Studies into Mg-preconditioning of Brewer's Yeasts

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I certify that this is the true and accurate version of the thesis approved by the examiners.

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Director of Studies

Studies into Mg-preconditioning of Brewer's yeasts: G D Smith

Abstract

This research was concerned with testing a hypothesis that brewer's yeast cells, which were physiologically "preconditioned" with Mg ions, had improved fermentative output and stress tolerance. Ale and lager yeast cells preconditioned in the presence of elevated levels of Mg-actate, yielded highest cellular Mg contents in malt extract broth and dilute brewer's wort media. Mg-acetate preconditioned lager yeast cells exhibited higher fermentative activities than non-preconditioned yeasts, resulting in higher yields of ethanol. The specific activity of the alcohologenic enzyme pyruvate decarboxylase was stimulated by Mg enrichment and a correlation between cellular Mg and PDC activity exists. This was especially evident in Mg acetate preconditioned cells. Mg ion homeostasis in Mg non-and preconditioned yeast cells was altered by temperature shock (both cold shock at 4°C and heat shock at 40°C) and toxic ethanol shock (at 10-20%). This resulted in significant cellular losses of Mg, which indicated that some of the extra Mg present in these cells was in a free (releasable) form rather than being tightly bound. Stress tolerance was found to be strain specific with lager yeast cells found to be more tolerant to low temperature stress (4°C) and ale yeast cells more tolerant to high temperature stress (40°C). The results of this study showed that intracellular Mg is beneficial to brewing yeasts at two levels: at the biochemical level to stimulate fermentative enzymes; and at the biophysical level to protect the structural integrity of the plasma membrane in stressed cells. This study has highlighted gaining a fundamental insight into aspects of yeast physiology may prove of direct practical value to industries concerned with production of ethyl alcohol by fermentation.

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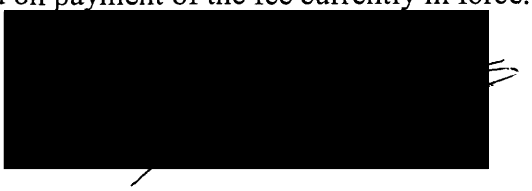
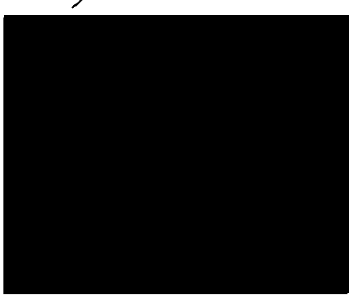
Signature 

Address 

November 7th 2001

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Contents

List of Figures and tables

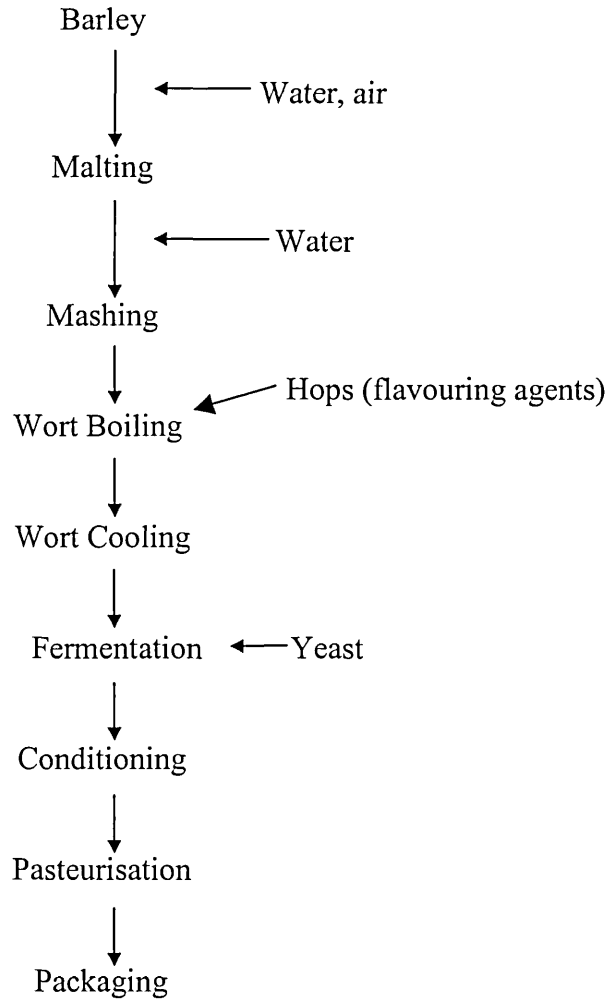
CHAPTER 1

INTRODUCTION

1.1 The Brewing Process

Brewing is the oldest known biotechnology and records show that it dates back to around 6,000 BC in Sumaria. Through time it has developed alongside/in partnership with baking. The term brewing is a bit misleading as it only describes one stage of the process (mashing) of converting the starch in malted barley into alcohol (see Fig 1.1).

Fig 1.1 The Brewing Process (adapted from Walker 1998a)



Brewing begins with malted barley (the barley is allowed to partially germinate before being dried and crushed to form grist), which is added to warm water to form the mash, triggering the conversion of the starchy endosperm to smaller sugar molecules. The

sweet liquor (or wort) is boiled in the presence of flavouring agents such as hops (other agents can be added for example honey, heather, pine and myrtle which are generally used in traditional ales), which impart a bitter flavour to the wort. Boiling the wort ensures the cessation of any further enzymatic breakdown of the starch and pasteurises the wort. The wort is then allowed to cool and is (partially) aerated (yeast requires oxygen at the start of fermentation). Yeasts are then added to convert the sugars into ethanol and carbon dioxide; the yeasts themselves release additional metabolites, which contribute to the final aroma and flavour of the final product. At the end of fermentation the yeasts are then removed and the green beer is allowed to mature or "lager". After this maturation period the beer is packaged into kegs, bottles or cans.

The role played by yeasts in fermentation was not appreciated until relatively recently in the long history of brewing, when Pasteur demonstrated that fermentation required the participation of living organisms. Hansen isolated brewing yeasts *per se* and propagated them in pure culture. Previously, in 1680, Antonie van Leewenhoek microscopically observed brewer's yeasts for the first time. Cagnaird de la Tour (France) and Schwann and Kutzing (both Germany), proposed that a microscopic form of life made the products of alcoholic fermentation (ethanol and carbon dioxide) (Abbott *et al.* 1993). Prior to these discoveries, brewers had used undefined mixed cultures of yeasts with somewhat unpredictable results (Abbott *et al.* 1993).

Brewing yeasts can be split into ale and lager types. Lager yeasts (*Saccharomyces cerevisiae var. carlsbergensis*) are employed in the production of lager and they differ from ale yeasts (*Saccharomyces cerevisiae*) in that they possess the ability to produce the extracellular enzyme α-galactosidase (melibiase), which breaks down the disaccharide

melibiose. These "bottom fermenting" yeasts flocculate and collect at the bottom of the fermenter vessel at the end of fermentation (conducted at 7-15°C). Ale yeasts can not produce melibiase, are "top fermenters" (18-22°C), forming loose clumps of cells, which are adsorbed to carbon dioxide bubbles and are carried to the surface of the wort at the end of fermentation. Beers can also be made using yeasts of non-*Saccharomyces* genus. Such examples are *Schizosaccharomyces pombe*, *pombe* being Swahili for beer and *Brettanomyces spp,* which are used in traditional Belgian beers; *Candida methanolovescens* has also been used in the production of non-alcoholic beers (van den Bremt *et al.* 2001). Low alcohol wines have been produced by strains of *Pichia* and *Willopsis* (Erten and Campbell 2001). A low alcohol beverage has been produced from whey permeate using *Kluyveromyces fragilis* (Parrondo *et al.* 2001a; 2001b).

1.1.1 Modern Brewing Practices

Modern breweries produce around 500 million litres of beer per annum (Linko *et al.* 1998). For this to occur in a cost efficient manner, and in order that they survive the ever-increasing competition of a global market, they must ensure that their beer is of a high and uniform quality.

In recent years, possibly the greatest innovation to enter the brewhouse has been the use of high gravity or very high gravity brewing (D'Amore *et al.* 1991). This practice is not all encompassing however, as a number of companies do not use this process through choice, due to product or legal reasons (Stewart 1999). High gravity brewing is the production and fermentation of wort (12-20° Plato) with a higher than normal sugar concentration than is required of the final beer. The final beer is adjusted to its required concentration (7-13° Plato) by the addition of deoxygenated water (Linko *et al.* 1998).

Very high gravity worts, those over 20° Plato (1080° Original Gravity, OG), are attained by adding syrups (e.g. glucose or maltose) to the wort kettle (McCaig *et al.* 1992).

Methods of reducing fermentation time are under investigation by brewers, as well as in the production of low alcohol or non-alcoholic beers using immobilised yeasts (Yamauchi *et al.* 1994; van Iersel *et al.* 1995; Bardi *et al.* 1997; Smogrovicova *et al.* 2000). Attempts have also been made to continuously produce wort with little success, however (Linko *et al.* 1998).

Improvements in the brewing industry have not solely been restricted to the brewhouse *per se.* For example, yeast strain selection to produce more ethanol and flavour compounds (esters) such as isoamyl acetate which impart "banana like" "apple like" or "solvent like" and phenylethyl acetate results in "rose or honey like" flavours (Lee *et al.* 1995), electric field in combination with chemical mutagens (Keun and Lee 1998) and recombinant DNA technology (Birol *et al.* 1998; Panoutsopoulou *et al.* 2001) have been investigated as means of improving brewing yeast productivity. Recombinant DNA technology has been viewed as having the potential to develop brewing yeast strains to ferment a greater variety of sugars, which are otherwise unfermentable, flocculate appropriately and sufficiently early, provide a greater tolerance of chemical and physical stresses in brewing and to produce a more stable beer with a greater flavour (Walker 1998a). Further examples of the usefulness of recombinant DNA technology are listed in Table 1.1.

Table 1.1 Practical applications of recombinant DNA technology and their potential applications to brewing yeasts (adapted from Walker 1998b .

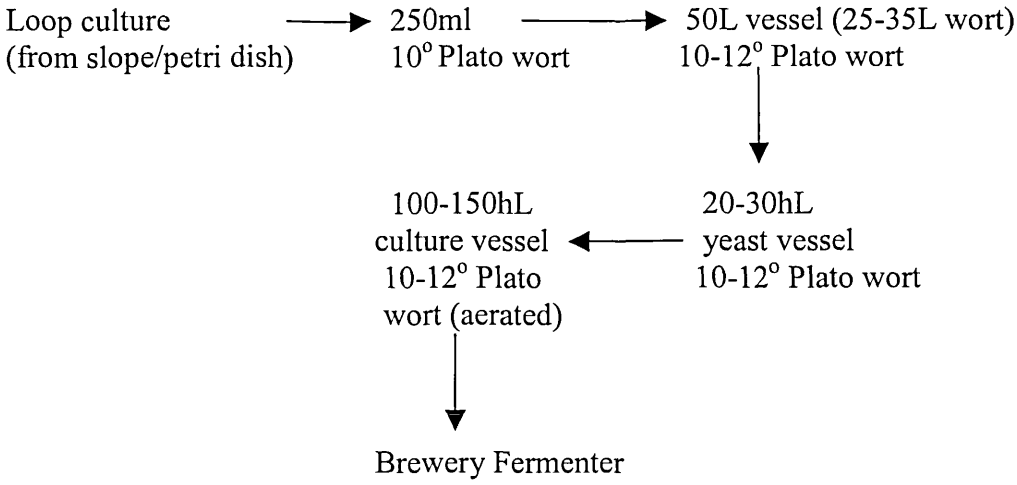
Current Strain Limitation	Desirable property of strain	Potential improvement through recombinant DNA technology
Low ethanol tolerance	Increase ethanol tolerance for high gravity and very high gravity fermentations	Transfer of acetoacetyl Co A thiolase (*ERG10*) gene from ethanol tolerant yeast
Fermentation of narrow sugar range	Fermentation of maltodextrins to produce low carbohydrate beer	Transfer of glucoamylase (*STA*) genes from *S.cerevisiae* var. *diastaticus* or *Aspergillus spp*
Sensitive to contamination by wild yeasts and bacteria	Anti microbial properties	Transfer of killer toxin plasmids by cytoinduction or electrotransformation
Inability to hydrolyse ß-glucans	Degradation of ß-glucan from adjunct worts and malted barley – improves wort filtration and eliminates beer haze	ß-glucanase genes cloned from bacteria, fungi and barley
Low proteolysis	Proteolysis would improve wort nitrogen assimilation and prevent beer haze	Transformation of *S.cerevisiae* to secrete a protease
Long beer maturation times	Reduction of maturation times through reducing levels/metabolism of off flavours	Cloning of genes encoding enzymes of the valine synthetic pathway (involved in diacetyl production). Transformation of cystathionine synthase genes (reduces H_2S production)
Uncontrolled flocculation	Proper timing of yeast flocculation of yeast to aid downstream processing of beer	Cloning of alcohol acetyl transferase (*AFT1*) gene into (non brewing) *S.cerevisiae* strain (responsible for acetate ester synthesis)
Instability of beer flavour	Increased SO_2 production to act as flavour stabilization and antioxidant	Deletion of sulphite reductase genes (*MET10*) resulting in increased SO_2 levels in beer
Repression of maltose utilization	Inhibition of glucose repression would improve maltose fermentation and allow glucose adjuncts to be used more	Transfer of maltose permease genes (*MAL6*) results in improved maltose utilization

A number of factors have and may continue to impede the use of recombinant DNA technology including: uncertain and lengthy government regulatory approval, availability of alternative solutions, patent applications (Walker 1998a) and probably the overriding factor at present is consumer acceptance of genetically modified organisms. A recombinant brewing strain together with a baking strain does however exist and has been cleared for commercial use in the UK (Walker 1998a). This strain (*S.cerevisiae*, No BRG 6050) possesses the ability to partially hydrolyse maltodextrins during wort fermentation through the incorporation of a plasmid containing the *STA2* gene from *S.cerevisiae* var. *diastaticus* (Walker 1998a).

1.1.2 Yeast cultivation and viability assessment

Brewing is unique amongst the biotechnology industries, in that the yeast used is generally cropped after fermentation and re-pitched into subsequent brews. Normal procedures in many breweries are such that fresh yeast is propagated (lager yeast in particular) every 8-10 generations (fermentation cycles) (Stewart and Russell 1998). Yeast propagation is generally carried out in standard brewery wort (Cahill *et al.* 2000) at only slightly higher temperatures than the fermentation with intermittent aeration, which stimulates yeast growth. Propagation of the master culture to plant fermentation scale is a series of fermentations increasing in size (5-20 times) until sufficient yeast is grown to pitch a full size commercial brew (see Fig 1.2) (Stewart and Russell 1998).

Fig 1.2 Yeast Propagation (adapted from Stewart & Russell 1998)



Yeast viability is routinely measured in brewery laboratories by microscopic means. This ensures that the pitching yeast is not contaminated and that it is viable. Methods of measuring viability can be divided into three categories: those, which use vital stains, those based on cell replication or those assessing the metabolic activity of the cell (Mochaba *et al.* 1998). Methylene blue is the most commonly used vital stain in breweries, in viable cells the dye is oxidised to a colourless compound in the cytoplasm of the cell whilst dead cells remain stained blue (O'Connor-cox *et al.* 1997). However, it has been reported as being unreliable when yeast viability drops below 95% (O'Connor-cox *et al.* 1997). Another viability dye currently being assessed is methylene violet (Smart *et al.* 1999), this works in similar manner to methylene blue, in that dead cells are stained pink. There are a number of fluorescent dyes available. For example, the magnesium salt of 1-anilino-8-naphthalene sulfonic acid (Mg-ANS) (McCaig 1990) and fluoroscein diacetate (FDA) (Mochaba *et al.* 1998), are two such dyes, both of which require fluorescent microscopes. Other methods of assessing viability include plate counting, whereby yeast suspensions are inoculated onto agar and incubated. Such a method is very accurate but also very slow. Vitality of brewing yeast is generally determined by measuring the metabolic activity of the cell (Mochaba *et al.* 1998), an example of such a method is the

measurement of intracellular ATP using firefly bioluminescence (Hysert and Morrison 1977). This has also been described as a viability method as it measures the ATP present in living cells (Stewart and Russell 1998). As ATP is degraded rapidly when cells die, an inference is therefore made between the ATP present in the biomass and the number of viable cells present (Stewart and Russell 1998). The Magnesium release test (Sigma) is another example of a vitality test, which is based on the observation that ions such as Mg^{2+}, K^{+} and PO_4^{2-} are released by yeast immediately after inoculation into glucose medium (Stewart and Russell 1998). In this case it allows yeast vitality to be measured by a quantative colourimetric measurement of Mg in wort before and immediately after yeast inoculation, the inference being drawn that the more magnesium the higher the yeast vitality (Stewart and Russell 1998).

1.1.3 Yeast Management

As modern beverage fermentation practices need to be cost effective, it is vitally important that the biological agent (*S.cerevisiae*) used to produce the potable alcohol, be it ale/lager, whisky, rum or neutral spirits, be looked after. Brewers and distillers are now looking at the way in which yeasts are handled both before and after fermentation with a particular interest being shown in storage conditions.

In an ideal world, yeast would be stored in an easily cleaned room, which contains a plentiful supply of sterile water and a separate sterile air supply whilst being maintained at 0°C (Stewart and Russell 1998). In the main, yeast is stored in less than six inches of beer, under water or 2% potassium dihydrogen phosphate solution (Stewart and Russell 1998). Sophisticated storage tanks with external cooling systems and equipped with low shear agitation devices are becoming more commonly used (Stewart and Russell 1998).

O'Connor-Cox (1997b; 1998a,b) has advocated that yeast handling be minimised in order to prevent both the loss of viability and vitality, as well as keeping the yeast cold. Morimura *et al* (1998) have concurred with these methods in relation to the storage of yeast used for whisky production. These authors found that yeasts stored at 5°C had a shorter lag time and increased viability compared to yeasts stored at 20°C which also showed a decreased rate in ethanol production. Long periods of storage can also have a detrimental effect on yeast resulting in sluggish fermentation rates and modifications to the flavour and stability of the final beer (Stewart and Russell 1998).

1.2 Yeast physiology and metabolism

It is very important to have a thorough knowledge of the relationship between the structure and function of the components which comprise the yeast cell, in order to properly exploit its metabolic activities (Rose 1993). To this end there is a great wealth of knowledge relating to the make-up of the yeast *S.cerevisiae* from the structure of the envelope layers (cell wall and cell membrane) to the cytoplasm, nuclear region, mitochondria, ribosomes, vacuoles, golgi, endoplasmic reticulum etc. For the purposes of this discussion the main components of the yeast cell (*S.cerevisiae*), which are affected by the rigours of its continued survival, will be explored further.

1.2.1 Yeast cell wall

The yeast cell wall is a rigid non-static structure, the permeability of which determines the passage of large and small molecules into and out of the cell (Stratford 1994). The cell wall itself is organized into two layers comprising cell wall proteins, glucans and chitin, all of which are interconnected by covalent bonds (Kapteyn *et al.* 1999). ß-1,6-glucans are small molecules comprised of 140 glucose residues and account

for 5% of the cell wall structure. ß-1,3-glucans are much larger accounting for over half of the cell wall structure and are its main structural component; together with chitin they are responsible for determining the cell walls' shape and strength (Kapteyn *et al.* 1999). Chitin accounts for 1-2% and is located in a ring at the base of the bud or is dispersed in the lateral walls (Kapteyn *et al.* 1999). The cell wall proteins have been divided into two groups according to the way they are attached to the structural network of the cell wall (see Table 1.2). The first group was considered to be noncovalently attached to the cell wall or retained by disulphide bridges, whilst the second group of cell wall proteins were assumed to be covalently linked to glucan.

Table 1.2 Cell wall proteins (Kokanj and Mrsa 2001)

Protein	**Bonding (covalent/noncovalent)**	**Function**
Cts1p	Noncovalent	Chitinase
Exg1p	Noncovalent	Exoglucanase
Bg12p	Noncovalent	Endoglucanase
Scw8p	Noncovalent	Pir-protein
Ccw13p	Covalent	Anaerobic growth
Flo1p	Covalent	Flocculation
Ccw5p	Covalent	Pir-protein
Ccw6p	Covalent	Pir-protein

Although much work has been carried out to elucidate the structure of the cell wall proteins, the function of many of these proteins remains a mystery requiring further study (Kokanj and Mrsa 2001).

The structure of the cell wall plays an important role in yeast cell flocculation. Flocculation as applied to brewer's yeast is "the phenomenon wherein yeast cells adhere in clumps and either sediment from the medium in which they are suspended or rise to the medium's surface" (Stewart and Russell 1998). Flocculation in yeasts is a genetically controlled characteristic (Jin and Speers 1998), encoded by four genes *FLO1, FLO5, FLO9*

and *FLO10,* a semi dominant (*flo3*) and two recessive genes (*flo6 and flo7*) have also been identified (Stewart and Russell 1998).

A number of hypotheses to explain yeast flocculation have been in existence for sometime now. The calcium-bridging hypothesis to explain cell-cell interactions was popular until the early 1980's (Jin and Speers 1998). This proposed that calcium ions formed bridges between flocculating cells by binding to negative charges on the cell surface. These bridges were stabilized by hydrogen bonding between carbohydrate hydrogen atoms and hydroxyl groups (Jin and Speers 1998). This has since been superseded by a more convincing mechanism to explain flocculation (Jin and Speers 1998). This hypothesis proposes that specific surface proteins known as zymolectins (named such, to distinguish them from lectins of other yeasts/microorganisms) bind to the mannose residues of mannan molecules on neighbouring cell surfaces. Calcium ions are believed to maintain the correct conformation of the zymolectin binding site (Jin and Speers 1998).

Flocculatability of brewing yeast strains varies greatly. Highly flocculant strains separate early from suspension in fermenting wort resulting in a less attenuated, sweeter and less than fully fermented beer (Stewart and Russell 1998). Non or strains, which are poorly flocculant, produce a dry, fully fermented beer, which is slow to clarify leading to difficulties in filtration and could acquire yeasty off flavours (Stewart and Russell 1998).

1.2.2 Yeast cell membrane

The plasma membrane in *S.cerevisiae* is comprised of a mixture of polar lipids and proteins which form a fluid mosaic (Walker 1998a) the primary function of which is to act as a barrier through which hydrophilic molecules must pass, preventing the contents of the cytoplasm of the cell mixing with the aqueous exterior of the cell (Rose 1993; Walker 1998a). It is comprised mainly of phospholipids (such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatydilserine and phosphatidylglycerol) and sterols (erogsterol and zymosterol) with the possibility that the phospholipids confer fluidity and the sterols rigidity (Walker 1998a). Unsaturated fatty acid and sterol synthesis requires oxygen. As fermentation is primarily anaerobic, oxygen must be added to wort prior to the onset of fermentation. A few hours post-pitching most of the oxygen in the wort has been removed by yeasts, which during this time will have, synthesised lipids (fatty acids and sterols) and reduced cellular glycogen levels (Stewart and Russell 1998), the glycogen having provided a carbon source for such synthesis (Boulton 2000). The proteins present include those involved in solute transport (ATPases, permeases, channels) cell wall biosynthesis (glucans and chitin synthases) transmembrane signal transduction (adenylate cyclase, G-protein) and cytoskeletal anchoring.

The cell membrane is an important target for disruption by ethanol, with the plasma membrane proteins associated with solute transport inhibited (Rose 1993). Increasing the fatty acyl chain length and the proportion of cis-mono-unsaturated fatty acids found in membrane lipids has been shown to increase ethanol tolerance (Ingram 1986; Rose 1993).

1.2.3 Yeast cytoplasm and vacuole

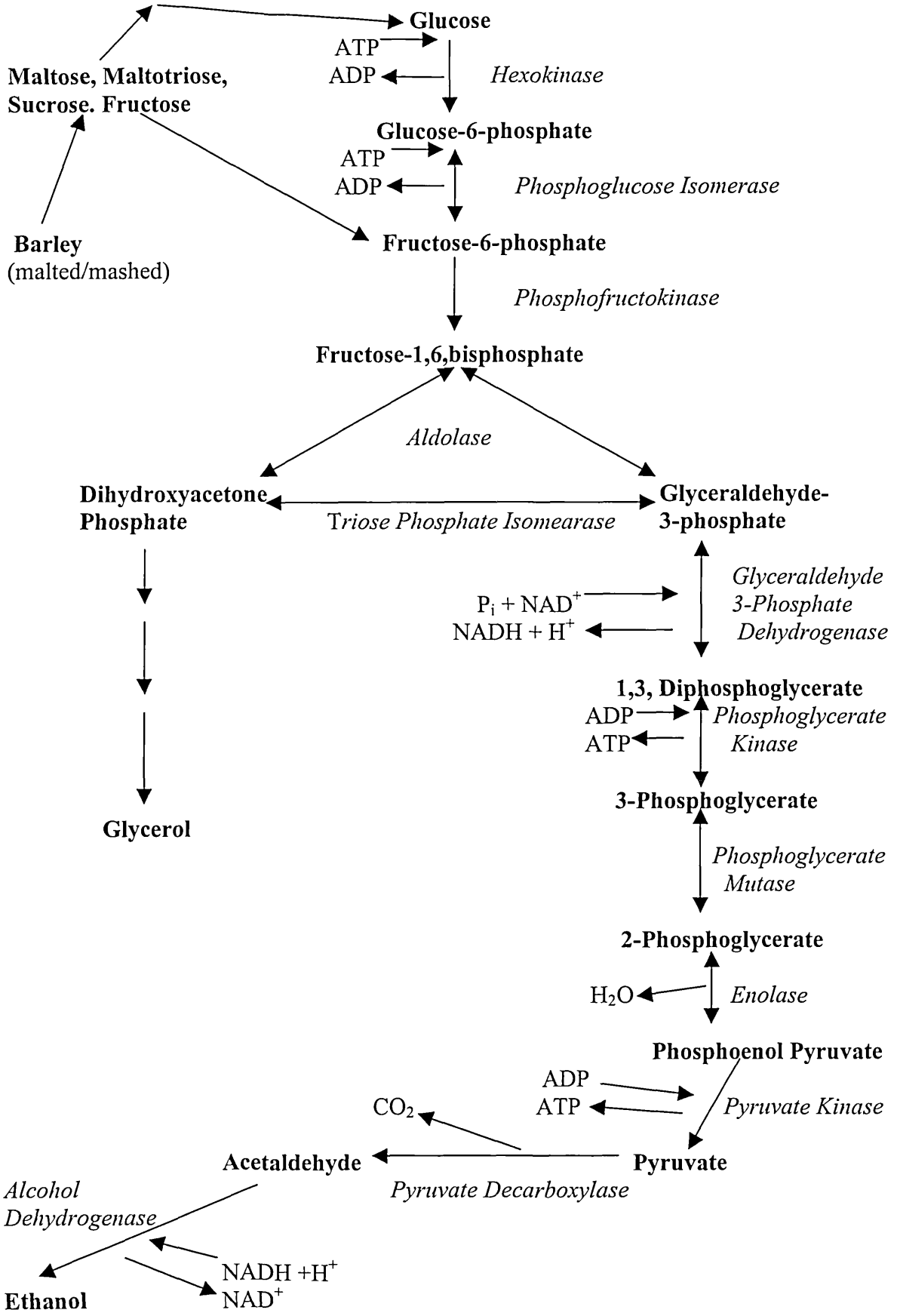
The yeast cytoplasm is an aqueous acidic colloidal fluid, which contains a variety of low and intermediate molecular weight compounds, dissolved proteins, glycogen and other soluble macromolecules (Walker 1998a). Glycogen, together with trehalose, is the main storage carbohydrate in yeast cells. It provides a source of biochemical energy during lag phase of fermentation, when energy demands for the synthesis of lipids is high and also during yeast storage between fermentations (Stewart and Russell 1998). During the later stages of fermentation glycogen reserves are restored with the maximal content dependent on yeast strain function, fermentation temperature, wort gravity and a variety of other factors (Stewart and Russell 1998).

The vacuole is the organelle involved in protein trafficking; it may exist as a single large entity or as a number of smaller entities known as provacuoles (Walker 1998a). The tonoplast is the structure, which encapsulates the vacuole and is made up of a variety of fatty acids and sterols, which differ from those that comprise the cell membrane; this enables it to have a greater degree of elasticity.

1.2.4 Yeast Metabolism

S.cerevisiae is a facultative anaerobe capable of utilising glucose under aerobic or anaerobic conditions. Brewer's wort (created from the malting and mashing of barley) is made up of the sugars sucrose, fructose, glucose, maltose and maltotriose with dextrins also present too. Ale and lager yeasts will normally ferment these sugars in the following sequence: sucrose, glucose, fructose, maltose and maltotriose (see Fig 1.3).

Fig 1.3 Glycolysis/Fermentative metabolism (adapted from Walker, 1998a)



Biological membranes are relatively impermeable to sugars (Lagunas 1993), yeasts possess a number of mechanisms capable of transporting sugars across the plasma membrane, these range from simple net and facilitated diffusion to active transport (Walker 1998a). *S.cerevisiae* possesses nearly twenty homologous genes encoding proteins belonging to the HXT sugar transporter family (Kasahara and Maeda 1998; Walker 1998a). The expression of the *HXT1* gene increases with increased glucose concentration whereas the high affinity *HXT2* (and *HXT4*) genes are expressed under low glucose concentrations and repressed under high concentrations (Horak 1997). The *HXT3* gene expression on the other hand appears to be induced by glucose irrespective of its concentration (Horak 1997). Galactose is transported through the expression of a single transporter gene (*GAL2*), which is repressible, by glucose (Horak 1997).

Two transport mechanisms are available in yeasts for the uptake of disaccharides (maltose and α-glycosidase), both of which are H^+-symporters, relying on electrochemical proton gradients (Lagunas 1993). Maltose, the main sugar in malt and important to brewers, is taken up by its own transporter encoded by the *MAL* genes and hydrolysed intracellularly by maltase to from two molecules of glucose. These genes are organised as five unlinked, homologous loci (*MAL1-MAL4, MAL6*) on different chromosomes. Each of the loci comprises three genes, which encode maltose permease, maltase and a transcriptional activator (Dickinson 1999). Sucrose is hydrolysed extracellularly by the enzyme invertase to glucose and fructose (Mwesigye and Barford 1996). This enzyme is encoded by the *SUC* genes, which are also a homologous family organised at six loci (*SUC1-SUC5, SUC7*) on different chromosomes (Dickinson 1999). The utilisation of both maltose and sucrose are repressible by glucose (Lagunas 1993; Dickinson 1999). In the case of maltose it is the *MAL* genes which are repressed when glucose concentrations are

higher than 1% (w/v), only once 40-50% of the glucose has been taken up does maltose and maltotriose uptake begin to occur (Stewart and Russell 1998) and in sucrose utilisation the enzyme invertase (Dickinson 1999) is repressed. This repression of sugar utilisation by glucose results in a reduction in wort fermentation rate.

1.3 Brewing yeast stress

As previously discussed, high gravity brewing involves the use of wort with a higher than normal sugar concentration (16-18° Plato). Such a practice has detrimental effects on not only the fermenting yeasts but also the flavour of the final beer.

Stress is an important factor, which can affect the performance of yeast in both high gravity and in normal gravity brewing. Such stresses can exert themselves in a variety of ways examples of which are shown in Table 1.3.

Table 1.3 Brewing yeast stresses (Smart *et al.* 1995; Walker and Birch 1998).

Stress	Type of stress
Cell ageing (serial repitching)	Biological
Microbial contaminants	Biological
Ethanol toxicity	Chemical
pH	Chemical
Acid wash	Chemical
Nutritional starvation	Chemical
Cold Shock	Physical
CO_2/hydrostatic/top pressure	Physical
Mechanical sheer (centrifugation)	Physical
Temperature fluctuations	Physical

It is generally known that non-growing (stationary phase) cells have a greater tolerance to stress than cells, which are actively growing (Lewis *et al.* 1993; Mager and Varela 1993; van Dijck *et al.* 1995; Swan and Watson 1997). This is thought to be due to cells which are actively growing on rapidly fermentable sugars (e.g. glucose) being stress sensitive and having low levels of trehalose (Lewis *et al.* 1993; van Dijck *et al.* 1995). However, cells undergoing respiratory growth, on ethanol and other non-fermentable

carbon sources, and stationary phase cells have higher trehalose contents and are therefore more stress tolerant (Lewis *et al.* 1993; van Dijck *et al.* 1995).

Ethanol is well known as being toxic to yeast cells even in ethanol producing species (Hallsworth 1998). Ethanol has an adverse affect on cell membranes, proteins and other hydrated cellular components (Hallsworth 1998), by affecting the structural integrity of these components, which are maintained by water molecules. Described by Slater *et al* (1993) as being a hydrogen-bonded network, which extends between lipids. Ethanol also inhibits glucose, maltose, ammonium and amino acid uptake, endocytosis and causes potassium ions, amino acids and nucleotides to leak out of the plasma membrane (Piper 1995; Lucero *et al.* 2000). Heat stress like ethanol stress increases the permeability of membranes, thus having an adverse affect on membrane associated processes (Piper 1995).

When exposed to a rapid decrease in temperature the yeast cell membrane cools. If this rate of cooling is faster than the rate at which the cell is able to adjust its cell membrane composition the membrane fatty acids and sterols undergo a phase transition from fluid to 'gel' states (Fargher and Smith 1995). The fatty acids/sterols may then be fixed in a random manner, which compromises the integrity of the cell membrane causing it to leak (Fargher and Smith 1995). The cold shock itself may be lethal or sublethal, sublethal injury causes damage to vacuoles resulting in the decompartmentalisation of some enzymes and impairment of protein synthesis (Fargher and Smith 1995; Zhang *et al.* 2001).

Starvation has an affect on the morphology of the cell wall. In non-starved cells a rough surface due to the presence of fine protrusions is observable, whilst in starved cells there may be a reduction in the cell surface charge and a modification in the cell surface topography due to the restructuring of the cell wall (Smart *et al.* 1995).

Since Pasteur's discovery that bacterial contamination causes the off flavours in beer, acid washing has been employed by breweries to remove bacterial contamination from pitching yeasts, without having an undue effect on their fermentation performance (Cunningham and Stewart 2000). Although they tolerate acid conditions *per se,* yeast cells grow well between pH 4.5-6.5. Nearly all yeast species can, however, grow in pH 3 media to a lesser extent (Walker 1998a). The physiological condition of the yeasts often determining whether or not the acid wash is detrimental to their continued well being (Cunningham and Stewart 1998).

1.3.1 Mechanisms of alleviating yeast stress

Yeasts possess a number of mechanisms designed to alleviate the effects of stress. The cells themselves may be exposed to a mild stress and thus develop tolerance not only to higher doses of the same stress but also to stress from other agents (Estruch 2000). This is known as *cross protection* and Estruch (2000) has proposed that an integrating mechanism, which recognises and responds to stress in various forms, may exist. The cyclic AMP-dependent Protein Kinase (cAMP-PKA) pathway is involved in the regulation of a number of cellular processes, including nutrient sensing, regulating yeast cell proliferation, carbon storage and responses to stress (Estruch 2000). This pathway is activated by the synthesis of cAMP by adenylate cyclase which is itself activated by a number of pathways involving the G-protein coupled receptor system or the GTPases

Ras1p and Ras2p (Igual and Estruch 2000). cAMP activates the cAMP-PKA pathway which causes transient changes in a number of systems including trehalose and glycogen metabolism, glycolysis and gluceogenesis.

Heat shock proteins are evolutionarily conserved proteins, which were originally characterised on the basis of their induction by heat shock; they are also induced by a number of chemicals including ethanol (Piper 1995). Two gene promoter elements, the Heat Shock Element (HSE) and the general Stress Responsive Element (STRE) have been shown to direct the activation of heat shock genes by heat stress (Piper 1995). The two elements themselves differ in the diversity of stresses, which cause their activation. HSE is activated by heat shock only in vegetative cells, whereas STRE (under negative regulation by cAMP-PKA) is induced by ethanol and a number of other stresses (Piper 1995).

As well as heat shock proteins, *S.cerevisiae* possesses the ability to induce two cold shock genes *TIP1* and *NSR1* (Zhang *et al.* 2001), which encode a modified membrane, associated protein and a nucleolin like protein which recognises the nuclear localisation sequence of other proteins respectively (Graumann and Marahiel 1996). Five LOT (low-temperature-inducible) genes have recently been identified (Zhang *et al.* 2001), the expression of which is induced by a downshift in temperature from 30-10°C. The *LOT* genes are however not uniformly regulated in response to temperature downshifts (Zhang *et al.* 2001). This suggests that multiple signalling pathways are involved in regulating their expression.

In addition to these mechanisms of stress alleviation, yeasts can also produce trehalose, which is a non-reducing disaccharide of glucose found in the cytosol. Trehalose

was considered for a long time to be a storage carbohydrate (Wiemken 1990; Mansure *et al.* 1997). In order for trehalose to confer stress resistance upon yeast cells it must be present on both sides of the plasma membrane (Kim and Alizadeh 1996). This occurs through the action of a trehalose specific transporter present on the plasma membrane, moving trehalose from the cytosol to the extracellular environment (Kim and Alizadeh 1996). It is the hydroxyl groups of trehalose forming hydrogen bonds with the polar head groups of the lipid bilayers which stabilises the cell membrane against phase transitions, thus aiding the maintenance of its structural integrity (Kim and Alizadeh 1996; Hallsworth 1998). Trehalose also protects cytosolic components against desiccation, heat and frost (Wiemken 1990; Mansure *et al.* 1997). In response to an increase in wort osmotic pressure in a high gravity brewery wort, trehalose levels in yeast were elevated (Majara *et al.* 1996a). This effect was shown to be independent of the concentration of ethanol produced during the fermentations (Majara *et al.* 1996a).

As ethanol is produced during fermentation yeast cells also protect themselves from its toxic effects by producing glycerol, which helps to maintain both the function and integrity of enzymes along with lipid bilayers (Hallsworth 1998). High glycerol levels have been found in yeasts during high gravity fermentations (Majara *et al.* 1996a).

1.4 The role of metal ions in brewing yeast nutrition

Yeasts, like all other organisms, require an optimal amount of a wide variety of nutrients for them to remain viable. It is therefore necessary to have a complete understanding of the nutritional requirements of these organisms, in order to optimise their growth and metabolic activities (Jones and Gadd 1990). This concept is fine in theory. In practice, however, brewer's wort is a complex medium and may not always contain the

optimum concentrations of these nutrients. This especially relates to metal ions (see Table 1.4), the bioavailability of which is regulated by soluble and insoluble chelators such as nitrogenous and polyphenolic compounds as well as phytic acid, which are found in wort (Mochaba *et al.* 1996; Walker *et al.* 1996). This has been highlighted by Bromberg *et al.*, (1997), who compared the metal ion concentrations of worts brewed from the malt of two successive years. These authors found that supplementing worts with extra metal ions (Ca^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+}) had no effect on yeast fermentation performance. Other studies (Walker *et al.* 1996; Rees and Stewart 1997a; 1997b) on the other hand have shown an opposite effect, especially with regard to Mg ions.

