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Extra-cellular chromate-reducing activity of the yeast cultures

Helena P. Ksheminska, Taras M. Honchar, Galyna Z. Gayda, Mykhailo V. Gonchar*

Department of Cell Regulatory Systems, Institute of Cell Biology, National Academy of Sciences of Ukraine, Lviv 79005, Ukraine

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Abstract: This paper reports on the experimental data supporting an essential role of extra-cellular reduction in chromate detoxification by baker's and non-conventional yeasts. A decrease of chromate content in the yeast culture coincides with an increase of Cr(III) content in extra-cellular liquid. At these conditions, cell-bound chromium level was insignificant and a dominant part of extra-cellular Cr(III) species was detected in the reaction with chromazurol S only after mineralization of the cell-free samples. This phenomenon of chromium "disappearance" can be explained by the formation of Cr(III) stable complexes with extra-cellular yeast-secreted components which are "inaccessible" in the reaction with chromazurol S without mineralization. It was shown that increasing sucrose concentration in a growth medium resulted in an increase of chromate reduction. A strong inhibition of chromate reduction by 0.25 mM sodium azide, a respiration inhibitor and a protonophore, testifies that extra-cellular chromate detoxification depends on energetic status of the yeast cells. It was shown that Cr(III)-biochelates produced in extra-cellular medium are of a different chemical nature and can be separated into at least two components by ion-exchange chromatography on anionit Dowex 1x10. A total yield of the isolated Cr(III)-biocomplexes is approximately 65 % (from initial level of chromate) with a relative molar ratio 8:5.

Keywords: Chromate, chromium(III), chromium(III)-biocomplexes, extra-cellular medium, reduction, yeast

* E-mail: gonchar@biochem.lviv.ua

1 Introduction

Chromium can exist in several states of oxidation; Cr(III) and Cr(VI) are the most stable and widely used species of this element. The main representatives of chromium compounds in hexavalent state - chromates/dichromates are very toxic, display mutagenic and carcinogenic activity [1, 2], and their pollution poses a serious environmental problem. Thus, the development of efficient methods for detoxification and remediation of this hazardous chromium oxy-anion is of great importance. It is generally accepted that chromium (III) species are not highly toxic and due to their limited solubility and biosorption, they can be easily removed by chemical and microbial approaches. The main unsolved problem for chromate detoxification is reduction to chromium (III). For this purpose, microbial systems seem to be the most promising, although genetic and biochemical aspects of this process are poorly studied. For microbial cells, it is known that chromate anions are transported into cells through sulfate-specific permease(s) coded in the case of baker's yeast, by the genes *SUL1* and *SUL2* [3], and can be reduced to Cr (III) by cellular reducing systems which can include enzymatic and non-enzymatic pathways. The most powerful non-enzymatic chromate reductants could be glutathione and cysteine for microbial cells, and ascorbate for higher organisms.

Supposively after chromate enters cells as a powerful oxidative agent, it should be reduced in non-enzymatical and/or enzymatical pathways. Bacterial reduction of chromate is well-established and runs through the different enzymatic pathways functioning under aerobic [4] and anaerobic [5] conditions. Anaerobic bacteria can reduce chromate using hydrogenase [6], cytochrome *c*-dependent electron transfer chains [7, 8]. Little is known about mechanisms of chromate reduction for aerobic bacteria, except some enzymes which are shown to catalyze this reaction *in vitro*, for example, nitrate reductase [9], flavin reductase [10], ferrireductase [11], and some flavoproteins [12, 13]. Among bacterial chromate-reducing enzymes are NADH and NAD(P)H-dependent reductases [14]. For eukaryotic microbial cells, especially yeasts, the knowledge of the chromate-reducing systems are more obscure. Generally it is not known what system, enzymatic or non-enzymatic, intracellular or extra-cellular, plays a leading role in chromate detoxification processes. Specific yeast mutants with a modified tolerance to chromate and/or impaired in activity of some enzymes are used to study the mechanisms of chromate toxicity and pathways of its detoxification. Using such models, some metabolites and enzymes have been shown to be responsible for the modulation of yeast cell tolerance to chromate, *e.g.* glutathione and glutathione reductase [15–17], glucose-6-phosphate dehydrogenase [15], mitochondrial Mn-dependent superoxide dismutase [17], cytosolic Cu,Zn-superoxide dismutase and methionine sulfoxide reductase [18]. NADH-dependent chromate reductase was found in a chromate-resistant strain of *Candida maltosa* [19]. Chromate reduction and distribution of the metal in the cells and medium was studied for the yeast *Candida utilis* [20].

