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Review

Role of magnesium ions on yeast performance during very high gravity fermentation

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The advent of highly efficient, environmentally friendly and cost effective fermentation technology has given impetus to research in the field of optimizing nutritional parameters for optimum yeast fermentative performance. Very high gravity fermentation is a novel fermentation technology that provides an increased production capacity from same size fermentation facilities, with outstanding benefits that includes: high ethanol yield per fermentable mash, considerable savings in energy and process water usage, and effluents with low biological oxygen demand amongst others. Limitations to full commercialization of the technology have been attributed to deleterious effects of the fermentation condition on yeast physiology which include high osmotic stress and ethanol toxicity amongst others. The impact of these physiological stresses on yeast cells during high substrate fermentation manifest as sluggish and incomplete fermentation with high residual sugars in beer, reduced ethanol yield, disproportionate synthesis of esters and generation of respiratory deficient yeast crop. However, compelling evidence has implicated magnesium ions with numerous biological processes and more importantly, with the role of curtailing the impact of these stress conditions. Hence, this review highlights two potential stress conditions of very high gravity fermentation; their mechanism of inhibition versus yeast stress response mechanism, role of magnesium ions in yeast physiology and its impact on fermentation processes. The knowledge emphasized herein will be of practical importance to industrial fermentation processes, as it provides feasible clues to improving yeast fermentative performance under high substrate conditions - with perspectives to precise magnesium regulation in yeast.

Key words: Very high gravity fermentation, osmotic stress, ethanol stress, yeast stress tolerance, magnesium ion.

INTRODUCTION

Alcoholic fermentation technology has a long history, yet it is still at the forefront of biotechnological development (Linko et al., 1998; Bamforth, 2003; Puligundla et al., 2011). Presently, there has been an increasing global concern on how to efficiently maximize the production of ethanol for alcoholic beverage production and biofuel. Production costs, environmental issues, dwindling fossil fuel reserves and increasing costs of gas (Bai et al., 2004; Hall et al., 2006; Bleick et al., 2007; Silva et al., 2008) have been identified as major drivers for the search for efficient ethanolic fermentation methods. Amongst various methods of ethanolic fermentation is high gravity (HG) fermentation - a technically sophisti-cated brewing practice which has been affirmed to be

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highly resourceful and environmentally friendly (Briggs et al., 2004; Pradeep and Reddy, 2010; Yu et al., 2012). This technology provides an increased production capacity from same size brewhouse and fermentation facilities (Blieck et al., 2007; Huuskonen et al., 2010; Biofuels business, 2009) and has been widely adopted for brewing standard beer, and for bioethanol production in developed countries, such as the United States of America, Canada, China and Brazil (Walker, 2004; Gibson et al., 2007; Li and Chan-Halbrent, 2009; Vidgren and Londesborough, 2011).

In recent years, the concept of HG fermentation (initiated in the 1980s) (Bai et al., 2008; Puligundla et al., 2011) was further extended to very high gravity (VHG) fermentation, with the intension of maximizing the benefits of high gravity fermentation which include high ethanol yield per fermentable mash (Agu, et al., 1993), reduced energy and water consumption (Zhao et al., 2009), time and money savings (Verbelen et al., 2009), improved smoothness, flavour and haze stability of beer (Sigler et al., 2009; Huuskonenet al., 2010; Yu et al., 2012), and effluents with low biological oxygen demand (Bajaj et al., 2003; Pradeep and Reddy, 2010). However, problem of flavour match in beer have been associated with HG fermentation as modified flavour and aroma compound profile in final beer produced is pronounced due to elevated levels of ethyl acetate and isoamyl acetate (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 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 and Micheal, 2001). This theoretical fact, practically allows a final ethanol yield of 15 to 18% (v/v) (Bai et al., 2008; Pradeep and Reddy, 2010). Figure 1 shows a sketch of the production of alcoholic beverage and fuel ethanol employing VHG technology, with Table 1 showing differences between normal gravity, HG and VHG fermentation methods. Limitation of applying VHG technology, 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 (Lin and Tanaka, 2006; Cao et al., 2011; Gibson, 2011; Yu et al., 2012). The high osmotic pressure caused by high concentration of dissolved carbohydrates leads to physiologic changes in yeast cells and a reduction in the cells' vitality, which further reduces sugar conversion and ethanol production (Phisalaphong et al., 2006; Sigler et al., 2009). 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 the fermentation efficiency (Narendranath et al., 2001; 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, and increased uptake of nutrients and high levels of viable biomass. In exposure to VHG fermentation condition, these desirable traits are incapacitated due to very high dissolved sugar (>18 g per 100 g mash) employed (Gibson, 2011; Puligundla et al., 2011), increased ethanol concentrations (15 to 18%), high temperatures and propensity to nutrient starvation (Banat et al., 1998; Cot et al., 2007; Verbelen et al., 2009; Gao et al., 2011; Puligundgla et al., 2011). Under such condition, the yeast cells tend to display unusual fermentative behaviours which is often referred to as yeast stress (Graves et al., 2007; Gibson, 2011; Yu et al., 2012).

The resultant effects of such stress on yeast cells during fermentation manifests as slow fermentation rate (reduced sugar uptake), poor attenuation of sugar, inordinate flocculation behaviour, and generation of petite (respiratory deficient) yeast strains (Blieck et al., 2007; Gao et al., 2010; Gibson, 2011). Other setbacks include poor foam stability (Cooper et al., 2000), haze development in beer (Malcorps et al., 2001) and disproportionate synthesis of esters (higher alcohols) which often result in poor flavour profile of the beer (Munroe, 1995; Blieck et al., 2007). Hence, this review highlights two yeast stress conditions:ethanol stress and osmotic stress; their mechanism of inhibition and yeast tolerance. Furthermore, the literature emphasises on magnesium transport mechanisms in yeast, its role in yeast physiology as well as in fermentation processes.

