IMPACT OF MOLASSES QUALITY ON ETHYL ALCOHOL FERMENTATION BY THE YEAST *SACCHAROMYCES CEREVISIAE*

BY

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SYNOPSIS

The use of blackstrap cane molasses in industrial ethanol production is characterised by fluctuations in fermentation efficiency due to its compositional variation. Crucial to optimisation of ethanol production is thorough knowledge of the constituents and properties of molasses which affect fermentation, in order to implement measures to diminish negative and augment positive impacts. These include both major and minor constituents.

According to literature, aspects of fermentation media which affect yeast fermentation include nutritional availability, the presence of trace elements and growth factors such as vitamins, without which yeast proliferation is inhibited. High osmotic pressures, found in VHG (very high gravity) fermentations, have a detrimental effect on the fermentation process. Additionally, elevated concentrations of inorganic salts and ionic strength in industrial fermentation media have been implicated in negatively affecting fermentation.

Based on these limited literature findings, the study addressed the effect of three cation constituents of molasses (K^+, Mg^{2+} and Na^+), expressed in terms of overall concentration, osmotic pressure and ionic strength, on fermentation was investigated in molasses media and a sucrose-based media. The decrease in yeast growth and fermentation performance upon increasing the cation concentrations, and thereby osmotic pressure and ionic strength, was primarily a result of specific cation toxicity. The extent of the negative effect in both media was cation specific, in decreasing order: $Na^+ > K^+ > Mg^{2+}$. In molasses fermentations at cation concentration of 15 $g.l^{-1}$, the specific growth rates relative to the control $\mu/\mu_{control}$ were 0.77, 0.99 and 0.98 for Na^+, K^+ and Mg^{2+} respectively, while the relative rates of ethanol production $\omega/\omega_{control}$, were 0.31, 0.77, 0.98, respectively. At an ionic strength of 25 mS $\mu/\mu_{control}$ was 0.85, 1.0 and 0.99, while $\omega/\omega_{control}$ was 0.79, 0.90 and 0.99, for Na^+, K^+ and Mg^{2+}, respectively. At osmotic pressure 4.0 MPa $\mu/\mu_{control}$ was 0.88, 0.95 and 0.98, while $\omega/\omega_{control}$ was 0.54, 0.70 and

0.95, respectively. These results demonstrated that the negative impact was not a direct function of ionic strength or osmotic pressure, but ion specific.

The negative effects were more pronounced in the sucrose-based media than in molasses media. At a cation concentration of 15 $g.l^{-1}$, $\mu/\mu_{control}$ of 0.02, 0.39 and 1.0 were found for for Na^+, K^+ and Mg^{2+} respectively, while $\omega/\omega_{control}$ was 0.00, 0.03 and 0.082, respectively.

Mitigation of the cation effects on yeast growth and fermentation performance in molasses media, relative to sucrose-based media, was attributed to the presence of chelating agents in molasses. These act by reducing bioavailability of toxic compounds. The case for the presence of chelating agents in molasses was further strengthened when supplementation of sucrose-based media containing 15 $g.l^{-1}$ K^+ with 20% (v/v) molasses media of equal K^+ concentration resulted in a 68% increase in the cell specific growth rate.

The categorisation of commercial molasses samples provided as "bad" molasses appeared valid upon fermentation, with ethanol production losses of 8.2 to 8.5% relative to "good" molasses, being realised. Estimated anhydrous ethanol losses of 193 to 234 l per typical 30, 000 l fermenter were calculated. Atomic absorption spectroscopy analysis of the "bad" molasses revealed relatively high K^+ and Na^+ concentrations in samples. Sample 1 typifying poor quality molasses had K^+ concentration of 3.3%, which was 10% greater than that in "good" molasses. Sample 2 typifying poor quality molasses had Na^+ concentration of 0.14%, which was 60% greater than that in "good" molasses.

The initial sugar concentration, determined by the degree of dilution of molasses, affected yeast growth and fermentation performance. A negative effect was observed as the initial sugar concentrations was increased from 120 through 210 $g.l^{-1}$. The specific growth rate (μ), decreased from 0.45 to 0.40 and 0.37 hr^{-1} at initial sugar concentrations 120, 170 and 210 $g.l^{-1}$. While high initial sugar concentrations produced higher ethanol concentrations, this was at the expense of fermentation efficiency which decreased from 0.88 at an initial sugar concentration of 120 $g.l^{-1}$ to 0.82 ± 0.02 at 170 $g.l^{-1}$ and 0.74 at 210 $g.l^{-1}$. It is

postulated that the reduced fermentation was a result of increased metabolic flux towards the osmoregulator glycerol, and stress related compounds such as glycogen and trehalose.

To counteract suboptimal fermentations, several options were recommended. These included the use of molasses of relatively low K^+ and Na^+ concentration, increased dilution of molasses, fed batch systems with continual molasses addition, yeast recycling, increased nutritional supplementation and supplementation of chelating agents.

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NOMENCLATURE

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
DNS	dinitrosalicylic acid
EMP	Embden-Meyerhof-Parnas
GC	gas chromatography
F.ε	fermentation efficiency
HOG	high osmolarlity glycerol
HPLC	high performance liquid chromatography
HSP	heat stress protein
M.U.ε	molasses usage efficiency
NADH	nicotinamide adenine dinucleotide hydrogen
OUR	oxygen utilisation rate
ROS	reactive oxygen species
R.S	residual sugar
S.U.ε	sugar usage efficiency
TCA	tricarboxylic acid
TRS	total reducing sugar
TSAI	total sugar as invert
VHG	very high gravity
m/m	mass per mass
rpm	revolutions per minute
v/v	volume per volume

Symbols

C_A	toxic compound concentration ($g.l^{-1}$)
C_p	product concentration ($g.l^{-1}$)

C_E	ethanol concentration ($g.l^{-1}$)
$C_{E,20}$	ethanol concentration after 20 hours ($g.l^{-1}$)
$C_{E,max}$	maximum ethanol concentration ($g.l^{-1}$)
C_N	cell concentration ($cells.ml^{-1}$)
C_s	substrate concentration ($g.l^{-1}$)
C_x	biomass concentration ($g.l^{-1}$)
K_I	growth inhibition constant ($g.l^{-1}$)
K_p	product inhibition term ($g.l^{-1}$)
K_s	saturation constant ($g.l^{-1}$)
k_d	specific death rate (hr^{-1})
I	ionic strength (mS)
N_T	total cell count ($cells.ml^{-1}$)
Π	osmotic pressure (MPa)
r_p	specific product formation rate (hr^{-1})
r_s	specific substrate utilisation rate (hr^{-1})
φ	average sugar utilisation rate (hr^{-1})
μ	specific growth rate (hr^{-1})
μ_{max}	maximum specific growth rate ($g.l^{-1}.hr^{-1}$)
ω	average ethanol production rate ($g.l^{-1}.hr^{-1}$)
Y_{xs}	yield of biomass on substrate ($g.g^{-1}$)
Y_{ps}	yield of product on substrate ($g.g^{-1}$)
Y_{ES}	yield of ethanol on substrate ($g.g^{-1}$)

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND AND STUDY OBJECTIVES

Sugarcane molasses, a by-product of sugar refining, has long been used as a feedstock for bioprocesses such as production of yeast, potable and industrial ethanol. In South Africa, the biofuel production of ethanol from sugarcane molasses has attracted much attention recently in response to the search for alternative renewable fuel sources. Despite being a waste product, molasses is nutritionally suitable for yeast growth and bioethanol production. Its suitability as a fermentative media is quantified in terms of the quality of molasses. The quality of molasses depends on many variables including the maturity of the sugarcane from which it is derived, amount of sugar extracted, method of extraction and its total solids content.

As with many by-products used as raw materials, the challenge faced on using molasses as a feedstock for bioprocesses, including ethanol production, is its inconsistent composition. This varies from refinery to refinery and with progression through the sugarcane crushing season. In this thesis, the impact of this molasses quality on ethanol production is considered in the South African context. When the sugarcane crushing season in South Africa begins in May sugar extraction efficiency in the mills is low, resulting in a molasses of higher quality with greater fermentative potential. This molasses is generally characterised by having high total reducing sugar (TRS) and total sugar as invert (TSAI) content, and low inorganic ash (consisting mainly of inorganic salts) content. The high sugar content usually translates to greater ethanol yields from

fermentation. The reduced inorganic ash results in minimal osmotic stress on the yeast (*Saccharomyces cerevisiae*) enabling it to perform optimally. However, as the crushing season progresses the mills become more efficient at sugar extraction, with a subsequent reduction in molasses quality evidenced by lower TRS and TSAI content, and increased inorganic ash levels. Fermentation performance (in terms of rate and extent) has been observed to be reduced progressively throughout the year affecting plant throughput (Brakenridge, 2006). It is therefore important for ethanol distillers to understand the effect of molasses quality on fermentation in order to implement process modification to alleviate the negative impact of the changing molasses quality.

This study was formulated with the following objectives:

1. to understand the role and effect on fermentation of selected constituents of molasses to which suboptimal fermentations have been attributed;
2. to establish fermentation performance differences, under laboratory conditions, between selected "good" and "bad" molasses to confirm their categorisation as such, thereby eliminating the potential role of plant operations in bad fermentations;
3. where fermentation performance differences between "good" and "bad" molasses were observed, to attempt to account for them based on the findings of point (1); and
4. to recommend process modification to alleviate negative impact of bad molasses.

1.2 STRUCTURE OF THESIS

The thesis commences with Chapter 2 in which a literature review is presented. The first part of the literature review details the production and composition of molasses, and challenges associated with its use as a fermentation media base. Yeast metabolism and yeast quality are discussed next. Potential yeast stressors are introduced, with special emphasis on osmotic and salt stress. The various processes used to produce ethanol conclude the chapter. Chapter 3 deals with the characterisation and quantification of

fermentation performance. Here, the materials and methods used in experiments are detailed. In Chapter 4 a brief statistical analysis and assessment of experimental data reproducibility are presented. The experimental results are presented and discussed in Chapters 5 and 6. Research conclusions and recommendations are provided in Chapter 7.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Industrial ethanol is a traditional fermentation product of excess molasses from the sugar industry. Its production as biofuel has been of substantial interest over recent years. Many ethanol manufacturers use batch processes which are easier to operate and have low investment costs (Çaylak and Vardar Sukan, 1996; Weusthuis, 1994). Prior to fermentation, molasses is diluted with water to produce molasses mash containing 15 to 16% sugars (Patil *et al*., 1998). The pH of the mash is adjusted to pH 4 to 5 with mineral acid (Lin and Tanaka, 2005), and supplemented with a nitrogen source such as urea (Piggot, 2003). This mash is inoculated with yeast and fermented at a temperature of 20 to 32^{o}C for 1 to 3 days (Lin and Tanaka, 2005). A typical molasses batch fermentation process is summarised schematically in Figure 2.1.

Owing to the high dependence of process economics on substrate conversion, optimal fermentation performance is critical. Variation in fermentation performance with seasonal variation in molasses quality has been recognised as a challenge to the processing of excess molasses (Piggot, 2003). This literature review seeks to establish the knowledge basis on the growth and fermentative capacity of the yeast *Saccharomyces cerevisiae* in response to stress conditions related to low quality molasses. To provide background knowledge on which to build this study, in this review the production, composition and suitability of molasses as a fermentation media base is discussed. A review of yeast growth and metabolism is given. Factors that may cause yeast stress are identified. In

considering characteristics of the molasses, emphasis is placed on osmotic and salt stress. Fermentation processes used in the production of ethanol are introduced.

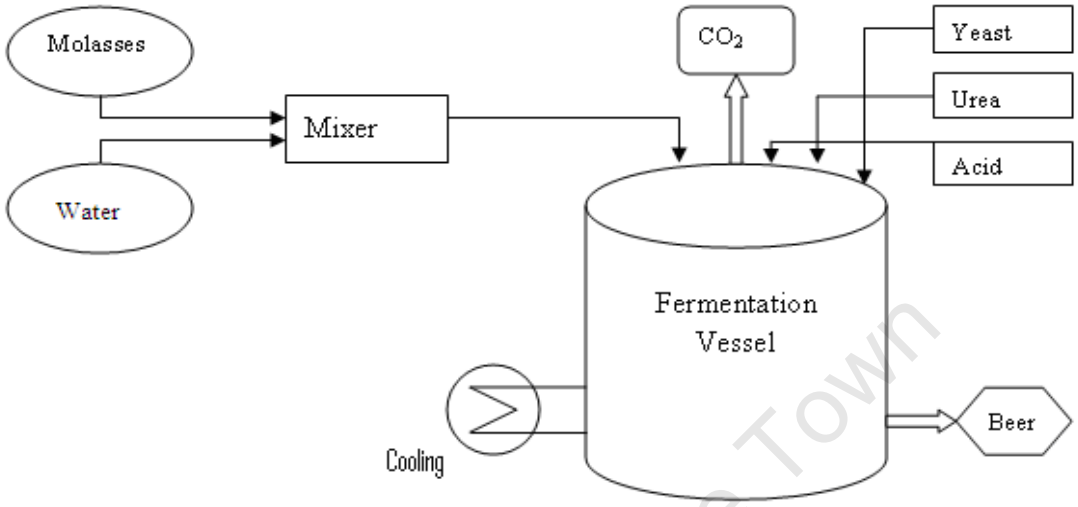

Figure 2.1 Schematic representation of batch fermentation process

2.2 MOLASSES

The term molasses generally refers to a syrup or pulp by-product of a manufacturing process such as sugar refining. Several types of molasses are defined by their sources and process as summarised in Table 2.1. In the context of this thesis, the literature review is limited to blackstrap molasses and high test molasses. Blackstrap molasses is the most

Table 2.1 Types of molasses (Piggot, 2003)

Blackstrap molasses	By-product of sugar production from sugar-cane
High-test (cane) molasses	Primary product: extracted from sugarcane
Beet molasses	By-product of sugar production from sugar beets
Citrus molasses	Juices extracted from manufacture of dried citrus pulp

commonly used feedstock in bioethanol production. High-test molasses is an alternative feedstock to blackstrap molasses due to its greater compositional consistency, and high fermentable sugar content.

2.2.1 Production and Composition of Molasses

Blackstrap Molasses Production

Blackstrap molasses is a by-product of either raw sugar manufacture or refining (Lavarack, 2003; Miranda *et al.,* 1999; Piggot, 2003). It is a brown, heavy and viscous liquid separated from low grade sugar syrup. No further sugar can be crystallised from it using conventional methods (James *et al*., 1993). Blackstrap molasses has traditionally been the most commonly used feedstock for the production of industrial ethanol by fermentation with *S. cerevisiae* (Lavarak, 2003; Ryan and Johnson, 2001). Demand has seen blackstrap molasses production increase markedly over the past 40 years, with approximately 37 million mT of cane molasses being produced worldwide in 2002 (Anon, 2002). For each metric tonne of sugar, 300 to 360 kg of blackstrap molasses is produced (Mosses and Springham, 1999).

Molasses production occurs concurrently with sugar production. During the sugarcane refining process, extracted juice is clarified by liming. This is followed by sulphitation and phosphatation. The addition of electrolytes promotes floc formation which precipitates impurities, including colouring agents (Kokugan and Kokugan, 1997). The clarified juice is boiled and concentrated before proceeding to the first "A" stage of sugar crystallisation. Crystallised sugar from this stage is termed "A" sugar and the uncrystallised solution called "A" molasses. "B" and "C" stages of further boiling and crystallisation normally follow, with the final by-product of uncrystallisable sugars and other solubles being known as "C" or blackstrap molasses (Scurlock *et al*., 1991). While any of the molasses from the sugar crystallisation process can be used for ethanol production, blackstrap molasses is the most commonly used (Çaylak and Vardar Sukan, 1996) for economic reasons (Ryan and Johnson, 2001). The production of blackstrap molasses from sugar cane is summarised in Figure 2.2.

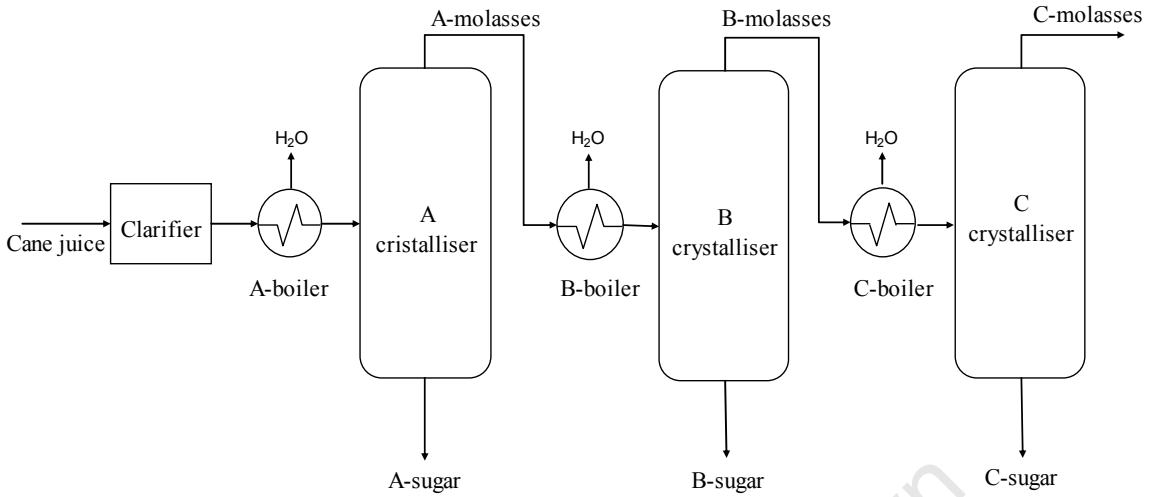

Figure 2.2 Simplified representation of molasses production from cane juice

Blackstrap Molasses Composition

Blackstrap molasses appears to be the ideal feedstock for ethanol production because it normally contains all the nutritional requirements for yeast growth (Wheals *et al*., 1999). However, molasses is a waste product of the sugar industry and its composition depends on the degree of optimisation achieved in the sugar refining process (Mosses and Springham, 1999). Hence, molasses composition can vary considerably, making it an inconsistent substrate. The variation in molasses composition is not affected by the sugar extraction process only. The age of the sugar cane, soil in which it is grown, fertilisation practices and climatic conditions further influence its composition and the subsequent molasses quality (Piggot, 2003).

The most crucial component of molasses is its fermentable sugar content, which acts as a carbon source for yeast. The amount of fermentable sugar present in a batch of molasses determines the amount of ethanol produced. The typical sugar composition of South African molasses is 28 to 32% (w/w) sucrose and 9 to 13% (w/w) reducing sugars (mainly glucose and fructose) (Illovo Sugar Limited). American (USA) molasses has 25 to 35% (w/w) sucrose and 20 to 35% (w/w) reducing sugars (Piggot, 2003). Approximately 35% (w/w) sucrose and 15% (w/w) reducing sugars have been reported

for Australian molasses (Ryan and Johnson, 2001). Growth factors such as biotin, folic acid and riboflavin which promote yeast growth, are also present (Piggot, 2003).

Molasses also contains impurities such as inorganic salts, unfermentable sugars, sulphated ash, as well as colouring agents. These impurities are a result of upstream processes (Kukugan and Kukugan, 1997). The inorganic impurities include significant amounts of Na, K, Mg, Ca, phosphates, and sulphates. Traces of the elements Cu, Mn and Zn have been recorded. Organic impurities include gums and unfermentable sugars. The detailed composition of American (USA) molasses (Piggot, 2003) and South African molasses (Illovo Sugar Limited) is summarised in Table 2.2.

Table 2.2 Comparative chemical composition of American & South African cane molasses (Piggot, 2003; Illovo Sugar Limited)

	American Molasses	**South African Molasses**
	Range or typical value (%)	
Total sugars	45-55	40-43
Sucrose	25-35	28-32
Reducing Sugars	20-35	9-13
pH	5-5.5	5.1-5.5
Ash	10-16	12-15
Starch/polysaccharides	0.5	0.1-0.3
Calcium	0.4-0.8	0.9-1.6
Sodium	0.1-0.4	0.1-1.0
Potassium	1.5-5	2.8-4.1
Magnesium	0.05-0.98	0.3-0.5
Phosphorus	0.03-0.1	0.05-0.14
Sulphur	0.3-0.8	0.06-0.6
	Range or typical value (ppm)	
Copper	2.2-38	Undetectable
Manganese	4-300	100
Zinc	4-48	10
Biotin	1.2-3.2	Data unavailable
Folic Acid	0.004	"
Riboflavin	2.5	"

High-Test Molasses Production

An alternative feedstock for bioethanol production is high-test molasses. Unlike other molasses, high-test molasses is not a by-product, but produced exclusively for bioethanol production. It is produced in the same way as raw sugar, except that a smaller amount of lime is added to the cane juice, giving it a pH of 6.0 to 6.3 (James *et al*., 1993). In factories that produce ethanol only, the cane juice is heated to about 110^{o}C to reduce microbial contamination, decanted and then concentrated by evaporation (Wheals *et al*., 1999). The sucrose in the resulting sugarcane syrup is partially inverted to glucose and fructose using the enzyme invertase. This process stabilises the syrup and prevents crystallisation in the final product (Piggot, 2003). The invertase method uses commercial granular yeast of a special strain. The dry yeast is mixed with water, acidified with HCl, to form a yeast cream. The yeast cream is then fed to syrup storage tanks. Inversion is carried out at 60^{o}C for 6 to 10 hours (James *et al.*, 1993). Alternatively, a mineral acid can be used for inversion. However, this approach is not favoured as it destroys up to 5% of fermentable sugars (Piggot, 2003).

High-Test Molasses Composition

Since high-test molasses is not a by-product, it is of controlled composition which makes it a more attractive feedstock for bioethanol production (Mosses and Springham, 1999). While high test molasses is more expensive than blackstrap molasses, it has numerous advantages. Its high reducing sugar to sucrose ratio allows faster initiation of fermentation process. Secondly, it has a lower inorganic ash content of 2.25% (m/m), compared with blackstrap molasses which can be as high as 16% (m/m) (Table 2.2). Additionally, it has fewer dissolved impurities and has a more controlled sugar profile and content. The typical composition of high-test molasses is given in Table 2.3.

Table 2.3 Typical composition of high test molasses

Sucrose, %	27.0
Reducing sugars, %	50.0
Calcium, %	0.2
Ash, %	2.25
Water, %	15.50

2.3 Yeast Quality

Brewers use the metabolic activity of the yeast *S. cerevisiae* to convert the fermentable sugars in molasses into ethanol, carbon dioxide and more yeast biomass. Upon exhausting fermentable sugars, or any of the available growth factors, the yeast flocculate and settle to the base of the fermentation vessel. The yeast can then be cropped and used in successive fermentations, reducing costs required for yeast replacement. It is imperative that yeast are cropped as quickly as possible to prevent loss in quality due to ethanol stress and nutrient deprivation. Yeast quality is often related to its fermentative potential and quantity of ethanol produced. The factors determining yeast quality have been defined by Basson (1996) to include:

- ability to grow and reproduce
- rate and extent of growth
- the metabolic rate
- flocculation and sedimentation characteristics
- dominant metabolic pathways
- cell envelope integrity
- ability to withstand stress

The above indicators of yeast quality are often used by ethanol producers to predict the yeast's ability to ferment molasses efficiently.

2.3.1 Yeast Growth and Reproduction

In the ethanol fermentation industry, yeast is cultured under for two different regimes. In the first, termed yeast propagation, increase in biomass is the target. The cells are maintained in an oxygen-rich nutrient medium through several batches of increasing volume to allow maximum cell growth and reproduction needed to attain the desired yeast quantities. This yeast biomass is used as the inoculum to pitch into fermentation vessels. In the second culture regime, termed fermentation, the yeast acts mainly as a biotransformation catalyst, converting the fermentable sugars to ethanol. Large inoculum concentrations are used to hasten the fermentation process and limit the activity of microbial contaminants. During fermentation, growth is limited to just a few cell cycles as conditions are selected to favour ethanol production (Priest and Campbell, 2003).

In general yeast can grow on and ferment relatively simple media. For optimal growth and fermentation, the overall requirements include (Hornsey,1999):

- a carbon source, such as fermentable sugars
- nitrogen source
- growth factors
- inorganic ions
- oxygen (especially in the early stages of fermentation)
- water

The composition of a typical synthetic medium used for optimal ethanol production by yeast was proposed by Atkinson and Mavituna (1991) and is provided in Table 2.4. While the fermentation media proposed by Atkinson and Mavituna (1991) for ethanol production contains all nutritional requirements for yeast growth and ethanol production, its use for industrial ethanol production would be uneconomical. Molasses (after adjusting the sugar concentration by water dilution producing mash) provides a more economically viable alternative (Wheals *et al*., 1999) as it only requires supplementation with nitrogen to provide adequate nutritional requirements for *S. cerevisiae*.

Table 2.4 Composition of a typical synthetic medium for ethanol production (Atkinson, 1991)

Component	Amount ($g.l^{-1}$)
Glucose	100.0
Ammonium sulphate	5.19
Potassium dihydrogen phosphate	1.53
Magnesium sulphate.$7H_2O$	0.55
Calcium chloride.$2H_2O$	0.13
Boric acid	0.01
Cobalt sulphate.$7H_2O$	0.001
Copper sulphate.$5H_2O$	0.004
Zinc sulphate.H_2O	0.010
Manganese sulphate.$7H_2O$	0.003
Potassium iodide	0.001
Ferrous sulphate.$7H_2O$	0.002
Aluminium sulphate	0.003
Biotin	0.000125
Pantothenate	0.00625
Inositol	0.125
Thiamin	0.005
Pyridoxine	0.00625
p-Aminobenzoic acid	0.001
Nicotinic acid	0.005

During the initial stages of cell growth, oxygen availability is crucial for the catabolism of reserve glycogen to produce sterols and fatty acids (Quain, 1988). These compounds are integral components of yeast cell membranes, and their availability determines the extent of yeast growth and membrane integrity (Pickerell *et al*., 1991). The reducing sugars in molasses (glucose and fructose) provide a readily available carbon source for biomass growth and energy requirements. Upon depletion of the reducing sugars, sucrose is hydrolysed extracellularly by yeast invertase to glucose and fructose (Westhuis, 1994).

Nitrogen, although present in small amounts in molasses, is usually supplemented using urea or any ammonium salts (Rose and Harrison, 1969). Nitrogen is required for the synthesis of nucleotides and amino acids, which form building blocks for nucleic acids and proteins respectively. Growth factors, including the vitamins such as biotin are present in molasses. Inorganic ions such as K, Mg, Ca, Fe, Cu, Fe, Zn, sulphates and phosphates provide a source of vital trace elements crucial in the metabolic activity necessary for cell growth.

Most yeast grow optimally in a temperature range of 20 to 30^{o}C, but can withstand temperatures of 35 to 43^{o}C. They generally prefer a pH of 4.5 to 6.5 (Walker, 1998).

2.3.2 Yeast Metabolism

When yeast cells are inoculated into fermentation media, such as molasses mash, a lag phase results during which the molecular reorganisation required for fermentation occur. In the initial stages oxygen availability is essential as the terminal electron acceptor for energy provision to stimulate yeast growth and cell replication to produce sufficient cells to drive fermentation. Enzymes required for the catabolism of metabolites are also synthesised (Rose and Harrison, 1969). Cellular carbohydrate reserves of glycogen are used up before cells begin to utilise the sugars in the fermentation media (Hammond, 1986). The carbohydrates directly available for immediate yeast metabolism in molasses are glucose and fructose. Sucrose, a non-reducing sugar, must be hydrolysed extracellularly by the enzyme invertase to glucose and fructose before uptake into cells (Weusthuis, 1994). The metabolic rate of *S. cerevisiae* determines the utilisation of these sugars and thus the rate of sugar depletion or attenuation. The metabolic rate of *S. cerevisiae* is dependent on its physiological state, oxygen and nutrient availability and can be quantified by the rate of fermentation. Although several metabolic pathways are used in parallel for catabolism of glucose and fructose, the Embden-Meyerhof-Parnas (EMP) pathway, leading to pyruvate formation dominates. This is followed by either the TCA cycle and aerobic metabolism or by fermentation to yield ethanol and carbon dioxide as the dominant products. Apart from ethanol and carbon dioxide, a variety of

other compounds, including alcohols, aldehydes, acids, esters and sulphur-containing compounds, may be produced by yeast metabolism and by interactions between metabolic products and media constituents (Macleod, 1977; Rose, 1977).

Embden-Meyerhof-Parnas (EMP) Pathway

The most dominant energy yielding metabolic pathway in fermentative yeasts is the Embden-Meyerhof-Parnas (EMP) pathway (Rose and Harrrison, 1969). The EMP pathway occurs in all major groups of organisms including filamentous fungi, yeasts and many bacteria. The pathway operates under both anaerobic and aerobic conditions. The EMP pathway is summarised in Figure 2.3.

Glycolysis is the initial process of carbohydrate catabolism occurring via a series of 10 enzyme catalysed reactions in the cytoplasmic matrix of cells, in which three key regulatory enzymes (hexokinase, phosphofructokinase and pyruvate kinase) work irreversibly. The other steps are freely reversible, which is important for the biosynthetic role of the pathway during glucose synthesis. The early stage of glucose break down results in the consumption of two ATP molecules in the three stage formation of fructose 1,6 biphosphate. The molecule is then cleaved through the action of aldolase to form glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DAP). Only GAP is directly processed in this pathway and DAP must be isomerised to GAP before it can be used. Oxidation of the resultant two GAP molecules to pyruvate generates energy in the form of four ATP molecules via substrate level phosphorylation reactions. Hence, for each glucose molecule oxidised to two pyruvate molecules, the net gain is two ATP molecules, due to ATP consumption in the earlier reaction (Priest and Campbell, 2003; Waites *et al.*, 2001).

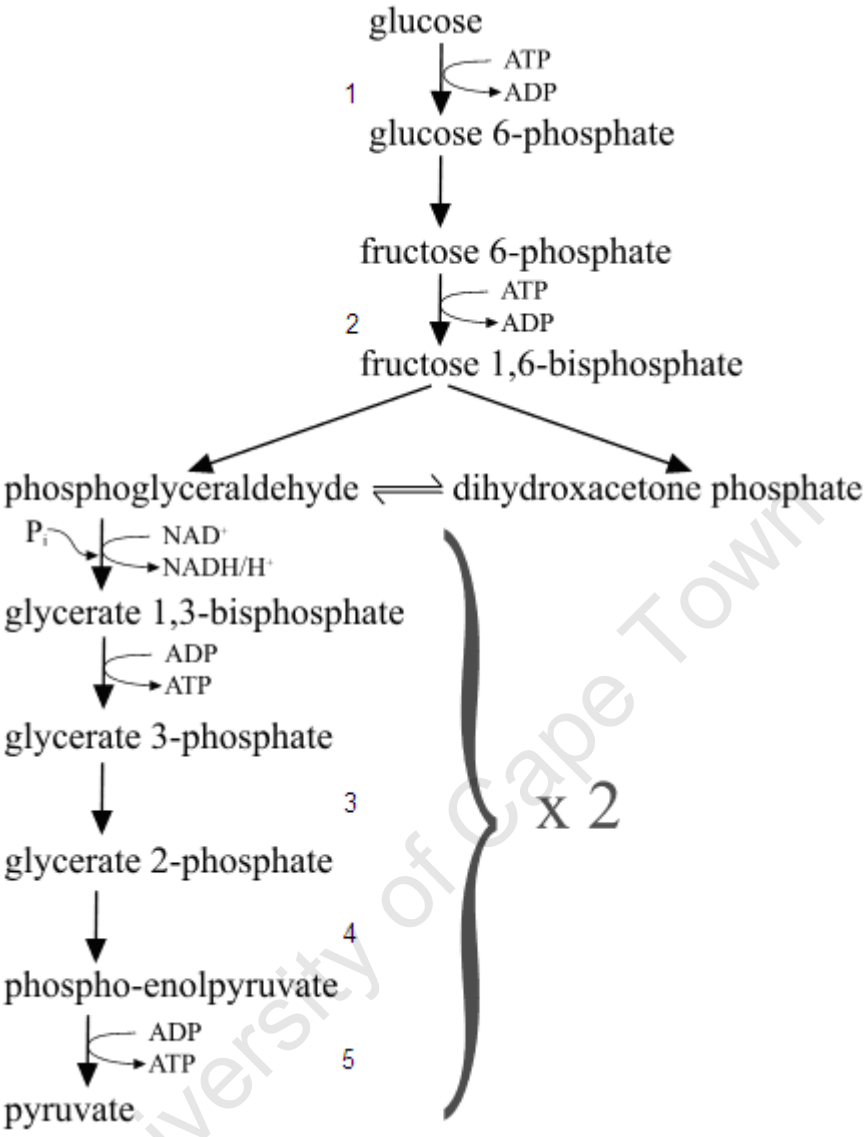

Figure 2.3 Embden-Meyerhof-Parnas pathway (Priest and Campbell, 2003)
Enzymes indicated; 1 Hexokinase, 2 Phosphofructokinase, 3 Glyceraldehyde 3-phosphate dehydrogenase, 4 Phosphoglycerate kinase, 5 Pyruvate kinase

Aerobic Respiration

The fate of pyruvate, formed during glycolysis, depends on the environmental conditions. Under aerobic conditions, pyruvate undergoes further catabolism. The first step involves the oxidative decarboxylation of pyruvate to form acetyl coenzyme A (acetyl CoA) as shown in reaction below:

$$\text{Pyruvate} + NAD^+ + \text{CoA} \rightarrow \text{acetyl CoA} + CO_2 + \text{NADH} + H^+$$

The acetyl CoA enters the tricarboxylic acid (TCA) cycle, which completes the oxidation of pyruvate to CO_2 and reduces electron carriers to produce NADH and $FADH_2$. These reduced coenzymes may then be used for further ATP synthesis. The TCA cycle is summarised in Figure 2.4. More ATP can be generated in the presence of oxygen from the oxidation of NADH and $FADH_2$. An electron transport system facilitates the transfer of electrons from NADH and $FADH_2$ to oxygen which is reduced to form water. The series of redox reactions in the electron transport system create a proton gradient required for ATP generation (Priest and Campbell, 2003).

For glucose sensitive yeast, fully aerobic respiration is only possible at low glucose concentrations for batch cultures and low dilution rates for chemostat cultures (Beck and von Meyenburg, 1968; Pham *et al*., 1998; Postma *et al*., 1989). At low dilution rates, yeast growth is characterised by high biomass yields of between 0.47 to 0.50 $g.g^{-1}$ and no ethanol production (Petrik *et al*., 1983; Postma *et al*., 1989). With successive increases in dilution rate, a point is reached beyond which there is a shift towards respiro-fermentative growth, characterised by low biomass yields (Kappeli, 1986 and Postma *et al*., 1989) and ethanol production (Petrik *et al*., 1983).

Fermentation

When oxygen is not present in sufficient quantity for normal cellular respiration, yeast use alternative mechanisms for the regeneration of coenzymes reduced during oxidation of glucose to pyruvate. In fermentation, pyruvate becomes the terminal electron acceptor with the regeneration of $NAD(P)^+$ and concomitant formation of reduced compounds such as ethanol. In fermentation to ethanol, pyruvate is decarboxylated to acetaldehyde:

$$\text{Pyruvate} \rightarrow \text{Acetaldehyde} + CO_2$$

Acetaldehyde is then reduced to ethanol:

$$\text{Acetaldehyde} + \text{NADH} + H^+ \rightarrow \text{Ethanol} + NAD^+$$

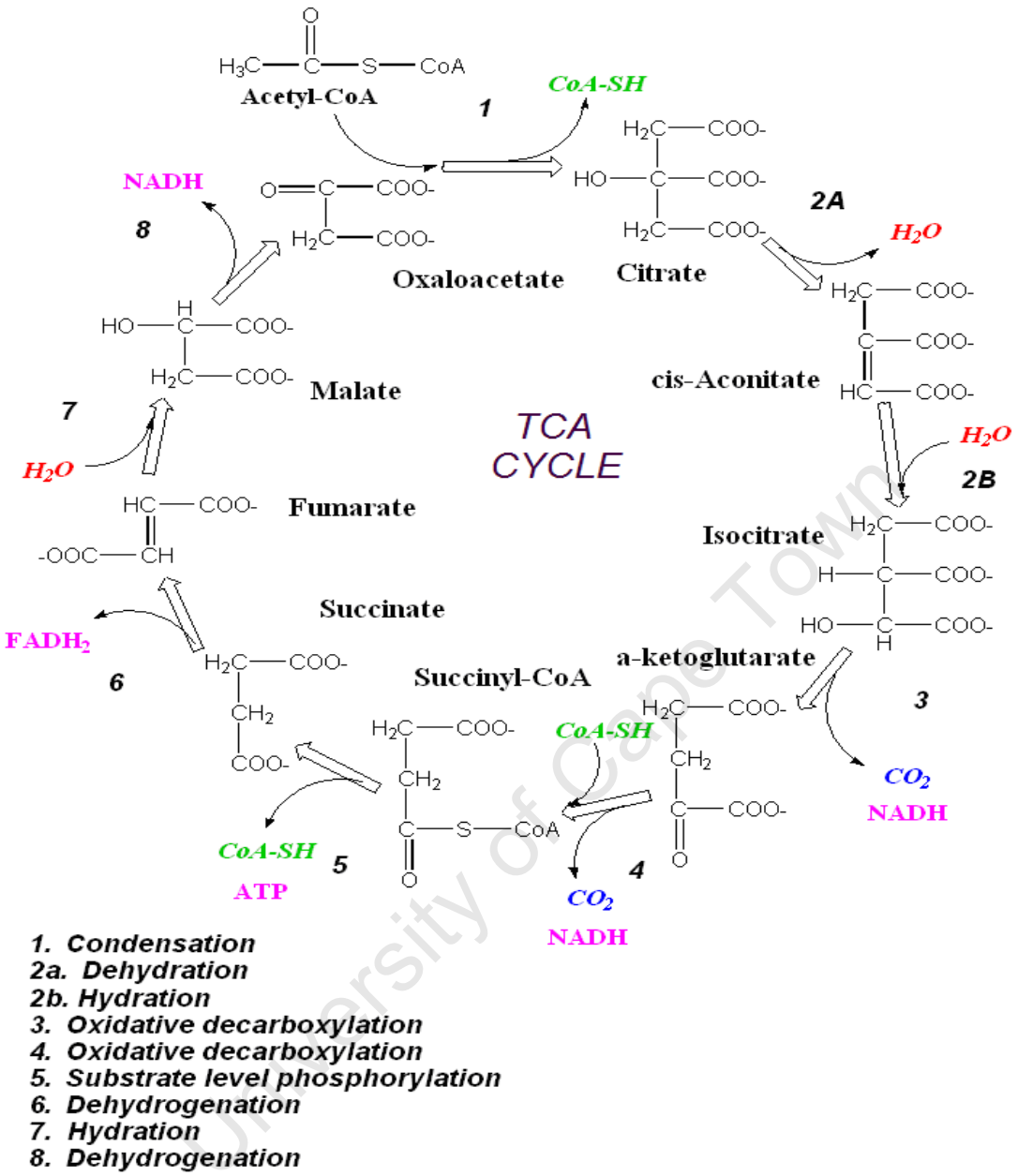

Figure 2.4 The tricarboxylic acid cycle

From an energy perspective the process is very wasteful, as only 2 ATP molecules are produced compared with complete oxidation which produces up to 36 ATP molecules per glucose molecule (Waites *et al.,* 2001). The biomass yield coefficient is reduced from 0.5 $g.g^{-1}$ under fully respirative conditions to below 0.1 $g.g^{-1}$ during fermentative growth. The lower biomass yield corresponds to a lower specific growth rate (Kappelli, 1986).

