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Uptake and Bioaccumulation of Cr(III) in Yeast *Saccharomyces cerevisiae*

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Summary

The chromium uptake and bioaccumulation were studied for selected yeast Saccharomyces cerevisiae ZIM-198 after standardised cultivation in designed substrate with adequate amount of Cr(III) in the media. The impact of Cr(III) on biomass accumulation, RNA, metal accumulation, and organically bound chromium was studied in vivo. Efficiency of Cr(III) in yeast biomass was primarily studied in vitro to define the impact of pH and energy source. A strong correlation between energy supplementation and chromium uptake was found. The optimal pH value was found to be approximately 4. Selected pH minimised also the possibility of Cr(III) precipitation in the media. High Cr(III) concentrations (> 1.9 mmol L^{-1}) intensify the pH decrease in the media. Further studies in vivo in standardised media confirmed the impact of Cr(III) on yeast cell metabolism. It was proved that Cr(III) has an important effect on cell components. Total chromium concentration in the yeast cell increased in Sacch. cerevisiae when continuous growth in a concentration of 96 μmol L^{-1} of Cr(III) in the medium was applied. The same tendency was detected for organically bound intracellular chromium where 43% higher concentration of chromium was determined under the same conditions of cultivation. The yeast detoxification capability for high intracellular chromium was reduced owing to the toxic action of environmental chromium loading pressure on yeast cell growth and viability. The cell detoxification system enables the yeast Sacch. cerevisiae to survive and grow at a moderate and higher concentration of inorganic chromium. In these processes, the distribution of excess inorganic chromium in the cell plays an important role.

Keywords: yeasts, nutrient, chromium, toxicity, uptake, accumulation

Introduction

Yeasts belong to the group of microorganisms which are predominantly used in the cultivation techniques of traditional, as well as in modern biotechnological industries. Optimization of their growth and metabolic activities requires a complete understanding of their nutrition. In this respect, the roles of ionic constituents of the medium such as inorganic and trace elements are often neglected. However, in general terms, they are known to be involved in a metabolic and a structural role. They stabilize the range of the biological structure from cell walls to protein conformations. They are often highly effective catalysts in a range of diverse biochemical processes and, in some cases, are able to trigger, moderate or inhibit reactions. It is important to note that elements may become toxic at higher concentrations, and that elements notorious for their toxicity may exert a beneficial effect at very low concentration levels. In high concentrations of Cd(II), Cu(II) or Zn(II) and other metal ions can be toxic while in low concentrations they stimulate growth and enzyme activity *(1–3)*. The demonstration that trace metal ions enhance the growth of an organism cannot always be unambiguously interpreted as evidence for its importance, as the element may function as a growth stimulant by substituting (albeit inefficiently) for another metal ion of similar chemical properties. Furthermore, the chelation of metal ions by organic components of the fermentation broth should not be neglected, e.g. molasses or corn steep liquor and other physicoche-

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mical attributes that can affect ionic availability such as pH or ionic strength *(4)*. In some cases the appropriate concentrations of essential elements can correct or diminish the deleterious effect of some more toxic elements. For example, Zn(II) and Mn(II) are able to antagonize the toxic effects of Mg(II), Cd(II) and Cu(II) *(5–7)*.

