Zinc accumulation and utilization by wine yeasts

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Abstract: The present study has focused on the accumulation of zinc by wine yeast strains of *Saccharomyces cerevisiae* during fermentation of both grape juice and chemically defined medium with different carbohydrates and at varying levels of zinc. The results have shown that zinc accumulation by wine yeast was very rapid with all zinc being removed from the medium by yeast cells within the first two hours. Zinc uptake was stimulated by the presence of sucrose. Zinc affected fermentation progress at defined levels, with optimal concentrations at 1.5–2.5 ppm, depending on yeast strain and zinc bioavailability. The bioavailability of metal ions in grape must and the roles of metals in wine yeast physiology are aspects poorly understood by enologists. In brewing, it has long been recognized that malt wort may be zinc deficient and brewers often carry out zinc supplementations to avoid sluggish and incomplete fermentations. In winemaking, zinc levels in grape musts may be compromised depending on the bioavailability of zinc ions in vineyard soils as well as treatments with fertilizers and fungicides during grape growing. As a consequence, sub-optimal zinc levels in grape musts may negatively influence the fermentative performance of yeasts. We believe that optimization of metal ion bioavailability will improve yeast fermentation performance in industrial processes and this study addresses some issues relating to zinc in enology.

Keywords: zinc, metal ions, micronutrients, *Saccharomyces cerevisiae*, winemaking

Introduction

Zinc is absolutely essential for yeast growth and metabolism. Zinc is required as a cofactor for the activity of all 6 enzymes classes, ie, oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases as identified by the International Union of Biochemistry and Molecular Biology (IUBMB).[1] It is also involved in the structure and function of proteins and nucleic acids.[2–4] The uptake and accumulation of zinc by yeast is biphasic and consists of a metabolism-independent and a metabolism-dependent stage.[5] During the first phase of metal ion accumulation, zinc is bound with the sulphydryl residues within the cysteine groups in the mannoprotein layer of the cell wall.[6] In *Saccharomyces cerevisiae* the proportion of zinc that is tightly bound to the cell wall is around 5% of total cellular zinc.[7] Zinc is rapidly transported inside the cell and primarily accumulated in the yeast vacuole.[7–10] Zinc sequestration may be influenced by numerous parameters including temperature, pH and presence of metabolic inhibitors.[5,11–14] The optimal concentration of zinc is yeast strain-dependent. Generally, for *S. cerevisiae*, 0.25–0.50 μg/mL appears to be optimal for cell growth, and 1–2 μg/mL for glycolysis.[15]

In brewing, malt wort is often zinc deficient, since most of the zinc precipitates with proteins during malt wort preparation and is therefore not bioavailable to yeast cells during fermentation. Therefore, it may be necessary to supplement wort with zinc salts, to avoid sluggish fermentations that may terminate prematurely. In the wine-making industry, it is unusual to carry out zinc analyses and specific zinc supplementations. Zinc concentrations in grape juice range from 0.04 to 7.8 μg/mL with an average of 0.9 μg/mL[16] and this is usually deemed satisfactory for the progress of the fermentation.

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Furthermore, part of this zinc may not be bioavailable to yeast cells due to chelation with proteins and polyphenols. Most susceptible to zinc deficiency are organic, calcareous soils or soils leveled for flood irrigation.[18] Sandy and acid soils have also been reported to be zinc deficient because of leaching, especially when carbonates are present.[19] In agronomy, several approaches have been proposed to overcome zinc deficiency: increasing the concentration of zinc in soils, reducing the amount of phytic acid (inhibitor of zinc absorption) and raising the concentration of sulphur-containing amino acids which promote zinc absorption.[17] Beneficial effects of zinc based fertilizers on levels of zinc in grapes have been reported by Christensen and Jensen[20] and Christensen.[21]

Very few publications are available on the influence of metal ions in grape must on yeast fermentation performance.[22–25] Commercial yeast food preparations based on a mixture of 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 fermentation. Although these actions aim to guarantee that yeast cells are healthy and active from the early stages of the fermentation, some of these supplements may be superfluous. However, specific zinc supplementations can be beneficial to yeast fermentation performance and the aim of this work was to gain further insight into zinc interactions with wine yeasts.

Material and methods

Yeast strains and growth media

Experiments were carried out with the wine yeast strains of *S. cerevisiae* L-2226 and L-2056 (Lallemand Inc., Montreal, Canada). The former is a strain particularly indicated for the fermentation of grapes with high sugars. The latter exhibits tolerance to alcohol and SO_2 and requires high levels of nutrients.

Zinc accumulation studies with various carbohydrates were conducted using yeast propagation medium (YPM), a modified version of Edinburgh minimal medium (EMM3):[26] the carbon sources supplied in the yeast growth medium were: either glucose, fructose, or sucrose, 30 g/L; ammonium sulphate, 5 g/l; ammonium dihydrogen phosphate, 2.84 g/l; potassium chloride, 2 g/l; magnesium sulphate heptahydrate, 1 g/l; calcium chloride dehydrate, 30 mg/l; potassium iodide, 0.15 mg/l; manganese sulphate, 0.6 mg/l; copper sulphate, 60 μg/l; citric acid. 1.5 mg/l; molybdic acid, 0.24 mg/l; ferric chloride, 0.3 mg/l; boric acid, 0.75 mg/l; nicotinic acid, 40 mg/l; inositol, 40 mg/l; calcium pantothenate, 4 mg/l; thiamine-HCl, 1.6 mg/l; pyridoxine-HCl, 1.6 mg/l; biotin 40 μg/l.

