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UPTAKE OF IRON BY YEAST CELLS AND ITS IMPACT ON BIOMASS PRODUCTION

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Procedures for the production of *Saccharomyces cerevisiae* biomass enriched with iron and the effects of the iron ions addition into the molasses medium on the yeast growth and the production of ethanol were studied. The growth of the yeast *S. cerevisiae* and the ethanol production in media with different concentrations of Fe were monitored in the batch process under semiaerobic and anaerobic conditions. The highest biomass concentration and ethanol production were achieved in the medium with 0.6–0.8 g l^{-1} of Fe under both (semiaerobic and anaerobic) conditions. Kinetics of the iron ions accumulation in yeast cells during 24 h of growth in the batch process under semiaerobic and anaerobic conditions were monitored. In anaerobic conditions the maximum uptake (10 mg g^{-1} d.m. yeast biomass) was obtained after 12 h of fermentation, while in semiaerobic conditions a four times lower uptake (2.5 mg g^{-1} d.m. yeast biomass) was obtained after 16 h of fermentation.

Keywords: fermentation, iron uptake, *Saccharomyces cerevisiae*, semiaerobic and anaerobic conditions

The presence of trace elements in substrates is neccesary for a normal functioning of biochemical processes in the microbial metabolism. Iron is the most versatile metal in cell redox-reactions. The high affinity of iron towards oxygen has also made iron an active site in heme, which is commonly involved in the oxygen binding and oxygen based enzymatic reactions (RADISKY & KAPLAN, 1999). The same properties of facile electron transport make iron potentially toxic as the free iron via Fenton and Haber-Weis reaction generates toxic radicals in the presence of oxygen derivates (EIDE et al., 1992). Although iron is the second most abundant metal in the earth's crust, it is found primarily in the ferric (Fe^{3+}) state, forming hydroxydes or salts of a very low solubility and, thus, biologically inaccessible by simple mechanisms. Regulation of iron status in *Saccharomyces cerevisiae* is mediated primarily by regulating the plasma membrane iron transport. It has multiple iron transport systems, all of which appear to require ferrous iron as a substrate. Extracellular Fe^{3+} is reduced to Fe^{2+} by the plasma membrane Fe^{3+} reductases encoded by *FRE1* and *FRE2* genes (GEORGATSOU & ALEXANDRAKI, 1994). The Fe^{2+} product is then taken up by either of the two transport systems. One system has a high affinity for iron (apparent K_m of 0.15 μM), which is necessary for iron-limited growth. The high affinity system is induced in iron-limited cells, and its components are transcriptionally regulated by the product of *AFT1* gene.

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Iron-replete yeast cells obtain iron through a second, low affinity uptake system with an apparent K_m of 30 μM. This system requires Fet4 which is the low affinity Fe^{2+} transporter (DIX et al., 1997).

Iron has an essential role in the human organism, too. Despite considerable efforts to decrease its prevalence, iron deficiency is the leading single-nutrient deficiency in the world (BEARD, 2000). The reason for it is the fact that the human organism cannot satisfy its needs for iron by absorption from food, especially from industrially manufactured food which has a negative effect on resorption, or from food with a low amount of iron. A lot of scientific research is concerned with the production of new, improved antianemic preparations. One possible approach is the production of yeast-based preparations, because yeast is capable, under certain conditions, of binding iron in concentrations several times higher than usual. Iron bound to an organic carrier has a better resorption quotient in the organism, it is less toxic, it has a pleasant taste and it is easier to dose (JANZSÓ et al., 1990).

Although molasses is a very useful medium for the growth of *S. cerevisiae* since it is a good source of nitrogen, inorganic constituents and vitamins, as well as of carbohydrates, some biogenic elements are not present in sufficient quantities. Iron, as a very important biogenic element for microbial metabolism (WRIGHT & HONEK, 1989; BERG et al., 2002), is in this substrate present in an insufficient concentration. The optimal concentration of iron in the substrate for the yeast growth and fermentation is 5–500 μg l^{-1} (JONES & GADD, 1990). In beet molasses the concentration of iron is 50–100 mg kg^{-1}, and in cane molasses the concentration is 150–500 mg kg^{-1} (MARIĆ, 2000). Nevertheless, the concentration available for the metabolism of yeast cells is much lower because of the binding of iron to organic carriers (JONES & GADD, 1990). The quantity of iron actually available in industrial molasses substrates is insufficient for the full satisfaction of the yeast's needs, and the addition of this element may contribute to the microbial growth.