The growth of yeasts in complex industrial media can be governed by metal ion limitations as well as by an excess of particular ionic species and by the interactions between these species. The availability or free concentrations rather than the total concentration of metal ions allowed growth or yield enhancement. The interaction complexity (metal-metal, metal-organic compound, metal-microbial cell, *etc.*) renders possible the determination of the concentrations of essentiality or toxicity. Especially for those elements to which trace amounts are likely to be present in the defined media (*e.g.* Na, Li, Ni(II), Pb(II), Al(III), Cr(III) ions, *etc.*) it seems easier to observe and determine the toxic effect, biosorption and bioaccumulation capacity than its essentiality. In the case of chromium, the role and beneficiality in yeasts is still not completely clear. On the contrary, its toxicity is often observed during the growth and fermentation of yeasts. The literature suggests chromium as an element which can stabilise tertiary structure of proteins and confirmation of the cell RNA and DNA *(8)*. However, it is well known that chromium is almost always present in industrial complex media (*i.e.* sulphite liquor, molasses, etc.) and defined (*i.e.* yeast nitrogen base, yeast malt base, *etc.*) growth media. Therefore, the behaviour of chromium action with regard to its uptake and accumulation as well as its consequences on cell growth and biomass production is vital for its activity. The chromium effect on the yeast cells is very significant, especially when the concentrations of chromium in the medium have elevated toxic level. In contrast, the yeasts are able to accumulate environmental chromium in the cell. The chromium concentration in the yeasts varied according to yeast species and medium used for cultivation (Table 1). In this article, we focused on biosorption, bioaccumulation and distribution processes which occur in chromium translocation from environment to the yeast cell. In addition the results of our work followed chromium ion uptake and accumulation in the yeast cell as well as its distribution and effect in the cell which has contributed to a better understanding of chromium ion uptake from the environment to the interior of the yeast cell.

The article also analyzes where the biosorption refers to non-active metal uptake by microbial biomass (living or dead) via physico-chemical mechanisms such as adsorption, ion exchange, complexation, coordination, chelation or inorganic microprecipitation, and it is assumed to be mainly controlled by the cell surface characteristics.

Table 1. Chromium mass fraction in dry yeast biomass

Yeast	Chromium mass fraction μg/g	Ref.
Sacch. cerevisiae	0.7 – 4.4	*(9–11,13)*
C. lipolytica	1.6 – 3.0	*(11)*
C. boidinii	1.1 – 5.1	*(11)*
Hor. platypodis	3.2	*(12)*
P. kluyveri	3.0	*(12)*
Y. lipolytica	0.22	*(12)*
Schizobl. starkeyi-henricii	0.19	*(12)*

The process of metal bioaccumulation depends on metal uptake and therefore on cell metabolism and physiology. While metal uptake can be used to refer the actual translocation of a metal ion across the plasmalemma.

Materials and Methods

I. Yeast

The yeast *Saccharomyces cerevisiae* ZIM-198 were obtained from the culture collection of industrial microorganisms (ZIM), and were stored on malt slants at 6 °C.

II. Growth media

The yeast growth took place in a semi-synthetic medium consisting of: yeast extract (1.0 g), $(NH_4)_2SO_4$ (2.0 g), K_2HPO_4 (3.98 g), KH_2PO_4 (2.72 g), $MgSO_4 \cdot 7H_2O$ (0.12 g), $FeSO_4 \cdot 7H_2O$ (0.002 g), $ZnSO_4 \cdot 7H_2O$ (0.004 g) and $CuSO_4 \cdot 5H_2O$ (0.004 g) per liter. Medium used for aerobic batch cultivation of yeast biomass production contained 20 g L^{-1} D(+) glucose. In the experiments of continuous cultivation of yeast the medium contained 5 g L^{-1} of D(+) glucose. Various concentrations of chromium(III) in the medium were obtained by the addition of appropriate amounts of stock solution of chromium chloride ($CrCl_3 \cdot 6H_2O$) ($c(Cr^{3+}) = 0.096$ mol L^{-1}). The initial pH of the media (4.0) was adjusted by 0.1 M HCl or NaOH.

III. Biomass production and determination

Two types of aerobic cultivation of yeast *Saccharomyces cerevisiae* ZIM-198 were performed for biomass production which is used in the experiments. Cultivation conditions in batch growth of yeast in bioreactor vessel were as follows: temperature 28 °C, revolution speed 250 min^{-1}, air flow 1.5 L min^{-1} and concentration of glucose 20 g L^{-1}. Yeast biomass from batch cultivation was centrifuged and used *in vitro* biosorption and uptake/extrusion of H^+ ions experiments.