Table 1.4 Optimum concentration of metal ions required by *Saccharomyces spp* and their roles played in successful growth and fermentation (Jones & Greenfield, 1984; Jones & Gadd, 1990; Walker 1998a)

Ion	Optimum Concentration	Role Played
Potassium	2-10mM	Regulation of divalent cation uptake
Magnesium	2-4mM	Stimulates fatty acid synthesis, alleviates inhibition by alkali metals, activates glycolytic enzymes
Calcium	0.5-5mM	Second messenger which modulates growth and metabolic responses
Zinc	5-15μM	Essential in activity of enzymes, enhances riboflavin synthesis, activates acid/alkaline phosphatases
Iron	1-10μM	Small amount required for haeme-enzyme function
Manganese	2-10μM	Stimulates increase in cellular nitrogen content, enhances free amino acid incorporation in proteins
Copper	1-10μM	Required by some enzymes, enhances protein content and activation of acid phosphatases
Cobalt	0.1-1μM	Protein and vitamin production enhanced, activates alkaline phosphatases
Nickel	1-50μM	Affects membrane and cytosolic enzymes, especially ADC, in their free forms
Molybdenum	1-10μM	Enhances dicarboxylic amino acid metabolism, activates acid and alkaline phosphatases

Yeasts possess a variety of transport systems designed to accumulate essential ions from their surrounding environment. Cations are taken up by yeasts to fulfil a number of roles. These include: regulation of intracellular pH homeostasis and the generation of a proton motive force; osmoregulation and charge balancing; function of enzyme cofactors and maintaining the structure of metalloenzymes (Walker 1998a). The proton pumping ATPases maintains cell pH and provides the driving force for nutrient uptake. Three classes of H^+-ATPase pump have been identified in yeasts, these are: the mitochondrial ATPase, the vacuolar ATPase and the plasma membrane ATPase. The latter two of which are involved in ion transport (Jones and Gadd 1990).

Metal ions may also be taken up and bound to the cell surface through a two-step process, the first being a rapid accumulation that is independent of temperature and metabolism. This is followed by a slower metabolism dependent step, which can accumulate higher amounts of cations than the first (Brady and Duncan 1994). Such an uptake system has been shown to accumulate heavy metals such as Co^{2+} and Cd^{2+} ions and appears to be a general one with only limited specificity (Brady and Duncan 1994). Transition metals are essential for the function of many proteins through the facilitation of redox reactions or by stabilising protein structure (Radisky and Kaplan 1999). Transport mechanisms for metals have been found to comprise high and low affinity transporters. High affinity transporters are selective for their target metals and are regulated closely according to their requirement (Radisky and Kaplan 1999). Low affinity transporters on the other hand are less responsive to metal needs and less selective for the metals transported (Radisky and Kaplan 1999). These dual uptake systems therefore allow metal ion homeostasis to be maintained under conditions of limitation or excess.

1.4.1 Role of calcium ions in yeast physiology

When calcium binds to the cell membrane, it plays an important role in regulating lipid-protein interactions whilst activating ATPases at 1mM. It also protects the structure of the cell membrane as well as maintaining its permeability barrier under duress (Jones and Greenfield 1984).

Like other eukaryotic cells, cytosolic free calcium ion concentrations in yeasts are actively maintained at micro-molar levels (Youatt 1993), across the plasma and intracellular membranes (Cunningham and Fink 1994). Calcium is also required as a secondary messenger in signal transduction in modulating the growth and metabolic responses to external stimuli (Walker 1998a). For example, when exposed to mating pheromones, *S.cerevisiae* responds by taking up calcium in a rapid and transient manner (Walker 1998a). Calcium ions have also been linked to cell cycle progression and implicated in the transition from lag phase to exponential phase in batch cultures of *S.cerevisiae* (Walker 1998a). Levels of calcium in cells are maintained by a variety of channels, antiporters and pumps (Cunningham and Fink 1994) as well as being sequestered by specific Ca-binding proteins such as calmodulin (Walker 1998a), with the yeast vacuole being a major sequesteror of calcium ions. Extracellular calcium is essential for α-amylase activity oxalate and phosphate precipitation as well as playing a role in wort pH control (Rees and Stewart 1997a). Calcium also plays a role in the induction of sporulation in yeasts (Suizu *et al.* 1994) and in the activation of lectins during flocculation (Soares and Seynaeve 2000).

High amounts of calcium may inhibit cell surface binding and membrane transport of Mg^{2+} and Mg-dependent ATPase activity (Ciesarova *et al.* 1996). Excess calcium has

also been shown to be antagonistic towards Mg dependent processes such as cell growth, cell division cycle progress and intermediary metabolism (Walker 1999). Elevated levels of calcium in wort have been shown to reduce fermentation rates and increase attenuation times in ale and lager fermentations in conventional and high gravity fermentations as well as reducing ethanol production (Rees and Stewart 1997b).

1.4.2 Role of magnesium ions in yeast physiology

Magnesium is the second most abundant metal ion within higher eukaryotic cells (Heaton 1993) and the most abundant divalent cation in yeast cells (Walker 1998a). This ion acts as an important cofactor for over 300 enzymatic reactions. It activates enzymes in two ways by either binding with the enzyme protein to produce allosteric activation or by forming part of the active substrate (Heaton 1993). Magnesium also maintains membrane integrity through its protein-protein membrane interaction, nucleic acid conformation, stabilises ribosomes (Jones and Greenfield 1984) as well as playing an important role in dictating cell cycle progress in yeast cells (Walker and Duffus 1980).

Magnesium uptake is thought to be driven by both the proton and potassium ion transmembrane gradients (Walker 1998a). Magnesium transport is understood to a much greater extent in other cells. For example, *Salmonella typhimurium* possesses three distinct magnesium transporter mechanisms encoded by the *CORA*, *MGTA* and the *MGTB* loci (Smith and Maguire 1993). More recently the yeast protein Alr1 was identified as the first candidate for a magnesium transporter in eukaryotic cells (MacDiarmid and Gardner 1998). This protein is a distant relation to the bacterial CorA proteins and Mg plays an important role in maintaining its stability, as this proteins' exposure to standard (1mM) and high (200mM) magnesium concentrations leads to a dramatic decrease in its stability

(Graschopf *et al.* 2001). The mechanism by which Alr1p mediates magnesium uptake into yeast cells has yet to be shown (Graschopf *et al.* 2001). Two candidate genes for mitochondrial magnesium transporters (*MRS2* and *LPE10*) have also been described (Wiesenberger *et al.* 1992; Bui *et al.* 1999; Gregan *et al.* 2001). The absence of either of these genes results in reduced magnesium levels in yeast mitochondria (Zsurka *et al.* 2001).

Much of the magnesium present within the cell may be stored in the vacuole. Beeler *et al.*, (1997) has provided evidence for this by showing that a 75% reduction in cellular magnesium had no effect on the growth rate of the cell, and it was suggested that vacuolar Mg may be utilised when external supply is limited (Beeler *et al.* 1997).

In a review by Jones & Greenfield (1984), magnesium is mentioned as being a central component in fermentative metabolism. Studies by Walker *et al.*, (1996); Walker & Maynard (1997); Walker & Smith (1998) and Smith & Walker (2000) have shown that there was a significant correlation between cellular magnesium uptake and alcoholic fermentation in industrial strains of *S.cerevisiae*. Rees & Stewart (1997a,b; 1999) have also shown a stimulatory effect in ethanol production by the supplementation of brewer's wort with magnesium. Magnesium may also exert a protective effect on yeast cells subjected to a variety of physical and chemical stresses (Walker 1998b). The above therefore highlights the need to expand upon the knowledge of Mg and its relationship with yeasts to date. This is especially true if it can be shown that Mg plays a beneficial role in yeast nutrition in the brewery situation, which is a major aim of the present work.

1.4.3 Role of Zinc ions in yeast physiology

Uptake of this metal ion occurs through two separate high and low affinity systems. The high affinity system is induced in zinc deficient cells and encoded by the *ZRT1* gene. The low affinity system, encoded by the *ZRT2* gene, is active in zinc-replete cells and is time, temperature, and concentration dependent with a preference to zinc over other metals as its substrate (Zhao and Eide, 1996a,b) . Like magnesium, this ion is stored in the yeast vacuole (Rees and Stewart 1998).

Brewers monitor this ion closely because concentrations of less than 0.1ppm (1.5μM) lead to sluggish and incomplete (stuck) fermentations (Rees and Stewart 1997a). It also has an important role in maintaining the structure of enzymes, such as alcohol dehydrogenase, cysteine desulphydrase, aldolase, RNA polymearase, alkaline phosphatase and Cu/Zn superoxide dismutase (Jones and Greenfield 1984; Magonet *et al.* 1992; Guerinot and Eide 1999). Deprivation of zinc in yeast cells also prevents budding and causes cell cycle arrest (Walker 1998a). The addition of zinc to wort has been shown to reduce attenuation time and enhance ethanol production in both conventional (12°P) and high gravity (20°P) wort, during ale and lager fermentations. However, these results were found to be strain dependent (Rees and Stewart 1998). Zinc supplementation in molasses has been shown to enhance growth of *S.cerevisiae* during fermentation in semi-aerobic and anaerobic conditions as well as increasing alcohol production (Stehlik-Tomas *et al.* 1997).

1.5 Aims and objectives

Studies by Walker *et al* (1996) , Walker & Maynard (1997), Walker & Smith (1998) and Smith & Walker (2000) have revealed that there was a significant correlation between cellular Mg uptake and alcoholic fermentation in industrial strains of *S.cerevisiae.* Rees & Stewart (1997a; 1997b; 1999) have also shown a stimulatory effect in ethanol production by the supplementation of brewer's wort with Mg. In addition Walker (1998b) has suggested that Mg plays a role in stress tolerance. The purpose of this Thesis was to test the hypothesis that brewer's yeasts, which were physiologically "preconditioned" with Mg ions, had improved fermentative output and stress tolerance. This was to be achieved through a number of specific aims:

1. Cellular Mg localisation. To determine where the extra Mg present in preconditioned cells was held with respect to the cell membrane and the vacuole using Mg fluorescent dyes.
2. Fermentative and pyruvate decarboxylase activity. To determine whether preconditioned yeasts were more fermentatively active in high gravity fermentations and whether there was a link between Mg-preconditioned yeasts and the activity of the key fermentative enzyme pyruvate decarboxylase.
3. Stress tolerance. To determine stress tolerance of Mg preconditioned yeasts by measuring viability and cellular release of Mg during their exposure to varying degrees of temperature and increasing concentrations of ethanol.

CHAPTER 2

MATERIALS AND METHODS

2.1 Yeast cultures employed

The yeasts used in this study were an industrial lager yeast *Saccharomyces cerevisiae* var. *carlsbergensis* obtained from Scottish Courage Brewing Ltd (courtesy of Dr B Taidi) and an ale yeast *S.cerevisiae* NCYC 1681 obtained from Brewing Research International, Nutfield (courtesy of Dr J Hammond). The cultures were maintained on Malt Extract Agar (MEA, Oxoid, Ltd, Basingstoke, Hampshire) slopes at 4°C and subcultured every 10-14 days with a master slope retained for 6 months.

2.2 Magnesium Preconditioning of Brewing Yeasts

2.2.1 Media Preparation

Synthetic wort medium (SWM)

A chemically defined medium, a modified version of Edinburgh Minimum Medium No.3 (EMM3 - see Fantes, 1977) was used in part of this study as the basis of a low gravity (1023°OG) synthetic wort medium (Chandrasena 1996), in order to simulate the sugar concentrations found in low gravity wort and to control the Mg levels in an accurate manner to allow the optimum Mg concentrations correlating to its maximum uptake by yeast to be determined. The components of SWM are listed in table 2.1.

Inoculum Preparation

SWM (100ml, containing 2mM Mg) was inoculated with a loopful of either ale or lager yeast stock cultures and propagated in an orbital incubator at 150rpm overnight at 25°C. These cultures were then used to seed experimental flasks containing sterile SWM with increasing Mg concentrations at a cell density of ~$5x10^6$ cells/ml.

Table 2.1 Synthetic Wort Medium Components

Element	**Amount/Litre**
Glucose	10g
Fructose	3.3g
Sucrose	5.3g
Maltose	40g
Ammonium Sulphate	5g
Sodium Hydrogen Phosphate	1.42g
Magnesium Sulphate	2.46-24.64g
Inorganic Salts:	
Potassium Hydrogen Phthallate	3.06g
Calcium Chloride	10mg
Potassium Chloride	1g
Di-Sodium Sulphate	300mg
Trace Elements:*	
Boric acid	0.50mg
Manganese sulphate	0.40mg
Zinc sulphate	0.40mg
Ferric chloride	0.20mg
Molybdic acid	0.16mg
Potassium iodide	0.10mg
Copper sulphate	0.04mg
Citric Acid	1.0mg
Vitamins:*	
Inositol	10mg
Nicotinic Acid	10mg
Calcium Pantothenate	1.0mg
Biotin	0.01mg

*Trace elements and vitamins were made up as sterile $1x10^3$ concentrated stock solutions and added at the requisite volume.

Magnesium Salts

Different magnesium anions were used, both organic and inorganic in order to determine whether varying the type of salt influenced the uptake of Mg^{2+} ions. Cells were propagated in Malt Extract Broth (Oxoid) supplemented with 50 and 100mM concentrations of the magnesium salts (Anala R and Sigma). The salts used are listed in Table 2.2.

Table 2.2 Mg salts used in this study.

Salt	Organic/Inorganic
Mg Chloride	Inorganic
Mg Sulphate	Inorganic
Mg Acetate	Organic
Mg Gluconate	Organic
Mg Lactate	Organic

Brewers Wort

Dilute brewer's wort was also used to investigate Mg uptake in a simulated brewery cultivation situation. Cells were propagated aerobically (using Schott 250ml baffled flasks) in hopped wort (high gravity 1st copper runnings) supplied by Scottish Courage Ltd (courtesy of Dr B Taidi), which had been previously autoclaved and then stored at 4°C until required.

Inoculum Preparation

100ml of dilute wort (1020°OG) was inoculated with a loopful of either ale or lager yeast stock cultures and propagated in an orbital incubator at 200rpm overnight at 25°C. These cultures were then used to seed experimental flasks containing sterile 1020°OG wort, which had been supplemented with or without increasing concentrations of the magnesium salts mentioned in table 2.2 above, prior to sterilisation.

2.3 Mg localisation studies

2.3.1 Inoculum preparation

Malt Extract Broth (100ml) was inoculated with a loopful of either ale or lager yeast stock cultures and propagated in an orbital incubator at 150rpm overnight at 25°C. These cultures were then used to seed experimental flasks containing sterile malt extract broth with or without 100mM supplements of Mg Acetate and Mg Sulphate at a cell density of ~$5x10^6$ cells/ml.

2.3.2 Fluorescent dye labelling of Mg preconditioned yeasts

Aliquots (1ml) of cell suspension were removed from cultures propagated for 24h or after 96h and centrifuged. The supernatant was removed and the pellet washed thrice-using Tris-HCl buffer solution, which contained 1.2M sorbitol, 135mM sodium chloride and 10mM Tris-HCl, pH 7.6 (Zhang *et al.* 1997). The cells were loaded with 5µM Magnesium Green and 5µM Mag Fura-2 acetoxymethylesters (Molecular Probes, Eugene, OR) in Tris-HCl buffer solution by incubation at 37°C for 30 min in a circulating water bath. The cells were then centrifuged and washed thrice in Tris-HCl buffer and incubated at 37°C for a further 30min to allow complete de-estrification of the intracellular AM esters. The cells were then centrifuged and resuspended in 100µl Tris-HCl buffer, 20µl of this cell suspension was pipetted onto a glass microscope slide and covered with a cover slip. Slides were viewed using a Leica Fluorescent microscope under oil using a X40 objective lens, images were captured using a Sony Video Camera and analysed using a Quantimet Q600S image analyser (Leica) and stored on an Iomega 2 GB Jaz disk.

2.4 Fermentative Behaviour of Preconditioned Yeast

2.4.1 Inoculum preparation

100ml of dilute wort (1020°OG) was inoculated with a loopful of either ale or lager yeast stock cultures and propagated in an orbital incubator at 200rpm overnight at 25°C. These cultures were then used to seed experimental (at a cell density of ~$5x10^6$ cells/ml) Schott baffled flasks containing sterile 1040°OG wort (supplied by Dr B Taidi, Scottish Courage Ltd) with or without 100mM supplements of Mg Acetate and Mg Sulphate, propagated for 24h (at 25°C, 200rpm) and used as the preconditioned inoculum for the subsequent fermentations with a pitching rate of ~$1x10^7$ cells/ml.

2.4.2 Experimental fermentations

Static wort fermentations were carried out using sterile wort (1080°OG) in 1L Imhoff cones (Nalgene) at 18°C in a circulating water bath (see Fig 2.1). Samples were removed at the depth of sterile 1ml plastic pastette pipettes on a daily basis thus ensuring samples were removed in a consistent manner for suspended yeast cell counts and chemical analysis.

Fig 2.1 Static wort fermentations in 1L Imhoff cones (Nalgene) at 18°C in a circulating water bath.

2.5 Response of yeast to stress

2.5.1 Effect of temperature stress

Cells (ale/lager yeasts) were preconditioned in malt extract broth supplemented with 100mM magnesium acetate or sulphate for 24h. 50ml of each culture was removed, centrifuged, washed thrice using ultra-pure deionised water (centrifugation 3,000 rpm, 5 mins followed by thorough vortexation) and resuspended in sterile universal bottles containing (15ml) deionised water. Cells were then exposed to varying temperatures (4°C, 25°C and 40°C) and samples removed at 3,6,24 and 48h time intervals.

2.5.2 Effect of ethanol stress

Cells (ale/lager yeasts) were preconditioned in malt extract broth supplemented with 100mM magnesium acetate or sulphate for 24h. 50ml of each culture was removed, centrifuged, washed thrice using ultra-pure deionised water (centrifugation 3,000 rpm, 5 mins followed by thorough vortexation) and resuspended in universal bottles (15ml total volume) containing increasing concentrations of ethanol (0, 10 and 20%). Samples were removed at 3,6,24 and 48h time intervals.

2.6 Analyses

2.6.1 Cell number

An Improved Neubauer haemocytometer (BS748) in conjunction with a phase contrast microscope was used. Cell viability was determined using citrate methylene blue (Institute of Brewing, 1991) . Dead cells were assessed by their blue colour due to their inability to oxidise the methylene blue.

2.6.2 Cell size

Cell size was carried out using a Coulter Multisizer II by diluting a 200µl of yeast suspension in 20ml Isoton II (Coulter Ltd, Luton, UK). Samples were passed through a 100µm diameter probe until 250,000 cells had been accumulated. The mean cell volume of the sample was calculated using the Coulter counter software (Accucomp, Coulter, UK) and expressed as cubic microns (μm^3) or femptolitres (fL).

2.6.3 Magnesium

A Perkin Elmer Atomic Absorption Spectrophotometer (AAS) 1100B was used to measure the magnesium present in both cell suspensions and supernatants, at 285.1nm. Calibration of the AAS was carried out by the dilution of a 1000ppm Mg stock ($Mg(NO_3)_2.6H_2O$ in HNO_3, Fluka) to produce standards within the linear calibration range (0.1-0.5ppm inclusive) of the AAS.

Cell magnesium was determined in triplicate 1ml cell suspension samples which were centrifuged in disposable microfuge tubes (10,000rpm, 3mins) and resuspended in deionised water to remove any loosely bound magnesium from the cell surface (carried out three times). The cells were resuspended in 2ml-deionised water and hydrolysed by the addition of 2ml concentrated nitric acid (15.8M) at room temperature, followed by a 10min boil. Hydrolysates were made up to 10ml and analysed by AAS Dilutions of the hydrolysates were made where necessary, using deionised water, as the diluent. Cell Mg content was expressed as femptograms/cell (fg/cell) and also by dry wt (mg Mg/g dry wt), protein (nmol Mg/mg protein) and cell volume (mM) (see Chapter 3, tables 3.1 and 3.2 respectively).

Magnesium concentrations in media and culture supernatants were determined in triplicate 1ml samples, which were resuspended in up to 10ml of deionised water. AAS analysis was carried out as per above with appropriate dilutions where necessary. Mg content was expressed as μg/ml in media and as fg/cell in culture supernatants.

2.6.4 Ethanol

This was determined in 1ml cell free supernatants using a Hewlett Packard Gas Chromatograph 5710A (oven temp 120°C, detector temp 200°C) with a Porapak Q column, together with a HP3396A integrator, with 5% isopropanol as the internal standard, ethanol was expressed as % v/v.

2.6.5 Specific Gravity

This was determined using a hydrometer (Nalgene) (original gravity) *in situ*, which was rinsed in sterile deionised water and 70% (v/v) ethanol between readings; specific gravity was expressed as °OG.

2.6.6 Biomass (by dry weight)

Aliquots (1ml) of cell suspension were removed in triplicate, centrifuged in disposable microfuge tubes (10,000rpm, 3mins) resuspended in deionised water and vacuum filtered through pre-weighed (using a Oertling NA164 four point balance) Whatman (GF/C, 47mm diameter with a pore size of 1.2μm) glass fibre filter discs. These were then dried in a Mettler LJ16 infrared moisture analyser and re-weighed (using a Oertling NA164 four point balance) dry weights were expressed as mg/ml.

2.6.7 Protein

Aliquots (1ml) of cell suspension were removed in triplicate, centrifuged in disposable microfuge tubes (10,000rpm, 3mins) and resuspended in deionised water and re-centrifuged, the resultant pellet was resuspended in 1ml of 1M NaOH and incubated for 1 hour at 60°C or until a clear hydrolysate formed. 100μl aliquots of the hydrolysate were neutralised with 100μl of 1M HCl, the neutralised aliquots (100μl) were added to 1ml Coomassie Blue (Pierce) this was mixed and left for 2/3 min before the absorption at

595nm was read against a water blank. A calibration curve was created using Bovine Serum Albumin, and protein content was expressed as mg/ml

2.6.8 Pyruvate Decarboxylase Activity (Van Urk *et al.* 1989).

Aliquots (10ml) of cell suspension were removed in triplicate, centrifuged (4,000rpm, 10mins) and resuspended in yeast homogenisation buffer, containing 75mM potassium phosphate (pH 6.5), 5mM magnesium sulphate, 1mM thiamine pyrophosphate and 1mM 2-mercaptoethanol. The cell suspension was disrupted with 0.5g glass beads (425-600 microns, Sigma) by vortexing for 30 sec (followed by cooling on ice) until each sample was disrupted for 5 min. The sample was centrifuged for 5 min at 4,000 rpm and the supernatant recovered and stored at 4°C until the reaction buffer, containing 5mM magnesium chloride, 10mM potassium phosphate, 50mM pyruvate, 88U alcohol dehydrogenase and 0.2mM thiamine pyrophosphate was prepared. The addition of 100µl of sample to the reaction buffer initiated the reaction; specific activity was expressed as µmol min/mg protein.

CHAPTER 3

PRECONDITIONING OF BREWERS' YEASTS

3.1 Introduction

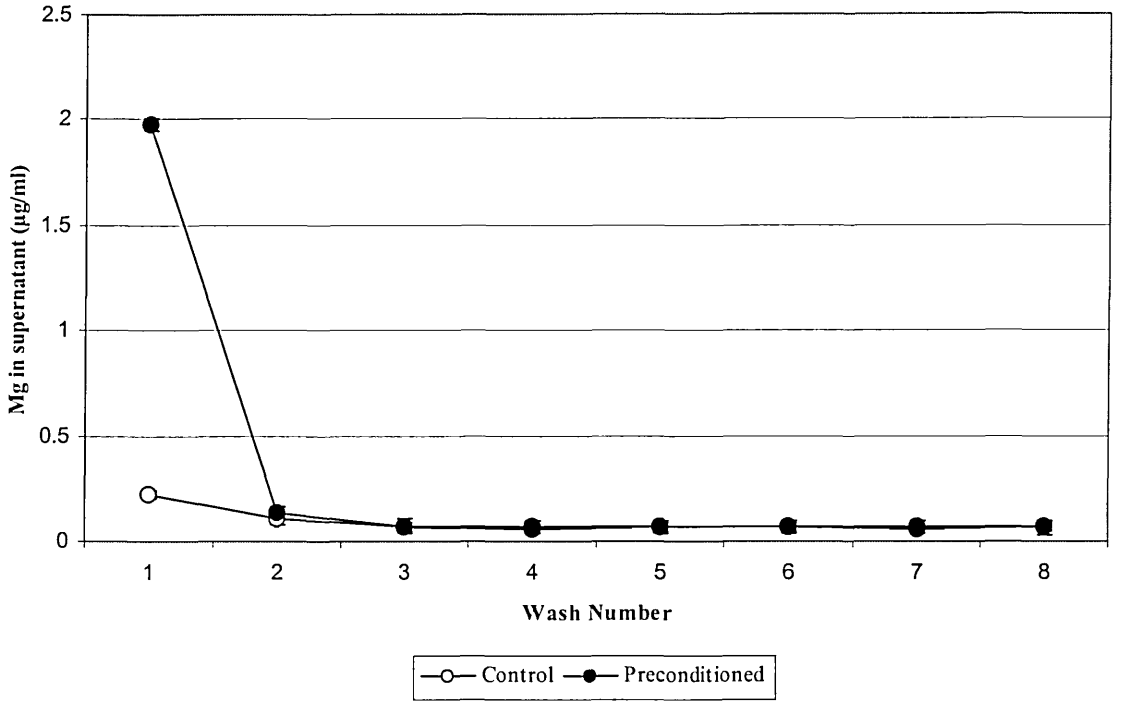
Previous studies (Saltukoglu and Slaughter 1983; Walker and Maynard 1997) have shown that magnesium present in the yeast cell is not directly proportional to the external magnesium concentration. Jones and Greenfield (1984) have suggested that the optimum magnesium concentration in culture media for yeast growth is in the range of 2-4mM. Such a concentration range is accurately achievable in chemically defined media, but in complex media, such as malt extract broth and media used in industry for the production of potable alcohol (molasses, brewers' wort), free (biologically available) Mg concentrations may not always be within this range. This is due to its regulation by soluble and insoluble chelators such as nitrogenous and polyphenolic compounds as well as phytic acid, found in wort (Mochaba *et al.* 1996; Walker *et al.* 1996). Free Mg in complex media may be increased by supplements with Mg salts, which has been shown to positively influence yeast fermentative metabolism (Walker *et al.* 1996; Rees and Stewart 1997a; Rees and Stewart 1997b; Rees and Stewart 1999). However, this research focuses on the role of intracellular, as opposed to extracellular, Mg in its affect upon yeast physiology. Prior to investigations of the behaviour of Mg enriched brewing yeasts in terms of fermentative metabolism and stress tolerance (see Chapters 5 and 6), initial studies were deemed necessary to establish conditions appropriate for effective "Mg preconditioning" of yeast. Preconditioning means the improvement of the physiological condition of yeast cells through increasing intracellular Mg, which is obtainable by propagation in elevated levels of magnesium ions. The aim of this chapter, therefore, was to investigate the relationship between Mg bioavailability and cell Mg content in ale and lager yeasts.

3.2 Experimental approach

An attempt was made to determine the optimal extracellular Mg concentration which would increase the intracellular Mg content in ale and lager yeasts, this was initially carried out using a chemically defined synthetic wort medium (SWM) as Mg levels could be manipulated in an accurate manner. This also allowed the number of washings pellets required to remove loosely bound Mg prior to metal ion analysis to be determined (see Fig 3.1). Once the optimum Mg concentrations for yeast growth and intracellular Mg were determined the focus of the study shifted to complex medium such as malt extract broth. A number of Mg salts were investigated to determine whether or not particular Mg salts would influence Mg uptake by the yeast cells. The final stage of this aspect of the study was to simulate the yeast propagation protocol employed by the brewing industry prior to fermentations. Baffled Erlenmeyer flasks used in a shaking incubator facilitated good oxygen transfer and allowed simulation of aerobic propagations to be carried out on a small scale. Mg uptake by yeasts preconditioned in the presence of the Mg salts used previously was observed and the results from this study could then be used to initiate the study of preconditioned yeast and their fermentative metabolism (in Chapter 5). Yeast growth media and inocula were prepared as described in section 2.2 and samples were removed on a regular basis for cell number, biomass and cellular Mg as described in section 2.6.

The results presented in Fig 3.1 below show that although there is a higher Mg content in the preconditioned supernatant than the control supernatant after 1 wash, the Mg content in the supernatants thereafter fall and plateau after 3 washes or more. Therefore three washes were deemed as being enough to remove any loosely bound Mg from the cell pellets, which may have otherwise influenced the metal ion analysis.

Fig 3.1 Mg released during yeast cell pellet washing. Yeast cells were preconditioned in SWM without (control) or with (preconditioned) Mg supplementation. Mg levels in the SWM were 1mM and 100mM respectively.



3.3 Results

3.3.1 Mg preconditioning in synthetic wort medium

Growth of ale yeast cells varied during propagation in SWM containing increasing Mg concentrations (Fig 3.2). Lager yeast cells, however, showed a higher growth rate in SWM containing 1-10mM Mg (Fig 3.5). Ale yeast biomass was higher in cells, which had been propagated in SWM containing 25-100mM Mg (Fig 3.3). Biomass in lager yeast cells propagated in SWM containing 1-100mM Mg varied (Fig 3.6). In both ale and lager yeast cells, the results (Figs 3.4 and 3.7) showed that as the Mg concentration in the medium increased the cellular Mg increased too. However, this was not directly proportional to the medium concentration as in both yeast strains cell Mg was highest after 24h growth in SWM containing 25mM Mg (Figs 3.4 and 3.7). Mg loss was observed in the ale yeast cells, with the greatest loss occurring in cells that had been propagated in 1mM Mg (Table 3.1). Lager yeast cells also lost some of their cellular Mg after 48h growth, especially when propagated in higher Mg concentrations (50-100mM, Fig 3.7). These losses were more clearly seen when Mg was expressed in terms of biomass (mg/g dry wt), protein (nmol Mg/mg protein) and cell volume (mM) as presented in Tables 3.1 and 3.2.

Fig 3.2 Growth of *S.cerevisiae* (ale yeast) propagated in synthetic wort medium supplemented with increased concentrations of Mg sulphate.

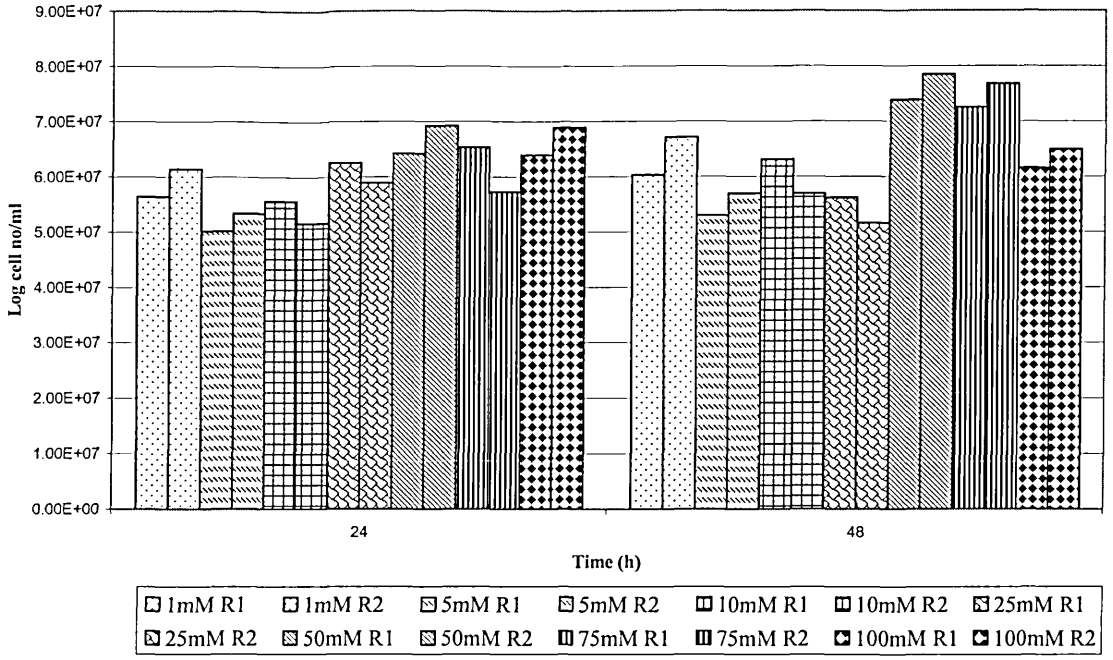


Fig 3.3 Biomass in *S.cerevisiae* (ale yeast) propagated in synthetic wort medium supplemented with supplemented with increased concentrations of Mg sulphate.

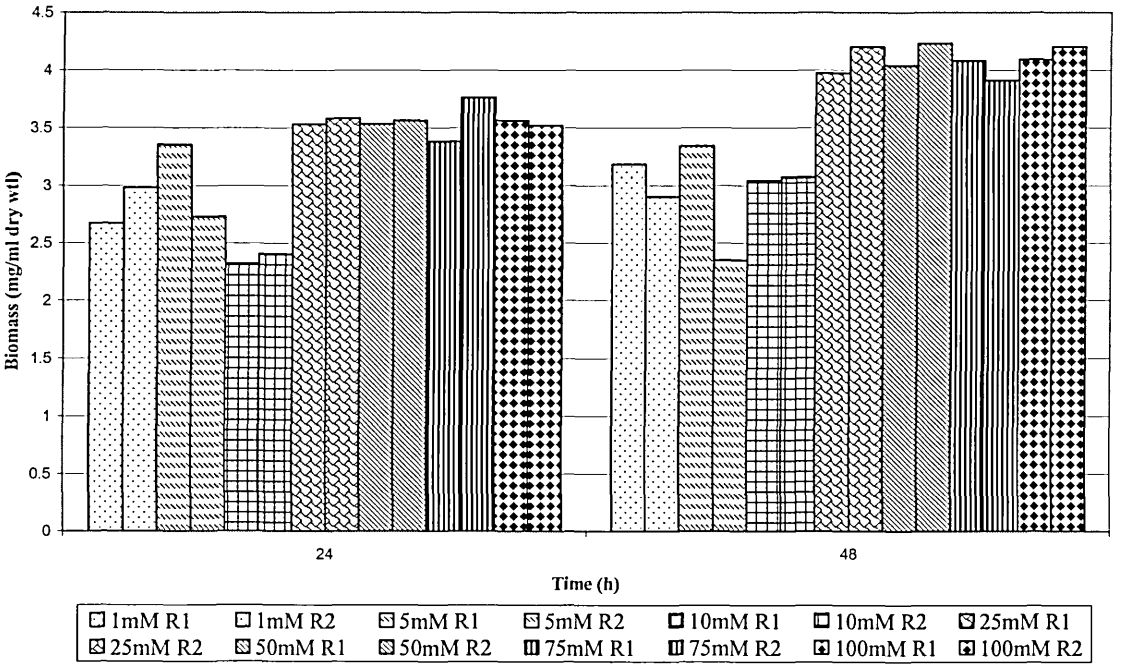


Fig 3.4 Cell Mg in *S.cerevisiae* (ale yeast) propagated in synthetic wort medium supplemented with increased concentrations of Mg sulphate. Cells were grown for the times indicated, then thoroughly washed prior to cell Mg analysis by atomic absorption spectrophotometry.

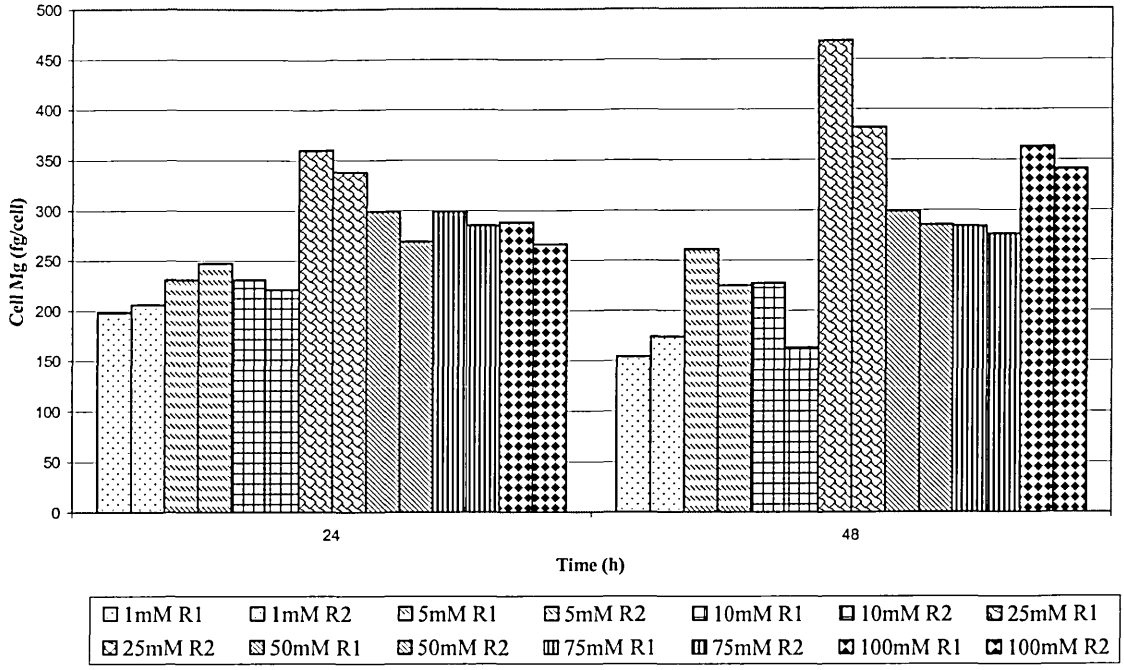


Fig 3.5 Growth of *S.cerevisiae* var. *carlsbergensis* (lager yeast) propagated in synthetic wort medium supplemented with increased concentrations of Mg sulphate.

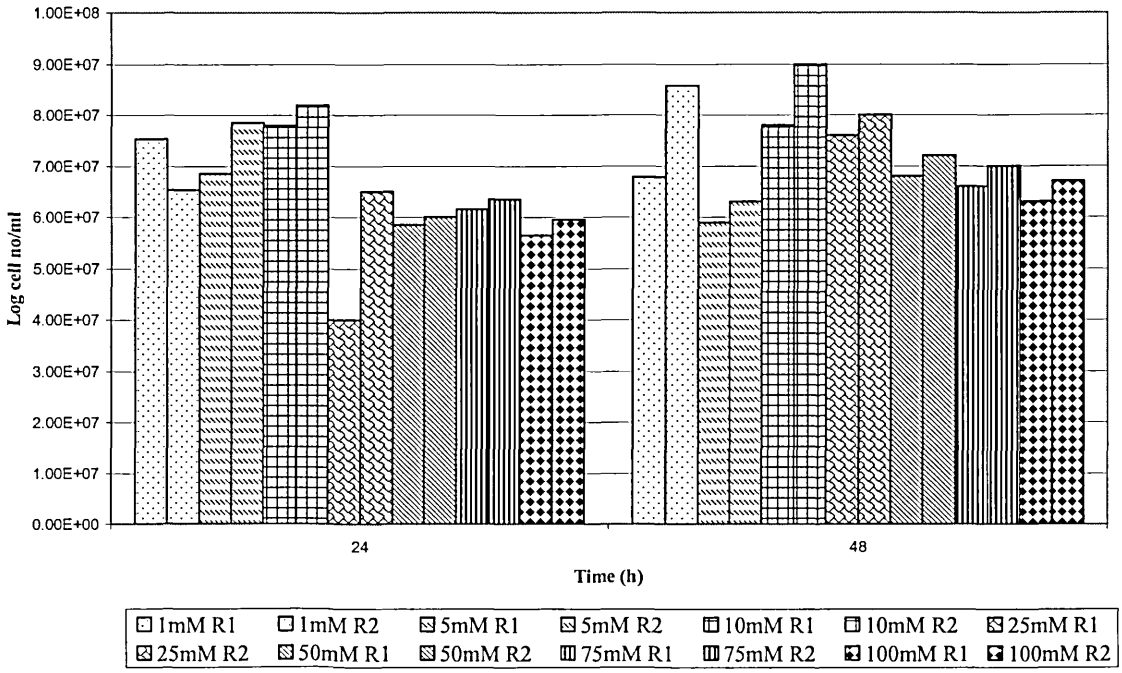


Fig 3.6 Biomass in *S.cerevisiae* var. *carlsbergensis* (lager yeast) propagated in synthetic wort medium supplemented with increased concentrations of Mg sulphate.

