

Accumulation and Release of Metal Ions by Brewer's Yeast During Successive Fermentations

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ABSTRACT

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Magnesium, zinc and calcium are the elements that are involved in the regulation of metabolic activity of yeast and/or other processes such as flocculation and cell division. The requirements for specific ions may be subject to changes in successive fermentations performed by the same biomass. The aim of this work was to analyze the changes in calcium, magnesium and zinc concentration in malt broth (9°P) and in the yeast over four successive fermentations. For all ionic species analysed, the qualitative uptake and release patterns observed during subsequent fermentations were similar. In the lag phase of the fermentation, yeast cells accumulated the metal ions under investigation, in order to liberate them later to an extent that depended on the specific metal and fermentation number. Some differences were noted when qualitative comparison of these processes was performed. During the first fermentation with the yeast culture, the maximum content of each ion in the yeast biomass was two- to threefold higher than in the subsequent fermentations. Calcium, magnesium and zinc levels in the biomass did not exceed 1, 6 and 0.6 mg/g yeast dry weight respectively.

Key words: bioaccumulation, brewer's yeast, calcium, magnesium, metal ions, release, zinc.

INTRODUCTION

The fermentation medium should contain, apart from the sources of carbon, nitrogen and phosphorus, also minerals and growth factors. Among the many cations present in yeast and wort, magnesium, calcium, zinc, manganese, potassium and copper are the ones most involved in the regulation of structure and metabolic activity of the cells during growth and fermentation^{1,2,9,15,18}. The rate of uptake and utilisation of these ions by the yeast biomass depends both on the ion concentration in the wort, as well as on its bioavailability. The latter is partly determined by the solubility of the ionic compounds and also by the presence or absence of complexing chelators³. It has been established that the accumulation and release of metal ions during ethanolic fermentation is a

dynamic process and that the intensity depends on the sugar and alcohol content in the fermentation medium^{8,9}. The distribution of ions to various regions of a cell is controlled at the cell membrane level, and this ensures a transport system to the intersections of the cell¹¹.

Brewer's yeast can be subjected to many stress factors of a physical, chemical, osmotic and/or hydrostatic nature. Serial repitching is a common brewing practice that applies additional stress on the yeast cells. Some brewers use the yeast slurry over fifteen times. Much information can be found in the literature regarding the function of metal ions, their optimal concentrations and mechanisms of accumulation and release, but these publications mainly describe yeast that has only been used a single time. Only a few reports have been based on the ionic nutrition of yeast used twice^{9,10,17,18} and therefore there is a lack of information regarding accumulation and release of metal ions in successive fermentations by the same biomass. In the present study, changes in the calcium, magnesium and zinc concentrations in wort, and in yeast biomass that was used over four successive fermentations, were investigated. Laboratory scale fermentations in a 9°P malt broth were conducted using the biomass obtained after each fermentation, to pitch the next portion of fresh wort.

MATERIALS AND METHODS

Yeast culture

The yeast strain used in this work was an industrial strain of *Saccharomyces cerevisiae*, W34/70 (Fermentis, Warszawa, Poland). Prior to propagation, the instant culture was reactivated on a 9°P wort-agar slopes (30°C, 24 h), to avoid the viability decrease caused by the sudden rehydration of a dry yeast culture. The biomass was then propagated using a 2-stage procedure: strains were first grown statically at 30°C in malt extract broth (9°P) for 24 h; next 10 mL of broth culture was transferred into the same medium (90 mL) and grown using an orbital shaker (120 rpm) at 30°C for 24 h. The yeast obtained was used to inoculate 500 mL Erlenmeyer flasks, containing 200 mL of culture medium, to achieve a biomass concentration of ca. 0.4 g per litre on a dry mass basis. Malt extract broth was produced by dilution of unhopped malt extract of 80°P to 9°P (Malt Extract Manufacturing Ltd., Wolsztyn, Poland) and sterilization of bulk quantities (ca. 2000 mL, 121°C, 20 min) followed by filtration to remove the precipitate. Next, the clear broth was standardised to 9°P, dispensed into 200 mL aliquots, sterilized again, cooled to

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4°C, and stored at this temperature for later use. The resulting broth was clear (absorbance at 620 nm < 0.1) so no compensation for cloudy wort was required when preparing the biomass growth calibration curve.