In *in vivo* study of chromium effect on yeast bioaccumulation and distribution, the continuous growth of yeast was at dilution rate (D) 0.08 h^{-1} the concentration of glucose was 5 g L^{-1} while the temperature, revolution speed and air flow were kept the same as in batch cultivation.

Biomass concentration, as optical density in all bioprocesses, was followed *on line* by spectrophotometer at 650 nm.

IV. Protein assay

A biuret reaction according to Stickland *(14)* was used for protein measurement. The calibration curve was prepared with bovine serum albumin (BSA) using the same procedure in a concentration range up to 4 g L^{-1}.

V. RNA assay

The extraction of RNA from yeast by 0.5 M $HClO_4$ was carried out at 37 °C (hydrolysed to nucleotides) and

ribose was estimated in the extracts by the Orcinol method according to Munro and Fleck *(15)*. Before the extraction of RNA the yeast cells were washed by ice-cold 0.5 mol L^{-1} perchloric acid to remove the low molecular mass substances. The calibration curve for RNA was prepared by the same procedure, employing standards of RNA from yeast *Rhodotorula (Candida)* γ(RNA) = 0–004 mg mL^{-1}).

VI. Chromium biosorption

The aqueous suspension with yeast dry biomass concentration of 1 mg mL^{-1} was inactivated at 80 °C for 15 minutes, and supplemented with 0.96 mmol L^{-1} of Cr(III). After 4 hours of yeast biomass exposure to Cr(III) at 28 °C in the shaker (200 mm^{-1}) the process of Cr(III) biosorption was stopped. Yeast biomass was then harvested by centrifugation and washed three times with distilled water. Washed yeast cells were treated with tris-HCl buffer (pH = 6.0) three times and the supernatant was collected after centrifugation at 14,000 mm^{-1} for 10 minutes. The concentration of chromium in the supernatant was measured by flame atomic absorption spectrometer (FAAS) at 357.87 nm, and expressed as biosorbed chromium on the yeast cell surface, which presented cell surface deposition capacity for chromium (CSDC-Cr).

VII. Chromium uptake

The chromium uptake process in aqueous solution with the yeast *Sacch. cerevisiae* was performed on a shaker, with and without addition of glucose in Erlenmeyer flasks filled with the chromium aqueous solutions containing 0.48, 0.96, 1.44, 1.92, 2.40, 2.88 and 3.85 mmol L^{-1}. The biomass concentration was adjusted to 1 mg mL^{-1}. Uptake conditions were as follows: initial pH = 4.0, temperature 28 °C and revolution speed 200 min^{-1} for 4 hours. At the end, yeast biomass was removed by centrifugation at 14,000 min^{-1} for 10 minutes. The concentration of chromium in the supernatant was measured by atomic absorption spectrometer (AAS) at 357.87 nm. In metabolism-dependent uptake experiments 10 mmol L^{-1} of glucose was added as a sole energy source. The experiment was performed in triplicate.

VIII. Chromium bioaccumulation/distribution assay

The harvested biomass from continuous bioprocess was separated by cross-flow filtration (pore size 15 μm) and centrifugation at 4,500 min^{-1} for 20 minutes. The wet biomass was washed three times by bidistilled water and dissolved in hot concentrated HNO_3 diluted with distilled water to volume (25 or 50 mL^{-1}). The content of total chromium in the yeast solution was measured at 357.87 nm by FAAS. Simultaneously, the dry weight of the biomass was determined by drying the yeast suspension to a constant weight at 105 °C. Results obtained by this measurement were treated as total chromium accumulation in yeast cell.

