Yeast-metal interactions: impact on brewing and distilling fermentations

Graeme M Walker*, Raffaele De Nicola, Starley Anthony and Robert Learmonth[1]

Yeast Research Group, University of Abertay, Dundee, Scotland.
[1] Centre for Systems Biology, University of Southern Queensland, Toowoomba 4350, Australia

Abstract
In brewing and distilling fermentations, key metal ions are magnesium and zinc, which act as co-factors for important glycolytic enzymes and also as modulators of yeast stress. In addition to their effects on enzymes, metal ions may have marked effects on stability and dynamics of cell membranes, leading to downstream effects on cell permeability and signalling systems. Occasionally, the bioavailability of certain key minerals may be limiting and this can adversely affect yeast fermentation processes. For example, zinc levels may decrease during mashing and wort boiling as the metal ion becomes complexed in precipitated trub. Consequently, zinc levels in wort may become compromised leading to impaired fermentation performance. This paper reviews previous work on the impact of magnesium and calcium on industrial yeasts and also describes the uptake and utilisation of zinc by brewing and distilling yeasts under conditions varying from deficit to oversupply. In addition, it discusses the impact of yeast-zinc interactions on the progress and efficiency of brewing and distilling fermentations, the status of cell membrane systems, and the influence of metals in governing yeast responses to environmental stress.

Key Words: *yeast, metal ions, zinc, membranes, fermentation, brewing, distilling*

Introduction
Yeast cells require a wide range of metals for their growth and metabolism. Although minerals in brewing and distilling fermentation processes are often overlooked as being important factors, metal ions can impact significantly on the progress and efficiency of industrial fermentations. For example, metal ions govern several important parameters including the rate of sugar conversion to ethanol, the degree of attenuation/ final ethanol yield, the amount of yeast produced, cell viability and stress tolerance, extent of foaming, and yeast flocculation behaviour. Bulk metals, such as magnesium and potassium, are generally required by growing yeast cells in the millimolar (i.e. hundreds of ppm) concentration range, whilst calcium and zinc are regarded as trace metals being required in the micromolar (sub-ppm) range. Magnesium is necessary for the activation of several glycolytic enzymes and, in practical terms, this means that if industrial media is magnesium-limited, the conversion of sugar to alcohol may be suppressed leading to slow or incomplete fermentation processes. Calcium requirements for yeast growth are very low (Walker, 1994; Youatt, 1993), however, calcium ions are acknowledged to play a key role in the important process of flocculation in brewing fermentations. Calcium antagonises uptake of magnesium and can block essential magnesium-dependent metabolic processes, so high levels would be detrimental to brewing efficiency. Other metals such as heavy metals, even at trace level concentrations, may be toxic to yeast (Jones and Gadd, 1990). Zinc is a trace element that is particularly important in fermentation with regard to its role as activator of the terminal alcohologenic Zn-metalloenzyme,

ethanol dehydrogenase. Media deficient in zinc may lead slow or incomplete fermentations, and this has long been recognised as an occasional problem in the brewing industry (e.g. Densky *et al*, 1966).

In addition to impacts on important metabolic enzymes, metal ions may also affect the stability and dynamics of cell membranes. However, the roles of yeast membranes in maintenance of nutrient transport, cell viability, permeability, stress tolerance and fermentation efficiency largely remain to be elucidated. When yeasts encounter changes in environment such as nutrient or ion depletion, metabolite accumulation or temperature variation, the plasma membrane must adapt prior to internal structures. Maintenance of membrane fluidity is a crucial factor in preservation of membrane functions. We believe that modulation of membrane fluidity is a primary response to environmental change (Learmonth and Gratton 2002).

Yeasts are able to very effectively accumulate essential minerals and exclude or detoxify non-essential minerals. Magnesium and zinc are actively translocated by yeast from wort into the cell in order to carry out essential physiological roles, and this may be usefully exploited in alcoholic beverage fermentations. For example, Smith and Walker (2000) have shown that Mg-preconditioned distiller's or brewer's yeast with elevated levels of cellular magnesium were more fermentatively active, compared with non-preconditioned cells with normal levels of cell magnesium. Mineral-enriched yeasts have potential in addressing the problem of insufficient bioavailable metal ions and some commercial products (e.g. zinc-enriched *S. cerevisiae*) are now available as fermentation supplements.

This paper firstly reviews previous research on the impact of magnesium and calcium ions on industrial yeast physiology and then focuses primarily on the roles of zinc in the growth and metabolism of industrial strains of the yeast, *Saccharomyces cerevisiae*, and discusses the impact of yeast-zinc interactions for brewing and distilling fermentation processes.

Experimental

Organisms, media and culture conditions

Four industrial yeast strains of *S. cerevisiae* were employed in this study: one ale brewing strain NCYC 1681, two lager strains LBA and LBB, and one Scotch whisky distilling strain DCLM. Experiments were carried out in both shake flasks and laboratory fermenters using freshly prepared malt wort (generally at OG 1060, 15°P). Conditions under which yeasts were subjected to environmental stress were essentially those described by Walker (1998b).

Lab-scale fermentations

Small scale brewing-simulated experiments offer the great advantages of more accurate control of parameters such as zinc concentration and avoidance of trace contaminants. An industrial lager yeast strain (LBB) was pitched at $5x10^6$ cells/mL into sterilised and clarified hopped malt wort. Yeast seed cultures were grown in shake flasks at 25°C, 180 rpm, for 24 hours. Simulated brewing fermentations were performed in conical Imhoff vessels (volume 1L, cone angle 74°) previously deionised with a nitric acid-EDTA-dH_20 wash. Wort was pre-aerated at 14°C for 2 hours using filtered air. Zinc concentrations in the range 0-23 ppm were tested using appropriate amounts of sterilised zinc acetate solution. Fermentation was performed at 14°C for 11 days and samples taken every 2 days. Yeast crop volumes were measured at end of fermentation and analysed for zinc cell content. In experiments with altered zinc levels, zinc acetate was added to the medium prior to cell inoculum from a sterile stock solution (1000 ppm zinc).

Microbiological and chemical analyses

Suspended yeast cell numbers were determined using a haemocytometer (improved Neubauer type) and a Coulter Counter. Zinc levels in cells and in wort were analysed after nitric acid digestion (90°C for 1 h) by atomic absorption spectroscopy (AAS). Wort Specific Gravity (SG) was detected by using an Anton Parr DMA 58 Density Meter and ethanol using a GCMS-QP2010 Gas Chromatograph Mass Spectrometer. Viability was evaluated using 2% (w/v) Methylene Violet 3 Rax (Sigma) in sodium citrate 0.01% according to the method of Smart *et al.* (1999).