Fermentation conditions and analytical procedures

All fermentations were conducted in triplicate, for 7 days at a constant temperature of 14°C, in an EQcell 240 thermostat (Wroclaw, Poland). They were monitored daily for biomass content and mass decrease. Growth was monitored by measuring the absorbance of the culture at 620 nm with a DU-650 Beckman spectrophotometer and the OD₆₂₀ values were converted to cell dry mass using a calibration curve established for this particular strain. Where necessary, samples were diluted to ensure that the OD₆₂₀ reading fell within the linear range of the calibration curve. Fermentation rate was measured by weighing the flasks and results were expressed as a decrease of initial nutrient mass as a percentage.

During the fermentation, the magnesium, zinc and calcium content of the medium or yeast biomass was analysed. Samples (3.5 mL) were withdrawn, in duplicate, out of well shaken fermentation mixtures, washed twice with deionised water to remove residues of the medium and centrifuged (735 × g, 5 min). Following this, HNO₃ (4 mL) was added to the resulting biomass pellet or to the clear broth (3 mL) and a wet-pressure digestion in a microwave oven for 15 min at 170°C (MARS Express, CEM, USA) was conducted. After cooling, the samples were diluted with deionised water to a final volume of 15 mL and supplied to the flame atomic absorption spectrometer with flame atomization (VARIAN 240FS), equipped with an air/acetylene burner, using a sample introduction pump (SIPS 20). Standard curves were prepared with samples obtained by dilution of Merck stan-

dard solutions (1000 mg/1000 mL) with deionised water.

All reagents used were per analysis purity Fluka products unless otherwise stated. Water was deionised to conductivity below 0.05 µS (HydroLab, Gdansk, Poland).

Serial repitching of biomass

The influence of serial repitching on the accumulation and release of certain metal ions was analyzed with the use of *Saccharomyces cerevisiae* strain W34/70. Fermentations of 9°P broth were conducted for 7 days in the laboratory. At the termination of a process, clear beer was decanted and the remaining yeast slurry was analyzed for dry weight content and cell viability. Viability did not drop below 90%. An appropriate portion of the slurry (to give a biomass concentration of 0.4 g d.m./1000 mL), corrected for the viability, was transferred with the use of a sterile pipette to a new batch of broth. During fermentation, samples of biomass, as well as of broth, were collected daily for 4 days after pitching and on the last day of fermentation (day 7) for the analysis of the yeast biomass ionic content before re-pitching.

RESULTS AND DISCUSSION

Biomass growth and fermentation performance

Figure 1 shows the biomass growth during four subsequent sets of all malt broth fermentations (labeled F1, F2, F3 and F4 on the figures), performed on a laboratory scale. For all sets, the yeast growth was significant until the fifth day of the process, and then after reaching the maximum, the biomass concentration remained at a constant level until the end of the fermentation. The growth during the first set of fermentations was significantly delayed compared to the following ones. At 24 h after pitching, the biomass content equalled about 50% of that reached in the subsequent fermentations. In the later stages of the process, the differences in the yeast growth were not as meaningful, although fermentations F2, F3 and F4 were constantly characterized by a higher biomass content (~15–25%) than fermentation F1.

Figure 2 shows the attenuation of the broth and the decrease of its initial mass expressed as a percentage. The slow attenuation in the first fermentation was in a good agreement with the biomass growth results for this set, reaffirming the concept that it is dividing cells that are responsible for fermentation activity and ethanol production. In the first set of fermentations, biomass growth was slower compared to the following ones, which can partially explain the poorer fermentation performance observed. Otherwise, regarding the mass decrease, there were no significant differences between the second and fourth fermentations, all of them reaching the end of fermentation after five days. Mochaba et al.^{9,10} analysed brewing yeast reuse in two subsequent fermentations and reported that contrary to our findings, growth in the second fermentation was reduced and delayed compared to their first fermentation. The fermentation process of both sets analyzed by Mochaba et al., in terms of broth attenuation or alcohol yield, did not differ significantly. The discrepancy between our findings and those of Mochaba