For determining the 0.2 M NH_4OH-extractable chromium the yeast biomass was prepared as before. The extraction procedure of cell organically-bound chromium with 0.2 M ammonium hydroxide was carried out on a shaker for 16 hours. The ratio between yeast biomass and ammonium hydroxide was 1:4. The ammonium hydroxide eluted organically bound chromium and precipitated the inorganic form (aquo, chloro, sulphato complexes) of chromium. The concentration of organically bound chromium was performed by an electrochemical atomisation atomic absorption spectrometer (ETA-AAS). Further, the separation of organically bound chromium in NH_4OH-extractable fraction proceeded by centrifugation through molecular sieves with 100,000, 10,000 and 3,000 nominal cut-off relative molecular masses. Relative error of chromium determination by FAAS and ETA-AAS was ±3.2 and ±4.9%, recpectively.

IX. Chromium organic incorporation factor (F_{Cr})

F_{Cr} is a ratio between chromium concentration in ammonium hydroxide extractable part separated by molecular sieves in yeast biomass grown in chromium loading media, and in control yeast biomass.

Results and Discussion

Inorganic nutrients play an important role in yeast cell metabolism and growth. In this respect, the metal ion uptake and translocation processes take a significant place in cell activity. In particular, those essential micronutrients which are involved in the known basic cellular function of yeasts (Zn, Cu, Fe, *etc.*) can be almost always found in the micromolar range *(16)*. A beneficial effect was found for most of them. On the contrary, the benefits of a certain group of ionic nutrients (Cr, Ni, Al, *etc.*) are still not clear, which cannot be unambiguously interpreted as evidence for non-essentiality. Further, metal ions are both essential and potentially toxic, and their intracellular concentrations are subject to precise homeostatic regulation *(17)*.

Until recently, a certain lack of results for tolerance to chromium in yeasts has been observed. In this respect, it was reported that tolerance to this element is distributed among different yeast species and genera *(18)*. Within the group of 23 yeast genera and 49 species studied, the tolerance varied from 1.9 to 6.9 mmol L^{-1} of Cr(III) in the environment *(18)*. The problem of toxicity is closely connected with chromium over-accumulation. A chromium ion has to pass different barriers from the environment to the cell interior. In the translocation process of metal ions to the yeast cell, the ions have to pass the cell wall and membrane. When the intracellular level of a metal ion rises to the critical level, it can interfere with vital processes resulting in cell death. Values for pH, temperature, metal biological availability, *etc.* are considered among the most important environmental parameters in the mechanism of metal ion translocation. The metal ion uptake is essentially a biphasic process consisting of a metabolism-independent and a metabolism-dependent step. The initial biosorption step for metal ions is rapid *(19,20)*, typically only a few minutes in duration *(21)*, and is temperature independent *(22)*. In the case of chromium this step was tested at different starting pH values in yeast *Sacch. cerevisiae* (Fig. 1). The initial binding step is thought to involve the microbial cell surface

components (*e.g.* cell wall components – polysaccharides). The results for chromium biosorption on the cell surface showed a correlation between environmental pH and chromium cell surface deposition (Fig. 1). Under the experimental conditions at different pH the cell surface deposition capacity for chromium (CSDC-Cr) of the yeast cells increased with the pH. At the pH = 6.0 CSDC-Cr in yeast *Sacch. cerevisiae* reached the value of 1.54 μmol of chromium per g of dry yeast cells (Fig. 1). The chromium concentration in supernatant at pH below 3.0 was below ETA-AAS detection limit. This agreed with the observation for other selected metal ions in the same pH range *(20)*. Complex media used in this study contain substances making the chromium ion biologically unavailable at pH = 6.0. Furthermore, in an aqueous solution at pH values higher than 6.0 Cr(III) became a subject of the olation process. To avoid the problem of Cr(III) olation in complex media the pH = 4.0 was selected for further investigation.

