Effects of mineral ions on yeast performance under very high gravity beer fermentation

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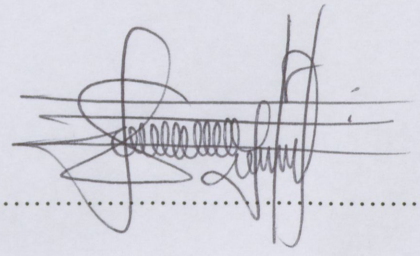
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Declaration

I , **Henry Okwudili Udeh**, hereby declare that this research project for Master of Science in Food Science and Technology submitted by me at the University of Venda in the School of Agriculture, Department of Food Science and Technology has not been previously submitted for any other degree at this or any other university. I further declare that the mini project is my own work in design, execution, all reference materials and work of others have been acknowledged.



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Dedication

I dedicate this study to God Almighty for his unconditional love and grace to have seen to the successful completion of this project. My special dedication goes to my family Mrs Teresa Udeh, Oliver Ifeanyi, Emmanuel Ejike, Ephraim Chukwuemeka, Rita Ngozi and Juliet Chinyere, for their love, financial and mental support. Also, to Prudence Masekela and to other students for their inspiring support during the course of this study.

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ABSTRACT

The effect of three divalent cationic concentrations of Zn^{2+}, Mg^{2+}, and Ba^{2+} on yeast (*Saccharomyces pastorianus*) fermentative performance was investigated at independent and four variable combinations in 24 experimental runs in accordance with the experimental design. Very high gravity wort of 21°P was prepared from barley malt, hops and water, to which the metal and their combinations were supplemented and subsequently pitched using lager brewing strain *S. pastorianus* and allowed for 96 h fermentation. After 96 h of fermentation, highest wort fermentability of %F = 30.16 and %F = 29.53 which were not significantly different ($p \geq 0.05$) were obtained for wort medium supplemented with 12, 900, and 4 ppm (Zn^{2+}, Mg^{2+}, and Ba^{2+}) and 900 and 4 ppm (Mg^{2+} and Ba^{2+}) respectively. Maximum ethanol yield of 8.4347% (v/v) was obtained in wort medium supplemented with the metal combination ratio of 12:900:4 (Zn^{2+}, Mg^{2+}, and Ba^{2+}). For the individual metal concentration levels tested, high wort fermentability of %F = 27.94 and %F = 26.03 which were not significantly different ($p \geq 0.05$) was obtained for wort media supplemented with 700 ppm Mg^{2+} and 2 ppm Ba^{2+}. High ethanol yield of 7.8844% (v/v) and 7.6245% (v/v) was recorded for Mg^{2+} and Ba^{2+} at concentration of 700 ppm and 2 ppm, respectively. Wort pH of 4.75 was found optimal for the yeast activity. Concentrations of 10 ppm Zn^{2+} and 4 ppm Ba^{2+}, were found to reduce yeast performance. Thus, the study established relationships between the metal ions on yeast fermentative performance and the impact of Ba^{2+} on yeast fermentation process – with concentrations of 1 and 2 ppm Ba^{+} found significant ($p \leq 0.05$).

Keywords: Very high gravity fermentation, brewer's yeast, yeast stress, yeast stress tolerance, yeast performance, mineral ions, fermentability, ethanol yield.

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List of abbreviations

NG	Normal gravity
HG	High gravity
VHG	Very high gravity
^{o}P	Degree Plato
^{o}Bx	Degree brix
UFAs	Unsaturated Fatty Acids
SFAs	Saturated Fatty Acids
SAB	South African Brewery
HOG	High Osmolarity Glycerol
MAP	Mitogen Activated Protein
HSR	Heat Stress Response
GSR	Global Stress Response
HSF	Heat Shock transcription Factor
STREs	Stress Response Elements
ZRE	Zinc Responsive Elements
ZIP	Zinc-Iron Permease
CDF	Cation diffusion facilitator
Zrt1	High affinity zinc transporter
Zrt2	Low affinity zinc transporter
Zap1	Zinc responsive activator protein1
Ubc	Ubiquitin conjugating enzyme
Rap5	UniProtKB gene
Mal61	Maltose permease gene
K_m	Michaelis Mentene constant
cAMP	Cyclic Adenosine Mono-phosphate
PKA	Protein Kinase A
TPS1	Trehalose 6-phosphate synthase encoding enzyme
TPS2	Trehalose 6-phosphatase encoding gene
TSH1	Trehalose 6-phosphate synthase
TSH2	Trehalose 6-phosphatase
NTH1	Neutral Trehalose Hydrolase degrading enzyme 1
NTH2	Neutral Trehalose Hydrolase – degrading enzyme 2

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ATH	Acidic Trehalose Hydrolase
OLE1	Desaturase (de-saturates saturated fatty acids to UFAs) encoding gene
ELO1	Encoding gene of the membrane-bound fatty acid elongation system
ALR1	Aluminium Resistance gene
ALR2	Aluminium resistance gene homologue
Alr1P	Aluminium resistance permease
Alr2P	Aluminium resistance permease homologue
CorA	Cobalt resistance protein
MIT	Membrane Integral Protein/ Metal Ion Transporter
mRNA	Messenger Ribonucleic Acid

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CHAPTER 1

INTRODUCTION

1.1 Background of research

Alcoholic fermentation technology has a long history and has evolved with precision for creating a more refined and organoleptically acceptable alcoholic beverage in the world today. It is believed that beer making has been in existence even before the Neolithic Revolution – a transition from nomadic to a stable farming lifestyle - that happened in several parts of the world between 9000-7000 BC (Hornsey, 2003). In the beginning of the fifth millennium BC, it was found that people of Sumeria of Northern Mesopotamia, were making beer which was in turn used as a form of currency for scarce natural resources like timber, metals and even precious stones (Rabin & Forget, 1998). Upon the fall of the Sumerian empire during the second millennium BC, the Babylonian took over the fertile Mesopotamia, and beer brewing was considered important and laws were issued to protect and preserve their beer brewing methods (Röllig, 1971). Today, the science of beer brewing is still in the forefront of biotechnological development (Linko *et al.,* 1998; Li & Chan-Halbrent, 2009), and the alcoholic beverage industry - taking a huge position among reputable industries that accounts for considerable economic growth of the globe.

Classically, beer is any maltose-based alcoholic beverage made essentially from malted barley, hops, and water (Nelson, 2005). Currently, the alcoholic beverage industry holds a striking statistical market prediction which unquestionably fits it among capital leading industries in the world. Globally, the alcoholic beverage industry is expected to exceed a market value of $ 1 trillion in 2014 with a predicted market volume of 210 billion litres (Anon, 2012). A typical example in Africa is the South Africa Brewery (SAB), which is currently the fifth world largest brewer, with 79 breweries in 21 countries and over 34,000 employees (Hayim *et al.,* 2001), with present beer consumption rate estimated at 2.6 billion litres annually. It is evident that the beverage is a blueprint for capital generation and also a multi-disciplinal employer, considering the hierarchy of labour force it employs.

Presently, there has been an increasing global concern on how to efficiently maximize the production of ethanol for alcoholic beverage production, and biofuel. Quality beer in short time, production cost, environmental issues, dwindling fusel fuel reserves and increasing cost

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of gas (Hall *et al.,* 2006; Silva *et al.,* 2008), have been identified among other factors, as major drivers for the search for efficient ethanol production methods. Amongst various forms of alcoholic fermentation is high gravity (HG) fermentation. HG fermentation is a technically sophisticated brewing practice which has been affirmed to be highly resourceful and environmentally friendly. This fermentation technology provides an increased production capacity from same size brewhouse and fermentation facilities (Blieck *et al.,* 2007; Huuskonen *et al.,* 2010) - thus a desirable approach to economic brewing. Being the best in class fermentation technology, HG fermentation has been widely adopted and successively applied for brewing standard beer, and for biofuel production in developed countries, such as the United States of America, Canada, China and Brazil (Walker, 2004; Li & Chan-Halbrent, 2009; Vidgren & Londesborough, 2011).

HG brewing as defined by Stewart and Russell, (1995), is a procedure that employs the preparation and fermentation to completion of wort with higher than normal sugar concentration (16 – 18 g per 100 g mash) which is subsequently diluted, after maturation, with deoxygenated water to normal strength 3 - 6 (v/v) before packaging. The concept of HG brewing was conceived in the 1980's, upon observation that thick mashes yield high fermentable sugars, and was successively applied for a final ethanol yield of 7 - 8 % (v/v) which is almost double of the ethanol yield of normal gravity (NG) brewing which range 3 - 4% (v/v) (Briggs' *et al.,* 2004; Bai *et al.,* 2008).

In recent years, the concept of HG fermentation was further extended to very high gravity (VHG) fermentation, with the intension of maximizing the benefits which include high ethanol yield per fermentable mash, reduced energy and water consumption, time and money savings, improved smoothness, flavour and haze stability of beer, and effluents with low biological oxygen demand (Agu, *et al.,* 1993; Sigler *et al.,* 2009; Verbelen *et al.,* 2009; Huuskonen *et al.,* 2010; Pulingundla & Reddy, 2010; Yu *et al.,* 2012). However, problem of flavour match in beer have been reported with HG fermentation as modified flavour and aroma compound profile in final beer produced is pronounced due to elevated levels of esters and higher alcohol (Blieck *et al.,* 2007; Piddocke *et al.,* 2009). Also, slow or stock fermentation due to negative effect of high osmotic pressure and increased ethanol concentration on yeast performance have been attributed to HG fermentation (Pátková *et al.,* 2000). VHG fermentation has been extensively used for biofuel production from several cereal extracts such as wort, corn steep liquor, and sweet sorghum juice (Li & Chan-Halbrent,

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2009; Cao *et al.,* 2011). However, this fermentation technology is yet to be aligned with the brewing of standard beer as there are significant setbacks associated with the technology.

Research findings have provided evidence on mechanisms through which VHG fermentation conditions affects yeast cells during fermentation. These mechanisms which are collectively called yeast stress, include; high osmotic, ethanol, thermal, nutrient and oxidative stress (Banat *et al.,* 1998; Bai *et al.,* 2008; Pulingundla *et al.,* 2011). It's been shown that the effect yeast stress on yeast performance is dependent on the yeast tolerance (i.e. ability to cope in harsh condition) - which in turn is dependent on the genetic make-up of the yeast strain, physiological state of the cell, physico-chemical nature of the fermentation medium and the presence of other competitive microbes like wild yeast and bacteria (Boulton & Quain, 2001; Walker, 2004; Xue *et al.,* 2008; Somda *et al.,* 2011).

Several authors (Briggs' *et al.,* 2004; Walker, 2004; Walker *et al.,* 2006) have affirmed that these stressful conditions experienced by yeast cells can be alleviated by modifying the factors which affects yeast tolerance. These modifications which includes strain selection and genetic engineering of yeast cell, optimization of system parameters, and medium supplementation with certain nutrients; have been evaluated and proved to be effective to improving yeast tolerance. Strain selection has been the technique applied to identifying high performing yeast strains and a base for genetic manipulation. A biodiversity study on yeast strain by Pereira *et al.* (2010), showed some strains of *Saccharomyces cerevisiae* exhibiting high fermentation performance with regards to ethanol production under VHG fermentation condition. Isolation of best performing yeast variants under VHG wort (> 22° P) condition has been successfully demonstrated by Blieck *et al.* (2007). Resulting variants where reported to exhibit faster rate of fermentation due to alterations in the expression of candidate genes which were affirmed by microarray gene analysis. Also, strain selection on mutagenized lager yeast strains was also demonstrated by Huuskonen *et al.* (2010); where mutants showed an appreciable fermentation performance and high viability over parent strains under high gravity condition. Global transcription machinery engineering (gTME), a novel and extremely complicated modification technique, has been used as a tool to re-program gene transcription factors for eliciting new phenotypes for improved yeast performance (Gao *et al.,* 2010).

On the other hand, system parameters such as temperature, pH, aeration and pitching rate, and the use of inorganic substances like acetaldehyde, vanadium pentoxide, urea and

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ammonium salt have been evaluated under high and very high gravity conditions (Jones & Ingledew, 1994; Barber *et al.,* 2002; Dragone *et al.,* 2008; Rosado *et al.,* 2009; Verbelen *et al.,* 2009). Optimal sugar utilization (> 75% dissolved solids) at 20-25°C has been observed with molasses with very high dissolved solid (Jones *et al.,* 1994), and an optimal temperature range of 27-30°C has been recommended for ethanol fermentation of VHG wheat mashes (36.5 g / 100 ml dissolved solids) to obtain more than 20% (v/v) ethanol within 55 h in the presence of 16 mM Urea (Jones & Ingledew, 1994). A significant reduction in fermentation time with negligible effect on yeast viability and vitality under wort gravity of 15°P was reported by Verbelene *et al.* (2009).

Wort aeration is considered an integral factor in predicting fermentation performance as yeast cells requires oxygen to synthesize essential lipids required for growth. Aeration rate of 0.82 l/min between early log and mid log phases of growth has been found to be effective in enhancing yeast viability and ethanol productivity under VHG conditions (Lin *et al.,* 2011). Increased ethanol production and enhanced yeast viability of both lager and ale yeast strain under normal (12°P) and high gravity conditions (20°P), has been reported by Rees and Stewart (1999). Reduced fermentation time with no effect on ethanol yield has also been reported by Barber *et al.* (2002) upon daily addition of acetaldehyde to high gravity fermentation medium. It was also found that these inorganic substances favours the production of other higher alcohols such as acetate, 2, 3-butanediol and 2-methylpropanol which are of brewing implications. These system parameters, although efficient in promoting ethanol productivity, are to be stringently controlled in order to minimize their effect on beer quality such as foam, flavour and taste.

Considerable body of evidence have revealed that yeast tolerance to stressful conditions is strongly dependent on the nutritional composition of the fermentation medium (Wang *et al.,* 2007, Xue *et al.,* 2008; Pulingundla & Reddy, 2010). It's been reported that essential yeast nutrients are compromised during the preparation of VHG wort (Briggs' *et al.,* 2004; Walker, 2004) and as such, the wort is unable to satiate the nutritional requirement for optimal yeast performance during fermentation. This nutritional imbalance not only affects the yeast cell but synergistically exacerbates other stressful conditions at the expense of the yeast cell. These nutrients which include carbon and nitrogen sources, vitamins and minerals have been evaluated under various conditions (including HG and VHG fermentation) with much attention to the impact of carbon and nitrogen sources on yeast performance. Unfortunately,

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the effects of mineral ions on yeast performance have been poorly elucidated, yet are vital co-components of the yeast biological machinery.

Mineral ions have been implicated with vital metabolic functions in yeast cells, which are of prime importance to brewing practice. Metal ions have been found to maintain structural integrity and functionality of intracellular organelles (Rees & Stewart, 1999; Walker, 2004; Aleksander *et al.,* 2009; Pisat *et al.,* 2009), induce cell-cell interaction such as flocculation (Walker, 2004; De Nichola *et al.,* 2009), govern gene expression and nutrient uptake mechanisms (Eide, 2006), activate arrays of enzymes intimately involved in metabolism (Walker *et al.,* 2006), and also, acting as stress-protectant (Birch & Walker, 2000). Of the myriads of cationic nutrients in existence; potassium, magnesium, zinc and calcium have been identified as important metals, and have been implicated with most of the aforementioned roles. In the presence of these metal ions, increased fermentability or rate of sugar conversion to ethanol, increased ethanol yield, improved yeast viability and vitality, desirable flocculation behaviour and enhanced stress tolerance (Rees & Stewart, 1999; Birch & Walker, 2000; Xue *et al.,* 2008; Vidgren & Londesborough, 2011), has been reported. But the interactive or combinatory effect of these metal ions in view of optimizing yeast performance under very high substrate condition has been poorly demonstrated, hence the approach of this study.

1.2 Problem statement

Slow fermentation due to reduced nutrient uptake, low production of ethanol as a result of the inhibitory effect of high ethanol concentration on yeast cells, high residual sugars in beer due to poor attenuation of extract and decreased viability of yeast cells are limitations associated with VHG fermentation, and are mostly attributed to sub-optimal levels of essential mineral ions in the fermentation medium (Briggs' *et al* 2004; Walker, 2004; Walker *et al.,* 2006; Gibson, 2011).

1.3 Justification of research

This study seeks to determine the independent and combinatory effects of increasing amounts of zinc, magnesium and barium ions on *Saccharomyces pastorianus* under very high concentrations of dissolved sugars. As metal ions are known to be compromised during VHG wort preparation, supplementing fermentation mediums with these metals will perhaps augment free metal ions necessary to maintain and enhance the yeast fermentative

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performance. Zinc and magnesium ions have been identified with functional enzymes intimately involved with most metabolic processes and as stress-protectants under VHG fermentation condition (Birch & Walker, 2000; Walker, 2004; Walker *et al.,* 2006); as such, their interactive effect with barium ion will be evaluated to ascertain the best possible combination(s) which will best promote yeast performance with respect to wort fermentability and ethanol productivity. In the same vein, the impact of barium on yeast cell will be ascertained, as little is known of the effect of the metal ion on yeast performance under VHG fermentation condition.

1.4 Hypothesis

Respectively, Mg^{2+} and Zn^{2+} promote rapid uptake of nutrients and acts as stress protectant, as such, they enhance the efficiency of substrate conversion and shield yeast cell under VHG condition. Suggestively, a high fermentability could be expected by augmenting Zn^{2+} and Mg^{2+} in VHG fermentation medium.

Zinc solely activates alcohol dehydrogenase which is essential for ethanol formation and Mg^{2+} activates and regulates numerous enzymes involved in glycolytic and bioenergetic pathways. Thus increasing Zn^{2+} and Mg^{2+} concentrations under VHG fermentation conditions will promote ethanol yield.

1.5 Aim: The aim of this research work is to determine the independent and combinatory effects of zinc, magnesium and barium at various concentration levels on yeast performance under VHG beer fermentation.

1.6 Objectives:

- To determine the effect of Zn^{2+} on yeast performance during VHG fermentation condition.
- To determine the influence of Mg^{2+} on yeast performance during VHG fermentation.
- To determine the impact of Ba^{2+} on yeast performance during VHG fermentation.
- To determine the combinatory effect of Zn^{2+}, Mg^{2+} and Ba^{2+} on yeast performance during VHG fermentation condition.

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1.7 Expected outcome

Despite the social perceptions on alcoholic beverage, which is a multi-disciplinal employer, the same product accounts for a tangible quota of the countries employment, revenue generation, and more conspicuously, the wellbeing of the citizens during occasional merriments. Considering the advantages of HG fermentation which include high ethanol yield, considerable saving on energy and optimal use of raw materials; actualizing and adopting VHG fermentation will further maximize the benefits, and increase production volume which will economically boost job creation, revenue generation, and also bringing the South African alcoholic beverage industry to limelight of the technology.

- Contribution to knowledge in the research field is anticipated in this study, as publications in the relevant journal of Brewing and Distilling, Food Science and Technology, Nutrition, Food Biotechnology, and Biotechnology; will accompany the successful completion of the research work.

- The outcome of this study will suggest a better nutrient supplement with good mineral combination that will enhance the performance of *S. pastorianus* in harsh fermentation conditions, thus promoting a high fermentation rates and ethanol productivity which is fundamental to alcoholic beverage and biofuel industries.

- This study can also be instrumental to improving products in bakery, meat, and also in pharmaceutical industries; where it could serve the purpose of bio-mineralization process for mineral deficient food products.

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CHAPTER 2

LITERATURE REVIEW

2.1 Brewer's yeast

The genus *Saccharomyces* represents a diverse group of microorganisms with biotechnological importance. For millennia, the abilities of *Saccharomyces* yeast have been utilized in leavening dough in baking and generating ethanol from starchy material for alcoholic beverage production (Briggs' *et al.,* 2004).

In taxonomical view point, yeast species of commercial interest falls under the genus *Saccharomyces* based on their structural and functional relatedness (Briggs' *et al.,* 2004). Presently the genus *Saccharomyces* is divided into 14 species. Sub-classification of these species has been the subject of intensive investigation, however over 10 of the 14 species has been assigned into two sub-groups i) *Saccharomyces sensuo lato* and ii) *Saccharomyces sensuo stricto* based on their ribosomal and mitochondrial similarities. Figure 1 is a part classification of the genus *Saccharomyces* with distinguishing physiological properties between ale and lager brewing yeast.

As shown in Figure 1, all the species of *Saccharomyces sensuo stricto* sub-group are exploited for commercial purpose except for *S. paradoxus*. *S. cerevisiae* is multifarious in application as it is widely used for brewing, baking and wine making. Strains of *S. bayanus* are specially employed for wine production due to their enhanced tolerance to high ethanol concentration at low temperatures (Blieck *et al.,* 2007). The *S. pastorianus* species, formally called *S. calsbergensis or S. uvarum* is strictly employed for brewing lager beer. But with the advent of cylindroconical fermentation vessels, lager strains are now employed for brewing ale beer, with no discriminatory differences in process behaviours such as buoyant-density of ale yeasts and flocculence of lager yeasts (Walker, 2000). It is apparent from Figure 1 that there is an impending metabolic hazard considering the physiological properties of brewing yeast species, their mode of sugar utilization and fermentation conditions of latest fermentation technologies.

