Metals in yeast fermentation processes

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Metals in Yeast Fermentation Processes

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I. Introduction

Yeast cells have been used for millennia in traditional fermentations of cereal mashes, grape musts and other naturally-derived substrates. These processes still represent very important industries pertinent to the brewing, baking, winemaking and distilling sectors. The substrates in question provide rich sources of fermentable carbohydrate, utilisable nitrogen, vitamins, other growth factors, and minerals. Unfortunately, the latter are often overlooked as important determinants of yeast fermentation performance (see Fig. 1), and it should be emphasised from the outset that the nature and concentration of metal ions supplied in growth media can have a significant impact on yeast-based industrial processes. After all, a pre-requisite for the success of any yeast biotechnology is a thorough understanding of the factors that regulate nutrition, growth, stress responses and metabolism in yeast cells. These include inorganic factors.

Fig. 1 Factors affecting yeast fermentation performance
Yeast cells require a wide range of metals for their growth and metabolic functions and the mineral nutrition of yeasts is thus very important in ensuring successful fermentation, particularly in alcohol production processes. The bioavailability of essential metal ions in industrial media can dramatically influence yeast fermentation performance. For ethanol fermentations, these ions include magnesium and zinc that act as co-factors for important fermentative enzymes and also as modulators of environmental stress. Some metals inhibit yeast growth and metabolism, either by antagonism with essential metals (for example, calcium against magnesium) or through direct toxicity effects (as with heavy metals). This Chapter reviews the mineral nutrition of yeasts employed in fermentation processes, with a particular focus on the roles of magnesium, calcium and zinc in the physiology of industrial strains of the yeast, Saccharomyces cerevisiae.

II. Overview of Yeast Fermentation Processes

The so-called “conventional” yeast, S. cerevisiae, represents the most exploited microbe known to mankind, being responsible for the production of many diverse commodities from beer to blood proteins. Following developments in recombinant DNA technology, S. cerevisiae is now widely employed to express foreign genes and synthesise a range of health-care proteins including hormones, serum albumin, enzymes, vaccines, and other pharmaceuticals. Table 1 provides an overview of some yeast products important in modern biotechnology.

In recent years, it has become increasingly apparent that S. cerevisiae may not be the best yeast species to use in the production of high-value biopharmaceuticals. Other “non-conventional” yeasts, notably, Schizosaccharomyces pombe, Kluyveromyces lactis, Pichia pastoris, Hansenula polymorpha and Yarrowia lipolytica, display distinct advantages over

Table 1. Diversity of some yeast fermentation products

<table>
<thead>
<tr>
<th>Yeast species</th>
<th>Examples of industrial fermentation products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>Beer, wine, distilled spirits, bioethanol, baked foods, probiotics/animal food supplement, organic chemical reductions, hepatitis B vaccine, human insulin, human serum albumin</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>Some bioethanol, rum, wine-deacidification, indigenous fermented beverages, recombinant proteins</td>
</tr>
<tr>
<td>Kluyveromyces spp.</td>
<td>Cheese whey fermentations, biomass protein, pectinases, recombinant chymosin</td>
</tr>
<tr>
<td>Pichia pastoris</td>
<td>Recombinant proteins</td>
</tr>
<tr>
<td>Hansenula polymorpha</td>
<td>Recombinant proteins</td>
</tr>
<tr>
<td>Yarrowia lipolytica</td>
<td>Recombinant proteins</td>
</tr>
<tr>
<td>Phaffia rhodozyma</td>
<td>Food and feed pigment (astaxanthin)</td>
</tr>
<tr>
<td>Candida utilis</td>
<td>Biomass protein</td>
</tr>
<tr>
<td>Zygosaccharomyces rouxii</td>
<td>Traditional oriental fermented food (e.g. soy sauce, miso)</td>
</tr>
</tbody>
</table>

Unfortunately, our knowledge of the cell physiology of non-Saccharomyces yeasts is still rudimentary, and this also refers to mineral nutrition aspects.

III. Nutrition of Yeasts Employed in Fermentation Processes

A. Yeast growth v. fermentation

The primary aim of a yeast cell is to produce more yeast cells. This is also the case during industrial fermentation processes and the metabolites secreted during yeast growth merely represent waste products as cells strive to maintain their redox balance. Many of these metabolites are valuable fermentation products, an important example being ethanol which is produced when cells regenerate NAD in an attempt to keep glycolysis going and
to make sufficient ATP for cellular biosyntheses. Ethanol cannot be produced without
significant yeast cell growth and non-growing yeast cells ferment only enough sugar to
produce energy for cell maintenance. Therefore, the 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, but it has been calculated (Ingledew, 1999) that growing cells produce
alcohol 33 times faster than non-growing cells! 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. Metal ions may play a role in this metabolic
regulation. For example, Walker et al (1982) have shown that the availability of
magnesium ions can dictate whether fermentation or respiration predominates under
certain conditions of yeast cultivation. This concept is discussed further by Walker
(1994), who has proposed that under respirofermentative conditions magnesium governs
the flow of carbon into fermentation or respiration based on the relative affinities of
pyruvate metabolising enzymes for intracellular free magnesium ions.

B. Carbon and nitrogen requirements

Walker (1999a) has reviewed industrial growth media commonly employed in traditional
yeast fermentation processes for the production of foods and beverages. Being
chemoorganotrophs, yeasts require organic substrates as carbon and energy sources. Most
yeasts employed in industrial fermentations, namely strains of *S. cerevisiae*, effectively
utilise sugars such as sucrose, glucose, fructose and maltose for their growth and metabolism. Sources of these sugars are extracted from sugar crops (cane and beet juice and molasses), fruit juices (wine must) and cereal starches (barley, maize, and wheat starch hydrolysates). Non-Saccharomyces yeasts can extend the range of carbon sources for industrial processes and these include lactose (fermented by *Kluyveromyces marxianus*), xylose (*Pichia stipitis*, *Candida shehatae*), methanol (*Pichia pastoris*, *Hansenula polymorpha*), starch (*Schwanniomyces occidentalis*), inulin (*Kluyveromyces marxianus*), and n-alkanes (*Yarrowia lipolytica*). Table 2 summarises the diversity of carbon sources available to yeasts for industrial fermentation processes.