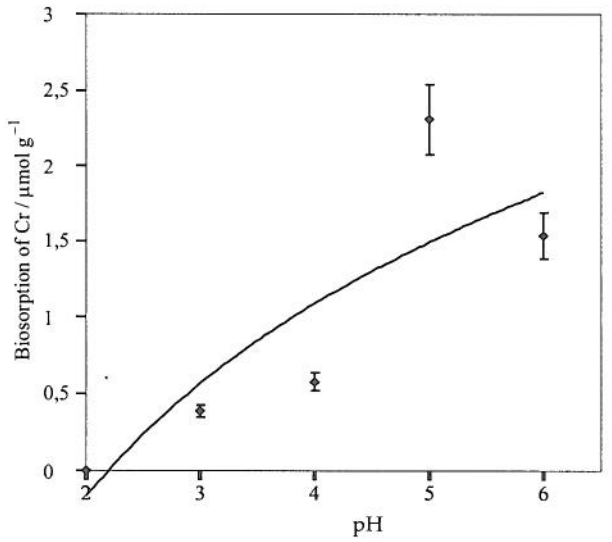

Fig. 1. Biosorption of Cr(III) on the surface of biomass of yeast *Sacch. cerevisiae* at different starting pH levels (28 °C, 200 min^{-1}, 4 h), expressed in μmol per g of dry mass.

Biosorption is exclusively responsible for metal ion accumulation by non-viable biomass owing to the absence of the metabolic activity necessary for intracellular metal ion accumulation *(19,23)*. Brady and Duncan *(20)* found that, when metal ion to biomass ratio was below 100 nmol/g, the metal ion accumulation was almost entirely dependent on biosorption of the metal ion to the cell wall. That was not the case in *Sacch. cerevisiae* where this ratio was 0.577 μmol of chromium per g of dry yeast cells at pH = 4.0 and temperature of 25 °C (Fig. 2). The results suggested the involvement of metabolic activity in Cr(III) accumulation, even though there is no other information on whether known metal uptake systems have the capacity to transport Cr(III) or whether specific chromium ion transport into the cell exists. The chromium ion presence in the environment induced the extrusion of H^+ ions (Table 2). Furthermore, concentrations of chromium ions higher than 2.40 mmol L^{-1} stimulate a more intensive extrusion of H^+ ions in yeast *Sacch. cerevisiae* (Table 2). It was also observed that the presence of glucose additionally stimulated H^+ ion extrusion (Table 2), which indicated active transport in response to electrochemical proton gradients, generated by membrane-bound H^+-ATPases, across the cytoplasmic and vacuolar membranes, which was the generally suggested mechanism for metal bioaccumulation *(16)*.

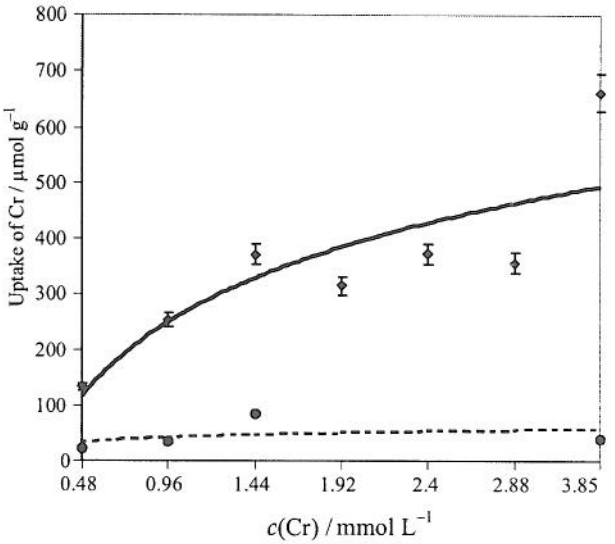

Fig. 2. Uptake of Cr(III) by yeast *Sacch. cerevisiae* at 25 °C and pH = 4.0 without (●) and with addition of 10 mM of glucose (◆), expressed in μmol per g of dry mass.

Toxicity results when metal ions with an unknown biological function compete with, or replace a function of metal ions *(24)*. During the continuous aerobic growth of yeast *Sacch. cerevisiae* at dilution rate (D) 0.08 h^{-1} chromium ions exert a harmful effect on yeast cell growth, cell protein and RNA concentration (Fig. 3). In yeast *Sacch. cerevisiae* the toxic effect of chromium ions include a 21% reduction of biomass, 9% reduction of protein and 22% of total cell RNA during continuous cultivation (Fig. 3). In batch cultivation, on the macro scale, the effect of chromium ions on the yeast *Sacch. cerevisiae* includes a reduction of growth rate and extension of lag phase (data not shown).