The zinc concentration of this medium was adjusted at 0, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, and 102.4 μg/mL, using a 4000 μg/mL sterile stock solution of zinc sulphate heptahydrate (Fluka Cheme AG, Switzerland).

In order to extend preliminary findings on zinc accumulation to more realistic industrial fermentation media, red grape juice (Welch Ltd, USA) at a sugar concentration 150 g/L was employed after clarification by centrifugation at 1500 g at 5 °C for 10 minutes. Medium was filter sterilized prior to inoculum. Zinc levels of originally 0.42 μg/mL (shake flask experiments) and 0.9 μg/mL (conical vessel experiments) were altered using a 1000 μg/mL zinc acetate stock solution (ACS Reagent, Sigma Aldrich, St. Louis, MO, USA). The use of zinc acetate or zinc sulphate was previously found not to influence zinc accumulation by yeast cells.[7]

Maintenance of zinc-free conditions

Prior to use, glassware, flasks, and conical vessels employed in this study were all deionized. This was necessary in order to remove any contaminant ions, including zinc. Glassware and conical vessels were soaked in 2% nitric acid for a period of 12 hours, then thoroughly washed with deionized and distilled water (ddH_2O), rinsed with 0.1 MEDTA and four times with ddH_2O prior to final drying.

Growth conditions

All seed cultures were prepared aseptically by inoculating a loopful of cells from refrigerated slope cultures into liquid media, in shake flasks (in orbital shaker at 25 °C, 200 rpm). After 24 hours of growth, a calculated amount of seed culture volume was centrifuged and cells washed once with sterile deionized water and resuspended in experimental media. YPM (0.8 ppm of Zn) was the seed media used for the experiments related to "Influence of carbohydrate sources on zinc accumulation by wine yeasts" and grape juice of 0.42 and 0.9 ppm of zinc was respectively used for the two experiments in "Zinc accumulation by wine yeast in grape juice".

Yeast cells were grown in orbital incubator at 200 rpm, 25 °C and initial cell densities of 5×10^6 cells/mL. Yeast cell numbers were determined using a hemocytometer (Neubauer Improved type), with a bright field microscope. Yeast cell viability was assessed using methylene violet staining according to the method of Smart and colleagues.[27]

Conical Imhoff vessels (Nalgene, Rochester, NY, USA) made of polycarbonate and of 1 L volume and cone angle 74°

were employed in small-scale fermentation experiments. The fermentations were protected from oxygen with fermentation locks. Yeast cells were inoculated into grape juice at 12×10^6 cells/mL and fermentation was carried out for five days at 25 °C, in static condition.

Samples were taken from the middle of the conical vessel (500 mL) with a sterile syringe. No homogenization was done prior to sampling.

Metal ion analyses

Zinc cell content was determined in yeast cells washed thrice in ddH_2O. Cells were hydrolyzed with concentrated nitric acid (69% AnaLar grade from Fisher Scientific, Loughborough, UK), at 90 °C for 1 h. Grape juice supernatants were also treated with nitric acid (1:1) at room temperature in order to hydrolyze possible metal-chelating proteins. Diluted yeast and medium hydrolyzates were analyzed for zinc content using a Perkin Elmer 1100B atomic absorption spectrophotometer. Each sample was analyzed in triplicate.

Ethanol analyses

In the conical vessel fermentation experiments, ethanol levels were analyzed using a Gas Chromatograph Mass Spectrometer GCMS-QP2010 (Shimadzu, Japan) fitted with an Agilent HP Blood Alcohol capillary column (ID: 0.32 mm, length: 7.5 m, film: 20 μm). Program conditions were as follows: column temperature 125 °C, injector temp 250 °C, split ratio 20:1, linear velocity 200 cm/sec, detector temperature 250 °C, temperature program: 125 hold 0.0 min rate 15 °C/min final temperature 150 °C hold 0.0 min.

Statistical analyses

All sample analyses were carried out in either duplicate or triplicate depending upon the experimental conditions. In order to verify the consistency of zinc concentration during fermentation, zinc levels were analyzed in cells and culture supernatants. When comparing the data of the two yeast strains and/or different experimental conditions appropriate statistical tests, eg, Students t test and ANOVA, were applied. In the figures presented, error bars are given where available to indicate the statistical significance of our observations.

Results and discussion

Influence of carbohydrate sources on zinc accumulation by wine yeasts

Wine yeast ferments grape must, the main sugars of which are fructose and glucose (typical total concentrations ~300 g/L), with a trace level of sucrose. Preliminary experiments on zinc accumulation by wine yeast were conducted using a defined culture medium (YPM) with glucose, fructose, or sucrose concentrations each at 30 g/L. Such concentrations differ from typical concentrations normally found in wine musts, but were chosen to provide insight into zinc accumulation by comparing different modes and energetics of sugar uptake by yeast in fermentation media with precisely defined zinc bioavailabilities. The ability of the wine yeast L-2226 to accumulate zinc ions when supplied with different zinc concentrations and carbohydrates was analyzed.

