Yeast encapsulation for bioethanol production

by

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Dissertation presented for the degree of

Master of Science (Biochemistry)

at

University of KwaZulu-Natal

School of Life Sciences

College of Agriculture, Engineering and Science

December 2014

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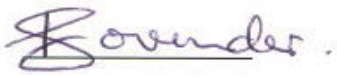
Signed:  Date: 28/01/2015

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SUMMARY

The bioethanol production industry is faced with hurdles such as uncovering cheap and abundantly available fermentation substrates, as well as yeast strains possessing high ethanol tolerance properties. Cane molasses is a substrate that sufficiently fits the aforementioned description and this has catapulted its use in bioethanol production. However, the downside to employing cane molasses as a fermentation substrate under laboratory conditions for comparative fermentation studies is nutrient composition variability in different batches. This has prompted the development of a standardised chemically defined molasses medium that facilitates the generation of more consistent and reliable fermentation data. In the first aspect of this study, a chemically defined molasses medium was formulated based on nutrient composition data of 10 different cane molasses batches as provided by the South African Sugar Research Institute (SASRI). The ability of laboratory and industrial *Saccharomyces cerevisiae* strains to ferment chemically defined molasses medium and industrially-derived cane molasses sourced from the Amatikulu, Felixton and Gledhow South African-based sugar mills was evaluated. The batch fermentation of the chemically defined molasses medium supplemented with yeast extract by BY4743 (laboratory strain), dry yeast (baker's yeast), Angel and cream yeast (distiller's yeast) were similar to those attained in batch fermentations of cane molasses in terms of fermentation kinetic profiles (sugar conversion, ethanol titer, yield and productivities). It was also observed irrespective of the fermentation substrate involved that cream yeast produced the highest ethanol output followed by angel yeast, dry yeast and then BY4743. These results seem to suggest that the chemically defined molasses medium containing yeast extract can be employed as a standardised laboratory medium.

Increased bioethanol yield is commercially attractive to relevant fermentation-based industries. In this regard, the immobilization of yeast by cell encapsulation has been touted as a tool which may increase the yeast's tolerance to higher ethanol levels. In this study, a strategy was developed in which the better performing Angel and cream yeast strains were immobilized in calcium alginate, alginate-chitosan, and low melting point agarose capsules. The fermentation efficiency in terms of ethanol production of encapsulated cells versus their free-suspended yeast cell counterparts was evaluated. The reusability of the capsules for more than one fermentation cycle was also investigated. The fermentation of Amatikulu and chemically defined molasses medium containing 10 g/L of yeast extract by Angel yeast encapsulated in low melting point agarose resulted in a 10% increase in bioethanol yields in comparison to their free-suspended Angel yeast counterparts. However, it was also observed that cream yeast fermentations with free-suspended and encapsulated cells generated

similar fermentation profiles and bioethanol yields. Only alginate-chitosan and low melting point agarose were used in the investigation of capsule reusability because of their superior stability over calcium alginate capsules. The low melting point agarose capsules remained stable and active for the three consecutive batch fermentations of Amatikulu cane molasses and synthetic CDM-YE molasses. The alginate-chitosan capsules remained active and stable for two cycles of fermentation and only showed signs of breakage during the third fermentation cycle. Fermentations with encapsulated Angel and cream yeast resulted in sustained ethanol outputs for the three fermentation cycles. The data seem to suggest that the cell encapsulation strategy may be beneficial to the bioethanol industry in that lower ethanol tolerant distillers yeast and other yet to be used strains which produce significantly less undesirable by-products such as acetic acid, ethyl aldehyde, n-propanol and methanol can be improved in terms of their bioethanol yield to meet the requirement of industry.

This thesis is dedicated to my family and the entire African youth trying to find their way through this maze which we call life

BIOGRAPHICAL SKETCH

Njabulo E. Nene was born on the 19th of May 1992 in Botha's Hill and relocated to Umlazi in the year 2000, a township on the outskirts of Durban. He matriculated in 2008 from Embizweni High School. In 2009, he enrolled for a BSc degree, majoring in Biochemistry and Microbiology at the University of KwaZulu-Natal. After attaining his Bsc degree, he pursued and attained a Bsc Honours degree in Biochemistry in 2010. Njabulo spends his spare time watching football, a proud supporter (most times) of Bafana Bafana, Orlando pirates FC, Arsenal FC and Real Madrid FC.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following people and institutions:

- All praises go to God, who gave me the strength, perseverance, courage and wisdom to see out this two year journey.
- My supervisor, Dr Patrick Govender for the guidance and faith that he showed in me, which in turn allowed me to grow as a budding researcher and person.
- My co-supervisors Dr Santosh Ramchuran and Dr Karen Pillay, for all the guidance and assistance
- Mr Ace Govender from NCP alcohol, for the invaluable support he offered that made this study possible
- My father, the greatest dad I could have ever possibly asked for. He has given me so much and I can only hope that this token makes him just as happy and proud.
- My mother, for being so supportive, understanding and patient with me.
- My older Sister Sithembile and younger brother Anele, all the jokes and sometimes fights that we share, always picked me up during tough times. I can only hope that this inspires them too.
- My lab colleagues, Ramesh, Spha, Khulekani, Lethu, Uraisha, Phili, Mel and Kamini for all the assistance and good times that we have shared.
- Dinesh, for the assistance and Monday football talks.
- Nkosi, Dj and Aminu, our talks about current events have really changed my outlook on life as a whole, for the better.
- And lastly, a very special lady in my life. She is the best.

PREFACE

This dissertation is presented as a compilation of five chapters.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**

Chapter 3 **Research Results 1**

Development of chemically defined molasses as a standardised laboratory yeast culture medium.

Chapter 4 **Research Results 2**

Encapsulation of industrial yeast for increased bioethanol yields.

Chapter 5 **General Discussion and Conclusion**

CONTENTS

ABBREVIATIONS

°Brix	Degrees Brix
°C	Degrees Celsius
B2	2% biodiesel: 98% diesel
BIS	Biofuels Industrial Strategy
C_2H_5OH	Ethanol
Ca^{2+}	Calcium cations
CFCs	Chlorofluorocarbons
CH_4	Methane
CHO	Carbohydrate
Cl	Chlorine
cm	Centimeter
CO	Carbon monoxide
CO_2	Carbon dioxide
Da	Dalton
E8	8% bioethanol: 92% petroleum
FAN	Free amino nitrogen
FFVs	Flex fuel vehicles
g/mol	Grams per mole
g/L	Grams per liter
GHGs	Greenhouse gasses
HPLC	High performance liquid chromatography
Hrs	Hours
IQ	Intellectual Quotient
km	Kilometer
λ	Lambda
M	Meter
mL	Milliliter
mm	Millimeter
MTBE	Methyl tertiary butyl ether
N_2	Nitrogen
NO_x	Nitrogen oxides
rpm	Revolutions per minute
SASRI	South African Sugar Research Institute
SO_2	Sulfur dioxide
S_f	Residual sugar
USA	United States of America
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
YEPD	Yeast extract peptone D-glucose
µm	Micrometer

LIST OF FIGURES

LIST OF TABLES

Chapter 1

INTRODUCTION AND STUDY AIMS

1.1 INTRODUCTION

The interest in bioethanol as an alternative fuel to currently used non-renewable energy resources has increased remarkably over the past few decades. The production of bioethanol from cheap sugar-rich biomass has proven to be the most attractive. In South Africa, the by-product of sugarcane processing; cane molasses is the most employed fermentation substrate. Mainly because of its abundance and low cost. Industrial bioethanol production utilises four main types of fermentation strategies, namely; batch, fed-batch, continuous, and semi-continuous operations. The most industrially employed fermentation strategy is batch fermentation, because of its low investment costs, simple controls, easy handling and sterilization of the substrate and a relatively simple operational principle that does not require specialised labour (Çaylak, 1998). The downside of batch fermentations is the accumulation of ethanol to inhibitory levels as the fermentation progresses, subsequently curtailing yeast performance and ethanol yields (Mannazzu *et al.*, 2008).

High ethanol concentrations have been reported to affect the composition and functional properties of cell membranes, leading to reduced cell viabilities and performance (Mannazzu *et al.*, 2008). The immobilization of yeast cells into capsules has been reported to improve the yeast's tolerance to high ethanol levels and improve ethanol yields. Cell encapsulation is defined as the physical isolation of the cell mass from the outside environment, while maintaining normal cell physiology within a barrier of desired permeability (Galazzo & Bailey, 1990; de Vos *et al.*, 2009; Westman *et al.*, 2012; Ylitervo *et al.*, 2013). Various theories have been put forward in an effort to elucidate the possible mechanisms behind encapsulated yeast putatively displaying increased ethanol yields than free-suspended cells. With some suggesting that the higher levels of saturated fatty acids in encapsulated cells compared to free cells confers upon them increased ethanol tolerance (Hilge-Rotmann & Rehm, 1991; Sun *et al.*, 2007). Others suggest that hydrogels tightly withhold water, leading to a highly structured water layer surrounding the yeast in the pores of the gel network (Israelachvili & Wennerstrijm, 1996). This water layer then prevents ethanol from dehydrating the encapsulated cells (Desimone *et al.*, 2002).

The possible use of capsules in batch fermentations could also be advantageous, due to the ease of separating the fermented substrate from the capsules, therefore negating the need for cell removal processes such as centrifugation which are very energy intensive. Meaning the process would consume less energy, a crucial factor considering the current energy crisis facing South Africa. The possible reusability of the capsules for consecutive fermentation cycles further makes this technology even more attractive when considering that most current bioethanol production industrial processes entail the purchasing of fresh inoculum for every single

fermentation cycle, one can only imagine the substantial economic benefits that would be associated with the successful industrial application of encapsulated cells.

1.2 AIMS OF THE STUDY

The main aims of the MSc study described within this dissertation are to:

1. Formulate a novel and chemically defined cane molasses medium and evaluate the ability of laboratory and industrial *Saccharomyces cerevisiae* strains to ferment chemically defined cane molasses medium *versus* industrially-derived cane molasses.
2. Encapsulate commercially available industrial *S. cerevisiae* strains employed in the South African bioethanol industry in hydrogels, and use the capsules as inoculum for cane molasses fermentations in an effort to increase their ethanol yields. The reusability of the capsules for more than one fermentation cycle will also be investigated.

This dissertation comprises of five chapters, the first is this introductory **Chapter 1**.

In **Chapter 2**, a comprehensive literature review encompassing the bioethanol production industry, cane molasses as a fermentation substrate, *S. cerevisiae* as the model fermentation organism and various cell immobilization strategies is presented.

In **Chapter 3**, a chemically defined molasses medium was formulated and supplemented with three different nitrogen sources. Chemically defined molasses and cane molasses sourced from three South African-based sugar mills (*i.e.* Amatikulu, Felixton and Gledhow) were subjected to batch fermentations with *S. cerevisiae* from a wide genomic background; BY4743 (lab strain), dry yeast (baker's yeast), Angel and cream yeast (distiller's yeast). This was conducted in order to evaluate the validity of the chemically defined cane molasses medium as a standardised yeast culture medium by comparing the fermentation ability of the yeast strains in the chemically defined molasses *versus* cane molasses.

In **Chapter 4**, only the two best performing industrial strains (Angel and cream yeast) in terms of fermentation ability were employed for this aspect of the study. Angel and cream yeast were encapsulated into calcium alginate, alginate-chitosan and low melting point agarose. The fermentation ability of the encapsulated yeast was measured against their free-suspended counterparts in the fermentation of chemically defined cane containing yeast extract and Amatikulu cane molasses. In addition, the reusability of the capsules for more than one fermentation cycle was investigated by reusing the yeast for three consecutive fermentation cycles.

Finally, **Chapter 5** comprises of a general discussion and conclusion as well as possible ideas for future research.

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Chapter 2

LITERATURE REVIEW

2. INTRODUCTION

The current world population is estimated to be well over 7 billion, a staggering figure when considering that for life to continue existing and grow, the security of a constant and sustainable energy supply is mandatory. In a South African context, the extent of our dependence on non-renewable energy (e.g. petroleum and coal) is typified by hardships encountered during energy shortages like load-shedding and mass actions by the transportation industry which leads to hindered fuel transportation across the country and stranded motorists. In the case of blackouts, all the added benefits of having electricity vanish along with the light and crucial public services like healthcare are compromised, and unfortunately in some instances leading to casualties. The aforementioned examples are all short term, but what would really happen if fuel (current fuel resources like oil, natural gas and coal) were to completely run out? Such questions have raised much speculation over the future of the human race, rightly so because a constant and reliable energy source is imperative for human existence (Dincer, 2000; Greene *et al.*, 2006; Rajapakse, 2007; Shafiee & Topal, 2009).

We are currently faced with a situation whereby the global oil supply is compromised due to diminished quantities of conventional oil in reserves (Laherrere, 2001; Greene *et al.*, 2006). This has given rise to much speculation and an on-going debate about when conventional oil will be depleted. Major oil-producing companies suggest that the current oil reserves should last for decades to come but these claims are refuted by pessimists who suggest that the event is imminent, and should happen within the next thirty to fifty years (Laherrere, 2001; Greene *et al.*, 2006; Rajapakse, 2007; Sthel *et al.*, 2013). More importantly, the sustainability of using conventional oil is a very sensitive and intricate issue, with serious possible repercussions for all parties involved (Owen *et al.*, 2010).

The on-going use of fossil fuel-derived energy has resulted in well reported negative environmental impacts (Dincer, 1999). This has prompted a global shift towards ‘Green Technologies’, that are able to produce alternative fuels (biofuels) from renewable sources and with no adverse effects on the environment (Thomas & Kwong, 2002; Hill *et al.*, 2006). Alternative fuels which possess superior environmental benefits over fossil fuels, an all-round viable substitution in terms of being economically competitive and producible in sufficient quantities in order to make a meaningful impact on energy demands, but more importantly; provide a net energy gain over the energy sources used to produce it (Hill *et al.*, 2006). One such alternative biofuel is bioethanol (commonly known as ethanol, ethyl

alcohol) which can be produced from energy crops, by-products of the sugar industry, lignocellulosic waste, etc. (Hahn-Hägerdal *et al.*, 2006). Bioethanol has already been introduced on a large scale in countries such as: Brazil, the United States of America (USA), India, and some European and African countries. These aforementioned countries use bioethanol as vehicle fuel and or as a fuel additive. The introduction of bioethanol in liquid fuels has driven the mitigation of greenhouse gas emissions and the eradication of harmful fuel additives like tetraethyl lead (Thomas & Kwong, 2002; Hansen *et al.*, 2005; Fargione *et al.*, 2008). However, bioethanol is yet to be introduced in liquid fuels in South Africa, and the continued high dependence of the South African economy on non-renewable energy resources (particularly coal and petroleum). This has resulted in the South African government prioritizing the in-house production of alternative green fuels, particularly bioethanol (BIS, 2007).

The bioethanol production industry faces fundamental bottlenecks like finding cheap and suitable (with easily assimilable sugars) fermentation substrates, and being able to produce economically viable bioethanol yields. The majority of current bioethanol production strategies use *Saccharomyces cerevisiae* as the fermenting yeast, which coincides with challenges such as restricted bioethanol yields. This is due to the toxic effects bioethanol exhibits upon the fermenting yeast as it accumulates in the fermentation substrate, leading to substantially diminished yeast fermentation performance and bioethanol yields (Lin & Tanaka, 2006; Sun *et al.*, 2007; Ylitervo *et al.*, 2011). This scenario beckons for scientific interventions that are able to improve bioethanol production processes for increased bioethanol outputs. One such innovative intervention is the immobilization of the yeast into semipermeable capsules, which has the potential to increase the yeast's tolerance to high bioethanol concentrations, thereby potentially increasing bioethanol yields. This review seeks to highlight the use of bioethanol as an alternative green fuel, cane molasses as a suitable substrate for bioethanol production and cell encapsulation as a possible means of increasing ethanol throughputs and different (past and present) cell encapsulation technologies reported to date.

2.1 NEGATIVE IMPACTS OF NON-RENEWABLE FUELS ON THE ENVIRONMENT

Fossil fuels have been the primary source of energy for the human population for centuries and their prolonged use has resulted in detrimental environmental effects. It is well documented that the use of fossil fuels is largely responsible for global climate change, stratospheric ozone depletion and acid rain (Dincer, 1999; Hill *et al.*, 2006).

2.1.1 Global climate change

The change in climate attributed to human activities such as the combustion of fossil fuels generating greenhouse gasses (GHGs) is defined as climate change (Dincer, 1999; Samimi & Zarinabadi, 2012). The change and increase of the atmospheric concentration of GHGs inevitably results in the alteration of the atmospheric energy balance, i.e. the greenhouse effect (Figure 2.1).

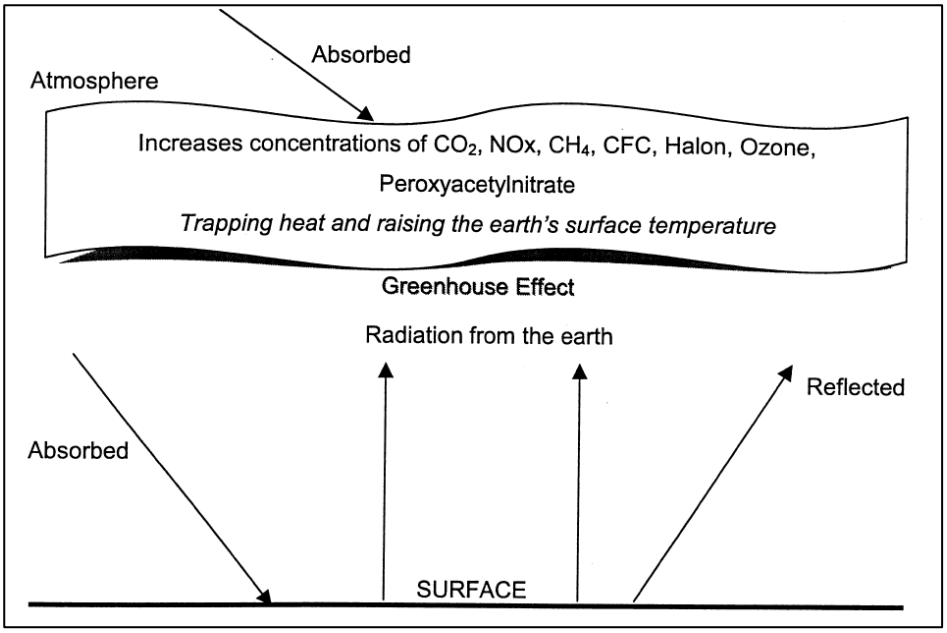

Figure 2.1 The greenhouse effect (Dincer, 1999)

The greenhouse effect is defined as an increase of the earth's temperature as a result of increased adsorption of the sun's radiation by GHGs. The GHGs include: carbon dioxide (CO_2, which is reported to contribute about 50% to the anthropogenic greenhouse effect), methane (CH_4), chlorofluorocarbons (CFCs), halons, nitrous oxide (N_2O), ozone (O_3) and peroxyacetylnitrate (Dincer, 1999; Dincer, 2000). The GHGs have been reported to have different warming capacities; CH_4 has 25 times the global warming potential of CO_2 and hydrofluorocarbons have more than 1 000 times the warming potential of CO_2. Over the last century, the earth's surface temperature has reportedly increased by 0.6°C, coupled to a 20 cm rise of the sea level (Dincer, 1999).

2.1.2 Stratospheric ozone depletion

Ozone (O_3) located in the stratosphere between altitudes of 12 and 25 km, plays a key role in naturally maintaining the equilibrium of the earth by adsorbing both ultraviolet (UV) (240±320 nm) and infrared radiation (Dincer, 1999). The emission of CFCs, halons and NO_x

(NO_2 or NO_3) has been demonstrated to cause the global distortion and regional depletion of the stratospheric ozone layer (Figure. 2.2). The depletion of ozone has led to increased levels of damaging UV radiation reaching the ground. This compromised protection of the earth's inhabitants from the deleterious effects of UV radiation has caused increased rates of skin cancer, eye damage and harm to many biological species (Rowland, 1991; Dincer, 2000).

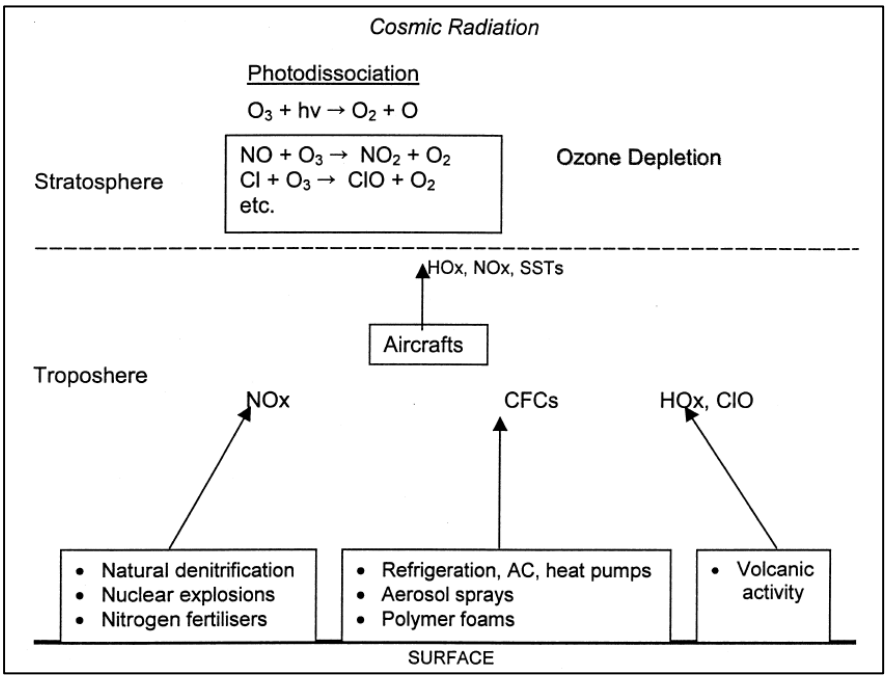

Figure 2.2 Schematic representation of stratospheric ozone depletion (Dincer, 2000)

The major contributors to ozone depletion in the stratosphere are CFCs (which are used in air conditioning and refrigeration equipment), and NO_x emissions produced by fossil fuel and biomass combustion processes, natural denitrification, nitrogen fertilizers and aircrafts (Dincer, 2000).