Table 2. Extrusion of H^+ ions in the Cr(III) uptake process of yeast *Sacch. cerevisiae* at 25 °C, with and without the addition of 10 mmol L^{-1} of glucose

$c(Cr^{3+})$ / mmol L^{-1}	pH_S	pH_E	pH_{GS}*	pH_{GE}*
0.48	3.92 ± 0.31	3.56 ± 0.28	3.97 ± 0.08	3.44 ± 0.45
0.96	4.03 ± 0.32	3.46 ± 0.07	4.00 ± 0.12	3.32 ± 0.22
1.44	3.96 ± 0.51	3.63 ± 0.00	4.01 ± 0.12	3.28 ± 0.11
1.92	3.98 ± 0.64	3.49 ± 0.07	4.00 ± 0.12	3.22 ± 0.11
2.40	3.97 ± 0.38	3.21 ± 0.06	3.99 ± 0.12	3.35 ± 1.55
2.88	4.01 ± 0.13	3.21 ± 0.10	4.04 ± 0.00	3.14 ± 0.00
3.85	3.94 ± 0.57	3.21 ± 0.00	4.01 ± 0.12	3.11 ± 0.00

S – start, E – end, GS – glucose addition start, GE – glucose addition end; * addition of glucose (10 mmol L^{-1})

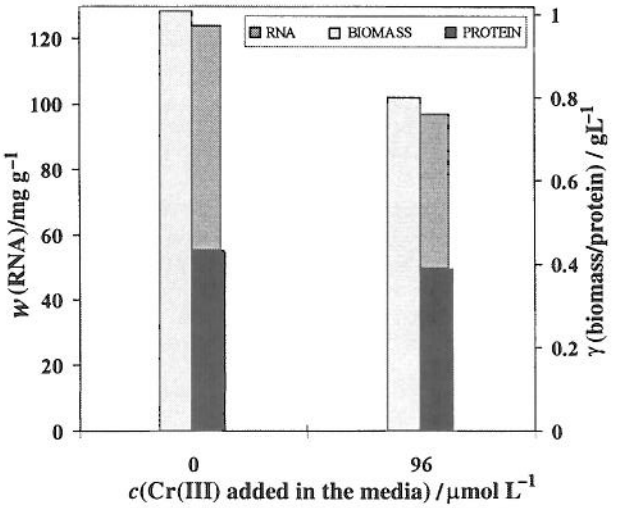

Fig. 3. Biomass, protein and RNA concentrations of yeast *Sacch. cerevisiae* grown in continuous culture with 28 mM of glucose at dilution rate 0.08 h^{-1} in chromium-free and chromium-loaded medium (96 μmol L^{-1})

The diversity of intracellular organelles and biomolecules provides a wide range of potential binding sites *(25)*. The total chromium concentration was 4.45 μg per gram of dry biomass of yeast *Sacch. cerevisiae* grown in the medium without the addition of chromium (Fig. 4). However, it must be pointed out that yeasts can normally contain chromium in their elemental biomass composition, even when chromium was not added in the medium (Table 1). When a higher concentration of chromium was present in the environment, a 47 times higher value was determined in the yeast biomass. This was reflected in the increase of organically bound chromium from 16% in control to 59% cultivated in a chromium loaded medium (Fig. 4). The Cr(III) inside the cell can bind to proteins and/or to small molecular mass (M_r) substances *(26)*. Speciation of organically bound chromium by a molecular sieve showed that the organic substances with a molecular mass (M_r) of between 100,000 and 10,000 expressed a high binding capacity to a chromium ion (Fig. 5). Under a normal condition (no addition of chromium) this peak was observed in the range of molecular mass between 10,000 and 3,000 (Fig. 5). Many cytoplasmic biomolecules have the ability to bind metal ions. The calculation of the chromium organic incorporation factor for different fractions showed that a molecular mass higher than 100,000 supports the mechanism of detoxification at high concentration levels of intracellular chromium in *Sacch. cerevisiae* (Fig. 6). It can

