Chapter 12
Interaction Between Yeasts and Zinc

Raffaele De Nicola and Graeme Walker

Contents

Abstract Zinc is an essential trace element in biological systems. For example, it acts as a cellular membrane stabiliser, plays a critical role in gene expression and genome modification and activates nearly 300 enzymes, including alcohol dehydrogenase. The present chapter will be focused on the influence of zinc on cell physiology of industrial yeast strains of *Saccharomyces cerevisiae*, with special regard to the uptake and subsequent utilisation of this metal. Zinc uptake by yeast is metabolism-dependent, with most of the available zinc translocated very quickly into the vacuole. At cell division, zinc is distributed from mother to daughter cells and this effectively lowers the individual cellular zinc concentration, which may become zinc depleted at the onset of the fermentation. Zinc influences yeast fermentative performance and examples will be provided relating to brewing and wine fermentations. Industrial yeasts are subjected to several stresses that may impair fermentation performance. Such stresses may also impact on yeast cell zinc homeostasis. This chapter will discuss the practical implications for the correct management of zinc bioavailability for yeast-based biotechnologies aimed at improving yeast growth, viability, fermentation performance and resistance to environmental stresses.

Keywords Zinc, gene expression, industrial yeast, vacuole, fermentative performance, homeostasis

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12.1 Introduction

Yeasts require mineral nutrients for cellular growth and metabolism (Jones and Greenfield, 1984). Bulk metals, such as potassium and magnesium, are required at millimolar concentrations (mg l^{-1}), whereas trace elements, such as calcium, zinc, manganese, iron and copper are required at micromolar concentrations (μg l^{-1}). Other ions may be toxic, even at very low concentrations (μg l^{-1}) and include: Pb, Cd, Cr, Hg, Ni, and Al. The utilisation of such ions by yeast cells is often influenced by many factors including metal ion deficiency or excess, chelating/absorbing material in the media and the presence of other ions acting as antagonists (Jones and Gadd, 1990). Metals play structural roles in cellular organelles, proteins and phospholipids and influence cell-cell interactions in the phenomenon of flocculation. Metals are also required for uptake of other nutrients (e.g. Mg-ATPase), gene expression, cell division, growth, fermentation and in energy maintenance. At defined concentrations, some metals may help cells to cope with environmental stresses (Walker, 2004). Table 12.1 summarises the principle functions of essential metal ions in yeast physiology.

Regarding fermentation, zinc is well known as an essential ion for alcohol dehydrogenase (ADH) (Magonet et al., 1992), which facilitates the conversion of acetaldehyde into ethanol at the end of fermentation. In addition, intracellular magnesium may stimulate the activity of pyruvate decarboxylase in brewing strains of *Saccharomyces cerevisiae* (Smith, 2001). If present at high concentration, calcium ions may be detrimental and inhibit both growth and fermentation, due to the antagonism of essential magnesium-dependent reactions (Walker et al., 1996). Yeast cells actively extrude calcium, a metal playing a key role in the phenomenon of flocculation by stabilising bridges between lectin proteins of the cell wall and carbohydrates receptors on another cell, thus facilitating adhesion between adjacent cells (Miki et al., 1982).

In brewing, such a capacity to form flocs facilitates the separation between cells from immature (green) beer at the end of fermentation with cold temperatures

Table 12.1 Functions of essential metal ions in yeast physiology (adapted from Walker, 2004)

Metal ion	Optimal concentration in growth medium*	Main cellular function
Macroelements:		
K	2–4 mM	Osmoregulation, enzyme activity
Mg	2–4 mM	Enzyme activity, cell division
Microelements:		
Ca	<μM	Second messenger, yeast flocculation
Fe	1–3 μM	Haem—proteins, cytochromes
Zn	4–8 μM	Enzyme activity, protein structure
Mn	2–4 μM	Enzyme cofactor
Cu	1.5 μM	Redox pigments

* Values depending on strain, culture conditions and presence of chelators in the media.

promoting this phenomenon (Briggs et al., 2004). It is an intriguing possibility that the rate of calcium ion extrusion could increase to promote the flocculation.

Yeast intracellular ionic composition is different from that of the external medium as is the case for most microorganisms. As a consequence, yeast cells have evolved a system of transporters in order to exploit environmental changes and survive when nutrients are limited or when they are in excess. The metabolic status of cells and the prevailing growth conditions will determine the cellular capacity of yeasts to take up metal ions. Other factors such as low temperature, metabolic inhibitors and absence of energy-yielding substrates are known to negatively influence this uptake. Metal ion uptake into the cell occurs in two stages: biosorption and absorption/translocation. The former is also referred to as the metabolic-independent phase while the latter as the metabolic-dependent phase (Table 12.2).

After an initial passive cell wall binding, the transport of divalent cations is dependent on the activity of the plasma membrane H±-ATPase (Jones and Gadd, 1990). The membrane potential influences ion transport, including metals. For example, the cell may extrude K^+ to increase polarisation of the plasma membrane in order to facilitate ion uptake. This has been documented in the lager yeast *S. carlsbergensis*, where uptake of Mn^{2+}, Mg^{2+} and Zn^{2+} was accompanied by K^+ efflux (Okorokov et al., 1983; Jones and Gadd, 1990). The transport of an ion across a membrane is mediated by specific sets of transporter proteins. Various metal ion transport systems are known and occasionally, several uptake systems with different affinities have been described for the same metal ion. The main genes responsible for encoding these metal ion transporters have been recently identified and they are listed in Table 12.3.

Once the ions have crossed the plasma membrane, many mechanisms work together to ensure that cellular ion homeostasis is maintained, through highly regulated processes of uptake, storage and secretion. The understanding of these mechanisms is fundamental for industrial fermentation design for processes aimed at maximising biomass (e.g. dried yeast starter cultures for winemaking and baker's yeast production) and for production of fermentation compounds such as beer, wine or fuel ethanol. More precisely, the object of these homeostatic mechanisms is two-fold: to prevent accumulation of the metals in the freely reactive form (metal detoxification pathways) and to ensure proper delivery of the ion to target metalloproteins (metal utilisation pathways). Some ions may be sequestered in the nucleus, or in the cytoplasm through polyphosphates or calmodulin, a protein serving as the major intracellular

Table 12.2 Two-stage metal uptake systems in yeast cells (adapted from Mowll and Gadd, 1983)

Phenomenon	Description	Energy requirement	Cell localisation
Biosorption	Unspecific binding of the ions to the cell wall. Cells may be dead.	No	Cell wall
Absorption	Transport is aided by a proton-pumping ATPase	Yes	Plasma membrane (then translocation to cytosol)