2.1.3 Acid rain

The combustion of fossil fuels, particularly from both stationary and mobile sources (e.g. smelters for nonferrous ores, industrial boilers, and transportation vehicles) generates pollutant gases that are transported great distances through the atmosphere and are deposited back on earth *via* precipitation, causing significant damage from excessive acidity to vulnerable ecosystems. Acid rain (Figure 2.3) has been reported to be the result of the emission of sulphur dioxide (SO_2) and NO_x, which react with water and oxygen in the atmosphere, resulting in the formation of sulfuric and nitric acids (Dincer, 1999).

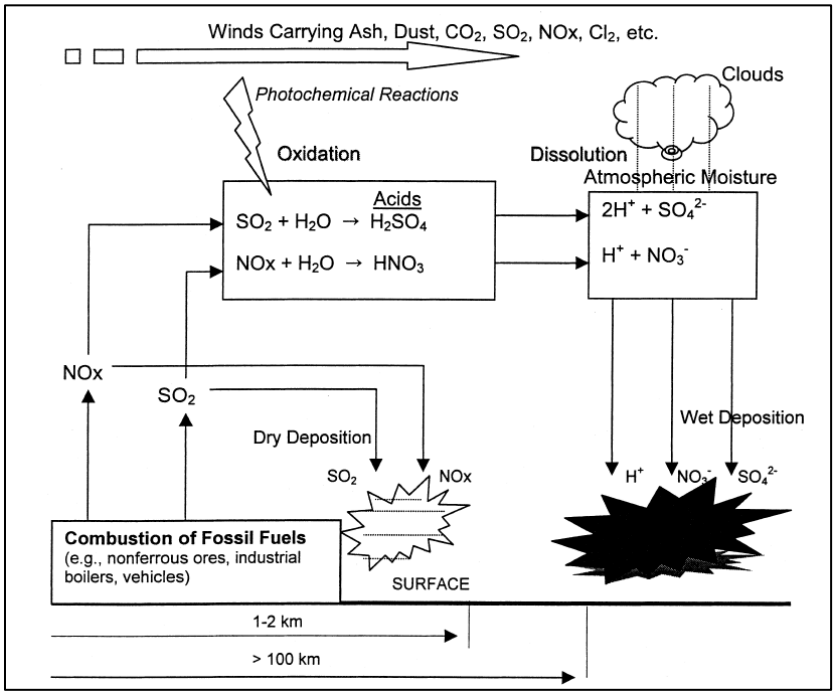

Figure 2.3 Schematic representation of the formation and distribution of acid rain (Dincer, 2000)

The energy generation sector is a major source of acid precipitation, with coal alone accounting for about 70% of SO_2 emissions. Road transport and fossil fuel combustion in stationery places are sources of the remaining NOx emissions (Dincer, 2000).

2.2 THE BIOFUELS INDUSTRIAL STRATEGY OF THE REPUBLIC OF SOUTH AFRICA

The Biofuels Industrial Strategy (BIS) aims to grow the South African biofuel market by creating a link between the first and second economies. This will be achieved by making use of underutilised arable land in South Africa, particularly in former homelands. Consequently creating jobs and an agricultural market in underdeveloped areas where agriculture was previously undermined by apartheid (BIS, 2007).

2.2.1 Underutilised land

In South Africa, only 14% of the total available land is arable and about 10% of this land is irrigated, consuming 60% of the national water supply. Conversely, there are 3 million hectares of underutilised, high potential land mainly in the former homelands. The utilization of 1 million ha of such land could produce biofuels equating to approximately 5% of national diesel expenditure. The strategy proposed a 2% (400 million litres per annum) initial biofuel penetration by 2013 that will have a producer support scheme requiring the use of local crops grown mainly on currently underutilised land and support from the commercial land. This will contribute 30% to the national renewable energy target for 2013 (BIS, 2007).

2.2.2 Feedstock for BIS biofuel production

Various plants have been initially suggested as possible feedstock for bioethanol production and these include: maize, sorghum, sugar cane and sugar beet. Because maize is a staple food in developing countries, concerns over the biofuels industry adversely affecting the price and availability of maize has led to its total exclusion as a potential feedstock, therefore leaving only sorghum, sugarcane and sugar beet as viable candidates. The total exclusion of maize from preliminary bioethanol production plans makes sense as the price of maize grown for human consumption and that grown for bioethanol production would have to be sold at similar prices. Any discrepancies could lead to “false procuring” of maize, in a sense that one could buy cheaper maize meant for human consumption and illegally channel it toward bioethanol production. Also, a majority of maize growers would opt to sell their produce at the highest available price for maximum profit, and inevitably significantly increasing maize prices. Therefore totally going against what the Biofuels Industrial Strategy set out to do, which is to ensure sustainable development and eradicate poverty (BIS, 2007).

2.2.3 Progress to date in implementing BIS

The initially suggested 4.5% fuels penetration levels has since been revised to 2% due to challenges encountered whilst developing the biofuels industry (Esterhuizen, 2009; Gazette, 2014). The BIS envisaged a five-year pilot phase, spanning from 2008-2013, during which the 2% penetration level of biofuels in the national liquid fuels (petrol and diesel) pool needed to be achieved. The suggested blending ration was B2 (2% biodiesel) and E8 (8% bioethanol), with sugar cane and sugar beet recommended feedstock for bioethanol production, as well as soybeans, canola, and sunflower recommended for biodiesel (Esterhuizen, 2009; Gazette, 2014).

To date, not a single large scale biofuels industry player has emerged, a snag that is a result of the unattractive nature of the prevailing feedstock and crude oil or liquid fuels prices. This has called on the government to take more drastic measures by introducing Mandatory Blending Regulations which seek to guarantee the total uptake of all biofuels supplied by licensed biofuels manufactures. This will be achieved by compelling licensed petroleum manufacturers and their wholesaling arms to buy and blend all the biofuels produced locally by licensed biofuels manufacturers (Gazette, 2014).

2.3 BIOETHANOL AS AN ALTERNATIVE FUEL

2.3.1 The basic chemistry ethanol

Ethanol is a colourless liquid under atmospheric conditions, largely found in alcoholic beverages and is denoted by the chemical formula C_2H_5OH. The ethanol molecule has a molecular weight of 46 g/mol and is small and tight when compared to most petrol (gasoline) components. In terms of electrochemistry, ethanol has both polar and nonpolar ends. Polarity refers to the distribution of electric load in a molecule and is a significant determinant of the physical and chemical properties a molecule possesses. The hydroxyl group in ethanol allows it to participate in relatively weak hydrogen bonding with other ethanol molecules or other polar substances. Although the H-bonds are weak, they are strong enough to make ethanol more viscous and less volatile than other similar but less polar substances. The polar end makes it possible for ethanol to be miscible with water; likewise, the nonpolar end makes ethanol miscible with nonpolar organic substances such as gasoline and, to a lesser degree, diesel fuel (Larsen *et al.*, 2009).

Ethanol often possesses small amounts of water due to its hygroscopic nature which allows it to easily absorb water. The method of production, improper storage, and accidental contamination can also lead to the introduction of water in ethanol. Fuel ethanol is produced in either of two purities: anhydrous and hydrous. Anhydrous ethanol (also known as pure, dry, or absolute alcohol) has a water content of less than 1%, whereas hydrous ethanol has a water content between 5 and 10%. The production of ethanol with purities above 95.6% (designated the azeotrope concentration) cannot be achieved by traditional distillation methods, but rather require separate costly dehydration equipment, a step that makes anhydrous ethanol approximately 20-25% more energy-demanding to produce than the ethanol/water azeotrope (Bernstein *et al.*, 2007; Larsen *et al.*, 2009).

Fuel ethanol is made undrinkable to avoid heavy taxation levied on spirits made for human consumption, by adding a foul-tasting or toxic substance (normally less than 10%) to the ethanol after distillation, and it is then called denatured alcohol. Examples of denaturants normally used are methanol, propanol, or acetone (Larsen *et al.*, 2009).

2.3.2 Ethanol as a petrol/diesel additive

A variety of ethanol-petrol and to a lesser extent, ethanol-diesel blends exist globally, and these are used as motor fuel. The amount of water present in the blends is dependent on factors such as: local legislation, vehicles, weather, consumer habits, and other conditions governing the local market in a given geographical location (Larsen *et al.*, 2009). Currently, a significant proportion of the world fuel ethanol market employs ethanol as a petrol additive, where ethanol constitutes 5-10% of the overall fuel mass in the blend and the rest is made up by petrol (Thomas & Kwong, 2002; Hansen *et al.*, 2005; Larsen *et al.*, 2009). The two major reasons for using ethanol as an additive are to:

i. Raise the octane number of the fuel blend, and replace the use of costly and harmful octane boosting components.
ii. The presence of oxygen in ethanol aids in the clean burning of ethanol-containing petrol, thus reducing the emissions of carbon monoxide (CO), particulates, and unburned petrol components.

Anhydrous ethanol is normally used as an additive in order to prevent the phase separation of water and petrol in the blend. Gasohol and E100 are the two major ethanol blends used in Brazil. Gasohol contains approximately 20% pure ethanol in petrol. The ethanol blend E100 is made up of hydrous ethanol without gasoline, and with a 7% (v/v) water content. The production of hydrous ethanol which constitutes a great fraction of E100 is relatively cheap when compared to anhydrous ethanol, a factor that makes E100 a cheaper alternative to gasohol. However, gasohol possesses a better cold starting capability, and a greater energy content per litre (De Oliveira *et al.*, 2005; Larsen *et al.*, 2009).

The low temperatures experienced in regions like the USA and Sweden, have prompted the widespread use of E85 in flex fuel vehicles (FFVs). E85 is an ethanol blend constituted by 71-85% anhydrous ethanol, with petrol making up the rest of the blend. The anhydrous ethanol in E85 prevents phase separation; the presence of water in the blend would otherwise lead to phase separation at low temperatures. A diesehol blend made up of diesel fuel and ethanol has also been produced for diesel engines. An example of a patented blend is E-diesel, which consists of 15% (v/v) anhydrous ethanol, additives (sometimes) and 85% (v/v) diesel fuel. Another blend available in the market is O_2-diesel, which is made up of 7.7% (v/v) anhydrous ethanol in diesel fuel. O_2-diesel has been successfully used in more than 5,000 buses in the Indian state of Karnataka (De Oliveira *et al.*, 2005; Hansen *et al.*, 2005; Larsen *et al.*, 2009).

2.3.3 Ethanol in the phasing out of Lead

Tetraethyl lead has for years been employed by the petroleum industry to boost the octane properties of petrol. Octane **rating** refers to the measurement of the tendency of an air/fuel mixture to resist spontaneous combustion (pre-ignition or engine knocking) as a result of being heated during the compression stroke in the engine cylinder. The compression strokes of engine cylinders generate high temperatures and pressure, which causes fuel molecules to break down into free radicals. Tetraethyl lead acts as a scavenger of these free radicals by reacting with them before they can build up the chain reaction that causes pre-ignition (Thomas & Kwong, 2002). The use of lead has since declined and fully eradicated in developed countries (Figure 2.4) due to reports from extensive research that lead induces adverse neurological effects (Fowler, 1993; Lanphear *et al.*, 2005). Schwartz (1994) and Lanphear *et al.* (2005) reported that increases in the levels of lead in the blood of school children led to a decreased Intellectual Quotient (IQ) and learning capacity. The phasing out of lead was reported to show a significant drop in the population's exposure to lead (Thomas & Kwong, 2002).

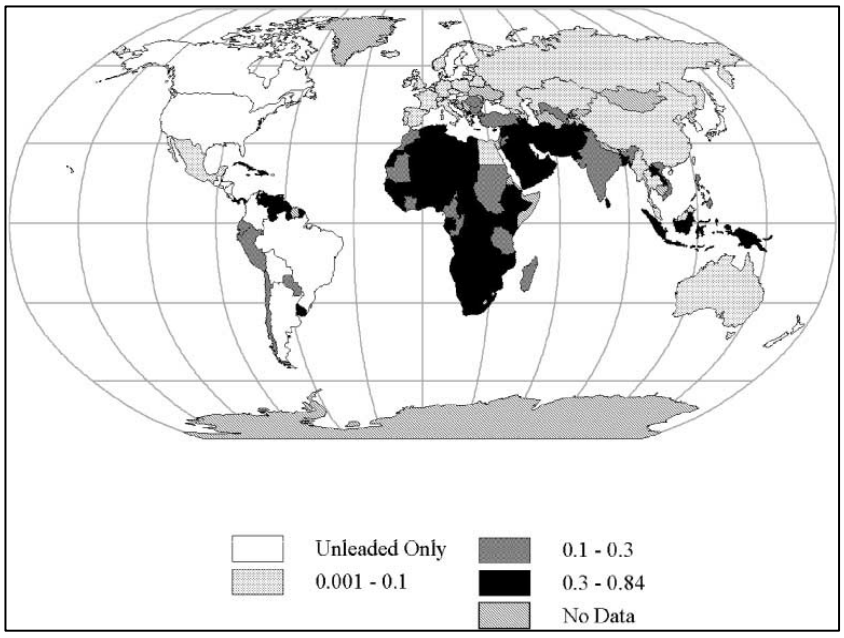

Figure 2.4 Concentration of Lead in petrol in the late 1990s (Thomas & Kwong, 2002)

At low levels, lead also causes hyperactivity, behaviour problems, impaired growth, and hearing loss in children. Lead poisoning in adults causes increased blood pressure, liver and kidney damage, and impaired fertility. Lead poisoning induced hypertension accounts for thousands of casualties a year, particularly of men between ages 35 and 50 (Thomas & Kwong, 2002).

The use of lead has since been curtailed, and was substituted with methyl tertiary butyl ether (MTBE). MTBE is a colourless liquid that possesses fuel oxygenating properties and is highly water soluble. The phasing out of lead significantly attributed to the increase in the demand for MTBE, until MTBE was reported to contaminate groundwater sources. Because MTBE is much more soluble than other petrol components, it can migrate faster and farther into the ground. This raised concerns over the possibility of MTBE contaminating public water systems and private drinking wells. The use of MTBE has since been banned in most developed countries and substituted with a cleaner alternative, ethanol (Thomas & Kwong, 2002). The use of ethanol as a petrol additive has some advantages compared to lead and MTBE. Ethanol has a higher oxygen content, and as a result less amount of additive is required. The increased percentage of oxygen afforded by ethanol in the fuel allows for a better oxidation of petrol hydrocarbons, and consequently, a reduced tailpipe emission of CO and aromatic compounds. Ethanol also has greater octane booster properties, is non-toxic, and does not contaminate water sources. In addition, the feasibility of using ethanol as a fuel oxygenate is supported by the fact that it would only incur a couple of cents more to the consumer, but a significant saving in terms of health (Thomas & Kwong, 2002; Hansen *et al.*, 2005).

2.3.4 Ethanol in the mitigation of GHG emissions

The mitigation of GHG emissions is dependent on the type of feedstock employed, cultivation method, conversion technologies, and energy efficiency assumptions. The level of GHG emission reduction that bioethanol can offer is still under debate. Some suggest that sugarcane-based bioethanol and cellulosic bioethanol (a second generation fuel) can best reduce the emission of GHGs. Bioethanol derived from maize has been reported to have the worst GHG emission performance, and in some instances, the GHG emissions can be greater than those obtained through the use of fossil fuels (Peskett *et al.*, 2007; Fargione *et al.*, 2008).

It has long been thought that biofuels are carbon neutral, suggesting that the carbon output is equal to the carbon input. However, it has been argued that such reports are due to flaws in carbon footprinting guidance and practice. Johnson (2009) suggested that in carbon footprints, rather than looking at carbon sequestered from the environment and that generated from combustion of the fuels, a ‘carbon-stock change’ line item could be applied. Suggesting that forests should be looked at as carbon stocks, subsequent deforestation for biofuel production results in a change in carbon stocks, the latter exceeding the former. This argument further reiterates that feedstocks have a characteristic GHG emission performance, and that no biofuel production strategy should be assumed as carbon neutral, unless proven (Johnson, 2009).

Chemical inputs, such as nitrogen fertilizers can also have a significant effect on GHG emissions. New research aiming at reducing chemical inputs, e.g. through the use of intercropping N_2 fixing soy beans with perennials, could aid reduce inputs and ensure an increased competitive edge of biofuel feedstocks in GHG terms (Peskett *et al.*, 2007).

2.4 SUGARCANE MOLASSES AS A FEEDSTOCK FOR BIOETHANOL PRODUCTION

2.4.1 The sugarcane crop

Sugarcane is a tall, perennial tropical grass that belongs to the genus *Saccharum. Saccharum* has two confirmed wild species (*S. spontaneum* and *S. robustum)* and four domesticated species (Bakker, 1999). *S. spontaneum* occurs largely on the tropical plains of Africa, Asia and Oceania. The occurrence of *S. robustum* is restricted to New Gunea and neighbouring islands. The modern cultivated sugarcane crops are hybrids that are derived from breeding between species of former commercial importance carrying desirable traits. The breeding process then generates sugarcane crops with the vigour and hardness of *S. spontaneum* and *S. sinense,* coupled with the high sugar content of *S. officinarum* and *S. barberi* (OECD, 2011).

The continued cultivation of sugarcane (which has traversed over millenniums) is owed to sugarcane possessing one of the most efficient solar energy collections systems (James, 2004). Sugarcane stores solar energy in the form of fibre and fermentable sugars (Sansoucy *et al.*, 1988). The sugarcane stalks (which reach an average maximum height of 3 to 4 m) are rich in sucrose, and account for an approximated 75% of the world's sucrose supply. At maturity i.e. when the sucrose content in the stalks is at its maximum concentration and that of invert sugar at its lowest concentration, the stem is capable of accumulating 12-16% of its fresh weight and approximately 50% of its dry weight as sucrose (Bull & Glasziou, 1963; Dillon *et al.*, 2007).

2.4.2 Sugarcane processing and its by-products

The sugar processing industry typically generates approximately 70% water, 15% bagasse, 10% sugar, 3% molasses, and, if produced, 2% filtercake (Fuller, 2004). The generated by-products i.e. molasses and filter cake are used as animal feed and as a soil conditioner, respectively (Fuller, 2004). The South African sugar industry generates ample amounts of cane molasses which is often commercialised as animal feed during drought season (i.e. to

improve the palatability of corncobs) and as a substrate for bioethanol production (Cleasby, 1963; Nel, 2010). Cane molasses has seen increased attention as a substrate for bioethanol production because it is a relatively cheap raw material, readily available, does not require any pre-treatment (e.g. starch hydrolysis of maize) and is already used for ethanol production (Patrascu *et al.*, 2009). It is because of the above mentioned factors that the fermentation of molasses to produce ethanol by *S. cerevisiae* has since been explored by major sugar-producing countries (Moreira & Goldemberg, 1999; Hahn-Hägerdal *et al.*, 2006; Amigun *et al.*, 2011). The fermentation of molasses offers the possibility of using molasses to its fullest capacity, thus generating profit and also alleviating fuel shortages (Nel, 2010).

2.4.3 Sugarcane molasses

When the term molasses was initially coined, it referred to the final effluent obtained in the preparation of sugar by repeated evaporation, crystallization and centrifugation of juices from sugar cane and sugar beets (Olbrich, 1963). The latter recognition of several types of molasses in modern times has led to the alteration of the definition, and now molasses refers to any liquid feed ingredient that contains in excess of 43% sugars (Curtin, 1983). It is imperative to understand the composition of molasses before employing it as a substrate for bioethanol production because yeast have specific needs for growth, and these have to be addressed accordingly in order for the fermentation process to be at optimum.

Molasses is made up of major components [water, carbohydrates (CHO), and non-sugar components] and minor components (trace elements, vitamins and growth substances) (Olbrich, 1963). The quality and composition (Table 2.1) of molasses is defined by a range of factors such as; soil type, ambient temperature, moisture, season of production, (cane) variety, sugar extraction technology at particular plant, and storage variables (Olbrich, 1963). The molasses trade industry commonly uses the term °Brix as an indicator of specific gravity to approximate total solid content. Brix was initially used as an indication of the percentage of sucrose in pure sucrose solutions on a weight basis. However, now °Brix is used to approximate the total solid content of molasses which encompasses sucrose, fructose, glucose and other non-sugar organic materials (Curtin, 1983).