It was demonstrated that ergosterol-less mutants with a modified plasma membrane structure exhibit Cr(VI) changed sensitivity [21]. The modified tolerance of the mu-

tant cells to chromate is accompanied with the changes in Cr(VI) uptake and chromium bioaccumulation [22]. It was shown that ascorbic acid influenced Cr(VI) toxicity both as an antioxidant, by decreasing intracellular reactive oxygen species, and as a reducing agent, by decreasing the level of Cr(V), a highly toxic intermediate in chromate reduction [23]. The effects of chromate on cell growth and lethality were studied using DNA repair-deficient *S. cerevisiae* mutants [24].

In summary, it can be concluded that the greater part of the recent research devoted to chromate detoxification in yeasts is focused on processes and systems of **intracellular** localization. Although, taking into account a high oxidative potential of Cr(VI) and competitive inhibition of chromate uptake by sulfate anions present in common microbial media, we suppose that some low-molecular products secreted by yeast cells can also be involved in the process of chromate detoxification. These products act as reducing agents enabling an extra-cellular reduction of chromate.

In this paper, experimental evidence is presented concerning an essential role of extracellular chromate reduction in its detoxification by yeast cells. It was revealed that a decrease of chromate content in a medium coincides with an increase of Cr(III) content in extra-cellular liquid, although some Cr(III) species are detected by the reaction with chromazurol only after mineralization of the cell-free samples. It was shown that Cr(III)-chelates, produced in extra-cellular medium, are of a different chemical nature and can be separated by an ion-exchange chromatography.

2 Experimental procedures

2.1 Yeast strains

An industrial strain of baker's yeast *Saccharomyces cerevisiae* "Enzym"; non-conventional yeast *Pichia guilliermondii* ATCC 20191 (L2), *Rhodotorula pilimanae* D-76, *Yarrowia lipolytica* VKM Y-917 and *Hansenula polymorpha* 356 from the Microbial Collection of the Institute of Cell Biology (Ukraine).

Cultivation was carried out in Erlenmeyer's flasks with aeration in a shaker (200 rpm) at 30 °C in the Burkholder's mineral medium [25] which usually contained 2 % sucrose and 0.1 % yeast extract, thiamine (0.2 mg/l), pyridoxine (4.5 mg/l), and pantothenic acid (4.5 mg/l). A sterile solution of chromate (K_2CrO_4) was added to the yeast culture with cell concentration 0.3 mg/ml (an early exponential phase of the growth) and incubation with aeration was continued at 30 °C. Biomass was measured by the optical density at 540 nm. Absolute dry mass expressed in mg/ml was calculated by the use of calibration curve obtained gravimetrically.

2.2 Chemical assays

The remaining chromate content in the extra-cellular liquid of yeast cultures was measured by colorimetric diphenylcarbazide method [26, 27].

The determination of Cr(III) content in the cultural liquid was carried out by the colorimetric method in our modification using the reaction of Cr(III) with chromazurol S [28] in the presence of SDS. This reaction is also positive to Fe(III) and Cu(II), but because of a much lower content of these ions in the standard Burkholder's medium (0.2 mg/l or 3.6 μM for Fe and 0.01 mg/l or 0.16 μM for Cu) compared to the analyzed Cr(III) levels (usually 0.1-0.6 mM), no interference from the aforementioned ions was observed. Moreover, at the starting point of a cultivation (high level of chromate, zero level of Cr(III) and the highest (initial) iron and cooper levels in a medium) a low optical density of photometric mixture was observed.

An aliquot which contained 1-4 μg Cr(III) was adjusted to the volume of 1 ml by water and a mixture of 1.0 ml 1 % dodecyl sulfate, 1 ml 0.5 M acetate buffer, pH 3.5 and 1 ml 0.015 % chromazurol S was added. The samples were put in a boiling water bath for 30 min, cooled and treated with 1 ml 1 M H_2SO_4. Absorbance of colored solutions was measured at 590 nm against the blank which did not contain chromium. Concentration of Cr(III) in the samples (in mM) were calculated using a calibration curve obtained for standard Cr(III) solutions.

To determine the correct concentration of Cr(III) produced by the yeast culture during chromate reduction, we tested possible contributions to this process of growth medium components during cultivation as well as during the analytical procedures. For this purpose, in parallels with the experimental variant, chromate was incubated at the same concentration with a growth medium but without inoculation of the cells. For the standard medium used in the experiments, the correction value based on such control was not higher than 5-7 % from the initial chromate level.