YEAST STRESS

Yeast, like any other living cell, is affected by natural factors such as gene type, environment and nutrition (Figure 2). 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 (Gibson, 2011; 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).

Stress conditions which include rapid temperature fluctuations, high osmotic pressure, increased ethanol concentration, and nutrient starvation (Ding et al., 2009; Zhao and Bai, 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. Figure 3 is a schematic illustration of the stressful conditions experienced by yeast cell under VHG fermentation, and these stressful conditions have been

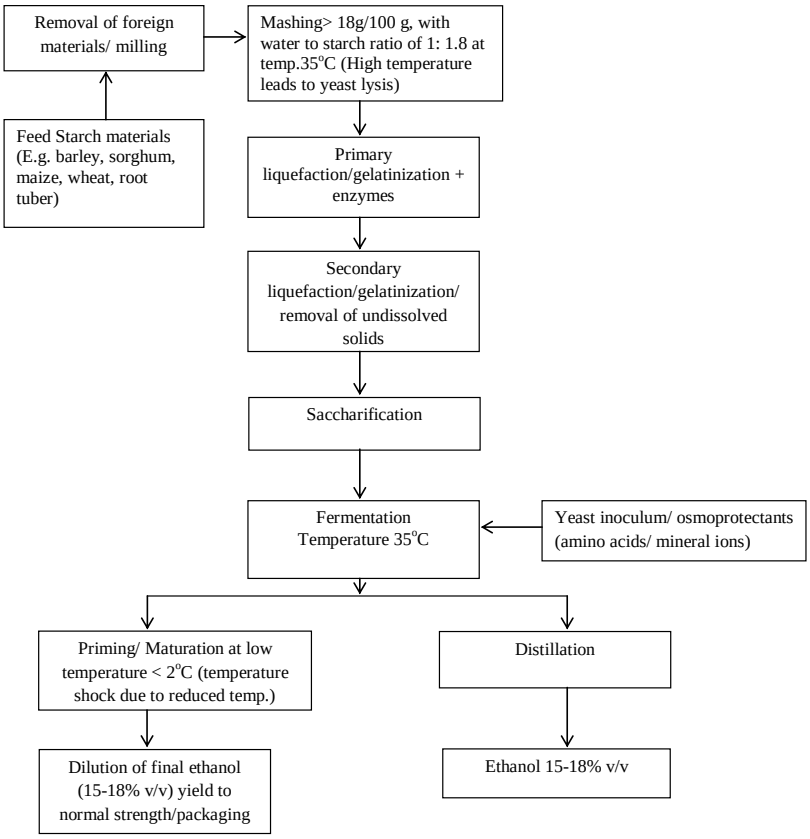

Figure 1. Schematic flow of the production of alcoholic beverage/fuel ethanol from starchy material using very high gravity fermentation method. Source:Pulingundla et al. (2011):Adapted.

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

Feature	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
Process water saving in terms of starch-to-water ratio)	1:3	1:2	1:1.8
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) and Cao et al. (2011).

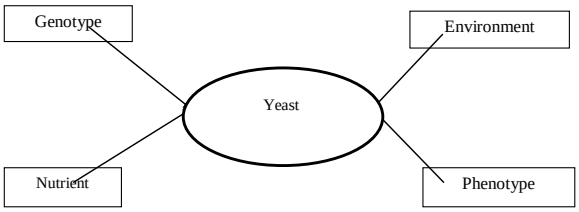

Figure 2. Factors affecting yeast behaviour.

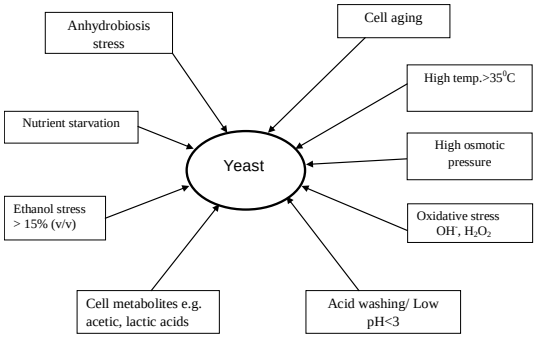

Figure 3. Major stresses experienced by yeast cell under very high gravity beer fermentation. Source:Walker (2004).

reported to be of relevance - as yeast cells are said to acquire an appreciable resistance trait after exposure (Blieck et al., 2007; Verbelene et al., 2009).

Ethanol stress

Use of sugar adjuncts in VHG fermentation, has led to a 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 and Walker, 2000). 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 and Ingram, 1985; Puligundla et al., 2011). At this stage, the yeast cell begins to displays various forms of physiological defects which ranges 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 (Osman and Ingram, 1985; Birch and Walker, 2000; Marza et al., 2002; Furukawa et al., 2004; Canetta et al., 2006; Ding et al., 2009; Trofimova et al., 2010).