Table 2. Carbon sources for major yeast fermentation processes

<table>
<thead>
<tr>
<th>Carbon form</th>
<th>Examples</th>
<th>Industrial source</th>
<th>Yeasts involved</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexose sugars</td>
<td>Glucose, fructose</td>
<td>Grape juice</td>
<td><em>S. cerevisiae</em></td>
<td>Wine, Recombinant proteins, pharmaceuticals</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>Starch hydrolysates</td>
<td><em>S. cerevisiae</em> and other yeasts</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentose sugars</td>
<td>Xylose, arabinose</td>
<td>Wood/cellulosic hydrolysates, corn steep loquor</td>
<td><em>Pichia stipitis, Candida. shehatae</em></td>
<td>Ethanol, biomass</td>
</tr>
<tr>
<td>Disaccharides</td>
<td>Sucrose</td>
<td>Sugar cane/beet juice and molasses</td>
<td><em>S. cerevisiae</em></td>
<td>Ethanol, baker’s yeast, food extracts</td>
</tr>
<tr>
<td></td>
<td>Maltose</td>
<td>Cereal mashes</td>
<td><em>S. cerevisiae</em></td>
<td>Beer, distilled spirits</td>
</tr>
<tr>
<td></td>
<td>Lactose</td>
<td>Cheese whey</td>
<td><em>Kluyveromyces marxianus</em></td>
<td>Ethanol, biomass</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>Starch</td>
<td>Cereals, tubers</td>
<td><em>Schwanniomyces Kluveromyces</em></td>
<td>Ethanol, biomass</td>
</tr>
<tr>
<td></td>
<td>Inulin</td>
<td>Tubers (Agave, artichoke)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aliphatic alcohols</td>
<td>Ethanol</td>
<td>Distilling residues</td>
<td><em>Candida utilis</em></td>
<td>Biomass protein, Recombinant proteins</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>Petrochemicals</td>
<td><em>Pichia pastoris</em>, <em>Hansenula polymorpha</em></td>
<td></td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>C₁₂-C₁₈ n-alkanes</td>
<td>Petrochemicals</td>
<td><em>Yarrowia lipolytica</em></td>
<td>Biomass, recombinant proteins</td>
</tr>
</tbody>
</table>
In terms of nitrogen sources, many of the plant-based fermentation media listed in Table 2 also provide yeasts with readily utilisable sources of nitrogen essential for cellular biosyntheses and enzyme/nucleic acid function. *S. cerevisiae* is non-diazotrophic (cannot fix nitrogen) and non-proteolytic (being unable to utilise proteins as nitrogen sources). Various types of hydrolysed proteins, for example, corn steep liquor, casein, soybean, barley malt, and yeast extract provide mixtures of amino acids and small peptides that are able to support *S. cerevisiae* growth during fermentation. Additional forms of inorganic nitrogen, such as ammonium salts and urea, may be required as supplements for some natural complex yeast media. For distillery yeasts, levels of ammonium ions, urea and free alpha-amino nitrogen (FAN) are assimilable, but can be growth limiting. Ingledew (1999) has reported that the growth of distilling strains of *S. cerevisiae* increases almost linearly with FAN levels up to 100mg/L. Some types of molasses may be deficient in assimilable nitrogen (e.g. total N-compounds are only 2-3% in cane molasses) and must be supplemented with ammonia or urea (Walker, 1999a).

Many of the agriculturally-derived yeast media are considered complete in that they supply not just rich sources of carbon and nitrogen, but also a range of other nutrients, including vitamins and minerals. Mineral requirements of yeasts will now be addressed with the following discussions focusing primarily on *S. cerevisiae* alcoholic fermentation processes.
C. Mineral requirements

1. Why do yeasts need metals?

Metals are very important in several areas of yeast cell physiology. For example, yeast cells need metals for maintaining cell and organelle structural integrity, for cell-cell interactions such as flocculation, for gene expression, cell division and growth, for nutrient uptake mechanisms, for enzyme action in metabolism, for osmoregulation, and for energy maintenance and cell survival. Additionally, yeast cells need metals as stress-protectants in the face of environmental insults (refer to section VI. E).

Bulk metals, such as potassium and magnesium, are generally required by growing yeast cells in the millimolar concentration range, and the trace metals such as calcium, manganese, zinc, iron and copper, are required in the micromolar range. These essential metals play numerous structural and functional roles in yeast cell physiology. Other metals, even at trace level concentrations, may be toxic to yeast and these include heavy metals (see below). Jones and Gadd (1990) have reviewed yeast inorganic nutrition.

2. Essential metals for yeast growth and metabolism

In general terms, metal ions can impact on yeast growth and metabolic processes during fermentation by influencing several important parameters. For alcohol fermentations, these include 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. All of these parameters can impact significantly on the efficiency of industrial yeast fermentations. Table 3 lists those metals and their approximate concentrations generally required for cellular growth and reproduction of *S. cerevisiae*. The figures quoted are approximate because precise metal
requirements will differ depending on the particular strain of yeast and the cultivation conditions.

Table 3 Metals required for yeast cell growth and metabolic functions

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Concentration supplied in growth medium*</th>
<th>Main cellular functions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macroelements</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>2-4mM</td>
<td>Osmoregulation, enzyme activity</td>
</tr>
<tr>
<td>Mg</td>
<td>2-4mM</td>
<td>Enzyme activity, cell division</td>
</tr>
<tr>
<td><strong>Microelements</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>2-4µM</td>
<td>Enzyme cofactor</td>
</tr>
<tr>
<td>Ca</td>
<td>&lt;µM *</td>
<td>Second messenger, yeast flocculation</td>
</tr>
<tr>
<td>Cu</td>
<td>1.5µM</td>
<td>Redox pigments</td>
</tr>
<tr>
<td>Fe</td>
<td>1-3µM</td>
<td>Haem-proteins, cytochromes</td>
</tr>
<tr>
<td>Zn</td>
<td>4-8µM</td>
<td>Enzyme activity, protein structure</td>
</tr>
<tr>
<td>Ni</td>
<td>~10µM</td>
<td>Urease activity</td>
</tr>
<tr>
<td>Mo</td>
<td>1.5µM</td>
<td>Nitrate metabolism, vitamin B12</td>
</tr>
<tr>
<td>Co</td>
<td>0.1µM</td>
<td>Cobalamin, coenzymes</td>
</tr>
</tbody>
</table>

* Figures relate to *S. cerevisiae* growth stimulation, but are dependent on the yeast species/strain and precise conditions of growth
* See text for further discussion on calcium requirements for yeast growth

Potassium, magnesium, calcium and zinc are cationic nutrients which play essential structural and functional roles in yeast cells and are particularly significant in fermentation processes. Potassium is the most abundant cellular cation in yeast, constituting 1-2% of yeast cell dry weight, and is the main electrolyte essential for osmoregulation, charge-balancing of macromolecules, and regulation of phosphate and divalent cation uptake (Jones & Greenfield, 1994). Potassium additionally acts as a major cofactor for enzymes involved in oxidative phosphorylation, protein biosynthesis and carbohydrate catabolism.

Sodium is the other main monovalent cation, but it is important to note that although yeast cells may sometimes contain quite high levels of sodium, and fermentation media are also
often high in sodium, this metal appears to be non-essential for yeast. For example, under normal growth conditions, S. cerevisiae actively excretes sodium (via a sodium-proton antiporter) to maintain intracellular sodium at very low, sub-toxic levels. Although certain halotolerant and marine yeasts (e.g. Debaryomyces hansenii) grow well in saline environments, there is no evidence to suggest that S. cerevisiae needs sodium for cellular growth, even at very low concentrations. If sodium is present at high concentrations it may prove toxic to yeast, possibly by antagonising essential potassium-dependent functions.