For determination of the total chromium up-taken/absorbed by the cells and the sum of chromium species remaining in the extra-cellular liquid, aliquots of the washed cells or cell-free cultural liquid were mineralized by burning in a mixture of sulfuric and nitric acids (1:2.5; v/v). Samples were put in the test-tubes, placed in an aluminum block, and concentrated by heating (2-5 min), and then a 0.15 ml H_2O and 0.2 ml acid mixture were added. The burning process was carried out at 260 °C until samples charred and nitrous vapors were removed. The samples were cooled and 0.15 ml H_2O and drops of concentrated nitric acid were added. The burning procedure was repeated until a visual discoloring of the samples was observed. Finally, 0.5 ml H_2O was added and samples were heated until the gases disappeared. This procedure was repeated twice to remove nitroso-sulfuric acid.

3 ml H_2O and 0.2 ml 5 mM potassium permanganate were added to the cooled samples and then placed for 20 min in the boiling water bath to allow the oxidation of chromium species to Cr(VI). In the case of discoloring, additional drops of permanganate solution were added, and an excessive amount of the oxidant was removed by adding drops of 0.25 % sodium azide solution. Further assay of the formed chromate was carried out by the reaction with diphenylcarbazide.

For convenience, chromium(III) in the cultural liquid, which is determined in the reaction with chromazurol S will be referred to as "accessible" and Cr(III) which is assayed

only after previous mineralization of the liquid will be called "chelated." Chromium determined in the mineralized cells will be called "cellular" whereas the chromium determined in the mineralized cultural liquid will be "total extra-cellular."

Analytical data was collected from 3 replicated experiments with each assay at least in duplicate.

2.3 Fractionation of the Cr(III)-complexes from a cultural liquid

A cultural liquid taken from a 2 day-culture of *P. guilliermondii* L2 yeast cultivated in the presence of 1 mM chromate was fractionated using ion-exchange chromatography. The procedure was carried out on a column (8x1 cm) with anion resin Dowex 1x10 (200/400 mesh) equilibrated in 20 mM acetate bufer, pH 5.0. Cultural liquid (160 ml) adjusted to pH 5.0 was put in the column followed by 20 mM acetate buffer of pH 5.0 to remove the un-binding components. A green-blue colored ring on the top of the column was clearly observed. Elution of the chromium(III) complexes was carried out gradually, first by 1 M NaCl in the starting buffer, and then by 50 mM Tris-HCl buffer, pH 8.8. Determination of Cr(III) in chromatographic fractions was carried out by diphenylcarbazide method after mineralization of the aliquots and transformation of Cr(III) to chromate by permanganate as described above.

3 Results and discussion

During incubation of the yeast *Saccharomyces cerevisiae* cells (strain "Enzym," 0.3 mg/ml) with 0.6 mM chromate in sucrose growth medium, a decrease of chromate level and a concomitant increase of Cr(III) amount in extra-cellular medium were observed (Fig. 1). On the fourth day of incubation, Cr(VI) was completely reduced and was not detected in a cultural liquid even in traces, but the extra-cellular content of "accessible" Cr(III) was only 13% from the initial chromate level. During further cultivation of the culture, the level of the "accessible" Cr(III) continuously decreased. Cell biomass during the incubation increased insignificantly and was close to a value 1.1-1.2 mg/ml (at an initial level of 0.3 mg/ml). The total chromium content in the cells at these conditions was 0.03 micromoles/mg (of abs. dry cell weight) which amounts to approximately 6% of the initial level of chromate. Thus, a phenomenon of chromium "disappearance" is observed in the cultural liquid. We suppose that chromium (III) generated during chromate reduction, forms stable complexes with some components of the culture medium. While considering a kinetic inertness of Cr(III) ions, the formed complexes become masked ("inaccessible") in the reaction with chromazurol S. The most expressed chromium "disappearance" was observed in the late phase of the cells growth (Fig. 1).

We determined the content of different chromium forms in the cultural liquid during the incubation of the yeast cells in the presence of 0.6 mM chromate (Fig. 2). At the 7th hour of incubation, 80% of the initial chromate level in the cultural liquid remained while the rest was reduced to the "accessible" Cr(III) form (Fig. 2, parts A and B). At