These cellular dysfunctions result from inhibitory effect of high ethanol concentrations on integral glycolytic enzymes (e.g. phosphofructokinase) and the alteration in cellular membrane lipid and protein composition (Birch and Walker, 2000; Ding et al., 2009). The effects of ethanol stress reflects in the fermentation process as sluggish fermentation, reduced cell growth and viability, generation of petite yeast crop which consequently affects successive fermentation performance (Cahill et al., 2000; Stewart, 2001). Loss of cell viability has also been reported to ensue from other factors such as fermentation temperature and nutrient starvation (Gibson et al., 2007). Other metabolites like aliphatic alcohols also exert adverse effects on the yeast cell, and the gravity of their effect is reported to be dependent on the chain length of the molecules - such that the longer the chain (higher alcohols, acetate) the more toxic they are (Brigg's et al., 2004). Also, poor yeast performance at high temperature and ethanol concentration has been reported - as the impact of ethanol stress on yeast cells was enhanced in a progressive fashion under elevated fermentation temperatures (van Uden, 1989; Birch and Walker, 2000).

Several mechanisms have been proposed through which the toxic effect of ethanol may be exerted. The non-specific osmotic effect (Jones and 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 has been suggested that the effect of ethanol on membranes is due to its insertion into the hydrophobic interior and the resultant effects on polarity, exchange of polar molecules and the position of membrane proteins (Weber and de Bont, 1996).

The resultant effect of these alterations on the plasma membrane includes leakage of cellular components, inhibition of transport systems (essential for nutrient uptake e.g. Mg-uptake) (Converti et al., 1992), alteration in membrane structure and increased membrane fluidity (Lloyd et al., 1993). Another target site of ethanol toxicity is the mitochondria membrane, where it causes a mutagenic change on the mitochondria DNA which result in respiratory deficient (petite) strains (Walker, 2004; Gibson, 2011). The potential impact of this effect (petite strain) is a decrease in successive fermentation performance when the same crop of strain is successively used. However, the yeast cell portrays an unusual attitude towards this stressful condition in order to survive.

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'. This stress response is characterised by the synthesis of heat shock proteins (hsps), increase in intracellular storage carbohydrates (glycogen and trehalose) (Neves and Francois, 1992; Parsell and Lindquist, 1994; Vianna et al., 2008), active production of compatible solutes (for example glycerol) (Briggs' et al., 2004), modulation of ion homeostasis (Walker, 2004), biosynthesis of chaperone proteins that stabilizes denatured proteins, and the production of glutathione and stress enzymes such as ATPase, superoxide dismutase (Osta et al., 1993; Lei et al., 2007). Several studies have revealed that permu=-tations 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 and 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 physio-logical status of the cell and physico-chemical nature (process parameters and bioavailability of essential nutrients) of the fermentation medium (Boulton and Quian, 2001; Zhao and Bai, 2009).

These responses which are often triggered upon exposure to sub-lethal and lethal limits of ethanol stress often result in concomitant acquisition of tolerance(s) to various forms of stressful conditions (Briggs et al., 2004; Verbelene et al., 2009). For example, barotolerance (osmotolerant) and thermotolerance (heat-tolerant) are acquired by yeast cells during ethanol stress. Several 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 said to be mediated by two major stress response pathways namely; heat shock response (HSR) pathway, which is activated at sub-lethal heat stress by heat shock transcription factor (HSF), and the global stress response (GSR) pathway, which is activated by lethal level of ethanol concentrations (Schmitt and McEntee, 1996; Verbelene et al., 2009; Puligundla et al., 2011). 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 to respond to array of stressful conditions in a non-specific manner; by simul-taneously activating specific responses to several stress conditions while maintaining cellular fitness (Martinez-Pastor et al., 1996; Galhardo et al., 2007). Under stress condition, 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 zinc-finger transcriptional activators, Msn2p and Msn4p, which has been implicated with the mediation of the transcription of a separate zinc regulon which is expressed during zinc starvation in a strain of *Saccharomyces cerevisiae* (Martinez-Pastor et al., 1996; Treger et al., 1998; Lyons et al., 2000; Gauci et al., 2009).

These transcriptional activators are known to be the principal mediator(s) of the GSR pathway (Martinez-Pastor et al., 1996; Smith et al., 1998; Gauci et al., 2009). The STRE element which is present in many genes associated with most stress responses is an active inducer of most genes which are 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 adeno-sine monophosphate-protein kinase A (Ras-cAMP-PKA) pathways whose activity is important in the biosynthesis and degradation of glycogen (Verbelene et al., 2009), and also under conditions of zinc deficiency or stress (Gauci et al., 2009). The importance of this regulatory machinery is observed in the variation of storage carbohydrates: glycogen and trehalose.

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). The performance of yeast cells during fermentation is dependent on the concentration level of glycogen in the cell - as its catabolic end-product (glucose) provides energy that drives the formation of essential lipids (sterols) required for reproduction (Quian and Tubb, 1982; Bolat, 2008). Glycogen concentration level in yeast is an indicator of cell cycle progression and stress adaptation by yeast cell (Gibson et al., 2007). It is regulated by enzymatic reaction(s) which involves the activity of glycogen synthase phosphatase (glycogen degrading enzyme) on glucose, under the control of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) pathway - having glucose 6-phosphate and glucose 1-phosphate as precursors for its biosynthesis (Boulton, 2000).

During reverse glycolysis (gluconeogenesis) in which the cell have to feast on oxidative carbon sources (ethanol), 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; Bolat, 2008). 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 and Quain, 2001). The aftermath of this cellular events, is the decline in glycogen concentration which is consistent with the sustenance of the cell during stress (elevated ethanol concentration and nutrient starvation) condition.