2.3.2.1 Factors Affecting Metabolic Pathway

The Crabtree and Pasteur Effects

The choice of metabolic pathway used by *S. cerevisiae* is known to be sensitive to both glucose and oxygen availability, as illustrated in the Crabtree and Pasteur effects respectively. These effects manifest themselves through catabolic repression mechanisms to determine which catabolic pathway dominates.

At high glucose concentrations under aerobic conditions, *S. cerevisiae* metabolism is fermentative rather than oxidative (Aon and Cortassa, 1998, Beck and von Meyenburg, 1968; Kappeli, 1986; Petrik *et al*., 1983; Postma *et al*., 1989). This phenomenon, known as the Crabtree effect, was named after Crabtree whose studies with tumour cells revealed that the glycolysis was excessive compared with the expected respiration rate. De Deken (1966) further illustrated the Crabtree effect when he showed that under aerobic conditions the fermentation rate increased as the glucose concentration increased (above 1 $g.l^{-1}$). In studies of glucose–limited aerobic continuous cultures Furukawa *et al.* (1983) reported that, above a critical dilution rate, the rate of fermentative growth dominates, while the oxidative pathway is repressed. Under the conditions used, the region of transition was found to lie at a dilution range (D) $0.2 < D < 0.3\ hr^{-1}$. At dilution rates below 0.2 hr^{-1}, glucose concentration was less than 50 $mg.l^{-1}$ and the ethanol less than 1 $mg.l^{-1}$. At dilution rates greater than 0.3 hr^{-1} glucose concentration exceeded 150 $mg.l^{-1}$ and ethanol 1 $mg.l^{-1}$. Fed-batch cultures of bakers yeast exhibited similar trends. Woehrer and Roehr (1981) showed that, at high feed rates, yeast biomass yields decreased in favour of ethanol production.

Traditionally the Crabtree effect was attributed to repression of respiration due to the presence of glucose (De Deken, 1966; Beck and von Meyenburg, 1968). More recently, the repression of respiration under Crabtree positive conditions has been challenged. The Crabtree effect is increasingly attributed to overflow of substrate from the glycolysis pathway, resulting in fermentative metabolism (Barford and Hall, 1979; Petrik *et al.*, 1983). Research has shown that the capacity for glucose transport is significantly higher than glucose utilisation via glycolysis (van Urk *et al*., 1989), resulting in potential for

substrate overflow. It is suggested that this substrate overflow overwhelms the oxidative metabolic pathway, allowing initiation of fermentative metabolism (Petrik *et al*., 1983; van Urk *et al*., 1989).

Also observed in yeast metabolism is the Pasteur effect. It has been defined as the inhibition of the sugar consumption rate by oxygen (Lagnus, 1986) (as cited in Weusthuis, 1994). In 1861, Pasteur found that fermentation in yeast was inhibited in the presence of oxygen. Under aerobic conditions, yeast growth was accelerated while uptake of sugar was diminished. As with the Crabtree effect many reasons have been suggested for this. The explanation given in literature has generally been the preferential utilisation of pyruvate in mitochondrial respiration, leading to reduced alcoholic fermentation and higher ATP and biomass yields. However, in her review of the Pasteur effect, Lagnus (1986) criticised the role of mitochondrial respiration in inhibition of fermentation. Lagnus (1979) pointed out that the growth media used by Pasteur to culture yeast lacked the extra requirements necessary for growth under anaerobic conditions, namely sterols and unsaturated fatty acids.

Figure 2.5 summarises the Crabtree and Pasteur effects by illustrating the influence of glucose and oxygen on the growth and ethanol production of *S. cerevisiae*.

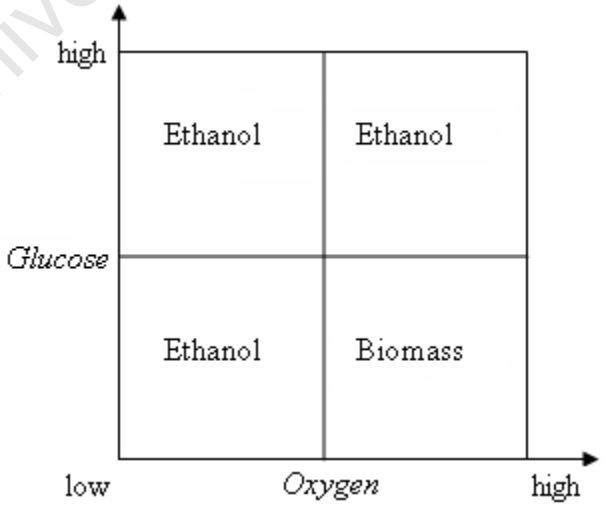

Figure 2.5 Influence of low and high concentrations of glucose and oxygen on the growth and ethanol production of *S. cerevisiae*.

2.3.3 Yeast Flocculation

The depletion of fermentable sugars is associated with the formation of flocs of yeast cells which fall out of suspension and settle at the base of fermentation vessels. This process, flocculation, is important in the brewing industry, facilitating both ethanol and biomass recovery. Early onset of flocculation may result in 'hanging fermentations' in which incomplete utilisation of fermentable sugars results in lower than theoretical ethanol yields. Therefore, the flocculation characteristics of a yeast strain or culture should be considered before brewing (Stewart and Russell, 1981).

Carbon dioxide evolution aids in the suspension of yeast cells during fermentation (van Hamersveld *et al*., 1998; Rose and Harrison, 1969). According to van Hamersveld *et al*. (1998) carbon dioxide induced velocities can reach up to 50 $cm.s^{-1}$. These velocities produce the turbulence instrumental in keeping yeast cells in suspension.

Various authors (Dengis *et al*., 1995; Khin *et al*., 1988; Stratford, 1989) have proposed a yeast flocculation mechanism, involving lectin resembling proteins called flocculins. The flocculins extend from flocculent cells and adhere to mannose residues found on neighbouring cells. Calcium ions have been reported by Bidard *et al*. (1995) and Bony *et al*. (1997) as essential in the activation of flocculins. Many other factors have been shown to affect flocculation of yeast, including nutritional availability, oxygen content, ethanol concentration, temperature, pH and cellular age. Since different yeast strains are affected differently, it is important for the brewer to know how these variables affect their strain (Sampermans *et al*., 2005; Verstrepen *et al*., 2003).

2.4 YEAST STRESS AND RESPONSE

Industrial yeasts such as S. *cerevisiae* are subjected to a variety of non-optimal physical, biological and chemical conditions in the process environment, collectively referred to as stress. The main stresses encountered by yeast include nutrient deprivation, heat stress, salt stress, osmotic stress and oxidative stress. The survival of yeast under stressful

conditions depends on its ability to adapt to a changing environment. Like most organisms yeast has acquired the ability to respond to unfavourable environmental conditions by induction of molecular mechanisms referred to as stress responses. In yeast, a marked increase in the stress response protein Hsp12p occurs when yeast are subjected to various forms of stress including nutritional limitations, high ethanol concentrations and high osmotic pressures (Karreman and Lindsey, 2005; Nisamedtinov *et al*., 2008). The induction of the molecular mechanisms results in a shift of cellular and metabolic processes to a new status (Birch and Walker, 2000; Hohmann and Mager, 1997). The role of these mechanisms is to protect cells from the potentially lethal effects of stressors and repair any cellular and molecular damage. These stress responses result in improved stress tolerance, preventing further cellular and molecular damage (Hohmann and Mager, 1997). Typically the onset of stress responses represent a metabolic burden to the cell.

2.4.1 Typical Stressors

A brief review on nutritional stress, thermal stress and oxidative stress is provided in this section. More detailed reviews of osmotic and salt stress are given in Section 2.5 and 2.6.

Yeast often experience nutritional limitation and starvation. They respond to nutritional limitations by a metabolic shift from utilisation of a richer to that of a poorer nutrient source. For instance, usually the first limiting nutrient in yeast cultures growing on a rich nutrient media is fermentable sugar. Yeast respond to this by reprogramming their metabolic capacity to allow utilisation of products such as ethanol and acetate as carbon sources. This change in metabolism is referred to as the diauxic shift and allows continuation of growth (de Winde *et al*., 1997). However, in the absence of nutritional substitutes (starvation) the shutdown of cellular proliferation is triggered, with subsequent entry into stationary phase. Growth ceases, and metabolic activity is kept minimal. The ultimate aim is the survival of the starvation period (Winderickx, *et al*., 2003).

Yeast are also sensitive to temperature, with suboptimal temperatures affecting numerous aspects of yeast physiology. These include cell viability, cell division and growth, plasma membrane structure and function, cellular metabolism, protein synthesis and chromosomal structure (Walker, 1998). Yeast response to sub-lethal temperature shock is the induction of the production of heat stress proteins (HSPs) encoded by stress response genes. However, certain preconditions must be met before induction of the heat shock response. Stationary phase cells, which are intrinsically thermotolerant, do not show a rapid response to temperature shock (Kirk and Piper, 1999; Piper, 1997). The response is induced over a narrow temperature range. Temperature upshifts, up to the maximum temperature at which yeast can grow (37 to 39^{o}C), result in strong induction of HSPs. Above this temperature, upshifts of 1 to 2^{o}C result in strong responses (Piper, 1997). The induction of HSPs results in a simultaneous suppression of synthesis of most proteins made prior to the stress (Shama *et al.*, 1998). Many HSPs function as protein molecular chaperones, binding destabilised or partially unfolded proteins and therefore protecting them from further degradation (Trot and Morano, 2003). A major stress response gene is the one coding for the heat shock protein Hsp104. Mild heat pre-treatment of yeast cells strongly induce this protein resulting in tolerance to higher temperatures. Wild type cells have been shown to have 100 to 1000 times the survival rates of hsp104 mutants when given short pretreatments (30 mins at 37^{o}C) followed by a severe heat shock (Sanchez and Linduist, 1990). After heat shock, Hsp104 promotes the resolubilisation of proteins that have unfolded and aggregated after heat shock (Parsell *et al*., 1994).

Oxidative stress occurs when yeast cells come into contact with reactive oxygen species (ROS) (Santoro and Thiele, 1997). ROS are produced during the reduction of molecular oxygen to water through acceptance of four electrons (Lu *et al*., 2005). Examples of ROS include the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). ROS attack most cell components including DNA, protein and lipid membrane (França *et al.*, 2006). However, treatment of cells with sub-lethal doses of ROS results in them being able to withstand higher magnitudes of oxidative stress (Farr and Kogoma, 1991). Lu *et al.* (2005) demonstrated this by pretreating yeast with 0.2 mM H_2O_2 prior to exposing them to 10 mM H_2O_2. After 45 hours, the pretreated cells had

approximately 82% viability while those that were not pretreated had a viability of about 22%. Yeast have evolved enzymatic mechanisms to protect themselves from oxidative stress. For instance, superoxide dismutases (SODs) are a group of metaloenzymes that catalyse the conversion of the superoxide anion to hydrogen peroxide. The hydrogen peroxide formed from SODs is scavenged by catalases which break it down to water and molecular oxygen (Santoro and Thiele, 1997).

2.5 YEAST OSMOTIC STRESS

Water plays a pivotal role in life as a solvent for many cellular biochemical structures, and provides an environment for biochemical reactions. Also, the cellular water content dictates turgor and therefore the shape and size of the cell. It is therefore vital for all cells to maintain their water levels within certain critical limits (Hohmann and Mager, 1997). Yeast, like all cells, experience changes in the water potential in their environment. When experiencing osmotic equilibrium, the intracellular and extracellular water potentials in yeast cells are equal (de Maranon *et al*., 1996). However, changes in the concentration of dissolved molecules in the medium surrounding yeast cells, alters water availability, potentially exposing yeast to high osmotic stress (Tamás and Hohmann, 2003; Myers *et al.*, 1997). Osmotic pressure effects on *S. cerevisiae* have been widely studied, and have been shown to affect cell growth and viability (Beney *et al.*, 2001; Laroche *et al.*, 2001; Myers *et al.*, 1997).

The optimum osmotic pressure for yeast viability has been reported to be 1.38 MPa at 25 ^{o}C by Beney *et al.* (2000) and Laroche *et al.* (2001). A standard laboratory medium containing about 50 g.l^{-1} of sugar, yeast extract and peptone has a solute concentration of 0.5 M (Bloomberg and Alder, 1992). This corresponds to an osmotic pressure of 1.24 MPa, which is slightly lower than the optimum value. However, Marechal and Gervais (1994) previously illustrated that *S. cerevisiae* can survive very high levels of osmotic pressure up to 100 MPa, provided the osmotic pressure was increased gradually. The slow linear increase in osmotic pressure allowed the cells to adjust to the changes in water potential without significant losses in viability. However, instantaneous increases

of the osmotic pressure proved lethal, resulting in significantly reduced yeast viability. Experiments by Beney *et al.* (2000) quantified these observations and showed that a steady 1.6 MPa.min^{-1} increase in osmotic pressure from 1.38 MPa to 100 MPa resulted in a final yeast viability of 70 ± 9%. A 100 MPa osmotic shock exposure reduced final yeast viability to 28 ± 7%, validating that a gradual osmotic pressure increase allows yeast to adapt to the osmotic pressure increases. These observations are summarised graphically in Figure 2.6.

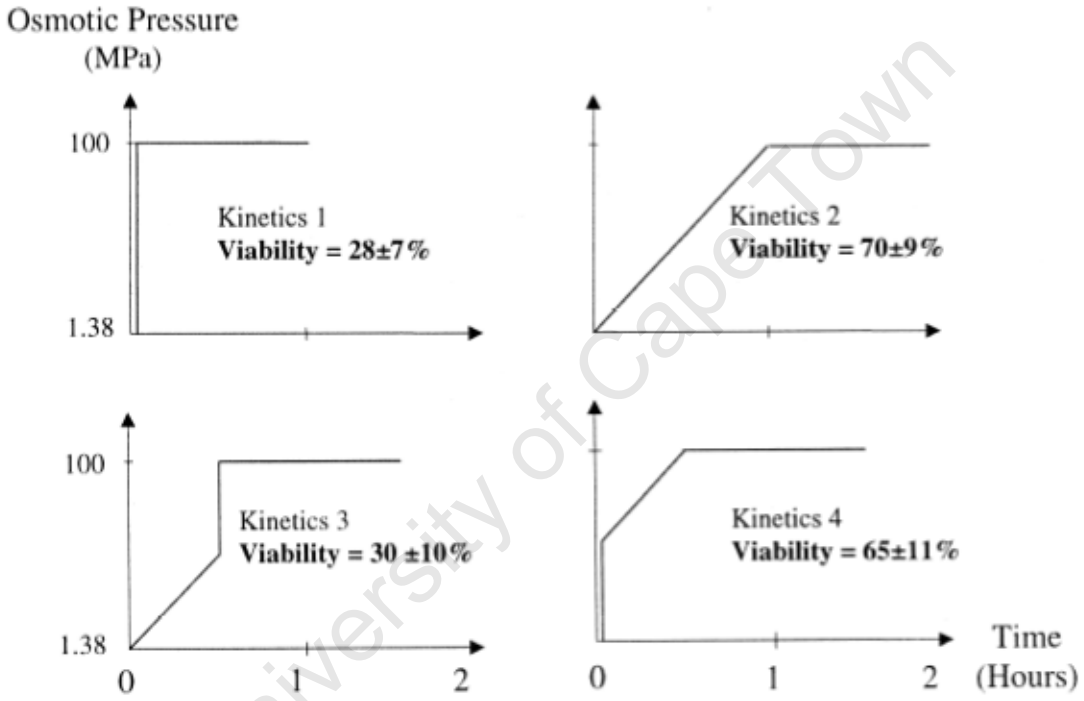

Figure 2.6 Viabilities of *S. cerevisiae* after different osmotic shifts from 1.38 to 100 MPa (Glycerol) (Beney *et al.,* 2000)

2.5.1 The Potential for Osmotic Stress in Yeast Fermentation

High concentrations of dissolved solutes in fermentation media such as brewers wort and molasses mash impose osmotic stress on yeast, affecting yeast growth and fermentation (Takeshige and Ouchi, 1995; Reddy and Reddy, 2006). While traditional beer brewing is done using normal gravity wort, which contains 11 to 12% dissolved solids, recent trends have been towards higher gravity fermentations (Thomas *et al*., 1995). By using more

concentrated worts, beers of greater strength are produced, which can be diluted back to specification levels (Murray and Stewart, 1991; Priest and Stewart, 2006). This approach results in increased plant output and cheaper beer production (Casey and Ingledew, 1983; Hammond, 1993; Stewart *et al*., 1988). Distillers and fuel alcohol manufacturers typically ferment mashes with dissolved solids concentrations which exceed 200 $g.l^{-1}$. Fermentations at these concentrations are considered high gravity fermentations. Their greatest benefits are that less process water is used and higher ethanol concentrations are achieved in the product stream. These benefits are important considering that close to 90% of energy consumption in ethanol production comes from ethanol purification by distillation, and stillage treatment (Bai *et al*., 2004). However, high gravity fermentations present some disadvantages. The disadvantages include loss in vital and viable yeast (Stewart, 2001) (as cited in Gibson *et al*., 2007), prolonged fermentation times, incomplete fermentations attributable to increased product inhibition and high osmotic pressures (Casey *et al*., 1984) (as cited in Barber *et al*., 2002).

Despite the disadvantages of high gravity fermentations, VHG (very high gravity) fermentations have attracted many studies. VHG fermentations are defined as fermentations of mashes with dissolved solids concentrations greater than 270 $g.l^{-1}$ (Banfrncová *et al*., 1999; Bayrock and Ingledew, 2001). In studies of yeast chemostat cultures under high glucose conditions, Zhao and Lin (2003) reported a decline in yeast biomass concentrations as glucose concentrations were increased from 100 to 300 $g.l^{-1}$. The biomass concentration was reduced from 0.91 $g.l^{-1}$ to 0.65 $g.l^{-1}$. They attributed the trend to increased osmotic stress contributed by the high glucose concentration, resulting in reduced yeast proliferation. The ethanol yield coefficient, in the same experiments, was reduced from 0.39 $g.g^{-1}$ at 10 $g.l^{-1}$ glucose to 0.24 ± 0.03 $g.g^{-1}$ at 100 $g.l^{-1}$ glucose, and maintained at that yield at the higher glucose concentrations. Bai *et al*. (2004) also report the deleterious osmotic effects of high glucose concentrations, but implicate high ethanol concentrations (>13%) as contributing both osmotic and toxic effects. Jones *et al.* (1994) showed that VHG fermentations of cane molasses produced higher ethanol concentrations, but at the expense of ethanol yield, which is of significance in process optimisation. Their findings are summarised in Table 2.5.

Fermentative performance of VHG systems, however, can be improved by yeast nutritional supplementation. Alfenore *et al*. (2002) showed that a vitamin mixture containing biotin was instrumental in enhancing fermentative performance in fed-batch cultures with initial glucose concentration of 100 $g.l^{-1}$. Doubling of the supplement resulted in increased growth rates (from 0.39 to 0.44 hr^{-1}) and higher final ethanol concentrations (126 to 132 $g.l^{-1}$). In similar studies, Barber *et al*. (2002) showed that acetaldehyde addition to VHG fermentations also improved fermentation performance. Daily additions of 0.2 $g.l^{-1}$ acetaldehyde to yeast fermentations of 300 $g.l^{-1}$ glucose reduced the time taken to consume the first 250 $g.l^{-1}$ glucose from 790 hours to 585 hours. Banfrncová *et al*. (1999) were able to improve final ethanol concentrations in 300 $g.l^{-1}$ glucose fermentation using urea supplementation. However, O'Connor-Cox and Ingledew (1991) showed that reduced fermentation performance as a result of nutrient limitation maybe overcome by increased pitching rates. These studies highlight the importance of rigorous understanding of limiting substance in VHG fermentations on rate, yield and concentration of biomass and product. Further, they may implicate yeast quality, affected by nutrient balance as a key parameter in response to osmotic stress.

Table 2.5 Effects of initial dissolved solids on ethanol yield of diluted molasses (Jones *et al.* (1994)

Initial dissolved Solids (g/100 ml)	Available fermentable Sugar (g/100 ml)	Dissolved solids consumed (g/100 ml)	Ethanol (% v/v)	Ethanol yield (% theoretical)
10.4	5.9	7	4	101
21.5	12.2	13.7	7.7	94
33.8	19.2	20.9	11.5	90
47.6	27.1	26.6	13.3	74

.

2.5.2 Osmotic Pressure Effects and Yeast Response

The exposure of yeast to high osmolarity results in rapid loss of intracellular water as it moves from a high water potential (yeast cytoplasm) to a lower water potential (surrounding media). Subsequently, yeast experience a loss of cell turgor, resulting in shrinkage of cells (Mager and Siderius, 2002). Cell shrinkage occurs rapidly, taking

approximately 1 minute (Bloomberg, 1999; Hohmann and Mager, 1997), with the final cell volume attained being inversely proportional to medium osmotic pressure (Hohmann and Mager, 1997). This cell volume fluctuation results in changes in the yeast cell structure. For instance, a rapid loss of actin fibres from mother cells occurs. Also, yeast actin cytoskeleton polarisation, which is essential for yeast budding, is lost. Consequently, cell growth is arrested (Tamás and Hohmann, 2003). Osmotic pressure also affects plasma membrane structure and its permeability, causing loss in cell viability (Wood, 1999). Cell membrane damage is temperature dependent and a consequence of water flow across an unstable membrane in phase transition (Laroche *et al*, 2001). Intracellularly, the loss of water from the cells results in water recruitment from the vacuole into the cytoplasm for the partial compensation of water loss (Mager and Siderius, 2002). The genetic response to water efflux is the induction of the high-osmolarlity glycerol (HOG) response pathway (Myers *et al*., 1997; Nevoigt and Stahl, 1997). The HOG response results in the increased production of glycerol which is retained by the cells to act as a osmoregulator (André *et al.,*1991; Mager and Siderius, 2002; Myers *et al*., 1997; Siderius *et al*., 2000). Glycerol production results in reduced metabolic flux towards pyruvate production. Consequently, glycerol production deprives cells of pyruvate, which is necessary for growth and ethanol production. Glycerol is synthesised by the reduction of dihydroxyacetone phosphate to glycerol 3-phosphate by glycerol 3-phosphate dehydrogenase, followed by dephosphorylation of glycerol 3-phosphate to glycerol. This is partly enhanced by over expression of *GDP1* which encodes glycerol-3-phosphate dehydrogenase. Glycerol is the main compatible solute produced by *S. cerevisiae* to counteract high osmotic pressure (Bloomberg, 1999; Elke and Stahl, 1997; Wodja *et al*., 2003). Its concentration has been reported to be proportional to the magnitude of the osmotic stress to which cells are subjected during growth (Lages *et al*., 1999). When quantifying glycerol levels in yeast under varying osmotic stress, André *et al*. (1991) measured glycerol levels of 16% dry weight in yeast grown in 0.68M NaCl media, while recording less than 1% in control cultures. Reed *et al*. (1987) found intracellular glycerol concentrations of 112 $g.l^{-1}$ in yeast grown in 0.86M NaCl media, while estimating it at less than 1 $g.l^{-1}$ in control cultures.

The increased glycerol levels in the cells lower its intracellular water potential, allowing water to influx back into the cell. The cell volume is increased, enabling regain of turgor pressure. The actin cables are reformed allowing cell polarisation and resumption of cell growth (Chant, 1999). However, the cells never reach their initial volume, their final size being inversely related to medium concentration (Hohman and Mager, 1997). The osmotic pressure response is summarised in Figure 2.7.

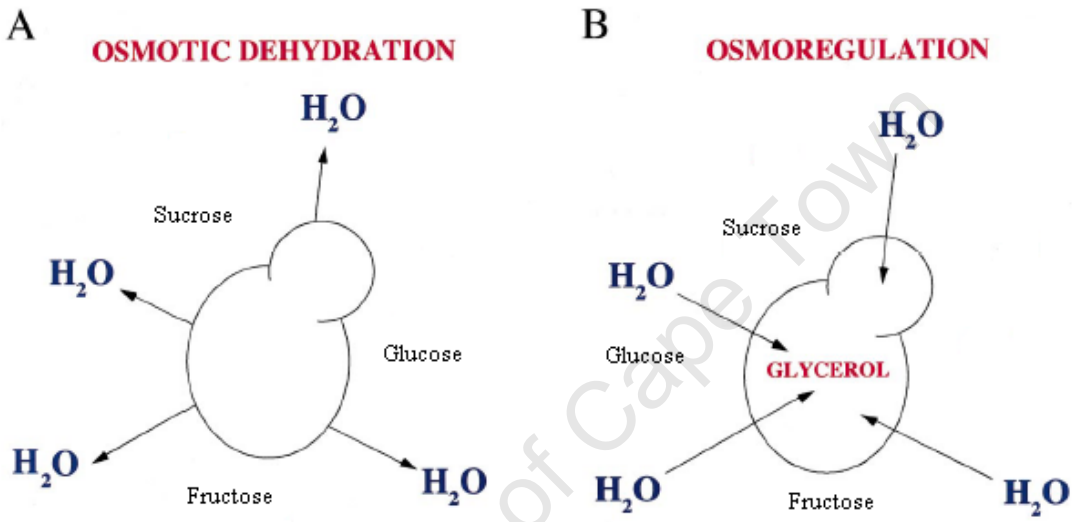

Figure 2.7 Key features of cellular response to high osmolarlity. A: Initial response is water efflux. B: The main osmoregulatory strategy of yeast is accumulation of glycerol, regain of water and turgor pressure (adapted from Bloomberg, 1999)

2.6 SALT STRESS

The presence of inorganic salts in fermentation media can affect yeast and fermentation performance. While some inorganic elements are required as trace elements for normal cell growth, exposure of yeast to high salt concentration subjects it to combined stresses due to reduced water potential, salt toxicity and ionic strength (Wadskog and Alder, 2003). To eliminate or reduce these stresses, the composition of typical fermentation media is carefully controlled (Table 2.4). However, many industrial processes such as ethanol production use waste products, like blackstrap molasses, as their fermentation

media. Such waste products can have high salt concentrations that inhibit cell growth and product formation (Table 2.2).

Blackstrap molasses has an inorganic salt concentration of 10 to 16% (m/m) (Table 2.2), which produces molasses mash with a salt content of 2.9 to 4.6%. These salt concentrations are high compared to typical fermentation media whose salt concentration is approximately 0.7% (Table 2.4). Consequently, yeast used to ferment blackstrap molasses is likely to experience salt stress. In this thesis the effect of three cations abundant in molasses, namely K^+, Mg^{2+} and Na^+, on fermentation of molasses by the yeast *S. cerevisiae* was investigated. A brief review of the role of each cation in yeast is given in this section.

K^+ is the most abundant cation (3.6% m/m) in South African molasses (Table 2.2). It is the preferred intracellular cation (Wadskog and Alder, 2003), with yeast having an absolute requirement for it for growth and fermentation (Suomalainen and Oura, 1971). Camacho *et al*. (1981) showed that yeast could not grow in a synthetic medium containing less than 0.2 mM K^+. Increases of media concentrations of K^+ from 0.2 mM to 0.35 mM K^+ resulted in a linear increase in the specific growth rate with a maximum specific growth rate of 0.22 hr^{-1} being achieved. Further increases above 0.35 mM did not result in a corresponding increase in the specfic growth rate. At growth limiting K^+ concentrations other alkali cations such as Na^+, Rb^+, Li^+ and Cs^+ can substitute for K^+, stimulating growth and fermentation (Camacho *et al*., 1981; Suomalainen and Oura, 1971). In their study Ryan and Johnson (2001) reported the optimum K^+ concentration for ethanol production by *S. cerevisiae* using artificial molasses as 10.5 $g.l^{-1}$ (0.27 M). This optimum K^+ concentration value is significantly greater than that reported by Camacho *et al*. (1981). The discrepancy is possibly due to the different fermentation media used in the experiments. The results of experiments performed in this thesis support this idea. The results of Chapter 5 show that the type of media influences the effect of cations on yeast growth and fermentation performance.

Na^+, which constitutes 0.28% (m/m) of molasses, is toxic (Gómez *et al*., 1996) with yeast having no absolute requirement for it. (Wadsjkog and Alder, 2003). Its presence in molasses is therefore undesirable due to its potential as a salt and osmotic stressor. The toxicity of Na^+ is manifested during inorganic sulphate assimilation in yeast. Inorganic sulphate activation results in the accumulation of sulphate as 3-phosphoadenosine-5-phosphosulphate (pAps). The use of pAps, either by reduction to sulphite or transfer to other molecules, produces nucleotide 3-phosphoadenosine-5-phosphate (pAp) (Murguía *et al*., 1996). This nucleotide, which is toxic to yeast, is hydrolysed to AMP to recycle adenosine. The nucleotidase (Ha12p), which catalyses this reaction is inhibited by Na^+, allowing pAp accumulation in the presence of Na^+ (Gómez *et al*., 1996; Murguía *et al*., 1996; Wadsjkog and Alder, 2003). Yeast response to high intracellular Na^+ concentrations includes increased expression of genes encoding Na^+ efflux (Gómez *et al*., 1996) and vacuole compartmentalisation of Na^+ through increased expression of NHX1 gene (Wadsjkog and Alder, 2003).

Mg^{2+} (0.46% m/m in molasses) plays a vital role in ethanol fermentation, that cannot be met by other metal ions. Its intracellular concentration is maintained at the millimolar level. It plays a crucial role in DNA replication, transcription and translation (Dombek and Ingram, 1986). Its importance in the maintenance and regulation of numerous growth and metabolic processes is absolute (Walker *et al*., 1996). Walker (1998) showed that extracellular Mg^{2+} in fermentative media provides physiological protection for yeast against stresses such as ethanol and temperature, in terms of enhancing yeast growth and viability. For example, when actively growing cells were transferred to a 10% ethanol solution containing 20 mM Mg^{2+}, their viability steadily decreased to 53% after 24 hours. Those that were placed in 2 mM Mg^{2+} had zero viability after the same duration. Yeast growing at 30^{o}C subjected to heat shock at 42^{o}C in the presence of 2 mM and 20 mM Mg^{2+} solution also illustrated the protective properties of Mg^{2+}. After 24 hours, viability in 2 mM Mg^{2+} solution was 4% while that in 20 mM Mg^{2+} solution was 25% (Walker, 1998). The above observations illustrate the importance of Mg^{2+} in maintaining high yeast viability and consequently fermentation performance at the millimolar level.

However, the effects of Mg^{2+} at high Mg^{2+} concentrations, such as those encountered in molasses fermentations are not well understood, and are investigated in this thesis.

2.7 TREHALOSE AND GLYCOGEN AS STRESS PROTECTANTS

Two carbohydrates have been implicated in playing important roles as stress protectants, namely trehalose and glycogen (Silljé *et al.,* 1999; Wiemken, 1990). Quantification of trehalose and glyocogen in experiments with stressed yeast was not performed due to unavailability of required equipment. Trehalose and glycogen are discussed in this section.

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a non-reducing disaccharide of glucose present in all forms of life except mammals (Basu *et al*., 2006; Conlin and Nelson, 2007; Van Dijck *et al*., 1995). It has historically been regarded as a storage carbohydrate, but has attracted more interest recently due to its role a stress protectant (Basu *et al*., 2006; Hounsa *et al*., 1998, Quain, 1991). Trehalose accumulation in yeast is observed under various stress conditions including nutrient stress, oxidative stress, osmotic stress and heat stress. For example, under heat stress trehalose and heat shock protein (HSP) levels rise rapidly (Conlin and Nelson, 2007). The high trehalose levels reportedly stabilise enzymatic activities (Felix *et al*., 1999; Zancan and Sola-Penna, 2005) and prevent exogenous protein aggregation from heat shock (Davies *et al*., 2006; Singer and Lindquist, 1998a). Under high osmotic pressure, it is also observed that those cells with higher trehalose levels have higher survival rates (Hounsa *et al*., 1998). Yeast growing exponentially on glucose has been shown to have low trehalose levels which correspond to limited stress resistance. Those in stationary phase show elevated trehalose levels (up to 20% of yeast dry weight) and increased stress resistance (Singer and Lindquist, 1998b; Van Dijck *et al*., 1995). This characteristic emphasises the importance of inoculating stationary phase yeast cells into a potentially stressful environment as it increases proliferation. Benaroudj *et al*. (2001) reported the protective role of trehalose during oxidative stress. When yeast cells, having trehalose levels of ~5 $mmol.g^{-1}$, were exposed to H_2O_2 for 15 to 20 minutes, there was no observable loss in yeast viability.

However, for cells with undetectable trehalose levels the viabilities decreased to approximately 55%, illustrating the importance of trehalose as a stress protectant.

Glycogen is a branched glucose polysaccharide found in yeast, consisting of linear α (1,4)-glucosyl chains with α (1,6)-linkages (François and Parrou, 2001). It has traditionally been regarded as a storage carbohydrate (François and Parrou, 2001; Lille and Pringle, 1980; Parrou *et al*., 1997) representing up to 23% of dry weight of cells (Lille and Pringle, 1980). Intracellular glycogen concentrations in pitching yeast are used routinely as an indicator of potential fermentative performance, with high levels being desirable. High glycogen levels are essential in the initial stages of fermentation, following inoculation with stationary phase yeast. Here glycogen is mobilised for lipid synthesis (Murray *et al*., 1984). Murray *et al*. (1984) also showed a decrease in fermentation rates with decreasing initial glycogen levels in *S. cerevisiae*. During brewing, the accumulation of glycogen during fermentation shows three distinct phases: rapid depletion during the initial hours of fermentation; accumulation during fermentation, followed by a gradual depletion towards the end of fermentation (Quain and Tubb, 1982; Murray *et al*., 1984) (as cited in Basson, 1996). Together with its role as a storage carbohydrate, glycogen is also considered a stress protectant. Intracellular glycogen accumulates not only during carbon starvation, but also other stress conditions such as nitrogen or sulphur starvation, osmotic stress and heat shock (Lillie and Pringle, 1980; Siljé *et al*., 1998). In support of glycogen being a stress protectant, Lille and Pringle (1980) showed that cells cultured in a nitrogen limited medium had higher intracellular glycogen (22% dry weight) concentrations than those cultured in the control medium (2.5% dry weight).

2.8 ETHANOL PRODUCTION

Industrial ethanol is produced by large-scale yeast fermentation of sugars derived from agricultural products such as sugarcane, followed by separation of the ethanol by distillation. While industrial ethanol has been produced by fermentation of agricultural waste products such as molasses for more than half a century, Brazil pioneered the first

major ethanol biofuel programme (ProAlcohol) in 1975 before the USA initiated its own programme in 1978 (Wheals *et al*., 1999). Various reactor configurations are used in bioethanol production and include batch, continuous and fed-batch systems. A detailed discussion of batch operations is provided in Section 2.8.1, while fed-batch and continuous systems are discussed briefly in Sections 2.8.2.

2.8.1 Ethanol Production in Batch Reactors

Most of the bioethanol has been produced by batch operation (Çaylak, and Vardar Sukan, 1996). In South Africa, production of ethanol from molasses is exclusively done in batch reactors, while in Brazil 70% of distilleries use batch processes (Wheals *et al*., 1999). In typical batch fermentations, mechanically agitated reactors are filled with diluted molasses to approximately 70% of their volume. After pH adjustment, reactor contents can be sterilised then cooled to fermentation temperature. A yeast culture is then inoculated into the fermentation vessel (Shuler and Kargi, 2002). The yeast extract available nutrition in the molasses converting it to biomass and ethanol. The fermentation cycle typically lasts 1 to 3 days (Lin and Tanaka, 2006).

Reasons for batch fermentations remaining the most common in industry include (Çaylak and Vardar Sukan, 1996; Shuler and Kargi, 2002):

- Low investment costs
- Ease of operation, eliminating the need for skilled labour
- Use of bioreactors for various product specification
- Genetic stability of the yeast

Kinetics of Growth and Ethanol Formation

Experimental data from batch cultures show that the rate of biomass formation is proportional to the amount of biomass and thus follows first order kinetics. This can be expressed mathematically as shown:

$$\frac{dC_x}{dt} = \mu C_x \qquad 2.1$$

where C_x is the biomass concentration (g.l^{-1}), t (hr) the elasped time and μ is the specific growth rate (hr^{-1}). The value of μ varies depending on the yeast strain and reactor conditions, and can range from 0.1 to 0.78 hr^{-1} (Philasopeng *et al*., 2006). Most values for batch fermentations fall in the 0.35 to 0.5 hr^{-1} range (Najafpour *et al*., 2004; Win *et al*., 1996) The specific growth rate is itself a function of the rate limiting substrate concentration as expressed by the Monod equation (Equation 2.2):

$$\mu = \left(\frac{\mu_m C_s}{K_s + C_s} \right) \qquad 2.2$$

where μ_m is the maximum specific growth rate, C_s (g.l^{-1}) the rate limiting substate concentration and K_s (g.l^{-1}) the saturation constant. Mathematically, K_s corresponds to the rate limiting substrate concentration at which the specific growth rate is half of its maximum value as shown in Figure 2.8.

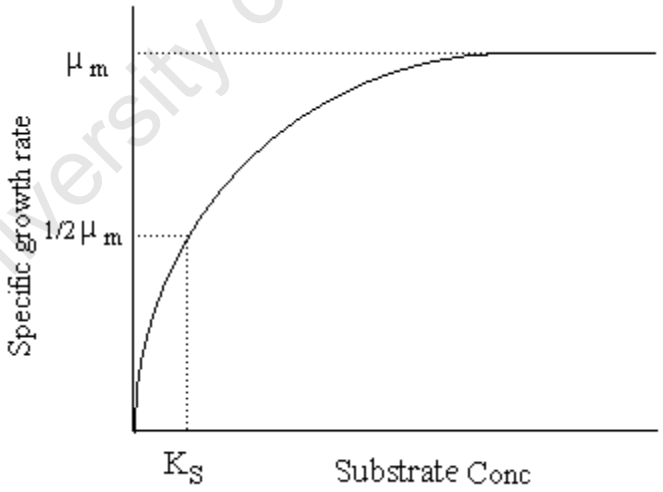

Figure 2.8 The relationship between μ_{max} and K_s (Waites *et al*., 2001)

Yeasts are constantly subjected to a variety of non-optimal conditions in the process environment. Under stressful conditions yeast death can occur, thus reducing the

effective specific growth rate. This is accounted for by introducing the specific death rate, k_d (hr^{-1}), as shown in Equation 2.3:

$$\mu = \left(\frac{\mu_m C_s}{K_s + C_s} \right) - k_d \qquad 2.3$$

High substrate concentrations, as encountered in VHG fermentations, can result in microbial growth inhibition by the substrate. Assuming that the inhibition is non-competitive and neglecting cell death Equation 2.2 becomes:

$$\mu = \frac{\mu_m}{\left(1 + \frac{K_s}{C_s}\right)\left(1 + \frac{C_s}{K_I}\right)} \qquad 2.4$$

where K_I is the inhibition constant.