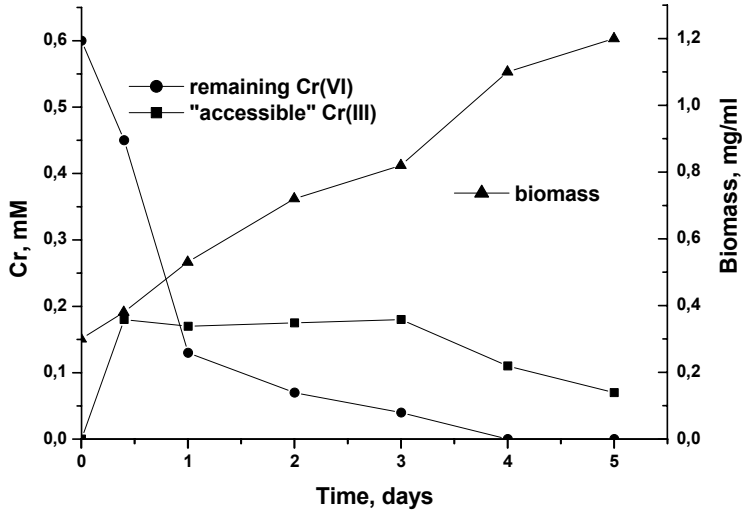

Fig. 1 The growth curve and dynamics of changes in chromate content and in "accessible" Cr(III) pool in a cultural liquid during cultivation of the yeast *S. cerevisiae* "Enzym" (initial cell concentration 0.3 mg/ml) with 0.6 mM chromate in sucrose (2%) growth medium.

this stage, the incubated yeast culture was separated into two portions: one part was left for further incubation, and the other part was used to obtain a cell-free supernatant. For this objective, cells were removed by centrifugation and the supernatant was passed on through the Amicon 0.3 μm-filter for "cold" sterilization. This supernatant, as an initial culture, was further incubated at 30 °C.

The calculation of the balance between initially inoculated chromate and the content of different chromium forms in a culture after 48-hour of incubation (we analyzed the remaining Cr(VI) and "accessible" Cr(III) in cultural liquid, as well as "cellular" chromium after cell mineralization) revealed that the sum of all measured chromium forms was not higher than 75% of the initial chromate content (Fig. 2, part C). We suppose that Cr(III) ions, at the moment of their formation during chromate reduction, form a stable complex/complexes with metabolites secreted by the cells thus becoming "inaccessible" for the reaction with chromazurol S. To evaluate this hypothesis, cultural liquid was mineralized and total chromium content was determined in the colorimetric reaction with diphenylcarbazide after oxidation to chromate. The sum of the total chromium in the cultural liquid (after its mineralization) and the cellular chromium was close to the initial level of Cr(VI) (Fig. 2, **C**), that indirectly confirms the hypothesis about the formation of Cr(III)-complexes with the components of cultural liquid.

The chromate-reducing ability observations of the cell-free supernatant, taken at the 7th hour of yeast culture incubation (Fig. 2, **D**), showed that this activity is peculiar not only to the whole culture, but also to the cell-free cultural liquid which confirms an extracellular mechanism of chromate reduction. A cultural liquid taken from a chromate-free culture also displayed the reduction ability, while the control experiment did not show any remarkable reduction when chromate was added to the initial nutrition medium

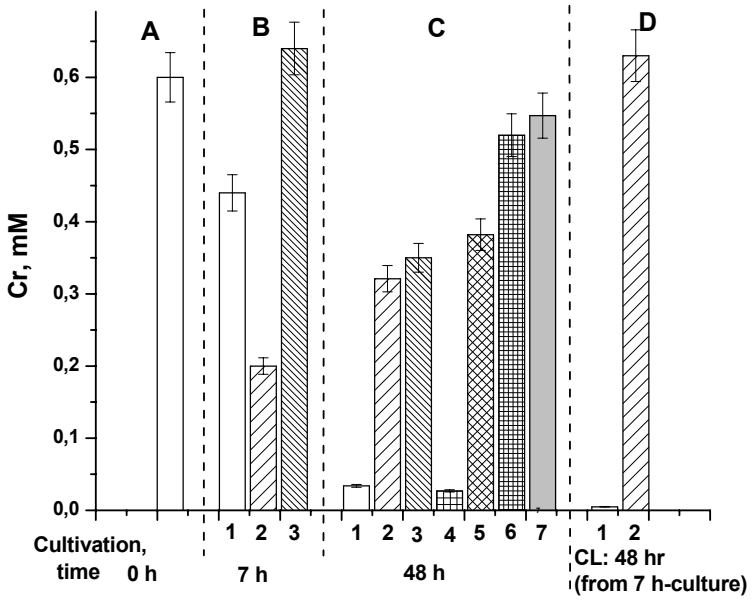

Fig. 2 The content of different chromium species in a cultural liquid (CL) during cultivation of the yeast *S. cerevisiae* "Enzym" (0.3 mg/ml) in the presence of 0.6 mM chromate in sucrose (2%) growth medium. The growth curve is similar as shown in Fig.1.
Abbreviations: **A** – an initial chromate level; **B** and **C** – chromate levels determined in a cultural liquid of 7- and 48-hour yeast culture, respectively; **D** – chromium species determined in a cell-free cultural liquid (CL), taken out from 7-hour culture and incubated without cells during 48 h.
1 – remaining Cr(VI); **2** – "accessible" Cr(III); 3 – sum of the remaining Cr(VI) and "accessible" Cr(III) in CL ("1"+"2"); **4** – cellular chromium (determined after mineralization of the cells); **5** – chromium in a culture calculated as a sum "1"+"2"+"4"; **6** – total chromium in CL (determined in a mineralized sample); **7** – total chromium in the culture (sum "4" + "6").