Table 2.1 Average nutrient composition of beet and cane molasses (Olbrich, 1963)

Constituent	Beet molasses (%)	Cane molasses (%)
Water	16.5	20.0
Organic constituents		
Sugars: Saccharose	51.0	32.0
Glucose	-	14.0
Fructose	- (53.0)	16.0 (62.0)
Or Invert sugar	1.0	-
Raffinose	1.0	-
Nonsugars: Nitrogenous materials, acids, soluble gummy substances	19.0	10.0
Inorganic constituents (ash)		
SiO_2	0.1	0.5
K_2O	3.9	3.5
CaO	0.26	1.5
MgO	0.16	0.1
P_2O_5	0.06	0.2
Na_2O	1.31 (11.5)	- (8.0)
Fe_2O_3	0.02	0.2
Al_2O_3	0.07	-
Soda and carbonate residue (as CO_2)	3.5	-
Sulfate residue (as SO_3)	0.55	1.6
Chlorides	1.6	0.4
Total	**100.0**	**100.0**

2.4.4.1 Major Constituents

2.4.4.1.1 Water

Water in molasses in present mostly in an unbound form, but a fraction is held as hydration water or hydrate water. The original end-product molasses in sugar mills contains 12-17 % water and is diluted to produce commercial molasses with an average water content of 20% The dilution of molasses (in addition to economic reasons) serves to partly dissolve microscopic sugar crystals and makes it easier for molasses to be moved *via* pumps (Olbrich, 1963).

2.4.4.1.2 Carbohydrates

Carbohydrates make up a significant fraction of molasses (greater than 60% in some instances), and they contribute toward the feeding value of molasses. The most predominant carbohydrate in cane molasses is sucrose and accounts for 25-40% (w/v) of molasses. Molasses also contains invert sugar (glucose and fructose) which result from sucrose hydrolysis during manufacture and also invertase activity during storage. The rate of hydrolysis increases with a decline in pH and an increase in temperature. Invert sugar accounts for 12-30% (w/v) of molasses. The amount of invert sugar present in molasses signifies how much sucrose has been lost to hydrolysis. The carbohydrates; sucrose, glucose and fructose collectively serve as assimilable carbon sources to yeast and other microorganisms (Olbrich, 1963; Curtin, 1983).

2.4.4.1.3 Non-sugar components

In terms of sugar technology, the non-sugar components consist of all constituents of cane molasses except sucrose. Non-sugar constituents can be classified as organic and inorganic non-sugars.

I. *Organic non-sugars*: These are further classified into nitrogen containing and nitrogen-free. The nitrogen containing fraction (responsible for buffer properties of molasses) is made up of amides, albuminoids, amino acids and other simple nitrogenous compounds (Curtin, 1983). The alkaline conditions that prevail during clarification causes amino acids to dissolve, hence all the amino acids in raw juice pass through the manufacturing processes almost unaltered and enter the molasses (Olbrich, 1963). A significant portion of nitrogen present in molasses is in the form of amino acids (account for greater than 1/3 of total nitrogen) and the rest is made up of low amounts of amide, nitrates, and ammonia nitrogen. The Nitrogen-free fraction consists of organic acids (Olbrich, 1963). The quantity and variety of organic acids present in molasses differ from batch-to-batch based on the micro flora present in the molasses and manufacture and storage conditions. Cane juice purification leads to the removal of significant amounts of organic acids, and those that remain, do so in minute concentrations. Examples of organic acids found in cane molasses are: volatile organic acids [formic acid, acetic acid (0.1%)] and non-volatile organic acids [aconitic acid (0.2%), lactic acid (0.8%) and to a lesser degree, malic and citric acid] (Nelson, 1929; Olbrich, 1963).

II. *Inorganic non-sugars*: The mineral content of molasses is very broad and serves to increase the solubility of sucrose. Salts in molasses are important for their molasses-producing characteristics. Sodium and potassium occur in relatively higher levels than the other salts, and have been reported to be more responsible for increased sucrose solubility. Cane and beet molasses are comparatively high in potassium, magnesium, sodium, chlorine and sulphur (Curtin, 1983). Approximately 80% of the crude ash is made up of potassium and sodium carbonate, the other 20% represents chlorides, sulfates, phosphates and silicates (Olbrich, 1963). Defecation with lime also results in the formation of insoluble calcium salts. The amount of salts present in a given molasses sample is dependent on soil type, and also manufacturing practises. An example is the addition of soda ash, which subsequently results in high sodium levels. Potassium has been found to be highly crucial for the metabolism of minerals by yeast, reports suggesting it should be 4% in molasses (Olbrich, 1963)

2.4.4.2 Minor components

Minor components are those present in relatively small amounts, and these are trace elements and vitamins.

2.4.4.2.1 Trace elements

Several heavy metals (Table 2.2) have been reported in molasses, the ones that have been commonly reported are: Iron, zinc, manganese, copper, boron and molybdenum. The levels of trace elements in molasses are indicative of the location in which the cane was grown (Curtin, 1983).

Table 2.2 Trace minerals in cane, beet and citrus molasses (Curtin, 1983)

Mineral (mg/kg)	Cane	Beet	Citrus
Copper	36	13	30
Iron	249	117	400
Manganese	35	10	20
Zinc	13	40	-

2.4.4.2.2 Vitamins

The broad range of vitamins and growth substances present in molasses is influenced by many factors, extending from the growth of the cane, to the manufacturing process. Molasses has a substantial amount of vitamins belonging to the Vitamin B complex (Table 2.3).

Table 2.3 Vitamins in different molasses (Curtin, 1983)

Vitamin (mg/kg)	Cane	Beet	Citrus
Biotin	0.36	0.46	-
Choline	745.0	716.0	-
Pantothenic acid	21.0	7.0	10.0
Riboflavin	1.8	1.4	11.0
Thiamine	0.9	-	-

The vitamins and growth substances present in cane molasses are: Thiamine (B1), Riboflavin (B2), Niacin (B3), Pantothenic acid (B5), Pyridoxine (B6), Folic acid (B9), Choline, Biotin (B7 or H), and Inositol (which is a CHO). The purification and clarification of cane juice results in a considerable loss of vitamins, the ones that survive collate in the molasses (Olbrich, 1963; Curtin, 1983)

2.5 *Saccharomyces cerevisiae* AS A MODEL FERMENTATION ORGANISM

The most employed microorganisms in fermentations are yeasts, particularly *S. cerevisiae*. Much success with *S. cerevisiae* is owed to its capability to hydrolyse sucrose into glucose and fructose and the yeast's increased tolerance to ethanol in comparison to other microorganisms like bacteria (Almeida *et al.*, 2007). Furthermore, *S. cerevisiae* has the ability to produce ethanol levels in excess of 18% (w/v) of the fermentation broth (Lin & Tanaka, 2006; Sanchez & Cardona, 2008). *S. cerevisiae* is generally recognised as safe (GRAS) and is predominantly used as a food additive for human consumption and is therefore ideal for producing alcoholic beverages and bread leavening (Lin & Tanaka, 2006).

2.5.1 Bioethanol production with *S. cerevisiae*

The production of bioethanol is a two-step biological process which entails the bioconversion of sugar-containing biomass by microorganisms to simpler sugars; these sugars are then fermented to produce ethanol and CO_2. The inherent ability of *S. cerevisiae* to produce invertase (biological enzyme which facilitates the breakdown of sucrose into glucose and fructose) and a relatively superior robustness when compared to other bioethanol producing microbes such as *Zymomonas mobilis* has made it the prime choice in bioethanol production (Ikram-Ul-Haq & Sikander, 2005; Lin & Tanaka, 2006). Various processes and substrates have been reported in the production of bioethanol by *S. cerevisiae*. The selection of the substrate mostly based on its local availability, and relative ease of substrate conversion to bioethanol, that is, without high capital investments. This has resulted in the development of technologies able to use non-food plant resources such as agricultural residues (corn cobs and stalks), sugarcane waste, energy crops and discards from the pulp and paper industry (Lin & Tanaka, 2006; Sanchez & Cardona, 2008). *S. cerevisiae* has also been successfully employed in high gravity batch fermentations, with final ethanol concentrations reaching as high as 16% (v/v) from an initial 35% (w/v) sucrose concentration (Breisha, 2010).

2.5.2 Shortfalls encountered in fermentations with *S. cerevisiae*

Bioethanol is mostly produced in large scale operations by batch fermentations. The downside to batch fermentations is the depletion of oxygen and accumulation of ethanol as the fermentation progresses, resulting in adversely restricted yeast performance. Under anaerobiosis, yeast cells fail to complete the biosynthesis of unsaturated fatty acids and ergosterols resulting in the accumulation of intermediary components of lipid metabolism. *S. cerevisiae* then progressively alters the composition of their cell walls, resulting in the reduction of organelle membranes surface areas and constant dilution of their lipid content until the limit of their viability (Mannazzu *et al.*, 2008; Bardi *et al.*, 1999). The altered and compromised wall permeability characteristics lead to decreased ethanol tolerance, which in turn results in severely diminished yeast viability and fermentation ability (Mannazzu *et al.*, 2008). A high ethanol concentration is a single factor amongst a host of other exogenous or environmental factors that lead to decreased yeast fermentation activity such as low pH, accumulation of toxins and nutrient limitation (Park & Chang, 2000).

2.6 CELL IMMOBILIZATION

The viability and sustainability of any bioprocessing operation is determined by its level of production. Low volumetric productivities greatly affect profit and need to be addressed accordingly. Microbial cell immobilization has been reported (in some instances) to solve this challenge (Park & Chang, 2000; Westman *et al.*, 2012). Whole cell immobilization is defined as the physical confinement or localization of intact cells to a certain region of space while

preserving their viability (Karel *et al.*, 1985). Immobilization eradicates low volumetric productivity by building up higher cell densities in the bioreactor, resulting in increased medium conversion to products and decreased operation times (Park & Chang, 2000; Westman *et al.*, 2012).

Over the years, cell immobilization has gained much attention and momentum. Cell immobilization is currently employed in fields like; pharmaceutics, food processing, environmental protection, biofuel production, agricultural biotechnology and waste water treatment (Rathore *et al.*, 2013). The increasingly growing application of immobilization is owed to the development of different methods for cell immobilization, with each one best suited to a specific application (Figure 2.5).

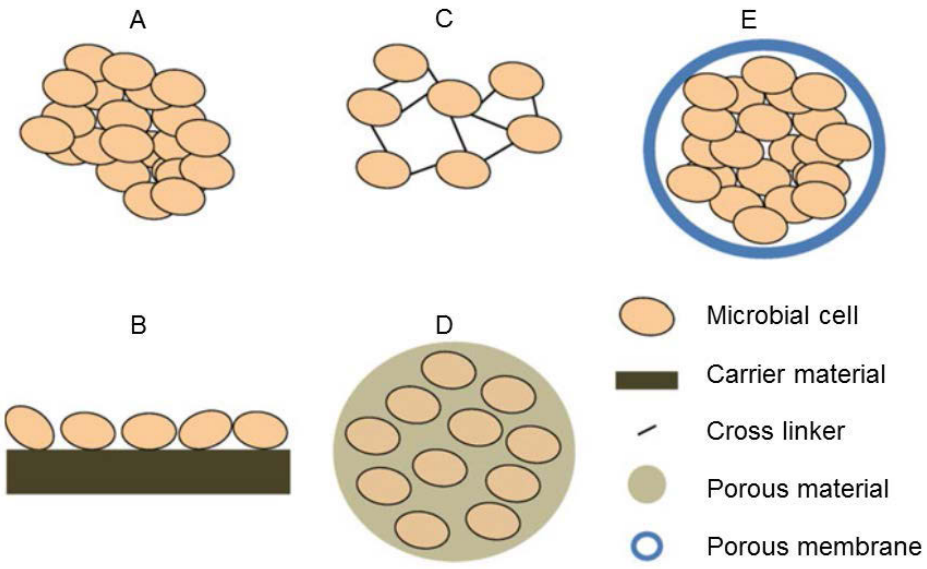

Figure 2.5 The common immobilization techniques for microbial cells are; (a) flocculation, (b) adsorption to a solid support, (c) cross-linking of cells, (d) immobilization in a porous matrix, and (e) encapsulation in a selectively permeable membrane (Westman *et al.*, 2012).

These methods are divided into five categories based on the physical mechanism of cell localization and the nature of support: cell flocculation (Purwadi *et al.*, 2007), sedimentation or filtration and cell recycling (Brandberg *et al.*, 2007), surface adsorption (Kubota *et al.*, 2005), cross-linking of cells (Abelyan, 2000), entrapment in a matrix (Taherzadeh *et al.*, 2001), and encapsulation in a polymer gel membrane (Talebnia & Taherzadeh, 2006).

2.6.1 Cell encapsulation

Cell encapsulation refers to the enclosure of cells within a polymeric capsule membrane that is permeable to nutrients, oxygen and metabolites (Galazzo & Bailey, 1990; de Vos *et al.*, 2009). In essence, encapsulation seeks to isolate the cell mass physically from the outside environment, while maintaining normal cell physiology within a barrier of desired permeability (de Vos *et al.*, 2009). The coating material is usually composed of natural or synthetic polymers. The choice of coating material employed greatly determines what characteristics a capsule possesses and subsequently, the conditions (application) that the capsule will be subjected to (Park & Chang, 2000).

The encapsulation of microbes offers significant advantages over employing freely suspended cells. In a hydrogel matrix (capsule), the microbial cells retain prolonged activity and are protected from an array of harsh environmental conditions such as temperature, pH, organic solvents, and toxins (Park & Chang, 2000). In a biotechnological process where the elimination of any step directly influences profits, the easy handling and recovery of the capsules further makes the technology more attractive. This is because it would eliminate high energy dependent processes like centrifugation which are used for product recovery (Park & Chang, 2000). However, encapsulation also has some shortcomings, like concerns over ensuring a sterile environment in large scale production, a coating material possessing all desired characteristics and a technology for mass capsule production are yet to be discovered. The advantages of encapsulation perhaps outweigh the negatives, but in order for encapsulation to be economically and industrially viable, some significant strides have to be made in addressing the negatives (Park & Chang, 2000; Westman *et al.*, 2012; Rathore *et al.*, 2013). Of all the techniques that have been developed for encapsulation, the most significant ones are: coacervation, interfacial polymerization (emulsion), pregel dissolving and liquid droplet formation.

2.6.1.1 Coacervation

In coacervation, the material (cells) to be encapsulated is either suspended in an organic or aqueous liquid, to which a polymeric solution is added drop wise. The polymer employed is dependent on the hygroscopic characteristics of the cell suspension. If cells are suspended in a water soluble solution, the polymer employed should be hydrophobic, and *vice versa*. This ultimately leads to a minimized surface area between the two solutions (i.e. cell suspension and polymer), triggering the formation of capsules by the polymer called coacervate. The alteration of any one of the parameters of the system e.g. pH, temperature, or composition, leads to the cross-linking and solidification of the polymer. The downside to coacervation is its expensive nature, which substantially dampens its practicability as a tool for encapsulation (Chang, 1964; Park & Chang, 2000; Westman *et al.*, 2012).

2.6.1.2 Interfacial polymerization

Interfacial polymerization, also known as emulsion polymerization entails the dispersion of a reactive water-soluble monomer into the aqueous phase of an emulsion. Constantly stirring the emulsion generates droplets of very narrow size distribution in the aqueous phase. The addition of a suitable solvent-soluble monomer to the organic phase results in the instantaneous formation of a polymeric membrane at the interface between the aqueous drop and the organic phase (Chang, 1964; Park & Chang, 2000; Westman *et al.*, 2012).

Chang (1964) reported the first successful encapsulation of cells by interfacial polymerization. Chang (1964) dispersed aqueous droplets containing the monomer 1,6-hexanediamine and erythrocyte hemolysate in a mixture of chloroform and cyclohexane. The addition of the solvent-soluble sebacoyl chloride to the organic phase resulted in the formation of capsules of substantial integrity and narrow size distribution. Albeit the interfacial polymerization technique generates thin and mechanically stable capsules, the conventional monomers are toxic to cells and severely affect cell viability (Chang, 1964; Green *et al.*, 1996; Park & Chang, 2000).

2.6.1.3 Pregel dissolving

This technique is also called the two-step method (Figure 2.6). A polyanion solution e.g. sodium alginate, is dripped (or extruded) into a gelling bath containing calcium cations (Ca^{2+}), forming hydrogel beads. An extra coat is added around the beads by retreating them with a polycation e.g. poly-L-lysine or chitosan, and a polyanion. The cores of the resultant capsules are dissolved by treatment with a chelating agent e.g. sodium citrate. The chelating agent forms a complex with Ca^{2+}, thus destabilizing the interactions between alginate and Ca^{2+} and generating a liquid core (Park & Chang, 2000; Wen-Tao *et al.*, 2005; Westman *et al.*, 2012).

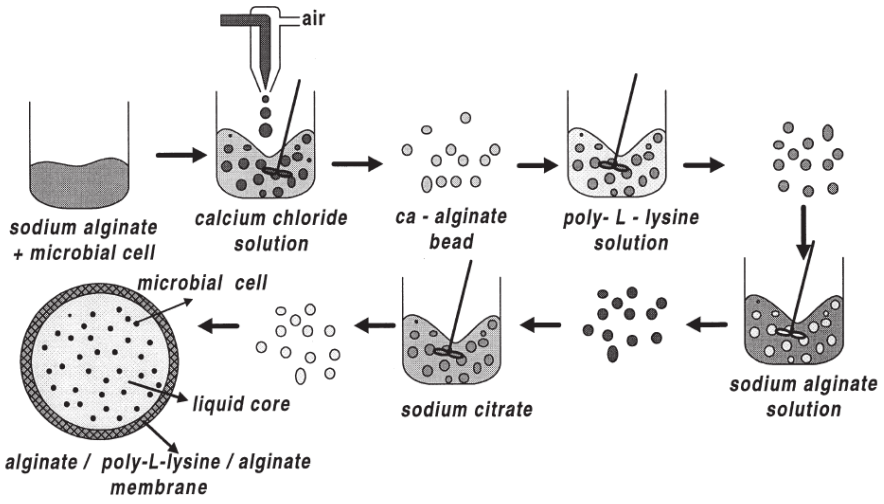

Figure 2.6 A schematic representation of the pregel dissolving two-step method (Park & Chang, 2000)

2.6.1.4 Liquid droplet formation

Also referred to as the one step method, it entails the formation of calcium alginate capsules by mixing cells in a calcium chloride solution and dripping the cell suspension into a swirling sodium alginate gelling bath (Figure 2.7) (Klein *et al.*, 1983). A resultant calcium alginate membrane is formed around the cells by ionic interaction.

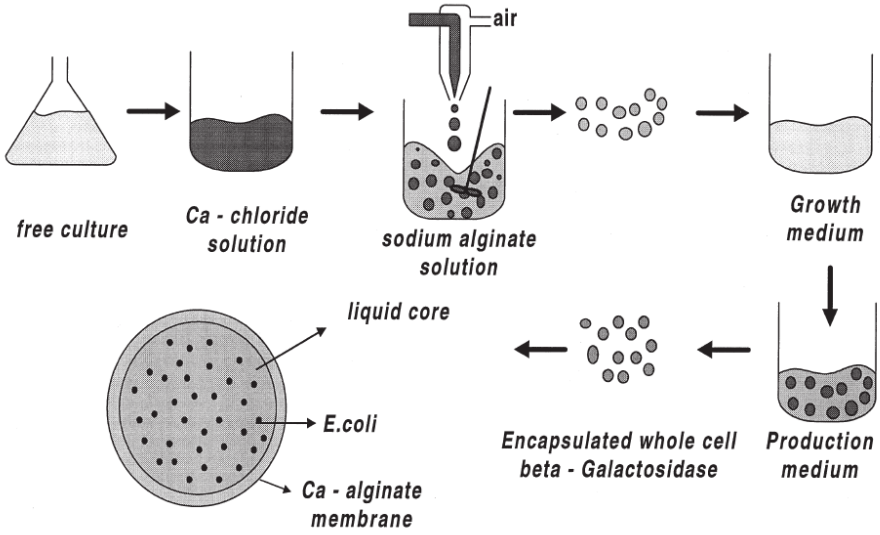

Figure 2.7 A schematic representation of capsule formation by the liquid droplet (one step) formation method (Park & Chang, 2000)

The alteration of the concentration of alginate, calcium and gel-forming polymer allow for the control and modulation of capsule properties i.e. wall thickness, pore size, surface charge and mechanical strength. This technique has been successfully employed to encapsulate yeast and bacteria (Chang *et al.*, 1996; Park & Chang, 2000; Lee *et al.*, 2011).

2.6.2 Microbial encapsulation in bioethanol production

A study conducted by Lee *et al.* (2011) found that yeast cells immobilized in alginate beads resulted in higher ethanol yields and shorter fermentation times (10-14 hrs) when compared to free cells (24hrs), under the same batch-fermentation conditions. Whereby the encapsulated cells displayed nearly a 100% ethanol yield, compared to an 88% ethanol yield for freely suspended cells when fermenting media with 10% glucose. In other instances the encapsulation of cells only results in slight increases in bioethanol yields. Such as the study reported by Behera *et al.* (2011), whereby fermentations with yeast cells encapsulated in agar agar and calcium alginate resulted in slightly higher bioethanol yields than the free-suspended cells (<4%).

This observed increase in ethanol yields for encapsulated cells is postulated to be from the larger proportion of the yeast cell population's catabolism being used for energy maintenance as the growth decreases, leading to higher ethanol yields and lower glycerol yields (Westman *et al.*, 2012). Some authors suggest that the higher levels of saturated fatty acids found in encapsulated cells compared to free cells confers upon them increased ethanol tolerance (Hilge-Rotmann & Rehm, 1991; Sun *et al.*, 2007). Others suggest that hydrogels tightly withhold water, leading to a highly structured water layer surrounding the yeast in the pores of the gel network (Israelachvili & Wennerstrijm, 1996). This water layer then prevents ethanol from dehydrating the encapsulated cells (Desimone *et al.*, 2002).