Determination and visualisation of intracellular zinc in yeast

Zinc concentrations in mother and daughter cells from model brewing fermentations were determined after isolation of the cells as follows. Cells were harvested after 16 hours of growth in malt extract with zinc at 0.25 ppm, washed with dH_2O, carefully layered onto the surface of a lactose gradient solution (15 to 30%) and centrifuged at 1000 rpm for 10 minutes. Cells at various size bands were removed with a syringe. Cell size was detected by Coulter Multisizer II. Cells were stained with 10µM Rhod-Zn 1 (Molecular Probes) and analysed using a Partec Cyflow Flow Cytometer.

For visualisation of zinc within cells, yeast were grown in EMM3 minimal medium prepared by modifying the Edinburgh Minimum Medium N°3 (Fantes and Nurse, 1977) with zinc concentration at 10 ppm. Cells were inoculated at $5x10^6$ cells/mL and collected after one hour of growth. Vacuolar and intracellular zinc were stained, respectively, with 10µM Cell Tracker B and 10µM Fluo-Zn 3 (both from Molecular Probes) and visualised with a fluorescent microscope LEICA using blue and green filters respectively.

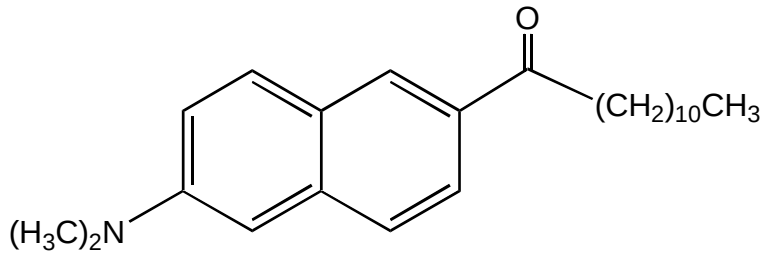
Membrane fluidity determinations

We used the fluorescent membrane probe Laurdan (Molecular Probes, 6-lauroyl-2-dimethylamino naphthalene – see Fig 1), which exhibits a 50 nm red shift in emission spectrum over the gel to liquid crystalline phase transition of lipid bilayer systems. The Generalised Polarization (GP) parameter is used as an index of membrane fluidity. GP is determined from relative fluorescence intensities at wavelengths representing gel and liquid crystalline phases, using the following equation (Parasassi *et al.* 1990):

$$GP = \frac{Igel - Ilc}{Igel + Ilc}$$

Where Igel and Ilc indicate relative fluorescence intensities at wavelengths representing gel (440nm) and liquid crystalline (490nm) phases of bilayer systems, respectively. Cells to be analysed were centrifuged, washed with dH_2O, resuspended in dH_2O at 0.1 OD_{600nm}, and labelled by mixing with laurdan stock in ethanol (1 µL per mL cells, final laurdan concentration 5µM) and incubating for 1 h in the dark. This negligible addition of ethanol was previously shown not to affect yeast. GP of Laurdan was measured as previously described (Learmonth and Gratton 2002) using a Perkin-Elmer LS-3B Fluorescence Spectrometer.

Fig. 1 Laurdan chemical structure



Results & Discussion

Impact of Mg, Ca and Zn on yeast fermentation

The mineral nutrition of yeasts is relevant to brewers, winemakers, distillers and bioethanol producers as they seek to increase fermentative capacity, improve ethanol yields and maintain product consistency. The bioavailability of metal ions in fermentation media are indeed important factors that influence yeast cell physiology and production of yeast fermentation products. The most important metals that influence yeast fermentation processes are potassium and magnesium (as bulk metals), and calcium, manganese, iron, copper and zinc (as trace metals). Stewart and Russell (1998) and Boulton and Quain (2001) have discussed the roles of bulk and trace metals in relation to brewing yeast fermentation processes. In relation to brewing, most interest to date has focused on the roles of zinc and calcium in influencing wort attenuation and yeast flocculation, respectively. Until relatively recently, little attention has be paid to the roles of magnesium in yeast physiology and fermentation performance. For industrial yeasts such as *S. cerevisiae*, magnesium is absolutely essential for growth and metabolism and the bioavailability of this cation in media such as malt wort (Walker *et al* , 1996), molasses (Chandrasena *et al*, 1997) and wine must (Birch *et al*, 2003) is now recognised as being very important for efficient industrial fermentations with this yeast. In alcohol fermentations, magnesium ions can directly influence the rate of yeast growth, sugar consumption and ethanol production (Saltokoglu and Slaughter, 1983; Walker *et al*, 1996; Rees and Stewart, 1999). For industrial strains of *S. cerevisiae* interacting with magnesium and calcium, Table 1 summarises salient points from previous studies in this laboratory.

Table I Influence of metal ions on yeast physiology and fermentation performance

Research findings	Industrial relevance	References
Metal ion *bioavailability* (esp. Mg) influences fermentation and Mg can dictate switch between fermentation and respiration in yeast	Increasing free Mg in fermentation media by supplementation stimulates alcohol production	Walker et al (1982); Walker (1994); Walker et al (1996); Walker & Maynard (1997); Smith & Walker (2000); Walker & Smith (1999)
Yeasts have high demand for Mg, but not Ca and high calcium levels antagonisie essential Mg-dependent functions	Important to maintain high Mg:Ca ratios in fermentation feedstocks for maximal fermentation performance	Saltukoglu & Slaughter, (1983); Walker et al (1996); Walker (1999b)
Statistical response surfaces show that Mg, K, Ca and Zn interact significantly in fermentation	Modelling of metal ion interactions can predict fermentation performance	Chandrasena et al (1997)
Mg protects cells from stress caused by ethanol	Mg-preconditioned yeasts may counteract	Walker (2004)