Trehalose alteration - a response to ethanol stress

The second storage carbohydrate, trehalose, is an important stress protectant and a metabolic signal molecule in yeast. Its concentration in yeast cell is believed to be related to stress tolerance and adaptation (Colin and Nelson, 2007). Trehalose has been reported to be responsible for repairing denatured proteins (Gibson et al., 2007; Ding et al., 2009), and in maintaining the integrity of the yeast plasma membrane by displacing the interaction of water and ethanol. This interaction establishes hydrogen bonds with polar groups of lipids that stabilize the membrane structure against ethanol and osmotic stress (Leao and van Uden, 1984; Neves and Francois, 1992).

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 and Parrou, 2001) which in turn confer stability to the plasma membrane (Mansure et al., 1994), enzymes (Sola-Penna and 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 (Majara et al., 1996).

The degradation of trehalose is also STRE regulated; which entails a series of reactions in which the fully glucose-activated cAMP/PKA pathway induces a post-transcriptional activation on genes (NTH and ATH) responsible for the synthesis of trehalose degrading enzymes (Zahringer et al., 2000b; Briggs et al., 2004; Bolat, 2008). These genes encode two forms of enzymes; the cytosolic hydrolase (NTH1, NTH2) and the vacuolar trehalase (ATH) - the earlier whose neutral form called the neutral trehalase (NTH1) is implicated in the degradation of intracellular trehalose (Zahringer et al., 2000a; Bolat, 2008). Upon reduction in concentration of trehalose, a normal cellular activity is re-established. The timely degradation of trehalose has been shown to be vital to maintaining a prompt resumption of metabolic activity in yeast cell after exposure to ethanol stress; 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).

Through the above mechanisms, 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 forms of alterations in cell component exist, which play prime role to the health of the yeast cell during harsh fermentation conditions.

Yeast ethanol stress response via lipid modification

Lipid is a compendium of molecules with structural function in yeast physiology. Their variability in concentration, 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 lipids present in yeast, fatty acids e.g. unsaturated fatty acid (UFAs), phospholipids (glycerophospholipids) and sterols (ergosterol) 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 to ethanol stress.

During ethanol stress, yeast cell tends to show specific membrane responses which include an increasing unsaturation index (palmitoleic and oleic acids) and a proportional increase in long chain fatty acids (You et al., 2003; Aguilera et al., 2006). These alterations are mediated through a gene-regulated series of enzymatic reactions which involves desaturation of saturated fatty acids (SFAs) by the action of ∆-9- fatty acid desaturase encoded by the desaturase OLE1 gene, and the elongation of medium assimilated short-chain fatty acids (C10-C12 to C16-C18) through a membrane-bound fatty acid elongation system encoded by ELO1 genes (Briggs et al., 2004; Ding et al., 2009).

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 effect of ethanol on the membrane as it is suggested by Weber and de Bont (1996) to antagonise the stereo-chemical effect of hydrophilic ethanol head

groups on lipid components of the plasma membrane (Weber and de Bont, 1996; Alexandre et al., 2001). 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 (You et al., 2003; Alexandre et al., 2001; Moonjai et al., 2002), with oleic acid been the most effective component of ethanol stress than palmitoleic acid (Ding et al., 2009). This modification also extends to other lipids of prime importance, which include sterol and phospholipids. Cellular ergosterol and inositol content has also been shown to correlate with ethanol tolerance in yeast *S. cerevisiae*, as cells grown in elevated levels of both sterols showed a high tolerance to ethanol stress (Ding et al., 2009).

Osmotic stress

High osmotic pressure, which is typical of high and VHG fermentation conditions, is among the deleterious stress conditions experienced by yeast cells during fermentation (Querol et al., 2003). This osmotic effect tends to alter the physiological fitness of the yeast cell, by extruding essential cell components that maintains cellular fecundity (Gibson, 2011).

Osmotic stress is a cellular imbalance that occurs when cells are suspended in medium with high osmotic potential than the intracellular content, which consequently results in loss of cellular water and tugor fall (Klipp et al., 2005). This osmotic change is often accompanied by a shrunken shaped 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). Correlatively, the impact of water activity (a_w) on yeast cell, regard as osmotic perturbations, is said to be an inverse function of the amount of dissolved solute; with the greatest potential damage anticipated with rapid change in a_w (Briggs et al., 2004). However, upon arrival of an osmo-balanced environment, the yeast cells tend to re-establish cell volume which possibly has some irreversible physiological changes that could negatively affect subsequent fermentation performance.

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 hyperosmotic condition is known as osmostress tolerance (Briggs et al., 2004). The yeast cell exhibit two forms of osmostress tolerance namely, osmotolerance and osmoadaption (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 (Sharma et al., 1996), proper vacuolar functioning (Nass and Rao, 1999) and storage carbohydrate- trehalose, confer some degree of protection to the cell. Enhanced osmotolerance is reported in the abundance of osmoprotectant molecules (for example, proline, methione, isoleucine, phenylalanine) whose functions are to stabilize cellular membranes, enzymes and other proteins (Singer and Lindquist, 1998; Verbelen et al., 2009). Amino acid especially aromatic amino acids have been identified as important osmoprotectant molecules. The importance of aromatic amino acids, as osmoprotectant molecules in yeast osmostress response as well as other stress responses 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 and Takagi, 2008; Marks et al., 2008).