The presence of growth inhibition factors in fermentation of waste industrial products such as molasses provides the need for further inhibition kinetics. The specific growth rate variation in the presence of toxic compounds, including salts, is a function of the toxic compound concentration C_A. Carvalheiro *et al*. (1999) have expressed this in terms of:

$$\frac{d\mu}{dC_A} = K_I \mu \qquad 2.5$$

where the growth inhibition constant, K_I is a term characteristic of each toxic compound. By integrating Equation 2.5:

$$\int_{\mu_o}^{\mu_i} \frac{d\mu}{\mu} = K_i \int_{A_0}^{A_i} dC_A \qquad 2.6$$

where μ_i is the specific growth rate under the concentration of toxic compound, C_A, and μ_0 is the specific growth rate at the minimum salt toxic concentration, C_{A0}, a linear equation results. A line function can be drawn from which slope K_I is determined as shown in equation 2.7:

$$Ln(u) = Ln(u_0) + K_I(C_{A_i} - C_{A_o}) \quad 2.7$$

Under anaerobic conditions yeast shift to respiro-fermentative growth, which is characterised by significantly reduced yeast growth and increased ethanol production. As fermentation progresses, the ethanol concentration in batch reactors increases, and further inhibits growth and viability of yeast. This phenomenon is termed product inhibition and affects the specific growth rate as shown in Equation 2.8:

$$\mu = \left(\frac{\mu_m C_s}{K_s + C_s}\right)\left(1 - \frac{C_p}{K_p}\right) \quad 2.8$$

where C_p is the ethanol concentration, and K_p is the ethanol inhibition term for growth. K_P represents the minimum ethanol concentration at which inhibition occurs. It is a function of both temperature and initial sugar concentration. K_p is reported at ~80 $g.l^{-1}$ at 30^oC and an initial sugar concentration of 22% (w/v) (Pilasopeng *et al*., 2006). Combinations of Equations 2.2 to 2.8 can be developed to describe the prevailing conditions in the yeast fermentation appropriately.

Yield Coefficients

The yield coefficients are important design parameters which quantify the amount of substrate recovered in the biomass and the metabolic products. These coefficients are given as ratios of the specific rates, e.g. for the yield of biomass on a substrate, Y_{xs}:

$$Y_{xs} = \frac{\mu_x}{r_s} \quad 2.9$$

where μ_x is the biomass specific growth rate and r_s is the specific substrate utilisation rate. Y_{xs} ranges from 0.5 $g.g^{-1}$ during aerobic growth to below 0.2 $g.g^{-1}$ during fermentation (Kappeli, 1986; Kryzystek and Ledakowicz, 1998). Similarly, for the yield of a metabolic product, in our case ethanol, on substrate, Y_{ps}:

$$Y_{ps} = \frac{r_p}{r_s} \qquad 2.10$$

where r_p is the rate of product formation. In the production of ethanol, it is important to optimise the yield of product on substrate by directing more carbon towards product formation than biomass.

For aerobic processes the yield of CO_2 from O_2, termed the respiratory quotient (RQ), is often used to characterise the metabolism of the cells. It is readily seen from a stoichiometric analysis that with complete respiration the RQ is close to 1, whereas if a metabolite is formed it is greater than 1 (Ratledge and Kristiansen, 2001).

2.8.2 Ethanol Production in Fed-batch and Continuous Systems

In fed-batch operations of ethanol production, yeast culture, molasses mash and required nutritional supplements are fed continuously or semicontinuously into the fermenter. Where a "fill and draw" regime is maintained the effluent is also removed discontinuously. The main advantage of fed-batch operation is prevention of substrate inhibition or catabolite repression by intermittent feeding of substrate. Where the substrate is inhibitory, fed-batch operations improve fermentation productivity by maintaining low substrate concentration (Çaylak and Vardar Sukan, 1996; Shuler and Kargi, 2002). Also, fed-batch operations can be used to produce product of higher alcohol content than batch processes, thus reducing plant hydraulic loading (Piggot, 2003).

Recent trends in ethanol production have seen substitution of batch fermentation processes with continuous processes. Approximately 30% of ethanol manufacturers in

Brazil now use continuous processes (Alegre *et al*., 2003; Wheals *et al*., 1999). Here the feed, containing molasses mash and nutritional supplements is continuously pumped into an agitated vessel (Çaylak and Vardar Sukan, 1996) or series of vessels (Alegre *et al*., 2003) where yeast are active. The product, containing ethanol, yeast cells and residual sugar is continuously removed. While continuous processes are more difficult to operate, requiring highly skilled labour, they offer numerous advantages over traditional batch processes. These include (Shuler and Kargi, 2002):

- A constant culture environment for yeast growth and ethanol formation resulting in a product of uniform quality.
- Reduced down time.
- Improved productivity. Approximately, 95% of sugar conversion to ethanol can be achieved with a residence time of 21 hours, as compared to batch operation of 40 hours.
- Optimisation of continuous systems by yeast recycling further improves ethanol productivity.

While continuous systems have numerous advantages, they may not be ideal for developing countries due to challenges presented by high capital costs and lack of skilled labour.

2.9 HYPOTHESIS

Based on the analysis of both the industrial fermentation of molasses to ethanol and of the relevant literature, the following hypotheses are put forward for investigation in this thesis:

1. Variation in inorganic ash content of molasses encountered through the sugar crushing season results in a variation in fermentation performance.

2. Reduced fermentation performance as a result of increased inorganic ash is a consequence of high salt concentration impacting yeast metabolism through concentration of specific ions, high ionic strength or osmotic stress on yeast.
3. The high K^+ concentration in molasses is the main contributor to the yeast stress and subsequent loss in fermentation performance.
4. Molasses mash concentration, and thereby sugar concentration further influences osmotic stress and thereby fermentation performance.

CHAPTER 3

CHARACTERISATION AND QUANTIFICATION OF FERMENTATION PERFORMANCE

3.1 INTRODUCTION

The experimental procedure in the chapter describes the anaerobic cultivation of *S. cerevisia*e in molasses media (mash) and sucrose-based media batch fermentations. The procedure allows comparisons of yeast growth and fermentation performance between plural media with different cation concentrations, ionic strengths, osmotic pressures and sugar concentrations. The ability of yeast to grow and reproduce and its physiological state during and after fermentation were used as indicators of yeast performance. Kinetic parameters, such as sugar utilisation rate, ethanol production rate, and fermentation efficiency, were used to assess fermentation performance. The materials and methods employed to assess and quantify fermentative performance are introduced and discussed.

3.2 MATERIALS

3.2.1 Yeast Strain

The commercial strain of the yeast *S. cerevisiae*, IL1ZK was supplied by Illovo Sugar Refineries (Merebank, KwaZulu Natal, South Africa). The yeast culture was available in two forms:

1. The yeast was provided on agar slants which were stored at 4°C for up to three months, after which new slants were requested. An inoculum culture was produced by aseptically transferring a loop of yeast from the agar slants into sterile YPD media. The composition of the YPD media is shown in Table 3.1. The pre-culture was cultivated for 24 hours at 30°C on a rotary shaker at 160 rpm, producing a final cell concentration of 10^8 cells.ml^{-1}. To produce an initial cell concentration of 10^5 cells.ml^{-1}, in shake flask fermentations, 0.3 ml of inoculum was added to 300 ml of fermentation media.

Table 3.1 Pre-inoculum culture media composition

Component	Composition (g.l^{-1})
Glucose	10
Peptone	5
Yeast extract	3
Malt extract	3

2. The yeast was provided as a cream slurry, having a yeast dry weight of 200 g.l^{-1}. The yeast cream was stored in sterile 250 ml Erlenmeyer bottles at 4°C for up to 1 month. This yeast was used to inoculate fermentations with high initial cell concentrations ($\geq 10^7$ cells.ml^{-1}). Approximately 3.5 ml of yeast cream per litre of fermentation media was required to produce an initial cell concentration of 10^8 cells.ml^{-1}.

Varying initial yeast concentrations, ranging from 10^5 to 10^8 cells.ml^{-1}, were used during fermentations depending on the aim of the experiment. Ethanol producers use large inocula of typically 10^8 cells.ml^{-1} (Alegre *et al*., 2003). However, the costs of the large inoculum can be weighed against batch cycle time and ethanol yield. Manipulation of inoculum size in research applications is recognised to alter sensitivity to stress response.

3.2.2 Fermentation Media and Preparation

Two fermentation base media were used to investigate the effect of cation concentration, ionic strength and osmotic pressure on yeast growth and fermentation performance. The media used were molasses media (mash) and a sucrose-based media. The salts KH_2PO_4, $MgSO_4$ and Na_2SO_4 were added to the media prior to fermentation in varying amounts to adjust K^+, Mg^{2+} and Na^+ concentration, ionic strength and osmotic pressure, such that their fermentative effects may be quantified.

Molasses media (mash)

Molasses media (MM1 and MM2) were prepared by mixing tap water and blackstrap molasses in a defined ratio, according to Table 3.2. Approximately 25 ml of 1M H_2SO_4 solution per litre of molasses media was required to adjust media pH from pH 5.2 to the desired pH 4.6. Exactly 1.25 g urea per litre of molasses media was added as a nitrogen supplement. Where necessary, suspended solids in the media were removed by centrifugation at 10,000 rpm for 10 minutes, and the supernatant used as fermentation media. A few drops of antifoam 204 (Sigma Aldrich) were added to the media to minimise foaming. The total sugar concentration of the media as reducing monosaccharides was measured by DNS following acid hydrolysis. The method is detailed in Appendix A1. The DNS-measured total sugar concentration overestimates the available fermentable sugar as it also measures unfermentable monosaccharides.

Table 3.2 Fermentation media used in fermentations

Media	Water: molasses ratio	pH	Total sugar concentration ($g.l^{-1}$) (DNS)	Fermentable sugar concentration ($g.l^{-1}$)
MM1	1:3	4.6	140-150	120-130
MM2	1:2.5	4.6	170-180	150-160
SM1	-	4.8	150-160	150-160

Sucrose-based media (SM1)

The use of a sucrose-based media as fermentation media was instrumental in comparing yeast and fermentation performance in molasses media to a standard yeast propagation media. The media contained (per litre) 145 g sucrose, 15 g glucose, 20 g peptone and 10 g yeast extract (manufactured by Merk Biolab). To buffer the media at pH 4.8, 3.06 g sodium acetate and 1.2 ml acetic acid were used. A few drops of antifoam 204 were added to minimise foaming.

3.3 EQUIPMENT

Batch fermentations were carried out in either 500 ml shake flasks or 5 l New Brunswick (Bioflo 110) bioreactors.

3.3.1 Shake Flask Fermentations

The majority of fermentations were performed in 500 ml Erlenmeyer flasks under anaerobic conditions. The flask openings and base had a diameter of 5 cm and 10 cm, respectively. Silicone bungs were used to close the flasks to prevent exposure of fermentation media to air. Two 0.125 cm diameter holes were cast into the silicone bungs and cylindrical glass tubes of equal diameter inserted to act as either a sample port or carbon dioxide exit point. The sample port tube extended into the fermentation medium, while the outlet was clamped. A 45 micron filter was placed on the exit of the carbon dioxide port. This tube extended into the headspace only. The shake flask fermentation set up is illustrated in Figure 3.1. Approximately 300 ml of media were used for each fermentation. A yeast slurry was inoculated at a cell concentration of 10^5 to 10^8 cells.ml^{-1} depending on the experimental objective. Fermentations were performed in a 30^{o}C incubator at an agitation speed of 160 rpm. At regular intervals samples of 5 ml were collected for analysis.

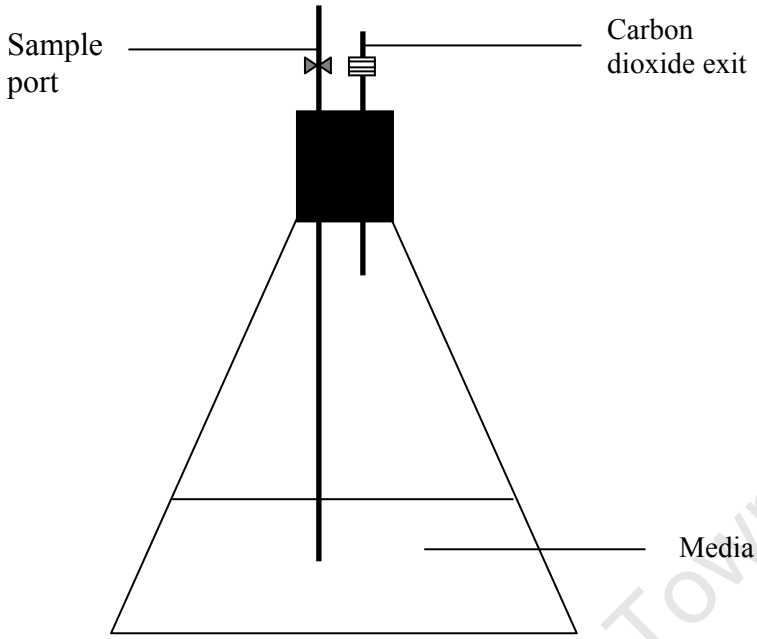

Figure 3.1 Schematic representation of shake flask system used for fermentations

3.3.2 Bioreactor Fermentations

Comparative fermentations of "good" and "bad" molasses were performed in New Brunswick Bioflo 110 bioreactors. The experimental set up used is shown in Figure 3.2. The New Brunswick Bioflo 110 bioreactor has a total internal volume of 7 litres with a working volume of 5 litres. It is equipped with two six-blade impellers in combination with four baffles to ensure efficient mixing. The temperature of the reactors were automatically controlled at 30^{o}C using a cooling water heat exchange and heated jacket system. pH control was unnecessary as the media pH remained constant at pH 4.6 throughout the fermentation process. Agitation was set at 300 rpm.

Figure 3.2 Bioreactor set up used in bad and good molasses fermentations

3.4 ANALYTICAL TECHNIQUES

3.4.1 Methods to Monitor Fermentation

Cell Counts

Cell counts, together with viability measurements, were performed by light microscopy. Cell viability is discussed in greater detail under yeast quality assessments in Section 3.4.2. Samples to be counted were diluted with methylene blue staining solution to achieve a cell concentration of approximately 10^6 cells.ml^{-1}. After 10 minutes of incubation at room temperature, a drop of the diluted sample was placed on the grid of a haemocytometer (Improved Neubauer, BS. 748, Weber Scientific International, England). A glass cover slide was placed over the haemocytometer. Cell counting was achieved using the light microscope (Model: BX40, Olympus Optical co. Ltd, Japan) at 20 x magnification with bright field optics. The new and improved Neubauer counting chamber comprised of 25 identical big square blocks, each containing 16 smaller ones.

The area of each small square is 0.0025 mm^2 and the depth is 0.1 mm. The cells in 5 (randomly chosen) of the 25 large squares were counted, and the number used to determine cell concentration. The cell concentration was calculated as shown in Equation 3.1:

$$Cell\ \ conc\ \ (cells\ .ml^{-1}) = \frac{N_T \times DF\ \times 1000}{0.0025\ \times 0.1 \times 16 \times 5} \qquad 3.1$$

where N_T is the total cells in 5 big blocks and DF is the dilution factor. The coefficient of variance for replicate samples was 10% at an average cell concentration of 1.2 x 10^8 cells.ml^{-1}.

Sugar Concentration

Two different methods were used to monitor sugar concentration: spetrophotometric determination of reducing sugars using DNS (3,5-dinitrosalicylic acid) and HPLC (high pressure liquid chromatography) to determine individual sugars.

a. DNS Method

The DNS method was used to determine the total sugar concentration as reducing monosaccharides following acid hydrolysis. This method was chosen as it is simple to use and relatively inexpensive. It is a colorimetric method originally developed by Miller (1959) to estimate glucose concentration in culture media. In the presence of reducing sugars DNS is reduced to 3-amino, 5-nitrosalicylic acid, which gives a reddish-brown colour. Using a spectrophotometer the intensity of the colour can be related to reducing sugar concentration. The coefficient of variance was 2.2% for measurements at a sugar concentration of 160 g.l^{-1} (158 ± 3.5 g.l^{-1}). The procedure is detailed in Appendix A1.

Owing to limitations of DNS in sugar analysis of molasses, it is recognised that these analyses are best interpreted on a relative basis. DNS measurements are influenced by a variety of salts, which result in an over or underestimation of true reducing sugar concentration (Sinegani and Emtiazi, 2006). Since salts are present in molasses, and some were used as part of the media formulation, some interference was expected. Also, DNS

total sugar measurements of molasses include non fermentable sugars, resulting in an over estimation of fermentable sugar content. Based on the above challenges associated with DNS use, calibration of a subset of sugar analysis was conducted using HPLC.

b. HPLC Method

The use of HPLC for sugar analysis is relatively expensive, however it is more accurate than DNS measurement. Further, the measurement of specific sugar concentrations (e.g sucrose, glucose and fructose), rather than total sugar concentration, is possible. The total sugar concentration can then be determined by addition of individual sugar concentrations. This approach also allows determination of the fermentable sugar content by removing non-fermentable sugars from the total sugar concentration. Reverse phase HPLC was used for determination of sugar concentration using a Merck Hitachi L-700 series HPLC equipped with an Agilent 1100 series refractive index (RI) detector. Detection was performed at 280 nm. An HPX-87 ion exclusion column was used (300mm by 7.8 mm; BioRad, California). An example of an HPLC chromatogram obtained is shown in Figure 3.3, illustrating individual sugar peaks and their respective retention times. The coefficient of variance was 0.6% for measurements at a sugar concentration of 130 $g.l^{-1}$ (131 ± 1 $g.l^{-1}$). The detailed method is given in Appendix A1.

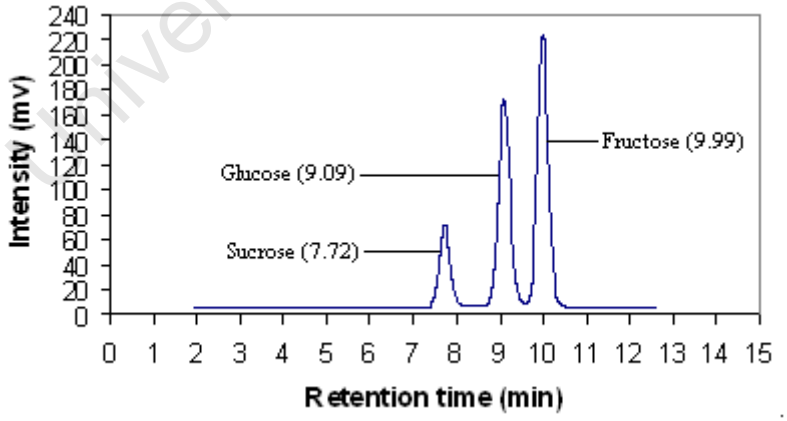

Figure 3.3 HPLC chromatogram showing sucrose, glucose and fructose peaks and their respective retention times

Ethanol Analysis

Ethanol was analysed by gas chromatography (GC). The Perkin Elmer Autosystem GC with a flame ionisation detector was used. The column used was a BP-20 column with polyethylene glycol packing. Helium was the carrier gas. An example of a GC chromatogram obtained is shown in Figure 3.4. Here the ethanol and 1,4 dioxane (internal standard) peaks are identified. The coefficient of variance was 2.5% for measurements at an ethanol concentration of 5.0% (5.0 ± 0.1%). Details of the method used in the analysis of ethanol are provided in Appendix A1.

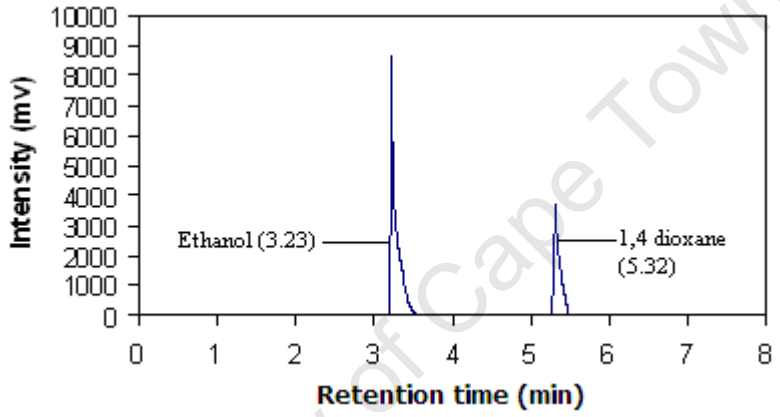

Figure 3.4 GC chromatogram showing 2% ethanol and 1% 1,4 dioxane

3.4.2 Yeast Quality Assessments

The assays used for the identification and quantification of yeast quality included the specific growth rate, yeast viability and oxygen utilisation rate. While other yeast quality assays such as intracellular reserve compounds and stress indicators were recommended, the instrumentation and methods required for them were not available for this research.

Specific growth rate (μ)

Microbial growth is dependent on their concentration and is therefore more appropriately defined in specific terms. The specific growth rate (μ) was introduced in Equation 2.1 in Section 2.6. By replacing biomass concentration, C_X, in Equation 2.1 with cell concentration, C_N, and rearranging it, we can define μ as:

$$\frac{dC_N}{dt}\frac{1}{C_N} = \mu \qquad 3.2$$

Further rearrangement followed by Integration of Equation 3.2, as shown in equation 3.3, results in a linear equation, Equation 3.4, from which μ can be determined.

$$\int_{C_{N,o}}^{C_{N,t}} \frac{dC_N}{C_N} = \mu \int_{t_0}^{t_t} dt \qquad 3.3$$

$$Ln(C_{N,t}) = Ln(C_{N,o)}) + \mu(t_t - t_0) \qquad 3.4$$

Cell counts were used to determine the specific growth rate as described above.

Cell Viability by Methylene Blue Staining

Cell viability is a measure of the cells' ability to reproduce. It is commonly referred to as a "live-dead" assay. Cell viability is assessed using staining techniques such as methylene blue staining used in this study. Methylene blue is historically the most commonly used staining technique in the brewing industry (Lentini, 1993), being recommended by European Brewing Convention (EBC) (1962), the Institute of Brewing (IOB) (1970) and the American Society of Brewing Chemists (ASBC) (1980). However, its continued use in industry has been criticised as it is regarded as representing a rough guide to yeast viability. The use of methylene violet has been proposed as a more accurate alternative (Smart *et al*., 1999). A slow trend to the use of methylene violet is beginning. However, methylene blue remains the industry standard for which benchmark comparison is readily available. It is for this reason that methylene blue was selected for use in this study.

Samples to be analysed were diluted to a cell concentration of ~10^6 cell.ml^{-1} using methylene blue, then incubated at room temperature for 10 minutes to allow the dye to stain non-viable or dead cells blue, while live or viable cells remained colourless. A small

drop of the sample was placed on a Neubauer counting chamber and viewed under microscope. Cell viability was then calculated using Equation 3.5:

$$Viability\ \ \% = \frac{N_T - N_{blue}}{N_T} \times 100 \qquad 3.5$$

where N_T is the total cell number and N_{blue} is the number of blue stained cells. At an average viability of 96 % a coefficient of variance of 2% was observed.

There are varying theories concerning the mechanisms of methylene blue. The European Brewing Convention (1962), American Society of Brewing Chemists (1976) and McCaig (1990) support the idea of living cells with intact cell membranes excluding the dye from their cytoplasm, while non living cells are unable to do so resulting in them being stained. Lentini (1993) suggests that methylene blue penetrates all cells, but only living cells are able to reduce the stain to a colourless compound.

Oxygen Utilisation Rate (OUR)

The physiological state of yeast determines the rate at which it takes up oxygen (Daoud and Searle, 1986), with yeast of poorer physiological condition showing low oxygen utilisation rates. The oxygen utilisation rate (OUR) of yeast can therefore be used as a measure of yeast activity. Yeast use oxygen for oxidation of carbohydrates for energy generation, biomass formation and manufacture of fatty acids and sterols (Boulton and Quain, 1987). The apparatus used to measure OUR consisted of the YSI dissolved oxygen electrode and an OUR meter with online data monitoring. The set up is shown in Figure 3.5, while the method used detailed in Appendix A1.

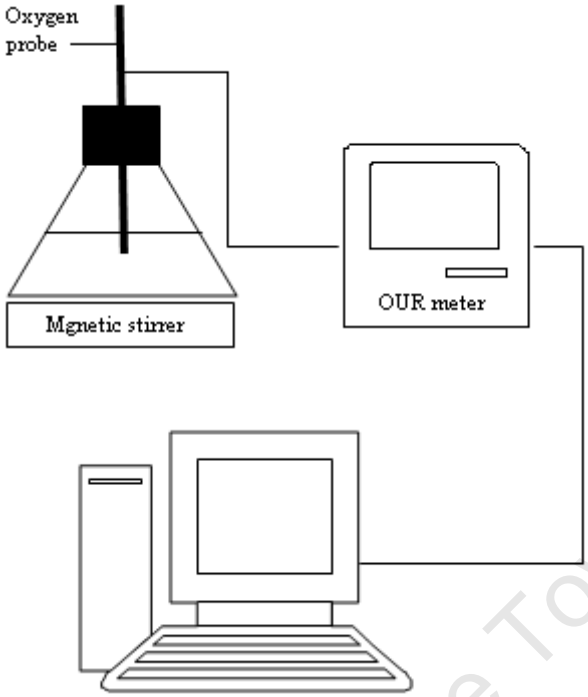

Figure 3.5 Set up and apparatus for OUR measurements

3.4.3 Media Characterisation

The physical properties of molasses vary as a result of its compositional variation. In this thesis, yeast growth and fermentation performance were related to the variation in some of the physical properties of molasses. These included sugar concentration (Section 3.4.1), ionic strength, osmotic pressure and cation composition. In this section the methods and apparatus used to measure ionic strength, osmotic pressure and cation composition are detailed.

Ionic Strength

The ionic strength of media can interfere with transport of nutrients in an out of cells, cellular metabolism and solubility of nutrients such as oxygen, resulting in a negative impact on fermentation (Shuler and Kargi, 2002). The ionic strength is a function of the charge and concentration of ions in solution. It is defined mathematically as:

$$I = \tfrac{1}{2}\sum_{B=1}^{n} c_B z_B^2 \qquad 3.6$$

where C_B is the concentration of ion B (mol.l^{-1}), Z is the charge of ion B, and the sum is taken over all the ions in solution. The unit of ionic strength is Siemens (S). Where the concentration and charge of ions in solution are unknown, the ionic strength of the solution can be measured using a conductivity meter. The ionic strength was measured using a CD-4301 Lutron conductivity meter. Media ionic strength is temperature dependent and was measured at 25 ^{0}C.

Cation Composition

Concentrations of the metal ions, K^+, Mg^{2+}, Na^+ and Ca^{2+} were determined using AAS (atomic absorption spectroscopy). In their elemental form, metals absorb ultraviolet light when excited by heat, with each metal absorbing a characteristic wavelength. AAS detects a particular metal by focusing a beam of ultraviolet light at a specific wavelength through a flame and into a detector. The sample is injected into the flame. If the metal of interest is present in the sample, it will absorb some of the light, thus reducing its intensity. The change in intensity is measured by a detector, which gives a signal relative to concentration of the metal. Samples to be analysed were first digested using acid before AAS analysis.

Osmotic Pressure

High osmotic pressures of media are known to affect fermentation negatively (Section 2.5). The osmotic pressure of the media was determined with the aid of the Osmometer 800 CL. The osmometer measures the total solutes using a cryometric method. The measuring range covers 0 to 2000 mOsm per kg of water, with a coefficient of variance of ± 1%. The osmolarlity is then used to calculate the osmotic pressure (Π) of the solution, which is given by:

$$\pi = iMRT \qquad 3.7$$

where, *i* is the Van't Hoff factor, *M* is the molarity, *R* the universal gas constant, and *T* the thermodynamic temperature.

3.5 FERMENTATION EFFICIENCIES COMMONLY USED IN INDUSTRY

Various efficiency calculations are used by ethanol producers to determine the quality of molasses from a fermentative perspective. These are described briefly below:

Fermentation efficiency (F.ε) is the ratio of the ethanol produced ($C_{E,max}$) to the theoretical amount determined using the stoichiometry of reducing sugar conversion to ethanol by yeast. The ideal fermentation efficiency of 100%, is rarely achieved.

$$F.\varepsilon = \frac{C_{E,Max}}{TSAI \times 0.511} \tag{3.8}$$

Molasses usage efficiency (M.U.ε) is the ratio of molasses used to the ethanol produced. The units are kg molasses/bulk l of ethanol, where bulk ethanol has a 96.4% purity. A small molasses usage efficiency is desirable indicating that less molasses is used for the production of a specific amount of alcohol. Usually this results from molasses of high sugar concentration.

$$M.U.\varepsilon = \frac{Molasses\,used\ (kg)}{Bulk\ ethanol\ produced\ (l)} \tag{3.9}$$

***Sugar usage efficiency* (*S.U.ε*)** is the ratio of TSAI in molasses to ethanol produced, given in units of kg TSAI/ l ethanol. Low sugar usage efficiencies are desirable as they indicate that the available sugar is used more efficiently in the production of ethanol.

$$S.U.\varepsilon = \frac{TSAI\,in\,molasses\,(kg)}{Bulk\ ethanol\ produced\,(l)} \tag{3.10}$$

Residual sugar (R.S) content is the ratio of the final TSAI content to the initial TSAI. A high residual sugar content (>1%) is undesirable and is an indication of incomplete fermentation. This is common for "bad" molasses.

$$R.S = \frac{TSAI_{final}}{TSAI_{initial}} \qquad 3.8$$

The above performance efficiencies were used in the characterisation of "good" and "bad" molasses.

3.6 EXPERIMENTAL APPROACH

The impact of molasses quality on ethanol fermentation was investigated through a three fold approach:

1. The role of three cations (K^+, Mg^{2+} and Na^+) on yeast growth and fermentation performance was investigated in two different media (molasses media and sucrose-based media). The cations were added as KH_2PO_4, $MgSO_4$ and Na_2SO_4 such that cation concentration ranged from 0 to 18.5 $g.l^{-1}$. Addition of the above salts, also resulted in corresponding increases in ionic strength and osmotic pressure of the media, with possible implications on yeast and fermentation performance.
    - Yeast performance was monitored in terms of specific growth rate, cell viability, and OUR (oxygen utilisation rate).
    - Sugar utilisation rate, ethanol production rate, fermentation efficiency, molasses usage efficiency and sugar usage efficiency were used as fermentation performance indicators.
2. The potential for molasses concentration to affect yeast and fermentation performance was also investigated. The molasses media was prepared as detailed

in Section 3.2.2 except that the molasses: water ratio concentration was varied to produce fermentable sugar concentrations ranging from 120 to 210 $g.l^{-1}$. The media was then fermented under standard conditions and yeast and fermentation performance monitored.

3. Having understood the effects of cations (1) and molasses media concentration (2) on yeast and fermentation performance, comparative fermentations of "good" and "bad" molasses were performed. Performance of the "good" and "bad" molasses fermentations was related to cation concentration, ionic strength, osmotic pressure and sugar concentration in an attempt to identify characteristics that can be used to distinguish between "good" and "bad" molasses.

CHAPTER 4

STATISTICAL ANALYSES AND REPRODUCIBILITY OF RESULTS

4.1 INTRODUCTION

In this chapter the methods used for statistical analyses and reproducibility of experimental data are discussed. A brief review of statistical theory and the statistical tools used for data analyses is followed by results to investigate data reproducibility. The reproducibility of results for both sucrose-based and molasses media fermentations were assessed using the shake flask setup. Analyses of results were done with the aid of the 'Data Analysis' tool available in Microsoft Excel, which utilises the statistical theory discussed in the chapter.

4.2 STATISTICAL THEORY

The standard deviation (s) of a set of experimental measurements is a measure of precision or agreement between the measurements. The standard deviation is defined in terms of the arithmetic mean as follows:

$$s = \sqrt{\frac{\sum_{i=1}^{N} \left(x_i - \bar{x}\right)^2}{N-1}} \qquad 4.1$$

where x_i is the i th measurement in the sample set, $\bar{x}$ is the set arithmetic mean and N is the number of measurements in the sample set.

Precision can also be expressed in terms of the coefficient of variance (*CV*), defined as:

$$CV = \frac{s}{\bar{x}} \times 100\% \qquad 4.2$$

The sample mean $\bar{x}$, determined when *N* is small, represents the mean of a sample drawn from the population. In contrast, the population mean (α) is the true mean of the population as *N* approaches infinity. The true standard deviation (σ) is therefore defined as:

$$\sigma = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \alpha)^2}{N}} \qquad 4.3$$

In most practical settings the true standard deviation cannot be obtained and a good approximation is obtained by performing numerous replicate measurements. Alternatively, data from a series of samples accumulated over time can be used to determine a pooled standard deviation (s_{pooled}) which is superior to the value of an individual subset. The pooled standard deviation is defined as:

$$s_{pooled} = \sqrt{\frac{\sum_{i=1}^{N_1}(x_i - \bar{x})^2 + \sum_{j=1}^{N_2}(x_j - \bar{x})^2 + \sum_{k=1}^{N_3}(x_k - \bar{x})^2 + ...}{N_1 + N_2 + N_3 + ... - N_s}} \qquad 4.4$$

where N_1 is the number of measurements in set 1, N_2 is the number of measurements in set 2 and so on. The term N_s is the number of data sets pooled. The denominator in the equation is referred as the 'number of degrees of freedom' (Napier-Munn, 1995).

4.2.1 Confidence Limits

Using statistical theory, limits can be set around the determined sample mean such that the true mean will lie within these limits with a given degree of probability. These limits are called confidence limits (*CL)* and the interval they define is known as confidence interval. Confidence limits can be illustrated by plotting relative frequency of deviations from the mean as a function of a quantity *z*, defined as:

$$z = \frac{(x - \alpha)}{\sigma} \qquad 4.5$$

The plot results in a normal error curve resembling the one shown in Figure 4.1.

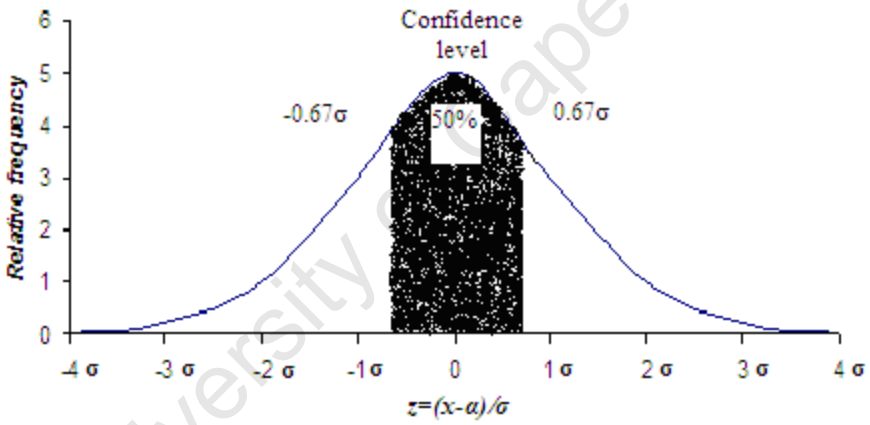

Figure 4.1 Graph showing area under curve at 50% confidence level

In Figure 4.1 50% of the area under the normal error curve is shown. For any normal error curve this area (50%) lies between -0.67σ and +0.67σ, while 95% lies between -1.96σ and +1.96σ. Therefore, it may be assumed that 95 of 100 times the true mean will be within ±1.96σ of any of the measurements made. In this case, the confidence level is 95%, while the confidence limit is ±1.96σ.

The calculation of *s,* which is based on a small set of data, is uncertain and hence confidence limits are necessarily broader. Analogous to *z*, a value *t* is defined as follows to account for the variability of *s*:

$$t = \frac{(x - \alpha)}{s} \qquad 4.6$$

The value of *t* depends on the desired confidence level and the number of degrees of freedom. As the number of degrees of freedom becomes infinite, t→z.

In the absence of a good estimate of σ, the confidence limits for the mean *x* of *N* replicate measurements can be derived from *t* using Equation 4.7:

$$CL \ for \ \alpha = \bar{x} \pm \frac{ts}{\sqrt{N}} \qquad 4.7$$

4.2.2 Comparison of Two Experimental Means

Determination of significant difference between the means of two sets of data is done by hypothesis testing. It is assumed that the samples are identical (i.e null hypothesis: $\bar{x}_1 - \bar{x}_2 = 0$) and hence observed difference is the result of indeterminate errors. To test this hypothesis against the alternative hypothesis ($\bar{x}_1 - \bar{x}_2 \neq 0$), the following equation can be used:

$$\bar{x}_1 - \bar{x}_2 = \pm ts_{pooled} \frac{\sqrt{N_1 + N_2}}{N_1 N_2} \qquad 4.8$$

Equation 4.8 assumes the standard deviations of the samples are not significantly different. If the experimental difference ($\bar{x}_1 - \bar{x}_2$) is smaller than value computed in the right hand side (RHS) of the equation, the null hypothesis cannot be rejected, hence no significant difference between the two means is demonstrated at selected confidence level. An experimental difference greater than the value computed from *t* indicates a significant difference between the means.

Paired T-test

When dealing with data collected before and after an intervention, paired-sample t-testing can be used. Here the signed differences (δ's) between the paired data are regarded as a random sample from the population for which α = δ. The null hypothesis α = 0 is tested against the alternative α ≠ 0. The critical value of rejection for the null hypothesis is calculated as follows:

$$\bar{x} - \alpha = \pm \frac{ts}{\sqrt{N}} \qquad 4.9$$

If the mean of the differences exceeds the critical value (RHS of equation) the null hypothesis is rejected and the differences between the before and after situation can be regarded as a real (Miller and Freud, 1984).

4.3 REPRODUCIBILITY OF EXPERIMENTAL RESULTS

To assess reproducibility of results in sucrose-based and molasses media, fermentations were performed in triplicate under standard conditions. Samples were collected at regular intervals and measurements of cell concentration, specific growth rate, cell viability, sugar concentration, ethanol concentration, ionic strength and osmotic pressure used to calculate the pooled standard deviation and coefficient of variance. The paired t-test was used to investigate if apparent differences in measurements were real or a result of random error.