Table 12.3 Main genes encoding transporters for metal uptake in yeast cells

Element	Genes	Comments
Zn	*ZRT1/2, ZRT3*	High and low affinity system. Also vacuolar transport
Fe	*FRE1/2/3/4/5/6/7 FET3, FTR1*	Fet4p is a metal transport also for Zn^{2+}, Mn^{2+} and Cu^{2+}
Mn	*SMF1/2/3*	Also for iron. Only high affinity system
Cu	*CTR1, 2, 3*	High and low affinity system
Mg	*ALR1/2, MRS2*	Membrane and mitochondrial transporters
Ca	*PMR1, PMC1*	Golgi and vacuole Ca^{2+}-ATPase

Information from Van Ho et al., 2002; Walker, 2004.

receptor for calcium and which mediates many effects of this ion (Cyert, 2001). Metallothioneins, cysteine-rich proteins of low molecular weight, are also known to bind various metals (especially copper) by means of their cysteine group, minimising their toxicity. Metallochaperones are soluble proteins that ensure the safe transfer of ions (e.g. copper and probably iron) to intracellular sites where they are required (O'Halloran and Culotta, 2000). Some ions may be compartmentalised in organelles for later utilisation. Other ions, such as zinc, magnesium and manganese can be stored in the vacuole. In some cases, ions may remain free in the cytoplasm at very low concentrations to become available for metabolic functions. In industry, the capacity to accumulate and store metals in the vacuole or other compartments can be used to pre-condition yeast cells with metal ions prior to inoculation.

This chapter focuses on yeast interactions with zinc and discusses implications of this for yeast-based industrial processes.

12.2 Critical Review

Zinc is a transition and group II element with atomic number 30 and an atomic weight 65.37 Daltons. Under physiological conditions zinc is very stable and exists in the divalent state. It is not redox active, since neither the potential oxidised form Zn^{3+}, nor the potential reduced form Zn^{+} occurs in cells. Zinc is able to form bonds with many molecules including sulphur from cysteine, nitrogen from histidine and oxygen from glutamate, aspartate and water (Berg and Shi, 1996). The characteristics of zinc's coordination sphere enables a variety of complex geometries to be formed and to allow zinc to participate in enzymatic oxido-reduction reactions in coordination with organic cofactors (Vallee and Auld, 1992).

In biological systems, zinc may be present in the bound form or as cellular free zinc depending on the type (neurons, blood cells, yeasts, etc.) and status of the cell. Its cellular concentration usually ranges from femtomolar to millimolar but this former level may occasionally drop in the range of picomolar to nanomolar concentrations (Outten and O'Halloran, 2001).

In biological systems, zinc is an essential trace element, estimated to be required for almost 3% of the yeast proteome function (Eide, 1998). Its abundance as a transition metal is second only to that of iron (Vallee, 1988). It is now known to be an integral component of a large number of proteins and enzymes, being indispensable for the function of nearly 300 of them (Vallee and Auld, 1990): e.g. acid and alkaline phosphatase, aldolases, Cu, Zn-superoxide dismutase and alcohol dehydrogenase (Leskovac et al., 2002). Zinc also plays a purely structural role in few enzymes (Berg and Shi, 1996). Zinc participates in a wide variety of metabolic processes including carbohydrate, lipid, protein and nucleic acid synthesis or degradation. Zinc ions can form bridges between lipid molecules reducing the capacity of the phosphate groups bound to zinc to take up water. These bonds make the membrane surface more hydrophobic and rigid (Binder et al., 2001), thus influencing membrane fluidity (Garcia et al., 2005). Excess zinc can influence respiration at the level of mitochondrial aconitase activity (Rhodes and Klug, 1993; Costello et al., 1997). Zinc is also the main constituent of the zinc finger proteins that bind specific DNA sequences, playing a role in gene expression (Rebar and Miller, 2004). This property is mainly due to the lack of redox activity of zinc which stabilises DNA and RNA molecules and prevents radical reaction resulting in nucleic acid damage (Berg and Shi, 1996). Zinc is also critically placed to control apoptosis, by suppressing major pathways leading to this phenomenon. Apoptosis is an active energy dependent process of programmed cell death, closely linked with oxidative stress. Zinc acts as a cytoprotectant and minimises oxidative damage by stabilising both lipids and proteins, increasing glutathione, the main cellular antioxidant, and by suppressing the activation of caspase-3, a protease that cleaves to substrates causing critical morphological changes (Truong-Tran et al., 2001).

Zinc plays a central role in the enzyme function in yeast cells, especially alcohol dehydrogenase (ADH). Zinc is also required for endoplasmic reticulum function in *S. cerevisiae* (Ellis et al., 2004). The average zinc cellular content in baker's yeast cells has been defined to be 0.12 g kg^{-1} dry weight (Jones and Greenfield, 1984), this value being strain and growth condition dependent. Zinc deficiency depresses yeast growth with cells tending to swell and to form clusters (Obata et al., 1996). Zinc depletion in *S. cerevisiae* also results in a decrease of the activity of phospholipid synthesis and subsequent alteration of phospholipid composition (Iwanyshyn et al., 2004).

Zinc is a hydrophilic, highly charged ion, which cannot cross biological membranes by passive diffusion and so must be translocated by transporter proteins (Guerinot and Eide, 1999). In *S. cerevisiae*, zinc uptake and homeostasis are controlled both at transcriptional and post-translational levels. The transcriptional system operates at moderate intracellular zinc levels, between 0.01 and 0.07 nmol Zn/million cells, and the post-translational level operates when intracellular zinc levels are above 0.07 nmol Zn/million cells (Zhao et al., 1998; Gitan et al., 1998). At the transcriptional level**,** three or more uptake systems are known to control zinc uptake. One system has a high affinity (K_d = 10 nmol l^{-1}) for extracellular zinc and is active in zinc limited cells (Zhao and Eide, 1996a). A second system has a lower affinity (K_d = 100 nmol l^{-1}) for extracellular zinc, and is active in zinc-replete cells

(Zhao and Eide, 1996b). These systems are very specific for zinc and are not involved in uptake of other metals. One transport protein works for each of the two systems: Zrt1 for the high affinity system and Zrt2 for the low affinity system. These proteins are localised in the plasma membrane and share 44% of identity in the amino acid sequence and 67% of similarity (Eide, 2003). A third system is also known to take part to zinc uptake: the transport protein Fet4. Fet4 is not only zinc specific but is also involved in iron and copper uptake (Waters and Eide, 2002). This protein, together with Zrt1 and Zrt2, belongs to the ZIP family of metal ion transporters. *ZRT1* and *ZRT2* are the genes encoding for Zrt1 and Zrt2, respectively and *FET4* for the transporter Fet4. The *ZAP1* gene, through its encoded activator protein Zap1, strictly controls the functioning of these genes and its own functioning, thanks to a mechanism of auto-regulation. Any controlled gene has one or more zinc responsive elements (ZRE) in their promoters. Zap1 binds to ZRE to maximise the expression of the target genes. Zinc levels play an important role in this mechanism. For example, under severe zinc deficiency, Zap1 is produced at high levels and the affinity for the *ZRT1* is kept very high. The affinity for *ZRT2* is reduced, maybe because other proteins bind with the promoter of this gene, which consequently becomes unavailable for Zap1 (Eide, 2003). This results in up-regulation of *ZRT1* and down-regulation of *ZRT2* expression.

