases (Fig 4.17). From day 3 to day 5 sugar consumption and ethanol production continues (Fig 4.17). Yeast viability is maintained at approximately 8.00 Log CFU/mL and it appears the cells are functioning in stationary phase for the duration of run 1 (Table 4.2;Fig 4.17). Kwan et al. (2007) reported a cell count of 8.51 Log CFU/mL under anaerobic conditions and fermentation temperature of 30ºC, which is similar to the yeast counts obtained during this fermentation run. Between day 5 and day 6 the glucose supply is depleted and this results in the rapid consumption of fructose (Fig 4.17), the ethanol concentration increases and there is yeast cell growth (Fig 4.17). Additionally, the glycerol concentration has increased from 0g/L to 1.14g/L (Fig 4.17) which coincides with the chromatogram peak detected by HPLC (Fig 4.16f). At day 7 there are changes in the fructose and ethanol concentrations, whilst no drastic changes are recorded for

the yeast viability counts and glycerol concentration (Fig 4.17). The day 8 trend is similar to day 7, with an increase in the glycerol concentration (Fig 4.17). Between day 9 to day 11 fructose consumption and ethanol production continues, the glycerol concentration increases, (Fig 4.17) and there is a decline yeast viability (Table 4.2;Fig 4.17). At this stage the minimal sugar concentration and ethanol accumulation contributes to the lack of viability. Moreover, the increase in glycerol concentration coincides with the decrease in viability. Yeast cells produce glycerol as a by-product (Alfenore et al., 2002) and in this study the production is probably in response to stress. There is also a slow progression in ethanol accumulation at day 9 to day 11 compared to day 1 to day 7 (Fig 4.17). From day 12 to day 14 no further change in fructose and ethanol concentrations were recorded. There is a decline in yeast viability and increase in glycerol (Table 4.2;Fig 4.17). Run 1 continued for a total of 14 days and the fermentation can be termed as "sluggish" (Malherbe et al., 2007). The data recorded for day 12 to day 14 shows that the ethanol steady state was reached by day 11 (Fig 4.17). During the course of fermentation glucose and fructose were the dominant sugars utilized by strain Y2084 and maltose was not consumed (Fig 4.17). A maximum ethanol concentration of 65.17g/L was obtained within 11 days. The concentration of residual sugars (fructose and maltose) was 8.38g/L. On the basis of the high ethanol concentration, the selected fermentation conditions were suitable for the yeast strain Y2084 to adapt to the CAJ medium and ferment the available sugars. The prolonged duration resulted in the decline in yeast viability and the rise in glycerol (Fig 4.17) which could be due to the lack of aeration prior to yeast addition and during the process. "However, since nitrogen gas was not passed

through the system to displace air in the vessel, the fermentation environment of run 1 can be considered as semi-anaerobic. This is advantageous as the yeast cells were able to survive for a longer time period by acquiring the available air. Other than the available sugars, nutrients such as proteins and minerals acquired from the CAJ (Table 4.1) and external supplements such as ammonium sulphate provide support for the growth and functioning of the yeast" (Deenanath et al., 2013). Ammonium and magnesium is known to stimulate yeast growth (Alfenore et al., 2002, Malherbe et al., 2007) both components are present in the CAJ. Furthermore, Vitamin C is probably sequestered from the CAJ (Table 4.1), as *S.cerevisiae* utilize vitamins as co-factors, together with minerals for the enzyme reactions of the glycolysis pathway during fermentation (Soubeyrand et al., 2005; Malherbe et al., 2007; Walker, 2011). Based on the experimental results of run 1, the ANOVA analysis demonstrated significance at an F-value of 21.86 and p-value<0.0001.

Fermentation Run 2

The qualitative data for fermentation run 2 is depicted in Figure 4.18a-f.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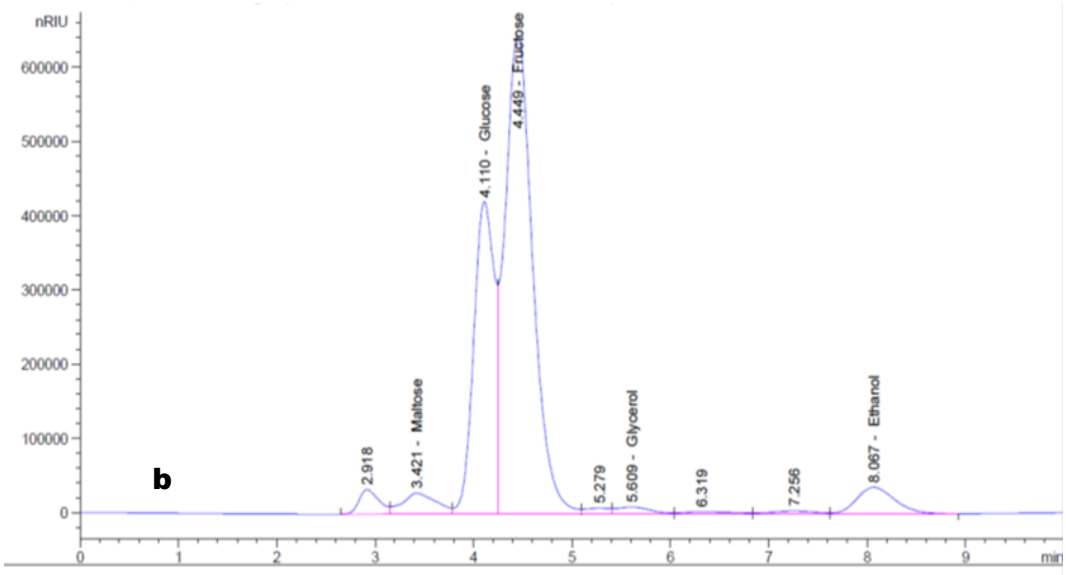
6
7
8
9
min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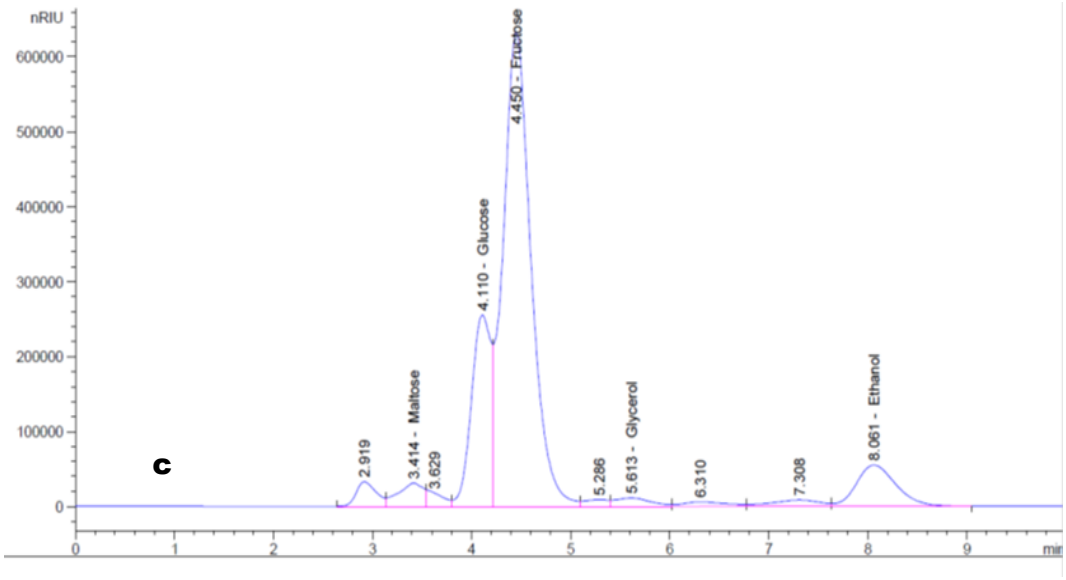
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5.286
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3
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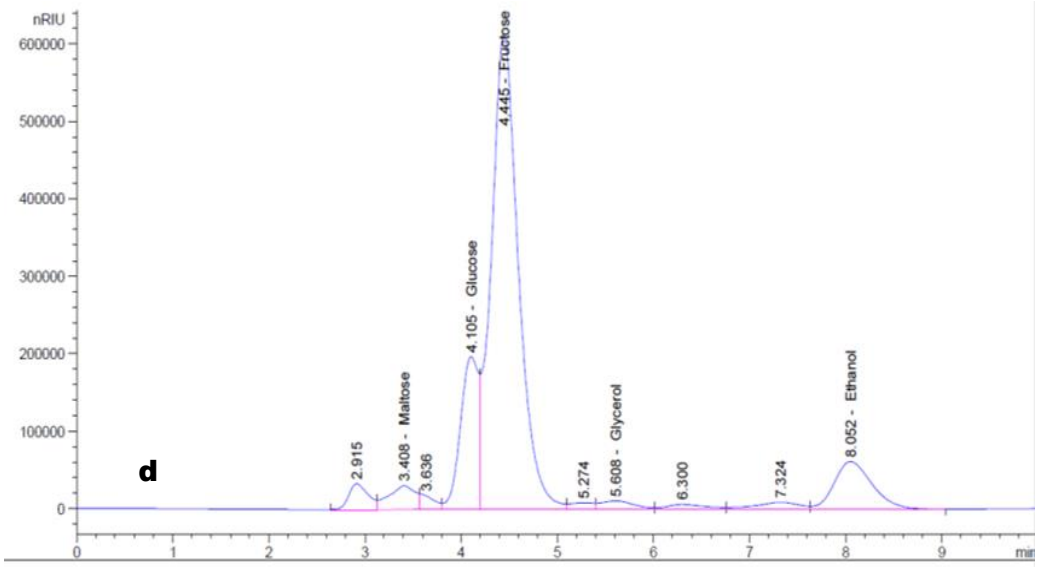
7
8
9
min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5.274
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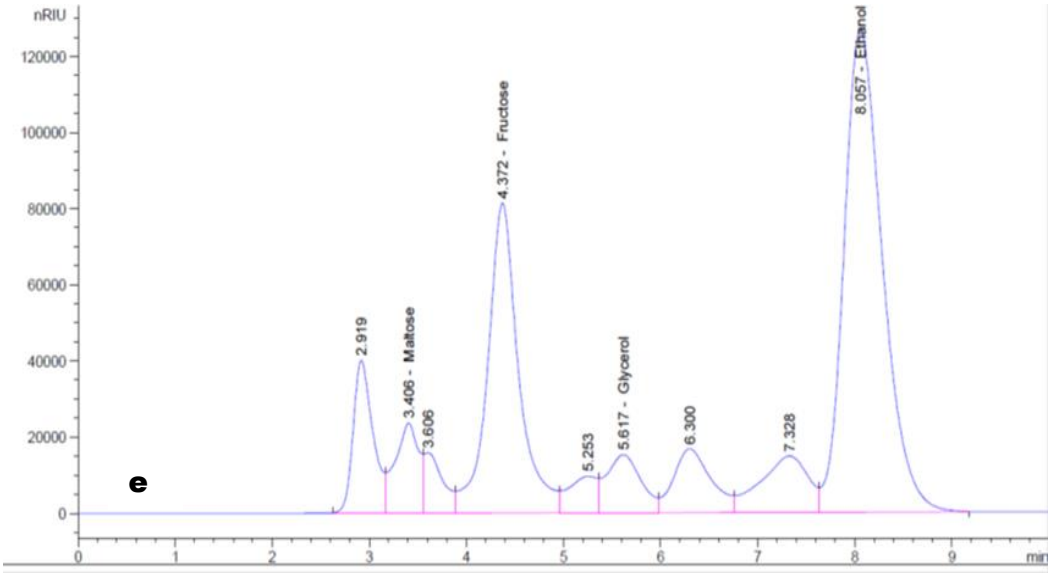
3
4
5
6
7
8
9
min



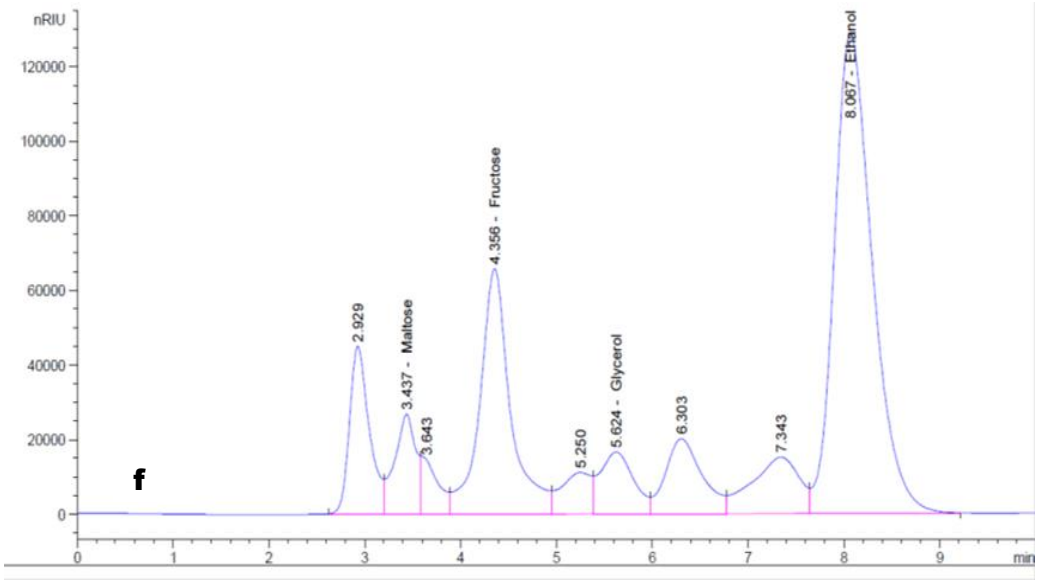


Figure 4.18a-f HPLC chromatograms of sugars, ethanol and glycerol from day 0 to day 5 of run 2. Peak identification based on the refractive index and retention time is indicated on the chromatograms.

The HPLC chromatograms of run 2, like run 1 reveal the order of elution as maltose, glucose, fructose, glycerol and ethanol (Fig 4.18a-f). Glucose is utilized

first, followed by fructose (Fig 4.18b-f). The ethanol peak is visible from day 1 to day 5 (Fig 4.18b-f) and the glycerol peak is visible at day 5 (Fig 4.18f).

The fermentation profile of run 2 is presented in Figure 4.19 from the quantitative HPLC data, which includes the yeast viability counts.

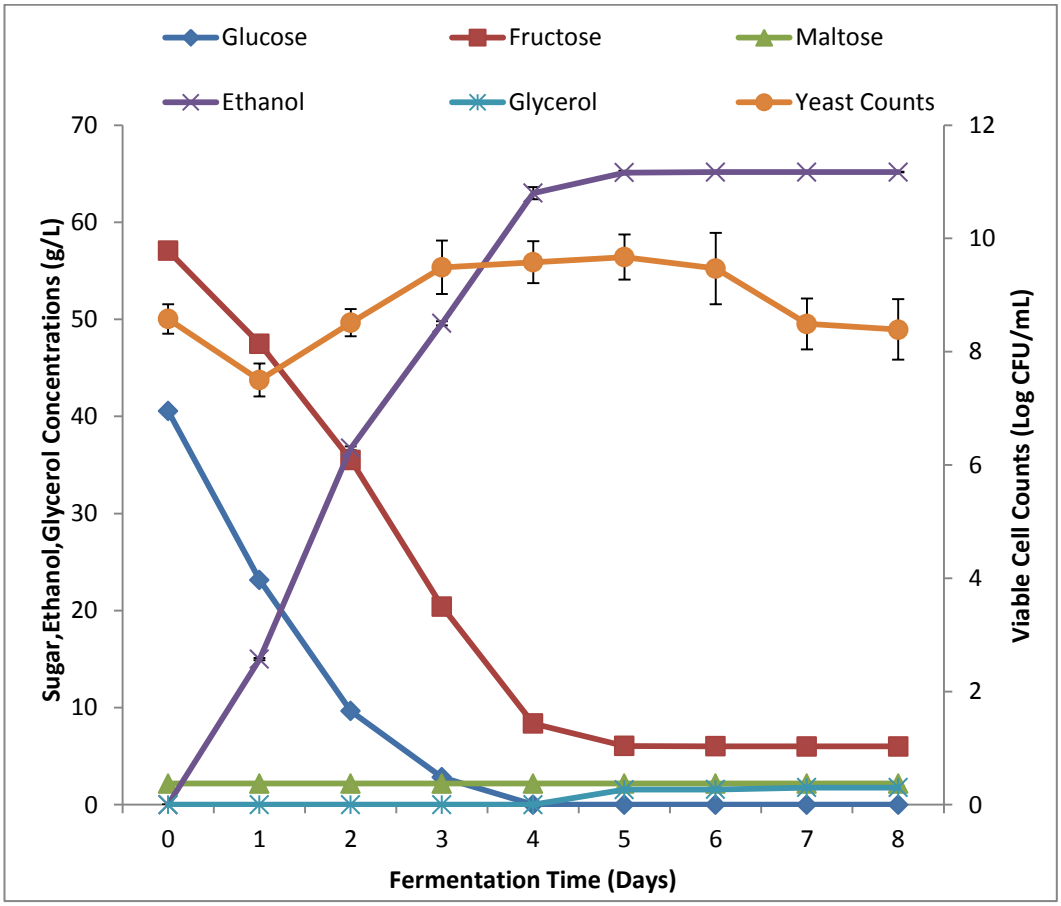


Figure 4.19 Fermentation profile of Y2084 at temperature: 30°C; pH: 4.50; oxygen saturation: 50%; stirrer speed: 150rpm; yeast inoculum concentration: 8.58 Log CFU/mL. The values plotted are an average from six replicates. Error bars represent ±SD, where not seen, they lie within the plot symbol.

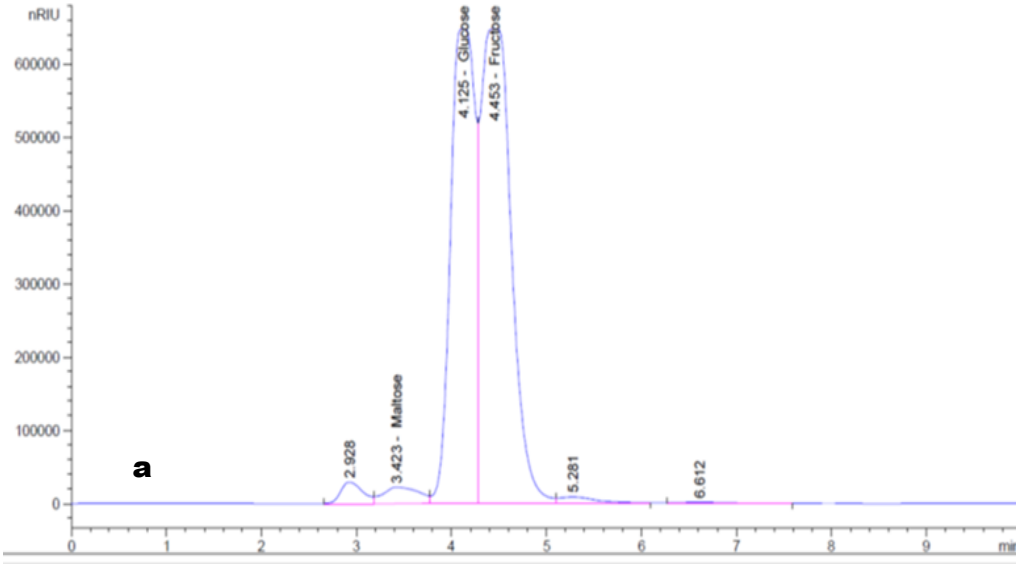
From day 0 to day 1 the glucose concentration is halved and fructose rapidly decreases (Fig 4.19). The utilization of sugars resulted in an increase in ethanol

concentration from 0g/L to 15.00g/L (Fig 4.19). Yeast cells enter a temporary lag phase from day 0 to day 2. At day 2 there is an increase in yeast viability (Table 4.2), the ethanol concentration is doubled and the glucose concentration almost depleted (Fig 4.19). The day 3 trend is similar to day 2, where the sugar consumption and ethanol production continues (Fig 4.19) with an increase in yeast viability from 8.00 to 9.00 Log CFU/mL. This show the cells have entered the exponential phase (Table 4.2;Fig 4.19). Between day 3 to day 4 the glucose supply is depleted (Fig 4.19), fructose consumption and ethanol production continues and there is an increase in viable cells (Fig 4.19). At day 5, similar to day 4 the decrease in fructose, increase in ethanol and an increase in yeast viability is noticed (Fig 4.19). In addition, the glycerol concentration spiked from 0 to 1.56g/L (Fig 4.19). From day 6 to day 8 no further change in fructose and ethanol concentrations were recorded. There is a decline in yeast viability and a slight increase in glycerol (Table 4.2;Fig 4.19). Run 2 continued for a total of 8 days. The data recorded for day 6 to day 8 shows the ethanol steady state was reached at day 5. The maximum ethanol concentration was 65.12g/L and the concentration of residual sugars was 8.19g/L. Maltose is not utilized (Fig 4.19). The fermentation conditions were suitable for ethanol production. Oxygen saturation allowed rapid growth of Y2084 and the cells entered the exponential growth phase. This contributed to a shorter duration of the fermentation and faster production of ethanol. The fermentation time of 5 days for this run was similar to the fermentation of CAJ by the yeast strains *S. cerevisiae* SCP and SCT (Araujo et al., 2011). The research reported on the evaluation of volatiles during CAJ fermentation for cashew wine production (Araujo et al., 2011). Fermentation

conditions such as temperature:28ºC and pH:4.00 (Araujo et al., 2011) were similar to the present study. Thus, CAJ fermentation at similar operating conditions can exhibit similar trends in the fermentation time. Based on the experimental results of run 2, the ANOVA analysis demonstrated significance at an F-value of 13.59 and p-value<0.0001.

Fermentation Run 3

The qualitative data for fermentation run 3 is depicted in Figure 4.20a-c.



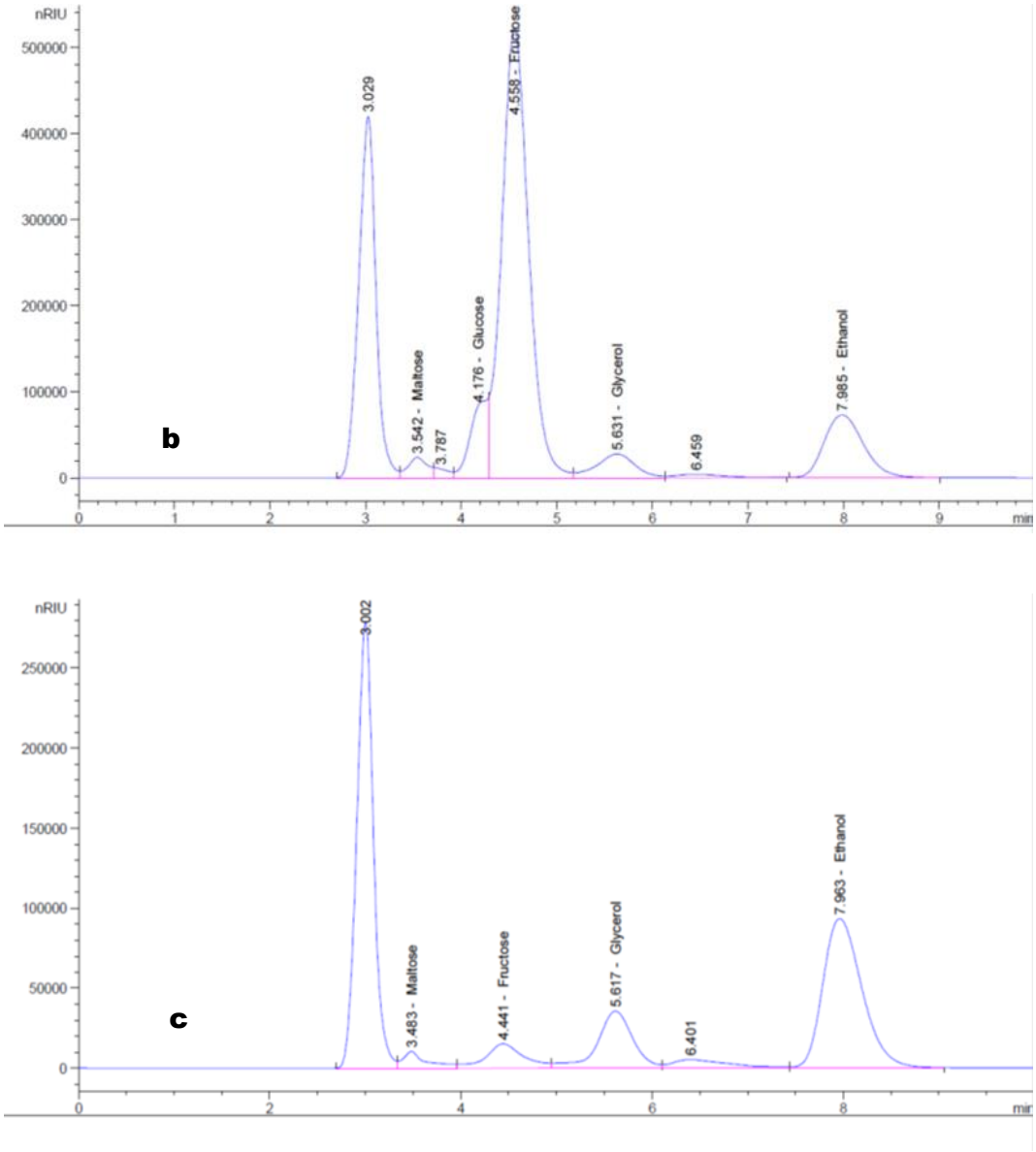


Figure 4.20a-c HPLC chromatograms of sugars, ethanol and glycerol from day 0 to day 2 of run 3. Peak identification based on the refractive index and retention time is indicated on the chromatograms.

The HPLC chromatograms of run 3 (Fig 4.20a-c) revealed the same order of elution as seen for run 1 and run 2 (Figs 4.16;4.18). Glucose and fructose peaks changed rapidly from day 0 to day 2 (Fig 4.20a-c). Ethanol and glycerol peaks are visible from day 1 (Fig 4.20b-c).

The fermentation profile of run 3 is presented in Figure 4.21 from the quantitative HPLC data, which includes the yeast viability counts.

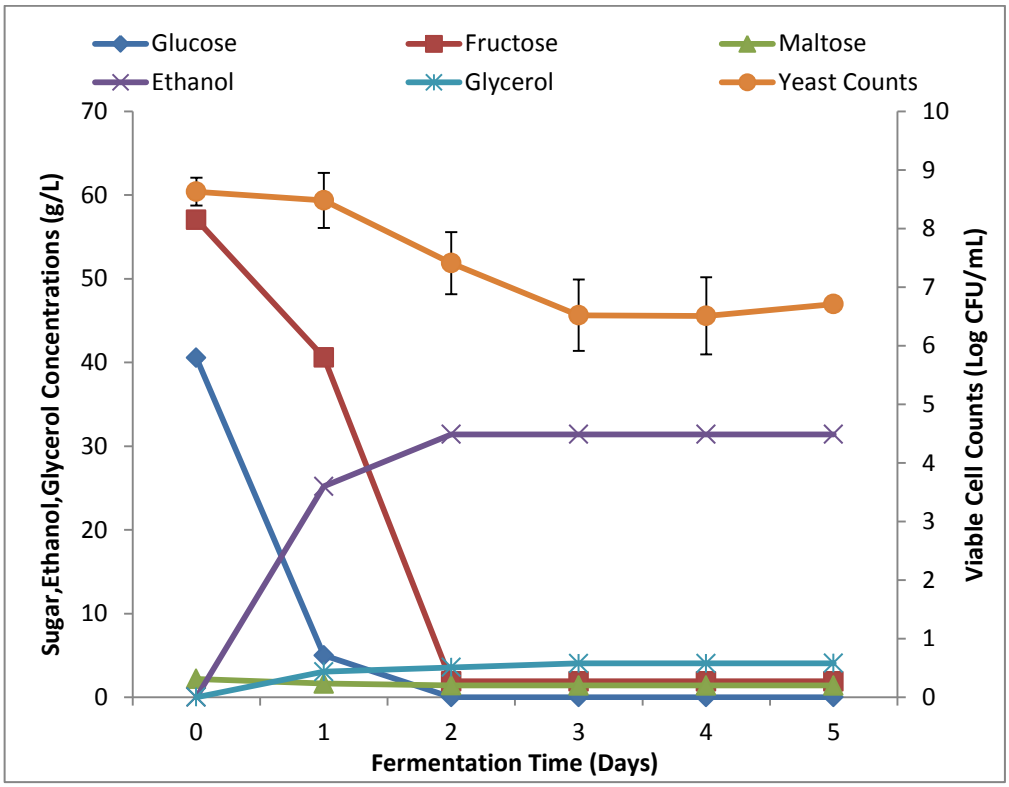


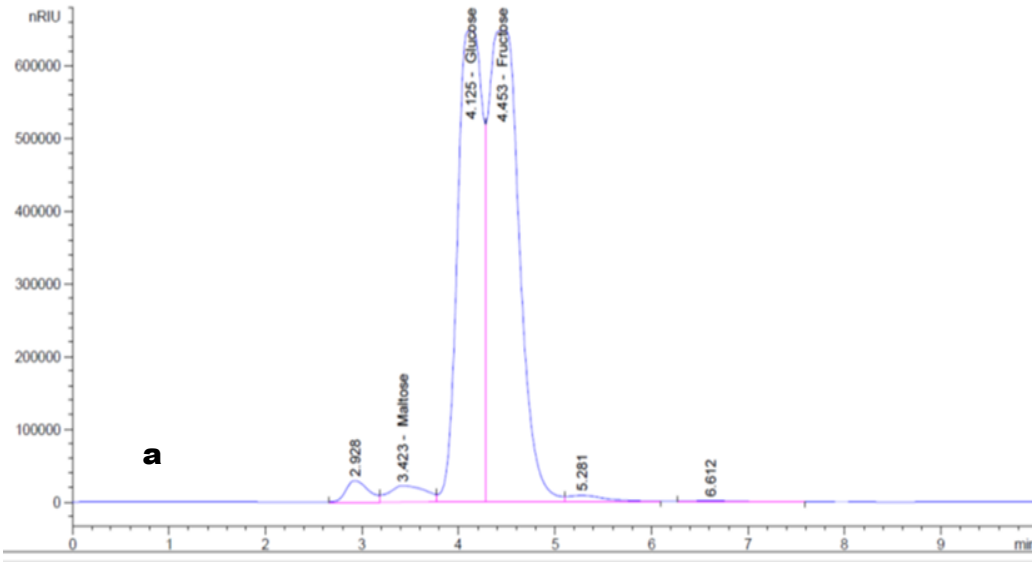
Figure 4.21 Fermentation profile of Vin13 at temperature: 20°C; pH: 4.50; oxygen saturation: 0%; stirrer speed: 150rpm; yeast inoculum concentration: 8.64 Log CFU/mL. The values plotted are an average from six replicates. Error bars represent ±SD, where not seen, they lie within the plot symbol.