The aim of this study was to find the optimal concentrations of iron ions for alcoholic fermentation with the yeast *S. cerevisiae* on a standard molasses substrate with an iron content that varies below the threshold of the yeast's needs (JONES & GADD, 1990). Besides that, we wanted to investigate the possibility of obtaining an organic Fe^{+2}-complex with the yeast *S. cerevisiae* by the uptake of iron ions in yeast cells with the addition of $FeCl_3$ into the standard molasses substrate, with the purpose of using the complex as an antianemic preparation.

1. Material and methods

1.1. Yeast strain

The yeast used in this study was *Saccharomyces cerevisiae* strain TVG_4 obtained from the Collection of Microorganisms of the Laboratory for Fermentation Technology, Faculty of Food Technology and Biotechnology, University of Zagreb. The culture was

maintained on a solid yeast medium (YM) containing (in g l^{-1}): D-glucose, 20; Bacto peptone, 10; Yeast extract, 5; Agar, 20.

1.2. Inoculum preparation

For the preparation of inocula the yeast *S. cerevisiae* TVG_4 was reinoculated 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 the obtained liquid yeast culture and flasks were shaken on a rotary shaker at 30 °C for 24 h.

1.3. Batch process

The composition of the basal medium for yeast cultivation was (in g l^{-1}): beet molasses, 90 (corresponding to 50 g l^{-1} sucrose); $(NH_4)_2HPO_4$, 2; $(NH_4)_2SO_4$, 1; $MgSO_4$, 0.5. The pH of medium was adjusted to 5.0 with H_2SO_4 (c = 0.5 mol l^{-1}). The medium was sterilised at 120 °C for 10 min, and after cooling to 30 °C it was centrifuged at 2000 g for 10 min. The clear supernatant was used as the basal medium (BM). For batch processes 500 ml Erlenmeyer flasks with 200 ml of BM were used. Cultivation was performed in BM with and without different amounts of iron-chloride (Fe in g l^{-1}: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9) in semiaerobic (shaker) and anaerobic (thermostat) conditions at 30 °C for 24 h. Samples were analysed in three replicates for biomass (expressed as biomass dry matter) and ethanol concentrations. The obtained data were statistically analysed using *t*-test (t follows Students' distribution) and Analysis of variance (STATSOFT, 2000).

1.4. 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 2000 g for 10 min on a portable centrifuge. Ethanol concentration in the medium was determined by the kit from Boehringer-Mannheim GmbH (Mannheim, Germany) and iron ions concentration in yeast cells was analysed by using a "Varan" Spectra AA 300 atomic absorption spectrophotometer, fitted with a 10 cm single slot burner head, and by using an air-acetylene flame. Iron concentration was determined by reference to an appropriate standard metal solution.

2. Results and discussion

In order to determine the optimal concentrations of iron ions, which stimulate the growth of yeast cells and the production of alcohol, batch fermentations were performed by applying semiaerobic and anaerobic growth conditions, with addition of different amounts of $FeCl_3 \times 6H_2O$ to the molasses medium (BM). Data presented in the following figures were the average of three simultaneous measurements, and obtained

variances for biomass yield and ethanol production in all experiments were small, the maximum level was about 0.051. The influence of Fe ions addition in the medium on the growth of *S. cerevisiae* TVG_4 and on the production of ethanol under anaerobic and semiaerobic conditions are shown in Fig. 1.

As can be seen in Fig. 1, a concentration of 0.6–0.8 g l^{-1} of Fe added to a molasses substrate (BM) enhanced the growth of yeast cells, as well as the synthesis of ethanol. The biomass yield increased by as much as 26% in semiaerobic conditions and 37% in anaerobic conditions by the addition of 0.8 g l^{-1} of Fe, compared to the yield in a substrate without the added Fe, which was a statistically significant ($t_{0.05}$) increase for both, calculated by *t*-test. The biosynthesis of ethanol in semiaerobic conditions increased by 15.6% and in anaerobic conditions by 24% by the addition of 0.8 g l^{-1} of Fe which was a statistically significant ($t_{0.05}$) increase for both, calculated by *t*-test.

It is noticeable that the concentrations under 0.6 g l^{-1} of Fe added into the molasses substrate in semiaerobic conditions did not improve the growth rate of yeast significantly, while a concentration of Fe higher than 0.8 g l^{-1} significantly inhibited the growth of the yeast cells. Figure 1 also shows the more positive influence of the addition of much lower quantities of Fe ions in anaerobic conditions, than in semiaerobic conditions.