At the post-translational level, zinc uptake is controlled by a mechanism of degradation of the protein Zrt1. At high zinc concentrations, Zrt1 is transferred to the vacuole and inactivated through a mechanism of endocytosis and proteolysis (Gitan et al., 1998). A summary of the zinc transporters is reported in Table 12.4.

Their localisation is depicted in Fig. 12.1 Once zinc is taken up into the cell, this metal is utilised for metabolic functions in the cytoplasm and in several organelles including mitochondria and endoplasmic reticulum. If zinc exceeds the requirements needed by the yeast cells, several mechanisms may be activated in order to store zinc until it may be required. The yeast vacuole is known to accumulate several divalent cations, such as Zn^{2+} (White and Gadd, 1987), Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Sr^{2+}, Ni^{2+} and the monovalent cations K^{+}, Li^{+}, and Cs^{+} (Okorokov et al., 1985; Ramsay and Gadd, 1997). Even zinc transport into the vacuole is controlled at the

Table 12.4 Summary of main transporters involved in zinc homeostasis

Transporter protein	Location	Transporter family	Zinc cell status
Zrt1	Plasma membrane	ZIP	Depleted
Zrt2	Plasma membrane	ZIP	Replete
Fet4	Plasma membrane	ZIP	Normal
Zrt3	Vacuole	ZIP	From replete to depleted
Zrc1	Vacuole	CDF	From depleted to replete
Cot1	Vacuole	CDF	From depleted to replete
Msc2	Endoplasmic reticulum	CDF	Normal
Zip7	Golgi apparatus	ZIP	Depleted

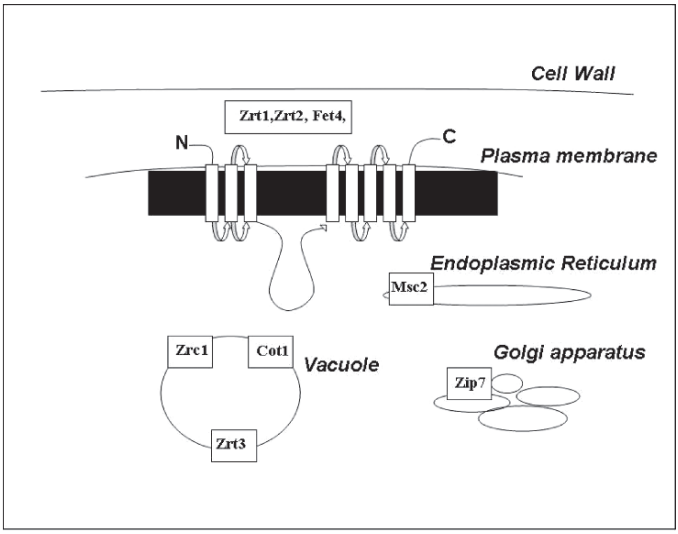

Fig. 12.1 Localisation of the main zinc transporters in the yeast cell. Information from Li and Kaplan (1998), Miyabe et al. (2001), MacDiarmid, et al. (2000, 2002), Waters and Eide (2002), Eide (2003), Ellis et al. (2004), Huang, et al. (2005) and relates to *S. cerevisiae*. See text for further information

transcriptional level. Three transporters are known to be localised in the vacuole: Zrc1 (Miyabe et al., 2001), Cot1 (Li and Kaplan, 1998) and Zrt3 (MacDiarmid et al., 2000, 2002). While the role of Zrc1 and Cot1 is to retain zinc brought in by endocytosis, the role of Zrt3 is to mobilise zinc stores out of the vacuole when zinc replete cells are exposed to zinc limiting conditions. A fourth transporter Msc2, already known to influence zinc homeostasis (Li and Kaplan, 2001), has been recently localised in the endoplasmic reticulum where it is involved in supplying zinc to this compartment (Ellis et al., 2004). The transporter Zip7, associated with the Golgi apparatus, has been found to have a role in zinc homeostasis, transporting zinc from the Golgi apparatus to the cytoplasm when yeast cells are grown in zinc depleted medium (Huang et al., 2005). When zinc starved cells are suddenly exposed to high zinc concentrations, a "zinc-shock" occurs (MacDiarmid et al., 2003). Zinc rapidly crosses the plasma membrane to accumulate in the cytoplasm first and then into the vacuole.

As a consequence, the transcription of genes encoding for the plasma membrane transporters is down regulated and the Zrt1 protein is inactivated by endocytosis. This system prevents excessive zinc uptake. At the same time, the genes encoding for the vacuolar transporters are up regulated. Studies with mutants have given very interesting information on the way zinc transport mechanisms act during zinc shock.

In *S. cerevisiae*, other systems have been described to play a role in zinc homeostasis. For example, Devirgiliis et al. (2004) have used specific fluorophores to detect zinc localisation and have been able to observe very small vesicles in the cytoplasmic periphery they named yeast *zincosomes*. These vesicles appear very

rapidly when zinc depleted cells are transferred to a medium containing micromolar zinc concentrations and disappear very slowly when the same cells are placed in zinc-depleted medium. They may play a role as transporters of zinc ions to the sites quickly requiring this metal and to the vacuole to storage. In *S. cerevisiae* there is no evidence of metallothioneins acting in zinc sequestration (Palmiter, 1998) although in *Schizosaccharomyces pombe* the gene *zym1*, encoding for one metallothionein, may play role in zinc tolerance and zinc storage (Borrelly et al., 2002).

The high capacity of *S. cerevisiae* to sequester heavy metals may be employed in bioremediation to clean up polluted industrial sites (Volesky and May-Phillips, 1995). A more comprehensive study has been recently published by Mapolelo et al. (2005) who studied the heavy metal uptake properties of various strains of *S. cerevisiae* in dam water, stream water, treated wastewater and industrial effluents. This potential of yeasts as tools for decontamination, including zinc, would be another novel application for this most useful microorganism.

12.3 Analysis

In industrial fermentation media, several divalent cations such as zinc, magnesium, manganese, calcium are known to play important roles in yeast cell physiology and in dictating the progress of fermentation. Zinc media concentrations in the range 0.25–0.50 ppm have been reported to be optimal for cell growth and 1–2 ppm for glycolysis (Jones and Greenfield, 1984; De Nicola, 2006). The concentration of this element is variable in different industrial media such as wine must, cane molasses or brewer's wort. Part of zinc may also not be available (bio-available) to yeast cells because it is bound to some compounds present in the media, for example, hop acids. Sometimes zinc may be present as zinc oxide, depending on the pH and the level of oxidation of the medium (Pourbaix, 1963). Zinc in this form is not taken up by yeast cells.