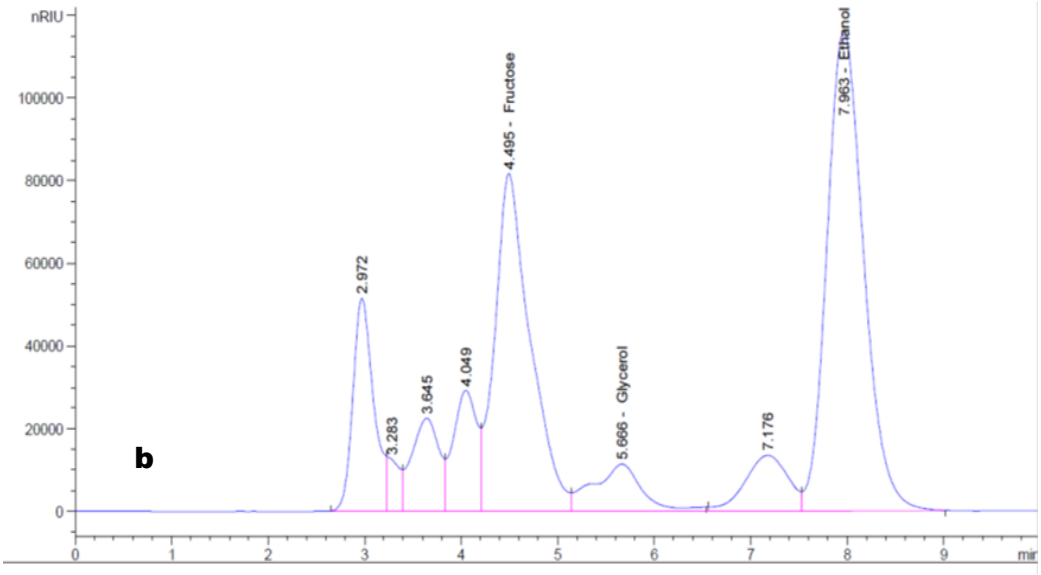
From day 0 to day 1 there is consumption of glucose and fructose (Fig 4.21). However, the results show minimal ethanol production with a decline in yeast viability (Fig 4.21). Additionally, maltose is utilized and glycerol is produced (Fig 4.21). At day 2 the glucose supply is depleted and fructose and maltose concentrations decrease Fig 4.21). The ethanol and glycerol concentrations increase whilst the yeast counts decreased (Fig 4.21). From day 3 to day 5 the

fructose, maltose and ethanol concentrations were unchanged (Table 4.5). The yeast viability declined and glycerol increased (Table 4.2;Fig 4.21). Run 3 continued for a total of 5 days. The data recorded for day 3 to day 5 show that ethanol steady state was reached at day 2. During the progression of fermentation there was a major reduction in the sugar concentration but minor production of ethanol (Fig 4.21). The maximum ethanol concentration of 31.40g/L was achieved at day 2 (Fig 4.21) and the fermentation can be regarded as an "abrupt arrest" (Malherbe et al., 2007). The concentration of the residual sugars was 3.30g/L (Fig 4.21). The fermentation conditions selected for run 3 were not efficient but satisfactory as ethanol production was possible. Low ethanol concentration could be due to slow metabolism of the yeast strain Vin13. However, the yeast was able to utilize the sugar for the growth, as seen from the cell counts and even though the cell count declined from the initial high count, viability was maintained between 7.00-8.00 Log CFU/mL (Table 4.2). The semi-anaerobic environment was not beneficial during run 3 and the yeast was unable to adapt to the environment. Glycerol production at day 1 is proof that the cells are stressed. Other products such as hexanoic, octanoic or decanoic fatty acids may have been produced, contributing to a slower metabolism and resulting in an arrest of the fermentation (Lafon-Lafourcade et al., 1984; Fleet, 2003; Malherbe et al., 2007, Beltran et al., 2008). Based on the experimental results, the ANOVA analysis demonstrated significance at an F-value of 2.92 and p-value of 0.0412.

Fermentation Run 4

The qualitative data for fermentation run 4 is depicted in Figure 4.22a-c.





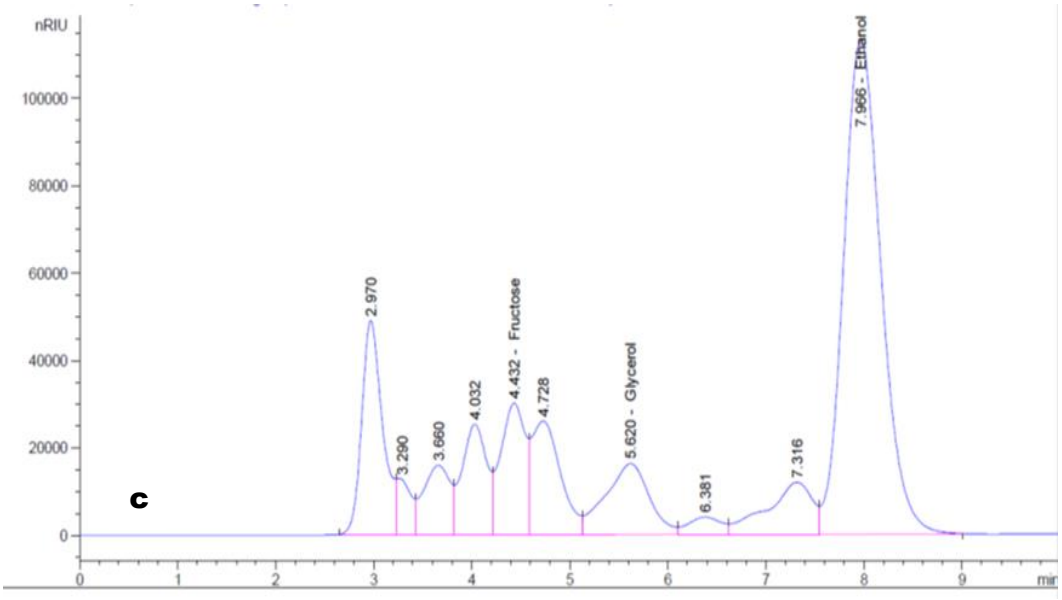


Figure 4.22a-c HPLC chromatograms of sugars, ethanol and glycerol from day 0 to day 2 of run 4. Peak identification based on the refractive index and retention time is indicated on the chromatograms.

The HPLC chromatograms of run 4 (Fig 4.22a-c) revealed the same elution trends as runs 1, 2 and 3 (Figs 4.16; 4.18; 4.20). A change in glucose and fructose peaks is visible from day 1 to day 2 (Fig 4.22b-c). Ethanol and glycerol peaks are visible from day 1 to day 2 (Fig 4.22b).

The fermentation profile of run 4 is presented in Figure 4.23 from the quantitative HPLC data, which includes the yeast viability counts.

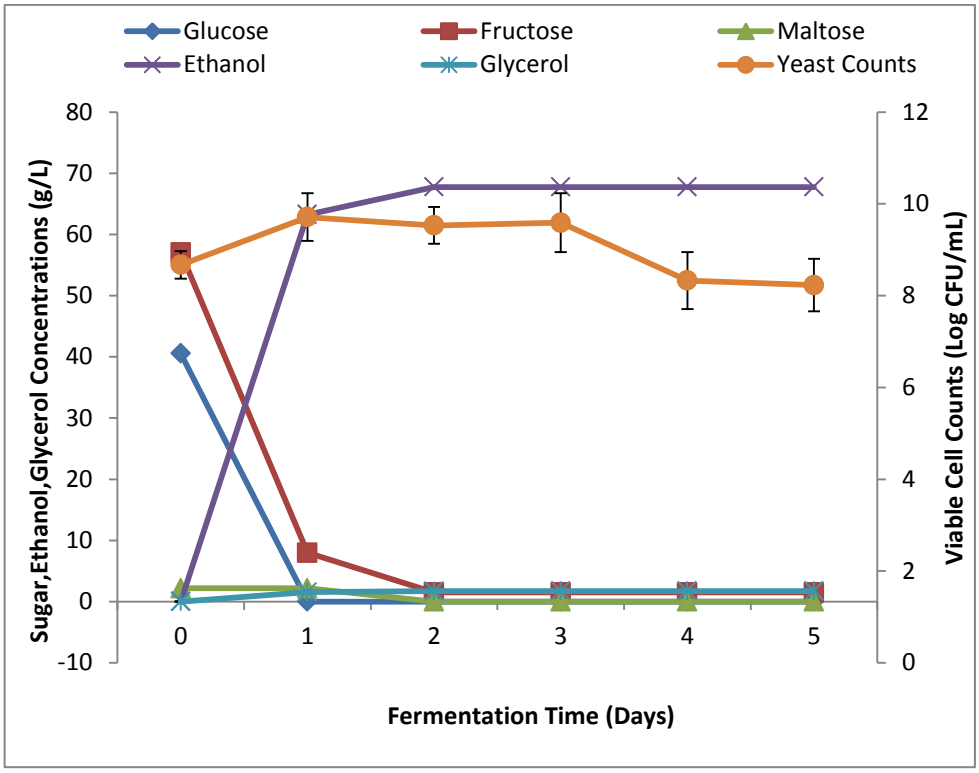


Figure 4.23 Fermentation profile of Vin13 at temperature: 20°C; pH: 4.50; oxygen saturation: 50%; stirrer speed: 150rpm; yeast inoculum concentration: 8.67 Log CFU/mL. The values plotted are an average from six replicates. Error bars represent ±SD, where not seen, they lie within the plot symbol.

From day 0 to day 1 the glucose and maltose supply is depleted, fructose is consumed, the ethanol concentration increases from 0g/L to 63.25g/L and yeast viability increases (Table 4.2; Fig 4.23). At day 1 no lag phase is noticed and the yeast cells are in the exponential growth phase (Fig 4.23). Additionally, a low concentration of glycerol is recorded (Fig 4.23). By day 2, fructose is at its lowest concentration (Fig 4.23). Ethanol and glycerol concentrations increase and yeast cells remain in the exponential phase (Fig 4.23). At day 3 to day 5, fructose and ethanol concentrations are unchanged. Yeast viability decreases and glycerol concentration increases (Table 4.2;Fig 4.23). Run 4 continued for a total of 5

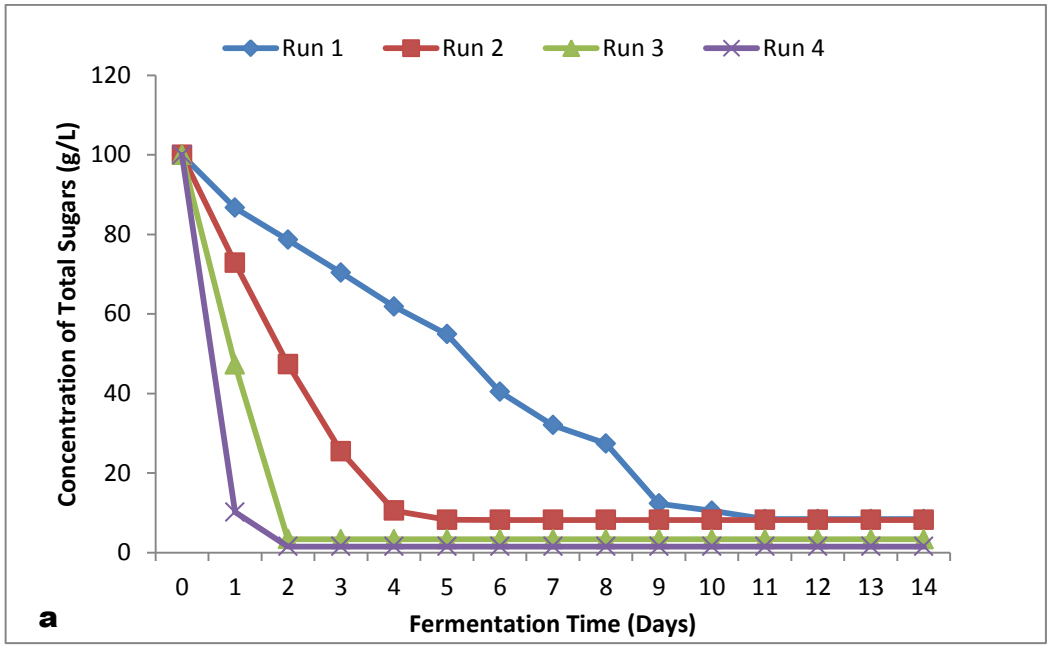
days. The data recorded for day 3 to day 5 show that ethanol steady state was reached at day 2. During the fermentation there was a major reduction in sugars and increase in ethanol (Fig 4.23). A maximum ethanol concentration of 67.74g/L was achieved at day 2 and the residual sugar concentration was 1.51g/L (Fig 4.23). From the high ethanol concentration obtained, the selected fermentation conditions were successful for ethanol production using Vin13 with the CAJ substrate. Oxygen saturation greatly improved the ethanol concentration and it is assumed that Vin13 is largely dependent on oxygen for CAJ fermentation. Based on the experimental results, the ANOVA analysis demonstrated significance at an F-value of 10.47 and p-value<0.0001.

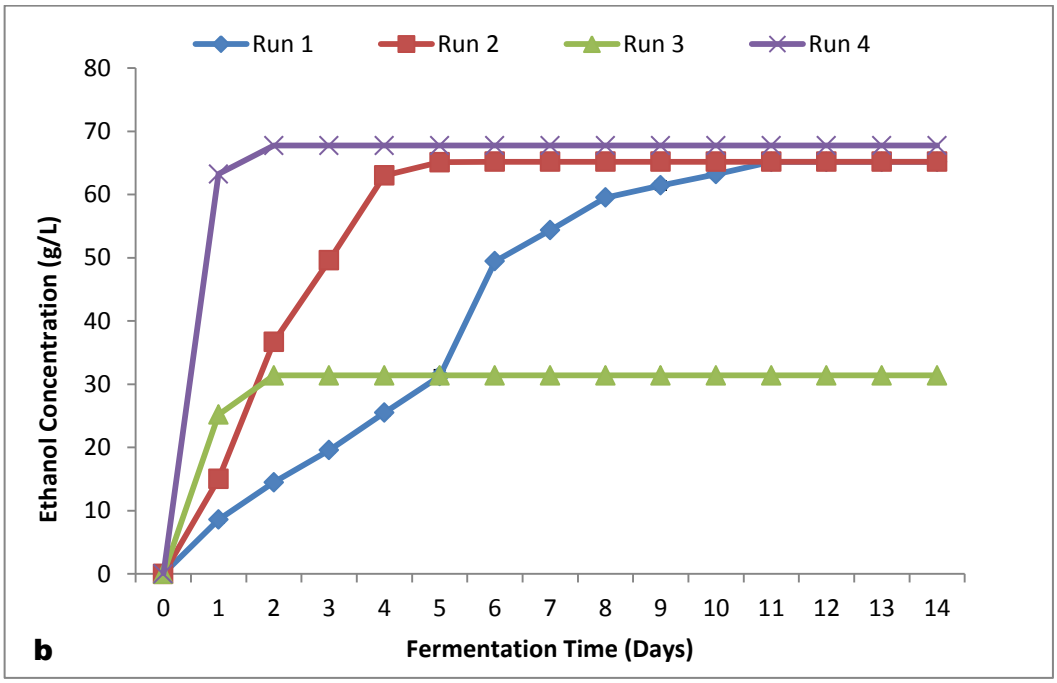
Comparative Analysis of the fermentations

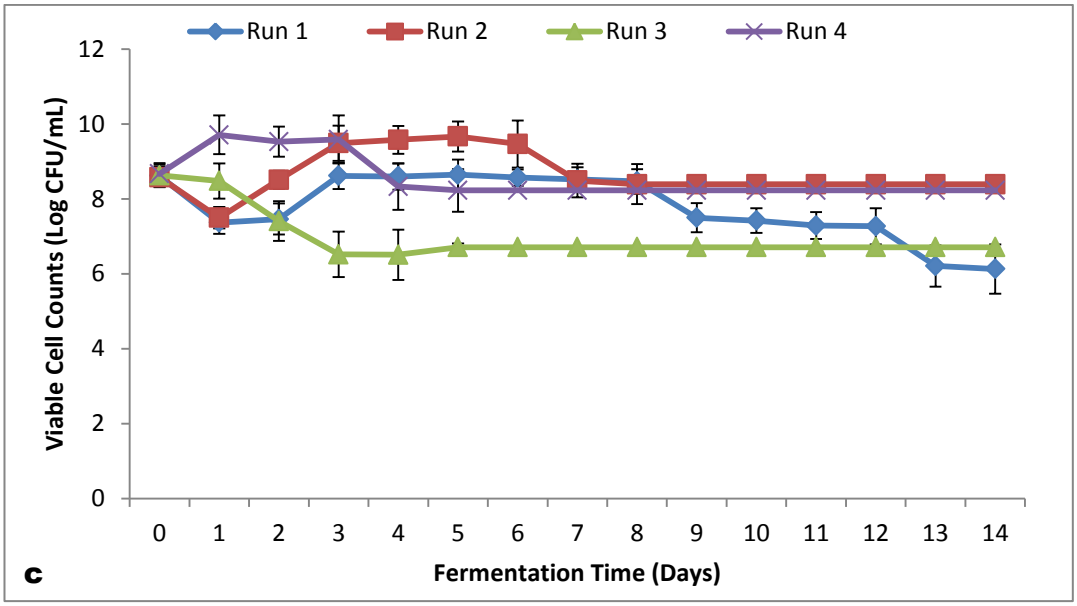
The BIOSTAT® Bplus fermenter was operated in batch mode for runs 1-4. Batch fermentation was the choice for CAJ fermentation to prevent contamination. CAJ is a sugar-rich substrate and no preservatives were added to the juice after the extraction process, hence contamination is likely. Furthermore the equipment was suited for batch fermentation and other modes of fermentation would require additional equipment and costs. The fermentation conditions were selected for the following reasons: (i) temperature: based on previous research on fermentation using brewers yeast and CAJ fermentation a temperature of 30ºC was generally used and brewers yeast is easily adaptable to high temperatures (Alfenore et al., 2002; Brosnan et al., 2002; Kwan et al. 2007; Pinheiro et al., 2008; Asli, 2010; Pacheco et al., 2010). Hence, 30ºC was the chosen temperature for the yeast strain Y2084. For the strain Vin 13, a temperature of 20ºC was used as wine yeasts exhibit better fermentative activity at lower temperatures (Ciani et al., 2006;

Molina et al., 2007; Beltran et al., 2008). The lowest possible temperature the BIOSTAT® system could be set at was 20ºC, as the cooling water passing through the jacket is from the laboratory supply and if a temperature <20ºC is required, the system will need to be connected to a chiller functioning at 4ºC and this specific strain is active at 20ºC and higher; (ii) pH: most CAJ fermentation studies were conducted at a pH of 4.50 (Alfenore et al., 2002; Kwan et al. 2007; Pinheiro et al., 2008; Asli, 2010; Pacheco et al., 2010) and in this study the pH used was close to the natural pH environment of the CAJ. *S. cerevisiae* yeasts are known to function better at pH ranges <6.00 (Malherbe et al., 2007) and the yeast glycolytic enzymes are optimal at a pH range of 4.50-5.00 (Reddy and Reddy, 2011); (iii) stirrer speed: the speed of 150rpm was used as the yeasts were cultured and conditioned to grow at this speed. Previous studies have used 150rpm as the optimal stirring speed for Saccharomyces yeasts (Alfenore et al., 2002; Kwan et al. 2007; Pinheiro et al., 2008; Asli, 2010; Pacheco et al., 2010) The medium was stirred continuously to keep the yeast in suspension, distribute the oxygen and expel the gases; (iv) oxygen saturation: oxygen concentrations between 5-10ppm is required for fermentation and if the concentration is too high the uptake of oxygen in decreased (Salmon et al., 2006). Optimal growth of *S. cerevisiae* at 5ppm has been researched and recommended (Fornairon-Bonnefond et al., 2003; Rosenfeld et al., 2003; Salmon et al., 2006; Gomez-Plaza and Cano-Lopez, 2011). Therefore, 50% saturation was chosen which equates to 5ppm.

Figure 4.24a-d depicts the comparative analysis of total sugars, ethanol, glycerol and yeast viability counts of both yeast strains. Table 4.3 represents the ethanol yield (E_y) and ethanol productivity (E_p).


Run 1
Run 2
Run 3
Run 4
Concentration of Total Sugars (g/L)
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Fermentation Time (Days)
a


Run 1
Run 2
Run 3
Run 4
Ethanol Concentration (g/L)
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Fermentation Time (Days)
b



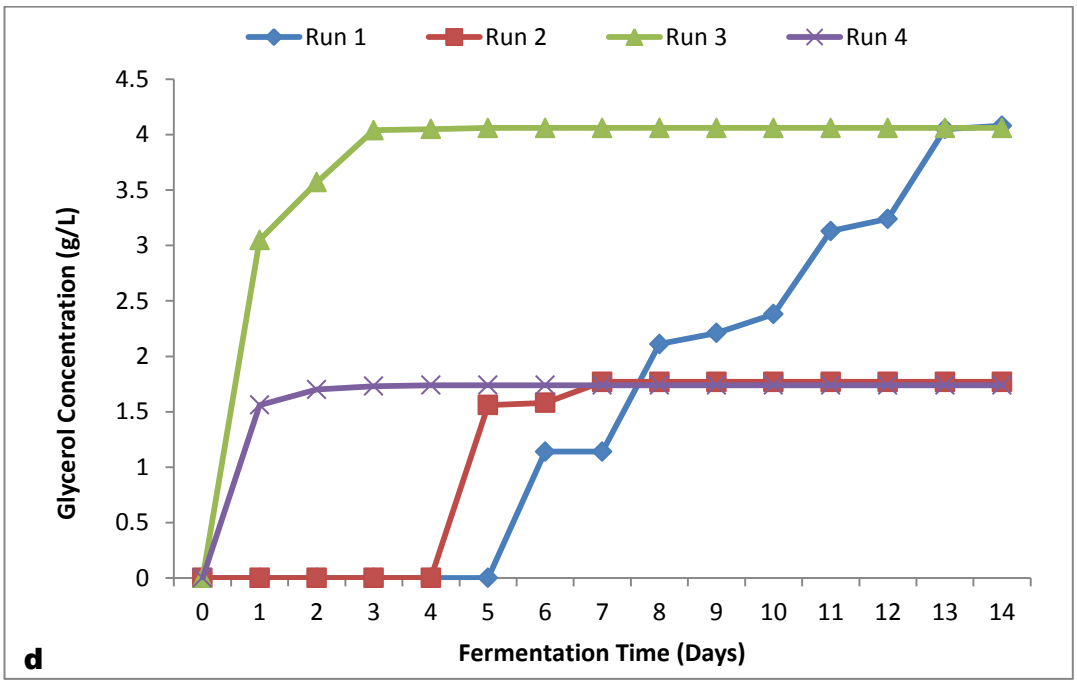


Figure 4.24a-d Combined fermentation profiles of the concentration of total sugars (a), ethanol concentration (b), viable cell counts (c) and glycerol concentration (d) of runs 1, 2, 3, and 4. The values plotted are an average from six replicates. Error bars represent SD, where not seen, they lie within the symbol (*Source:* Deenanath et al., 2013).

Table 4.3 Ethanol yield (E_y) and productivity (E_p) of fermentation runs 1-4.

Fermentation Run	Ethanol Concentration (g/L)	Ethanol Yield (g/g)	Ethanol Productivity (g/L/h)
Run 1	65.17	0.71	0.24
Run 2	65.12	0.71	0.54
Run 3	31.40	0.32	0.65
Run 4	67.74	0.70	1.41

"Both yeast strains exhibit the same patterns of sugar consumption, where glucose was first utilized followed by fructose, irrespective of oxygen saturation (Figs 4.17;4.19;4.21;4.23;4.24a). This was expected as Saccharomyces yeast easily assimilates monosaccharides such as glucose and fructose and disaccharides such as maltose. Moreover, these results correspond to the normal pattern of CAJ. The difference in sugar consumption is Vin 13 showed an affinity for maltose, whereas Y2084 did not (Figs 4.21;4.23) and the residual sugar concentration of run 3 and run 4 is lower (Fig 4.24a) due to the consumption of maltose and higher consumption of fructose by the yeast strain Vin13. High sugar concentrations are known to cause osmotic stress to yeast which is not the case in this study as the yeast strains were able to withstand the sugar concentration of the CAJ. The maximum ethanol concentration produced by Y2084 was 65.17g/L ($p<0.0001$) for run 1 and 65.12g/L ($p<0.0001$) for run 2 (Fig 4.24b). The results are significantly similar" (Deenanath et al., 2013). The semi-anaerobic or aerobic environment did not influence the ethanol concentration but the fermentation time was affected (Fig 4.24b). This is not unusual as fruit juice fermentation can be sluggish (Wang

et al., 2004, Malherbe et al., 2007). In the present study, the results showed that this situation can be overcome with oxygen addition. “For run 3 and run 4 the maximum ethanol concentrations were 31.40g/L ($p=0.0412$) and 67.74g/L ($p<0.0001$), respectively (Fig 4.24b). The results were significantly different” (Deenanath et al., 2013). The semi-anaerobic or aerobic environment influenced the ethanol concentration but the fermentation time was unchanged (Fig 4.24b). The E_y for run 1 and run 2 are the same as the ethanol concentrations were similar (Table 4.3). The E_p is lower for run 1 compared to run 2 (Table 4.3) as the fermentation time was longer. Thus, Y2084 has a greater productivity at 50% oxygen saturation. For run 3 and run 4, the E_y of run 4 was higher (Table 4.3). It is expected as the ethanol concentration is higher than run 3. The E_y of run 4 is similar to run 1 and run 2 because the ethanol concentration, although higher is similar. The E_p of run 4 is the highest (Table 4.3) as the maximum ethanol concentration was achieved in the shortest time. Yeast strain Vin13 has a better E_p than Y2084 mainly due to the fermentation time. “Hence, Y2084 is a slow fermenting yeast compared to Vin13 and requires a longer adaptation period when inoculated into the CAJ substrate. The strain Y2084 has an advantage over Vin13, as in the absence of oxygen Y2084 growth was persistent and it is likely the strain is able to acquire air, even when the cell numbers are low to sustain growth. The strain Y2084 was isolated from brewers yeast and these yeasts can easily adapt and grow within stressful conditions. Whereas Vin13 was unable to multiply and oxygen is a major contributing factor to achieve a high ethanol concentration (Fig 4.24b). Oxygen stimulates the production of unsaturated fatty acids and lipids, which contribute to the maintenance of the cell membrane structure and

metabolism during fermentation. In the absence of oxygen, the lipid components of the yeast cell plasma membrane is solubilized and utilized as an additional growth source and low cell viability in the absence of oxygen is normal as the available lipids are easily exhausted" (Deenanath et al., 2013). However, this can affect the integrity of the membrane and survival of the cells. In addition to lipid solubilisation, the cells are able to acquire phytosterols from the fermentation medium to maintain growth (Luparia et al., 2004; Soubeyrand et al., 2005; Salmon et al., 2006; Beltran et al., 2008). Phytosterols were previously identified as components of the skin of fruits and during fermentation of grape must for wine production (Luparia et al., 2004; Soubeyrand et al., 2005; Beltran et al., 2008). In this study, the peels of the CAs were not removed prior to the juice extraction and phytosterols could have entered the juice and during the semi-anaerobic fermentations it is assumed that phytosterols could have been utilised by the yeasts to sustain its growth and ferment. "Viable cell counts show that Y2084 enters a lag phase when introduced into the CAJ (Fig 4.24c). This phase is shorter when the medium is saturated with oxygen like run 2 (Fig 4.24c). Under semi-anaerobic conditions, Y2084 progresses from the lag phase to a short exponential phase followed by the stationary phase and eventually enter the death phase (Fig 4.24c). The exponential phase is short lived and most of the fermentation occurs when the cells are in the stationary phase (Fig 4.24c). Lack of nutrients and oxygen forces the cells into the death phase. Under aerobic conditions, lag phase is short and the Y2084 cells mainly function in exponential phase and enter into the stationary phase (Fig 4.24c). When the ethanol steady state is reached the cells are in stationary phase (Fig 4.24b-c). Viable cell counts

of Vin13 showed no distinct changes in the growth phases during the course of fermentation (Fig 4.24c) unlike Y2084. Under semi-anaerobic conditions, the cell viability decreases and the cells remain in the stationary phase (Fig 4.24c). In an aerobic environment, the cells are maintained in the exponential phase (Fig 4.24c). For both yeast strains, the by-product glycerol was produced and the concentrations were significantly lower under aerobic conditions (Run 2:$p<0.0001$; Run 4:$p<0.0001$) compared to the semi-anaerobic conditions (Run 1:$p<0.0001$; Run 3:$p=0.0412$) (Fig 4.24d). High glycerol concentration is common for anaerobic fermentations. In the semi-anaerobic environment of run 1 and run 3 the yeast cells released glycerol as a stress response and the glycerol is produced when there is change in enzyme activity from alcohol dehydrogenase to glycerol-3-phosphate dehydrogenase in order to reoxidize NAD^+ when oxygen is not available. Vin13 experienced stress at day 1 and Y2084 at day 6 (Fig 4.24d). The glycerol production is in accordance with sugar depletion and increased ethanol concentrations (Figs 4.24a-b;d). It is known that high ethanol concentrations greater than 4% (v/v) are toxic to yeast and can cause osmotic stress. In the current study, toxic-ethanol shock is a possibility under semi-anaerobic conditions for strain Y2084, as in a related study it was suggested the accumulation of ethanol was responsible for the decrease in yeast efficiency as the fermentation approached day 15. For the strain Vin13, stress is most likely due to the lack of oxygen. The low glycerol concentrations of run 2 and run 4 (Fig 4.24d) is due to sugar deprivation and the provided oxygen assists with survival of the cells and the ability to adapt to the stress. The accumulation of glycerol towards the endpoint of fermentation in the current study did not correspond with a

previous study" (Deenanath et al., 2013). In the study by Alfenore et al. (2002), fed-batch fermentation of glucose by the yeast *S. cerevisiae* strain CBS8066 was carried out. The fermentation conditions were: temperature:30ºC; pH:4.0; glucose substrate:100g/L; stirrer speed:400rpm; and air saturation:0.2vvm (Alfenore et al., 2002). Based on these conditions, temperature, pH and substrate concentration were similar to this research, however Alfenore et al. (2002) reported the glycerol was produced as the yeast cells multiplied during the process. The intermittent supply of glucose and increased stirrer speed could stimulate early production of glycerol. "Oxygen saturation has a major effect on both yeast strains as previously mentioned. Other than oxygen the temperature could also play a role in the fermentation. At a temperature of 30ºC and oxygen saturation of 0%, the fermentation duration was 11 days, whilst at 50% saturation the duration was 5 days (Figs 4.17;4.19;4.24a-d). Since oxygen dependence is not an issue for Y2084, based on the significantly similar ethanol concentrations, a temperature change could possibly affect the fermentation time. The ability of Y2084 to ferment at 30ºC is not unusual as *S. cerevisiae* yeasts can withstand temperatures up to 35ºC. At a temperature of 20ºC for run 3 and run 4, the duration of fermentation was shorter (Figs 4.21;4.23;4.24a-d). On the basis of the ethanol results, temperature may or may not influence the concentration as a high ethanol concentration is possible, provided the substrate is saturated with oxygen. Thus, Y2084 can be manipulated at different temperatures to evaluate the effect of fermentation time. For Vin13, different temperatures can be tested to determine the effect on time and ethanol concentrations, specifically under semi-anaerobic conditions. From the present data, optimal conditions for Y2084 are at

temperature:30ºC and 0% or 50% oxygen, as the ethanol concentration is high. Optimal conditions for Vin13 are temperature:20ºC and 50% oxygen saturation, as a higher ethanol concentration was obtained. Overall, the fermentation runs 1-4 showed sugar consumption, ethanol production and yeast viability. With oxygen saturation, the cells surpassed the initial cell count at the point of inoculation. For run 2 and run 4, cells entered the exponential phase which resulted in faster ethanol production which coincides with the statement by Bellissimi and Ingledew. (2005), "that ethanol is produced 30-33 times faster when yeasts are in log phase" (Bellissimi and Ingledew, 2005). In the absence of oxygen, nitrogen is beneficial to yeasts. For run 1, the nitrogen from the ammonium supplement and other components of the CAJ proved useful as Malherbe et al. (2007) suggests nitrogen is necessary to produce proteins that transport sugars for the substrate in to the yeast cells. The maximum ethanol produced in the current study for runs 1, 2 and 4 is higher than the value of 44.40g/L obtained by Pinheiro et al. (2008) for bioethanol from CAJ. Fermentation conditions were identical to this study, with the exception of oxygen saturation and the yeast strain. Pinheiro et al. (2008) also reported a higher glycerol concentration (5.80g/L) and suggested that the production is in response to external osmotic stress. Furthermore, no pre-treatment of the CAJ was carried out. From the results of the present study, pre-treatment to reduce tannins were useful and another contributing factor in obtaining a high ethanol concentration" (Deenanath et al., 2013). The detection of tannins was necessary because these are known to hinder fermentation. Condensed tannins have an affinity for various sites on proteins and in turn affect enzymes by binding and forming irreversible complexes which in turn causes a reduction in the

activity of enzymes (Mullins and Lee, 1991; Duodo et al., 2003). These tannins mainly affect enzyme activity during hydrolysis (Duodo et al., 2003). Hydrolysis was not performed in this investigation but enzymatic reactions are a feature of yeast functioning during fermentation and to avoid the reduction in yeast performance, the CAJ was pre-treated to decrease the amount of tannins. The pre-treatment method used proved successful as the tannin content was reduced (Table 4.1). Other studies using CAJ as a fermentation substrate for bioethanol production involved the use of immobilized yeast cells (Neelakandan and Usharani, 2009; Pacheco et al., 2010). Neelakandan and Usharani. (2009) showed that 7.62% (v/v) of ethanol is possible at a temperature of 32.5ºC; pH:6.0; and ~8.00 Log CFU/mL of immobilized *S. cerevisiae* cells. This result is higher than the current reported ethanol concentration. Pacheco et al. (2010) showed an ethanol concentration of 37.83g/L is achieved at temperature:30ºC; pH:4.0 and 12.5g immobilized cells. This result is lower than the ethanol concentration of runs 1, 2 and 4 and similar to the value reported for run 3. Attri. (2009) showed that a maximum ethanol concentration of 8.90% is possible from CAJ using the yeast *S. cerevisiae* var. *ellipsoideus*, for cashew wine production. The fermentation time was 15 days (Attri, 2009) similar to the time of run 1. The CAJ was pre-treated with gelatine and this resulted in an improved yeast capacity and a higher ethanol concentration (Attri, 2009). In another study, the *S.cerevisiae* wine yeast strain DBVPG 1014 was used to ferment grape juice for potable alcohol at the following conditions, temperature:20ºC, pH:3.10; initial sugar:270g/L; oxygen saturation:0% (Ciani et al., 2006). An ethanol concentration of 15.9% was obtained by day 14 (Ciani et al., 2006). The outcome of the ethanol result was

better than run 3 and run 4 with the exception of fermentation time. This study (Ciani et al., 2006) showed other than fermentation conditions the starting sugar concentration is crucial in the outcome of the ethanol concentration. "From the research data, fermentation conditions, fermentation substrate concentration and yeast strain collectively contribute to the ethanol output and different yeast strains behave differently under certain conditions with each *S. cerevisiae* strain being unique" (Deenanath et al., 2013). The analysis of the fermentation data was purely based on instrumentation analysis and thus kinetics was not investigated as it is out of scope as defined by the objectives. The effect of the experimental conditions on the bioethanol output is represented and discussed in terms of the total organic carbon (section 4.4.5).