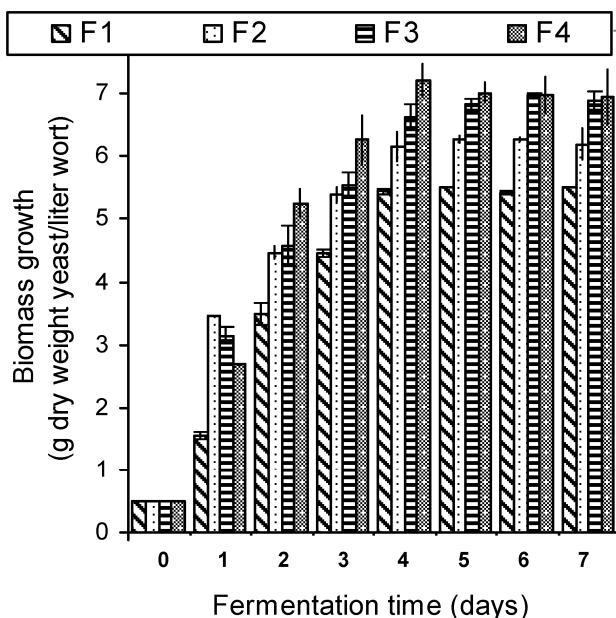


Fig. 1. Growth of biomass serially re-pitched into four subsequent fermentations (F1, F2, F3, F4) of a 9°P malt broth incubated at 14°C. Bars represent standard deviations.

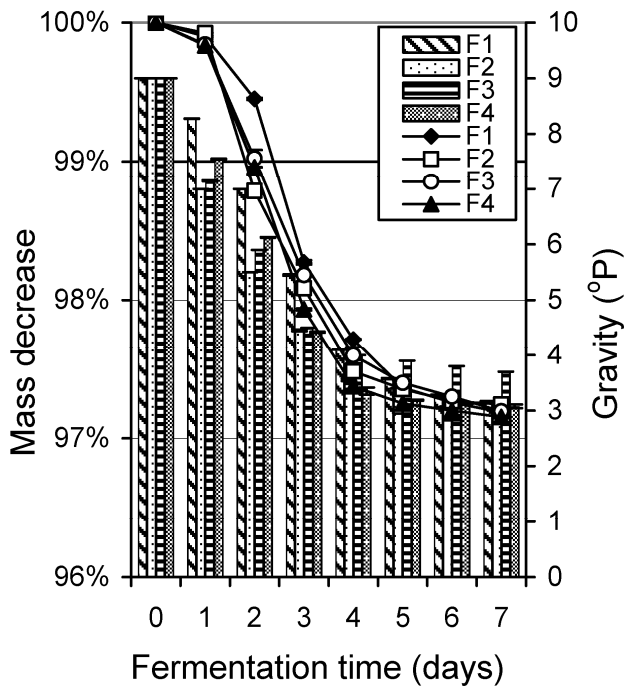


Fig. 2. Fermentation rate of a 9°P broth serially re-pitched (F1, F2, F3, F4) with biomass of *Saccharomyces cerevisiae* strain W34/70 incubated at 14°C. Bars represent standard deviations.

et al.^{9,10} could be because biomass growth was expressed as cell count, and this is not always proportional to biomass on a dry mass basis.

Calcium ion uptake and release

Figure 3 shows the total calcium concentration in the yeast biomass during four subsequent fermentations. A general trend of uptake of the ions in the beginning of the process can be noted in all fermentations, followed by calcium ion release 48 h after pitching. It was suggested in a previous report by Kellermayer et al.⁴ that starved cells subjected to carbohydrate re-addition transiently elevate their cytosolic calcium content. Thus, a rise of the biomass calcium directly after pitching into fresh broth can be due to the starvation that the cells undergo at the end of propagation or a previous fermentation. An earlier report by Saavedra-Molina et al.¹² suggested that yeast cells exhibit an increased calcium uptake when they start to bud, then the uptake returns to basal values, which are maintained until the end of the cell cycle. They concluded that after the starting point of cell division, calcium must reach its minimum level to ensure continuous budding. Our results on biomass growth appear to confirm this principle. Cells during the first fermentation maintained their calcium content at a higher level throughout the process. This was associated with poorer biomass growth compared to growth observed during the following fermentations. During the first use of the biomass, the accumulation of calcium ions at the beginning of the process was much more evident than in the following batches. In our research, it was observed that serial reuse of the biomass resulted in lower calcium ion uptake.