4.3.1 Reproducibility in Sucrose-based Media

The cell concentration, cell viability, sugar concentration and ethanol concentration of triplicate runs in sucrose-based media are shown in Figure 4.1. Good reproducibility was shown in sucrose-based media in all the above variables except for cell concentration, in

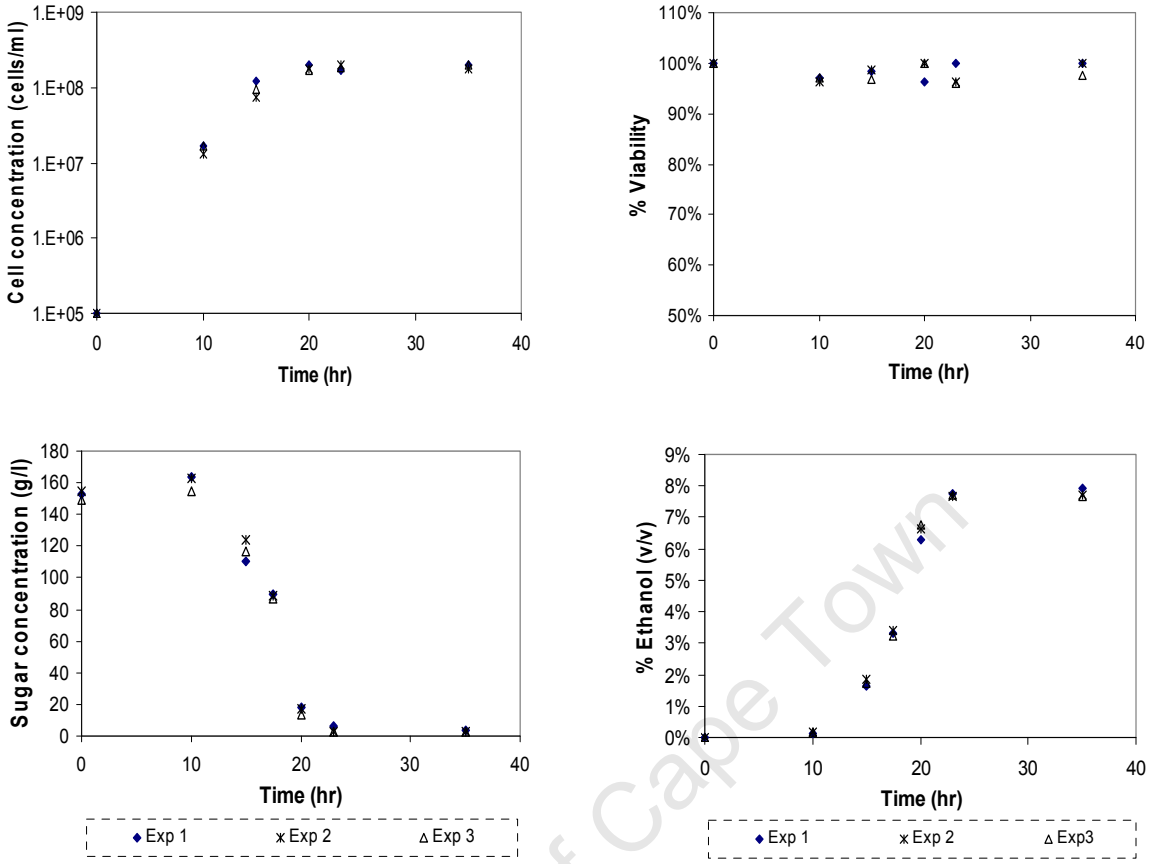

Figure 4.2 Reproducibility of cell concentration, cell viability, sugar concentration and ethanol concentration in sucrose-based media

Table 4.1 Reproducibility of results in sucrose-based media

Method	Significant difference	% confidence	Standard deviation	Coeff of variance
Cell concentration	No	95	1.4 E+07 cells.ml^{-1}	13%
Specific growth rate	No	95	0.02 hr^{-1}	3%
Cell viability	No	95	2%	2%
Sugar concentration	No	95	4 g.l^{-1}	5%
Ethanol concentration	No	95	0.1%	3%
Ionic strength	No	95	0.1 mS	2%
Osmotic pressure	No	95	0.1 MPa	2%

which the coefficient of variance was relatively high at 13%. The paired t-test, performed at 95% confidence level, showed no significant difference in replicates. Table 4.1 summarises this reproducibility.

Table 4.2 shows raw cell concentration data for the triplicate fermentations, the calculated s_{pooled}, *CV* and *DF* as an example of reproducibility analyses performed.

Table 4.2 Cell concentration data and reproducibility in sucrose-based media

	Cell concentration (cells.ml^{-1})		
Time	**Experiment 1**	**Experiment 2**	**Experiment 3**
0	1.0 E+05	1.0 E+05	1.0 E+05
10	1.7 E+07	1.3 E+07	1.7 E+07
15	1.2 E+08	7.4 E+07	9.4 E+07
20	2.0 E+08	1.8 E+08	1.7 E+08
23	1.7 E+08	2.0 E+08	1.9 E+08
35	2.0 E+08	1.8 E+08	2.0 E+08
		s_{pooled}	1.4 E+07
		CV	13%
		DF	12

An example of the paired t-test using the cell concentration data in Table 4.2 is shown in Figure 4.3. The rest of the results are available in Appendix B1. Here each combination of the three replicate runs were paired and analysed using the paired t-test application available in the Data Analysis package found in Microsoft Excel.

Yeast cell concentration

t-Test: Two-Sample Assuming Equal Variances

	Run 1	*Run 2*
Mean	117350000	107016666.7
Variance	7.99048E+15	8.01342E+15
Observations	6	6
Pooled Variance	8.00195E+15	
df	10	
t Stat	0.200079777	
P(T<=t) two-tail	0.845428456	
t Critical two-tail	2.228138842	

t-Test: Two-Sample Assuming Equal Variances

	Run 1	*Run 3*
Mean	117350001.5	111350000
Variance	7.99048E+15	7.73932E+15
Observations	6	6
Pooled Variance	7.8649E+15	
df	10	
t Stat	0.117183245	
P(T<=t) two-tail	0.909035003	
t Critical two-tail	2.228138842	

t-Test: Two-Sample Assuming Equal Variances

	Run 2	*Run 3*
Mean	107016666.7	111350000
Variance	8.01342E+15	7.73932E+15
Observations	6	6
Pooled Variance	7.87637E+15	
df	10	
t Stat	-0.08457066	
P(T<=t) two-tail	0.934271873	
t Critical two-tail	2.228138842	

P(T<=t) two-tail gives probability that apparent differences are due to random error

Figure 4.3 Cell concentration reproducibility using paired t-test assuming unequal variances

4.3.2 Reproducibility in Molasses Media

Reproducibility in molasses mash was analysed in a similar way to sucrose-based media. The cell concentration, cell viability, sugar concentration and ethanol concentration for triplicate runs in molasses media are shown in Figure 4.4. Good reproducibility was shown in all cases with the coefficient of variance ≤ 7%. The paired t-test, performed at 95% confidence level, showed no significant difference in replicates. Table 4.3 summarises this reproducibility.

Table 4.4 shows raw sugar concentration data for triplicate fermentations in molasses media, the calculated s_{pooled}, *CV* and *DF* as an example of reproducibility analyses performed. The paired t-test results are shown in Figure 4.5 and show a high degree of reproducibility for sugar concentration.

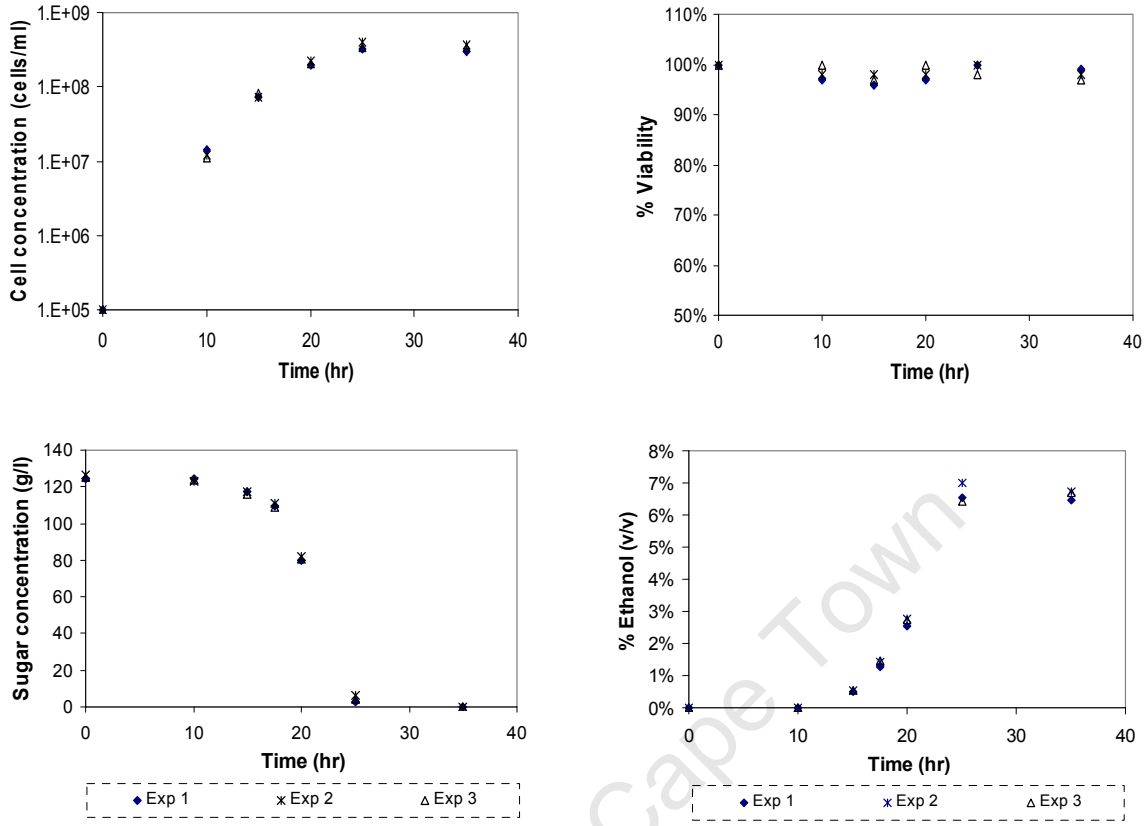

Figure 4.4 Reproducibility of cell concentration, cell viability, sugar concentration and ethanol concentration in molasses media

Table 4.3 Reproducibility of results in molasses media

Method	Significant difference	% confidence	Standard deviation	Coeff of variance
Cell concentration	No	95	2.3E+07 cells.ml^{-1}	7%
Specific growth rate	No	95	0.1 hr^{-1}	2%
Cell viability	No	95	1%	1%
Sugar concentration	No	95	2 g.l^{-1}	2%
Ethanol concentration	No	95	0.1%	6%
Ionic strength	No	95	0.2 mS	1%
Osmotic pressure	No	95	0.1 MPa	2%

Table 4.4 Sugar concentration data and reproducibility in molasses media

	Sugar concentration ($g.l^{-1}$)		
Time	**Experiment 1**	**Experiment 2**	**Experiment 3**
0	124	126	124
10	125	123	123
15	118	117	114
17.5	109	111	106
20	80	82	79
25	3	6	4
35	0	0	0
		s_{pooled}	2
		CV	2
		DF	14

Sugar concentration

t-Test: Two-Sample Assuming Equal Variances

	Run 1	*Run 2*
Mean	79.79192029	80.81440714
Variance	3091.244438	3030.231599
Observations	7	7
Pooled Variance	3060.738018	
df	12	
t Stat	-0.03457631	
P(T<=t) two-tail	0.972986081	
t Critical two-tail	2.178812827	

t-Test: Two-Sample Assuming Equal Variances

	Run 1	*Run 3*
Mean	79.79192029	78.547644
Variance	3091.244438	2969.730363
Observations	7	7
Pooled Variance	3030.4874	
df	12	
t Stat	0.042285805	
P(T<=t) two-tail	0.966966312	
t Critical two-tail	2.178812827	

t-Test: Two-Sample Assuming Equal Variances

	Run 2	*Run 3*
Mean	80.81440714	78.547644
Variance	3030.231599	2969.730363
Observations	7	7
Pooled Variance	2999.980981	
df	12	
t Stat	0.077424947	
P(T<=t) two-tail	0.939561487	
t Critical two-tail	2.178812827	

Figure 4.5 Sugar concentration reproducibility using paired t-test assuming unequal variances

4.4 DISCUSSION AND CONCLUSIONS

The reproducibility study showed acceptable experimental reproducibility in both sucrose-based media and molasses media. All variables measured had coefficients of variance of 6% or less, except for cell concentration which had a coefficient of variance of 7% and 13% in molasses media and sucrose-based media, respectively. Comparison of experimental data sets using the paired t-test showed good reproducibility in both media at 95% confidence level.

CHAPTER 5

THE EFFECT OF CATIONS K^+, Mg^{2+} and Na^+ ON YEAST GROWTH AND FERMENTATION PERFORMANCE

5.1 INTRODUCTION

The cations K^+, Mg^{2+}, and Na^+ are abundant in molasses relative to their requirement by yeast for fermentation (Camacho *et al*., 1981; Gómez *et al*., 1996, Ryan and Johnson, 2001; Wadskog and Alder, 2003; Walker *et al*., 1996). Their presence in excess can potentially affect yeast growth and fermentation performance by inducing salt, ionic, and osmotic stress in yeast. In this chapter the salt concentration, ionic and osmotic effects of the above cations on yeast growth and fermentation performance were investigated. A special emphasis was placed on K^+ ions as they are the largest inorganic constituent of molasses at 3.6 % (m/m). First, the effect of K^+ was investigated at varying concentrations in a nutrient rich, sucrose-based media, whose composition is detailed in Section 3.2.2. Yeast performance in the media was quantified by monitoring its specific growth rate (μ), viability and oxygen utilisation rate (OUR). The sugar utilisation rate (φ), ethanol production rate (ω) and fermentation efficiency (F.ε) were used to quantify fermentation performance.

Next, fermentations were performed in molasses mash (prepared as shown in Section 3.2.2), in which the K^+ concentration was supplemented. The results were compared with those from the sucrose-based media. Molasses usage efficiency (M.U.ε) and sugar usage efficiency (S.U.ε) were included in the analyses. Differing yeast growth and fermentation performance in sucrose-based media and molasses mash necessitated further comparison

of the effects on yeast growth and fermentation performance of the two remaining cations (Mg^{2+} and Na^+). An attempt to explain performance differences in sucrose-based media and molasses mash was made by performing further fermentations in sucrose-based media supplemented with molasses mash at a constant K^+ concentration of 15 $g.l^{-1}$. The above experiments were conducted using the shake flask set up described in Section 3.3.1. The results of these experiments are detailed in this chapter.

5.2 EFFECT OF K^+ ON ETHANOL FERMENTATION

The requirement for K^+ by *S. cerevisiae* is strict with no growth possible in media containing less than 0.2 mM K^+ (0.008 $g.l^{-1}$ K^+) (Camacho *et al.*, 1981). However, the optimum concentration for growth is not certain with Ryan and Johnson (2001) reporting an optimum K^+ of 10.5 $g.l^{-1}$ for ethanol production in synthetic molasses, while Camacho *et al.*, 1981 report the optimum concentration for cell growth at 0.014 $g.l^{-1}$ K^+. These conflicting reports necessitate an investigation of the role and effect of K^+ on yeast and fermentation performance. To achieve that, the effect of K^+ concentration was studied in both a well defined, complex sucrose-based media and molasses mash. Results from experiments were analysed and compared.

5.2.1 Effect of K^+ on Ethanol Fermentation in Sucrose-based Media

Experimental Setup and Data Collection

To study the effect of K^+ ions in sucrose-based media, experiments were conducted in 500 ml shake flasks at conditions shown in Table 5.1. The K^+ concentration was varied from 0 to 15 $g.l^{-1}$, by addition of KH_2PO_4 to the fermentation media. To achieve the desired K^+ concentration, KH_2PO_4 was added as shown in Table 5.2. Upon yeast inoculation of the fermentation media, samples were taken to determine initial conditions. Samples were taken at 0 and 10 hours and thereafter at five hour intervals. Cell concentration, cell viability, sugar concentration and ethanol concentration data obtained in the investigation are provided in Figure 5.1.

Table 5.1 Operating conditions for fermentations in sucrose-based media

Flask volume	500 ml
Working volume	300 ml
Initial sugar concentration	~150 $g.l^{-1}$
Inoculum concentration	10^5 $cells.ml^{-1}$
Media pH	4.8
Operating temperature	30 ^oC
Agitation speed	160 rpm

Table 5.2 KH_2PO_4 added to working volume to achieve desired K^+ concentration

K^+ concentration ($g.l^{-1}$)	KH_2PO_4 added to working volume (g)
0	-
5	5.1
10	10.2
12.5	12.8
15	15.4

Specific Growth Rate, Viability and Vitality in Sucrose-based Media of Varying K^+ Concentration

The yeast cell concentration, as a function of time, is shown in Figure 5.1a. A reduction in yeast cell concentration was observed with increasing K^+ concentration. For instance, 10 hours after inoculation the cell concentration in the control experiment (0 $g.l^{-1}$ K^+) was 8 x 10^7 $cells.ml^{-1}$, while that in media containing 10 $g.l^{-1}$ K^+ was 2 x 10^7 $cells.ml^{-1}$. In media containing 15 $g.l^{-1}$ K^+, the cell concentration was 7 x 10^6 $cells.ml^{-1}$. The latter represented a ten fold reduction in cell concentration relative to the control. The cell concentration profiles were used to evaluate the respective specific growth rates which are presented in Figure 5.2. The specific growth rate of yeast decreased from 0.49 hr^{-1} for the control to 0.41 hr^{-1} at 10 $g.l^{-1}$ K^+ and 0.19 hr^{-1} at 15 $g.l^{-1}$ K^+.

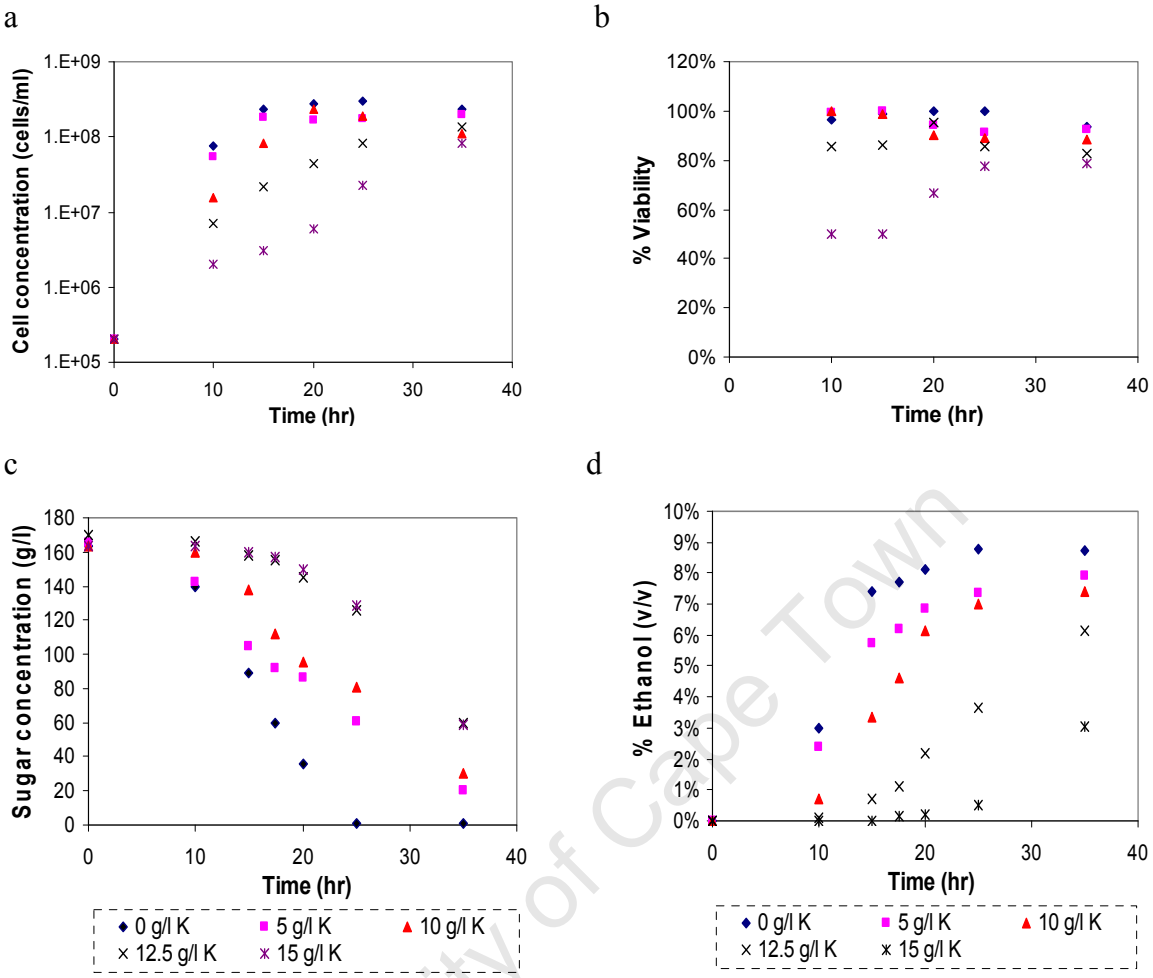

Figure 5.1 Cell concentration, viability, sugar concentration and ethanol concentration profiles of fermentations in sucrose-based media on varying K^+ concentration

The cell viability profile (Figure 5.1b) revealed reduced cell viability with increasing K^+ concentration. This reduction was more pronounced 10 hours into fermentation, after which it steadily increased as the yeast adapted to the high K^+ concentration. Yeast are known to initially lose viability when exposed to sub-lethal stress conditions (Woods, 1999). However, the loss in viability is temporal as yeast have stress response mechanisms to repair and prevent further cellular damage (Section 2.4). Figure 5.2 shows viability at 25 hours after inoculation. This time was chosen to coincide with the conclusion of fermentation in the control experiment. The yeast viability, after 25 hours of fermentation, was reduced from 100% for the control to 89% at 10 $g.l^{-1}$ K^+ and 78 % at K^+ concentration of 15 $g.l^{-1}$.

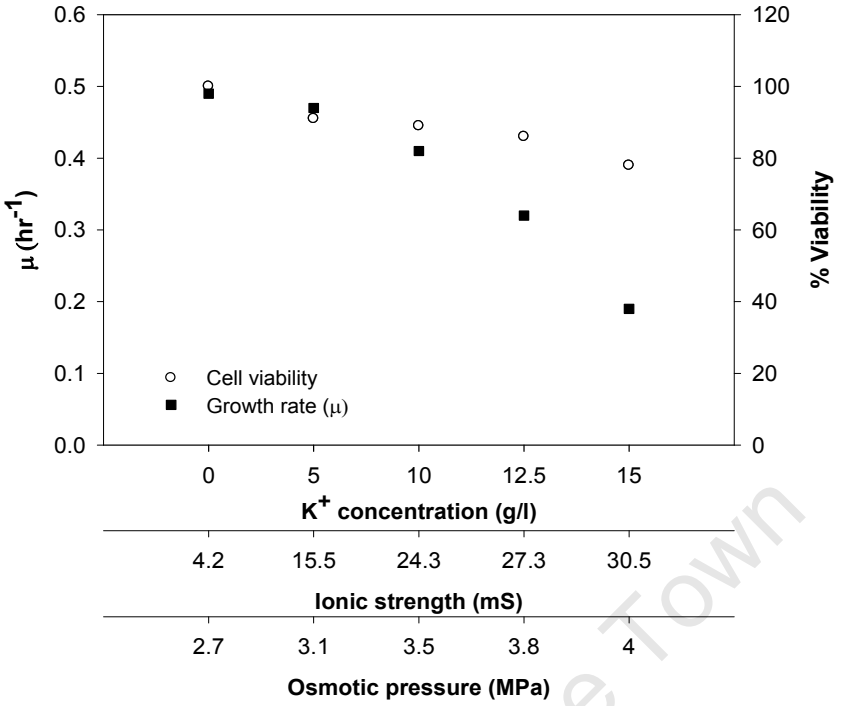

Figure 5.2 Specific growth rate and viability of *S. cerevisiae* in sucrose-based media on varying K^+ concentration, ionic strength and osmotic pressure

Yeast vitality was assessed by measuring its oxygen utilisation rate (OUR), giving an indication of the metabolic activity of the yeast. A sample, taken 20 hours after inoculation, was used to determine the rate at which oxygen was utilised in oxygen saturated YDP media at 30°C. The OUR measured at 0 and 15 $g.l^{-1}$ K^+ is shown in Table 5.3. The reduced OUR in sucrose-based media with K^+ concentration of 15 $g.l^{-1}$ indicated compromised metabolic activity. Daoud and Searle (1986) observed a correlation between yeast metabolic activity and its physiological state. Yeast subjected to stressful conditions showed reduced oxygen utilisation rates compared to yeast of presumably good physiological condition. Thus, yeast cells subjected to the high K^+ concentration had reduced vitality and were of poorer physiological state, and therefore likely to negatively affect fermentation performance.

Table 5.3 OUR of *S. cerevisiae* in sucrose-based media containing 0 and 15 g.l^{-1} K^+

K^+ concentration (g.l^{-1})	OUR (mg O_2 per 10^8 cells)
0	0.018 ± 0.002
15	0.012 ± 0.001

Fermentation Performance

Sugar utilisation, ethanol production

During batch fermentation of fermentable sugars to ethanol, rapid sugar utilisation and ethanol production are desirable to reduce batch cycle time. The sugar utilisation and ethanol production rates are therefore important indicators of fermentation performance. The sugar and ethanol concentration profiles for fermentation of sucrose-based media showed a decrease in sugar utilisation and ethanol production rate with increasing K^+ concentration (Figure 5.1c,d). Table 5.4 illustrates the above providing values of initial sugar concentration ($C_{s,o}$), sugar concentration after 20 hours ($C_{s,20}$) and ethanol concentration after 20 hours ($C_{E,20}$) of fermentation in sucrose-based media containing 0, 10 and 15 g.l^{-1} K^+.

Table 5.4 $C_{s,o}$, $C_{s,20}$ and $C_{E,20}$ for sucrose-based media containing 0, 10 and 15 g.l^{-1} K^+

Media	$C_{s,o}$ (g.l^{-1})	$C_{s,20}$ (g.l^{-1})	$C_{E,20}$ (g.l^{-1})
Sucrose based (0 g.l^{-1} K^+)	163	36	64
Sucrose based (10 g.l^{-1} K^+)	164	96	48
Sucrose based (15 g.l^{-1} K^+)	164	150	2

To quantify the sugar utilisation and ethanol production rates, the respective sugar and ethanol concentrations 20 hours after inoculation were divided by the elapsed time, giving an average rate over this period. These rates are represented graphically in Figure 5.3. Sugar utilisation rate decreased from 6.4 g.l^{-1}.hr^{-1} at 0 g.l^{-1} K^+ (control) to 3.4 g.l^{-1}.hr^{-1} at 10 g.l^{-1} K^+ and 0.7 g.l^{-1}.hr^{-1} at 15 g.l^{-1} K^+, representing a final value of 11% of initial value. The ethanol production rate decreased from 3.2 g.l^{-1}.hr^{-1} to 2.4 g.l^{-1}.hr^{-1}

and 0.1 $g.l^{-1}.hr^{-1}$ over the same K^+ concentration range, representing a final value of 3% of the original. The above results illustrate the potential for high K^+ concentrations to negatively affect fermentation. In similar studies, Ryan and Johnson (2001) reported the optimal K^+ concentration for fermentation of defined artificial molasses as 10.5 $g.l^{-1}$. In their studies, an increase in K^+ concentration up to 10.5 $g.l^{-1}$ K^+ had stimulatory effects on the ethanol production rate. However, beyond this concentration marked decreases in the ethanol production rate were reported. This is contrary to our results where decreases in ethanol production are observed with any increase in K^+ concentration. Contrary to Ryan and Johnson (2001), Camacho *et al.* (1981) reported an optimum K^+ concentration of 0.014 $g.l^{-1}$, while working at a K^+ concentration range of 0.008 to 0.020 $g.l^{-1}$. Above 0.014 $g.l^{-1}$ K^+, no stimulatory effects were reported.

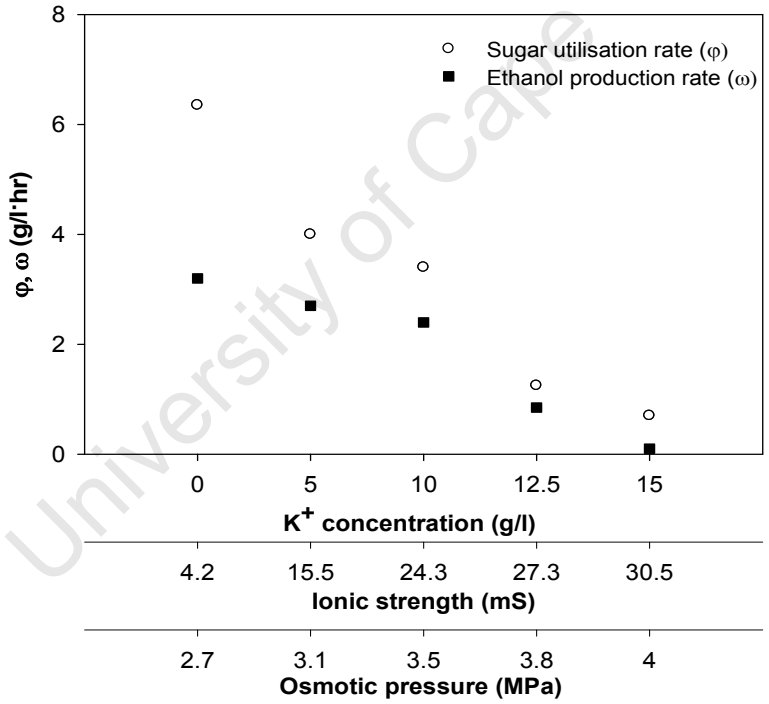

Figure 5.3 Sugar utilisation and ethanol production rate in sucrose-based media on varying K^+ concentration, ionic strength and osmotic pressure

The overall fermentation efficiency of *S. cerevisiae* in sucrose-based media, as defined in Equation 3.8, was determined 25 hours after inoculation. Fermentation efficiency decreased with increasing K^+ concentration as illustrated in Figure 5.4. The fermentation

efficiency ranged from a maximum of 0.83 in the control to a minimum of 0.29 at 15 $g.l^{-1}$ K^+. The reduced fermentation efficiency at high K^+ concentration was attributed to reduced sugar utilisation rate, which resulted in high residual sugars and limited ethanol production (Figure 5.1c,d).

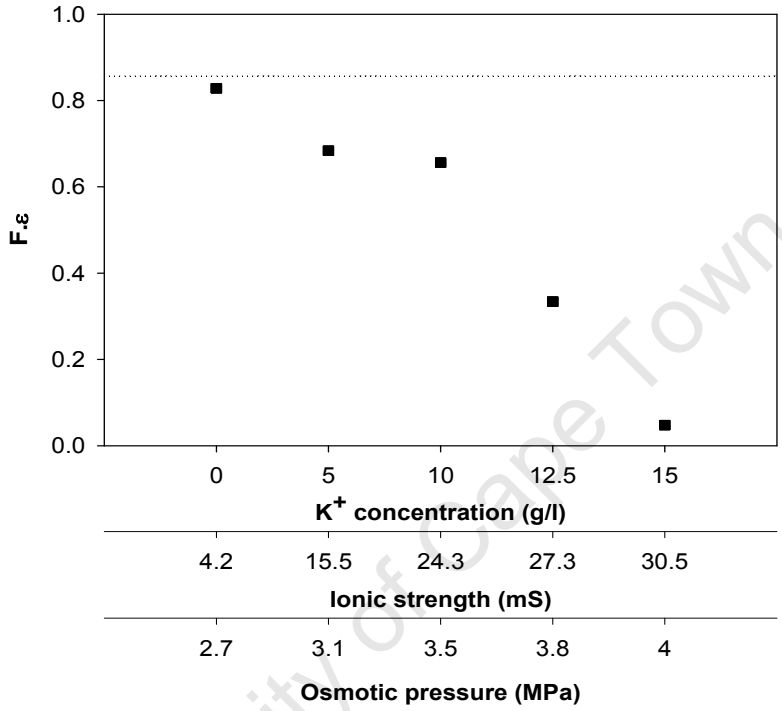

Figure 5.4 Fermentation efficiency in sucrose-based media on varying K^+ concentration, ionic strength and osmotic pressure

5.2.2 Effect of K^+ on Ethanol Fermentation in Molasses Mash

Similar experiments to the ones performed in Section 5.2.1 were conducted using molasses mash and are discussed in this section. The K^+ concentration ranged from 9.5 $g.l^{-1}$ K^+ (control) to 18.5 $g.l^{-1}$ K^+. Figure 5.5 is a graphical representation of the results. A comparison of Figure 5.1 and Figure 5.5 shows significant differences in fermentations of sucrose-based media and molasses mash as a function of K^+ concentration. A more pronounced negative effect of K^+ concentration on yeast growth and fermentation performance was observed in sucrose-based media than in molasses mash. However, the

small variation noted in molasses mash as K^+ concentration was increased could be significant in industrial settings where optimal operations are desirable.

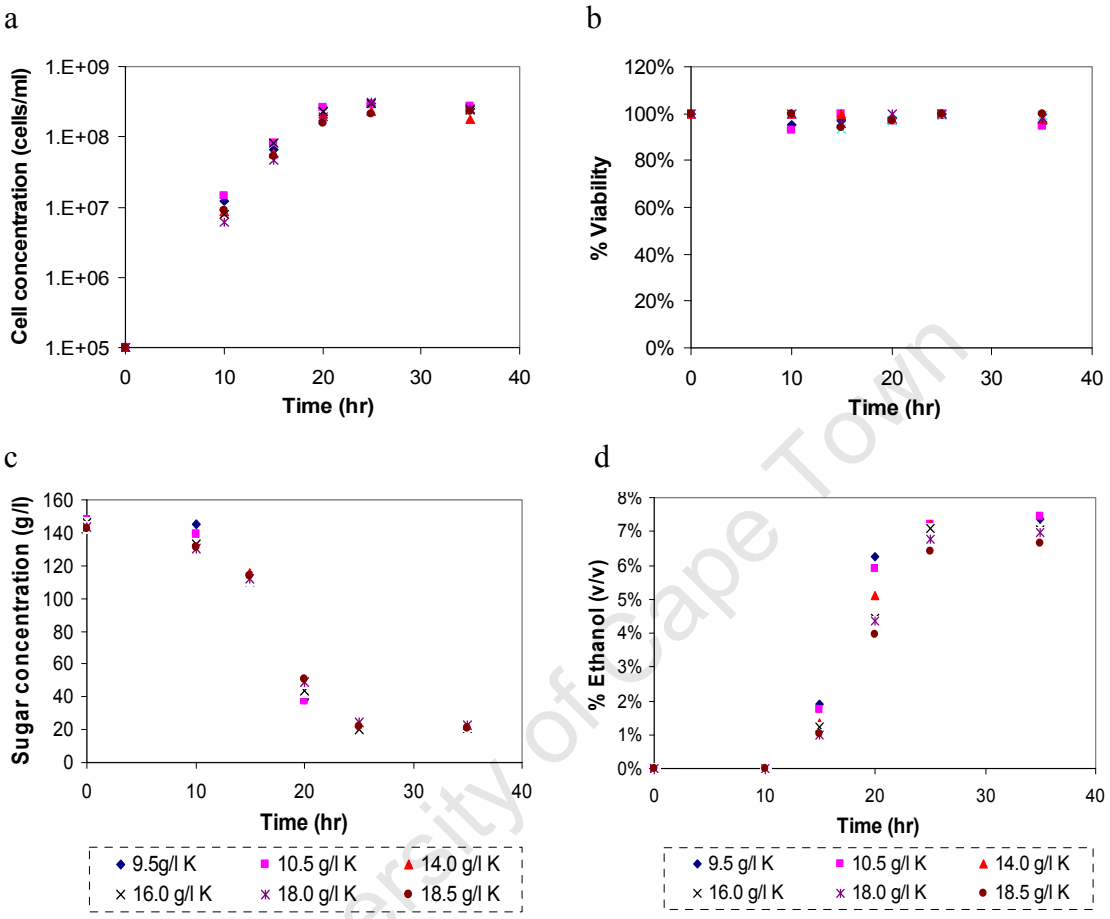

Figure 5.5 Cell concentration, cell viability, sugar concentration and ethanol concentration profiles of fermentations in molasses mash on varying K^+ concentration

Specific Growth Rate, Cell Viability and Vitality in Molasses Mash of Varying K^+ Concentration

The decrease in the specific growth rate and cell viability (Figure 5.6) on increasing K^+ concentration was less pronounced than observed in the sucrose-based media (Compare Figure 5.2). The specific growth rate ranged from a maximum 0.45 ± 0.1 hr^{-1} at 10.5 $g.l^{-1}$ K^+ to a minimum of 0.41 ± 0.1 hr^{-1} at 18.0 $g.l^{-1}$ K^+. The cell viability remained relatively high, (≥94%) over the K^+ concentration range investigated.

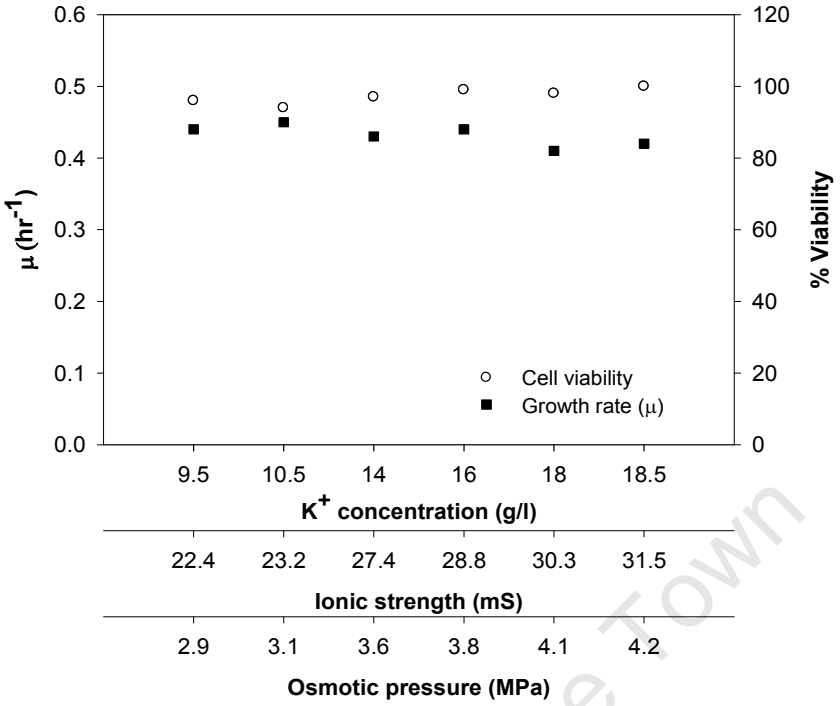

Figure 5.6 Specific growth rate and viability of *S. cerevisiae* in molasses mash on varying K^+ concentration

The OUR in molasses mash is given in Table 5.5. No significant change in OUR of yeast was observed as K^+ concentration was increased. The results, which indicate no measurable loss in yeast vitality, differ from those from the sucrose-based media fermentations, where a significant reduction in OUR was observed upon increasing K^+ concentration.

Table 5.5 OUR of *S. cerevisiae* in molasses mash of 9.5 and 15 $g.l^{-1}$ K^+

K^+ concentration ($g.l^{-1}$)	OUR (mg O_2 per 10^8 cells)
9.5	0.016 ± 0.002
15	0.015 ± 0.002

Fermentation Performance

Sugar Utilisation and Ethanol Production rate

A steady reduction in the sugar utilisation and ethanol production rate was observed with increasing K^+ concentration as illustrated in Figure 5.7. However, the reduction in both rates was not as pronounced as that in the sucrose-based media (Figure 5.3). The sugar utilisation rate decreased from 5.3 ± 0.1 $g.l^{-1}.hr^{-1}$ at K^+ concentration of 9.5 $g.l^{-1}$ to 4.6 ± 0.1 $g.l^{-1}.hr^{-1}$ at K^+ concentration of 18.5 $g.l^{-1}$, representing a final value of 87% of the original. The ethanol production rate reduced from a maximum of 2.5 ± 0.2 $g.l^{-1}.hr^{-1}$ to a minimum of 1.6 ± 0.1 $g.l^{-1}.hr^{-1}$ over the same K^+ concentration range, representing a final value of 64% of the initial. The relatively greater decrease in ethanol production rate compared to sugar utilisation rate suggests reduced metabolic flux towards pyruvate production (necessary for ethanol production) and increased glycerol production (André *et al*., 1991; Mager and Siderius, 2002). Glycerol, the main compatible solute in yeast, is produced to counteract increased osmotic pressure. Also, a shift towards production of stress related compounds such as trehalose could account for the reduced ethanol production rate (Housa *et al*., 1998).