4.4.3 Gas Analysis

"Carbon dioxide (CO_2) and residual oxygen (O_2) gas output signals were recorded continuously by the BCP-sensors during the course of fermentation" (Deenanath et al., 2013). The results of the volume percentage of each gas passing through the sensor are summarized in Table 4.4.

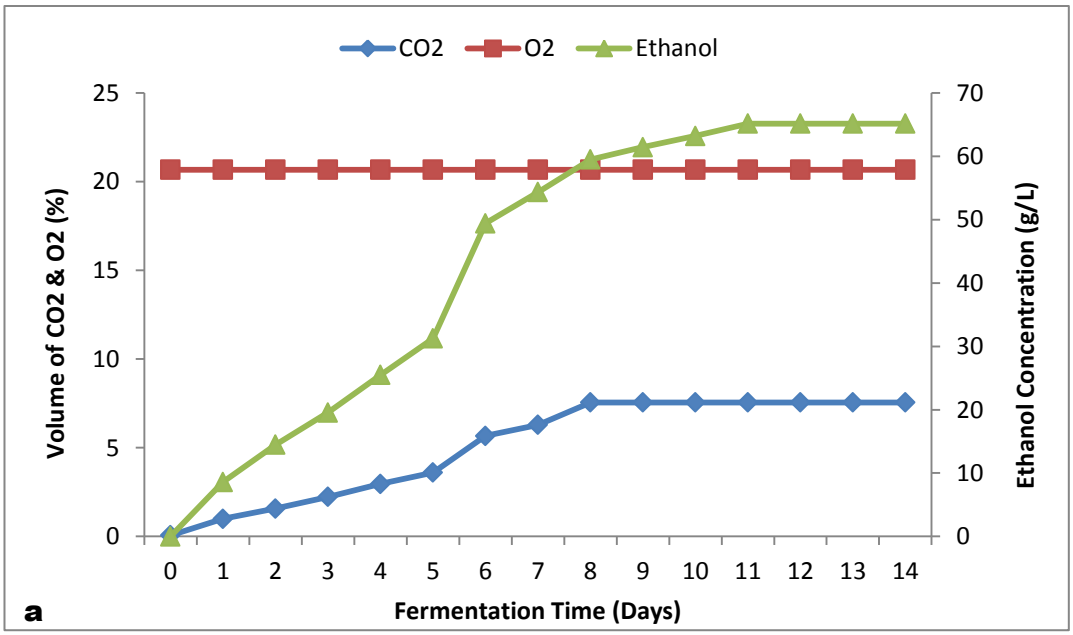
Table 4.4 Carbon dioxide (CO_2) and oxygen (O_2) gas volumes (%) of runs 1-4. The values are an average of duplicate fermentations.

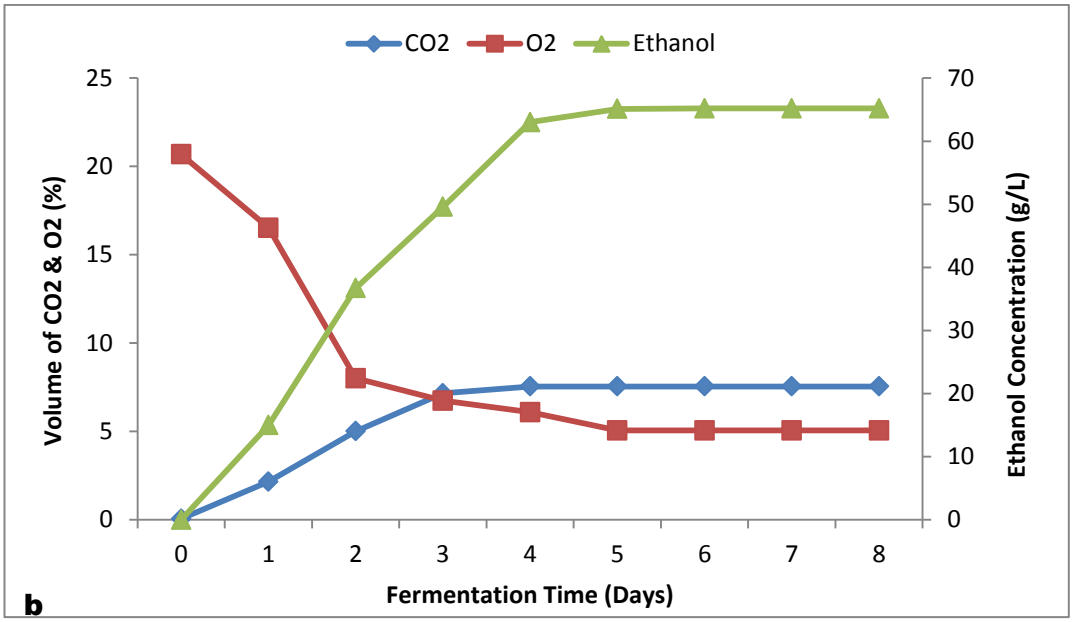
Fermentation Time (Days)	Run 1		Run 2		Run 3		Run 4	
	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2
0	0.03	20.68	0.03	20.68	0.03	20.68	0.03	20.68
1	0.99	20.68	2.16	16.53	0.90	20.68	7.55	6.11
2	1.57	20.68	5.01	8.01	2.27	20.68	7.55	5.76
3	2.23	20.68	7.16	6.74	2.27	20.68	7.55	5.76
4	2.96	20.68	7.55	6.09	2.27	20.68	7.55	5.76
5	3.60	20.68	7.55	5.06	2.27	20.68	7.55	5.76
6	5.67	20.68	7.55	5.06	-	-	-	-
7	6.29	20.68	7.55	5.06	-	-	-	-
8	7.55	20.68	7.55	5.06	-	-	-	-
9	7.55	20.68	-	-	-	-	-	-
10	7.55	20.68	-	-	-	-	-	-
11	7.55	20.68	-	-	-	-	-	-
12	7.55	20.68	-	-	-	-	-	-
13	7.55	20.68	-	-	-	-	-	-
14	7.55	20.68	-	-	-	-	-	-

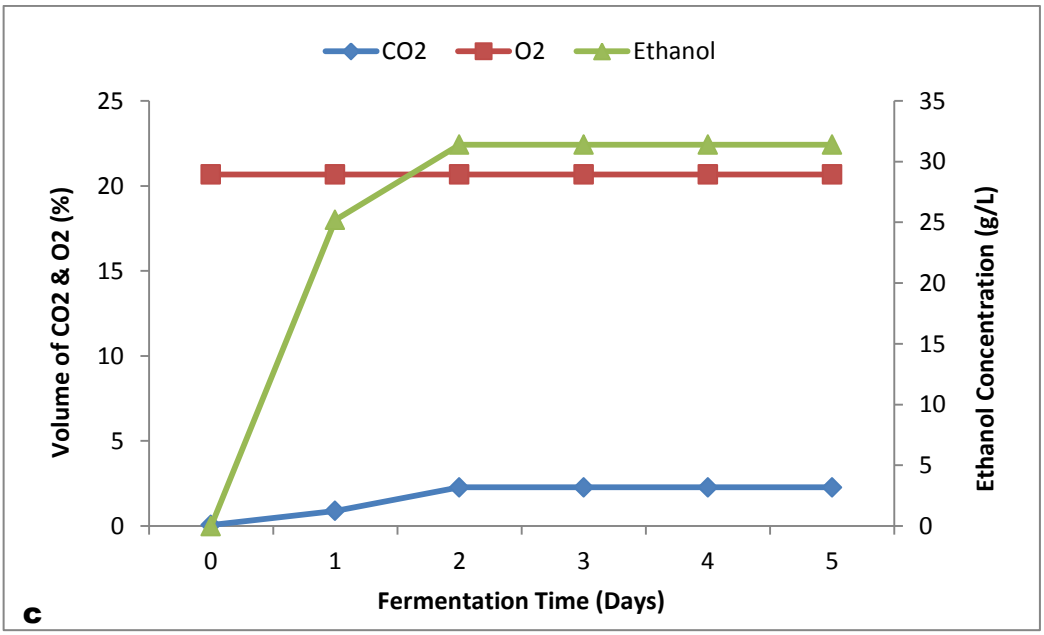
CO_2 volume increased as time progressed for runs 1, 2 and 4 (Table 4.4). These results are in accordance with the increase in ethanol concentration. For run 3, the low CO_2 volume (Table 4.4) was expected as the ethanol concentration was low. Volume of O_2 for run 1 and run 3 were unchanged (Table 4.4) as no oxygen was added during these fermentations. This is the opposite for run 2 and run 4 where

the O_2 volume decreases (Table 4.4) because during aerobic fermentations the yeast utilise the oxygen for growth and the residual oxygen is released (Table 4.4). Overall, as O_2 decreases the CO_2 increases which coincides with: (i) ethanol production, (ii) CO_2 production, (iii) oxygen uptake, and (iv) yeast growth.

The relationship between the gases and ethanol production is depicted in Figure 4.25a-d.




CO2
O2
Ethanol
Volume of CO2 & O2 (%)
Ethanol Concentration (g/L)
Fermentation Time (Days)
b


CO2
O2
Ethanol
Volume of CO2 & O2 (%)
Ethanol Concentration (g/L)
Fermentation Time (Days)
c

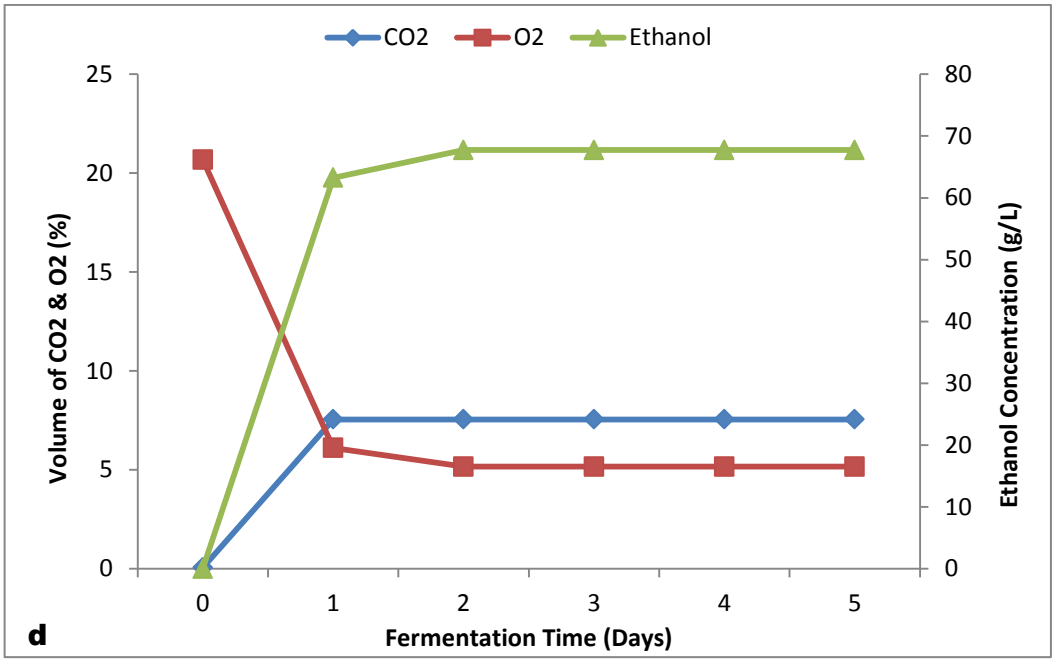


Figure 4.25a-d Volume of CO_2 and O_2 to changing ethanol concentrations during the course of fermentation of run 1 (a), run 2 (b), run 3 (c), and run 4 (d) (*Source:* Deenanath et al., 2013).

"From Figure 4.25a-d, it is evident that ethanol concentration and CO_2 volume are in sync and the values converge towards the endpoint of fermentation. For run 1, there is a slow increase, followed a rapid increase in both CO_2 and ethanol (Fig 4.25a). This coincides to the yeast lag phase and the gradual increase in yeast viability and ethanol production as fermentation progresses (Fig 4.17). At day 6 there is a spike in CO_2 (Table 4.4;Fig 4.25a), caused by the decline in fructose and rise in ethanol concentration (Fig 4.17). At day 8 CO_2 readings reaches steady state (Table 4.4;Fig 4.25a), whereas ethanol steady state is at day 11 (Figs 4.17; 4.25a). This is probably due to the slower production and lower accumulation of ethanol from day 8 to day 11, and the CO_2 volume produced is also lower which is not registered by the sensor. The O_2 volume is unchanged (Table 4.4;Fig 4.25a) as run 1 was carried out at 0% oxygen. Run 2, similar to run 1 showed a link

between ethanol concentration and CO_2 volume (Fig 4.25b). Between day 1 and day 2 the CO_2 volume increases as ethanol increases (Fig 4.25b) and yeast cell metabolism is active (Table 4.2;Fig 4.19). From day 3 to day 4 there is a gradual increase in CO_2 and at day 4, steady state is reached (Fig 4.25b). During run 2, a decrease in the O_2 volume is recorded at day 1 and day 2 as the yeast cells enter the exponential phase resulting in an increase in oxygen consumption from the CAJ substrate and a decrease in the volume of total oxygen added (Table 4.4;Fig 4.25b). The decline in O_2 coincides with yeast growth (Table 4.2;Fig 4.19), CO_2 production (Fig 4.25b) and ethanol production (Fig 4.25b). For run 3, the abrupt arrest of the fermentation (Fig 4.21) resulted in a low volume of CO_2 (Table 4.4; Fig 4.25c). The O_2 volume is unchanged (Table 4.4;Fig 4.35c) like run 1.

For run 4, the maximum CO_2 volume was recorded at day 1 (Table 4.8;Fig 4.25d). This coincides with the rapid increase in ethanol concentration, oxygen consumption and yeast activity (Tables 4.2;4.4;Figs 4.23;4.25d). A higher volume of CO_2 is a possibility as a higher ethanol concentration was obtained for run 4. However, the maximum reading volume is 10%. When the output signal on the DCU (section 3.4.1) reads as 10%, the threshold concentration for the BCP-CO_2 sensor is reached and volumes greater than 10% is not recorded. The O_2 volume is similar to run 2 (Fig 4.25b;d), hence the uptake of oxygen by both strains is similar. Based on the gas analysis there is an agreement between the data, where the CO_2 and ethanol increase are in proportion. This is expected because CO_2, like ethanol is released by yeast cells during alcoholic fermentation. The CAJ is a sufficient substrate for the yeast strains Y2084 and Vin13 as the fermentation follows a normal course where sugar consumption, ethanol production, CO_2

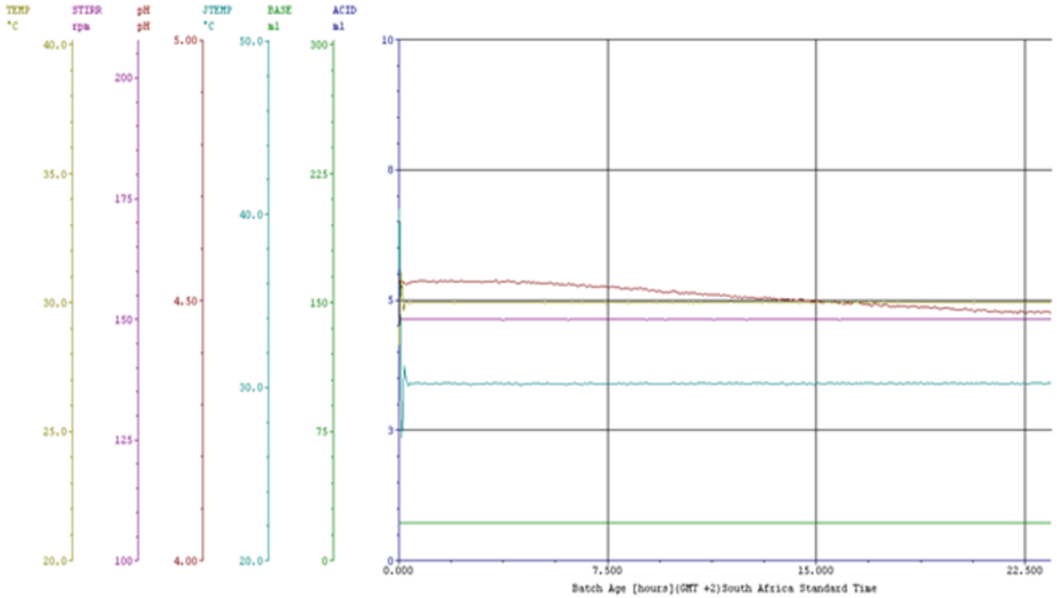
production, oxygen consumption and yeast metabolism are in accordance with that" (Deenanath et al., 2013).

4.4.4 MFCS/DA Analysis

"The software programme BioPAT® MFCS/DA 3.0 was used for the MFCS/DA analysis. This programme allowed the storage and visualization of the operating conditions as process data of the batch fermentations. MFCS/DA data analysis was performed every 24hrs. The software plots, for the respective fermentation runs are depicted in Figures 4.26-4.29. These plots show the operating conditions or process variables (y-axis) against the batch time or age (x-axis). In general, the plots are: a visual description of the fermentation process; input and output setpoint values; shows deviations from the setpoint values; and re-alignment of the process on par with the setpoint values is visible" (Deenanath et al., 2013).

Figure 4.26a-k shows the MFCS/DA plots of run 1.

a


TEMP °C
STIRR rpm
pH pH
JTEMP °C
BASE ml
ACID ml
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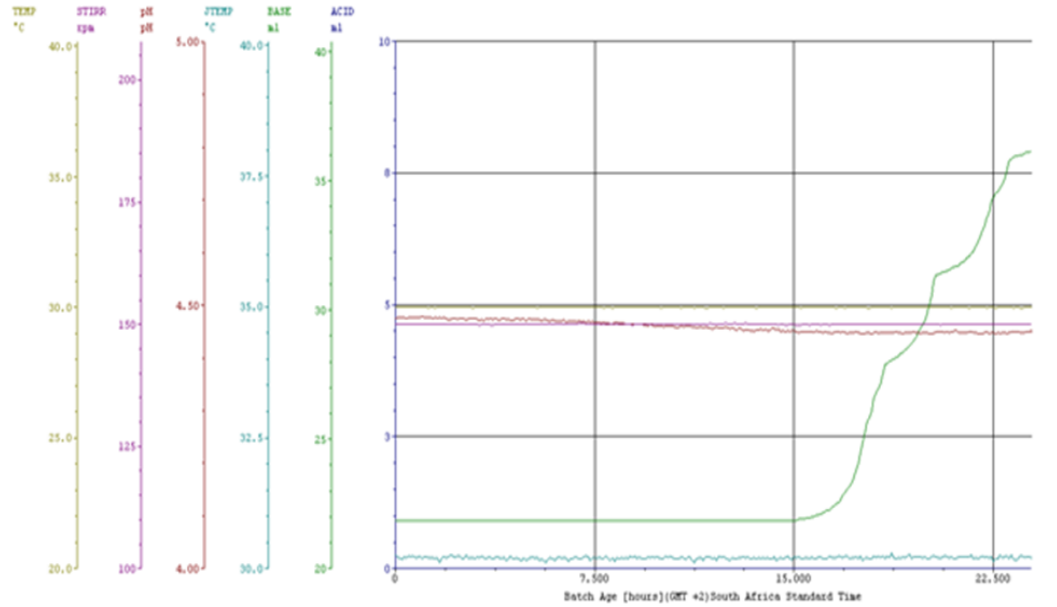
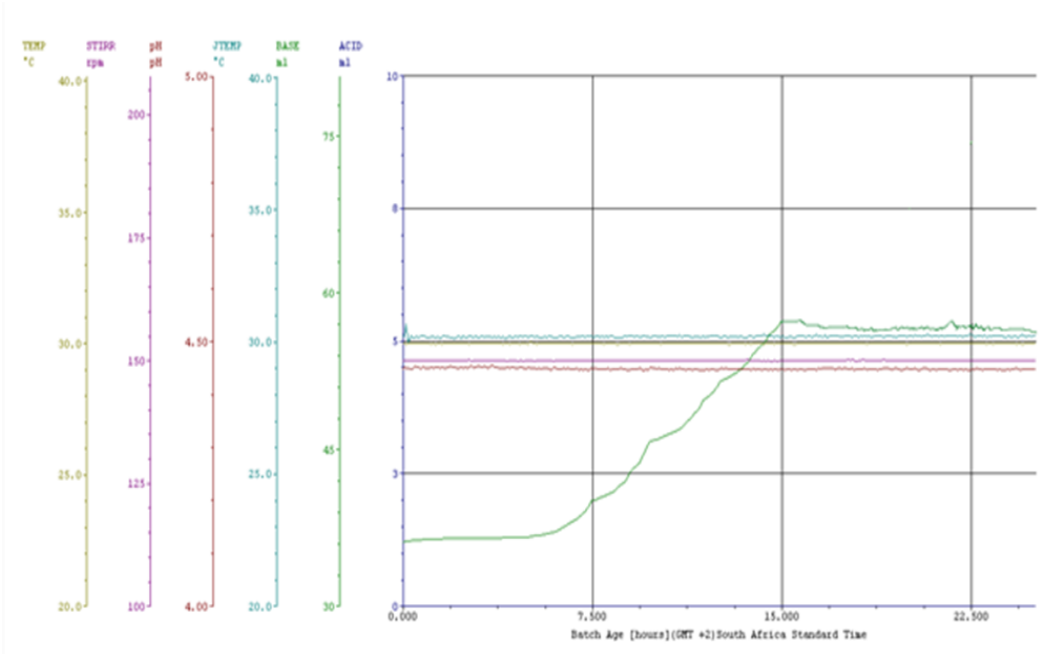
35.0
30.0
25.0
20.0
200
175
150
125
100
5.00
4.50
4.00
50.0
40.0
30.0
20.0
300
225
150
75
0
10
8
5
3
0
0.000
7.500
15.000
22.500
Batch Age [hours](GMT +2)South Africa Standard Time

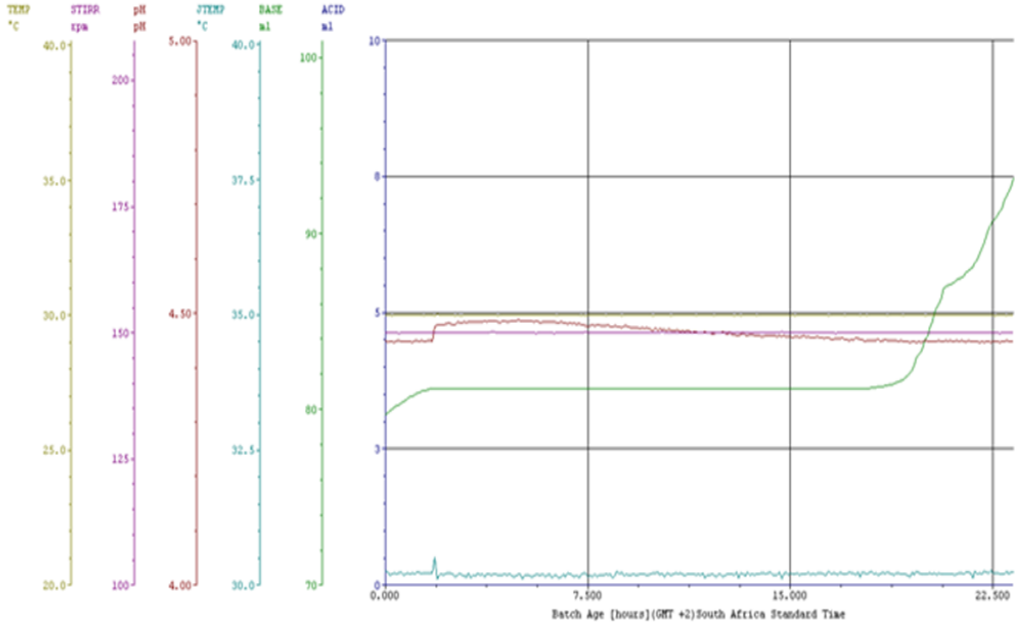
b


TEMP °C
STIRR rpm
pH pH
JTEMP °C
BASE ml
ACID ml
40.0
35.0
30.0
25.0
20.0
200
175
150
125
100
5.00
4.50
4.00
40.0
37.5
35.0
32.5
30.0
40
35
30
25
20
10
8
5
3
0
7.500
15.000
22.500
Batch Age [hours](GMT +2)South Africa Standard Time

c


TEMP
°C
STIRR
rpm
pH
pH
JTEMP
°C
BASE
ml
ACID
ml
40.0
35.0
30.0
25.0
20.0
200
175
150
125
100
5.00
4.50
4.00
40.0
35.0
30.0
25.0
20.0
75
60
45
30
10
8
5
3
0
0.000
7.500
15.000
22.500
Batch Age [hours](GMT +2)South Africa Standard Time

d


TEMP
°C
STIRR
rpm
pH
pH
JTEMP
°C
BASE
ml
ACID
ml
40.0
35.0
30.0
25.0
20.0
200
175
150
125
100
5.00
4.50
4.00
40.0
37.5
35.0
32.5
30.0
100
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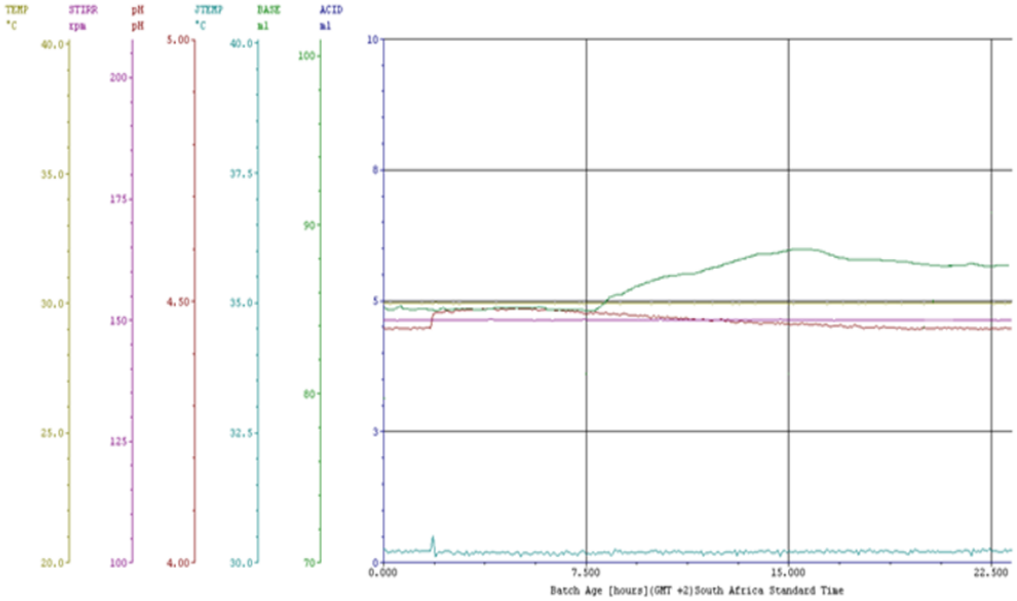
Batch Age [hours](GMT +2)South Africa Standard Time

e



f-k

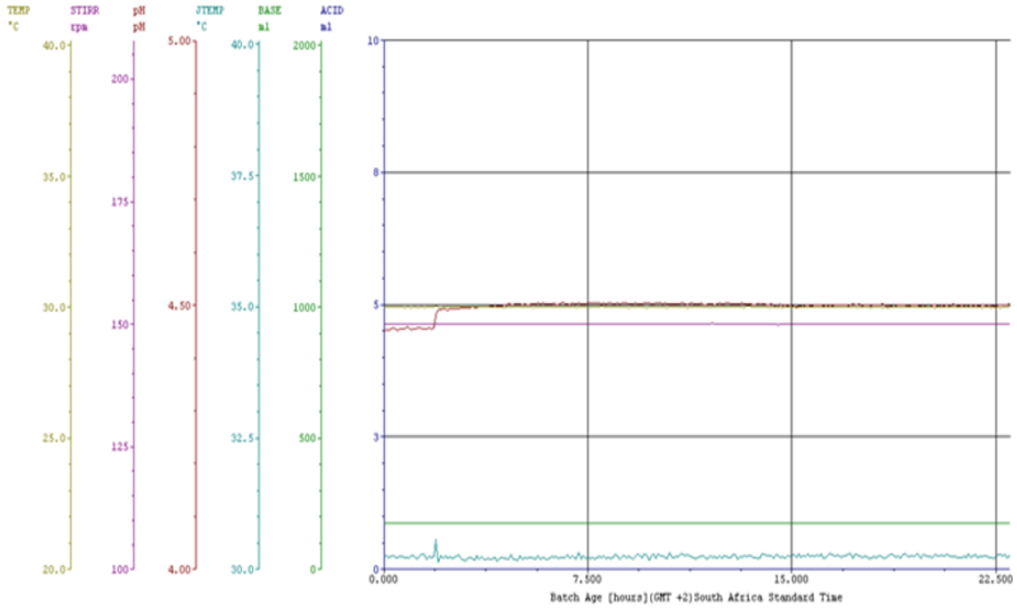
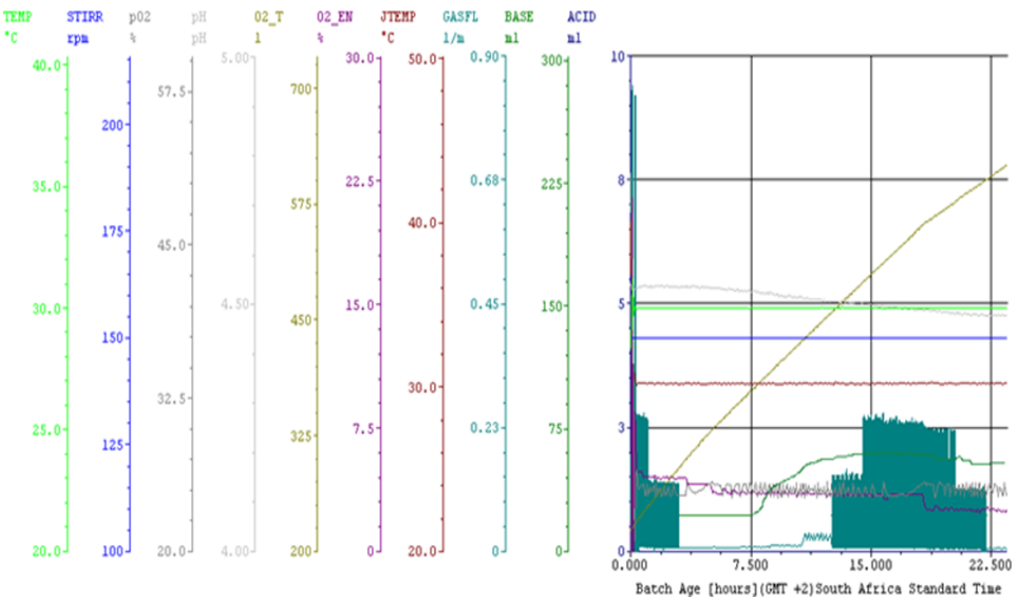


Figure 4.26a-k MFCS/DA plots of run 1 showing multiple process variables of temperature (TEMP); stirrer speed (STIRR); pH (pH); jacket temperature (JTEMP); acid (ACID) and base (BASE) dosing volumes from day 1 to day 11 (a-k).