Magnesium is the most abundant intracellular divalent cation in all living cells and is absolutely essential for yeast growth. Magnesium-deficient cells will not complete mitosis and no other metal in the periodic table can substitute for magnesium in this role (reviewed by Walker, 1994). Magnesium constitutes around 0.3% of yeast cell dry weight and acts an essential cofactor for over 300 enzymes intimately involved in many metabolic and bioenergetic pathways (e.g. magnesium is an absolute requirement for the synthesis of DNA and ATP). Changes in intracellular magnesium concentration can dramatically influence enzyme activity and Grubbs and Maguire (1987) have proposed a key regulatory function for magnesium ions in eukaryotic cell metabolism. Together with potassium, magnesium can neutralise the electrostatic forces in nucleic acids, polyphosphates, and proteins. Concerning the latter, magnesium maintains the tertiary structure of proteins and the general structural integrity of cells and organelles. Magnesium can also shield charged phospholipids and in doing so can maintain the structure of membranes, especially when cells are stressed. In short, magnesium plays multifaceted roles in yeast cell physiology at the cytological, biochemical and biophysical levels. Importantly with regard to industrial fermentation processes, magnesium is necessary for the activation of several glycolytic enzymes (e.g. all those involved in
transfer of phosphate moieties). 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 has long been ascribed a pivotal role as a second messenger of external stimuli in eukaryotic cells. Minute changes in intracellular calcium trigger cascades of protein kinase activity leading ultimately to initiation of key events such as the onset of mitosis. However, these changes in calcium are extremely small, and levels of intracellular free calcium are maintained at very low (sub-micromolar) levels. This, in turn, means that calcium requirements for cell division and growth are also very low. For yeast growth, we should therefore consider calcium to be a trace metal. Calcium binds to yeast cell walls and plays a key role in flocculation which is important in brewing fermentations. Calcium also antagonises uptake of magnesium and can block essential magnesium dependent metabolic processes. Calcium-magnesium antagonism, especially as it relates to yeast fermentation processes is discussed further below.

As for other trace elements, iron, zinc, nickel, copper, cobalt, manganese and molybdenum are required in metalloenzymes, redox pigments, haem-proteins and vitamins as structural stabilisers and as essential cofactors. For enzymes, some of these metals bind to catalytic active sites and this is the case with zinc. In alcoholic fermentations, zinc is particularly important 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 (as discussed below).
IV. Interaction of Yeasts with Metals

A. Mineral contents of yeast cells

Table 4 Shows the mineral content of a “typical” yeast cell. As with Table 3, the figures quoted are approximations because precise values of cellular minerals will depend on the particular yeast strain in question and its growth conditions. In addition, the phase of yeast growth and the position of cells in the cell division cycle may result in different cellular metal concentrations. For example, Walker and Duffus (1980) have shown that the magnesium content of dividing yeast cells varied in a temporal manner with cell cycle progress. For the fission yeast, *Schizosaccharomyces pombe*, it was revealed that intracellular magnesium levels fell during growth until a point just prior to mitosis, when a large influx of magnesium took place. This ensured that daughter cells at cell division received the same magnesium content as their mother cells had originally at the start of their cell cycle. Magnesium influx just before cell division was proposed to govern the disassembly of the mitotic spindle, specifically by de-polymerisation of tubulin, the major structural protein of microtubules. Walker (1986) has further discussed this role of magnesium in cell cycle control.
Table 4 Average elemental composition of *Saccharomyces* (g/kg dry wt)

<table>
<thead>
<tr>
<th>Element</th>
<th>Concentration (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium</td>
<td>22</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>16</td>
</tr>
<tr>
<td>Sulphur</td>
<td>3</td>
</tr>
<tr>
<td>Magnesium</td>
<td>2.7</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.6</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.5</td>
</tr>
<tr>
<td>Barium</td>
<td>0.15</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.12</td>
</tr>
<tr>
<td>Iron</td>
<td>0.1</td>
</tr>
<tr>
<td>Copper</td>
<td>0.05</td>
</tr>
<tr>
<td>Manganese</td>
<td>0.03</td>
</tr>
<tr>
<td>Cobalt</td>
<td>0.005</td>
</tr>
<tr>
<td>Nickel</td>
<td>0.0025</td>
</tr>
<tr>
<td>Arsenic</td>
<td>0.0018</td>
</tr>
<tr>
<td>Lead</td>
<td>0.0015</td>
</tr>
<tr>
<td>Iodine</td>
<td>0.00125</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>0.0007</td>
</tr>
<tr>
<td>Boron</td>
<td>0.0005</td>
</tr>
<tr>
<td>Aluminium</td>
<td>0.0001</td>
</tr>
<tr>
<td>Chromium</td>
<td>$10 \times 10^{-25}$</td>
</tr>
<tr>
<td>Vanadium</td>
<td>$5 \times 10^{-25}$</td>
</tr>
</tbody>
</table>

The metal content of yeast cells also depends on the phase of growth during cultivation in liquid medium and will vary between lag, logarithmic and stationary phases of the batch growth cycle. Walker and Duffus (1980) have shown changes in yeast cell magnesium levels during transitions between the lag and logarithmic growth phases and Walker and Maynard (1997) showed that *S. cerevisiae* cells released magnesium at the onset of the stationary phase.

Yeasts display differential affinities for certain metal ions. For example, *S. cerevisiae*, *Schizo. pombe* and *Candida utilis* possess high growth affinities for magnesium, with respective $K_s$ (saturation coefficients) values of 36μM (Walker and Maynard, 1996), 20μM (Walker, Maynard and Johns 1990) and 15μM (Shkidchenko, 1977). These micromolar values reflect the high growth demands that yeasts have for magnesium. This means that it is feasible to prepare Mg-limited growth media for yeast, and to grow cells
under Mg-limited conditions in a chemostat. Walker and Maynard (1996) accomplished this for *S. cerevisiae* and were able to facilitate studies of yeast cell physiology under conditions in which cell growth was dictated solely by magnesium ion availability. Such experiments would not be feasible with calcium because yeasts have a low growth demand for this metal (i.e. high Ks value) and it is not possible to cultivate cells in a chemostat with calcium as the sole growth-limiting nutrient.