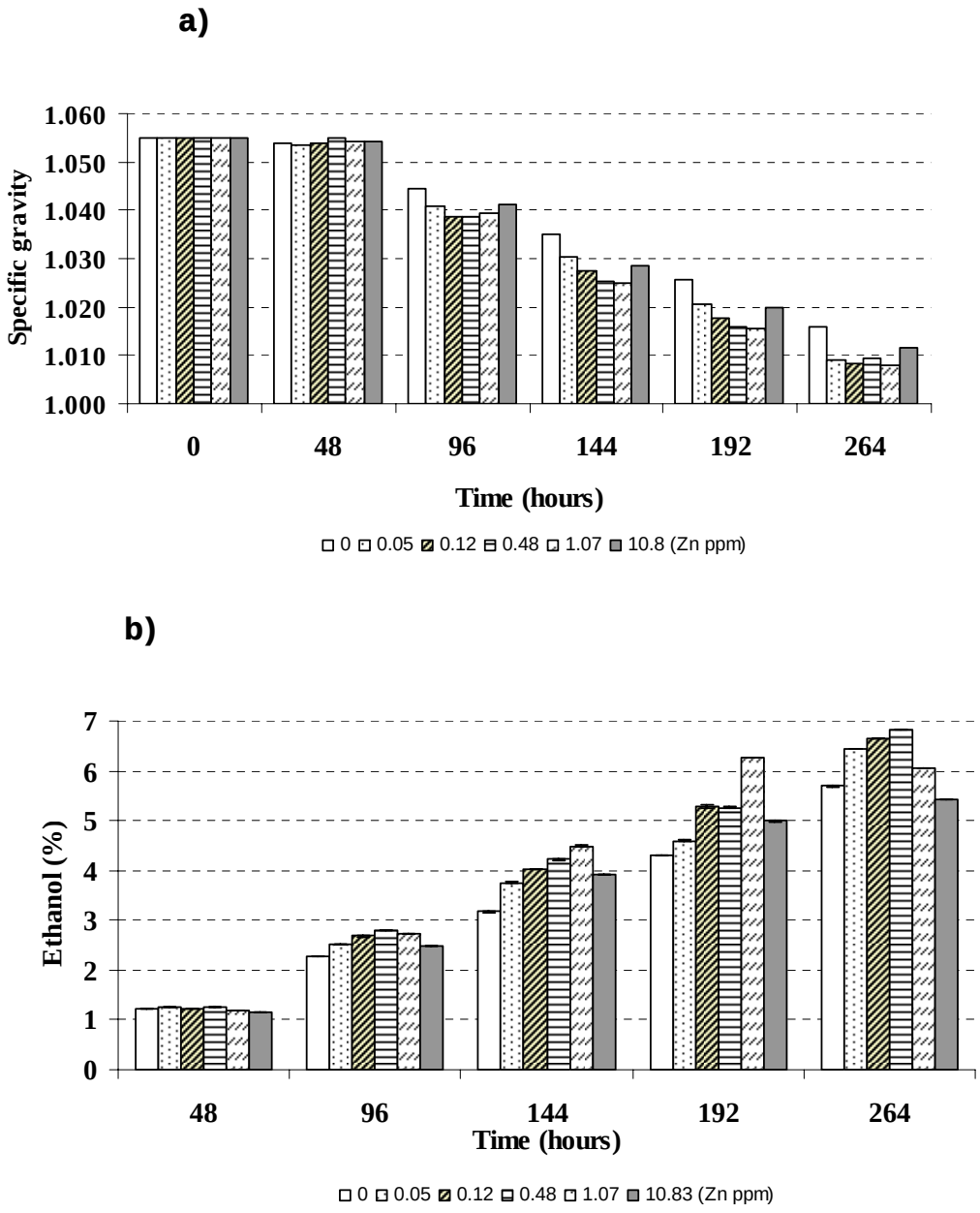
toxicity, temperature shock, heavy metals and oxidative stress	physiological stress and improve fermentative metabolism	

A dilemma facing distillers, brewers and winemakers is one of supplying sufficient nutrients to yeast to carry out fermentation whilst minimising yeast growth. For industrial alcohol producers, excess yeast represents alcohol loss. However, growing cells produce alcohol 33 times faster than non-growing cells (Ingledew, 1999). Compromise efforts are made to keep yeast under conditions that do not lead to low growth rates or to cell death. Minimising yeast growth during alcoholic fermentation may be accomplished by employing: high yeast cell densities/cell re-cycle systems, continuous/semi-continuous fermentations, or immobilised yeast bioreactors. In addition, it would be desirable to encourage a predominantly fermentative, rather than respiratory, mode of metabolism in the yeast strains employed for alcohol production. We have found that metal ions may play a role in this metabolic regulation, especially magnesium (see Table 1 and Walker *et al*, 1982; Walker, 1994).

It is noteworthy that zinc is an essential micronutrient for yeast and occasionally brewer's wort may be Zn-deficient, resulting in impaired fermentation performance (Densky *et al*, 1966; Desmartez, 1993; Bromberg *et al*, 1997; Stehlik-Thomas *et al*, 1997; Rees and Stewart, 1998). This phenomenon, which can lead to slow, or so-called "sluggish", fermentations in breweries, is yeast strain-dependent but may encountered when wort zinc levels are generally below 0.1ppm. Zinc plays a major role in yeast fermentative metabolism not only because it is essential for ethanol dehydrogenase activity (the terminal Zn-metalloenzyme in alcoholic fermentation – see Magonet *et al*, 1992), but also because it can stimulate uptake of maltose and maltotriose into brewing yeast cells, thereby augmenting fermentation rates. Furthermore, elucidation of possible effects of zinc on cell membrane stability and dynamics may define further fermentation improvements. In some instances zinc bioavailability may be limiting due to decrease of zinc levels during mashing, lautering and boiling through complexing in precipitated trub. We have studied the impact of varying zinc levels in a model brewing fermentations, utilising wort initially depleted of zinc. Wort zinc was depleted by bio-chelation following a short-term exposure to yeast cells, providing a wort normal in all aspects except zinc levels (De Nicola, PhD thesis University of Abertay Dundee). A lager brewing strain of *S. cerevisiae* (LBB) was extensively cultured in low zinc medium to deplete cellular zinc levels, and then pitched into wort containing zinc concentrations ranging from 0 to 10 ppm (provided as zinc acetate).

Results shown in Fig 2 indicate that zinc availability significantly affected fermentation performance. Additionally, we found that even at very high Zn levels studied (23ppm) zinc was not toxic to the yeast cells. The lack of toxicity may have been due to presence of manganese ions, although Mn levels in our wort (0.20ppm) were half of that previously reported to be required for yeast to tolerate levels of zinc above 2 ppm (Jones and Greenfield 1984). This level may be strain dependent and the lager strain used in this experiment may have high zinc tolerance. Malt wort medium is rich in nutrients, some of which may have led to increased yeast zinc tolerance. Bromberg *et al.* (1997) found that zinc requirements are higher in poor quality malt wort assuming an interaction among trace metals and zinc.

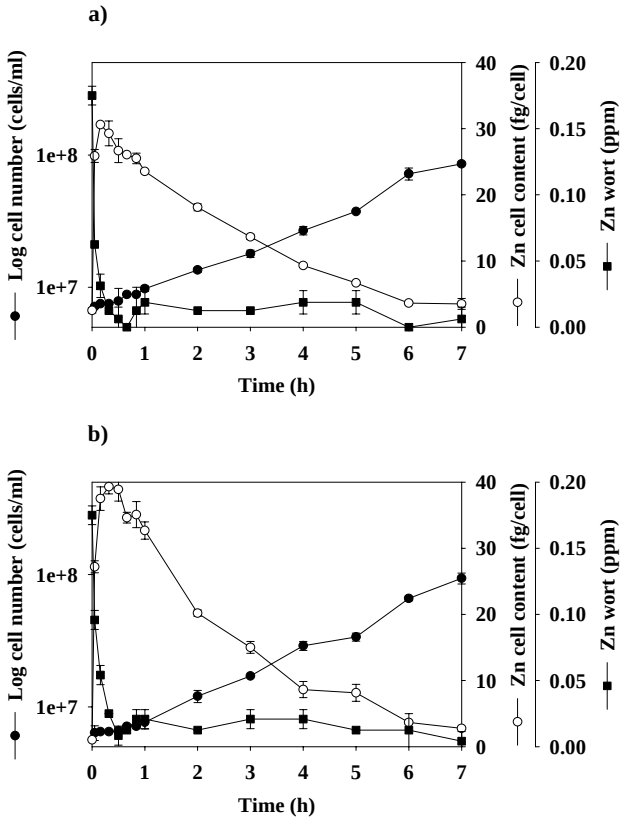
Fig. 2 *Impact of zinc bioavailability on yeast fermentation performance.*
A lager yeast strain (LBB) of *S. cerevisiae* was grown in malt wort (OG 1055), at 14ºC, for 11 days, in Imhoff conical vessels (1 L, cone angle 74°). Zinc levels were altered with zinc acetate supplementations. Fermentation performance was evaluated by analysing specific gravity (a) and ethanol levels (b) at regular intervals.