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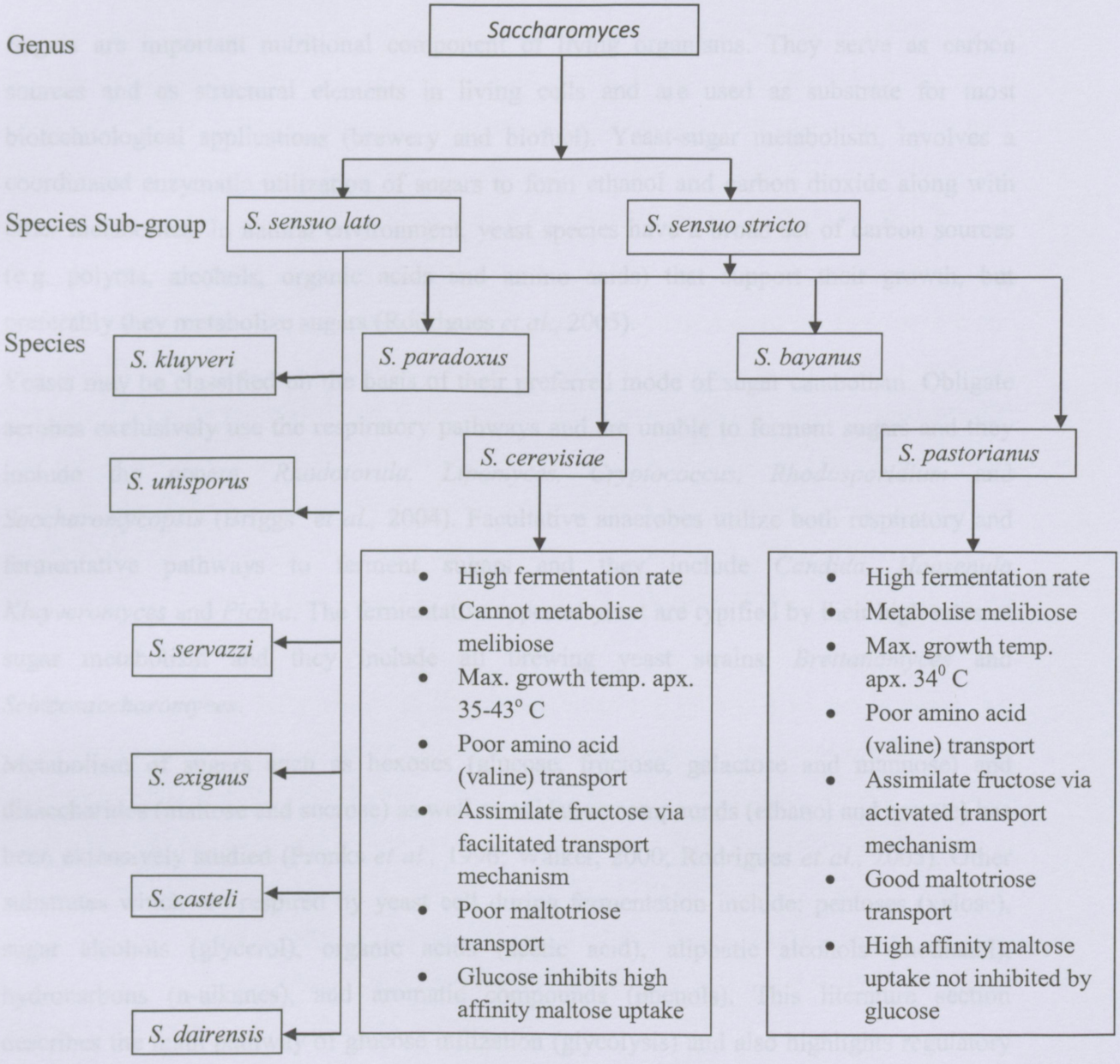


Figure 1. Part taxonomical chart for the genus *Saccharomyces*
Adapted from Briggs' *et al.* (2004).

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2.2 Yeast-sugar metabolism

Sugars are important nutritional component of living organisms. They serve as carbon sources and as structural elements in living cells and are used as substrate for most biotechnological applications (brewery and biofuel). Yeast-sugar metabolism, involves a coordinated enzymatic utilization of sugars to form ethanol and carbon dioxide along with other metabolites. In natural environment, yeast species have a broad set of carbon sources (e.g. polyols, alcohols, organic acids and amino acids) that support their growth, but preferably they metabolize sugars (Rodrigues *et al.,* 2005).

Yeasts may be classified on the basis of their preferred mode of sugar catabolism. Obligate aerobes exclusively use the respiratory pathways and are unable to ferment sugars and they include the genera, *Rhodotorula, Lipomyces, Cryptococcus, Rhodosporidium* and *Saccharomycopsis* (Briggs' *et al.,* 2004). Facultative anaerobes utilize both respiratory and fermentative pathways to ferment sugars and they include *Candida, Hansenula Kluyveromyces* and *Pichia*. The fermentative types of yeast are typified by their high rates of sugar metabolism and they include all brewing yeast strains, *Brettanomyces* and *Schizosaccharomyces*.

Metabolism of sugars such as hexoses (glucose, fructose, galactose and mannose) and disaccharides (maltose and sucrose) as well as oxidative compounds (ethanol and acetate) has been extensively studied (Pronks *et al.,* 1996; Walker, 2000; Rodrigues *et al.,* 2005). Other substrates which are respired by yeast cell during fermentation include: pentoses (xylose), sugar alcohols (glycerol), organic acids (acetic acid), aliphatic alcohols (methanol), hydrocarbons (n-alkanes), and aromatic compounds (phenols). This literature section describes the main pathway of glucose utilization (glycolysis) and also highlights regulatory phenomena which affect sugar utilization in yeast.

Sugar utilization in yeast involves series of pathways notably, the glycolytic pathway, pentose phosphate pathway and tricarboxylic acid pathway. Glycolysis, also referred to as Embden-Meyerhof-Panas Pathway, is an ancient pathway in which glucose is broken-down to form pyruvate. Utilization of glucose in yeast cells is fated to be dissimilatory and / or assimilatory by metabolic processes (Pronk *et al.,* 1996). Once inside the yeast cell, glucose is phosphorylated by kinases to form glucose 6-phosphate which is then isomerized to fructose 6-phosphate by phosphoglucose isomerase. In the reaction, fructose 6-phosphate is further phosphorylated to fructose 6-bisphosphate by phosphofructo 1-kinase, which is

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subject to regulation by several metabolites. Several flux analyses have shown that approximately 50% of glucose 6-phosphate is metabolized via glycolysis and 30% via the pentose phosphate pathway in Crabtree negative yeasts. However, about 90% of the carbon going through the pentose phosphate pathway re-enters the glycolytic pathway at the level fructose 6-phosphate or glyceraldehyde 3-phosphate (Gonzalez-Siso *et al.,* 2000). These reaction steps are the first phase of glycolysis which requires energy in the form of ATP.

The subsequently acting enzymes are aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and pyruvate kinase (Pronk *et al.,* 1996). The last reaction block of glycolysis leads to pyruvate formation which is associated with a net production of two molecules of ATP and reducing equivalent (NAD^+). Figure 2 schematically illustrate glycolysis and the fate of pyruvate in yeast. In the case of yeast utilizing oxidative phosphorylation, the ATP value increases to approximately 30 molecules per molecule of glucose (Briggs' *et al.,* 2004). The regeneration of NAD^+ is important in glycoslysis to maintain redox balance and prevents stalling of the glycolytic pathway (Walker, 2000).

At the pyruvate (the end product of glycolysis) branching points, it's been reported that pyruvate can follow three different metabolic fates depending on the yeast species and the environmental conditions (Pronk *et al.,* 1996). In the presence of oxygen, aerobic organisms completely oxidize pyruvate to CO_2 and H_2O. In fermentative yeast cells or absence of oxygen, pyruvate is converted to acetaldehyde by a multi-enzyme complex pyruvate dehydrogenase, which is then reduced by NADH to form ethanol through the mediation of the enzyme alcohol dehydrogenase. CO_2 is formed in a decarboxylation reaction, in which an organic acid losses a carboxyl group as CO_2.

Insight into the utilization of pyruvate in other higher organisms shows that pyruvate can also be converted to acetylCoA in the cytosol via acetaldehyde and to acetate by pyruvate dehydrogenase bypass pathway (Pronk *et al.,* 1996). The pyruvate dehydrogenase bypass pathway has been shown to require the activity of three different enzymes namely: i) pyruvate dehydrogenase which converts pyruvate to acetaldehyde ii) acetaldehyde dehydrogenase which converts acetaldehyde to acetate and iii) acetylCoA which converts acetate to cytosolic acetylCoA (Kispal *et al.,* 1991). However, it is still a matter of debate if the flux distribution during pyruvate metabolism determines the pyruvate flux through the pyruvate dehydrogenase complex or through the pyruvate dehydrogenase bypass and thereby the split between respiration and fermentation (Pronk *et al.,* 1996). One of the mechanisms

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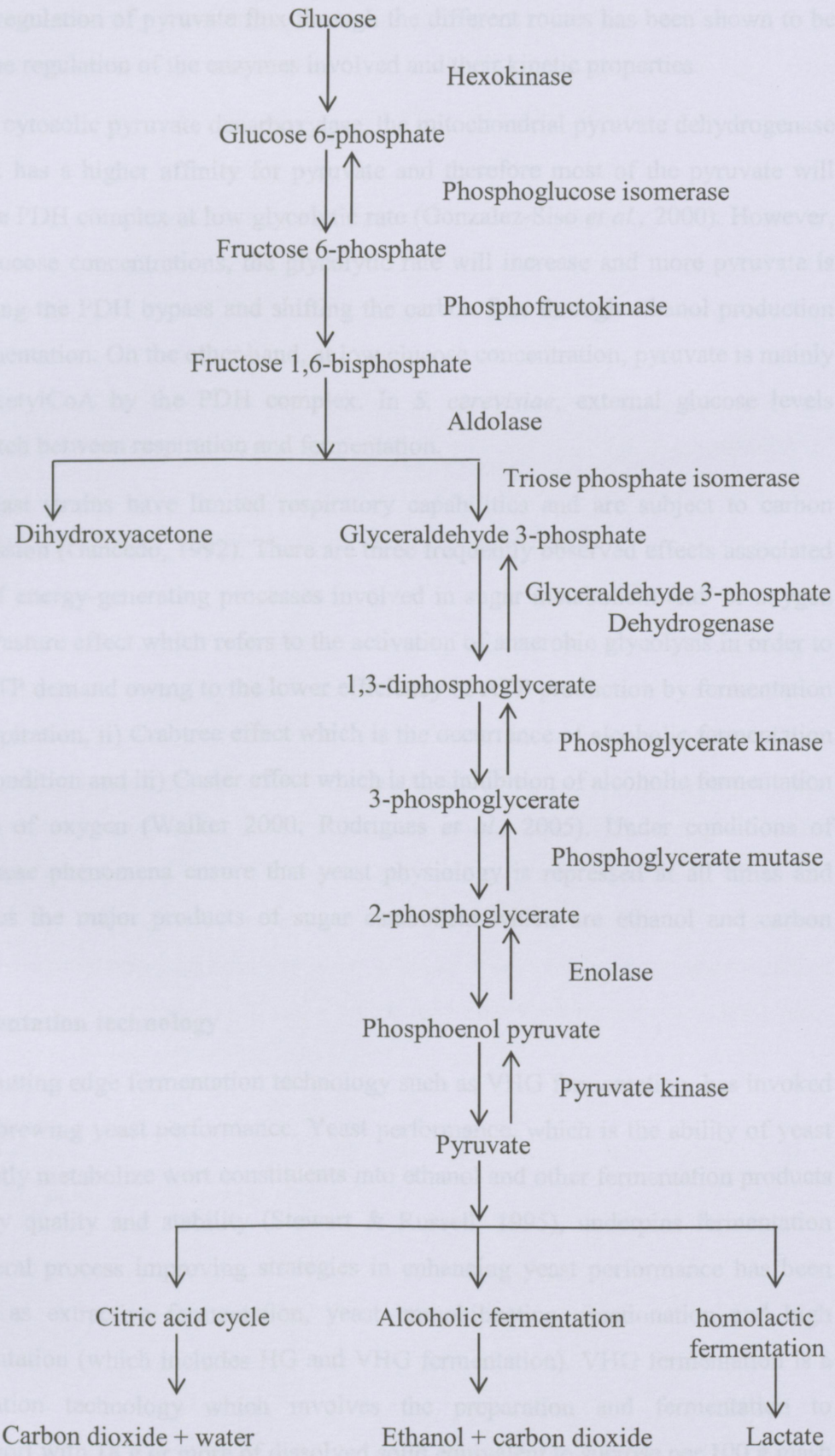


Figure 2. Glycolysis and fate of pyruvate in yeast.
Source: Walker 2000: Adapted.

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underlying the regulation of pyruvate flux through the different routes has been shown to be at the level of the regulation of the enzymes involved and their kinetic properties.

Compared with cytosolic pyruvate decarboxylase, the mitochondrial pyruvate dehydrogenase (PDH) complex has a higher affinity for pyruvate and therefore most of the pyruvate will flow through the PDH complex at low glycolytic rate (Gonzalez-Siso *et al.,* 2000). However, at increasing glucose concentrations, the glycolytic rate will increase and more pyruvate is formed, saturating the PDH bypass and shifting the carbon flux through ethanol production and initiate fermentation. On the other hand, at low glucose concentration, pyruvate is mainly converted to acetylCoA by the PDH complex. In *S. cerevisiae*, external glucose levels controls the switch between respiration and fermentation.

All brewing yeast strains have limited respiratory capabilities and are subject to carbon catabolite repression (Gancedo, 1992). There are three frequently observed effects associated with the type of energy-generating processes involved in sugar metabolism and/ or oxygen availability: i) Pasture effect which refers to the activation of anaerobic glycolysis in order to meet cellular ATP demand owing to the lower efficiency of ATP production by fermentation compared to respiration, ii) Crabtree effect which is the occurrence of alcoholic fermentation under aerobic condition and iii) Custer effect which is the inhibition of alcoholic fermentation by the absence of oxygen (Walker 2000; Rodrigues *et al.,* 2005). Under conditions of fermentation, these phenomena ensure that yeast physiology is repressed at all times and therefore ensures the major products of sugar catabolism which are ethanol and carbon dioxide.

2.3 Novel fermentation technology

The advent of cutting edge fermentation technology such as VHG fermentation, has invoked the nemesis of brewing yeast performance. Yeast performance, which is the ability of yeast cell to consistently metabolize wort constituents into ethanol and other fermentation products with satisfactory quality and stability (Stewart & Russell, 1995), underpins fermentation efficiency. Several process improving strategies in enhancing yeast performance has been evaluated such as extractive fermentation, yeast immobilization, fractionation and high substrate fermentation (which includes HG and VHG fermentation). VHG fermentation is a novel fermentation technology which involves the preparation and fermentation to completion of wort with 18 g or more of dissolved solid equivalent to sucrose per 100 g mash (Bayrock & Micheal, 2001). This theoretical fact, practically allows a final ethanol yield of

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15 - 18% (v/v) (Bai *et al.,* 2008; Pulingundla & Reddy, 2010). Figure 3 illustrates the production of alcoholic beverage and biofuel using VHG fermentation technology, with Table 1 showing differences between normal gravity (NG), HG and VHG fermentation methods.

Limitation of applying VHG fermentation, which has been the constraining factor to its full commercialization, is the high initial concentration of glucose and other sugars formed by the enzymatic hydrolysis (Cao *et al.,* 2011). The high osmotic pressure caused by high concentration of dissolved sugars leads to physiologic changes in yeast cells which consequently result in reduction in the cells' vitality, which further reduces sugar conversion and ethanol production (Phisalaphong *et al.,* 2006). Moreover, the high concentration of glucose also causes an inhibitory feedback effect on the hydrolase and hinders the mash from complete saccharification which consequently lowers fermentation efficiency (Graves *et al.,* 2007).

Under normal wort strength (12°Plato), high performing brewing yeast exhibit high sugar-ethanol conversion rate, low production of secondary metabolites, increased nutrient uptake and high levels of viable biomass. In exposure to VHG fermentation condition, these desirable traits are incapacitated due to very high dissolved sugar, increased ethanol concentration, high temperatures and propensity to nutrient starvation (Cot *et al.,* 2007; Gao *et al.,* 2011). Under such conditions, the yeast cells tend to display unusual fermentative behaviours which are often referred to as yeast stress. The resultant effect of these stress conditions during fermentation manifests as slow or stock fermentation, poor attenuation of sugar, inordinate flocculation behaviour, and generation of petite (respiratory deficient) yeast strains (Gibson, 2011). Other setback include poor foam stability (Cooper *et al.,* 2000), haze development in beer due to released glycogen by lysed or disrupted yeast cells (Malcorps *et al.,* 2001) and disproportionate synthesis of esters and higher alcohols which often result in poor flavour profile of the beer (Munroe, 1995).

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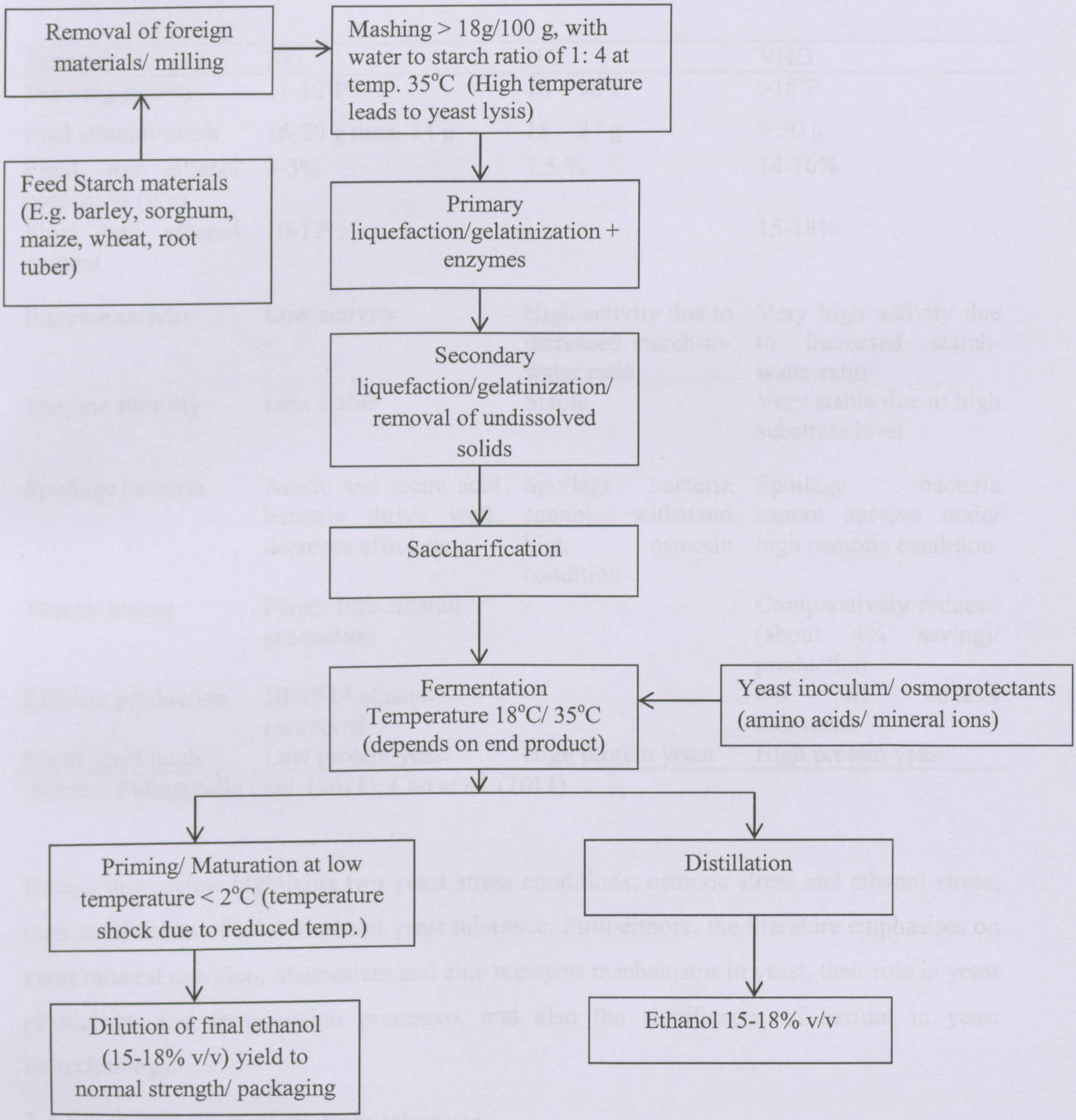


Figure 3. Schematic flow of the production of alcoholic beverage/ fuel ethanol from starchy material using very high gravity fermentation method.
Source: Pulingundla *et al.* (2011); Udeh and Kgatla (2013)

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Table 1. Differences between normal gravity (NG), high gravity (HG), and very high gravity (VHG) fermentation

Features	NG	HG	VHG
Brewing gravity	11-12°P	16 – 18°P	>18°P
Fuel ethanol-mash	16-20 g max. 24 g	18 – 27 g	> 30 g
Final beer ethanol content % (v/v)	4-5%	7.5 %	14-16%
Final fuel ethanol content	10-12%	-	15-18%
Enzyme activity	Low activity	High activity due to increased starch-to-water ratio	Very high activity due to increased starch-water ratio
Enzyme stability	Less stable	Stable	Very stable due to high substrate level
Spoilage bacteria	Acetic and lactic acid bacteria thrive well, decrease efficiency	Spoilage bacteria cannot withstand high osmotic condition	Spoilage bacteria cannot survive under high osmotic condition
Energy saving	Fixed/ litre ethanol production	-	Comparatively reduced (about 4% saving)/ production
Effluent production	10-15 l/l ethanol recovered	-	6-9 l/l ethanol recovered
Spent yeast quality	Low protein yeast	High protein yeast	High protein yeast

Source: Pulingundla *et al.* (2011); Cao *et al.* (2011)

Hence, this review highlights two yeast stress conditions: osmotic stress and ethanol stress; their mechanism of inhibition and yeast tolerance. Furthermore, the literature emphasises on yeast mineral nutrition, magnesium and zinc transport mechanisms in yeast, their role in yeast physiology and fermentation processes, and also the significance of barium in yeast biotechnology.

2.4 Yeast stress and yeast stress tolerance

Yeast, like any other living cell, is affected by natural factors such as gene type, environment and nutrition (Figure 4). Overexposure of yeast cells to any of these factors may result in the yeast cells struggling to maintain their metabolic processes. In industrial situation, yeast stress often results from its metabolic by-products (ethanol, higher alcohols) and the nature of the brew system (Rautio *et al.,* 2007). Stressed yeast cells are identified by an increase in their acidifying ability upon transfer into a nutrient rich medium (Briggs *et al.,* 2004).

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Stress conditions which include rapid temperature fluctuations, high osmotic pressure, increased ethanol concentration, and nutrient starvation (Ding *et al.,* 2009), adversely affect yeast cell dynamics; thus resulting in inefficient fermentation process. Although recent study by Belloch *et al.* (2008) has shown low pH not to be much of a stress to the yeast cell, nevertheless; it exacerbates the impact of other stress factors (Udeh & Kgatla, 2013). Figure 5 is a schematic illustration of the stressful conditions experienced by yeast cell during VHG fermentation, and these stressful conditions have been reported to be of relevance since yeast cells are said to acquire an appreciable resistance trait after exposure (Blieck *et al.,* 2007).

2.4.1 Osmotic stress

High osmotic stress, which is typical of high and VHG fermentation, is among the deleterious stress condition experienced by yeast cells during fermentation (Querol *et al.,* 2003). It is a cellular imbalance that occurs when cells are suspended in medium with high osmotic potential (high solute concentration) than the intracellular content, which consequently results in loss of cellular water and tugor fall (Klipp *et al.,* 2005). This osmotic effect tends to alter the physiological fitness of the yeast cell, by extruding essential cell components that maintains cellular fecundity. This osmotic change is often accompanied by a shrunken yeast cell with conspicuous plasma membrane invaginations (Rapoport *et al.*, 1995), which consequently deter the physiological state of the yeast cell, culminating in loss of cell viability and reduced cell growth (D' Amore, 1992).

The impact of water potential on yeast cell, regard to osmotic perturbation, is said to be an inverse function of the amount of dissolved solute - with the greatest potential damage occurring with rapid change in water activity (Briggs *et al.,* 2004). It's been found that majority of yeast cells display moderate growth tolerance under low water potential, with some been able to grow well at very low water potential (Barnett *et al.,* 1990).

However, upon arrival of an osmo-balanced environment, the yeast cells tend to re-establish cell volume which is dependent on temperature and permeability of osmoticum (i.e. buffering cellular solutes).