Figure 1a shows that after 24 h, zinc accumulation by yeast is generally dependent on the type of the metabolizable energy source, with sucrose leading to the highest zinc uptake.

Although an active transport mechanism has been found in yeast for sucrose,[29] this sugar is mainly hydrolyzed extracellularly by the periplasmic enzyme invertase (α-glucosidase) to its component monosaccharides (1 and fructose) before being available to yeast cells. The slower process of hydrolysis, uptake, and metabolism of sucrose results in prolonged availability of glucose and fructose compared with their supply as individual sugars. As a consequence, the energy source for zinc uptake may also be available for the prolonged accumulation of this metal. On the contrary, fructose uptake was probably very quick and energy derived from this sugar was immediately available to completely uptake zinc from the medium. This would explain the entire uptake of zinc from the medium when the initial concentration of zinc was 0.8 μg/mL.

The hypothesis that different sugars tested in this experiment supplied extra zinc is excluded since media samples at time 0 proved that all detected zinc was only provided with the added zinc solution. In addition, since the medium was defined, it is excluded that high concentrations of other heavy metals (eg, copper) could have affected zinc uptake by yeast cells.

Influence of zinc on wine yeast growth and viability

Yeast cell viability remained unchanged at the range of zinc concentrations studied. High yeast cell numbers were found when cells were grown in glucose and fructose based YPM. This was probably due to the immediate availability of these readily metabolized sugars in the growth medium (Figure 1b). Maximal growth was achieved after 24 h at a cell density of $270 \times 10^6 \pm 9 \times 10^6$ cells/ml with sucrose, at zinc concentration of 102.4 μg/mL (data not shown). Wine yeast growth on sucrose was greater when compared with growth on