In the wine-making industry, for example, zinc concentration in grape wine must be between 0.04 and 7.8 ppm and the average is 0.90 ppm (Cabanis and Flanzy, 1998). Usually in winemaking such zinc levels are satisfactory for the progress of fermentation. Average zinc concentration in beet molasses is 40 ppm while in sugar cane molasses is 13 ppm (Curtin, 1973) with optimal concentration for ethanol production in the range 1–5 ppm. In brewing, the mineral content in malt is usually 2–3% of the dry weight depending on the agronomic conditions and the trace element content of soil (zinc, manganese, iron, calcium and copper). During malt wort preparation, a minor amount of metals is extracted, for example, less than 5% of zinc, iron and strontium (Jacobsen and Lie, 1979; Jacobsen et al., 1982). Jacobsen and Lie (1977) have analysed the degree of extraction of various elements and found an interesting correlation between zinc concentration in wort and the peptide and amino acid level, concluding that the cysteine groups may be active in the process of zinc sequestration. Other compounds are also known to take part to the process of ion chelation: phenols (Fe), α-amylase (Ca) and phytic acid (Ca, Zn and Fe). The level of these compounds in wort may depend on the technical processes used during its

preparation. Zinc levels usually decrease during the wort mashing, lautering and boiling (Daveloose, 1987; Jacobsen and Lie, 1977; Jacobsen et al., 1982; Kreder, 1999). For example, a concentrated mash has a decreased protein level and consequently a lower level of zinc-binding compounds. During lautering or filtering of the mash, some ions may be washed out. In this respect, zinc ions become complexed in precipitated trub during the wort boiling and cooling and may become unavailable to yeast cells. Zinc binding with trub is loose. Kreder (1999) has demonstrated that keeping part of the trub into the wort during fermentation is beneficial, because zinc ions are slowly released into the medium. Since the trub has other nutritional and physical qualities, it is also needed for proper yeast growth and fermentation performance. As a result, zinc levels in malt wort may be below 0.1 ppm.

Zinc deficiencies during fermentation may cause serious problems in terms of diminished yeast cell growth and reduced ethanol production. Generally speaking, when zinc levels fall below around 0.1 ppm, then fermentation problems may be encountered (Helin and Slaughter, 1977; Jacobsen and Volden, 1981; Bromberg et al., 1997) and this may lead to slow and incomplete fermentations, which are termed "sluggish" (Jacobsen et al., 1982).

The excess of zinc in brewer's wort is rare and synergistic effects with manganese have been reported (Helin and Slaughter, 1977; Jones and Greenfield, 1984). It is clear that it is very difficult to determine the limits of zinc tolerance since this may depend on the interaction with other elements. Generally speaking, in the range of 5–50 ppm, several divalent cations influence the uptake of other divalent cations (Helin and Slaughter, 1977). High gravity wort may help yeast cells to tolerate extremely high zinc concentrations up to 1310 ppm. This effect has been demonstrated in some lager yeast strains that keep the same attenuation time and maintain a relatively good viability (about 50%) compared to the viability they would have in normal gravity worts. This may depend on the different tolerance to zinc of various strains as well as the greater buffering capacity of high gravity wort (Rees and Stewart, 1998). In relation to other ions, zinc is known to have the following interaction properties (Jones and Greenfield, 1984): prevention of Cu^{2+} and Cd^{2+} toxicity, enhancement of the beneficial growth properties of Ca^{2+} and Mn^{2+}, Cu^{2+} level stimulation of Zn^{2+} absorption, stimulation of growth in communion with Mn^{2+} and Cu^{2+}.

Glucose, as a source of energy, strongly stimulates zinc uptake. In yeast peptone dextrose medium (YPD) with various sugar concentrations (Hall, 2001), it was shown that industrial yeasts growing and fermenting in high sugar concentration (5%), accumulated higher levels of zinc compared to cells growing and respiring in low sugar concentration (0.1%). Most likely, high levels of glucose provided enhanced energy to drive accumulation of intracellular zinc. The stimulatory effect of glucose on zinc uptake was inhibited by the following metabolic inhibitors: antimycin A (Mowll and Gadd, 1983), potassium cyanide, 2,4-dinitrophenol (DNP), DCCD, diethylstilboestrol (DES) and 2-deoxyglucose (White and Gadd, 1987). Although these compounds showed a clear metabolic dependent mechanism of zinc uptake, unfortunately they may interfere with various metabolic pathways or may have non specific effects. Therefore, they do not prove any specific mechanism implicated in zinc uptake. In *Candida utilis,* zinc uptake was strongly impaired in starved cells by the presence of

the protein synthesis inhibitor cycloheximide (Failla et al., 1976). This demonstrated that *de novo* proteins were involved in zinc uptake.

Zinc uptake exhibits Michaelis-Menten kinetics. A variety of dissociation constants have been found for yeasts (Borst-Pauwels, 1981). This variation has been found even within the same yeast species and may be attributed to different techniques used to measure zinc or to the presence in the medium of buffers or complexing anions. More precisely for *S. cerevisiae*, two systems have been found: one high affinity system with K_d = 10 nmol, active in zinc-limited cells (Zhao and Eide, 1996a) and one low affinity system with K_d = 100 nmol, active in zinc-replete cells (Zhao and Eide, 1996b).

Growth medium pH may affect zinc uptake suggesting some sort of proton symport mechanism. In *C. utilis*, zinc uptake rate decreased while pH increased from 4.8 to 8.2 (Failla et al., 1976; Failla and Weinberg, 1977). This may have also depended on the reduced bioavailability of zinc which may form complexes with polyphosphates, carbonates and hydroxides at pH values over 6.8 (Ross, 1994) or the formation of zinc oxide, which depends on the level of oxidation of the medium (Pourbaix, 1963). Uptake of heavy metals like cadmium is usually accompanied by release of K^+ (Norris and Kelly, 1977; Lichko et al., 1982; Mowll and Gadd, 1983). This phenomenon is not present in *S. cerevisiae* during zinc uptake, when zinc extra-cellular levels are at normal concentrations but it can be observed at very high zinc concentrations (Mowll and Gadd, 1983).