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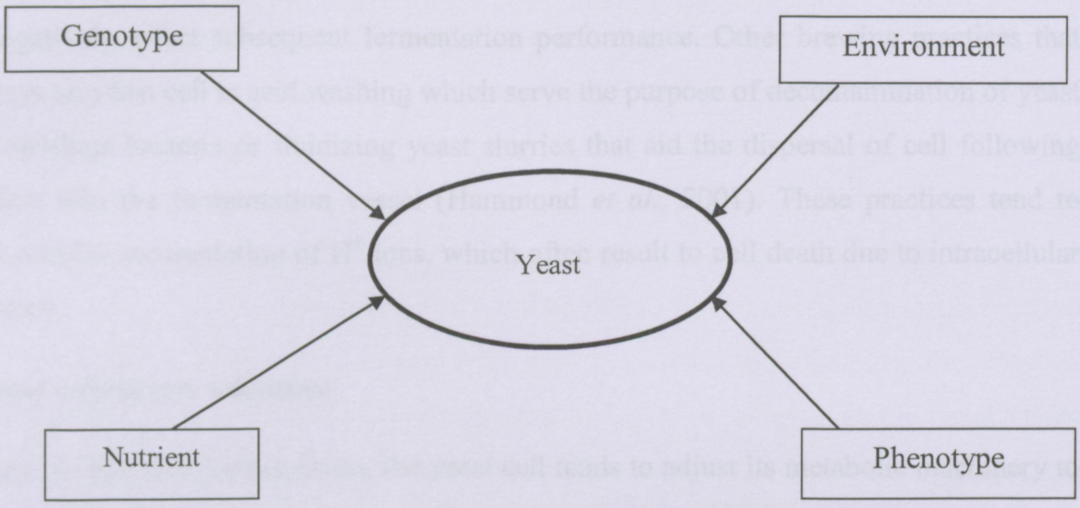


Figure 4. Factors affecting yeast behaviour

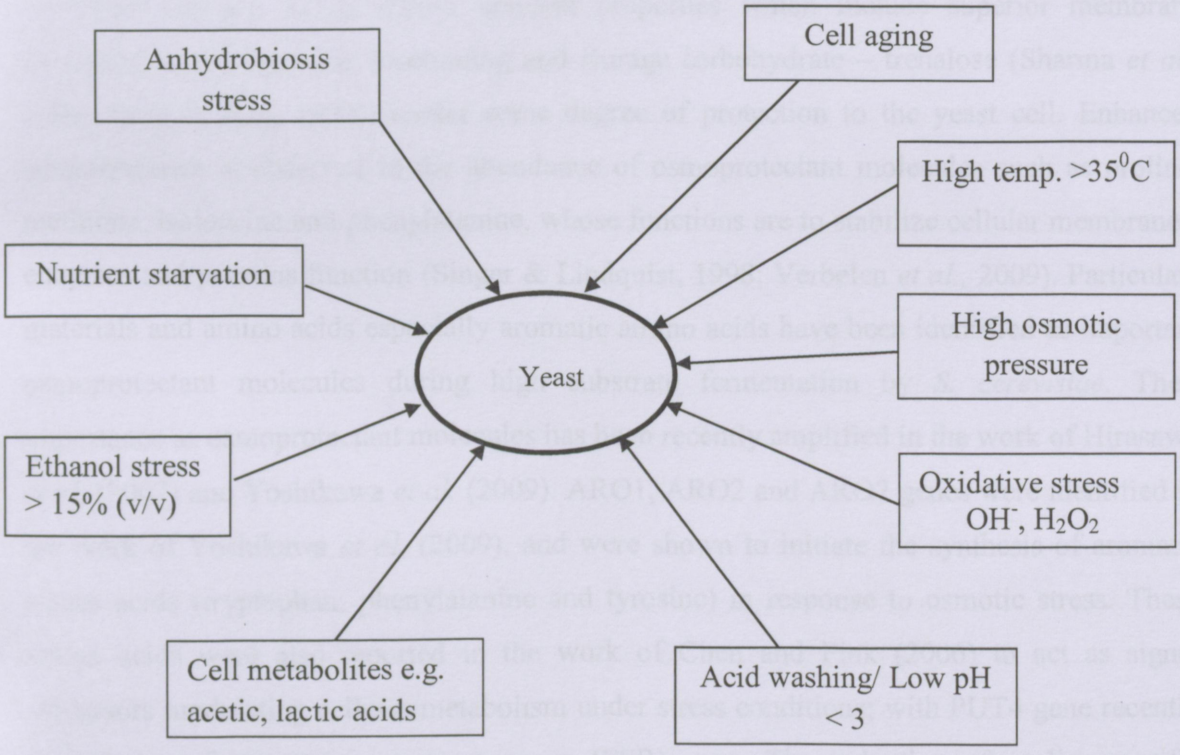


Figure 5. Major stresses experienced by yeast cell during very high gravity fermentation.

Source: Walker, (2004): Adapted.

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These physiological changes perhaps have some irreversible effects on the yeast cells that could negatively affect subsequent fermentation performance. Other brewing practices that exert stress on yeast cell is acid washing which serve the purpose of decontamination of yeast cells of spoilage bacteria or fluidizing yeast slurries that aid the dispersal of cell following inoculation into the fermentation vessel (Hammond *et al.,* 2001). These practices tend to increase cellular accumulation of H^+ ions, which often result to cell death due to intracellular acidification.

2.4.2 Yeast osmostress tolerance

In response to this abusive condition, the yeast cell tends to adjust its metabolic machinery to contain the effects of the stress. The ability of yeast cell to adapt to osmotic condition is known as osmostress tolerance and it exhibit two forms namely, osmotolerance and osmoadaption (Briggs *et al.,* 2004; Gibson *et al.,* 2007).

Osmotolerance is an adaptation due to inherent properties which enables the cell to cope with sub-lethal osmotic stress. These inherent properties which include superior membrane structure, proper vacuolar functioning and storage carbohydrate – trehalose (Sharma *et al.,* 1996; Nass & Rao, 1999), confer some degree of protection to the yeast cell. Enhanced osmotolerance is observed in the abundance of osmoprotectant molecules such as proline, methione, isoleucine and phenylalanine, whose functions are to stabilize cellular membranes, enzymes and proteins function (Singer & Lindquist, 1998; Verbelen *et al.,* 2009). Particulate materials and amino acids especially aromatic amino acids have been identified as important osmoprotectant molecules during high substrate fermentation by *S. cerevisiae*. Their importance as osmoprotectant molecules has been recently amplified in the work of Hirasawa *et al*. (2007) and Yoshikawa *et al.* (2009). ARO1, ARO2 and ARO7 genes were identified in the work of Yoshikawa *et al.* (2009), and were shown to initiate the synthesis of aromatic amino acids (tryptophan, phenylalanine and tyrosine) in response to osmotic stress. These amino acids were also reported in the work of Chen and Fink (2006) to act as signal precursors modulating cellular metabolism under stress conditions; with PUT4 gene recently identified as fermentation stress response (FSR) gene. The induction of proline-specific permease encoded by the gene (PUT4) was found to correlate positively upon ethanol and sorbitol shock (Kaino & Takagi, 2008; Marks *et al.,* 2008).

In dreaded osmotic condition, yeast cells tend to adopt a unique biochemical behaviour which alters its physiological state, in turn enhancing its tolerance to lethal osmotic stress condition.

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This adaptive behaviour which is known as osmoadaption involves the synthesis of buffering macromolecules or neutral polyols collectively called compatible solutes via the high osmolarity glycerol (HOG) signal transduction pathway (Hohmann, 2002; Gibson, *et al.,* 2007). Compatible solutes are cellular osmolytes which effectively substitute cellular water, restore cell volume and enable enzyme activity to continue (Walker, 2000). They include glycerol, arabitol, arabinitol, sorbitol, mannitol and erythritol. The most effective of the compatible solutes in yeast is glycerol, which under reduced water activity, are synthesized in order to control intracellular solute potential relative to medium, thus negating the effect of dehydration (Walker, 2000). Glycerol accumulation in *S. cerevisiae* upon salt-induced stress, have been shown to only take place after several hours of adaptation during which K, Na and trehalose contents increases (Singh & Norton, 1991). Glycerol has also been found to mitigate the detrimental effects of high ionic strength by stabilizing proteins within cell and the plasma membrane, and maintaining the liquid crystal state of membrane phospholipids during dehydration.

It has been found that changes in external osmotic pressure induce the transcription and expression of certain genes involved in the synthesis of compatible solutes. In *S. cerevisiae*, osmostress response is mediated by the HOG signal transduction cascade, whose function appears to be limited to high osmolarity (Ruis & Schuller, 1994; Briggs' *et al.*, 2004). As it will be discussed further in this literature, the transcriptional control elements called the stress response elements (STREs) which is activated by range of stress conditions activates the HOG pathway during osmotic stress. Involving various kinase cascades, the HOG is triggered upon hyperosmotic shock and in response to this; a metabolic signal is passed, which results in the transcription of genes SLN1 (encoding osmosensor) and HOG1 (whose gene product encodes the mitogen-activated protein kinase MAPK) (Ruis & Schuller, 1994). Upon expression, metabolic signal is sent through two separate osmosensors located in the plasma membrane to the mitogen-activated protein kinase signal transduction cascade (Maede *et al.,* 1994). This cellular events, initiates the synthesis of compatible solutes resulting in an osmo-balanced cell. The osmolarity balance is accompanied by the stability of enzymes, membrane proteins and phospholipids which further enhances tolerance (Mager & Varela, 1993). This characteristic is strain-dependent with some yeast strain out-performing others (Zhao & Bai, 2009).

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2.4.3 Ethanol stress

Use of sugar adjuncts in VHG fermentation, has led to higher ethanol concentrations which adversely affects the entire yeast physiology during fermentation. Amongst all stress conditions experienced by yeast cells during high gravity and VHG fermentation, ethanol stress is the most lethal and the ability or inability of the yeast to tolerate the stress is of commercial implication (Birch & Walker, 2000; Udeh & Kgatla, 2013). Under normal gravity fermentation, brewing yeast strains are tolerant to the usual ethanol concentration of 4 to 5% v/v but in VHG fermentation, the yeast cells are exposed to higher ethanol concentrations that often result in loss of most of the yeast's cellular functions. In VHG fermentation, ethanol concentrations can reach up to 18% v/v (Bai *et al.,* 2004) which exceeds the tolerable limit (11% v/v) of brewing yeast (Osman & Ingram, 1985; Puligundla *et al.,* 2011). At this stage, the yeast cell begins to displays various forms of physiological defects which range from loss of cellular ionic homeostasis due to dysfunctional H^+- ATPase activity, leakage of nucleotide and phosphate, increased membrane permeability, enzyme inactivation, growth inhibition, and loss of cofactors, which all often culminates to lysis (Furukawa *et al.,* 2004; Canetta *et al.,* 2006; Trofimova *et al.,* 2010). These cellular dysfunctions is said to result from the inhibitory effect of high ethanol concentrations on integral glycolytic enzymes and the alteration in cellular membrane lipid and protein composition (Birch & Walker, 2000; Ding *et al.,* 2009). However, glycolytic metabolism have been shown to be comparatively resistant to the inhibitory effects of ethanol, as little effect on denaturation of glycolytic enzymes was observed in *S. cerevisiae* around 13% w/v ethanol (Walker, 2000). Regard to growth inhibition, ethanol is considered a non-competitive inhibitor of yeast growth at relatively low concentrations.

The effects of ethanol stress during fermentation manifests as sluggish or stock fermentation, reduced cell growth and vitality, generation of petite yeast crop which consequently affects successive fermentation performance (Cahill *et al.,* 2000). Loss of cell viability has also been reported to ensue from other factors such as fermentation temperature and nutrient starvation (especially Mg^{2+} ions) (Gibson *et al.,* 2007). Functional overlap of heat shock and ethanol stress on yeast cells has been documented, where the impact of ethanol stress on yeast cells was enhanced in a progressive fashion under elevated fermentation temperatures (van Uden, 1989; Birch & Walker, 2000). Other metabolites like aliphatic alcohols, aldehydes, esters, organic acids, fatty acids, carbonyl and phenolic compounds, also exert adverse effects on the yeast cell, and the magnitude of their effect (especially aliphatic alcohols) is said to be

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dependent on the chain length of the molecules, such that the longer the chain the more toxic they are (Brigg's *et al.,* 2004).

Several mechanisms have been proposed through which the toxic effect of ethanol may be exerted. The non-specific osmotic effect (Jones & Gadd, 1990) and specific target sites (D' Amore *et al.,* 1988; Mishra, 1993) are the operative mode / area of ethanol toxicity. Of most implicated area of ethanol attack on the yeast cell, is the membrane structure (Piper, 1995). It's been generally known that exposure of yeast cells to ethanol increases membrane fluidity and consequentially decreases membrane structural integrity; which has been proposed to result from its insertion into the membrane hydrophobic interior and the resultant effect on polarity, exchange of polar molecules and position of membrane proteins (Weber & de Bont, 1996). The resultant effect of these membrane alterations include leakage of cellular components, inhibition of transport systems essential for nutrient uptake, alteration in membrane structure and increased membrane fluidity (Converti *et al.,* 1992; Lloyd *et al.,* 1993).

Another target site of ethanol toxicity is the mitochondria membrane, where it causes a mutagenic change on the mitochondria DNA (Walker, 2004; Gibson, 2011). This mutagenic change results in respiratory deficient strains, whose potential impact is a decrease in successive fermentation performance when the same crop of strain is successively used. Other area and membrane target sites of ethanol toxicity include; endoplasmic reticulum, hydrophilic soluble proteins of the cytoplasm, vacuolar, and nuclear membranes (Walker, 2000).

2.4.4 Yeast ethanol tolerance

In the face of this adversity, the yeast cell tends to portray a counteracting biochemical behaviour which is often referred to as yeast tolerance or 'domestication'. Yeast ethanol tolerance refers to the ability of specific yeast strain to withstand higher levels of ethanol without any deleterious effects on viability and metabolic activities (Walker, 2000). This stress response is characterised by the synthesis of heat shock and ethanol stress proteins, increase in intracellular storage carbohydrates (glycogen and trehalose) (Neves & Francois, 1992; Parsell & Lindquist, 1994; Vianna *et al.,* 2008), active production of compatible solutes (Briggs' *et al.,* 2004), modulation of ion homeostasis, biosynthesis of chaperone proteins that stabilizes denatured proteins, and the production of glutathione and stress enzymes such as ATPase and superoxide dismutase (Costa *et al.,* 1993: Lei *et al.,* 2007).

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Several studies have revealed that permutations of this cellular alteration confer tolerance to ethanol stress, of which their very significance depends on the type of stress and the physiological state of the yeast culture (Birch & Walker, 2000; Walker, 2004). With regards to ethanol stress, the extent of ethanol tolerance by yeast cell is usually considered to be genetically based and also dependent on the physiological status of the cell and physico-chemical nature (process parameters and bioavailability of essential nutrients) of the fermentation medium (Boulton & Quian, 2001). Numerous techniques have been employed in determining ethanol tolerance which include; growth suppression, variation in fermentation rate, rate of carbon dioxide evolution and extracellular acidification in the presence of exogenous ethanol. However, the final amount of ethanol produced by fermenting yeast cells has been shown to better reflect ethanol tolerance during fermentation.

Numerous studies have highlighted possible response mechanisms of yeast ethanol tolerance with two major stress pathways been highlighted herein. In the yeast cell, stress response mechanism is mediated by two major stress response pathways namely: i) Heat Shock Response pathway, which is activated at sub-lethal heat stress by the heat shock transcription factor and ii) the Global Stress Response (GSR) pathway, which is activated by lethal level of ethanol concentrations (Schmitt & McEntee, 1996; Verbelene *et al.,* 2009). These two stress pathways are not exclusive to ethanol stress but applicable to other environmental stresses such as thermal stress and osmotic stress.

The GSR pathway is believed to be an evolutionary adaptation that allows the yeast cell to respond to various stressful conditions in a non-specific manner by simultaneously activating specific responses while maintaining cellular fitness (Martinez-Pastor *et al.,* 1996; Galhardo *et al.,* 2007). During ethanol stress, the GSR pathway is up-regulated by set of c. 200 genes which have in their promotor region, a consensus sequence CCCCT known as stress response element (STRE) (Eide, 2003; Gibson *et al.,* 2007). The work of Gauci *et al.* (2009) identified the STREs consensus sequence to be a part regulatory element under condition of zinc starvation beside the zinc regulatory consensus sequence 5′-ACCYYNAAGGT-3′ known as zinc response elements (ZRE) (Van Ho *et al.,* 2002). The transcription of these STRE-driven genes is activated by the action of two transcriptional activators, Msn2p and Msn4p, which has been implicated with the mediation of the transcription of a separate zinc regulon besides the ZAP1 transcriptional activator during zinc starvation (Martinez-Pastor *et al.,* 1996; Lyons *et al.,* 2000). These transcriptional activators are known to be the principal mediators of the GSR pathway (Smith *et al.,* 1998).

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The STRE element which is present in many genes associated with stress response, is an active inducer of genes expressed under stress conditions - as it serve as the major interactive site for the GSR mediators (Msn2/4p) (Gibson *et al.,* 2007; Gauci *et al.,* 2009). The stress-induced expression, conferred by STRE is negatively regulated by the Ras cyclic adenosine monophosphate-protein kinase A (Ras-cAMP PKA) pathways whose activity is important in the biosynthesis and degradation of glycogen (Verbelene *et al.,* 2009). The importance of this regulatory scheme is observed in the variation of storage carbohydrates: glycogen and trehalose.

2.4.4.1 Glycogen alteration – a yeast ethanol stress response

Glycogen is the fourth minor cell wall component and most important reserve carbohydrate in yeast (Briggs *et al.,* 2004). Yeast performance during fermentation is dependent on the concentration level of glycogen - as its catabolic end-product (glucose) provides energy that drives the formation of essential lipids (sterols) required for reproduction (Quian & Tubb, 1982; Bolat, 2008). Glycogen level in yeast is an indicator of cell cycle progression and stress adaptation (Gibson *et al.,* 2007). It is regulated by the activity of glycogen synthase phosphatase (glycogen degrading enzyme), under the control of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) pathway – with glucose 6-phosphate and glucose 1-phosphate as precursors for its biosynthesis (Boulton, 2000).

During reverse glycolysis (gluconeogenesis) where the yeast cell feast on oxidative carbon sources (ethanol, glycerol, succinate, acetate), the intracellular cAMP levels increases – whose sole function is to activate the protein kinase A (PKA) pathway (Smith *et al.,* 1998). At this point (after lag phase), a maximum glycogen level is reached which is subsequently broken down by glycogen phosphorylase in the presence of phosphoric acid, and partly through a complex enzymatic reactions in which active protein kinase A (PKA) is liberated by the binding action of cAMP to inactive PKA (Boulton, 2000; Briggs *et al.,* 2004). These series of events culminate to cellular signalling for the mobilization of glucose, which is achieved by the action of the debranching enzyme (amylo-α-1-6-glucosidase) on α-1-6-glucosidic bonds (Boulton & Quain, 2001). The aftermath of this cellular events, is the decline in glycogen concentration which is consistent with the sustenance of the cell during fermentation.

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2.4.4.2 Trehalose alteration - a yeast ethanol stress response

The second storage carbohydrate, trehalose, is an important stress protectant and a metabolic signal molecule in yeast. This disaccharide represents one of the most effective sugars in the stabilization of biological membranes against osmotic shock (Walker, 2000) and other stress condition. Trehalose has been reported to be responsible for repairing denatured proteins (Ding *et al.,* 2009) and maintaining integrity of the yeast plasma membrane (Leao & van Uden, 1984; Neves & Francois, 1992). Its concentration level in yeast is believed to be related to stress tolerance and adaptation. This concept was evident in the report of Gadd *et al.* (1987) and MacKenzie *et al.* (1988); where trehalose accumulation increased in stationary phase *S. cerevisiae* cultures that were grown under low water potential, of which the cells displayed increased osmotolerance than actively dividing cells (Colin & Nelson, 2007).

Upon exposure to ethanol stress, the STRE regulated genes (TPS1, TPS2) become up-regulated and encodes the trehalose synthesizing enzymes trehalose 6-phosphate synthase (TPS1) and trehalose 6-phosphatase (TPS2) (Winderickx *et al.,* 1996; Zahringer *et al.,* 2000a). In the face of ethanol stress, the synthesized trehalose subsequently binds to the membrane structure and proteins (Francois & Parrou, 2001) which in turn confer stability to the plasma membrane (Mansure *et al.,* 1994), enzymes (Sola-Penna & Meyer-Fernandes, 1998), and repairs denature proteins (Simola *et al.,* 2000). These set of reactions enables the cell to withstand certain level of ethanol stress and is indicative of a stressed yeast cell, due to its detectable rapid accumulation. For example, in early fermentation phase of high and very high gravity fermentation, trehalose levels have been found to rise higher than glycerol and as such have been proposed as an important osmoprotectants and stress indicator (Majara *et al.,* 1996). Relevance of trehalose to osmotically stressed yeast cells have been reported (Trivedi & Jacobsen, 1986; van Dijck *et al.,* 1995).

The degradation of trehalose is also STRE regulated. It involves a series of reaction in which the fully glucose-activated cAMP/PKA pathway induces a posttranscriptional activation on NTH and ATH genes responsible for the synthesis of trehalose degrading enzymes (Zahringer *et al.,* 2000b; Briggs *et al.,* 2004). These genes encode two forms of enzymes; the cytosolic hydrolase (NTH1, NTH2) and the vacuolar trehalase (ATH). The earlier enzyme whose neutral form is called the neutral trehalase (NTH1) is implicated in the degradation of intracellular trehalose (Zahringer *et al.,* 2000a; Bolat, 2008). Upon reduction in concentration level of trehalose, a normal cellular activity is re-established. The timely degradation of

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trehalose has been shown to be vital to maintaining a prompt resumption of metabolic activity, as its accumulation is reported to have an inhibitory effect on the catalytic efficacy of essential enzymes like glutathione reductase and hexokinase (Sebollela *et al.,* 2004). For example, the report of Lewis *et al.* (1997) have shown high cellular trehalose content to confer no tolerance to stresses imposed by chemical (ethanol) and physical agents (heat shock, osmostress).

Through the above mechanism, the yeast cell is able to tolerate ethanol stress and other related stresses. This self-defensive response by yeast cell to adverse conditions, involves a whole system adjustment; that is to say, the variation of storage carbohydrate is just a part of an integrated cellular alteration. Other cellular changes which play significant role in yeast performance also exist.

2.4.4.3 Yeast ethanol stress response via lipid modification

Lipid is a compendium of molecules with structural function in yeast physiology. Their concentration level, which is dependent on the availability of oxygen, plays a stress mitigating role during the course of fermentation (Bai *et al.,* 2008). Of the various classes of lipid present in yeast; fatty acids, phospholipids and sterols are of utmost significance in ameliorating the fluidizing effect of ethanol stress on yeast plasma membrane. These lipids, which are synthesized via series of pathways, tend to undergo changes in concentration levels in response to ethanol stress (Lei *et al.,* 2007; Chi *et al.,* 1993). It has been reported by several authors (You *et al.,* 2003; Aguilera *et al.,* 2006), that these lipid molecules which are integral components of the plasma membrane plays a structural role in the yeast membrane and has been shown to confer tolerance against ethanol stress. In this regard, Mizoguchi and Hara (1997) have shown that palmitic acid-enriched cells exhibited enhanced tolerance to ethanol.