The general consensus amongst a majority of researchers is that encapsulated cells show increased robustness characteristics when faced with external stresses (such as toxins in hydrolyzates and elevated temperatures) during fermentation, allowing encapsulated yeast to thrive under conditions where free cells would not be able to (Talebnia & Taherzadeh, 2006; Pourbafrani *et al.*, 2007; Sun *et al.*, 2007; Talebnia, 2008; Ylitervo *et al.*, 2011; Ylitervo *et al.*, 2013).

2.6.3 Yeast encapsulation polymers

Encapsulation has evolved over the years, with many different encapsulating materials reported (Rathore *et al.*, 2013). The most commonly employed encapsulating materials are natural water-soluble polymers; alginate, agarose, k-carrageenan, starch and gelatin (Burgain *et al.*, 2011). Natural polymers are highly biocompatible, and allow for high permeability levels of low molecular weight nutrients and metabolites, thus ensuring the provision of optimal functioning conditions to the encapsulated microbial cells (Brun-Graeppi *et al.*, 2011; Rathore *et al.*, 2013). Synthetic polymers have also been employed, albeit they afford higher mechanical strength and better chemical stability when compared to natural polymers, their use is hampered by their characteristic poor biocompatibility (Rathore *et al.*, 2013). Examples of synthetic polymers include polyacrylamide and polyvinyl alcohol (Lozinsky & Plieva, 1998; Calvet *et al.*, 2004; Leskošek-Čukalović & Nedović, 2005).

The production of capsules by synthetic polymers is further dealt a blow by the required harsh conditions that affect cell viability and integrity (Lozinsky & Plieva, 1998; Leskošek-Čukalović & Nedović, 2005). Conversely, capsule production with natural polymers is done under mild and non-toxic conditions, therefore not affecting cell integrity and viability (Rathore *et al.*, 2013). Some have employed hybrid polymer matrices, in efforts of trying to take advantage of the beneficial properties possessed by both natural and synthetic

polymers. Even so, concerns about discovering a polymer robust enough to withstand harsh conditions encountered in industrial applications (or any other application), but also possessing substantial biocompatibility properties still exist, and can only be addressed with further research (Gill & Ballesteros, 2000; Gill & Ballesteros, 2000; Sakai *et al.*, 2001).

2.6.3.1 Alginate

Alginate (Figure 2.8) is an anionic linear polysaccharide of D-mannuronic and L-guluronic acids which makes up the cell linings of marine brown algae and readily forms hydrogels in the presence of divalent cations (Serp *et al.*, 2000; Talebnia, 2008). The use of alginate as an encapsulating material has substantially increased in recent years when compared to other natural polymers. This is because natural polymers such as agar, k-carrageenan, and starch are relatively expensive, toxic to cells due to excess potassium employed for cross-linking and are susceptible to acidic conditions, respectively (Rathore *et al.*, 2013). Alginate on the other hand is relatively cheap, forms capsules under simple gelling conditions, is non-toxic and possesses excellent biocompatibility properties (Wen-Tao *et al.*, 2005). Although alginate is susceptible to capsule disruption or dissolution in the presence of antigelling ions (e.g. monovalent cations, phosphate and citrate), coating with a polycation such as chitosan solves this problem (Serp *et al.*, 2000; Wen-Tao *et al.*, 2005).

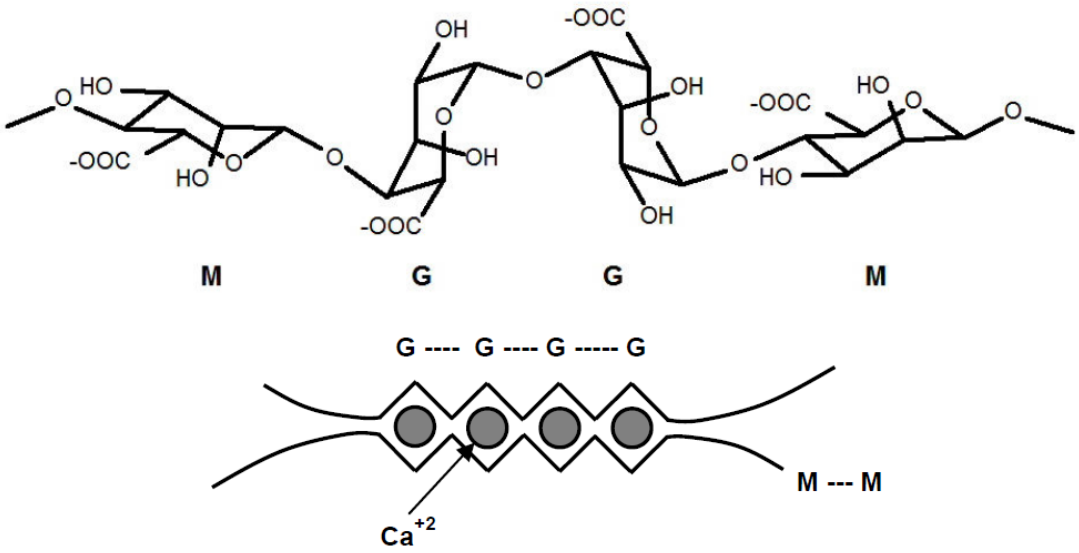

Figure 2.8 Inter-chain linkage between G blocks and Ca^{2+} (Talebnia, 2008)

Alginate possesses most of the desired characteristics in an encapsulating polymer when compared to other previously reported polymers, but its mechanical strength is still being questioned. While increasing the concentration of alginate does increase the mechanical

strength of the capsule produced, the current capsule production technologies simply cannot form capsules with highly viscous solutions. In essence, alginate can be seen as a stepping stone toward finding the elusive polymer of choice (Westman *et al.*, 2012).

2.6.3.2 Agarose

Agarose is a neutral (non-ionic) polysaccharide polymer extracted from seaweed. Agarose is made up of agarobiose repeating units, a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose. Agarose is one part of two major components of agar, and is purified from agar by removing agaropectin (Shoichet *et al.*, 1996; Nussinovitch, 2010). Agarose forms a gel when heated to its melting temperature ~90°C and then its temperature lowered to below 40°C. Agarose has been successfully used to encapsulate cells, whereby the cell suspension is added to agarose as it cools (Shoichet *et al.*, 1996). The downside to using agarose in cell encapsulation is its high gelation temperature 40°C, which leads to substantial loss of cell viability. However, low melting point agarose offers the possibility of retaining high cell viabilities of the encapsulated cells due to a low gelling temperature (below 30°C).

2.6.3.3 Chitosan

Chitosan is a linear, cationic polysaccharide composed of β(1-4) linked glucosamine units together with some N-acetylglucosamine units. Chitosan is obtained from the extensive deacetylation of chitin, a polysaccharide present in the shells of crustaceans, molluscs, the cell walls of fungi, and the cuticle of insects (Peniche *et al.*, 2003). Chitosan is biocompatible, biodegradable, and non-toxic. Its cationic nature allows it to react with polyanions to form polyelectrolyte complexes (Peniche *et al.*, 2003; Wen-Tao *et al.*, 2005). Chitosan is predominantly used as a coating material of Ca-alginate capsules, to increase the bead's stability in the presence of monovalent cations. The use of chitosan has since increased and displaced the use of expensive polycations like poly-L-lysine, because chitosan is cheap, in abundance and biocompatible (Wen-Tao *et al.*, 2005).

2.6.4 Mass production of capsules

Cell encapsulation promises to be the possible answer to several industrial setbacks in the near future, although there are still some issues that need to be addressed. In an industrial setting, the use of encapsulation technology will only be viable once capsules can be efficiently mass produced (Whelehan, 2011; Westman *et al.*, 2012). Different capsule production technologies have been designed to date, but none completely fit the bill in terms

of possessing all the desired characteristics, but with that said; positive strides are being made to address this concern (Whelehan, 2011).

An ideal encapsulation technology would have to be capable of producing small (<200 µm), mono-dispersed, homogenous and spherically shaped capsules, with a narrow size distribution, within a short production time, under sterile, mild and simple conditions (Whelehan, 2011). The equipment would have to possess high encapsulation efficiencies (i.e. % of encapsulated product) and production rates, with the capability of capsule production from highly viscous solutions, therefore affording more freedom and versatility in terms of the type of capsules (Figure 2.9) that can be produced. The cost of the technology would also have to be reasonable and be augmented by substantial advantages over simply using freely suspended cells (Whelehan, 2011).

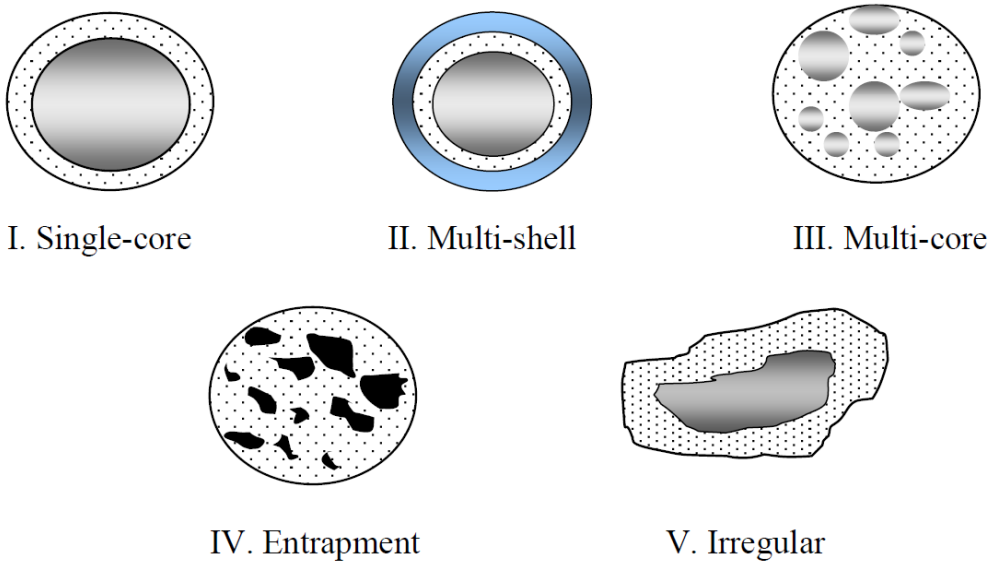

Figure 2.9 The main structural forms of microcapsules (Whelehan, 2011)

The encapsulation equipment that has been described to date is based on capsule production by mechanical means and work by extruding drops through a nozzle (orifice).

2.6.4.1 Spray-drying

Microspheres (microcapsules) are formed by atomizing a liquid product containing the material to be encapsulated and the shell material with a hot gas current in a drying chamber, expelling moisture in the process (Gharsallaoui *et al.*, 2007). This process is carried out in a double-sized nozzle, which facilitates the atomization of a liquid stream with air, forming fine micro particles encapsulating the product. The particles are then subjected to drying by flash evaporation and then collected in a holding chamber (Mokarram *et al.*,

2009). Spray-drying can generate very fine powder capsules (10-50 µm) to large size particles (2-3 mm), depending on the properties (viscosity, density, etc.) of the feed material and the operating conditions; atomization pressure and nozzle type (Gharsallaoui *et al.*, 2007).

The advantages of spray-drying are that it is capable of mass producing capsules with high encapsulation efficiencies in a continuous mode, under sterile conditions (if required) and at a relatively cheap cost. The major disadvantage of the technique is that it can only produce type 4 capsules and offers very limited control over the shape and size distribution of the produced capsules (Whelehan, 2011).

2.6.4.2 Jet cutting technique

The forcing of a polymer through a specialized nozzle at a high velocity generates a solid liquid jet. Passing the resultant solid liquid jet through a cutting tool breaks it up into equal cylindrical segments, which then conform into spheres due to exposure to surface tension when they traverse the air, i.e. from the cutting tool to the hardening solution (Pruesse & Vorlop, 2004). The diameter of the particles generated is dependent on the: number of cutting wires, number of rotations of the cutting tool, polymer flow rate and velocity through the nozzle and nozzle diameter (Pruesse & Vorlop, 2004). This technique is relatively simple and capable of producing small mono-dispersed homogenously shaped capsules. The size of capsules that can be generated ranges from > 200 µm to several mm in diameter, with a narrow standard size deviation using viscous fluids at high production rates (Prüße *et al.*, 1998; Pruesse & Vorlop, 2004).

The setback to this technique is the incurred loss of material with every cut of the liquid jet, referred to as cutting loss. Cutting loss can be reduced to negligible amounts when by recycling the material, or using smaller cutting wires. However, the production of large particles requires thicker wires, thus generating a substantial cutting loss which cannot be ignored if seeking to employ this technique in an industrial setting (Pruesse & Vorlop, 2004). Other disadvantages that further limit the application of this technique are the inability to produce beads < 200 µm in diameter and ensuring sterile conditions for large scale production. Furthermore, the invasive nature of the cutting process is speculated to impose cell damage upon the cells, which may then question the effectiveness of the recycling strategy (Pruesse & Vorlop, 2004; Whelehan, 2011). The employed high velocities also have the potential to deform the spheres upon impact in the hardening/collection solution, but this can be addressed by using the soft landing method (i.e. place the gelling bath closer to the

cutting tool) or a pre-gelling step. Such added measures further increase the complexity of the process. Presently, this technique is used to produce type 4 capsules, but has shown the capability to also produce type 1 capsules (Pruesse & Vorlop, 2004).

2.6.4.3 Electrostatic extrusion

This bead generation technique entails the augmentation of the normal dripping process with electrostatic forces which pull the droplets off the orifice (needle). This results in droplet formation at a considerably faster rate compared to the normal dripping process, which relies solely on gravitational force. The polymer is passed through a charged needle which produces droplets that subsequently fall into a collecting/hardening solution carrying an opposite charge or earthed (Halle *et al.*, 1993; Bugarski *et al.*, 1994; Bugarski *et al.*, 2004).

Electrostatic extrusion is capable of producing substantially smaller beads compared to normal dripping (≥ 50 µm in diameter), of uniform size and shape, under reproducible and sterile conditions (Manojlovic *et al.*, 2006). The disadvantage of this technique is low production rates due to low polymer flow rate through the needle. Increasing the number of needles has been reported to slightly improve capsule production, but not to industrially feasible levels (Bugarski *et al.*, 2004). This technique has currently shown the capability to produce type 4 microcapsules (Whelehan, 2011).

2.6.4.4 Coaxial air-flow

Is also referred to as the concentric air-jet technique, and is based on the acceleration of the normal dripping process by using a stream of compressed air to pull the liquid droplets form the nozzle at a faster rate compared to the normal dripping process (Anilkumar *et al.*, 2001; Prüsse *et al.*, 2008). This technique employs a coaxial concentric nozzle which consists of an inner orifice through which the polymer material is extruded, and an outer orifice, through which the compressed air flows and strips the droplets formed at the tip of the internal nozzle (Prüsse *et al.*, 2008). This technique is capable of producing uniformly shaped and sized capsules with a diameter > 200 µm, under reproducible, mild and sterile conditions (Anilkumar *et al.*, 2001). The disadvantage of this technique is low production rates due to low polymer flow rates through the needle. Futile attempts to address this issue have involved increasing the air flow rate, but this merely increases trajectories of the produced drops resulting in the requirement of a larger surface area, further complicating the process. This technique is capable of only produces type 4 microcapsules (Whelehan, 2011).

2.6.4.5 Vibrating-jet (nozzle) technique

The vibrating-jet technique is one of the most commonly used technologies for capsule production (Heinzen *et al.*, 2004). This technique is based on the application of a vibrational frequency of defined amplitude to an extruded jet to break up the jet into equal segments, which then conform to spheres due to surface tension. Naturally, jets have the capacity to freely break up into segments at certain flow rates when exposed to natural irregular disturbances above the threshold. Natural break up can be irregular and cannot be fully controlled leading to the formation of droplets of unequal size and shape (Haas, 1992; Heinzen *et al.*, 2004).

A study by Rayleigh (1882) demonstrated that it is possible to control the break-up of laminar jets to generate small, uniform droplets possessing identical characteristics. This was achieved by applying a permanent sinusoidal force at defined frequencies to a liquid jet, resulting in the formation of one droplet per hertz of frequency applied (figure 2.10). In order for this highly regular and reproducible jet break-up to occur, the vibrational frequencies have to be close to the natural jet break-up frequency (Rayleigh, 1882; Haas, 1992).

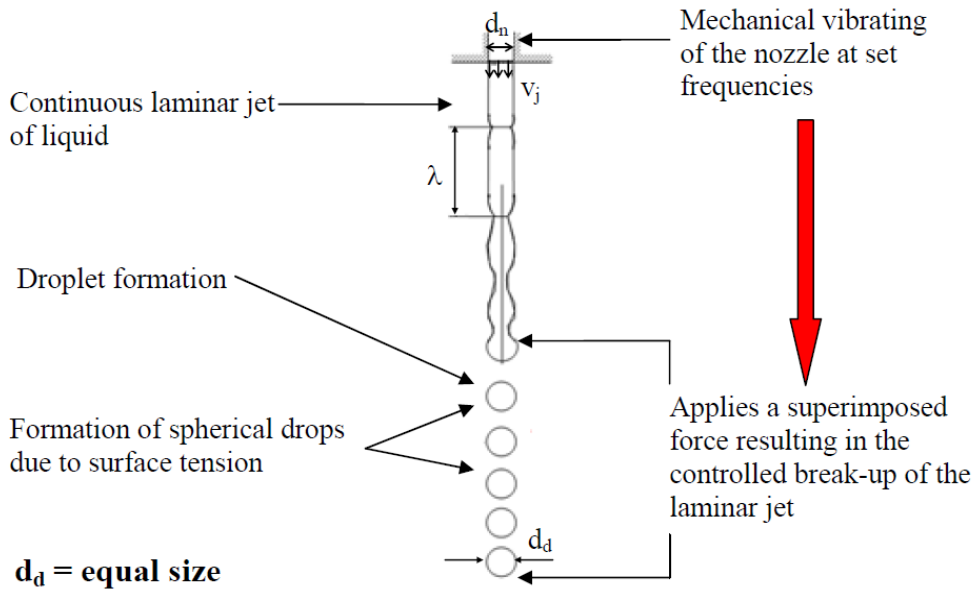

Figure 2.10 Mechanical vibration of a nozzle to break up liquid jet into droplets of equal size (Whelehan, 2011)

The properties of the resultant microcapsules are dependent on the nozzle diameter, laminar jet flow rate, size of the frequency at defined amplitude, and the viscosity of the extruded liquid (Serp *et al.*, 2000). The sinusoidal force can be applied upon the liquid jet by either vibrating the nozzle (vibrating nozzle technique), or by vibrating the chamber (vibrating

chamber technique) harbouring the polymer before extruding the polymer through a nozzle, or periodic alterations of the nozzle diameter during extrusion (Heinzen *et al.*, 2004; Whelehan, 2011). This technique is able to produce type 1-4 capsules.

2.7 CONCLUSION

Bioethanol promises to play a vital role in the liquid fuels industry, especially in the early transitional stages from fossil fuels to green fuels. The refinement of the currently existing bioethanol production strategies by encapsulating the fermenting yeast promises to substantially augment bioethanol outputs, which should coincide with improved feasibility of the currently employed bioethanol production strategies. Although the technology still faces challenges like the development of encapsulation equipment able to mass produce capsules and an encapsulating material robust enough to withstand the harsh exogenous conditions encountered during large scale fermentations, the technology is still nevertheless very attractive and very promising. It was noted that there is limited publications on fermentation studies of cane molasses.

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Chapter 3

RESEARCH RESULTS 1

Development of chemically defined molasses as a standardised laboratory yeast culture medium

Development of chemically defined molasses as a standardised laboratory yeast culture medium

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3.1 ABSTRACT

The variability of the nutrient composition of cane molasses has given rise to the need for a standardised laboratory medium which can substitute the use of industrially derived molasses in fermentation studies. This was addressed by formulating a chemically defined molasses medium supplemented with a different nitrogen source, *viz.,* 10g/L of casein hydrolyzate, peptone or yeast extract. The chemically defined molasses media and cane molasses sourced from three South African-based sugar mills (Amatikulu, Felixton and Gledhow), were subjected to batch fermentations in 250 mL Erlenmeyer flasks at 30°C for 48 hours with different *Saccharomyces cerevisiae* strains; BY4743, Angel yeast, cream yeast and dry yeast. Yeast fermentation profiles were monitored by measuring CO_2 evolution *via* flask weight measurements. Sugar attenuation, glycerol and ethanol formation profiles were monitored by High-Pressure Liquid Chromatography (HPLC). The chemically defined molasses medium containing yeast extract produced yeast fermentation profiles most similar to those attained in the fermentation of cane molasses. The fermentations of chemically defined molasses containing casein hydrolysate and peptone resulted in a twofold decrease in yeast fermentation activity and significantly diminished bioethanol yields. The results attained suggest that the novel chemically defined molasses medium containing yeast extract can therefore be employed as a standardised laboratory culture medium in yeast fermentation studies.

3.2 INTRODUCTION

The speculation surrounding the sustainability of using fossil fuels as energy sources has resulted in bioethanol production gaining substantial momentum in recent times; with the industry generating billions of litres annually (Laherrere, 2001; Greene *et al.*, 2006). The bioethanol industry is faced with challenges of finding cheap and abundantly available fermentation substrates possessing nutritional characteristics favorable for yeast proliferation and fermentation ability. As a result, bioethanol has been produced from an array of feedstocks such as sugar-based raw material (e.g. molasses), lignocellulosic material, and excess or by-products of wineries (Tang *et al.*, 2010; Bangrak *et al.*, 2011). Cane molasses is one of the predominantly employed substrates in bioethanol production, this because it is relatively cheap, available in abundance and contains sugars that cannot be extracted further by any economically feasible process (Patrascu *et al.*, 2009). The use of cane molasses for bioethanol production has been one of the key drivers behind the expansion of the South African bioethanol production industry (Van Zyl & Prior, 2009). With major bioethanol producing companies like NCP alcohol and Illovo sugar producing millions of liters of bioethanol per annum from cane molasses.