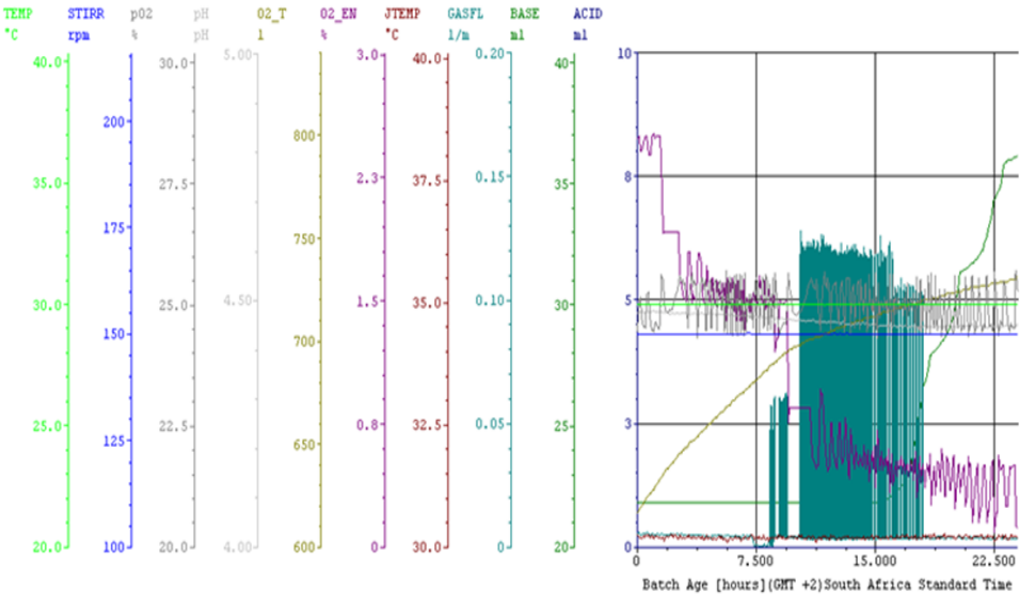
As seen from the plot, at day 1 TEMP and STIRR are stable at 30ºC and 150rpm, respectively (Fig 4.26a). The stability of the temperature is due to the water circulation through the jacket. JTEMP is unstable at first as the control function; to reach the setpoint of the TEMP value is active and once acclimatized it is steady (Fig 4.26a). TEMP, STIRR, JTEMP and ACID are stable throughout the process as seen in the plots (Fig 4.26a-k). During day 2, a decrease in pH is noticed followed by an increase in base dosing volume (Fig 4.26b). Once base is added the pH increases to the initial pH setpoint value (Fig 4.26a). As run 1 continues the pH and BASE variables are the most active (Fig 4.26a-k). "The decrease in pH is normal as most fermentation studies report a decrease in pH which is mainly due to the production of lactic acid or other organic acids" (Deenanath et al., 2013). From the HPLC chromatograms (Fig 4.16), other than the targeted sugars, ethanol and glycerol the smaller peaks may be organic acids.

Figure 4.27a-e shows the MFCS/DA plots or run 2.

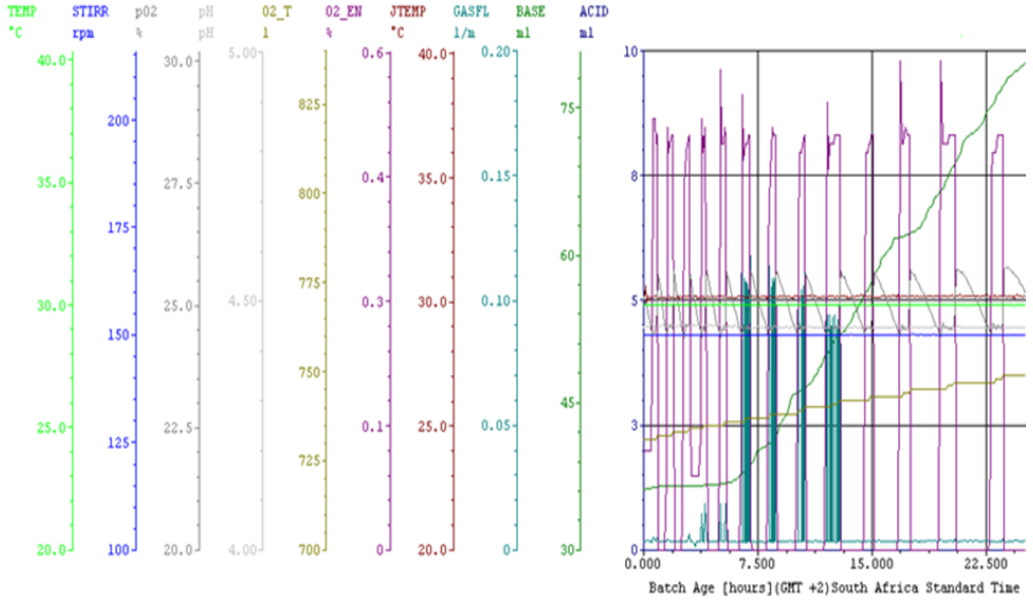
a



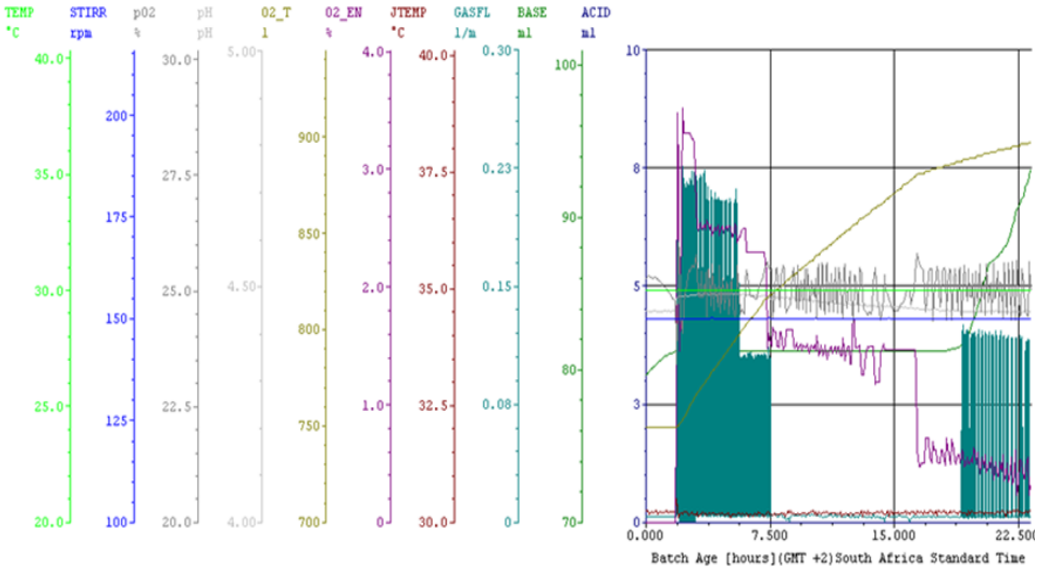
b



c


TEMP °C
STIRR rpm
p02 %
pH pH
02_T l
02_EN %
JTEMP °C
GASFL l/m
BASE ml
ACID ml
Batch Age [hours](GMT +2)South Africa Standard Time

d


TEMP °C
STIRR rpm
p02 %
pH pH
02_T l
02_EN %
JTEMP °C
GASFL l/m
BASE ml
ACID ml
Batch Age [hours](GMT +2)South Africa Standard Time

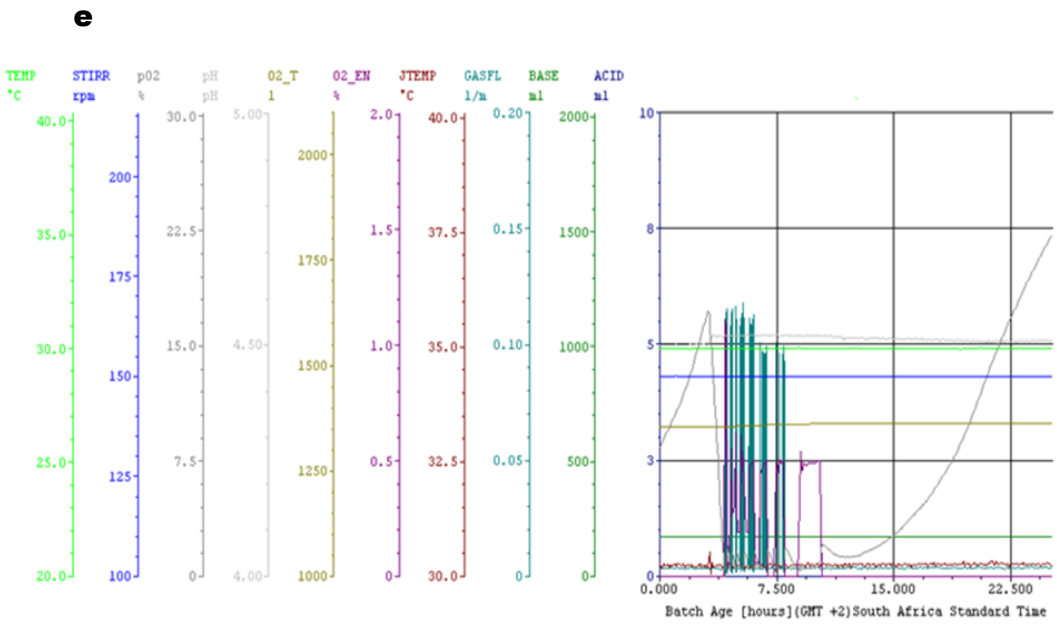


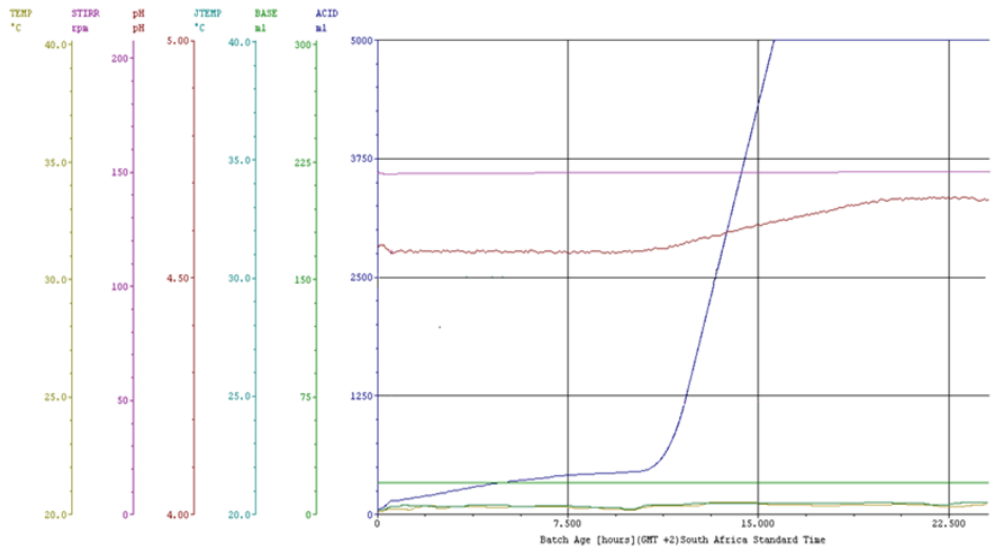
Figure 4.27a-e MFCS/DA plots of run 2 showing multiple process variables of TEMP; STIRR; pH; JTEMP; ACID/BASE dosing volumes; oxygen saturation (pO_2); volume of oxygen supply (O2_T); opening/closing of valve (O2_EN); flow rate of gas (GASFL) from day 1 to day 5 (a-e).

From the plots, TEMP; STIRR; JTEMP and ACID are stable (Fig 4.27a-e). The pH value and BASE volume fluctuate as the fermentation progresses (Fig 4.27a-e), similar to run 1. In accordance to the decrease in pH, base is dosed (Fig 4.27a-e). Under aerobic conditions when the pO_2 value drops below the setpoint value, regulation of this value is visible by a change in O2_T; O2_EN and GASFL (Fig 4.27a-e). "This is interpreted as follows: when oxygen is consumed by the yeast the pO_2 decreases (Fig 4.27a-e) and to re-align the process and ensure that CAJ is saturated at 50%, there is an addition of oxygen and the value is recorded and displayed as O2_T (Fig 4.27a-e). Simultaneously, for oxygen addition the valve is opened and closed as the addition is pulsed-flow and denoted by O2_EN (Fig

4.27a-e). The valve will open and close (O2_EN) at controlled intervals and the gas flow will not exceed the set flow rate of 1l/min as shown by the GASFL variable (Fig 4.27a-e)" (Deenanath et al., 2013). From the plots of run 2 when the pO_2 decreases, the yeast uptake oxygen and at these time points is when the yeast viability increases. It is noted that pO_2 readings do not show 50% on the plots (Fig 27a-e), as the yeast consume the oxygen immediately when added.

Figure 4.28a-b shows the MFCS/DA plots of run 3.

a



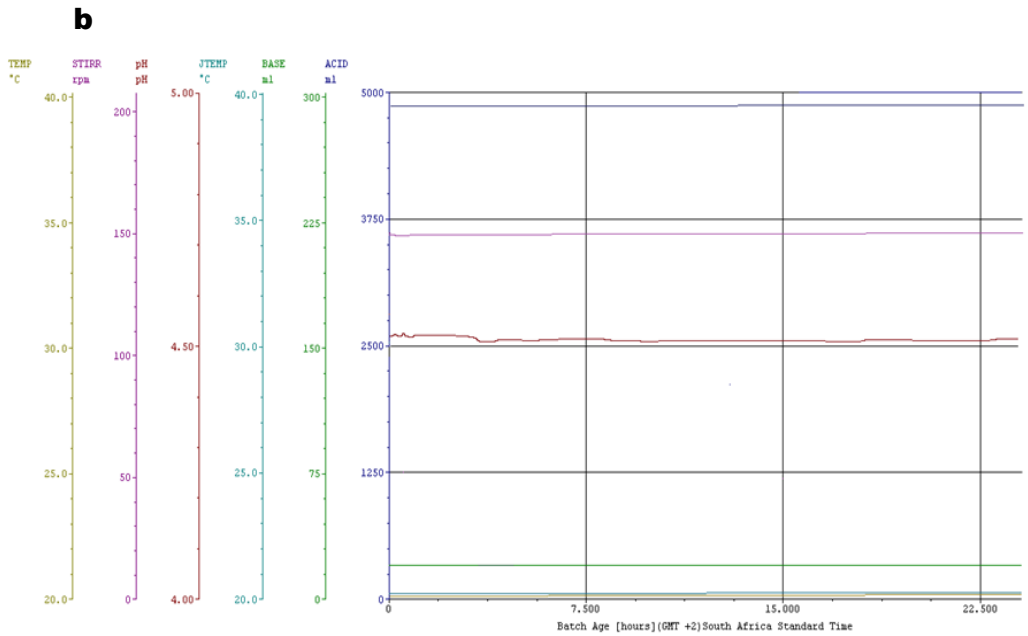
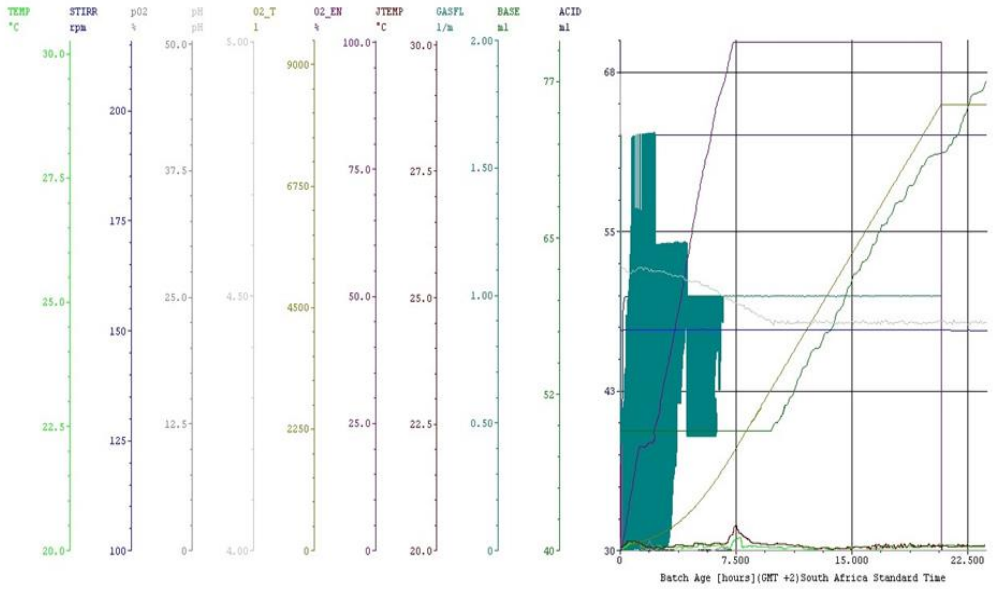


Figure 4.28a-b MFCS/DA plots of run 3 showing multiple process variables of TEMP; STIRR; pH; JTEMP; ACID/BASE dosing volumes from day 1 to day 2 (a-b).

Similar to run 1, the plots of run 3 shows the TEMP; STIRR; JTEMP and BASE variables are stable (Fig 4.28a-b). At day 1 there is an increase in pH and ACID (Fig 4.28a), "which was probably due to ethanol accumulation" (Deenanath et al., 2013). At day 2 all variables are stable (Fig 4.28b), as the fermentation ceased (Fig 4.21).

Figure 4.29a-b shows the MFCS/DA plots of run 4.

a



b

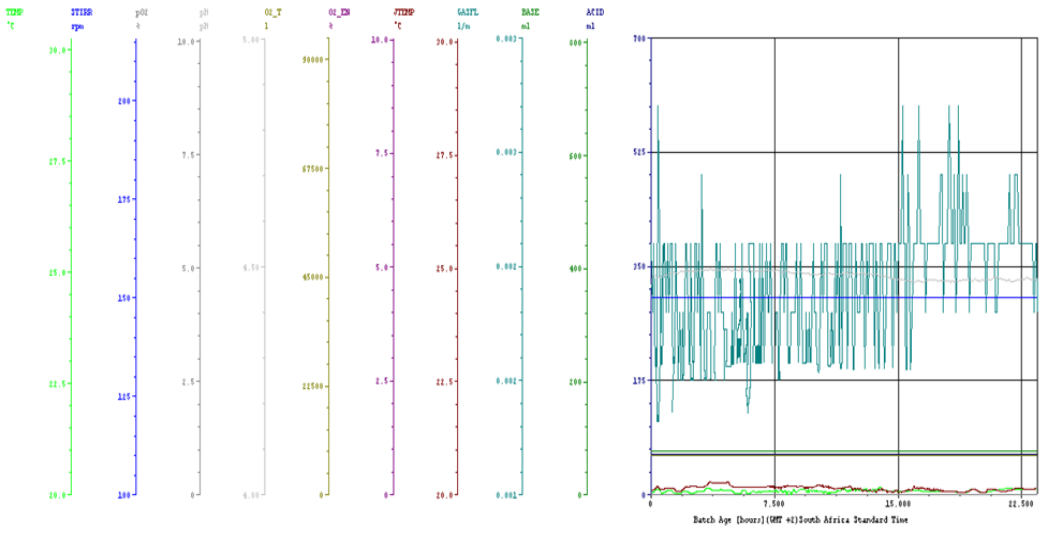


Figure 4.29a-b MFCS/DA plots of run 4 showing multiple process variables of TEMP; STIRR; pH; JTEMP; ACID/BASE dosing volumes; pO_2; O2_T; O2_EN; GASFL from day 1 to day 2 (a-b).

The plots of run 4 show the TEMP; STIRR; JTEMP and ACID variables are stable, like run 1. The changes in the pH and BASE are visible (Fig 4.29a-b). Similar to run 2 aerobic conditions are monitored by pO_2; O2_T; O2_EN and GASFL (Fig 4.29a-b). The sporadic changes are attributed to yeast oxygen uptake. During run 4 there is a rapid decline in pO_2 and increase in O2_T at day 1 (Fig 4.29a) which coincides with the yeast entering the exponential phase.

"Based on the MFCS/DA analysis, the potential of the equipment and the in-house control centre is a capable system for bioethanol production. Fermentation of CAJ using yeast strains Y2084 and Vin13 coupled with the BIOSTAT® Bplus system is a contributing factor towards obtaining a high bioethanol concentration. Visualization of the stable functioning of the system at small-scale is valuable as it enhances the understanding of how the process is controlled on a daily basis and to document the process control. At large-scale or industrial scale, fermentation stability among batches might not be possible, thus this type of data monitoring shows the potential that on par processes are possible" (Deenanath et al., 2013). It is important to note that TEMP is stable during the processes as a temperature increase could have resulted in runs 1, 2 and 4 to abruptly stop and reduced ethanol yields (Balakumar and Arasaratham, 2009).

4.4.5 Total Organic Carbon Analysis

"The results of the total organic carbon (TOC) of the CAJ and the fermented CAJ are presented in Table 4.5 and Table 4.6, respectively. Table 4.6 displays the TOC results of the final fermentation product of each run as "finished samples may be

more consistent in their organic loads" as suggested by Quayle et al. (2009)" (Deenanath et al., 2013).

Table 4.5 Carbon percentage (carbon input) of the cashew apple juice (*Source:* Deenanath et al., 2013).

Inputs	% Carbon
Total sugars (glucose, fructose, maltose)	88.08
Other unidentified compounds	11.92
Total Carbon Input	100

Table 4.6 Carbon percentage (carbon output) of fermentation runs 1-4 (*Source:* Deenanath et al., 2013).

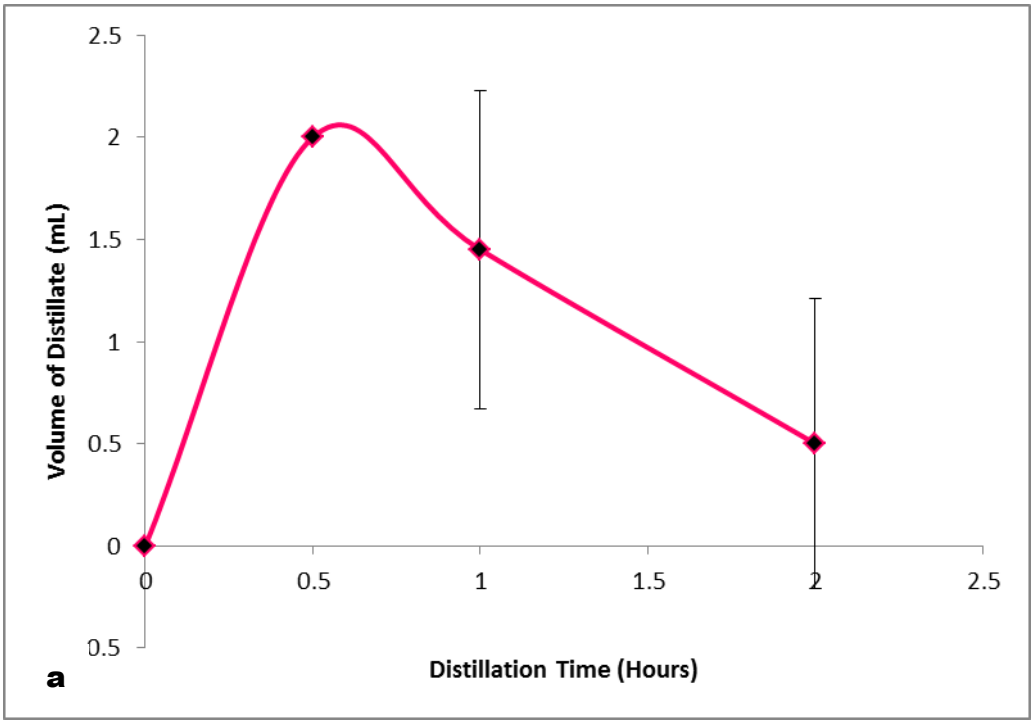
	% Carbon			
Outputs	**Run 1**	**Run 2**	**Run 3**	**Run 4**
Residual sugars	9.31	8.82	8.00	1.70
Ethanol	72.33	70.00	74.79	76.11
Carbon dioxide	7.55	7.55	2.28	7.55
Yeasts	4.46	9.02	5.93	9.62
Total Carbon Output	**93.65**	**95.39**	**90.70**	**95.00**

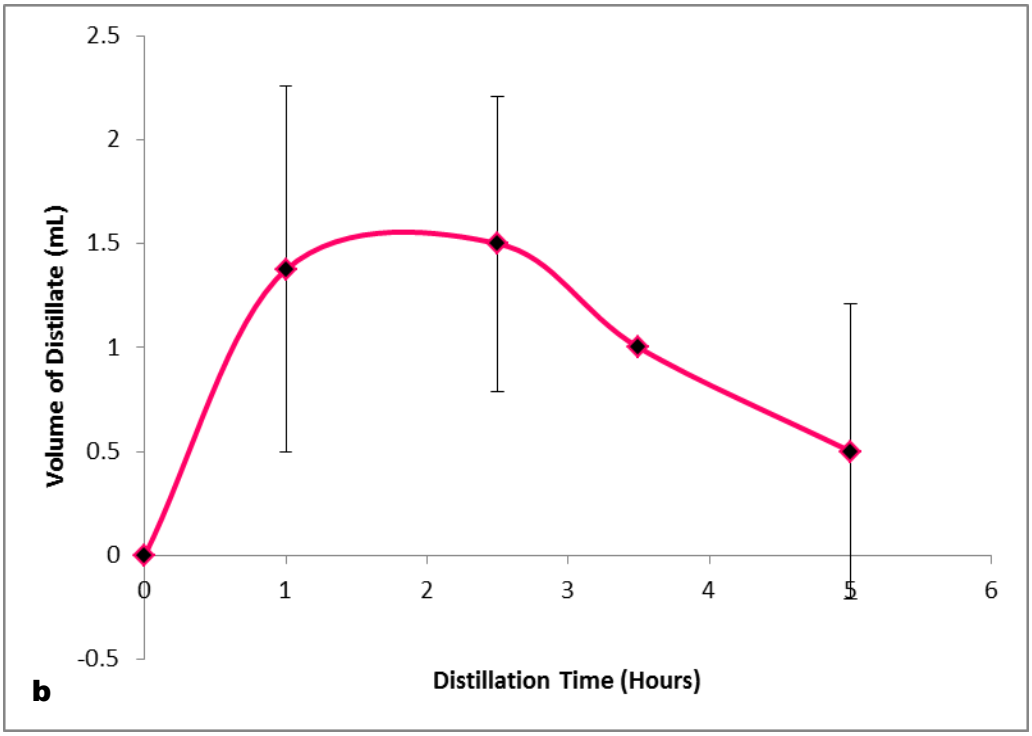
"Carbon output (Table 4.6) is a representation of the carbon recovery or carbon accounted for after the fermentation process from an initial carbon input of 100% (Table 4.5). The highest recovery is reported for run 2 and run 4 (Table 4.6) and the lowest for run 3 (Table 4.6). A high carbon output for run 2 and run 4 is

expected because of the high ethanol and yeast concentrations. The carbon output of run 1 is lower than run 2 and run 4 but higher than run 3 (Table 4.6) as the ethanol concentration is greater than run 3 and the yeast concentrations are lower than run 2 and run 4. Limited research is available for TOC and fermented products. A comparison of the TOC results in the present study can be made to a study by Wang and Wang. (2002). The research showed a carbon recovery of 98.60% and 98.31% for aerobic and anaerobic fermentation, respectively is possible from the fermentation of glucose by the bacteria *Bacillus cereus*. The results in this study are largely different probably because the conversion of sugar by yeast and bacteria during fermentation is different and fermentations conditions could play a role in determining the carbon output. Based on the TOC analysis, the dominant carbon constituent recovered is ethanol (Table 4.10). Thus the use of CAJ in conjunction with the selected yeast strains is suitable for bioethanol production as the majority of sugars were converted to ethanol. Moreover, the plant performance is efficient under the selected fermentation conditions as greater than 90% of carbon was recovered with the most efficient processes being run 2 and run 4 as less carbon was lost during the process. Based on the experimental results, the ANOVA analysis demonstrated significance as follows: (i) TOC Run 1: F-value=75659.78; p-value<0.0001; (ii) TOC Run 2: F-value=56109.73; p-value<0.0001; (iii) TOC Run 3: F-value=1.3E+06; p-value<0.0001; (iv) TOC Run 4: F-value=1.4E+05; p-value<0.0001. The results are significant" (Deenanath et al., 2013).

