Zinc, Copper and Manganese Enrichment in Yeast
Saccharomyces cerevisae

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Summary

The aim of the present work was to study the incorporation of some microelements in the yeast *Saccharomyces cerevisiae* and its impact on the physiological state of the yeast cells during the alcoholic fermentation. The cultivations were performed on molasses medium in anaerobic (thermostat) and semiaerobic (shaker) conditions, with and without the addition of zinc, copper and manganese sulphate (0.1 g/L of each) at 30 °C and different pH values of the medium (3.5–6.0) for 8 h. The addition of the mentioned salts in molasses medium enhanced the yield of the yeast biomass up to 30 % in semiaerobic conditions, but the ethanol yield was changed very little. On the other hand, in anaerobic conditions the yields of the yeast biomass were increased up to 10 % and alcohol yield up to 20 %. After the fermentations were performed, the concentration of metal ions in yeast cells was determined. Different values were achieved depending on the used growth conditions. The highest amount of Zn ions in dry matter (700 μg/g) was incorporated in the yeast biomass under anaerobic conditions. In contrast, the incorporation of Cu and Mn was preferred in semiaerobic conditions and the highest value of Cu^{2+} ions in dry matter (1100 μg/g) and Mn^{2+} in dry matter (300 μg/g) in yeast biomass were obtained. Optimal pH for all ion incorporations was between 4 and 5.

Key words: yeast enriched with microelements, *Saccharomyces cerevisiae*, alcoholic fermentation, zinc, copper, manganese, metal uptake

Introduction

The role of biogenic microelements in the metabolic state of microorganisms and higher organisms (human and animal) has become an especially interesting field for research (*1*). Microelements play an important role in the cellular metabolism, primarily due to their requirements as cofactors for a large number of enzymes (*2,3*). Apparently, metal ions are vital for all organisms, and therefore ion transporters play a crucial role in maintaining their homeostasis. The number of studies of the processes involved in the uptake of trace metals by the yeast *Saccharomyces cerevisiae* has increased considerably in recent years. This yeast has become a model microorganism for studying metal transporters and their accumulation in the cells (*4,5*). However, excess amounts of the same metal ions are toxic and can cause damage to the function that they serve.

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Zinc, copper and manganese ions are very interesting because they have a positive effect on the respiratory activity and the growth rate of *Saccharomyces cerevisiae.* Thus, the impact of these ions on the yeast growth and fermentation activity have been reviewed (*6*).

Zinc, in a biologically relevant form of Zn^{2+} ions, is essential as catalytic cofactor of many enzymes, including alcohol dehydrogenase, alkaline phosphatase, carbonic anhydrase and several carboxypeptidases. Zinc also plays a critical structural role in enzymes and many noncatalytic proteins (*3*). It has been pointed out that the presence of Zn^{2+} ions in optimal amounts in nutrient medium of 5–15 μM enhances the growth rate of yeast cells as well as the production of ethanol (*6*). In contrast, Zn^{2+} ion deficiency stops the cell growth and fermentation activity. On the other hand, high concentration of zinc ions in a nutrient substrate may be toxic, since zinc affects the permeability of membranes to potassium causing a decrease in both the yeast growth and the fermentation activity as well (*7*).

Copper is also a vital divalent cation in yeast cells, acting as a cofactor of some enzymes such as cytochrome c oxidase, lactase and Cu,Zn-superoxide dismutase (*8,9*). The optimal concentrations of Cu^{2+} ions in the nutritive medium for the yeast growth and fermentation activity are in the range of 1–10 μM (*6*). However, it is also toxic in excess amount (*10*).

Yeast cells require manganese as an essential trace element at a concentration of 2–10 μM for optimal yeast growth (*6*). The specific growth rate of *Saccharomyces cerevisiae* was higher in a continuous batch culture (*11*), if Mn^{2+} ions were present in optimal concentrations in the medium. Transport of Mn^{2+} ions into yeast cells can be passive and driven by the concentration gradient (*12*) or energy-dependent, stimulated by glucose (*13,14*). Manganese has an important role in the metabolism of *S. cerevisiae* as a part of some enzymes, *e.g.* pyruvate carboxylase.