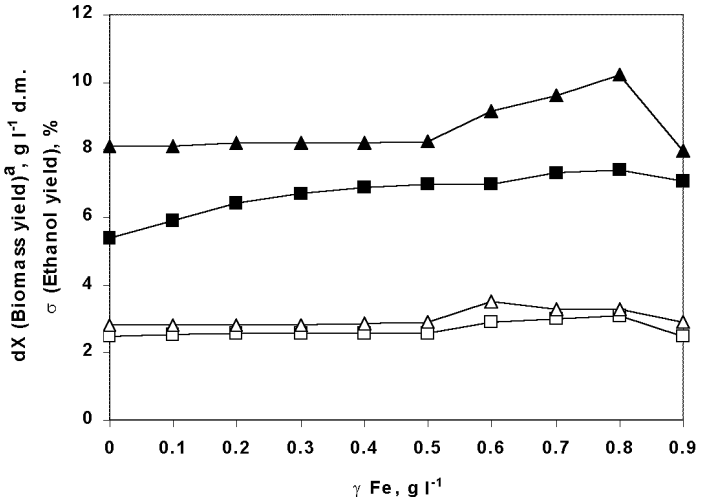

Fig. 1. The yeast biomass growth and ethanol yield after 24 h or fermentation in anaerobic and semiaerobic conditions with the addition of various concentrations of Fe into a molasses substrate. ■: Biomass yield in anaerobic conditions; ▲: biomass yield in semiaerobic conditions; □: ethanol yield in anaerobic conditions; Δ: ethanol hield in semiaerobic conditions. [a] dX: difference between final mass concentration of biomass and mass concentration of inoculum

The growth enhancement of the yeast *S. cerevisiae* when Fe was added into the molasses substrate can be explained by the important role iron plays in the reactions of the citric acid cycle, being the coenzyme of enzymes that catalyze the particular reactions. Iron deficiency in the yeast *S. cerevisiae*, and in other microorganisms as well, lowers the activity of the enzymes aconitase and isocitrate dehydrogenase (BERG et al., 2002).

Figure 2 shows the influence of the addition of 0.8 g l^{-1} of Fe into the molasses substrate in semiaerobic conditions on the kinetics of the yeast cell growth and ethanol production. As it can be seen, the addition of Fe (0.8 g l^{-1}) into the molasses substrate enhances the growth of the yeast biomass and alcoholic fermentation. The concentration of biomass after 24 h of fermentation was c. 10 g l^{-1} d.m., and the volumetric percentage of alcohol was c. 3.2%. The addition of Fe into the substrate significantly increased ($P<0.01$) the biomass concentration by 21%, and the production of alcohol by 16% as well.

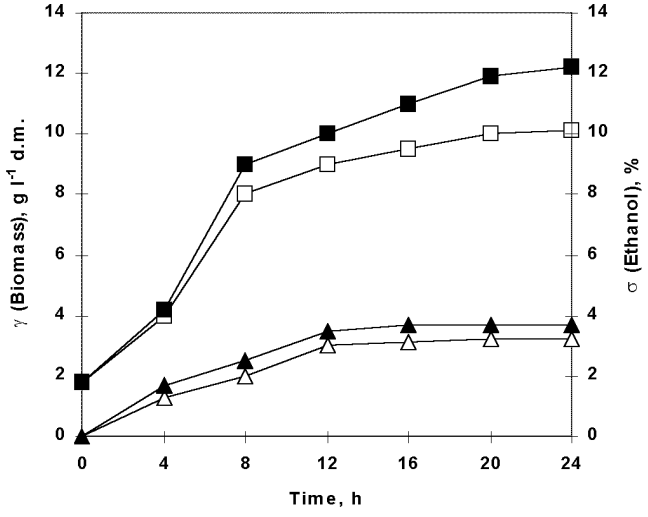

Fig. 2. The kinetics of yeast biomass and ethanol production during 24 h of fermetation in semiaerobic conditions by the addition of 0.8 g l^{-1} Fe into the molasses substrate. □: Biomass without addition of Fe; ■: biomass with addition of Fe; Δ: ethanol without addition of Fe; ▲: ethanol with addition of Fe

Figure 3 shows the influence of the addition of 0.8 g l^{-1} of Fe into the molasses substrate in anaerobic conditions on the kinetics of alcoholic fermentation. It is evident that the addition of Fe (0.8 g l^{-1}) into the molasses substrate enhances alcoholic fermentation. In a fermentation without the addition of Fe into the substrate, the biomass concentration after 24 h was c. 9.4 g l^{-1} d.m. and the volume percentage of

alcohol was c. 2.5%. The addition of Fe into the substrate increased the biomass concentration by 27% and alcohol production by 24%. Analysis of variance showed that both enhancements were statistically significant ($P<0.01$).