In dreaded osmotic stress condition, the yeast cell tends to adopt a unique behaviour which alters its physiological state, in turn enhancing its tolerance to lethal osmotic stress condition, unlike in osmotolerance adaptation. This adaptive behaviour which is known as osmoadaption (Gibson, et al., 2007) involves the synthesis of buffering macromolecules or neutral polyols collectively called compatible solutes through the high osmolarity glycerol (HOG) signal transduction pathway (Hohmann, 2002; O'Rourke et al., 2002; Saito and Tatebatashi, 2004).

In brewing yeast *S. cerevisiae*, the HOG pathway is triggered upon osmotic shock, which sends metabolic signals through two separate osmosensors located in the plasma membrane to the mitogen-activated protein (MAP) kinase signal transduction cascade (Maede et al., 1994). The osmolarity balance is also accompanied by the stability of enzymes, membrane proteins and phospholipids, which compatible solutes play a major role (Mager and Varela, 1994). This characteristic is said to be strain-dependent, with some yeast strain outperforming others (Zhao and Bai, 2009; Puligundla et al., 2011).

The above briefly accounts for mechanisms through which the yeast cell struggle to offset harsh conditions. However, yeast mineral nutrition amongst other nutritional stress alleviation strategy has been

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

Element/composition (g/kg dry weight)	Requirement (mM)	Function	Reference
Potassium 22	2-4	Maintains charge homeostasis, enzyme activity	Walker, 2004; Briggs, 2004
Magnesium 2.7	2-4	Cell division, enzyme activator, stress suppressor	Walker, 2004; Briggs, 2004
Zinc 0.12	4-8	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	Enzyme activity	Walker, 2004
Iron 0.1	1-3	Cytochrome, Haem-protein component	Walker, 2004
Cobalt 0.005	0.1	Enzyme activator	Walker, 2004
Molybdenum 0.0007	1.5	Nitrate metabolism, vitamin 12	Walker, 2004
Nickel 0.0025	~10	Urease activator	Walker, 2004
Copper 0.05	0.1	Enzyme activator, cobalamin	Walker, 2004

increasingly drawing scientific attention, as metal ions has been shown to be vital activators and regulators of most biological processes in all living organisms.

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. Recently, there has been an increasing concern on the role of mineral elements, as they influence numerous metabolic processes in all living organisms (Blazejack and Duszkiewicz-Reinhard, 2004; Aleksander et al., 2009; Okon and Nwabueze, 2009; Bose et al., 2011).

These elements which are often required at millimolar and micromolar concentrations (Jones and Greenfield, 1987) are classified into 'bulk' and 'trace' elements (Walker, 1994; Rees and 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; Walker et al., 2006). 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 to the yeast cell (Eide, 2000; Gaither and Eide, 2001). 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.

Yeast-mineral nutrition, which entails the preconditioning of yeast cells in metal-rich mediums or supplementation of fermentation medium with metals, 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. This literature emphasizes basically on the homeostasis, role(s) and fermentative significance of magnesium in yeast.

Magnesium

Magnesium ion is an important and most abundant divalent metal ion involved in wide range of biological processes (Elin, 1994; Hartwig, 2001; Lee and Gardner, 2005). This metal which constitutes about 0.3% of the yeast dry weight (Walker, 2004), activates over 300 enzymes (Rees and Stewart, 1998; Walker et al., 2006; Bose et al., 2011) intimately involved in metabolic and bioenergetic pathways; both in prokaryotes and eukaryotes (Walker, 1994). Maintenance of cellular integrity which includes structural stabilization of nucleic acids, polynucleotides, chromosomes, polysaccharides and lipids, have all been attributed to magnesium (Birch and Walker, 2000). It has been globally recognized in most living cells, that magnesium deprivation result in the

loss of cell conformational status (Walker, 1994). This attribute implicates the metal with structural function.

In humans, the 'intrinsic' regulatory role of magnesium ions is revealed in its ability to reduce the excitability of the nerve and muscle, by regulating the production of neurotransmitter, acetylcholine (Watson and Vaughan, 2001). Regulation of the production of cyclic adenosine monophosphates (cAMP), through the mediation of adenylate cyclase enzyme, has been ascribed to magnesium as it is reported that all adenylate cyclases possess an absolute requirement for Mg for their activities, invariably, Mg^{2+} influences various physiological processes whose operations are dependent on cAMP activity, for example, degradation of reserve energy substrate (glycogen), aggregation of thrombocytes, excretion and functioning of the parathyroid gland hormones (Stryer, 1997). Magnesium has also been implicated with lipid metabolism, where it influences the activity of the lipoprotein lipase; in turn regulating the concentration of cholesterol in the blood (Walker, 1994; Blazejack and Duszkiewicz-Reinhard, 2004). The structural, functional, heavy metal detoxification and stress protection roles of Mg^{2+} in yeast have been demonstrated (Pisat et al., 2009). Considering the diverse roles of Mg^{2+} ions in yeast physiology, understanding its homeostasis is of primary importance.

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 and Gardner, 2005). Mg^{2+} homeostasis in yeast cell is vital, as it is essential for maintaining growth and other metabolic functions of physiological significance. This regulation or homeostasis involves the localization, compartmentalization, sequestration (Walker, 2004) and subsequent release of the metal ion by specific membrane transport proteins present in specific intracellular organelles. The yeast periplasmatic, mitochondrial and more importantly the vacuolar membranes, are identified ionic channels and stores (MacDiarmid and Gardner, 1998; Koop et al., 2005) through which Mg^{2+} ions are regulated by the mediation of Mg^{2+} transport proteins, localized thereon. 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).