The nutrient composition of cane molasses varies between regions, seasons and cultivation conditions under which the cane was grown (Olbrich, 1963). This variability in cane molasses nutrient composition constitutes one of the major drawbacks of working with cane molasses as a fermentation substrate for fermentation studies under laboratory conditions. This has prompted the need for the development of a standardised chemically defined molasses medium that facilitates the generation of more consistent and reliable fermentation data. Tosun and Ergun (2007) have previously reported a synthetic cane molasses medium formulation based on the composition of cane molasses attained from a sugar company based in Turkey. While these authors sought to investigate the effects of zinc, sodium and potassium ion concentrations on yeast fermentation ability in the synthetic cane molasses, they omitted to study the fermentation kinetic profiles of the yeast in the synthetic cane molasses medium *versus* cane molasses.

Therefore, this has led to the present study, which seeks to develop a chemically defined molasses medium formulation based on nutrient composition data of 10 different cane molasses batches as provided by SASRI. The chemically defined molasses will be supplemented with yeast extract, peptone or casein hydrolysate as nitrogen source. The fermentation profiles of a laboratory strain and commercially available *S. cerevisiae* strains employed in the South African bioethanol industry will be evaluated in chemically defined molasses medium and cane molasses (sourced from three South African-based sugar mills). This was pursued in an effort to develop a standardized laboratory culture medium which can be routinely employed in molasses fermentation studies thereby overcoming the nutritional variance that is often encountered with different batches of industrially-derived cane molasses.

3.3 MATERIALS AND METHODS

Chemicals, solvents and media used in this study were of analytical grade and purchased from Merck (Pty) Ltd, South Africa, unless otherwise stated. Vitamins were purchased from Sigma Aldrich, Germany.

3.3.1 Strains

The yeast strains employed in this study are listed in table 3.1:

Table 3.1 *S. cerevisiae* strains employed in this study

Strain	Source
Angel yeast (sugar tolerant)	Angel Yeast Co., Ltd, China
Cream yeast	Lallemand Inc., Montreal, QC, Canada
Dry yeast (high sugar)	Premier, Guangxi Sungain Yeast technology Co., Ltd., China
BY4743	Euroscaf, Frankfurt, Germany

3.3.2 Media and cultivation conditions

The routine cultivation of yeast strains was carried out in 2% YEPD medium which contained 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose at 30ºC, for all solid media; 2% (w/v) Agar (Difco, Detroit, MI) was used. Single colonies from three-day old YEPD plates were used to inoculate 20 mL YEPD of experimental cultures contained in 100 mL Erlenmeyer flasks. These were incubated at 30ºC with shaking (160 rpm) for 16 hours in an Infors HT Multitron environmental shaker (United Scientific, South Africa). Yeast strains were stored at -80ºC as freeze cultures in YEPD supplemented with 15% (v/v) glycerol (Sigma Aldrich, Germany).

3.3.3 Chemically defined molasses medium

The composition of the chemically defined molasses medium was formulated based on chemical compositional data that was kindly furnished by SASRI. The data represented the composition of ten molasses samples sourced from different sugar mills in South Africa. The average of the ten samples was used to formulate the synthetic molasses in terms of essential components that facilitate yeast growth. The synthetic molasses comprised of (expressed per litre): sucrose (110 g), glucose (16 g), fructose (24 g), $MgSO_4.7H_2O$ (16.3 g), $FeSO_4.H_2O$ (0.0779 g), $CuSO_4.5H_2O$ (0.00277 g), $ZnSO_4.7H_2O$ (0.00815 g), K_2HPO_4 (33.44 g). NaH_2PO_4 (10.25 g), $CaCl_2.2H_2O$ (9.31 g), pantothenate (0.00324 g), thiamine (0.000868 g),

riboflavin (0.000529 g), biotin (0.0000713 g), pyridoxine (0.00147 g). Additionally as a nitrogen source either casein hydrolysate; peptone or yeast extract was added at 10g/L. All the chemicals were of analytical grade. From here on, the chemically defined molasses media supplemented with different nitrogen source will be referred to as: CDM-CH (casein hydrolyzate), CDM-P (peptone) and CDM-YE (yeast extract).

3.3.4 Sugar cane molasses

Molasses samples were obtained from three South African-based sugar mills; Amatikulu, Felixton and Gledhow. The molasses samples were stored at 4°C until use.

3.3.5 Batch fermentations

Pre-inoculum was prepared by inoculating sterile 50 mL aliquots of diluted cane molasses containing 5% (w/v) total initial sugar (fructose, glucose and sucrose) in 250 mL Erlenmeyer flasks with 1 mL starter-inoculum that was obtained from yeast cultures grown at 30ºC for 16 hours with shaking (160rpm) in YEPD. The diluted cane molasses precultures were then incubated at 30ºC with shaking (160 rpm) for 10 hrs. Thereafter the cell density of precultures was determined using a hemacytometer counting chamber (Bright-Line™, Buffalo, N.Y., USA). Batch fermentations were initiated by inoculating 50 mL aliquots of diluted cane molasses containing 15% (w/v) total initial sugar at a cell density of 1×10^8 cells mL^{-1} that was obtained from precultures. The fermentations were carried out in 250 mL Erlenmeyer flasks fitted with fermentation airlocks and incubated statically at 30ºC for 48 hours (Memmert incubator INE 600, Germany, Figure 3.1).

Figure 3.1 An illustration of the fermentation setup used for the fermentation of cane molasses (left) and chemically defined molasses (right)

To determine the progress of fermentations, carbon dioxide release was monitored at 8 hour intervals by measurement of fermenter weight loss. Additionally, at these intervals samples were withdrawn for analysis under aseptic conditions as swiftly as possible to limit the fermentations exposure to oxygen.

3.3.6 High-Pressure Liquid Chromatography (HPLC).

Prior to analysis, all fermentation samples were centrifuged for 5 minutes at 12 000 rpm (centrifuge 5417R, Eppendorf, Germany) and filtered through a 0.2 µm cellulose acetate filter (GVS Filter Technology Inc., USA). Chromatographic separations were performed on a Shodex KS-801 column (Shodex, Japan). The Shimadzu Prominence LC-20A Series system consisted of a binary pump system (LC-20AB), automatic injector furnished with a 100 µL loop, refractive index detector (RID-10A) maintained at 40°C (Shimadzu corporation, Kyoto, Japan), and LCSolution chromatography software (version 1.25). The thermostatically controlled column chamber was set at 75 ºC and elution was performed with 0.2 µm filtered distilled water at a flow rate of 1 mL min^{-1}. Quantification of glucose, fructose, sucrose, glycerol and ethanol was performed using external standards prepared from chemically pure compounds (Sigma-Aldrich, Missouri, USA).

3.3.7 Ethanol yield, productivity and sugar conversion

The ethanol yield, productivity and sugar conversion were calculated according to the following equations where S_0: initial sugar concentration (g/L), S_f: final sugar concentration (g/L), P_f: final ethanol concentration (g/L) and t: fermentation duration (h):

$$\text{Ethanol yield } (Y_{P/S}) = \frac{P_f}{(S_0 - S_f)}$$

$$\text{Ethanol productivity } (g.L^{-1}.h^{-1}) = \frac{P_f}{t}$$

$$\text{Sugar conversion } (\%) = \frac{(S_0 - S_f)}{S_0} \times 100$$

3.3.8 Statistical analysis

One-way analysis of variance (ANOVA) was employed to statistically compare fermentation data of all yeast strains employed in this study. In addition, the data sets that were acquired from the fermentations of chemically defined molasses and cane molasses were also evaluated. Analyses were performed using the statistical software package GraphPad Prism version 5.00 for Windows (GraphPad Software, Inc., San Diego California).

3.4. RESULTS

3.4.1 Fermentation profiles

The fermentation profiles of BY4743, Angel, cream and dry yeast strains in three different cane molasses batches were monitored by measuring the evolution of CO_2 as the fermentations progressed (Figure 3.2). The CO_2 release trends of all strains were similar in that fermentations were most active at 24 hours and subsided after 32 hours. It was also observed that the cream yeast strain displayed the highest fermentation rate followed by decreasing rates for the Angel, dry and BY4743 yeast strains.

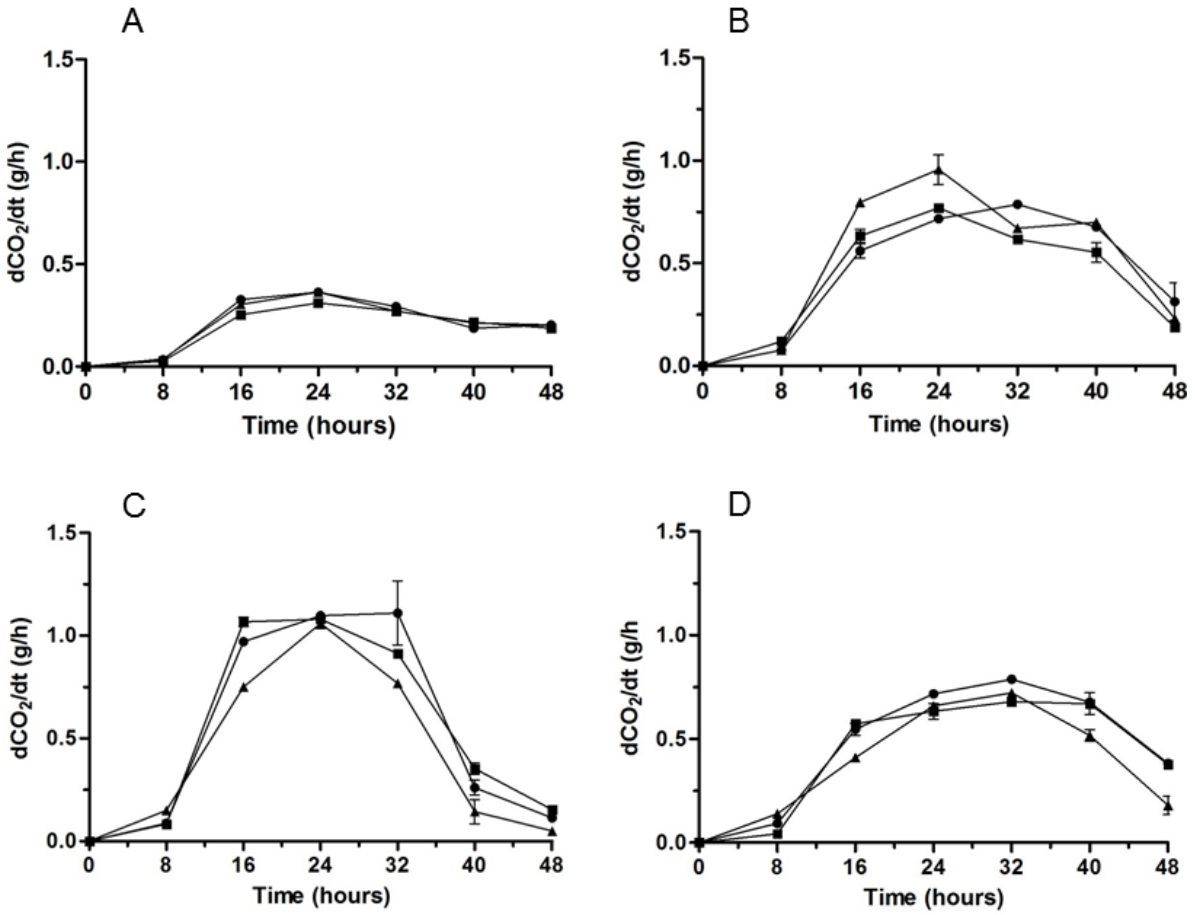

Figure 3.2 The fermentation profiles of BY4743 (A), Angel (B), cream (C) and dry (D) yeast strains in cane molasses sourced from Amatikulu (●), Gledhow (■) and Felixton (▲).Values represent the mean of experiments performed in triplicate, and error bars represent standard deviations.

The fermentation profiles of yeast strains when cultivated in chemically defined molasses substrates supplemented with different nitrogen sources are presented in Figure 3.3. The CO_2 release trends of all strains were similar in that fermentations were most active at 24 hours and subsided after 32 hours. In all synthetic cane molasses substrates, the cream yeast displayed superior fermentation rates followed by decreasing rates for Angel, dry and BY4743 yeast strains. It was observed that synthetic CDM-YE sustained the highest fermentation rates for all strains. Interestingly, the cream yeast also displayed the highest fermentation rate in synthetic CDM-YE which is congruent with that observed when industrially-derived cane molasses was utilised. However, all yeast strains displayed diminished fermentation efficiencies when grown in synthetic CDM-CH or CDM-P molasses. There was no statistically significant difference ($p > 0.05$) observed in yeast fermentation rates when cultivated in either CDM-YE or cane molasses.

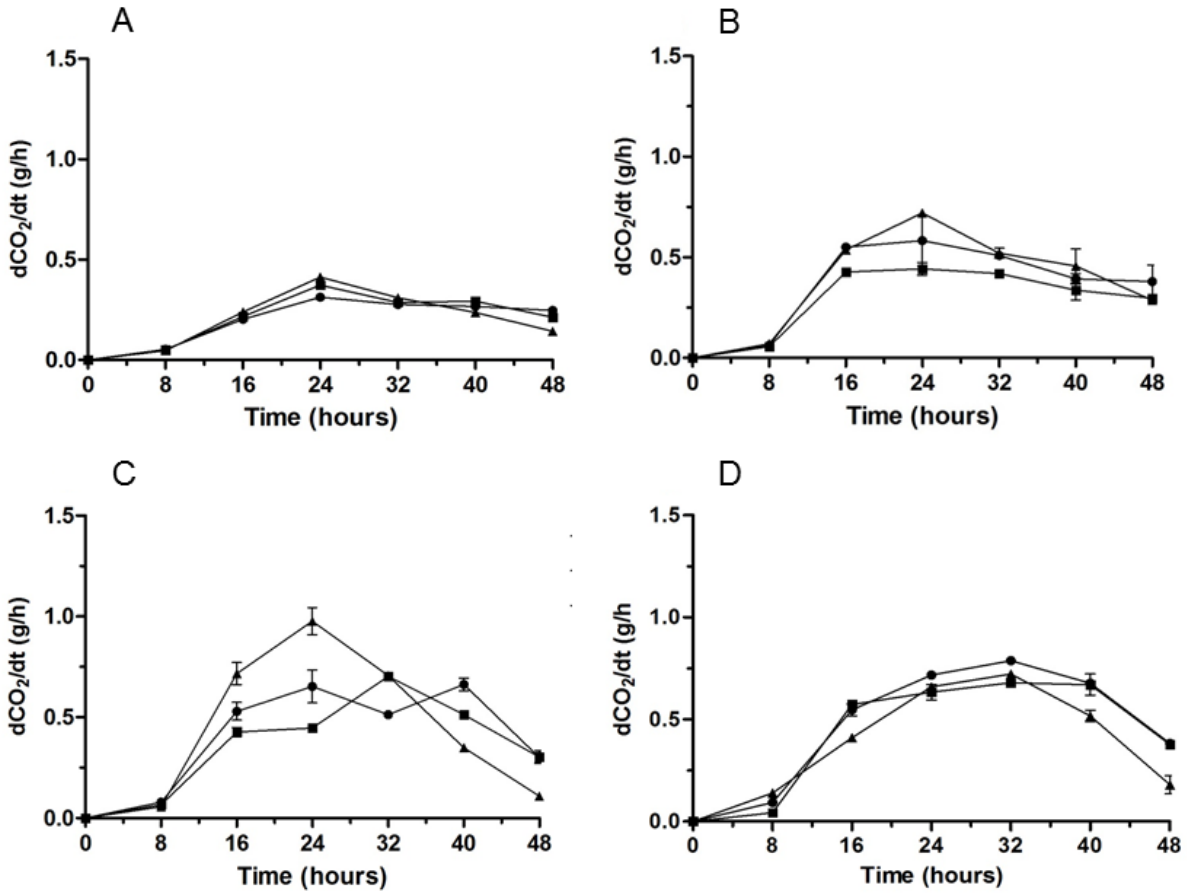

Figure 3.3 The fermentation profiles of BY4743 (A), Angel (B), cream (C) and dry (D) yeast strains in synthetic CDM-CH (●), CDM-P (■) and CDM-YE (▲) molasses. Values represent the mean of experiments performed in triplicate, and error bars represent standard deviations.

3.4.2 HPLC analysis

Prior to their use in batch fermentations, the total initial sugar content of the three cane molasses samples was adjusted to 150 g/L. The total sugar content of these fermentation substrates as determined by HPLC analysis comprised predominantly of sucrose (~110 g/L) and to a lesser extent glucose and fructose (~40 g/L). The high residual total sugars ranging from 60 to 67 g/L for the different cane molasses samples observed on completion of fermentations by laboratory yeast strain BY4743 implies that it has a markedly reduced sugar utilization ability when compared to other yeast strains employed in this study (Figure 3.4). This also resulted in the BY4743 strain yielding the lowest ethanol concentrations ranging from 45 to 53 g/L when fermenting the different cane molasses samples. Fermentations completed by the cream, Angel and dry yeast strains were characterized by lower residual total sugars. In this regard the cream yeast (3-8 g/L) was the most efficient followed by Angel yeast (11-15 g/L) and then the dry yeast strain (7-23 g/L). A similar trend in terms of ethanol production from different cane molasses fermentations by the industrial yeasts was observed viz. cream yeast (89-93 g/L), Angel yeast (85-88 g/L) and dry yeast (75-90 g/L).

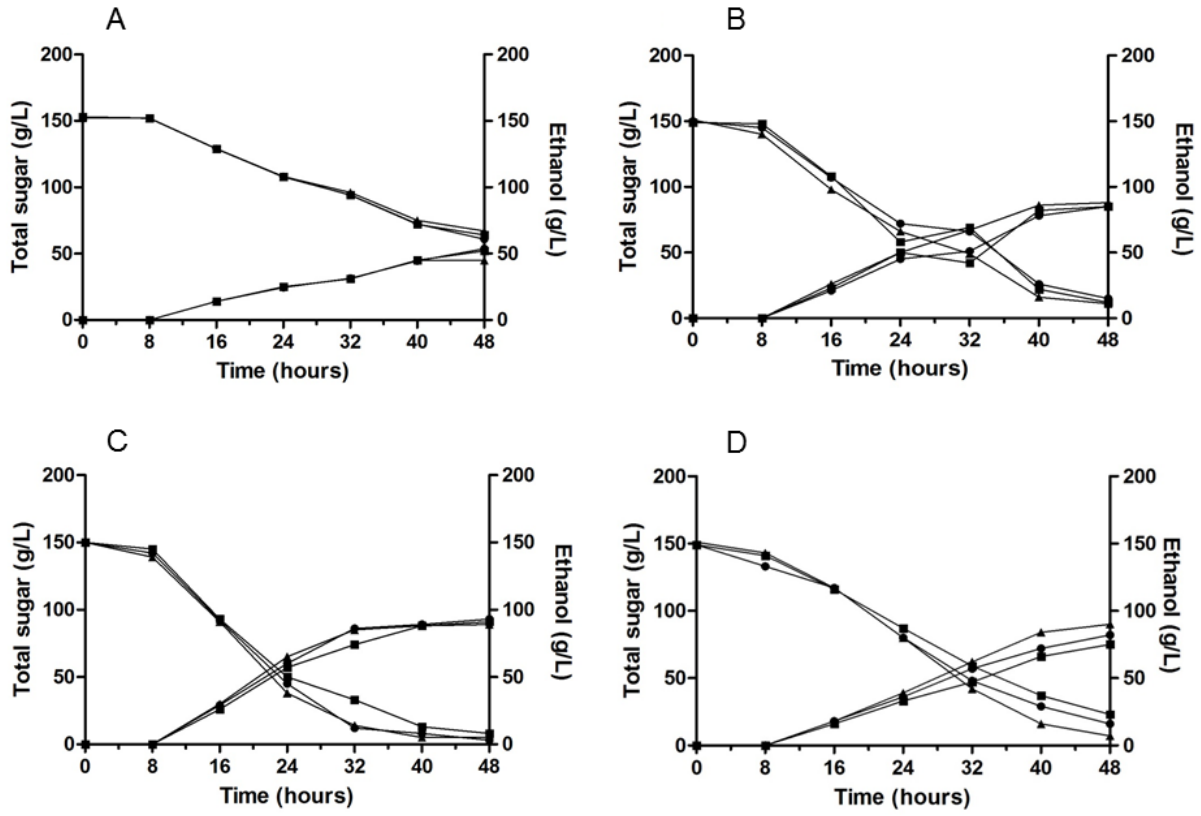

Figure 3.4 Total sugar utilization and ethanol production by BY4743 (A), Angel (B), cream (C) and dry (D) yeast strains fermenting cane molasses sourced from Amatikulu (●), Gledhow (■) and Felixton (▲) sugar mills. Values represent the mean of experiments performed in triplicate, and error bars represent standard deviations.

In terms of by-product formation, the BY4743 laboratory yeast strain produced the least amount of glycerol (6 g/L) across the three cane molasses samples when compared to the industrial yeast strains (Figure 3.5). It was observed that glycerol production by cream and dry yeast (10-11 g/L) were similar whilst the Angel yeast (8-10 g/L) yielded a lower amount. The accumulation of glycerol in the fermentation broth was noticeable after 8 hours, and steadily increased and peaked at the 40 hours.