4.5 Distillation

The method of choice to purify the bioethanol from the CAJ fermentation is distillation. A total volume of 2.9L was distilled. This volume was the remaining product of fermentation runs 1, 2 and 4, as a high ethanol concentration was obtained with a greater distillation potential for ethanol recovery. As described in section 3.6, a volume of 500mL for the duplicate fermentations were subjected to batch distillation. As the distillation process continued, aliquots of the distillate were collected and the time noted. The results are shown in Figure 4.30a-c. For run 1, the volume 3.95mL was collected over 2hrs (Fig 4.30a). For run 2, the volume 4.375mL was collected over 5hrs (Fig 4.30b) and for run 4 the volume 5.75mL was collected over 6hrs (Fig 4.30c).





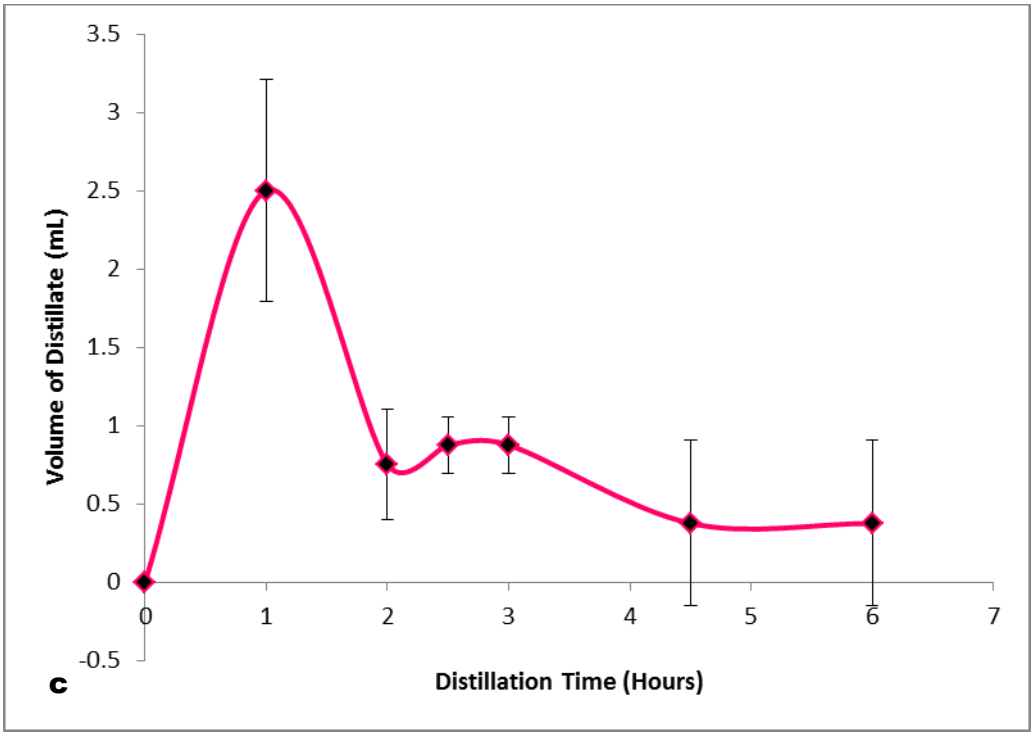


Figure 4.30a-c Distillation profile of run 1(a), run 2 (b) and run 4 (c). The values plotted are an average of the duplicate fermentations. ±SD.

In total, 14.075mL of distillate was obtained and the product was analysed for water content and concentration. The water content was determined by drying the distillate for 1hr. Post-drying, the remaining volumes were transferred to a measuring tube and the volume recorded was 10.50mL. Thus the product recovered is estimated as 75% ethanol and 25% water. The high water content could be attributed to the temperature of 90ºC used during the distillation process as this temperature is close to the boiling point of water, allowing more water to accumulate in the distillate. This temperature was used as at 78ºC, the oil was not at boiling point and as suggested by Tangka et al. (2011), distillation at 90ºC can be used to extract ethanol from fermented products. The ethanol recovered in this study was higher than a previous study, which resulted in 67% ethanol recovery from fermented banana waste (Coelho et al., 2012). These results do not agree with the quality of ethanol distillation products, where it is suggested that 93% to 95% recovery is possible (Dias et al., 2009;2011; Coelho et al., 2012; Errico and Rong, 2012). To achieve a product of approximately 95% ethanol, two stages of recovery is used. The first is simple distillation which results in 37% of ethanol, followed by a second rectification step to produce 95% ethanol (Coelho et al., 2012). In the present study, a single step resulted in 75% of ethanol and thus the process is considered satisfactory. The concentration, determined by HPLC revealed a pure product with a concentration of 44.02g/L (Fig 4.31). Based on the concentration the distillation process resulted in ethanol loss. A large amount of the ethanol could not be recovered and this could be due to an inefficient distillation system or as suggested by Coelho et al. (2012) impurities in the fermentation sample could affect the distillation. On the other hand, combination

of the distillate of runs 1, 2 and 4 could have contributed to a change in the initial ethanol concentration of the distillate. However, this is an assumption as the concentration of the distillate, prior to drying was not determined. Furthermore, the method used to dry the sample to determine the water content could have resulted in the evaporation of the ethanol.

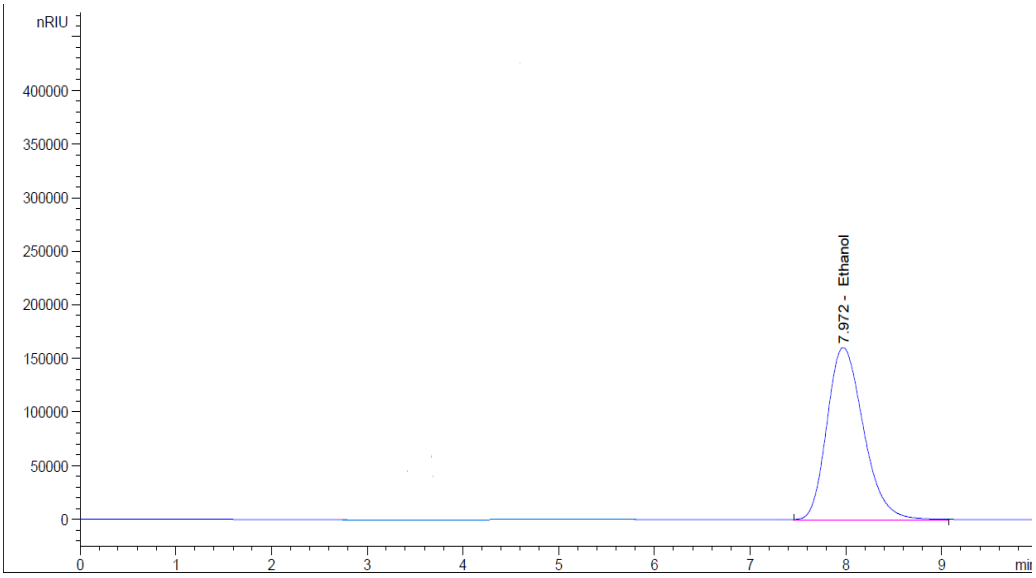


Figure 4.31 HPLC chromatogram of distillate. Peak identification based on the refractive index and retention time of the ethanol standard is indicated on the chromatogram.

From the distillation experiments, a minimal volume and concentration of ethanol was recovered. The choice of simple distillation and the apparatus used was satisfactory in obtaining a pure product but not efficient in extracting the total amount of bioethanol from the fermented CAJ. An improved recovery could be achieved by using other methods of ethanol recovery, for example vacuum extractive fermentation, double-effect distillation, membrane separation or

extractive distillation with solvents (Dias et al., 2009;2011; Errico and Rong, 2012; Pacheco-Basulto et al., 2012).

4.6 Ethanol/Petrol Blends

In this section the blending of ethanol with petrol evaluates the compatibility between two separate chemical compounds at various ethanol-to-petrol blend ratios. Moreover, generator runs with ethanol/petrol blends shows the effect of ethanol addition to petrol on fuel consumption.

4.6.1 Preparation of Blends

The E5 and E10 ethanol/petrol blends were prepared using absolute ethanol and neat petrol (95 unleaded SASOL). In addition, the $E_{distillate}$ or E4.4 blend was prepared based on the estimated water content and concentration of the distilled product (section 4.5). This solution was an ethanol/water/petrol blend, which is meant to mimic the bioethanol product obtained during the fermentation and distillation experiments. The blends are shown in Figure 4.32a-c. Figure 4.32b-c shows ethanol concentrations of 5% and 10% are fully miscible in petrol and the method of sonication was successful for mixing the two components as a single phase is visible (Fig 4.32b-c).

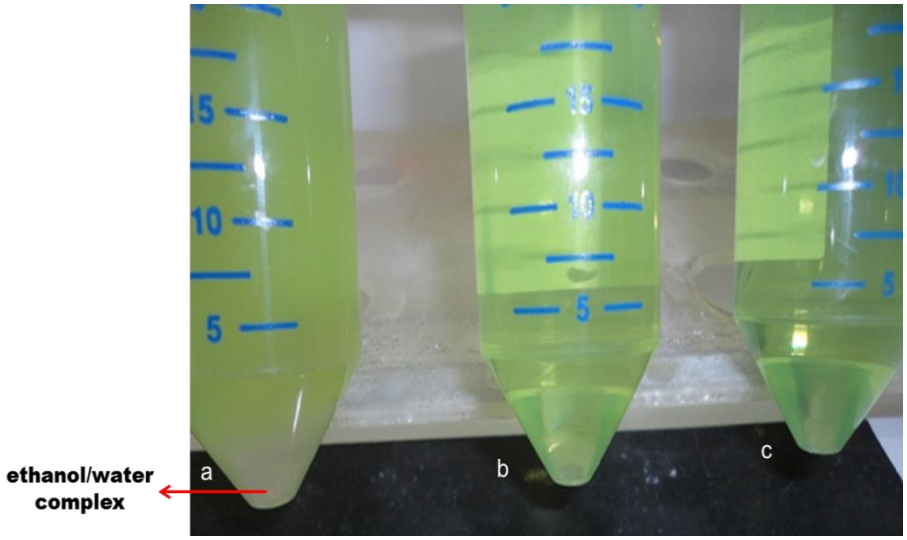


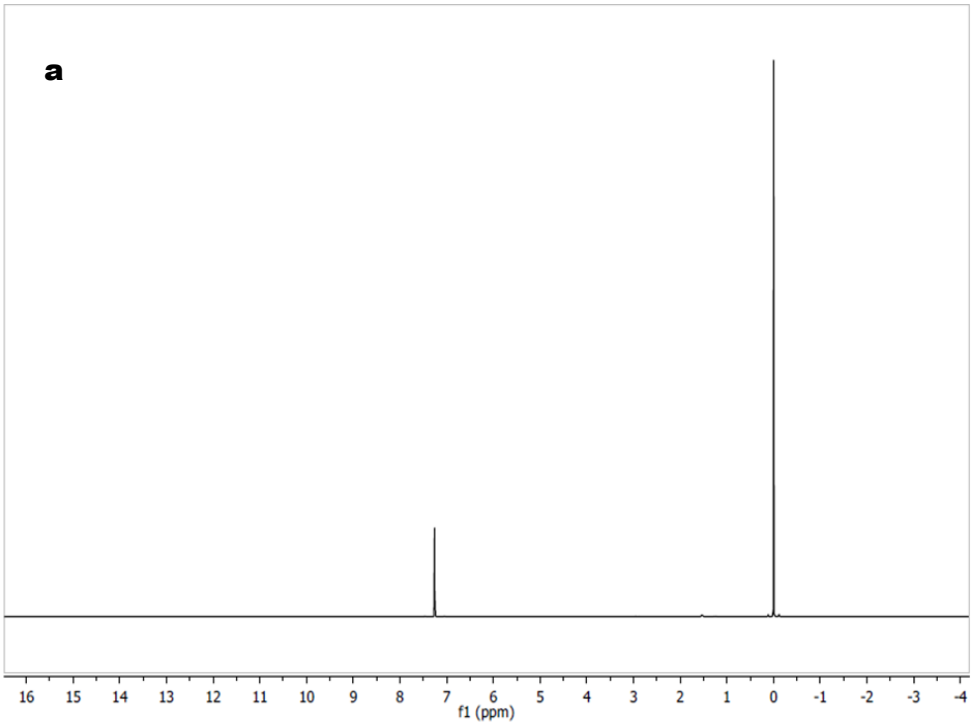
Figure 4.32a-c Ethanol/Petrol blends at room temperature showing the E4.4 (a), E5 (b) and E10 (c) blends.

This is expected as freshly prepared blends rarely exhibit phase separation. This phenomenon usually occurs after approximately 4 weeks due to the accumulation of water (Muzikova et al., 2009; Torres-Jimenez et al., 2011). Water accumulation is contributed by: (i) the concentration of ethanol; the higher the ethanol concentration the greater will be the ability to attract water molecules, (ii) temperature at which the blend is stored; as lower temperatures tend to decrease the solubility of water, (iii) final volume of the blend; the larger the volume the larger the storage vessel and the greater the possibility of moisture, (iv) storage time of the blend; the longer the blends are stored the greater the chance water or moisture will enter the storage vessel and separate the blend (Muzikova et al., 2009; Torres-Jimenez et al., 2011). In this study, water accumulation and phase separation was not evaluated as the blends were prepared and then utilized for other analysis. For the E4.4 blend, phase separation is visible due to the presence

of water (Fig 4.32a) and there is no cohesion between the ethanol and petrol. Ethanol as a polar molecule is easily soluble in water (Muzikova et al., 2009) and for this reason the miscibility in petrol is affected. It is clear the quality of a blend is decreased by the presence of water, shown by the cloudiness in the sample (Fig 4.32a). Hence, bioethanol produced by CAJ fermentation will require methods of ethanol recovery that drastically reduce the water content, if the product is to be used for other applications.

4.6.2 Analysis of Blends

Ethanol/petrol blends were subjected to qualitative NMR analysis with the intention to determine the presence or compatibility of the compounds in a blend. Neat petrol, absolute ethanol and the solvent ($CDCl_3$) were analysed as reference lines. The ^{1}H and ^{13}C spectra of these references are illustrated in Figures 4.33a-b, 4.34a-b and 4.35a-b.



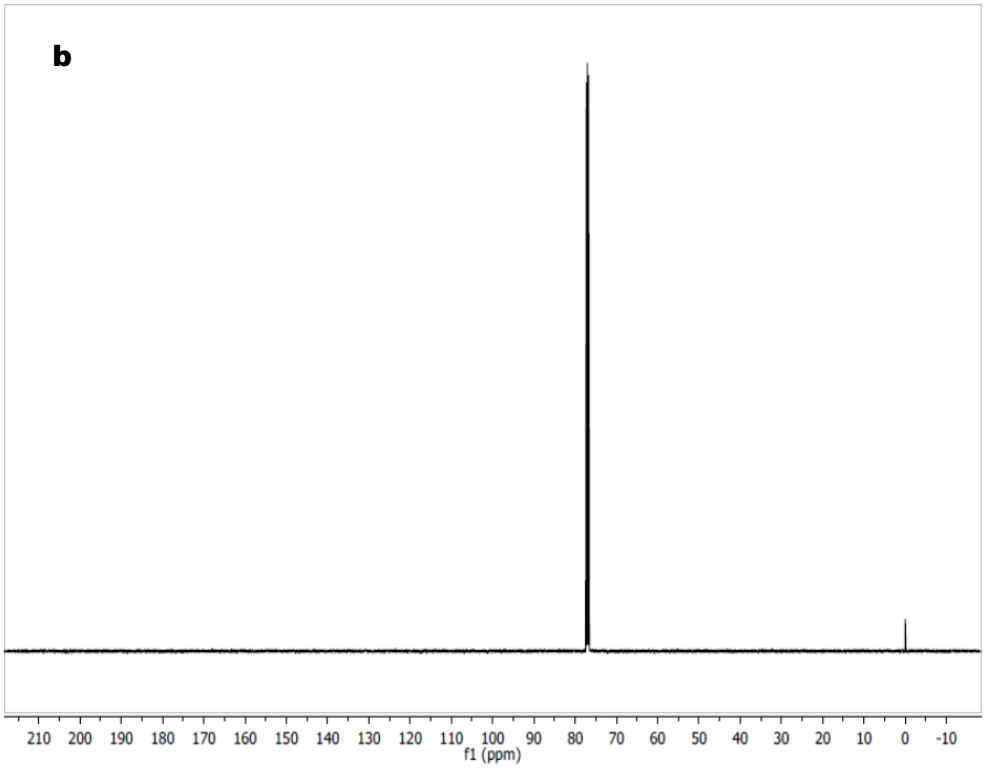
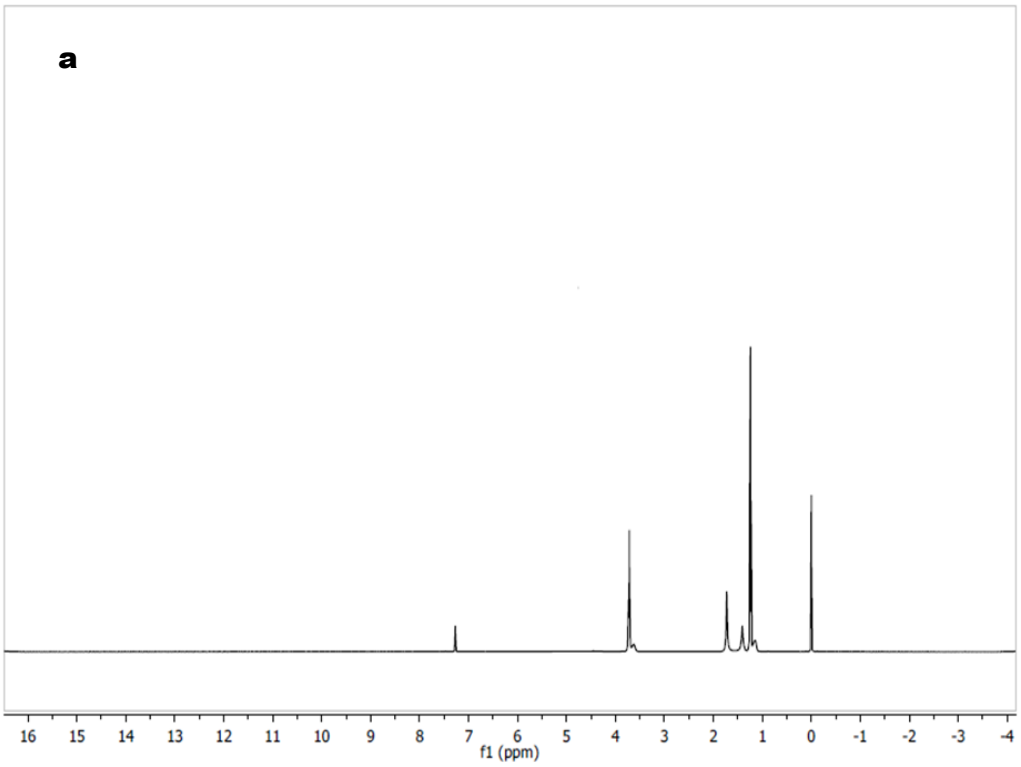


Figure 4.33a-b ^{1}H (a) and ^{13}C (b) spectra of the deuterated chloroform solvent, $CDCl_3$.



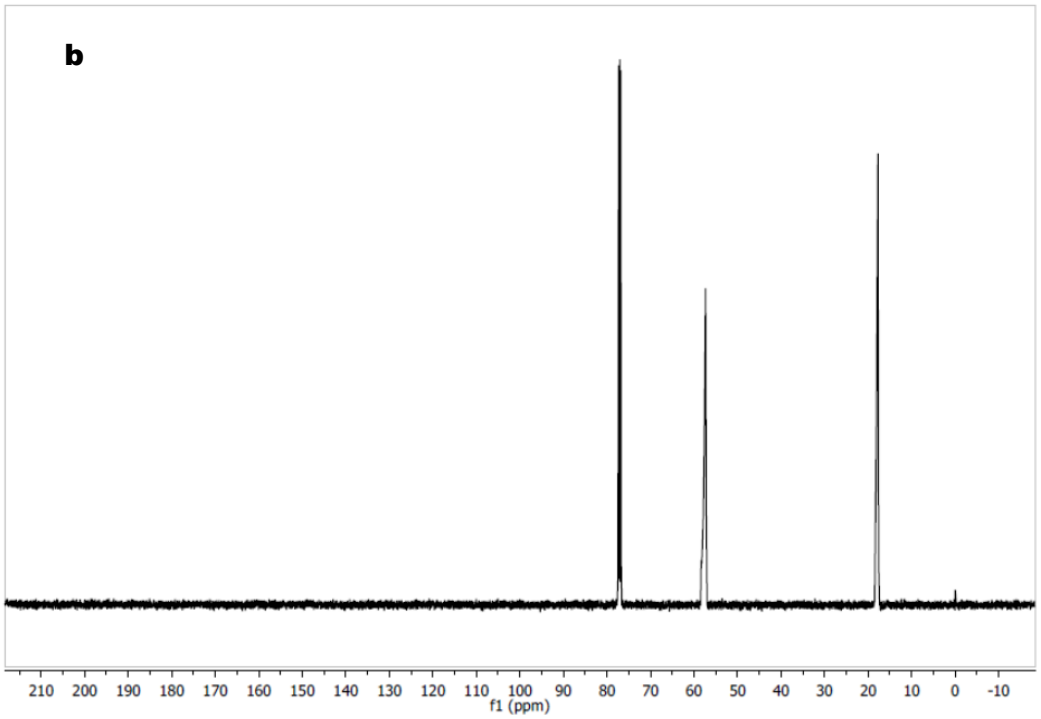


Figure 4.34a-b ^{1}H (a) and ^{13}C (b) spectra, in $CDCl_3$, of absolute ethanol.

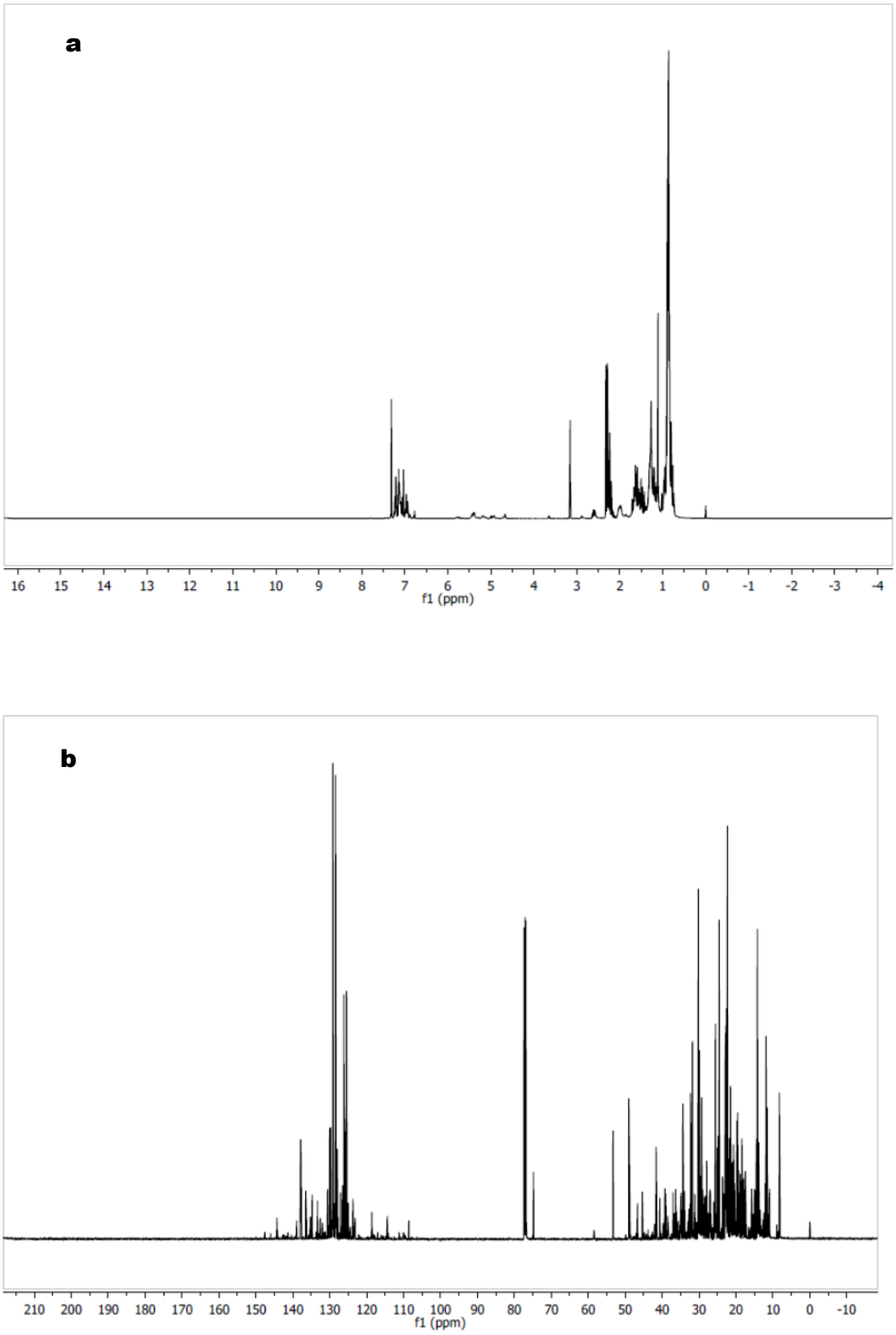


Figure 4.35a-b ^{1}H (a) and ^{13}C (b) spectra, in $CDCl_3$, of 95 unleaded petrol.

The chemical shift of the ^{1}H spectrum is from 0-16ppm and of the ^{13}C spectrum from 0-210ppm (Figs 4.33-4.35). The solvent resonance (Fig 4.33a-b) was detected at 7.26ppm (^{1}H) and 77.01ppm (^{13}C). The ethanol resonance (Fig 4.34a-b) was detected between 1-4ppm (^{1}H) and 18-58ppm (^{13}C). The petrol resonance (Fig 4.35a-b) was detected between 1-7ppm (^{1}H) and 8-150ppm (^{13}C). The chemical shifts obtained for the solvent corresponds to the normal pattern of NMR shifts for $CDCl_3$ which is usually around the 7-7.26ppm region of ^{1}H and 76-80ppm of ^{13}C (Gottlieb et al., 1997; Nicodem et al., 1998). For ethanol, ^{1}H shifts are between 1-3.58ppm and ^{13}C shifts are between 18-57.6ppm (Sarpal et al., 1997; Bansal et al., 2008) which is similar to the shifts obtained in this study. From the petrol NMR spectra it appears petrol is a complex structure with a number of carbon and hydrogen molecules. Based on the resonance, SASOL unleaded petrol is dominated by aliphatic carbons, aromatic carbons, aliphatic hydrogens and a benzene region. This is based on chemical shifts previously identified for petrol, where aliphatic carbons of ^{13}C lie between 10-50ppm, aromatic carbons of ^{13}C lie between 100-160ppm, aliphatic hydrogens of ^{1}H lie between 0.8-1.8ppm and benzene of ^{1}H is between 2.1-2.8ppm (Renzoni et al., 1985; Sarpal et al., 1997; Smirnov and Frolov, 1997; Nicodem et al., 1998; Bansal et al., 2008). Aromatic hydrogens are also present as there is resonance around 7ppm (Fig 4.35a), and it has been shown that ^{1}H shifts of aromatic hydrogens lie between 6-9ppm (Smirnov and Frolov, 1997; Nicodem et al., 1998; Bansal et al., 2008). The ^{1}H and ^{13}C spectra of the ethanol/petrol blends are illustrated in Figures 4.36a-b, 4.37a-b and 4.38a-b.

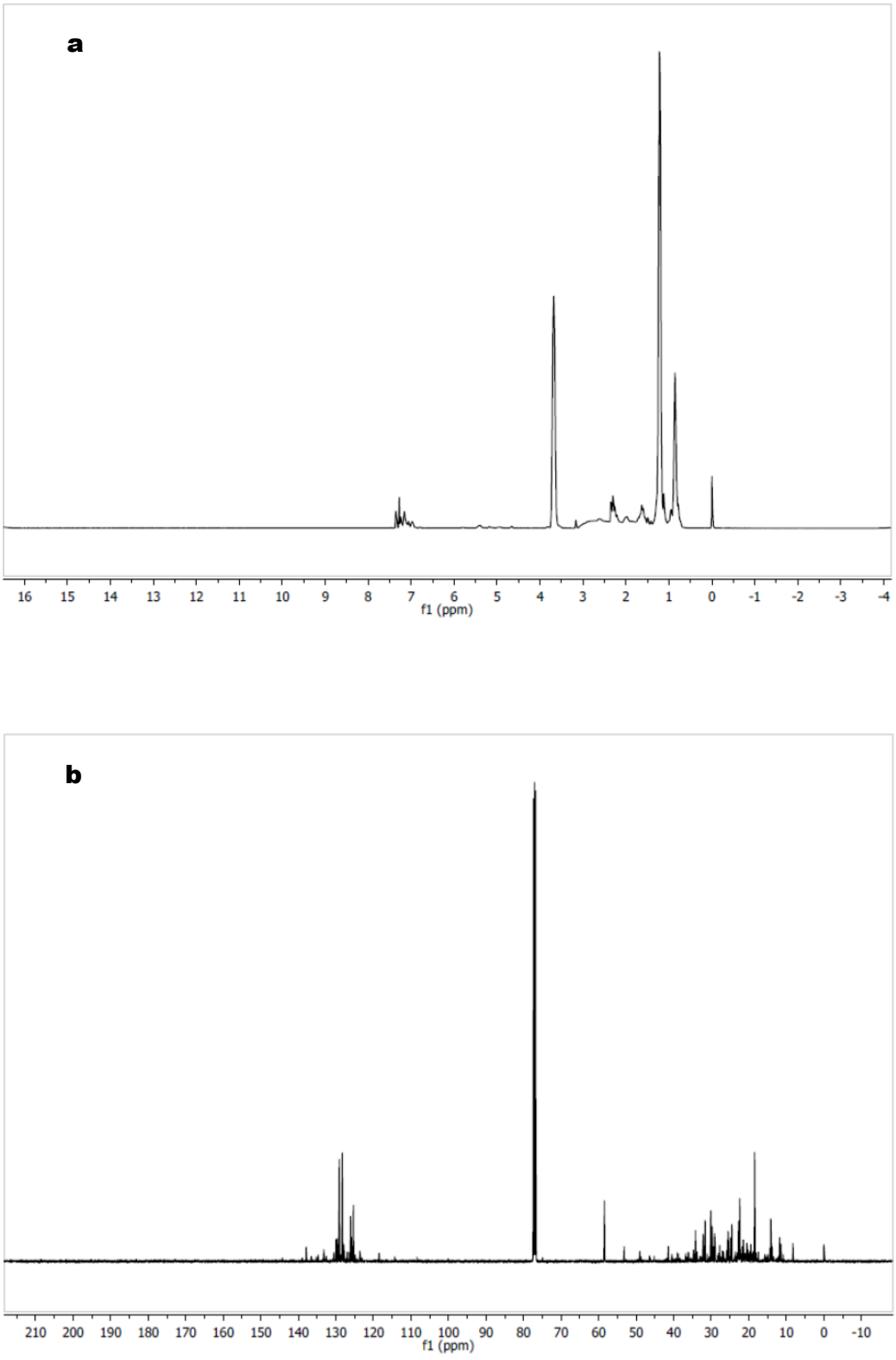
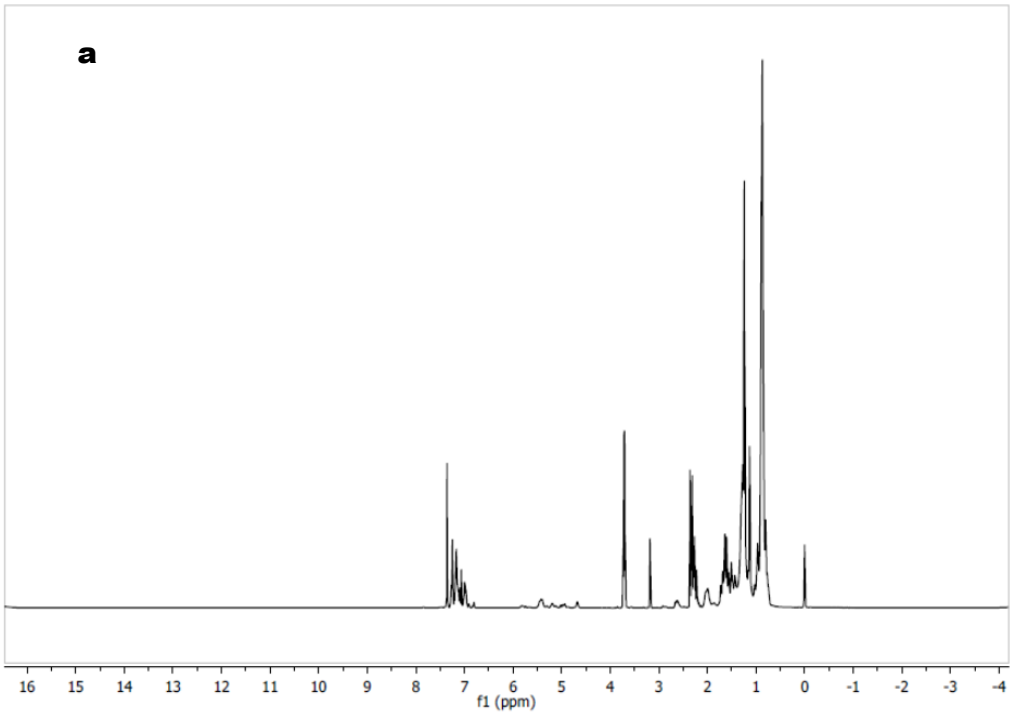


Figure 4.36a-b ^{1}H (a) and ^{13}C (b) spectra, in $CDCl_3$, of the E4.4 blend.