Chemisorption of magnesium ion in yeast

Chemisorption of Mg^{2+}, which is the first phase of its uptake or transport, is dependent on the physiological fitness of the yeast cell wall. The yeast cell wall, which consist mainly of β-1,3 glucan (up to 50% of wall d.m), mannoproteins (up to 40%), β-1,6-glucan (up to 10%) and chitin (1%) (Klis et al., 2002) serve as binding site for Mg2+ due to the presence of negatively-charged anionic groups (phosphomanate, carboxyl, hydroxyl and amines) present on the outer layer of the cell wall (Walker, 1994; Chmiel, 1998). Under depleting condition, Mg2+ actively bind to the anionic groups and are subsequently internalised through the action of negatively charged Mg2+ transport proteins (ALR1 and ALR2). This Mg-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+} ion.

Mg dynamism at the cell wall level has been reported to be significantly affected by 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 ranges of 5 to 25°C has been demonstrated; with temperature range below 5°C exerting an inhibitory effect on the binding process of metals to yeast cell wall (White and Gadd, 1987; Gniewosz et al., 2007). Medium pH has been reported by Tuszynski and Pasternakiewicz (1999), to have a predictory role on the binding competence between hydrogen ions and metal cation(s) for absorption sites on the yeast cell wall. Antagonistic correlations has been shown of magnesium to other metal ions such as strontium, where strontium has been reported to partially remove magnesium from yeast vacuole under enhanced cell activity (Avery and 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 (Blackwell et al., 1995). Another prime factor for magnesium uptake is the yeast viability. An inhibitory effect on metal binding process in high cell volumes has been reported by Park et al. (2003), although highly viable yeast cells have been shown to actively bind to magnesium (Blackwell et al., 1995). Also, enhanced magnesium accumulation in the presence of 1 to 2% mannose has been reported in the work of Blazejack et al. (2008), where cells (*Candida utilis*) grown in media enriched with magnesium and mannose, displayed higher amounts of absorbed magnesium when 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.

However, literature data (Gniewosz et al., 2007; Pisat et al., 2009) 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. The influence of variant conditions and source of carbon as well as nitrogen for augmenting magnesium bioavailability is of brewing implication as Mg2+ has been recurrently shown to be essential for yeast growth. However, under high and very high gravity fermentation condition, radical modification of these parameters is required to suitably adapt the yeast cell to adequately bind to Mg2+, considering the effect on quality of the end product as well as the yeast viability.

Bioaccumulation of magnesium ion in yeast cell

Bioaccumulation, which is the second phase of Mg transport, involves the uptake of magnesium via the cell wall and cytoplasmatic membranes into the cytosol of the yeast cell. The intracellular accumulation of magnesium is primarily mediated by the principal Mg transport proteins Alr1p and Alr2p (aluminium-resistance permeases) which are encoded by ALR1 and ALR2 genes (Blazejack and Duszkiewicz-Reinhard, 2004; Walker, 2004; Lee and Gardner, 2005). Both transport proteins belong to the membrane integral proteins or metal ion transporters (MIT) (Hirumura and Sakurai, 2001) and are found to share characteristic resemblance in terms of structure, molecular weight and isoelectric point (MacDiarmid and Gardner, 1998; Blazejack and Duszkiewicz-Reinhard, 2004); 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), which strongly suggests their specificity to Mg-uptake.

These membrane transport proteins has been reported by Lui et al. (2002) to serve as a like protein channels for Mg transport, as they tend to generate potential difference at both ends of the cytoplasmatic membrane (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 Mg^{2+}. However narrow variable regions (240 to 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 and Gardner, 2005).

These membrane proteins which shares homologue identity with the known Mg transport protein (CorA) in bacteria (*Salmonella typhimurium*), are expressed upon low concentration of Mg^{2+} ions on the cell wall (Lui et al., 2002; Hirumura and Sakurai, 2001; Pisat et al., 2009). Upon activation, basically Alr1p mRNA, the proteins are subsequently internalised and degraded (Graschopf et al., 2001) to release bounded Mg ion. This process has been reported to proceed at a slower pace as compared to the chemisorption process (Blazejack and Duszkiewicz-Reinhard, 2004). The released magne-sium ion, is subsequently sequestered by intracellular polyphosphates, RNA and ATP, leaving a minute quantity as free Mg^{2+} which is available for biochemical processes (Bui et al., 1998) such as enzyme activation, intracellular pH regulation, osmoregulation, protein and membrane stabilization, and signal transduction pathways (Walker, 2004).

The functionality of the Alr1p transporter has been reported to be dependent on the concentration of magne-sium ion - with the metal transporter losing its function at magnesium concentration exceeding 100 mM/L (MacDiarmid and Gardner, 1998; Graschopf et al., 2001). At this stage, the Alr1p is either modified for other functions or degraded in the vacuole, while Mg-uptake is undertaken by other mechanism(s) e.g. Alr2p (Blazejack and Duszkiewicz-Reinhard, 2004). 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 magnesium transporters (Alr1p and Alr2p), other envisaged transport proteins (Table 3) has been identified such as Mrs2p, Lpe10p and Mnr2 (Gadd, 1993; Pisat et al., 2009). The earlier transporter 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 (human liver cell) and lower (yeast cells) euka-ryote 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). Structural and functional similarity has been reported for Mrs2p and Lpe10p; where Lpe10p has been shown to undertake similar functional roles of Mrs2p - which could perhaps be active under condition of Mrs2p inactivation (Gadner, 2003). 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 magnesium ion, with the latter Mg-transporter (Mnr2) regulating the release of the metal ion (under both Mg-replete and Mg-deficient conditions) via the vacuolar membrane (tonoplast). It was further revealed, that the regulatory role of the protein transporter 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

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 and 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)
CCT	Fe^{2+}	Vacuolar membrane	Vanbelle and Andre (2001)

the primary Mg-transport proteins (Pisat et al., 2009).