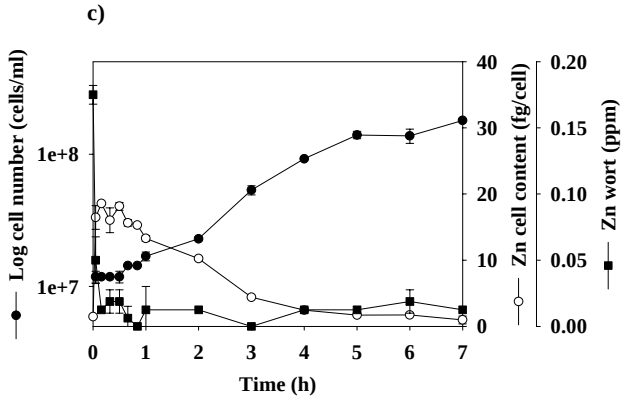


Zn uptake by brewing and distilling yeasts
Several physico-chemical constraints may impede metal ion uptake by yeast, including chelation, adsorption, and binding. In complex growth media like malt wort, this can lead to reduced metal bioavailability during fermentation. Zinc composition of wort will vary greatly depending on raw materials and process conditions. Therefore, any factor which reduces zinc bioavailability and compromises zinc uptake

will, in turn, adversely affect yeast growth and fermentative activity. A major aim of this study was to investigate zinc uptake kinetics of brewing and distilling yeast strains during malt wort fermentations. Zinc was measured by AAS in both yeast cells and wort in the first 7 hours when cells were actively dividing (early exponential phase) and after 24 hours (early stationary phase). Fig. 3 shows zinc uptake versus yeast cell growth. Zinc accumulation was very rapid and zinc in the media became depleted within the first hour or so of fermentation. From this data, it appears that yeast demand for zinc is very high and immediate during the initial stages of fermentation.

Fig. 3 *Zinc uptake by S. cerevisiae strains in malt wort.*
Three industrial strains of *S. cerevisiae* were cultivated in shake flasks in malt wort, OG (1060, with zinc at 0.175 ppm), at 25°C for 24 hour. Cell growth versus zinc cell content and residual zinc wort was determined for a lager brewing yeast LBA (a), ale brewing yeast NCYC 1681 (b) and whisky distilling yeast strain DCLM (c) Graphs show the first 7 hours of fermentation.





At higher concentrations, zinc accumulation was slower and 1-2 hours were required to uptake all zinc from wort. Over the same period of time, we noted that magnesium and calcium level fluctuations were not as dramatic as for zinc (not shown). At all zinc concentrations studied, average zinc cellular content decreased when cells started to actively divide. Depending on the extent of yeast growth during fermentation, this may result in the generation of zinc-depleted biomass at the time of yeast cropping with severe consequences for the following fermentations when cells are repitched.

In industry, zinc supplementations (if required) may be more conveniently applied at beginning of fermentation, when sources of energy are available for active zinc translocation across yeast membranes. In many breweries, yeast cells are often repitched for several cycles. After one fermentation and prior to the following, yeasts may be stored in storage tanks where they are kept starved at low temperature (4°C) and high cell density for variable periods of time. Cells may also be acid-washed to reduce contaminant bacteria. Zinc supplementations in yeast storage tanks would not be recommended considering that yeast slurries would have limited energy supplies available for zinc uptake. Addition of zinc during acid washing was investigated by Taidi *et al.* (2000) who did not find any improvement in fermentation performance after zinc supplementation at this stage. Zinc addition to hot wort was also not effective since bioavailable zinc would be lost by chelation to the trub. Physiologically speaking, the best time proposed for any zinc supplementations would be at pitching, where bioavailability would be higher.
Concerning the effects of temperature on zinc uptake by yeast, lower temperatures impair zinc accumulation due to reduced metabolic activity of zinc transporters in the cell membrane (Mowll and Gadd 1983). Since brewing fermentations are usually carried out at lower temperatures than in the whisky and allied distilling industries, we conducted fermentations at 25°C and 8°C. We found that yeast cells had a reduced capacity to uptake zinc at 8°C during initial stages of fermentation, but after 48 hours of fermentation, the final zinc cell content was the same at both the temperatures under study. All zinc was finally removed from the medium, irrespective of fermentation temperature. Data obtained from this study may be of relevance to the brewing industry since zinc uptake may be delayed during fermentation at low temperatures. Importantly, we have also found that dead yeast cells (killed by treatment at 65°C for 1 hour) were unable to actively uptake zinc. Some initial passive binding of zinc to dead cell walls was noted, however this biosorption was only short term and this zinc was subsequently completely released back to the medium.

Maintenance of high viability yeast for brewing and distilling will therefore ensure maximum effective zinc uptake during fermentation.

Determination and localisation of intracellular zinc in yeast

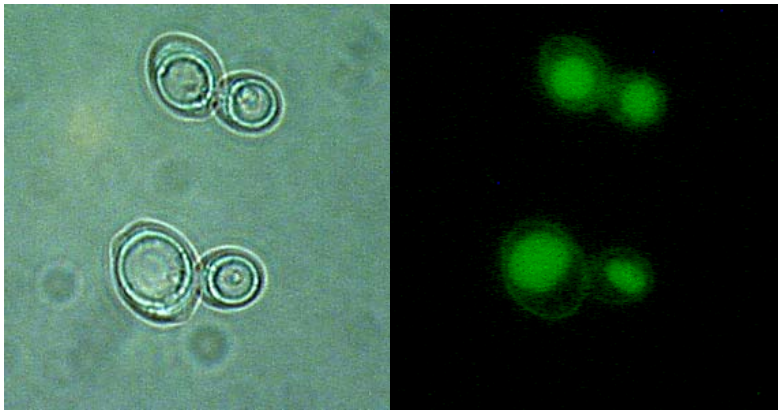
Specific transport mechanisms employed by yeast depend on the bioavailability of metal ions and the prevailing environmental conditions, but generally, most metals bind to yeast cells in a biphasic manner: firstly, by non-specific cell surface biosorption; and secondly, by selective transmembrane-mediated translocation into the cytosol. To facilitate the latter, diffusion channels and active transport are most likely to operate in *S. cerevisiae* with a proton-pumping ATPase-mediated mechanism prevailing for the majority of metal ions. Once transported into yeast cells, metals may end up in different cellular locations, including: free in cytoplasm at very low concentrations (often sub-μM); sequestration in cytoplasm (by metallothioneins, calmodulin, polyphosphates and polyamines); compartmentalised (in the cell wall, vacuole, Golgi apparatus, mitochondrion and nucleus), or detoxification / transformation (by reduction, methylation and alkylation). The yeast vacuolar membrane, called the tonoplast, is thought to play an important role in regulating ionic homeostasis and in detoxification of potentially toxic metals in yeast. MacDiarmid *et al* (2000) have shown that the vacuole plays a key role in regulating zinc homeostasis in yeast, with the *ZRT3* gene mediating vacuolar zinc uptake.