The initial changes in biomass calcium content were confirmed by the results obtained for the broth (Fig. 4). It

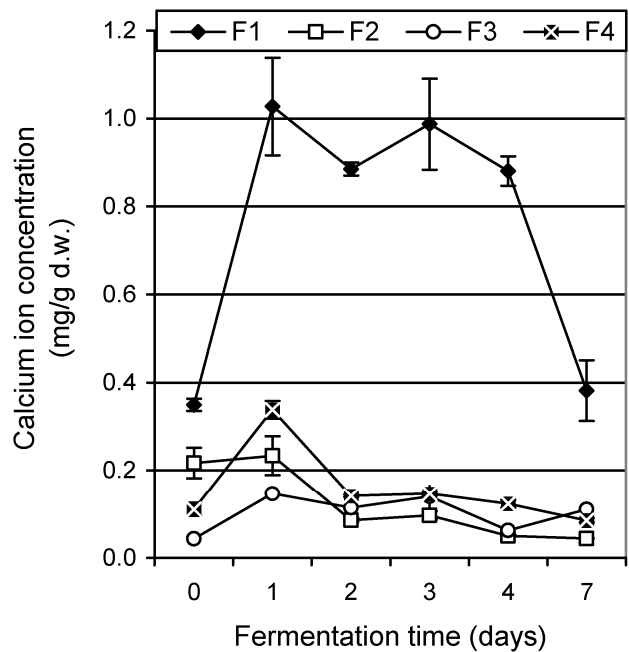


Fig. 3. Calcium ion concentration in yeast biomass during four fermentations (F1, F2, F3, F4) of a 9°P all-malt broth.

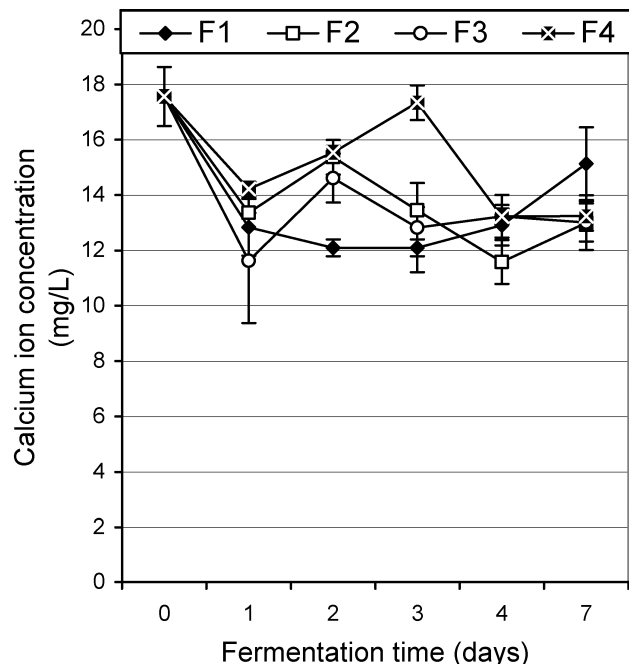


Fig. 4. Calcium ion concentration in broth during four fermentations (F1, F2, F3, F4) of a 9°P all-malt broth.

is worth remembering that when biomass concentration rises (Fig. 1), and the concentration of calcium in broth remains constant, a decrease of calcium ions present in the biomass may occur, as it is calculated per unit of dry mass.

Generally, at the beginning of the process, there is a decrease in the broth calcium concentration. There are also some fluctuations of broth calcium content during the logarithmic phase of the growth, which consists of another increase and decrease in calcium concentration.