**B. Yeast transport strategies for metals**

Yeast cells can transport, localise, compartmentalise and sequester metals required for various physiological functions and can neutralise metals that are potentially toxic. These functions include: intracellular pH homeostasis, osmoregulation, enzyme function, protein structure, membrane stabilisation and signal transduction. For yeast growth and survival, cellular concentrations of metals are maintained within relatively narrow ranges though a variety of homeostatic mechanisms (reviewed by Walker, 1998a) In order to take up metals from their growth environment, yeast cells must transport metals as free, ionised forms. Therefore, several physico-chemical constraints may impede metal ion uptake by yeast and these include chelation, adsorption, and binding. In complex growth media like sugarcane molasses or malt wort, this can lead to reduced metal bioavailability during fermentation. To increase metal bioavailability from their growth environment, some yeast species may mobilise metal ions by secreting low-molecular weight metal-sequestering compounds called siderophores (Van der Helm and Winkelmann, 1994) or organic acids such as citric acid (White, Sayer and Gadd, 1997). Although a few yeasts have been shown to excrete siderophores (for iron uptake), yeasts more commonly internalise essential metals through specific membrane transport systems. However, in order to be transported into the yeast cellular milieu, several barriers firstly need to be overcome by metals. These include the capsule (exopolysaccharide layer, if present), cell
wall, periplasm, plasma membrane and organellar membranes. 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, the following strategies may be adopted: free diffusion, facilitated diffusion, diffusion channels and active transport. Of these, the latter two are most likely to operate in *S. cerevisiae* with a proton-pumping ATPase mediated mechanism prevailing for the majority of metal ions. This enzyme is very important for metal accumulation in yeast but it also regulates growth and fermentation by excreting acidity and regulating cell pH (yeasts can lower external pH to ~1.5 and during fermentation, and around 30% of media acidity is attributed to ATPase activity). The primary driving force for the ATPase-mediated mode of metal uptake by yeast is the membrane potential and the transmembrane electrochemical proton gradient, generated by ATP-hydrolase activity. The latter extrudes protons using the free energy of ATP hydrolysis and enables metal ions to enter yeast cells either with influxed protons (as in symport mechanisms) or against effluxed protons (as in antiport mechanisms). Such a mechanism requires participation of metal-translocating permeases, of which there are several specific high-affinity types identified in *S. cerevisiae*. These processes can also operate in the opposite direction to facilitate controlled efflux of metals (e.g. calcium and copper) to maintain low intracellular levels. Some low-affinity, relatively non-specific, permeases operate to transport metals when they are present in high abundance extracellularly.

The other main mechanism for metal uptake by yeast cells involves diffusion channels that are voltage-dependent membrane proteins activated by membrane depolarisation to influx (or efflux) specific ions like potassium (Reid *et al*, 1996). In fermenting cells of *S.
cerevisiae, potassium accumulation is rapid.Mechanosensitive ion channels also exist in yeast cell membranes to control calcium ion homeostasis (Gustin et al, 1986).

C. Molecular biology of metal uptake by yeast

As discussed above, the majority of metals are taken up into yeast cells via specific active transport proteins. These transporters possess varying affinities for particular metals: high-affinity systems ensure that essential metals are accumulated under conditions of limited availability, whilst low-affinity systems control uptake when metals are present in excess. Recent molecular genetic studies with S. cerevisiae have revealed several genes encoding specific metal ion transporters (see Table 5).

Table 5 Some metal ion transporters in S. cerevisiae

<table>
<thead>
<tr>
<th>Genes</th>
<th>Transporters</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZRT1/2, ZRT3</td>
<td>Zn</td>
<td>high/low affinity; vacuolar</td>
</tr>
<tr>
<td>IRT1, FET, FTR, FRE</td>
<td>Fe</td>
<td>also transports Mn and Zn</td>
</tr>
<tr>
<td>SMF1/2, CDC1, PMR1, CCC1, ATX1</td>
<td>Mn</td>
<td>membrane, cytosol, Golgi transporters</td>
</tr>
<tr>
<td>CTR1, CCC2</td>
<td>Cu</td>
<td>cellular and Golgi transporters</td>
</tr>
<tr>
<td>ALR1/2, MRS2</td>
<td>Mg</td>
<td>membrane and mitochondrial</td>
</tr>
<tr>
<td>Channel protein-encoding gene</td>
<td>Ca</td>
<td>mechanosensitive ion channel</td>
</tr>
</tbody>
</table>

Eide (1998) has reviewed the molecular biology of metal transport in yeast and some of the S. cerevisiae genes have now been shown to encode proteins capable of transporting several metals. For example, the Smf proteins (encoded by the SMF family of transport genes) play a major role in regulating copper and manganese homeostasis and, under certain conditions, Smf1p may also function in iron assimilation by cells (Cohen, Nelson and Nelson, 2001). There are many similarities between human and yeast cells in terms of metal uptake mechanisms and genetic homology in Saccharomyces cerevisiae and Homo sapiens has now been demonstrated for Fe, Cu, Mn, and Mg transporters (e.g. Zsurka,
Gregan and Schweyen, 2001). This has lead to yeast being used to study the molecular bases of certain human genetic disorders linked to dysfunction of metal homeostasis, including Wilson’s and Menkes syndromes. These are diseases of copper overload and copper deficiency, respectively. Remarkably, the relevant human genes which regulate copper homeostasis can substitute for their S. cerevisiae counterparts, enabling their structure and function to be effectively studied in yeast (Nelson, 1999; Askwith and Kaplan, 1998).

For cellular magnesium transport by S. cerevisiae, two plasma membrane transporters, encoded by ALR1,2 genes and one mitochondrial transporter, encoded by the MRS2 gene, have been demonstrated. The latter shows homology with a human mitochondrial Mg transporter. Recent evidence (Lui et al, 2002) has been presented which implicates Alr1p as a Mg-channel transporter in yeast cells. ALR1 encodes a 96kDa membrane-spanning protein which transports Mg, and mutants lacking ALR1 contain much less Mg and need high Mg levels for growth (Graschopf et al, 2001). MacDiarmid and Gardner (1998) have also shown that ALR1 increases tolerance to Al^{3+} in yeast and that inhibition of magnesium uptake may be the main cause of aluminium toxicity in yeast. In acidic soil conditions, aluminium can be leached from insoluble forms and in certain industrial fermentation processes, notably those employing sugar cane molasses, aluminium may be toxic to yeast. Suppression of yeast fermentation performance by aluminium may possibly be ameliorated by magnesium.

D. Fate of intracellular metals in yeast

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 may become detoxified/transformed (following reduction, methylation and dealkylation). Considering compartmentalisation of metals in yeast cells, selective transport protein genes have now been identified that control organelar membrane transport. For example, in *S. cerevisiae* the *CCC2* encoded protein regulates export of copper into the lumen of the Golgi; the *PMR1* gene is involved with uptake and release of manganese from vacuoles; the *MRS* gene controls mitochondrial uptake of magnesium and the *ZRT3* gene mediates zinc uptake in the vacuole. 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. Tonoplast uptake mechanisms resemble those of the yeast plasma membrane with proton-pumping ATPases involved in transport of magnesium, manganese, iron, zinc, cobalt, calcium and nickel to the yeast vacuole. Beeler, Bruce and Dunn (1997) have shown that, in *S. cerevisiae*, the vacuole plays an important role in regulating intracellular magnesium levels, especially under magnesium-limited growth conditions. Similarly, MacDiarmid, Gaither and Eide (2000) have shown that the vacuole plays a key role in regulating zinc homeostasis in yeast.