Yeasts are known for their ability to accumulate metal ions from aqueous solutions by different physico-chemical interactions, *e.g.* by adsorption and absorption, or by a metabolism-dependent mechanism (*15,16*). Sorption processes are dependent on disposable functional groups on the cell surface and on the nature of metal ions. Thus, the concentration of free ions, ligand electronegativity, metal cation, ligand charge and the cavity size have a great influence on the selectivity of metal uptake (*17,18*). Furthermore, the composition of nutritive medium to which the microorganism is exposed affects the amount of metal uptake (*19*), because of the cell wall structure and the metabolic state of the cell (*15,20*). Therefore, it may be taken in consideration that the growth in different media can influence the capacity and the selectivity of metal uptake by creating other binding sites or diverse enzymatic systems within the cells.

The aim of the present study was the evaluation of optimal growth conditions for the enrichment of yeast biomass with divalent zinc, copper and manganese ions and their impact on the yeast growth and fermentation activity. The fermentations were carried out with *Saccharomyces cerevisiae* on a standard molasses substrate with zinc, copper and manganese content that varies below the threshold of the yeast's needs (*6*).

Materials and Methods

Microorganism

The yeast used in this study was *Saccharomyces cerevisiae* TVG_4 from the Collection of Microorganisms of the Laboratory of Fermentation Technology and Yeast, Faculty of Food Technology and Biotechnology, University of Zagreb. The culture was maintained on a solid yeast medium (YM) containing (in g/L): D-glucose 20, Bacto peptone 10, yeast extract 5 and agar 20.

Molasses

Mixture of beet molasses produced in Croatian factories was used in all the experiments. The concentration of metals (Cu, Zn and Mn) in the molasses used (expressed in g/L) was indeed low (Mn 6, Cu 3.5 and Zn 25) (*21*).

Inoculum preparation

For the preparation of inoculum, *S. cerevisiae* TVG_4 was transferred from agar slants into test tubes containing each 10 mL of sterile liquid YM and incubated in a thermostat at 30 °C for 24 h. Sterile 200 mL of liquid YM in 500-mL Erlenmeyer flasks were inoculated with 5 % of obtained liquid yeast culture and the flasks were shaken on a rotary shaker at 150 rpm and 30 °C for 24 h.

Batch process

The composition of the basal medium for yeast biomass cultivation was (in g/L): beet molasses 90 (corresponding to 50 g/L of sucrose), $(NH_4)_2HPO_4$ 2, $(NH_4)_2SO_4$ 2, $MgSO_4$ 0.5. The pH of the medium was adjusted from 3.5 to 6.0 with H_2SO_4 (c=0.5 mol/L). The medium was sterilized at 120 °C for 10 min and after cooling to 30 °C it was centrifuged at 4000 rpm for 10 min. Clear supernatant was used as the basal medium (BM). For batch processes 500-mL Erlenmeyer flasks with 200 mL of BM were used. Cultivations were performed in BM with and without the addition of zinc-sulphate ($ZnSO_4$ 0.1 g/L), copper-sulphate ($CuSO_4$ 0.1 g/L), or manganese-sulphate ($MnSO_4$ 0.1 g/L) in semiaerobic (shaker, OTR=5.06 mmol/(L·h)) and anaerobic (thermostat) conditions at 30 °C for 8 h. The number of runs referring to different Zn^{2+}, Cu^{2+} and Mn^{2+} concentrations was two. Samples were analyzed three times for both biomass (expressed as biomass dry matter) and ethanol concentrations.

Analysis

Dry matter of yeast biomass was determined by drying yeast biomass at 105 °C to a constant weight after centrifuging 5 mL of samples at 4000 rpm for 10 min on a portable centrifuge. Ethanol concentration in the medium was determined by alcohol dehydrogenase method (*22*) while zinc, copper and manganese ion concentrations in yeast cells were analyzed by using a »Varian« Spectra AA 300 Atomic Absorption Spectrophotometer, fitted with a 10-cm single slot burner head, and using an air-acetylene flame. Zinc, copper and man-

ganese concentrations were determined by reference to an appropriate standard metal solution (*23*).