Figure 12.2 shows typical zinc uptake kinetics by a distiller's yeast strain (*S. cerevisiae*) cultivated in malt wort. Zinc is accumulated in most yeast strains within the first hour of growth with cells reaching the maximum mean zinc cell

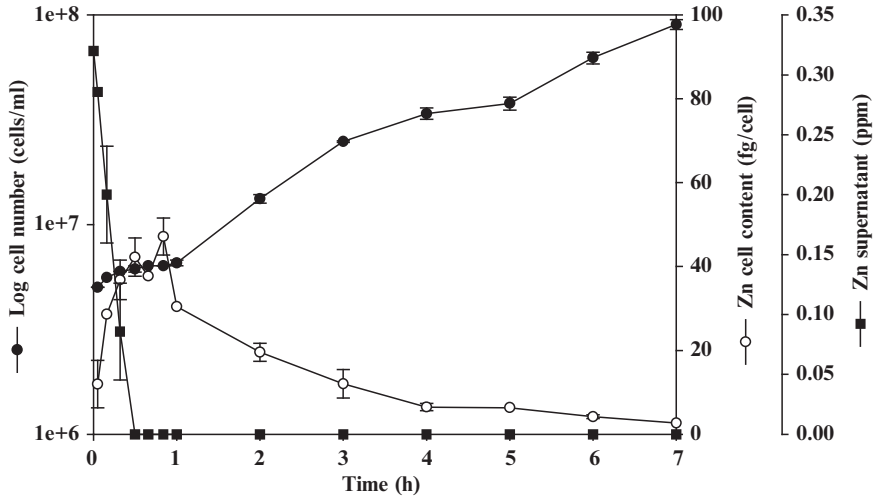

Fig. 12.2 Zinc uptake by a distiller's yeast strain of *S. cerevisiae* in industrial media. A distiller's yeast strain was cultivated in shake flasks in malt wort (zinc at 0.32 ppm), at 25°C for 24 h. Figure represents cell growth, zinc cell content and zinc supernatant concentrations in the first 7 h of growth (De Nicola, 2006)

content. After cell division, the average zinc gradually decreases (De Nicola, 2006). In the same study, zinc uptake was unaffected by calcium concentrations in the range 16–76 ppm, as well as various sources of zinc salts (Zn sulphate, Zn acetate and Zn chloride). In brewer's wort, zinc is occasionally present at sub-optimal concentrations for growth and fermentation. Zinc uptake studies by industrial yeast strains have been carried out by Mochaba et al. (1996) in small-scale 2 l fermenters and in 60 l fermenters, at 11°C, using malt wort with zinc levels up to 0.75 ppm. They found an immediate cellular zinc increase in the first hours after inoculation, followed by zinc level fluctuations in the remaining days of fermentation.

Zinc movement within the yeast cell occurs through various barriers. The first obstacle is represented by the cell wall. The cell wall constitutes 15–25% of the dry weight of the cell and it is essentially composed of a highly dynamic structure that is responsible for protecting the cell from rapid changes in external osmotic potential (Levin, 2005). Specific binding regions for metal ions are present on the cell wall which may change depending on the yeast strain or the media in use (Engl and Kunz, 1995). The zinc specific binding in the cell wall is the sulphydryl group of cysteine located in the mannoprotein fraction (Brady and Duncan, 1994; Mochaba et al., 1996). An energy dependent metabolism acting during zinc uptake has been reported in several studies. For example, White and Gadd (1987) determined the proportion of compartmented zinc within the cell: 56% in the soluble vacuolar fraction, 39% bound to insoluble components, probably the cell wall, and only 5% was found in the cytosol. An ATP-dependent zinc uptake system whose properties were consistent with a Zn^{2+}/H^{+} antiport was localised in isolated yeast vacuoles (White and Gadd, 1987). Lichko et al. (1982) have previously reported that intracellular K^{+} is concentrated in the vacuoles and that yeast cells may lose up to 30% of vacuolar K^{+} as they accumulate divalent cations. As previously mentioned, this phenomenon was only observed in *S. cerevisiae* when extra-cellular zinc levels were very high. In brewing experiments conducted in 60 l fermenters, Mochaba et al. (1996) have analysed yeast cellular zinc localisation throughout fermentation, finding high zinc concentrations in the mannoprotein and in the intracellular fractions. Further, at the end of the fermentation, zinc ions were translocated to the outer surface presumably to aid in maltotriose uptake. De Nicola (2006) showed that a low percentage of total cell associated zinc, in the range 5–30%, was bound to the cell wall.

In yeast cells killed by heat treatment, zinc uptake was minimal and probably reflected binding to the cell wall material. This association was loose and zinc was readily released back to the medium. It was concluded by De Nicola (2006) that biosorption by bond formation between zinc and the cell wall was not relevant during zinc uptake and an active translocation mechanism was likely. Free zinc was not visualised in the proximity of cell walls of yeast cells by using a specific zinc probe, probe Fura Zin-1 (MacDiarmid et al., 2003). Instead, zinc ions were quickly translocated into the yeast cell and stored into the vacuole (Fig. 12.3).

The yeast vacuole is a reservoir for several nutrients (Lichko et al., 1982) and vacuolar polyphosphate bodies, known as volutin granules, have already been shown to be associated with zinc (Jones and Gadd, 1990). Zinc is probably stored in this organelle to be mobilised under zinc-limiting conditions or to be directed to

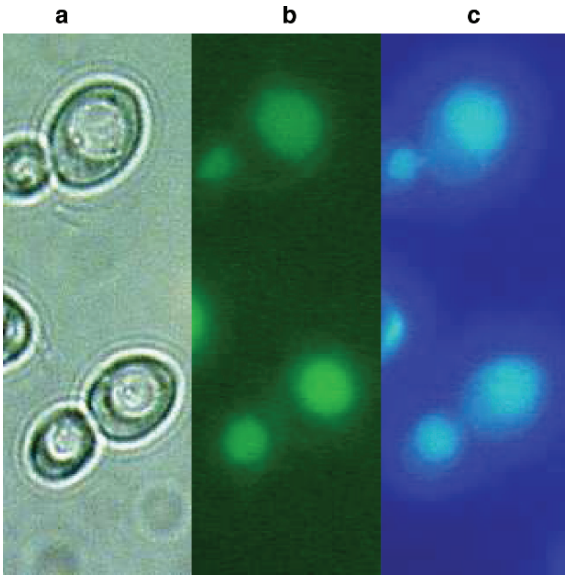

Fig. 12.3 Free zinc ions and vacuole localisation in yeast cells. Yeast cells of a brewing yeast strain were cultured in EMM3 medium (Zn at 10 ppm) and stained with both Fluo-Zn3 for zinc visualisation (**b**) and Cell-Tracker B for vacuole visualisation (**c**). Pictures were taken using a Leica microscope under bright field (**a**), using a green filter (**b**) and a blue filter (**c**) (De Nicola, 2006)

the daughter cell during budding and cell division (Eide, 2003). Zinc storage in the vacuole regulates the mechanisms of zinc distribution among the cellular population since zinc may be inherited by daughter cells during budding. The vacuole 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. Such a mechanism could generate heterogeneities in yeast populations with respect to their zinc status especially under conditions where zinc uptake from the medium was faster than the rate of cell division. In alcohol production processes by fermentation, a major effort is made to keep yeast cells under appropriate physical, chemical and nutrient conditions in order to minimise cell growth and stimulate metabolic pathways leading to optimal alcohol production. In laboratory conditions with laboratory strains, however, growing cells produce alcohol 33 times faster than non-growing cells (Ingledew, 1999). Compromise efforts are made to keep yeasts under conditions that do not lead to low growth rates or to cell death. Control and management of micronutrient levels, such as zinc, are vital for the correct progress of fermentation and to encourage a predominantly fermentative, rather than respiratory, mode of metabolism in the yeast strains employed in alcohol production.