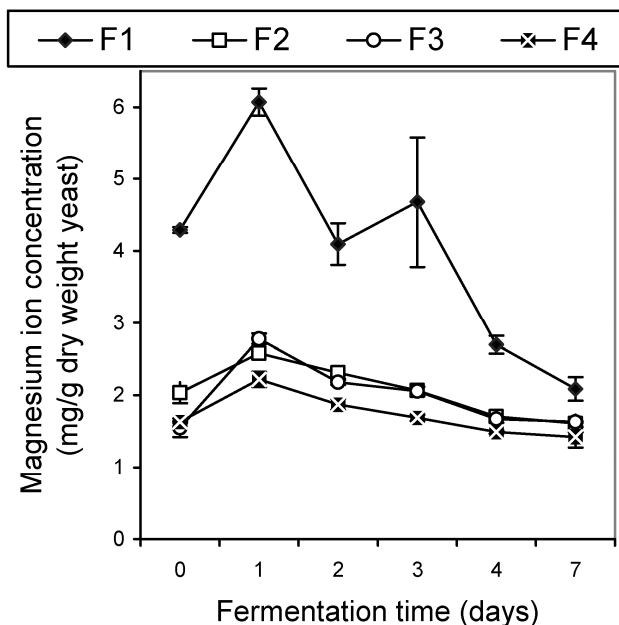


Fig. 5. Magnesium ion concentration in yeast biomass during four fermentations (F1, F2, F3, F4) of a 9°P all-malt broth.

The latter one would suggest that the ion is taken up by the biomass; this however, cannot be verified by the results of biomass calcium content. Many early workers suggested that there is a calcium requirement in flocculation⁷, confirming its uptake by the yeast during this process. Stratford¹⁴ however indicated that when cells flocculate, a large amount of calcium is bound to the cell wall, as it is required to maintain the lectin-like proteins in the correct conformation for the process to take place⁷. Considering this, it was speculated that as the calcium content in broth decreased, without a significant rise of the cellular calcium content, the ions were taken up by the biomass and bound loosely on the outer surface of the cells (this calcium was probably later washed off in the course of sample preparation). Thus, the calcium content in the supernatant liquid obtained from cell pellet washing was analyzed. Indeed, towards the end of fermentation, there was a significant rise (over threefold) of supernatant calcium, as calculated per dry weight of the biomass (results not shown). It was assumed that just before the termination of fermentation, the calcium ions were removed from the broth by the yeast biomass and absorbed on the cell surface, in order to be utilized in the flocculation process.

Magnesium

Figure 5 shows the intracellular magnesium ion content in yeast biomass used four times to ferment a 9°P all malt broth. As opposed to calcium or zinc, the content of magnesium ion in the yeast cells reached much higher levels, which was in good agreement with previous reports that magnesium is the most abundant divalent ion in yeast^{9,10,17}. It is worth noting that the biomass obtained from propagation contained relatively high amounts of magnesium ion, ca. two times higher than in the yeast at the end of the fermentation (Fig. 5). It is thought that the oxygenation of the broth during propagation influenced

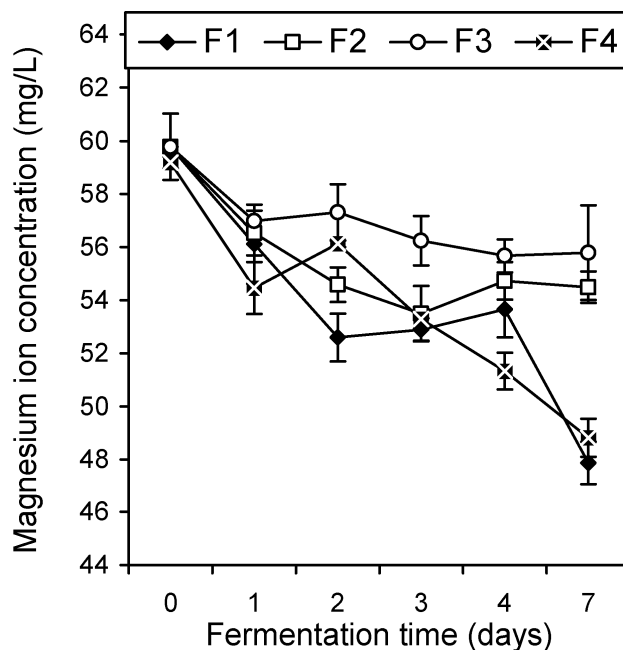


Fig. 6. Magnesium ion concentration in broth during four fermentations (F1, F2, F3, F4) of a 9°P all-malt broth.

the ability of the yeast cells to accumulate magnesium ions. During aeration, cell division is more efficient, and as magnesium is known to be essential for biomass growth¹⁶, the cells might have had a higher requirement for that ion, than during anaerobic metabolism.