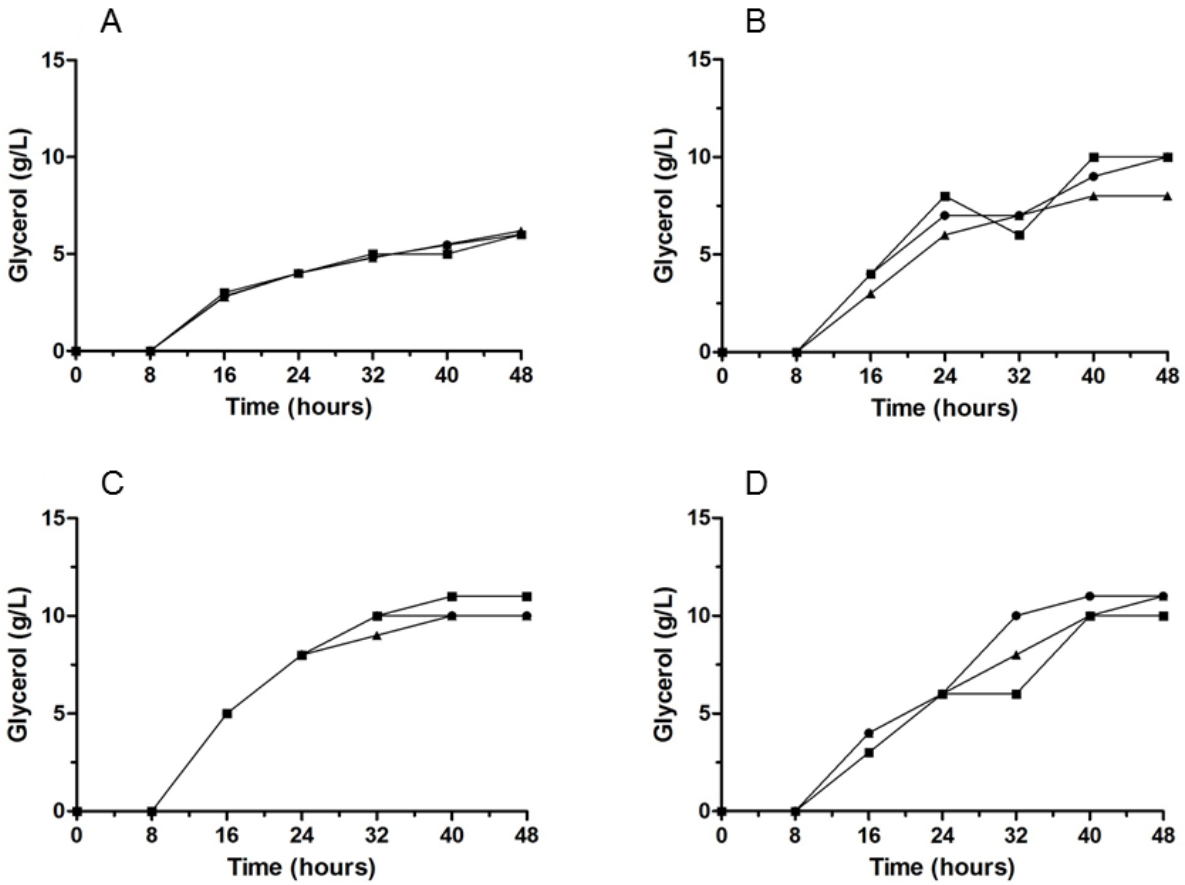

Figure 3.5 The formation of the glycerol by-product over time by BY4743 (A), Angel (B), cream (C) and dry (D) yeast strains in cane molasses sourced from Amatikulu (●), Gledhow (■) and Felixton (▲) sugar mills. Values represent the mean of experiments performed in triplicate, and error bars represent standard deviations.

After 48 hours of fermentation of the three types of chemically defined molasses (Figure 3.6), BY4743 showed a similar trend to that observed in cane molasses, with substantial residual sugars (54-63 g/L) and low final ethanol concentrations (41-51 g/L) at the end of fermentation. It was observed that in fermentations completed by cream, Angel and dry yeast strains were characterized by lower residual total sugars. The decreasing order of sugar utilization ability for industrial yeasts was as follows; cream yeast (3-17 g/L),

Angel yeast (8-13 g/L) and dry yeast (6-41 g/L). A similar trend in terms of ethanol production from the different chemically defined molasses fermentations by the industrial yeasts was observed viz. cream yeast (72-85 g/L), Angel yeast (67-78 g/L) and dry yeast (50-80 g/L). The yeast strains displayed a marginally faster rate of sugar utilization and ethanol formation in synthetic CDM-YE when compared to synthetic CDM-CH molasses. All fermentations completed by industrial yeast strains in synthetic CDM-P displayed 2-fold higher residual sugars when compared to the fermentation of cane molasses.

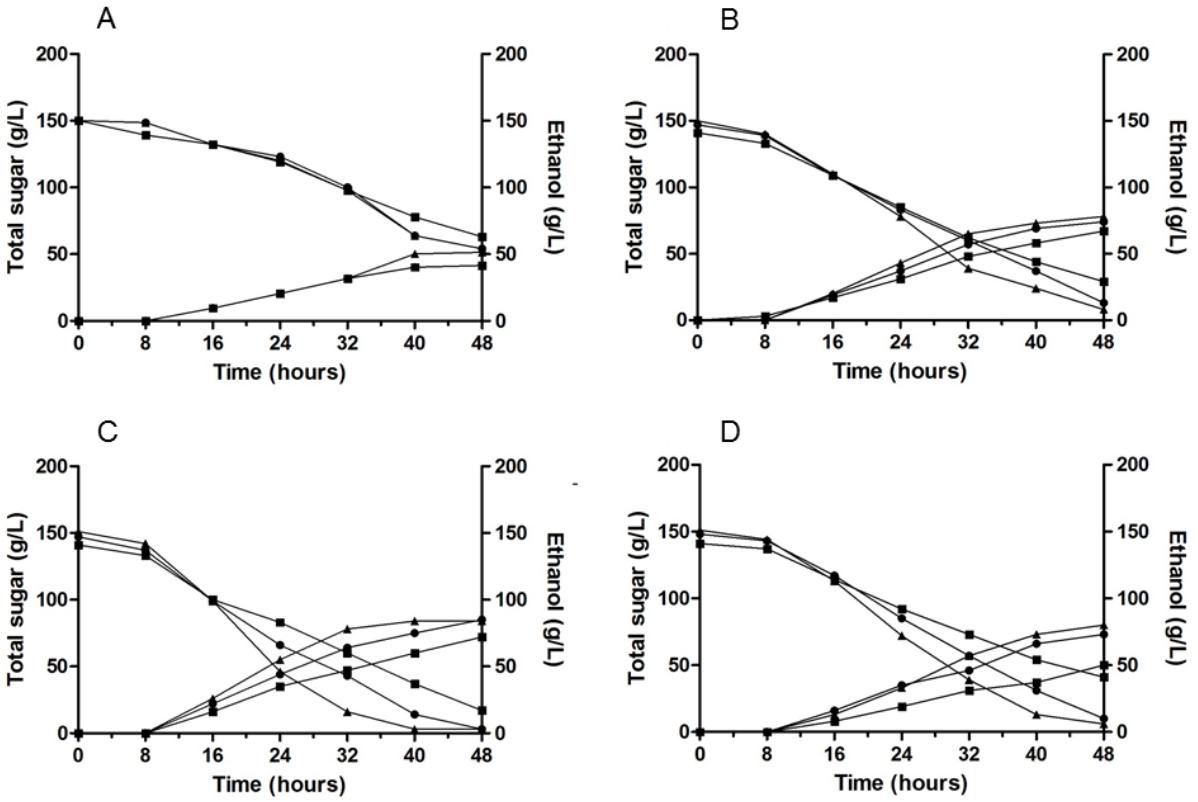

Figure 3.6 Total sugar utilization and ethanol production by BY4743 (A), Angel (B), cream (C) and dry (D) yeast strains in synthetic CDM-CH (●), CDM-P (■) and CDM-YE (▲). Values represent the mean of experiments performed in triplicate, and error bars represent standard deviations.

There was no significant difference ($p > 0.05$) in the final glycerol concentrations produced by the industrial yeast strains in all types of chemically defined molasses (Figure 3.7). Glycerol accumulation in the fermentation broths was noticeable after the 8 hour mark and peaked at 32 hours. The final glycerol levels attained with commercial industrial yeasts were higher than that of the BY4743 yeast strain (4-4.7 g/L). It was observed that the descending order of glycerol production was as follows; dry yeast (10-11 g/L), cream yeast (9-11 g/L) and then Angel yeast (8-9 g /L).

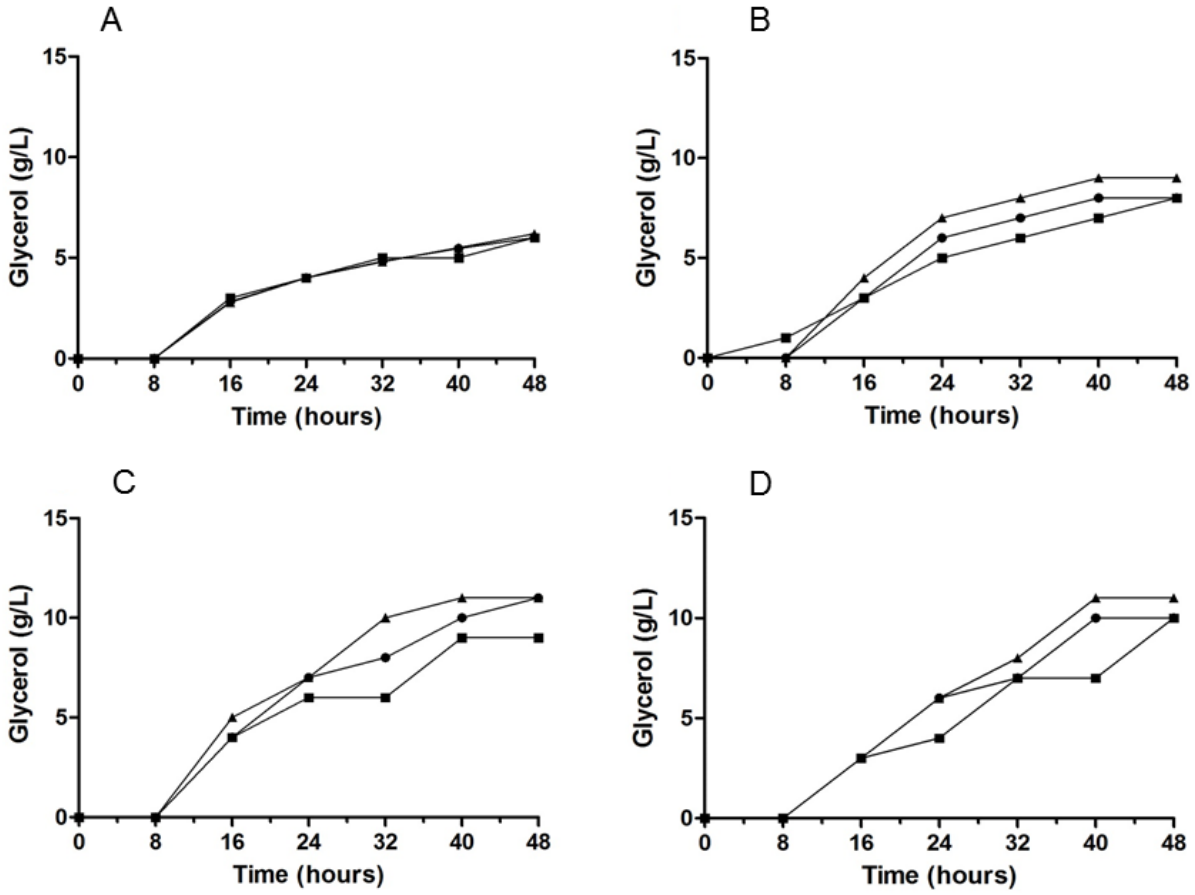

Figure 3.7 The formation of the by-product glycerol by BY4743 (A), Angel (B), cream (C) and dry (D) yeast strains in synthetic CDM-CH (●), CDM-P (■) and CDM-YE (▲).Values represent the mean of experiments performed in triplicate, and error bars represent standard deviations.

3.4.3 Ethanol yield, productivity and sugar conversion

The fermentation profiles observed in the fermentation of cane molasses and chemically defined molasses are presented in Table 3.2. The commercial yeast strains displayed similar fermentation abilities in the 3 different cane molasses. The BY4743 yeast strain catalysed sluggish fermentations, with substantial residual sugars at the end of the fermentation. Cream yeast generated slightly higher ethanol yields, productivity and sugar conversions when compared to Angel and dry yeast in the fermentation of Amatikulu and Felixton cane molasses. Interestingly, the fermentation profiles observed in both synthetic CDM-YE and cane molasses by all yeast strains were comparable in all evaluated parameters. The synthetic CDM-P was associated with relatively poor yeast fermentative performance in comparison to the other synthetic media. This was exemplified by substantial decreases in ethanol yields, especially with dry yeast which had the least ethanol titre (50 g/L) and yield (0.049).

Table 3.2 Fermentation of cane and chemically defined molasses

Molasses Substrate	Yeast strain	S_0 (g/L)	S_f (g/L)	Conversion (%)	Ethanol titre (g/L)	Ethanol yield ($Y_{P/s}$)	Ethanol productivity (g L^{-1} h^{-1})	Glycerol titre (g/L)
Amatikulu	BY4743	150	60	60	53	0.589	1.104	6
	Angel	150	15	90	85	0.630	1.771	10
	Cream	150	3	98	93	0.633	1.938	10
	Dry	150	16	89	82	0.612	1.708	11
Felixton	BY4743	150	64	57	52	0.605	1.083	6
	Angel	150	12	92	85	0.616	1.771	10
	Cream	150	8	95	91	0.641	1.896	11
	Dry	150	23	85	75	0.591	1.563	10
Gledhow	BY4743	150	67	55	45	0.542	0.938	6
	Angel	150	11	93	88	0.633	1.833	8
	Cream	150	5	97	89	0.614	1.854	10
	Dry	150	7	95	90	0.629	1.875	11
CDM-CH	BY4743	150	54	64	41	0.427	0.854	4
	Angel	150	13	91	74	0.540	1.542	8
	Cream	150	3	98	85	0.578	1.771	11
	Dry	150	10	93	73	0.521	1.521	10
CDM-P	BY4743	150	63	58	41	0.471	0.854	4
	Angel	150	10	93	67	0.479	1.396	8
	Cream	150	17	87	72	0.541	1.5	9
	Dry	150	41	73	50	0.459	1.042	10
CDM-YE	BY4743	150	54	64	51	0.531	1.063	4.7
	Angel	150	8	95	78	0.549	1.625	9
	Cream	150	3	98	84	0.571	1.75	11
	Dry	150	6	96	80	0.556	1.667	11

S_0: initial sugar concentration (g/L)

S_f: final sugar concentration (g/L)

3.5 DISCUSSION

This study reports on the formulation of a chemically defined molasses fermentation substrate as a standardised laboratory medium which can substitute the use of industrially derived molasses in fermentation studies. The development of such a synthetic substrate will overcome variations in the nutritional composition of different batches/sources of industrially derived cane molasses that results in inconsistent and sometimes erroneous laboratory fermentation data.

The control yeast strain, BY4743 is a well characterized diploid laboratory yeast strain. The fermentation of both cane molasses and chemically defined molasses with BY4743 resulted in sluggish fermentations, characterized by low ethanol yields and poor sugar attenuation. The cream and Angel yeasts outperformed dry yeast and BY4743 in terms of achieving higher ethanol titres and yields. This may lie in yeast strains possessing different ethanol tolerance capacities or thresholds, with dry yeast seemingly the least tolerant to high ethanol concentrations amongst the commercial yeast strains. These findings are supported by research findings of Della-Bianca and Gombert (2013) which showed that distiller's yeast were more tolerant to high ethanol levels when compared to laboratory and baker's yeast strains.

The inclusion of different nitrogen sources (casein hydrolysate, peptone and yeast extract) at 10 g/L in synthetic molasses was based on a previous study that reports nitrogen content of cane molasses ranges from 4-15 g/L (Olbrich, 1963). The fermentation profiles of the yeast strains employed in this study of synthetic CDM-YE molasses was most similar to those observed when cane molasses was used as substrate. All the yeast strains showed decreased fermentation rates and ethanol yields in synthetic CDM-CH and CDM-P. It has been reported that the tolerance of yeast to ethanol is not only governed by the genetic makeup of the yeast, but also the nutritional conditions the yeast is facing (Bely *et al.*, 1990; Thomas & Ingledew, 1990). Nitrogen has been extensively reported as one of the principal factors that plays a vital role in yeast growth, sugar assimilation and ethanol tolerance. Research studies have shown that when assimilable nitrogen is below optimum levels in the fermentation broth it leads to poor yeast growth and sugar metabolism (Bely *et al.*, 1990; Thomas & Ingledew, 1990; Beltran *et al.*, 2005). The data seems to suggest that 10 g/L of yeast extract is an effective nitrogen source in synthetic molasses which suitably mimics cane molasses as opposed to the inclusion of either casein hydrolyzate or peptone. There was no marked change in glycerol outputs across the different molasses media by individual yeast strains, suggesting that yeast metabolism responsible for glycerol production was not

affected. This finding is supported by the study conducted by Yalcin and Ozbas (2008) who reported that glycerol production is primarily affected by pH and temperature.

3.6 CONCLUSION

The individual yeast strains employed in this study displayed similar fermentation profiles across the three cane molasses samples. The fermentation profiles by individual yeast strains in synthetic CDM-YE closely mirrored profiles produced by the same yeast when fermenting cane molasses. In addition, it was also observed that irrespective of the fermentation substrate involved, cream yeast produced the highest ethanol output followed by angel, dry and BY4743 yeast strains. The attained results seem to suggest that the synthetic CDM-YE can be employed as a standardised laboratory culture medium in cane molasses fermentation studies. Thereby allowing for the generation of more consistant and reliable fermentation data.

3.7 ACKNOWLEDGEMENTS

This study was made possible through financial support from the National Research Foundation. The research facilities were provided by the University of KwaZulu-Natal. The South African Sugar Research Institute and Mr Ace Govender (NCP alcohol) also offered invaluable support.

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Chapter 4

RESEARCH RESULTS 2

Encapsulation of industrial yeast for increased bioethanol yields

Encapsulation of industrial yeast for increased bioethanol yields

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4.1 ABSTRACT

Increased bioethanol yield is commercially attractive to the relevant fermentation-based industries. In this study, cell encapsulation as a means of increasing bioethanol yields, along with the possibility of reusing the capsules for more than one fermentation cycle were investigated. The fermentation profiles of free-suspended industrial yeast cells were paralleled to their encapsulated derivatives. Angel and cream yeast strains were encapsulated into calcium-alginate, alginate-chitosan or low melting point agarose capsules. Amatikulu cane molasses and chemically defined molasses containing yeast extract (CDM-YE) were subjected to batch fermentations in 250 mL Erlenmeyer flasks at 30°C for 48 hours. The following parameters were used to monitor fermentations: evaluation of CO_2 evolution *via* flask weight measurements, sugar utilization and product formation (ethanol and glycerol). Batch fermentations with Angel yeast encapsulated into calcium alginate and alginate-chitosan capsules displayed a marginal increase in bioethanol outputs. However, fermentations with Angel yeast encapsulated in low melting point agarose resulted in significantly higher (~10%) ethanol yields when compared to their free-suspended cell counterparts. Fermentations with encapsulated cream yeast resulted in ethanol yields similar to those attained in fermentations with their free-suspended cell counterparts. Due to their stability in fermentation environments, the capsule reusability of alginate-chitosan and low melting point agarose capsules were assessed. The low melting point agarose capsules remained stable and active through the three sequential batch fermentations whilst maintaining the same ethanol output. On the contrary, the alginate-chitosan capsules showed signs of breakage during the third fermentation cycle.

4.2 INTRODUCTION

The bioethanol production industry produces copious amounts of bioethanol annually, and it does so by heavily relying on the inherent ability of *Saccharomyces cerevisiae* to biologically convert fermentable sugars into bioethanol (Lin & Tanaka, 2006). Over the years, various bioethanol production strategies have been designed and refined to maximize outputs, however some scientific innovation and intervention is still very much required in order to further increase the bioethanol production capacities of these industrial processes (Gray *et al.*, 2006; Wang *et al.*, 2007; Mariam *et al.*, 2009; Breisha, 2010). This is because in most large scale production processes, the fermenting yeast is exposed to a wide spectrum of environmental stresses which hinder optimal yeast performance, therefore restricting substrate conversion and subsequently, final product (bioethanol) formation (Park & Chang, 2000; Wang *et al.*, 2007). Such stresses have deleterious effects on cell growth, viability and performance and they include; high ethanol concentrations that accumulate as the fermentation progresses (especially in batch bioethanol fermentations), osmotic pressure, oxidative stress, heat and pH. Thus signaling the need for yeast with enhanced robustness characteristics that are able to perform optimally under the adverse conditions they encounter during fermentations (Lin & Tanaka, 2006; Sun *et al.*, 2007; Ylitervo *et al.*, 2011).