During ethanol stress, the yeast cell tend to show specific membrane responses which include increasing unsaturation index and proportionate increase in long chain fatty acids (Aguilera *et al.,* 2006). This reaction involves i) the desaturation of saturated fatty acids by the action of 9- fatty acid desaturase encoded by the desaturase OLE1 gene, and ii) the elongation of medium assimilated short-chain fatty acids through a membrane-bound fatty acid elongation system encoded by ELO1 genes (Briggs *et al.,* 2004). This reaction presumably accounts for the elevated UFAs, which are consequently incorporated into the membrane structure. The binding action of these UFAs is believed to have a counteracting response to the fluidizing

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effect of ethanol, as it antagonises the stereo-chemical effect of hydrophilic ethanol head groups on lipid components of the plasma membrane (Weber & de Bont, 1996; Alexandre *et al.,* 2001). It's been shown that *S. cerevisiae* cells grown in the presence of ethanol exhibited an increased monounsaturated fatty acids (especially oleic acid, 18:1) and a decrease in saturated fatty acids residues (palmitic acid, 16:1) (Walker, 2000). Also, increase in fatty acyl chain length and increase in the proportion of unsaturated fatty acids and sterols in the plasma membrane has been reported.

This phenomenon which is seen as a mechanism through which yeast cell tolerate ethanol stress, is supported by results of many researchers who observed a positive effect of increased UFAs index on ethanol stress (Moonjai *et al.,* 2002; You *et al.,* 2003). In the findings, oleic acid has been reported to be the most effective component against ethanol stress than palmitoleic acid. This modification also extends to other lipids such as sterol and phospholipids. Cellular ergosterol and inositol content has also been shown to positively correlate with ethanol tolerance in *S. cerevisiae* (Ding *et al.,* 2009). In combination with phospholipids, sterol confers fluidity on yeast plasma membrane which enables the cell to alter its membrane structure in a flexible and responsive way to ethanol stress and other related stresses.

The above briefly accounts for mechanisms through which the yeast cell struggle to offset harsh conditions during fermentation. However, yeast mineral nutrition amongst other nutritional stress alleviation strategy has been increasingly drawing scientific attention, as mineral ions has been shown to be vital activators and regulators of most biological processes in all living organisms.

2.5 Effect of mineral nutrition on yeast performance

Studies on yeast mineral nutrition probably ensued from the increasing evidence on genetic identity in most living organism, especially in human; such that the role of a metal in human could possibly play same role in lower organisms like yeast. Nutrients may generally refer to a collection of vital chemical substances essential for growth and vitality; mineral nutrient in this context, is indicative of all relevant chemical elements which are indispensably required by yeast cell for optimal performance. The need for mineral nutrition in the case of yeast cell includes; regulation of intracellular pH homeostasis, generation of proton motive force, osmoregulation and charge balancing, enzyme cofactor functions, metalloezyme structural functions and signal transduction functions (Walker, 2000).

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These elements which are often required at millimolar and micromolar concentrations (Jones & Greenfield, 1987) are classified into 'bulk' and 'trace' elements (Walker, 1994; Rees & Stewart, 1998; Walker, 2004), respectively. The bulk elements include potassium and magnesium, while the trace elements are zinc, calcium and manganese (Briggs' *et al.,* 2004). Other trace elements of physiological significance include Ba^{2+}, Fe^{3+}, Co^{2+}, Mo^{2+}, Ni^{2+} and Cu^{2+}. The trace elements are required in micromolar concentrations, as their excess is reported to be toxic.

These mineral nutrients (Table 2), although minute in amounts, are essential activators and modulators of numerous biological activities which are pivotal to yeast performance and survival. The functionality of the ATPase enzyme whose function drives the uptake of many yeast nutrients is dependent on the availability of hydrogen ions. Regard to pH homeostasis in yeast cells, the H^{+}- ATPase activity is important in modulating both intra and extracellular pH, which function is significant in the transport of nutrient via proton symporters or via the proton antiporter (Walker, 2000; Briggs' *et al.*, 2004). Potassium has been implicated with the maintenance of electrical neutrality as well as the activities of integral glycolytic enzyme (phosphofructokinase) in yeast cells. Manganese has been shown to be necessary for yeast growth and metabolism although at trace levels. The physiological and biotechnological significance of Ca^{2+} in yeasts is evident in its multifunctional role as a second messenger in the modulation of growth and metabolic responses of cells to external stimuli (Briggs' *et al.*, 2004). Copper and iron has also been shown to be essential nutrient for yeast, both acting as cofactor for several enzymes including the redox pigment of respiratory chain (Walker, 2000).

Yeast-mineral nutrition, which entails the preconditioning of yeast cells in metal-rich mediums or supplementation of fermentation medium with mineral ions, necessitates the understanding of the homeostasis of metals in yeast cell, their roles in yeast physiology and how they influence fermentation processes. Such knowledge, which is emphasized herein, will provide insight on how best to optimize these mineral nutrients with other system parameters under high substrate condition for high throughput while retaining high levels of yeast viabilities.

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Table 2. Average composition of mineral elements in *Saccharomyces cerevisiae*, requirements and functions.

Element/ composition (g/kg dry weight)	Requirement	Function	Reference
Potassium 22	2-4 mM	Maintains charge homeostasis, enzyme activity	(Walker, 2004; Briggs' 2004)
Magnesium 2.7	2-4 mM	Cell division, enzyme activator, stress suppressor	(Walker, 2004; Briggs' 2004)
Zinc 0.12	4-8 µM	Stress suppressor, enzyme activator, cell growth, protein and membrane maintenance	(Walker, 2004, Zhao *et al.,* 2009; Gauci *et al.,* 2009; Miyabe *et al.,* 2001)
Calcium 0.5	-	Cell interaction, membrane maintenance	(Walker, 2004; Trofimova *et al.,* 2010)
Barium 0.15	-	-	-
Phosphorus 16	-	-	-
Sulphur 3	-	-	-
Manganese 0.03	2-4 µM	Enzyme activity	(Walker, 2004)
Iron 0.1	1-3 µM	Cytochrome, Haem-protein component	(Walker, 2004)
Cobalt 0.005	0.1 µM	Enzyme activator	(Walker, 2004)
Molybdenum 0.0007	1.5µM	Nitrate metabolism, vitamin 12	(Walker, 2004)
Nickel 0.0025	~10 µM	Urease activator	(Walker, 2004)
Copper 0.05	0.1 µM	Enzyme activator, cobalamin	(Walker, 2004)

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2.5.1 Magnesium

Magnesium is an indispensible divalent metal ion involved in a wide range of biological processes (Elin, 1994; Bose *et al.,* 2011). Considered the most abundant intracellular divalent cation, this metal constitutes about 0.3% of the yeast dry weight and activates over 300 enzymes intimately involved in metabolic and bioenergetic pathways both in prokaryotes and eukaryotes (Rees & Stewart, 1998; Walker, 2004; Walker *et al.,* 2006). Maintenance of cellular integrity which includes structural stabilization of nucleic acids, polynucleotides, chromosomes, polysaccharides and lipids, has all been attributed to magnesium (Birch & Walker, 2000). It's been globally recognized in most living cells, that magnesium deprivation result in the loss of cell conformational status (Walker, 1994). Structural, functional, heavy metal detoxification and stress protection roles of Mg^{2+} in yeast have been demonstrated (Pisat *et al.,* 2009).

2.5.2 Magnesium homeostasis in yeast cell

Mg^{2+} is an essential enzyme cofactor and a key structural component of most biological molecules, yet the molecular identity of its regulation in yeast cell are still poorly elucidated (Lee & Gardner, 2005). Mg^{2+} homeostasis in yeast cell is important, as it is essential for maintaining growth and other metabolic functions. This regulation or homeostasis involves the localization, compartmentalization, sequestration (Walker, 2004) and subsequent release of the metal ion by specific membrane transport proteins. The yeast periplasmatic, mitochondrial and more importantly the vacuole are identified ionic channels and stores through which Mg^{2+} ions are regulated (MacDiarmid & Gardner, 1998; Koop *et al.,* 2005). This regulatory scheme which is often reported to be independent of metabolic energy though pH and membrane-gradient potentially driven, occurs in two phase namely; chemisorption and bioaccumulation (Blazejack *et al.,* 2008).

2.5.2.1 Chemisorption of magnesium ions in yeast

Chemisorption of Mg^{2+}, which is the first phase of its uptake, is dependent on the physiological fitness of the yeast cell wall. The yeast cell wall, which consist mainly of β-1, 3 glucan (*ca.* 50%), mannoproteins (*ca.* 40%), β-1, 6-glucan (*ca.* 10%) and chitin (1%) (Klis *et al.,* 2002) serve as binding site for Mg^{2+} due to the presence of negatively-charged anionic groups (phosphomanate, carboxyl, hydroxyl and amines) present on the outer layer of the cell wall (Chmiel, 1998). Under depleting condition, Mg^{2+} actively bind to the anionic groups and

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are subsequently internalised through the action of negatively charged Mg^{2+} transport proteins (Alr1 and Alr2). This uptake or binding process is reported by Blazejack and Duszkiewicz-Reinhard (2004) to proceed at a fast rate, which could presumably be as a result of the increasing number of cell wall components (as the yeast cell actively divides) and varying affinity of metal transporters to free Mg^{2+}. An earlier study on the order of affinity of divalent cation transport in *S. cerevisiae* was described by Fuhrmann and Rothstein (1968) as follows: $Mg^{2+} > Co^{2+} > Zn^{2+} > Mn^{2+} > Ni^{2+} > Ca^{2+} > Sr^{2+}$. It is apparent from the uptake sequence, that *S. cerevisiae* cells show an appreciable affinity and requirement for Mg^{2+} to other divalent cations, which is significant in most yeast cellular functions.

Mg dynamism at the cell wall level is significantly affected by factors such as temperature, pH, and number of viable yeast cells as well as the presence of other ions (Park *et al.,* 2003). A constant metal biosorption rate at temperature range of 5 - 25°C has been reported; with temperature range below 5°C exerting inhibitory effect on the binding process of metals to yeast cell wall (White & Gadd, 1987; Gniewosz *et al.,* 2007). Medium pH has been reported by Tuszynski & Pasternakiewicz (1999), to have a predictory role on the binding competence between hydrogen ions and metal cations for absorption sites on the yeast cell wall. Antagonistic relationship has been shown of magnesium to other metal ions. For example, strontium has been reported to partially remove magnesium from yeast vacuole under enhanced cell activity (Avery & Tobin, 1992). Cadmium has been shown to negatively affect the binding process of magnesium, while magnesium has been reported to incapacitate the yeast bioaccumulation capacity for zinc and manganese ions (Blackwell *et al.,* 1995; Briggs' *et al.,* 2004).

Another prime factor for magnesium uptake is yeast viability. Inhibitory effect on Mg binding process in high cell volumes has been reported by Park *et al.* (2003), although highly viable yeast cells have been shown to enhance Mg uptake (Blackwell *et al.,* 1995). Increased accumulation of Mg in the presence of 1 to 2% mannose has been demonstrated by Blazejack *et al.* (2008). In the report, cells that were grown in Mg and mannose-rich media, displayed higher amounts of absorbed Mg compared with the control which was enriched with magnesium alone. The influence of glucose was also shown to have no significant difference from the cells grown with mannose, suggesting same effect of both carbon sources on the binding capacity of yeast to magnesium.

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However, literature data has provided and recommended optimal temperature and pH ranges of 25 to 35°C and 4.0 to 8.0, respectively; with a low or dilute pitching concentration of viable yeast cells for enhanced absorption of Mg^{2+} ions (Gniewosz *et al.,* 2007; Pisat *et al.,* 2009).

2.5.2.2 Bioaccumulation of magnesium ions in yeast

Bioaccumulation, which is the second phase of Mg uptake, involves the transport of Mg via the cell wall and cytoplasmatic membranes into the cytosol of the yeast cell. The intracellular accumulation of Mg is primarily mediated by the principal Mg transport proteins Alr1p and Alr2p (aluminium-resistance permeases) which are encoded by ALR1 and ALR2 genes (Lee & Gardner, 2005). Both transport proteins belong to the membrane integral proteins or metal ion transporters (MIT) and are found to share characteristic resemblance in terms of structure, molecular weight and isoelectric point (MacDiarmid & Gardner, 1998; Hirumura & Sakurai, 2001); purportedly sharing same line of activity. The inactivation of both transport proteins has been reported to confer Mg-dependent growth and impaired Mg-uptake in yeast (Graschopf *et al.,* 2001).

These transporters serve as protein channels, as they generate potential gradient at both ends of the cytoplasmatic membrane which drives the flow of Mg^{2+} (Lui *et al.,* 2002; Schindl *et al.,* 2007). Although divergent in their basic sequence, the MIT proteins has been reported by Knoop *et al.* (2005) to be made up of a conserved structure with a pair of transmembrane domains close to the C-terminus and a triad of conserved residues (glycine-methionine-asparagine) that are essential for Mg uptake. The work of Lee and Gardner (2005), further reveals the genetics of the primary transport protein Alr1p; where large part of the N-terminal and C-terminals where shown not to be necessary for Mg-uptake. The hypothesis was justified, as mutants with truncations in amino acid (1 - 239) units of the N-termini and amino acid (1 - 53) units of the C-termini, displayed similar growth progress with the wide type grown under same Mg concentration of 4 mM. However narrow variable regions (240 - 396 units of the amino acids) have been shown to be essential for Mg-uptake with site mutagenesis on the amino acid units revealing conserved residues (Iluecine and methionine) to be critical for Mg-uptake (Lee & Gardner, 2005).

ALR1 and ALR2, which shares homologue identity with known Mg transport protein (CorA) in *Salmonella typhimurium*, are expressed upon low concentration of Mg^{2+} on the yeast cell wall (Hirumura & Sakurai, 2001; Pisat *et al.,* 2009). Upon activation, basically Alr1p mRNA,

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the proteins are subsequently internalised and degraded (Graschopf *et al.,* 2001) to release bounded Mg^{2+}. The released Mg^{2+} is subsequently sequestered by intracellular polyphosphates, RNA and ATP, leaving a minute quantity as free Mg^{2+} which is available for biochemical processes such as enzyme activation, intracellular pH regulation, osmoregulation, protein and membrane stabilization, and signal transduction pathways (Bui *et al.,* 1998; Walker, 2004). This process has been reported to proceed at a slower rate as compared to the chemisorption process (Blazejack & Duszkiewicz-Reinhard, 2004). A simultaneous uptake of Mg^{2+} and phosphate ions has been reported along with reserves of polymeric Mg-orthophosphate in the yeast vacuole during Mg transport (Okorokov *et al.,* 1974) and this scheme is believed to contribute to free Mg regulation in the yeast cytosol. Report by Beeler *et al.* (1997) have shown that cytosolic Mg^{2+} levels in *S. cerevisiae* are maintained within concentration range of 0.1 - 1.0 mM by regulatory fluxes across the vacuolar and plasma membrane.

The functionality of Alr1p is dependent on the concentration of Mg, with the metal transporter losing its function at Mg concentration exceeding 100 mM/L (MacDiarmid & Gardner, 1998). At this stage, the Alr1p is either modified for other functions or degraded in the vacuole, while Mg-uptake is undertaken by other mechanisms e.g. Alr2p. The Alr2p has been shown to compensate the loss of Alr1p under condition of over-expression and has been reported to physically associate with Alr1p *in vivo*, although not essential for growth (Wachek *et al.,* 2006).

Beside the principal Mg transporters, other envisaged transport proteins (Table 3) has been identified such as Mrs2p, Lpe10p and Mnr2 (Gadd, 1993; Pisat *et al.,* 2009). Mrs2p localized in the inner mitochondria membrane, regulate the entry of Mg into the mitochondria matrix, maintains magnesium concentration therein and ensure the proper functioning of the mitochondria (Bui *et al.,* 1998; Graschopf *et al.,* 2001). It has been shown for both higher and lower eukaryote that the mitochondria transport system for Mg^{2+} does not contribute significantly to the intracellular homeostasis of Mg^{2+} (Bygrave, 1976; Pisat *et al.,* 2009).

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Table 3. Some metal transporters in yeast

Gene	Metal	Localization	Reference
ALR1, ALR2	Mg^{2+}	Plasma membrane	(Walker, 2004; Knoop *et al.*, 2005)
MNR2	Mg^{2+}	Vacuolar membrane	(Lee & Gardner, 2005; Pisat *et al.*, 2009)
MRS2	Mg^{2+}	Mitochondria membrane	(Knoop *et al.*, 2005)
LPE10	Mg^{2+}	Mitochondria membrane	(Pisat *et al,*. 2009)
CCC1,ATX1	Mn^{2+}	Plasma membrane	(Walker, 2004)
ZRT1, ZRT2	Zn^{2+}	Plasma membrane	(Walker, 2004; Miyabe *et al.* 2001; Eide, 2006)
ZRT3	Zn^{2+}	Vacuolar membrane	(Eide, 2006)
COT1	Zn^{2+}	Vacuolar membrane	(Eide, 2003; Eide, 2006)
ZRC1	Zn^{2+}	Vacuolar membrane	(Miyabe *et al.*, 2001)
MSC2	Zn^{2+}	Endoplasmic recticulum	(Ellis *et al.*, 2004)
CTR1, CRT1, CCC2	Cu^{2+}	Plasma membrane, cytosol	(Walker, 2004)

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Structural and functional similarity has been reported for Mrs2p and Lpe10p, where Lpe10p undertake similar functional role of Mrs2p (Gadner, 2003) - which could perhaps be active under condition of Mrs2p inactivation. As hypothesized by Okorokov *et al.* (1974), the work of Pisat *et al.* (2009) has shown the yeast vacuole to be an intracellular store for excess Mg^{2+}, with the Mnr2 gene regulating the release of the metal ion via the vacuolar membrane (tonoplast). It was further revealed, that the regulatory role of Mnr2p is independent of the principal Mg-transport proteins Alr1p and Alr2p, as its over-expression independently suppressed the growth defects of mutants (*alr1 alr2*) of the primary Mg-transport proteins.

Other metal transport systems, although not specific to Mg^{2+} uptake, has also been reported to mediate Mg transport. Low affinity K^+ transport system has reported to enable Mg^{2+} uptake, although at a relatively slower rate, as it is only believed to mediate Mg^{2+} uptake under K^+-deficient condition (Borst-Pauwell, 1981). The trivalent metal chelator, siderophore, which is actively involved in the transport of iron, is also believed to exhibit affinity for Mg, although the process is reported to serve the purpose of Mg-uptake under high Mg deficient condition (Emelynova, 2001). Earlier studies on Mg homeostasis by Gunther *et al.* (1985) revealed a positive correlation of intracellular polyamines with free Mg^{2+}. Sodium and cadmium transport systems along with low-molecular proteins metallothioneins, and oligoamines like spermidine and putresine, have all been implicated with Mg^{2+} homeostasis (Truchlinski & Pasternak, 2002). It should be emphasized that this inter-metal homeostasis is not exclusive to Mg^{2+}, but extends to other divalent metals such as Co^{2+}, Zn^{2+}, Mn^{2+} and Ni^{2+} (MacDiarmid & Gardner, 1998), which all have relevance in yeast biotechnology.

2.5.3 Role of magnesium ions in yeast physiology

Increasing research interest in the role of mineral ions in living cells, has unfolded intriguing and novel cellular properties which are of biotechnological significance. Substantive insight into the importance of Mg in living cells has identified vital roles of magnesium in prokaryotes and eukaryotes. The work of Walker and Daffus (1980) reveals the un-substitutable role of Mg in the yeast cell cycle progression. Magnesium was found to re-establish growth in *Schizosaccharomyces pombe*, whose cells' division was initially inhibited with the metal chelator - ionophore A23187. It was further hypothesized in the study, that Mg act as a size transducer and growth enhancer, as its exhaustion and repletion was consistence with the initiation of tubulin polymerization and spindle breakdown which precedes cell division. This hypothesis is supported by the work of Maynard (1993), where entry of cells

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into the stationary phase and the time for maximum ethanol production and minimum sugar concentration coincided with maximum Mg^{2+} in the growth media. Positive correlations of cellular Mg^{2+} uptake and alcoholic fermentation have been reported for industrial strains of *S. cerevisiae* (Walker, 1996; Walker & Maynard, 1997). Similar phenomenon was also reported by Pironcheva, (1998). Although supportive phenomenon has been reported of Mg in yeast cell cycle progression, elucidation on the molecular mechanism of the proliferating and size transducing effect in yeast cells is still demanding - as it could be possible to maintain a constant viable population density which has been shown by Belloch *et al.* (2008) to be associated with high throughput and product uniformity.

The production of reactive oxygen species which are known to cause DNA damage are actively reduced upon elevation of Mg^{2+} ions, both in yeast and human cells (Maynard, 1993). The work of Giles and Czuprynski (2004) demonstrates the impact of Mg on yeast growth; where pathogenic fungi *Blastomyces dermatitidis* showed an enhanced growth at Mg^{2+} concentration of 0.1 mg/ml. The study of Blazejack (2006) has also implicated Mg^{2+} with growth-related stimuli such as nutrient uptake and growth factors and mating pheromones (Walker, 2000).

Genetic evidence on the growth stimulating effect of Mg has been shown in the work of MacDiarmid and Gardner (1998). In the report, mutants of the principal Mg transporter Alr1p was found to display enhanced growth at Mg^{2+} concentration of 50 to 500 mM. The work of Trofimova *et al.* (2010), showed a supportive phenomenon as exponentially growing cells were found to display maximum yeast biomass accumulation at Mg^{2+} level of 300 mg/L, although no effect was observed of Mg^{2+} supplementation in the stationary phase cultures. Mg^{2+} was further shown to have different effect on yeast viability for different growth phases; as cells obtained from exponential and stationary growth phases displayed moderate and high levels of viability, respectively. Improved tolerance to dehydration-rehydration treatments for stationary phase cells was also reported (Trofimova *et al.,* 2010). Walker (1998) reported improved cell viabilities of 0 and 5% for wine yeast which were subjected to 10 and 20% ethanol shock for 24 h under Mg concentrations of 50 and 500 ppm, respectively. Also, Hu *et al.* (2003) showed that 9 h exposure of yeast cell to 20% ethanol led to absolute cell death, unlike in the Mg-replete condition (85 ppm) where viabilities between 25 and 55% were reported.

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Anhydrobiosis study by Trofimova *et al.* (2010) implicated Mg with charge stabilization of membrane phospholipids, which are essential for maintaining membrane integrity. This conjecture was evident by the improved viability observed for slowly rehydrated cells which were pre-conditioned in various amounts of Mg^{2+}. Enhanced viability and reduced cell surface damage for Mg concentration of 20 mM after ethanol and thermal shock was reported in the work of Birch and Walker (2000). Unlike in the control, where aberrant cells and absolute cell death was recorded for Mg^{2+} concentration of 2 mM.