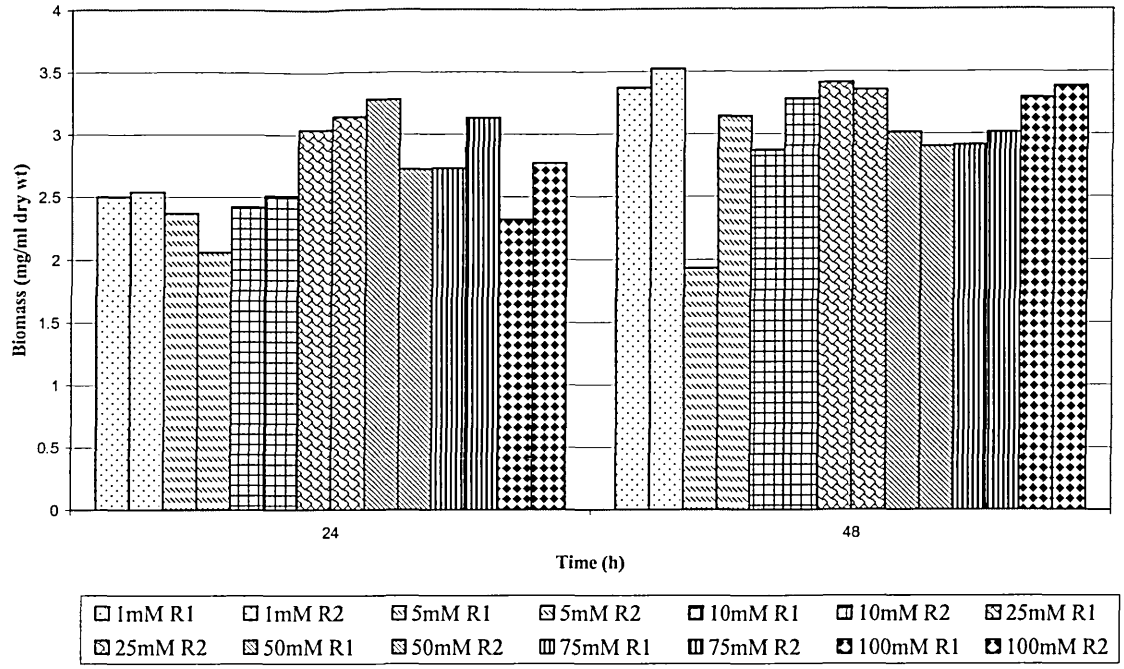


Fig 3.7 Cell Mg in *S.cerevisiae* var. *carlsbergensis* (lager yeast) propagated in synthetic wort medium supplemented with increased concentrations of Mg sulphate. Cells were grown for the times indicated, then thoroughly washed prior to cell Mg analysis by atomic absorption spectrophotometry.

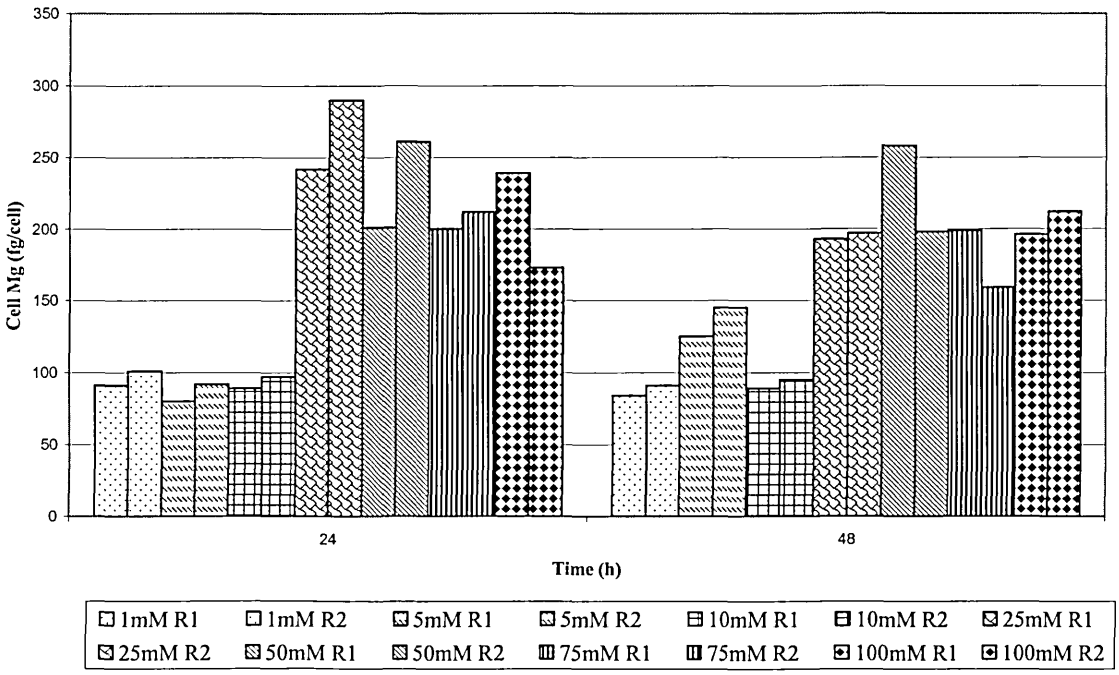


Table 3.1 Measurement of cellular Mg in *S.cerevisiae* (ale yeast) propagated in synthetic wort medium supplemented with increased concentrations of Mg sulphate. Cells were grown for the times indicated, then thoroughly washed prior to cell Mg analysis by atomic absorption spectrophotometry.

Mg conc. in SWM (mM)	Mg Mg/g dry wt		nmol Mg/mg protein		mM Mg*	
	24h	48h	24h	48h	24h	48h
1mM (1)	4.13	2.77	1297	641	67	67
1mM (2)	4.25	3.05	1379	705	85	117
5mM (1)	3.97	4.13	1329	955	93	81
5mM (2)	4.17	4.61	1361	989	111	95
10mM (1)	5.05	3.61	1417	997	97	67
10mM (2)	5.21	4.07	1499	1061	119	83
25mM (1)	5.87	5.53	1361	881	200	213
25mM (2)	6.05	5.71	1423	921	236	229
50mM (1)	5.00	5.21	817	801	143	76
50mM (2)	5.70	5.49	861	873	177	84
75mM (1)	4.87	5.13	668	781	171	100
75mM (2)	5.15	5.35	722	813	205	122
100mM (1)	5.01	5.24	731	793	97	91
100mM (2)	5.41	5.52	777	851	123	105

Table 3.2 Measurement of cellular Mg in *S.cerevisiae* var. *carlsbergensis* (lager yeast) propagated in synthetic wort medium supplemented with increased concentrations of Mg sulphate. Cells were grown for the times indicated, then thoroughly washed prior to cell Mg analysis by atomic absorption spectrophotometry.

Mg conc. in SWM (mM)	Mg Mg/g dry wt		nmol Mg/mg protein		mM Mg*	
	24h	48h	24h	48h	24h	48h
1mM (1)	2.62	1.57	441	496	68	63
1mM (2)	2.84	1.75	473	506	82	73
5mM (1)	2.71	3.17	467	539	63	87
5mM (2)	3.07	3.33	519	581	81	111
10mM (1)	2.98	2.49	348	401	70	63
10mM (2)	3.10	2.57	374	435	80	81
25mM (1)	4.49	4.47	977	821	198	147
25mM (2)	4.69	4.63	1045	871	214	161
50mM (1)	4.43	5.17	891	965	177	161
50mM (2)	4.61	5.23	871	1029	189	177
75mM (1)	4.31	4.05	1023	861	152	143
75mM (2)	4.47	4.19	1099	807	162	157
100mM (1)	4.87	3.94	1148	883	195	127
100mM (2)	5.01	4.06	1248	937	207	143

*mM Mg was calculated as follows:

cell Mg (fg/cell)/mean cell volume (fL) = fg Mg/fL = g/L/mwt Mg = mM Mg

3.3.2 Mg preconditioning in malt broth

When preconditioned in the presence of 50 and 100mM organic Mg salts ale and lager yeast cells yielded higher biomasses than either the unpreconditioned ale/lager yeast cells or the cells preconditioned in the presence of the inorganic Mg salts (Figs 3.10, 3.11, 3.14 and 3.15). Ale yeast cells preconditioned in the presence of 50mM Mg sulphate showed the highest cellular Mg contents (Fig 3.8) whereas the highest cellular Mg content in the presence of 100mM Mg salts was observed in Mg acetate preconditioned cells (Fig 3.9). Lager yeast cells preconditioned in the presence of 50 and 100mM Mg acetate yielded higher cellular Mg contents than either the unpreconditioned yeasts or the other Mg salts (Fig 3.12. and 3.13). When Mg content in yeast cells was expressed in terms of biomass (mg/g dry wt) Mg content was highest in both ale and lager yeast cells preconditioned in the presence of 50mM Mg acetate (Tables 3.3 and 3.4). This was also observed in ale/lager yeast cells preconditioned in the presence of 100mM Mg after 24h only, as in both yeast strains after 48h, cells preconditioned in the presence of 100mM Mg lactate yielded a higher Mg content (Tables 3.3 and 3.4).

Fig 3.8 Cell Mg in *S.cerevisiae* (ale yeast) preconditioned in malt broth supplemented without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the broth were 0.4mM and 50mM, respectively.

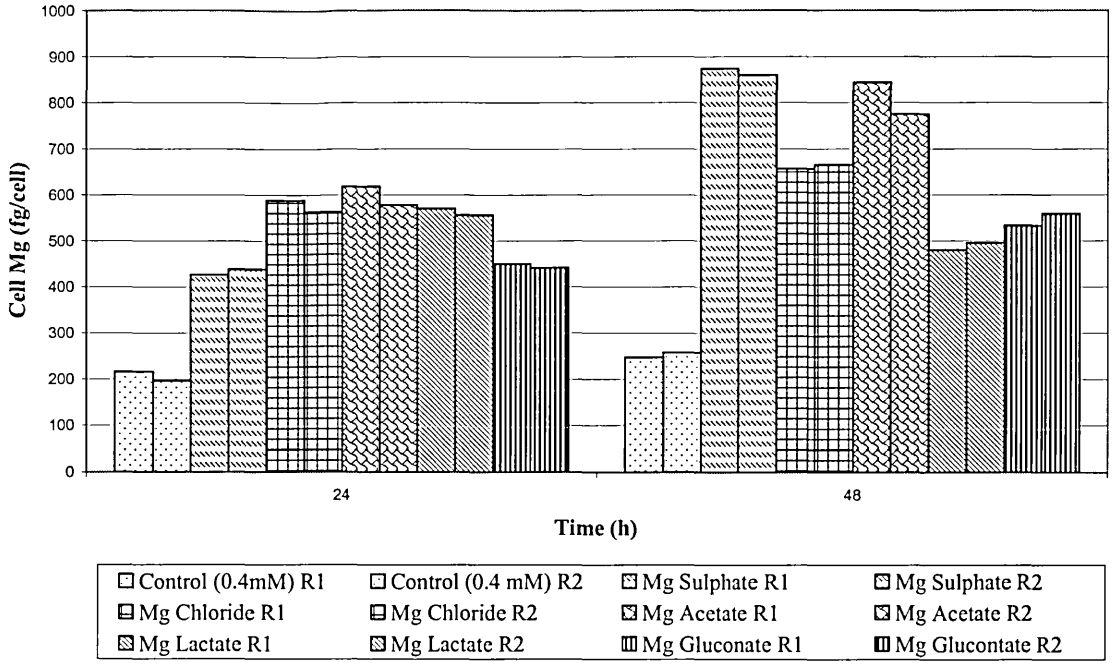


Fig 3.9 Cell Mg in *S.cerevisiae* (ale yeast) preconditioned in malt broth supplemented without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the broth were 0.4mM and 100mM, respectively.

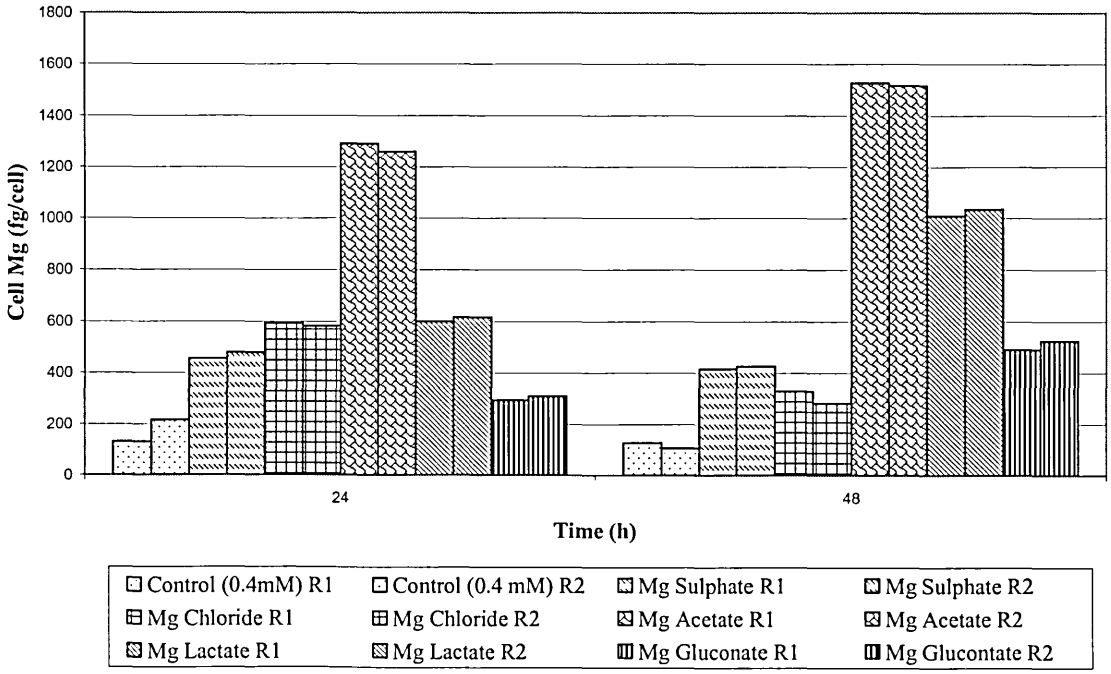


Fig 3.10 Biomass in *S.cerevisiae* (ale yeast) preconditioned in malt broth supplemented without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the broth were 0.4mM and 50mM, respectively.

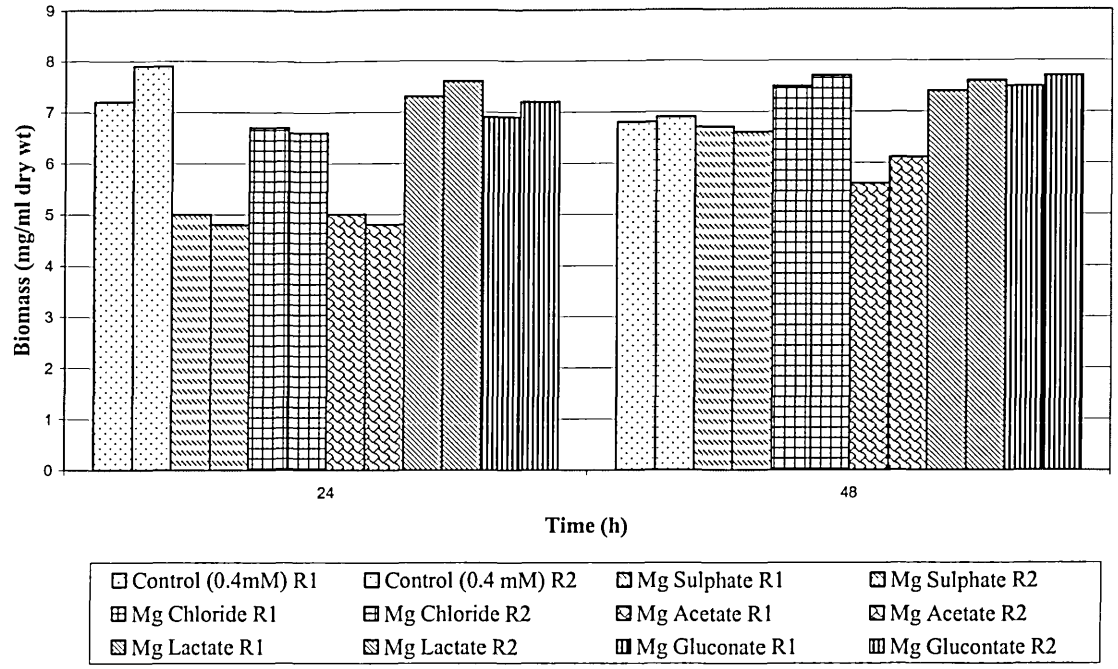


Fig 3.11 Biomass in *S.cerevisiae* (ale yeast) preconditioned in malt broth supplemented without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the broth were 0.4mM and 100mM respectively.

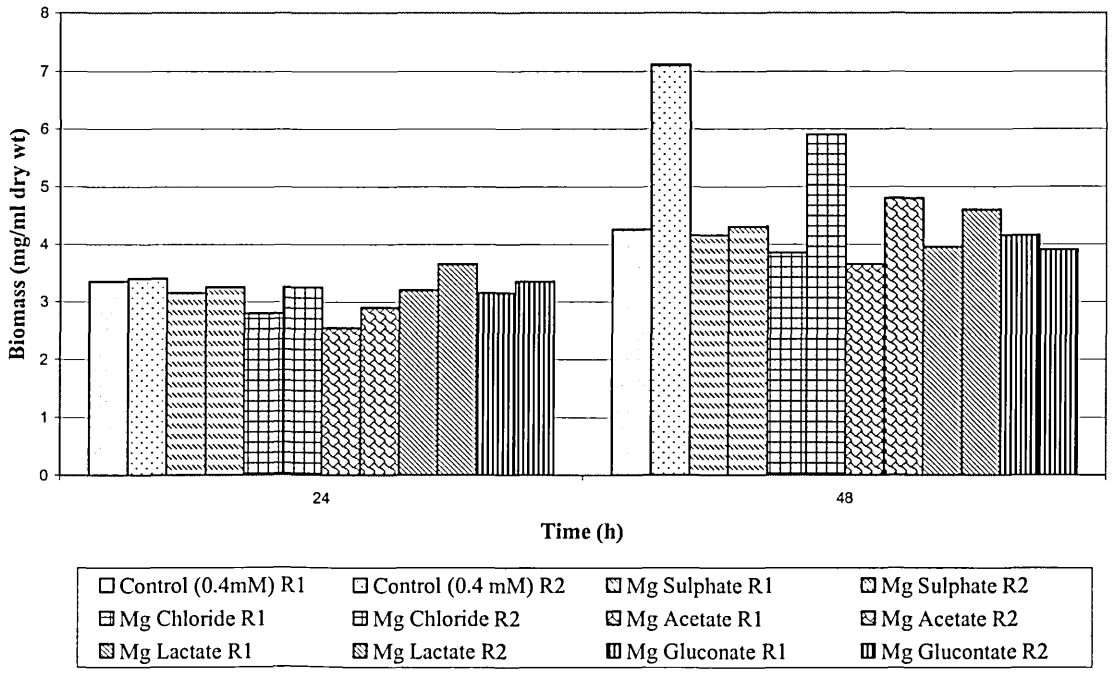


Fig 3.12 Cell Mg in *S.cerevisiae* var. *carlsbergensis* (lager yeast) preconditioned in malt broth without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the broth were 0.4mM and 50mM, respectively.

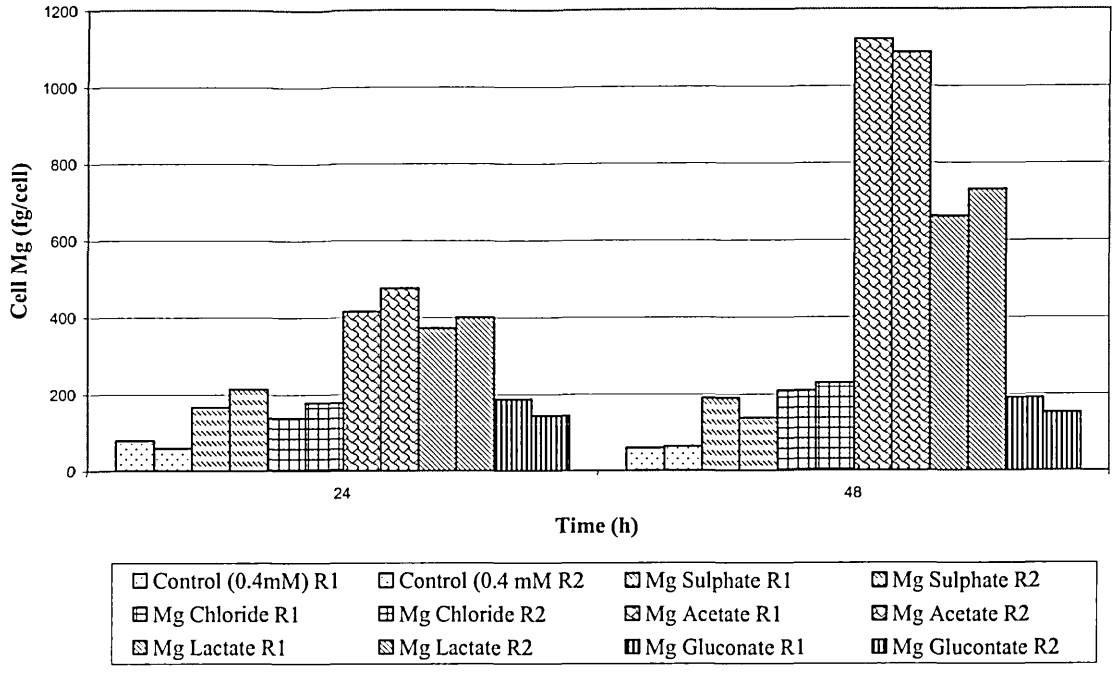


Fig 3.13 Cell Mg in *S.cerevisiae* var. *carlsbergensis* (lager yeast) preconditioned in malt broth without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the broth were 0.4mM and 100mM, respectively.

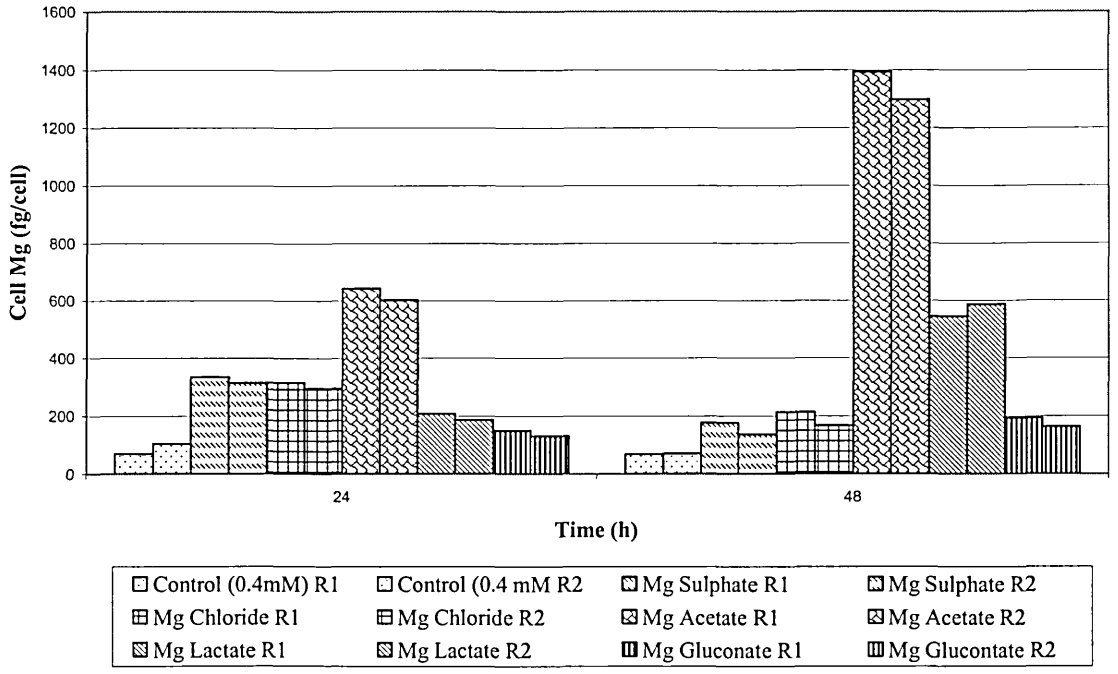


Fig 3.14 Biomass in *S.cerevisiae* var. *carlsbergensis* (lager yeast) preconditioned in malt broth without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the broth were 0.4mM and 50mM, respectively.

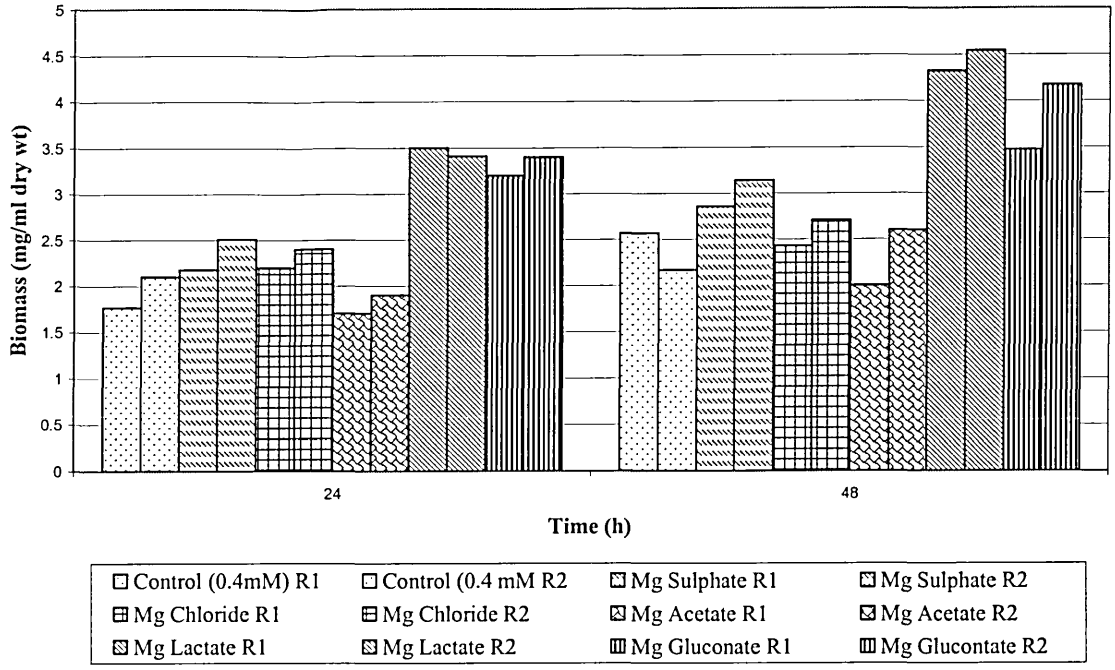


Fig 3.15 Biomass in *S.cerevisiae* var. *carlsbergensis* (lager yeast) preconditioned in malt broth without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the broth were 0.4mM and 100mM, respectively.

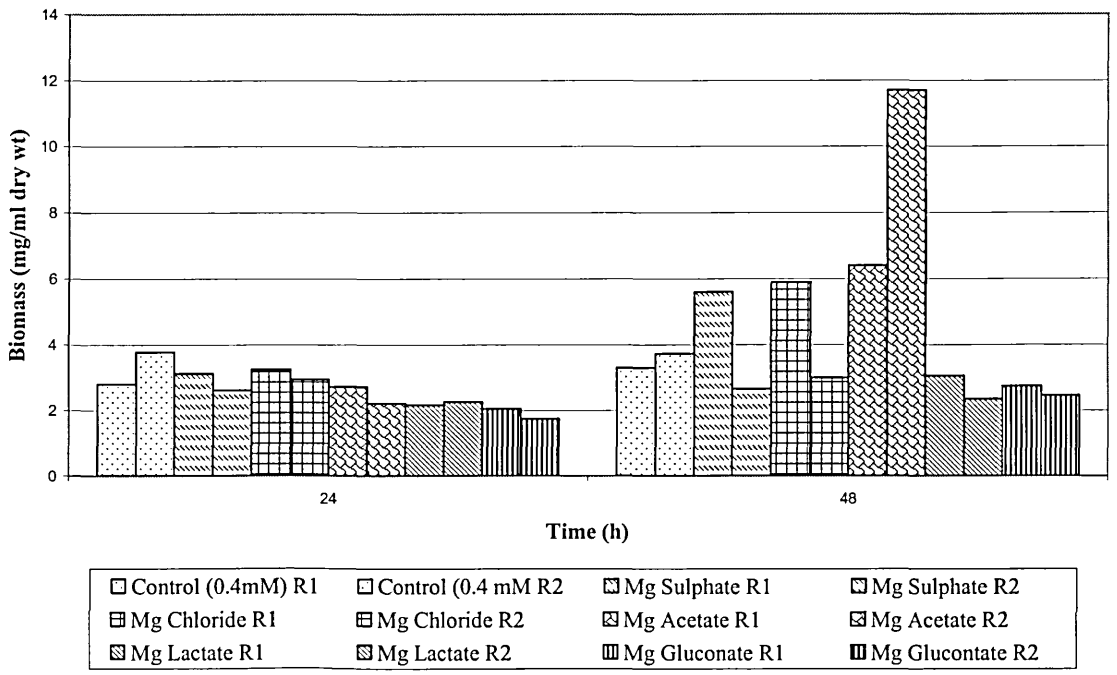


Table 3.3 Mg content (mg Mg/g dry wt) in *S.cerevisiae* (ale yeast) preconditioned in malt broth supplemented without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the broth were 0.4mM, 50mM and 100mM, respectively.

Mg Salt	50mM Mg		100mM Mg	
	24h	48h	24h	48h
Control (1)	1.92	2.31	2.56	2.08
Control (2)	2.06	2.47	2.68	2.18
Mg Sulphate (1)	5.25	5.97	8.43	6.88
Mg Sulphate (2)	5.53	6.13	8.59	7.06
Mg Chloride (1)	6.01	6.31	8.71	5.31
Mg Chloride (2)	6.37	6.57	8.99	5.65
Mg Acetate (1)	6.77	7.39	14.92	7.86
Mg Acetate (2)	6.91	7.55	15.10	8.00
Mg Gluconate (1)	5.58	5.87	7.39	7.15
Mg Gluconate (2)	5.72	5.97	7.75	7.25
Mg Lactate (1)	5.79	6.41	13.51	18.18
Mg Lactate (2)	5.93	6.57	13.87	18.30

Table 3.4 Mg content (mg Mg/g dry wt) in *S. cerevisiae* var. *carlsbergensis* (lager yeast) preconditioned in malt broth supplemented without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the broth were 0.4mM, 50mM and 100mM, respectively.

Mg Salt	50mM Mg		100mM Mg	
	24h	48h	24h	48h
Control (1)	1.98	2.17	0.98	1.39
Control (2)	2.42	2.27	0.46	1.55
Mg Sulphate (1)	3.81	2.81	3.97	2.51
Mg Sulphate (2)	4.09	3.15	4.27	2.81
Mg Chloride (1)	3.65	4.11	2.91	2.83
Mg Chloride (2)	3.81	3.87	3.15	2.69
Mg Acetate (1)	10.57	15.91	9.42	9.67
Mg Acetate (2)	10.79	16.09	9.64	10.97
Mg Gluconate (1)	5.49	3.43	4.00	3.63
Mg Gluconate (2)	5.81	3.61	4.24	3.85
Mg Lactate (1)	7.86	13.51	4.71	11.85
Mg Lactate (2)	8.10	13.75	5.07	12.09

3.3.3 Mg preconditioning in brewers' wort

Cellular Mg content was highest in aerobically preconditioned ale yeast cells in wort supplemented with 50mM Mg acetate after 48h (Fig 3.16). Fig 3.17 shows that ale yeast cells preconditioned in the presence of 100mM Mg lactate and gluconate yielded higher cellular Mg contents than Mg acetate, chloride and sulphate at time 24h (see also Table 3.5). However, after 48h cellular Mg content was highest in Mg acetate preconditioned ale yeast cells (Fig 3.17 and Table 3.5). Preconditioning ale yeast cells in the presence of 50mM Mg salts had an effect on biomass in that biomass yields were lower than that yielded by the unpreconditioned cells after 48h (Fig 3.18). Biomass of ale yeast cells preconditioned in the presence of 100mM Mg acetate was affected after 48h, with Mg gluconate preconditioned cells yielding the highest biomass (Fig 3.19).

Lager yeast cells aerobically preconditioned in the presence of 50 and 100mM Mg acetate yielded the greatest cellular Mg content after 48h (Figs 3.20, 3.21 and Table 3.6). Between time 24h and 48h lager yeast cells preconditioned in the presence of 50 and 100mM Mg gluconate lost cellular Mg (Figs 3.20 and 3.21). Biomass yields were higher in lager yeast cells preconditioned in the presence of 50mM Mg salts than 100mM Mg salts (Figs 3.22 and 3.23).

Fig 3.16 Cell Mg in *S.cerevisiae* (ale yeast) aerobically preconditioned in weak wort (1020°OG) without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the wort were 0.6mM and 50mM, respectively.

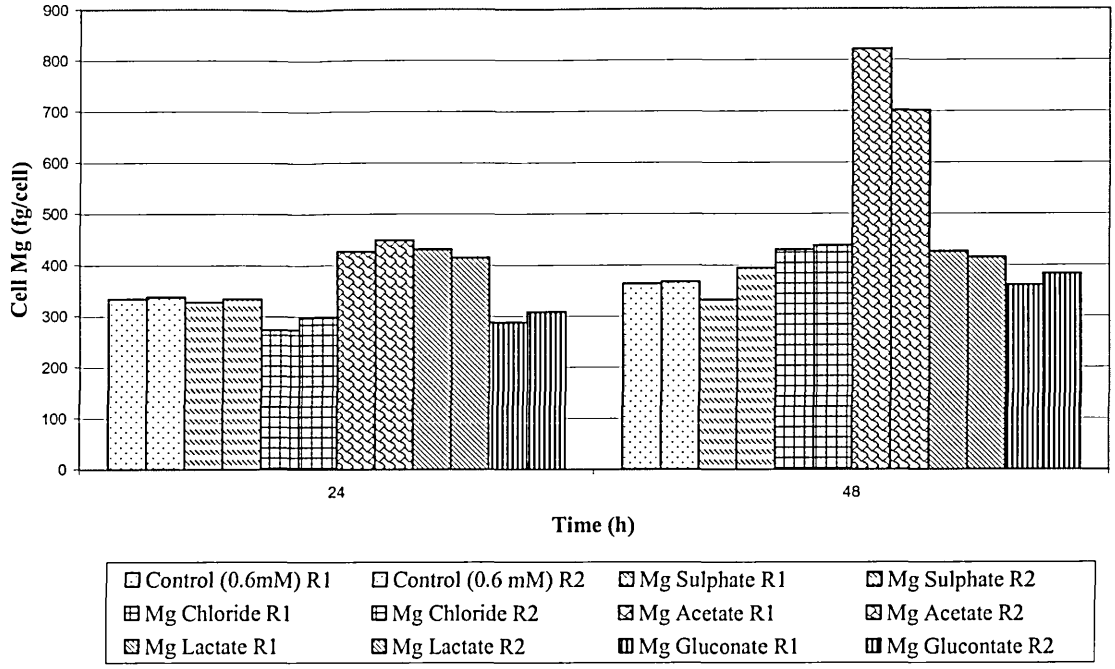


Fig 3.17 Cell Mg in *S.cerevisiae* (ale yeast) aerobically preconditioned in weak wort (1020°OG) without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the wort were 0.6mM and 100mM, respectively.

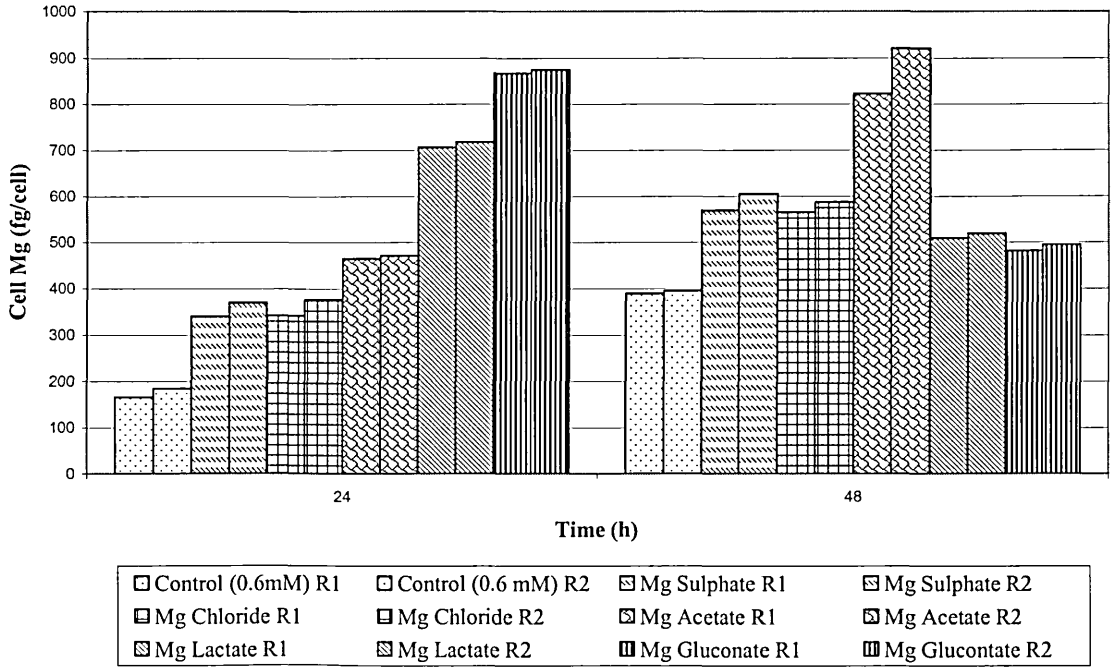


Fig 3.18 Biomass in *S.cerevisiae* (ale yeast) aerobically preconditioned in weak wort (1020°OG) without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the wort were 0.6mM and 50mM, respectively.