The fermentation efficiency profile illustrated in Figure 5.8 shows that fermentation efficiency remained relatively high across K^+ concentration, ionic strength and osmotic pressure range. It ranged from a maximum of 0.90 ± 0.02 at 14 $g.l^{-1}$ K^+ to a minimum of 0.83 ± 0.02 at 18.5 $g.l^{-1}$ K^+. Again, while there was a reduction in fermentation efficiency in molasses mash as K^+ concentration was increased, the extent was significantly lower than that observed in sucrose-based media (Compare with Figure 5.4).

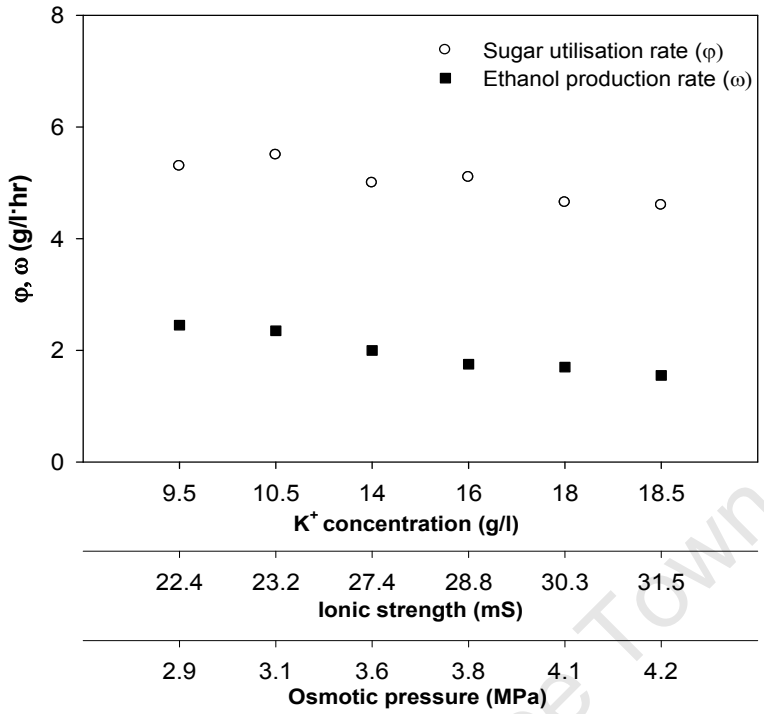

Figure 5.7 Sugar utilisation and ethanol production rate in molasses mash on varying K^+ concentration, ionic strength and osmotic pressure

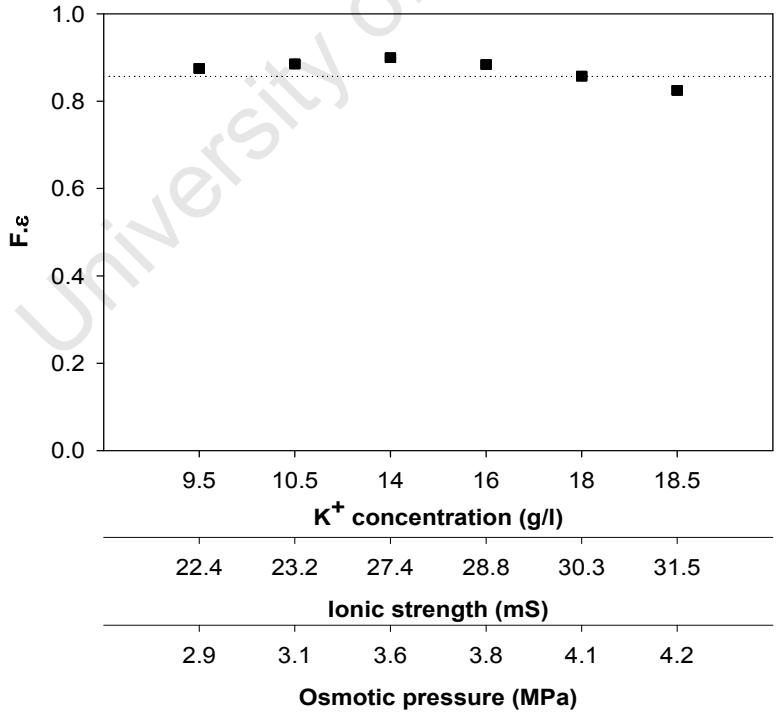

Figure 5.8 Fermentation efficiency in molasses mash on varying K^+ concentration, ionic strength and osmotic pressure

Molasses and sugar usage efficiency

Molasses usage efficiency (M.U.ε) and sugar usage efficiency (S.U.ε) were determined for the molasses mash. M.U.ε and S.U.ε are defined in Section 3.5 and are important parameters in the industrial production of ethanol. The graphical results presented in Figure 5.9 represent the results after 25 hours of fermentation.

The change in M.U.ε and S.U.ε with K^+ concentration was minimal. M.U.ε ranged from a minimum of 3.3 ± 0.2 $kg.l^{-1}$ at 10.5 and 14 $g.l^{-1}$ K^+ to a maximum of 3.7 ± 0.2 $kg.l^{-1}$ at 18.5 $g.l^{-1}$ K^+. The S.U.ε ranged from minimum of 1.7 ± 0.1 $kg.l^{-1}$ at 14 $g.l^{-1}$ K^+ to maximum of 1.9 ± 0 .1 at 18.5 $g.l^{-1}$ K.

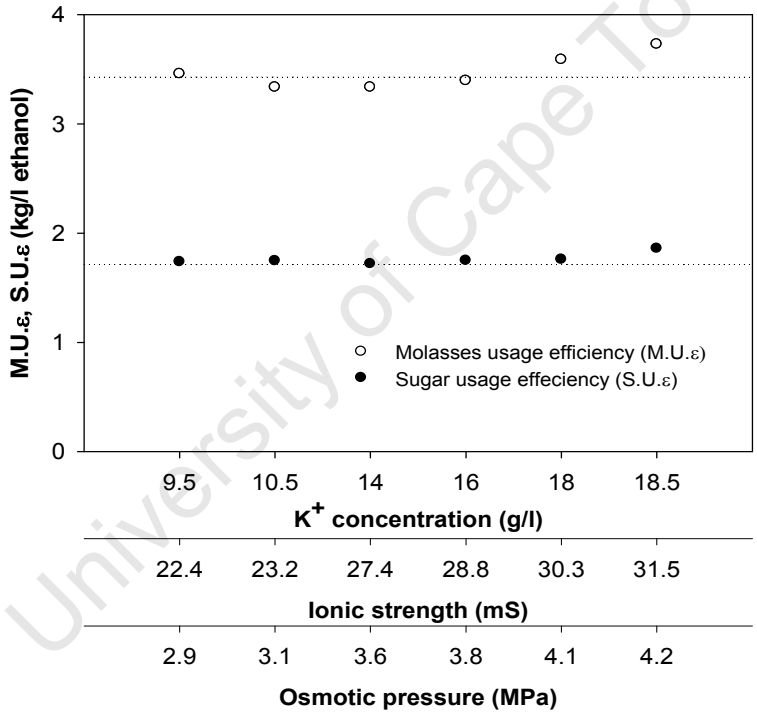

Figure 5.9 Molasses and sugar usage efficiency in molasses mash on varying K^+ concentration, ionic strength and osmotic pressure

5.2.3 Summary of the Effect of K^+ Concentration on Yeast Growth and Fermentation Performance

Increasing the K^+ concentration in sucrose-based media and molasses mash negatively affected yeast growth and fermentation performance. This reduction was more

pronounced in the sucrose-based media, where statistically significant differences were observed across the K^+ concentration range studied. Yeast growth and fermentation performance differences were less marked in molasses mash, with some apparent differences (e.g. OUR, M.U.ε and S.U.ε) being within the margin of experimental error. The parameters that revealed statistically significant differences were the specific growth rate, sugar utilisation rate and ethanol production rate.

The differences in yeast growth and fermentation performance observed in sucrose-based media and molasses mash were dependent on the media type used. Unknown constituents of molasses appeared to mitigate the negative effects of high K^+ concentration. It is possible that the mitigating effect was a result of the presence of chelating agents in the molasses. Ergun *et al*. (1997) and Oderinde *et al*. (1985) showed improved fermentation efficiency upon supplementation of chelating agents to fermentation media. To demonstrate the mitigating effect of molasses on K^+ inhibition, hypothesised to be due to chelation, the impact of low molasses concentration on the inhibition in sucrose media is studied in Section 5.6.

5.3 EFFECT OF Mg^{2+} ON ETHANOL FERMENTATION

Molasses has a typical Mg content of ~0.46 % (m/m) (Table 2.1), producing molasses mash with a Mg^{2+} concentration of 1.3 to 1.5 $g.l^{-1}$. Mg^{2+} is an essential nutrient in maintenance and regulation of metabolic processes. Its intracellular level is maintained at the millimolar level, as it plays a crucial role in DNA replication, transcription and translation (Dombek and Ingram, 1986). The presence of Mg^{2+} in fermentation media reportedly provides protection for yeast against ethanol and temperature stress (Walker, 1998). However, it is important to understand its effect on fermentation at levels in excess of the minimal requirement, as may be encountered in industrial ethanol fermentations.

In the study of the effect of Mg^{2+} on yeast growth and fermentation performance, a similar approach to that used to study the effect of K^+ concentration was adopted.

However, this time yeast growth and fermentation performance in sucrose-based media and molasses mash are compared simultaneously. The Mg^{2+} concentration was adjusted using $MgSO_4$.

5.3.1 Yeast Performance

Cell Growth Rate and Viability

Mg^{2+} reportedly plays an important role in the maintenance and regulation of growth processes (Walker *et al*., 1996) and stabilisation of biological membranes (Walker, 1998). The above appeared to be confirmed when the specific growth rate and viability of *S. cerevisiae* in sucrose-based media and molasses mash were studied at a Mg^{2+} concentration range of 0 to 15 $g.l^{-1}$ and 1.5 to 15 $g.l^{-1}$, respectively. The results are compared in Figure 5.10.

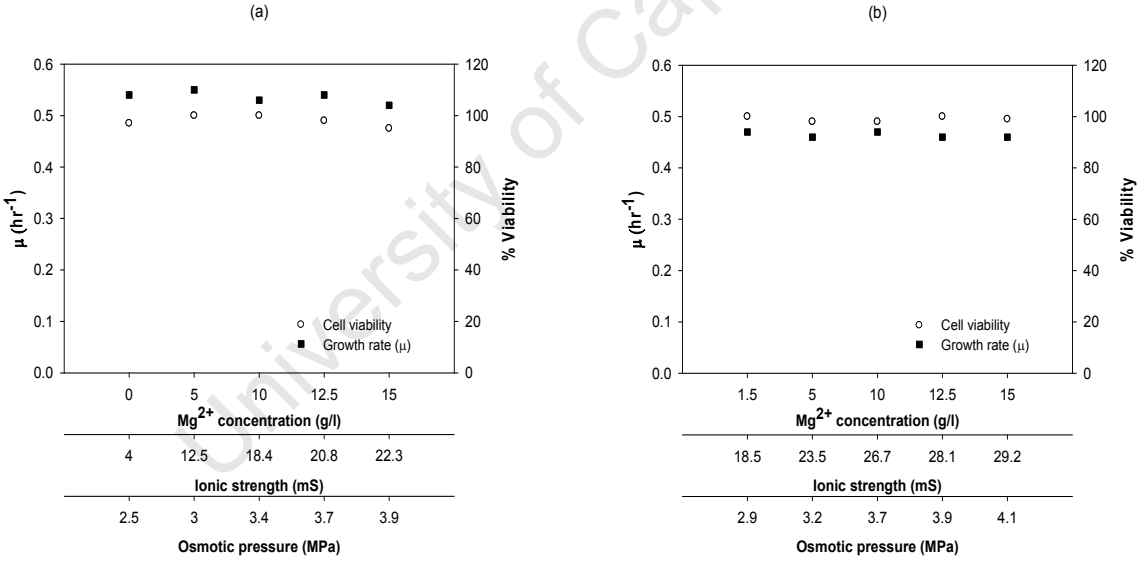

Figure 5.10 Specific growth rate and viability of S. *cerevisiae* in (a) sucrose-based media and (b) molasses mash on varying Mg^{2+} concentration, ionic strength and osmotic pressure

The specific growth rate and cell viability of *S. cerevisiae* in both media were relatively constant with increasing Mg^{2+} concentration. The specific growth rate in the sucrose based-media was a maximum of 0.55 ± 0.02 hr^{-1} at 5 $g.l^{-1}$ Mg^{2+} and a minimum of

0.52 ± 0.02 hr^{-1} at 15 $g.l^{-1}$ Mg^{2+}, while yeast cell viability remained greater than 95% in all cases. The specific growth rate in the molasses mash was constant at 0.47 ± 0.02 hr^{-1}, while yeast viability remained greater than 98% in the Mg^{2+} concentration range investigated. Thus, no measurable change in the specific growth rate and cell viability was observed in both sucrose-based media and molasses mash with increasing Mg^{2+} concentration, ionic strength and osmotic pressure.

The OUR of *S. cerevisiae* is given in Table 5.6. No significant change in OUR was observed in either case indicating preservation of yeast vitality.

Table 5.6 OUR of *S. cerevisiae* in sucrose-based media and molasses mash of varying Mg^{2+}

Media	Mg^{2+} concentration ($g.l^{-1}$)	OUR (mg O_2 per 10^8 cells)
Sucrose based (control)	0	0.018 ± 0.002
Sucrose based	15	0.017 ± 0.002
Molasses mash (control)	1.5	0.016 ± 0.002
Molasses mash	15	0.013 ± 0.001

5.3.2 Fermentation Performance

Sugar Utilisation and Ethanol Production Rate

The sugar utilisation and ethanol production rate are shown in Figure 5.11. In sucrose-based media a steady decrease in both was observed as Mg^{2+} concentration was increased. The sugar utilisation rate decreased from 5.5 $g.l^{-1}.hr^{-1}$ at 0 $g.l^{-1}$ Mg^{2+} to 4.9 $g.l^{-1}.hr^{-1}$ at 10 $g.l^{-1}$ Mg^{2+} and 4.3 ± 0.2 $g.l^{-1}.hr^{-1}$ at 15 $g.l^{-1}$ Mg^{2+}. This represented a final value of 78% of the initial. The ethanol production rate decreased from 2.5 $g.l^{-1}$ to 2.1 $g.l^{-1}.hr^{-1}$ and 2.0 ± 0.1 $g.l^{-1}.hr^{-1}$ over the same Mg^{2+} concentration range, representing a final value of 80% of the original.

In molasses mash fermentations, the decrease in sugar utilisation and ethanol production rate was less pronounced. The sugar utilisation rate decreased from 4.5 ± 0.1 $g.l^{-1}.hr^{-1}$ at 1.5 $g.l^{-1}$ Mg^{2+} to 4.0 ± 0.1 $g.l^{-1}.hr^{-1}$ at 15 $g.l^{-1}$ Mg^{2+}, representing a final value of 89% of the initial. The decline in the ethanol production rate was from 1.9 ± 0.1 $g.l^{-1}.hr^{-1}$ to 1.8 ± 0.1 $g.l^{-1}.hr^{-1}$ over the same Mg^{2+} concentration range, representing a final value of 95% of the original. So, while a statistically significant decrease in sugar utilisation rate was observed as Mg^{2+} concentration was increased from 1.5 to 15 $g.l^{-1}$ the reduction in ethanol production rate was statistically insignificant. Again, a possible reason for this is increased substrate flux towards glycerol (André *et al*.,1991; Mager and Siderius, 2002) and stress compounds production (Housa *et al*., 1998).

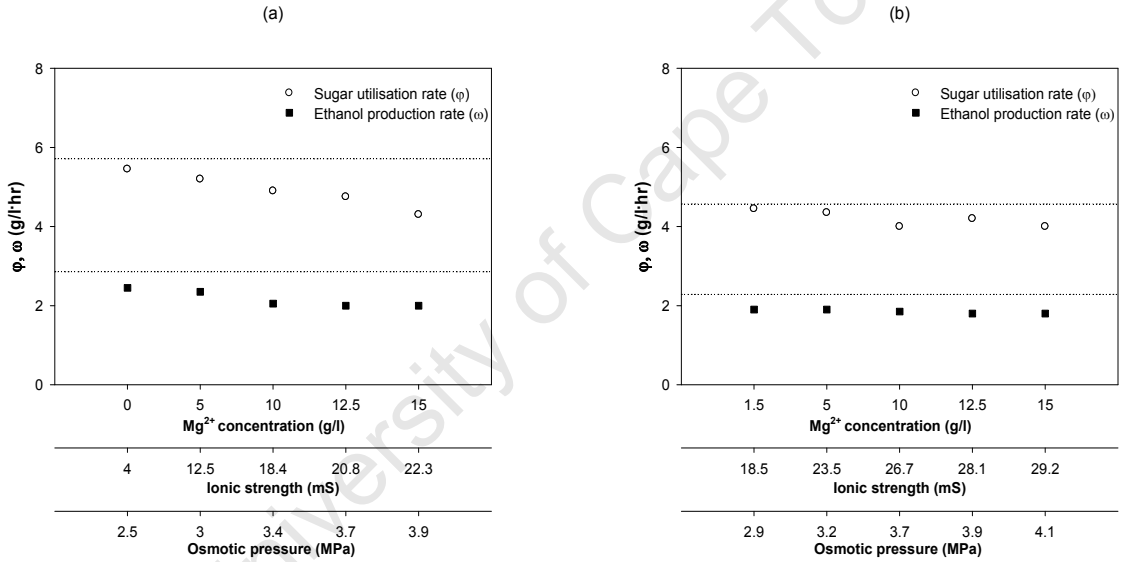

Figure 5.11 Sugar utilisation and ethanol production rate in (a) sucrose-based media and (b) molasses mash on varying Mg^{2+} concentration, ionic strength and osmotic pressure

The overall fermentation efficiency in sucrose-based media ranged from a maximum of 0.89 ± 0.4 at 5 $g.l^{-1}$ Mg^{2+} to a minimum of 0.74 ± 0.4 at 15 $g.l^{-1}$ Mg^{2+}. In the molasses mash fermentation efficiency was a maximum of 0.86 ± 0.5 at 10 $g.l^{-1}$ Mg^{2+} and a minimum of 0.78 ± 0.5 at 15 $g.l^{-1}$ Mg^{2+} (Figure 5.12). Again, a statistically significant difference in fermentation efficiency was observed in sucrose based-media, while the apparent difference in molasses mash was within the experimental margin of error.

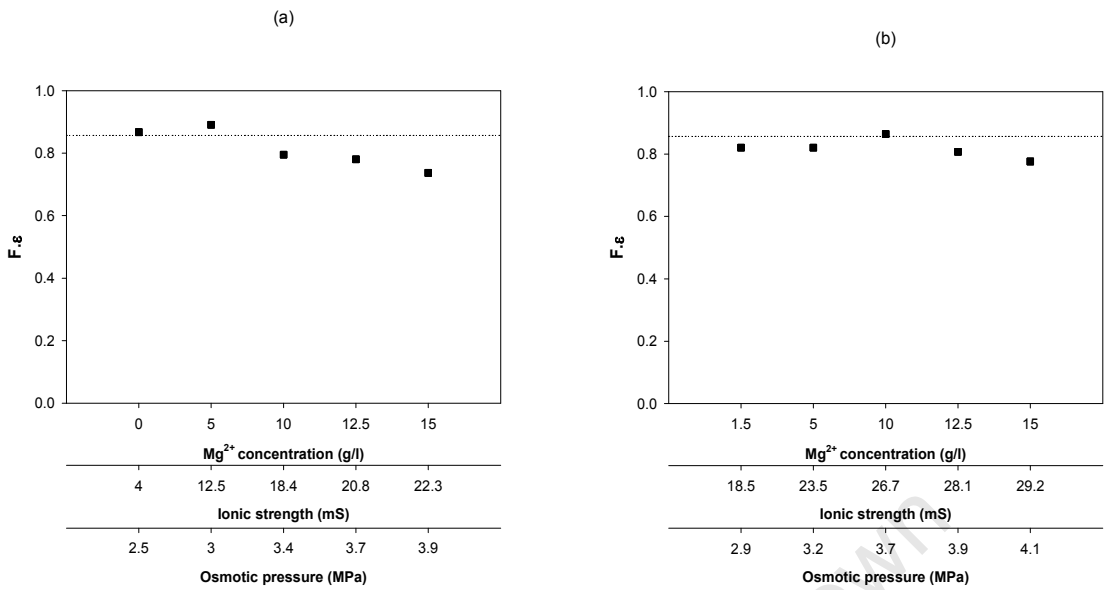

Figure 5.12 Fermentation efficiency in (a) sucrose-based media and (b) molasses mash on varying Mg^{2+} concentration, ionic strength and osmotic pressure

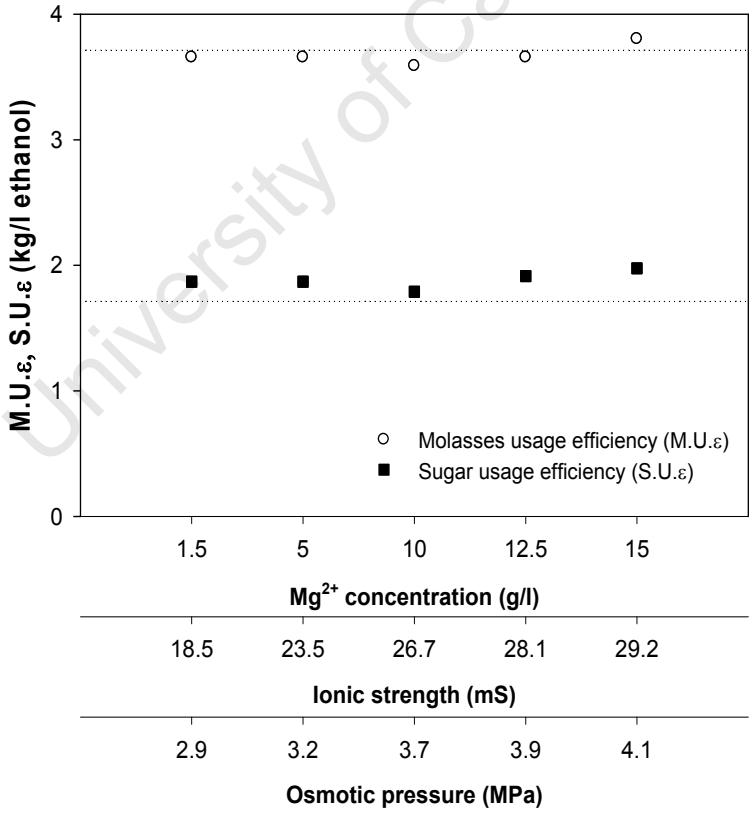

Figure 5.13 Molasses and sugar usage efficiency in molasses mash on varying Mg^{2+} concentration, ionic strength and osmotic pressure

The M.U.ε and S.U.ε were calculated for the molasses mash only and are presented in Figure 5.13. There was no significant difference in either parameter in the Mg^{2+} concentration range investigated.

5.3.3 Summary of the Effect of Mg^{2+} Concentration on Yeast Growth and Fermentation Performance

Increasing the Mg^{2+} concentration from 0 to 15 $g.l^{-1}$ in sucrose-based media did not result in a measurable change in specific growth rate, yeast viability or OUR. However, marginal but distinct reductions in sugar utilisation rate, ethanol production rate and fermentation efficiency were observed. Increasing the Mg^{2+} concentration from 1.5 to 15 $g.l^{-1}$ in molasses mash did not result in any significant differences in yeast growth and fermentation performance. The effect of Mg^{2+} on fermentation performance at the same concentration as K^+ was significantly lower.

5.4 EFFECT OF Na^+ ON ETHANOL FERMENTATION

Na constitutes 0.1 to 0.9% (m/m) of molasses, producing molasses mash with an Na^+ concentration of 0.3 to 2.5 $g.l^{-1}$ Na^+. It is toxic (Gómez *et al*., 1996, Murguía *et al*., 1996) with no requirement for it by yeast, (Wadskog and Alder, 2003) except as a replacement for K^+ ions. Its presence in molasses is therefore undesirable and expected to negatively impact fermentation. In this section, the effect of Na^+ on yeast growth and fermentation performance was studied in sucrose-based media (0 to 15 $g.l^{-1}$ Na^+), and molasses mash (0.3 to 15 $g.l^{-1}$ Na^+). Na^+ concentration was adjusted using Na_2SO_4.

5.4.1 Yeast Performance

Specific Growth Rate and Viability

The effect of Na^+ concentration on the specific growth rate and viability of *S. cerevisiae* is shown in Figure 5.14. In the sucrose-based media the specific growth rate was

0.54 ± 0.02 hr^{-1} at 0.3 and 1 $g.l^{-1}$ Na^+. On increasing the Na^+ concentration to 2.5 $g.l^{-1}$, the specific growth rate decreased to 0.40 ± 0.02 hr^{-1}, and continued to decrease with increasing Na^+ concentration to 0 hr^{-1} at 15 $g.l^{-1}$ Na^+. The cell viability followed a similar trend with the cell viability maintained at 100% as the Na^+ concentration was increased to 2.5 $g.l^{-1}$. Thereafter, cell viability decreased significantly to 44 ± 2% at 15 $g.l^{-1}$ Na^+.

Cell viability in molasses mash remained high at 100%, across the Na^+ concentration range studied. The specific growth rate was constant at 0.46 ± 0.2 hr^{-1} over the Na^+ concentration range 0.3 to 2.5 $g.l^{-1}$. Above 2.5 $g.l^{-1}$ Na^+, an appreciable decrease in the specific growth rate was observed. The specific growth rate was reduced from 0.46 ± 0.2 hr^{-1} at 2.5 $g.l^{-1}$ Na^+ to 0.37 ± 0.2 hr^{-1} at 15 $g.l^{-1}$ Na^+. This reduction of 24% in the specific growth rate was substantially greater than any decrease caused by the effects of K^+ and Mg^{2+} in the concentration ranges studied.

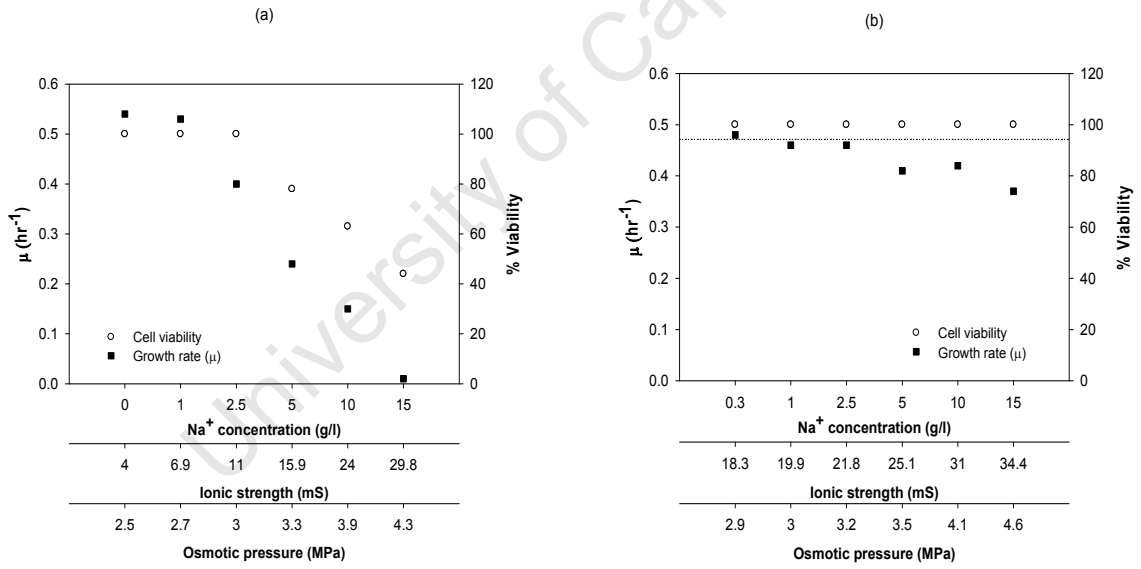

Figure 5.14 Specific growth rate and viability of *S. cerevisiae* in (a) sucrose-based media and (b) molasses mash on varying Na^+ concentration, ionic strength and osmotic pressure

These results are in agreement with Wadsjkog and Alder (2003) who predict cell shrinkage and cessation of growth with increased NaCl stress. The results also confirm the toxic effects of Na^+ ions (Gómez *et al*., 1996; Murguía *et al*., 1996). In related work

Carvalheiro *et al*, (1999) used NaCl to vary the Na^+ concentration in studying its effects on yeast cell growth in a defined media. The specific growth rate of yeast was reduced from 0.4 hr^{-1} to 0.2 hr^{-1} as the Na^+ concentration of the media was increased from 0 to 17 $g.l^{-1}$, also suggesting Na^+ toxicity. While the lag phase was not monitored in our experiments, Carvalheiro *et al*. (1999) reported an increase from 1.5 to 3 hours in the mentioned Na^+ concentration range.

The OUR is summarised in Table 5.7. The OUR in sucrose-based media containing 15 $g.l^{-1}$ Na^+ was reduced to undetectable levels, representing a complete loss in metabolic activity and thus yeast vitality. The results were similar in the molasses mash with the OUR significantly reduced at 15 $g.l^{-1}$ Na^+.

Table 5.7 OUR of *S. cerevisiae* in sucrose-based media and molasses mash on varying Na^+ concentration

Media	Na^+ concentration ($g.l^{-1}$)	OUR (mg O_2 per 10^8 cells)
Sucrose-based (control)	0	0.018 ± 0.001
Sucrose-based	15	-
Molasses mash (control)	0	0.016 ± 0.001
Molasses mash	15	0.003 ± 0.001

5.4.2 Fermentation Performance

Sugar Utilisation and Ethanol Production Rate

A rapid decrease in sugar utilisation rate in the sucrose-based media was observed as Na^+ concentration was increased. On increasing the Na^+ concentration from 0.3 $g.l^{-1}$ to 1 $g.l^{-1}$, sugar utilisation rate reduced from 5.5 ± 0.3 to 4.4 ± 0.3 $g.l^{-1}.hr^{-1}$. Thereafter, further increases in the Na^+ concentration resulted in substantial reductions in the sugar utilisation rate, which reached 0 $g.l^{-1}.hr^{-1}$ at 5 $g.l^{-1}$ Na^+. Similarly, ethanol production rate was reduced from 2.5 ± 0.1 $g.l^{-1}.hr^{-1}$ at 0.3 $g.l^{-1}$ Na^+ to 2.2 ± 0.1 $g.l^{-1}.hr^{-1}$ at 1 $g.l^{-1}$ Na^+. Thereafter, further increases in the Na^+ concentration resulted in substantial reduction in the ethanol production rate, with no ethanol production occurring at Na^+ concentrations

greater than 5 $g.l^{-1}$. The decrease in sugar utilisation and ethanol production rate in molasses mash was less pronounced. Sugar utilisation rate remained in the range 4.3 ± 0.2 to 3.9 ± 0.2 $g.l^{-1}.hr^{-1}$ as the Na^+ concentration was increased from 0.3 to 5 $g.l^{-1}$ Na^+. On further increase, the rate declined rapidly with the sugar utilisation rate reaching 1.5 $g.l^{-1}.hr^{-1}$ at 15 $g.l^{-1}$. The ethanol production rate followed a similar trend and was reduced from 2.0 ± 0.1 $g.l^{-1}$ at 0.3 $g.l^{-1}$ Na^+ to 1.7 ± 0.1 at 5 $g.l^{-1}$ Na^+ and 0.6 ± 0.0 $g.l^{-1}$ at 15 $g.l^{-1}$ Na^+. These results are summarised in Figure 5.15.

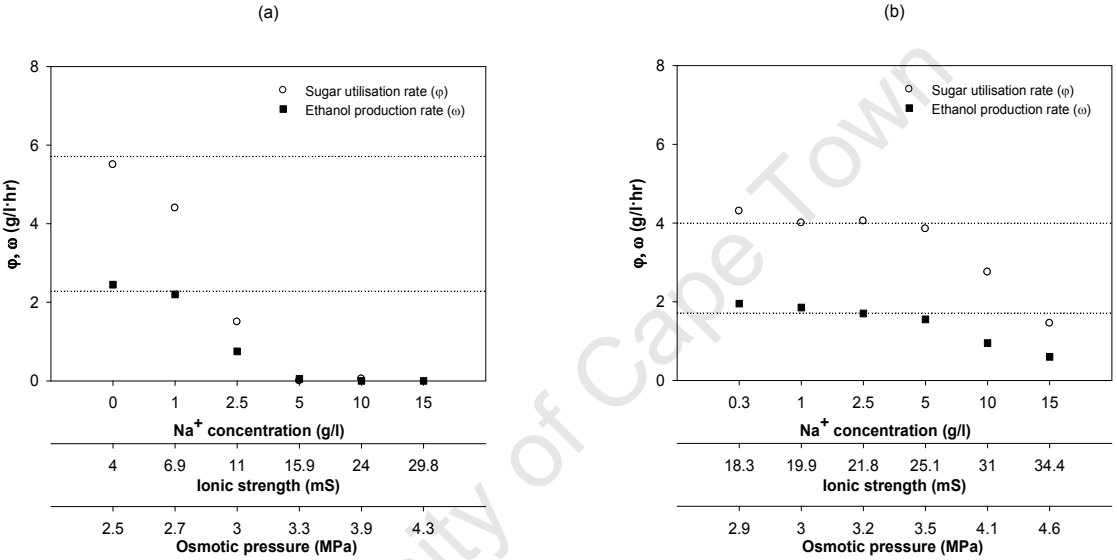

Figure 5.15 Sugar utilisation and ethanol production rate in (a) sucrose-based media and (b) molasses mash on varying Na^+ concentration, ionic strength and osmotic pressure

A reduction in fermentation efficiency was observed with increasing Na^+ concentration in both media (Figure 5.16). Again, the reduction was more pronounced in the sucrose-based media where fermentation efficiency was reduced from 0.85 at 0 $g.l^{-1}$ Na^+ to 0.33 at 2.5 $g.l^{-1}$ Na^+. At 5 $g.l^{-1}$ Na^+ the fermentation efficiency was 0.03, while at 10 $g.l^{-1}$ Na^+ it approached 0.00 $g.l^{-1}$. In the molasses mash the fermentation efficiency was constant at 0.86 ± 0.04 up to Na^+ concentration of 2.5 $g.l^{-1}$. At 5 $g.l^{-1}$ the F.ε decreased to 0.79 ± 0.04 and continued to decrease to 0.32 ± 0.02 at 15 $g.l^{-1}$ Na^+.

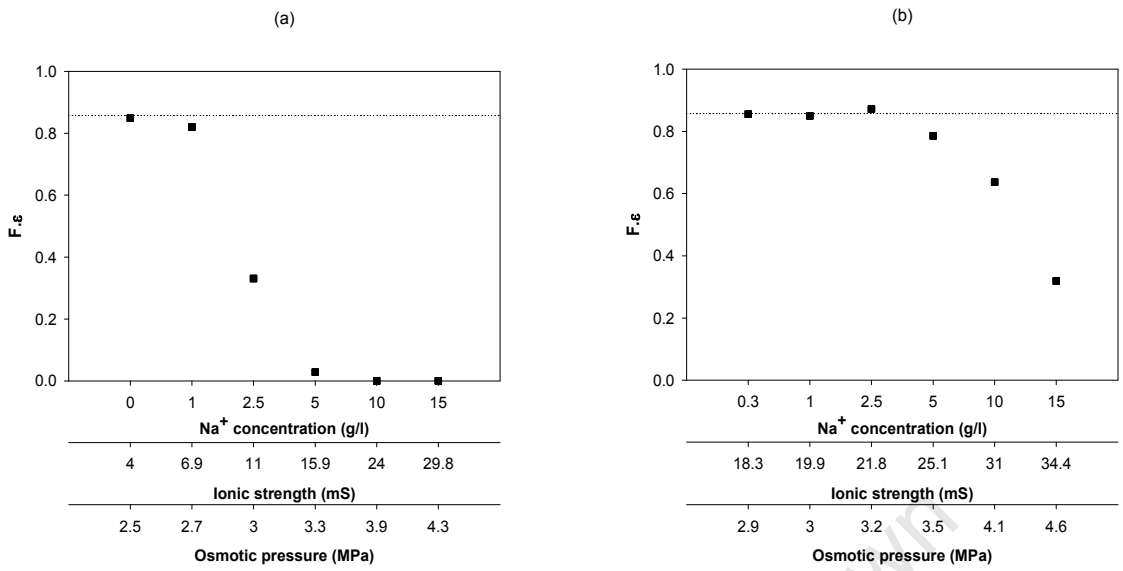

Figure 5.16 Fermentation efficiency in (a) sucrose-based media and (b) molasses mash on varying Na^+ concentration, ionic strength and osmotic pressure

The M.U.ε and S.U.ε for molasses mash was relatively stable at Na^+ concentrations less than 5 $g.l^{-1}$ Na^+. At concentrations above 5 $g.l^{-1}$ Na^+, an exponential increase in both M.U.ε and S.U.ε was observed. M.U.ε was 3.7 ± 0.1 $kg.l^{-1}$ in the range 0 to 5 $g.l^{-1}$ Na^+. At 10 $g.l^{-1}$ Na^+, M.U.ε increased to 4.6 ± 0.1 $kg.l^{-1}$, before peaking at 8.6 ± 0.3 $kg.l^{-1}$ at 15 $g.l^{-1}$ Na^+. The S.U.ε followed a similar trend and was 1.8 ± 0.2 $kg.l^{-1}$ in the range of 0 to 5 $g.l^{-1}$ Na^+. At 10 $g.l^{-1}$ S.U.ε increased to 2.5 $kg.l^{-1}$ before peaking at 4.9 ± 0.3 $kg.l^{-1}$ at 15 $g.l^{-1}$ Na^+. The large M.U.ε and S.U.ε values at high Na^+ concentrations are attributed to large residual sugar concentrations at 25 hours of fermentation.