Statistical analysis

Results are expressed as the average values of two runs. Statistically significant differences between the results achieved with and without the addition of given ions (Mn^{2+}; Zn^{2+}; Cu^{2+}) were established using analysis of variance (*24*).

Results and Discussion

Molasses, which have been used as the main substrate for the yeast and ethanol production, have most of the necessary microelements, but not in the optimal concentrations. Zinc, copper and manganese are present in very small concentrations, and the addition of these elements may enhance the cell growth (*6*). Nevertheless, the amount of microelements available for the metabolism of yeast cells in molasses is much lower because of the binding of metal ions to organic carriers (*25*). Jones and Gadd (*6*) have suggested concentrations of many microelements which can be optimal for the yeast growth and the ethanol production. Recent studies have indicated somewhat lower or higher concentrations for some biologically important microelements which may be applicable in nutritive medium (*6,26,27*).

Following these recommendations, the addition of 0.1 g/L of each $ZnSO_4$, $CuSO_4$ and $MnSO_4$ has been chosen in this work for the alcoholic fermentations by *S. cerevisiae* on beet molasses, applying the above mentioned conditions. As can be seen in Fig. 1, the addition of the mentioned salts in the molasses medium increased the yield of yeast biomass up to 30 % in semi-

aerobic conditions, but the ethanol yield changed very little. The yield of yeast biomass and ethanol was higher when cultivations were performed with the addition of each Mn, Cu and Zn sulphate 0.1 g/L in semiaerobic conditions but statistically significant differences ($p \leq 0.05$) were established whether the cultivation was performed with or without the addition of metal salts.

With the addition of 0.1 g/L of each $ZnSO_4$, $CuSO_4$ and $MnSO_4$, the highest biomass yield per dry matter (*i.e.* 6.3 g/L) was achieved at pH=5.0 in semiaerobic conditions. The results indicate that the stimulatory effect is greatly affected by the pH of the medium. This effect could be explained by ATPase-dependent H^+ efflux, which is considered to be involved in the cation uptake into cells by establishing the plasma-membrane potential (*13*). The highest ethanol fraction (*i.e.* 1.6 %) was achieved at the same pH=5.0.

In Fig. 2 it can be seen that these effects were lower in anaerobic conditions. The biomass yield per dry matter was 3.75 g/L and ethanol yield was 2.1 %. The addition of Zn, Cu and Mn ions enhanced the biomass yield for up to 10 % and alcohol yield for 20 %, which is in accordance with some previous results (*6,27*). It is evident that the addition of salts in the concentration of 0.1 g/L stimulates the growth of *S. cerevisiae* in anaerobic and semiaerobic conditions. The yield of yeast biomass and ethanol in anaerobic conditions was lower but there were also statistically significant differences ($p \leq 0.05$) taking into consideration whether the cultivation was performed with or without the addition of salts. Significant differences ($p \leq 0.05$) were found in the yield of yeast biomass under anaerobic and semiaerobic conditions of cultivation as well as in the production of ethanol under anaerobic conditions in the presence of mineral salts.
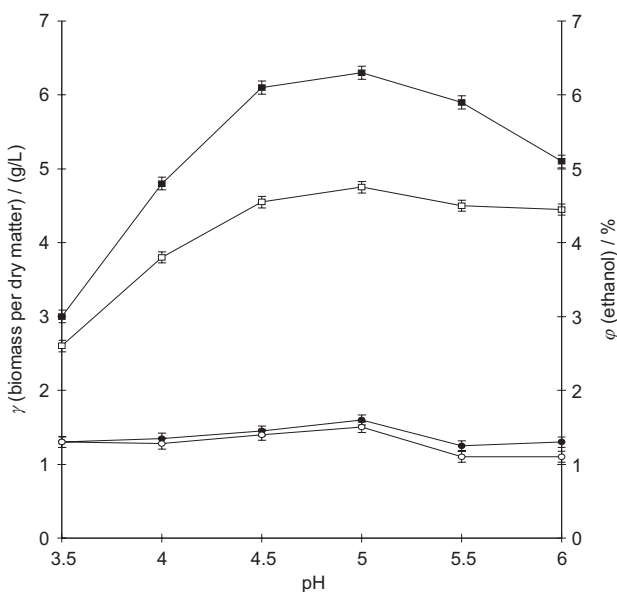