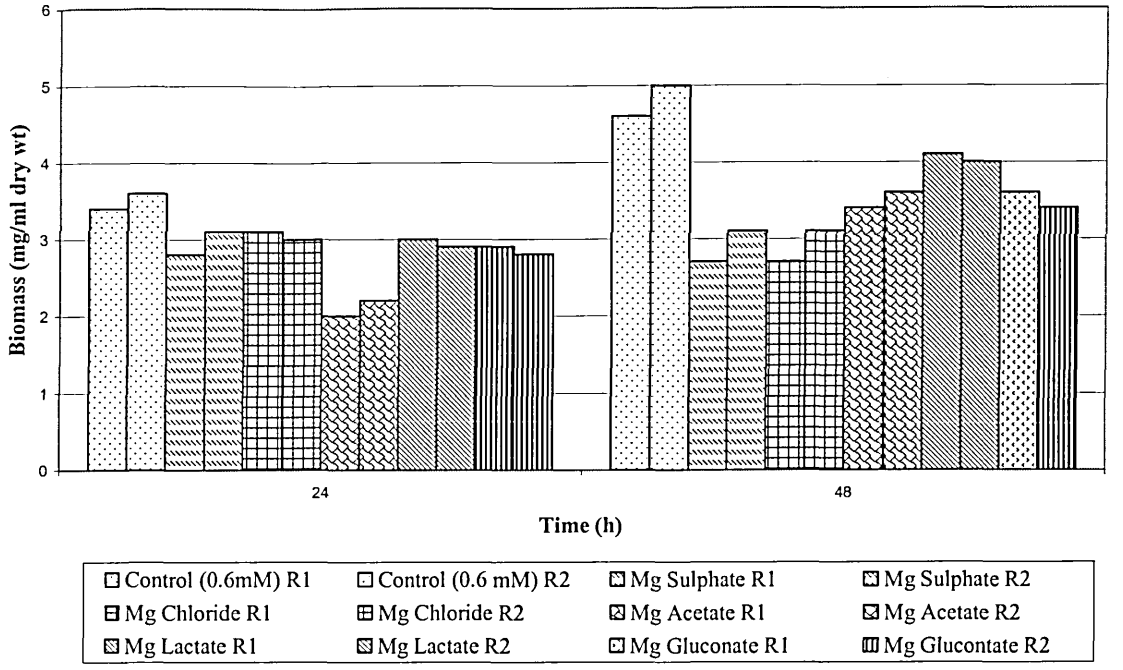


Fig 3.19 Biomass in *S.cerevisiae* (ale yeast) aerobically preconditioned in weak wort (1020°OG) without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the wort were 0.6mM and 100mM, respectively.

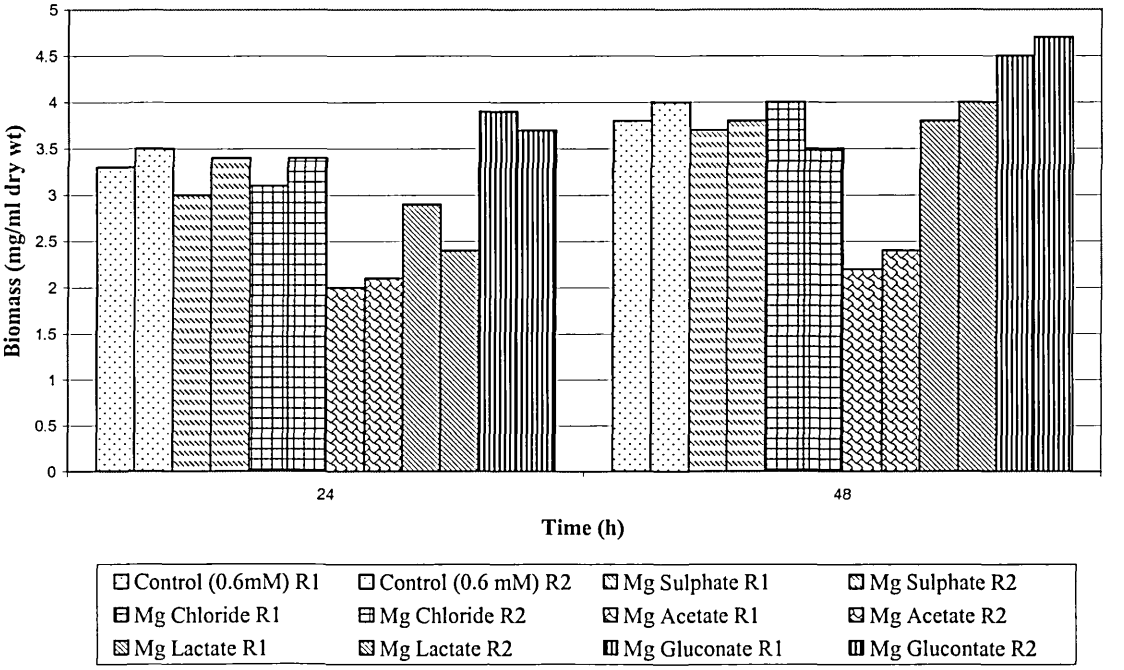


Fig 3.20 Cell Mg in *S.cerevisiae* var. *carlsbergensis* (lager yeast) aerobically preconditioned in weak wort (1020°OG) without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the wort were 0.6mM and 50mM, respectively.

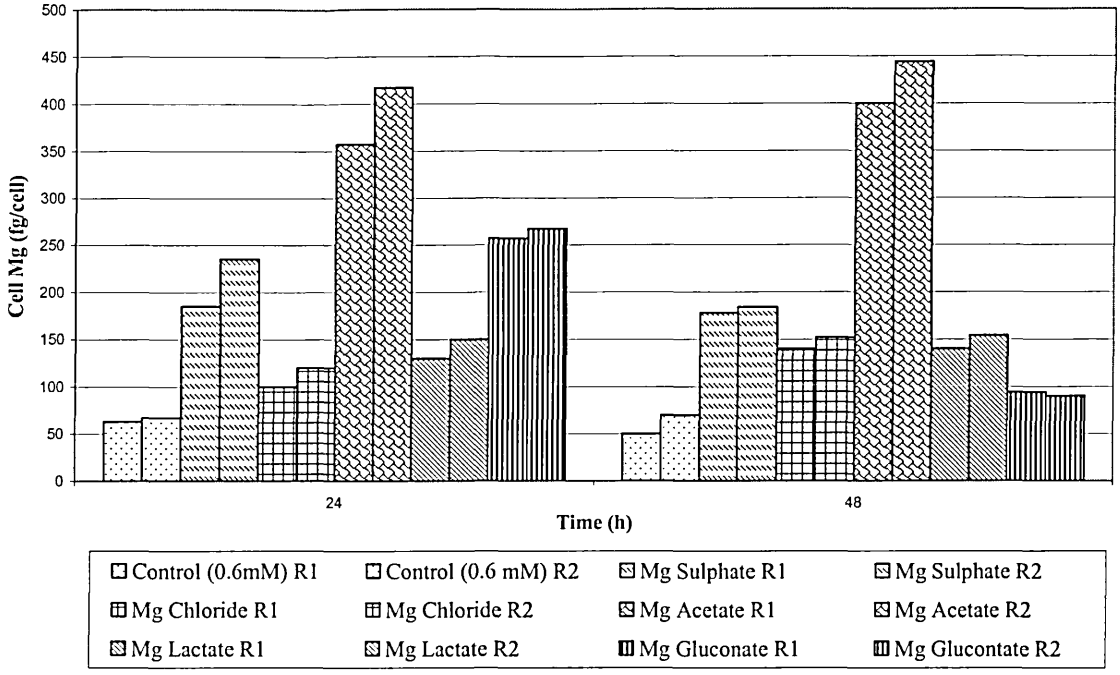


Fig 3.21 Cell Mg in *S.cerevisiae* var. *carlsbergensis* (lager yeast) aerobically preconditioned in weak wort (1020°OG) without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the wort were 0.6mM and 100mM, respectively.

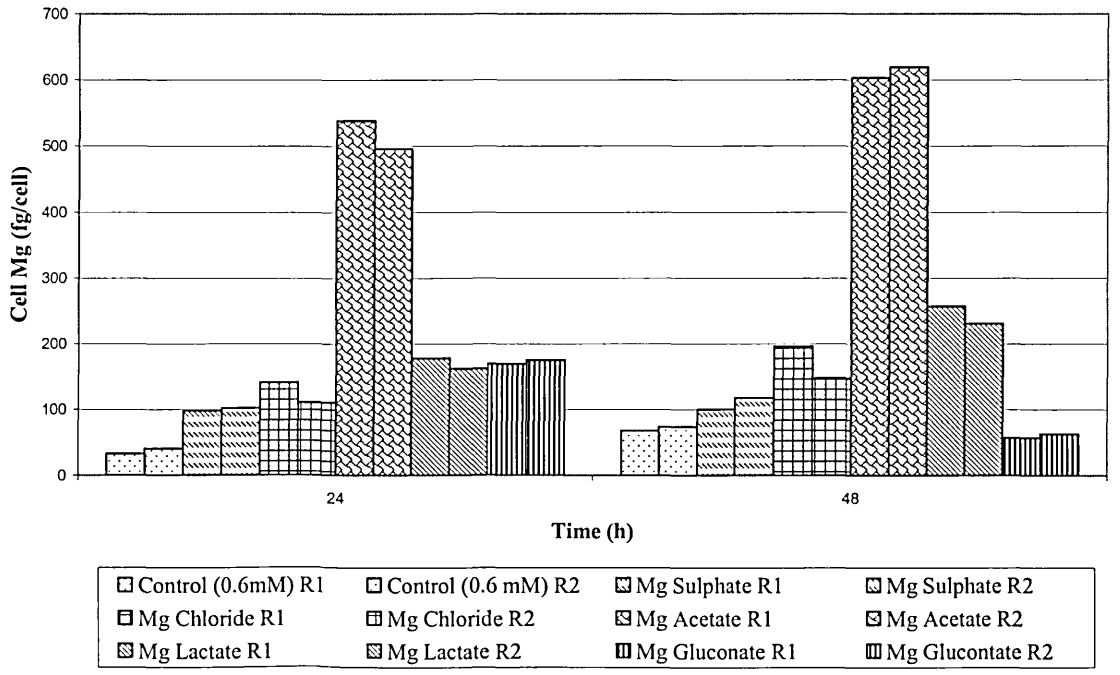


Fig 3.22 Biomass in *S.cerevisiae* var. *carlsbergensis* (lager yeast) aerobically preconditioned in weak wort (1020°OG) without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the wort were 0.6mM and 50mM, respectively.

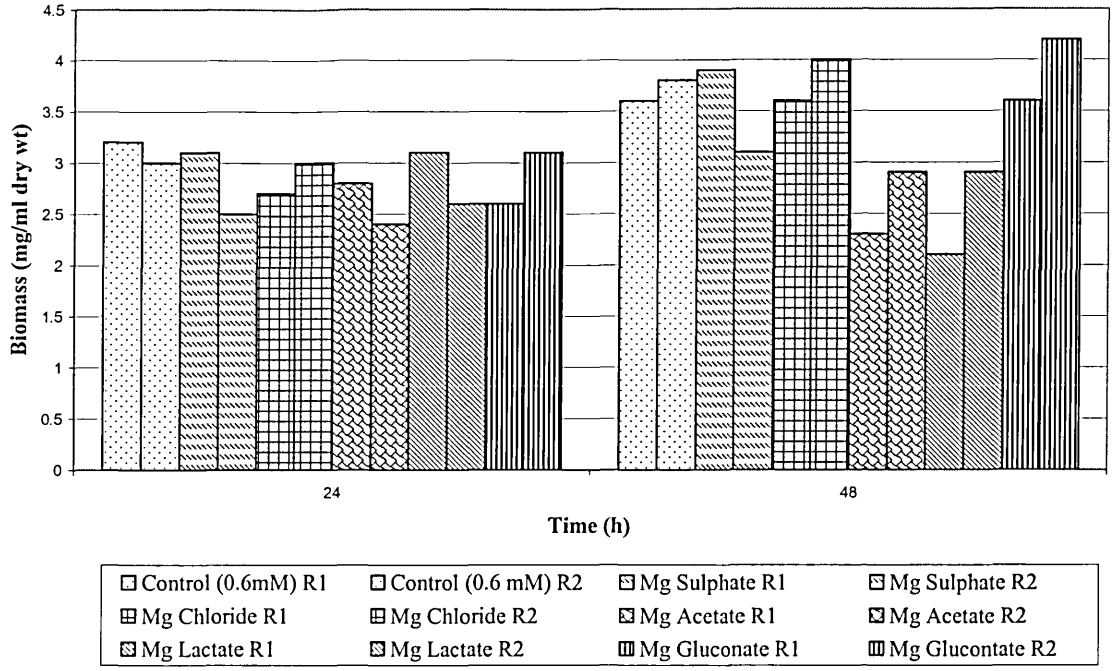


Fig 3.23 Biomass in *S.cerevisiae* var. *carlsbergensis* (lager yeast) aerobically preconditioned in weak wort (1020°OG) without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the wort were 0.6mM and 100mM, respectively.

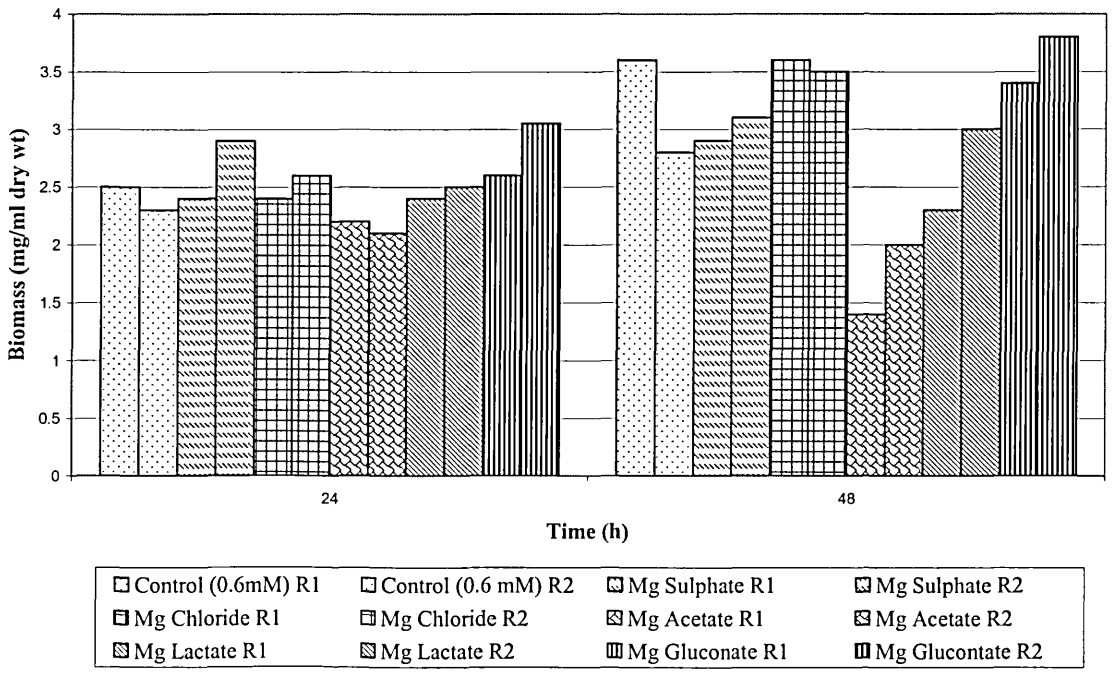


Table 3.5 Mg content (mg Mg/g dry wt) in *S.cerevisiae* (ale yeast) aerobically preconditioned in weak wort (1020°OG) supplemented without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the wort were 0.6mM, 50mM and 100mM, respectively.

Mg Salt	50mM Mg		100mM Mg	
	24h	48h	24h	48h
Control (1)	4.70	4.46	3.97	4.93
Control (2)	4.90	4.66	4.11	4.93
Mg Sulphate (1)	5.49	5.02	6.07	7.08
Mg Sulphate (2)	5.77	5.30	6.37	7.32
Mg Chloride (1)	4.81	6.78	14.21	23.76
Mg Chloride (2)	4.95	7.00	14.39	24.02
Mg Acetate (1)	10.77	10.73	6.11	6.38
Mg Acetate (2)	10.95	10.93	6.29	6.52
Mg Gluconate (1)	5.79	6.15	13.17	5.97
Mg Gluconate (2)	5.93	6.37	13.41	6.13
Mg Lactate (1)	6.21	5.93	15.46	7.13
Mg Lactate (2)	6.39	6.11	15.70	7.39

Table 3.6 Mg content (mg Mg/g dry wt) in *S.cerevisiae* var. *carlsbergensis* (lager yeast) aerobically preconditioned in weak wort (1020°OG) supplemented without (control) or with supplementation of Mg salts, for the times indicated. Mg levels in the wort were 0.6mM, 50mM and 100mM, respectively.

Mg Salt	50mM Mg		100mM Mg	
	24h	48h	24h	48h
Control (1)	1.27	0.96	0.65	1.17
Control (2)	1.51	1.06	0.89	1.39
Mg Sulphate (1)	3.28	2.90	2.41	2.76
Mg Sulphate (2)	3.32	3.12	2.63	3.00
Mg Chloride (1)	2.31	2.18	2.31	2.27
Mg Chloride (2)	2.57	2.34	2.53	2.41
Mg Acetate (1)	7.93	10.13	10.21	17.04
Mg Acetate (2)	8.15	10.33	10.45	17.48
Mg Gluconate (1)	3.46	0.91	2.63	0.77
Mg Gluconate (2)	3.66	1.07	2.77	0.93
Mg Lactate (1)	3.00	3.78	3.85	5.33
Mg Lactate (2)	3.20	3.98	4.07	5.53

3.3.4 Reproducibility

The data presented herein is data of duplicated experiments (R1and R2), which showed a close relationship between the two data sets. Differences were observed in data presented for lager yeast cells grown in SWM supplemented with 25mM Mg (Fig 3.5).

These differences between the two data sets could be due to error during the cell count or fluctuations in the cell growth of the lager yeast cells during the two experiments.

Differences were also observed in data presented for ale yeast cell biomass in malt extract broth (control and 100mM Mg chloride, Fig 3.11) and lager yeast cell biomass (100mM, Mg sulphate, Mg chloride and Mg acetate, Fig 3.15). The observed variations may be due to culture conditions and/or yeast cell growth. Further replicates of the experiments would have allowed greater statistical accuracy, thus reducing variations between data sets.

3.4 Discussion

When grown in SWM with increasing Mg concentrations, lager yeast cells showed an increase in cellular Mg content. However, this was not directly proportional to the Mg concentration in the medium, in that after 24h both yeast strains yielded the highest intracellular Mg when preconditioned in the presence of 25mM Mg, which has also been shown by previous authors (Saltukoglu and Slaughter 1983; Walker and Maynard 1997). Optimal growth of the lager yeast cells occurred in the presence of 10mM Mg with optimal growth of ale yeast cells occurring in the presence of 50 and 75mM Mg.

The results (Tables 3.1 and 3.2) showed that when Mg was expressed in terms of biomass (mg/g dry wt), data from both ale and lager yeast cells concurred with data presented by Obata *et al* (1996) (3.42 mg Mg/g dry wt) and were higher (100mM) than data presented by Graschopf *et al* (2001) who presented results for yeast cells grown in the presence of 200mM Mg (3.20 mg Mg/g dry wt). When Mg was expressed in terms of protein (nmol Mg/mg protein) the results (Table 3.1 and 3.2) were higher than data presented by Dombek and Ingram (1986b) and Beeler *et al* (1997) (100-200 nmol Mg/mg

protein and 100-400 nmol Mg/mg protein respectively). The Mg data expressed in mM (Table 3.1 and 3.2) represented total Mg and was therefore much higher than data presented by (Zhang *et al.* 1997) (0.93-1.2mM Mg), which represented free Mg in *S.pombe* cells rather than total Mg.

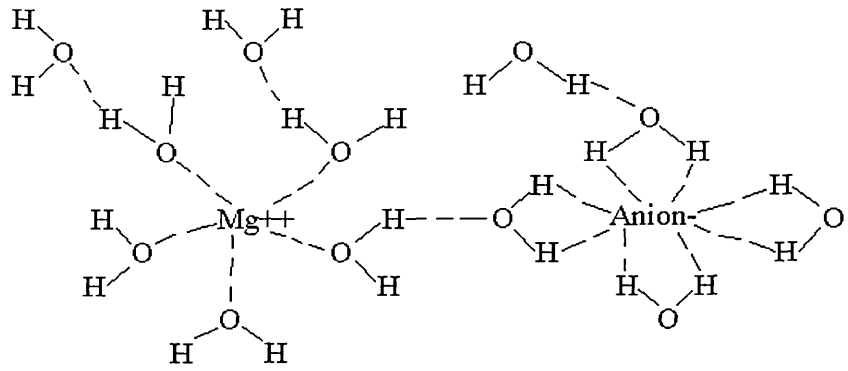
Ale and lager yeast cells preconditioned in the presence of 50 and 100mM Mg salts yield the highest cellular Mg contents in the presence of Mg acetate. When Mg was expressed in terms of biomass, lager yeast cells showed lower Mg contents for Mg acetate at 100mM. This might have been due to a combination of low biomass results after 24h and a high biomass results after 48h influencing the net result. Cell Mg content results for Mg lactate showed that ale yeast cells preconditioned in the presence of 50 and 100mM were similar, this would suggest that the uptake of this salt was not directly proportional to its bioavailability. In preconditioned ale and lager yeast cells, when Mg content was expressed in terms of biomass, the results (Table 3.3 and 3.4) were higher than data presented by Obata *et al* (1996)(3.42 mg Mg/g dry wt) and Graschopf *et al* (2001) who presented results for yeasts grown in the presence of 200mM (3.20 mg Mg/g dry wt). These apparent discrepancies may be due to strain differences, in that Obata *et al* (1996) and Graschopf *et al* (2001) used laboratory strains of *S.cerevisiae* whereas the strains used in this study were brewing strains used in industry.

Aerobically preconditioning ale and lager yeast cells in dilute wort in the presence of 50 and 100mM Mg acetate yielded the highest cellular Mg content. When lager yeast cells were preconditioned in the presence of 50 and 100mM Mg gluconate, Mg was lost after 24h, this was also observed in ale yeast cells aerobically preconditioned in the presence of 100mM of this salt, these results suggest that in both yeast strains uptake of

this salt was not directly proportional to its bioavailability. The results would suggest that maximal Mg uptake might be dependent on the salt used and the mechanism of transport used to take it into the cell. When expressed in terms of biomass produced the resultant Mg contents (Table 3.5 and 3.6) were higher than data presented by Obata *et al* (1996)(3.42 mg Mg/g dry wt) and Graschopf *et al* (2001) who presented results for yeasts grown in the presence of 200mM (3.20 mg Mg/g dry wt). Such apparent discrepancies may be due to strain differences, in that Obata *et al* (1996) and Graschopf *et al* (2001) used laboratory strains of *S.cerevisiae* whereas the strains used in this study were brewing strains used in industry.

Variable levels of cellular Mg obtained when yeast cells were preconditioned in the presence of different Mg salts may be explained by the following theoretical considerations. Water consists of two hydrogen atoms covalently bonded to an electronegative oxygen atom. Both the hydrogen and oxygen atoms carry small positive and negative charges respectively. When Mg salts dissolve in water, they dissociate and form hydrogen bonds with the oxygen and hydrogen atoms, the two ions (Mg and its anion) each become surrounded by a 'shell' of water molecules (see Fig 3.24) (Wood and Smith 1991). This indicates that the Mg ions are freely available for transportation across the cell membrane.

Fig 3.24 Schematic representation of Mg salt in solution (adapted from Wood and Smith 1991).



Results have shown that Mg acetate dramatically increases cellular Mg in both malt broth and dilute wort. This suggests that the anion of the salt directly promotes the uptake of Mg ions into the cell. Several mechanisms may account for this. For example, this could be due to the anion altering the pH of the medium. When the Mg salts (100mM) used in this study were dissolved in malt broth, Mg gluconate, lactate and acetate were found to increase the pH of the medium from pH 5.5 to pH 5.6, 5.7 and pH 6.3, respectively. Mg sulphate and chloride were found to lower the pH from pH 5.5 to pH 5.0 and 4.7, respectively. Similar alterations were observed when pH measurements were made using 50mM of the Mg salts. As it was not possible to measure the effect the Mg salts had on the pH of the dilute brewer's wort, it can only be assumed that the Mg salts were exerting a similar influence on the wort pH as they did with the malt broth. Given that the Mg acetate salt increased the medium pH to nearer neutrality, it might be possible that a greater proportion of the acetate molecules were in their undissociated state, which would allow more of the acetate to pass through the cell membrane by diffusion (Stratford and Anslow 1996; 1998). This would in turn lower the intracellular pH, which might influence cell membrane permeability, which could be having a knock on effect on the H^+-pumping ATPase and the putative Mg transporter *ALR1* (MacDiarmid and Gardner 1998).

In addition, acetate can be used as an alternative carbon source where it is oxidised in the glyoxylate cycle, which bypasses two decarboxylation steps in the TCA cycle (Walker 1998a). Such metabolism may result in the co-translocation of acetate and Mg when conditions favouring the glyoxylate cycle prevail (sugar limitation, oxidative growth).

The identification of a putative low affinity Mg transporter gene (*ALR1*) (MacDiarmid and Gardner 1998) as well as the putative mitochondrial Mg transporters (*LPE10* and *MRS2*) (Wiesenberger *et al.* 1992; Bui *et al.* 1999; Gregan *et al.* 2001) would suggest that Mg may also be transported into yeasts by a high affinity transport system. This is especially pertinent, since such dual transport mechanisms exist in yeasts for metals such as Zn and Mn (Zhao and Eide, 1996a,b Walker, 1998a) .

Differences in cellular Mg content in ale and lager yeast cells may also be due to their having different nutritional requirements and growth demands for Mg as well as there being genetic differences between the two strains. The proteins encoded by the Mg transporter genes may therefore consist of slightly different subunits derived from the genetic information of their respective genomes. This would suggest that lager yeasts might possess transporter proteins, which are more efficient at regulating Mg uptake into the cell hence the lower cellular Mg contents. Conversely the differences in Mg uptake could be due to there being a greater diversity of ale yeast strains in general which may in part be due to top cropping yeasts being used (mostly unknowingly) for at least 3000 years (Boulton and Quain 2001). Through this strain selection, ale yeast strains may have evolved with subtle changes in their Mg requirements, especially in geographical terms where worts can vary in ionic composition. Conversely, bottom cropping lager yeasts, had been used exclusively by Bavarian brewers until relatively recently (~1840). They were

then smuggled to Czechoslovakia and Denmark, and soon became used on a worldwide basis through increased trade and travel (Boulton and Quain 2001). In terms of diversity, therefore, lager yeasts are relatively new to the brewing process. It must be noted that much of this is purely speculative in order to try and explain the differences in cellular Mg, which resulted from this particular aspect of the study.

CHAPTER 4

LOCALISATION OF INTRACELLULAR Mg In PRECONDITIONED YEASTS

4.1 Introduction

The ability to measure the concentration of intracellular free Mg^{2+} (Mg^{2+}_i) is extremely desirable in order to allow its physiological role in a variety of intracellular processes to be fully understood. This can be achieved through the use of *in vivo* nuclear magnetic resonance (NMR) and fluorescence spectroscopy, both of which have provided a useful means for determining intracellular Mg (London 1991).

NMR can be used to provide information on the levels of magnesium in the cell using endogenous indicators such as the small metabolites found in cells, which exist in equilibrium between uncomplexed and Mg^{2+} complexed states (London 1991). Exogenous indicators have also been used to measure cytosolic Mg ion levels; the main advantage of this strategy is that the indicators can be designed with a greater degree of sensitivity (London 1991). A number of other methods exist to measure intra and extracellular Mg, these include atomic absorption spectrophotometry, ion chromatography, spectrophotometry using metallochromic dyes (e.g. Eriochrome blue, arsenazo, methyl thymol blue, calmagite magnon) (Walker 1994).

Fluorescence (spectroscopy) using dyes such as Mg Green and Mag Fura-2 have been used to measure the intracellular Mg in a variety of mammalian cells (Leyssens *et al.* 1996; Budinger *et al.* 1998; Schweigel *et al.* 1999; Kang *et al.* 2000; 2000) as well as yeast cells (Zhang *et al.* 1997). This Chapter concerns the use of such dyes in the determination of intracellular Mg in brewing yeast cells.

4.2 Experimental approach

An attempt was made to visualise the cell Mg in ale and lager yeast cells that had been preconditioned, compared to those that had not. This was accomplished using the magnesium specific, fluorescent dyes Mag-fura-2 and Magnesium Green, as described in section 2.3. Ale and lager yeast cells were stained and visualised using fluorescence microscopy and image analysis as described in section 2.3.2. Inoculum and medium preparation are detailed in section 2.3.1. Cellular Mg was measured by AAS as described in section 2.6.3.

4.3 Results

4.3.1 Mg preconditioned ale yeast

Mg acetate preconditioned ale yeast cells had a higher cellular Mg content than Mg sulphate preconditioned and unpreconditioned cells after 24h (Table 4.1). Both preconditioned and unpreconditioned ale yeast cells fluoresced in the presence of Mag-fura-2 (Table 4.2). However, far fewer cells were observed fluorescing in the presence of Mg Green (Table 4.3, Fig 4.1a-c). After 96h propagation, preconditioned cells had further accumulated Mg (Table 4.1) and the mean number of Mg Green fluorescing cells had also increased (Table 4.3). Ale yeast cells preconditioned in the presence of Mg acetate had also shown signs of sporulation after 96h growth (Fig 4.2c) with vegetative cells containing 2-3 ascospores observed. The Mg Green dye appeared to have highlighted Mg being present on the outside of each ascospore. Due to the lower fluorescence intensity exhibited by cells stained with Mag-fura-2 the image analysis equipment was unable to capture images of a sufficient quality to be worthy of presentation here in.

Table 4.1 Cellular Mg (measured by AAS) in *S.cerevisiae* (ale yeast)

Mg Salt	Cell Mg (fg/cell)	
	24h	96h
Control (1)	143	189
Control (2)	232	229
Mg Sulphate (1)	217	727
Mg Sulphate (2)	300	380
Mg Acetate (1)	670	2004
Mg Acetate (2)	590	2065

Table 4.2 Mean *S.cerevisiae* (ale yeast) cells fluorescing in the presence of Mag-Fura-2

Mg Salt	Mean cells counted		Mean cells fluorescing	
	24h	**96h**	**24h**	**96h**
Control (1)	117	66	117	66
Control (2)	137	42	137	42
Mg Sulphate (1)	135	52	135	52
Mg Sulphate (2)	110	87	110	87
Mg Acetate (1)	203	62	203	62
Mg Acetate (2)	113	36	113	36

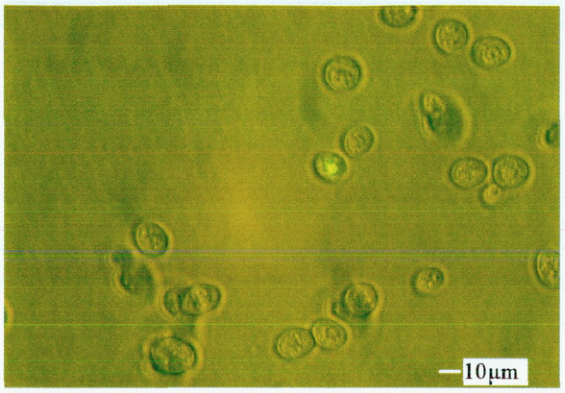
Table 4.3 Mean *S.cerevisiae* (ale yeast) cells fluorescing in the presence of Mg Green

Mg Salt	Mean cells counted		Mean cells fluorescing	
	24h	**96h**	**24h**	**96h**
Control (1)	92	100	1	2
Control (2)	56	68	1	2
Mg Sulphate (1)	79	91	1	2
Mg Sulphate (2)	103	63	1	5
Mg Acetate (1)	81	62	1	62
Mg Acetate (2)	113	36	2	36

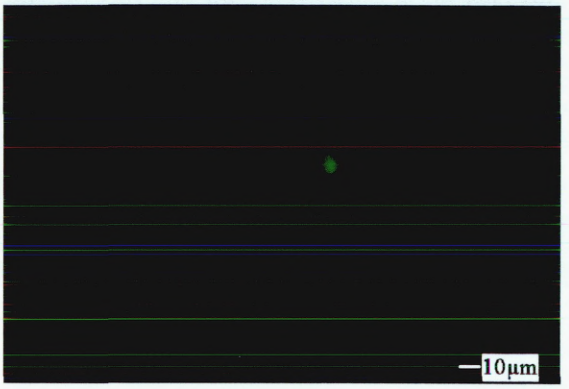
Fig 4.1 Mg Green fluorescent images: *S.cerevisiae* (ale yeast) after 24h growth (X40 Magnification)

a) Control

Transmitted light

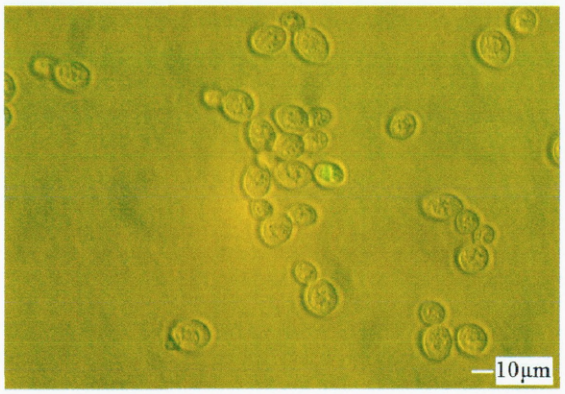


fluorescence

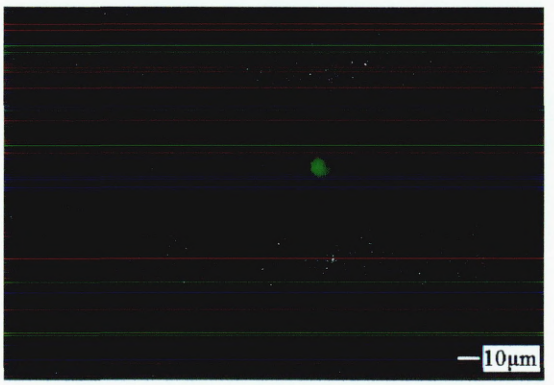


b) Mg sulphate preconditioned

Transmitted light

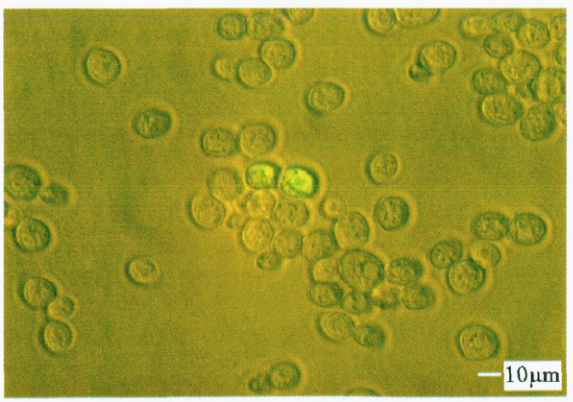


fluorescence



c) Mg acetate preconditioned

Transmitted light



fluorescence

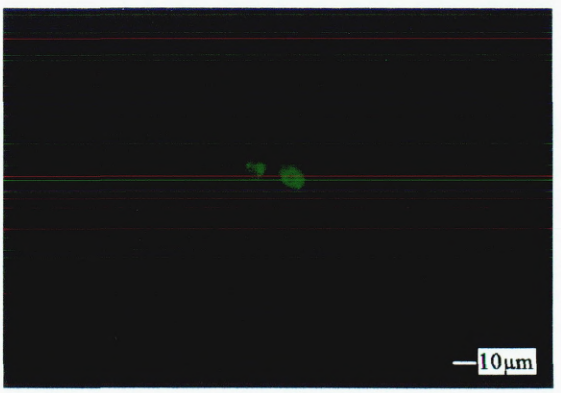
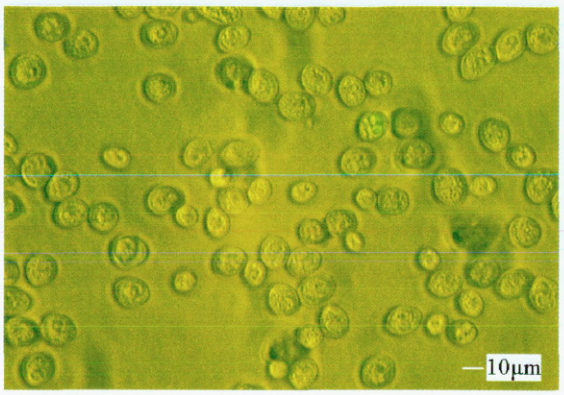


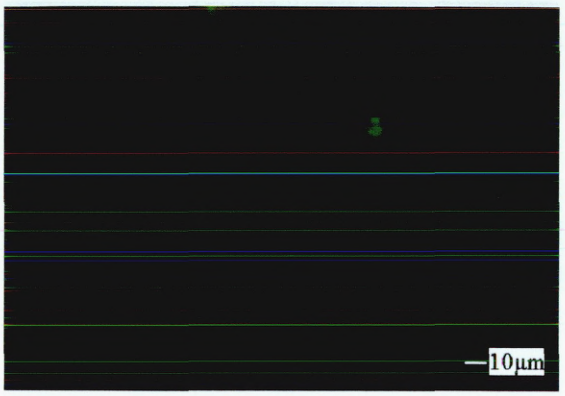
Fig 4.2 Mg Green fluorescent images: *S.cerevisiae* (ale yeast) after 96h growth (X40 Magnification)

a) Control

Transmitted light

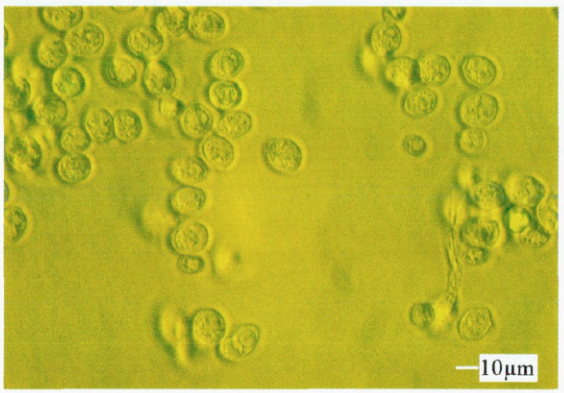


fluorescence

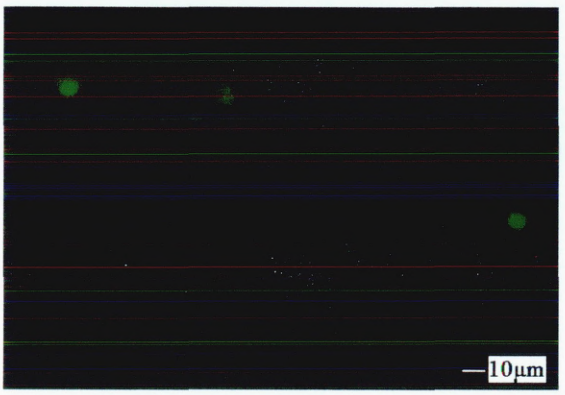


b) Mg sulphate preconditioned

Transmitted light

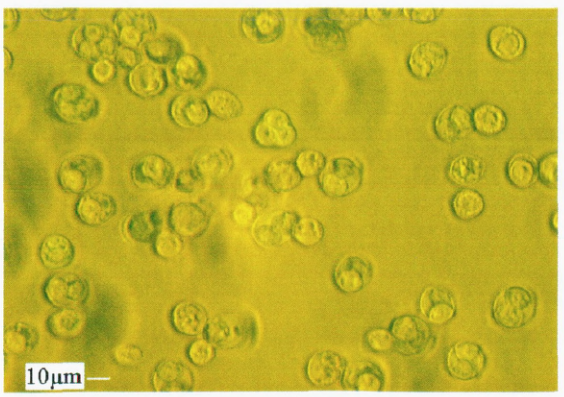


fluorescence

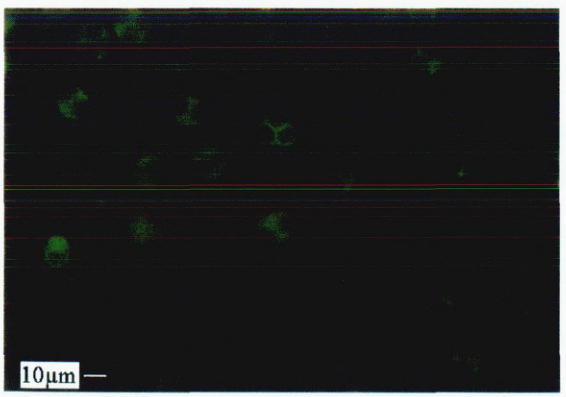


c) Mg acetate preconditioned

Transmitted light



fluorescence



4.3.2 Mg preconditioned lager yeast

The results presented in Table 4.4 show that after 24h growth Mg acetate preconditioned cells had four times the amount of cell Mg of that present in Mg sulphate preconditioned and unpreconditioned cells. This was also reflected in the number of cells observed fluorescing in the presence of Mg Green (Table 4.6), however this was less than those observed fluorescing in the presence of Mag-fura-2 as all cells showed fluorescence when stained with this dye (Table 4.5). Fig 4.3c shows that the Mg contained in the Mg acetate preconditioned cells is distributed throughout the cell, with the magnesium in Mg sulphate and unpreconditioned cells appearing to be more localised (Figs 4.3a-b). After 96h propagation both Mg acetate and Mg sulphate preconditioned cells showed further uptake in Mg in the intervening 72h period, this was also reflected in the number of cells observed fluorescing in the presence of Mg Green (Table 4.6 and Figs 4.4b-c). Due to the lower fluorescence intensity exhibited by cells stained with Mag-fura-2 the image analysis equipment was unable to capture images of a sufficient quality to be worthy of presentation here in.