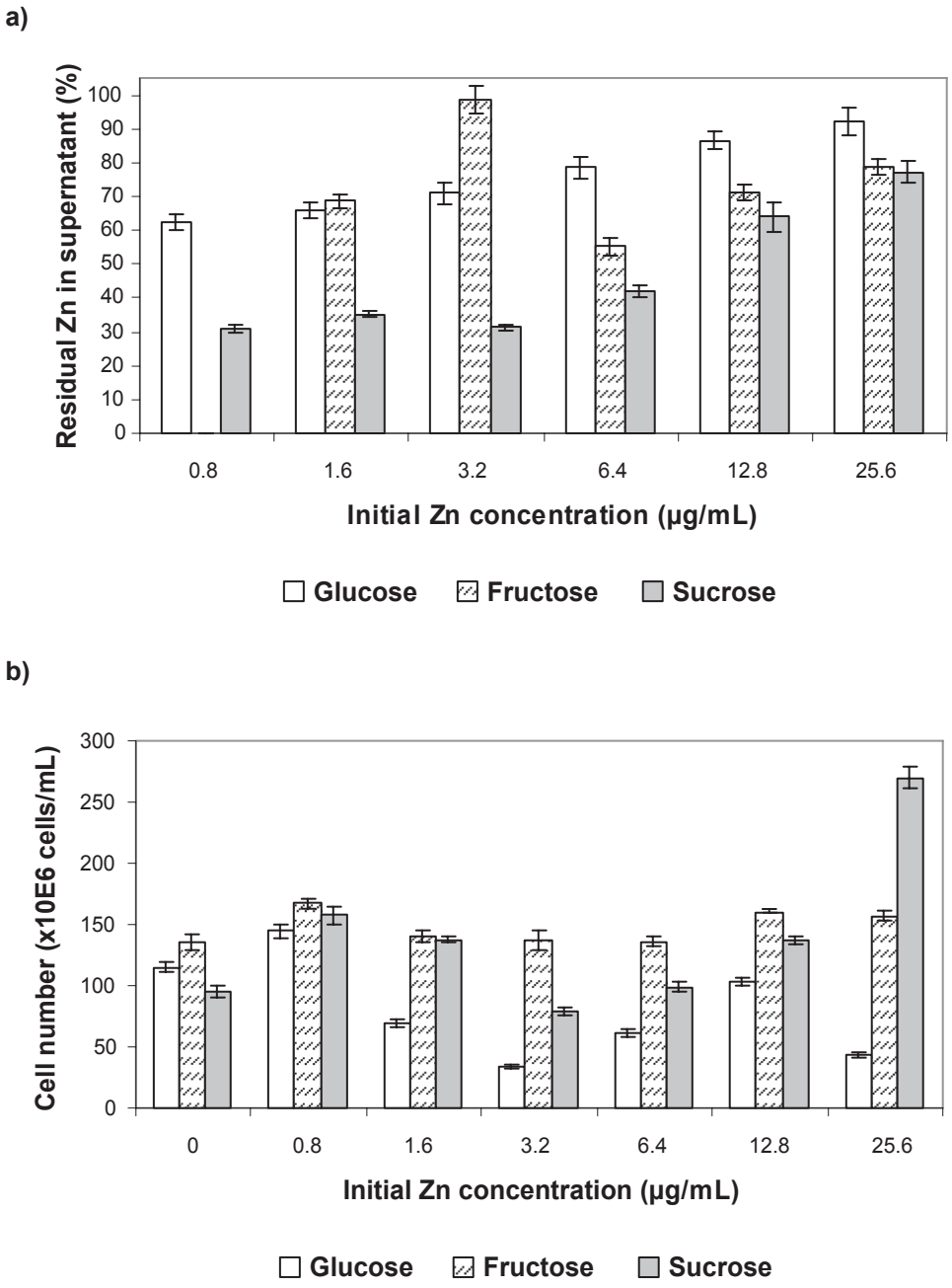

Figure 1 Zinc accumulation and growth of wine yeast strain L-2226 grown in YPM with variable zinc and carbohydrate sources. Residual zinc levels **a**) in media were calculated as percentages considering the initial zinc levels in media and the zinc accumulated by yeast cells after 24 h of growth in YPM in shake flasks, at 25 °C for 24 h. Cell number **b**) was assessed to evaluate the influence of variable zinc levels on cell growth.
Note: Error bars denote standard deviation and show which differences are statistically relevant.
Abbreviation: YPM, yeast propagation medium.

individual sugars: glucose, 145 x $10^6 \pm 6 \times 10^6$ cells/mL (with 0.8 µg/mL Zn) and fructose, 167 x $10^6 \pm 4 \times 10^6$ cells/mL (with 0.8 µg/mL Zn) after 24 h (Figure 1b).

The maximal cell densities achieved in defined media, when containing the monosaccharides glucose and fructose, were therefore evident at the low end of the zinc concentration (0.8 µg/mL). Excessive zinc (>1.6 µg/mL) appeared to have a detrimental effect on wine yeast growth only when grown in glucose-based YPM. Hammond[30] reported that excessive zinc could depress the growth of brewing yeast unless the concentrations of manganese were similarly high. In the present study, we did not determine the relationship between zinc and manganese on wine yeast growth. Nevertheless, we have shown that growth of wine yeast in the presence of different metabolizable energy sources differs with variable zinc availability.

Industrial fermentation media for wine yeasts is grape must, which is rich in fructose and glucose. Therefore, if must was also to be used for yeast propagation (as opposed to fermentations) then it would be advisable to determine the amount of zinc in the must. If winemakers are concerned about levels of zinc being suboptimal for fermentation, various commercial yeast foods are available that contain zinc. For example, one of the products available is "*Servomyces*" from Lallemand Inc. *Servomyces* is a dried single-strain brewing yeast, available as dead or viable cells, pre-enriched with zinc during the propagation process, whose zinc concentration reaches 50,000–55,000 ppm.

Zinc accumulation by wine yeast in grape juice

Zinc accumulation by the wine strain of *S. cerevisiae*, L-2226, in grape juice at 25 °C was analyzed in the first 7 h (early exponential phase) and after 24 h (early stationary phase) of fermentation (data not shown). Figure 2 shows zinc accumulation versus yeast cell growth in the first 7 h. Zinc accumulation was very rapid with cells reaching zinc contents 35 times higher in the first 2 h following inoculation. During this period, cell number slightly increased. The zinc accumulated by the yeasts in the first 2 h of growth and grape juice consequently became rapidly zinc-depleted. It is clear that all zinc present in the medium was bioavailable and no chelators were present in the medium to bind this metal ion. The same patterns of zinc accumulation were also described in cultures of *Candida utilis* by Failla and colleagues[11] and in other brewing and distilling strains of *S. cerevisiae* in malt wort with similar zinc medium concentrations by De Nicola.[7]

In the remaining hours of fermentation, mean zinc cellular content gradually decreased while cells were dividing. Most likely, in the first period of fermentation, actively growing yeast cells may have had sufficient intracellular carbohydrate reserves (eg, glycogen) to be utilized immediately as energy required for active zinc accumulation. Subsequently, sugar sources in the growth medium may have had a stimulatory effect on zinc cell uptake most likely stimulating plasma membrane ATPase activity and generating a transmembrane proton gradient, which mediates zinc uptake into the cell.[31] In grape juice, this energy was mainly provided by glucose and fructose. The use of shake flasks kept cells homogeneously in suspension and cells were effectively exposed to zinc ions present in the medium. In wineries, although some agitation may be implemented, fermentations are infrequently kept agitated. Therefore, in these experiments, agitation may have been an important factor contributing to rapid kinetics of zinc uptake. Moreover, other factors that have been reported to influence zinc uptake in yeasts, such as pH and