Fig. 1. The yield of yeast biomass and ethanol after 8 h of fermentation by *Saccharomyces cerevisiae* in the molasses medium with and without the addition of each Mn, Cu and Zn sulphate 0.1 g/L in semiaerobic conditions at 30 °C and different pH values: ■ biomass with the addition of Mn, Cu, Zn; □ biomass without the addition of Mn, Cu, Zn; ● alcohol with the addition of Mn, Cu, Zn; ○ alcohol without the addition Mn, Cu, Zn
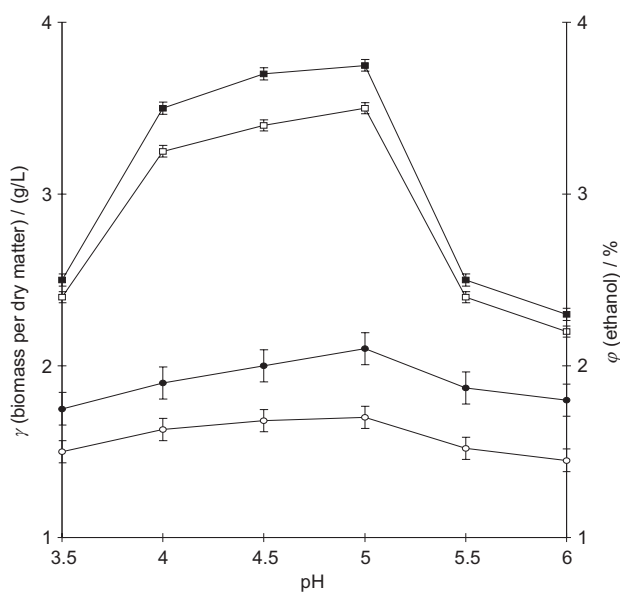

Fig. 2. The yield of yeast biomass and ethanol after 8 h of fermentation by *Saccharomyces cerevisiae* in the molasses medium with and without the addition of 0.1 g/L of each Mn, Cu and Zn sulphate in anaerobic conditions at 30 °C and different pH values: ■ biomass with the addition of Mn, Cu, Zn; □ biomass without the addition of Mn, Cu, Zn; ● alcohol with the addition of Mn, Cu, Zn; ○ alcohol without the addition of Mn, Cu, Zn

Increased alcohol fermentation caused by the addition of zinc sulphate to the medium may be explained by the fact that zinc stimulates the binding of acetaldehyde to alcohol dehydrogenase and its reduction to ethanol. Furthermore, zinc ions promote the synthesis of riboflavin, the lack of which arrests the cell growth (*28*). Anyway, the explanations on the genetic level (*3*) give a good insight in the metabolic and structural roles of metal ions. The structural role is particularly relevant to the function of plasma membrane and H^+ bond in the DNA structure (*3*). Thus, copper in cooperation with Zn^{2+} ions acts in the structure of Cu,Zn-superoxide dismutase, enzyme which is responsible for the detoxification of yeast cells. Also, the respiration is enhanced by Cu^{2+} ions (*29,30*). In the metabolism of *S. cerevisiae*, manganese has also an important role because it is incorporated in some enzymes, such as pyruvate carboxylase, glutamine synthetase, and arginase (*31*) and it is essential for the bud growth. Manganese enhances the yeast growth, especially in aerobic conditions (*3*). Mn^{2+} is also present in the Golgi, where it activates glycosyltransferases, which are involved in the processing of the secreted proteins. Mitochondrial Mn^{2+} is required by the enzymes of the citric acid cycle as well as proteases involved in mitochondrial protein import (*32*).