Table 4.4 Cellular Mg (measured by AAS) in *S.cerevisiae* var. *carlsbergensis* (lager yeast)

Mg Salt	Cell Mg (fg/cell)	
	24h	96h
Control (1)	214	192
Control (2)	379	218
Mg Sulphate (1)	289	399
Mg Sulphate (2)	329	326
Mg Acetate (1)	1042	2180
Mg Acetate (2)	1666	2154

Table 4.5 Mean *S.cerevisiae* var. *carlsbergensis* (lager yeast) fluorescing in the presence of Mag-Fura-2

Mg Salt	Mean cells counted		Mean cells fluorescing	
	24h	**96h**	**24h**	**96h**
Control (1)	88	98	88	98
Control (2)	117	66	117	66
Mg Sulphate (1)	76	97	76	97
Mg Sulphate (2)	100	74	100	74
Mg Acetate (1)	64	62	64	62
Mg Acetate (2)	100	62	100	62

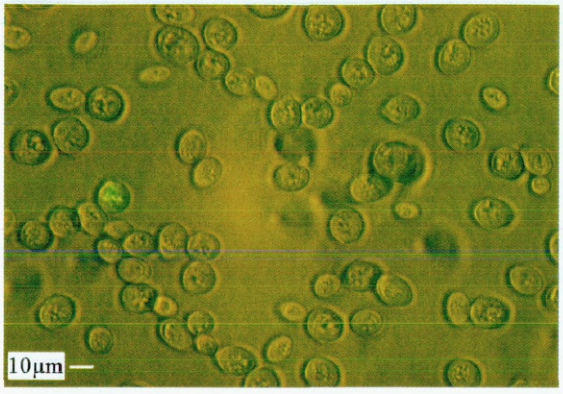
Table 4.6 Mean *S.cerevisiae* var. *carlsbergensis* (lager yeast) fluorescing in the presence of Mg Green

Mg Salt	Mean cells counted		Mean cells fluorescing	
	24h	**96h**	**24h**	**96h**
Control (1)	86	93	2	2.2
Control (2)	102	85	0.8	1.2
Mg Sulphate (1)	100	91	1.4	3.8
Mg Sulphate (2)	74	69	1.2	1.2
Mg Acetate (1)	87	83	3.2	7.6
Mg Acetate (2)	71	91	4.4	8.6

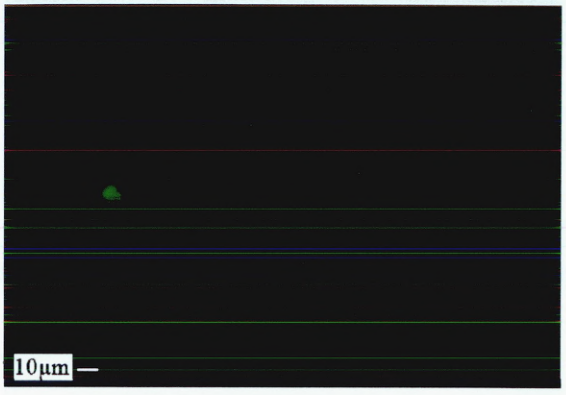
Fig 4.3 Mg Green fluorescent images: *S.cerevisiae* var. *carlsbergensis* (lager yeast) after 24h growth (X40 Magnification)

a) Control

Transmitted light

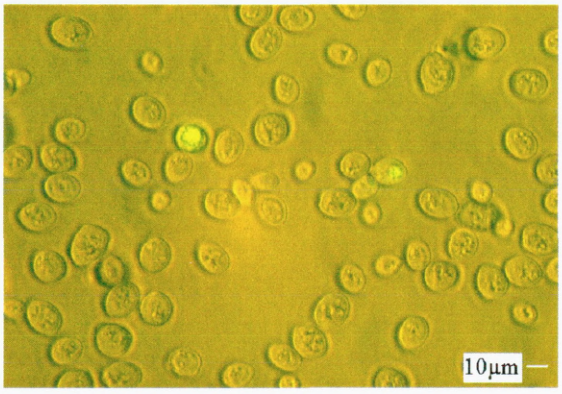


fluorescence

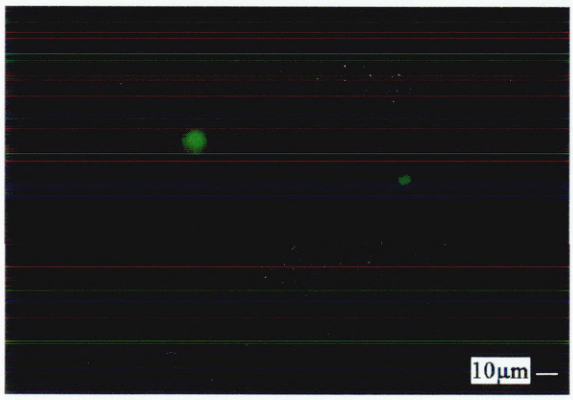


b) Mg sulphate preconditioned

Transmitted light

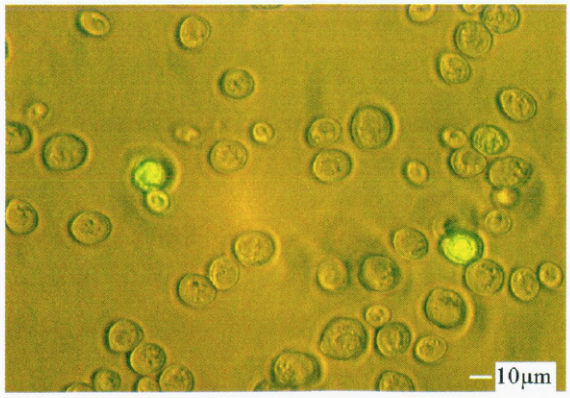


fluorescence



c) Mg acetate preconditioned

Transmitted light



fluorescence

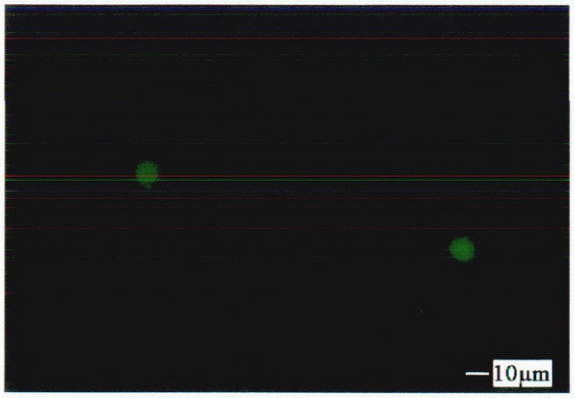
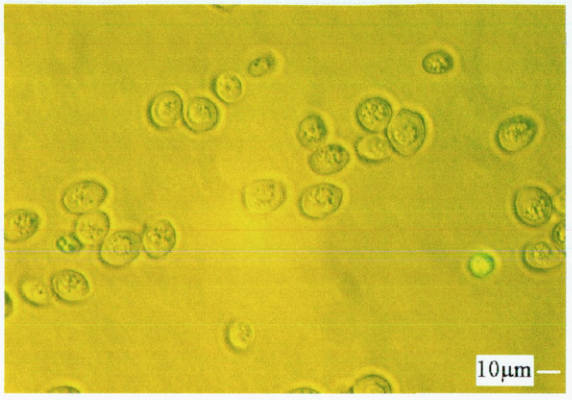


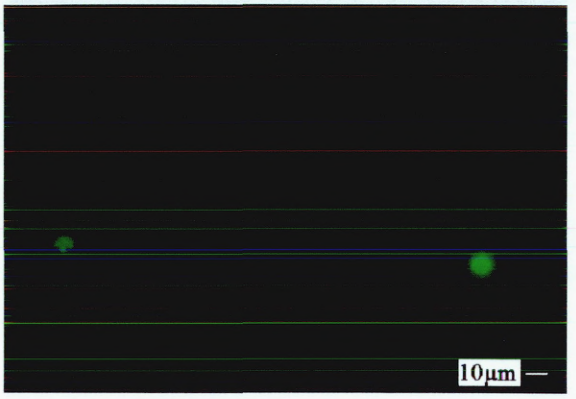
Fig 4.4 Mg Green fluorescent images: *S.cerevisiae* var. *carlsbergensis* (lager yeast) after 96h growth (X40 Magnification)

a) Control

Transmitted light

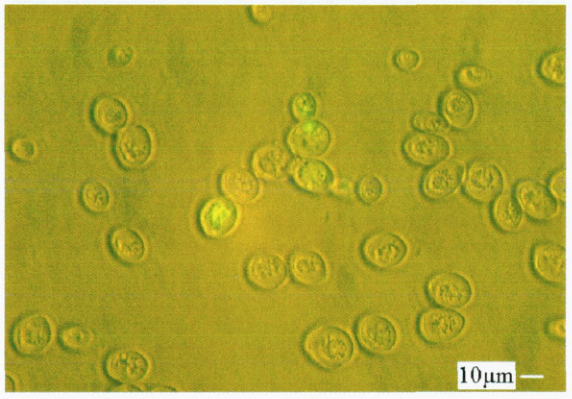


fluorescence

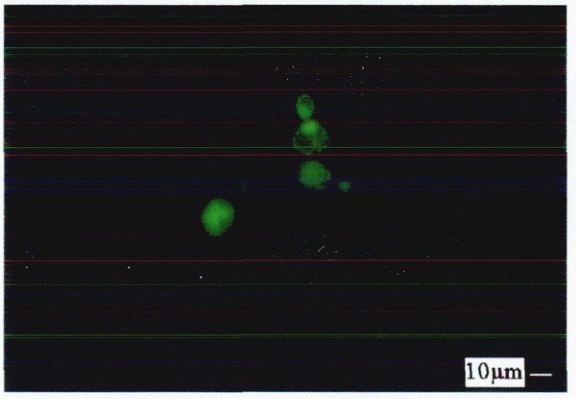


b) Mg sulphate preconditioned

Transmitted light

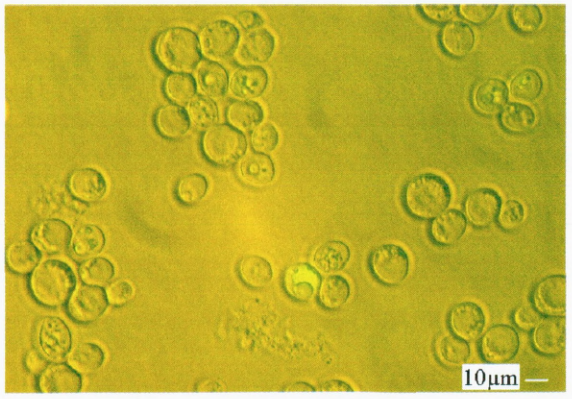


fluorescence

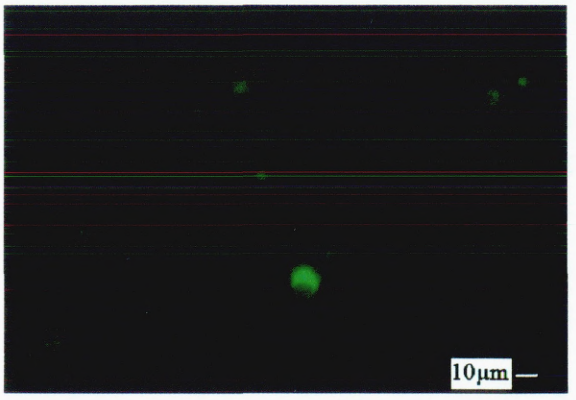


c) Mg acetate preconditioned

Transmitted light



fluorescence



4.3.3 Reproducibility

The data presented herein is data of duplicated experiments (R1 and R2), which showed a close relationship between the two data sets. Differences in the mean cells counted fluorescing were however observed in both ale and lager yeast cells. Further replicates of the experiments would have allowed greater statistical accuracy, thus reducing variations between data sets.

4.4 Discussion

Through the use of Mg fluorescent dyes it was hoped that the visualisation of the intracellular Mg would provide a clearer picture as to where the extra Mg contained in preconditioned ale and lager yeast cells was. This was however not entirely possible due to the limitations in the equipment used to capture images of the fluorescing cells. Variation in the number of fluorescing cells observed using Mag-fura-2 and Mg Green might be due in part to the spectral differences between the two dyes, in that Mag-fura-2 is excited by UV light and Mg content is measured by the spectral difference between excitation (330nm) and emission (370nm). Mg Green however, is excited by visible light and upon binding with magnesium it exhibits an increase in fluorescence intensity without a shift in wavelength at 480nm. This would suggest that the Mg Green data presented here in might be more accurate. Previous studies by Zhang *et al* (1997) showed that the cellular distribution of intracellular Mg was not homogeneous and increased as a consequence of increments in extracellular Mg concentration. Results presented here support this view, as preconditioned ale and lager yeast cells had a greater fluorescence capacity, which, was concomitant with a higher cellular Mg content. The variations in the number of fluorescing cells might also be due to the ale and lager yeast cells not being synchronised; since low Mg in the cell is accompanied by an increase in cell size during which time

tubulin is polymerised and the spindle pole bodies are formed (Walker and Duffus 1980). Once the chromosomes separate an influx of Mg causes the spindle to breakdown whereupon nuclear and cell division ensue (Walker and Duffus 1980). Such changes in the Mg present in the cells could, however, only account for variations in Mg green fluorescence in the 24h data. After 96h the cells would be expected to be in late stationary phase (or G_0 phase) and in some cases may have entered programmed cell death.

The overall low Mg green fluorescence may be due in part to inactivity or denaturation of the esterase enzymes occurring during the 37°C incubation of the cells in the presence of the Mg fluorescent dyes. Intracellular esterases are required to breakdown the ester bonds present in the dye; this then allows the dye to bind to free Mg present in the cell. Differences in the optimal growth temperatures of lager and ale yeasts may also provide an explanation for this, especially since lager yeasts have an optimal growth temperature of below 30°C and ale yeasts have an optimal growth temperature of above 30°C (Boulton and Quain 2001).

Lager yeast cells, which had been preconditioned in the presence of Mg sulphate or Mg acetate, showed a higher cellular Mg content than ale yeast cells. However this was not reflected in the number of cells fluorescing in the presence of Mg Green especially after 96h. This may be due to the Mg in the cell being bound rather than in its free state or suggesting differences in the inorganic nutritional requirements of the two strains. Ale yeast cells preconditioned in the presence of Mg acetate were also observed to have formed spores with 2-3 asci observed. However, it was not determined whether these spores were viable or not. The presence of spores suggests that the ale yeast cells have used the acetate as a carbon source having exhausted the sugars present in the medium. This is significant,

as yeast cells grown on acetate as a carbon source, have been shown to sporulate (Aon and Corassa 1996; McCammon 1996; Paiva *et al.* 1999). As brewing yeast strains are not usually haploid or diploid but are polyploid, and as such possess little or no mating ability and have a poor sporulation capacity, it is doubtful if the spores, which had formed, would have been viable (Stewart and Russell 1998).

The identification of some putative Mg transporter genes (*ALR1, LPE10* and *MRS2*) (Wiesenberger *et al.* 1992; MacDiarmid and Gardner 1998; Bui *et al.* 1999; Gregan *et al.* 2001) would suggest that a high affinity Mg transport mechanism might exist in yeasts. This is especially pertinent since such dual transport mechanisms exist in yeasts for metals such as Zn and Mn (Zhao and Eide 1996a; 1996b; Walker 1998a).

The well-documented genetic differences between ale and lager yeasts may mean that they have different nutritional requirements and growth demands. These genetic differences could also mean that the proteins encoded by the Mg transporter genes may therefore consist of slightly different subunits derived from the genetic information of their respective genomes. Such differences may aid the explanation of the observed differences in cellular Mg content in ale and lager yeast cells.

The use of Mg fluorescent dyes in this study has allowed some visualisation of intracellular Mg in preconditioned yeasts to occur with the results presented in this chapter showing that much of the extra Mg contained in preconditioned ale and lager yeast cells may be contained in the vacuole. This could be further determined using preconditioned yeast cell cultures synchronised with respect to cell cycle. Using enhanced image analysis techniques it may be therefore possible to determine the intracellular compartmentalisation

of Mg, which has previously been achieved in cultures of *S.pombe* (Zhang *et al.* 1997). These authors found that Mg was concentrated along a gradient from the sub-plasma membrane region > cytoplasm > nucleus and that it increased in a heterogeneous manner when the extracellular Mg concentration increased. The data presented here showed a similar effect in that as the extracellular Mg concentration increased cellular Mg increased in a concomitant manner. Mg present in the nucleus could also be visualised with DAPI (4,6-diamidino-2-phenylindole) in conjunction with either of the Mg fluorescent dyes used herein.

Other means of establishing the intracellular localisation of metal ions in yeast cells include cell fractionation, whereby the cells are fractionated into their component parts. Thus allowing the determination of cell membrane bound Mg, mitchondrial Mg and nuclear Mg using AAS analysis. X-ray microprobe analysis could also be used to measure intracellular localised Mg (Walker 1998a). Spectrophotometry with metallochromic dyes such as Eriochrome blue (used to measure mitochondrial Mg concentrations Gregan *et al* 2001) and NMR spectroscopy using ^{28}Mg could also be used to determine intracellular free Mg (Walker 1998a).

CHAPTER 5

FERMENTATIVE BEHAVIOUR OF Mg-PRECONDITIONED YEASTS

5.1 Introduction

As previously discussed in Chapter 1, brewers are striving to attain maximum fermentation efficiency and to ensure a uniform product brand. Previous studies (Walker *et al.* 1996; Walker and Maynard 1997; Walker and Smith 1998; Smith and Walker 2000) have shown a significant correlation between cellular Mg uptake and alcoholic fermentation in industrial strains of *S.cerevisiae.* Rees and Stewart (1997a; 1997b; 1998; 1999) have also shown that supplementing wort with Mg and Zn ions stimulates ethanol production.

The cytosolic enzyme pyruvate decarboxylase, PDC, (EC 4.1.1.1) was first described in yeasts by Newberg and Karczag (1911 cited by Killenberg-Jabs *et al* 1996) is encoded by three structural genes (*PDC1, PDC5* and *PDC6,* (Schmitt and Zimmerman 1982; Hohmann and Cederberg 1990; Hohmann 1991a). The active PDC is a tetramer of 250kDa with four identical subunits and requires the two co-factors thiamine diphosphate and Mg for its activity. Under high glucose conditions the intracellular pyruvate not degraded by pyruvate dehydrogenase is metabolised to produce ethanol by PDC and alcohol dehydrogenase (ADH) (Hohmann and Cederberg 1990; Pronk *et al.* 1994).

The purpose of this chapter was to investigate the influence of Mg-preconditioned ale and lager yeast cells and their fermentation performance in high gravity fermentations. The hypothesis was that elevation of intracellular, as opposed to extracellular, bioavailability of Mg to brewing yeast would stimulate fermentative metabolism and the activity of pyruvate decarboxylase.

5.2 Experimental approach

The effects of Mg-preconditioning on fermentation performance were studied through the setting up of small-scale fermentations using (1L) Imhoff sedimentation cones (with a top diameter of 10cm, bottom diameter with removable cap of 1cm and a total length of 47cm). It was hoped that use of these cones would simulate larger scale cylindro-conical fermenter vessels, in terms of hydro-dynamics, used by the brewing industry. Inoculum preparation, yeast preconditioning and analyses were carried out according to the methods outlined in sections 2.4 and 2.6. 1040°OG wort was used as the preconditioning medium since Cahill *et al* (2000) recommended that the propagation wort be half the gravity of the fermentation wort.

The effects of Mg-preconditioning on the PDC activity in ale and lager yeast cells were studied through the previously described propagation methods (see section 2.4.1). Inoculum preparation, yeast propagation and analyses were carried out according to sections 2.6.

5.3 Results

5.3.1 Fermentation performance of Mg-preconditioned ale yeast

Results in Fig 5.2 show that ale yeast viability in the 24h period after pitching drops to between 30-40% in preconditioned and unpreconditioned ale yeast cells. This low viability was also reflected in the lower suspended cell numbers in the unpreconditioned cells at 24h (Fig 5.1). Viability was, however, higher in preconditioned cells at the end of the fermentations (Fig 5.2). Cell volume analysis showed that mean cell volume increased up to 72h of the fermentations (Fig 5.3). In all cells a decline in cell volume was observed thereafter. A steady increase in suspended cell biomass (Fig 5.4) was observed in R2 un/preconditioned cells up to 72h of fermentation, compared to a more rapid increase in biomass in R1 un/preconditioned cells up to 48h (Fig 5.4). This was concomitant with observed increases in cell number and cell volume (Fig 5.1 and 5.3).

Cell Mg was highest immediately after pitching (Fig 5.5), thereafter release occurred and cell Mg remained steady throughout the course of the fermentations. At the end of the fermentations cell Mg was observed to be highest in Mg sulphate and acetate preconditioned cells (Fig 5.5).

Fig 5.6 shows a steady decline in specific gravity, which was concomitant with an increase in ethanol production (Fig 5.7). After 24h, Mg acetate preconditioned cells showed a faster rate of fermentation, which was reflected in lower final specific gravity values compared with control and Mg sulphate preconditioned cells (Figs 5.6 and 5.7). The rate of fermentation by control cultures and Mg sulphate preconditioned cells was similar. However, Mg sulphate preconditioned cells produced less ethanol compared to Mg acetate and control cells, which did not correlate with an expected lower specific gravity in the case of the latter (Figs 5.6 and 5.7).

Fig 5.1 Growth of *S.cerevisiae* (ale yeast) during fermentation in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.

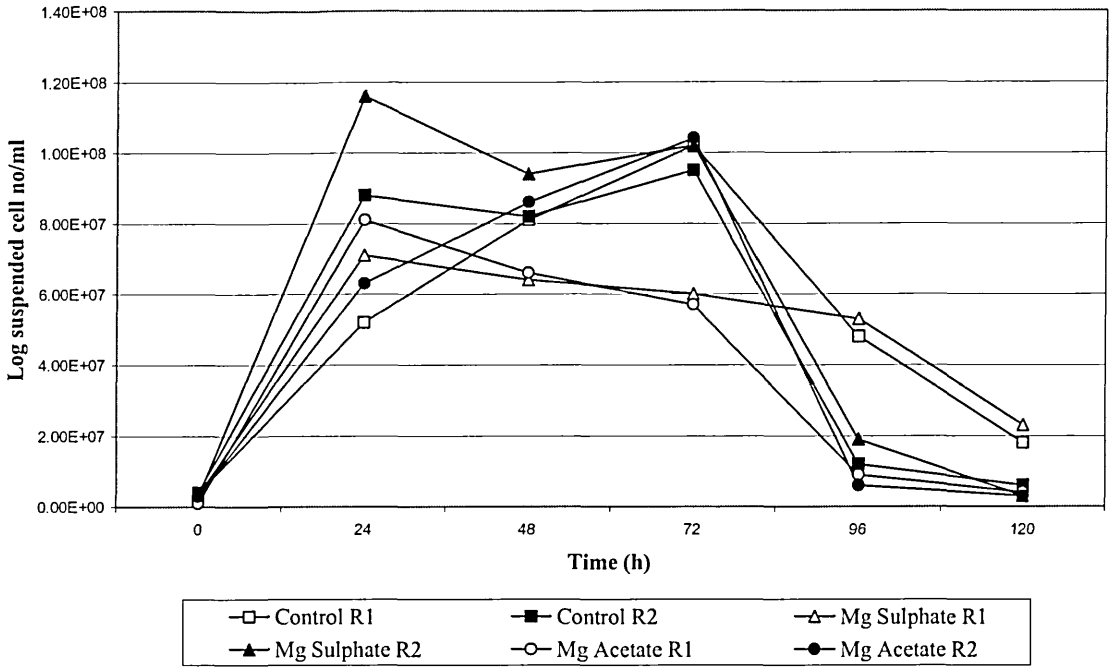


Fig 5.2 Viability of *S.cerevisiae* (ale yeast) during fermentation in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.

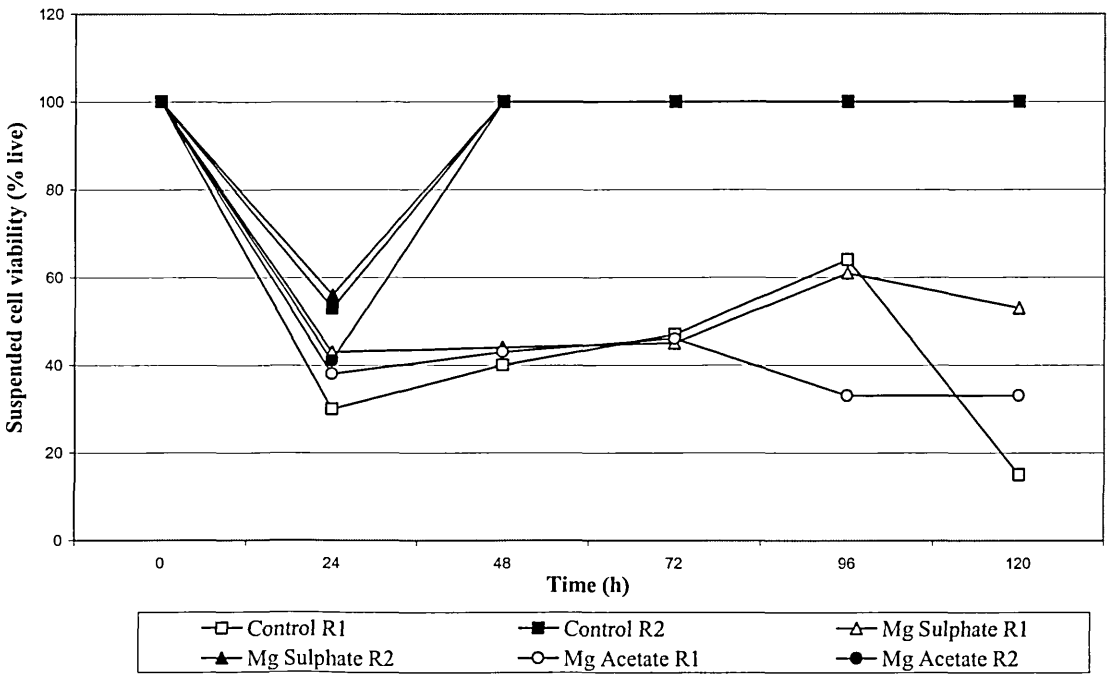


Fig 5.3 Mean cell volume of *S.cerevisiae* (ale yeast) during fermentation in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.

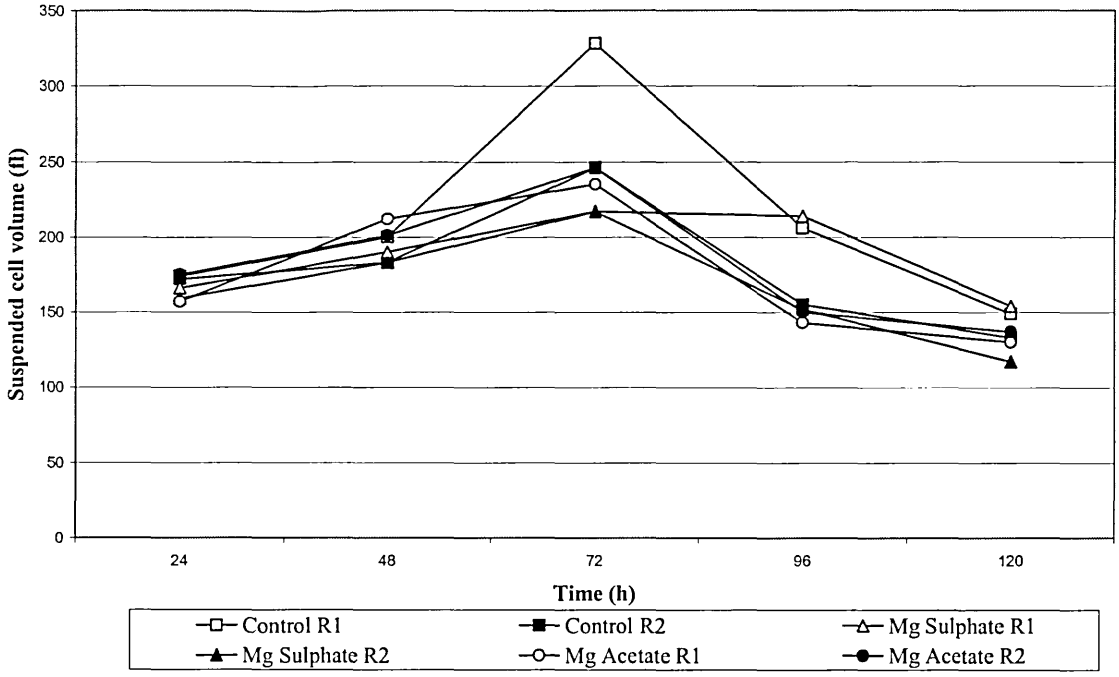


Fig 5.4 Biomass in *S.cerevisiae* (ale yeast) during fermentation in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.

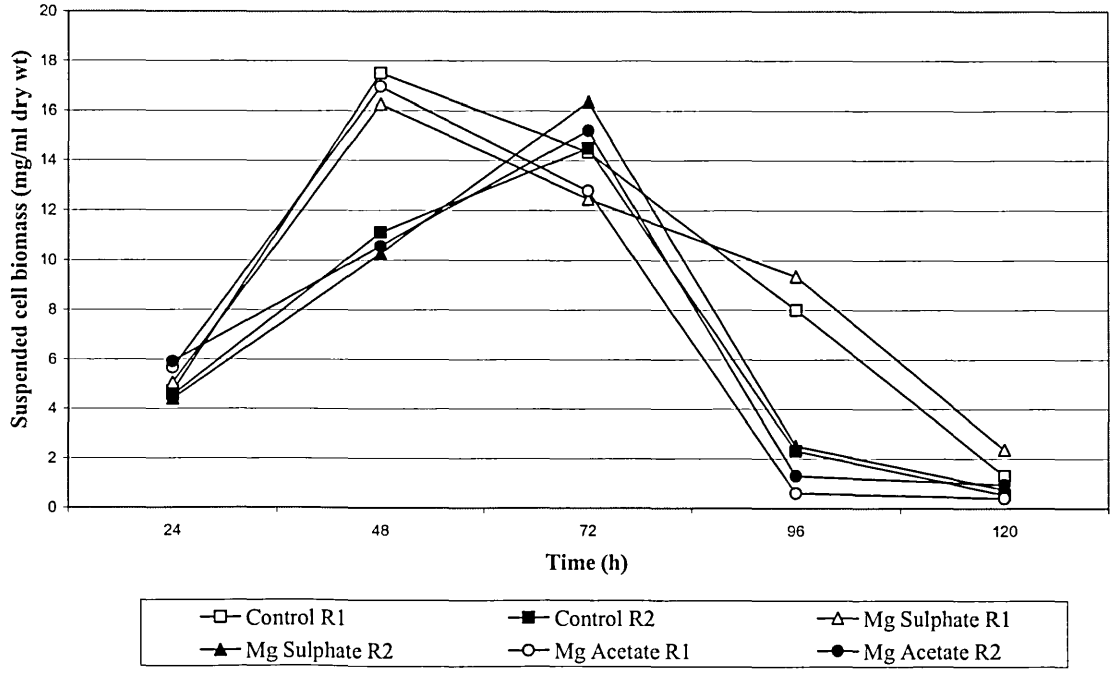


Fig 5.5 Cell Mg in *S.cerevisiae* (ale yeast) during fermentation in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.

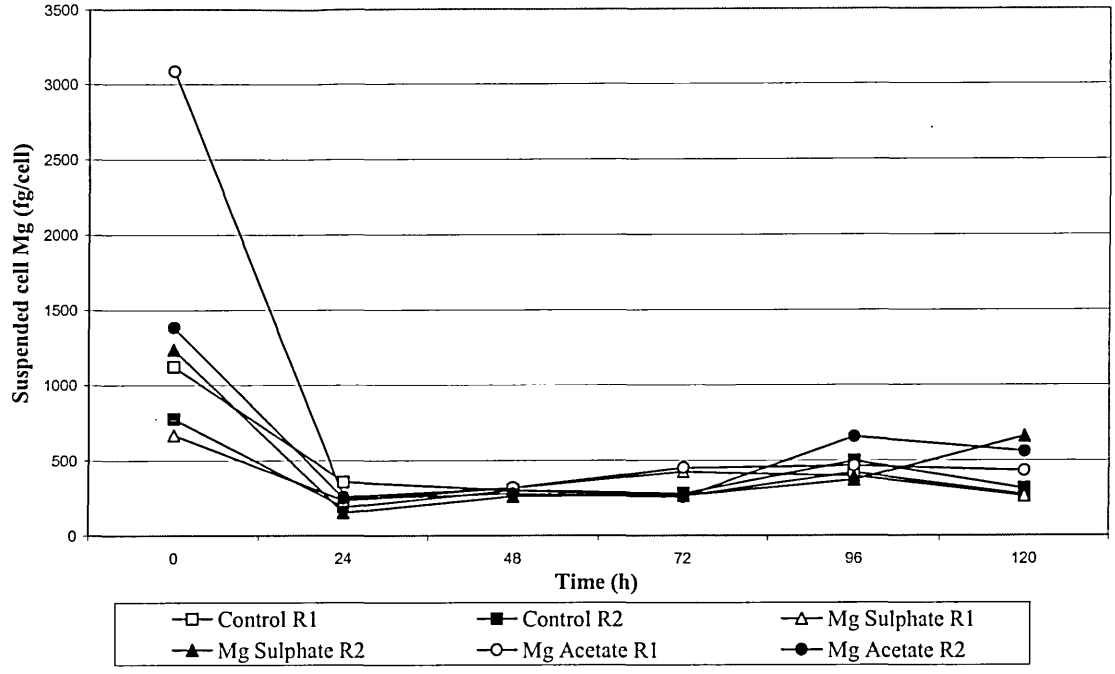


Fig 5.6 Specific Gravity during fermentation of *S.cerevisiae* (ale yeast) in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.