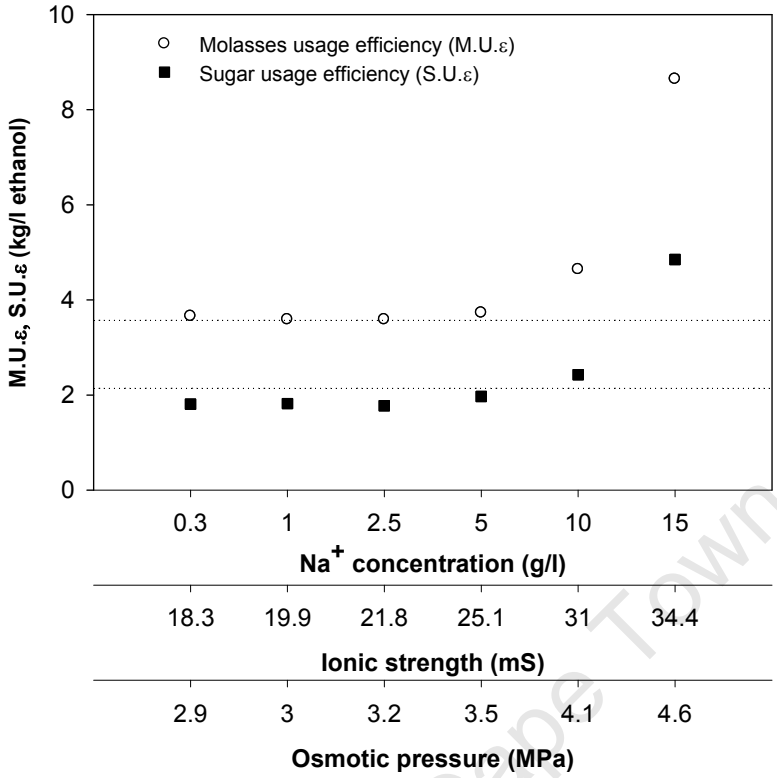

Figure 5.17 Molasses and sugar usage efficiency in molasses media of varying Na^+ concentration, ionic strength and osmotic pressure

5.4.3 Summary of the Effects of Na^+ Concentration on Yeast Growth and Fermentation Performance

Increasing the Na^+ concentration, in sucrose-based media and molasses mash, resulted in a decrease in yeast growth and fermentation performance. The results confirmed the toxic effects of Na^+ (Gómez *et al*., 1996, Murguía *et al*., 1996). During inorganic sulphate assimilation the toxic nucleotide, 3-phosphoadenosine-5-phosphate is produced. The nucleotidase (Ha12p) which hydrolyses the nucleotide is inhibited by Na^+ ions, which explains the high toxicity of Na^+. Again, the reduction in performance was less pronounced in molasses mash. Importantly, the negative effects of Na^+ in molasses mash were significantly greater than those observed for K^+ and Mg^{2+}. For example, in fermentations of molasses mash containing 15 $g.l^{-1}$ K^+ or Mg^{2+} the specific growth was equal to that of control. However, in molasses mash containing 15 $g.l^{-1}$ Na^+ the specific growth rate was 78% of the control.

5.5 COMPARATIVE EFFECTS OF CATION CONCENTRATION, IONIC STRENGTH AND OSMOTIC PRESSURUE ON FERMENTATION

In Sections 5.2 to 5.4 yeast growth and fermentation performance were quantified as a function of the specific ion concentration, namely K^+, Mg^{2+} and Na^+, by varying these individually in a sucrose-based media and molasses mash. The corresponding ionic strength and osmotic pressure of the media were also measured. A general decrease in yeast growth and fermentation performance was observed with increasing cation concentration, ionic strength and osmotic pressure for a specified cation. In this section, the variation in performance, in terms of overall cation concentration effect, ionic strength effect and osmotic pressure effect, are considered. To achieve this, it was necessary to normalise performance parameters to enable comparison of data between experiments. The specific growth rate and ethanol production rate were selected to quantify yeast growth and fermentation performance, respectively. Normalisation was done by dividing the experimental specific growth rate and ethanol production rates by their respective control values.

5.5.1 Effect of Cation Concentration on Specific Growth Rate and Ethanol Production Rate

The normalised specific growth rate and ethanol production rate as a function of cation concentration are shown in Figures 5.18 and 5.19, respectively. A general decrease in $\mu/\mu_{control}$ and $\omega/\omega_{control}$ was observed with increasing cation concentration. However, the extent of decrease appeared to be cation species dependent, and was not simply a function of cation concentration. For instance, in sucrose-based media with a cation concentration of 15 $g.l^{-1}$ $\mu/\mu_{control}$, was 1.0, 0.39 and 0.02 where the contributing cations were Mg^{2+}, K^+ and Na^+, respectively. The values of $\omega/\omega_{control}$ were 0.82, 0.03 and 0.00 respectively. The values appear to indicate that reduced $\mu/\mu_{control}$ and $\omega/\omega_{control}$ are a result of cation toxicity rather than cation concentration. Na^+ toxicity is well documented (Gómez *et al*., 1996; Murguía *et al*., 1996; Wadsjkog and Alder, 2003), while K^+ is considered toxic at high concentrations (Ryan and Johnson, 2001). While no literature

was available on Mg^{2+} toxicity, its important role in yeast growth is well documented (Dombek and Ingram, 1986; Walker *et al*., 1996; Walker, 1998). Also, of importance was the fermentation media used. The toxicity effects of the cations was mitigated in molasses mash, suggesting possible presence of chelating agents in molasses.

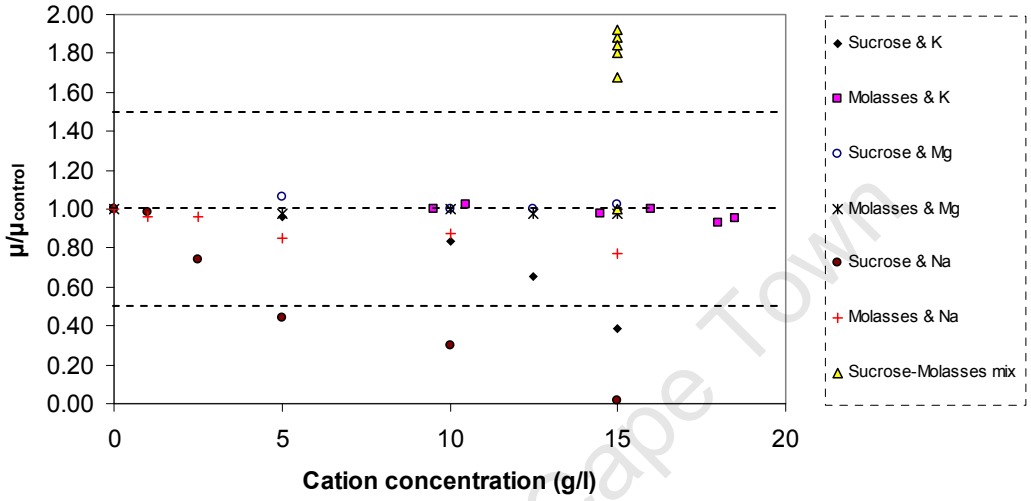

Figure 5.18 Normalised specific growth rate of yeast with varying cation concentration

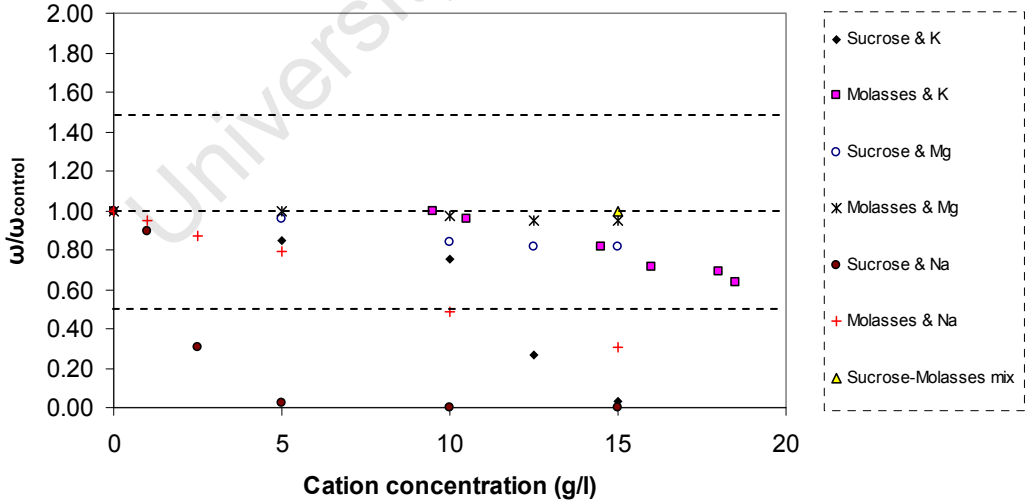

Figure 5.19 Normalised ethanol production rate with varying cation concentration

Included in Figures 5.18 and 5.19 are data points for experiments in which sucrose-based media supplemented with molasses mash was used as the fermentation media. These

experiments are detailed later in section 5.6. Of note is the omission of some ω/ω_{contol} values, for sucrose-based media supplemented with molasses mash, from Figure 5.19 due to the values lying outside the scale of Figure 5.19 (Table 5.4). Incorporating these results into Figure 5.19 would distort the observed trends. This applied to Figure 5.21 and Figure 5.23 as well.

Table 5.8 Variation of normalised growth and ethanol production rate of *S. cerevisiae* at 15 g.l^{-1} K^+ concentration

Cation concentration (g.l^{-1})	Cation	Media base	$\mu/\mu_{control}$	$\omega/\omega_{control}$
15	K^+	Sucrose	0.39	0.03
15	K^+	Molasses	0.99	0.77
15	Mg^{2+}	Sucrose	1.0	0.82
15	Mg^{2+}	Molasses	0.98	0.98
15	Na^+	Sucrose	0.02	0.00
15	Na^+	Molasses	0.77	0.31
15	K^+	Sucrose- molasses mix	1.7-1.9	21-28

5.5.2 Effect of Ionic Strength on Specific Growth Rate and Ethanol Production Rate

A general decrease in both $\mu/\mu_{control}$ and $\omega/\omega_{control}$ was observed with increasing ionic strength (Figure 5.21 and Figure 5.22). However, the extent of decrease was cation species dependent, and not simply a function of ionic strength. For instance, in sucrose-based media with an ionic strength of 25 mS $\mu/\mu_{control}$ was 0.80 where K^+ was the contributing cation, while $\mu/\mu_{control}$ was 0.25 where Na^+ was the contributing species. The values for $\omega/\omega_{control}$ were 0.64 and 0.00, respectively. These results indicate that while ionic strength influences yeast growth and fermentation performance (Shuler and Kargi, 2002) it is ultimately the toxicity of cation species that determines the magnitude of the negative impact. Also, as previously observed, the negative impact is mitigated in molasses mash.

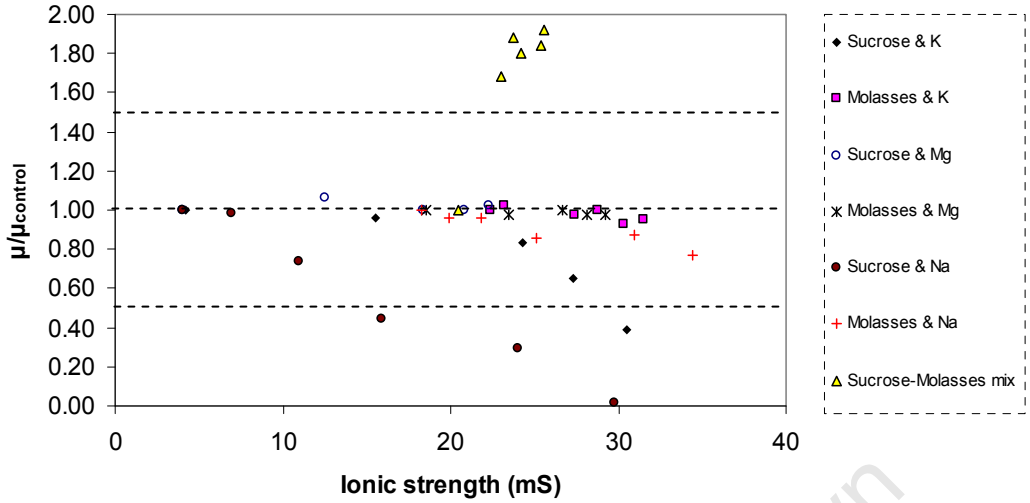

Figure 5.20 Normalised specific growth rate of yeast with varying osmotic pressure

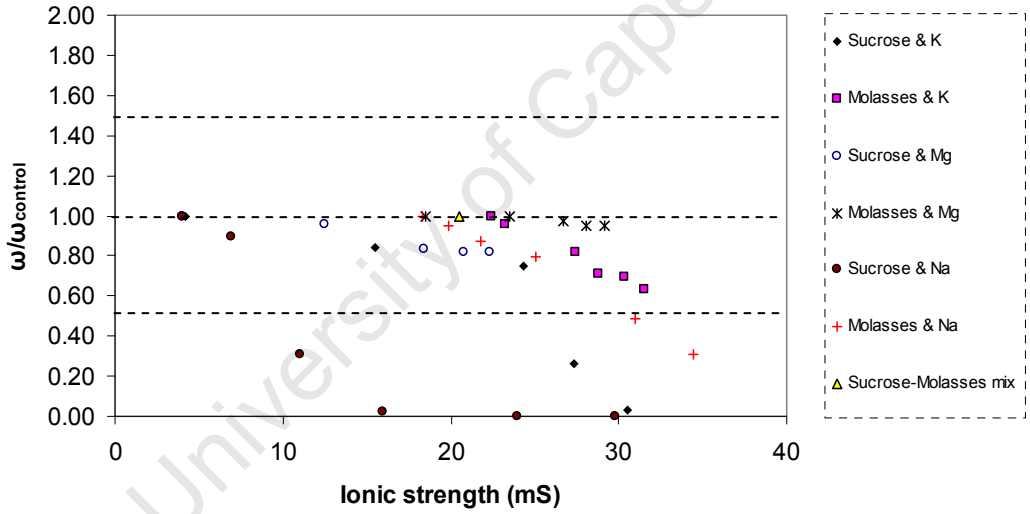

Figure 5.21 Normalised ethanol production rate with varying ionic strength

Table 5.9 Variation of normalised specific growth rate and ethanol production rate of *S. cerevisiae* at ionic strength of 25 mS

Ionic strength (mS)	Cation	Media base	$\mu/\mu_{control}$	$\omega/\omega_{control}$
25	K^+	Sucrose	0.80	0.64
25	K^+	Molasses	1.0	0.90
25	Mg^{2+}	Sucrose	-	-
25	Mg^{2+}	Molasses	0.99	0.99
25	Na^+	Sucrose	0.25	0.00
25	Na^+	Molasses	0.85	0.79

5.5.3 Effect of Osmotic Pressure on Specific Growth Rate and Ethanol Production Rate

Variation of $\mu/\mu_{control}$ and $\omega/\omega_{control}$ with increasing osmotic pressure is illustrated in Figure 5.22 and Figure 5.23, respectively. Again, while osmotic pressure appeared to be responsible for the decrease in $\mu/\mu_{control}$ and $\omega/\omega_{control}$ the cation species responsible for the osmotic pressure plays a greater role. In sucrose-based media with an osmotic pressure of 4.0 MPa, $\mu/\mu_{control}$ was 1.0, 0.90 and 0.23 where the contributing species was Mg^{2+}, K^+ and Na^+, respectively. A similar trend was observed with $\omega/\omega_{control}$ (Table 5.10). Although yeast grow optimally at osmotic pressure 1.38 MPa (Beney *et al.*, 2000 and Laroche *et al.*, 2001) they are known to survive at up to 100 MPa (Marechal and Gervais, 1994). This together with previous observation of cation species playing a greater role than cation concentration and ionic strength suggests that osmotic pressure plays a smaller role in determining yeast growth and fermentation performance. The negative effects were also more pronounced in sucrose-based media.

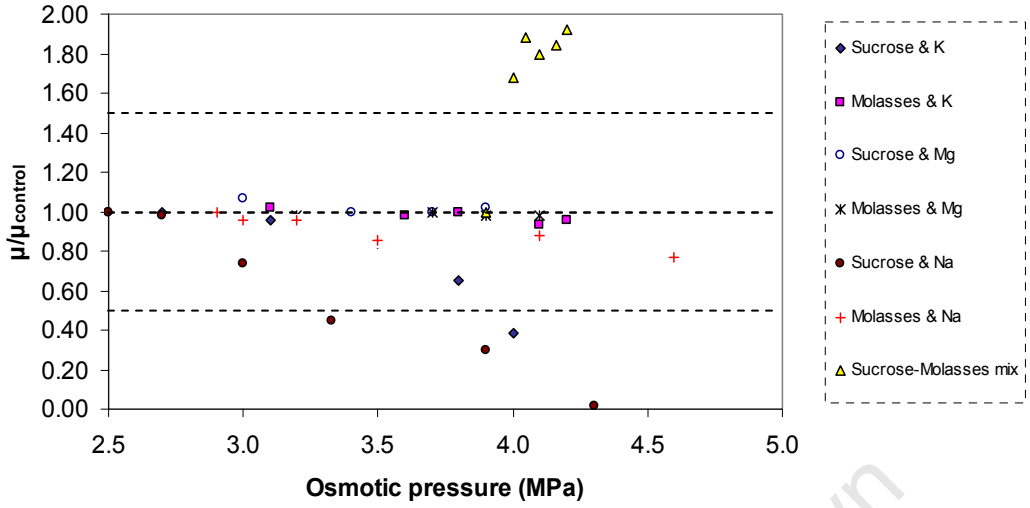

Figure 5.22 Normalised specific growth rate of yeast with varying osmotic pressure

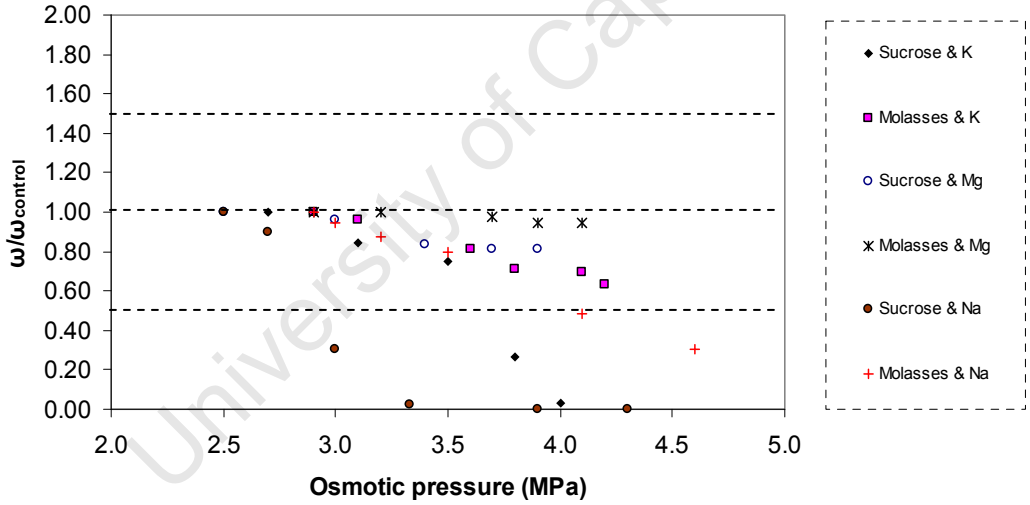

Figure 5.23 Normalised ethanol production rate with varying osmotic pressure

Table 5.10 Variation of normalised specific growth and ethanol production rate of S. *cerevisiae* at osmotic pressure of 4.0 MPa

Osmotic pressure (MPa)	Cation	Media base	$\mu/\mu_{control}$	$\omega/\omega_{control}$
4.0	K^+	Sucrose	0.90	0.03
4.0	K^+	Molasses	0.95	0.70
4.0	$Mg2^+$	Sucrose	1.02	0.82
4.0	$Mg2^+$	Molasses	0.98	0.95
4.0	Na^+	Sucrose	0.23	0.00
4.0	Na^+	Molasses	0.88	0.54

5.5.4 Summary of Cation Concentration, Ionic Strength and Osmotic Pressure

Section 5.5 showed that yeast and fermentation performance do not correlate as a function of overall cation concentration, ionic strength or the osmotic pressure of the media. Instead, the toxicity of the specific cation responsible for increasing the cation concentration, ionic strength, and osmotic pressure of the media is important. The results of this section show that at a specified cation concentration, ionic strength or osmotic pressure, the negative effects on yeast growth and fermentation performance increase with specific cation according to $Na^+ > K^+ > Mg^{2+}$. Also of importance in the determination of the effects of cation concentration, ionic strength and osmotic pressure was the fermentation media. The effects were more pronounced in sucrose-based media than molasses, suggesting the presence of compounds in molasses mash that provide protection against the negative effects.

5.6 SUPPLEMENTATION OF MOLASSES MASH TO SUCROSE-BASED MEDIA FOR IMPROVED YEAST AND FERMENTATION PERFORMANCE

The results summarised in Section 5.2 to 5.5 revealed a decrease in yeast growth and fermentation performance with increasing cation concentration in molasses mash and sucrose-based media. However, a more pronounced decrease was observed in the sucrose-based media than in the molasses mash, suggesting the possible presence of chelating agents in molasses. In this section, an attempt was made to better understand this phenomenon. To achieve this, sucrose-based media containing high K^+ concentration was supplemented with varying amounts of molasses mash and fermented. Yeast growth and fermentation performance were monitored and related to the amount of molasses mash supplemented. Before supplementation, the separate media were prepared as outlined in Section 3.2.2 and the K^+ concentration in each media adjusted to 15 $g.l^{-1}$ K^+. Fermentation media were prepared as shown in Table 5.11. The specific growth rate, cell viability, sugar concentration and ethanol profiles of the fermentations are shown in Figure 5.24.

Table 5.11 Composition of fermentation media

Experiment	K^+ concentration ($g.l^{-1}$)	% Sucrose media component (v/v)	% Molasses mash component (v/v)
A	15	100	-
B	15	80	20
C	15	60	40
D	15	40	60
E	15	20	80
F	15	-	100

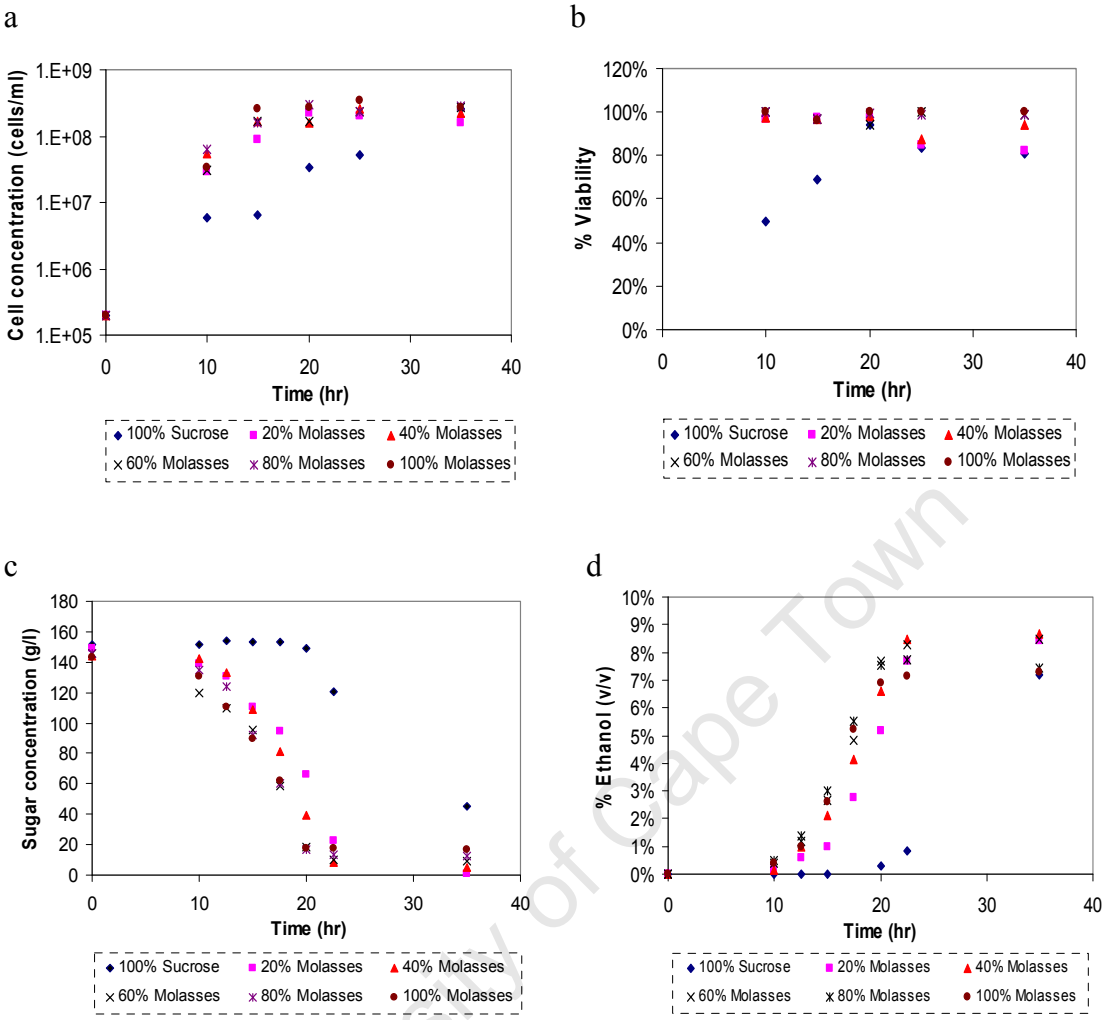

Figure 5.24 specific growth rate, viability, sugar concentration and ethanol concentration profiles of fermentations of molasses mash supplemented sucrose-based media of equal K^+ concentration

5.6.1 Specific Growth Rate and Viability

The cell concentration as a function of time is shown in Figure 5.24a. An increase in yeast growth was observed as the molasses mash component of sucrose-based media was increased. For instance, 10 hours after inoculation the cell concentration in 100% sucrose- based media was 6 x 10^6 cells.ml^{-1}, while that supplemented with 20% (v/v) molasses mash had a cell concentration of 3 x 10^7 cells.ml^{-1}. This represented a five fold increase in cell concentration. The specific growth rate in 100% sucrose-based media was 0.25 ± 0.1 hr^{-1} and increased to 0.42 ± 0.2 hr^{-1} upon supplementation with 20% molasses

mash, representing a 68% increase (Figure 5.25). Increasing the molasses mash component of the fermentation media to 40%, further increased the specific growth rate to 0.47 ± 0.2 hr^{-1}. Above a molasses mash component of 40% no significant improvement in the specific growth rate was observed. The highest specific growth rate was 0.48 hr^{-1} and occurred in 100% molasses mash.

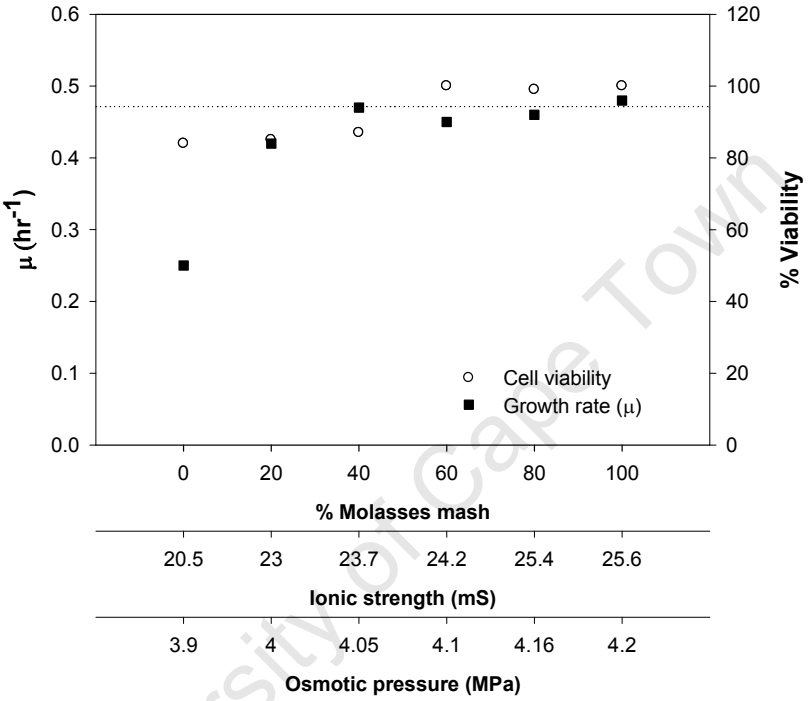

Figure 5.25 Specific Growth rate and viability of *S. cerevisiae* in molasses mash supplemented sucrose-based media of equal K^+ concentration (15 $g.l^{-1}$)

Yeast cell viability followed a similar trend, increasing when the molasses mash component of fermentation media was increased. Ten hours after inoculation yeast in 100% sucrose-based media had a cell viability of 50 ± 2%, while that in sucrose-based media supplemented with 20% (v/v) molasses mash had a significantly higher viability of 97% (Figure 5.24b). After ten hours, a gradual increase in yeast viability with time was observed in 100% sucrose-based media. This phenomenon is common when yeast is exposed to sub-lethal stresses. Initially cell damage occurs and yeast respond by inducing cellular responses that result in cell repair, adaptation and resumption of growth (Hohmann and Mager, 2003).

Figure 5.25 shows yeast cell viabilities at the standard time of 25 hours after fermentation. In 100% sucrose-based media the yeast cell viability was 84%. When molasses mash composition was 60% of the fermentation media, viability had improved to 100%, representing a 19% improvement in cell viability.

The improved yeast growth in sucrose-based media, as a result of molasses mash supplementation, corresponded with increases in the both the ionic strength and osmotic pressure of the medium (Figure 5.25). These results appear to be contrary to previous observations where decreases in yeast growth were observed with increasing ionic strength and osmotic pressure. However, the results support the idea that chelating agents may be present in molasses resulting in mitigation of salt effects.

5.6.2 Sugar Utilisation and Ethanol Production Rate

The sugar and ethanol concentrations as a function of time are provided in Figure 5.24c,d. A long lag phase, approximately 20 hours, was observed in 100% sucrose-based media, thus negatively affecting both the sugar utilisation and ethanol production rates. As the molasses mash composition of the fermentation media was increased, sugar utilisation and ethanol production rate increased as illustrated in Figure 5.26. In 100% sucrose-based media the sugar utilisation rate was 0.2 $g.l^{-1}.hr^{-1}$, while ethanol production rate was 0.1 $g.l.hr^{-1}$. Supplementation with 20% molasses mash increased the sugar utilisation and ethanol production rate to 4.2 ± 0.3 and 2.1 ± 0.1 $g.l^{-1}.hr^{-1}$, respectively (Figure 5.26). This increase in fermentation performance, again, occurred despite a corresponding increase in both the ionic strength and osmotic pressure of the fermentation medium. Subsequent supplementation with molasses mash increased both the sugar utilisation and ethanol production rate, before a slight decline. The maximum recorded sugar utilisation rate was 6.5 ± 0.3 $g.l^{-1}.hr^{-1}$ at molasses mash component of 80% of the fermentation media. The maximum ethanol production rate was 2.8 ± 0.1 $g.l^{-1}.hr^{-1}$ at molasses mash component of 60% of the fermentation media. Sugar utilisation and ethanol production rate in 100% molasses mash was 6.3 ± 0.3 and

2.4 ± 0.1 $g.l^{-1}.hr^{-1}$, respectively. Also, refer to Table 5.4 which shows variation of normalised ethanol production rate at a constant K^+ concentration.

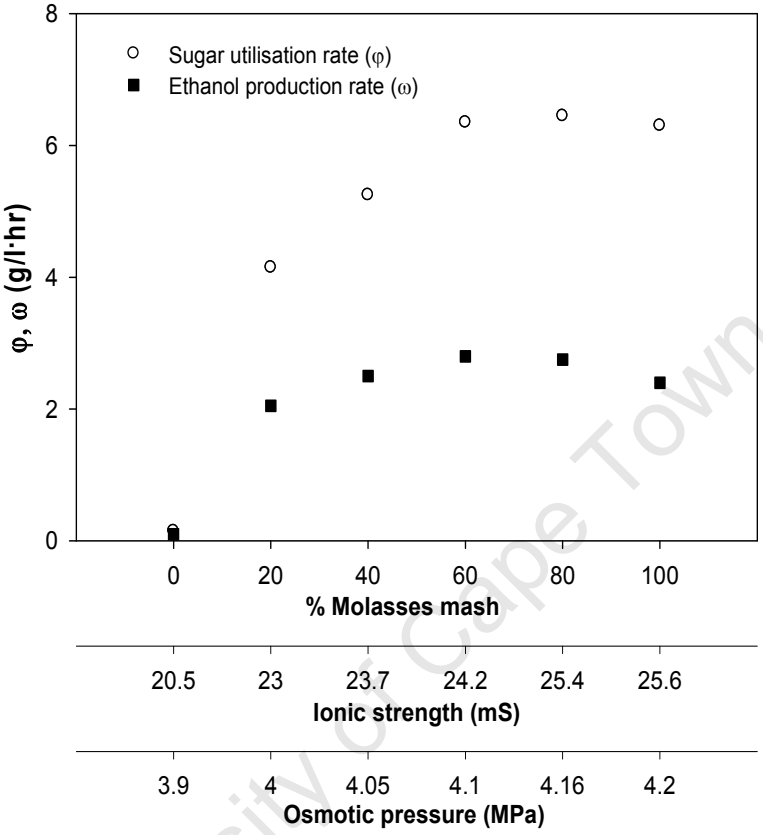

Figure 5.26 Sugar utilisation and ethanol production rate of *S. cerevisiae* in molasses mash supplemented sucrose-based media with constant K^+ concentration (15 $g.l^{-1}$)

The fermentation efficiency followed an expected trend and increased significantly from 0.09 in 100% sucrose-based media to 0.79 ± 0.05 in sucrose-based media supplemented with 20% molasses mash. Fermentation efficiency peaked at 0.92 ± 0.05, where the fermentation media had a 40% molasses mash composition. Further increases in the molasses mash composition of the fermentation media resulted in a steady reduction in fermentation efficiency, which reduced to 0.79 when media composition was 100% molasses mash. The results are presented graphically in Figure 5.27.

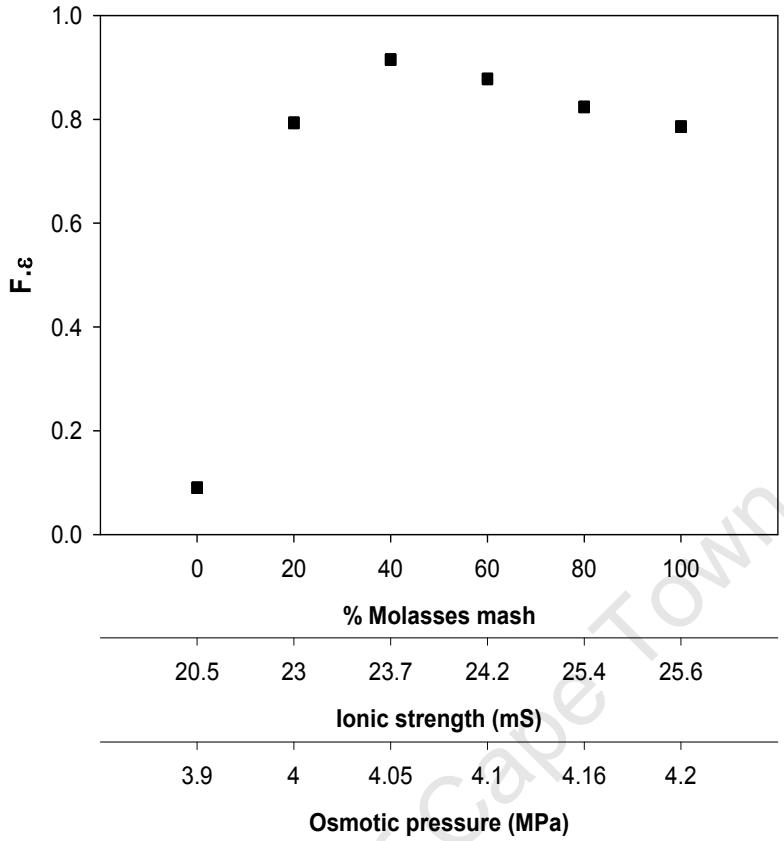

Figure 5.27 Fermentation efficiency of *S. cerevisiae* in molasses mash supplemented sucrose-based media with constant K^+ concentration (15 g.l^{-1})

5.6.3 Summary of Effect of Molasses Supplementation to Sucrose-based Media

Supplementation of sucrose-based media with a high K^+ concentration with a small amount of molasses mash (20% v/v) of equal K^+ concentration, resulted in significant increases in yeast growth and fermentation performance. Subsequent supplementation above 20% resulted in further improvements, although these were less pronounced. Yeast growth and fermentation performance peaked in media whose molasses mash composition was between 60 and 80%. A slight decline in performance was observed in 100% molasses mash. The increases in yeast growth and fermentation performance occurred despite increases in media ionic strength and osmotic pressure, suggesting that ionic strength and osmotic pressure of the media played a smaller role than the cation toxicity in determining ultimate yeast growth and fermentation performance. These

results also suggest the presence of chelating agents in molasses mash. This would explain why fermentations of molasses mash are less affected by monovalent cation concentrations than those of sucrose-based media. The results also highlight the important role chelating agents can play in improving yeast growth and fermentation performance in media containing high concentrations of toxic cations.

5.7 SUMMARY AND DISCUSSION OF RESULTS

In this chapter the growth and fermentation performance of *S. cerevisiae* in molasses mash and sucrose-based media of varying cation (K^+, Mg^{2+}, Na^+) concentration, ionic strength, and osmotic strength were evaluated and compared. Analytical parameters, namely specific growth rate, cell viability, sugar utilisation rate, ethanol production rate and fermentation efficiency were used to quantify yeast and fermentation performance. In the case of fermentations using molasses mash, molasses usage efficiency (M.U.ε) and sugar usage efficiency (S.U.ε) were also evaluated. Based on the experimental results presented in the chapter, some important deductions were made:

- Increasing the cation concentration (and consequently, ionic strength and osmotic pressure) of K^+ and Na^+ in fermentation media across the range 0 to 18.5 $g.l^{-1}$ had a negative impact on yeast and fermentation performance in sucrose-based media and molasses mash. The decrease in performance was due to a combination of salt, ionic and osmotic stress caused by the particular cation. With Mg^{2+}, no significant changes in yeast and fermentation performance were detected in molasses mash. However, in sucrose-based media, marginal decreases in fermentation performance were observed with increasing Mg^{2+} concentration in the range 0 to 15 $g.l^{-1}$
- The extent of the decrease in fermentation performance did not correlate with ionic strength or osmotic pressure across the range of salts used, but were cation specific. The magnitude of the decrease in fermentation performance at the same ion concentration increased in the order: $Na^+ > K^+ > Mg^{2+}$. These results suggest that inhibitory effects or toxicity of specific cations played a bigger role in

determining yeast and fermentation performance than ionic strength or osmotic pressure.

- The effect of cation concentration on yeast growth and fermentation performance was dependent on the fermentation media used. The effects were more pronounced in sucrose-based media than in molasses mash. This observation was further strengthened when supplementation of sucrose-based media of 15 $g.l^{-1}$ K^+ concentration with 20% (v/v) molasses mash of equal K^+ concentration significantly increased yeast and fermentation performance, relative to sucrose media at 15 $g.l^{-1}$ K^+ in the absence of molasses.

The effect of Na^+ and K^+ on yeast metabolism has been reported previously. The studies in the chapter confirm that high concentrations of these cations, as may be encountered in South African cane molasses, can affect fermentation performance. Although it was envisaged that ionic strength and osmotic pressure may also be implicated in compromised yeast and fermentation performance, it has been shown that these effects are not dominant in the fermentation system used. It was not anticipated that the type of fermentation media would play a significant role. However, this observation allowed us to expand on factors that could contribute to suboptimal fermentations. It is hypothesised that the presence of chelating or complexing agents in molasses mitigate monovalent cation toxicity, the idea being that the chelating agents act as metal ion buffers releasing the metal cations at low concentrations matching yeast requirements (Ergun *et al*., 1997; Oderinde *et al*., 1985). Therefore, molasses quality should be defined based not only on cation concentration, ionic strength and osmotic pressure, but also on the presence or absence of chelating agents in fermentation media. The effects of chelating agents on molasses fermentation and consequent improved fermentation have been demonstrated previously (Ergun *et al*., 1997; Oderinde *et al*., 1985).