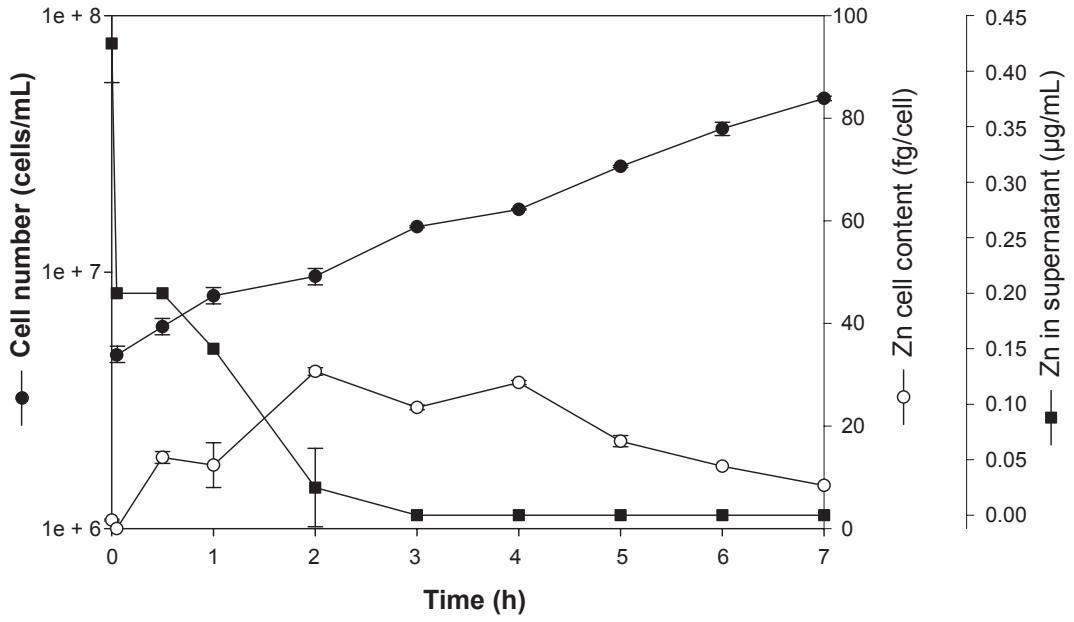

Figure 2 Zinc accumulation by wine yeast strain L-2226 in grape juice medium. The wine yeast strain L-2226 was cultivated in shake flask, in grape juice (zinc at 0.42 µg/mL), at 25 °C, 200 rpm and for 24 h. Figure represents cell growth, zinc cell content, and zinc supernatant concentrations in the first 7 h of growth. Cell growth was determined by hemocytometer. Zinc cell content was expressed on a per cell basis after cell hydrolysis and analysis by atomic absorption spectrophotometer (AAS). Medium residual zinc levels were also analyzed by AAS.
Note: Error bars denote standard deviations.

temperature, could influence zinc uptake by yeasts in wine fermentations. In *C. utilis*, zinc uptake rates decreased while pH increased from 4.8 to 8.2,[11,12] although these levels are far from values normally found in wine. Mowll and Gadd[5] have observed a decrease in zinc accumulation in cells of *S. cerevisiae* when the temperature was reduced from 25 °C to 4 °C. The influence of temperature on zinc uptake was also found in beer fermentation studies[7] where lager brewing strains decreased zinc cellular accumulation when exposed to lower temperatures (8 °C compared to 25 °C). Most likely, lower temperatures reduced yeast metabolism and ATPase activity, although all zinc was removed from the medium after 24 h.

Influence of zinc on wine fermentations

Zinc accumulation patterns by the wine yeast strains L-2226 and L-2056 were different in small scale conical fermentation vessels (Figures 3a, 3b) from those described above in shake flasks. These experiments were carried out without agitation and for longer periods of time (five days compared to