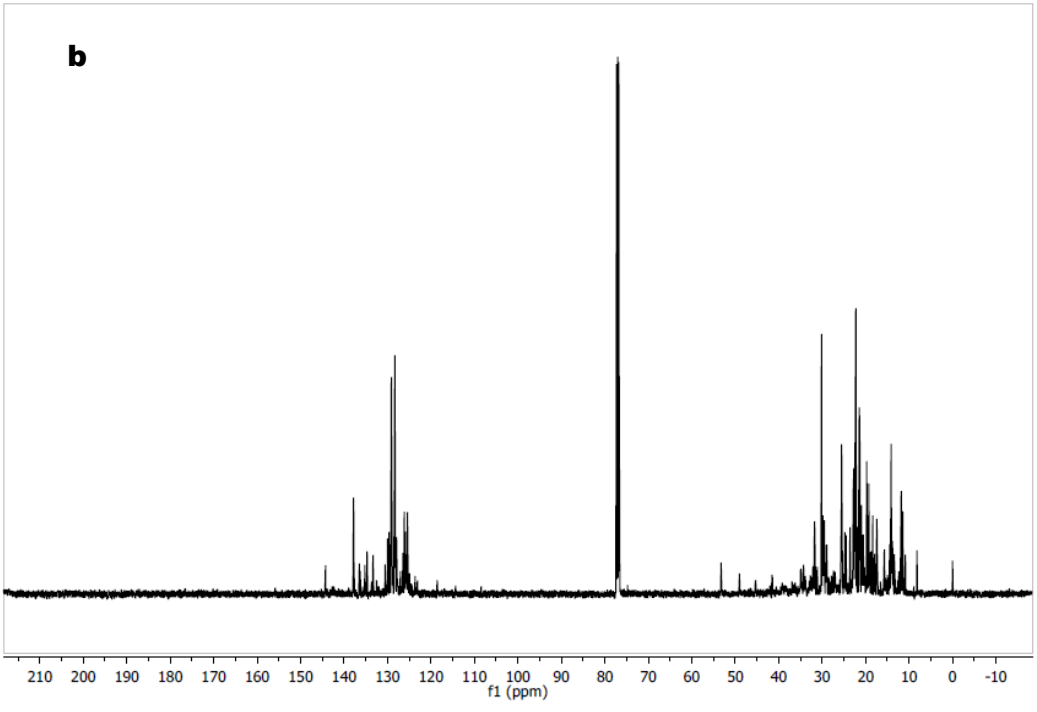
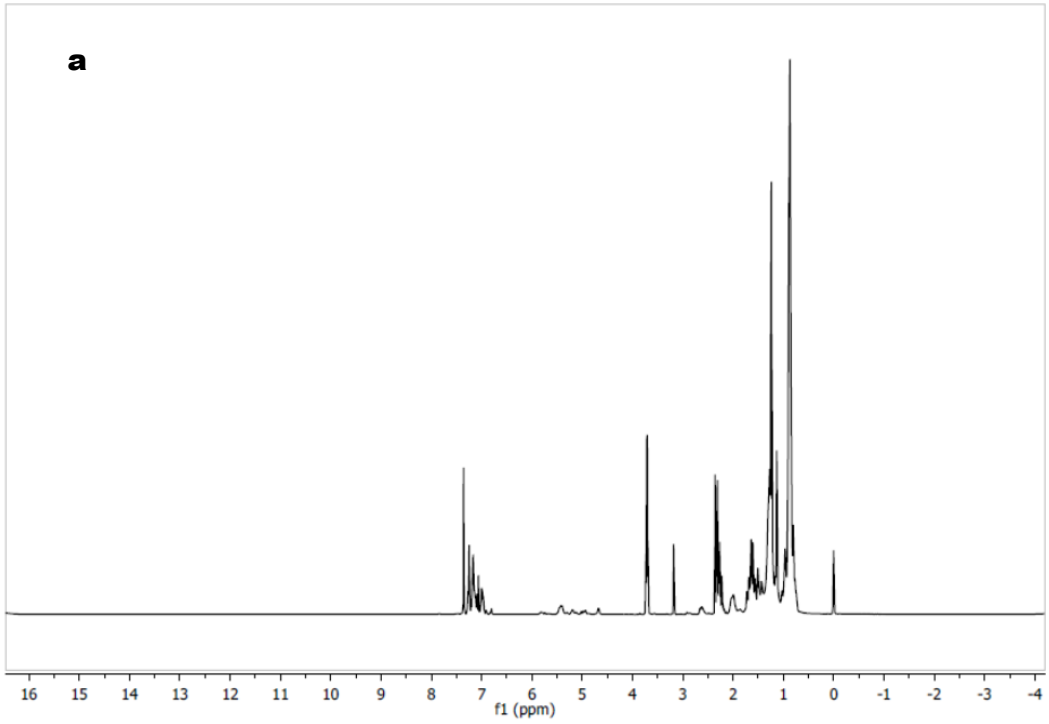


Figure 4.37a-b ^{1}H (a) and ^{13}C (b) spectra, in $CDCl_3$, of the E5 blend.



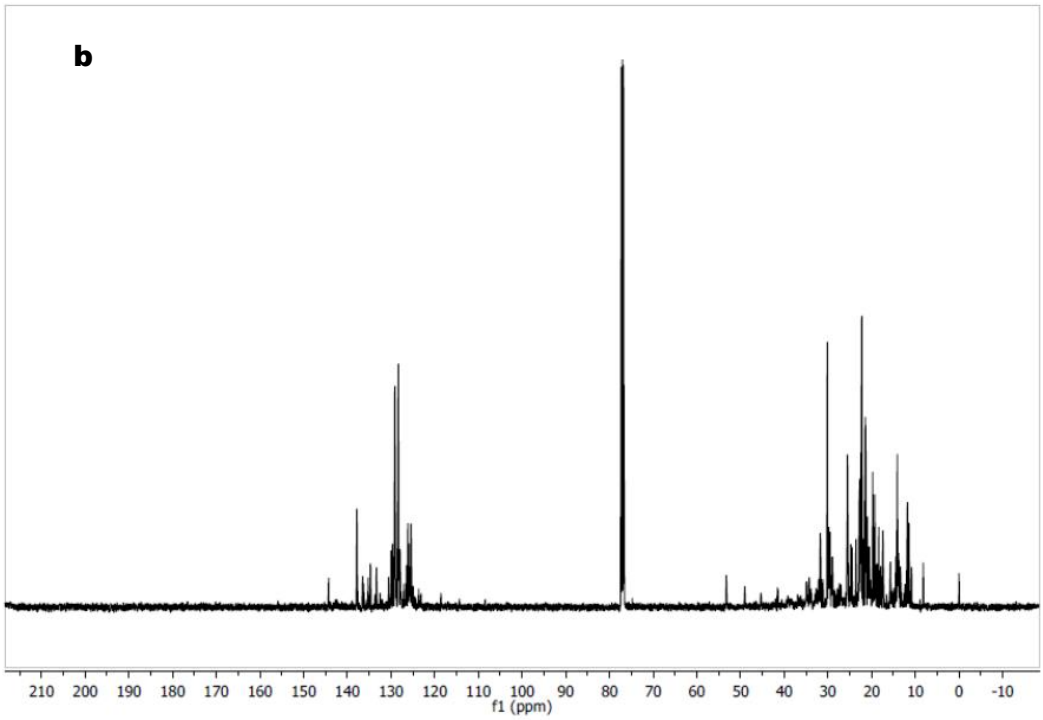
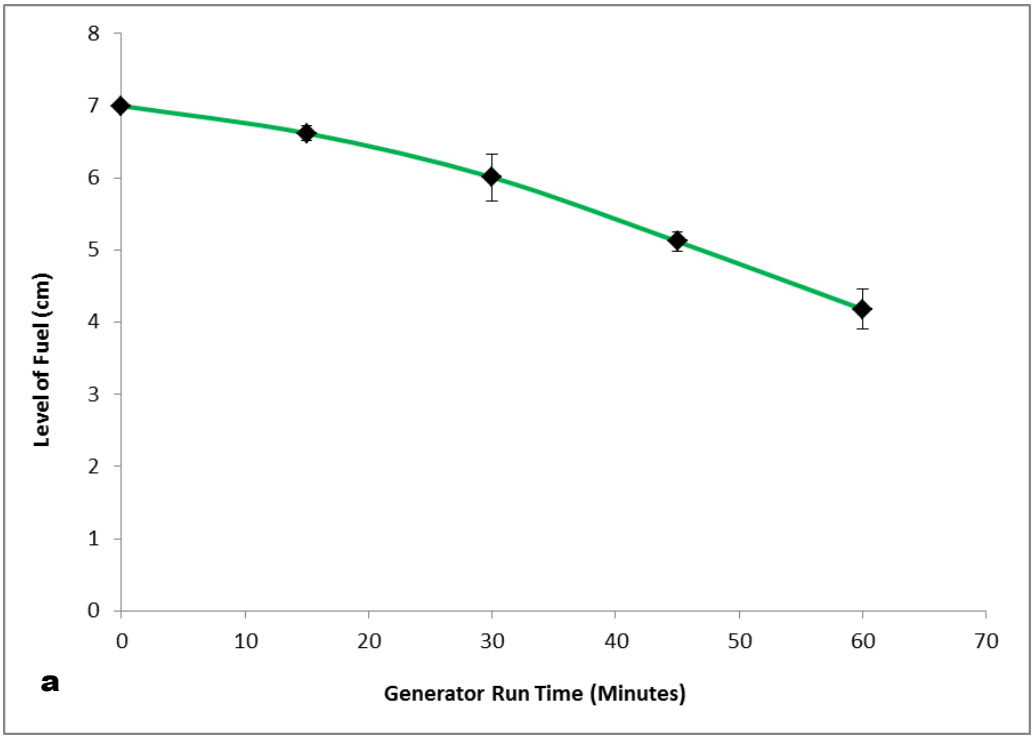


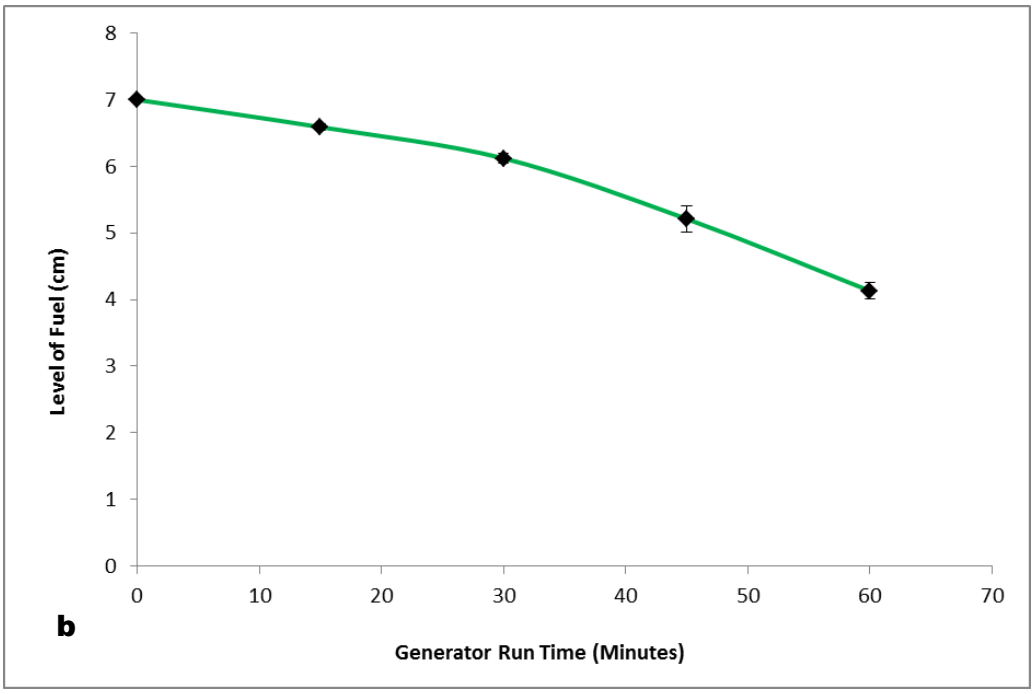
Figure 4.38 a-b ^{1}H (a) and ^{13}C (b) spectra, in $CDCl_3$, of the E10 blend.

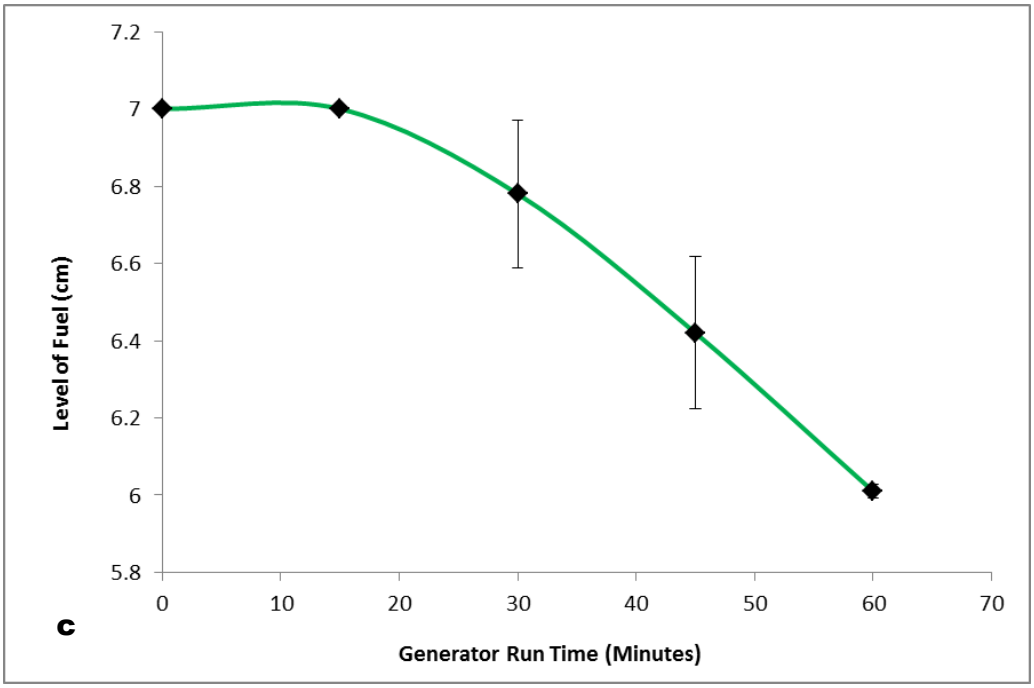
By comparison to the reference lines (Figs 4.33-4.35) the NMR spectra of the blends (Figs 4.36-4.38) revealed they are comprised of compounds consistent to ethanol and petrol, are independent of the solvent and the structure and composition of the individual components are unchanged. For the E5 and E10 blends (Figs 4.37a-b; 4.38a-b) the ethanol is visible in the portion of the petrol close to 4ppm (^{1}H) and at 18.42 and 58.45ppm (^{13}C) which is in agreement with the general ethanol resonance (Fig 4.34a-b), present between 1-4ppm (^{1}H) and 18-58ppm (^{13}C). For the E4.4 blend, the ^{13}C spectrum is consistent with the E5 and E10 blends (Figs 4.36b-4.38b), whereas the ^{1}H spectrum appears to be slightly different (Figs 4.36a-4.38a). The ethanol is visible close to the 4ppm region as seen in Figures 4.37a and 4.38a, with a lower intensity in the hydrogen component of the petrol for the E4.4 blend (Fig 4.36a) and this could be attributed to the water content of the blend. It is assumed the mixing of ethanol/water hinders the miscibility of ethanol/petrol. Based on the NMR analyses, the general structure or chemical fingerprint of the ethanol/petrol blends could be determined. From the structures the ethanol/petrol blend spectra visually displayed that each individual component is unaffected and when there is a change in ethanol concentration, the spectra are identical and hence the structure is intact. NMR also allowed the recognition of the ethanol in a blend, as the ethanol peaks are in the region where there are no petrol. The presence of ethanol shows it is substituted in a blend with petrol and ethanol/petrol blends are compatible and exist as a cohesive compound. These analyses also showed the chemical pattern or fingerprint of ethanol blended specifically with SASOL petrol, which will assist in differentiating from other petrol patterns and whether or not ethanol is a component in petrol as a blend.

4.6.3 Testing of Blends

Four sets of generator experiments were performed to evaluate the fuel consumption over time. First the generator was run with neat petrol to obtain a reference level of the fuel consumed to compare against that of the blends. The generator was then run using the E5 and E10 blends, followed by the E4.4 blend which is the mimic blend of the distillate (section 4.6.1). The result of the generator experiments, to test the ability of the blends is presented in Figure 4.39a-d. From Figure 4.39a-d the change in fuel level is reported over a specified time period. During the generator run time it is evident there is a decrease in the fuel level of all tested samples (Fig 4.39a-d). For the neat petrol (Fig 4.39a) there is a change in the fuel level from 7cm to 6.62cm within 15min. As time progresses, a continued decrease in the fuel level occurs and the final level is noted as 4.18cm (Fig 4.39a). A similar trend, to the neat petrol, is noticed for the E5 blend (Fig 4.39b), where the fuel level decreases from 7cm to 6.59cm in 15mins. The final level for the E5 blend is noted as 4.13cm (Fig 4.39b). The E10 blend (Fig 4.39c) is different compared to the neat petrol and E5 blend. A decrease in the fuel level from 7cm to 6.78cm occurred in 30min, with the final level noted as 6.01cm (Fig 4.39c). The E4.4 blend (Fig 4.39d) did not differ greatly from the neat petrol and E5 blend, with the exception of the generator run time. At 15min, the fuel level was 6.69cm with the final level noted as 6.16cm at 30min (Fig 4.39d) as the engine of the generator switched off during this particular run.


Level of Fuel (cm)
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
70
Generator Run Time (Minutes)
a


Level of Fuel (cm)
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
70
Generator Run Time (Minutes)
b



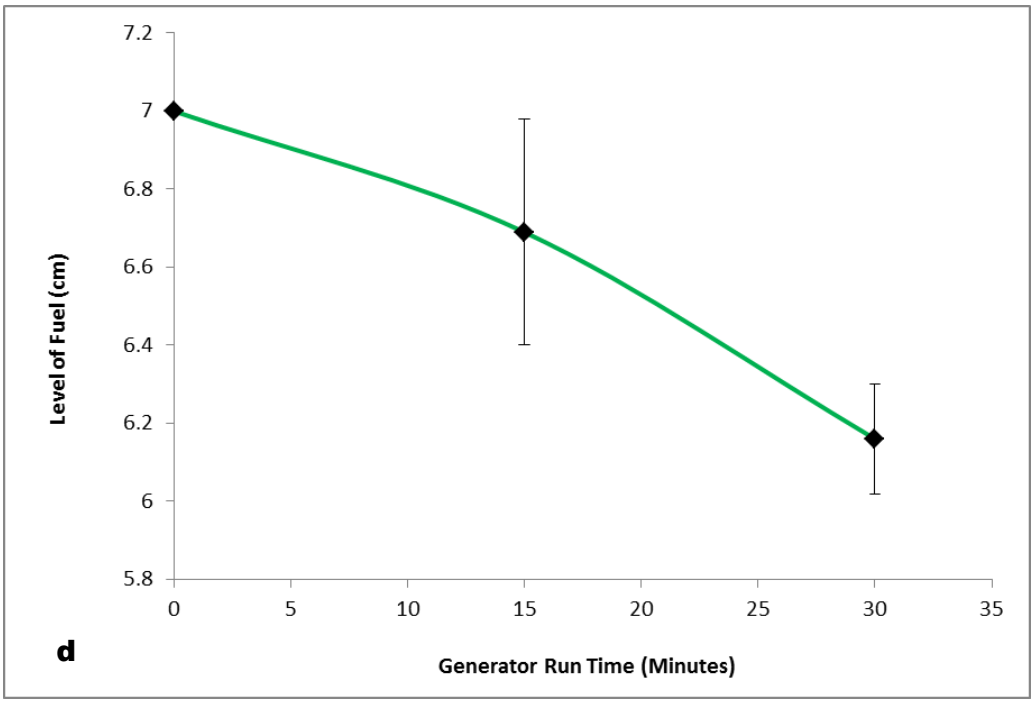


Figure 4.39a-d Fuel consumption profile of neat petrol (a), E5 blend (b), E10 blend (c) and E4.4 blend (d) at time intervals of 0, 15, 30, 45, 60 minutes. The values plotted are an average of triplicate experiments. ±SD.

The addition of ethanol at a concentration of 5% (v/v) did not influence the consumption of fuel since neat petrol and the E5 blend exhibit similar fuel

consumption patterns. When the concentration of ethanol was increased to 10% (v/v), the fuel consumption decreased and it is assumed, ethanol at a concentration greater than 5% (v/v) has a positive effect when blended with petrol on the engine performance. A possible reason for this is due to ethanol having a higher heat of vaporization which causes less heat production and in turn fuel is used at a slower rate (Megaritis et al., 2007; Costagliola et al., 2012). The presence of water in the E4.4 blend did not affect the fuel consumption during the initial stages of the generator run time from 15 to 30 minutes; however it did pose a major problem with the ability of the generator engine to consume the total volume of fuel added to the fuel tank in order to run to completion. Furthermore, once the generator started a cloud of white smoke was visible, which persisted during the run. This is probably due to inefficient combustion and shows water in a blend is undesirable. At present no supporting evidence from previous research studies is used to compare the data obtained as the method used in the current research is independent of research conducted on engine analysis using ethanol/petrol blends.

Chapter 5

Summary of the Findings, Concluding Remarks and Future Recommendations

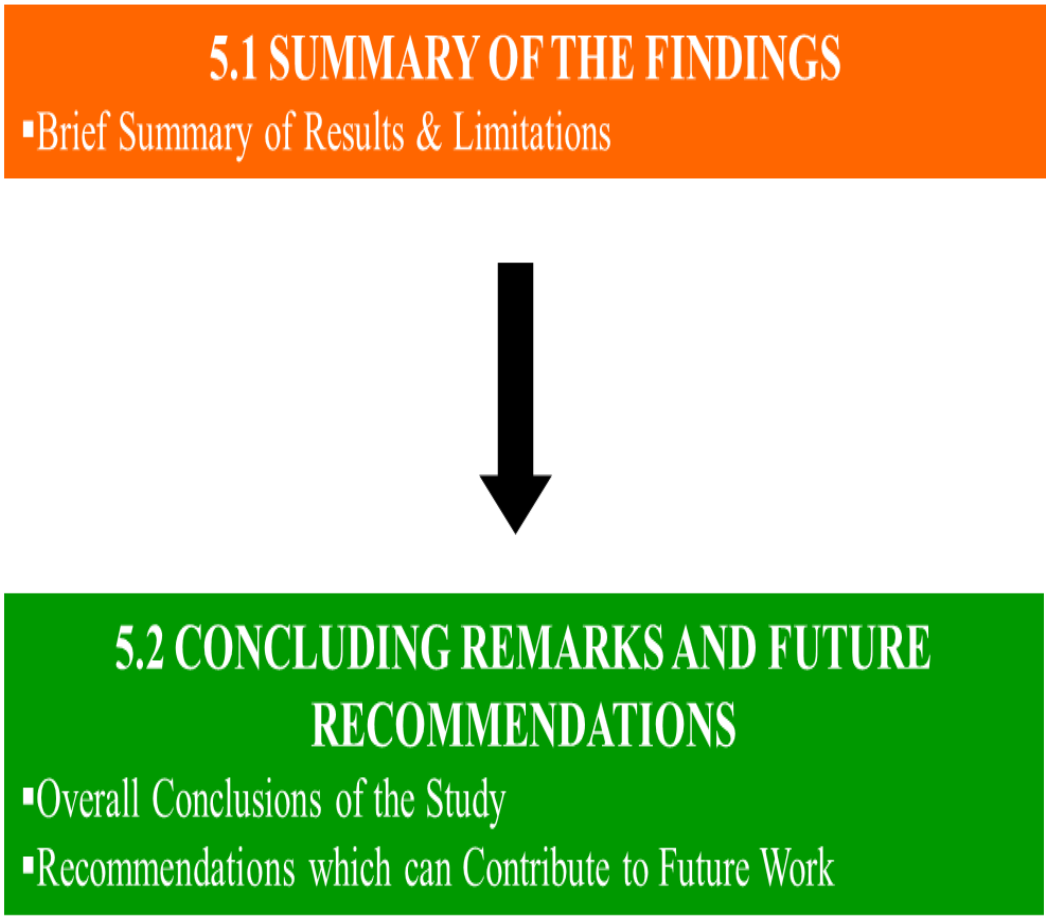


Figure 5.1 A diagram representing the outline of Chapter 5.

In the previous chapters theoretical information and experimental results were presented to share knowledge of the investigation conducted and shed light on bioethanol, the production of bioethanol, and the application of bioethanol. This chapter provides a brief summary of the research findings and limitations to the study. Lastly, concluding remarks are drawn from the investigation and recommendations for future research is proposed.

5.1 Summary of the Findings

In the study, at total of 33kg of cashew apples (CAs) were collected from the Coastal Cashew plantation at the KwaNgwanase region. The apples collected show a mixed variety of colours with an average length of 58mm and width of 44mm. The colour, shape and size were similar to CAs growing in other world regions. From the apples, a volume of 8.75L of cashew apple juice (CAJ) was extracted. The percentage yield of juice was 26.52%; extraction capacity was 2.9L/hr and percentage extraction efficiency was 33.14%. Physical characteristics of the CAJ were 1.050 and 4.52 for specific gravity and pH, respectively. The specific gravity offered a preliminary prediction of the ethanol concentration achievable, which was between 6-7% (v/v). The pH value was similar to the pH range of CAJ extracted from CAs growing in Nigeria, Ghana and Brazil. Chemical characteristics of CAJ were total sugar: 100g/L; condensed tannins: 55.34mg/L; total minerals: 15.89ppm; vitamin C: 112mg/100mL; total proteins: 1.78g/L. These attributes are indicative of the CA growth region. The extracted CAJ was fermented using *Saccharomyces cerevisiae* yeast strains NRRL Y2084

and Vin13 to produce bioethanol and evaluate the concentration of ethanol, sugar consumption, glycerol concentration and yeast viability under controlled fermentation conditions. The maximum ethanol concentration of 65.17g/L was achieved by yeast strain NRRL Y2084 within 11 days under semi-aerobic conditions, whilst under aerobic conditions a maximum ethanol concentration of 65.12g/L was achieved within 5 days. Yeast strain Vin13 produced 31.40g/L of ethanol under semi-aerobic conditions and 67.74g/L under aerobic conditions. These concentrations were obtained within 2 days. Both yeast strains rapidly consumed glucose and fructose sugars with Vin13 showing an affinity for maltose and the glucose supply was depleted. Higher glycerol concentrations were reported under semi-aerobic conditions of both yeast strains and yeast viability was rapidly increased under aerobic conditions of both yeast strains. Selected fermentation conditions were ideal for bioethanol production. During the fermentation process, carbon dioxide and oxygen gas were monitored. Carbon dioxide percentage increased as fermentation progressed in relation to the increase in ethanol concentration. Oxygen percentage was unchanged for the semi-aerobic fermentations and decreased during the aerobic fermentations. Additionally, *real time* monitoring of the fermentation parameters showed a decrease in pH, with the exception of Run 3. The change in pH triggered the acid/base dosing control function. Temperature, jacket temperature and stirrer parameters were stable throughout the process. Oxygen-related parameters exhibited continuous fluctuations during the aerobic fermentations. The bioethanol produced was recovered by distillation. Results showed a total volume of 14.075mL of distillate was collected with a water content of 25% and ethanol concentration of 44.04g/L.

The distillation process was satisfactory. The second part of the study focused on the application of ethanol blended with commercial petrol. Ethanol/petrol blends were prepared and the chemical compatibility evaluated. These results revealed: E5 and E10 blends showed no phase separation; the E4.4 blend displayed cloudiness due to the presence of water and NMR analysis showed compatibility of ethanol/petrol blends as all the individual components of petrol and ethanol was present in the blend. Following chemical analysis, the blends were tested in a generator to determine the fuel consumption and the results were: (i) neat petrol and the E5 blend show similar change in the fuel level over a time period of 1hr from 7cm to 4cm, (ii) for the E10 blend the level decreased from 7cm to 6cm and (iii) the total run time for the E4.4 blend was 30min, as the engine switched off and the fuel level changed from 7cm to 6cm.

The main limitations experienced during the research were for distillation and the generator testing.

For distillation, the equipment used was likely not suitable for low ethanol concentration extraction. The concentration of the distillate could have been affected by the drying method used and by combining the various distillate products. However, this is an assumption, as the concentration before drying of the distillate was not determined due to the limited volume recovered. Minimal distillate volume of bioethanol-derived-CAJ prevented the use of the product for subsequent analysis. For the generator testing, according to manufacturers instruction, a maximum ethanol concentration of 10% can be used for this specific

generator model as a higher concentration causes engine damage. This limited testing blends greater than 10% ethanol.