The need for more robust yeast has since attracted considerable global scientific interest and research resources. It is hypothesized that yeast with increased robustness characteristics might display heightened survival and biomass conversion efficiencies, resulting in higher ethanol yields and a substantial augmentation of the economic gains for the bioethanol industry. The industrial use of genetically modified microbial strains is still viewed negatively by the public, therefore signifying the need for other possible strategies to increase ethanol yields without any genetic manipulation (Talebnia, 2008; Lee *et al.*, 2011; Ylitervo *et al.*, 2011; Westman *et al.*, 2012; Rathore *et al.*, 2013; Ylitervo *et al.*, 2013). The immobilization of yeast cells by cell encapsulation into semi-permeable hydrogels has been touted as the possible solution to increasing ethanol yields. Cell encapsulation is defined as the physical isolation of the cell mass from the outside environment, while maintaining normal cell physiology within a barrier of desired permeability (Galazzo & Bailey, 1990; de Vos *et al.*, 2009; Westman *et al.*, 2012; Ylitervo *et al.*, 2013). The encapsulation of cells is a multifaceted technique and has been employed across various fields like; biomedicine, food production, agriculture, waste water treatment, environmental decontamination, production of therapeutic agents, enzymes and biofuels (de Vos *et al.*, 2009; Burgain *et al.*, 2011; Whelehan, 2011; Rathore *et al.*, 2013; Ylitervo *et al.*, 2013).

Cell encapsulation has been previously reported to increase yeast robustness and therefore allow for encapsulated cells to thrive and ferment in conditions where otherwise free-suspended cells would not (Pourbafrani *et al.*, 2007; Talebnia, 2008). The encapsulation of *S. cerevisiae* has also been reported to increase bioethanol yields in the fermentation of mahula flowers (Behera *et al.*, 2010) and a glucose-based substrate (Lee *et al.*, 2011). However research studies are yet to be initiated in the assessment of the encapsulation of *S. cerevisiae* into calcium alginate, alginate-chitosan and low melting point agarose as viable tools for increasing ethanol yields in cane molasses fermentations. Thus giving rise to this present study, which seeks to encapsulate commercial yeast that are commonly employed in the South African bioethanol production industry (Angel and cream yeast strains) into capsules produced by ionotropic (calcium alginate and alginate-chitosan) and thermal (low melting point agarose) gelation. The encapsulated yeast cells will then be used in the fermentation of chemically defined molasses and Amatikulu sugarcane molasses supplemented with yeast extract. This will investigate whether encapsulated yeast cells produce superior bioethanol yields in comparison to their free-suspended cell counterparts in molasses fermentations. The reusability of the capsules for more than one fermentation cycle will also be evaluated.

4.3 MATERIALS AND METHODS

Chemicals, solvents and media used in this study were of analytical grade and purchased from Merck (Pty) Ltd, South Africa, unless otherwise stated. Vitamins were purchased from Sigma Aldrich, Germany.

4.3.1 Strains

The yeast strains employed in this study are listed in table 4.1:

Table 4.1 *S. cerevisiae* strains employed in this study

Yeast strain	Source
Angel yeast (sugar tolerant)	Angel Yeast Co., Ltd, China
Cream yeast	Lallemand Inc., Montreal, QC, Canada

4.3.2 Media and cultivation conditions

The routine cultivation of yeast strains was carried out in 2% YEPD medium which contained 1% (w/v) yeast extract, 2%(w/v) peptone, and 2% (w/v) glucose at 30ºC, for all solid media; 2% (w/v) Agar (Difco, Detroit, MI) was used. Single colonies from three-day old YEPD plates were used to inoculate 20 mL YEPD of experimental cultures contained in 100 mL Erlenmeyer flasks. These were incubated at 30ºC with shaking (160 rpm) for 16 hours in an Infors HT Multitron environmental shaker (United Scientific, South Africa). Yeast strains were stored at -80ºC as freeze cultures in YEPD supplemented with 15% (v/v) glycerol (Sigma Aldrich, Germany).

4.3.3 Chemically defined molasses medium

The composition of the chemically defined molasses medium was formulated based on chemical compositional data that was kindly furnished by SASRI. The data represented the composition of ten molasses samples sourced from different sugar mills in South Africa. The average of the ten samples was used to formulate the synthetic molasses in terms of essential components that facilitate yeast growth. The synthetic molasses comprised of (expressed per litre): sucrose (110 g), glucose (16 g), fructose (24 g), $MgSO_4.7H_2O$ (16.3 g), $FeSO_4.H_2O$ (0.0779 g), $CuSO_4.5H_2O$ (0.00277 g), $ZnSO_4.7H_2O$ (0.00815 g), K_2HPO_4 (33.44 g). NaH_2PO_4 (10.25 g), $CaCl_2.2H_2O$ (9.31 g), pantothenate (0.00324 g), thiamine (0.000868 g), riboflavin (0.000529 g), biotin (0.0000713 g), pyridoxine (0.00147 g). Additionally as a nitrogen source yeast extract was added at 10g/L. All the chemicals were of analytical grade. This chemically defined molasses will hereon be referred to as CDM-YE.

4.3.4 Sugar cane molasses

The molasses sample employed in this aspect of the study was attained from the Amatikulu sugar mill was stored at 4°C.

4.3.5 Cell encapsulation

4.3.5.1 Calcium alginate

Calcium alginate capsules were prepared as described by Lee *et al.* (2011) and Najafpour *et al.* (2004). Yeast cultures were grown in 50 mL aliquots of YEPD to mid-exponential phase (16 hours) at 30ºC with shaking (160 rpm) in an Infors HT Multitron environmental shaker (United Scientific, South Africa). The optical density of the yeast cell cultures were adjusted to an OD_{600nm} of 4 (Analytik Jena Specord 210, Germany). To prepare the calcium alginate capsules, yeast cells were harvested from 16 hr yeast cultures by centrifugation at 1000 rpm

for 1 minute (centrifuge 5810R, Eppendorf, Germany) and then resuspended to 50 mL in 2% (w/v) sodium alginate (Sigma Aldrich, Germany). The alginate-cell mixture was extruded through a 23 gauge needle fitted to a peristaltic pump into 0.1 M calcium chloride (Sigma Aldrich, Germany). The calcium alginate capsules were allowed to form for 20 minutes, and then washed with 0.9% (w/v) saline to remove excess calcium chloride from the capsules.

4.3.5.2 Chitosan coating of calcium alginate capsules

Calcium alginate capsules were prepared as described above. The calcium alginate capsules were coated with a 0.040 M acetate buffer solution containing 0.2% (w/v) low molecular weight chitosan (Sigma Aldrich, Germany) and 300 mM $CaCl_2$ (Sigma Aldrich, Germany) at pH 4.5 for 24 hours at 4ºC. Excess chitosan was removed by washing the capsules with 0.9% (w/v) saline (Ylitervo *et al.*, 2011).

4.3.5.3 Low melting point agarose

Yeast cultures were grown in 50 mL aliquots of YEPD to mid-exponential phase (16 hours) at 30ºC with shaking (160 rpm). The optical densities of yeast cell cultures were adjusted to an initial OD_{600nm} of 4. To prepare the calcium alginate capsules, yeast cells were harvested from 16 hr yeast cultures by centrifugation at 1000 rpm for 1 minute (centrifuge 5810R, Eppendorf, Germany) and then resuspended to 50 mL in 4% (w/v) TopVision low melting point agarose (Thermo Scientific, USA) cooled to room temperature. The agarose-cell mixture was extruded through a 23 G needle with the aid of a peristaltic pump into ice cold sterile distilled water to facilitate rapid capsule formation.

4.3.6 Batch fermentations

Batch fermentations of Amatikulu cane molasses and synthetic CDM-YE molasses were performed with free-suspended and encapsulated yeast cells. For free-suspended yeast fermentations, pre-inoculum was prepared by inoculating sterile 50 mL aliquots of diluted cane molasses containing 5% (w/v) total initial sugar (fructose, glucose and sucrose) in 250 mL Erlenmeyer flasks with 1 mL starter-inoculum that was obtained from yeast cultures grown at 30ºC for 16 hours with shaking (160rpm) in YEPD. The diluted cane molasses precultures were then incubated at 30ºC with shaking (160 rpm) for 10 hrs. Thereafter the cell density of precultures was determined using a hemacytometer counting chamber (Bright-Line™, Buffalo, N.Y., USA). Batch fermentations were initiated by inoculating 50 mL aliquots of diluted cane molasses containing 15% (w/v) total initial sugar at a cell density of 1×10^8 cells mL^{-1} that was obtained from precultures. The fermentations were carried out in 250 mL Erlenmeyer flasks fitted with fermentation airlocks and incubated statically at 30ºC for 48 hours (Memmert incubator INE 600, Germany, Figure 3.1). For encapsulated yeast

fermentations, 50 mL aliquots of the fermentation substrates were inoculated with 2 g (wet weight) of the capsules, i.e. calcium alginate, alginate–chitosan and low melting point agarose. The batch fermentations using encapsulated yeast were carried out as described for free-suspended cells above. To determine the progress of fermentations, carbon dioxide release was monitored at 8 hour intervals by measurement of fermenter weight loss. Additionally at these intervals samples were withdrawn for analysis under aseptic conditions as swiftly as possible to limit the fermentations exposure to oxygen. Capsule reusability was assessed by harvesting the capsules after fermentation, washing them with 0.9% saline and reusing them as inoculum for two subsequent fermentation cycles.

4.3.7 HPLC analysis

Prior to analysis, all fermentation samples were centrifuged for 5 minutes at 12 000 rpm (centrifuge 5417R, Eppendorf, Germany) and filtered through a 0.2 µm cellulose acetate filter (GVS Filter Technology Inc., USA). Chromatographic separations were performed on a Shodex KS-801 column (Shodex, Japan). The Shimadzu Prominence LC-20A Series system consisted of a binary pump system (LC-20AB), automatic injector furnished with a 100 µL loop, refractive index detector (RID-10A) maintained at 40°C (Shimadzu corporation, Kyoto, Japan), and LCSolution chromatography software (version 1.25). The thermostatically controlled column chamber was set at 75 ºC and elution was performed with 0.2 µm filtered distilled water at a flow rate of 1 mL min^{-1}. Quantification of glucose, fructose, sucrose, glycerol and ethanol was performed using external standards prepared from chemically pure compounds (Sigma-Aldrich, Missouri, USA).

4.3.8 Ethanol yield and sugar conversion

The ethanol yield and sugar conversion were calculated according to the following equations, where S_0: initial sugar concentration (g/L), S_f: final sugar concentration (g/L) and P_f: final ethanol concentration (g/L):

$$\text{Ethanol yield } \left(Y_{\frac{P}{S}}\right) = \frac{P_f}{(S_0 - S_f)}$$

$$\text{Sugar conversion } (\%) = \frac{(S_0 - S_f)}{S_0} \times 100$$

4.3.9 Statistical analysis

One-way analysis of variance (ANOVA) was employed to statistically compare data obtained from the fermentation of the Amatikulu cane molasses and synthetic CDM-YE with free-suspended Angel and cream yeast strains to that of their counterparts encapsulated in Ca-alginate, alginate-chitosan and low melting point agarose. Analyses were performed using the statistical software package GraphPad Prism version 5.00 for Windows (GraphPad Software, Inc., San Diego California).

4.4 RESULTS

4.4.1 Fermentation profiles

The fermentation profiles of Angel and cream yeast strains; free-suspended and encapsulated in calcium alginate, alginate-chitosan and low melting point agarose were evaluated in Amatikulu (Figure 4.1) and synthetic CDM-YE molasses (Figure 4.2). Generally it was observed that after an initial lag period of 8 hours in the two growth substrates both yeast strains in either form (free or encapsulated) displayed increasing fermentation activities. The free-suspended yeast strains displayed a slightly faster fermentation activity in comparison to their encapsulated derivatives. Calcium alginate encapsulated Angel and cream yeast displayed lower activities throughout the fermentations when compared to their free or other encapsulated forms. However, an interesting exception was observed in that cream yeast encapsulated in low melting point agarose displayed an extended lag phase of approximately 16 hours in both Amatikulu and synthetic CDM-YE molasses.

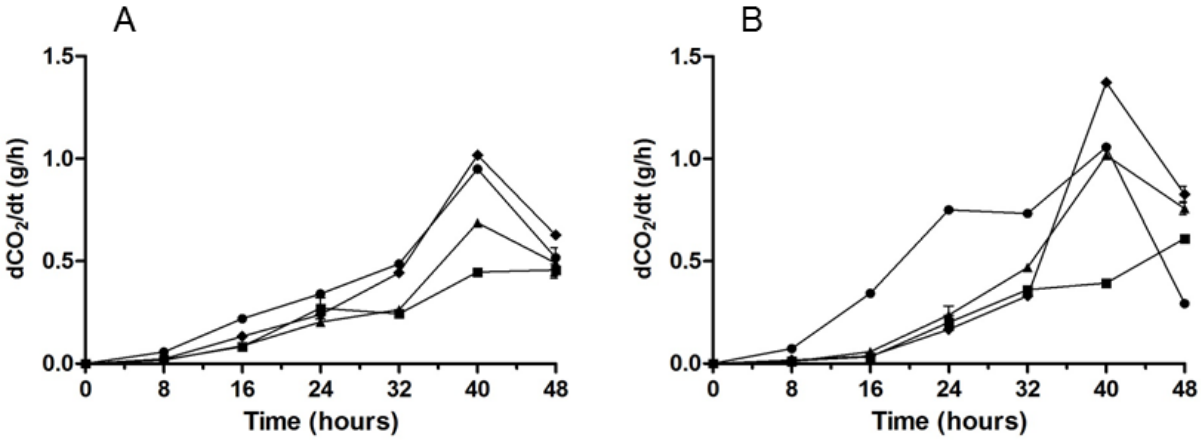

Figure 4.1 The fermentation profiles of Angel (A) and cream (B) yeast strains in Amatikulu cane molasses; free-suspended (●), encapsulated in calcium alginate (■), alginate-chitosan (▲) and low melting point agarose (♦). Values represent the mean of experiments performed in triplicate, and error bars represent standard deviations.

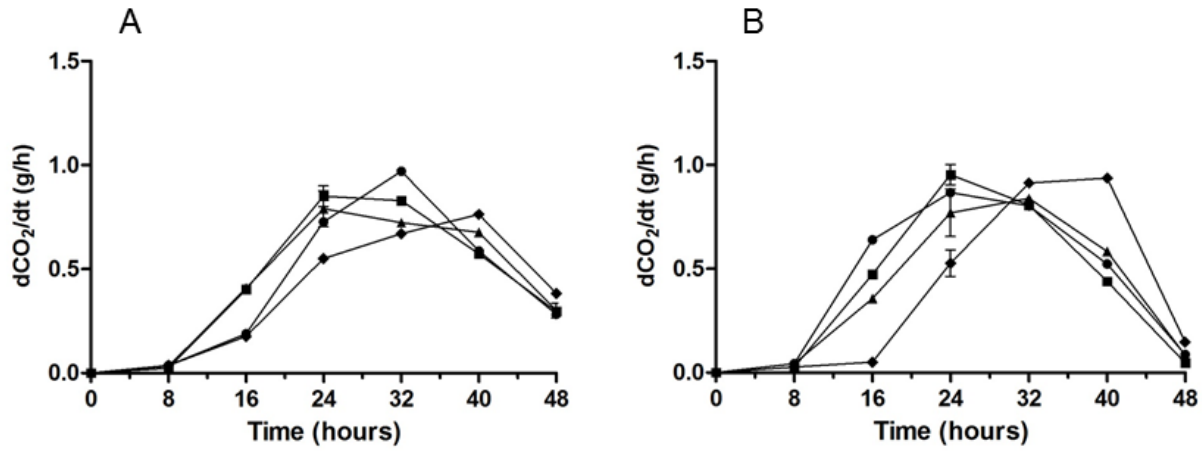

Figure 4.2 The fermentation profiles of Angel (A) and cream (B) yeast strains in synthetic CDM-YE; free-suspended (●), encapsulated in calcium alginate (■), alginate-chitosan (▲) and low melting point agarose (♦). Values represent the mean of experiments performed in triplicate, and error bars represent standard deviations.

4.4.2 HPLC analysis

In fermentations of Amatikulu cane molasses, the Angel yeast strain encapsulated in alginate-chitosan displayed the highest sugar utilization ability (lowest total residual sugars) in comparison to its free-suspended cell counterparts and its other encapsulated forms (Figure 4.3). The descending order of the sugar utilization ability of Angel yeast is as follows; alginate-chitosan (20.3 ± 0.757 g/L), low melting point agarose (26.5 ± 0.09 g/L), free-suspended (29.2 ± 0.07 g/L) and calcium alginate (52.3 ± 0.728 g/L). It was also observed that Angel yeast encapsulated in low melting point agarose produced the highest final ethanol concentration (100.1 ± 0.099 g/L), followed by alginate-chitosan (92.4 ± 0.520 g/L), free-suspended (91.4 ± 0.45 g/L) and then calcium alginate (72.2 ± 0.191 g/L). Free-suspended cream yeast showed the most efficient sugar assimilation capacity when fermenting Amatikulu cane molasses. This was typified by substantially lower residual total sugars at the end of the fermentations. The descending order of sugar utilization ability by the cream yeast strain is as follows; free-suspended (11.4 ± 0.152 g/L), alginate-chitosan (15.6 ± 0.067 g/L), low melting point agarose (16.8 ± 0.15 g/L) and then calcium alginate (22.3 ± 0.114 g/L). The final ethanol concentrations produced by cream yeast were; low melting point agarose (103.9 ± 0.101 g/L), alginate-chitosan (103.8 ± 0.174 g/L), free-suspended (103.4 ± 0.419 g/L) and calcium alginate (92.8 ± 0.124 g/L).

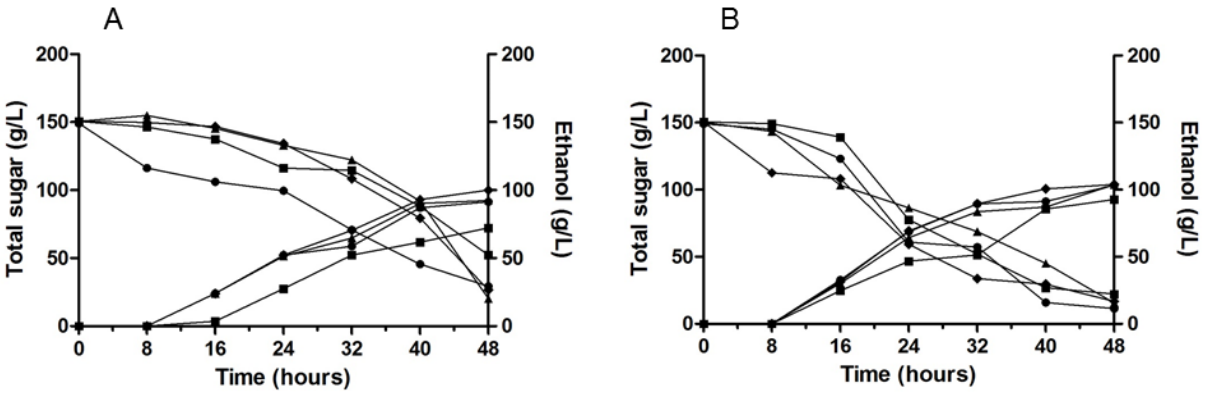

Figure 4.3 Total sugar utilization and ethanol production by Angel (A) and cream (B) yeast strains in Amatikulu cane molasses; free-suspended (●), encapsulated in calcium alginate (■), alginate-chitosan (▲) and low melting point agarose (♦). Values represent the mean of experiments performed in triplicate, and error bars represent standard deviations.

In terms of glycerol byproduct formation when fermenting Amatikulu cane molasses, the free-suspended yeast cells of both Angel and cream yeast showed higher glycerol formation rates when compared to their encapsulated cell counterparts (Figure 4.4). Both Angel and cream yeast strains when encapsulated in calcium alginate displayed significantly lower final glycerol titres ($p < 0.05$) when compared to cells encapsulated in alginate-chitosan and low melting point agarose. The descending order of glycerol production by the Angel yeast strain was as follows; low melting point agarose (10.7 ± 0.101 g/L), alginate-chitosan (10.5 ± 0.122 g/L), free-suspended (9.8 ± 0.015 g/L) and then calcium alginate (8.1 ± 0.012 g/L). Of note, a similar glycerol byproduct generation trend was observed for cream yeast strain in Amatikulu cane molasses fermentations. The descending order of glycerol ouput by the cream yeast strain was; low melting point agarose (13.6 ± 0.069 g/L), alginate-chitosan (11.4 ± 0.035 g/L), free-suspended (10.2 g/L) and then calcium alginate (9.5 ± 0.01 g/L).

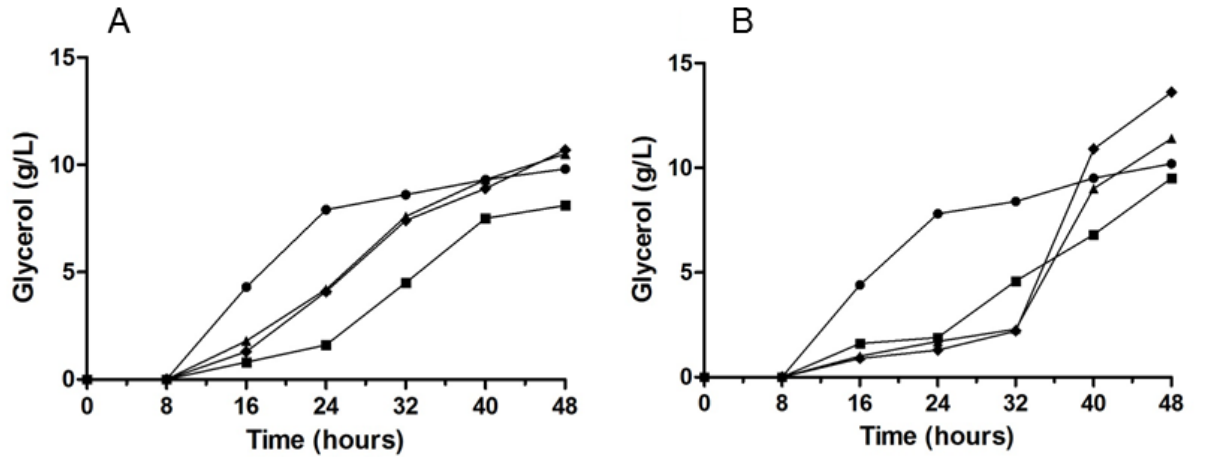

Figure 4.4 The formation of the glycerol by-product over time by Angel (A) and cream (B) yeast strains in Amatikulu cane molasses; free-suspended (●), encapsulated in calcium alginate (■), alginate-chitosan (▲) and low melting point agarose (♦). Values represent the mean of experiments performed in triplicate, and error bars represent standard deviations.