As the yeast vacuole is known to be a reservoir for many nutrients (Lichko *et al.*, 1982), we investigated the intracellular localisation of zinc using a combination of flow cytometery and fluorescence miscroscopy. Fig 4 shows that zinc in cells was primarily located in the vacuole.

Fig 4 *Free zinc ion localisation in yeast cells.*
Yeast cells (lager brewing strain LBB) were stained with both Fluo-Zn3 (for zinc visualisation) and cell-tracker B (for vacuole visualisation). Pictures were taken using a LEICA microscope in bright field (A), using a green filter (B) and a blue filter (C).

A: Control **B: Cells stained with Fluo-Zn3 to** **C: Cells stained with Cell-tracker B**

visualise free Zn ions **to visualize the vacuole**

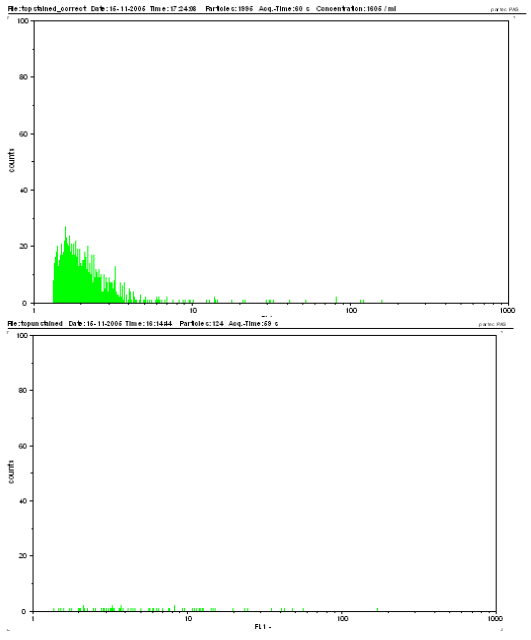


The vacuole in *S. cerevisiae* is actively divided between mother and daughter cells. This inheritance initiates early in the cell cycle and ends in G_2, just prior to nuclear migration (Weisman, 2003). A portion of the vacuole extends into the emerging bud

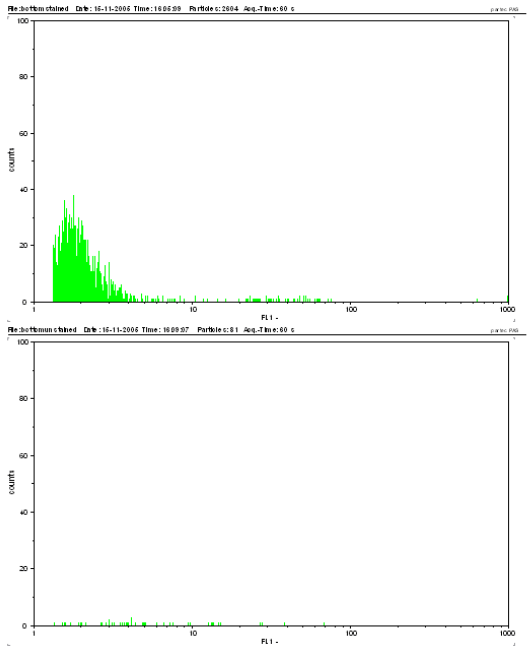
enabling continued exchange of vacuole contents and therefore zinc between mother and daughter vacuoles. We attempted to determine the exact proportion of zinc shared from mothers to daughters during cell division. After 16 hours of growth in malt extract broth (with Zn at 0.25 ppm), yeast cells were heterogeneous with respect to size. Prior to cell division, all zinc was completely taken up in the first hour. After division, the zinc cell content was shared from mothers to daughters. The use of fluorescent probes for zinc and the determination of the fluorescence emitted by cells of different sizes, by flow cytometry gave an estimate of intracellular free zinc. Fig. 4 shows that the intensity emitted by large mother cells (mean cell volume 132 fL) was higher than small daughter cells (57 fL).

Fig. 5 *Zn fluorescence in yeasts of different sizes analysed by flow cytometry*. Cells of lager brewing strain LBB were grown up in wort (Zn at 0.25 ppm), in shake flasks for 16 hours, harvested and separated using a lactose gradient solution. Samples were taken from the top of the gradient solution: mean cell volume, 57fL and from the bottom: mean cell volume132fL. Cells were stained with Rhod-Zn 1 and analysed by a Partec Cyflow flow cytometer. Stained cells (a) were compared to unstained (b) to determine the level of cellular autofluorescence (which was minimal).

a) Zn fluorescence in small cells (57 fL) b) Autofluorescence in small cells (57 fL)



a) Zn fluorescence in large cells (132 fL) b) Autofluorescence in large (132 fL)



These data indicated higher zinc concentration in mothers than in daughters and it is conceivable that mother cells possess a higher capacity to accumulate intracellular zinc levels when the zinc bioavailability is higher. It may also indicate uneven distribution of zinc between mother and daughter vacuoles prior to cell division. This may have practical applications in brewing where at end of fermentation, cells flocculate at the bottom of the fermenter cone. Powell *et al* (2004) have shown heterogeneity in yeast age, viability and fermentation performance of the yeast cone. Perhaps different levels of yeast cell zinc may also be expected; therefore the practice of removal of part of the yeast cone may deplete yeast biomass of an important reservoir of zinc.