Of critical importance of Mg^{2+} in yeast is its stress counteractive property. The work of Birch and Walker (2000) simplistically demonstrates the protective effect of Mg to both heat and ethanol stress in yeast cells. Mg^{2+} was found to negate the synthesis of heat shock proteins (Hsps) whose sole function is to counter the damaging and fluidizing effects of thermal and ethanol stress. This protective role of Mg^{2+} was evident as very few heat shock proteins were expressed in actively growing cells which were conditioned in elevated Mg concentration (5 to 20 mM) prior to a 60 min heat shock treatment. Unlike in the control where most of the Hsps (150, 104, 90, 83, 70, 60, 46 and 30 kDa) and other lower molecular weight were expressed. It is apparent from these reports, that high-temperature fermentations such as HG and VHG fermentation could be improved by controlling the bioavailability of Mg^{2+}.

Unlike the basic antagonistic relationship of Mg^{2+} and Ca^{2+}, the work of Giles and Czuprynski (2004) has reported a novel relationship for both metals. Complete restoration of cell growth and enhanced membrane stability for *B. dermatitidis* and *S. cerevisiae* respectively were recorded in the presence of Mg^{2+} and Ca^{2+} (Trofimova *et al.,* 2010). It was hypothesized in the report, that both metals cooperatively stabilize and reduce fluctuation in plasma membrane fluidity which has been correlatively found to be an inverse function of yeast survival (Simonin *et al.,* 2008). However, Mg^{2+} have been shown to modulate the kinetics of Ca^{2+}, as it destabilizes Ca^{2+} complexes and actively counter the binding action of Ca^{2+} to intracellular and extracellular ligands (Levine & Coburn, 1984). Reports from other authors has identified Ca^{2+} as the dominating metal in the relationship due to its high affinity for binding sites on ligands such as ATP and nucleic acids (Walker, 1994).

With recent literatures demonstrating inverse correlation between Mg^{2+} and Ca^{2+}, it is apparent that the competence for dominance between both metals is hugely dependent on their concentration levels, with high level of Mg:Ca ratio having best impact on yeast

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physiology and in fermentation processes (Rees & Stewart, 1999; Birch *et al.,* 2002; Okon & Nwabueze, 2009). Also, metal detoxification role of Mg^{2+} has been revealed in the work of MacDiarmid and Gardner (1998), where overexpression of ALR gene conferred increased tolerance to aluminium and gallium. Although increased sensitivity to other divalent metal ions such as Co^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, La^{2+}, Cu^{2+} and Ca^{2+} was observed, it is apparent from the result that Mg counters the impact of aluminium toxicity in yeast, as the transporter Alr1p is basically specific to Mg- uptake.

2.5.4 Role of magnesium ions in ethanolic fermentation

Considering the central role of fermentation in biotechnology, it is apparent that Mg^{2+} has a great deal in predicting the performance of fermentation processes. Pyruvate kinase, phosphofructokinase, hexokinase, phosphoglycerate kinase and enolase have all been reported to show an absolute requirement for Mg^{2+} (Walker, 2004). Walker *et al.* (1990) hypothesized that Mg^{2+} exhibit dictating role in the metabolism of pyruvate; such that its availability controls metabolic carbon flux either into the pyruvate dehydrogenase or the pyruvate decarboxylase, in turn controlling switch between respiro-fermentative processes in yeast (Walker *et al.,* 2006). Smith (2001) also reported a close relationship between intracellular Mg^{2+} and activity of pyruvate decarboxylase. However, it is still unclear and demanding at the cellular level on how Mg^{2+} dictates the respiro-fermentative switch in the presence of oxygen.

Studies on the role of metal ions in fermentation processes have implicated magnesium with high throughput regards to ethanol and biomass yield. The work of Slininger *et al.* (2006) has shown the impact of Mg^{2+} on the fermentation of lignocellulosic material. Mg^{2+} was found to increase biomass and ethanol yield with xylose as substrate. Increased fermentation rate, cell viability and ethanol yield for both ale and lager yeast strains under normal (12°P) and high gravity (20°P) wort has been reported for Mg^{2+} concentration of 500 ppm, with best throughput for ethanol under oxygenated condition (Rees & Stewart 1999; Aleksander *et al.,* 2009). Villen *et al.* (2009) has shown that yeast cells requires a constant narrow range of Mg^{2+} in other to sustain high ethanol productivity, rather than its excessive accumulation, which has been believed to be proportionate to fermentation efficiency. This proposition is supported by the report of Pironcheva (1998) that showed higher concentration (>700 µM) of Mg levels, to have no further influence on ethanol yield. A steady biomass accumulation in the presence of Mg^{2+} has also been reported by Aleksander *et al.* (2009), which also point to

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the importance of Mg^{2+} to cell growth.

Mineral optimization study by Okon and Nwabueze (2009) has reported an optimum ethanol yield of 12.53% (v/v) for metal combinations of Mg^{2+}, Zn^{2+} and Ca^{2+}, with Mg^{2+} to Ca^{2+} ratio approximately 2:1. Another metal combination study under conventional and very high gravity wort condition by Rees and Stewart (1997) reported an increased fermentation rate for Mg:Ca ratio of 17:1 and 11:1 for 12 and 20°P, respectively, as compared to the control containing basal concentrations of Mg:Ca ratio of 3:1. It has also been shown that increasing amounts of Mg exerts positive influence on high substrate ethanolic fermentation, as it alleviates the impact of osmotic stress on the yeast cells (D'Amore *et al.,* 1988). Ordinate flocculation behaviour during fermentation has been shown in the presence of Mg^{2+}, with *ca.* 10 ppm substituting Ca^{2+} under experimental conditions (Stewart & Goring, 1976; Gibson, 2011). Also, increased fermentability and repression of the synthesis of stress proteins under condition of heat and ethanol stress was reported in the work of Thanonkeo *et al.* (2007). It is understandable from these viewpoints that Mg^{2+} availability is an index of yeast fermentative performance; as such supplementation of Mg^{2+} should be done with precision considering other counteractive parameters that could impede its bioavailability.

2.5.5 Zinc

Zinc is an essential nutritional component of all living organisms (Guerinot & David, 1999; Mayibe *et al.,* 2001). The discovery of the zinc-binding cytosolic metallothionein (MT) protein by Margoshes and Vellee in 1957 has fostered the interest of many researchers in the cell biology of zinc and of its transport mechanisms (Eide, 2006). Zn has been shown to be an essential co-catalytic factor for over 300 enzymes including alcohol dehydrogenase, Cu/Zn superoxide dismutase, carbonic anhydrase and many proteases (Guerinot & David, 1999; Eide, 2003). More importantly, Zn^{2+} serves as a structural component of zing-binding motifs of many transcriptional activators (Zhao *et al.,* 1998). However, its excess accumulation is potentially toxic (MacDiarmid *et al.,* 2000).

The terminology, 'zinc quota', which was first proposed by O'Halloran (2001), is used to infer the total amount of Zn sufficient to support the optimum growth of a cell (Eide, 2006; Simm *et al.,* 2007). In yeast cell, the Zn quota is approximately 1.5×10^7 atoms per cell, with over 20% being crucial for the proper conformation of ribosomes and functioning of major enzymes involved in ethanol formation and stress tolerance (Walker, 2004; Simm *et al.,*

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2007). Study of MacDiarmid *et al.* (2000) has shown that the yeast cell requires a minimum amount of 5 x 10^6 Zn atoms per cell to support growth. Several studies have also revealed that the function of about 3 – 5% of the yeast proteome are Zn-dependent, with over 2% of the yeast *Saccharomyces cerevisiae* genome containing Zn-dependent DNA binding domains (Schjerling & Holmberg, 1996; Bohm *et al.,* 1997). It is apparent from these cellular estimations, that the yeast cell possesses a tight regulatory mechanism that maintains the availability of this important metal.

2.5.6 Zinc homeostasis in yeast

The yeast cell has been a remarkable model system in the study of metal transport in prokaryotic and eukaryotic cells. Several studies have presented compelling evidence of the regulatory mechanisms that help sustain nutritional requirements as well as environmental stress (Bird *et al.,* 2000). Zn^{2+} homeostasis involve the transport of Zn^{2+} across the plasma membrane, binding of the metal to sequestering ligands (metallothioneins; phytochelatin and ferritin), storage in intracellular compartments (endoplasmic recticulum, mitochondria, vacuole, Golgi apparatus) and subsequent efflux from the cell (Gitan *et al.,* 1998; Eide, 2006). This scheme is mediated by set of transport proteins belonging to two known eukaryotic metal transport families namely the ZIP (Zinc-Iron Permease) and the CDF (Cation diffusion facilitator) (Eide, 2006).

2.5.6.1 Control mechanisms of zinc uptake in yeast

In yeast cell, Zn uptake is tightly regulated to ensure adequate level is acquired while preventing excess accumulation (Guerinot & Eide, 1999). Zn uptake in yeast, which is energy dependent and driven both by proton and potassium gradients, is primarily mediated by two Zn-specific uptake systems; the Zrt1-high affinity and Zrt2-low affinity system (MacDiarmid *et al.,* 2000; Dainty *et al.,* 2008). These uptake systems are controlled by the transcription of Zrt1 and Zrt2 transport proteins which are encoded by the ZRT1 and ZRT2 genes, respectively (Eide, 2003). Both proteins share 44% sequence identity and 67% similarity, and approximately 30 – 35% identity and 54 – 65% similarity to the IRT proteins (Guerinot & Eide, 1999). The Zrt1-high affinity for Zn have an estimated K_m of 10 nM Zn^{2+} which is active under zinc-limiting condition, while the Zrt2-low affinity for zinc is about 100 nM Zn^{2+} under zinc-replete condition (Zhao & Eide, 1996; Guerinot, 2000). The functionality of these uptake systems are regulated at the transcriptional and posttranslational level by the Zap1 transcriptional activator which is controlled by ZAP1 gene (Gitan & Eide, 2000). Under

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zinc limiting condition, the Zap1 induces the transcription of ZRT1 and ZRT2 gene as well as its own gene (ZAP1), through an autoregulatory mechanism (Rutherford & Bird, 2004).

2.5.6.1.1 Transcriptional control of zinc uptake in yeast

The transcriptional control of Zn uptake in yeast is regulated by the ZAP1 gene, whose function is dependent on Zn availability (Bird *et al.,* 2004). Zn inhibits ZAP1 function and down-regulate the expression of Zap1 target genes (ZRT1, ZRT2) under zinc-replete condition (MacDiarmid *et al.,* 2003). The work of Bird *et al.* (2000) revealed that the Zap1 is localized in the cell nucleus and its localization is unperturbed during Zn fluctuation. DNA sequence analysis of the ZAP1 and its target genes (ZRT1, ZRT2) have identified in their promoter regions, the presence of an 11 base pair sequence (5′-ACCYYNAAGGT-3′) called the zinc responsive elements (ZRE) (Gauci *et al.,* 2009). This consensus sequence was found to be sufficient and necessary for Zn responsiveness or transcriptional activity of Zap1 target genes as well as its own gene (ZAP1). Sequence analysis also revealed at least three ZREs in the ZRT1 gene, two in ZRT2 and one in the ZAP1genes (Zhao *et al.,* 1998). This ZRE profile of the Zap1 target genes was shown to be consistent with the function and differential sensitivity of ZRT1, ZRT2 and ZAP1 to zinc availability (Eide, 2003). It was shown that ZAP1 gene directly binds to the ZREs of ZRT1 and ZRT2 genes in a sequence-specific fashion, and their functionality was found to be unaffected regardless of their orientation to the start site of transcription (Bird *et al.,* 2004). This cellular circuitry is repressed at high intracellular Zn levels; although the repressive effect of Zn on ZRT2 gene was found at higher concentration unlike the ZRT1 gene. Thus, the transcriptional mechanism mediates the uptake of zinc ions from the environment via the plasma membrane, by the transcription and subsequent binding of the zinc transport proteins to the metal, which is consequently internalized in the posttranslational control.

2.5.6.1.2 Post-translational control of zinc uptake in yeast

The post-translational control of zinc uptake is induced upon exposure of yeast cells to high extracellular zinc levels or upon saturation of the yeast plasma membrane with zinc ions. It essentially involves the controlled removal of the transport proteins from the plasma membrane through endocytosis, and its subsequent degradation in the vacuole (Guerinot & Eide, 1999; Gitan & Eide, 2000; Mayibe *et al.,* 2000). The work of Gitan *et al.* (1998) showed that the Zrt1 protein is a stable plasma membrane protein in Zn^{2+} limited cells, as immunoblot analysis under Zn^{2+} limiting condition detected the protein in the subcellular

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fraction of the cell which is enriched with cellular membranes. It was envisaged that plasma membrane proteins are subjected to posttranslational modification (glycosylation and ubiquitination) which equips them for the endocytic or internalization phase (Guerinot & Eide, 1999). Analysis on the ubiquitination process or ubiquitin conjugation in yeast by Rotin *et al.* (2000) identified ubiquitin(s) Ubc4, Ubc5, Rsp5 to be responsible for the regulated endocytosis of Zrt1 as well as other plasma membrane proteins such as Ste2, Mal61 (Gitan *et al.,* 2003). The post-translational modification was apparent, as Zrt1 protein showed a marked increase in molecular mass (from 45 kDa to 53 – 63 kDa) following the addition of 8 kDa ubiquitin proteins (Gitan & Eide, 2000).

Immunoblot analysis on the Zrt1 proteins by Gitan *et al.* (2003) mapped the metal-responsive ubiquitination site to lysine residue (lys-195) found between TM 3 and 4 loop. This metal-responsive domain (MRD) was found to be necessary and sufficient for the ubiquitination process, as Lysine-195 to Arginine mutation abolished ZRT1 inactivation response. In response to yet an unidentified signal, the ubiquitinated Zrt1 protein accumulates in a clathrin-coated pit or vesicles which is subsequently engulfed into newly formed endosomes (Gitan *et al.,* 1998). The proteins are then transferred to the late endosome which is subsequently delivered to the vacuole where it is degraded by vacuolar proteases and subsequently releasing the metal ion. Although this Zn-induced endocytosis of Zrt1 was reported to be Zn-specific; cadmium and cobalt was also shown to induce similar response albeit in a less effective fashion. This effect was attributed to similar coordination chemistries of the metals to Zn, which allows maximum inactivation of the protein upon exposure to excess Zn. This cellular event consequently shuts off Zn uptake activity, thereby preventing excess accumulation of the metal. Thus, the post-translational mechanism constitutes the major pathway of Zn uptake and serving as a stress tolerance mechanism.

It is clear that the transcriptional and post-translational control systems function separately although harmoniously, to maintain intracellular Zn levels; with the transcriptional control system serving the purpose of Zn acquisition under condition of moderate Zn fluctuation while the post-transcriptional system responds to high level of Zn by inducing the endocytosis of the transport protein. However, some key questions still remains unanswered at these levels of Zn control i) how does Zn regulate the transcriptional and post-translational activity of the ZAP1 gene? ii) The mechanism that initiates the ubiquitination of Zrt1 protein in response to Zn and, iii) forms in which zinc is sensed by the Zrt1 protein.

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2.5.7 Role of zinc ions in ethanolic fermentation

Zinc is the most commonly utilized mineral supplement in fermentation processes and has been associated with crucial role in wort fermentation. Its availability is paramount to fermentation efficiency, as it plays diverse roles in yeast metabolism which drives important biochemical reactions that ensures efficient fermentation process. Zn has been implicated with gene expression machinery that regulates the metabolism of storage carbohydrates, biogenesis of mitochondria and flavour development in beer (De Nicola *et al.,* 2007). Differential regulation of genes has been reported in Zn deficient conditions (Higgins *et al.,* 2003). Redox balancing within cells has been shown to be dependent on Zn availability, of which its limitation result in overproduction of reactive oxygen species (ROS), which consequently causes DNA damage (Wu *et al.,* 2007). Zn-deprivation has also been reported to prevent budding and arrest cell growth in *S. cerevisiae* (Walker, 1998). Structural functions and enzyme activation properties of Zn in yeast cell have been documented (Leskovac *et al.,* 2002; Regalla & Lyon, 2006).

Yeast-zinc requirement is influenced by several factors such as presence of metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, K^{+}, and Na^{+}), concentration and time. Wort Zn^{2+} levels are variable, with value of 0.2 ppm Zn been optimum for standard gravity wort fermentation (Bromberg *et al.*, 1997), while concentrations below 0.1 ppm result in sub-optimal fermentation (Gibson, 2011). Antagonistic relationship between Zn^{2+} and Mn^{2+} has been documented (Helin & Slaughter, 1977). It's been shown that yeast-Zn requirement is influenced by the concentration of Mn^{2+} present in wort, with 0.6 ppm Zn having an inhibitory effect on fermentation when Mn levels are below 0.01 ppm. Although dependent on Mn^{2+} availability, concentrations as high as 65, 65.5, 325.7, 500 and 1300 ppm have been shown to impact positively on fermentation time, uptake of fermentable trisaccharides and ethanol yield (Jones & Greenfield, 1984; Rees & Stewart, 1998). Under high substrate condition (20°P), lager strains were found to cope better with excessive levels of Zn^{2+} than ale strains. This was attributed to either lower levels of Zn in the initial wort or Mn^{2+} availability. Also, it's been suggested that Zn^{2+} supplementation during pitching time ensure optimal utilization by yeast for improved performance (Gibson, 2011).

The rapid uptake of Zn^{2+} by yeast cells in the first hours of fermentation has been shown to reflect its importance in brewery fermentation processes (Poreda *et al.* 2009). Stuck or sluggish fermentation is characteristic of Zn^{2+}-deplete media. Improved fermentation rates,

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increased yeast growth and viability, high ethanol productivity and enhanced stress tolerance have been reported upon Zn supplementation (Tosun & Ergun, 2007). Increased ethanol yield at Zn concentration of 0.30-0.48 mg/l was reported in metal optimization study of by Okon and Nwabueze (2009). The study also showed that high levels of Mg^{2+} and Zn^{2+} to Ca^{2+} supports high ethanol productivity.

Improved cell viability under high gravity condition was reported in the work of Zhao *et al.* (2009). Viability of flocs after heat (45°C) and ethanol (20% v/v) shock increased from 60.2% in the control to 82.1%, 84.1% and 84.5% for $ZnSO_4$ concentrations of 0.01, 0.05 and 0.1 g/L, respectively. In the same study, enhanced ethanol production was observed in all concentrations, with 0.05 g/l showing highest ethanol yield of 114.5 ± 1.9 g/l in contrast to 104.1 ± 2.3 g/l in the control culture. High ethanol yield of 110.1 g/l against 101.6 g/l in the control was reported in the work of Xue *et al.* (2010) at Zn^{2+} concentration of 50 mg/l. Also, addition of Zn^{2+} to growth media was found to protect self-flocculating yeast cells against ethanol shock (30 min. exposure in 18% v/v ethanol solution), with a value of 0.02 g/L $ZnSO_4$ found to be optimal (Xue *et al.,* 2008). In all of the studies, reduced glycerol (the main contributor to chemical oxygen demand of waste water) was reported (Cibis *et al.,* 2002). However, the underlying mechanism of Zn^{2+} on ethanol and thermal tolerance of yeast *S. cerevisiae* still remains unclear and demanding.

It's been shown that average floc size has implication on yeast cellular metabolism and ethanol tolerance (Lei *et al.* 2007). The effect of Zn ions on yeast flocculating behaviour was shown in the work of Tarlor and Orton (1973) where 0.1 M $ZnCl_2$ (6540 ppm) was found to inhibit yeast flocculation at pH 7.6. Raspor *et al.* (1990) observed enhanced flocculation up to a concentration of 2.6 ppm, with reduced flocculation occurring thereafter. On the contrary, Wackerbauer *et al.* (2004) found that propagation of yeast with Zn^{2+} supplements led to enhanced flocculation, which probably resulted from increased growth rate and more rapid depletion of sugars from the system. Recent studies by Zhao *et al.* (2009) have reported an inverse correlation between yeast flocculence and zinc availability. It was found that all concentration of zinc (0.01, 0.05 and 0.1 g/L) resulted in reduced floc size which was accompanied with improved tolerance to ethanol and thermal stress. Although several *in vitro* studies have identified effects of Zn^{2+} on yeast flocculation properties, it is unlikely that these effects will be apparent during wort fermentation (Gibson, 2011).

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Regard to the influence of Zn ions on beer flavour; increased synthesis of esters (ethyl butyrate, isobutyl acetate, isopentyl acetate, ethyl hexanoate, ethyl acetate and isoamyl acetate) and higher alcohols (isobutanol and amyl alcohol) has been reported for Zn supplementation (*ca.* 0.5 ppm) (Seaton *et al.* 1990; Quilter *et al.* 2003). The work of De Nicola *et al.* (2007) on the effects of Zn-pre-conditioning on flavour profile of distillates produced from fermented distillery malt wort, reported an increase in higher alcohol with a reduced ethanol yield of 1.8% which is contrary to the role of Zn^{2+}. Considering the process impact of Zn^{2+} supplementation, if extra Zn^{2+} supplementation is to be carried out in effort to improve yeast ethanol productivity and tolerance during high substrate fermentation, the potential effects on ester synthesis and overall flavour profile of the finished product should be considered.

2.5.8 Barium

Barium, a highly alkaline, silvery-white metal is found naturally as barite ($BaSO_4$). It is very light and occurs in combined state with other elements, such as sulphur, carbon and oxygen. Ba^{2+} oxidizes in air, reacts vigorously with water to form hydroxide liberating hydrogen. Ba^{2+} reacts with almost all non-metals, often forming poisoning compounds, and has found application in X-ray photographic and fluoroscopic view of internal organs.

2.5.9 Role of barium ions on yeast and yeast fermentation processes

The effects of Ba on living cells have been generally recorded as toxic (Iqbal & Naz, 1989). Relatively, there has been no much work done on the effect of Ba on yeast physiology and its effects in fermentation processes, however other related study have been reported. Study on the effects of various cations on the flocculating behaviour of yeast, showed Ba^{2+} to inhibit yeast flocculation after La^{3+} and Sr^{2+} (Kuriyama *et al.,* 1991). Enzyme immobilization study by Meena and Raja (2004) reported a good enzymatic activity when Ba^{2+} was combined with strontium ions, although a lower activity was recorded for Ba^{2+} alone. Similar effect was reported in the work of Park and Khang (1995), where Ba alginate was found to exhibit best impact on cell activity and gel stability. Experimental study on the effect of cations on endogenous chloride and potassium current in *Xenopus oocytes,* revealed Ba effect on depolarization of cells to be concentration and time dependent (Tokimasa & North, 1996). Peak amplitude of depolarization on the sampled cells followed a concentration range of 1 mM, 100 µM and 10 µM where concentration of 1 µM was found to have no effect on the cells.