CHAPTER 6

MOLASSES QUALITY AND FERMENTATION PERFORMANCE

6.1 INTRODUCTION

Blackstrap molasses is the most commonly used feedstock for ethanol production in South Africa. It is a by-product, typically gathered from different sugar refineries; its composition is known to vary (Piggot, 2003). This variation results in molasses mash that ferments differently from batch to batch. Fermentation of good quality molasses typically results in speedy fermentations with high ethanol yields, while that of poor quality or "bad" molasses is sluggish with reduced ethanol yields. It is important to note that it is not only poor quality molasses that is responsible for suboptimal fermentations, but also the age of the refinery and osmotic loading. Lavarack (2003) defined good fermentation yields for Australian molasses as ranging from 87 to 94%, while those lower than 87% were typical for plants designed in the 1970's and 1980's. In Indian batch fermentations, 7 to 8% (v/v) ethanol is produced from diluted molasses with 15 to 16% sugars. These fermentations result in fermentation efficiencies of 80 to 85% (Patil *et al*., 1998). Many ethanol producers experience fluctuations in fermentation efficiencies within their plants and desire to pinpoint whether "bad" fermentations are a consequence of low quality molasses or inefficient plant operation.

In this chapter an attempt was made to establish performance differences as a function of molasses quality, using molasses samples identified as "good" and "bad" on the basis of fermentation at Illovo Sugar (Merebank, South Africa). The categorisation of molasses as

"good" or "bad" was investigated by performing laboratory scale fermentations in low volume shake flasks (500 ml) and higher volume (5 l) bioreactors. The intention was to verify if variations in fermentation performance were a consequence of molasses quality or plant operation. Performance was assessed in terms of specific growth rate, cell viability, sugar utilisation rate, ethanol production rate, fermentation efficiency, molasses usage efficiency and sugar usage efficiency. Where suboptimal fermentations were observed, attempts were made to identify possible causes. This was done by relating performance to available fermentable sugar concentration, cation concentration, and ionic strength of the molasses mash. The potential for the dilution ratio of molasses to impact yeast growth and fermentation efficiency negatively was also investigated.

6.2 FERMENTATIONS OF "GOOD" AND "BAD" MOLASSES IN SHAKE FLASKS

Initial experiments in the investigation of "good" and "bad" molasses were performed in shake flasks. Molasses samples of differing quality were fermented simultaneously under conditions shown in Table 6.1. The "good" molasses was used as the control.

Table 6.1 Operating conditions for "good" and "bad" molasses fermentations in shake flasks

Flask volume	500 ml
Working volume	300 ml
Initial sugar concentration	120-140 $g.l^{-1}$
Inoculum concentration	10^5 $cells.ml^{-1}$
Media pH	4.6
Operating temperature	30 ^oC
Agitation speed	160 rpm

The fermentation media was prepared by mixing molasses and water in a 1:3 ratio, with the addition of urea and sulphuric acid, as detailed in Section 3.2.2. A smaller yeast inoculum, relative to that used in industry, was employed. Typical industrial

fermentations use high initial cell concentrations of 10^8 cells.ml^{-1} (Algre *et al*., 2003; Illovo Sugar, 2007). However, it was envisaged that low cell concentrations would magnify the fermentation differences between "good" and "bad" molasses. In this section, fermentation performance in "good" molasses (Batch # 212302007) and "bad" molasses (Batch # 232022008) was established. Fermentations were performed simultaneously in triplicate and the resulting fermentation performances compared.

6.2.1 Specific Growth Rate and Cell Viability

The cell concentration and viability were monitored regularly during fermentation. The average cell concentration and viability as a function of time are represented in Figure 6.1. The cell growth in the "good" and "bad" molasses was similar. The maximum cell concentration in "good" molasses (reached 25 hours after inoculation) was 3.5 ± 0.2 x 10^8 cells.ml^{-1}, while that in the "bad" molasses was 3.6 ± 0.2 x 10^8 cells.ml^{-1}. The specific growth rate in the "good" molasses was 0.45 ± 0.2 hr^{-1}, while that in the "bad" molasses was 0.44 hr^{-1}. No loss in cell viability was observed in either "good" or "bad" molasses throughout the duration of fermentation. Viability remained high at 100% during fermentation.

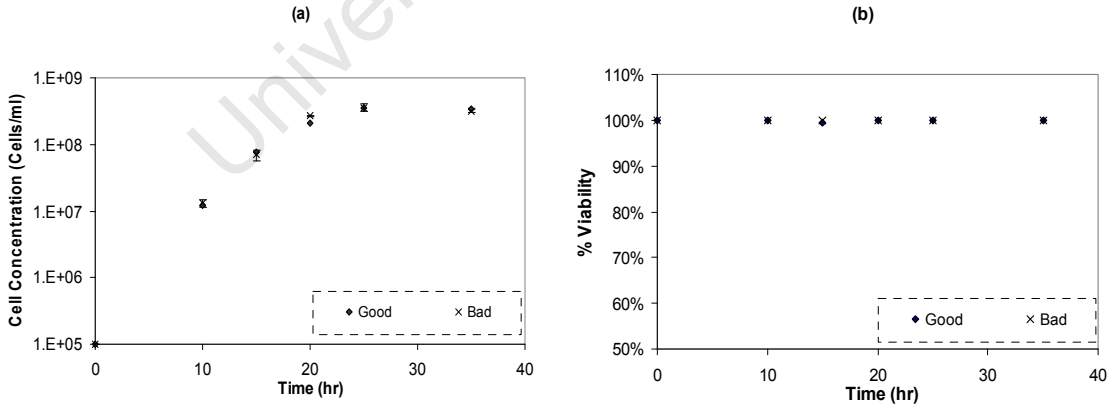

Figure 6.1 Cell concentration (a) and cell viability (b) in "good" and "bad" molasses

6.2.2 Sugar and Ethanol Concentration

Sugar analyses, showed that molasses mash produced from the "good" and "bad" molasses had significantly different initial sugar concentrations. This implied different sugar concentrations in the original molasses batches, since both molasses mashes were similarly prepared. Interestingly, the "bad" molasses mash had a higher initial sugar concentration of 142 ± 2 $g.l^{-1}$, while that from the "good" molasses mash had a lower sugar concentration of 125 ± 2 $g.l^{-1}$. Fermentation of the molasses mash resulted in sugar and ethanol profiles shown in Figure 6.2.

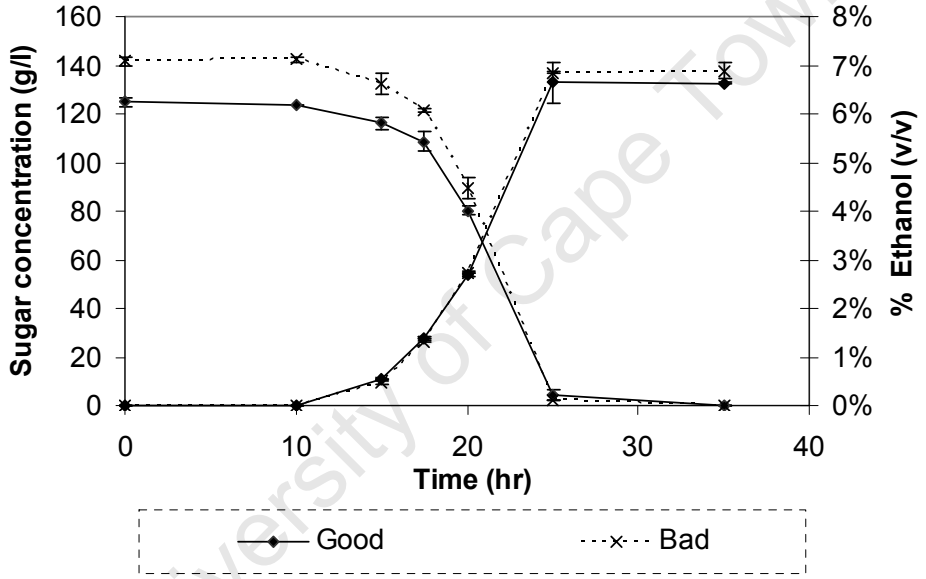

Figure 6.2 Sugar and ethanol concentration profiles from "good" and "bad" molasses fermentations

Sugar utilisation by yeast in the first ten hours after inoculation was negligible, with less than 1 $g.l^{-1}$ being used up in each case. This was attributed mainly to the low inoculum concentration of 10^5 $cells.ml^{-1}$. After the first 10 hours, the sugar utilisation rate increased appreciably as more biomass was available for biotransformation of the sugar into ethanol. The average sugar utilisation rate in the first 20 hours was 2.6 ± 0.2 $g.l^{-1}.hr^{-1}$ for the "bad" molasses mash, while that for "good" molasses was 2.2 $g.l^{-1}.hr^{-1}$, illustrating faster metabolism in the "bad" molasses mash than the "good" molasses mash. However, the ethanol production rate, over the same period, was similar in the "good" and "bad"

molasses mash at 1.1 ± 0.1 $g.l^{-1}.hr^{-1}$. This suggests possible diversion of fermentable sugar in the "bad" molasses to side products such as glycerol, succinic acid and acetic acid (Murtagh, 1999; Paturau, 1989) (as cited in Lavarack, 2003). It is estimated that up to 10% of fermentable sugar in molasses can be diverted to the formation of these side products (Alves, 1994) (as cited in Wheals *et al*., 1999). The final ethanol concentration (after 25 hours) was higher in the "bad" molasses mash than in the "good" molasses mash. The final ethanol concentration was 6.9 ± 0.1% for the "bad" molasses mash and 6.6 ± 0.4% for the "good" molasses.

The above results appear to contradict the general understanding of "bad" molasses as having lower fermentable sugars and producing low ethanol concentrations. However, consideration of fermentation efficiencies, presented in Table 6.2, reveal why this molasses may have been termed "bad" molasses.

Table 6.2 Fermentation efficiencies of "good" and "bad" molasses

Molasses	F. ε	M.U.ε (kg/ l ethanol)	S.U.ε (kg/ l ethanol)	Y_{ES}
"Good"	0.82 (± 0.03)	3.6 (± 0.2)	1.8 (± 0.1)	0.44 (± 0.2)
"Bad"	0.75 (± 0.01)	3.5	2.0	0.39

"Good" molasses resulted in a higher overall fermentation efficiency (F.ε) of 0.82 ± 0.03 than "bad" molasses, whose fermentation efficiency was 0.75 ± 0.01. The molasses usage efficiency in the "good" and "bad" molasses was statistically similar despite the "bad" molasses having higher fermentable sugar content. Sugar utilisation efficiency was higher in the "bad" molasses at 2.0 $kg.l^{-1}$ compared to 1.8 ± 0.1 $kg.l^{-1}$ in the "good" molasses. Previously this was attributed to possible diversion of some fermentable sugar to side product production and cell maintenance. This was also confirmed by the higher ethanol yield on substrate exhibited by the "good" molasses of 0.44 ± 0.2 $g.g^{-1}$ compared to 0.39 $g.g^{-1}$ for the "bad" molasses.

6.2.3 Cation Composition of "Good" and "Bad" Molasses

Previously, in Chapter 5, we showed a general decrease in both yeast growth and fermentation performance with increasing cation concentration. Thus, the "good" and "bad" molasses were analysed for the four most abundant cations (K^+, Mg^{2+}, Na^+ and Ca^{2+}), and attempts were made to relate their relative concentrations to fermentation performance. The results are tabulated in Table 6.3.

Table 6.3 Cation composition and ionic strength of "good" and "bad" molasses

Molasses	K^+ %	Mg^{2+} %	Na^+ %	Ca^{2+} %	I (mS)
"Good"	3.0	0.47	0.09	0.68	18.6
"Bad"	3.3	0.49	0.07	0.69	19.3

The most significant difference in cation concentrations of the "good" and "bad" molasses was the K^+ concentration, which was 10% higher in "bad" molasses. It is envisaged that higher K^+ concentration in the "bad" molasses contributed to salt stress on the yeast. Also of significance was the higher ionic strength in the "bad" molasses mash (19.3 mS) compared to the "good" molasses (18.6 mS). The above could be contributing factors to the classification of molasses (Batch # 232022008) as "bad" molasses. It can be argued that "good" molasses had a Na^+ concentration of 29% greater than the "bad" molasses and that too should contribute to less efficient fermentation. However, the difference in K^+ concentration in "good" and "bad" molasses accounts for 3000 ppm K^+. The difference in Na^+ concentration accounts for only 200 ppm. While Na^+ has a more deleterious effect on ethanol fermentation on a mass per mass basis, it is postulated that the K^+ concentration had a greater impact due to its significantly higher concentration.

6.2.4 Implications of "Bad" Molasses Fermentations in Industrial Settings

Suboptimal fermentations are undesirable as they result in a loss in production capacity and increased raw material requirements. Typically, ethanol producers target specific

fermentation efficiencies to meet production targets. When fermentation efficiencies fall below optimal ranges, production losses occur. To illustrate this, the above fermentation of "good" and "bad" molasses was analysed for ethanol losses. This was achieved by calculating the potential ethanol production based on the sugar content of the "bad" molasses, while comparing the fermentation efficiencies achieved for "good" and "bad" molasses. The calculations were performed for a typical 30 000 l industrial fermenter and are presented in Table 6.4.

Table 6.4 shows potential anhydrous ethanol losses as a result of reduced fermentation efficiency of "bad" molasses. To calculate ethanol losses, 100% ethanol recovery during separation was assumed. The ethanol density used was 0.789 $g.l^{-1}$ at 20°C (Perry and Green, 1984). In this case, fermentation of poor quality molasses can result in a total ethanol loss of 193 l per batch, relative to "good" molasses. This corresponds to a reduction in production of 8.5%.

Table 6.4 Ethanol loss as a result of "bad" fermentations in 30 000 l fermenter

Molasses	$C_{s,0}$ ($g.l^{-1}$)	F.ε	Anhydrous ethanol produced (l)
"Bad"	142	0.75	2069
"Good"	142	0.82	2262
	Total ethanol lost		**193 l**

6.3 FERMENTATIONS OF "GOOD" AND "BAD" MOLASSES IN BIOREACTORS

"Good" and "bad" molasses fermentations were also performed in New Brunswick bioreactors as described in Section 3.3.2. In this section the yeast and fermentation performance of "good" molasses (Batch # 212302007) and "bad" molasses (Batch # A130322007) were considered. The operating conditions were adjusted to resemble industrial conditions as shown in Table 6.5. In these experiments the molasses: water

ratio was adjusted to 1: 2.5 (m/m) producing molasses mash of approximately 150 to 160 $g.l^{-1}$ total sugar as invert. The initial yeast concentration was increased to ~6 x 10^7 $cells.ml^{-1}$ to resemble industrial conditions.

Table 6.5 Operating conditions for "good" and "bad" molasses fermentations in New Brunswick bioreactors

Reactor volume	7 l
Working volume	5 l
Initial sugar concentration	150-160 $g.l^{-1}$
Inoculum concentration	6 x 10^7 $cells.ml^{-1}$
Media pH	4.6
Operating temperature	30 ^oC
Agitation speed	300 rpm

6.3.1 Growth Rate and Cell Viability

The "good" and "bad" molasses mash was inoculated with 3.4 ml of yeast cream per liter of mash, resulting in an initial yeast concentration of 6 x 10^7 $cells.ml^{-1}$ in the reactor. The cell concentration and viability as a function of time for "good" and "bad" molasses fermentations in bioreactors are given in Figure 6.3. At 10 hr the cell concentration in the "good" molasses exceeded that of "bad" molasses with values of 2.9 x 10^8 $cells.ml^{-1}$ and 2.2 x 10^8 $cells.ml^{-1}$, respectively. Thereafter, a convergence in the cell concentration of the "bad" molasses towards that of the "good" molasses was observed. The final cell concentration, taken at 17 hours, was 3.5 ± 0.2 x 10^8 $cells.ml^{-1}$ for the "good" molasses and 3.3 ± 0.2 x 10^8 $cells.ml^{-1}$ for the "bad" molasses. The specific growth rate in the "good" molasses was 0.11 hr^{-1}, while that in the bad molasses was 0.10 hr^{-1}. Cell viability in both "good" and "bad" molasses remained high at 100% throughout the fermentations.

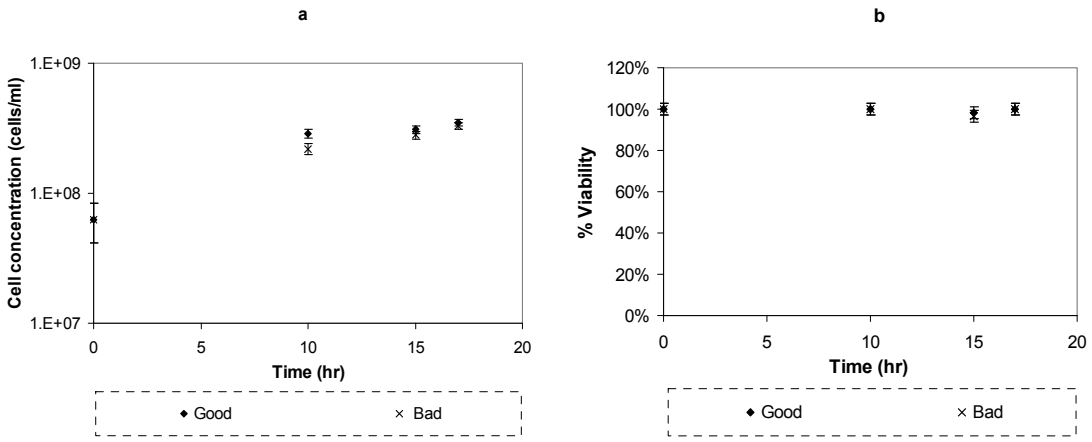

Figure 6.3 Cell concentration (a) and cell viability (b) in "good" and "bad" molasses

6.3.2 Sugar and Ethanol Concentration

The initial sugar concentration in "good" and "bad" molasses mash was similar, being 160 $g.l^{-1}$ and 159 $g.l^{-1}$, respectively. Fermenting the two media simultaneously, resulted in the sugar and ethanol concentration profiles illustrated in Figure 6.4. Comparison of the two profiles revealed a 1 to 2 hour fermentation lag of "bad" molasses relative to the "good" molasses. Sugar depletion in the "good" molasses occurred after 15 hours of fermentation, while that in "bad" molasses occurred after 17 hours, despite both mashes having similar initial sugar concentrations. The maximum ethanol concentration produced in both "good" and "bad" molasses fermentations was reached at time 15 hours. The former had a maximum ethanol concentration of 74 ± 2 $g.l^{-1}$ (9.4 ± 0.3%), while the latter had a maximum concentration of 69 ± 2 $g.l^{-1}$ (8.7 ± 0.3%). It was noted that the maximum recorded ethanol concentration in the "bad" molasses was at time 15 hours, before sugar depletion. However, at time 17 hours sugar depletion had occurred. This coincided with a slight reduction in ethanol concentration in both "good" and "bad" molasses fermentations. The ethanol concentration in the "good" molasses was reduced to 70 ± 2 $g.l^{-1}$, while that in the "bad" molasses mash was reduced to 67 ± 2 $g.l^{-1}$. A possible reason for the reduction in ethanol concentration was the diauxic shift in yeast metabolism, which resulted in ethanol consumption. A summary of the sugar utilisation and ethanol production are given in Table 6.6. The maximum sugar utilisation rate (φ_{max}) and ethanol production rate (ω_{max}) were taken between 10 and 15 hr.

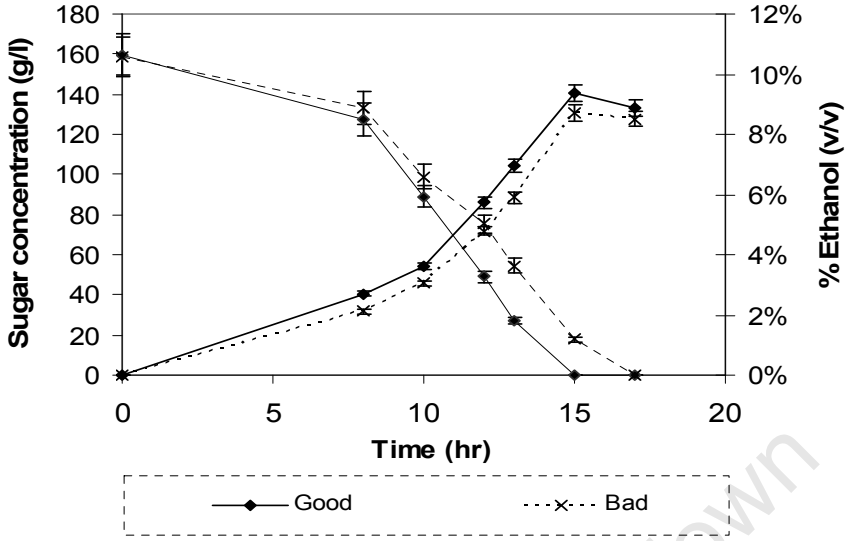

Figure 6.4 Sugar and ethanol concentration profiles from "good" and "bad" molasses fermentations

Table 6.6 Summary of sugar, ethanol concentration, maximum sugar utilisation and ethanol production rate in "good" and "bad" molasses

Molasses	$C_{s,i}$ ($g.l^{-1}$)	φ_{max} ($g.l.hr^{-1}$)	ω_{max} ($g.l.hr^{-1}$)	E_{max} ($g.l^{-1}$)	E_{final} ($g.l^{-1}$)
"Good"	160	18 (R^2=0.99)	9.1 (R^2=1.0)	74 ± 2	70 ± 2
"Bad"	159	16 (R^2=0.98)	8.9 (R^2=0.98)	69 ± 2	67 ± 2

The fermentation efficiencies of the good and bad molasses were determined and are summarised in Table 6.7. The F.ε, based on the maximum ethanol concentration, was 0.92 ± 0.3 for good molasses and 0.85 ± 0.3 for the bad molasses. The M.U.ε in good molasses was 3.1 ± 0.1 $kg.l^{-1}$, while that in bad molasses was 3.3 ± 0.1 $kg.l^{-1}$. The lower molasses usage efficiency in good molasses indicates that more bad molasses is needed to produce the same amount of ethanol as good molasses. Similarly, the lower sugar utilisation efficiency in good molasses implied more fermentable sugar was channelled by yeast to ethanol production.

Table 6.7 Fermentation efficiencies of "good" and "bad" molasses

Molasses	F. ε	M.U.ε (kg/ l ethanol)	S.U.ε (kg/ ethanol)	$Y_{E,s}$
"Good"	0.92 ± 0.3	3.1 ± 0.1	1.7	0.47
"Bad"	0.85 ± 0.3	3.3 ± 0.1	1.8	0.44

6.3.3 Cation Composition and Ionic Strength of "Good" and "Bad" Molasses

The "good" and "bad" molasses were analysed for the four most abundant cations (K^+, Mg^{2+}, Na^+ and Ca^{2+}), and attempts were made to relate their relative concentrations to fermentation performance. The ionic strength of the fermentation media was also measured. The results are tabulated in Table 6.8.

Table 6.8 Cation composition and ionic strength of "good" and "bad" molasses

Molasses	K^+ %	Mg^{2+} %	Na^+ %	Ca^{2+} %	I (mS)
"Good"	3.0	0.47	0.09	0.68	20.3
"Bad"	3.0	0.49	0.14	0.70	20.4

The K^+, Mg^{2+} and Ca^{2+} composition of "good" and "bad" molasses was very similar. The recorded differences in the concentrations of these elements were all less than or equal to 4%. The only significant difference observed was the Na^+ concentration. The Na^+ concentration in "bad" molasses was 60% higher than that in the "good" molasses. Considering that Na^+ is toxic (Gómez *et al*., 1996, Murguía *et al*., 1996), with yeast cells and having no absolute need for it (Wadskog and Alder, 2003), the poor performance can be linked to the relatively high Na^+ concentration. The ionic strength in the "good" and "bad" molasses was similar measuring 20.3 mS and 20.4 mS, respectively.

6.3.4 Implications of "Bad" Molasses Fermentations

The "good" and "bad" molasses fermentation efficiency in 5 l bioreactors was used for scale up to typical 30 000 l industrial sized batch fermenters as discussed in Section 6.2.4.

The potential for ethanol losses in a 30 000 l fermenter as a result of "bad" molasses are shown in Table 6.9. A potential loss of 234 l of anhydrous ethanol was calculated per batch cycle due to reduced fermentation efficiency of "bad" molasses. This value represents an 8.2 % reduction in expected production.

Table 6.9 Ethanol loss as a result of "bad" fermentations in 30 000 l fermenter

Molasses	$C_{s,0}$ ($g.l^{-1}$)	F.ε	Anhydrous ethanol produced (L)
"Good" (21230207)	160	0.92	2860
"Bad" (A130322007)	159	0.85	2626
	Total ethanol lost		**234 L**

6.4 THE EFFECT OF SALT ADDITION TO "GOOD" MOLASSES IN BIOREACTOR FERMENTATIONS

In Chapter 5, a reduction in yeast and fermentation performance was observed on increasing salt concentration over a wide range in shake flask fermentations. In Section 6.2 and 6.3, performance differences between "bad" and "good" molasses were related to differences in the K^+ and Na^+ concentrations. While "bad" molasses was shown to contain increased concentrations of K^+ and Na^+ and resulted in some 8% reduction in ethanol yields, the direct relationship between bad fermentations and cation concentrations could not be shown. To show this, two fermentations using the same "good" molasses were performed simultaneously in the bioreactors. However, the K^+ and Na^+ concentrations in one reactor was increased by 20%. To achieve this, 35 g of KH_2PO_4 and 0.74 g Na_2SO_3 were added to 5 l of good molasses mash. The resultant K^+ and Na^+ concentrations in the mashes were 3.0 and 3.6 $g.l^{-1}$ K^+ and 0.09 and 0.11 $g.l^{-1}$ Na^+, respectively. The molasses used in this investigation was Batch # 212302007.

6.4.1 Cell Growth and Viability

The cell concentration and cell viability as a function of time are given in Figure 6.5. The initial cell concentration in the "good" molasses mash and salt augmented mash was 6 x 10^7 cells.ml^{-1}. The maximum cell concentration was reached after 16 hours of fermentation. The maximum cell concentration in good molasses mash was 3.4 x 10^8 cells.ml^{-1}, representing a 5.7 fold increase. The maximum cell concentration in the salt augmented mash was 2.0 x 10^8 cells.ml^{-1}, representing a 3.3 fold increase. The specific growth rates were 0.11 hr^{-1} and 0.10 hr^{-1}, respectively. The cell viability during fermentation ranged between 99 and 100% in the good molasses mash while that in salt augmented mash ranged from 93 to 100%.

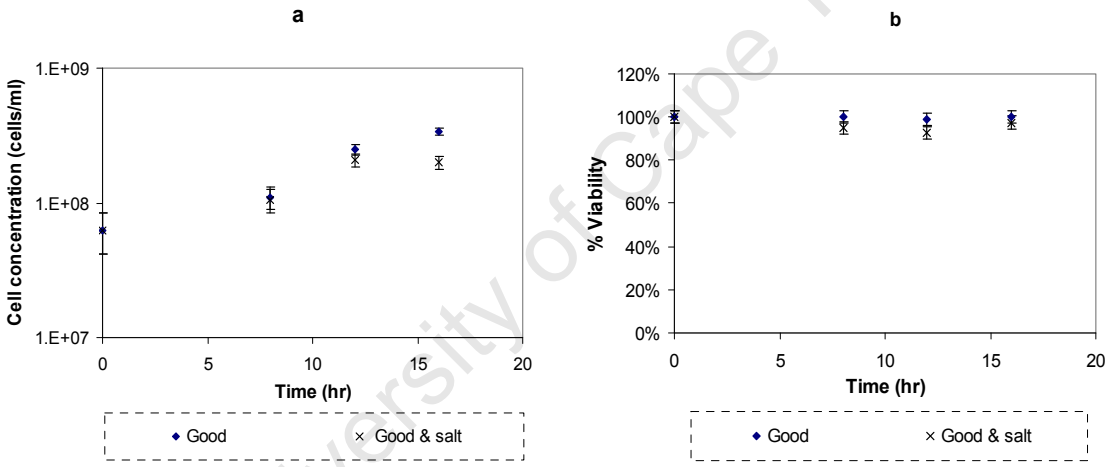

Figure 6.5 Cell concentration (a) and cell viability (b) in "good" molasses and "good" molasses with salt addition

6.4.2 Sugar and Ethanol Concentration

The sugar and ethanol concentration as a function of time for "good" molasses and the salt augmented molasses are shown in Figure 6.6. The sugar and ethanol profiles of the salt augmented mash lag those of the "good" molasses by ~1 to 2 hours. Sugar depletion in "good" molasses mash occurred after 16 hours, while 17 hours was required for sugar depletion in the salt augmented mash. The maximum sugar utilisation and ethanol production rates were evaluated between 8 and 15 hours and are shown in Table 6.10.

The φ_{max} was statistically similar at 13 ± 1 $g.l^{-1}.hr^{-1}$ for the "good" molasses mash and 14 ± 1 $g.l^{-1}.hr^{-1}$ the salt augmented mash. The ω_{max} was significantly higher in the "good" molasses mash at 7.1 ± 0.2 $g.l^{-1}.hr^{-1}$ compared to 6.0 ± 0.2 $g.l^{-1}.hr^{-1}$ for the salt augmented mash. The maximum ethanol concentration produced was 8.7 ± 0.3% (68 ± 2 $g.l^{-1}$) and 8.2 ± 0. 2% (65 ± 2 $g.l^{-1}$), respectively.

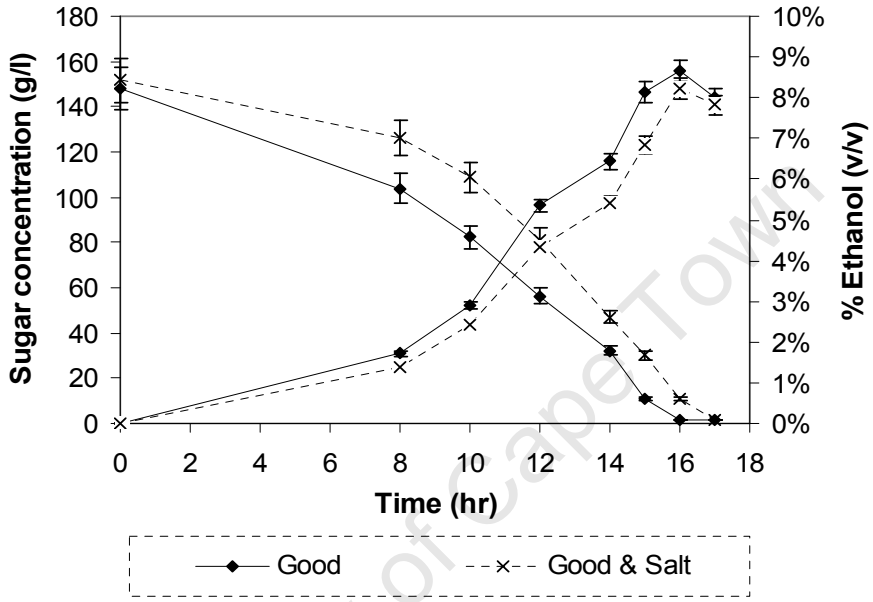

Figure 6.6 Sugar and ethanol concentration in "good" molasses and "good" molasses with salt addition

Table 6.10 Summary of sugar, ethanol concentration, maximum sugar utilisation and ethanol production rate in "good" molasses and "good" molasses with salt added

Molasses	$C_{s,i}$ ($g.l^{-1}$)	φ_{max} ($g.l.hr^{-1}$)	ω_{max} ($g.l.hr^{-1}$)	E_{max} ($g.l^{-1}$)	E_{final} ($g.l^{-1}$)
Good	148	13 ± 1	7.1 ± 0.2	68 ± 0.2	63 ± 0.2
Good & salt	152	14 ± 1	6.0 ± 0.2	65 ± 0.2	62 ± 0.2

The fermentation efficiencies are summarised in Table 6.11 and show reduced fermentation performance in the salt augmented molasses mash. For instance F.ε in the "good" molasses mash was 0.90 ± 0.3, while that in the salt augmented mash was

0.84 ± 0.3. Similarly, the yield of ethanol from sugar decreased with increasing salt concentration.

Table 6.11 Fermentation efficiencies of "good" molasses and "good" molasses with salt added

Molasses	F. ε	M.U.ε (kg/ l ethanol)	S.U.ε (kg/ l ethanol)	$Y_{E,s}$
"Good"	0.90 ± 0.3	3.4 ± 0.1	1.7 ± 0.1	0.46
"Good" & salt	0.84 ± 0.3	3.5 ± 0.1	1.8 ± 0.1	0.43

The results confirm the negative impact of increases in the concentration of K^+ and Na^+ salts on both cell growth and fermentation performance. They also highlight the importance of selecting, for fermentation, molasses of low inorganic ash.

6.5 EFFECT OF INITIAL SUGAR CONCENTRATION ON ETHANOL FERMENTATION OF MOLASSES MASH

Since molasses is derived from different sugar mills, its sugar content is expected to vary. It is therefore envisaged that, during dilution, molasses mash of differing initial sugar concentration can be produced if a constant ratio of molasses to water is used. In this section, the effect of initial sugar concentration on yeast growth and fermentation performance was investigated. Molasses mash with initial sugar concentrations of ~120 $g.l^{-1}$, 170 $g.l^{-1}$ and 210 $g.l^{-1}$ was prepared by using molasses to water dilution ratios of 1:3, 1:2.3 and 1:1.7 (m/m), respectively. The fermentations were performed in 500 ml shake flasks.

6.5.1 Specific Growth Rate and Cell Viability

The cell concentration and cell viability as a function of time are represented in Figure 6.7. An inoculum concentration of 10^5 $cells.ml^{-1}$ was used. A reduction in the rate of cell growth and the final cell concentration was observed with increasing initial sugar

concentration. For instance, after 15 hours the yeast concentration in molasses mash containing 120 $g.l^{-1}$ sugar was 8.7 x 10^7 $cells.ml^{-1}$ compared with 1.9 x 10^7 $cells.ml^{-1}$ in molasses mash containing 210 $g.l^{-1}$ sugar, representing a 4.6 fold reduction in the cell concentration. The specific growth rate in molasses mash containing 120 $g.l^{-1}$ sugar was 0.45 hr^{-1}, while that in molasses mash containing 170 and 210 $g.l^{-1}$ sugar was 0.40 and 0.37 hr^{-1}, respectively. The reduced growth was attributed increased osmotic stress from the high sugar concentration (Tamás and Hohmann, 2003) and increased salt toxicity that results from low dilution ratio. In studies of yeast chemostat cultures at steady state, Zhou and Lin (2003) reported a decline in yeast biomass concentrations as the glucose concentrations ranged from 100 to 300 $g.l^{-1}$. They too attributed the decline to increased osmotic stress contributed by the high glucose concentrations.

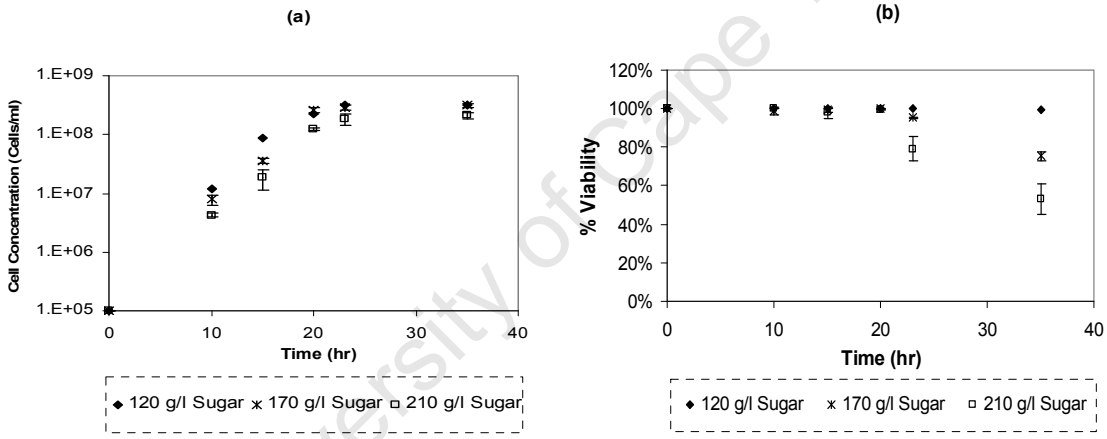

Figure 6.7 Cell concentration (a) and cell viability (b) in molasses mash of varying initial sugar concentration

Cell viability remained close to 100% at all initial sugar concentrations in the first 20 hours of fermentation. Thereafter yeast viability at the higher sugar concentrations began to decrease. In molasses mash with an initial sugar concentration of 170 $g.l^{-1}$, cell viability was 95% after 23 hours, before decreasing to 75 ± 2% after 35 hours. In molasses mash with an initial sugar concentration of 210 $g.l^{-1}$ cell viability was 79 ± 6% after 23 hours, before decreasing to 53 ± 8 % after 35 hours of fermentation. These results are expected and supported by Beney *et al.* (2001), Laroche *et al.* (2001) and

Myers *et al.* (1997), who all showed that growth and viability of S. *cerevisiae* was negatively affected by high osmotic pressure.

6.5.2 Sugar and Ethanol Concentration

The sugar concentration as a function of time is shown in Figure 6.8. A reduction in sugar utilisation was observed with increasing initial sugar concentration. The average sugar utilisation rate in the first 20 hours after inoculation decreased as the initial sugar concentration was increased. The sugar utilisation rate was 3.2, 2.8 ± 0.3 and 1.6 ± 0.2 $g.l^{-1}.hr^{-1}$ at 120, 170 and 210 $g.l^{-1}$ initial sugar concentrations, respectively.

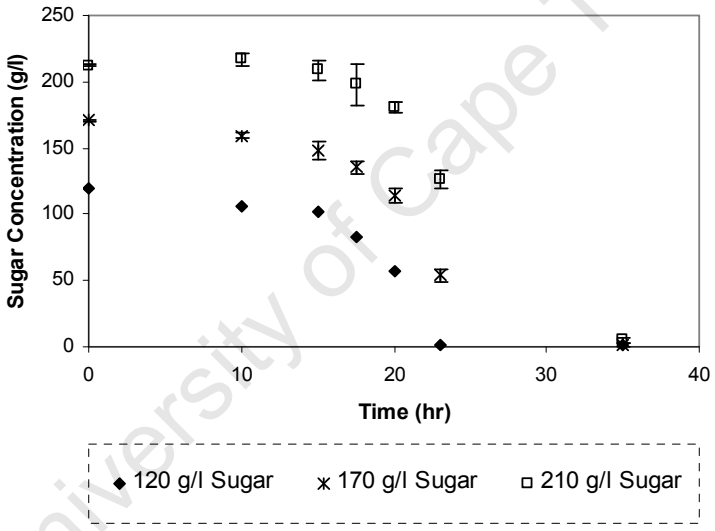

Figure 6.8 Sugar concentration in molasses mash fermentations of varying initial sugar concentration

The maximum sugar utilisation rates are provided in Table 6.12. The maximum sugar utilisation rate decreased from 12.4 to 10.1 ± 0.4 $g.l^{-1}.hr^{-1}$ as the initial sugar concentration was increased from 120 to 210 $g.l^{-1}$, respectively. The reduction in the sugar utilisation rates was related to reduced yeast growth which resulted in less biomass available to catalyse fermentation.