Other metal transport systems, although not specific to magnesium uptake, has also been reported to mediate magnesium influx. Potassium transport system was 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 magnesium which could possibly partake in the bioaccumulation of magnesium, although the process is reported to serve the purpose of Mg-uptake under high magnesium deficient condition (Emelynova, 2001).

Earlier studies on magnesium homeostasis by Gunther et al. (1985) revealed a positive correlation of intracellular polyamines with free Mg^{2+} ions; which was believed to result from the mediation action of polyamines to the liberation of bounded magnesium, supposedly from the magnesium transport proteins, thus implicating the compound in the bioaccumulation process of the metal. Sodium and cadmium transport systems along with low-molecular proteins called the metallothioneins, and oligoamines like spermidine and putresine, have all been implicated with magnesium homeostasis (Truchlinski and Pasternak, 2002). It should be emphasized that this inter-metal homeostatic machinery is not exclusive to magnesium, but extends to other divalent metals of physiological significance basically Co^{2+}, Zn^{2+}, Mn^{2+} and Ni^{2+} (MacDiarmid and Gardner, 1998).

Role of magnesium in yeast physiology

Increasing research interest in the role of metal ions in living cells, has unfolded intriguing and novel cellular properties which are of biotechnological significance. Substantive insight into the importance of magnesium in living cells has identified pivotal roles of magnesium in prokaryotes and eukaryotes such as yeast as well as in human cells. The study of Walker and Daffus (1980) reveals the un-substitutable role of magnesium ions 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 A_{23187} (Walker, 2004).

Magnesium was further hypothesized in the study to act as a size transducer and growth enhancer - as its exhaustion and repletion was consistence with the initiation of each phase (tubulin polymerization and spindle breakdown) which precedes cell division in the yeast cell cycle (Walker and Daffus, 1980; Wolf et al., 2008). This hypothesis was supported by the work of Maynard (1993)- where entry of cells into the stationary phase and the time for maximum ethanol production and minimum sugar concentration coincided with maximum Mg^{2+} ions in the growth media.

The above report is consistent with the work of Pironcheva, (1998), where similar phenomenon was reported. Recent development in human genetics has also implicated Mg^{2+} with cell cycle control, as Mg^{2+} ions were shown to actively modulate cell cycle inhibitory proteins such as $p27^{kip1}$ and $p21^{cip1}$ of the mammary epithelial cells (Wolf et al., 2008).

Although, supportive phenomenon has been reported for the role of magnesium ions in yeast cell cycle progression; elucidation on the molecular mechanism of the cell proliferating and size transducing effect of magnesium ions in yeast cells is still demanding - as it could be possible to maintain a constant viable population density which has been shown in the work of Bellochet al. (2008) to be associated with high throughput and product uniformity.

Mg^{2+} has been reported to be essential for DNA duplication, as polymerases and ligases showed a requirement for the metal. The production of reactive oxygen species which are known to cause DNA damage has been shown to be actively reduced upon elevation of Mg^{2+} ions, both in yeast and human cells (Maynard, 1993;

Maede et al., 2004). The work of Giles and Czuprynski (2004) demonstrates the impact of magnesium ions on yeast growth - where pathogenic fungi *Blastomyces dermatitidis* showed an enhanced growth at Mg^{2+} ion concentration of 0.1 mg/ml. The study by Blazejack (2006) has also implicated magnesium indirectly with the activation of the nutrient uptake and growth machinery-where magnesium supplementation at levels of 1.25 g/L increased the binding capacity of the metal.

Genetic evidence on the growth stimulating effect of magnesium was indirectly shown in the work of MacDiarmid and Gardner (1998), as mutants' *alr1* of the principal magnesium transporter Alr1p displayed an enhanced growth upon addition of Mg^{2+} (50 to 500 mM of $MgSO_4$) to the growth medium. As demonstrated in the work of Trofimova et al. (2010), magnesium was found to stimulate growth, as exponentially growing cells displayed a maximum yeast biomass accumulation at magnesium level of 300 mg/L, although no effect was observed for magnesium supplementation to stationary phase cultures.

Magnesium ions 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, with improved tolerance to dehydration-rehydration treatments for stationary phase cells (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 magnesium 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 for same medium.

Anhydrobiosis study on yeast cell by Trofimova et al. (2010) has also implicated magnesium ions with charge-stabilization of membrane phospholipids, which are essential for maintaining membrane integrity. This conjecture was evident in the improved viability for slowly rehydrated cells which were pre-conditioned in variant amounts of Mg^{2+} ions. Unlike in the control reduced cell viability and aberrant cell surface was recorded, although cells were rapidly rehydrated. Enhanced viability and reduced cell surface damage was also reported in the work of Birch and Walker (2000), where magnesium concentration was elevated to 20 mM upon initiation of the stress conditions (ethanol and thermal shock).

Unlike in the control, aberrant cells (damaged cell surface) and absolute cell death was recorded for magnesium concentration of 2 mM. Of critical importance of magnesium in yeast is its stress counteractive property. The work of Birch and Walker (2000) simplistically demonstrates the protective effect of magnesium ions to both heat and ethanol stress in yeast cells. Magnesium was reported to negate the synthesis of heat shock proteins whose sole function is to counter the damaging and fluidizing effects of thermal and ethanol stress, respectively (Birch and Walker 2000; Gibson, 2011).