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Causes of growth inhibition may be multivariate. For example, imbalance in oxidation energy and phosphate potential has been shown to significantly affect ATP levels in cells hence affecting cell division and expansion (Minton and Wilson, 1973). In morphogenetic view point, cell division and expansion have been shown to be impaired by Ba toxicity (Debrath & Mukerji 1982). Functional comparison of plant inward-rectifier channels expression in yeast by Bertl *et al.* (1997), revealed potassium channels genes KAT1 and AKT1 to be impeded by Ba^{2+} with moderate effectiveness of 30-50% at 10 mM. Peroxidase, an iron porphyrin enzyme whose precise role in plant metabolism is still vague, is implicated with various plant functions. In the study of Iqbal and Naz (1989), Ba was found to increase peroxidase activity in the concentration range of 0.1 to 40 mM. It is apparent from these results that Ba exhibits an inhibitory effect on the functionality of most yeast cellular functions. However, the effect of Ba^{2+} in VHG medium have shown some positive effect regards to ethanol yield and substrate fermentability during a trial experimental run using synthetic medium. The effect of Ba^{2+} during fermentation of malt wort has not been reported and is the focus of the present study.

2.6 Methods of determining ethanol content in alcoholic beverages

Ethanol content is an index of quality and taxation factor (US Internal Revenue Service, 1970) for alcoholic beverages. Rapid, accurate, highly sensitive, and reproducible method of determining ethanol content is of utmost importance to alcoholic beverage industries, as precision is principal to product consistency. Table 4 is a list of methods for determining ethanol content in alcoholic beverages. Of the list of methods of determining ethanol content in alcoholic beverages is gas chromatography (GC), which is best in class regards to rapid determination time (7-8 min) of ethanol content, high sensitivity and resolution, and relatively obtainable device (Wang *et al.,* 2003).

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Table 4. Methods for determining ethanol content in alcoholic beverages

Methods	Remarks	Reference(s)
Dichromate oxidation spectrophotometry	Reliable but limited due to large sample volume and toxic nature of reagents	(Caputi *et al.*, 1968)
Oxidation of the distillate	Reliable but limited due to large sample volume and toxic nature of reagents	(Caputi & Wright, 1969)
Densimetric analysis	Relatively accurate and reproducible but limited due to complicated pre-treatment protocols and requires large sample volume	(Anonymous, 1992; AOAC, 1990).
Enzymatic method	Low accuracy, stability and reproducibility	(Jones, 1995)
Potentiometry	Low accuracy, stability and reproducibility	(Kokovkin & Smolyakov, 1995).
Biosensor	Low accuracy, stability and reproducibility	
Gas chromatography	Rapid, accurate, small sample volume, high resolution and high sensitivity, but time consuming pre-treatments	(Navilio *et al.*, 2001)
Capillary electrophoresis	High reproducibility and accuracy, but expensive to use	(Collin *et al.*, 1997)
Near Infra-Red spectroscopy	Low accuracy, time consuming in creating calibration curve	(Van der Berge *et al.*, 1997;
High performance liquid chromatography	Very low sensitivity and complicated pre-treatment procedures	Martin *et al.*, 1986)
Modular Raman spectrometry	High reproducibility and accuracy, but expensive to use	(Sanford & Mantooth, 2001)
Beer analyser	Low accuracy, time consuming in creating calibration curve	(AOAC, 1990)

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CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection

The malted barley [well modified (containing well developed enzyme)], hops and yeast (*Saccharomyces pastorianus*) used in the experiment were purchased from Brewmasters Incorporation, Johannesburg. Reagents were ordered from Rochelle chemicals and Mercks chemical stores. The experiment was carried out in the Food Science and Technology Department, School of Agriculture, University of Venda.

3.2 Yeast cultivation

Materials: Dry lager yeast (*S. pastorianus*), electronic weighing balance (Mettler PJ12), Incubator (Plant growth chamber MGC-250), centrifuge (Hettich Zentrifugen D-78532), glucose, yeast extract powder, peptone water buffered, nutrient broth, 250 ml conical flask (x3) and distilled water.

Method: Yeast propagation was done prior to the mashing process. Approximately 1 g of the dried yeast cells (*S. pastorianus*) was weighed and dissolved in 10 ml distilled water. The cells were first grown in a synthetic medium composed (g/l); glucose (20), yeast extract powder (5), peptone water buffered (3) and distilled water (100 ml) for 2 days at 18°C (Briggs' *et al.*, 2004). Upon observation of active fermentation (which was indicative by increased foam formation), the cells were harvested at 4000 rpm for 5 min. The harvested cells was washed with distilled water and emptied in nutrient broth composed of (g/l): Lab-lemco powder 1, yeast extract 2, peptone 5, and sodium chloride 5, and allowed to grow for 24 h at 18°C. This was done in order for the cells to reach their active fermentative fitness.

3.3 Wort preparation

Materials: Two thousand four hundred grams (2400 g) malt, seven grams (7 g) Saaz hops, Lugol's iodine, portable refractometer (Sper Scientific, 300003), thermometer (GH Zeal 76 mm 1mm), centrifuge (Bench top centrifuge, NF 1200), pH meter (Crison instrument E-08328), water bath (Labcon, WBH601), weighing balance (Mettler PJ 12), Autoclave (Speedy autoclave, WM-340), refrigerator, ceramic mortar, stainless bowel, sieve (aperture

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µm 2000), filter papers, aluminium foil, stirrer rod, dropper, 5000 ml conical flask (x1), 1000 ml conical flasks (x3), 10 ml test tube (x2) and distilled water.

Method: Wort gravity of 21°P was prepared by first removing the debris, thereafter milling the malt into grist using the ceramic mortar. A high ratio of fine to coarse grist was maintained during the milling. Care was also taken to ensure the milling process did not destroy the husks. After milling, the malt grist was mixed with warm water (50°C) in the ratio 1:4, allowed to stand for 20 min before raising the temperature to 65°C. The temperature was maintained for 1 h at 65°C with variations between 65 - 67°C. At 10 - 15 min intervals, the mash was stirred by swirling. After the first hour of mashing, the mash temperature was further raised to 76°C and allowed to stand for another 1 h at 76°C with variations between 76 - 78°C. Again 10 - 15 min intervals, the mash was stirred by swirling (Bamforth, 2003). The stirring process was to ensure a uniform temperature throughout the mash. Upon completion of the set time, the mashing process was discontinued and the mash was tested for the presence of fermentable sugar (saccharification).

Saccharification test

Two millilitres (2 mℓ) of the mash was drawn from the mash and emptied in a test tube with the aid of a dropper. Two drops of Lugol's iodine were added and gently mixed. A positive result was indicated of a pale yellow colouration, which stood for more than 30 min. After attaining saccharification, the wort was cooled to 20°C and decanted into 500 ml conical flasks. Clearer wort for the hop boiling process was obtained by centrifuging the wort solution at 5000 rpm for 3 min.

Hop boiling was done at 100°C, during which the grounded Saaz hops was added. The wort was allowed to boil for 1 h with temperature variations of 95 - 100°C, while stirring 15 - 20 min interval. This was done in order to further concentrate the wort. After the hop boiling period, the hoped wort was allowed to cool to 50°C and then sterilized at 121°C for 5 min. The wort was allowed to cool to 20°C in which white precipitates were observed and was subsequently filtered off using filter papers. After filtration, the clear hopped wort was stored at 4°C until use.

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3.4 Ethanolic fermentation

Materials: Hopped wort, zinc sulphate heptahydrate ($ZnSO_4$. $7H_2O$), magnesium sulphate heptahydrate ($MgSO_4$. $7H_2O$), barium chloride ($BaCl_2$. $2H_2O$), nutrient broth containing inoculums of *S. pastorianus*, electronic weighing balance (Mettler PJ12), incubator (Plant growth chamber MGC-250), refrigerator, micropipette, spatula, 50 ml volumetric flask (x 66) and distilled water.

Method: A total working volume of 1980 ml of the hopped wort (pH 5.25) was dispensed per 30 ml into 50 ml volumetric flasks. Quantified amounts of the salts (Table 3.1 and Appendix A) were added to the wort and subsequently pitched with 1 ml of the yeast inoculum. The control (C) medium which contained no added $ZnSO_4.7H_2O$, $MgSO_4.7H_2O$ and $BaCl_2.2H_2O$ salts was used to measure the effects of the mineral ions on wort fermentability. A total of 24 experimental runs with increasing concentrations and combinations of the salts were performed in triplicate. After mixing gently, the setup was aerated for 5 min before initiating fermentation. A static fermentation condition was maintained for 24, 48, 72 and 96 h at 18°C (Lima *et al.*, 2011).

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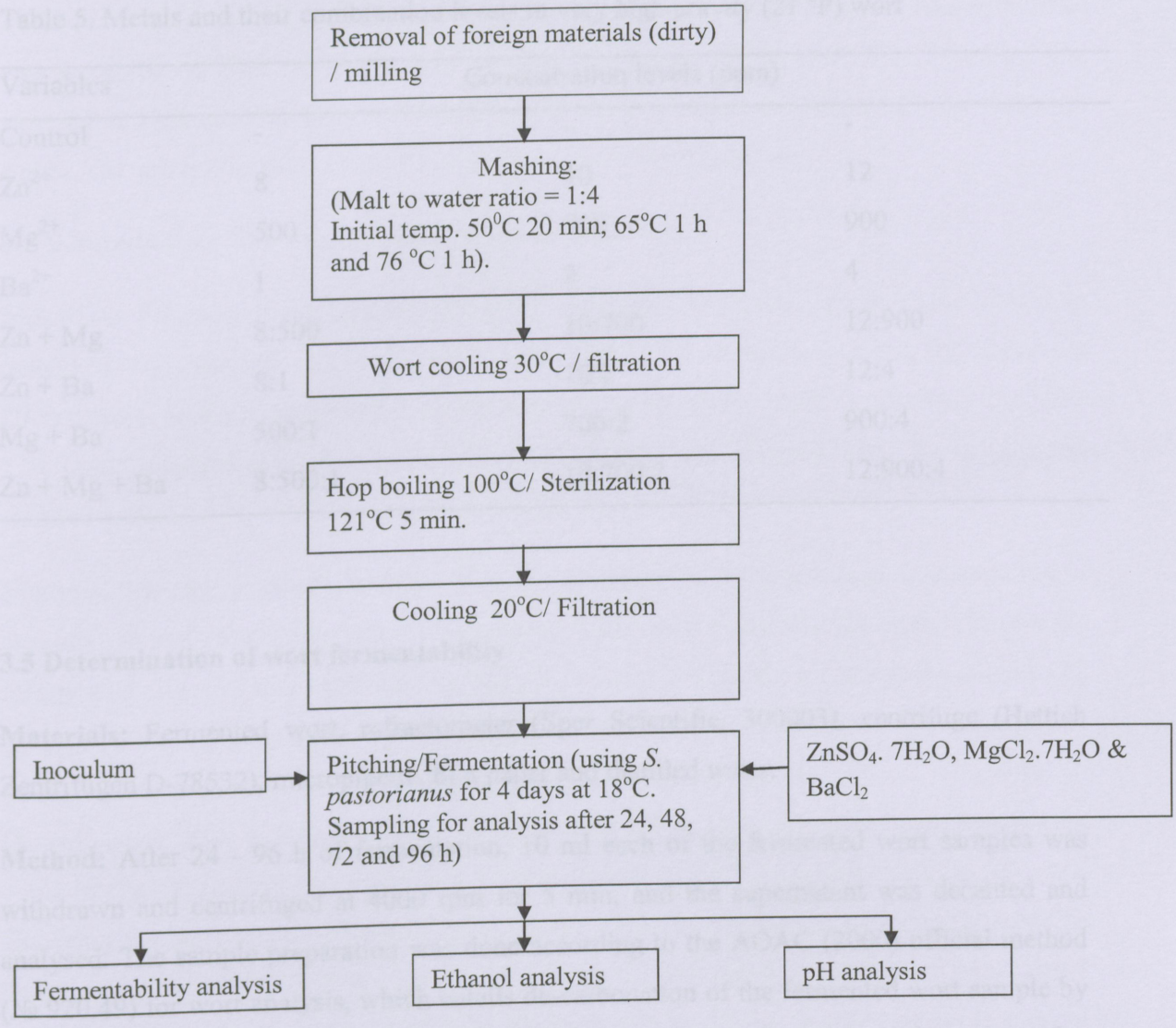


Figure 6. Experimental flow chart for analysing very high gravity fermented wort.

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Table 5. Metals and their combination levels in very high gravity (21 °P) wort

Variables	Concentration levels (ppm)		
Control	-	-	-
Zn^{2+}	8	10	12
Mg^{2+}	500	700	900
Ba^{2+}	1	2	4
Zn + Mg	8:500	10:700	12:900
Zn + Ba	8:1	10:2	12:4
Mg + Ba	500:1	700:2	900:4
Zn + Mg + Ba	8:500:1	10:700:2	12:900:4

3.5 Determination of wort fermentability

Materials: Fermented wort, refractometer (Sper Scientific, 300003), centrifuge (Hettich Zentrifugen D-78532), micropipette, blot paper and distilled water.

Method: After 24 - 96 h of fermentation, 10 ml each of the fermented wort samples was withdrawn and centrifuged at 4000 rpm for 5 min, and the supernatant was decanted and analysed. The sample preparation was done according to the AOAC (2000) official method (№ 920.49) for wort analysis, which entails de-carbonation of the fermented wort sample by shaking gently at first and then vigorously. This was accompanied with foam formation, after which 0.2 ml each of the clear decarbonated samples was placed on the optical part of the refractometer to obtain the brix. The brix value obtained which is °P equivalent was used to calculate the fermentability (Brigg's *et al.,* 2004) using the equation.

$$\text{Fermentability (\%F)} = \frac{\text{Original gravity} - \text{Final gravity} \times 100}{\text{Original gravity}}$$

3.6 Determination of pH of fermented wort

Material: Fermented wort, pH meter (Crison instrument, E-08328), pipette, 10 ml test tube (x 66) and distilled water.

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Method: After 24 - 96 h of fermentation, 5 ml of the fermented wort sample was withdrawn and degassed by shaking gently at first, then vigorously. Afterward, the pH probe was lowered into the mixture and the pH reading was recorded when the displayed value becomes stable. The probe was rinsed with deionised water after each analysis.

3.7 Determination of ethanol content of fermented wort

Materials: Fermented wort sample, Gas chromatography [GC specification: flame ionization detector (FID), stainless steel capillary column containing chromosorb mesh, N_2 carrier gas 20 mL /min, injector temperature 250°C, column temperature 220°C isothermal, run time 2.71 min], standard ethanol solution (absolute), pipette, 100 ml volumetric flasks (x4) and distilled water.

Method: Standard ethanol solutions of 6, 10, 16 and 20% were prepared from dilution of absolute ethanol solution in 100 ml volumetric flasks with distilled water and subsequently refrigerated. The GC was calibrated by injecting 10 µml of the ethanol standards into the GC column twice and peak height was generated. The peak area of the ethanol standards was determined and calibration curve of each standard was set against ethanol concentration (Navilio *et al.,* 2001.). Ethanol content of the fermented samples was determined by drawing 5 ml of the fermented mesh which was centrifuged at 4000 rpm for 5 min at 4°C and analysing the supernatant. Five millilitres (5 ml) of each supernatant was decarbonated as described in the AOAC (2000) official method (№ 920.49). From the fermented wort, 10 µℓ was injected into the GC and the ratio of the peak area of ethanol content of the fermented wort to the peak area of the ethanol standards was measured. Each of the analysis was carried out in triplicate. The ethanol content was calculated from the slope of the line equation derived from the calibration curve.

3.8 Statistical analysis

Statistical analysis was carried out on the triplicate data obtained from the fermentations. A Duncan multiple comparison test was used to analyse the data using SPSS version 9, with statistical significance accepted at 5% probability levels ($p \leq 0.05$).

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CHAPTER 4

RESULTS

4.1 Effect of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations on wort fermentability

Fermentability is the measure of the rate of total sugar utilization by yeast during fermentation. It is an important parameter in determining yeast performance during fermentation. The effect of three cationic concentrations of Zn, Mg, Ba ions and their combinations (Zn + Mg, Zn + Ba, Mg + Ba and Zn + Mg + Ba) on wort fermentability were determined and are presented in Table 6.

Sugar utilization from the medium is an essential parameter to be observed in fermentation. Table 6 illustrates the effect of three different concentration levels of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations (Zn + Mg, Zn + Ba, Mg + Ba and Zn + Mg + Ba) on wort fermentability with time. During the set period (24 – 96 h) of fermentation, a general increase in wort fermentability with time was observed for the entire media.

During the first 24 h of fermentation for the individual metal ions, the effect of Mg^{2+} at the evaluated concentration was found to have a more significant effect on wort fermentability compared to Zn^{2+} and Ba^{2+}. At the end of 96 h of fermentation, wort (media) supplemented with 500 ppm Mg^{2+} , 1 ppm Ba^{2+} and 8 ppm Zn^{2+}, was found with high (wort) fermentability of %F = 25.08, %F = 23.81 and %F = 23.17, which was not significantly different ($p \geq 0.05$), respectively. In the control medium, a lower fermentability of %F=10.90 was recorded.

In media containing the metal combinations, the cationic combinations of (Zn^{2+} and Mg^{2+}), (Mg^{2+} and Ba^{2+}) and (Zn^{2+}, Mg^{2+} and Ba^{2+}) displayed a rapid increase in fermentability for the first 24 h of fermentation unlike in the media supplemented with (Zn^{2+} and Ba^{2+}), where low fermentability was observed. After 96 h of fermentation, high wort fermentability of %F = 25.71 was recorded for wort (media) supplemented with 8:500:1 ppm (Zn^{2+}, Mg^{2+} and Ba^{2+}). Low wort fermentability of %F = 14.29 and %F = 13.65 were obtained for wort (medium) supplemented with the combination ratios of 500:1 ppm (Mg^{2+} and Ba^{2+}) and 8:500 ppm (Zn^{2+} and Mg^{2+}), respectively. The least wort fermentability of %F = 10.48 was obtained

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for wort (medium) containing the combination ratio of 8:1 ppm (Zn^{2+} and Ba^{2+}), which is not significantly different ($p \geq 0.05$) with the fermentability of the control medium (%F = 10.90).

For the second concentration level tested for the metal ions and their combinations high wort fermentability of %F = 27.94 and %F = 26.03 ($p \geq 0.05$) were obtained after 96 h of fermentation, in wort (media) supplemented with 700 ppm Mg^{2+} and 2 ppm Ba^{2+} respectively, compared to the control (%F = 10.90). A low wort fermentability of %F = 11.75 was recorded for wort (medium) supplemented with 10 ppm Zn^{2+}.

In wort (media) containing the metal combinations, high wort fermentability of %F = 27.00, %F = 27.00 and %F = 26.35 were obtained for wort (media) supplemented with the combination ratios of 10:700:2 ppm (Zn^{2+}+ Mg^{2+} + Ba^{2+}), 700:2 ppm (Mg^{2+} + Ba^{2+}) and 10:2 ppm (Zn^{2+} + Ba^{2+}) respectively. The least wort fermentability of %F = 15.87 was obtained for wort (medium) supplemented with 10:700 ppm (Zn^{2+} + Mg^{2+}).

For the third concentration level tested for the metal ions and their combinations; increased wort fermentability of %F = 27.38 and %F = 25.40 ($p \geq 0.05$) were recorded after 96 h of fermentation in wort (media) supplemented with 900 ppm Mg^{2+} and 12 ppm Zn^{2+}, respectively, compared to the control medium %F = 10.90. A low wort fermentability of %F = 10.80 was obtained in wort (medium) supplemented with 4 ppm Ba^{2+}.

In media containing the metal combinations, the highest wort fermentability of %F = 30.16 and %F = 29.53 were obtained in media supplemented with 12:900:4 ppm (Zn^{2+} + Mg^{2+} + Ba^{2+}) and 900:4 ppm (Mg^{2+} + Ba^{2+}) respectively. A relatively high wort fermentability of %F = 27.30 was obtained for wort (medium) containing 12:900 ppm (Zn^{2+} and Mg^{2+}). The least wort fermentability of %F = 22.54 ($p < 0.05$) was obtained for wort (medium) containing 12:4 ppm (Zn^{2+} + Ba^{2+}).

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Table 6. Effect of Zn, Mg, Ba and their combinations on wort fermentability

Period (h)	Fermentability (%F)							
	Control	Zn^{2+}	Mg^{2+}	Ba^{2+}	Zn + Mg	Zn +Ba	Mg + Ba	Zn + Mg + Ba
24	4.76 ± 0.0^{a} (0 ppm)	4.13 ± 0.55^{a} (8 ppm)	6.67 ± 0.00^{b} (500 ppm)	3.17 ± 1.10^{c} (1 ppm)	10.48 ± 0.00^{d} (8:500 ppm)	5.71 ± 0.00^{e} (8:1 ppm)	10.80 ± 0.55^{d} (500:1 ppm)	7.62 ± 0.00^{f} (8:500:1 ppm)
		5.24 ± 0.48^{a} (10 ppm)	10.16 ± 0.55^{b} (700 ppm)	4.76 ± 0.00^{a} (2 ppm)	12.38 ± 0.00^{c} (10:700 ppm)	3.49 ± 0.55^{d} (10:2 ppm)	9.20 ± 0.55^{e} (700: 2 ppm)	9.20 ± 0.55^{e} (10:700: 2 ppm)
		9.52 ± 0.00^{b} (12 ppm)	12.06 ± 0.55^{c} (900 ppm)	5.71 ± 0.00^{d} (4 ppm)	11.43 ± 0.00^{e} (12:900 ppm)	3.49 ± 0.55^{f} (12:4 ppm)	11.43 ± 0.00^{g} (900:4 ppm)	11.43 ± 0.00^{g} (12:900:4 ppm)
48	5.39 ± 0.55^{a} (0 ppm)	7.62 ± 0.00^{b} (8 ppm)	11.75 ± 0.55^{c} (500 ppm)	7.62 ± 0.00^{b} (1 ppm)	10.48 ± 0.00^{d} (8:500 ppm)	6.99 ± 0.55^{b} (8:1 ppm)	10.81 ± 0.58^{d} (500:1 ppm)	10.80 ± 0.55^{d} (8:500:1 ppm)
		6.03 ± 0.55^{a} (10 ppm)	14.92 ± 0.55^{b} (700 ppm)	10.48 ± 0.00^{c} (2 ppm)	13.33 ± 0.00^{d} (10:700 ppm)	8.89 ± 0.55^{e} (10:2 ppm)	12.70 ± 0.55^{d} (700:2 ppm)	13.02 ± 1.10^{d} (10:700:2 ppm)
		11.43 ± 0.00^{b} (12 ppm)	16.19 ± 0.00^{c} (900 ppm)	6.03 ± 0.55^{a} (4 ppm)	16.19 ± 0.00^{c} (12:900 ppm)	7.94 ± 1.10^{d} (12:4 ppm)	15.62 ± 0.49^{c} (900:4 ppm)	14.29 ± 0.00^{e} (12:900:4 ppm)
72	8.57 ± 0.95^{a} (0 ppm)	11.75 ± 1.10^{b} (8 ppm)	15.87 ± 0.55^{c} (500 ppm)	12.38 ± 0.00^{b} (1 ppm)	12.00 ± 0.00^{b} (8:500 ppm)	8.57 ± 0.00^{d} (8:1 ppm)	12.38 ± 0.00^{b} (500:1 ppm)	17.46 ± 0.55^{e} (8:500:1 ppm)
		8.89 ± 0.55^{a} (10 ppm)	20.32 ± 0.55^{b} (700 ppm)	16.82 ± 0.55^{c} (2 ppm)	14.29 ± 0.00^{d} (10:700 ppm)	14.61 ± 0.55^{d} (10:2 ppm)	17.46 ± 1.98^{c} (700:2 ppm)	18.73 ± 0.55^{f} (10:700:2 ppm)
		16.82 ± 0.55^{b} (12 ppm)	20.95 ± 0.00^{c} (900 ppm)	8.10 ± 0.74^{a} (4 ppm)	19.37 ± 0.55^{d} (12:900 ppm)	13.97 ± 0.00^{e} (12:4 ppm)	20.63 ± 0.55^{c} (900:4 ppm)	20.63 ± 0.55^{c} (12:900:4 ppm)
96	10.90 ± 0.48^{a} (0 ppm)	23.17 ± 3.07^{b} (8 ppm)	25.08 ± 3.85^{b} (500 ppm)	23.81 ± 1.65^{b} (1 ppm)	13.65 ± 0.55^{c} (8:500 ppm)	10.48 ± 0.00^{a} (8:1 ppm)	14.29 ± 0.00^{c} (500:1 ppm)	25.71 ± 1.65^{b} (8:500:1 ppm)
		11.75 ± 1.45^{a} (10 ppm)	27.94 ± 1.10^{b} (700 ppm)	26.03 ± 0.55^{b} (2 ppm)	15.87 ± 0.55^{c} (10:700 ppm)	26.35 ± 2.20^{b} (10:2 ppm)	27.00 ± 0.58^{b} (700:2 ppm)	27.00 ± 1.99^{b} (10:700:2 ppm)
		25.40 ± 1.46^{b} (12 ppm)	27.38 ± 1.15^{b} (900 ppm)	10.80 ± 0.55^{a} (4 ppm)	27.30 ± 0.55^{b} (12:900 ppm)	22.54 ± 2.74^{c} (12:4 ppm)	29.53 ± 2.52^{d} (900:4 ppm)	30.16 ± 1.46^{d} (12:900:4 ppm)

Values in the same row having different superscript for different concentration of each metal are significantly different ($p < 0.05$)

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4.2 Effect of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations on wort pH

Wort pH is the measure of the drift to acidity or alkalinity of the medium during fermentation. Although not considered much as a critical factor affecting yeast performance, wort pH during fermentation plays a dictating role in metal uptake in yeast cells, and as such controls the impact of the metal ions on the fermentation progress. The effect of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations (Zn + Mg, Zn + Ba, Mg + Ba and Zn + Mg + Ba) on wort pH as affected by yeast performance were determined.