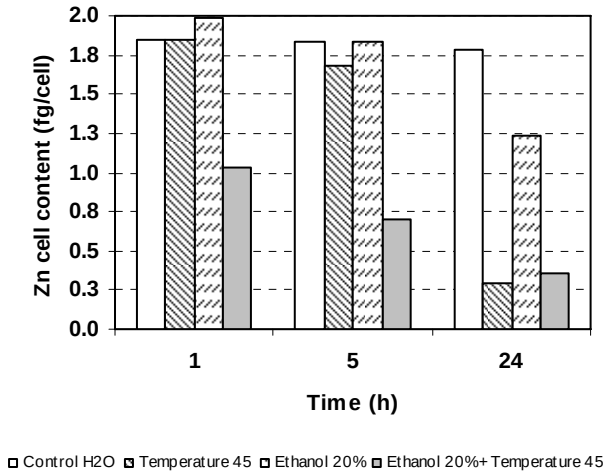
Zn and yeast stress

During fermentation, yeast cells take up metals for growth, cell division, energy transduction, and survival in the face of stress. In the fermentation industries, yeasts may be subject to a variety of chemical, physical and biological stresses which impact adversely on yeast growth and metabolic activity (reviewed by Walker, 1998a). The major stresses encountered by industrial yeasts are temperature shock, osmotic stress and ethanol toxicity.

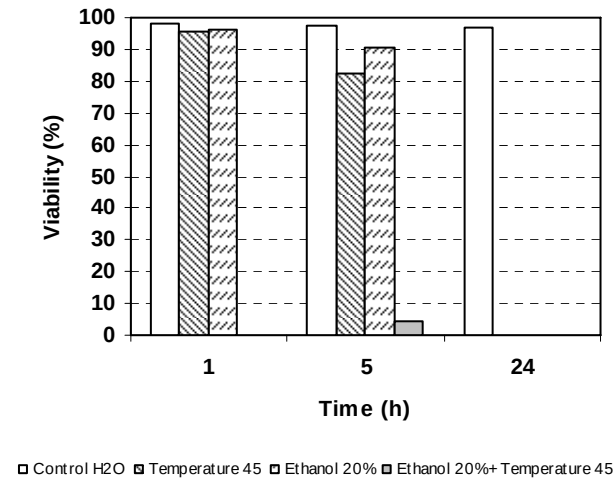
Fig. 6 *Influence of temperature and ethanol stress on intracellular zinc in yeast*.

A lager brewing yeast strain (LBB) was cultured in malt wort, resuspended in dH_2O or ethanol and stressed at 45ºC or in ethanol for 24 hours. Zinc cell content (a) and cell viability (b) were analysed.

a)



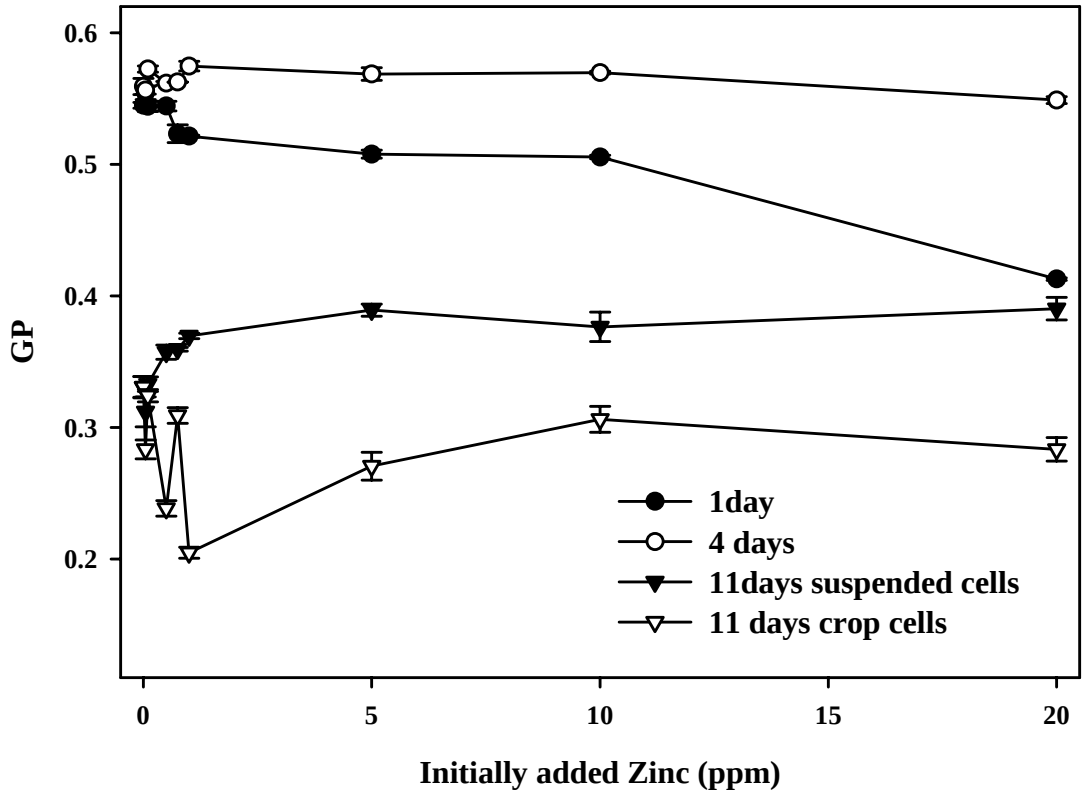
b)



We investigated the relationship between zinc in yeast cells and the response to various environmental insults. Fig 6 shows that stresses induce significant leakage of zinc ions from brewing strains of *S. cerevisiae*, and such leakage correlated to loss of culture viability. After 1 and 5 h exposure, cells retained zinc and viability following heat or ethanol stress, although the synergistic effects of heat and ethanol combined overwhelmed cellular defence systems. After 24 h exposure, all cells died, although some residual zinc was associated with the dead cell mass (Fig 6). We previously found that cellular magnesium enrichment (or preconditioning) conferred physiological protection on cells exposed to otherwise lethal heat shock or toxic ethanol (Walker, 1998b). It is conceivable that metals like magnesium and zinc may be exerting a general stress-protective role in yeast cells by charge-neutralisation of membrane phospholipids resulting in a stabilisation of the lipid bilayer and a decrease in membrane fluidity (Walker, 1999b). We therefore assessed membrane fluidity of the *S. cerevisiae* lager brewing strain LBB by measuring Generalized Polarization (GP) of the membrane-localising fluorescent probe Laurdan. We observed that zinc levels impacted upon growth rate, ethanol yields and membrane fluidity (Fig 7). We found that membrane fluidity varied with culture age and ethanol accumulation, as well as in relation to cellular zinc levels.

***Fig 7*. Generalised Polarisation at different levels of zinc during the fermentation process**

Cell GP of the lager brewing strain LBB under fermentation in conical Imhoff vessels (volume 1L, cone angle 74°) at 14°C. Results represent mean and standard deviation of 3 measurements. Where error bars are not shown they are less than the symbol size.



When different levels of zinc addition were compared on each day (Fig 7), some variation was seen indicating a complex relationship between membrane fluidity and initially added zinc levels. However, for each curve most points fell within 10% of the zero zinc GP value. It must be noted that membrane fluidity will relate to the actual level of zinc in cells at the time of measurement. The initially added levels provide a useful guide, but do not necessarily reflect cellular zinc levels which will also relate increase in biomass and non-biological removal of zinc. Further experiments will include determination of cellular zinc levels at each sampling time.