The cell wall is also a major site for metal localisation in yeast, and this mode of metal binding is often referred to as biosorption or bioaccumulation (Fuhrmann and Rothstein, 1974; Norris and Kelly, 1977; Walker, 1985; Brady and Duncan, 1994; Engl and Kunz, 1995). This represents a biophysical attachment of metals to negatively-charged cell wall moieties (e.g. carboxyl groups) and is the first step in the biphasic uptake of metals by yeast (the second being transmembrane uptake). Metal binding to yeast cell walls is an immediate, fairly non-specific event. With regard to yeast fermentation processes, the cell wall binding of calcium ions is important in flocculation mechanisms. This phenomenon
is particularly relevant for brewing strains of S. cerevisiae. Calcium is thought to participate in yeast flocculation by activating cell wall α-mannan residues, thus enabling lectin proteins to facilitate adhesion between adjacent yeast cells (Miki et al, 1982). Another yeast cell-cell interaction phenomenon which involves metal ions is agglomeration. This is also called yeast “grittiness” and is occasionally experienced following the growth of baker’s yeast (S. cerevisiae) on molasses. Agglomeration is detrimental to yeast quality for baking because cells fail to resuspend in water and this adversely affects subsequent fermentation performance. Although it is a type of yeast cell adhesion, agglomeration is distinct from flocculation (which is a reversible process). Guinard and Lewis (1993) have proposed that calcium ions were involved in promoting baker’s yeast agglomeration, whilst more recently, Birch, Dumont and Walker (2002) have shown that magnesium acted antagonistically against calcium-induced agglomeration, possibly by blocking calcium binding to cell surface receptors.

E. Metals toxic to yeast and detoxification strategies

Many metals are toxic to yeast cells, but the degree of toxicity depends on the actual metal in question, its concentration and its bioavailability. Heavy metals generally adversely affect yeast growth at concentrations greater than around 100µM (Rose, 1976). Metal-induced toxicity towards yeast is expressed at the levels of both cytotoxicity and genotoxicity through damage inflicted on cellular proteins and DNA, respectively. Those metals that may occasionally prove toxic to yeast during fermentation processes include: copper, cobalt, aluminium, manganese, cadmium, zinc, nickel, mercury, arsenic and lead. For example, copper is an essential metal for yeast respiratory pigments, but above certain threshold concentrations may be toxic.
Some yeasts, together with filamentous fungi have the ability to carry out heavy metal detoxification using a variety of strategies including chemical transformation, sequestration, cell wall biosorption, immobilisation, and protection (e.g. binding competition or membrane stabilisation by beneficial metals). Potentially toxic levels of calcium ions are maintained at very low levels (often sub-micromolar) by intracellular binding to Ca-specific proteins such as calmodulin, which has been identified in *S. cerevisiae* and other yeasts. In certain yeasts and in filamentous fungi, intracellular sequestration of metals may also be achieved by binding to metallothioneins and phytochelatins (Winkelmann and Winge, 1994). Metallothioneins are small cysteine-rich polypeptides that bind to essential metals such as copper and zinc, as well as to toxic metals such as cadmium. Copper-resistance in *S. cerevisiae* is conferred by induction of copper-metallothionein biosynthesis. Phytochelatins are D-glutamyl peptides derived from glutathione which are involved in heavy metal detoxification in some yeasts and fungi, as well as in plants and animals. Yeasts can also chemically transform metals to reduce their toxic effects (see Gadd and Sayer, 2000). Such transformations involve reduction (e.g. Cu (II) to Cu (I); Fe (III) to Fe (II); and Se (VI) to Se (IV) to elemental Se), methylation (e.g. of arsenic and selenium) and dealkylation (e.g. of organotin compounds to Sn (II) and organomercury compounds to Hg).

Magnesium has been shown to alleviate the toxic effects of several heavy metals, including aluminium (McDiarmid and Gardner, 1996), cadmium (Kessels *et al*, 1985), cobalt (Aoyama *et al*, 1986), copper (Karamushka and Gadd, 1994), manganese (Blackwell *et al*, 1997), and zinc (Karamushka *et al*, 1996). These protective effects of magnesium are thought to be mediated by membrane stabilisation (e.g. charge neutralisation of phospholipids) and competitive membrane binding in the face of heavy
metal toxicity. This may have some practical implications for yeast fermentation processes (see below).

V. Practical Significance of Metal Uptake by Yeast

A. Bioremediation. This relates to removal of heavy metals in industrial wastewaters which may be accomplished using yeasts and other microorganisms (reviewed by Gadd, 2000). For example, *S. cerevisiae* is very effective in sequestering zinc and the potential exists to use yeast, including residual yeast from fermentation industries, to biosorb zinc from effluents (e.g. from the electroplating industry). It may be possible to recover/recycle zinc from yeast. The cell wall plays an important role in zinc sequestration by yeast (White and Gadd, 1987). For example, Hall (2001) has shown that in actively-dividing, viable cells of *S. cerevisiae*, most zinc is soluble (vacuolar) whilst in starved or non-viable cells most zinc is insoluble (cell wall). This means that dead yeast cells, or even yeast cell wall preparations could potentially be used in bioremediation of zinc from industrial process effluents.

B. Biomineral nutrition. This relates to the use of yeast in human dietary supplements as sources of trace minerals. *S. cerevisiae* possesses several attributes as a biomineral nutrient including it’s safety/non-pathogenicity; availability/economy; well developed technology; public acceptability and nutritional value. Regarding the latter, yeast cells comprise the following cellular constituents: proteins (~50%), carbohydrates (~30%), lipids (~5%), nucleic acids (~10% RNA), vitamins, antioxidants and minerals. Easily-grown and readily available yeasts such as baker’s or brewer’s strains of *S. cerevisiae* represent excellent natural sources of essential metals such as K, Mg, Ca, Fe, Mn, and Zn and this yeast can be further artificially enriched with several other inorganic micronutrients including selenium, molybdenum and chromium. Such yeasts are now
commercially produced as effective carriers of these trace elements for use in alleviation of dietary deficiencies in humans and animals.

C. Beverages. The ability of yeast cells to accumulate metals may be usefully exploited in alcoholic beverage biotechnology. For example, Smith and Walker (2000) have investigated the potential of using metal-enriched *S. cerevisiae* to improve fermentation performance. They 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 and also displayed increased tolerance to stress. Mineral-enriched yeasts have potential in addressing the problem of insufficient bioavailable metal ions for optimal fermentation performance by yeast and some commercial products (e.g. zinc-enriched *S. cerevisiae*) are now available as fermentation supplements. Such products may also be acceptable for use in German breweries and Scotch Whisky distilleries that do not allow mineral supplements (in the form of inorganic salts) due to national legislative restrictions.