Fig. 3 shows the specific growth rate at different pH with and without the addition of Zn^{2+}, Cu^{2+} and Mn^{2+} ions. The addition of metal ions enhanced the specific growth rate in both semiaerobic and aerobic conditions. The specific growth rates were enhanced for 7–10 % in semiaerobic conditions and for about 6 % in anaerobic conditions, respectively. Statistical analysis of data from Fig. 3 pointed out that specific growth rates were significantly different ($p \leq 0.05$) in both semiaerobic and anaerobic cultivation in the presence of mineral salts or their absence.
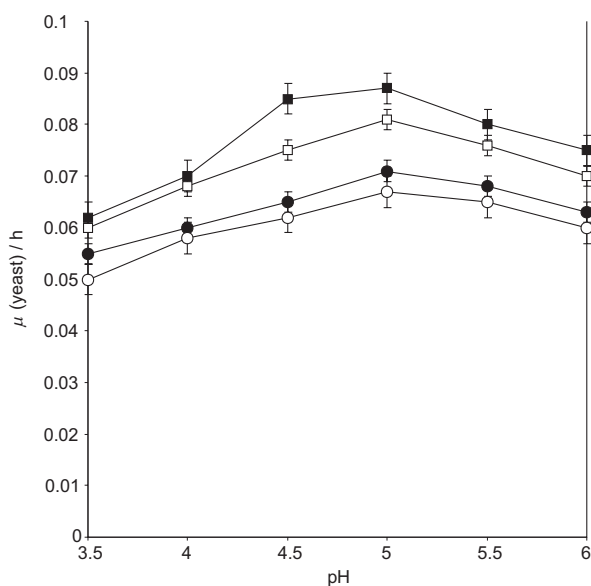

Fig. 3. Influence of Mn, Cu and Zn sulphate on the specific growth rate of *Saccharomyces cerevisiae* in semiaerobic and anaerobic conditions at 30 °C at different pH values: ■ with the addition of Mn, Cu, Zn (semiaerobic conditions); □ without the addition of Mn, Cu, Zn (semiaerobic conditions); ● with the addition of Mn, Cu, Zn (anaerobic conditions); ○ without the addition of Mn, Cu, Zn (anaerobic conditions)

The accumulation of Zn^{2+}, Cu^{2+} and Mn^{2+} in the molasses medium during fermentative processes is shown in Figs. 4a, 4b and 4c. The accumulation of Mn and Cu in yeast biomass was significantly higher ($p \leq 0.05$) during the semiaerobic fermentation in molasses medium as opposed to the anaerobic conditions. Different cultivations (semiaerobic or anaerobic) with the addition of Zn salt did not have any significant influences on Zn accumulation in yeast biomass ($p \geq 0.05$) although the highest amount of Zn^{2+} ions was incorporated in the yeast biomass under anaerobic conditions. When cultivation was performed without the addition of the mentioned salts there were no significant differences ($p \geq 0.05$) in the amount of minerals in yeast biomass in either cultivation, semiaerobic or anaerobic. pH value significantly influenced ($p \leq 0.05$) biomass yield and ethanol production, specific growth rate and the amount of incorporated ions in yeast biomass in both anaerobic and semiaerobic cultivations.

The concentrations of the incorporated ions were measured after 8 h of fermentation since the previous results (*28,29*) showed that the accumulation ended after the log-phase. The results suggest that *S. cerevisiae* TVG_4 needs quite a high concentration of Cu^{2+} and Zn^{2+}, but lower amount of Mn^{2+}. Under anaerobic conditions greater amount of zinc was incorporated. On the other hand, the uptake of copper and manganese was preferred in semiaerobic conditions.

Fig. 4a shows the uptake of zinc ions into the yeast cells. It can be seen from the results that during the fermentation under anaerobic conditions the accumulation of zinc ions reaches the value of 700 µg/g of yeast at pH=4. These results are in agreement with the previous findings (*28*), which have established that, in semiaerobic conditions, yeast accumulates lower amount of zinc ions (regardless of the added zinc ions in the medium) than in anaerobic conditions. Failla *et al.* (*33*) claimed that at lower pH values higher amounts of zinc ions were accumulated because zinc created complexes with poliphosphates, carbonates and hydroxides (insoluble residues) at around pH=6.8.