The GP of the cell membranes slightly increased (indicating a fluidity decrease) until the 4th day of fermentation and then gradually decreased throughout the remaining period. When GP values and ethanol levels were averaged for each day over all

ferments (containing the different initial zinc concentrations), compared to an average GP of 0.4176 and ethanol less than 1% after 1 day, there was a 9 % GP increase with average of about 2.5% ethanol on day 4, and thereafter decreased GP of 10% on day 6 and 24% on day 8 with ethanol levels averaging around 4 and 5%, respectively. By the end of fermentation at day 11, around 6% ethanol had accumulated, with a 31% decrease in GP in suspended cells and 45% decrease in crop cells. This indicates that membrane fluidity changes with cell aging and with the nutrient availability. The lower GP values indicate higher membrane fluidity, which likely reflects the higher ethanol levels as fermentation progresses, placing the cells under ethanol stress. At the end of fermentation, cells remaining in suspension had higher GP values than cells in yeast crop, which may relate to higher stress status of cells in the crop and locally increased ethanol concentrations.

Summary

There are several industrial implications arising from the research discussed in this paper. It is evident that many metals, particularly magnesium, calcium and zinc, strongly influence yeast fermentation performance and brewers and distillers should pay more careful attention to such minerals in malt wort than has hitherto been the case. The findings reported here clearly show the impact of zinc on yeast fermentation performance and stress physiology and the following conclusions may be drawn. Zinc is completely and rapidly taken up by yeast cells during fermentation; as a consequence beer is zinc depleted and cellular zinc content is variable. Zinc concentrations in the range 0.5 to 1 ppm appear appropriate in terms of efficient fermentation. However, in brewing and distilling processes it is important to determine in each situation the optimal zinc concentration for best fermentative performance by the yeast strains and wort formulation chosen. Irrespective of zinc availability, yeast membrane fluidity varies with culture age which may relate to metabolic status, increasing ethanol and decreasing sugar/nutrient levels. There is a complex relationship between yeast cell zinc status and membrane fluidity, but it appears that cells with low zinc content exhibit low GP levels indicating high membrane fluidity. Such cells would be more susceptible to membrane fluidisation by ethanol. Overall, the findings from this study provide new insight into yeast-metal interactions that are pertinent to optimisation of fermentation processes in the brewing and distilling industries.

Acknowledgements

We acknowledge with gratitude the work of the Abertay yeast research group, past and present, who have contributed greatly to the information discussed in this article.

References

Birch, R.M. and Walker, G.M., Influence of magnesium ions on heat shock and ethanol stress responses of *Saccharomyces cerevisiae*, *Enzyme and Microbial Technology*, 2000, 26, 678-687.

Birch, R.M., Ciani, M. and Walker, G.M.., Magnesium, calcium and fermentative metabolism in wine yeasts, *Journal of Wine Research*, 2003, 14(1), 3-15.

Birch, R.M., Dumont, A. and Walker, G.M., The role of magnesium and calcium in governing yeast agglomeration, *Food Technology and Biotechnology*, 2002, 40, 199-205.

Blackwell, K.J., Tobin, J.M. and Avery, S.V., Manganese uptake and toxicity in magnesium-supplemented and unsupplemented Saccharomyces cerevisiae, *Applied Microbiology and Biotechnology,* 1997, 47, 180-184.

Boulton, C.A, and Quain, D. E., Brewing Yeast & Fermentation. Blackwell Science, Oxford.

Bromberg, S.K., Bower, P.A., Duncombe, G.R., Fehring, J., Gerber, L. and Tata, M., Requirements for zinc, manganese, calcium and magnesium in wort, *Journal of the American Society of Brewing Chemists*, 1997, 55, 123-128.

Chandrasena, G., Walker, G.M. and Staines, H.J., Use of response surfaces to investigate metal ion interactions in yeast fermentations, *Journal of the American Society of Brewing Chemists*, 1997, 55, 24-29.

De Nicola, R., Interaction of zinc with industrial strains of the yeast *Saccharomyces cerevisiae*, PhD Thesis. University of Abertay Dundee, UK, 2006.

Densky, H., Gray, P.J. and Buday, A., Further studies on the determination of zinc and its effects on various yeasts, *Proceedings of the American Society of Brewing Chemists*, 1966, 93-94.

Desmartez, B., The suitability of some sugar preparations for the production of beer with a high alcohol content, *Cerevisiae and Biotechnology*, 1993, 18, 9-10.

Dombek, K.M. and Ingram, L.O., Magnesium limitation and its role in apparent toxicity of ethanol during yeast fermentation, *Applied and Environmental Microbiology*, 1986, 52, 975-981.

Eide, D.J., The molecular biology of metal transport in *Saccharomyces cerevisiae*, *Annual Review of Nutrition*, 1998, 18, 441-469.

Fantes, P. and Nurse, P., Control of cell size at division in fission yeast by a growth-modulated size control over nuclear division, *Experimental and Cellular Research,* 1977, 107, 377-386.

Gadd, G.M., Bioremedial potential of microbial mechanisms of metal mobilization and immobilization, *Current Opinion in Biotechnology*, 2000, 11, 271-279.

Hall, N., The influence of zinc on the physiology of industrial strains of *Saccharomyces cerevisiae*, PhD Thesis, University of Abertay Dundee, UK. 2001.

Hughes, M.N. and Poole, R.K., Metal speciation and microbial growth – the hard (and soft) facts, *Journal of General Microbiology*, 1991, 137, 725-734.

Ingledew, M. G., Alcohol production by Saccharomyces cerevisiae: a yeast primer. In: The Alcohol Textbook, 3rd ed., T.P. Lyons and D.R. Kelsall, Eds., Nottingham University Press: Nottingham, 1999, pp. 49-87.

Jones, R.P. and Gadd, G.M., Ionic nutrition of yeast – physiological mechanisms involved and implications for biotechnology, *Enzyme and Microbial Technology*, 1990, 12, 133-140.

Jones, R.P. and Greenfield, P.F., A review of yeast ionic nutrition, I.: Growth and fermentative requirements, *Process Biochemistry*, 1994, 4, 48-59.

Learmonth R. P. and Gratton E., Assessment of membrane fluidity in individual yeast cells by Laurdan Generalised Polarisation and Multi-photon Scanning Fluorescence Microscopy, In: Fluorescence Spectroscopy, Imaging and Probes- New Tools in Chemical, Physical and Life Sciences, Springer Series on Fluorescence: Methods and Applications, Springer, Heidelberg, 2002, (2), pp. 241-252.

Lentini, A., Jones, R.D., Wheatcroft, R., Lim, Y.H., Fox, C. Hawthorne, D.B. and Kavanagh, T.E., Metal ion uptake by yeast, *Proceedings of the Institute of Brewing (Australia and New Zealand Section)*, 1990, 158-163.