Optimal zinc concentrations reduce the attenuation time considerably compared with zinc-limited conditions (Skanks et al., 1997). Sometimes the specific fermentation rate changed only after several successive fermentations (Bromberg et al., 1997).

In the brewing industry, due to technological processes employed to prepare malt wort that may lead to lack of zinc in the medium, zinc levels may be determined, throughout the fermentation process and in the final beer. Zinc supplements may be employed when zinc levels are very low to avoid sluggish and incomplete fermentations. Moreover, although present, some of the zinc may be not bioavailable to the yeast cells.

In the literature, various levels of zinc have been proposed for optimal fermentation performance in brewing: between 0.1 and 0.15 ppm (Bromberg et al., 1997), 0.18 ppm (Helin and Slaughter, 1977) and between 0.05 and 0.30 ppm (Jacobsen et al., 1981). Precise zinc cellular requirements are obviously yeast strain dependent (Rees and Stewart, 1998) but the malt wort quality and the fermentation conditions also cause variations in zinc demand by yeast cells.

Taylor and Orton (1973) demonstrated zinc to be an inhibitor of flocculation only at very high concentrations, above 6500 ppm and pH 7.5, concluding that the role of zinc in flocculation is unimportant in brewing as these conditions are unrealistic. An experiment *in vitro* showed that in terms of flocculation, lager strains are not affected by the presence of zinc, while some ale strains flocculated when the zinc concentration reached the so-called saturation point at 2.6 ppm (Raspor et al., 1990). Above this level, de-flocculation occurred. This phenomenon may be explained by differences in the cell surface structures and may be employed to differentiate between ale and lager flocculating strains. In recent years, a study on yeast propagation demonstrated that only the addition of 0.30 ppm of zinc may have a consistent effect on flocculation (Wackerbauer et al., 2004). During fermentation, zinc may interact with other cations such as calcium, involved in the flocculation process, thus promoting this phenomenon. The hypothesis that zinc is involved in flocculation is also supported by zinc limitation studies in aerobic continuous culture (De Nicola, 2006). The gene *MUC1*, was found 50 and 30 times more up-regulated in zinc-limitation conditions compared to, respectively, carbon and nitrogen limitation albeit in a non-flocculent laboratory strain. This gene is involved in yeast flocculation (Guo et al., 2000) through the synthesis of the protein Flo11p, member of the second group of the Flo family proteins, uniformly localised around the cell surface of the haploid cells but only in few surface areas of diploid yeast cells. Zinc depleted cells were found to form clusters (Obata et al., 1996).

Positive influences of zinc on the synthesis of esters and higher alcohols as well as the decrease of acetaldehyde levels have been widely documented (Hodgson and Moir, 1990; Seaton et al., 1990; Skanks et al., 1997; Quilter et al., 2003). With regard to production of volatiles and higher alcohols by yeast, Skanks et al. (1997) have shown that elevated zinc concentrations increased the levels of higher alcohols and esters but reduced acetaldehyde levels. Lager yeast cells preconditioned with zinc produce distillates with higher alcohols (2-methyl-1-buthanol, 3-methyl-1-buthanol and isobutanol), esters and aldehydes and without iso-amyl acetate (Melville, 2003). Although addition of 0.5 ppm of zinc increased volatile organic compounds, it may also increase the concentration of medium chain fatty acids (MCFA) which can cause unwanted soapy, fatty and rancid taste (Villa et al., 1999) and reduce foam stability (Lange et al., 2004).

In the bioethanol (fuel alcohol) industry, zinc levels in beet molasses are much higher than in grape must or brewers wort and zinc deficiencies are rare. In the wine-making industry, as in the bioethanol industry, it is unusual to carry out analyses of zinc levels since the metal concentration in grape must is usually deemed satisfactory for the progress of the fermentation. In winemaking, research on zinc interaction with yeasts is mostly performed by companies involved in the commercialisation of yeast supplements. Commercial preparations, based on nutrients such as organic and inorganic nitrogen, fatty acids, sterols, vitamins and mineral salts, including zinc, are usually added during yeast rehydration and propagation to ensure that yeast cells are supplemented with satisfactory levels of nutrients prior to cell inoculation. These actions aim to guarantee that yeast cells are healthy and active from the early stages of the fermentation. Unfortunately, in wine making, most of the research has been focused only on agronomic studies of the vineyard soils or grape fertilizers based on zinc (Christensen, 1980; Christensen and Jensen, 1978) and there are very few studies available on the influence of zinc with wine yeast strains. In a wine making simulated experiment, zinc uptake patterns by a wine yeast strain showed high zinc uptake in the first two days of fermentation, followed by release or leakage back into the medium after 48 h (Fig. 12.4).

Most likely when ethanol levels were above 6% (Fig. 12.5), yeast cells continuously exposed to such a relatively high concentration may have had altered plasma membrane permeability, resulting in the loss of zinc ions. In this regard, (Learmonth and Gratton, 2002) reported that ethanol stress increased the membrane fluidity of the yeast cells. Yeast growth of the two wine strains was not dramatically affected by the zinc concentrations of this study. At the end of the fermentation, the yeast

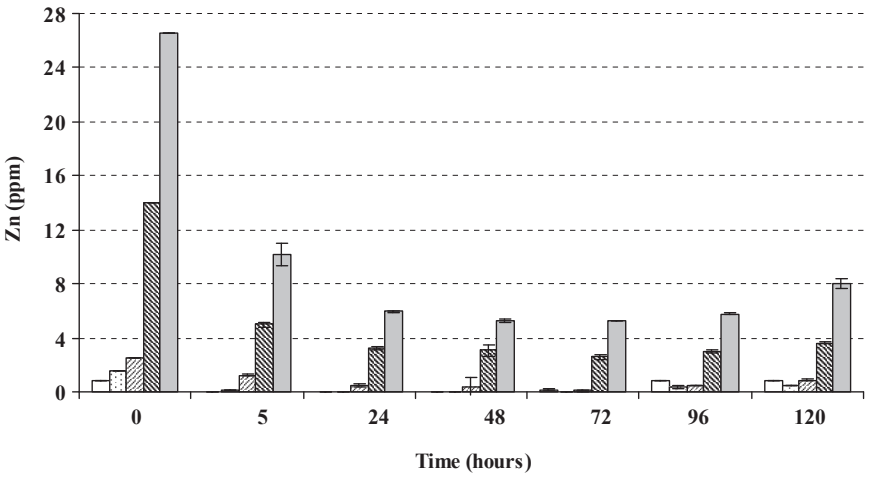

Fig. 12.4 Zinc uptake during grape juice fermentation by a wine yeast strain. Yeast cells of a wine strain were inoculated in 1 l Imhoff conical vessels, in grape juice with variable zinc levels. Fermentation was carried out at 25°C for 120 h (5 days). Zn residual levels in supernatants were analysed throughout fermentation by atomic absorbance spectrophotometry. Concentrations of zinc tested were as follows: 0.9 (□), 1.5 (□), 2.5 (▨), 14 (▩), 26.5 (▤) ppm