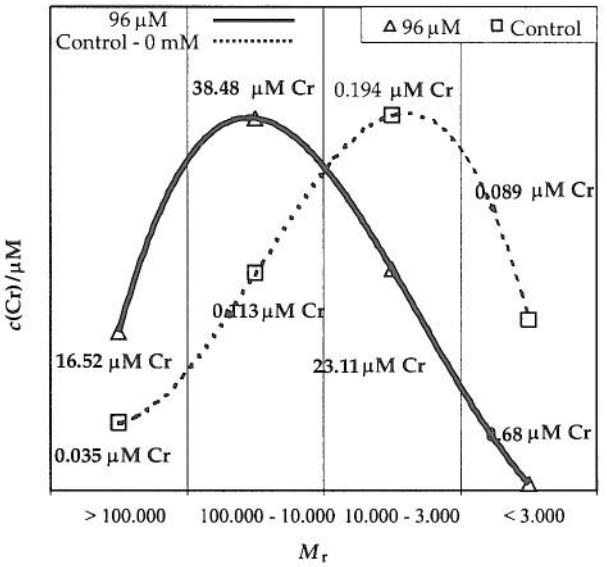

Fig. 5. Speciation of organically bound chromium among different molecular mass organic fractions (e.g. proteins) in yeast *Sacch. cerevisiae* grown in continuous culture with 28 mM of glucose at dilution rate 0.08 h^{-1} in chromium-free (- - -) and chromium-loaded medium (96 μM) (——)

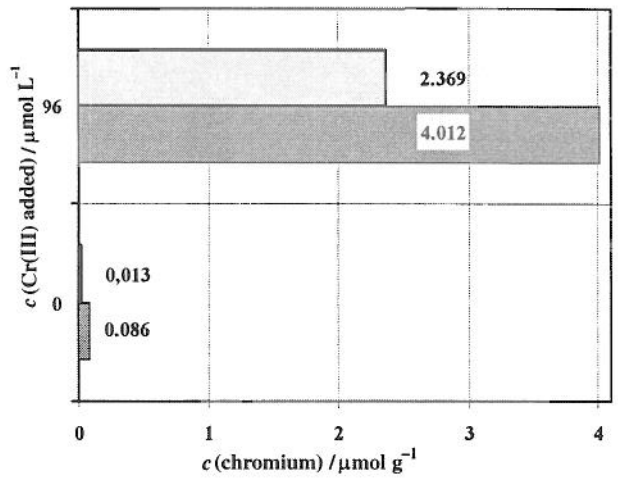

Fig. 4. Total and organically bound chromium in yeast biomass of *Sacch. cerevisiae* grown in continuous culture with 28 mM of glucose at dilution rate 0.08 h^{-1} in chromium-free and chromium-loaded medium (96 μmol L^{-1}); ▨ organically bound Cr, ■ total Cr.

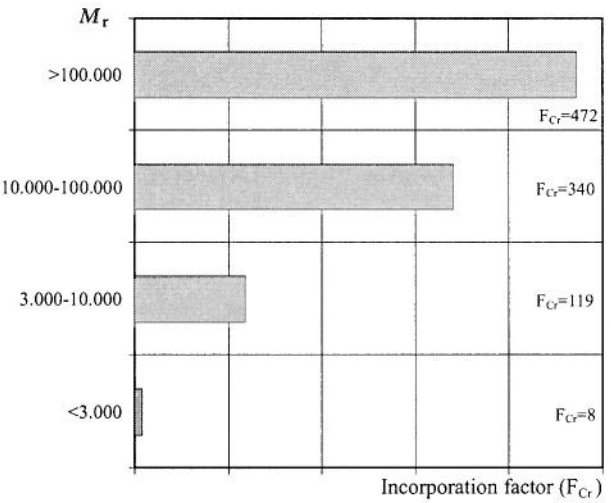

Fig. 6. Chromium organically bound incorporation factor of different molecular mass fractions of yeast *Sacch. cerevisiae* grown in continuous culture with 28 mM of glucose at dilution rate 0.08 h^{-1} in medium loaded with 96 μM of chromium