The enhancement of alcoholic fermentation can be indirectly attributed to the addition of iron into the substrate, because it is a consequence of a faster yeast cell growth. A higher yield and a higher specific rate of the product biosynthesis with the addition of 0.6–0.8 g l^{-1} of Fe into the molasses substrate in semiaerobic conditions are due to the fact that iron has an important role in the reactions of the citric acid cycle, being a part of the Fe-S coenzyme of the enzymes that catalyze particular reactions, and to the important role of iron in many parts of the respiration chain (BERG et al., 2002).

To specify the conditions in which the best uptake of iron ions in yeast cells was obtained, fermentations were carried out with the addition of 0.8 g l^{-1} Fe into the basic substrate in anaerobic and semiaerobic conditions. During fermentation the kinetics of iron ions uptake in yeast biomass was monitored. The results are shown in Fig. 4.

From Fig. 4 it is possible to see that the iron ions uptake in yeast biomass was significantly better in anaerobic than in semiaerobic conditions. The same was shown by analysis of variance ($P<0.01$). In anaerobic conditions the maximum amount of Fe taken up (10 mg g^{-1} d.m yeast biomass) was reached after 12 h of fermentation, while in semiaerobic conditions a four times lower uptake (2.5 mg g^{-1} d.m yeast biomass) was obtained after 16 h of fermentation.

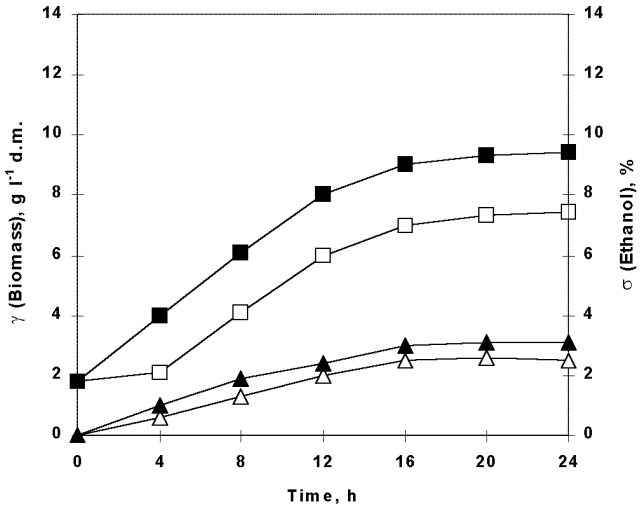

Fig. 3. The kinetics of yeast biomass and ethanol production during 24 h of fermetation in anaerobic conditions with the addition of 0.8 g l^{-1} Fe into the molasses substrate. □: Biomass without addition of Fe; ■: biomass with addition of Fe; Δ: ethanol without addition of Fe; ▲: ethanol with addition of Fe

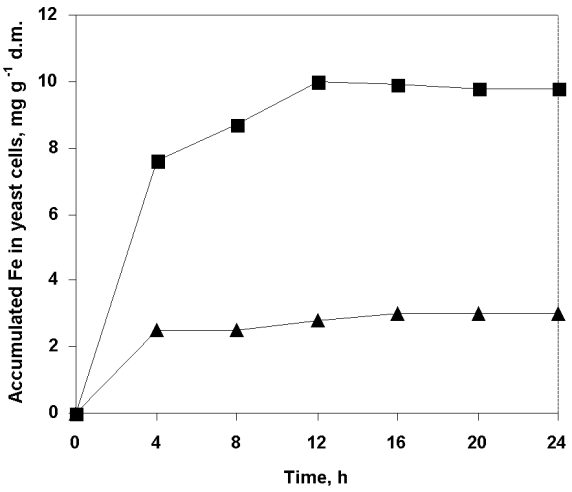

Fig. 4. Accumulation of iron ions in yeast biomass with the addition of 0.8 g l^{-1} Fe into the molasses substrate in anaerobic and semiaerobic conditions. ■: Fe uptake in anaerobic conditions; ▲: Fe uptake in semiaerobic conditions