In the yeast of the first fermentation, the magnesium content was as high as 6 mg/g d.m. In the following repitches, it stayed at a lower level, not exceeding 3 mg/g d.m. There was a general trend, where in the beginning of the process cells took up magnesium ions, and after the first day of fermentation, the cells slowly began to release it. At the end of the process, yeasts of the fermentations F3 and F4 contained the same amount of magnesium as at the time of pitching. Saltukoglu and Slaughter¹³ reported that yeast accumulates a constant amount of magnesium per cell, rather than proportionally to its availability. This was later confirmed in two subsequent sets of fermentations by Mochaba et al.¹⁰

The content of magnesium ions in the broth (Fig. 6) appeared to stay in an agreement with results obtained for the biomass. Generally, in all fermentations, the broth magnesium dropped by about 3–6 mg/1000 mL, shortly after the addition of yeast biomass into the medium. As expected, the most substantial decrease was noted during the first set of fermentations, as the biomass of the first fermentation accumulated the largest amount of magnesium. The decrease of broth magnesium in fermentations F1 and F2 proceeded until the 48 h in the process, whereas in fermentation F3, the magnesium level was stable until the termination of fermentation.

Zinc ion uptake and release

Zinc uptake by yeast is shown in Fig. 7 and the corresponding broth zinc ions concentrations are shown in Fig. 8. Zinc ions were rapidly taken up by the biomass at the beginning of the process. In the first set of fermentations,

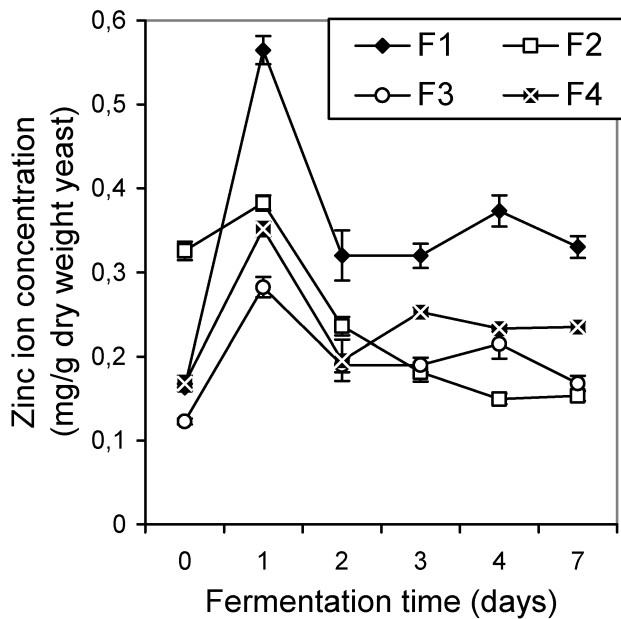


Fig. 7. Zinc ion concentration in yeast biomass during four fermentations (F1, F2, F3, F4) of a 9°P all-malt broth.