D. Bioethanol. Over 30 billion litres of ethanol are produced per annum, and around 60% of this is for fuel use. Bioethanol, that is fermentation alcohol destined for fuel use (as both an extender and as an additive to gasoline), is already produced on a large scale in Brazil and North America, and is set to increase significantly in the UK and in Europe. The substrates currently employed are sucrose (juice & molasses), and starch (cereals), but there is potential in exploiting lactose (from cheese whey), fructose (from plant tuber inulin), and cellulose/lignocellulose (from forestry and agriculture) in the future. The “ideal” yeast for bioethanol production would possess the following characteristics: rapid and efficient fermentation (with minimal yeast growth and foaming characteristics); consistently low production of secondary fermentation metabolites (glycerol, fusel oils);
stress-tolerance (ethanol, osmotic, temperature, acid, bacteria); appropriate flocculation characteristics; high viability and vitality for re-cycling/pitching and genetic stability. It may be possible, using metal-enriched yeast seed cultures, to improve some yeast physiological characteristics (such as fermentation efficiency and stress-tolerance) which would benefit bioethanol producers. Walker and Smith (2000) have already shown that magnesium preconditioned S. cerevisiae exhibit improved fermentation performance and increased stress-resistance. Smith (2001) further showed that elevated cellular magnesium content of preconditioned yeast correlated with increased activity of pyruvate decarboxylase, a key enzyme of fermentative metabolism. On a similar vein, Hall (2001) showed a correlation between cell zinc content and alcohol dehydrogenase activity in industrial strains of S. cerevisiae. Such physiological cell engineering of yeasts holds promise for the fermentation industries at a time when there is reluctance to embrace genetic engineering (at least for food and potable alcohol producers). It is clear, however, that further exploitation of metal-enriched yeast for alcohol fermentation processes requires more research in terms of metal uptake, cellular localization and utilisation.

VI. Metals and Yeast Fermentation Processes

A. Metals important in 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 nature and concentration of metal ions in fermentation media are indeed important factors that influence yeast cell physiology and production of yeast fermentation commodities. 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.

Zinc is an essential micronutrient for yeast and occasionally brewer’s wort may be Zn-deficient resulting in impaired fermentation performance (Densky, Gray and Buday, 1966; Desmartez, 1993; Bromberg et al, 1997; Stehlik-Thomas, Grba and Runjic-Peric, 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 below around 0.1ppm. Zinc plays a major role in yeast fermentative metabolism because it is essential for ethanol dehydrogenase activity (the terminal Zn-metalloenzyme in alcoholic fermentation – see Magonet et al, 1992), but it can also stimulate uptake of maltose and maltotriose into brewing yeast cells, thereby augmenting fermentation rates. Table 6 summarises important roles for zinc in yeast physiology.
Table 6 Roles for zinc in yeast physiology pertinent to fermentation processes

<table>
<thead>
<tr>
<th>Role</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme activity</td>
<td>Dehydrogenases (eg. alcohol dehydrogenase, glutamate dehydrogenase, glyceraldehyde 3-phosphate, aldehyde dehydrogenase, cysteine desulphydrase, carbonic anhydrase, carboxypeptidase A &amp; B, alkaline phosphatase, α-mannosidase, aldolase, superoxide dismutase, DNA/RNA polymerase, ribonuclease)</td>
</tr>
<tr>
<td>Protein structure maintenance</td>
<td>Zn-finger DNA binding proteins</td>
</tr>
<tr>
<td>Cell surface integrity</td>
<td>Promotes yeast flocculation, stabilises cell membranes</td>
</tr>
<tr>
<td>Sugar uptake</td>
<td>Stimulation of maltose and maltotriose uptake</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Activation of riboflavin synthesis</td>
</tr>
</tbody>
</table>

Calcium requirements for yeast fermentation are arguable. Certainly, a clear-cut requirement for external calcium ions for growth of yeast cells (and other microbial cells) has yet to be demonstrated (Youatt, 1993). Cells actively exclude calcium to maintain sub-toxic cytosolic levels and intracellular calcium concentrations are further controlled by specific Ca-binding proteins such as calmodulin. Calcium’s role in yeast fermentation processes appears to be mainly as an extracellular cation. For example, calcium may act as a protector of certain secreted proteins (such as hydrolytic enzymes) and as a facilitator of yeast-yeast interaction during flocculation (Miki et al, 1982) and agglomeration (Guinard and Lewis, 1993). The presence of excess calcium in fermentation media (e.g. in molasses, malt wort etc.) can inhibit yeast growth (Saltukoglu and Slaughter, 1983) and fermentative activity (Walker et al, 1996). These effects of calcium may be expressed at the level of direct inhibition, or through antagonism with other essential cations, notably magnesium. Calcium can detrimentally affect yeast physiological functions by
antagonising magnesium uptake and by suppressing magnesium-dependent enzymes. Calcium-magnesium antagonism with respect to yeast fermentation processes is discussed further below (section VI. C).

Other trace metals that may influence fermentation include manganese, copper and iron. These are required for yeast metabolism as enzyme cofactors (especially Mn), and in yeast respiratory pathways as components of redox pigments (especially Fe and Cu).

Until relatively recently, little attention has been paid to the roles of magnesium in yeast physiology and fermentation performance. Magnesium ions participate in a myriad of physiological processes in yeast cells including cell division cycle progression, intermediary and biosynthetic metabolism environmental stress-protection and the general maintenance of cell viability and vitality (see Table 7). 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, Ciani and Walker, 2003) is now recognised as being very important for efficient industrial fermentations with this yeast.

### Table 7 Roles for magnesium in yeast physiology pertinent to fermentation processes

<table>
<thead>
<tr>
<th>Role</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme action</td>
<td>Essential cofactor for numerous (over 300) enzymes, especially those required for glycolysis (including pyruvate decarboxylase)</td>
</tr>
<tr>
<td>Cell viability and growth</td>
<td>Magnesium absolutely required for cell division cycle progress in yeast (stimulates DNA synthesis and onset of mitosis). Yeasts have high growth demands for magnesium (low Ks values- see text). Cells can be synchronised into division using a Mg starve-feed regime</td>
</tr>
<tr>
<td>Cell and organelle structure</td>
<td>Membrane ribosome and mitochondria stabilisation</td>
</tr>
<tr>
<td>Stress-protectant</td>
<td>Counteracts stresses caused by temperature, osmotic pressure, oxygen free radicals, heavy metals (see Table 8)</td>
</tr>
</tbody>
</table>
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).

However, important questions remain regarding magnesium and other metals in industrial fermentation processes. For example: Do growth media metal ion levels remain constant? Is there sufficient bioavailable metal ion for optimal enzyme action/fermentation? Do levels of certain metals antagonise beneficial effects of others? The following sections attempt to provide some insight into these questions.

B. Bioavailability of metals in industrial media
The major factors which impact on yeast fermentation performance, particularly for the production of ethanol, are: yeast strain (genotype), nutrients, physical conditions and competitive microbes (notably wild yeasts and bacteria). Mineral nutrients should be given careful attention because efficient conversion of carbon source (e.g. sugar) to desired product (e.g. ethanol) by fermentation depends not solely on the available fermentable carbon, but also on the bioavailability of essential metal ions. Metal composition of fermentation media will vary greatly depending on raw materials and process conditions. Therefore, any factor which reduces metal bioavailability and compromises metal ion uptake will, in turn, adversely affect yeast growth and fermentative activity. An important question which thus arises is: are the minerals supplied in industrial fermentation media bioavailable for yeast cell assimilation? Bioavailability depends on metal solubility and the properties of metal-complexing ligands. Generally, industrial fermentation feedstocks such as molasses and malt wort contain many metal chelating and absorbing components which can reduce bioavailability. The levels of un-complexed, un-absorbed and un-sequestered metals in
yeast growth media represent biologically free levels and are much more meaningful than total levels (as discussed by Hughes and Poole, 1991). Free metals represent bioavailable metals and attention to metal bioavailability may prevent slow and premature fermentations conducted by yeast. For magnesium ions in fermentation, the following considerations are important:

1. Yeast demand for Mg during fermentation is high (for glycolytic enzyme activity)
2. Free (biologically available) Mg may not be sufficient to meet this demand
3. Ca antagonism reduces Mg uptake and Mg bioavailability
4. Increasing free Mg in stimulates fermentation.