As it can be seen in Fig. 4b copper uptake into yeast cells is higher in semiaerobic conditions at pH=4. This high uptake of copper ions into yeast cells can be explained by the fact that copper induces the metallothionein synthesis (MT), which bounds metals and protects *S. cerevisiae* from heavy metal poisoning (*30,34*). As a response to high copper concentrations, metallothionein expression is activated with the initiation level of gene transcription (*30*).

When Cu^{2+} ions increase further in the environment, they are accumulated in excess through a low affinity transport system (*35*). In *Saccharomyces cerevisiae* resistance to Cu^{2+} is associated with the production of a metal-binding protein (metallothionein), mineralization (*35*) and sequestration to the vacuoles (*36*), leading to a reduction in the cytoplasmic concentration of a free copper ion. One of the major copper detoxification mechanisms in *Saccharomyces cerevisiae* is the Cu-induced synthesis of metallothionein (MT), which leads to a decreased binding of free copper ions in the cytosol (*37*). Cu-MT has been well characterised as a unique cysteine-rich protein hav-
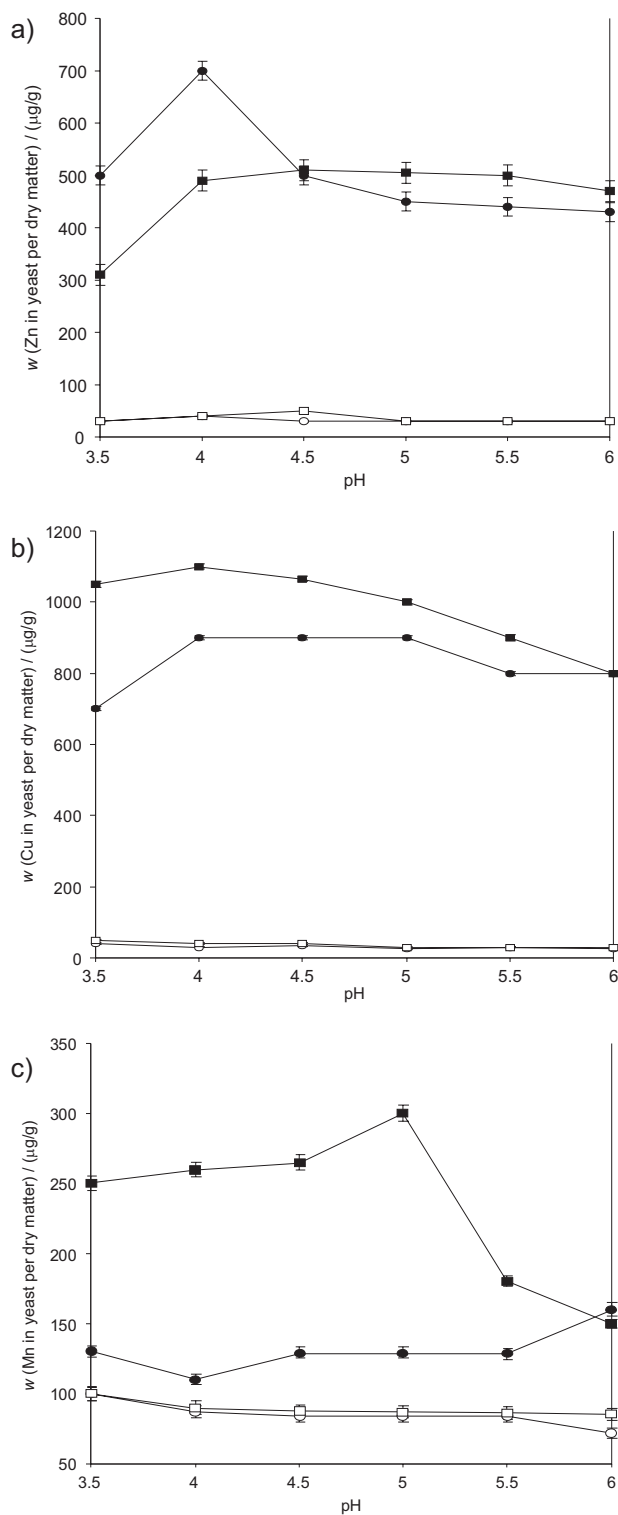
Fig. 4. The uptake of Mn, Cu and Zn sulphate into yeast biomass in semiaerobic and anaerobic conditions at 30 °C at different pH values:
a) ■ with Zn^{2+} (semiaerobic conditions); □ without the addition of Zn^{2+} (semiaerobic conditions); ● with Zn^{2+} (anaerobic conditions); ○ without the addition of Zn^{2+} (anaerobic conditions)
b) ■ with Cu^{2+} (semiaerobic conditions); □ without the addition of Cu^{2+} (semiaerobic conditions); ● with Cu^{2+} (anaerobic conditions); ○ without the addition of Cu^{2+} (anaerobic conditions)
c) ■ with Mn^{2+} (semiaerobic conditions); □ without the addition of Mn^{2+} (semiaerobic conditions); ● with Mn^{2+} (anaerobic conditions); ○ without the addition of Mn^{2+} (anaerobic conditions)