5.2 Concluding Remarks and Future Recommendations

Increasing greenhouse gas emissions, depleting fossil fuel reserves, climate change, sustainability and renewable resources are constantly highlighted in the field of bioethanol research. With these keywords in mind, the current research was undertaken to contribute to existing information on bioethanol. The purpose of this investigation was to determine the level of bioethanol possible from the fermentation of CAJ by the action of Saccharomyces yeasts in a lab-scale fermentor, followed by analysis and testing of ethanol/petrol blends for its use in ICEs. The evidence reported supports the idea that sufficient bioethanol can be produced using CAJ as a substrate. Moreover, CAJ extracted from CA varieties growing in the KwaNgwanase region has various beneficial characteristics, both physical and chemical, that assist to enhance yeast growth to easily convert the CAJ substrate to ethanol. The fermentation experimental approach used in this research proved successful for bioethanol production and the BIOSTAT® Bplus plant is efficient in terms of maintaining the conditions for optimal functioning and ultimately contributing to a high bioethanol yield. Thus, CAJ extracted from South African specific CAs can be successfully utilized as a biomass-to-bioethanol feedstock. Another factor considered for this research was analysis and testing of ethanol/petrol blends. This application aspect of the study represented taking bioethanol to the next level, rather than limiting the research to production. Ethanol/petrol blends proved compatible and a fingerprint or pattern unique to

using SASOL unleaded grade petrol in a blend was shown. Hence, ethanol can be easily mixed or blended with this type of petrol to serve as an alternative additive and furthermore, the testing of the blends for fuel consumption revealed the addition of ethanol can decrease fuel consumption.

Based on the evidence provided, future recommendations are as follows:

1. **Assessing the effect of different fermentation parameter values.**

Motivation for Recommendation 1: The fermentation conditions selected in this study were suitable in achieving a high ethanol concentration, however testing a wider range of parameter values will determine if a higher bioethanol concentration is possible as well as if a change in fermentation time will occur.

2. **Testing different methods of ethanol recovery.**

Motivation for Recommendation 2: The rotary evaporator system was satisfactory in extracting the ethanol; however since ethanol loss occurred, other methods might be useful in extracting the total available ethanol.

3. **Analysing the effect of ethanol concentrations from 20% to 90% for fuel consumption in a blend.**

Motivation for Recommendation 3: Ethanol concentrations from 4% to 10% was used in this study for reasons mentioned above and the results proved the addition of 10% ethanol to a specific type of petrol can decrease the fuel consumption. The testing of other ethanol concentrations will further show the benefit of ethanol blended in petrol.

4. **Determining biochemical kinetics.**

Motivation for Recommendation 4: The data obtained can be used to determine the reaction rates of yeast growth, substrate consumption and product formation. This will assist in showing the effect of the selected experimental parameters on the product output. Furthermore the kinetics data can be used for mathematical modelling to improve the bioethanol yield.

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APPENDIX A:

RESEARCH OUTPUTS AND ACTIVITIES

1. Peer reviewed publication. Deenanath, E.D., Rumbold, K. and Iyuke, S. (2013). The Production of Bioethanol from Cashew Apple Juice by Batch Fermentation Using *Saccharomyces cerevisiae* Y2084 and Vin13. *ISRN Renewable Energy*. **Volume 2013, Article ID 107851, 11 pages**.

2. Peer reviewed publication. Deenanath, E.D., Iyuke, S. and Rumbold, K. (2012). The Bioethanol Industry in Sub-Saharan Africa: History, Challenges, and Prospects. *Journal of Biomedicine and Biotechnology*. **Volume 2012, Article ID 416491, 11 pages**.

3. Attended and Paper Presentation. The Third International Conference on BIOTECHNOLOGY ENGINEERING (ICBioE'13), Berjaya Times Square Hotel, Kuala Lumpur, Malaysia, 02nd-04th July 2013.

4. Attended and Paper Presentation. The South African Institution of Chemical Engineers (SAIChE) Conference for INNOVATIVE TECHNOLOGIES, Champagne Castle, Drakensburg, South Africa, 16th-19th September 2012.

5. Interviewed by Dr. Lisberg-Haag (Trio-Medien, Germany) and Mr. Mirco Lomoth (Textetage, Germany) for a short research profile-DAAD publication as part of the German-South African Year of Science 2012/13. Interview held at DAAD offices, Senate House, WITS University, 30th November 2012.

6. Attended the 2nd DAAD-NRF Joint In-Country Scholarship Holders Beneficiary Conference held at Techno Park, Stellenbosch, South Africa, 23rd-25th November 2012.

7. Attended the WITS Writing Centre Wartenweiler Library/Faculty of Engineering and the Built Environment Postgraduate Writing Workshop Retreat held at Ekala Guest Farm, Eikenhof Johannesburg, South Africa, 19th-21st November 2012.

8. Attended and Poster Presentation at the 4th Cross-Faculty Postgraduate Symposium held at Professional Development Hub, WITS University, 19th-22nd October 2012. Awarded 3rd Place for Best Poster.

9. Attended the Jesuit Institute South Africa/OSI-APES WITS University Workshop on "Self Awareness for Sustainable Development-The Ultimate Response Ability" held at WITS Club, 3rd August 2012.

10. Attended the South African Nanoscience and Nanotechnology-Nanoscience Characterization Techniques Course Summer School held at the Nelson Mandela Metropolitan University (NMMU), Port Elizabeth, South Africa, 27th-2nd December 2011.

11. Attended the 1st DAAD-NRF Joint In-Country Scholarship Holders Meeting held at Techno Park, Stellenbosch, South Africa, 25th-27th November 2011.

12. Attended the South African Chemical Institute Young Chemist's/Nanotechnology Young Researcher's Symposium (Gauteng Region) held at the Vaal University of Technology, The Vaal, South Africa, 14th October 2011.

13. Participated and Attended the 4th Annual South African Breweries (SAB) Intervarsity Brewing Competition held at SAB-TI Kyalami, Johannesburg, South Africa, 26th-28th August 2011.

14. Attended the Carbon Nanotechnology and Biomimicry Course held at the School of Chemical & Metallurgical Engineering, WITS University, 1st-5th August 2011.

15. Traveled to the KwaNgwanase region to collect cashew apples and extract cashew apple juice, 13th-17th February 2011.

16. Attended and Poster Presentation at the 3rd Cross-Faculty Postgraduate Symposium held at Senate House Concourse, WITS University, 25th-29th October 2010. Awarded 1st Place for Best Poster.

17. Attended the Labotec EXPOSED 2010-50th Year Birthday Bash Seminars and Product Exhibitions held at Labotec, Halfway House Midrand, South Africa, 11th-15th October 2010.

APPENDIX B:
RECIPES FOR MEDIA AND SOLUTIONS

Information on the preparation of media and solutions used for experimental purposes are provided below. Premixed powder and liquids (acids etc) were purchased from Merck and Sigma Aldrich.

Yeast Peptone Dextrose Liquid Broth

Used as a liquid growth medium for the culturing of *S. cerevisiae* yeasts for fermentation.

Yeast Extract…… 10g

Peptone……. 20g

Glucose……. 20g

Distilled Water……. 950mL

Dissolve yeast extract powder and peptone powder in 950mL of distilled water. Sterilize by autoclaving at 121°C for 20 minutes. Dissolve glucose powder in 50mL of distilled water. Once sterilized broth is cool, filter sterilize the glucose solution in to the broth culture to a final volume of 1L.

Buffered Peptone Water

Used as a diluent for the tenfold serial dilutions to determine yeast viability counts post-culturing and during the course of fermentation.

Buffered Peptone Water……. 20g

Distilled Water……. 1000mL

Dissolve buffered peptone water powder in 1000mL of distilled water and sterilize at 121°C for 20 minutes.

Malt Extract Agar

Used a solid medium for petri plates to confirm pure cultures of *S. cerevisiae* yeast strains and as a growth medium for yeast viability counts.

Malt Extract Agar……. 50g

Distilled Water…… 1000mL

Dissolve malt extract agar powder in 1000mL of distilled water and sterilize at 121°C for 20 minutes.

1M Hydrochloric Acid

Used as an acidic reagent for pH control during fermentation.

37% Hydrochloric Acid…….. 86.2mL

Distilled Water……. 913.8mL

Measure 913.8mL of sterilized distilled water into a schott bottle; add 86.2mL of acid to a final volume of 1L. Prepare in a fume hood.

1M Sodium Hydroxide

Used as a basic reagent for pH control during fermentation.

Sodium Hydroxide…… 40g

Distilled Water…… 1000mL

Dissolve 40g of sodium hydroxide pellets in 850mL of distilled water, when pellets have dissolved make up to a final volume of 1L. Sterilize at 121ºC for 20 minutes.

Digestion Solution

Used for the total organic carbon assay.

Potassium Dichromate…… 2.6g

Mercuric Sulphate…….. 8.33g

98% Sulphuric Acid…… 42mL

Distilled Water……. 208mL

Weigh potassium dichromate and mercuric sulphate into a 500mL schott bottle and dissolve these powders in 42mL of sulphuric acid. Once dissolved, slowly add 208mL of distilled water to a final volume of 250mL. Prepare in a fume hood and if necessary, use a funnel for the addition of water to control the flow.

Catalyst Solution

Used for the total organic carbon assay.

98% Sulphuric Acid…… 500mL

Silver Sulphate……. 5.06g

Measure 500mL of sulphuric acid, using a glass measuring cylinder into a 1L schott bottle and add the silver sulphate powder to dissolve. Prepare in fume hood.

Catechin Stock Solution

Used as the standard to detect condensed tannins by the Vanillin-HCl assay.

Catechin…… 120mg

Distilled Water……. 1000mL

Dissolve catechin powder in 1L of sterilized distilled water.

Vanillin Solution

Used to detect condensed tannins by the Vanillin-HCl assay.

Vanillin Reagent…….. 4g

99.8% Methanol…….. 100mL

Dissolve vanillin reagent plus 99% in methanol.

Ascorbic Acid Stock Solution

Used as the standard to detect Vitamin C by the Iodine Titration assay.

Ascorbic Acid……... 5g

Distilled Water……. 1000mL

Dissolve ascorbic acid powder in 1L of sterilized distilled water.

Iodine Solution

Used to detect Vitamin C by the Iodine Titration assay.

Potassium Iodide……. 5g

Potassium Iodate……. 0.27g

Distilled Water……. 200mL

3M Sulphuric Acid…….. 30mL

Weigh potassium iodide and potassium iodate and dissolve in sterilized distilled water, to this add a pre-prepared 3M sulphuric acid solution. Prepare in fume hood.

3M Sulphuric Acid Solution

Used to detect Vitamin C by the Iodine Titration assay.

98% Sulphuric Acid……. 8.34mL

Distilled Water……. 41.66mL

Measure distilled water into a schott bottle; add the acid to a final volume of 50mL. Prepare in a fume hood.

1% Starch Indicator Solution

Used to detect Vitamin C for the Iodine Titration assay.

Litner's Starch……. 0.5g

Distilled Water……. 50mL

Weigh Litner's Starch and dissolve in 50mL of near-boiling distilled water. Mix the solution well. Add starch slowly to water to prevent formation of globules.

Bovine Serum Albumin Stock Solution

Used as the standard to detect proteins for the Coomassie Blue assay.

Bovine Serum Albumin…….. 250mg

Distilled Water……. 1000mL

Dissolve bovine serum albumin powder into 1L of sterilized distilled water.

Coomassie Blue Dye

Used to detect proteins by the Coomassie Blue assay.

Coomassie Brilliant Blue Dye……. 100mg

99.8% Ethanol……. 50mL

99.99% Phosphoric Acid…… 100mL

Distilled Water……. 850mL

Weigh G250 Coomassie Brilliant Blue Dye and dissolve in ethanol. Add phosphoric acid to the dye-ethanol solution and then add distilled water to a final volume of 1L. Prepare in fume hood, add distilled water slowly to dye-ethanol-acid solution. Prior to use of the dye, filter the volume of dye required using filter paper.

APPENDIX C:
DATA
AND STATISTICAL
ANALYSIS

Data presented in this section were used for the construction of graphs depicted in Chapter 4: Results and Discussion. Statistical analysis for the data is included and outputs from the STATA programme for the fermentation and TOC results are shown.

Catechin Standard Curve Data:

Catechin Concentration (mg/L)	Replicates (A_{500nm})			Mean
	1	2	3	
0	0	0	0	0
10	0.165	0.171	0.170	0.169
20	0.203	0.197	0.210	0.203
30	0.346	0.341	0.340	0.342
40	0.486	0.501	0.495	0.494
50	0.531	0.526	0.510	0.522
60	0.615	0.629	0.623	0.622
70	0.713	0.704	0.700	0.706
80	0.827	0.819	0.811	0.817
90	0.915	0.912	0.908	0.912
100	1.000	1.000	1.001	1.000

Cashew Apple Juice Tannin Data:

Sample	Replicates (A_{500nm})			Mean
	1	2	3	
Un-treated	0.570	0.573	0.568	0.570
Pre-treated	0.157	0.155	0.161	0.158

Cashew Apple Juice Mineral Data:

Minerals	Replicates (ppm) 1	2	3	Mean
Zinc	1.39	1.39	1.38	1.39
Copper	2.16	2.19	2.19	2.18
Manganese	1.24	1.25	1.24	1.24
Iron	1.30	1.35	1.32	1.32
Magnesium	0.27	0.27	0.26	0.27*
Sodium	0.38	0.31	0.33	0.34*

*Readings of 6.25% dilution

Ascorbic Acid Standard Curve Data:

Ascorbic Acid Concentration (g/L)	Replicates (mL) 1	2	3	Mean
0	0	0	0	0
0.5	16.80	17	17	17
1	30	31	31	31
1.5	48.5	45	46.5	47
2	55	55	55	55

Cashew Apple Juice Vitamin C Data:

Sample	Replicates (mL) 1	2	3	Mean
CAJ	32.5	32	34.50	33

Bovine Serum Albumin Standard Curve Data:

BSA Concentration (mg/L)	Replicates (A_{595nm})			Mean
	1	2	3	
0	0.000	0.000	0.000	0.000
5	0.216	0.216	0.215	0.216
10	0.345	0.335	0.336	0.339
15	0.510	0.515	0.513	0.513
20	0.650	0.650	0.663	0.654
25	0.790	0.791	0.785	0.789
30	0.910	0.901	0.905	0.905

Cashew Apple Juice Protein Data:

Sample	Replicates (A_{595nm})			Mean
	1	2	3	
CAJ	0.853	0.840	0.843	0.845*

*Reading of 1.5% dilution

Yeast Viability Data:

Fermentation Run 1

Time (days)	Dilution Factor	Replicates						Mean
		1	2	3	4	5	6	
0	1*E-05	264	225	251	271	269	231	251.83
	1*E-06	66	59	51	71	85	75	67.83
1	1*E-04	165	115	171	103	159	135	141.33
	1*E-05	35	47	43	43	33	29	38.33
2	1*E-04	158	171	152	159	109	135	147.33
	1*E-05	45	58	51	67	60	53	55.67
3	1*E-05	198	264	232	259	163	283	233.17
	1*E-06	67	69	75	73	78	79	73.50
4	1*E-05	298	215	203	135	217	271	223.17
	1*E-06	73	55	93	63	45	101	71.67
5	1*E-05	220	189	235	271	225	249	231.50
	1*E-06	89	93	72	87	79	88	84.67
6	1*E-05	269	248	257	273	220	293	260
	1*E-06	48	79	33	59	52	43	52.33
7	1*E-05	206	193	159	189	210	239	199.33
	1*E-06	59	63	54	51	53	59	56.50
8	1*E-05	166	179	174	159	167	172	169.50
	1*E-06	43	59	49	57	53	47	51.33
9	1*E-04	178	157	173	169	163	171	168.50
	1*E-05	51	55	59	69	73	52	59.83
10	1*E-04	149	153	177	151	148	141	153.17
	1*E-05	38	41	44	51	48	47	44.83
11	1*E-04	101	110	125	99	103	111	108.17
	1*E-05	37	35	31	33	39	37	35.33
12	1*E-04	71	79	81	89	95	99	85.67

	1*E-05	45	49	43	37	33	39	41
13	1*E-03	65	53	61	75	71	69	65.67
	1*E-04	39	33	35	41	49	39	39.33
14	1*E-03	55	49	47	35	48	39	45.50
	1*E-04	31	47	35	43	30	45	38.50

Time (days)	**Dilution Factor**	**CFU/mL (Mean)**	**Log CFU/mL**	**Mean Log**	**±SD**
0	1*E-05	2.52E+08	8.4		
	1*E-06	6.80E+08	8.83	8.62	0.304056
1	1*E-04	1.41E+07	7.15		
	1*E-05	3.80E+07	7.58	7.37	0.304056
2	1*E-04	1.47E+07	7.16		
	1*E-05	5.56E+07	7.75	7.46	0.417193
3	1*E-05	2.33E+08	8.37		
	1*E-06	7.35E+08	8.87	8.62	0.353553
4	1*E-05	2.23E+08	8.35		
	1*E-06	7.16E+08	8.85	8.60	0.353553
5	1*E-05	2.31E+08	8.36		
	1*E-06	8.46E+08	8.93	8.65	0.403051
6	1*E-05	2.60E+08	8.41		
	1*E-06	5.23E+08	8.72	8.57	0.219203
7	1*E-05	1.99E+08	8.29		
	1*E-06	5.65E+08	8.75	8.52	0.325269
8	1*E-05	1.70E+08	8.23		
	1*E-06	5.13E+08	8.71	8.47	0.339411
9	1*E-04	1.69E+07	7.22		
	1*E-05	5.98E+07	7.78	7.50	0.39598

10	1*E-04	1.53E+07	7.18		
	1*E-05	4.48E+07	7.65	7.42	0.33234
11	1*E-04	1.08E+07	7.03		
	1*E-05	3.53E+07	7.55	7.29	0.367696
12	1*E-04	8.56E+06	6.93		
	1*E-05	4.10E+07	7.61	7.27	0.480833
13	1*E-03	6.57E+05	5.82		
	1*E-04	3.93E+06	6.59	6.21	0.544472
14	1*E-03	4.55E+05	5.66		
	1*E-04	3.85E+06	6.59	6.13	0.657609

Fermentation Run 2

Time (days)	Dilution Factor	Replicates						Mean
		1	2	3	4	5	6	
0	1*E-05	239	241	233	249	253	246	243.5
	1*E-06	57	61	69	53	49	51	56.67
1	1*E-04	282	193	135	189	168	192	193.17
	1*E-05	43	39	72	40	69	41	50.67
2	1*E-05	210	225	215	209	229	223	218.50
	1*E-06	49	55	57	47	41	39	48
3	1*E-06	131	227	126	129	153	107	145.50
	1*E-07	58	93	67	61	61	59	66.50
4	1*E-06	293	197	203	189	171	210	210.50
	1*E-07	63	97	59	71	59	68	69.50
5	1*E-06	225	213	273	217	281	253	243.67
	1*E-07	98	111	57	101	103	71	90.17
6	1*E-06	106	113	101	121	93	92	104.33
	1*E-07	39	85	88	91	125	63	81.83
7	1*E-05	198	191	107	74	115	198	147.17

	1*E-06	65	43	47	69	79	75	63
8	1*E-05	84	98	121	103	108	108	103.67
	1*E-06	49	63	67	57	66	53	59.17

Time (days)	Dilution Factor	CFU/mL (Mean)	Log CFU/mL	Mean Log	±SD
0	1*E-05	2.44E+08	8.39		
	1*E-06	5.70E+08	8.76	8.58	0.26163
1	1*E-04	1.93E+07	7.29		
	1*E-05	5.06E+07	7.70	7.50	0.289914
2	1*E-05	2.19E+08	8.34		
	1*E-06	4.80E+08	8.68	8.51	0.240416
3	1*E-06	1.45E+09	9.16		
	1*E-07	6.65E+09	9.82	9.49	0.46669
4	1*E-06	2.10E+09	9.32		
	1*E-07	6.95E+09	9.84	9.58	0.367696
5	1*E-06	2.43E+09	9.39		
	1*E-07	9.01E+09	9.95	9.67	0.39598
6	1*E-06	1.04E+09	9.02		
	1*E-07	8.18E+09	9.91	9.47	0.629325
7	1*E-05	1.47E+08	8.17		
	1*E-06	6.30E+08	8.80	8.49	0.445477
8	1*E-05	1.03E+08	8.01		
	1*E-06	5.91E+08	8.77	8.39	0.537401

Fermentation Run 3

Time (days)	Dilution Factor	Replicates						Mean
		1	2	3	4	5	6	
0	1*E-05	299	289	288	285	293	290	290.67
	1*E-06	59	61	65	63	60	69	62.83
1	1*E-05	128	124	125	131	225	115	141.33
	1*E-06	42	95	54	69	41	83	64
2	1*E-04	79	103	99	105	129	125	106.67
	1*E-05	53	35	110	57	58	45	59.67
3	1*E-03	128	111	105	113	131	152	123.33
	1*E-04	69	60	73	108	113	117	90
4	1*E-03	129	138	128	96	80	83	109
	1*E-04	123	83	91	61	106	110	95.67
5	1*E-03	63	58	55	69	53	66	60.67
	1*E-04	47	39	43	41	47	45	43.67

Time (days)	Dilution Factor	CFU/mL (Mean)	Log CFU/mL	Mean Log	±SD
0	1*E-05	2.97E+08	8.47		
	1*E-06	6.28E+08	8.8	8.64	0.233345
1	1*E-05	1.41E+08	8.15		
	1*E-06	6.40E+08	8.81	8.48	0.46669
2	1*E-04	1.06E+07	7.03		
	1*E-05	5.96E+07	7.78	7.41	0.53033
3	1*E-03	1.23E+06	6.09		
	1*E-04	9.00E+06	6.95	6.52	0.608112
4	1*E-03	1.09E+06	6.04		
	1*E-04	9.56E+06	6.98	6.51	0.66468
5	1*E-03	6.06E+06	6.78		
	1*E-04	4.36E+06	6.64	6.71	0.098995

Fermentation Run 4

Time (days)	Dilution Factor	Replicates						Mean
		1	2	3	4	5	6	
0	1*E-05	287	291	290	286	288	283	287.50
	1*E-06	73	69	75	75	78	77	74.50
1	1*E-06	201	298	179	199	205	239	220.17
	1*E-07	183	97	95	139	94	108	119.33
2	1*E-06	156	251	123	145	157	203	172.50
	1*E-07	63	66	66	63	62	67	64.50
3	1*E-06	139	114	153	137	127	131	133.50
	1*E-07	97	91	58	110	159	139	109
4	1*E-05	81	69	65	63	80	107	77.5
	1*E-06	53	52	37	67	69	67	57.5
5	1*E-05	79	97	50	53	55	64	66.33
	1*E-06	43	40	51	35	38	49	42.67

Time (days)	Dilution Factor	CFU/mL (Mean)	Log CFU/mL	Mean Log	±SD
0	1*E-05	2.87E+08	8.46		
	1*E-06	7.45E+08	8.87	8.67	0.289914
1	1*E-06	2.20E+09	9.34		
	1*E-07	1.19E+10	10.07	9.71	0.516188
2	1*E-06	1.72E+09	9.24		
	1*E-07	6.45E+09	9.81	9.53	0.403051
3	1*E-06	1.35E+09	9.13		
	1*E-07	1.09E+10	10.04	9.59	0.643467
4	1*E-05	7.75E+07	7.89		
	1*E-06	5.75E+08	8.76	8.33	0.615183
5	1*E-05	6.63E+07	7.82		
	1*E-06	4.26E+08	8.63	8.23	0.572756

High Performance Liquid Chromatography Data:

Fermentation Run 1

Time (days)	Glucose Replicates						Mean	±SD
	1	2	3	4	5	6		
0	40.42	40.81	40.80	40.84	40.42	40.04	40.56	0.318732
1	28.05	30.04	28.19	28.2	29.84	28.05	28.73	0.942919
2	22.96	23.04	23.15	23.52	22.45	23.44	23.09	0.38469
3	16.8	16.83	16.89	16.11	17.01	16.42	16.68	0.341272
4	10.72	10.72	10.74	10.86	10.12	10.67	10.64	0.261719
5	4.87	4.70	4.98	5.93	4.71	4.22	4.90	0.566866
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0

Time (days)	Fructose Replicates						Mean	±SD
	1	2	3	4	5	6		
0	56.76	57.36	57.36	57.25	56.80	56.84	57.06	0.290545
1	55.45	55.12	55.55	55.85	56.12	56.36	55.74	0.457271
2	53.56	53.86	53.14	53.04	53.26	53.18	53.34	0.310097
3	51.45	51.17	52.10	52.05	51.03	51.26	51.51	0.45865
4	48.14	49.56	49.96	49.14	48.66	48.79	49.04	0.65478
5	47.80	47.69	47.65	47.83	48.01	47.70	47.78	0.132061
6	38.12	38.02	38.28	38.30	38.42	38.19	38.22	0.142045
7	30.90	30.17	30.75	31.01	31.10	30.95	30.81	0.336135
8	25.27	25.20	25.38	25.09	25.15	25.25	25.22	0.101127
9	9.96	10.17	10.05	10.31	10.01	10.23	10.12	0.136589
10	8.27	8.09	8.17	8.45	8.45	8.46	8.32	0.161957
11	6.27	6.09	6.30	6.10	6.35	6.15	6.21	0.110815
12	6.13	6.31	6.35	6.11	6.09	6.19	6.20	0.109301
13	6.19	6.12	6.12	6.33	6.10	6.35	6.20	0.111609
14	6.03	6.07	6.21	6.29	6.31	6.31	6.20	0.125007

Time (days)	Maltose Replicates						Mean	±SD
	1	2	3	4	5	6		
0	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
1	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
2	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
3	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
4	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
5	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
6	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
7	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
8	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
9	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
10	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
11	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
12	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
13	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
14	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0

Time (days)	Ethanol Replicates						Mean	±SD
	1	2	3	4	5	6		
0	0	0	0	0	0	0	0	0
1	8.00	9.15	8.15	8.19	9.00	9.06	8.59	0.529959
2	14.45	14.55	14.36	14.98	14.10	14.35	14.47	0.293309
3	19.14	19.37	19.27	20.01	19.89	19.76	19.57	0.359704
4	25.80	25.65	24.9	24.83	25.70	25.81	25.45	0.456395
5	30.10	31.11	31.02	33.11	31.10	31.09	31.26	0.990207
6	49.65	49.63	48.73	49.55	49.55	49.50	49.44	0.349843
7	54.85	54.62	53.98	54.28	54.39	53.98	54.35	0.347333
8	59.60	59.42	59.43	59.54	59.59	59.51	59.52	0.077136
9	61.73	60.01	61.81	61.60	61.71	61.83	61.45	0.709378
10	63.83	63.10	63.11	62.89	63.15	63.25	63.22	0.320401
11	65.21	65.39	64.73	65.15	65.35	65.21	65.17	0.235768
12	65.21	65.19	65.25	65.11	65.15	65.13	65.17	0.052789
13	65.20	65.05	65.09	65.27	65.17	65.23	65.17	0.084004
14	65.15	65.23	65.11	65.16	65.15	65.22	65.17	0.046043

Time (days)	Glycerol Replicates						Mean	±SD
	1	2	3	4	5	6		
0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	1.17	1.15	1.15	1.10	1.13	1.11	1.14	0.026646
7	1.27	1.10	1.11	1.09	1.10	1.12	1.13	0.068532
8	2.13	2.13	2.11	2.11	2.10	2.09	2.11	0.016021
9	2.25	2.21	2.19	2.16	2.21	2.25	2.21	0.034881
10	2.30	2.37	2.41	2.47	2.35	2.39	2.38	0.057417
11	3.17	3.14	3.03	3.12	3.19	3.13	3.13	0.055498
12	3.20	3.23	3.19	3.25	3.27	3.31	3.24	0.044907
13	4.01	4.05	4.10	4.03	4.03	4.09	4.05	0.036009
14	4.10	4.07	4.19	4.04	4.05	4.05	4.08	0.056451

Fermentation Run 2

Time (days)	Glucose Replicates						Mean	±SD
	1	2	3	4	5	6		
0	40.42	40.81	40.80	40.84	40.42	40.04	40.56	0.318732
1	23.17	23.29	23.25	22.91	23.15	23.21	23.16	0.134263
2	9.55	9.63	9.71	9.56	9.75	9.71	9.651	0.084479
3	2.93	2.71	2.75	2.91	2.84	2.81	2.83	0.08666
4	0	0	0	0	0	0	0	0
5	0	0	0		0	0	0	0
6	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0

Time (days)	Fructose Replicates						Mean	±SD
	1	2	3	4	5	6		
0	56.76	57.36	57.36	57.25	56.80	56.84	57.06	0.290545
1	47.46	47.50	47.39	47.49	47.51	47.5	47.48	0.045056
2	35.91	35.57	34.83	35.81	35.49	35.51	35.52	0.378365
3	20.03	20.15	20.13	20.11	21.01	21.01	20.41	0.469113
4	8.58	8.39	8.44	8.23	8.21	8.35	8.37	0.137792
5	6.05	6.10	6.04	6.04	6.06	6.05	6.06	0.022509
6	5.97	6.04	5.98	6.03	6.10	6.01	6.02	0.047081
7	6.13	6.10	6.00	5.91	6.02	5.89	6.01	0.09704
8	6.15	6.09	5.71	6.05	6.03	6.07	6.02	0.155778

Time (days)	Maltose Replicates						Mean	±SD
	1	2	3	4	5	6		
0	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
1	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
2	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
3	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
4	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
5	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
6	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
7	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
8	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0

Time (days)	Ethanol Replicates						Mean	±SD
	1	2	3	4	5	6		
0	0	0	0	0	0	0	0	0
1	14.90	15.01	15.05	14.89	14.97	15.15	15.00	0.097929
2	36.53	37.10	36.77	36.66	36.71	36.57	36.72	0.204516
3	49.45	49.56	49.35	50.01	49.53	49.61	49.59	0.22731
4	63.83	62.89	62.71	62.23	62.68	63.71	63.01	0.629934
5	64.89	64.91	65.17	65.21	65.23	65.31	65.12	0.176522
6	65.23	65.11	65.17	65.13	65.25	65.21	65.18	0.056095
7	65.25	65.19	65.23	65.18	65.13	65.10	65.18	0.057271
8	65.16	65.19	65.20	65.19	65.23	65.16	65.18	0.026394

Time (days)	Glycerol Replicates						Mean	±SD
	1	2	3	4	5	6		
0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	1.51	1.50	1.51	1.77	1.52	1.53	1.56	0.105008
6	1.53	1.61	1.59	1.61	1.58	1.56	1.58	0.030984
7	1.89	1.54	1.83	1.75	1.79	1.81	1.77	0.121065
8	1.91	1.93	1.88	1.61	1.67	1.59	1.77	0.158209

Fermentation Run 3

Time (days)	Glucose Replicates						Mean	±SD
	1	2	3	4	5	6		
0	40.42	40.81	40.80	40.84	40.42	40.04	40.56	0.318732
1	5.03	4.91	5.02	5.03	5.05	4.89	4.99	0.069402
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0

Time (days)	Fructose Replicates						Mean	±SD
	1	2	3	4	5	6		
0	56.76	57.36	57.36	57.25	56.80	56.84	57.06	0.290545
1	40.57	40.56	40.61	40.59	40.63	40.62	40.60	0.028048
2	1.90	1.92	1.89	1.92	1.96	1.92	1.92	0.024014
3	1.89	1.91	1.94	1.93	1.90	1.87	1.91	0.02582
4	1.95	1.95	1.90	1.86	1.88	1.86	1.90	0.041473
5	1.93	1.90	1.88	1.91	1.89	1.90	1.90	0.017224

Time (days)	Maltose Replicates						Mean	±SD
	1	2	3	4	5	6		
0	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
1	1.64	1.64	1.62	1.63	1.60	1.63	1.63	0.015055
2	1.42	1.41	1.42	1.43	1.42	1.42	1.42	0.013784
3	1.36	1.36	1.45	1.43	1.43	1.37	1.40	0.040988
4	1.37	1.43	1.39	1.46	1.40	1.40	1.41	0.031885
5	1.41	1.35	1.38	1.47	1.40	1.45	1.41	0.044272