In the fermentation of synthetic CDM-YE molasses, the Angel yeast strain encapsulated in alginate-chitosan displayed a marginally higher sugar assimilation ability when compared to its free-suspended cell counterparts and those encapsulated in calcium alginate (Figure 4.5). However, Angel yeast encapsulated in low melting point agarose displayed substantially increased total residual sugars when fermenting synthetic CDM-YE molasses. The descending order of sugar utilization ability of Angel yeast was follows; alginate-chitosan (3.3 ± 0.012 g/L), free-suspended (3.9 ± 0.092 g/L), calcium alginate (3.9 ± 0.012 g/L) and then low melting point agarose (7.1 ± 0.01 g/L). In contrast and of interest Angel yeast encapsulated in low melting point agarose produced the highest final ethanol concentrations (88 ± 0.106 g/L), followed by alginate-chitosan (84 ± 0.231 g/L), calcium alginate (83 ± 0.124 g/L) and free-suspended (81.5 ± 0.15 g/L). There was no significant difference ($p>0.05$) in the sugar assimilation abilities displayed by the free-suspended and encapsulated cream yeast in the fermentation of synthetic CDM-YE. The sugar assimilation abilities of cream yeast were closely related i.e. free-suspended (2.9 ± 0.012 g/L), calcium alginate (3 g/L), alginate-chitosan (3 ± 0.006 g/L) and low melting point agarose (3 g/L). Neglible differences were also noted in the ethanol production by cream yeast i.e. free-suspended (86.9 ± 0.081 g/L), low melting point agarose (86.55 ± 0.081 g/L), calcium alginate (84.6) and then alginate-chitosan (83.7 ± 0.162 g/L).

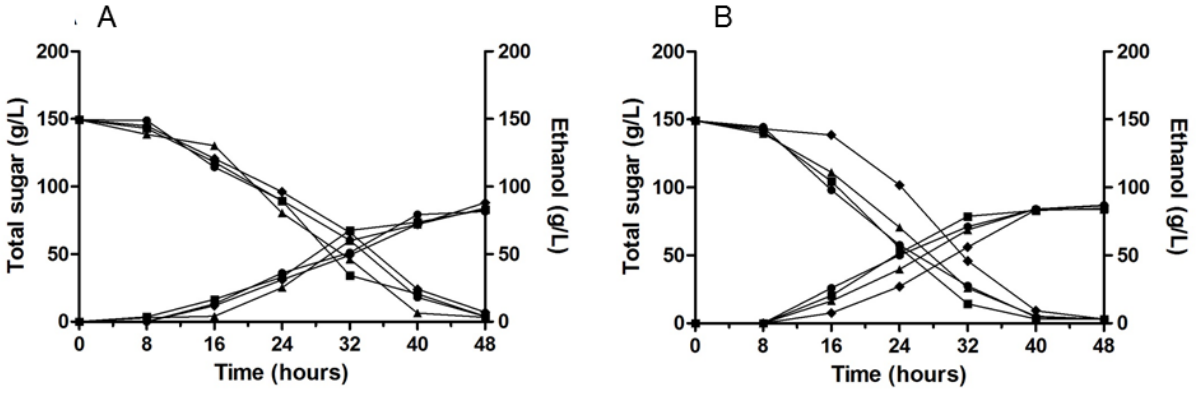

Figure 4.5 Total sugar utilization and ethanol production by Angel (A) and cream (B) yeast strains in synthetic CDM-YE; free-suspended (●), encapsulated in calcium alginate (■), alginate-chitosan (▲) and low melting point agarose (♦). Values represent the mean of experiments performed in triplicate, and error bars represent standard deviations.

The profiles of glycerol production displayed by free-suspended and encapsulated Angel yeast in the synthetic CDM-YE molasses were very similar. in (Figure 4.6). The final glycerol concentrations associated with Angel yeast strains were as follows; alginate-chitosan (8.9 ± 0.023 g/L), free-suspended (8.6 ± 0.02 g/L), calcium alginate (8.1 ± 0.042 g/L) and then low

melting point agarose (8 ± 0.021 g/L). The fermentation of synthetic CDM-YE by cream yeast encapsulated in low melting point agarose displayed the slowest glycerol formation profile when compared to free-suspended and other encapsulated forms. The glycerol production by the cream yeast strain was free-suspended (8.5 ± 0.058 g/L), alginate-chitosan (8.2 ± 0.02 g/L), calcium alginate (8.2 ± 0.012 g/L) and low melting point agarose (7.87 ± 0.058 g/L).

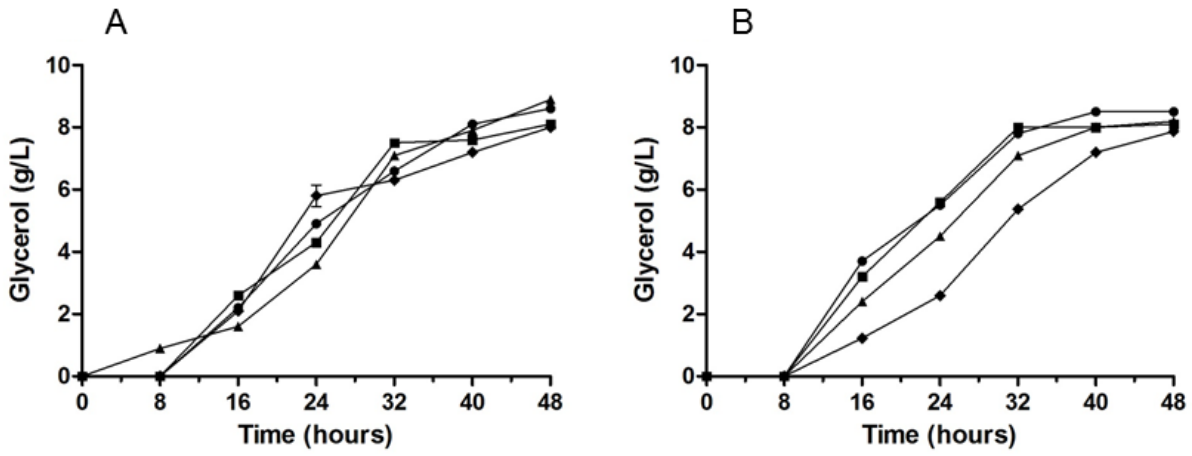

Figure 4.6: The formation of the glycerol by-product over time by Angel (A) and cream (B) yeast strains in synthetic CDM-YE; free-suspended (●), encapsulated in calcium alginate (■), alginate-chitosan (▲) and low melting point agarose (♦). Values represent the mean of experiments performed in triplicate, and error bars represent standard deviations.

4.4.3 Capsule reusability

Capsule reusability was assessed by harvesting the capsules after batch fermentations and reusing them as inoculum for two subsequent fermentation cycles. For this aspect of the study, only alginate-chitosan and low melting point agarose capsules were employed. This selection was based on the tendency of calcium alginate capsules to be disrupted under conditions of cane molasses batch fermentations. In contrast, the low melting point agarose capsules remained stable and active throughout the three consecutive batch fermentations of Amatikulu cane molasses and synthetic CDM-YE molasses. The alginate-chitosan capsules remained active and stable through two cycles of fermentation and only showed signs of breakage during the third fermentation cycle (Table 4.2 and 4.3). The final ethanol and glycerol titres produced by the reused alginate-chitosan and low melting point agarose capsules remained unchanged across three cycles of fermentation. There was no apparent sign of increased or diminished fermentation activity with the recycling of capsules. The

fermentation of both Amatikulu and synthetic CDM-YE molasses with Angel yeast encapsulated in low melting point agarose resulted in significantly higher ethanol titres and yields for all three cycles of batch fermentations when compared to single use of free-suspended yeast cells counterparts.

Table 4.2 Fermentation of three batches of Amatikulu cane molasses by reusing original encapsulated yeast inoculum

Yeast strain	Coating material	Ferm. Cycle	S_f (g/L)	Sugar conversion (%)	Ethanol titre (g/L)	Ethanol yield ($Y_{P/S}$)	Glycerol titre (g/L)
Angel	AC	1	20.3	86	92.4	0.712	10.5
		2	20.07	87	92.73	0.714	10.13
		3	20.03	87	92.43	0.711	10.13
	LMPA	1	26.5	82	100.1	0.811	10.7
		2	25.87	83	99.9	0.805	10.73
		3	25.47	83	99.87	0.802	10.43
Cream	AC	1	15.6	90	103.8	0.751	11.4
		2	16.03	89	103.6	0.773	11.37
		3	16.03	89	103.8	0.775	11.37
	LMPA	1	16.8	89	103.9	0.780	13.6
		2	17.47	88	103.87	0.784	13.83
		3	17.57	88	103.73	0.783	13.83

AC: alginate-chitosan; LMPA: low melting point agarose; S_f: final sugar concentration (g/L)

Table 4.3 Fermentation of three batches of synthetic CDM-YE molasses by reusing original encapsulated yeast inoculum

Yeast strain	Coating material	Ferm. Cycle	S_f (g/L)	Sugar conversion (%)	Ethanol titre (g/L)	Ethanol yield ($Y_{P/s}$)	Glycerol titre (g/L)
Angel	AC	1	3.3	98	84	0.573	8.9
		2	13	91	80.67	0.589	6.57
		3	17.57	88	83.7	0.632	8.27
	LMPA	1	7.1	95	88	0.616	8
		2	3.4	98	88.35	0.603	8.2
		3	5.23	97	89.07	0.615	8.93
Cream	AC	1	3	98	83.7	0.569	8.2
		2	3.47	98	81.13	0.554	10.3
		3	3.43	98	81.9	0.559	9.4
	LMPA	1	3	98	86.5	0.588	7.87
		2	3.1	98	82.27	0.560	8.07
		3	3.2	98	86.47	0.589	9.4

AC: alginate-chitosan, LMPA: low melting point agarose, S_f: final sugar concentration (g/L)

4.5 DISCUSSION

In this study; Angel yeast and cream yeast were encapsulated into three different coating materials; calcium alginate, alginate-chitosan and low melting point agarose. This was done on the basis that different coating materials form capsules possesing different properties, thereby resulting in capsules with intracapsular conditions which elicit distinctive physiological responses from the encapsulated microbial cells (Qi *et al.*, 2006; Sun *et al.*, 2007). The fermentation of Amatikulu and synthetic CDM-YE molasses with encapsulated Angel and cream yeast was postulated to lead to encapsulated yeast cells displaying superior bioethanol yields than free-suspended yeast cells. This was achieved in fermentations with encapsulated Angel yeast. Angel yeast encapsulated in calcium alginate and alginate-chitosan resulted in slightly higher final bioethanol titres when compared to free-suspended Angel yeast. A finding similar to that reported by (Behera *et al.*, 2010), whereby fermentations of glucose with yeast cells encapsulated in agar-agar and calcium alginate resulted in slightly higher bioethanol yields than the free-suspended cells (<4%).

However, in this study, an increase in bioethanol yields was attained in fermentations with Angel yeast encapsulated in low melting point agarose. The fermentation of Amatikulu and synthetic CDM-YE molasses with Angel yeast encapsulated in low melting point agarose resulted in a statistically significant increase in bioethanol yields (~10%) when compared to their free-suspended cell counterparts. According to the best of our knowledge, this is the first time that the fermentation of cane molasses with yeast encapsulated in low melting point agarose has been reported. To date, previous research studies have employed standard agarose and agar as encapsulating materials. However, the shortcoming of these encapsulating materials is their high gelation temperatures (>30°C) which substantialy decreases yeast cell viability (Nigam *et al.*, 1998; Behera *et al.*, 2010; Singh *et al.*, 2013).

The fermentation of Amatikulu and CDM-YE by cream yeast encapsulated in calcium alginate, alginate-chitosan and low melting point agarose resulted in bioethanol yields very similar to those attained by the free-suspended yeast cells.This finding is supported by Qi *et al.* (2006), who reported that free-suspended yeast cells and encapsulated cells achieved similar sugar assimilation and ethanol formation profiles. In another study conducted by Mariam *et al.* (2009), free-suspended cells and cells encapsulated in calcium alginate also achieved similar fermentation profiles and final ethanol titres. The findings of the latter study is compromised by inferior capsule stability, which resulted in the release of cells into the fermentation medium. Thereby relinquishing any protective effects that the capsules might have had on the previously encapsulated microbial cells.

The fermentation data of this study suggests that the Angel and cream yeast strains responded differently to encapsulation. Cream yeast displayed inherently superior bioethanol titres compared to Angel yeast that also maintained encapsulation. On the contrary, cell encapsulation of Angel yeast into low melting point agarose capsules resulted in increasing the ethanol producing capability of the yeast strain. Thus far, two theories have been proposed that attempts to explain the mechanism of improved bioethanol yields behind cell encapsulation. Some authors suggest that the higher levels of saturated fatty acids in encapsulated yeast cells confers upon them increased ethanol tolerance (Hilge-Rotmann & Rehm, 1991; Sun *et al.*, 2007). Other studies suggest that hydrogels tightly withhold water, leading to a highly structured water layer surrounding the yeast in the pores of the gel network (Israelachvili & Wennerstrijm, 1996). This water layer then prevents ethanol from dehydrating the encapsulated cells (Desimone *et al.*, 2002).

An increase in glycerol titres for Angel yeast and cream yeast encapsulated in alginate-chitosan and low melting point agarose was observed in Amatikulu cane molasses. Glycerol has been reported to play a pivotal role in osmoregulation. As such yeast increase glycerol production in response to decreased water activity thereby allowing yeast to proliferate over a wide range of external water activities (Sun *et al.*, 2007).

Of note, calcium alginate capsules displayed the least stability when compared to alginate-chitosan and low melting point agarose capsules. Calcium alginate has been reported to be susceptible to destabilisation in the presence of antigelling agents such as monovalent cations, phosphates and citrate (Serp *et al.*, 2000; Wen-Tao *et al.*, 2005). Cane molasses possesses high amounts of cations, which decreases the stability of the calcium alginate capsules, thus rendering this matrix as an unsuitable encapsulation agent for the fermentation of molasses. However calcium alginate capsules when coated with the polycation chitosan results in increased capsule stability (Wen-Tao *et al.*, 2005). This is the first study to demostrate the use of chitosan coated alginate capsules in molassess batch fermentations. Additionaly it was also shown that a single inoculum preparation of alginate-chitosan capsules remained active and stable through two cycles of fermentation and only showed signs of breakage during the third fermentation cycle. The latter observation, may have resulted from ineffiecient CO_2 transport across the capsules which can be improved by

the inclusion of surfactants such as Tween 20 which been previously reported to improve capsule permeability to CO_2 (Chang *et al.*, 1996). However, it is suitable for use in fermentations of other substrates as a previous study has reported that these capsules can be reused for as many as 21 cycles in the fermentation of cellulosic biomass (Talebnia & Taherzadeh, 2006). The data clearly demonstrates that Angel yeast encapsulated in low melting point agarose still maintained higher ethanol yields in comparison to free-suspended cells for the three consecutive batch fermentations cycles using a single inoculum prepared from this encapsulation material. As such this study is the first to report that low melting point agarose is a suitable yeast cell encapsulation matrix for the fermentation of molasses.

4.6 CONCLUSION

The fermentation of Amatikulu and synthetic CDM-YE with Angel yeast encapsulated in low melting point agarose resulted in a significant increase in bioethanol yields when compared to its free-suspended yeast cell counterparts. The stability and reusability of the alginate-chitosan and especially low melting point agarose offers attractive and offers new directives in terms of future research prospects. The bioprocessing industry currently invests heavily in fresh cream or dry yeast inoculum for their bioprocesses and the reusability of encapsulated yeast is attractive as a cost-saving measure in this regard. It should be noted that encapsulation technology although very promising, still faces hurdles like feasibility and economic viability when paralleled to currently employed bioprocessing technologies.

4.7 ACKNOWLEDGEMENTS

This study was made possible through financial support from the National Research Foundation. The research facilities were provided by the University of KwaZulu-Natal. The South African Sugar Research Institute and Mr Ace Govender (NCP alcohol) also offered invaluable support.

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Chapter 5

General discussion and conclusion

5.1 GENERAL DISCUSSION AND CONCLUSION

The first aspect of this study reports on the formulation of a chemically defined molasses fermentation substrate as a standardised laboratory medium that can substitute the use of industrially derived molasses in fermentation studies. In the fermentation of three industrially derived and chemically defined molasses, it was observed that distiller's yeast strains (Angel and cream) outperfomed the laboratory (BY4743) and baker's (Dry) yeast strains in terms of ethanol outputs. This latter observation was supported by research studies reported by Della-Bianca and Gombert (2013). The fermentation of synthetic CDM-YE by the individual yeast strains resulted in closely mirrored profiles to those produced by the same yeast when fermenting cane molasses. The data seem to suggest that the newly formulated synthetic CDM-YE can be employed as a standardised laboratory culture medium in cane molasses fermentation studies. Thereby allowing for the generation of more consistent and reliable fermentation data which is not compromised by the variabiity of cane molasses nutrient composition across different batches.

For the second aspect of the study, Amatikulu cane molasses and synthetic CDM-YE were used as the fermentation substrates. Only the two better performing industrial yeast strains were selected, *i.e*. Angel and cream yeast strains. The fermentation of Amatikulu and synthetic CDM-YE with Angel yeast encapsulated in calcium alginate and alginate-chitosan resulted in a marginal increase of bioethanol titers. This finding was in line with that reported by Behera *et al.* (2011), whereby the authors reported that the fermentation of mahula flowers with *S. cerevisiae* encapsulated in agar-agar and calcium alginate resulted in 1% and 4% respective increases in final bioethanol titers when compared to their free-suspended cell counterparts. In this study, the most significant increase in bioethanol yields was achieved in fermentations with Angel yeast encapsulated in low melting point agarose, which resulted in a ~10% increase of bioethanol yields. Although yeast encapsulation with other coating materials have been previously reported to increase bioethanol yields, this is the first time that fermentation of cane molasses with industrial yeast encapsulated in low melting point agarose is being reported to result in increased bioethanol yields. An example is a recent study conducted by Lee *et al.* (2011) that showed fermentation of a laboratory medium with 10% (w/v) glucose content with *S. cerevisiae KCTC 7906* cells encapsulated in calcium alginate resulted in a 12% increase in ethanol yield in comparison to free cells.

On the contrary, fermentations with encapsulated cream yeast did not result in superior bioethanol producing abilities. Fermentations with encapsulated cream yeast resulted in fermentation profiles and bioethanol yields very similar to those attained with their free-

suspended cell counterparts. This observation was in line with a research study reported by Qi *et al.* (2006). The authors had encapsulated *S. cerevisiae* cells in alginate-chitosan-alginate (ACA) capsules, and reported that encapsulated and the free-suspended yeast cells achieved similar sugar assimilation, ethanol formation and final bioethanol concentrations.

The results attained in this study perhaps suggest that a strain-by-strain approach should be adopted when seeking to encapsulate yeast instead of a holistic approach buoyed by the notion that the encapsulation of any yeast, will lead to increased bioethanol yields. As the results of this study suggest, only the encapsulation of Angel yeast abetted bioethanol yields. Of note, Angel yeast had an inferior bioethanol producing capacity when compared to cream yeast, before encapsulation. High ethanol levels have been reported to thwart the cell's lipid biosynthesis mechanisms, leading to cells with altered and compromised cell wall characteristics. Subsequently leading to decreased fermentation activity and cell viability (Mannazzu *et al.*, 2008). It may be suggested that low melting point agarose seemingly provided the encapsulated Angel yeast with intracapsular conditions permissive of optimal yeast performance, by acting as a protective barrier to the external environment where bioethanol levels were exponentially accumulating toward inhibitory levels as fermentation progressed.

Additionaly, it was also shown that a single inoculum preparation of alginate-chitosan capsules remained active and stable through two cycles of fermentation and only showed signs of breakage during the third fermentation cycle. However, low melting point agarose capsules remained stable through the three cylces of fermentations whilst maintaing the same levels of bioethanol production. The successful reusability of capsules for successive fermentation cycles has been reported for other fermentation substrates. Behera *et al.* (2011) reported the successful and stable use of *S. cerevisiae* encapsulated in agar-agar and calcium alginate capsules for three consecutive fermentation cycles of mahula flowers. Talebnia and Taherzadeh (2006) also reported the successful use of calcium alginate capsules for 21 cycles of cellulosic biomass fermentations.

The single-strain approach adopted by most researchers (Wen-Tao *et al.*, 2005; Qi *et al.*, 2006; Talebnia, 2008; Mariam *et al.*, 2009; Lee *et al.*, 2011; Ylitervo *et al.*, 2013) leaves a common question unanswered i.e. if yeast selected from a wider genetic pool would also result in increased bioethanol tolerance and yields?. Therefore future study prospects to improve the bioethanol fermentation capabilities of yeast may entail the encapsulation of strains from a broader genetic background.

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