be concluded that in the case of chromium, cytosolic biomolecules with high molecular masses exist that have the ability to bind chromium and support the fast reduction of toxic intracellular chromium ion concentration (Fig 6). Besides, the small reduction of cellular proteins (Fig. 3), which was observed during cultivation in a chromium-enriched media, suggested the presence of binding molecules with a high chromium ion binding capacity (Fig. 5) whose synthesis can be induced by the presence of a chromium ion. This is the generally accepted mechanism for the action of other heavy metals *(25)*.

Conclusions

The effect of environmental pH was found to correlate positively with the chromium deposition on the cell wall of the yeast *Sacch. cerevisiae*. Furthermore, the yeast cell does not just absorb chromium on the cell surface, but it can also be conveyed into the cell by a metabolically dependent system. The high concentration of chromium in the environment always causes a reduction of RNA and protein concentrations on the cellular level. On the macro-scale, chromium causes a reduction of growth rate and biomass production, and an extension of lag phase. In *Sacch. cerevisiae* organic compounds with a molecular mass from 100,000 to 10,000 showed the highest intracellular chromium binding capacity. The overall fast reduction of intracellular chromium was also dependent on cell biomolecules with molecular masses greater than 100,000. Chromium essentiality in yeast should be further investigated by identifying the cell biomolecules with a high chromium binding capacity and the effect of chromium on metabolic activity.

Acknowledgements

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Prihvat i bioakumulacija Cr(III) u kvascu *Saccharomyces cerevisiae*

Sažetak

Ispitan je prihvat i akumulacija Cr(III) u odabranom kvascu Sacch. cerevisiae ZIM-198 nakon standardnog uzgoja u definiranoj podlozi koja je sadržavala određenu količinu Cr(III). Praćen je in vivo utjecaj Cr(III) na akumulaciju biomase, na RNA, na akumulaciju metala i na nastanak organski vezanog kroma. Prethodno je in vitro ispitan utjecaj pH i izvora energije na djelovanje Cr(III) u biomasi kvasca. Ustanovljena je velika međusobna povezanost između opskrbe energijom stanice i prihvata kroma. Optimalna pH-vrijednost bila je približno 4,0. Pri toj je pH-vrijednosti smanjena i mogućnost taloženja Cr(III) u podlozi. Relativno velike koncentracije Cr(III) (1,9 mmol L^{-1}) pojačavaju pad pH u podlozi. Daljnja in vivo ispitivanja u standardnoj podlozi potvrdila su utjecaj Cr(III) na metabolizam stanica kvasca. Dokazalo se da Cr(III) znatno utječe na sastav-

ne dijelove stanice. Kada se proveo kontinuirani uzgoj s 96 μmol L^{-1} Cr(III) u podlozi, povisila se ukupna koncentracija kroma u stanicama Sacch. cerevisiae. Pod istim uvjetima uzgoja utvrđeno je da se i koncentracija organski vezanog intracelularnog kroma povisila za 43%. Sposobnost kvasca da ukloni veliku intracelularnu količinu kroma bila je umanjena zbog toksičnog djelovanja kroma u podlozi na stanični rast i preživljavanje. Stanični sustav detoksifikacije omogućava stanicama Sacch. cerevisiae da prežive i rastu pri umjerenim i većim koncentracijama anorganskog kroma. U tim procesima važnu ulogu ima razdioba viška anorganskog kroma u stanici.