Table 7 demonstrates the effect of the three cationic concentration level tested for Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations (Zn + Mg, Zn + Ba, Mg + Ba and Zn + Mg + Ba) on wort pH. During 24 – 96 h of fermentation, a general decrease in wort pH was observed in the entire wort (media). It was observed for all concentration levels tested after 24 h that the addition of Zn^{2+} and Ba^{2+} resulted in an initial increase in wort pH and thereafter a decrease (increasing acidity).

For the first concentration level tested, significant decrease in wort pH of 4.78 was recorded after 96 h of fermentation in wort (medium) supplemented with 500 ppm Mg^{2+}, compared to wort pH of 4.88 and 4.83 recorded for wort (medium) supplemented with 8 ppm Zn^{2+} and 1ppm Ba^{2+} ions, respectively. For wort media supplemented with the metal combinations, it was found that the pH of the medium supplemented with 8:500:1 ppm (Zn^{2+} + Mg^{2+} + Ba^{2+}) decreased significantly (pH 4.77) compared to wort pH of other combinations. Also, reduced pH of 4.82, 4.85 and 4.90 was recorded for wort medium supplemented with the combination ratios of 500:1 ppm (Mg^{2+} and Ba^{2+}), 8:500 ppm (Zn^{2+} and Mg^{2+}) and 8:1 ppm (Zn^{2+} and Ba^{2+}) respectively.

After 96 h of fermentation for the second concentration level tested, a significant decrease in wort pH of 4.75 was recorded for wort (medium) supplemented with 700 ppm Mg^{2+} compared to wort pH of 4.96 and 4.87 for wort (medium) containing 10 ppm Zn^{2+} and 2 ppm Ba^{2+}, respectively. In wort (media) supplemented with the metal combinations, pH of 4.77, 4.77 and 4.78 was obtained for wort media supplemented with the metal combination ratios of 10:700:2 ppm (Zn^{2+}, Mg^{2+} and Ba^{2+}), 700:2 ppm (Mg^{2+} and Ba^{2+}) and 10:700 ppm (Zn^{2+} and Mg^{2+}) respectively. The least effect on wort pH was recorded for wort (medium) supplemented with 10:2 ppm (Zn^{2+} and Ba^{2+}) with pH of 4.87.

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Table 7. Effect of Zn, Mg, Ba and their combinations on wort pH

Period (h)	Wort pH							
	Control	Zn^{2+}	Mg^{2+}	Ba^{2+}	Zn + Mg	Zn + Ba	Mg + Ba	Zn + Mg + Ba
24	5.32 ± 0.02^{a} (0 ppm)	5.30 ± 0.01^{b} (8 ppm)	5.23 ± 0.02^{c} (500 ppm)	5.32 ± 0.01^{c} (1 ppm)	5.19 ± 0.01^{d} (8:500 ppm)	5.31 ± 0.01^{a} (8:1 ppm)	5.18 ± 0.02^{d} (500:1 ppm)	5.15 ± 0.01^{e} (8:500:1 ppm)
		5.30 ± 0.06^{a} (10 ppm)	5.17 ± 0.03^{b} (700 ppm)	5.26 ± 0.00^{c} (2 ppm)	5.17 ± 0.01^{b} (10:700 ppm)	5.25 ± 0.01^{c} (10:2 ppm)	5.15 ± 0.02^{b} (700:2 ppm)	5.13 ± 0.01^{b} (10:700: 2 ppm)
		5.26 ± 0.05^{b} (12 ppm)	5.21 ± 0.02^{c} (900 ppm)	5.32 ± 0.03^{a} (4 ppm)	5.17 ± 0.03^{c} (12:900 ppm)	5.27 ± 0.01^{b} (12:4 ppm)	5.13 ± 0.01^{d} (900:4 ppm)	5.11 ± 0.01^{d} (12:900:4 ppm)
48	5.14 ± 0.04^{a} (0 ppm)	5.08 ± 0.02^{b} (8 ppm)	5.01 ± 0.03^{c} (500 ppm)	5.00 ± 0.03^{c} (1 ppm)	5.02 ± 0.02^{c} (8:500 ppm)	5.10 ± 0.03^{b} (8:1 ppm)	4.99 ± 0.02^{c} (500:1 ppm)	4.91 ± 0.04^{d} (8:500:1 ppm)
		5.08 ± 0.02^{b} (10 ppm)	5.00 ± 0.04^{b} (700 ppm)	5.04 ± 0.08^{b} (2 ppm)	4.98 ± 0.03^{b} (10:700 ppm)	5.03 ± 0.03^{b} (10:2 ppm)	4.93 ± 0.02^{c} (700:2 ppm)	4.91 ± 0.03^{c} (10:700:2 ppm)
		5.05 ± 0.03^{b} (12 ppm)	4.94 ± 0.03^{c} (900 ppm)	5.11 ± 0.02^{a} (4 ppm)	4.99 ± 0.02^{d} (12:900 ppm)	5.07 ± 0.02^{b} (12:4 ppm)	4.91 ± 0.02^{c} (900:4 ppm)	4.91 ± 0.02^{c} (12:900:4 ppm)
72	5.01 ± 0.01^{a} (0 ppm)	4.93 ± 0.01^{b} (8 ppm)	4.89 ± 0.02^{b} (500 ppm)	4.91 ± 0.02^{b} (1 ppm)	4.94 ± 0.02^{c} (8:500 ppm)	4.97 ± 0.02^{c} (8:1 ppm)	4.92 ± 0.04^{b} (500:1 ppm)	4.83 ± 0.02^{d} (8:500:1 ppm)
		5.03 ± 0.06^{a} (10 ppm)	4.85 ± 0.07^{b} (700 ppm)	4.91 ± 0.05^{c} (2 ppm)	4.90 ± 0.04^{c} (10:700 ppm)	4.92 ± 0.01^{c} (10:2 ppm)	4.86 ± 0.02^{b} (700:2 ppm)	4.84 ± 0.01^{b} (10:700:2 ppm)
		4.91 ± 0.05^{b} (12 ppm)	4.87 ± 0.04^{c} (900 ppm)	5.01 ± 0.02^{a} (4 ppm)	4.86 ± 0.02^{c} (12:900 ppm)	4.89 ± 0.02^{b} (12:4 ppm)	4.84 ± 0.01^{c} (900:4 ppm)	4.84 ± 0.02^{c} (12:900:4 ppm)
96	4.99 ± 0.05^{a} (0 ppm)	4.88 ± 0.03^{b} (8 ppm)	4.78 ± 0.03^{c} (500 ppm)	4.83 ± 0.02^{d} (1 ppm)	4.85 ± 0.05^{d} (8:500 ppm)	4.90 ± 0.01^{b} (8:1 ppm)	4.82 ± 0.02^{d} (500:1 ppm)	4.77 ± 0.02^{c} (8:500:1 ppm)
		4.96 ± 0.12^{a} (10 ppm)	4.75 ± 0.02^{b} (700 ppm)	4.87 ± 0.05^{c} (2 ppm)	4.78 ± 0.01^{b} (10:700 ppm)	4.87 ± 0.01^{c} (10:2 ppm)	4.77 ± 0.02^{b} (700:2 ppm)	4.77 ± 0.02^{b} (10:700:2 ppm)
		4.88 ± 0.03^{b} (12 ppm)	4.78 ± 0.03^{c} (900 ppm)	4.98 ± 0.03^{a} (4 ppm)	4.79 ± 0.01^{c} (12:900 ppm)	4.79 ± 0.01^{c} (12:4 ppm)	4.78 ± 0.02^{c} (900:4 ppm)	4.75 ± 0.02^{c} (12:900:4 ppm)

Values in the same row having different superscript for different concentration of each metal are significantly different ($p < 0.05$)

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For the third concentration level tested for the metal ions and their combinations, a significant decrease in wort pH of 4.78 was recorded for wort (medium) supplemented with 900 ppm Mg^{2+}; compared to wort (medium) supplemented with 12 ppm Zn^{2+} and 4 ppm Ba^{2+} ions. The least effect on wort pH of 4.98 was observed for wort (medium) containing 4 ppm Ba^{2+}. For wort (media) containing the metal combinations, there were no significant difference ($p \geq 0.05$) in pH; with wort pH of 4.75, 4.78, 4.79 and 4.79 recorded in the order of the metal combinations Zn + Mg + Ba, Mg + Ba, Zn + Mg and Zn + Ba.

4.3 Effect of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations on ethanol yield

Ethanol yield indicates the amount of ethyl alcohol produced by yeast during fermentation. It is a critical parameter in determining yeast performance during ethanol fermentation processes. The effect of increasing amounts of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations (Zn + Mg, Zn + Ba, Mg + Ba and Zn + Mg + Ba) on ethanol yield was determined.

Table 8 shows the effect of three different concentration levels tested for Zn, Mg, Ba and their combinations (Zn + Mg, Zn + Ba, Mg + Ba and Zn + Mg + Ba) on ethanol yield with time. A general increase in ethanol yield was observed during the fermentation period. After 96 h of fermentation for the first concentration level tested for the individual metal ions and their combinations, high ethanol yield(s) of 6.6815% (v/v), 6.3526% and 6.2492% were obtained for wort (media) supplemented with 1 ppm Ba^{2+}, 500 ppm Mg^{2+}, and 8 ppm Zn^{2+} respectively, unlike in the control where ethanol yield of 4.7980% (v/v) was recorded.

In wort (media) containing the metal combinations, the highest ($p < 0.05$) ethanol yield of 6.8783% (v/v) was recorded for wort (medium) containing the metal combination ratios of 8:500:1 ppm (Zn^{2+} + Mg^{2+} + Ba^{2+}), followed by 5.9720% (v/v) for wort (medium) supplemented with 500:1 ppm (Mg^{2+} and Ba^{2+}). Lower ethanol yields of 4.7838% and 4.4635% (v/v) compared to the control (4.79% v/v) was obtained for wort (media) supplemented with 8:500 ppm (Zn^{2+} + Mg^{2+}) and 8:1 ppm (Zn^{2+} + Ba^{2+}).

Within the first 24 h of fermentation for the second concentration level tested, a significant increase in ethanol yield was observed for wort (medium) supplemented with 700 ppm Mg^{2+} compared to wort (medium) containing 10 ppm Zn^{2+} and 2 ppm Ba^{2+}.

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Table 8. Effect of Zn, Mg, Ba and their combinations on ethanol yield

Period (h)	Ethanol yield (% v/v)							
	Control	Zn^{2+}	Mg^{2+}	Ba^{2+}	Zn + Mg	Zn +Ba	Mg + Ba	Zn + Mg + Ba
24	0.6489 ± 0.00^{a} (0 ppm)	0.5412 ± 0.93^{a} (8 ppm)	1.1278 ± 0.17^{b} (500 ppm)	0.5077 ± 0.49^{a} (1 ppm)	1.4302 ± 0.00^{c} (8:500 ppm)	0.9175 ± 0.00^{d} (8:1 ppm)	1.4647 ± 0.58^{c} (500:1 ppm)	1.1833 ± 0.37^{b} (8:500:1 ppm)
		0.6489 ± 0.00^{a} (10 ppm)	1.3020 ± 0.54^{b} (700 ppm)	0.4703 ± 0.05^{a} (2 ppm)	1.7953 ± 0.00^{c} (10:700 ppm)	0.4876 ± 0.00^{a} (10:2 ppm)	1.2055 ± 0.00^{b} (700:2 ppm)	1.2187 ± 1.22^{b} (10:700:2 ppm)
		1.3057 ± 0.00^{b} (12 ppm)	1.6837 ± 0.09^{c} (900 ppm)	0.8842 ± 0.01^{d} (4 ppm)	1.5337 ± 0.00^{e} (12:900 ppm)	0.5287 ± 0.52^{f} (12:4 ppm)	1.4302 ± 0.00^{e} (900:4 ppm)	1.5337 ± 0.00^{e} (12:900:4 ppm)
48	1.8380 ± 0.16^{a} (0 ppm)	2.1147 ± 0.12^{a} (8 ppm)	3.2837 ± 0.66^{b} (500 ppm)	2.5148 ± 0.56^{c} (1 ppm)	3.0968 ± 0.58^{b} (8:500 ppm)	2.0828 ± 0.88^{a} (8:1 ppm)	3.2980 ± 0.32^{b} (500:1 ppm)	3.3313 ± 0.26^{b} (8:500:1 ppm)
		2.9103 ± 0.00^{b} (10 ppm)	4.3723 ± 0.47^{c} (700 ppm)	3.8266 ± 0.58^{d} (2 ppm)	4.0365 ± 0.12^{d} (10:700 ppm)	3.3985 ± 1.02^{d} (10:2 ppm)	4.4922 ± 0.50^{c} (700:2 ppm)	4.8722 ± 0.07^{c} (10:700:2 ppm)
		2.9103 ± 0.00^{b} (12 ppm)	4.9491 ± 0.88^{c} (900 ppm)	2.5895 ± 0.54^{d} (4 ppm)	4.1081 ± 0.21^{b} (12:900 ppm)	3.9944 ± 0.21^{b} (12:4 ppm)	4.9564 ± 0.05^{c} (900:4 ppm)	5.9720 ± 0.00^{e} (12:900:4 ppm)
72	2.9040 ± 0.60^{a} (0 ppm)	4.7984 ± 0.38^{b} (8 ppm)	5.4239 ± 0.53^{c} (500 ppm)	4.9838 ± 0.00^{c} (1 ppm)	4.2578 ± 0.00^{b} (8:500 ppm)	3.2249 ± 0.58^{d} (8:1 ppm)	4.7838 ± 0.58^{c} (500:1 ppm)	6.2855 ± 0.58^{e} (8:500:1 ppm)
		3.0818 ± 0.52^{a} (10 ppm)	5.9506 ± 0.37^{b} (700 ppm)	4.9410 ± 0.04^{d} (2 ppm)	4.5310 ± 0.58^{d} (10:700 ppm)	4.6592 ± 0.54^{d} (10:2 ppm)	5.5933 ± 0.39^{b} (700:2 ppm)	5.4871 ± 0.23^{b} (10:700:2 ppm)
		5.2300 ± 0.58^{b} (12 ppm)	6.2962 ± 0.01^{c} (900 ppm)	3.6131 ± 0.01^{d} (4 ppm)	5.7908 ± 0.11^{c} (12:900 ppm)	5.0413 ± 0.06^{b} (12:4 ppm)	6.1946 ± 0.01^{c} (900:4 ppm)	7.1871 ± 0.19^{e} (12:900:4 ppm)
96	4.7980 ± 0.64^{a} (0 ppm)	6.2492 ± 0.93^{b} (8 ppm)	6.3526 ± 0.53^{b} (500 ppm)	6.6815 ± 0.24^{b} (1 ppm)	4.7838 ± 1.00^{a} (8:500 ppm)	4.4635 ± 0.00^{a} (8:1 ppm)	5.9720 ± 0.00^{c} (500:1 ppm)	6.8783 ± 0.40^{b} (8:500:1 ppm)
		4.9867 ± 0.64^{a} (10 ppm)	7.8844 ± 0.02^{b} (700 ppm)	7.6245 ± 0.54^{b} (2 ppm)	5.0808 ± 0.05^{a} (10:700 ppm)	7.4668 ± 0.49^{b} (10:2 ppm)	7.2880 ± 0.53^{b} (700:2 ppm)	7.3603 ± 0.23^{b} (10:700:2 ppm)
		7.0164 ± 0.57^{b} (12 ppm)	7.3529 ± 0.03^{b} (900 ppm)	4.8000 ± 0.64^{a} (4 ppm)	7.1313 ± 0.52^{b} (12:900 ppm)	6.1757 ± 0.50^{c} (12:4 ppm)	7.3491 ± 0.38^{b} (900:4 ppm)	8.4347 ± 0.39^{d} (12:900:4 ppm)

Values in the same row having different superscript for different concentration of each metal are significantly different ($p < 0.05$)

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At the end of 96 h, the highest ethanol yields of 7.8844% (v/v) and 7.6245% (v/v) were obtained for wort (media) supplemented with 700 ppm Mg^{2+} and 2 ppm Ba^{2+} respectively. A low ethanol yield of 4.9867% (v/v) was obtained for wort (medium) supplemented with 10 ppm Zn^{2+} which was not significantly different ($p \geq 0.05$) from the control (4.7980% v/v). In wort (media) containing the metal combinations, high ethanol yield of 7.4668%, 7.3603% v/v and 7.2880% were obtained for the metal combination ratios of 10:2 ppm (Zn + Ba), 10:700:2 ppm (Zn + Mg + Ba) and 700:2 ppm (Mg + Ba) respectively, compared to the control (4.7980% v/v). A lower ethanol yield of 5.0808% (v/v) was obtained for wort (medium) supplemented with 10:700 ppm (Zn + Mg).

At the end of 96 h of fermentation for the third concentration level tested, high ethanol yields of 7.0164% (v/v) and 7.3529% (v/v) were obtained for wort media supplemented with 12 ppm Zn^{2+} and 900 ppm Mg^{2+}, respectively. Compared to the ethanol yield (4.7980% v/v) of the control medium, a low ethanol yield of 4.8000% (v/v) was obtained for wort medium supplemented with 4 ppm Ba^{2+}.

In wort (media) supplemented with the metal combinations, the highest ($p < 0.05$) ethanol yield of 8.43% (v/v) was recorded for wort medium supplemented with 12:900:4 ppm (Zn + Mg + Ba). Ethanol yields of 7.3491% and 7.1313% (v/v) were obtained for wort (medium) supplemented with 900:4 ppm (Mg + Ba) and 12:900 ppm (Zn + Mg) respectively. A low ethanol yield of 6.1757% (v/v) was obtained for wort medium containing 12:4 ppm (Zn^{2+} and Ba^{2+}).

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CHAPTER 5

DISCUSSION

This study evaluates the effect of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations (Zn + Mg, Zn + Ba, Mg + Ba and Zn + Mg + Ba) on yeast fermentative performance during VHG beer fermentation. Being considered key parameters for determining yeast performance during fermentation, degree of wort fermentability (attenuation), ethanol productivity as well as pH variation was adopted to determine the effects of the metal ions and their combinations on the yeast (*S. pastorianus*) performance.

5.1 Effect of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations on wort fermentability

In this study, supplementation of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations (Zn:Mg, Zn:Ba, Mg:Ba and Zn:Mg:Ba) at three concentration levels on wort fermentability was carried out. Standard method of determining wort fermentability according to Briggs' *et al.* (2004) was adopted in determining the wort fermentability using a hand held refractometer. Results obtained were descriptive of the event during the fermentation - as it presents the changes in the wort strength with time in Brix (^{o}Bx) which is Plato equivalent (^{o}P).

In the experiment, zinc supplementation in the concentration range of 8 ppm, 10 ppm and 12 ppm was found to have variable effect on wort fermentability during the course of fermentation. Supplementation of the metal ion resulted in high fermentability of %F = 25.40 at concentration of 12 ppm Zn^{2+}, followed by 8 ppm (%F = 23.17); with 10 ppm Zn^{2+} having a lesser effect on wort fermentability (%F = 11.75) at 96 h fermentation. This finding agrees with the report of Walker *et al.* (2006) where Zn^{2+} levels between 0.05 - 1.07 ppm Zn^{2+} were found to enhance wort fermentability of lager yeast strain of *S. cerevisiae*, compared to 10 ppm Zn^{2+}. Also, Zn^{2+} levels as high as 65.5, 327.5 and 1300 ppm have been reported to improve fermentation rates (Jones & Greenfield, 1984; Rees & Stewart, 1999), which is in favour of the optimal value obtained at 12 ppm Zn^{2+}. The cause of variation in the wort fermentability at concentration of 10 ppm Zn^{2+} could be attributed to the repressive effect of Zn^{2+} on Zn-uptake systems (the Zrt1-high affinity and Zrt2-low affinity system) at the set concentration or the presence of other counteractive metal ions (Mn^{2+}, Mg^{2+}) that are present in the wort. This observation agrees with the report of Gitan & Eide (2000) and MacDiarmid *et al.* (2003), where high intracellular Zn^{2+} levels was said to inhibit the function of ZAP1

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gene (a zinc uptake regulatory gene in yeast) which consequently down-regulate the expression of Zap1 target genes (ZRT1, ZRT2).