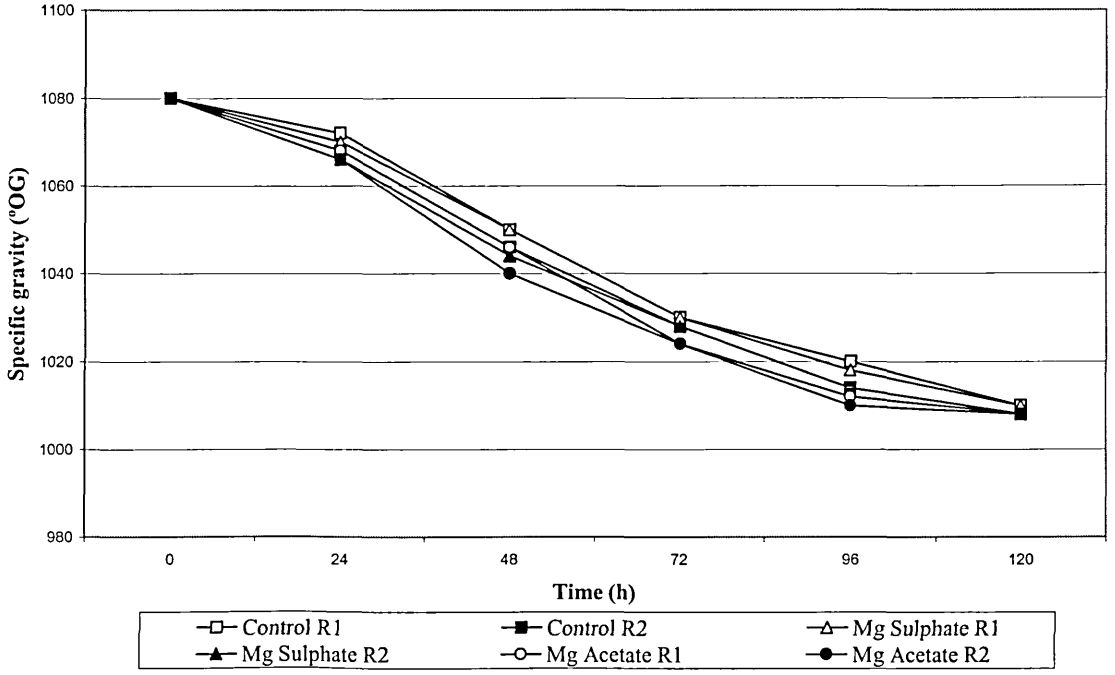
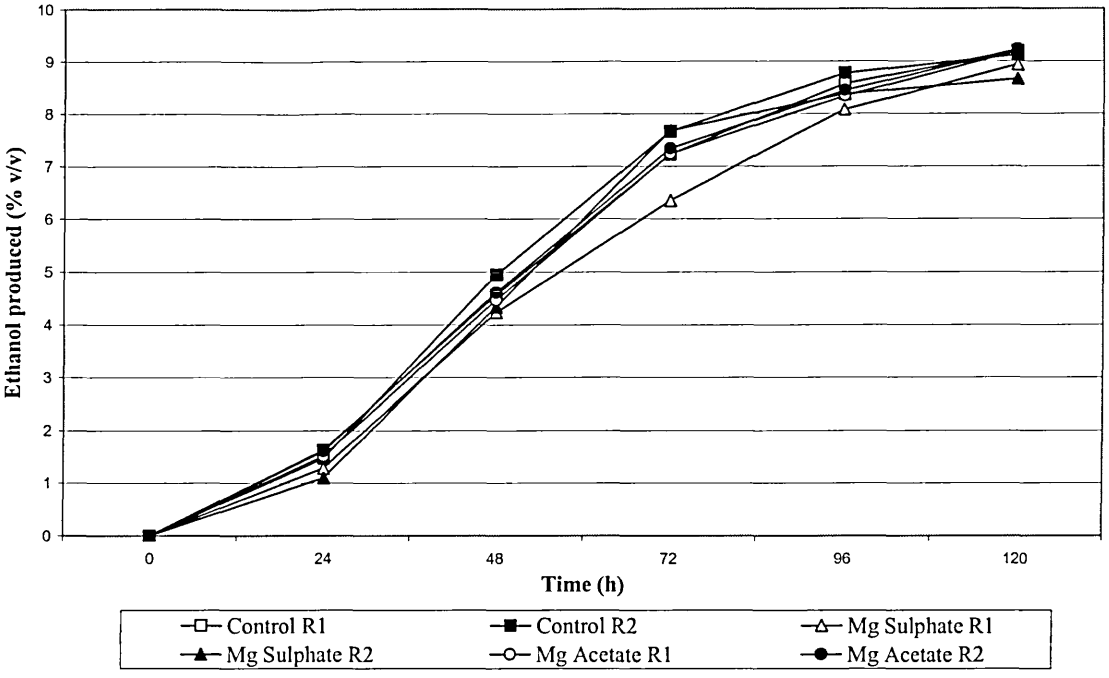


Fig 5.7 Ethanol produced by *S.cerevisiae* (ale yeast) during fermentation in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.



5.3.2 Fermentation performance of Mg-preconditioned lager yeast

The results of the duplicated fermentations show that cell number increases up to 72h of fermentation, with the highest observed in Mg sulphate preconditioned cells, after 120h cell number starts to decline steadily due to flocculation (Fig 5.8). Yeast cell viability was deleteriously affected in un/preconditioned lager yeast cells in the 24h period after pitching (Fig 5.9). The viability of un/preconditioned cells in R1 was lower than that of cells in R2, with Mg acetate preconditioned cells most adversely affected (Fig 5.9). The viability of preconditioned cells recovered after 24h and was higher than the unpreconditioned cells at the end of the fermentations (Fig 5.9). Mean cell volume data shows an increase in cell volume up to 120h where upon a maximum was reached (Fig 5.10), after 144h a steady decline occurred, which was concomitant with a decline in cell numbers (Figs 5.8 and 5.10). A steady increase in biomass was observed, with Mg acetate preconditioned cells showing consistently higher biomass over the course of the fermentation up to 192h (Fig 5.11). As the fermentations continued past 120h biomass started to fall concomitant with cell number and cell volume (Figs 5.8, 5.10 and 5.11).

Cell Mg was highest immediately after pitching (Fig 5.12). Thereafter, an Mg uptake and release pattern occurred during the course of the fermentations, with Mg acetate preconditioned cells having highest cell Mg at the end of the fermentations (see Fig 5.12).

Results show steady decline in specific gravity concomitant with an increase in ethanol produced (Figs 5.13 and 5.14). After 96h Mg acetate preconditioned cells showed a faster rate of fermentation than cells preconditioned in Mg sulphate and control cells and ultimately produce more ethanol (Fig 5.14).

Fig 5.10 Mean cell volume of *S.cerevisiae* var. *carlsbergensis* (lager yeast) during fermentation in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.

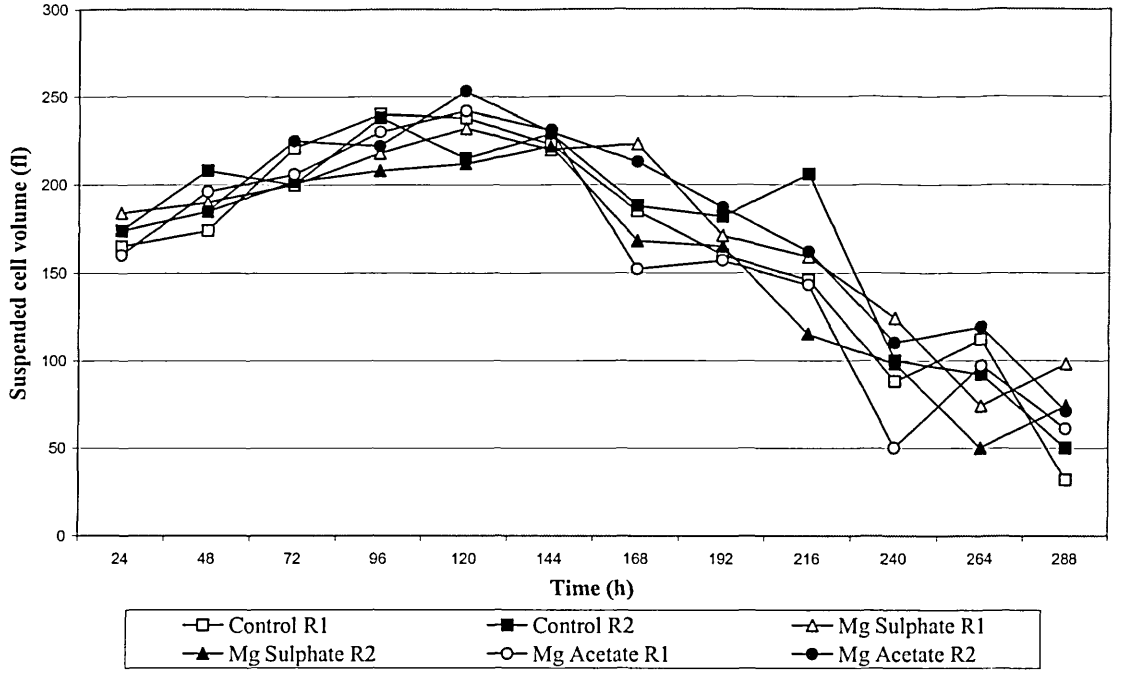


Fig 5.11 Biomass in of *S.cerevisiae* var. *carlsbergensis* (lager yeast) during fermentation in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.

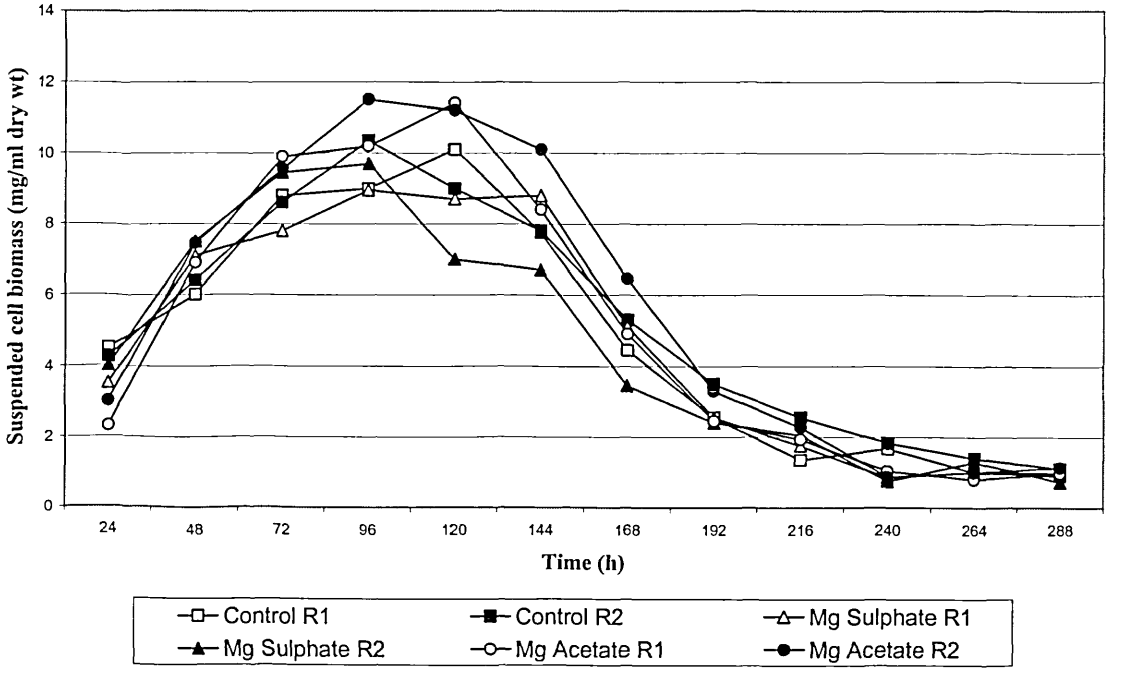


Fig 5.8 Growth of *S.cerevisiae* var. *carlsbergensis* (lager yeast) during fermentation in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.

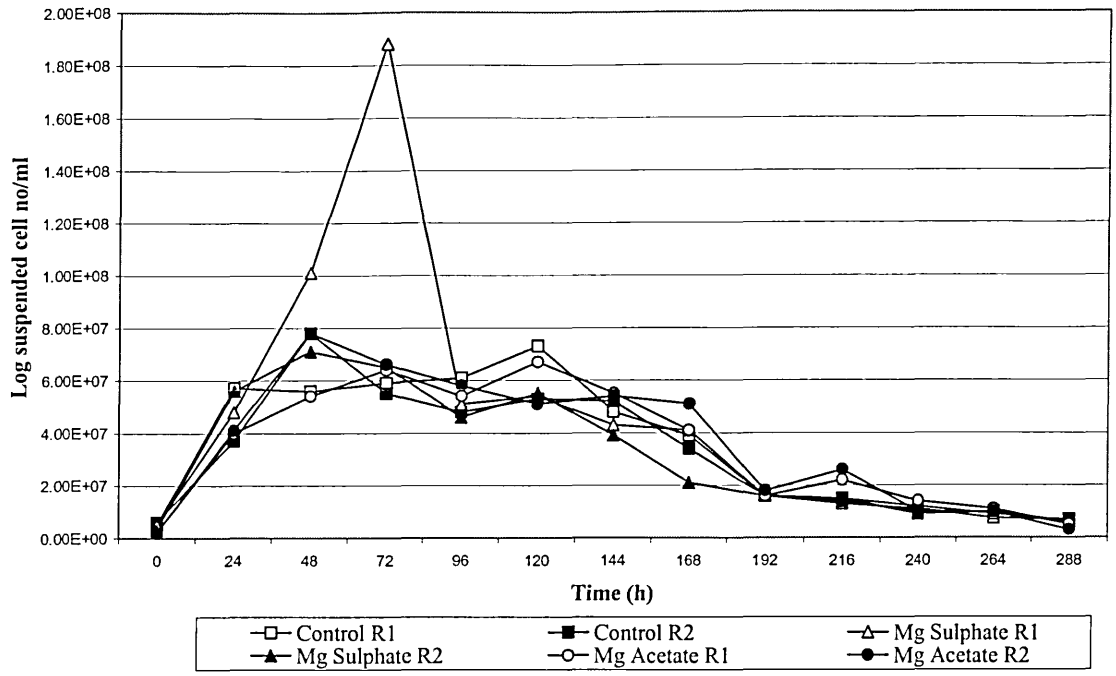


Fig 5.9 Viability of *S.cerevisiae* var. *carlsbergensis* (lager yeast) during fermentation in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.

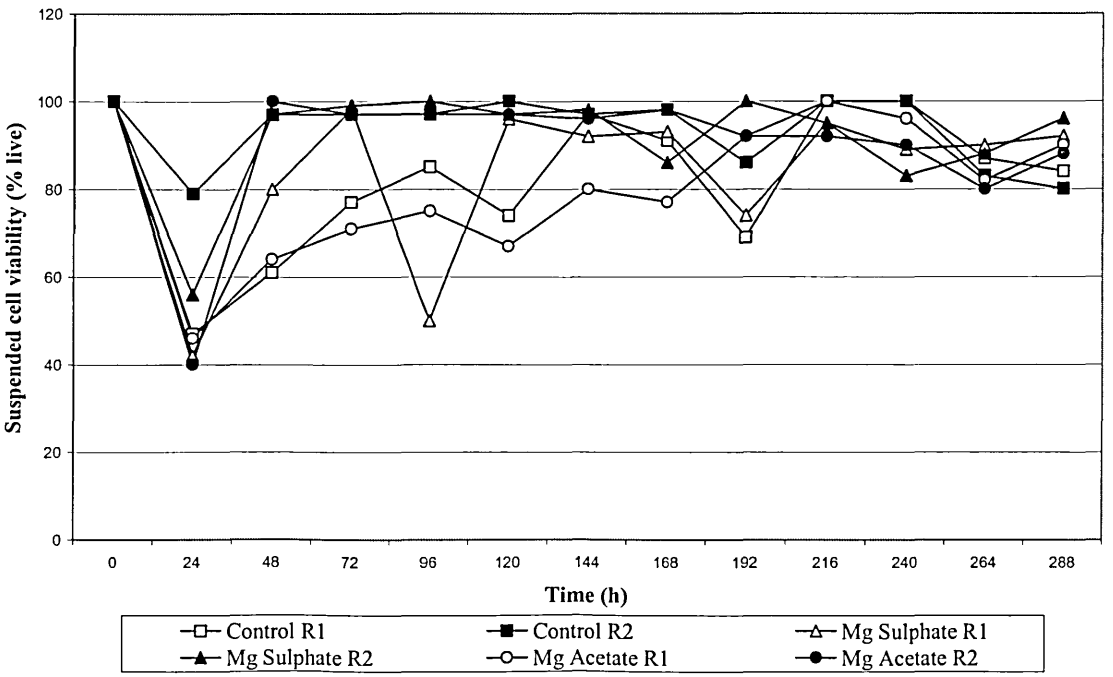


Fig 5.12 Cell Mg in of *S.cerevisiae* var. *carlsbergensis* (lager yeast) during fermentation in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.

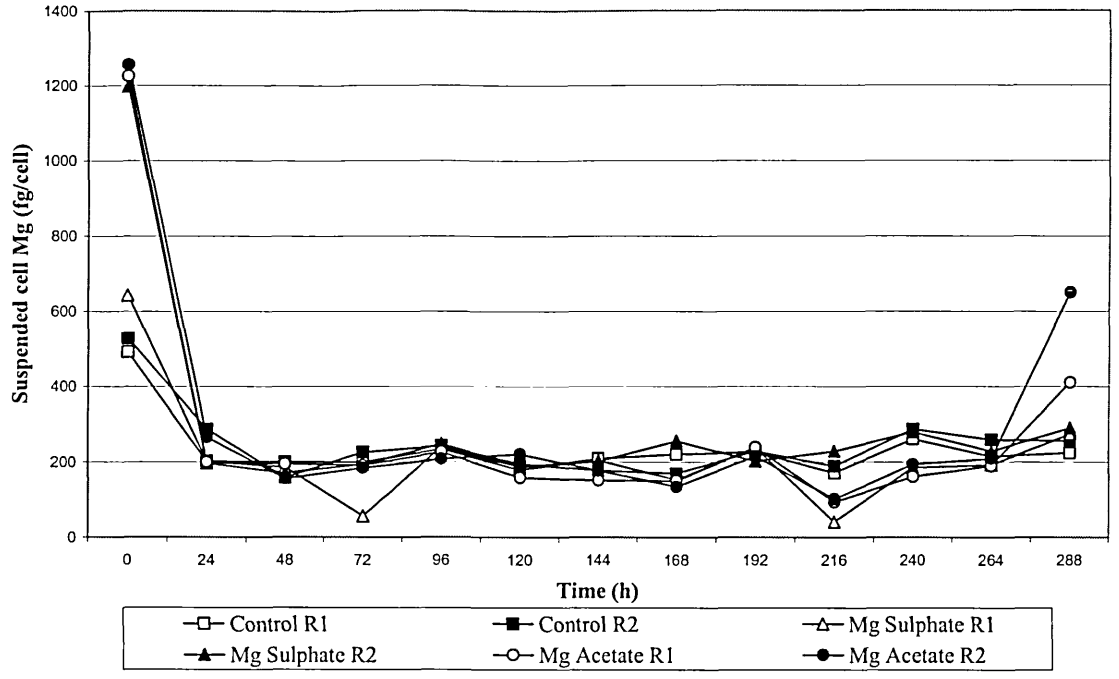


Fig 5.13 Specific Gravity during fermentation of *S.cerevisiae* var. *carlsbergensis* (lager yeast) in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.

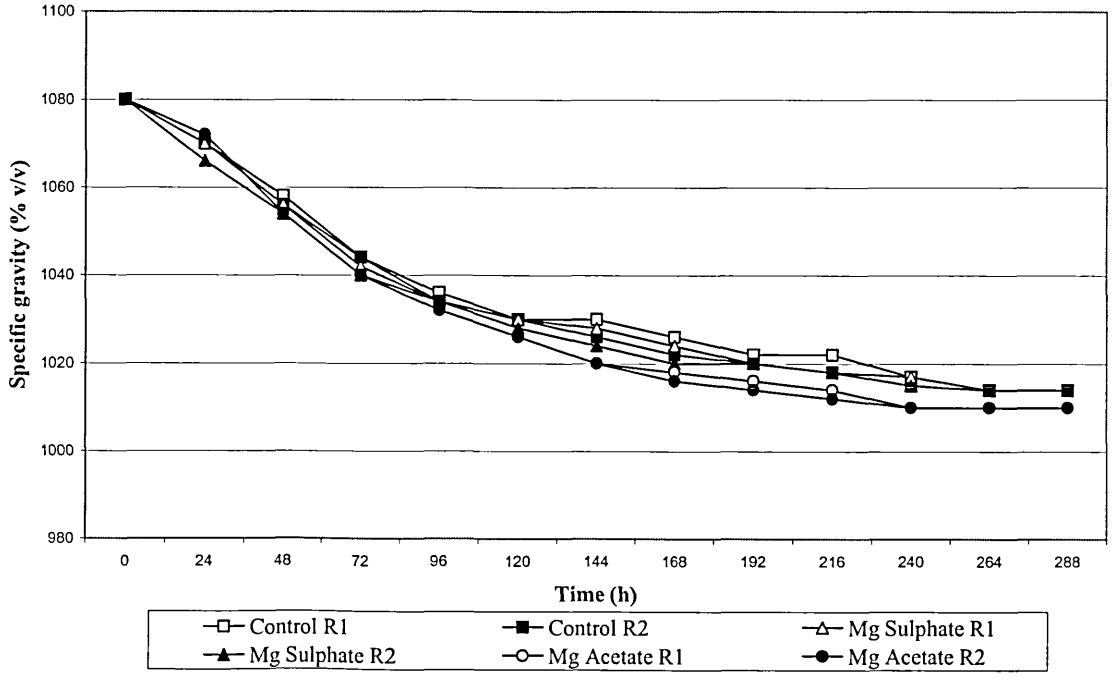
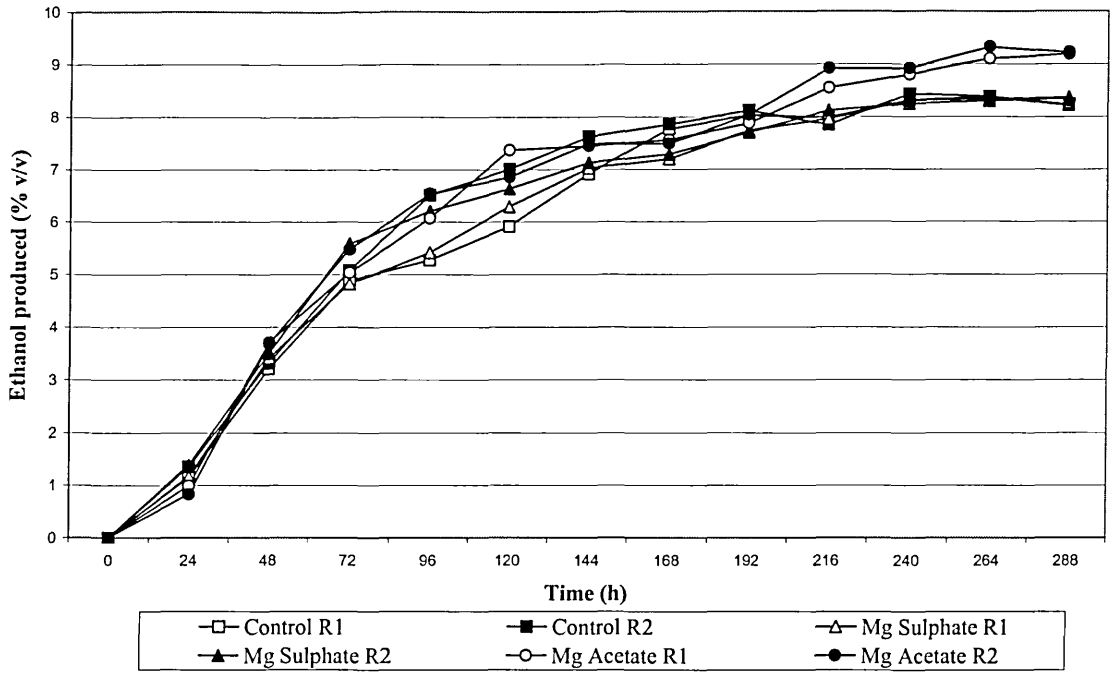


Fig 5.14 Ethanol produced by *S.cerevisiae* var. *carlsbergensis* (lager yeast) during fermentation in 1080°OG wort. Cells were aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 2.5mM and 100mM respectively.



5.3.3 Pyruvate Decarboxylase activity and Mg-preconditioned ale yeast

The results (Table 5.1) show the control and Mg sulphate preconditioned cells having a higher biomass than the Mg acetate preconditioned cells. Mg preconditioned cells had a higher cell Mg concentration than the unpreconditioned cells, in all cases the cell Mg increased after 48h of growth which was concomitant with an increase in biomass (Table 5.1).

Protein concentrations were lower in preconditioned cells compared to unpreconditioned cells (Table 5.2). Total and specific activities of PDC were highest in Mg acetate preconditioned cells, with unpreconditioned cells having the lowest activities. However, PDC activities fell after 48h in both the preconditioned and unpreconditioned cells (Table 5.2). Fig 5.15 shows that a close relationship exists between cellular Mg and specific PDC activity in the preconditioned ale yeast cells.

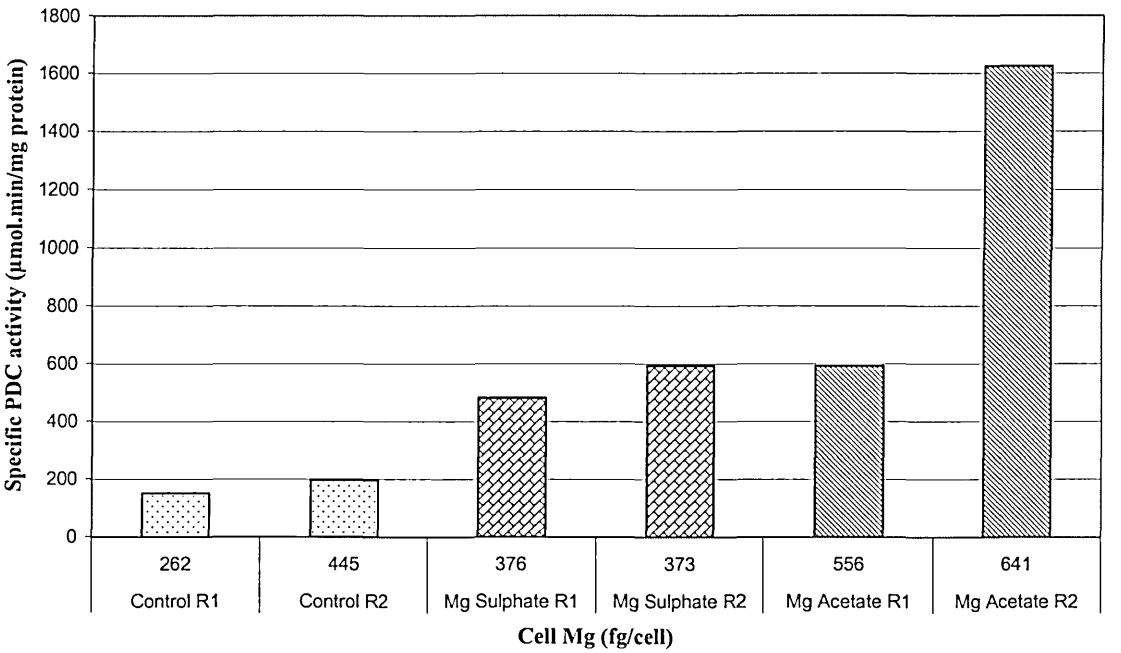
Table 5.1 Cell Mg (fg/cell) and biomass (mg/ml dry wt) in *S.cerevisiae* (ale yeast) aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 1.7mM and 100mM respectively.

Mg Salt	Cell Mg		Biomass (mg/ml dry wt)	
	24h	48h	24h	48h
Control 1	262	304	6.65	6.05
Control 2	445	514	5.40	6.40
Mg Sulphate 1	376	400	6.45	7.65
Mg Sulphate 2	373	542	5.55	6.75
Mg Acetate 1	556	703	4.70	5.25
Mg Acetate 2	641	579	3.80	4.60

Table 5.2 PDC activity in *S.cerevisiae* (ale yeast) aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 1.7mM and 100mM respectively.

Mg Salt	Protein conc. (µg/ml)		Total activity (µmol/min^{-1})		Specific activity (µmol/min/mg protein)	
	24h	48h	24h	48h	24h	48h
Control 1	49	44	7.39	2.41	151	55
Control 2	55	47	10.85	6.43	197	137
Mg Sulphate 1	20	13	9.65	1.23	482	95
Mg Sulphate 2	23	35	13.66	4.39	593	125
Mg Acetate 1	38	26	22.51	18.48	592	711
Mg Acetate 2	37	41	60.13	24.27	1625	591

Fig 5.15 Relationship between PDC activity and cell Mg in *S.cerevisiae* (ale yeast) aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 1.7mM and 100mM respectively.



5.3.4 Pyruvate Decarboxylase activity and Mg-preconditioned lager yeast

Table 5.3 shows that control and Mg sulphate preconditioned cells had a higher biomass than Mg acetate preconditioned cells, both the control and Mg sulphate preconditioned cells showed an increase in biomass after 48h, with the Mg acetate cells losing some biomass. Mg preconditioned cells had a higher cellular Mg concentration than unpreconditioned cells, with Mg acetate and to a lesser extent Mg sulphate preconditioned cells showing an increase in cell Mg after 48h (Table 5.3).

Protein concentrations were lower in preconditioned cells compared to unpreconditioned cells (Table 5.4). Total and specific activities are highest in Mg acetate preconditioned cells, with unpreconditioned cells having the lowest activities after 24h (Table 5.4). However, PDC activities fell after 48h in both the preconditioned unpreconditioned cells (Table 5.4). Fig 5.16 shows that a close relationship exists between cellular Mg and specific PDC activity in the preconditioned lager yeast cells.

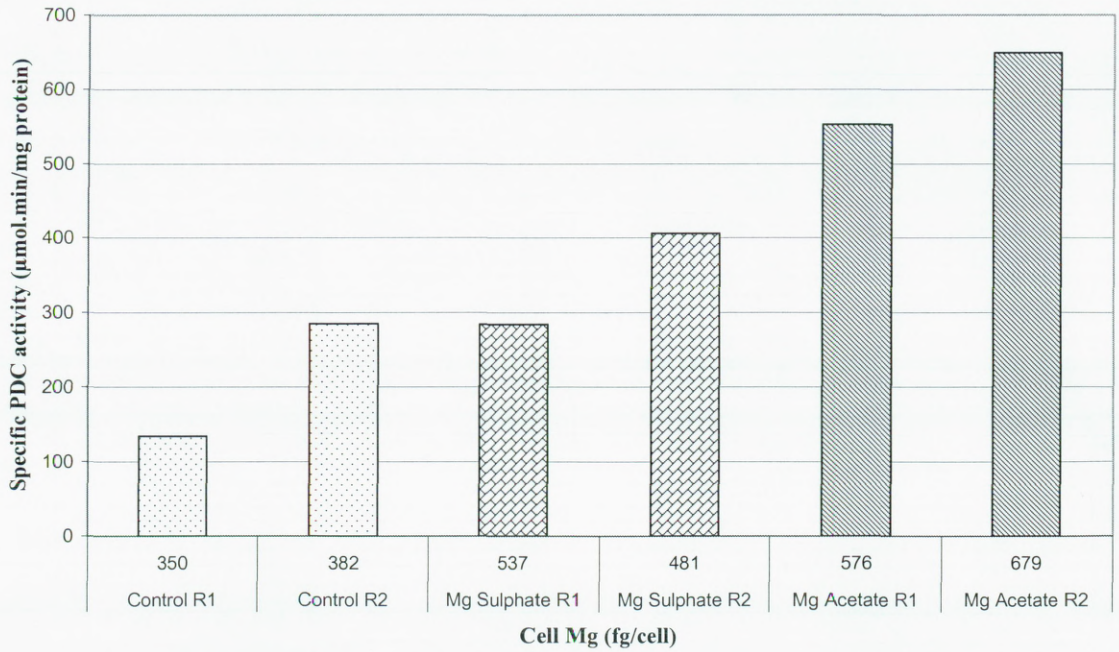
Table 5.3 Cell Mg (fg/cell) and biomass (mg/ml dry wt) in *S.cerevisiae* var. *carlsbergensis* (lager yeast) aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 1.7mM and 100mM respectively.

Mg Salt	Cell Mg		Biomass (mg/ml dry wt)	
	24h	48h	24h	48h
Control 1	350	333	4.40	5.85
Control 2	382	375	4.00	5.10
Mg Sulphate 1	537	421	4.85	6.50
Mg Sulphate 2	481	608	5.05	6.50
Mg Acetate 1	576	658	4.30	2.32
Mg Acetate 2	679	877	3.60	4.20

Table 5.4 PDC activities in *S.cerevisiae* var. *carlsbergensis* (lager yeast) aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 1.7mM and 100mM respectively.

Mg Salt	Protein conc. (µg/ml)		Total activity (µmol/min^{-1})		Specific activity (µmol/min/mg protein)	
	24h	48h	24h	48h	24h	48h
Control 1	43	13	5.78	1.67	134	128
Control 2	42	23	12.00	6.10	285	265
Mg Sulphate 1	43	14	12.22	3.69	284	263
Mg Sulphate 2	34	48	12.80	9.65	406	201
Mg Acetate 1	80	34	44.21	19.29	553	567
Mg Acetate 2	58	104	37.62	40.35	649	388

Fig 5.16 Relationship between PDC activity and cell Mg in *S.cerevisiae* var. *carlsbergensis* (lager yeast) aerobically preconditioned in wort (1040°OG) without (control) or with Mg acetate or Mg sulphate supplementation. Mg levels in the propagation wort were 1.7mM and 100mM respectively.



5.3.5 Reproducibility

The data presented herein is data of duplicated experiments (R1 and R2), which showed a close relationship between the two data sets. Differences were observed however in data presented for ale and lager yeast cell number and viability (Figs 5.1, 5.2, 5.8 and 5.9). The very high cell number observed in Mg sulphate R1 at 72h could have been due to a high cell concentration in the sample taken at that particular time point, an additional cell count or sample may have determined whether this anomaly was a true indication of the cell number. The higher specific PDC activities observed in R2 samples of ale and lager yeast cells could have been due to greater efficiency in the homogenisation procedure. Further replicates of the experiments would have allowed greater statistical accuracy, thus reducing variations between data sets even further.

5.4 Discussion

Previous authors have shown that yeast cells which had not been preconditioned took up Mg from their surrounding environment soon after they were pitched into either defined growth medium or brewer's wort (Walker and Smith 1998; Smith and Walker 2000). During the 24h period after pitching there appears to be a release in cell Mg by non-and preconditioned ale and lager yeast cells. Thereafter, these yeast cells demonstrated patterns of Mg uptake and release over the course of the fermentations, a phenomenon also observed by Mochaba *et al* (1996) . This release of Mg after pitching has been associated with changes in the permeability of the yeast cell membrane in preparation for sugar uptake (Mochaba *et al.* 1996). Uptake and release is not solely restricted to Mg, since Mochaba *et al* (1996) observed that zinc is also released upon pitching followed by a gradual reabsorption.

It was hoped that by Mg-preconditioning yeasts during propagation, the need for the uptake of Mg shown by previous authors (Walker and Smith 1998; Smith and Walker 2000) during the early stages of fermentation (Mochaba *et al.* 1996) may have been reduced, thus allowing much of the Mg accumulated within the cell to be utilised for the transport and metabolism of sugars for alcohol production, rather than for growth and reproduction. Basal levels of cell Mg may therefore be retained for cell cycle progression (Walker and Duffus 1980). The use of Mg-preconditioned yeast for brewing would also circumvent the need to add Mg salts to wort, which is prohibited in some countries (e.g. Rheinheigstbot beer purity laws in Germany).

Release of Mg has also been suggested as an indicator of yeast vitality (Mochaba *et al.* 1996). These authors found that Mg excreted into wort after pitching (which was subsequently reabsorbed during the initial period of active fermentation and yeast growth) occurred only when yeast quality was good. During a subsequent fermentation these authors found that yeast of a poorer quality did not excrete Mg into wort after pitching, which the authors suggested was indicative of the cell membranes not undergoing changes in permeability in preparation for sugar uptake. The results presented here would therefore suggest that both the preconditioned ale and lager yeast cells may have had a higher vitality than those which had been unpreconditioned, as they appear to have released more Mg. However, this was not borne out by the viability results in the early stages of fermentation, especially those of the ale yeast cells (both preconditioned and non preconditioned), which, although having lower viabilities, appeared to have a shorter lag phase than the lager yeast cells and ultimately had a shorter attenuation time. The poor viabilities in both the ale and lager fermentations could be attributed to a combination of an osmotic shock and a low pitching rate. The latter could be quite significant since Casey

and Ingledew (1983) observed that low pitching rates in high gravity wort fermentations (1080-1092°OG) resulted in the early death of a significant portion of the yeast population. This in turn reduced the amount of yeast able to utilize oxygen in the wort for lipid production, limiting the potential for cell mass synthesis, thus lowering the rate of fermentation (Casey and Ingledew 1983). Suihko *et al* (1993) have supported these findings, and suggested an optimal pitching rate of $5.8x10^7$ counted cells/ml (8g/L wet wt) for a 1080°OG fermentation.

In terms of fermentation performance, both the Mg acetate preconditioned ale and lager yeasts showed faster rates of fermentation, this was observed in the faster specific gravity reductions over the period of the fermentations. Mg acetate preconditioned lager yeast cells produced the greatest yields of alcohol (9.2 and 9.3%). This represented a 1.0 and 1.1% increase in yield when compared to the unpreconditioned lager yeast cells (8.21 and 8.23% respectively). In the case of the ale yeast fermentations there was little or no difference in the alcohol yielded by the unpreconditioned and Mg acetate preconditioned cells. These results, therefore, partially concur with previous results (Walker *et al.* 1996; Walker and Smith 1998; Smith and Walker 2000) in normal gravity, high gravity and defined media fermentations using Mg-preconditioned yeasts.

Mochaba *et al* (1996) observed that yeast repitched into wort for a second fermentation had a reduced vitality. This is a common phenomenon in breweries, especially following high gravity wort fermentation. It is therefore hoped that preconditioned cells will retain their cell Mg and thus have an enhanced vitality upon repitching. Especially since Mochaba *et al* (1996) have suggested a link between increased wort Mg and yeast vitality upon repitching. Should this theory be correct the results

presented in Figs 5.5 and 5.12 would suggest that Mg-acetate preconditioned ale and lager yeast cells may have had a higher vitality at the end of their respective fermentations.

Pyruvate decarboxylase is a key fermentative enzyme, which requires Mg as a co-factor. Mg preconditioning may help to achieve this by providing an enhanced intracellular pool of Mg for the activity of this enzyme. A number of studies have investigated PDC activity in *S.cerevisiae* and *C.utilis* in terms of regulation of specific activities of the enzyme (Van Urk *et al.* 1989; Sims *et al.* 1991; Derrick and Large 1993; Pronk *et al.* 1994; Flikweert *et al.* 1996). The results presented here attempted to quantify the effect additional Mg in preconditioned yeasts had on PDC activity. Both preconditioned ale and lager yeast cells had higher PDC activities than unpreconditioned yeast cells after 24h propagation in 1040°OG wort. Higher PDC activities in the preconditioned yeast cells would suggest that the much of the extra Mg taken up by the cells was being utilised as the cofactor for enzymes such as PDC instead of for growth and cell proliferation. This could especially be true in Mg acetate preconditioned cells. Results also showed that after 48h propagation, both preconditioned and non-preconditioned ale and lager yeast cells had reduced PDC activities, this reduction being more pronounced in preconditioned yeast cells. This observed reduction in PDC activity after 48h suggests that PDC activity is tightly regulated and the cells were respiring rather than fermenting, with more of the pyruvate being converted to acetyl-Co A and fed into the TCA cycle to provide energy for cell growth, as seen by the increase in biomass after 24h (Table 5.1 and 5.3). Cell Mg in Mg sulphate preconditioned lager yeast cells would appear not to enhance the PDC activity after 24h. This could be due to the yeast cells absorbing a constant amount of the additional Mg available in the wort, rather than in proportion to its availability (Saltukoglu and Slaughter 1983). Walker and Maynard (1997) have shown

that high concentrations of Mg had no effect on ethanol productivity at a cellular level. The additional Mg supplied by Mg sulphate may be being utilised in cell growth, as biomass results for cells preconditioned in this Mg salt were higher than that of Mg acetate preconditioned and non-preconditioned cells. However there appeared to be a correlation between cell Mg and PDC specific activity. This was especially evident in Mg acetate preconditioned yeast cells (Figs 3.15 and 3.16). These results suggest that a relationship exists between the particular Mg salt and its uptake mechanism. This is seen in the lower biomass yielded by Mg acetate preconditioned cells compared to that of the non-and Mg sulphate preconditioned cells. Protein results in ale yeast cells (Table 5.2) were lower in preconditioned cells compared to that of unpreconditioned cells and also lower than that of preconditioned lager yeast cells. This could be due to there being a lower protein content in the preconditioned ale yeast cells. This does not, however, correlate with the preconditioned ale yeast cells having higher PDC activities than the preconditioned lager yeast cells. The additional Mg present in the preconditioned ale yeast cells could have suppressed protein biosynthesis instead of aiding it thus lowering the total protein concentrations in these cells. Such anomalies in protein content could also be due to the method used to analyse cell protein (see section 2.6.7) in that protein was measured in whole cell pellets obtained at the same time as samples were removed for PDC analysis. For the results to be potentially more accurate and therefore more meaningful, protein analysis of the homogenate used for PDC activity should have been carried out.