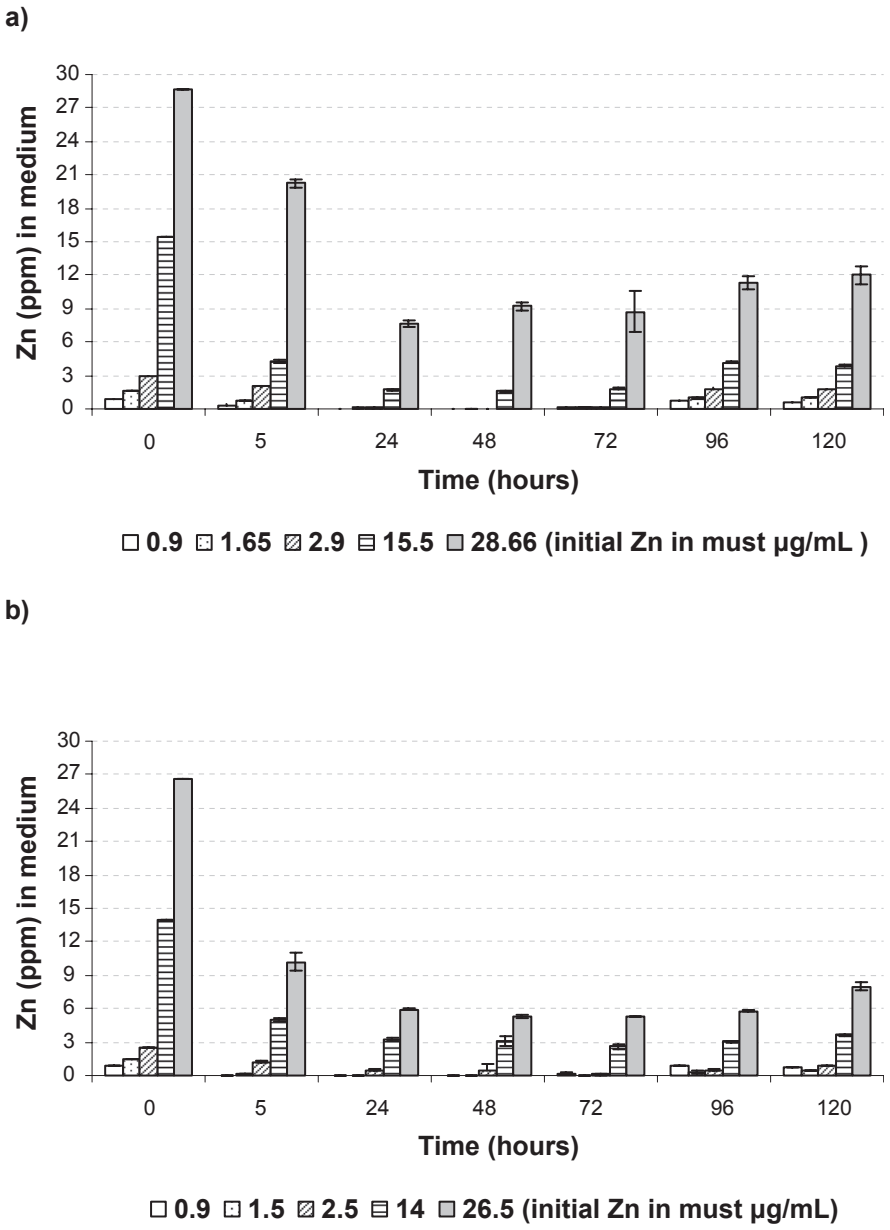

Figure 3 Influence of zinc supplementations on zinc accumulation during grape must fermentation by wine yeasts. Yeast cells of the strains L-2226 **a**) and L-2056 **b**) were inoculated in Imhoff conical vessels, in grape juice with zinc levels adjusted adding Zn acetate. Fermentation was carried out at 25 °C for 120 h (5 days). Zn residual levels in supernatants were analyzed throughout fermentation by atomic absorption spectrophotometer.
Note: Error bars denote standard deviations.

24 hours). Although zinc accumulation was high in the first two days of fermentation, the wine strains tended to release zinc back into the medium after, respectively, 48 (Figure 3a) and 72 hours (Figure 3b). This phenomenon was not related to any loss of viability. Zinc did not have any toxic effect on yeast cells at any zinc concentration tested and the viability of both strains was unaltered. At end of fermentation cell viability was 84% for L-2226 and 83% for L-2056 at the highest zinc concentrations.

Zinc release was therefore time-dependent and perhaps due to alcohol stress. For example, when L-2226 and L-2056 yeast cells were continuously exposed to ethanol concentrations above 6% v/v (Figures 4a, 4b) they may encounter altered plasma membrane permeability, resulting in loss of zinc ions. In this regard, Learmonth and Gratton[32] have previously reported that ethanol stress increased membrane fluidity of yeast cells.

Yeast growth of the two wine strains was not dramatically affected at the zinc concentrations studied (Figure 5). At the end of the fermentation, the yeast crop formed at the bottom of the fermenters was generally the same volume in all fermenters. Only when the wine strain L-2056 was grown in medium with 0.9 µg/mL of zinc, was a lower yeast crop measured. However, during L-2226 fermentation, zinc at variable concentrations