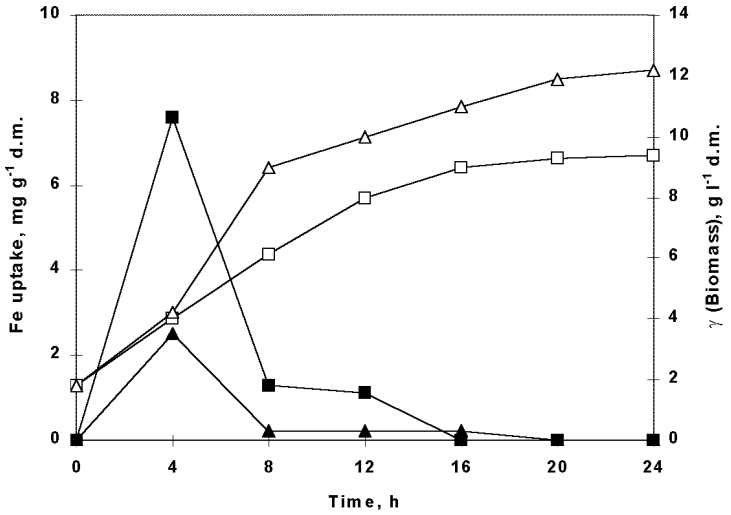

Fig. 5. The rate of iron ions uptake in yeast biomass with the addition of 0.8 g l^{-1} Fe into the molasses substrate in anaerobic and semiaerobic conditions and kinetics of yeast biomass production in anaerobic and semiaerobic conditions, during 24 h of fermentation. ■: Fe uptake in anaerobic conditions; ▲: Fe uptake in semiaerobic conditions; □: biomass in anaerobic conditions; Δ: biomass in semiaerobic conditions

Figure 5 shows that the maximum iron uptake occurred within the first 4 h of incubation in both anaerobic and semiaerobic conditions.

3. Conclusions

The monitoring of the yeast biomass growth kinetics in semiaerobic and anaerobic conditions (Figs 2 and 3) showed that the increase in the growth rate of yeast cells with the addition of iron ions was visible from the beginning of the process. That is in accordance with the hypothesis that the activity of iron uptake is strongly enhanced by cell growth and that the maximum rate of iron uptake is already obtained before the exponential growth phase. EIDE and co-workers (1992) claim that cells in the stationary growth phase have a low capability of iron ions uptake and that a rapid increase in iron accumulation is obtained when these cells are inoculated into a fresh substrate. According to them, the iron uptake rate grows and reaches its maximum before cells reach the exponential growth phase. By monitoring the kinetics of iron ions uptake in the yeast biomass we have confirmed that hypothesis. The results have shown that the activity of the iron uptake system has reached its maximum during the first 4 h. The uptake rate has significantly decreased during the exponential growth phase, and the uptake process has almost completely stopped in the stationary phase.

The results presented in this paper have shown that the iron uptake is much higher in anaerobic conditions than in semiaerobic ones, which is in accordance with the hypotheses of HASSETT and co-workers (1998). Under the conditions of an iron-rich medium (0.8 g l^{-1} = 14.3 mM Fe), the iron ions uptake is carried out by Fet4 mediated low affinity iron uptake (DIX et al., 1994). Fet4 mediated iron uptake is likely to be 2–3 fold greater from anaerobic, in comparison to aerobic medium. Fe^{2+} will predominate in the former condition, while Fe^{3+} will predominate in the latter one. Fet4 uptake is strongly dependent on the redox state of the exogenous metal (HASSETT et al., 1998).

The comparison of the growth kinetics of the yeast cells in anaerobic and aerobic conditions shows that the positive influence of the addition of iron on the yeast growth is greater in anaerobic than in aerobic conditions. That is the consequence of a significantly larger iron uptake by a low-affinity uptake in anaerobic conditions.

Based on the experimental results one can conclude that the addition of 0.6–0.8 g l^{-1} of Fe to molasses medium enhances the growth of yeast cells during alcoholic fermen-tation under both semiaerobic and anaerobic conditions, while the production rate of alcohol is accelerated by the addition of 0.6 g l^{-1} in aerobic and 0.6–0.8 g l^{-1} in anaerobic conditions of Fe. An examination of the kinetics of iron-ions incorporation into yeast cells by addition of 0.8 g l^{-1} Fe to the molasses medium has proved that the greatest amount of iron ions is incorporated into yeast cells in anaerobic conditions when batch fermentation is applied.

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