Ethanol production also decreased as the initial sugar concentration was increased. The ethanol concentration as a function of time is shown in Figure 6.9. The average ethanol production rate in the first 20 hours of fermentation was 1.6, 1.1 and 0.8 $g.l^{-1}.hr^{-1}$ at initial sugar concentrations of 120, 170 and 210 $g.l^{-1}$, respectively. The maximum ethanol production rates are also provided in Table 6.12 and follow a similar trend. The reduced ethanol production rate was a consequence of reduced sugar utilisation. The final ethanol concentration (after 35 hours of fermentation) was 6.9%, 9.3 ± 0.3% and 10.2% in molasses mash with an initial sugar concentration of 120, 170 and 210 $g.l^{-1}$, respectively.

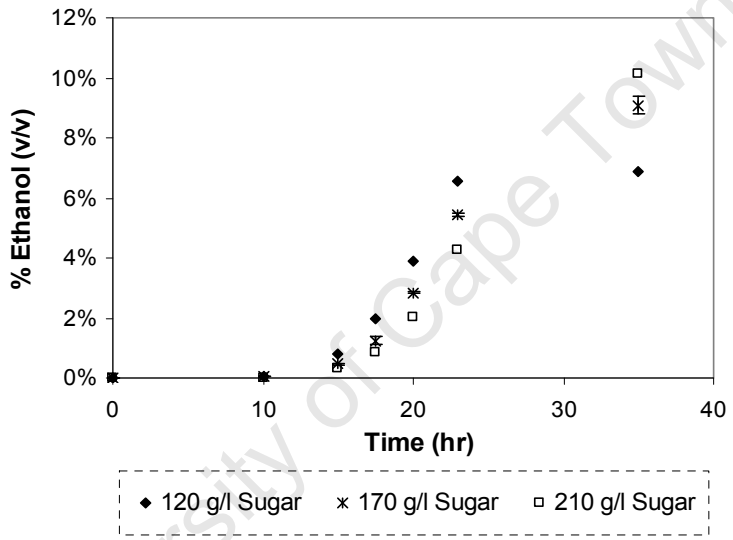

Figure 6.9 Ethanol concentration in molasses mash fermentations of varying initial sugar concentration

Table 6.12 Summary of sugar, ethanol concentration, maximum sugar utilisation and ethanol production rate in mash of different initial sugar concentration

$C_{s,i}$ ($g.l^{-1}$)	φ_{max} ($g.l.hr^{-1}$)	ω_{max} ($g.l.hr^{-1}$)	E_{final} ($g.l^{-1}$)
120	12.4	5.8	54
170 ± 1	11.6 ± 0.6	5.0 ± 0.1	72 ± 2
210 ± 1	10.1 ± 0.4	3.9 ± 0.0	80

Figure 6.10 shows fermentation efficiency as a function of initial sugar concentration. A reduction in fermentation efficiency was observed as the initial sugar concentration was

increased from 120 to 210 $g.l^{-1}$. The fermentation efficiency reduced from 0.88 at initial sugar concentration of 120 $g.l^{-1}$ through 0.82 ± 0.2 at 170 $g.l^{-1}$ to 0.74 at 210 $g.l^{-1}$.

The effect of high sugar concentrations on ethanol yields has been confirmed by previous researchers. Zhou and Lin (2003) showed a reduction in ethanol coefficient yield from 0.39 $g.g^{-1}$ at 10 $g.l^{-1}$ glucose to 0.24 $g.g^{-1}$ at 100 $g.l^{-1}$ glucose. Jones *et al*. (1994) showed that the ethanol yields in molasses mash with a fermentable sugar content 59 $g.l^{-1}$ were 27 % greater than those of molasses mash with a fermentable sugar concentration of 270 $g.l^{-1}$. In both work osmotic stress was identified as a contributor to suboptimal fermentations.

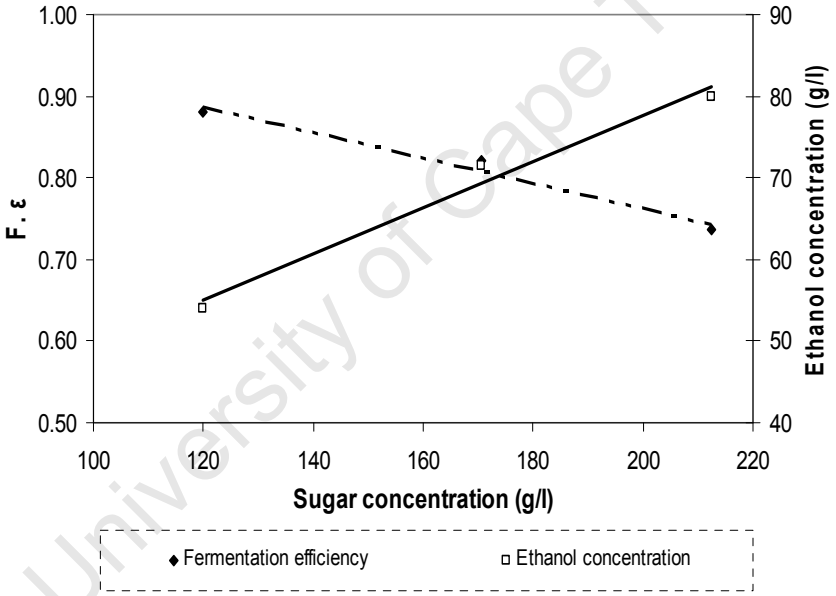

Figure 6.10 Effect of initial sugar concentration on fermentation efficiency and final ethanol concentration

6.6 SUMMARY OF RESULTS

In this chapter yeast and fermentation performance in "good" and "bad" molasses was compared to confirm its classification as such. Yeast performance was assessed in terms of specific growth rate and viability. Fermentation performance was assessed in terms of sugar utilisation rate, ethanol production rate, fermentation efficiency and sugar and

molasses usage efficiency. Where fermentation differences were exposed, attempts were made to account for them by considering available fermentable sugar concentration, the molasses cation concentration and the ionic strength of the fermentation media. Also, the possible role of media preparation in bad fermentations was investigated by fermenting the same molasses at different initial sugar concentrations.

The classification of molasses as "good" or "bad" appeared valid from a fermentation efficiency perspective. So, while some molasses had a high fermentable sugar content and produced more ethanol during fermentation, it was regarded as "bad" molasses due to reduced fermentation efficiency. This was the case in molasses Batch # 23222008. Fermentation efficiency for this molasses was 0.75 ± 0.01, while that for "good" molasses was 0.82 ± 0.03. The initial sugar concentration in the "bad" molasses mash was 142 ± 2 $g.l^{-1}$, while that in the "good" molasses was 125 ± 2 $g.l^{-1}$. The final ethanol concentration in the "bad" molasses fermentation was 6.9 ± 0.1%, while that in the "good" molasses was 6.6 ± 0.4%. The "bad" molasses had a K^+ concentration 10% greater than the "bad" molasses, resulting in increased salt, osmotic, and ionic stress. The ionic strength in the "bad" molasses mash was 19.3 mS while that in the "good" molasses mash was (18.6mS). The increased K^+ cation concentration which resulted in increased ionic strength was responsible for the reduced fermentation performance.

Reduced fermentation efficiency was also observed in the "bad" molasses mash (Batch # A13032007) relative to "good" molasses (Batch # 212302007). Fermentation efficiency for the "good" molasses was 0.92 ± 0.03 while, that in the "bad" molasses was 0.85 ± 0.03. The initial sugar concentration in the "good" and "bad" molasses mashes was 160 $g.l^{-1}$ and 159 $g.l^{-1}$, respectively. The most significant difference in the two molasses was the higher Na^+ concentration in the "bad" molasses. The Na^+ concentration in the "good" molasses was 0.09%, while that in the "bad" molasses was 0.14%. This represents a 60% increase. The ionic strength in this good and bad molasses was similar at 20.3 and 20.4 mS, respectively. The categorisation of the bad molasses as such was attributed to increased salt stress as a resulted of elevated Na^+ concentrations.

In verifying that reduced fermentation was indeed a consequence of salt stress, the K^+ and Na^+ concentrations of a "good" molasses were increased by 20% and fermented. A 1 to 2 hour lag in sugar and ethanol concentration was observed in the molasses mash with increased salt relative to the control. Sugar depletion occurred after 16 hours in the control, while that in the molasses mash with increased salt concentration occurred after 17 hours. A final ethanol concentration of 8.7 ± 0.3%, representing a fermentation efficiency of 0.90 ± 0.03, was recorded for the control. In the molasses mash with increased salts, the final ethanol concentration was 8.2 ± 0.2%, representing a fermentation efficiency of 0.84 ± 0.03.

The dilution ratio of molasses: water affected yeast and fermentation performance. A reduction in performance was observed as initial sugar concentration in molasses mash was increased from 120 to 170 to 210 $g.l^{-1}$. The specific growth rate decreased from 0.45 to 0.40 to 0.37 hr^{-1} over the same range. Cell viability after 23 hours of fermentation was 100%, 95% and 79 ± 6%, respectively. Fermentation efficiency was reduced from 0.88 to 0.82 ± 0.2 to 0.74. The reduction in yeast growth was attributed to a combination of increased osmotic, salt and ionic strength.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 INTRODUCTION

The thesis sought to investigate the effect of varying molasses quality on yeast growth and fermentation performance with the objective of identifying responsible factors. This knowledge would then be available to manage differences in efficiencies of the industrial fermentation process. Specifically the inorganic ash content of molasses and its dilution were investigated. Variation in the inorganic ash content of molasses affects the cation concentration, osmotic pressure and ionic strength of the media. In the investigation special emphasis was placed on the effect of K^+ ions owing to their abundance in molasses. The effect on fermentation of the use of different dilution ratios in producing molasses mash, thereby affecting both sugar concentration and concentration of other dissolved solids, was also investigated. In this chapter conclusions of the investigations are presented and recommendations for improved yeast growth and fermentation performance discussed.

7.2 CONCLUSSIONS

Variation in the inorganic ash component of fermentation media, with respect to K^+, Mg^{2+} and Na^+ cation concentration resulted in changes in media cation concentration, ionic strength and osmotic pressure. The effect of the variation on yeast growth and fermentation performance was found to be strongly dependent on the cation species. The

effect of the cations K^+, Mg^{2+} and Na^+ was therefore found to be more strongly influenced by cation inhibition or toxicity rather than osmotic pressure or ionic strength. In molasses fermentations at cation concentration of 15 $g.l^{-1}$, the specific growth rates relative to the control $\mu/\mu_{control}$ were 0.77, 0.99 and 0.98 for Na^+, K^+ and Mg^{2+} respectively, while the relative rates of ethanol production $\omega/\omega_{control}$ were 0.31, 0.77, 0.98, respectively. At an ionic strength of 25 mS $\mu/\mu_{control}$ was 0.85, 1.0 and 0.99, while $\omega/\omega_{control}$ was 0.79, 0.90 and 0.99, for Na^+, K^+ and Mg^{2+}, respectively. At osmotic pressure 4.0 MPa $\mu/\mu_{control}$ was 0.88, 0.95 and 0.98, while $\omega/\omega_{control}$ was 0.54, 0.70 and 0.95, respectively. In sucrose-based media fermentations at a cation concentration of 15 $g.l^{-1}$, $\mu/\mu_{control}$ was 0.02, 0.39 and 1.0 for Na^+, K^+ and Mg^{2+} respectively, while $\omega/\omega_{control}$ was 0.00, 0.03 and 0.82, respectively These results demonstrated that the negative impact was not a direct function of ionic strength or osmotic pressure, but ion specific.

The magnitude of the inhibiting effects of the cations studied presented in the order: $Na^+ > K^+ > Mg^{2+}$. Na^+ toxicity in yeast is well documented (Gómez *et al*., 1996, Murguía *et al*., 1996; Wadskog and Alder, 2003), while intermediate toxicity has been observed for K^+ (Ryan and Johnson, 2001). Toxicity or inhibition of yeast by Mg^{2+} is not reported in the literature. Conversely, a positive role in fermentation is documented (Dombek and Ingram, 1986; Walker *et al*., 1996; Walker, 1998).

Comparing fermentations of sucrose-based media and molasses mash, cation toxicity was found to be more pronounced in the former. It is hypothesised that the presence of chelating agents in molasses reduced the bioavailability of the cations, thereby limiting their inhibitory effect. This hypothesis was supported by the experiments in which supplementation of sucrose-based media containing 15 $g.l^{-1}$ K^+ concentration with 20% (v/v) molasses mash of equal K^+ concentration significantly improved yeast and fermentation performance relative to that in a sucrose-based medium in the absence of molasses. A 68% increase in the specific growth rate was recorded, while the ethanol production rate increased from 0.1 to 2.1 $g.l^{-1}.hr^{-1}$. The increase in yeast growth and ethanol production corresponded to increases in the ionic strength and osmotic pressure of the media, illustrating also that ionic strength and osmotic pressure play a lesser role in

the ultimate determination of yeast growth and fermentation performance. Yeast growth and fermentation performance peaked in media whose molasses mash composition was between 60 and 80%, before a slight decline in performance was observed in 100% molasses mash.

The effect of increased cation toxicity was also observed in comparison of fermentation performance with "good" and "bad molasses". Here losses in ethanol production in "bad" molasses (relative to "good" molasses) of 8.2 to 8.5% were recorded. The loss in ethanol production was related to increased K^+ and Na^+ concentrations in two molasses batches of lower quality. In molasses batch # 232022008 losses were attributed to a high K^+ concentration of 3.3% (m/m), 10% higher than the control molasses. Loss in ethanol production in molasses batch # A130322007 was attributed to its relatively high Na^+ concentration of 0.14% (m/m), 60% higher than in the control molasses. Increase of the K^+ and Na^+ concentration of good molasses by 20% to 3.6% (m/m) and 0.11% respectively, resulted in reduced fermentation performance, confirming the negative role played by these cations.

While K^+ is most abundant cation in molasses, the Na^+ concentration demonstrates a greater inhibitory effect, contributing to yeast stress and subsequent losses in ethanol production at lower concentrations. Hence the role of both cations must be considered. In investigation of "good" and "bad" molasses, an increase of 3000 ppm (representing 10% of original concentration) in K^+ concentration had a similar effect on the specific growth rate and loss in ethanol production of the yeast as an increase of 500 ppm (representing 60% of original concentration) in Na^+ concentration. In both cases, μ was reduced from 0.11 to 0.10 hr^{-1}. Ethanol production losses of 8.5 and 8.2 % were observed, respectively. In molasses fermentations at cation concentrations of 15 $g.l^{-1}$ Na^+, K^+ and Mg^{2+} $\mu/\mu_{control}$ was 0.77 0.99, 0.98, respectively. The values of $\omega/\omega_{control}$ were 0.31, 0.77 and 0.98, respectively. The above results show that while high K^+ concentrations result in yeast stress and subsequent losses in fermentation performance, lower concentrations of Na^+ can have an equal or larger effect due to their greater toxicity.

Yeast growth and ethanol production were related to the dilution ratio of molasses, which determines the initial sugar and inorganic ash concentrations in molasses mash. By varying the dilution ratio of the molasses stock from 1:3.5 to 1:1.7, a range of sugar concentrations from 120 to 210 $g.l^{-1}$ were achieved, with proportional variation in the ash content. Reduced specific growth rate and cell viability were observed with increasing molasses mash concentration across this range. The specific growth rate μ, decreased from 0.45 through 0.40 to 0.37 hr^{-1} with initial sugar concentrations 120, 170 and 210 $g.l^{-1}$, respectively. Cell viability at 35 hours was 100%, 75 ± 2% and 53 ± 8%, respectively. While higher ethanol concentrations were produced at high sugar concentrations, the ethanol productivity and fermentation efficiency (F.ε) were lower. F.ε reduced from 0.88 at 120 $g.l^{-1}$ through 0.82 ± 0.02 at 170 $g.l^{-1}$ to 0.74 at 210 $g.l^{-1}$. The decrease in yeast growth and fermentation efficiency was attributed to both increased osmotic pressure (due to sugar concentration and other dissolved solids) and cation toxicity (due mainly to elevated K^+ and Na^+ concentration). High osmotic pressures are known to reduce cell growth and viability (Beney *et al*., 2001, Laroche *et al*., 2001; Myers *et a*l., 1997) by inducing loss of cytoskeleton polarisation, which is essential for yeast budding (Tamás and Hohmann, 2003). The yeast respond to the high osmotic pressure by diverting more reducing sugars towards production of the osmoregulator glycerol and stress compounds such as trehalose and glycogen. This shift in metabolic flux reduces the amount of reducing sugar available for ethanol production, resulting in reduced fermentation efficiency.

7.3 RECOMMENDATIONS

The findings of the study allow specific recommendations to be made, with the objective to improve molasses fermentation efficiency. These are detailed below:

1. The use of molasses of relatively low K^+ and especially Na^+ concentration is recommended for reduction of cation toxicity, identified as a main contributor to sub-optimal fermentations. Where selection and blending of molasses is not possible, increased dilution to reduce their concentrations in the molasses mash is expected to

result in improved fermentation efficiency. However, this approach requires optimisation as it would result in increased plant hydraulic loading and separation costs. Alternatively, the use of chelating agents such as EDTA, as demonstrated by Ergun *et al*., (1997) and Oderinde *et al*. (1986) can reduce the bio-availability of excess cations and thus reduce their toxic effects. Identification of the naturally occurring chelating agents present in molasses may aid this approach.

2. The use of fed-batch operation or incremental feeding could also reduce the negative effect of cation toxicity. With batch operation, molasses mash and yeast cream are added into the fermenter simultaneously, exposing the yeast to high sugar and salt concentrations. However, fed-batch operation provides the opportunity to initiate fermentations at low concentrations, which is the preferred fermentation environment for yeast. Under these conditions, yeast quality can be maintained over an extended portion of the fermentation and will only reach inhibitory levels in the latter stages of fermentation at which stage yeast growth is complete. In Section 6.5, an increased yeast specific growth rate, sugar utilisation rate and ethanol production rate were demonstrated at low sugar and salt concentrations.

3. Nutritional supplementation of fermentation media has been shown to increase fermentation performance in VHG systems. These are proposed as a possible avenue for improving fermentation performance in "bad" molasses. Alfenore *et al*. (2002) were able to increase yeast specific growth rate in 100 $g.l^{-1}$ glucose fermentation using a vitamin mixture containing biotin, while Barber *et al*. (2002) showed that acetaldehyde addition to VHG improved fermentation performance. Banfrncová *et al*. (1999) were able to enhance fermentation performance using urea.

4. The recycling of yeast in successive fermentations also provides an opportunity to optimise the fermentation process. While most processes in batch industrial ethanol production do not recycle yeast, yeast recycling in the beer brewing industry is popular resulting in significant savings in the cost of yeast. In some Brazilian batch processes, yeast is separated from the medium by centrifugation, washed with dilute

sulphuric acid to reduce bacterial contamination and reused in subsequent fermentations. This practice allows the use of very high cell densities (8 to 17% v/v) to enhance ethanol productivity, resulting in the production of high ethanol concentrations (8 to 11%) at high ethanol yields (90 to 92%) in very short fermentation times (6 to 10 hours) (Wheals *et al*., 1999).

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APPENDIX A

A.1 ASSAYS AND CALIBRATION CURVES

A.1.1 Sugar Concentration Using DNS

Preparation Method

- Place 2 g of sucrose in 2 l volumetric flask
- Fill volumetric flask with distilled water to the 2 litre mark.
- Stir solution using a magnetic bar until sucrose is completely dissolved.
- From volumetric flask take 3000 µl, 1500 µl, 750 µl and 0 µl samples and place in 4 different 10 ml test tubes.
- Fill each test tube to 3 ml using distilled water.
- Add 50 µl of 32% HCl to each test tube.
- Lightly cap each test tube and place in 90 ^{o}C water-bath for 5 minutes.
- After heating add 3 ml of DNS reagent (Table A1).
- Recap the test tubes and place in 90^{o}C water bath for 10 minutes.
- After heating add 1 ml of 40% (w/v) sodium tartrate solution.
- Cool to room temperature.
- Measure U.V absorbance at 575 nm using a spectrophotometer.
- Develop calibration curve by plotting the absorbance against corresponding glucose concentration.

Table A.1 Composition of medium DNS reagent

Component	Concentration (g.l^{-1})
Dinitrosalicylic acid	10
Phenol	2
Sodium sulphite	0.5
Sodium hydroxide	10

An example of a calibration curve obtained using linear regression is shown in Figure A1. The curve can be used to calculate the unknown sugar concentration in a sample. The reproducibility of the assay at 160 $g.l^{-1}$ is shown in Table A.2.

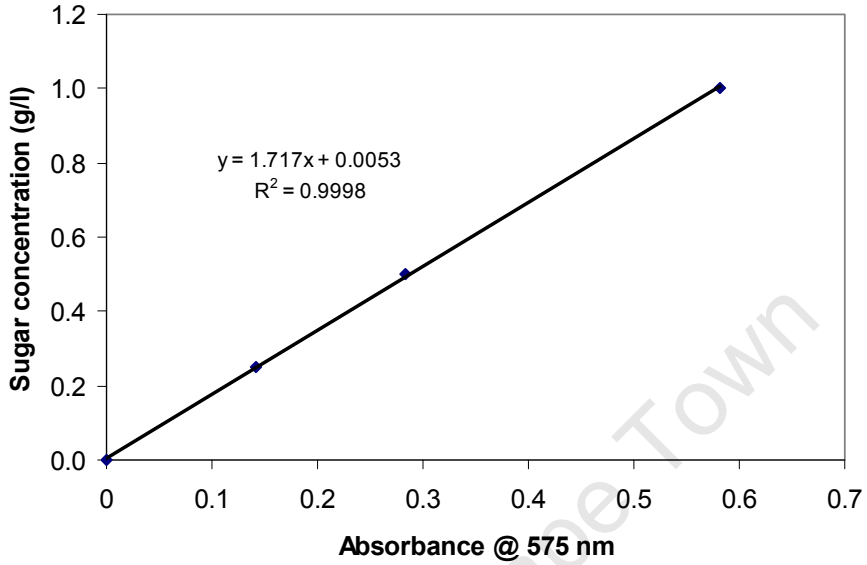

Figure A.1 Calibration curve for determining sugar concentration using DNS at wavelength of 575 nm

Table A.2 Reproducibility of DNS sugar concentration assay

Sample	Absorbance	Dilution	TSAI (g/l)
1	0.769	119	157.8
2	0.75	119	153.9
3	0.793	119	162.7
4	0.761	119	156.1
5	0.783	119	160.6
Average			**158.2**
STNDV			**3.5**
Coefficient of variance			**2.2%**

A.1.2 Sugar Concentration Using HPLC

Preparation Method

- Prepare a 150 $g.l^{-1}$ (m/v) sugar solution in a 1 l volumetric flask. The sugar solution should consist of 120 $g.l^{-1}$ sucrose, 15 $g.l^{-1}$ glucose and 15 $g.l^{-1}$ fructose.
- Prepare 100 ml solutions with sugar concentrations of 150 $g.l^{-1}$, 37.5 $g.l^{-1}$, 18.75 $g.l^{-1}$ and 0 $g.l^{-1}$ using the 150 $g.l^{-1}$ sugar solution as shown in Table A4.

Table A.3 Standards preparation for total sugar analysis using HPLC

	Sugar solution (150 $g.l^{-1}$) (ml)	**Distilled water (ml)**
Sugar solution (150 $g.l^{-1}$)	100	-
Sugar solution (37.5 $g.l^{-1}$)	25	75
Sugar solution (18.75 $g.l^{-1}$)	12.5	87.5
Sugar solution (0 $g.l^{-1}$)	-	105

- Mix 500ul of each standard solution with 500 µl of 10 mM H_2SO_4 solution and place in separate HPLC vials.
- Place the vials in the HPLC machine and start HPLC runs at a mobile phase flow rate of 0.6 $ml.min^{-1}$.
- Using results obtained form HPLC runs develop standard curve by plotting the total sugar peak area against the sugar concentration as shown in Figure A2.

The reproducibility of the assay at sugar concentration of 130 $g.l^{-1}$ is shown in Table A4.

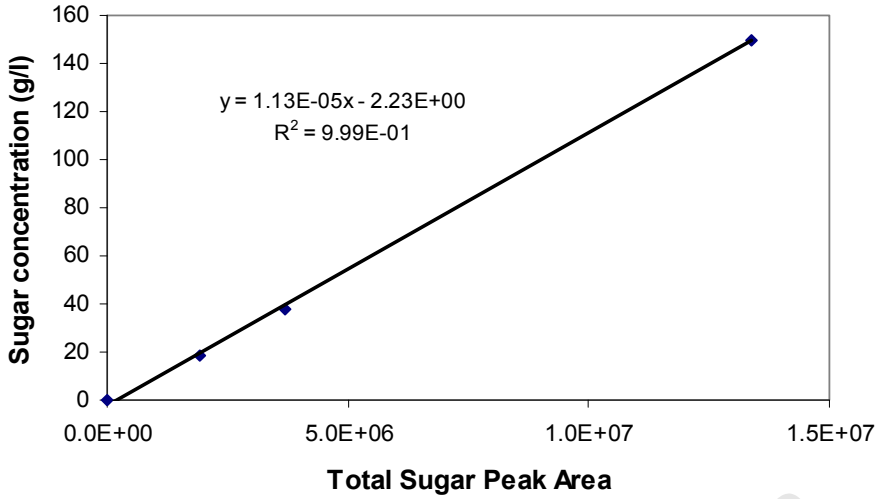

Figure A.2 Calibration curve for determining sugar concentration using HPLC

Table A.4 Reproducibility of HPLC sugar concentration assay

Sample	Total Sugar Peak Area	TSAI (g/l)
1	11701701	130
2	11791265	131
3	11790355	131
4	11891769	132
Average		**131**
STNDV		**1**
Coefficient of variance		**0.6%**

A.1.3 Ethanol Concentration Using GC

Preparation Method

- Prepare 0%, 2%, 6% and 10% (v/v) ethanol solutions using distilled water.
- Place 300 µl of each into 1.5 ml eppendorf tubes, then mix with 300 µl of 1, 4 dioxane (internal standard).
- Vortex mixture to ensure it is homogeneous.
- Set up GC method such that the column temperature is held at 40^{o}C for 4 minutes, then steadily increased to 220oC at a rate of 15^{o}C per minute.

- From each eppendorf inject 1 µl into the GC and run the method.
- Develop standard curve from resulting chromatograms by plotting the ratios of the ethanol peak areas to 1,4 dioxane peak area against ethanol concentration.

Figure A3 represents a standard curve used to determine ethanol concentrations in samples.

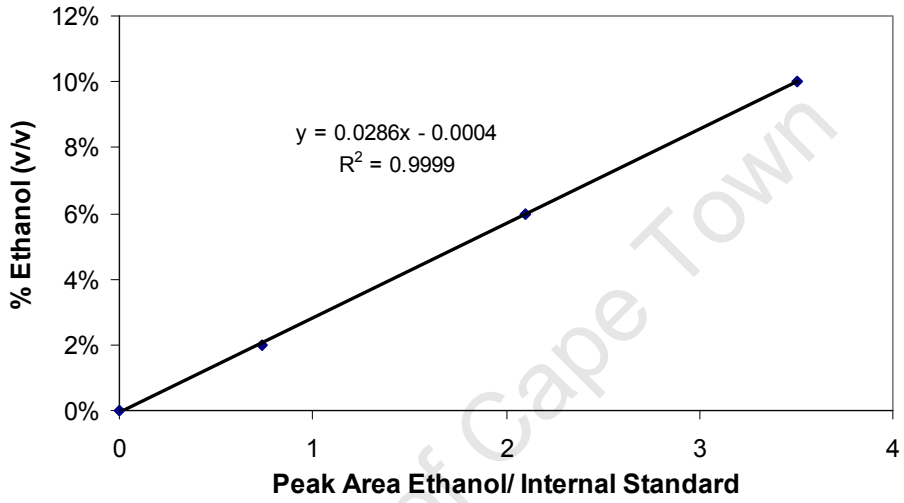

Figure A.3 Calibration curve relating ethanol concentration to the peak area ratio of ethanol to 1, 4 dioxane

Table A5 shows the method error at an ethanol concentration of 5%.

Table A.5 Reproducibility of ethanol concentration using GC

Sample	Ethanol/Internal Standard	% Ethanol
1	1.88	5.0%
2	1.84	4.9%
3	1.92	5.1%
4	1.90	5.0%
5	1.82	4.8%
Average		**5.0%**
STNDV		**0.1%**
Coefficient of variance		**2.4%**

A.1.4 Oxygen Utilisation Rate

Preparation Method

- First calibrate oxygen probe by placing it in a continuously stirred saturated sodium sulphite solution for 10 minutes.
- Zero OUR meter reading by adjusting right side knob to zero.
- After thoroughly rinsing probe, place it in 30^{o}C air-saturated water for 5 minutes and then adjust left side knob to 7.53 mg and allow to equilibrate.
- Switch on computer and type in c:\dolog and then type in a file name. Do not press enter as this will initiate program
- Place yeast sample of determined concentration in flask containing air saturated YPD media at 30 ^{o}C such that it constitutes 10% (v/v).
- Insert probe in the mixture and press enter to initiate readings.

APPENDIX B

B.1 REPRODUCIBILITY OF DATA

B.1.1 Sucrose Based-Media

Table B.1 Cell concentration and cell viability reproducibility data in sucrose based media

	Cell concentration (cells.ml^{-1})			*Cell viability (%)*		
Time	**Exp 1**	**Exp 2**	**Exp 3**	**Exp 1**	**Exp 2**	**Exp3**
0	1.00E+05	1.00E+05	1.00E+05	100%	100%	100%
10	1.65E+07	1.30E+07	1.65E+07	97%	96%	97%
15	1.20E+08	7.40E+07	9.40E+07	98%	99%	97%
20	2.00E+08	1.75E+08	1.73E+08	96%	100%	100%
23	1.70E+08	2.03E+08	1.85E+08	100%	96%	96%
35	1.98E+08	1.78E+08	2.00E+08	100%	100%	98%

Yeast cell concentration

	Run 1	*Run 2*
Mean	117350000	107016666.7
Variance	7.99048E+15	8.01342E+15
Observations	6	6
Hypothesized Mean Difference	0	
df	10	
t Stat	0.200079777	
P(T<=t) two-tail	0.845428456	
t Critical two-tail	2.228138842	

	Run 1	*Run 3*
Mean	117350001.5	111350000
Variance	7.99048E+15	7.73932E+15
Observations	6	6
Hypothesized Mean Difference	0	
df	10	
t Stat	0.117183245	
P(T<=t) two-tail	0.909035003	
t Critical two-tail	2.228138842	

	Run 2	*Run 3*
Mean	107016666.7	111350000
Variance	8.01342E+15	7.73932E+15
Observations	6	6
Hypothesized Mean Difference	0	
df	10	
t Stat	-0.084570659	
P(T<=t) two-tail	0.934271873	
t Critical two-tail	2.228138842	

Figure B.1 Cell concentration reproducibility in sucrose-based media using paired t-test assuming equal variances

Yeast cell viability

	Run 1	*Run 2*
Mean	0.985921717	0.985164652
Variance	0.000282623	0.000342624
Observations	6	6
Hypothesized Mean Difference	0	
df	10	
t Stat	0.0741623	
P(T<=t) two-tail	0.942343788	
t Critical two-tail	2.228138842	

	Run 1	*Run 3*
Mean	0.985921717	0.985164652
Variance	0.000282623	0.000342624
Observations	6	6
Hypothesized Mean Difference	0	
df	10	
t Stat	0.0741623	
P(T<=t) two-tail	0.942343788	
t Critical two-tail	2.228138842	

	Run 2	*Run 3*
Mean	0.985164652	0.978706923
Variance	0.000342624	0.000297
Observations	6	6
Hypothesized Mean Difference	0	
df	10	
t Stat	0.625450659	
P(T<=t) two-tail	0.545683361	
t Critical two-tail	2.228138842	

Figure B.2 Cell viability reproducibility in sucrose-based media using paired t-test assuming equal variances

Table B.2 Sugar and ethanol reproducibility data in sucrose based media

	Sugar concentration ($g.l^{-1}$)			*Ethanol concentration (%)*		
Time	**Exp 1**	**Exp 2**	**Exp 3**	**Exp 1**	**Exp 2**	**Exp3**
0	153	155	149	0.0%	0.0%	0.0%
10	163	162	154	0.1%	0.2%	0.2%
15	110	124	117	1.7%	1.9%	1.7%
20	90	88	87	3.3%	3.4%	3.2%
23	18	17	14	6.3%	6.6%	6.8%
35	6	3	3	7.8%	7.6%	7.7%

Sugar concentration

	Variable 1	*Variable 2*
Mean	77.6988	78.94915714
Variance	4720.416225	5038.724704
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	-0.033487082	
P(T<=t) two-tail	0.97383673	
t Critical two-tail	2.178812827	

	Variable 1	*Variable 2*
Mean	77.6988	75.2481
Variance	4720.416225	4626.159887
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	0.06706762	
P(T<=t) two-tail	0.947632362	
t Critical two-tail	2.178812827	

	Variable 1	*Variable 2*
Mean	78.94915714	75.2481
Variance	5038.724704	4626.159887
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	0.09960393	
P(T<=t) two-tail	0.922303416	
t Critical two-tail	2.178812827	

Figure B.3 Sugar concentration reproducibility in sucrose-based media using paired t-test assuming equal variances

Ethanol concentration

	Run 1	*Run 2*
Mean	0.038661194	0.03910788
Variance	0.001191946	0.001159387
Observations	7	7
Pooled Variance	0.001175667	
Hypothesized Mean Difference	0	
df	12	
t Stat	-0.024372198	
P(T<=t) two-tail	0.980956324	
t Critical two-tail	2.178812827	

	Run 1	*Run 3*
Mean	0.038661194	0.038983772
Variance	0.001191946	0.00118506
Observations	7	7
Pooled Variance	0.001188503	
Hypothesized Mean Difference	0	
df	12	
t Stat	-0.017505277	
P(T<=t) two-tail	0.986321213	
t Critical two-tail	2.178812827	

	Run 2	*Run 3*
Mean	0.03910788	0.038983772
Variance	0.001159387	0.00118506
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	0.006781555	
P(T<=t) two-tail	0.99470058	
t Critical two-tail	2.178812827	

Figure B.4 Ethanol concentration reproducibility in sucrose-based media using paired t-test assuming equal variances

B.1.2 Molasses Based-Media

Table B.3 Cell concentration and cell viability reproducibility data in molasses-based media

	Cell concentration (cells.ml^{-1})			*Cell viability (%)*		
Time	**Exp 1**	**Exp 2**	**Exp 3**	**Exp 1**	**Exp 2**	**Exp3**
0	1.00E+05	1.00E+05	1.00E+05	100%	100%	100%
10	1.40E+07	1.20E+07	1.12E+07	97%	98%	100%
15	7.10E+07	7.30E+07	8.10E+07	96%	98%	97%
20	1.95E+08	2.25E+08	2.08E+08	97%	98%	100%
25	3.23E+08	4.00E+08	3.38E+08	100%	100%	98%
35	2.95E+08	3.63E+08	3.45E+08	99%	98%	97%

Cell concentration

	Run 1	*Run 2*
Mean	149600000	178766666.7
Variance	2.00025E+16	3.11518E+16
Observations	6	6
Hypothesized Mean Difference	0	
df	10	
t Stat	-0.315879475	
P(T<=t) one-tail	0.379294081	
t Critical two-tail	2.228138842	

	Run 1	*Run 3*
Mean	149600000	163716666.7
Variance	2.00025E+16	2.4371E+16
Observations	6	6
Hypothesized Mean Difference	0	
df	10	
t Stat	-0.164151959	
P(T<=t) one-tail	0.436440983	
t Critical two-tail	2.228138842	

	Run 2	*Run 3*
Mean	178766666.7	163716666.7
Variance	3.11518E+16	2.4371E+16
Observations	6	6
Hypothesized Mean Difference	0	
df	10	
t Stat	0.156450259	
P(T<=t) two-tail	0.878791538	
t Critical two-tail	2.228138842	

Figure B.5 Cell concentration reproducibility in molasses-based media using paired t-test assuming equal variances

Yeast cell viability

	Run 1	*Run 2*
Mean	0.981666667	0.986666667
Variance	0.000296667	0.000106667
Observations	6	6
Hypothesized Mean Difference	0	
df	10	
t Stat	-0.609836721	
P(T<=t) two-tail	0.555573075	
t Critical two-tail	2.228138842	

	Run 1	*Run 3*
Mean	0.981666667	0.986666667
Variance	0.000296667	0.000226667
Observations	6	6
Hypothesized Mean Difference	0	
df	10	
t Stat	-0.535372958	
P(T<=t) two-tail	0.604093227	
t Critical two-tail	2.228138842	

t-Test: Two-Sample Assuming Equal Variances

	Run 2	*Run 3*
Mean	0.986666667	0.986666667
Variance	0.000106667	0.000226667
Observations	6	6
Hypothesized Mean Difference	0	
df	10	
t Stat	2.97904E-14	
P(T<=t) two-tail	1	
t Critical two-tail	2.228138842	

Figure B.6 Cell viability reproducibility in molasses based media using paired t-test assuming equal variances

Table B.4 Sugar and ethanol reproducibility data in molasses based media

	Sugar concentration ($g.l^{-1}$)			*Ethanol concentration (%)*		
Time	**Exp 1**	**Exp 2**	**Exp 3**	**Exp 1**	**Exp 2**	**Exp3**
0	124	126	124	0.0%	0.0%	0.0%
10	125	123	123	0.0%	0.0%	0.0%
15	118	117	114	0.5%	0.6%	0.6%
20	109	111	106	1.3%	1.4%	1.5%
23	80	82	79	2.5%	2.8%	2.7%
35	3	6	4	6.5%	7.0%	6.4%

Sugar concentration

	Run 1	*Run 2*
Mean	79.79192029	80.81440714
Variance	3091.244438	3030.231599
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	-0.03457631	
P(T<=t) two-tail	0.972986081	
t Critical two-tail	2.178812827	

	Run 1	*Run 3*
Mean	79.79192029	78.547644
Variance	3091.244438	2969.730363
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	0.042285805	
P(T<=t) two-tail	0.966966312	
t Critical two-tail	2.178812827	

	Run 2	*Run 3*
Mean	80.81440714	78.547644
Variance	3030.231599	2969.730363
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	0.077424947	
P(T<=t) two-tail	0.939561487	
t Critical two-tail	2.178812827	

Figure B.7 Sugar concentration reproducibility in molasses based media using paired t-test assuming equal variances

Ethanol concentration

	Run 1	*Run 2*
Mean	0.024707731	0.026386833
Variance	0.000827852	0.000923215
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	0.106163351	
P(T<=t) two-tail	0.917206841	
t Critical two-tail	2.178812827	

	Run 1	*Run 3*
Mean	0.024707731	0.02546596
Variance	0.000827852	0.000838285
Observations	7	7
Hypothesized Mean Difference	0	
df	12	
t Stat	-0.049146643	
P(T<=t) two-tail	0.96161097	
t Critical two-tail	2.178812827	

	Run 2	*Run 3*
Mean	0.026386833	0.02546596
Variance	0.000923215	0.000838285
Observations	7	7
Pooled Variance	0.00088075	
Hypothesized Mean Difference	0	
df	12	
t Stat	0.058050681	
P(T<=t) two-tail	0.954663728	
t Critical two-tail	2.178812827	

Figure B.8 Ethanol concentration reproducibility in molasses based media using paired t-test assuming equal variances