By increasing magnesium ion bioavailability, either extracellularly with media supplements (Walker et al, 1996), or intracellularly by yeast cell preconditioning (Walker and Smith, 1999; Smith and Walker, 2000), certain improvements become evident in yeast fermentation performance and in cellular stress-protection. In industrial fermentations, magnesium bioavailability may be augmented by supplementing media with magnesium salts (e.g. magnesium sulphate), by using magnesium-enriched (or preconditioned) yeast, or by using proprietary yeast “foods”. The latter have multifunctional roles such as: alleviation of CO₂ inhibitory effects, provision of extra sources of assimilable nitrogen (hydrolysed protein), vitamins and metal ions (to increase bioavailability). Magnesium supplements have been shown to improve fermentation in the following industrial feedstocks: molasses, malt wort, cheese whey, wine must (Walker et al, 1996).

In summary, several factors may reduce the bioavailability of essential metal ions in yeast fermentation processes, including the makeup of the media employed, processing conditions and the presence of antagonistic and toxic metals. However, several relatively straightforward strategies can be adopted to counteract such reduction.
C. Metal-metal interactions

Interactions between metals in fermentation media can influence essential metal ion bioavailability and, consequently, yeast physiology. An imbalance of mineral nutrition, particularly with respect to metal-metal antagonism, can result in complex alterations in yeast growth and metabolism. Metals may compete with each other for binding sites on and in yeast cells and they may act antagonistically toward each other in terms of biochemical functions. Knowledge of metal-metal interactions is important in media optimisation studies. Chandrasena, Walker and Staines (1997) have investigated metal ion interactions in yeast fermentations, particularly with regard to K, Mg, Ca and Zn interactive effects on alcohol production by *S. cerevisiae*. Fermentation media were designed to simulate high, intermediate and low levels of K, Mg and Ca in molasses and similarly for Mg, Ca and Zn in malt wort. Subsequent ANOVA (analysis of variance analysis) of fermentations with these levels of metals showed that alcohol production by yeast depended on complex interactions among the relevant metals. It was found that for a fixed level of Mg in molasses, ethanol production varied with changing levels of Ca and K in a predictable way (a response surface model fitted). In addition, for high levels of Mg, the model showed that certain combinations of K and Ca could maximise ethanol production following molasses fermentations. In malt wort, Mg, Ca and Zn were found to exert significant interactive effects on fermentation and it was concluded that statistical modelling using response surfaces had the potential to predict fermentation performance in media with variable levels of metal ions.

In terms of antagonistic interactions, the biochemical antagonism between magnesium and calcium may have practical implications for yeast fermentation industries. Many
enzymes, particularly several transphosphorylases of glycolysis, have specific and essential requirements for magnesium and these enzymes are inhibited by calcium ions which bind competitively to them (Heaton, 1990; Kaim and Schwederski, 1994; Walker, 1999b). Magnesium is absolutely required as a cofactor for numerous enzymes in cells, but relatively few enzymes by comparison need calcium. Other physiological differences between magnesium and calcium include the active cellular inclusion of magnesium, but the active exclusion of calcium. This is reflected in major cellular concentration differences between the two cations; intracellular free magnesium being around 0.5-1.0mM, whist calcium is maintained at sub-micromolar levels (around 100nM).

Unfortunately, this differential cellular demand for magnesium and calcium is not met by industrial yeast growth media many of which contain calcium levels that are much higher than magnesium (Walker, 1994). This physiologically anomalous situation can be signified by the following concept:

For example, cellular Mg:Ca ratios may be as high as 1000:1 (for intracellular free ions), but media Mg:Ca ratios may be as low as 0.1:1 (for some types of molasses). In other words, some industrial fermentation media may not be satisfying yeast physiological
requirements for these particular metals. Walker (1999b) has discussed the biotechnological significance of magnesium-calcium antagonism, which in yeast fermentation processes is manifest by calcium counteraction of magnesium stimulatory effects. Basically, by increasing Mg:Ca ratios in fermentation media, Walker et al (1996) found it possible to improve alcohol production. This was presumably due to suppression of the inhibitory effects of calcium on magnesium uptake and cellular utilisation. Careful adjustments of external magnesium and calcium concentrations are therefore viewed as a relatively simple means of manipulating yeast fermentation performance.

C. Demand for metals by yeast during growth and fermentation

During fermentation, yeast cells take up metals in order to satisfy various physiological needs. Such needs are nutrient uptake, growth, cell division, energy transduction, and survival in the face of stress. Cellular uptake and subsequent metabolic utilisation of metal ions are prerequisites for maximising fermentation performance by yeast. This is especially evident for metals which are essential cofactors for glycolytic and alcohologenic enzymes. Magnesium and zinc are two such metals.

For magnesium, Walker and Maynard (1997) have shown that a close relationship exists between fermentative activity of S. cerevisiae and magnesium accumulation from growth media. Cellular demands for magnesium during fermentation were reflected at different stages of fermentation, such that entry of cells into stationary phase (coinciding with the time of maximum ethanol and minimal sugar concentrations) correlated with periods of maximal magnesium uptake. Lentini et al (1990) have shown similar patterns in brewing fermentations. Walker and Maynard (1997) further proposed that magnesium taken up, and subsequently released by yeast during fermentation represented cytosolic free magnesium required as a metabolic cofactor.
For zinc, Hall (2001) and De Nicola and Walker (unpublished observations) have shown that fermenting cells of *S. cerevisiae* take up this metal very rapidly from their growth medium. Fig 2. shows a typical pattern of zinc uptake observed by industrial strains of this yeast.

**Fig. 2. Zinc uptake by a brewing strain of *S. cerevisiae*.**

Cells of an industrial ale yeast were inoculated into malt wort (original gravity, OG 1060) at 28°C. Zinc was analysed during the initial stages of fermentation (first 7 hours) in both cells (♦) and supernatant (■) using atomic absorption spectrophotometry.
From this data, it appears that yeast demand for zinc is immediate during the initial stages of fermentation. It is most likely that a large proportion of this zinc is simply cell wall-bound (biosorbed), and Hall (2001) has provided evidence of such an interaction during fermentation.