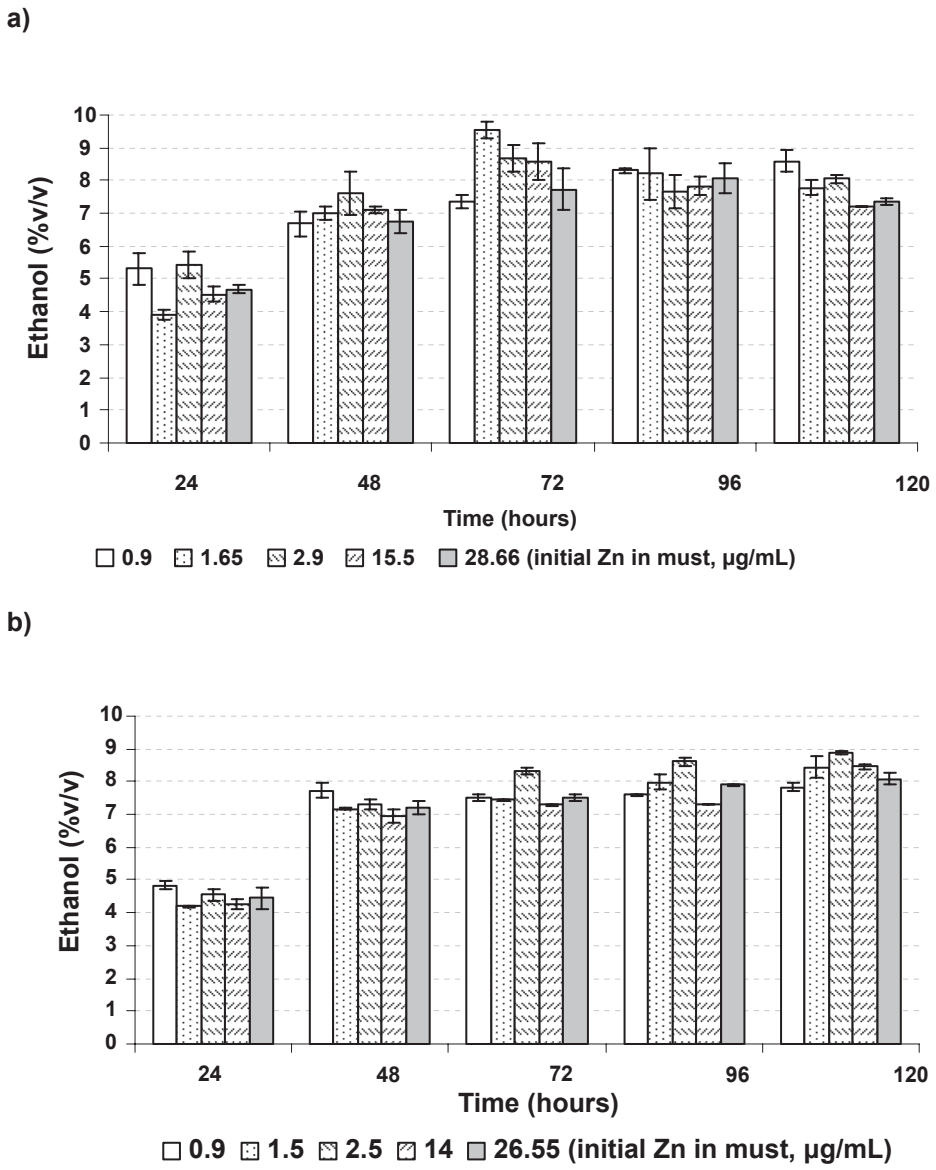

Figure 4 Influence of zinc on wine yeast fermentation of grape must Fermentation performance of the wine strain L-2226 **a**) and L-2056 **b**) in conical vessels, at variable levels of zinc were evaluated by analyzing ethanol production from 1 to 5 days.
Note: Error bars denote standard deviations.

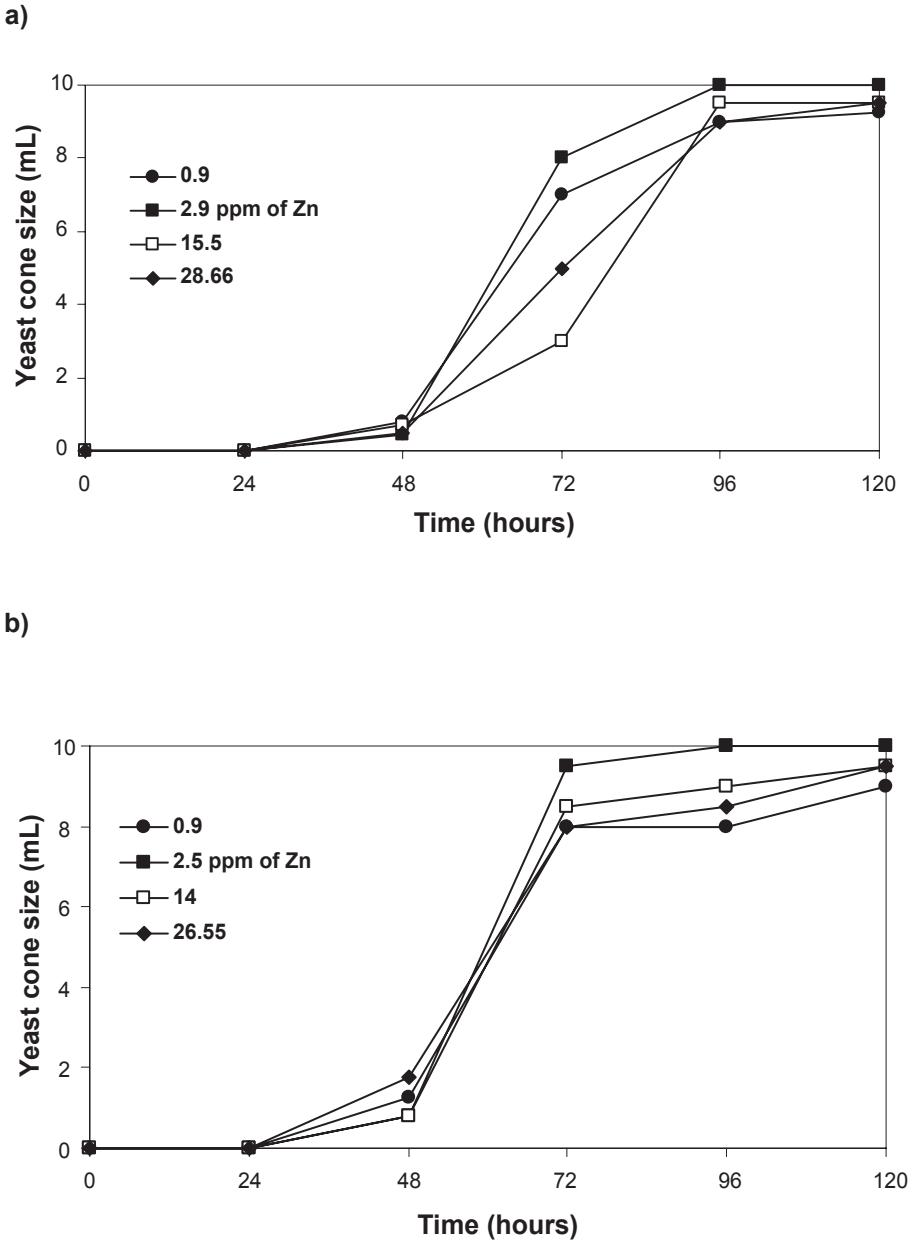

Figure 5 Influence of zinc on growth of wine yeast strain L-2226 and L-2056 during fermentation of grape must. Cell growth of L-2226 **a**) and L-2056 **b**) was checked daily by evaluation of the yeast crop size formed at the bottom of the conical vessels by sedimentation of the cells. No homogenization was done prior to sampling. For clarity, standard deviations were not included in the graphs.