ing about 30 % of total amino acids and binding to metals other than Cu^{2+}, such as Cd^{2+}, Zn^{2+} and Co^{2+}, *in vitro* (*38*).

Manganese uptake into yeast cells was also higher in semiaerobic conditions (Fig. 4c). Mochaba *et al.* (*39*) claimed that manganese ions were absorbed and distributed inside the yeast cell depending on yeast physiology, growth phase and the role of manganese in the yeast metabolism. The authors explain that manganese was absorbed mainly in cell wall mannoprotein and less in citosol. The amounts of Mn^{2+} incorporated in yeast cells were lower than those of Cu^{2+} and Zn^{2+} ions, which means that optimal concentration of Mn^{2+} ions in the medium should be lower because they can be toxic in excess amounts.

Because the yeast biomass is an exceptionally important material for humans and animals with therapeutic effects, this study attempted at least in part to define more clearly the accumulation of these ions in yeast cells with reference to cultivation conditions and to elucidate the influence of these metal ions on the biomass and alcohol yield as well.

Conclusion

On the basis of the experimental results we can conclude that the addition of each Cu, Zn and Mn sulphate into the molasses medium enhances the kinetics of alcoholic fermentation by *Saccharomyces cerevisiae* under both semiaerobic and aerobic conditions. The enhancement was higher in semiaerobic conditions. On the other hand, the accumulation of metal ions in yeast cells during fermentative processes in molasses media ended after 8 h of alcoholic fermentations. The accumulated amounts of the examined microelements in yeast biomass are different. Presented results can also indicate that the optimal amounts of Mn^{2+} ions in growth medium could be lower than it was previously suggested.

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Obogaćivanje kvasca *Saccharomyces cerevisiae* cinkom, bakrom i manganom

Sažetak

U ovom je radu istražena ugradnja nekih mikroelemenata u kvasac *Saccharomyces cerevisiae* i njihov utjecaj na fiziološko stanje kvaščevih stanica tijekom alkoholne fermentacije. Uzgoj je proveden u melasnoj podlozi u anaerobnim (termostat) i semiaerobnim (tresilica) uvjetima s dodatkom 0,1 g/L cinkova, bakrena i manganova sulfata i bez njega pri 30 °C i različitim pH-vrijednostima medija (3,5–6,0) tijekom 8 sati. Dodatkom spomenutih soli u melasnu podlogu povećan je prinos kvaščeve biomase do 30 % tijekom alkoholne fermentacije u semiaerobnim uvjetima, a pritom se prinos etanola nije bitno mijenjao. S druge strane, u anaerobnim uvjetima prinosi kvaščeve biomase povećani su do 10 %, a prinosi etanola do 20 %. Nakon provedenih fermentacija određene su koncentracije iona metala u kvaščevim stanicama. Postignute su različite vrijednosti, ovisno o primijenjenim uvjetima rasta. Najviše cinkovih iona (700 μg/g) ugrađeno je u kvaščevu biomasu u anaerobnim uvjetima. Nasuprot tome, veća ugradnja bakra i mangana postignuta je u semiaerobnim uvjetima, u kojima je postignuto Cu 1100 μg/g, te Mn 30 μg/g kvaščeve biomase. Optimalna pH-vrijednost za ugradnju svih iona bila je između 4–5.