Time (days)	Ethanol Replicates						Mean	±SD
	1	2	3	4	5	6		
0	0	0	0	0	0	0	0	0
1	25.21	25.18	25.20	25.23	25.18	25.18	25.20	0.020656
2	31.37	31.39	31.41	31.39	31.40	31.41	31.40	0.015166
3	31.40	31.40	31.37	31.39	31.40	31.43	31.40	0.033862
4	31.41	31.38	31.38	31.42	31.43	31.39	31.40	0.042308
5	31.29	31.41	31.33	31.46	31.43	31.49	31.40	0.107781

Time (days)	Glycerol Replicates						Mean	S±D
	1	2	3	4	5	6		
0	0	0	0	0	0	0	0	0
1	3.05	3.03	3.06	3.04	3.05	3.05	3.05	0.010328
2	3.58	3.57	3.57	3.55	3.56	3.56	3.57	0.010488
3	4.00	4.03	3.97	4.10	4.07	4.07	4.04	0.04899
4	4.01	4.10	4.08	4.05	4.03	4.03	4.05	0.034059
5	4.05	4.06	4.01	4.08	4.06	4.10	4.06	0.030332

Fermentation Run 4

Time (days)	Glucose Replicates						Mean	±SD
	1	2	3	4	5	6		
0	40.42	40.81	40.80	40.84	40.42	40.04	40.56	0.318732
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0

Time (days)	Fructose Replicates						Mean	±SD
	1	2	3	4	5	6		
0	56.76	57.36	57.36	57.25	56.80	56.84	57.06	0.290545
1	7.97	7.94	7.99	8.00	7.98	7.97	7.98	0.020736
2	1.51	1.48	1.49	1.53	1.53	1.52	1.51	0.020976
3	1.48	1.48	1.53	1.53	1.50	1.51	1.51	0.022583
4	1.55	1.48	1.46	1.52	1.49	1.50	1.50	0.031623
5	1.51	1.51	1.47	1.50	1.51	1.48	1.50	0.017512

Time (days)	Maltose Replicates						Mean	±SD
	1	2	3	4	5	6		
0	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
1	2.18	2.18	2.18	2.18	2.18	2.18	2.18	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0

Time (days)	Ethanol Replicates						Mean	±SD
	1	2	3	4	5	6		
0	0	0	0	0	0	0	0	0
1	63.21	63.23	63.29	63.25	63.23	63.29	63.25	0.033466
2	67.73	67.79	67.69	67.71	67.75	67.75	67.74	0.035024
3	67.74	67.77	67.75	67.75	67.71	67.73	67.74	0.020412
4	67.69	67.69	67.73	67.75	67.79	67.73	67.73	0.037947
5	67.73	67.75	67.76	67.70	67.78	67.71	67.74	0.030605

Time (days)	Glycerol Replicates						Mean	±SD
	1	2	3	4	5	6		
0	0	0	0	0	0	0	0	0
1	1.56	1.56	1.55	1.55	1.55	1.56	1.56	0.005477
2	1.71	1.70	1.69	1.71	1.68	1.71	1.70	0.012649
3	1.69	1.75	1.73	1.77	1.70	1.71	1.73	0.030822
4	1.73	1.73	1.74	1.76	1.78	1.70	1.74	0.027568
5	1.77	1.79	1.75	1.72	1.72	1.70	1.74	0.034303

Gas Analysis Data:

Fermentation Run 1

Time (days)	CO2 (%) 1	CO2 (%) 2	Mean	O2 (%) 1	O2 (%) 2	Mean
0	0.03	0.03	0.03	20.68	20.68	20.68
1	1.32	1.30	1.31	20.68	20.68	20.68
2	2.15	2.00	2.08	20.68	20.68	20.68
3	2.92	3.00	2.96	20.68	20.68	20.68
4	3.91	3.92	3.92	20.68	20.68	20.68
5	4.77	4.77	4.77	20.68	20.68	20.68
6	7.53	7.49	7.51	20.68	20.68	20.68
7	8.36	8.30	8.33	20.68	20.68	20.68
8	10	10	10	20.68	20.68	20.68
9	10	10	10	20.68	20.68	20.68
10	10	10	10	20.68	20.68	20.68
11	10	10	10	20.68	20.68	20.68
12	10	10	10	20.68	20.68	20.68
13	10	10	10	20.68	20.68	20.68
14	10	10	10	20.68	20.68	20.68

Fermentation Run 2

Time (days)	CO2 (%) 1	CO2 (%) 2	Mean	O2 (%) 1	O2 (%) 2	Mean
0	0.03	0.03	0.03	20.68	20.68	20.68
1	2.87	2.85	2.86	16.00	17.05	16.53
2	6.65	6.63	6.64	8.01	8.00	8.01
3	9.45	9.51	9.48	6.78	6.70	6.74
4	10	10	10	6.10	6.07	6.09
5	10	10	10	5.02	5.10	5.06
6	10	10	10	5.02	5.10	5.06
7	10	10	10	5.02	5.10	5.06
8	10	10	10	5.02	5.10	5.06

Fermentation Run 3

Time (days)	CO2 (%) 1	CO2 (%) 2	Mean	O2 (%) 1	O2 (%) 2	Mean
0	0.03	0.03	0.03	20.68	20.68	20.68
1	1.14	1.21	1.18	20.68	20.68	20.68
2	3	3	3	20.68	20.68	20.68
3	3	3	3	20.68	20.68	20.68
4	3	3	3	20.68	20.68	20.68
5	3	3	3	20.68	20.68	20.68

Fermentation Run 4

Time (days)	CO2 (%) 1	CO2 (%) 2	Mean	O2 (%) 1	O2 (%) 2	Mean
0	0.03	0.03	0.03	20.68	20.68	20.68
1	10	10	10	6.16	6.05	6.11
2	10	10	10	5.76	5.76	5.76
3	10	10	10	5.76	5.76	5.76
4	10	10	10	5.76	5.76	5.76
5	10	10	10	5.76	5.76	5.76

Total Organic Carbon Data:

Cashew Apple Juice-Carbon Input

	Replicates: Concentration Range: 1500-5000mg/L						
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**
CAJ	3408	3404	3405	3406	3410	3401	3406*

*Reading of 3% dilution

Fermentation Run 1-Carbon Output

With Yeasts (Insoluble TOC)

	Replicates: Concentration Range 1500-5000mg/L						
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**
Run 1	2828	2826	2826	2824	2823	2819	2824*

*Reading of 3% dilution

Without Yeasts (Soluble TOC)

	Replicates: Concentration Range 1500-5000mg/L						
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**
Run 1	2703	2703	2699	2705	2700	2709	2703*

*Reading of 3% dilution

Fermentation Run 2-Carbon Output

With Yeasts (Insoluble TOC)

	Replicates: Concentration Range 1500-5000mg/L						
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**
Run 2	3056	3053	3055	3060	3057	3059	3057*

*Reading of 3% dilution

Without Yeasts (Soluble TOC)

	Replicates: Concentration Range 1500-5000mg/L						
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**
Run 2	2809	2804	2801	2801	2803	2803	2804*

*Reading of 3% dilution

Fermentation Run 3-Carbon Output

With Yeasts (Insoluble TOC)

	Replicates: Concentration Range 25-1500mg/L						
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**
Run 3	1331	1329	1329	1333	1335	1330	1331*

*Reading of 3% dilution

Without Yeasts (Soluble TOC)

	Replicates: Concentration Range 25-1500mg/L						
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**
Run 3	1259	1260	1257	1257	1255	1256	1257*

*Reading of 3% dilution

Fermentation Run 4-Carbon Output

With Yeasts (Insoluble TOC)

	Replicates: Concentration Range 1500-5000mg/L						
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**
Run 4	2929	2925	2927	2924	2930	2928	2927*

*Reading of 3% dilution

Without Yeasts (Soluble TOC)

	Replicates: Concentration Range 1500-5000mg/L						
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**
Run 4	2669	2670	2668	2671	2671	2670	2670*

*Reading of 3% dilution

Distillation Data:

Fermentation Run 1

	Duplicate Fermentations			
Time	**1**	**2**	**Mean**	**±SD**
0	0	0	0	0
0.5	2	2	2	0
1	0.9	2	1.45	0.777817
2	1	0	0.5	0.707107

Fermentation Run 2

	Duplicate Fermentations			
Time	**1**	**2**	**Mean**	**±SD**
0	0	0	0	0
1	2	0.75	1.375	0.883883
2.5	1	2	1.5	0.707107
3.5	1	1	1	0
5	1	0	0.5	0.707107

Fermentation Run 4

Time	Duplicate Fermentations		Mean	±SD
	1	2		
0	0	0	0	0
1	2	3	2.5	0.707107
2	0.5	1	0.75	0.353553
2.5	0.75	1	0.875	0.176777
3	0.75	1	0.875	0.176777
4.5	0.75	0	0.375	0.53033
6	0.75	0	0.375	0.53033

Generator Data:

Neat Petrol (95 Unleaded SASOL)

Run 1: Triplicate Dipstick Readings

Time (mins)	Readings (cm)			Mean
	1	2	3	
0	7	7	7	7
15	6.50	6.60	6.90	6.67
30	6.00	6.00	5.60	5.87
45	5.30	5.00	5.50	5.27
60	4.50	4.50	4.50	4.50

Run 2: Triplicate Dipstick Readings

Time (mins)	Readings (cm)			Mean
	1	2	3	
0	7	7	7	7
15	6.49	6.50	6.50	6.50
30	5.55	5.80	6.00	5.78
45	5.00	5.00	5.00	5.00
60	4.10	4.00	4.00	4.03

Run 3: Triplicate Dipstick Readings

Time (mins)	Readings (cm)			Mean
	1	2	3	
0	7	7	7	7
15	6.90	6.60	6.53	6.68
30	6.10	6.50	6.53	6.38
45	5.30	5.00	5.00	5.10
60	4.00	4.00	4.00	4.00

Runs 1-3: Mean Dipstick Readings

Time (mins)	Mean Readings (cm)			Mean	±SD
	Run 1	Run 2	Run 3		
0	7	7	7	7	0
15	6.67	6.50	6.68	6.62	0.10
30	5.87	5.78	6.38	6.01	0.32
45	5.27	5.00	5.10	5.12	0.14
60	4.50	4.03	4.00	4.18	0.28

E5 Blend

Run 1: Triplicate Dipstick Readings

	Readings (cm)			
Time (mins)	**1**	**2**	**3**	**Mean**
0	7	7	7	7
15	6.30	6.80	6.80	6.63
30	6.00	6.30	6.30	6.20
45	5.00	5.00	5.00	5.00
60	4.50	4.00	4.20	4.23

Run 2: Triplicate Dipstick Readings

	Readings (cm)			
Time (mins)	**1**	**2**	**3**	**Mean**
0	7	7	7	7
15	6.50	6.50	6.80	6.60
30	6.10	6.20	6.00	6.10
45	5.00	5.50	5.60	5.37
60	4.00	4.00	4.50	4.17

Run 3: Triplicate Dipstick Readings

	Readings (cm)			
Time (mins)	**1**	**2**	**3**	**Mean**
0	7	7	7	7
15	6.60	6.50	6.50	6.53
30	6.00	6.00	6.20	6.07
45	5.00	5.50	5.30	5.27
60	4.00	4.00	4.00	4.00

Runs 1-3: Mean Dipstick Readings

Time (mins)	Mean Readings (cm)			Mean	±SD
	Run 1	Run 2	Run 3		
0	7	7	7	7	0
15	6.63	6.60	6.53	6.59	0.05
30	6.20	6.10	6.07	6.12	0.06
45	5.00	5.37	5.27	5.21	0.20
60	4.23	4.17	4.00	4.13	0.12

E10 Blend

Run 1: Triplicate Dipstick Readings

Time (mins)	Readings (cm)			Mean
	1	2	3	
0	7	7	7	7
15	7	7	7	7
30	7	6.50	6.50	6.67
45	6.10	6.00	6.50	6.20
60	6.00	6.00	6.00	6.00

Run 2: Triplicate Dipstick Readings

Time (mins)	Readings (cm)			Mean
	1	2	3	
0	7	7	7	7
15	6.80	7	7	6.93
30	7	7	7	7
45	6.60	6.60	6.50	6.57
60	6.10	6.00	6.00	6.03

Run 3: Triplicate Dipstick Readings

Time (mins)	Readings (cm)			Mean
	1	2	3	
0	7	7	7	7
15	7	7	7	7
30	7	7	6.00	6.67
45	6.50	6.50	6.50	6.50
60	6.00	6.00	6.00	6.00

Runs 1-3: Mean Dipstick Readings

Time (mins)	Mean Readings (cm)			Mean	±SD
	Run 1	Run 2	Run 3		
0	7	7	7	7	0
15	7	6.93	7	7	0.04
30	6.67	7	6.67	6.78	0.19
45	6.20	6.57	6.50	6.42	0.20
60	6.00	6.03	6.00	6.01	0.02

E4.4 Blend

Run 1: Triplicate Dipstick Readings

Time (mins)	Readings (cm)			Mean
	1	2	3	
0	7	7	7	7
15	6.50	7	6.40	6.63
30	6.50	6.00	6.10	6.20
45	-	-	-	-
60	-	-	-	-

Run 2: Triplicate Dipstick Readings

Time (mins)	Readings (cm)			Mean
	1	2	3	
0	7	7	7	7
15	6.30	6.50	6.50	6.43
30	6.60	6.00	6.20	6.27
45	-	-	-	-
60	-	-	-	-

Run 3: Triplicate Dipstick Readings

Time (mins)	Readings (cm)			Mean
	1	2	3	
0	7	7	7	7
15	7	7	7	7
30	6.00	6.00	6.00	6.00
45	-	-	-	-
60	-	-	-	-

Runs 1-3: Mean Dipstick Readings

Time (mins)	Mean Readings (cm)			Mean	±SD
	Run 1	Run 2	Run 3		
0	7	7	7	7	0
15	6.63	6.43	7	6.69	0.29
30	6.20	6.27	6.00	6.16	0.14
45	-	-	-	-	-
60	-	-	-	-	-

Statistical Analysis STATA Output Data:

The Statistics/Data Analysis was done using STATA 21.1 (StataCorp LP, College Station, Texas 77845 USA).

Table 1: Fermentation Run 1

Values	Summary of Ferm1 Mean	Std. Dev.	Freq.
Ethanol	43.199222	23.904408	15
Fructose	30.132111	21.217447	15
Glucose	8.3062222	13.048642	15
Glycerol	1.5652222	1.563831	15
Maltose	2.1800001	0	15
Total	17.076556	22.542578	75

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	20884.0322	4	5221.00805	21.86	0.0000
Within groups	16720.3875	70	238.862679		
Total	37604.4197	74	508.167834		

```
Bartlett's test for equal variances:  chi2(3) =  58.5514  Prob>chi2 = 0.000

note: Bartlett's test performed on cells with positive variance:
      1 multiple-observation cells not used
```

Comparison of Ferm1 by Values (Bonferroni)

Row Mean-Col Mean	Ethanol	Fructose	Glucose	Glycerol
Fructose	-13.0671 0.235			
Glucose	-34.893 0.000	-21.8259 0.002		
Glycerol	-41.634 0.000	-28.5669 0.000	-6.741 1.000	
Maltose	-41.0192 0.000	-27.9521 0.000	-6.12622 1.000	.614778 1.000

```
. cii 75 17.08 22.54, level(95)
```

Variable	Obs	Mean	Std. Err.	[95% Conf.	Interval]
	75	17.08	2.602695	11.89402	22.26598

Table 2: Fermentation Run 2

```
. oneway Ferm2 Values, tab bonf
```

Values	Summary of Ferm2 Mean	Std. Dev.	Freq.
Ethanol	47.220371	24.803573	9
Fructose	21.437037	20.209336	9
Glucose	8.4661112	14.306065	9
Glycerol	.74111111	.88166233	9
Maltose	2.1800001	0	9
Total	16.008926	22.960601	45

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	13363.7227	4	3340.93067	13.59	0.0000
Within groups	9832.60257	40	245.815064		
Total	23196.3253	44	527.18921		

```
Bartlett's test for equal variances:  chi2(3) =  41.1158  Prob>chi2 = 0.000

note: Bartlett's test performed on cells with positive variance:
      1 multiple-observation cells not used
```

Comparison of Ferm2 by Values (Bonferroni)

Row Mean-Col Mean	Ethanol	Fructose	Glucose	Glycerol
Fructose	-25.7833 0.012			
Glucose	-38.7543 0.000	-12.9709 0.869		
Glycerol	-46.4793 0.000	-20.6959 0.078	-7.725 1.000	
Maltose	-45.0404 0.000	-19.257 0.128	-6.28611 1.000	1.43889 1.000

Variable	Obs	Mean	Std. Err.	[95% Conf.	Interval]
	45	16	3.422675	9.102052	22.89795

Table 3: Fermentation Run 3

```
. oneway Ferm3 Values, tab bonf
```

Values	Summary of Ferm3 Mean	Std. Dev.	Freq.
Ethanol	25.123611	12.554552	6
Fructose	17.543889	24.776863	6
Glucose	7.5888886	16.267962	6
Glycerol	3.1269445	1.5831875	6
Maltose	1.575	.30881138	6
Total	10.991667	16.212266	30

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	2428.4991	4	607.124775	2.92	0.0412
Within groups	5193.79071	25	207.751628		
Total	7622.28981	29	262.83758		

Bartlett's test for equal variances: chi2(4) = 51.1783 Prob>chi2 = 0.000

Comparison of Ferm3 by Values
(Bonferroni)

Row Mean- Col Mean	Ethanol	Fructose	Glucose	Glycerol
Fructose	-7.57972 1.000			
Glucose	-17.5347 0.453	-9.955 1.000		
Glycerol	-21.9967 0.140	-14.4169 0.955	-4.46194 1.000	
Maltose	-23.5486 0.091	-15.9689 0.665	-6.01389 1.000	-1.55194 1.000

Variable	Obs	Mean	Std. Err.	[95% Conf.	Interval]
	30	10.99	2.959528	4.937087	17.04291

Table 4: Fermentation Run 4

values	Summary of Ferm4 Mean	Std. Dev.	Freq.
Ethanol	55.706389	27.349667	6
Fructose	11.837778	22.295289	6
Glucose	6.7574997	16.552426	6
Glycerol	1.4102778	.69445696	6
Maltose	.72666669	1.1257472	6
Total	15.287722	26.484701	30

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	12737.6595	4	3184.41487	10.47	0.0000
Within groups	7604.08281	25	304.163312		
Total	20341.7423	29	701.439389		

Bartlett's test for equal variances: chi2(4) = 49.2637 Prob>chi2 = 0.000

Comparison of Ferm4 by values
(Bonferroni)

Row Mean- Col Mean	Ethanol	Fructose	Glucose	Glycerol
Fructose	-43.8686 0.002			
Glucose	-48.9489 0.001	-5.08028 1.000		
Glycerol	-54.2961 0.000	-10.4275 1.000	-5.34722 1.000	
Maltose	-54.9797 0.000	-11.1111 1.000	-6.03083 1.000	-.683611 1.000

Variable	Obs	Mean	Std. Err.	[95% Conf.	Interval]
	30	15.29	4.834564	5.402205	25.17779

Table 5: TOC1 (Fermentation Run 1)

```
. oneway TOC1 values, tab bonf
```

values	Summary of TOC1 Mean	Std. Dev.	Freq.
Chasew	3405.5	3.2710854	6
NoYeast	2703.1667	3.6009258	6
Yeast	2824.3333	3.1411251	6
Total	2977.6667	315.44404	18

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	1691416.33	2	845708.167	75659.78	0.0000
Within groups	167.666667	15	11.1777778		
Total	1691584	17	99504.9412		

Bartlett's test for equal variances: chi2(2) = 0.0918 Prob>chi2 = 0.955

Comparison of TOC1 by values
(Bonferroni)

Row Mean- Col Mean	Chasew	NoYeast
NoYeast	-702.333 0.000	
Yeast	-581.167 0.000	121.167 0.000

Variable	Obs	Mean	Std. Err.	[95% Conf.	Interval]
	18	2977.67	74.34992	2820.805	3134.535

Table 6: TOC 2 (Fermentation Run 2)

```
. oneway TOC2 var2 , tab bonf
```

var2	Summary of TOC2 Mean	Std. Dev.	Freq.
Chasew	3405.5	3.2710854	6
NoYeast	2804.3333	3.4448028	6
Yeast	3056.6667	2.5819889	6
Total	3088.8333	253.63968	18

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	1093516.33	2	546758.167	56109.73	0.0000
Within groups	146.166667	15	9.74444444		
Total	1093662.5	17	64333.0882		

Bartlett's test for equal variances: chi2(2) = 0.4087 Prob>chi2 = 0.815

Comparison of TOC2 by var2
(Bonferroni)

Row Mean-Col Mean	Chasew	NoYeast
NoYeast	-601.167 0.000	
Yeast	-348.833 0.000	252.333 0.000

Variable	Obs	Mean	Std. Err.	[95% Conf.	Interval]
	18	3088.83	59.78352	2962.698	3214.962

Table 7: TOC 3 (Fermentation Run 3)

```
. oneway TOC3 var2 , tab bonf
```

var2	Summary of TOC3 Mean	Std. Dev.	Freq.
Chasew	3405.5	3.2710854	6
NoYeast	1257.3333	1.8618987	6
Yeast	1331.1667	2.4013885	6
Total	1998	1024.5791	18

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	17845860.3	2	8922930.17	1.3e+06	0.0000
Within groups	99.6666667	15	6.64444444		
Total	17845960	17	1049762.35		

Bartlett's test for equal variances: chi2(2) = 1.4502 Prob>chi2 = 0.484

Comparison of TOC3 by var2
(Bonferroni)

Row Mean-Col Mean	Chasew	NoYeast
NoYeast	-2148.17 0.000	
Yeast	-2074.33 0.000	73.8333 0.000

Variable	Obs	Mean	Std. Err.	[95% Conf.	Interval]
	18	1998	241.4958	1488.488	2507.512

Table 8: TOC 4 (Fermentation Run 4)

```
. oneway TOC4 var2 , tab bonf
```

var2	Summary of TOC4 Mean	Std. Dev.	Freq.
Chasew	3405.5	3.2710854	6
NoYeast	2669.8333	1.1690452	6
Yeast	2927.1667	2.3166067	6
Total	3000.8333	313.66379	18

Analysis of Variance

Source	SS	df	MS	F	Prob > F
Between groups	1672457.33	2	836228.667	1.4e+05	0.0000
Within groups	87.1666667	15	5.81111111		
Total	1672544.5	17	98384.9706		

Bartlett's test for equal variances: chi2(2) = 4.2084 Prob>chi2 = 0.122

Comparison of TOC4 by var2
(Bonferroni)

Row Mean-Col Mean	Chasew	NoYeast
NoYeast	-735.667 0.000	
Yeast	-478.333 0.000	257.333 0.000

Variable	Obs	Mean	Std. Err.	[95% Conf.	Interval]
	18	3000.83	73.93037	2844.851	3156.809

Inferential Statistics

Title	P Value	Comment	95% CI	
Fermentation Run 1	<0.0001	Significant	11.89402	22.26598
Fermentation Run 2	<0.0001	Significant	9.102052	22.89795
Fermentation Run 3	0.0412	Significant	4.937087	17.04291
Fermentation Run 4	<0.0001	Significant	5.402205	25.17779
TOC1	<0.0001	Significant	2820.805	3134.535
TOC2	<0.0001	Significant	2962.698	3214.962
TOC3	<0.0001	Significant	1488.488	2507.512
TOC4	<0.0001	Significant	2844.851	3156.809

APPENDIX D: IDENTIFICATION OF *SACCHAROMYCES CEREVISIAE* STRAIN NRRL Y2084

Information provided on the identification of strain NRRL Y2084 was extracted from Deenanath, E.D. (2009). Enzymatic Hydrolysis of Bitter Sorghum for Bioethanol Production. Masters Dissertation. 113pages.

Isolation

One packet of dry brewers yeast was added to 200mL of YPD liquid broth, pure colonies were obtained by two successive streak plates onto MEA plates.

Identification

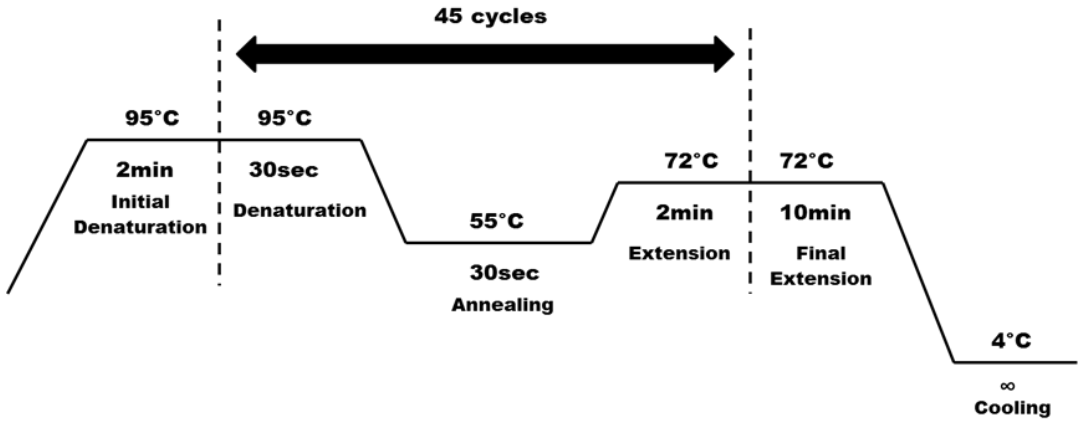
Polymerase Chain Reaction (PCR) and Automated Sequencing were carried out for the identification. These procedures were performed by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. For PCR, ITS 1 and ITS 4 primers were used and for sequencing, ITS 4 primer was used.

The primer sequences were as follows:

ITS 1 (5`-TCCGTAGGTGAACCTGCGG-3`)

ITS 4 (5`-TCCTCCGCTTATTGATATGC-3`)

The PCR cycles were programmed as shown as below:



For Automated Sequencing, the sequence output file was edited using Finch TV v1.4 software programme. The edited or "query" sequence was submitted for analysis using the Basic Local Alignment Search Tool (BLAST) software programme. Similarity of ≥98% to the "query" sequence allowed identification of the isolate to this specific strain.

APPENDIX E:
DOCUMENTS

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2891		SJ	OUR VAN	3192150		J08985	29/11/2010	1	509815

PROD. CODE PROD. KODE	CATALOGUE NO. KATALOGUS NR.	DESCRIPTION BESKRYWING	QTY. ORDER HOEV. BESTEL	QTY. SUPPLIED HOEV. VERSK.	TO FOLLOW TE VOLG	UNIT EENH.	PRICE PRYS	% DISC. AFSL.	NETT AMOUNT NETTO BEDRAG
RBP1M02LOTRDG2		BIOSTAT B-PLUS-MO 2 LITER SER/NO 22000749	1	1	0	EA	330000.00		500325000.00

Received 1/12/2010
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VAT BTW	14.00	45500.00
TOTAL TOTAAL		370500.00

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Coastal Cashews

Legal name: Dotcom Trading 25
VAT # 4560208888
P.O Box 330, KwaNgwanase, 3973 KwaZulu-Natal
Mobile Telephones: 079-281-5255, 071-228-0000
Email: coastal.cashews@gmail.com

Banking Details
FNB
Manguzi
Branch code # 260151
Acc no# 62150165089

Tax invoice for Evanie Deenanath
Wits University , Johannesburg

Invoice Date: 2/14/2011

Dear Sir / Madam

The customer's signature below confirms delivery of the goods

Signature: _____

Pease find below the billing details for your recent order of cashew apples
Thank you very much for your business.

Best,
Ms. Thully Thwala
Coastal Cashews

Description of goods and services provided

	Kilograms	Type fruits	Price per kg	Net (ZAR incl)
	33	Cashew apples	33.00	990.00
Labour cost			630.00	630.00
Management cost			250.00	250.00
Total			1870.00	1870.00
VAT 14%			261.00	261.00
Total Amount				2131.00

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Journal of Biomedicine and Biotechnology
Volume 2012 (2012), Article ID 416491, 11 pages
doi:10.1155/2012/416491

Review Article

The Bioethanol Industry in Sub-Saharan Africa: History, Challenges, and Prospects

Evanie Devi Deenanath,[1] Sunny Iyuke,[1] and Karl Rumbold[2]

[1]School of Chemical and Metallurgical Engineering, University of the Witwatersrand, 1 Jan Smuts Avenue, Braamfontein, Johannesburg 2000, South Africa
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Volume 2013 (2013), Article ID 107851, 11 pages
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Research Article

The Production of Bioethanol from Cashew Apple Juice by Batch Fermentation Using Saccharomyces cerevisiae Y2084 and Vin13

Evanie Devi Deenanath,[1] Karl Rumbold,[2] and Sunny Iyuke[1]

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in the 3rd International Conference on Biotechnology Engineering (ICBioE 2013), in Conjunction with IIUM Engineering Congress 2013 held at

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This is to certify that

Evanie Deenanath

Attended the Nanoscience Characterization Techniques Course

Dr Daniel Adams
Chief Director: DST

Prof Emmanuel Iwuoha
University of the Western Cape

From Cashew Apples to Biofuel

Basic research on a new renewable

Biologist Evanie Deenanath wants to use the previously unused juice of cashew apples to produce bioethanol. She aims to transform a waste product into a source of sustainable mobility in Africa.

At the moment, it is still all happening in the lab: Evanie Deenanath ferments the juice of the cashew apple to produce bioethanol, which she then combines with conventional petrol to create ethanol fuel. It is the subject of her doctoral thesis at the University of the Witwatersrand in Johannesburg and – so she hopes – will also help to promote more sustainable mobility on the entire continent. "Biofuels are not very common in Africa yet. The industry is extremely conservative and still campaigns for traditional resources," explains Deenanath. "I want my research to draw attention to the enormous potential of cashew apples as a renewable resource."

Cashew fruit is cultivated in many parts of Africa to produce the ever-popular cashew nut. In South Africa the main areas of cultivation are on the Mozambique border. However, at present, producers hardly make any use of the cashew apple, the large, swollen fruit of the cashew tree, from which the cashew nut hangs. "They're used to make jam, wine and ice cream, but otherwise most of the fruit is thrown away," says Evanie Deenanath, who discovered the valuable waste product when she was doing a brewery project at university.

Just one large cashew plantation in South Africa produces some 380 tonnes of waste cashew apples every year. Evanie Deenanath discovered that the juice is excellent for producing bioethanol. "The complexity of the juice supported yeast growth during the fermentation process very well. I was able to produce a high concentration of ethanol," she says, summing up her results. "But you would have to concentrate production on the short period from January to March because this is when the fruit is ripe."

With an eye on food security

Bioethanol plants already existed in South Africa back in the 1990s, mostly processing maize and sugarcane for biofuels. But they were soon closed down, because the raw materials were needed for human food consumption. "Using cashew apples for biofuel is not a food security issue in Africa," the DAAD-NRF scholarship holder notes. She has just submitted her doctoral thesis and is hoping a South African company will recognise the potential of this easily-accessible resource; she wants to test bioethanol production on a larger scale and make it commercially viable. A research stay or postdoc position in Germany would also interest her. "Research on biofuels in Germany is much more advanced than it is here. It would be a great opportunity," says the biologist. Whatever happens, sooner or later she would like to return to university to pass on her knowledge to others and train the next generation of South African scientists working on sustainable biofuels.