influenced yeast cell sedimentation: at 72 h, most of the cells exposed at 2.9 μg/mL precipitated (Figure 5a). Zinc affected yeast sedimentation at defined concentrations and this could be related to a possible role of this ion in cell flocculation, although this phenomenon was not investigated in this study. In this study for example, zinc concentrations of 2.5 μg/mL and 2.9 μg/mL, respectively, affected wine strains L-2056 and L-2226 by enhancing flocculation of the yeast cells and giving a slightly higher final yeast crop (Figures 5a, 5b). Various zinc concentrations did not dramatically affect fermentation performance, although wine strain L-2056 showed higher ethanol titers at 1.5 and 2.5 μg/mL of zinc (Figure 4b). This result, associated with a final higher biomass, indicated that this concentration range had a general stimulatory effect on fermentation performance with this strain. Zinc requirements for optimal fermentative performance appear to be higher in wine yeast strains (eg, 2.5 μg/mL) compared with brewing yeast strains (0.4–1 μg/mL).[7] These ranges are only indicative since they may be strain- and media-dependent. The former has been clearly shown in the present study and the latter

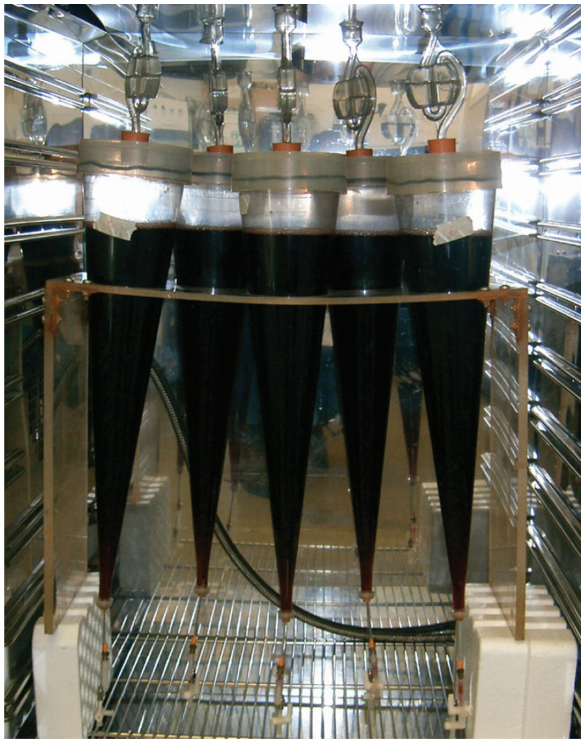

Figure 6 Imhoff conical vessels used in small-scale wine fermentation. Wine yeast strains were grown in graduated conical vessels (1 L, cone angle 74°) made of polycarbonate and closed with fermentation locks. Cones were washed previously with nitric acid, ddH_2O, EDTA (as described in Materials and methods) and filled with grape juice. Yeast cells were inoculated at initial cell density of 12×10^6 cells/mL and fermentation was carried out at 25 °C for five days.

may depend on the sequestration of zinc by cysteine groups of peptides and amino acids present in malt wort that may alter zinc bioavailability.[33]

Conclusions

This study has generated new information on zinc accumulation by wine yeast strains. Zinc accumulation was affected by various carbohydrates with sucrose enhancing accumulation of zinc compared to glucose and fructose. In grape juice, yeast accumulated zinc very rapidly, resulting in concomitant rapid lowering of zinc from the medium (eg, during the first 2 h when cells were grown in shake flasks). In small scale static fermentations with grape must, zinc accumulation was slower and some zinc was released back into the medium. This could be associated to the increased ethanol levels. Although zinc concentrations did not significantly affect yeast growth and cell viability, optimal fermentation performance was found at 1.5 and 2.5 μg/mL of zinc with the wine strain L-2056 showing higher ethanol titers.

Our results showed that zinc influences wine yeast physiology and fermentation performance. Previous work from this laboratory[23] has already shown important effects of magnesium ions on wine yeast physiology, affecting yeast growth, sugar consumption and ethanol yields. Whilst magnesium is required as an essential mineral for wine yeast at much higher levels compared with zinc (eg, 100 vs 1 μg/mL), the latter is nevertheless very important for grape fermentations.

The Imhoff conical vessels employed in this study (Figure 6) allowed us to carry out this study in small scale and to ensure anaerobic condition throughout the fermentation, although the shape of these vessels could not be representative of typical wine fermentations as other vessels (eg, cylindrical vessels). The quantitative findings of this work need to be confirmed in a panel of grape juices and musts, which would better represent the conditions used in commercial wine production.

Very little research on the role of metal ions in wine yeast fermentations has been published.[31–33] However, this study has demonstrated that the correct management of zinc in industrial fermentation processes could be beneficial in terms of improved yeast growth, viability, fermentation performance and resistance to environmental stresses. For winemaking, we advise close attention being paid to metal ion levels in grape must to ensure consistently good progress of wine yeast fermentations.

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Disclosure

The authors report no conflicts of interest in this work.

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