Lichko, L.P., Okorov, L.A. and Kulaev, I.S., Partecipation of vacuoles in regulation of levels of K^+, Mg^{2+} and orthophosphate ions in the cytoplasm of the yeast *Saccharomyces carlsbergensis*, *Archives of Microbiology*, 1982, 132, 289-293.

MacDiarmid, C.W., Gaither, L.A. and Eide, D., Zinc transporters that regulate vacuolar zinc storage in *Saccharomyces cerevisiae, EMBO Journal*, 2000, 19, 2845-2855.

Magonet, E., Hayen, P., Delforge. D., Delaive, E. and Remacle, J, Importance of the structural zinc atom for the stability of yeast alcohol dehydrogenase, *Journal of Biochemistry*, 1992, 287, 361-365.

Mowll, M.L. and Gadd, G.M., Zinc uptake and toxicity in the yeasts *Sporobolomyces roseus* and *Saccharomyces cerevisiae*, *Journal of General Microbiology*, 1983, 129, 3421-3425.

Parasassi, T., De Stasio, G., d'Ubaldo, A. and Gratton, E. Phase fluctuation in phospholipid membranes revealed by laurdan fluorescence. *Biophysical Journal*, 1990, 57, 1179-1186.

Powell, C.D., Quain, D.E., Smart, K.A., The impact of sedimentation on cone yeast heterogeneity, *Journal of American Society Brewing Chemistry,* 2004, 62 (1), 8-17.

Rees, E.M.R. and Stewart, G.G., Strain specific response of brewer's yeast strains to zinc concentrations in conventional and high gravity worts, *Journal of the Institute of Brewing*, 1998, 104, 221-228.

Rees, E.M.R. and Stewart, G.G., The effects of increased magnesium and calcium concentrations on yeast fermentation performance in high gravity worts, *Journal of the Institute of Brewing*, 1999, 103, 287-291.

Saltokoglu, A. and Slaughter, J.C., The effect of magnesium and calcium on yeast growth, *Journal of the Institute of Brewing*, 1983, 89, 81-83.

Smart, K., Chambers, K.M., Lambert, I., Jenkins, C. and Smart, C.A., Use of Methylene violet staining procedures to determine yeast viability and vitality, *Journal of American Society Brewing Chemistry,* 1999, 57 (1), 18-23**.**

Smith, G.D., Studies into Mg-preconditioning of brewer's yeast, PhD Thesis. University of Abertay Dundee, UK, 2001.

Smith, G.D. and Walker, G.M.., Fermentation performance of Mg-preconditioned yeast, In: Brewing Yeast Fermentation Performance, K.A. Smart, Ed., Blackwell Scientific Publications: Oxford, 2000, pp. 92-95.

Stehlik-Thomas, V., Grba, S. and Runjic-Peric, V., Zinc uptake by *Saccharomyces cerevisiae* and its impact on alcoholic fermentation, *Chemical and Biochemical Engineering Quarterly*, 1997, 11, 147-151.

Stewart, G.G. and Russell, I., An Introduction to Brewing Science and Technology. Series III. Brewer's Yeast. The Institute of Brewing, London. 1998.

Taidi, B., Hoogenberg, B., Kennedy A.I. and Hodgson, J.A., Pre-treatment of pitching yeast with zinc, Poster presented at the World Brewing Conference, Orlando, Florida, USA, 2000.

Van Uden, N., Temperature profiles of yeasts, *Advances in Microbiol Physiology*, 1984, 25, 195-251.

Walker, G.M., Accumulation of magnesium ions during the cell cycle. In: Cell Membranes and Cancer, T. Galeotti, A. Cittadini, G. Neri, S. Papa and L.A. Smets., Eds., Elsevier Applied Sciences: Amsterdam, 1985, pp. 161-165.

Walker, G.M., Magnesium and cell cycle control – an update, *Magnesium*, 1986, 5, 9-23.

Walker, G.M., The roles of magnesium in biotechnology, *Critical Reviews in Biotechnology,* 1994, 14, 311-354.

Walker, G.M., Yeast Physiology and Biotechnology. J. Wiley & Sons, Eds., Chichester, 1998a.

Walker, G.M., Magnesium as a stress-protectant for industrial strains of *Saccharomyces cerevisiae*, *Journal of the American Society of Brewing Chemists*, 1998b, 56, 109-113.

Walker, G.M., Media for Industrial Fermentation, In: Encyclopedia of Food Microbiology, J. Lederberg, Ed., Academic Press, New York, 1999a, pp. 674-683.

Walker, G.M., Biotechnological implications of the interactions between magnesium and calcium. *Magnesium Research*. 1999b, 12, 303-309.

Walker, G.M. Metals in yeast fermentation processes. Adv. in App. Micrbiol. 2004, 54, 197-229.

Walker, G.M. and Duffus, J.D., Magnesium ions and the control of the cell cycle in yeast, *Journal of Cell Science,* 1980. 42, 329-356.

Walker, G.M. and Maynard, A.I., Magnesium-limited growth of *Saccharomyces cerevisiae*, *Enzyme and Microbial Technology*, 1996, 18, 455-459.

Walker, G.M. and Maynard, A.I., Accumulation of magnesium ions during fermentative metabolism in *Saccharomyces cerevisiae*, *Journal of Industrial Microbiology and Biotechnology*, 1997, 18: 1-3.

Walker, G.M. and Smith, G.D., Metal ion preconditioning of brewers yeast. In: Proceedings of the 5th Aviemore Conference on Malting, Brewing and Distilling, I. Campbell., Ed., Institute of Brewing, London, 1999, pp. 311-315.

Walker, G.M., Birch-Anderson, A., Hamburger, K and Kramhoft, B., Magnesium-induced mitochondrial polymorphism and changes in respiratory metabolism in the fission yeast, *Schizosaccharomyces pombe*, *Carlsberg Research Communications* 1982, 47, 205-214.

Walker, G.M., Maynard, A.I. and Johns, C.W., The importance of magnesium ions in yeast biotechnology, In: Fermentation Technologies. Industrial Applications, P.K. Yu., Ed., Elsevier Applied Science, London, 1990, pp. 233-240.

Walker, G.M., Birch, R.M., Chandrasena, G. and Walker, G.M., Magnesium, calcium and fermentative metabolism in industrial yeasts, *Journal of the American Society of Brewing Chemists*, 1996, 54, 13-18.

Weisman, L.S. 2003. Yeast vacuole inheritance and dynamics. *Annual Review of Genetics*, 37, 435-460.

White, C. and Gadd, G.M., The uptake and cellular distribution of zinc in *Saccharomyces cerevisiae*, *Journal of General Microbiology*, 1987, 133, 727-737.

Youatt, J., Calcium and microorganisms, *Critical Reviews in Microbiology*, 1993, 19, 83-97.