A number of studies based on kinetic modelling of the glycolytic pathway have been carried out into increasing metabolic flux (Smits *et al.* 2000). These studies have suggested that flux control is at the point of sugar uptake, at hexokinase, phosphofructokinase or at pyruvate kinase (Galazzo and Bailey 1990; Cortassa and Aon

1995). Glycolytic enzymes including those previously mentioned have been over-expressed individually and in combinations without increasing glycolytic flux (Schaaff *et al.* 1989) which points to a very tight control of the individual glycolytic enzymes (Smits *et al.* 2000). The results of Schaaff *et al* (1989) led these workers to conclude, "Overproduction of different glycolytic enzymes had no effect on the rate of ethanol production". Recombinant strains of *S.cerevisiae* have been created to express the lower glycolytic enzymes (Smits *et al.* 2000). This resulted in phosphoglyceromutase having the greatest increase in activity and PDC with the least increase in activity (Smits *et al.* 2000). This led Smits *et al* (2000) to surmise that an increase in glycolytic capacity by the over production of enzymes may only lead to a flux increase when the cell needs the extra capacity to produce additional ATP. In terms of PDC activity it therefore remains unlikely that the results presented in Tables 5.2 and 5.4 would be translated into a massive increase in ethanol output during fermentation, especially since Smits *et al* (2000) have implied that for a substantial enhancement of the glycolytic flux to occur all the enzymes would need to be simultaneously over expressed. This was thought to be unlikely however due to the excessive protein burden placed upon the cell (Smits *et al.* 2000). As previously mentioned, the active PDC is a tetramer of 250kDa with four identical subunits and requires the two co-factors thiamine diphosphate and Mg for its activity. Both of these co-factors bind very tightly but noncovalently to the enzyme (Hohmann 1997). Pyruvate binds covalently to thiamine diphosphate and becomes decarboxylated (Hohmann 1997). After decarboxylation of the bound pyruvate, a α-carbonion is formed as an intermediate, which is followed by the addition of a proton with the subsequent release of acetaldehyde (Hohmann 1997). The extra Mg available in preconditioned cells may therefore aid in the function of the PDC active site. The following scheme (Fig 5.17) summarises the PDC

reaction and Fig 5.18 elaborates on the catalysis of thiamine diphosphate, which is, along with Mg, an important co-factor for the activity of this important enzyme.

Fig 5.17 Summary of PDC reaction (pyruvate-ethanol adapted from Voet *et al*, 1999).

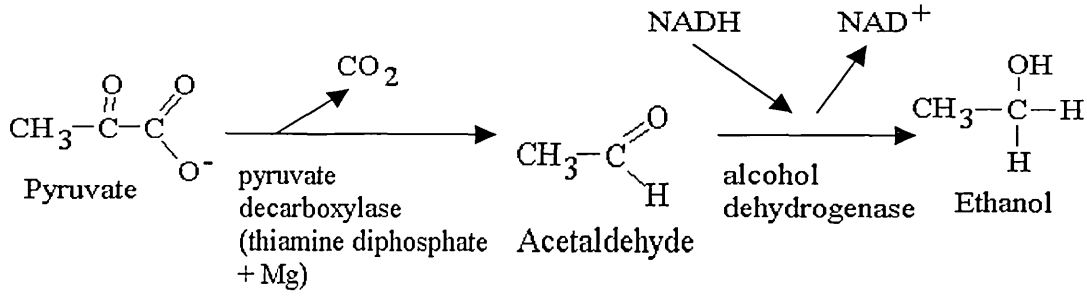
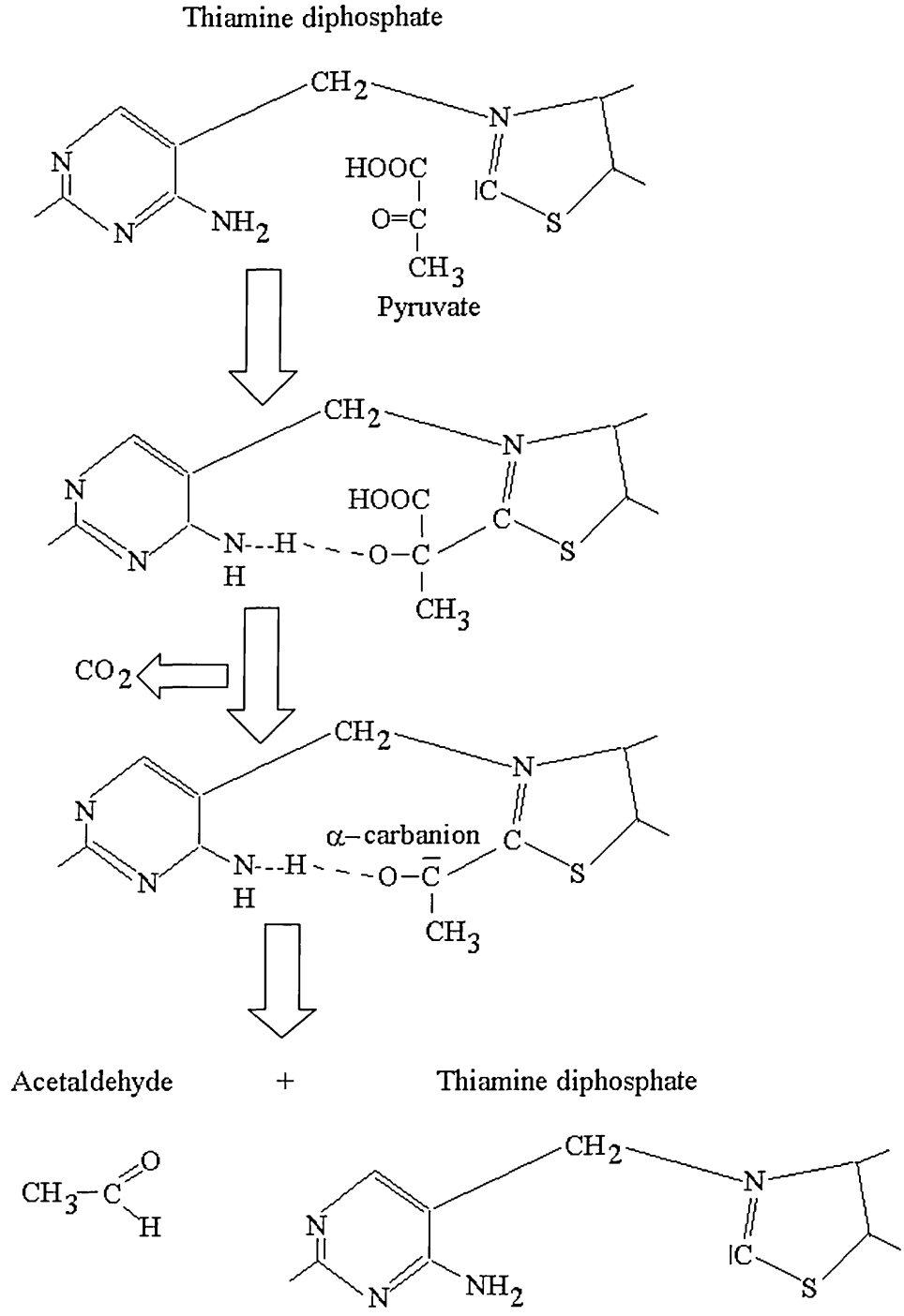


Fig 5.18 Thiamine diphosphate catalysis (adapted from Hohmann 1997)



The genetic differences between ale and lager yeast strains may also account for differences in the PDC activities presented herein for ale and lager yeast cells (Tables 5.2 and 5.4). Especially since southern blot analysis has shown an extensive restriction

fragment length polymorphism at the PDC structural gene loci (Hohmann 1997). The PDC enzyme in ale and lager yeasts may therefore consist of slightly different subunits derived from the genetic information of their respective genomes (Hohmann 1997).

Ultimately the activity of any of these enzymes will depend on the energy requirements of the cell. Further work studying the enzymes further up the glycolytic pathway with regard to Mg preconditioned yeasts would need to be carried out to determine whether the extra Mg is stimulating these enzymes too. These results could be translated into a further study to look at the possibility that Zn preconditioned yeasts may also be more fermentatively active on the basis of zinc stimulation of the terminal fermentative enzyme alcohol dehydrogenase, which, requires zinc for its structure (Walker 1998a). In this context Hall (2001) found that YPDM (Yeast Propagation Defined Medium) supplemented with increasing concentrations of zinc stimulated ADH activity, which was concomitant with enhanced ethanol output.

Chapter 6

STRESS TOLERANCE OF Mg-PRECONDITIONED YEASTS

6.1 Introduction

As previously discussed in Chapter 1, yeasts encounter a wide variety of fluctuations in their environment; to cope with these changes an adaptation to their metabolism occurs. Such metabolic adaptations include the accumulation of the reserve carbohydrates trehalose and glycogen, and the induction of heat shock proteins. When yeast cells are exposed to a mild stress, this results in tolerance to exposure to a higher (normally lethal) dose of the same stress and also to other stresses (Estruch 2000). Heat shock or chemical agents such as ethanol, heavy metals, sodium arsenate, amino acid analogues and sodium azide have all been shown to induce tolerance to stresses in yeasts (Parsell and Lindquist 1993; Rikhvanov *et al.* 2001). The ability of *S.cerevisiae* to tolerate high concentrations of ethanol and elevated temperatures are two very desirable characteristics of a brewing yeast strain (Odumeru *et al.* 1993).

Brewing and baking yeast strains of *S.cerevisiae* have been shown to be less ethanol tolerant than distilling and sake strains, this is reflected in the higher ethanol yields obtained at the end of fermentation (D'Amore *et al.* 1990). Preconditioning wine and distilling yeasts in the presence of Mg ions have been shown to enhance tolerance to ethanol (Chandrasena 1996; Birch 1997). Elevating intracellular Mg appears to reduce cell mortality, preventing cell surface damage and repress stress protein biosynthesis (Birch and Walker 2000). Following on from Chapter 5, which indicated improvements in fermentation performance of Mg preconditioned brewing yeasts; the purpose of the present Chapter was to investigate if Mg-preconditioned ale and lager yeast cells exhibited enhanced tolerance to ethanol and heat stresses.

6.2 Experimental approach

An attempt was made to determine whether or not Mg-preconditioned ale and lager yeast cells had an increased tolerance to ethanol and temperature stresses. The ethanol concentrations chosen were 10% and 20% because these represented ethanol concentrations that brewers yeasts are likely to encounter in a fermentation environment with 20% representing an above maximum ethanol concentration, which would be likely to induce a lethal shock, the temperatures chosen were 4°C, 25°C and 40°C because these too represented a range of temperatures likely to be encountered by yeast cells especially with regards cold shock and a lethal heat shock. Inocula and media preparation was detailed in sections 2.1 and 2.5 with samples removed on a regular basis for cell number/viability and Mg analysis as per sections 2.6.

6.3 Results

6.3.1 Effect of yeast Mg-preconditioning on ethanol stress

Ale yeast cells

The viability of unpreconditioned ale yeast cells was greatly affected by exposure to increased ethanol concentrations (Table 6.1), with low viabilities being observed across the ethanol concentration ranges in non-preconditioned yeast cells, after only 3h. However, some recovery thereafter was observed. With 20% ethanol deleteriously affecting yeast cell viability over time (Table 6.1). Cell Mg in unpreconditioned ale yeast cells remained largely unaffected by exposure to increased concentrations of ethanol (Figs 6.1-6.3) with very little Mg measured in the culture supernatants over the range of the ethanol concentrations (Table 6.4).

Ale yeasts preconditioned in the presence of Mg sulphate and exposed to increased ethanol concentrations also showed low viabilities after just 3h (Table 6.1). After 3h a

recovery is shown by the Mg sulphate preconditioned ale yeast cells exposed to increasing ethanol concentrations with recovery observed in the presence of 20% ethanol up to 24h followed by a complete loss in viability thereafter. During exposure to increased ethanol concentrations, cell Mg in Mg sulphate preconditioned ale yeasts remained largely unaffected with very small amounts of Mg measured in the culture supernatants (Figs 6.1-6.4).

Mg acetate preconditioned ale yeast cells exposed to increased ethanol concentrations showed low viabilities after 3h, with the highest viability observed in those preconditioned cells exposed to 20% ethanol (Table 6.1). Viability recovers in Mg acetate in Mg acetate preconditioned ale yeast cells exposed to 20% ethanol and was subsequently lost in Mg acetate preconditioned ale yeast cells (Table 6.1). Cell Mg in Mg acetate preconditioned cells was affected when these cells were exposed to 10 and 20% ethanol (Figs 6.2 and 6.3). This was also observed in the culture supernatants, with the greatest loss of cell Mg observed in Mg acetate preconditioned cells exposed to 20% ethanol (Figs 6.3 and 6.4).

Table 6.1 Viability in ethanol stressed ale yeast cells (% live). Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to varying ethanol concentrations. Mg levels in the medium were 0.4mM and 100mM.

Time (h)	Unpreconditioned			Mg Sulphate preconditioned			Mg Acetate preconditioned		
	0%	**10%**	**20%**	**0%**	**10%**	**20%**	**0%**	**10%**	**20%**
3 (1)	2	4	25	6	8	20	6	16	35
3 (2)	4	6	35	10	12	24	10	20	41
6 (1)	15	9	8	20	10	36	20	23	48
6 (2)	23	15	12	28	16	46	26	35	60
24 (1)	25	9	8	21	20	45	22	25	50
24 (2)	35	15	10	27	22	55	28	33	60
48 (1)	40	13	0	31	19	0	26	30	0
48 (2)	56	21	0	41	23	0	38	38	0

Fig 6.1 Cell Mg in ethanol stressed ale yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to 0% ethanol. Mg levels in the medium were 0.4mM and 100mM.

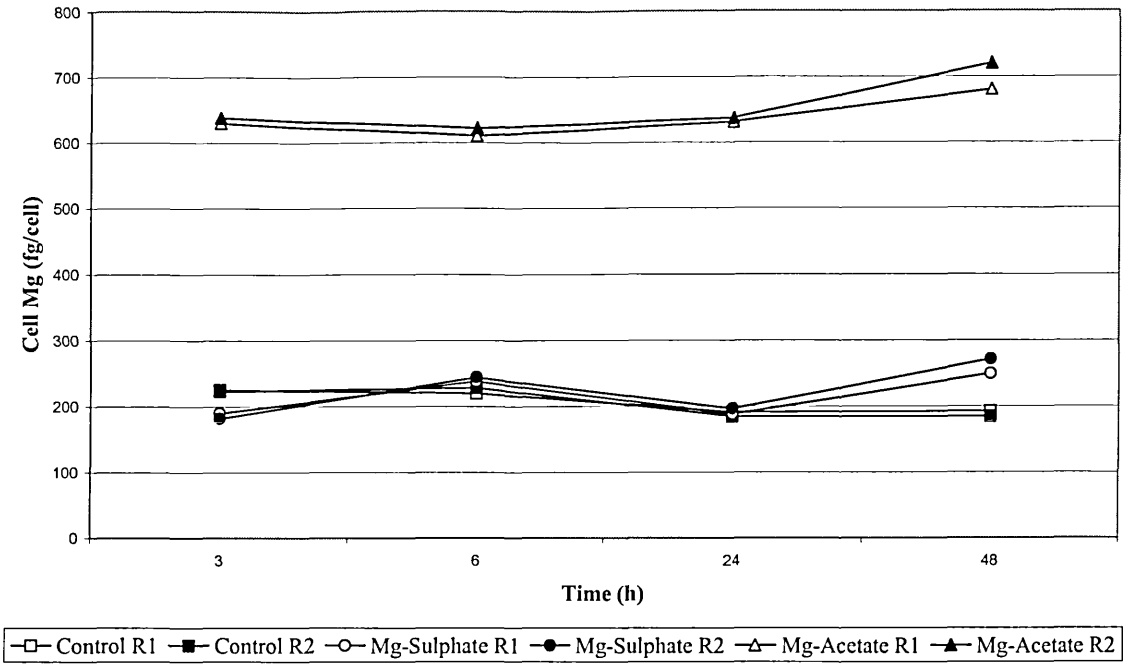


Fig 6.2 Cell Mg in ethanol stressed ale yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to 10% ethanol. Mg levels in the medium were 0.4mM and 100mM.

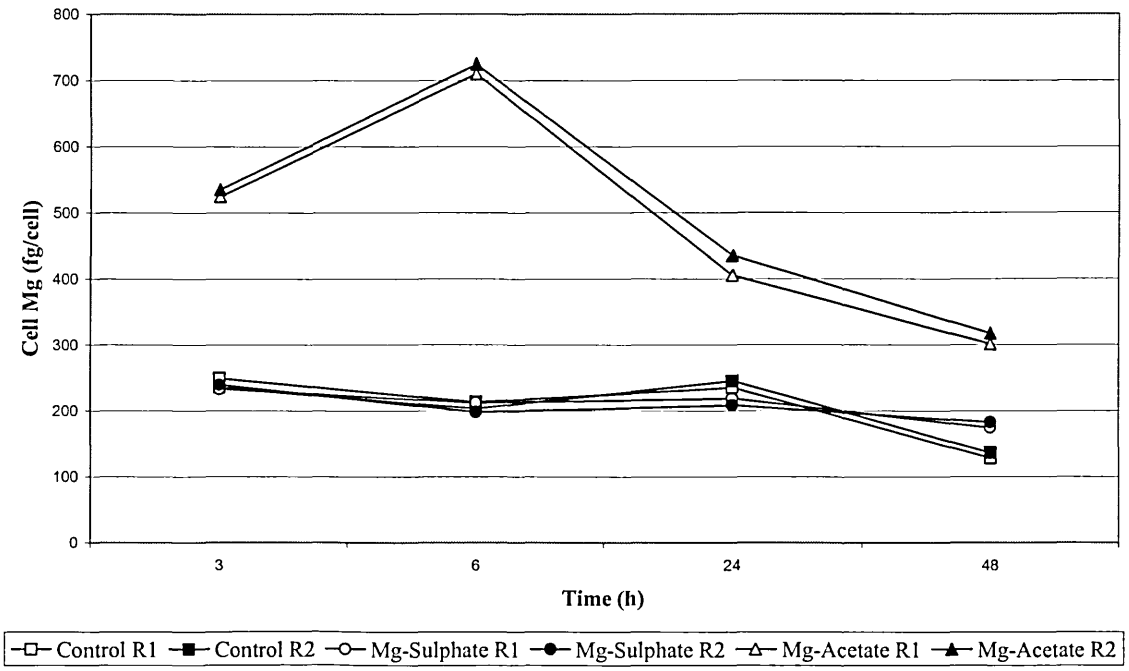


Fig 6.3 Cell Mg in ethanol stressed ale yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to 20% ethanol. Mg levels in the medium were 0.4mM and 100mM.

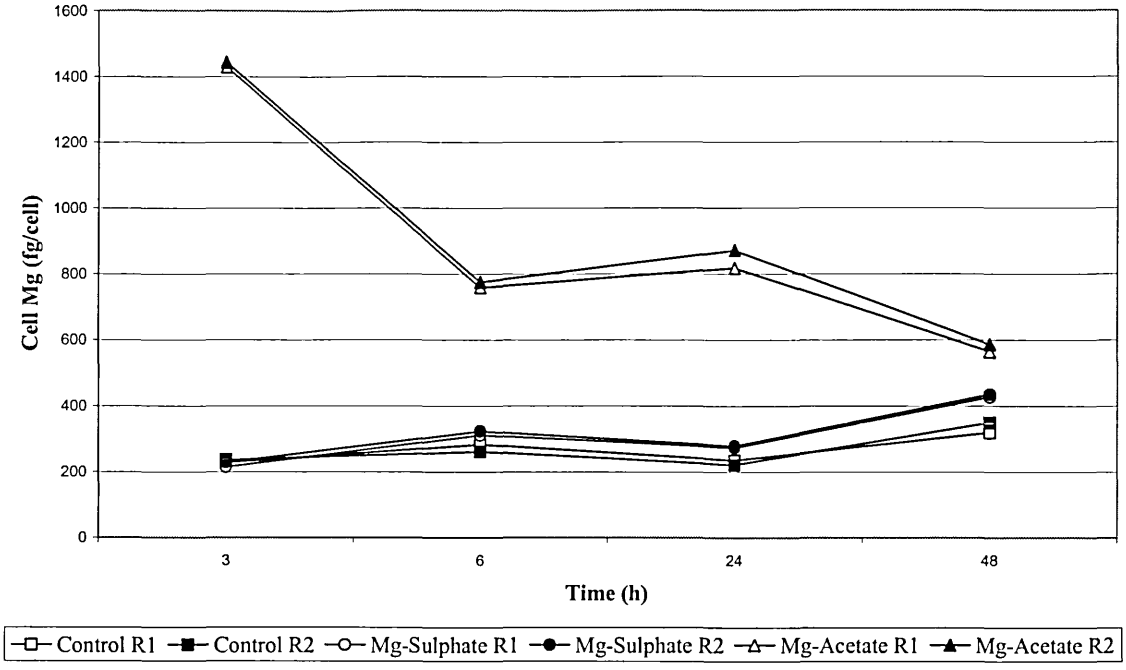
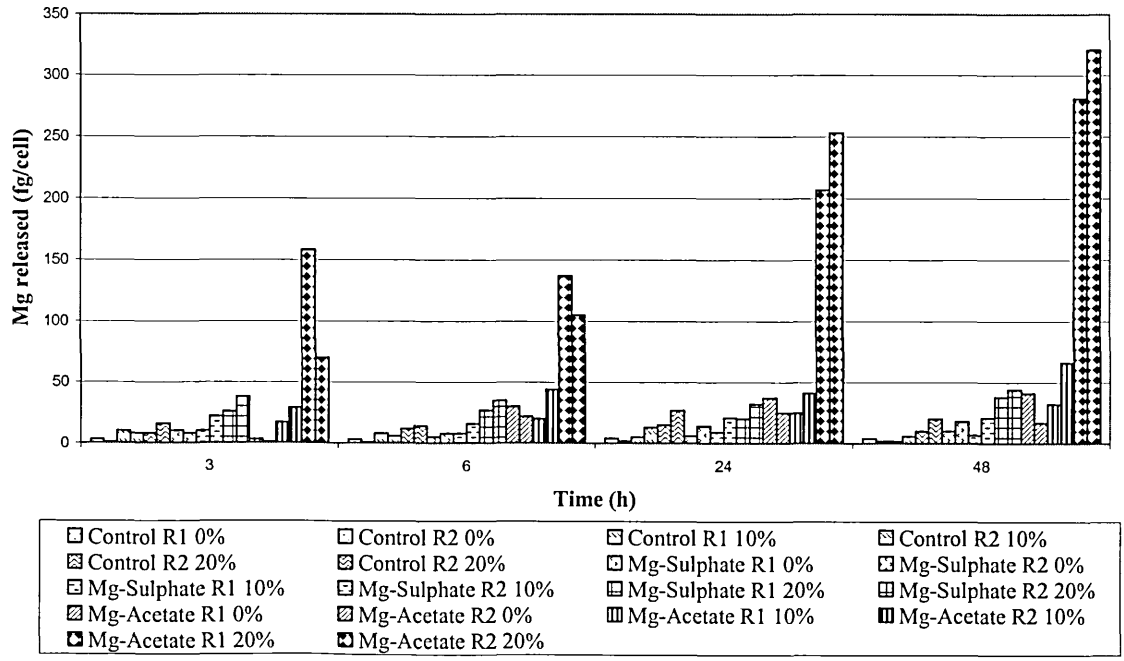


Fig 6.4 Mg released in ethanol stressed ale yeast cells. Cells were preconditioned in malt extract broth without (control) or one of two Mg salts present and exposed to varying ethanol concentrations. Mg levels in the medium were 0.4mM and 100mM respectively.



Lager yeast cells

Unpreconditioned lager yeast cell viability remained relatively unaffected by exposure to 10% ethanol (Table 6.2) with little concomitant change in their cell Mg (Fig 6.5-6.7). However, after 24h viability was affected during exposure to 20% ethanol (Table 6.2). Mg sulphate-preconditioned lager yeast cells showed some release of Mg during exposure ethanol with the highest Mg release observed during exposure to 20% ethanol after 48h (Fig 6.8). Viability remained relatively unaffected during exposure to ethanol over 48h. However, viability was lost after 24h in cells exposed to 20% ethanol (Table 6.2). Lager yeast cells preconditioned in Mg acetate also remained unaffected by exposure to 10% ethanol. When exposed to 20% ethanol, these cells showed a loss in viability after just 3h, viability was then halved after 6h with a complete loss after 24h (Table 6.2). There was also a concomitant loss in cell Mg (Fig 6.8).

Table 6.2 Viability in ethanol stressed lager yeast cells (% live). Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to varying ethanol concentrations. Mg levels in the medium were 0.4mM and 100mM.

Time (h)	Unpreconditioned			Mg Sulphate preconditioned			Mg Acetate preconditioned		
	0%	10%	20%	0%	10%	20%	0%	10%	20%
3 (1)	95	96	97	97	97	97	97	97	85
3 (2)	99	98	99	99	99	99	99	99	93
6 (1)	96	93	97	96	83	93	96	97	37
6 (2)	98	97	99	98	93	97	98	99	45
24 (1)	97	97	92	96	96	25	97	97	0
24 (2)	99	99	90	98	98	35	97	99	0
48 (1)	97	97	2	97	99	0	97	95	0
48 (2)	99	99	4	99	99	0	99	97	0

Fig 6.5 Cell Mg in ethanol stressed lager yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to 0% ethanol. Mg levels in the medium were 0.4mM and 100mM.

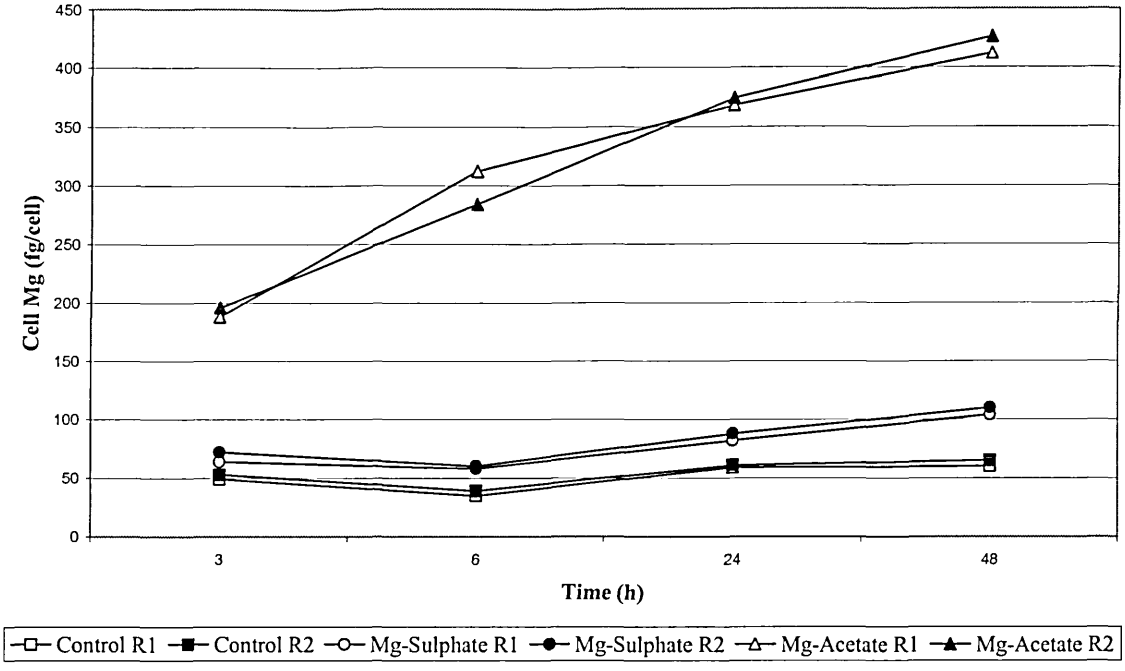


Fig 6.6 Cell Mg in ethanol stressed lager yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to 10% ethanol. Mg levels in the medium were 0.4mM and 100mM.

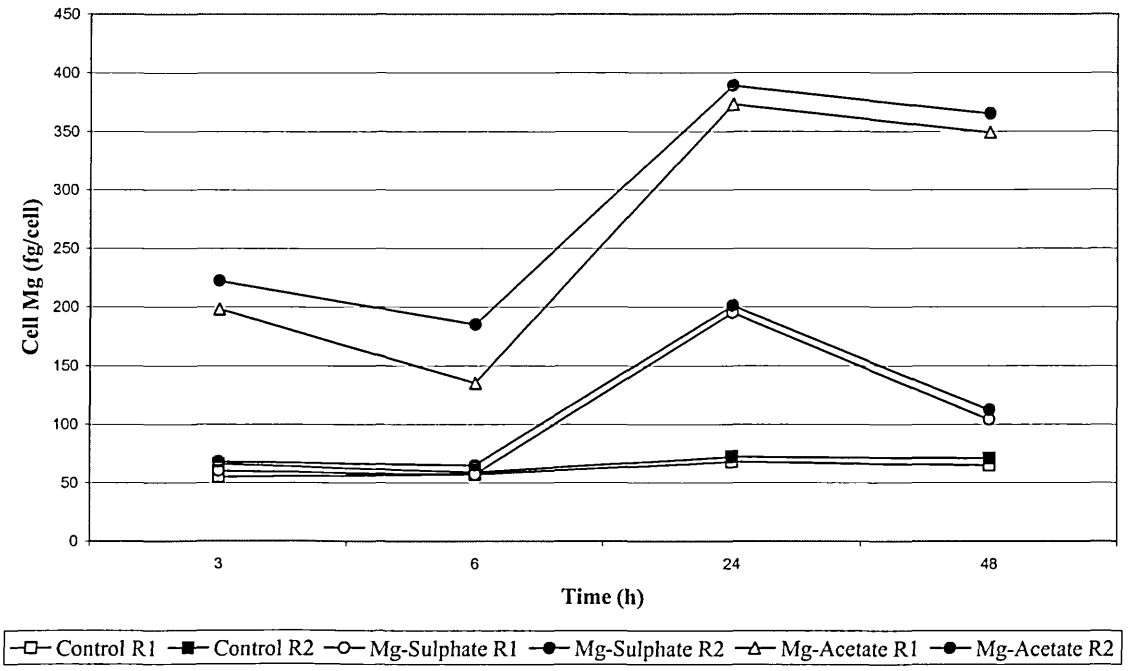


Fig 6.7 Cell Mg in ethanol stressed lager yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to 20% ethanol. Mg levels in the medium were 0.4mM and 100mM.

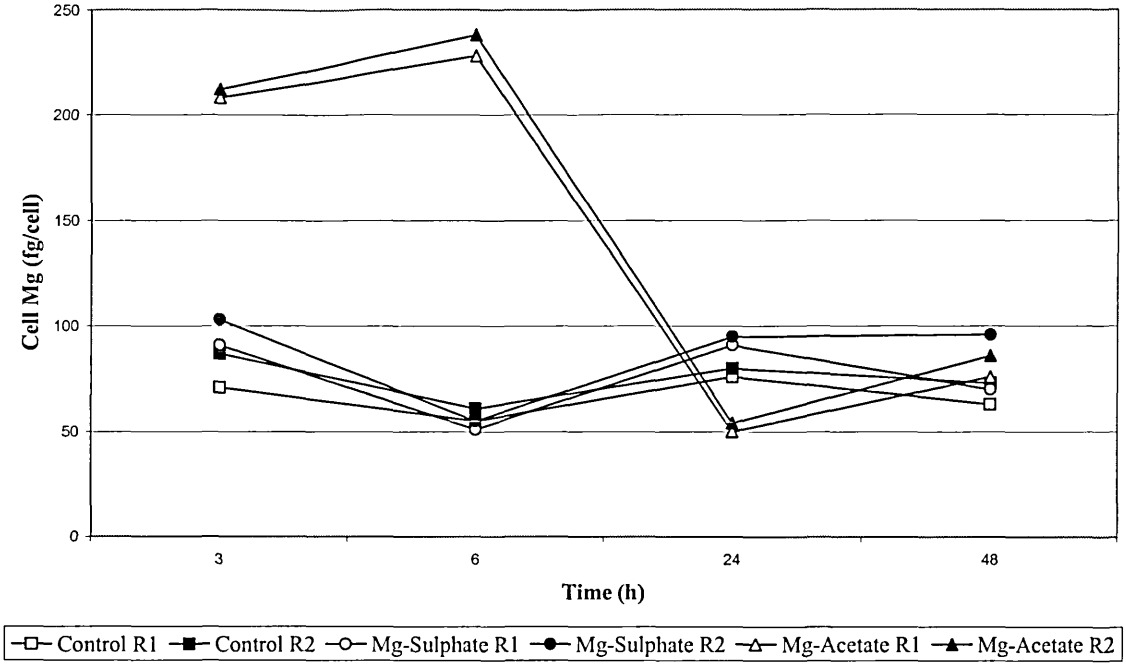
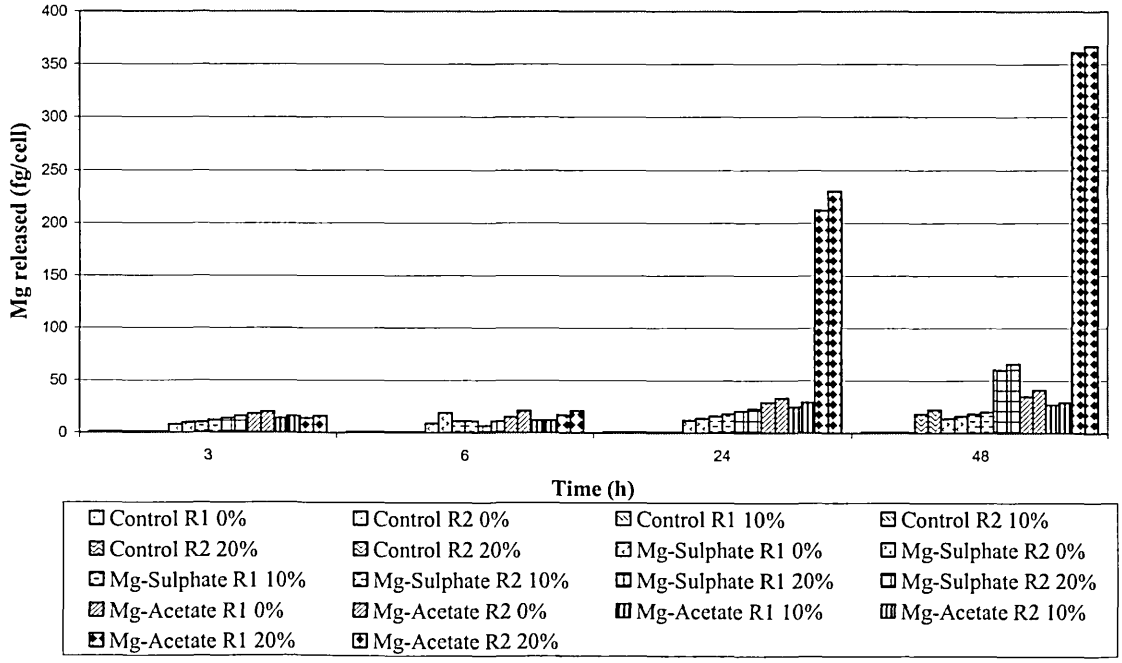


Fig 6.8 Mg released in ethanol stressed lager yeast cells. Cells were preconditioned in malt extract broth without (control) or one of two Mg salts present and exposed to varying ethanol concentrations. Mg levels in the medium were 0.4mM and 100mM respectively.



6.3.2 Effect of Mg-preconditioning on temperature stress

Ale yeast cells

The viability of unpreconditioned ale yeast cells was deleteriously affected during exposure to 40°C after just 3h (Table 6.3). Exposure to 4°C and 25°C has little observable affect on cell Mg (Figs 6.9 and 6.10). Viability of Mg sulphate-preconditioned cells was also affected by exposure to 4-40°C (Table 6.3). For example, viability remained consistently low over the 48h period of exposure to 4 and 25°C (Table 6.3). Cell Mg, however, remained largely unaffected in cells exposed to 4°C (Fig 6.9), whereas there was an observable decrease in cellular Mg (Fig 6.9) and a concomitant increase in Mg release after 24h in cells exposed to 25°C (Fig 6.12). Exposure to 40°C also resulted in a loss of viability after 3h exposure, which remained significantly low until after 24h where it was lost completely (Table 6.3). This correlated with a significant loss of cell Mg and a concomitant increased release of Mg (Fig 6.11 and 6.12).

Mg acetate preconditioned ale yeast cells also showed a loss of viability after only 3h when exposed to temperatures ranging from 4-40°C (Table 6.3). The greatest loss was in those cells exposed to 4°C, these cold-shocked cells show a recovery in viability after 48h, this was also observed in cells exposed to 25 and 40°C. However, viability was completely lost in cells exposed to 40°C for longer than 24h (Table 6.3). Cell Mg fluctuated in cells exposed to 4°C and 25°C during the 48h period of exposure (Fig 6.9 and 6.10). During exposure to 40°C an increase in cell Mg was observed between 3h and 6h, which then stabilised (Fig 6.11). However, a loss in Mg was observed after 24h, which correlated with a loss of viability (Table 6.3 and Fig 6.12).

Table 6.3 Viability in temperature stressed ale yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to varying temperatures. Mg levels in the medium were 0.4mM and 100mM.

Time (h)	**Unpreconditioned**			Mg Sulphate preconditioned			**Mg Acetate preconditioned**		
	4°C	25°C	40°C	4°C	25°C	40°C	4°C	25°C	40°C
3 (1)	34	20	1	30	23	14	9	17	10
3 (2)	40	14	1	34	27	20	7	19	12
6 (1)	9	15	18	28	21	9	13	17	13
6 (2)	13	11	26	32	25	11	19	25	19
24 (1)	65	37	6	24	17	27	12	26	36
24 (2)	73	43	4	30	25	31	18	30	44
48 (1)	39	7	0	32	16	0	40	35	0
48 (2)	45	7	0	42	20	0	44	43	0

Fig 6.9 Cell Mg in temperature stressed ale yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to 4°C. Mg levels in the medium were 0.4mM and 100mM.