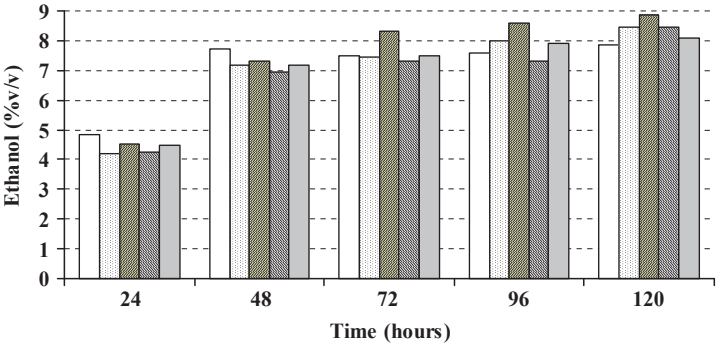

Fig. 12.5 Influence of zinc on ethanol production during wine fermentation. Conditions employed were as described in Figure 12.4. Fermentation performance of the wine strain was evaluated by analysing the ethanol produced daily. Concentrations of zinc tested were as follows: 0.9 (□), 1.5 (▨), 2.5 (■), 14 (■), 26.5 (▤) ppm

cone formed at the bottom of the fermenters was approximately of the same size in all the fermenters with the exception of 0.9 ppm of zinc, having a slower growth rate and lower final biomass. Zinc concentrations of 2.5 ppm accelerated the flocculation process of the yeast cells and gave a slightly higher final biomass. The viability was not affected by any of the zinc concentrations tested and at the end of the fermentation was 84%. Various zinc concentrations tested did not dramatically affect the fermentation rate. Higher ethanol production was determined at 2.5 ppm of zinc (Fig. 12.5). This result, together with a higher growth rate, appeared to indicate that this concentration had a general beneficial effect on this strain.

The physico-chemical properties of the environment can strongly influence the physiology of yeast cells, affecting both viability and vitality. In the fermentation alcohol industries, at any stage of the process, yeast cells can encounter a variety of stresses which can have a significant impact on cell growth and fermentation performance. Yeasts respond to stresses by changing their metabolic activities and by adapting their physiology in order to protect the cellular components from damage, to survive and to recover when optimal environmental conditions are re-established. The understanding and the correct management of the interactions between environmental stresses and yeasts is fundamental for brewers, wine-makers and distillers who want to optimise cell growth, viability and fermentation performance. In industrial processes, the stresses most commonly encountered are chemical (e.g. ethanol and other metabolite toxicity, oxidative, anaerobiosis, pH changes and acid wash treatments, nutrient limitation/starvation, metal ion toxicity/limitation), physical (e.g. osmostress, changes in temperatures, dehydration, rehydration, mechanical sheer, hydrostatic pressure) and biological (e.g. cell aging, genotypic changes or competition from other organisms) (Walker, 1998).

With regard to chemical and physical stresses encountered during industrial fermentation processes, magnesium is already known to protect cells from osmostress (D'Amore et al., 1988), ethanol (Birch and Walker, 2000), toxic metals (Blackwell

et al., 1997; Karamushka and Gadd, 1994) and the oxidant effect of free radicals (Szantay, 1995). As a consequence, Mg-enriched cells retain viability and vitality under stress. Magnesium ions may protect the integrity of cell plasma membrane during stress insults by stabilising its lipid bilayer and decreasing its membrane fluidity (Walker, 1999). Although an antioxidant role has been ascribed to zinc (Truong-Tran et al., 2001) and charge-neutralisation properties of membrane phospholipids have been assigned to this metal ion (Binder et al., 2001), to date, no information has been provided on how this metal may protect yeast cells from industrial stresses.

Experiments carried out with a brewing strain of *S. cerevisiae* under temperature, ethanol and combined temperature/ethanol stresses (Fig. 12.6), showed a significant release of zinc ions accompanied by loss of culture viability. After 1 and 5 h exposure, cells retained zinc and viability following heat or ethanol stress, although the combination of these insults had a synergistic dramatic effect on the viability of the cells which were all dead after only one hour from the beginning of the stress. After 24 h exposure, all cells died, although some residual zinc was associated with the dead cell mass probably due to tight cell wall biosorption (de Nicola, 2006).

The influence of ethanol on membrane permeability has already been observed by Learmonth and Gratton (2002). In that study, the authors used the probe Laurdan to determine the Generalised Polarization (GP) parameter, as an index of cellular

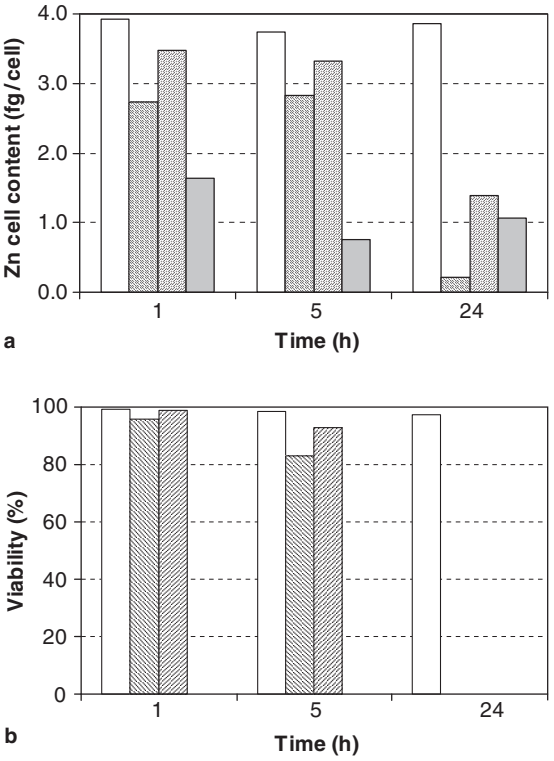

Fig. 12.6 Influence of temperature and ethanol stress on intracellular zinc in yeast. A lager brewing yeast strain was cultured in malt wort, resuspended in distilled water and stressed at 45 °C or in ethanol for 24 h. Zinc cell content (**a**) and cell viability (**b**) were analysed. Stress conditions tested were as follows: control in distilled water at 25 °C (□), temperature 45 °C (■), ethanol 20% at 25 °C (v/v) (■), temperature 45°C + ethanol 20% (v/v) (■)