This protective role of magnesium was evident, as very few heat shock proteins (Hsps) were expressed on actively growing cells which were conditioned in elevated magnesium 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 (Birch and Walker, 2000; Vianna et al., 2008). It is apparent from this report, that high-temperature fermentations such as HG and VHG fermentation could be improved by controlling the bioavailability of magnesium ions.

Unlike the basic antagonistic relationship of Mg^{2+} and Ca^{2+}, the work of Giles and Czuprynski (2004) and Trofimova et al. (2010) has reported a novel relationship for both metals, as 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+}. It was hypothesized in the report, that both metals cooperatively stabilizes and reduces fluctuation in plasma membrane fluidity which has been correlatively found to be an inverse function of yeast survival (Simonin et al., 2008).

However, magnesium ions have been shown to modulate the kinetics of calcium, as it destabilizes calcium complexes and actively counter the binding action of calcium ions to intracellular and extracellular ligands (Levine and Coburn, 1984). Reports from other authors has identified calcium as the dominating metal in the antagonistic relationship, as a result of calcium's high affinity for binding sites on ligands like the ATP and nucleic acids (Walker, 1994; Gibson, 2011).

With recent literatures demonstrating inverse correlation between Mg and Ca, 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 physiology as well as in fermentation processes (Rees and Stewart, 1999; Birch et al., 2002; Okon and Nwabueze, 2009). Also, metal detoxification role of magnesium has been indirectly revealed in the work of MacDiarmid and Gardner (1998), where over expression of the principal magnesium transport gene ALR conferred increased tolerance to aluminium and gallium. Although, increased sensitivity to other divalent metal ions Co^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, La^{2+}, Cu^{2+} and Ca^{2+} was observed. It is apparent from the result that magnesium counters the impact of aluminium toxicity in yeast, as the transporter Alr1p is basically specific to Mg-uptake.

Role of magnesium in ethanolic fermentation

Considering the central role of fermentation in biotechnology, it is apparent that magnesium have a great deal in

predicting the performance of fermentation processes, as it influences numerous metabolic processes of comer-cial yeast strains. Pyruvate kinase, hexokinase, phosphofructokinase, phosphoglycerate kinase and enolase have all been reported to show an absolute requirement for magnesium ions (Walker, 2004). Walker et al. (1990) hypothesized that magnesium ions 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 magnesium and activity of pyruvate decarboxylase. However, it is still unclear and demanding at the cellular level on how magnesium dictates the respiro-fermentative switch in the presence of oxygen and glucose.

Studies on the role of metal ions in fermentation processes have implicated magnesium with high throughput with 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 in the work of Rees and Stewart (1999) upon supplementation of 500 ppm $Mg^{2+}$with best throughput for ethanol under oxygenated condition (Aleksander et al., 2009).

Villen et al. (2009) has shown that yeast cells requires a constant narrow range of magnesium amounts 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 magnesium levels, to have no further influence on ethanol yield. A steady biomass accumulation in the presence of Mg^{2+} was also reported in the work of Aleksander et al. (2009), which also point to the importance of magnesium ions to cell growth - an active phase for ethanol formation.

Mineral optimization study by Okon and Nwabueze (2009) has reported an optimum ethanol yield of 12.53% (v/v) for metal combinations of Mg, Zn and Ca, with Mg to Ca ratio approximately 2:1. Another metal combination study under conventional and very high gravity wort condition by Rees and Stewart (1997) has 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 availability of magnesium exerts positive influence on high substrate ethanolic fermentation, as it alleviates the impact of osmotic pressure on the yeast cells (D'Amore et al., 1988). Ordinate flocculation behaviour of yeast cells during fermentation has been shown in the presence of Mg^{2+}, with *ca*. 10 ppm of Mg^{2+} ions substituting Ca^{2+} under experimental conditions (Stewart and 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 magnesium availability is an index of yeast fermentative performance; as such, supplementation of the metal should be done with precision considering other counteractive parameters that could impede its bioavailability.

CONCLUSION

Although, great achievements have been reported for cell engineering in view of enhancing yeast performance under high substrate fermentation, the feasibility of VHG fermentation in the radius of alcoholic beverage production still remain problematic. Knowledge on stress conditions, their mechanism of inhibition as well as stress tolerance in yeast, as reviewed in part in this work as well as in other authors reviews, strive at unveiling underlying yeast metabolic machinery for the purpose of cellular modification for enhanced yeast performance under challenging fermentation condition.

Single cell manipu-lation and the impact of variant nutritional supplements, such as nature of carbon and nitrogen sources, vitamins, and minerals in enhancing yeast performance under high gravity fermentation has been reported; however, a holistic approach, is required as to streamline their influence on the best possible condition which will sustain yeast performance while ensuring a high throughput and quality of the end product, which is of utmost importance. With weight of evidence implicating magnesium ion in numerous biological processes, which are pivotal to sustaining and enhancing yeast fermentation perfor-mance, it is important that critical modification should be made with respect to cell engineering for phenotypes with high binding ability to the metal (magnesium), while developing credible techniques for maintaining relatively constant levels of magnesium ions during ethanolic fermentation processes.

Considering the increasing global embrace of VHG fermentation and recent biotechnological developments such as global transcription machinery engineering (gTME), and the availability of 'omic' tools; development of desirable phenotypes which are pertinent to VHG brewing fermentation should be explored in-depth, with due attention to cellular optimization processes that integrates mineral fortification.

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