It is important to remember that *S. cerevisiae* is able to perform fermentation or respiration and this yeast has therefore been described as a facultative organism in terms of sugar catabolism. Fermentative or respiratory modes of metabolism in this yeast will predominate depending on the availability of oxygen and glucose. Two regulatory phenomena describe how *S. cerevisiae* responds to alterations in oxygen and glucose availability: The Pasteur Effect and The Crabtree Effect. Basically, the former states that fermentation is faster in the absence of O$_2$ (i.e. cells respond to energetic discrepancies (lack of ATP) by increasing the rate of glucose catabolism under anaerobic conditions), and the latter states that fermentation predominates, even in presence of O$_2$, because high sugar levels suppress respiration. This means that in industrial fermentations when sugar levels are high (generally when they exceed 0.1%w/v for *S.cerevisiae*), the Crabtree effect is much more relevant than the Pasteur effect. Several possible reasons for the Crabtree effect have been proposed: catabolite repression (Gancedo, 1992); catabolite inactivation (Wills, 1990); limited respiratory capacity (Käppeli and Sonnleitner, 1986), and magnesium availability (Walker, 1994). Considering the latter, it has been shown that magnesium ions dramatically affect mitochondrial structure and control switches from respiration to fermentation in Crabtree-positive yeasts. Magnesium may therefore influence expression of Crabtree effect and Walker (1994) has hypothesised that
intracellular magnesium may control metabolic flux at level of pyruvate. In essence, this hypothesis proposes that pyruvate decarboxylase (which channels carbon down the fermentative pathway) and pyruvate dehydrogenase (which channels carbon down the respiratory pathway) possess low and high affinities for intracellular free magnesium ions, respectively. Smith (2001) has provided some support for such a hypothesis by demonstrating a close relationship between intracellular magnesium and pyruvate decarboxylase activity in brewing strains of *S. cerevisiae*. Such a model has practical implications for industrial fermentation processes because it may be feasible to promote either respiration (for maximising yeast biomass) or fermentation (for maximising ethanol) solely on the basis of manipulating magnesium bioavailability.

D. Metals and yeast stress during fermentation

In the fermentation industries, the viability and vitality of the culture yeasts are crucially important for ensuring process efficiency and product quality. Unfortunately, yeasts used for industrial fermentation processes 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 yeast are summarised in Fig. 3, and for *S. cerevisiae* alcohol fermentations, the principal stress factors are temperature shock, osmostress and ethanol toxicity.
an understanding of stress physiology in yeast cells is necessary to counteract the deleterious effects of stress on fermentation performance. Depending on the particular stress, yeast cells evoke stress responses in an effort to ensure survival when they are exposed to environmental insults, and these include the following:

1. Increased synthesis of trehalose and glycerol
2. Induction of heat/cold shock protein biosynthesis
3. Stress enzyme induction (e.g. ATPase, superoxide dismutase)
4. Cell membrane structural changes
5. Production of glutathione
6. Modulation of ionic homeostasis
Concerning the latter, both heat shock and ethanol can lead to disruption of cellular ionic homeostasis and this can lead to yeast cell death. Walker (1998b) has shown that these stresses induce significant leakage of magnesium ions from brewing strains of *S. cerevisiae* and such leakage correlated to loss of culture viability. It has further been shown that increasing magnesium ion availability, either through external media supplementations, or through cellular magnesium enrichment (or preconditioning), resulted in physiological protection being conferred on cells exposed to otherwise lethal heat shock or toxic ethanol (Walker, 1998b). In a study with wine yeasts, Birch and Walker (2000) showed that cultures propagated in elevated levels of magnesium (20mM, as opposed to 2mM) lead to repression of heat shock protein biosynthesis following thermostress or ethanol toxicity. Several yeast studies have now implicated magnesium as a cellular protectant against osmostress (D’Amore et al, 1988), ethanol (Dombek and Ingram, 1986; Ciesarova, Smogrovicova and Domeny, 1996; Walker, 1998b; Birch and Walker, 2000), and toxic metals like manganese (Blackwell, Tobin and Avery, 1997), copper (Karamushka and Gadd, 1994), cadmium (Kessels et al, 1985), aluminium (MacDiarmid and Gardner, 1996), cobalt (Aoyama, Kudo and Veliky, 1986). There is also evidence from animal cells that magnesium can act as an antioxidant by neutralising the effects of oxygen free radicals and by increasing levels of intracellular glutathione (Durlach, 1988; Rayssiguier et al, 1993; Szantay, 1995). Table 8 summarises anti-stress functions of magnesium.
Table 8. Anti-stress functions of magnesium

<table>
<thead>
<tr>
<th>Stress</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>High and low temperatures</td>
<td>Magnesium maintains cell viability when cells are heat or cold shocked. Magnesium prevents synthesis of heat-shock proteins.</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>Magnesium counteracts stress caused by reactive oxygen species. Magnesium deficit contributes to cellular ageing linked to free radical cellular damage. Mg-deficient cells are more susceptible to in vivo oxidative stress causing lipid peroxidation (magnesium causes a significant fall in malonyl dialdehyde and increase in reduced glutathione) by neutralising $O_2^\cdot$ free radicals</td>
</tr>
<tr>
<td>Ethanol toxicity</td>
<td>Ethanol increases yeast cell permeability to magnesium. Magnesium increases tolerance to otherwise toxic levels of ethanol</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>Magnesium counteracts the toxic effects of Cd, Co, Cu, Al</td>
</tr>
</tbody>
</table>

Magnesium 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).

The practical implications of this for yeast fermentation industries is that magnesium-replete cultures are much more likely to withstand the rigours of industrial processes that magnesium-limited cultures.

VII. Conclusions and Future Prospects

This review has highlighted the important roles of metals in yeast fermentation processes. In yeast cell physiology, these roles are multifarious and can impact significantly on the progress and efficiency of industrial fermentations. For S. cerevisiae cell physiology, the following are some of the salient points that have been raised herein: metal ion bioavailability in fermentation media is more important that total levels of metals; high calcium levels are detrimental; metal-preconditioned yeasts may improve fermentative
metabolism; stress affects metal ion (e.g. Mg) homeostasis and some metals can counteract physiological stress.

There are several industrial implications arising from the research discussed in this paper. Firstly, it is evident that many metals strongly influence yeast fermentation performance and more careful attention should be paid to minerals in fermentation feedstocks than has hitherto been the case. This author is of the opinion that metals are as equally important as carbon and nitrogen sources in industrial media used for optimisation of yeast fermentation processes. Secondly, by physiologically adapting starter yeast cultures, for example using metal – preconditioning, benefits may accrue in terms of improved fermentations. For brewers, winemakers and distillers such an approach may circumvent any reluctance, or necessity, to supplement fermentation media with additional mineral salts. Thirdly, industrial yeast fermenters represent stressful environments for yeast cells. However, certain metals may minimise such stress, particularly caused by extremes in temperature and ethanol concentrations, by conferring a degree of cell membrane protection. Magnesium is the prime candidate for a yeast stress-protectant in fermentation processes.

This review has focussed on *S. cerevisiae* and traditional fermentations such as alcohol production. Nowadays, many non-*Saccharomyces* yeasts are employed in bioreactors for production of high-value pharmaceutical commodities. Whilst we are gradually accumulating useful fundamental information on the mineral nutrition and metabolism of *S. cerevisiae*, which may prove of practical value, unfortunately, we have only scratched the surface of similar knowledge for the massed ranks of non-conventional yeasts. Only when we understand how metals interact with these organisms will biotechnologists be able to fully exploit yeast biodiversity.
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VII. References


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