membrane fluidity. It is plausible that highest membrane fluidities at high ethanol concentrations influenced the permeability of the tonoplast, the vacuolar membrane. Since most cellular zinc is stored in the vacuole when accumulated in large amounts (Fig. 12.3 and MacDiarmid et al., 2003), changes in fluidity of the tonoplast may have determined zinc release. This theory is also supported by recent studies on the influence of zinc on phospholipid synthesis (Iwanyshyn et al., 2004; Han et al., 2005; Carman, 2005). Zinc depletion was found to highly affect the *DPP1* gene encoding DGPP phosphatase (Lyons et al., 2000) an enzyme associated with the vacuolar membrane and involved in the synthesis of phosphatidate and diacylglycerol. Lack of zinc reduced these two compounds and phosphatidylserine, increasing the production of phosphatidylinositol (Han et al., 2005). In general, the mechanisms by which zinc influences phospholipid synthesis appear to be complex and associated not only with the *DPP1* gene.

As for ethanol, temperature is known to influence the plasma membrane by increasing its fluidity (Learmonth and Gratton, 2002). The yeast plasma membrane has been recently suggested to be the primary cellular compartment controlling heat stress tolerance (Guyot et al., 2005). Interestingly, magnesium and zinc cell contents as well as ATP levels were affected with similar patterns, suggesting that the phenomenon of compound release was more general and not only specific for zinc (De Nicola, 2006). There is evidence that ethanol and temperature stresses influenced plasma membrane fluidity allowing a general release of metal ions. It is conceivable that a suitable level of zinc may stabilise the plasma membrane and help yeast cells to alleviate the effects of some environmental stresses.

Several genes have been determined to be up- and down regulated during zinc deficiency stress. For example, Lyons et al. (2000) have determined 15% of differentially expressed genes, 46 of which regulated by Zap1. Higgins et al. (2003) have shown that 76 genes were induced or repressed fivefold in a brewing yeast strain grown in conditions of zinc depletion with two genes, *YOR387c* and *YGL258w*, found to be induced 2 h after zinc starvation. Both studies were performed in batch cultures, with continuously changing environmental conditions. Experimental replication and acceptable levels of reproducibility are fundamental conditions in transcriptome analyses as shown by an inter-laboratory study of comparison between chemostat cultures with that in shake-flasks (Piper et al., 2002). Recent studies of zinc limitation in chemostat continuous culture have revealed a more robust set of genes involved during zinc deficiency. Chemostat culture allowed to control specific growth rate and prevented the occurrence of specific-growth-rate-related responses. The transcriptional regulation of this gene set was independent of the oxygen supply (De Nicola et al., 2007).

12.4 Future Perspectives and Conclusions

Zinc interaction with yeast, its transport and utilisation, during brewing and other industrial processes, are phenomena still not completely understood. To date, most of the efforts of the applied research have been concentrated towards the determination

of zinc in industrial media and specifically in malt wort and the discovery of new technologies to improve such availability. Most of the studies on zinc uptake kinetics were merely aimed at describing variation of zinc wort levels during fermentation and in determining the zinc concentration for the best fermentative performance.

With regard to zinc uptake, optimal zinc accumulation occurred using active and healthy cells, with high levels of available sugars as source of energy and at relatively high temperatures. Therefore, zinc supplementations to industrial media may be appropriate at the moment of inoculum additions and not during yeast slurry cold storage or acid-washing.

Since no major differences were found in terms of zinc uptake using different zinc salts, it is therefore advisable to use an economically convenient salt as a source of supplementary zinc, such as zinc sulphate. Although high zinc concentrations may delay fermentation rates, they may increase the synthesis of some esters and higher alcohols, altering taste and aroma of fermented alcoholic beverages. Further studies are required to elucidate the role of zinc in these pathways. The range of zinc levels in the media reported in the literature are merely indicative as conditions may change in relation to the yeast strain used, media composition and environmental characteristics. Industries employing yeasts in fermentation processes would be advised to perform tests aimed at verifying the optimal zinc concentration for their strains and processes employed. The practice to pre-enrich yeast cells with zinc may be implemented as demonstrated previously for magnesium (Walker and Smith, 1999). This may be very useful for industries adhering to food purity laws such as the German Reinheitsgebot which stipulates that the brewing of beer must only employ malted barley, hops, water and yeast. Experimental studies in yeast propagation vessels may give useful indications on how to optimise intracellular zinc accumulation. During the fermentation process, at any stage, brewers should control the viable status of their yeast cells and avoid any undue stresses. These may lead to diminished fermentation performance and loss of viability, as well as cellular zinc and magnesium. Yeast cells may concomitantly encounter more than a single stress and it would be useful to determine the degree of zinc release during a combination of more stresses at the same time, as shown for combined temperature/ethanol stresses in this paper. For example, in brewing, after a fermentation process and prior to pitching into a subsequent fermentation vessel, yeast cells can be kept in storage tanks at high cell density, starved of nutrients, exposed to high ethanol and low temperature, for variable periods of time. The stress caused by the combination of these factors can influence cell viability as well as zinc cellular homeostasis.

The practice to recycle yeast biomass for several fermentations (as well as in some fuel alcohol processes), should be carried out carefully. A fraction of the yeast cone is usually discarded. Heterogeneity in age and fermentation performance was found in cells from different yeast crop fractions (Powell et al., 2004) and mother cells may retain high levels of vacuolar zinc. The combination of these two conditions may cause the loss of a part of the yeast biomass representing an important reservoir of zinc. A specific set of over-expressed genes during fermentation process may be utilised as zinc-responsive molecular biomarker to determine zinc cellular

status. This approach may be used in those industries using yeasts, as a modern tool to determine the condition of zinc cellular deficiency and to gain deeper insight into metabolic responses to zinc bioavailability. Although some potential genes have already been identified, further research is required to confirm these findings, using approaches with higher levels of reproducibility, for example, in Zn-limited chemostat continuous culture. This has been the focus of our recent research efforts (De Nicola et al., 2007).

Further investigations are needed on the interaction of zinc with other metal ions such as Mg, Ca, Mn and Cu. For example, during industrial processes, zinc may exert a synergistic effect with Mg on plasma membrane stability. It is conceivable that an optimal level of intracellular zinc is required for plasma membrane stability during chemical and physical stresses. In addition, zinc may alleviate the inhibitory effects produced by ions such as Cu. Industries having problems with fermentation due to high residual levels of copper, may conceivably employ supplementary zinc as an efficient antagonist.

This study has highlighted the need to improve our fundamental understanding of yeast nutrition and cell physiology that will ultimately prove to be of practical benefit to yeast-based industries.

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