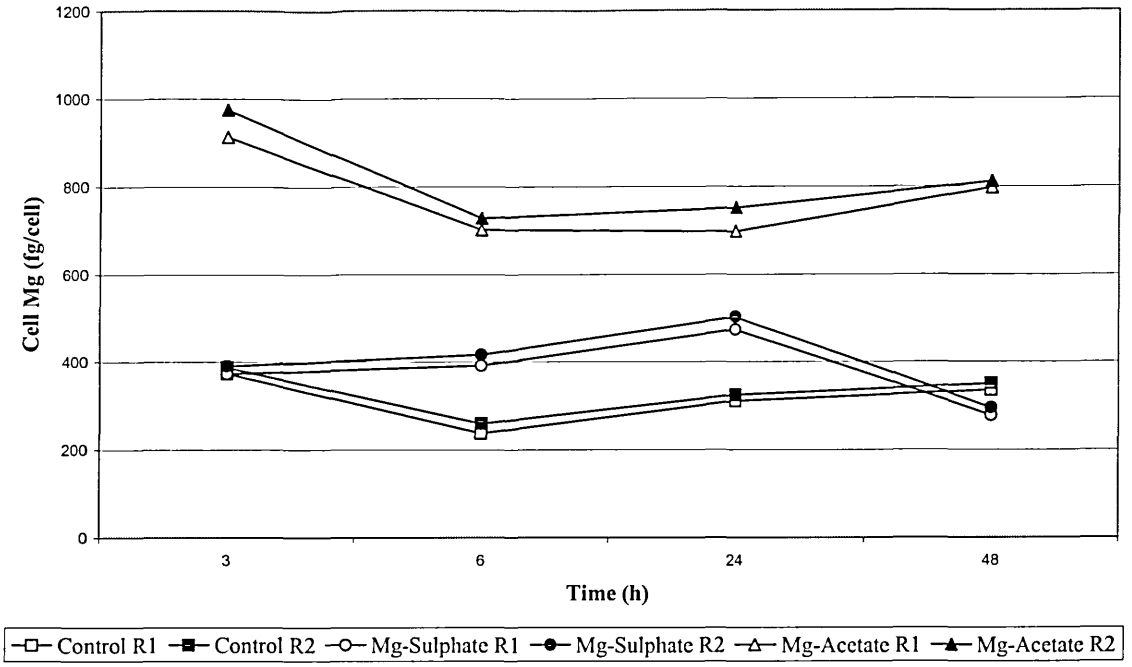


Fig 6.10 Cell Mg in temperature stressed ale yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to 25°C. Mg levels in the medium were 0.4mM and 100mM.

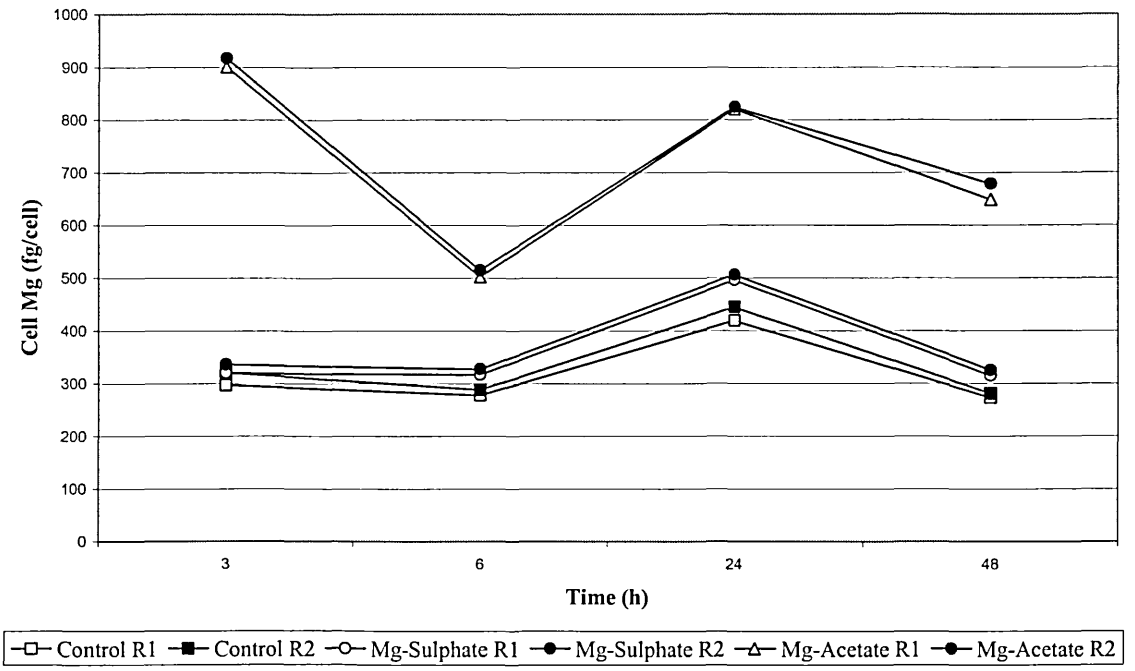


Fig 6.11 Cell Mg in temperature stressed ale yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to 40°C. Mg levels in the medium were 0.4mM and 100mM.

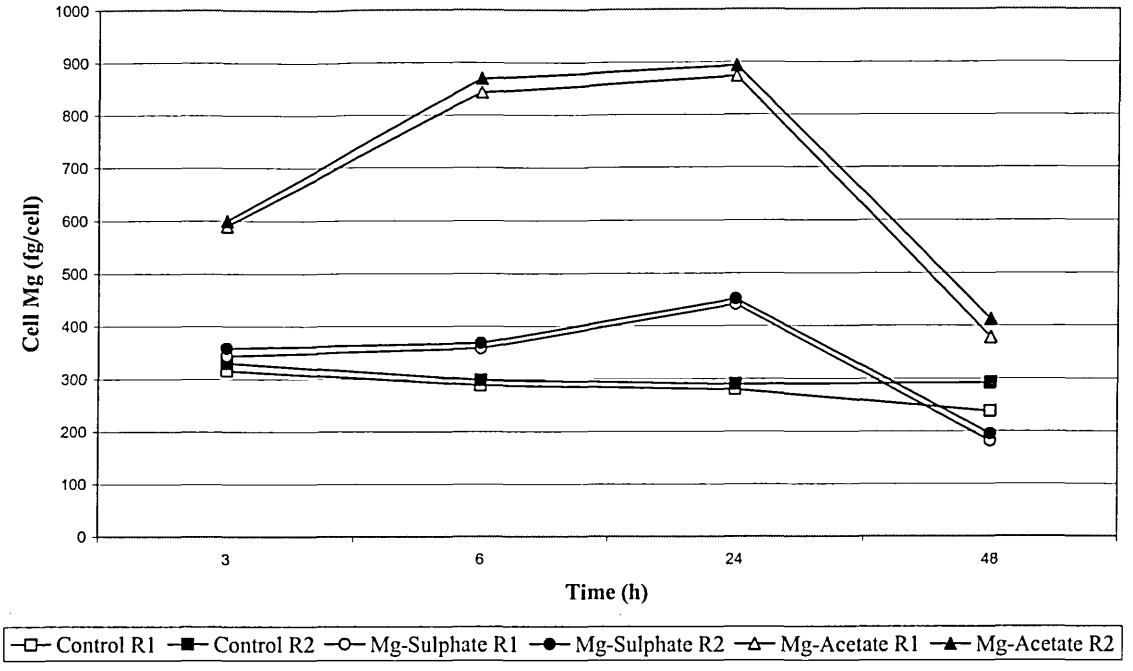
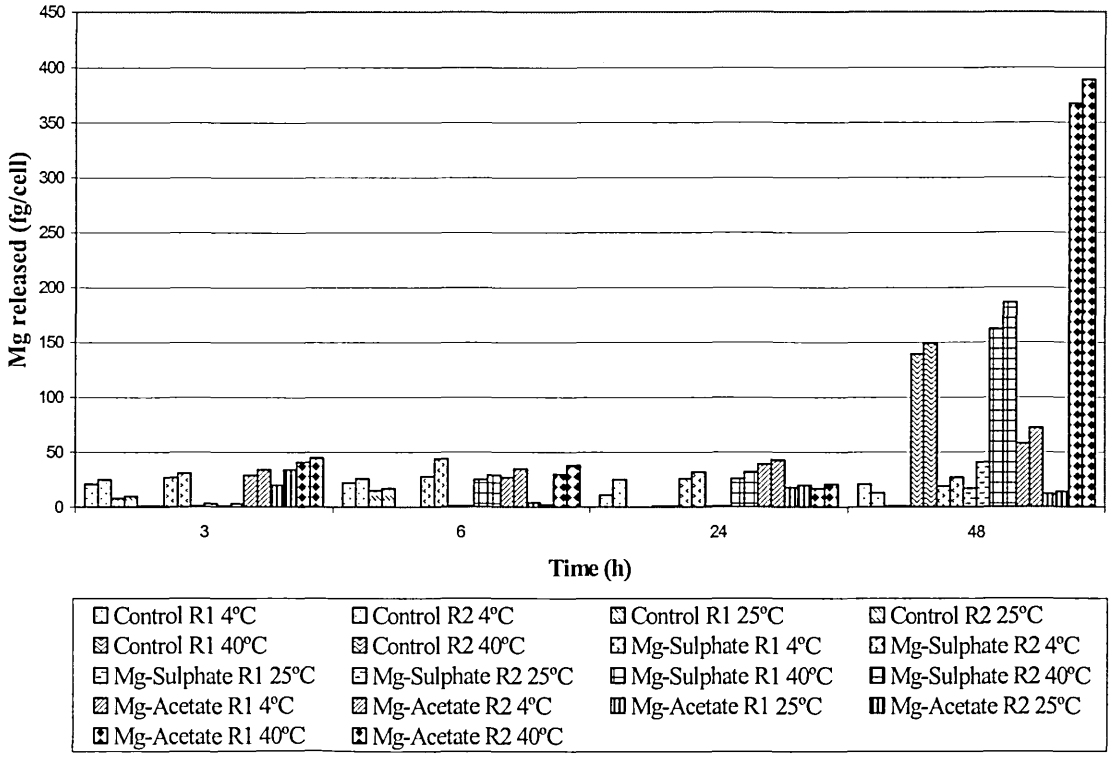


Fig 6.12 Mg released in temperature stressed ale yeast cells. Cells were preconditioned in malt extract broth without (control) or one of two Mg salts present and exposed to varying temperatures. Mg levels in the medium were 0.4mM and 100mM respectively.



Lager yeast cells

Unpreconditioned lager yeast cells remained largely unaffected in terms of exposure to temps of 4 and 25°C (Table 6.4, Figs 6.13 and 6.14). However, viability was affected by exposure to 40°C for more than 6h. This correlated with an increase in Mg release and a decrease in cellular Mg (Figs 6.15 and 6.16).

The viability of Mg-sulphate preconditioned lager yeast cells also remained relatively unaffected by prolonged exposure to temperatures of 4°C and 25°C (Table 6.4, Figs 6.13 and 6.14 respectively). Exposure to 40°C however, resulted in a decrease in viability after 6h (Table 6.4) and a total loss after 48h, which correlated with a decrease in cell Mg (Figs 6.15 and 6.16).

Mg acetate preconditioned lager yeast cells remained unaffected during exposure to 4 and 25°C with viability remaining constant (Table 6.4). In both cases a decrease in cell Mg was observed after 24h and 6h, respectively (Figs 6.13 and 6.14). Exposure of these cells to 40°C for longer than 6h resulted in a significant decrease in viability with a complete loss after 48h (Table 6.4). This also correlated with a significant loss of cellular Mg (Figs 6.15 and 6.16).

Table 6.4 Viability in temperature stressed lager yeast cells (% live). Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to varying temperatures. Mg levels in the medium were 0.4mM and 100mM.

Time (h)	**Unpreconditioned**			Mg Sulphate preconditioned			**Mg Acetate preconditioned**		
	4°C	25°C	40°C	4°C	25°C	40°C	4°C	25°C	40°C
3 (1)	98	97	96	99	98	99	98	99	98
3 (2)	94	95	92	99	96	97	94	97	94
6 (1)	97	95	97	98	97	100	99	99	99
6 (2)	95	91	95	100	95	96	97	99	97
24 (1)	96	97	0	98	98	7	98	99	10
24 (2)	90	95	0	96	94	11	96	97	6
48 (1)	94	97	0	97	98	0	99	98	0
48 (2)	90	95	0	93	96	0	99	98	0

Fig 6.13 Cell Mg in temperature stressed lager yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to 4°C. Mg levels in the medium were 0.4mM and 100mM.

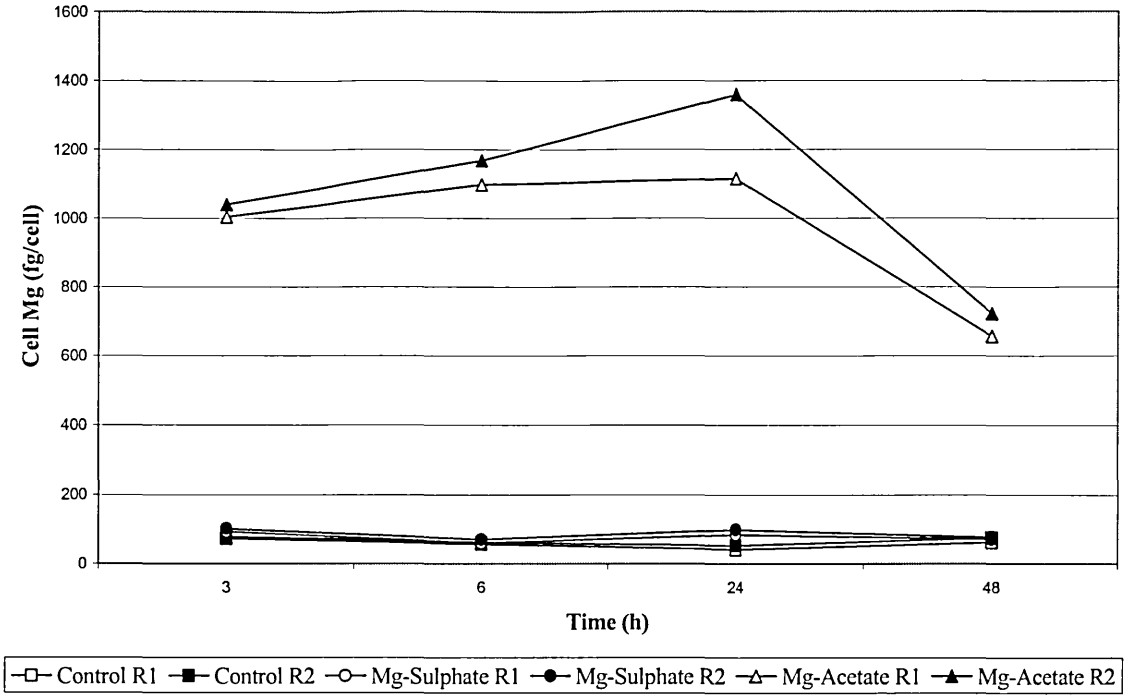


Fig 6.14 Cell Mg in temperature stressed lager yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to 25°C. Mg levels in the medium were 0.4mM and 100mM.

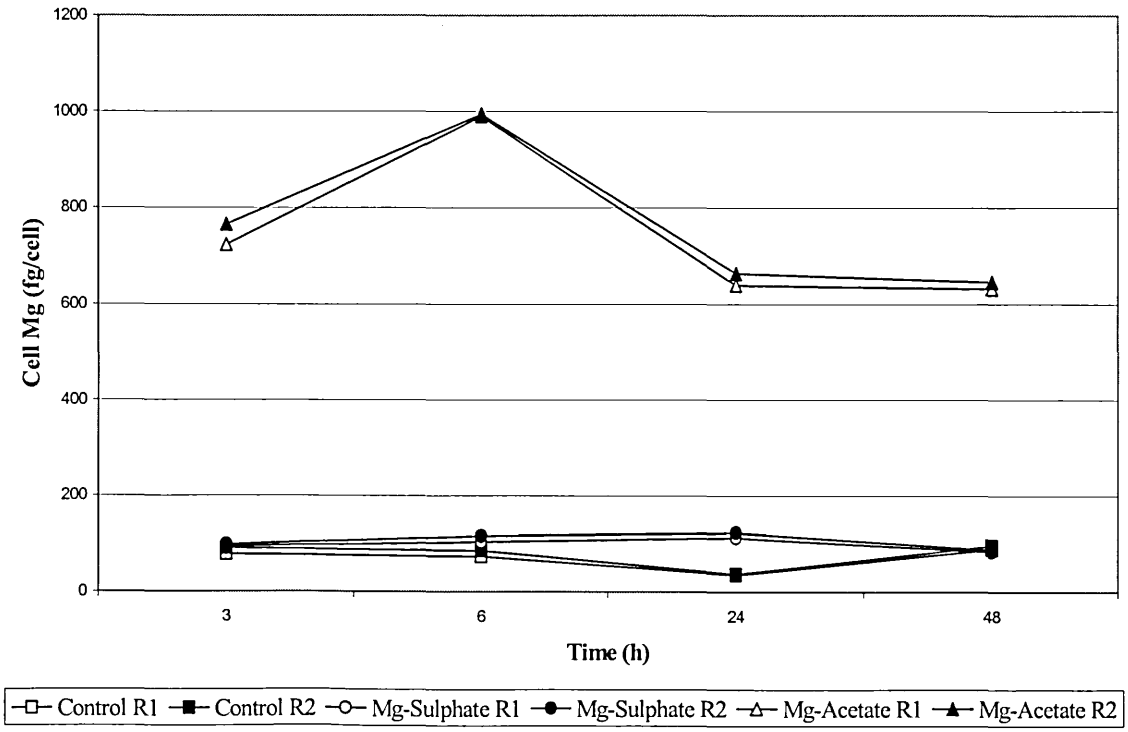


Fig 6.15 Cell Mg in temperature stressed lager yeast cells. Cells were preconditioned in malt extract broth without (control) or with one of two Mg salts present and exposed to 40°C. Mg levels in the medium were 0.4mM and 100mM.

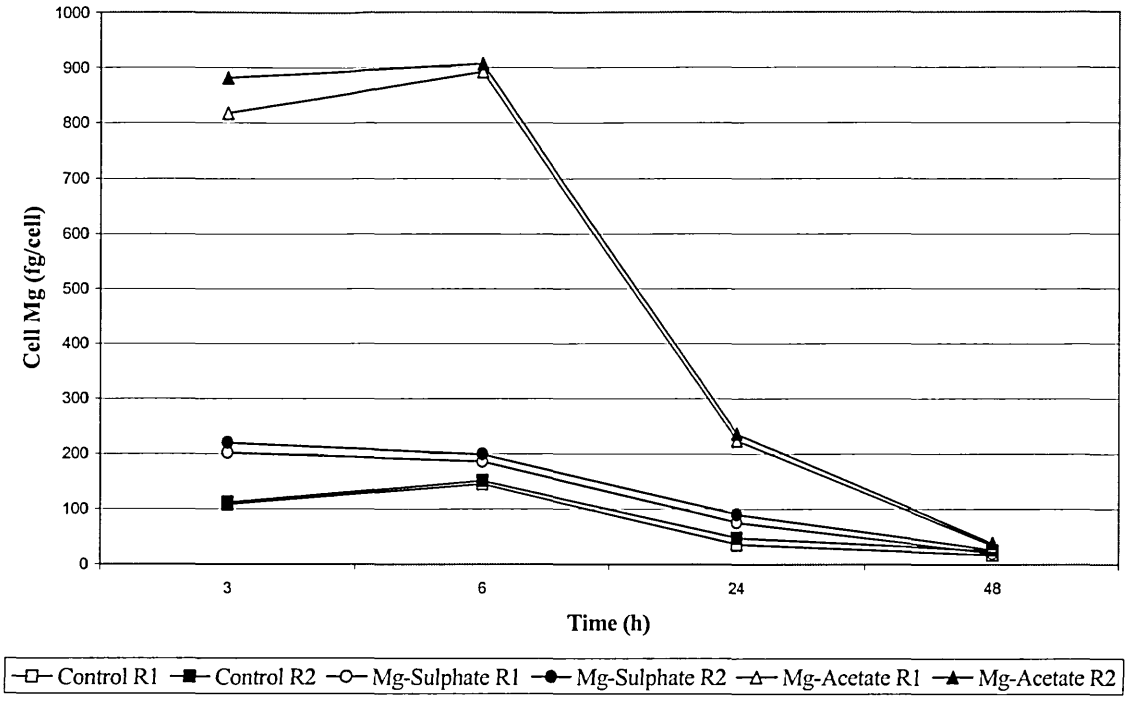
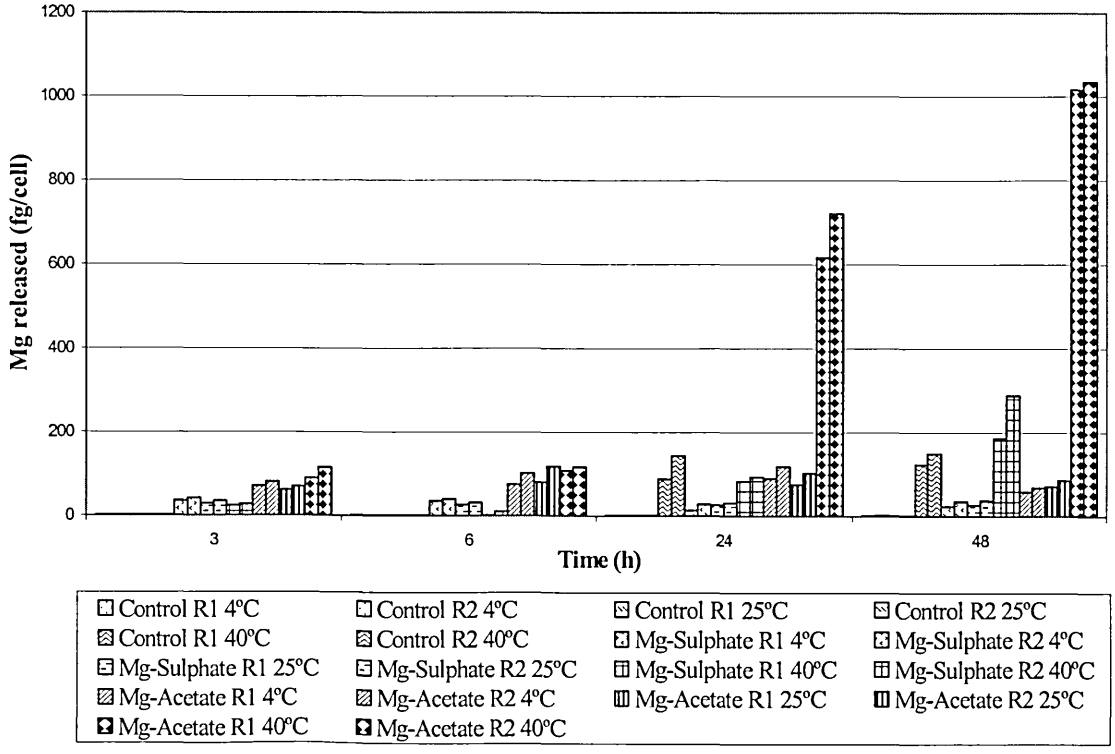


Fig 6.16 Mg released in temperature stressed lager yeast cells. Cells were preconditioned in malt extract broth without (control) or one of two Mg salts present and exposed to varying temperatures. Mg levels in the medium were 0.4mM and 100mM respectively.



6.3.3 Reproducibility

The data presented herein is data of duplicated experiments (R1and R2), which showed a close relationship between the two data sets. Further repeats of the experiments would have allowed greater statistical accuracy.

6.4 Discussion

Previous studies have suggested that Mg can moderate the inhibitory effects of ethanol and temperature stresses in yeasts (Dombek and Ingram 1986b; Ciesarova *et al.* 1996; Walker 1998b; Birch and Walker 2000). However, magnesium's' ability to moderate the harmful effects of ethanol and temperature stresses has not been so clearly revealed in the present study in ale yeast cells when compared with lager yeast cells. This may be due in part to doubt cast over the accuracy of the methylene blue method for determining yeast viability (O'Connor-cox *et al.* 1997). Questions have been raised surrounding the methylene blue test's accuracy when yeast cell viability falls below 90% (O'Connor-cox *et al.* 1997). This may be due to the instability of the polychromic methylene blue dye itself (which can be oxidatively demethylated) rather than its interaction with the yeast cell *per se* (Smart *et al.* 1999).

Lager yeast cells, irrespective of Mg preconditioning, were found to be less sensitive to prolonged exposure to 10% ethanol, this suggests that augmenting the cellular Mg may not be offering as much of a stress protective benefit to cells. Preconditioned ale and lager yeast cells released more Mg than those which were unpreconditioned, this would suggest that some of the extra Mg present in these cells is in a free (releasable) form rather than being tightly bound. Previous work has shown that brewer's yeasts lose Mg and higher amounts were lost in preconditioned yeasts (Walker 1998b). This was thought

to be attributed to stress induced cell autolysis as opposed to membrane leakage (Walker 1998b).

Mg acetate preconditioned lager yeast cells may be more sensitive to a high (20%) ethanol stress due to excess acetate in the cell lowering the intracellular pH of the yeast (Stratford and Anslow 1996; Stratford and Anslow 1998). Cytosolic acidification may potentially exacerbate any deleterious effects of ethanol on cell membrane fluidity. Previous authors (Chandrasena 1996; Birch 1997) have shown that Mg-preconditioned cultures of wine and distilling yeasts showed an enhanced tolerance to ethanol. However, the results obtained here with brewing yeast strains do not appear to support such findings. Studies (Dombek and Ingram 1986b) have also shown that supplementing glucose medium with 0.5mM Mg resulted in extended exponential growth with a lower reduction in fermentation rate. These findings were supported by the investigation carried out by Ciesarova *et al* (1996) into the effects of Ca and Mg on ethanol tolerance. The conclusions of that study were that Ca and Mg exerted a protective effect on stationary phase yeast cells in the presence of 10% ethanol (Ciesarova *et al.* 1996). Mg was also found to reverse the ethanol induced leakage of ions and small metabolites (Ciesarova *et al.* 1996). This is thought to be due to the Mg ions decreasing the proton and, especially, the anion permeability of the plasmalemma (Petrov and Okorokov 1990), by interacting with membrane phospholipids resulting in stabilization of the membrane layer (Birch and Walker 2000). This also translated into a reduction in the gross cellular damage, in that preconditioned wine yeast cells appeared smoother and less wrinkled when compared to unpreconditioned cells after a 1h heat shock (Birch and Walker 2000).

The results (Table 6.1-6.4) would suggest that the lager yeast cells used in this study were more tolerant to ethanol and temperature stresses than the ale yeast cells. This would suggest that the cell membrane of ale yeasts is more sensitive to ethanol and low temperature stresses even in the presence of additional Mg ions, which have been shown to promote stress tolerance in wine making and distilling yeasts (Walker 1998b; Birch and Walker 2000). Conversely the results (Table 6.3 and 6.4) also show that both preconditioned and unpreconditioned ale yeast cells were able to withstand exposure to 40°C for a longer period of time than the lager yeast cells. Such differences in tolerance to high and low temperatures may be due in part to differences in the maximum growth temperatures of the two species, especially since lager yeasts as a 'rule' perform ideally at low temperatures (8-15°C with a maximum operating temperature of 31.6-34°C) whereas ale yeasts as a 'rule' operate at higher temperatures (~20°C with a maximum operating temperature of 37.5-39.8°C) (Boulton and Quain 2001). In terms of overall stress tolerance, lager yeast cells would be expected to be more stress tolerant, especially since they are traditionally seen as 'bottom fermenting yeasts' and have therefore evolved to cope with stresses such as prolonged exposure to ethanol, lowering of pH during fermentation, CO_2 and hydrostatic pressure. Whereas, with the recent introduction of cylindro-conical fermenter vessels ale yeasts would appear to be handicapped in evolutionary terms having traditionally been 'top fermenting yeasts'.

Whilst brewing yeasts may not be exposed to a shock such as 40°C during normal brewing practice, it was worthwhile determining whether or not Mg preconditioning offered a greater degree of protection to cells exposed to such temperatures. Birch and Walker (1996), Birch (1997) and Birch and Walker (2000) have shown in wine strains of *S.cerevisiae* that, Mg plays a thermoprotective role, and that biosynthesis of heat shock

proteins is repressed in cells incubated in the presence of 10mM Mg. When exposed to a rapid decrease in temperature, the cell membrane cools. If this rate of cooling is faster than the rate at which the cell can adjust its membrane composition, the membrane passes through a temperature zone in which the membrane lipids undergo a phase transition from liquid to 'gel states' (Fargher and Smith 1995). The lipids then become fixed in a random manner thus compromising not only the integrity of the cell membrane causing it to leak but also allowing protein synthesis to be impaired (Fargher and Smith 1995; Zhang *et al.* 2001). The induction of the *LOT* (low temperature inducible) genes (expression of which is induced by a down shift in temperature) together with the synthesis of trehalose and glycerol are mechanisms whereby yeasts, and brewer's yeasts in particular, could cope with the onset of a cold stress usually associated with cooling process (and storage), which is initiated at the end of fermentation to enhance yeast flocculation prior to yeast cropping.

CHAPTER 7

CONCLUDING DISCUSSION

The original hypothesis set out at the beginning of this research was that Mg-preconditioning would have a beneficial effect on brewing yeast physiology, specifically in terms of enhanced fermentative metabolism and stress resistance. The experimental plan was designed to test aspects of this hypothesis. Firstly Chapter 3 set out to establish conditions appropriate for effective "Mg-preconditioning". Secondly Chapter 4 set out to visualise the cell Mg contained in ale and lager yeast cells, which had been preconditioned, compared to those, which had not. Chapters 5 and 6 were designed to investigate the behaviour of Mg enriched brewing yeasts in terms of their fermentative metabolism and stress tolerance.

7.1 Mg-preconditioning of brewer's yeasts

Preconditioning ale and lager yeast cells in synthetic wort medium showed that although cellular Mg content increased with elevated Mg concentrations, the uptake of the ion was not proportional to its availability, which has also been shown by previous authors (Saltukoglu and Slaughter 1983; Walker and Maynard 1997). Expressing Mg in terms of biomass and protein resulted in data that concurred with or was higher than that which has been previously been published (especially in preconditioned yeasts) (Dombek and Ingram 1986b; Obata *et al.* 1996; Beeler *et al.* 1997; Graschopf *et al.* 2001).

When ale and lager yeast cells were preconditioned in malt extract broth supplemented with a variety of Mg salts, it was found that the organic Mg salts elevated cellular Mg contents higher than that of the inorganic salts, with Mg-acetate in particular being very efficient at elevating cell Mg contents. When these results were transferred

across to a wort propagation situation, cellular Mg was highest in Mg acetate preconditioned ale and lager yeast cells. The results from both the malt extract broth and dilute wort propagation studies showed that Mg uptake was still not directly proportional to its availability in the case of Mg lactate and Mg gluconate.

The variation in cellular Mg when yeast cells were preconditioned in the presence of different Mg salts may be explained by the following theoretical considerations:

- Dissociation of the Mg salts in water, which would indicate that the Mg was freely available to the yeast cells for transportation across the cell membrane.
- Alteration of medium pH, which in the case of Mg acetate may mean that the acetate molecules were lowering intracellular pH thus influencing membrane permeability, which in turn could affect the H^+-ATPase and the putative Mg transporter Alr1p.

Although much has still to be learnt about the mechanisms of Mg transport in yeast cells (and indeed in other eukaryotes) some putative Mg transporter genes have been identified (*ALR1, LPE10* and *MRS2*) (Wiesenberger *et al.* 1992; MacDiarmid and Gardner 1998; Bui *et al.* 1999; Gregan *et al.* 2001). The results obtained from these studies suggest that Mg may also be transported by a high affinity mechanism. This is of great relevance since dual transporter systems exist in yeasts for metals such as zinc and manganese (Zhao and Eide, 1996a,b Walker, 1998a) .

The genetic differences between ale and lager yeasts as well as their differing nutritional requirements and growth demands for Mg, may help to explain the differences in cellular Mg in ale and lager yeast cells observed herein.

7.2 Localisation of intracellular Mg in preconditioned yeasts

Through the use of Mg fluorescent dyes it was hoped that the visualisation of intracellular Mg would provide a clearer picture as to where the extra Mg contained in preconditioned yeasts was. This proved to be less fruitful than was hoped due to limitations in the equipment to capture images of the fluorescing cells. Previous studies by (Zhang *et al.* 1997) have shown that the cellular distribution of intracellular Mg was not homogeneous and increased as a consequence of increments in extracellular Mg conc. The results presented in Chapter 4 support this view as preconditioned ale and lager yeast cells had a greater fluorescence capacity, which was concomitant with a higher cellular Mg content. Variations in the number of fluorescing cells may be due to a lack of synchrony on the part of the ale and lager yeast cells especially as cells in different stages of the cell cycle are thought to contain different amounts of Mg (Walker and Duffus 1980). Of particular interest was the observation that Mg-acetate preconditioned ale yeast cells had sporulated after four days propagation in Mg-acetate supplemented medium, especially since brewing yeasts have a poor sporulation capacity (Stewart and Russell 1998). The result of this was asci being observed, with the Mg Green fluorescent dye highlighting the presence of Mg on the outside of each ascospore. This suggests that a lot of Mg was present in the asco-spore wall; it was also observed that these sporulating cells still had high intracellular Mg contents.

The observed differences in cellular Mg content and consequently Mg Green fluorescence in the ale and lager yeast cells, could be explained by their having different nutritional requirements and growth demands for Mg. The genetic differences between the two strains may also mean that the proteins encoded by the recently identified putative Mg transporter genes may therefore consist of slightly different subunits derived from the

genetic information of their respective genomes. This is especially pertinent since Hohmann (1997) suggested that differences might exist in the structure of the enzyme pyruvate decarboxylase in ale and lager yeasts.

7.3 Fermentative behaviour of Mg-preconditioned yeasts

Non-and preconditioned pitching yeast inocula for high gravity fermentations appeared to release Mg during the 24h period after pitching. This release of Mg has been associated with changes in membrane permeability of the yeast cell in preparation for sugar uptake (Mochaba *et al.* 1996). This release of Mg has also been suggested as an indicator of yeast vitality, with yeasts with good vitality releasing more Mg than those of a poorer vitality (Mochaba *et al.* 1996). The results of this study showed that despite a high release of Mg, yeast cell viability in the 24h after pitching had been deleteriously affected by preconditioning. In terms of fermentation performance however both Mg acetate preconditioned ale and lager yeast cells showed faster rates of fermentation, this was reflected in faster reductions of specific gravity and higher yields of ethanol.

In terms of the activity of the fermentative enzyme pyruvate decarboxylase the results presented attempted to quantify the effect additional Mg in preconditioned yeasts had on its activity. The results presented in Chapter 5 showed that a close relationship existed between yeast cell Mg and PDC activity. In such cases, the additional intracellular Mg may be directly influencing substrate binding to the PDC tetramer and may also be acting in synergy with the other essential co-factor of the PDC reaction, thiamine diphosphate.

Although cells with increased cell Mg displayed enhanced PDC activity, this was not reflected in a dramatic increase in the yield of ethanol during fermentation. This is not entirely surprising since flux through glycolysis is influenced by a number of enzymes rather than a single enzyme. Other workers using recombinant DNA technology have shown that the overproduction of glycolytic enzymes fails to influence the rate of ethanol production (Schaaff *et al.* 1989). The differences in response to PDC enzyme activation by Mg in the ale and lager yeast cells might also be explained by the differences in PDC quaternary structure as suggested by (Hohmann 1997). Nevertheless this work on Mg-preconditioning, as an example of physiological cell engineering (as opposed to genetic engineering) has indicated potential benefits for improved ethanol production in industrial cells of *S.cerevisiae.*

7.4 Stress tolerance of Mg preconditioned brewing yeasts

The results of ethanol and temperature stress experiments show that the lager yeast cells used in this study appeared to be more stress tolerant than the ale yeast cells, irrespective of whether or not they had been preconditioned. This would suggest that the ale yeast cell membrane might be more sensitive to ethanol and low temperature stresses even in the presence of Mg ions, which have previously been shown to promote stress tolerance in wine making and distilling yeasts (Walker 1998b; Birch and Walker 2000). Conversely the ale yeast cells used in this study were, irrespective of Mg-preconditioning, found to be more tolerant to exposure to 40°C heat stress than the lager yeast cells.

It is therefore unsurprising that the lager yeast cells were shown to be more stress tolerant, as in evolutionary terms, lager yeasts in general have been exposed to a wider variety of stresses, such as CO_2 and hydrostatic pressures, prolonged exposure to ethanol

and lowering of pH during fermentation. With modern breweries employing cylindro-conical fermenter vessels, ale yeasts are now being exposed to an alien environment, especially since they have been selected by centuries of brewing to be top fermenting yeasts. The differences in tolerance to temperature stresses by ale and lager yeast cells could be explained by the differences in their maximum growth temperatures. Lager yeasts ideally operate at low temperatures (8-15°C) whilst ale yeasts operate at higher temperatures (~20°C).

The results have also shown that preconditioned yeast cells released more Mg than those which had not been preconditioned during ethanol and temperature stress, this suggests that some of the extra Mg present in such cells is in a free (releasable) form rather than being tightly bound. Previous work in brewer's yeasts has shown a similar release in Mg-preconditioned yeasts (Walker 1998b). This was thought to have been attributed to stress induced cell autolysis as opposed to membrane leakage.

Over all the work described in this thesis has shown the following:

- 'Preconditioning' can augment the intracellular Mg content of brewing yeast cells and the actual degree of augmentation is dependent on the Mg salt used in the yeast growth medium.
- Mg fluorescent dyes were found to be of limited value in localising Mg in brewing yeast cells.
- High intracellular Mg content in preconditioned lager yeast cells stimulates ethanol production and increases ethanol yield.
- A close relationship exists between yeast cell Mg and pyruvate decarboxylase activity.

- Lager yeast cells used in this study may be intrinsically more stress tolerant than the ale yeast cells used in this study, irrespective of their intracellular Mg contents.
- Ale and lager yeast cells exposed to ethanol and temperature stresses released Mg.

7.5 Reproducibility

The data presented in this thesis was of duplicated experiments, a close relationship between the two sets of data was observed. Differences observed between data sets could be accounted for through fluctuations in cell growth/culture conditions between experiments or a greater efficiency in analytical techniques. Further repeats of the experiments would have allowed greater statistical accuracy, thus reducing any variations between the data sets even further.

7.6 Future work

Future work could be designed to scale up the propagation of pitching yeasts in relation to preconditioning thus providing a larger preconditioned pitching inocula. Investigations into the effect of acid washing (commonly employed to remove bacterial wild yeast contamination from pitching yeasts) on preconditioned yeasts after fermentation could be made. The release of Mg as well as other ions after pitching in relation to yeast vitality could be studied. The usefulness of preconditioned yeasts in relation to normal and high gravity fermentations over a number of serial repitchings could be investigated. Studies into the interaction of yeasts with other ions such as Zn could be made. The feasibility of producing a Zn-preconditioned yeast or combined Mg/Zn preconditioned yeasts could be determined.

Recently a number of mineral enriched yeast products (Lallemand, Inc) have become commercially available. These include Mg and Zn-enriched dried yeasts for the brewing industry to stimulate fermentation, these products are added during wort boiling to release the ions into the wort for yeast utilisation during fermentation (J.McLaren, Lallemand Inc, personal communication). Also available are selenium, chromium, molybdenum and copper ion enriched yeasts designed for the human health and nutrition market (J.McLaren, Lallemand Inc, personal communication). Although not metal ion preconditioning per se, these products show that yeast ion enrichment is practical on an industrial scale.

CHAPTER 8

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Appendix 1

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Appendix 2

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