In this work, magnesium supplementation in the concentration range of 500, 700 and 900 ppm was found to impact positively on wort fermentability, with high fermentability of %F = 27.94 obtained at concentration of 700 ppm Mg^{2+} after 96 h of fermentation. No significant increase in wort fermentability was observed above 700 ppm, as 900 ppm showed no significant increase in fermentability (%F = 27.38) after 96 h. This could indicate that the yeast cell have reached its optimum requirement for the mineral ion. The finding of 700 ppm (700 mg/L $MgSO_4.7H_2O$) as optimal concentration for yeast fermentative performance; agrees with the report of Pirocheva (1998) and Slininger *et al.* (2006) who observed optimal yeast attenuating strength at concentration ≥700 mM. Also, the result supports the report of Rees and Stewart (1999), who observed increased yeast performance upon supplementation of 500 ppm Mg^{2+}. Thus, maintaining Mg^{2+} at concentration of 700 ppm in VHG wort would allow for improved yeast attenuating (fermentability) strength during very high gravity beer fermentation.

Literature on the role of Ba^{2+} on yeast physiology as well as in fermentation processes is grossly lacking. The impact of Ba^{2+} on yeast fermentative performance have not been reported and in this present work, Ba^{2+} was found to enhance wort fermentability. Barium ions were found to improve wort fermentability at concentration range of 1 ppm and 2 ppm. High wort fermentability of %F = 26.03 and %F = 23.81 was recorded at 2 ppm and 1 ppm Ba^{2+} respectively. This impact could be as a result of the effect of the metal ions on sugar uptake through yet an undefined mechanism at the set concentration. Concentrations of 4 ppm Ba^{2+} was found to slow sugar utilization (%F = 5.71) with time. This process impact could be as a result of the metal ion having reached its toxic level, whereby the cells are unable to maintain active cellular metabolism to efficiently ferment the sugars. Also, it could be as a result of the negative effect of the metal ions on the gating kinetics of essential metal ions such as potassium - as is shown in the work of Bert *et al.* (1997) where 10 mM of Ba^{2+} blocked about 53% of the potassium transport protein. This finding supports the hypothesis of Debrath and Mukerji (1982) who demonstrated that cell division and expansion can be impaired by Ba^{2+} toxicity. Thus supplementing Ba^{2+} in the concentration range of 1 – 2 ppm during VHG beer fermentation will allow for improved fermentation rate, with 4 ppm having a negative influence.

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The effect of metal interactions on yeast fermentative performance have been reported, with much elucidation on the interactive effect of Mg^{2+} and Ca^{2+} on yeast fermentation performance (Walker, 1994; Rees & Stewart, 1999; Gibson, 2011). The combined effects of Zn^{2+}, Mg^{2+} and Ba^{2+} on yeast performance have never been reported and in this study, an increase in wort fermentability was found for the metal combinations at varied concentration levels except for Zn:Mg:Ba, which showed increased wort fermentability for all concentration levels tested. Addition of 12:900:4 ppm (Zn^{2+} + Mg^{2+} + Ba^{2+}), was found to impact optimally (%F = 30.16) on wort fermentability after 96 h of fermentation compared to the individual metal ions as well as for other combinations except for Mg:Ba at concentration of 900:4 ppm with fermentability of %F = 29.53. This effect could be as a result of yet an unknown relationship between the metal ions (Mg + Ba), which cooperatively enhances the fermentative ability of the yeast cell. It is apparent from the result that the metal combination (Zn + Mg + Ba) exhibits 'best' impact on wort fermentability with time. However, their impact on beer quality as well as yeast viability should further be investigated.

The interactive effect of Mg and Ba ions was established in this study and was found to have same effect with Zn:Mg:Ba on wort fermentability at concentration of 900:4 ppm (Mg^{2+} and Ba^{2+}). This finding yet, suggests a higher amount of Mg^{2+} to trace metal for optimal yeast performance. This report supports the view of Walker, (1996), Chandrasena *et al.* (1997) and Okon and Nwabueze (2009); in which higher ratio of Mg^{2+} to trace element was found to enhance fermentation rate.

A lesser effect on wort fermentability (%F = 13.65) was observed at 500:8 ppm (Zn + Mg) compared to (%F = 14.29) at 500:1 ppm (Mg + Ba). A similar effect was also observed at concentration level of 10:700 ppm (Zn^{2+} + Mg^{2+}) with %F = 15.87 compared to 700:2 ppm (Mg^{2+} + Ba^{2+}) with %F = 27. This observation could imply a counteractive relationship between Mg^{2+} and Zn^{2+} on *S. pastorianus* compared to Mg^{2+} and Ba^{2+}. Although report on the interactive effect of Mg^{2+} and Ba^{2+} is lacking, the observation for Mg^{2+} and Zn^{2+} accord with Helin and Slaughter (1977), Blackwell *et al.* (1995) and Briggs' *et al.* (2004), who demonstrated counteractive relationship between Mg^{2+} and Zn^{2+}.

Though the wort medium containing Zn^{2+} and Ba^{2+} displayed a comparable fermentability at concentration level of 10:2 ppm (%F = 26.35), reduced wort fermentability (%F = 10.48 and %F = 22.54) was observed for other concentration levels tested (i.e. 8:1 ppm and 12:4 ppm)

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respectively. The result suggests a strong inverse relationship between Zn^{2+} and Ba^{2+} at somewhat higher or lower concentration on yeast fermentative performance.

5.2 Effect of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations on wort pH

As shown in Table 9, pH range of 4.75 – 4.88 was found to enhance the fermentative performance of *S. pastorianus* during fermentation of the VHG wort; with pH of 4.75 found to be optimal for the yeast activity. With regards to Zn^{2+} and Ba^{2+}, addition of the salts was found to slightly increase the wort pH during the first 24 h of fermentation. This effect can be as a result of the highly alkaline nature of the salts.

These results indicate that the stimulatory effect of the mineral ions on yeast performance is greatly affected by pH. This effect could be explained by the ATPase-dependent H^+ activity, which is known to be associated with cation uptake in cells (Okorokov *et al.,* 1985; Park *et al.,* 2003; Walker, 2004). Also, medium pH has been reported by Tuszynski and Pasternakiewicz (1999), to have a predictory role on the binding competence of metal cations for absorption sites on the yeast cell wall. These results agreed with the work of Stehlik-Thomas *et al.* (2004) and Maurice (2011); where yeast optimal ability to ferment sugars, although with different medium composition was found at pH range of 4.5 – 5.0. Considering the optimal wort fermentability [%F = 30.16 (12:900:4 ppm Zn + Mg + Ba), %F = 29.53 (900:4 ppm Mg + Ba), and %F = 27.94 (700 ppm Mg^{2+})] and highest ethanol yield [8.4347% v/v (12:900:4 ppm Zn + Mg + Ba)] obtained for the metal ions and the combination ratios; it can be concluded that pH range of 4.75 is optimal for the fermentative performance of *S. pastorianus* during VHG wort fermentation.

5.3 Effect of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations on ethanol yield

The effect of three different concentration levels of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations on ethanol yield was determined. The ethanol yield was obtained using the method according to Navilio *et al.* (2001). Precision in the result obtained was advantageous, although it is time consuming in terms of standard preparation and run time (2.71 min).

In this study, supplementation of Zn^{2+} in the concentration range of 8 ppm, 10 ppm and 12 ppm resulted in enhanced ethanol formation, with concentration of 8 ppm (6.2492% v/v) and 12 ppm (7.0164% v/v) having beneficial effect on ethanol productivity. This result supports

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the works of Densky *et al.* (1966) and Rees and Stewart (1998) in which lager brewing strains were found with enhanced ethanol production upon Zn supplementation. Also, the result agrees with the reports of Okon and Nwabueze (2009) and Xue *et al.* (2010) in which increased ethanol yield was reported at increasing minute (0.01 - 1 g/L and 0.30 – 0.48 mg/L) amounts of Zn^{2+}. The low ethanol yield (4.98% v/v) recorded at Zn^{2+} concentration of 10 ppm could be attributed to the amount of Mg^{2+} and Mn^{2+} originally present in the wort, which could have imposed a counteractive effect on Zn^{2+} during ethanol production. This observation agrees with the report of Walker *et al.* (2006) in which Zn^{2+} at 10 ppm was found to have a lesser effect on ethanol productivity. Also, the report supports the view of Helin and Slaughter (1977) who demonstrated that yeast-Zn requirement is affected negatively by the availability of Mn^{2+} in wort. Thus, taking into account of counteractive metals, supplementing Zn^{2+} in somewhat lower or higher concentration would allow for improved yeast fermentative process.

For each concentration tested for Mg^{2+}, a rapid initial increase ethanol yield after 24 h of fermentation was recorded compared to Zn^{2+} and Ba^{2+}. This observation can be explained by the growth stimulating effect of the metal on yeast cells, which has a direct impact on ethanol productivity. As shown in the results, addition of Mg^{2+} in the concentration range of 500 ppm, 700 ppm and 900 ppm was found to promote ethanol productivity. This result agrees with the report of Rees and Stewart (1997 and 1999) in which ethanol productivity was enhanced for both standard and high gravity wort upon Mg^{2+} supplementation. High ethanol yield of 7.8844% and 7.3529% (v/v) was recorded for Mg^{2+} at concentrations of 700 ppm and 900 ppm respectively. The result supports the report of Pironcheva (1998), who demonstrated that $Mg^{2+} \geq 700$ µM had no further effect on ethanol production. Also, the result agrees with the result of Thanonkeo *et al.* (2007), who reported enhanced ethanol tolerance and ethanol productivity upon addition of 10-20 mM of Mg^{2+}. Therefore, maintaining concentration of 700 ppm Mg^{2+} allows for a desired yeast fermentative output.

The effect of Ba^{2+} on yeast fermentation performance has never been reported and in this study it was found that the metal ions have beneficial effect on fermentation process. Supplementation of Ba^{2+} in the concentration range of 1 ppm and 2 ppm was found to impact positively on ethanol productivity – with concentration of 2 ppm exerting optimal effect (7.6245% v/v) on ethanol yield. This result suggests that Ba^{2+} has an enhancing effect on ethanol formation. This observation could be explained in part by the enhancing effect of Ba^{2+} on protein synthesis, which may have impacted positively on the yeast sugar

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metabolism. This observation supports the report of Park and Khang (1995) who observed increased protein production for *Cephalosporium acremonium* cells that were entrapped in Ba-alginate. However, the report of Iqbal and Naz (1989) stated otherwise the effect of Ba^{2+} on protein synthesis in seedling growth. Addition of 4 ppm was found to reduce ethanol productivity (4.8000% v/v), which implies that the metal could be toxic to the yeast cells at concentrations ≥4 ppm. Retardation in cell division and expansion has been shown to be key factors to Ba^{2+} mediated growth inhibition (Debnath and Mukerji, 1982; Iqbal and Naz, 1989). This process impact can be attributed to the toxic effect of high amounts of Ba^{2+} on yeast cells in terms of decreasing its protein synthesis and increasing peroxidase activity (which is an indication of toxicity).

The combinatory effect of Zn^{2+}, Mg^{2+} and Ba^{2+} has never been reported and in this study, novel relationships between the metal ions were established. Maximum ethanol yield of 8.4347% (v/v) was recorded for wort medium supplemented with 12:900:4 ppm (Zn + Mg + Ba). The results (Table 8) demonstrate that the addition of Zn, Mg and Ba ions in the concentration of 12 ppm, 900 ppm and 4 ppm stimulates ethanol production. The increased ethanol formation could be explained by the combined effect of Zn^{2+} on alcohol dehydrogenase, activation of glycolytic enzymes by Mg^{2+} and yet undefined promotory effect of Ba^{2+}; which synergistically enhances ethanol formation. Thus it can be stated that the metal combinations impact optimally on ethanol yield, although their supplementation should be done with consideration on yeast viability and vitality as well as on the beer quality.

A synergistic relationship was also observed for Mg^{2+} and Ba^{2+} in which high ethanol yield of 7.3491% (v/v) was recorded for the metal combination at concentration level of 900:4 ppm (Mg^{2+} and Ba^{2+}). This could imply that Ba^{2+} have a promotory effect on yeast performance in the presence of high Mg^{2+} concentration.

The interactive effect of Zn^{2+} and Mg^{2+}, and Zn^{2+} and Ba^{2+} was found to have variable impact on ethanol yield. Low ethanol yield was observed more in combinations of Zn^{2+} to Mg^{2+} and Ba^{2+}, although at high concentration of Mg^{2+} to Zn^{2+} and Ba^{2+}; an enhanced ethanol production was observed. It was observed that high concentration of Mg^{2+} to the trace metals had beneficial impact on ethanol yields. This observation supports the view of Walker *et al.* (1996), Chandrasena *et al.* (1997) and Okon and Nwabueze (2009), who have reported enhanced ethanol productivity upon high concentration of Mg^{2+} to trace metals. It is understandable from the results that a counteractive relationship exists at somewhat higher

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concentration of Zn^{2+} to Mg^{2+} and Ba^{2+}. This could be explained by the competitive reactivity of Zn^{2+} with Mg^{2+} for binding sites either on chelating molecule in the wort or on the yeast membrane, which may have reduced the uptake of the ions by the yeast cells. This observation supports the views of Tosun and Ergun (2007) and Chandrasena and Walker (1997) on ion-ion interaction of Zn^{2+}, in which metal ions such as Ca^{2+}, K^{+}, Mn and Mg^{2+} were found to influence the effect of Zn on yeast fermentation performance. However, the relationship between Zn^{2+} and Ba^{2+} on yeast fermentative activity as observed in the study awaits further investigation.

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CONCLUSION AND RECOMMENDATION

In this study, the effects of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations (Zn + Mg, Zn + Ba, Mg + Ba and Zn + Mg + Ba) on the fermentative performance of *S. pastorianus* during VHG wort fermentation was determined as well as the best possible conditions that could enhance yeast performance. It is evident from the results that Mg^{2+} has a profound effect on yeast fermentative performance, with 700 ppm Mg^{2+} concentration found optimal for yeast fermentative output.

The effects of Zn^{2+} on yeast performance have been reported by several authors, however in this study it was found that increasing amount of Zn^{2+} does not necessarily translate into high yeast fermentative performance. Therefore, the need for monitoring Zn^{2+} content during wort fermentation is crucial, as variable concentration range may reduce yeast fermentation progress.

The toxic effect of Ba^{2+} on yeast cell is grossly lacking. However, it is noteworthy from the results that Ba^{2+} has beneficial effect on yeast fermentation process at certain concentration levels. The effect was established at concentration range of 1 and 2 ppm, with concentration of 4 ppm Ba^{2+} found to reduce yeast performance with time. Thus, the need to elucidate the effect of Ba^{2+} on yeast physiology with regards to its enhancing and inhibitory mechanism on yeast performance during fermentation is required, as it could suggest a biotechnological significance not only to the brewing and biofuel industry but as well as health at its trace levels.

It is apparent from the results that maintaining high concentration of Mg^{2+} to trace elements in the combinations enhances the fermentative performance of lager yeast *S. pastorianus* during very high gravity wort fermentation. The metal combination ratio of 12:900:4 ppm (Zn + Mg + Ba) had 'best' impact on the evaluated parameters. With regard to wort pH, the effects of Zn^{2+}, Mg^{2+}, Ba^{2+} and their combinations were found at pH range of 4.75 – 4.88, with pH of 4.75 found suitable for the fermentative performance of *S. pastorianus*.

It is therefore necessary to optimize the mineral ions in their beneficial concentration range and combinations as identified in this study and also adjusting the wort pH at range which would allow for improved yeast performance during VHG wort fermentation. Nevertheless, their impact on beer character requires further investigation.

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Appendix A

Amount of metal salts

The amount of each metal ions used in the experiment was considered in parts per millions (ppm) which was calculated in grams of the salt, such that a defined amount dissolved in water will produce the required concentration.

Stock solutions of the metal salts were prepared using the equation:

$$X \text{ ppm} = \frac{X \text{ g x relative molecular mass of salt}}{\text{Molar mass of metal}}$$

Example 5000 ppm $MgSO_4.7H_2O$ was prepared as follows:

$$5000 \text{ ppm} = \frac{5 \text{ x } 246.48}{24.31}$$

Relative molecular mass of magnesium sulphate = 246.48 g / mol.

Molar mass of magnesium = 24.31 g/ mol.

Quantified amounts of the metal ions were calculated using the equation:

$$C_1V_1 = C_2V_2$$

C_1 = Concentration of stock solution (ppm)

V_1 = Volume of stock solution (cm^3)

C_2 = Required concentration (ppm)

V_2 = Required volume (cm^3)

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Appendix B

Conference papers

Udeh HO, Kgatla TE and Jideani AIO (2014). Effects of mineral ions on yeast performance under very high gravity beer fermentation. World Congress on Engineering and Technology (CET) 2014. 26th – 28th October, 2014, Wuhan, China.

Udeh HO, Kgatla TE, and Jideani AIO (2013). Role of magnesium ions on yeast performance under very high gravity ethanolic fermentation. 20th Biennial International SAAFoST Congress and Exhibition, 7-10th October, 2013, CSIR Convention Centre, Pretoria.

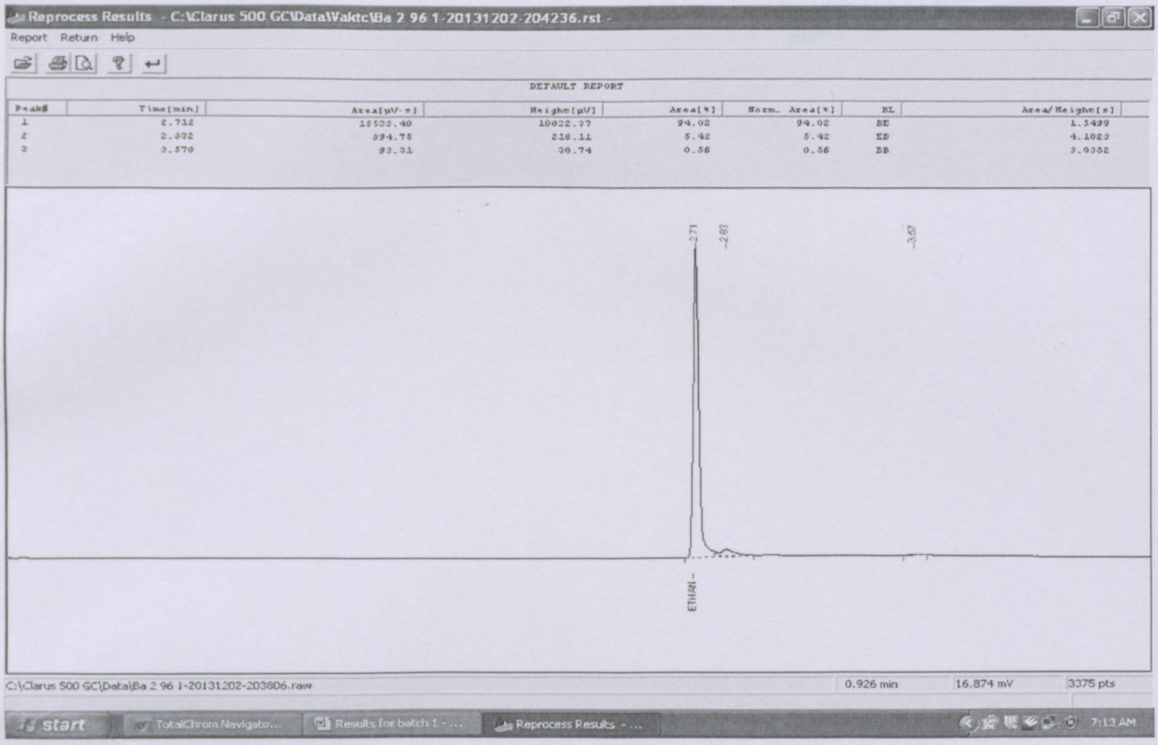
Publication in scientific journal

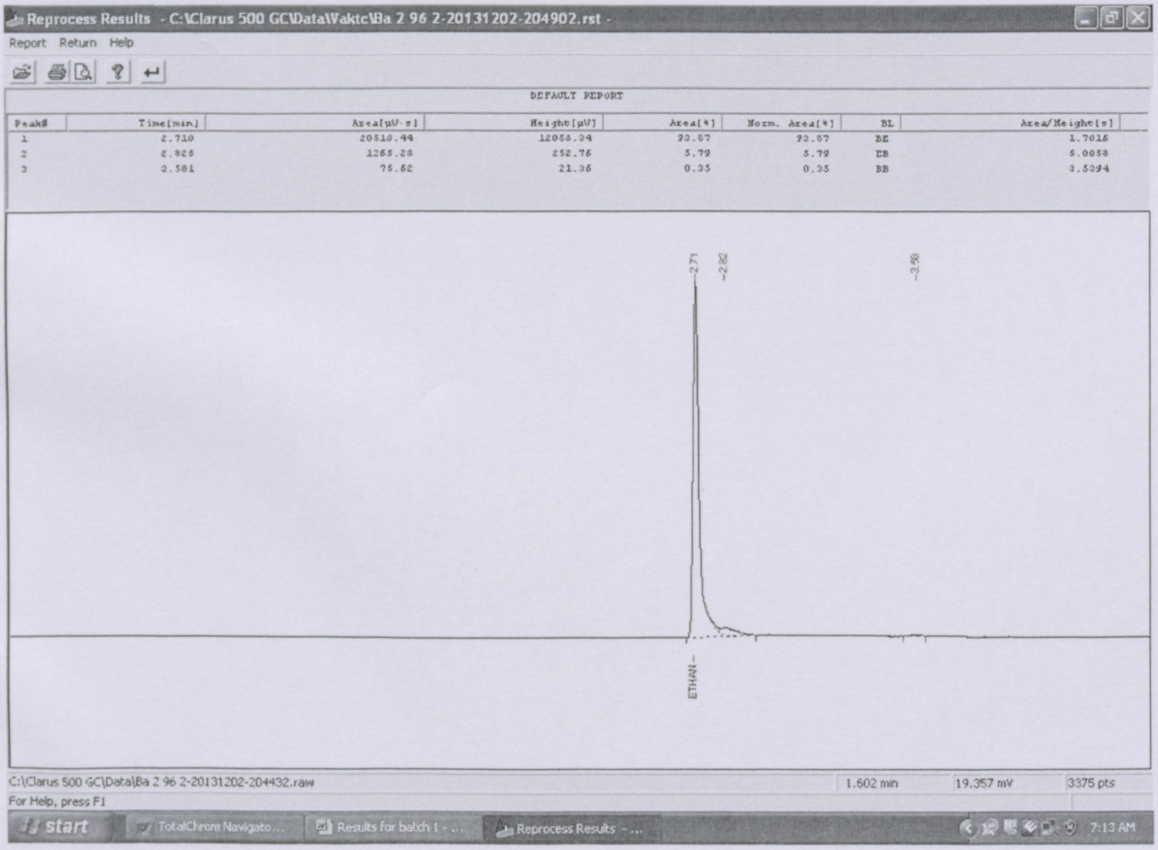
Udeh HO and Kgatla TE (2013). Role of magnesium ions on yeast performance during very high gravity fermentation - Review. Journal of Brewing and Distilling. 4(2):19-45.

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Appendix C

Gas chromatography graph sample result





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