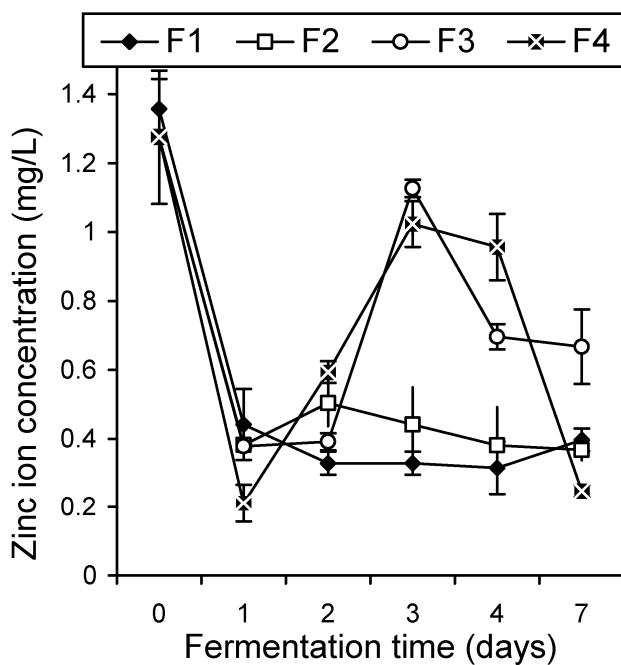


Fig. 8. Zinc ion concentration in broth during four fermentations (F1, F2, F3, F4) of a 9°P all-malt broth.

the yeast contained as much as 0.55 mg zinc per gram of dry biomass. This was in an agreement with previous reports that zinc is absorbed by the yeast biomass, even before the fermentation commences^{5,6,9,10} (i.e., just after the cells are dispensed into broth). The biomass obtained after the first fermentation, pitched into fresh broth of presumably the same composition (broth was prepared in the same batch for all fermentations), exhibited a lower ability to accumulate the zinc ions. The trend of decreasing ability to absorb zinc was also observed in the next fermentations. The broth zinc content dropped rapidly from

more than 1 mg/1000 mL to 0.3–0.5 mg/1000 mL. This phenomenon was not dependent on the fermentation number. It was however observed that the zinc ion content in broth remained at its minimal level in the first and the second fermentation (F1 and F2), while in the next two fermentations (F3 and F4), it increased as the process progressed, then dropped again at the end. The findings of Mochaba et al.¹⁰ suggested that in the second fermentation, cells appeared to be less active in regard to zinc ion uptake compared to the first fermentation, based on experiments with a double use of a yeast slurry. In our work, the yeast biomass was used four times, and based on the results obtained, it can be concluded that after the second repitch, the ability of the yeast to accumulate zinc ions decreases even further. This can be associated with either a lower requirement for the ion, or a poorer state of the biomass, namely a lower ability to perform an active uptake of zinc ions. The former conclusion may be reinforced by the results of biomass growth and fermentation performance, which indicated that the overall condition of the biomass did not worsen with an increasing number of biomass repitchings.

The increase of zinc in the broth after the initial drop (Fig. 8), may be due to a drop in pH during the fermentation process, as that drop decreases the apparent stability constant on the zinc ions metal binders⁶. Such an increase in zinc ion concentration in broth was noted for batches F2 to F4, this being more considerable the more times the yeast slurry was used.

CONCLUSIONS

Yeast, when used many times in an anaerobic process, exhibited some variations regarding biomass growth and broth attenuation. A major difference was noted for fermentation performed by the biomass used for the first time, when biomass growth together with extract utilization was delayed, as compared to later usage. The metabolic performance of the cells was correlated with the dynamics of metal ion accumulation and release. For all ionic species analyzed (magnesium, calcium and zinc) the qualitative uptake and release patterns observed during subsequent fermentations were similar. In the lag phase of the process, before the fermentation commenced, yeast cells accumulated the metal ions under investigation, in order to liberate them later to an extent that depended on the specific metal and fermentation number. Some differences were noted when qualitative comparison of these processes was performed. During the first fermentation, the maximum content of each ion in the yeast biomass was two- to threefold higher than in the subsequent fermentations.

It can be concluded that the management of yeast ionic composition, reflects the metabolic and physiological changes that the biomass undergoes with each serial reuse. During oxygenation at the propagation stage, when there is a considerable biomass growth, the magnesium content in the yeast is much higher (threefold) than at the end of an anaerobic process, which confirmed its crucial role for yeast growth. Calcium ions, known to play an important part in flocculation, were taken up at the end of the logarithmic phase of the process.

The results of this work encourage the performance of further experiments on a larger scale. A project is being prepared in which the ionic content of both wort and yeast biomass will be monitored during successive fermentations in a commercial brewery.

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