without inoculation of the cells. So, chromate-reducing activity of the yeast culture is in a tight relation with the cellular metabolism, but runs extra-cellularly possibly through the reaction with metabolites secreted by cells.

It is worth mentioning that at the 48th hour of cell-free supernatant incubation, after continued chromate reduction, the phenomenon of Cr(III) "disappearance" was not observed. It was found that almost all quantity of Cr(III) were present in the cultural liquid even without its mineralization. It can be concluded that the compounds which reduce chromate and the Cr(III)-complexing substances are of a different nature and are secreted by the cells at different phases of the culture growth.

Since the flow of cell-secreted metabolites which can possess a chromate-reducing ability is dependent on the concentration of carbon substrate in the growth medium, we studied the influence of sucrose concentration in a nutrition medium (from 2 to 20 g/l) on the chromate reduction during 3 days-cultivation (Fig. 3). It was shown that an increase in sucrose concentration resulted in improvement of chromate reduction. At

the optimal sucrose concentration in the medium, cells completely metabolized 0.5 mM chromate during 3 days (Fig. 3). The "productivity of the reduction," defined as an amount of Cr(VI) metabolized by 1 mg of cells was three times higher at sucrose content of 20 g/l when compared to 2 g/l. This might be explained by the enhancement of cell secretory activity when grown at higher sucrose concentrations.

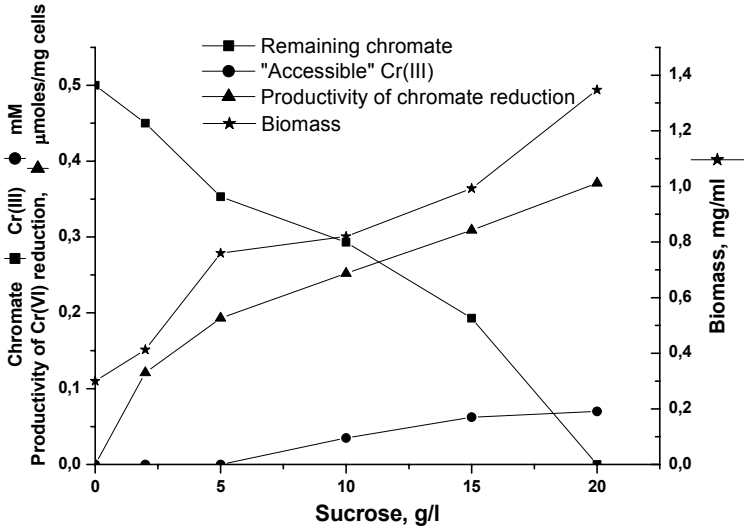

Fig. 3 Biomass, remaining chromate concentration, and "accessible" Cr(III) levels in a cultural liquid during 3 days-cultivation of the yeast *S. cerevisiae* "Enzym" in the media with different sucrose concentrations (initial cells concentration – 0.3 mg/ml; 0.5 mM chromate). "Reduction productivity" is defined as an amount of Cr(VI) metabolized by 1 mg cells under standard cultivation conditions.

Chromate reduction efficiency and formation of stable Cr(III)-complexes depend on energetic status of the cells. This was confirmed in the experiments with sodium azide, a respiration inhibitor and a protonophore. 1 mM sodium azide suppressed chromate reduction by the yeast culture up to 60 % (60 % of the initial amount of chromate remained un-reduced in a culture supplemented with NaN_3, while all added chromate (0.5 mM) was completely reduced in the corresponding control without addition of azide). This inhibition is significantly less expressed (only near 15 %) for cell-free supernatant taken from the culture. Similar results were received in the presence of 1 mM rotenone, an inhibitor of mitochondrial electron transport.

To conclude the presented data, it can be suggested that the reduction of Cr(VI) to Cr(III) and chelation of the latter species can run extra-cellularly, probably in the reaction with metabolites secreted by cells. To reveal whether this phenomenon is unique for baker's yeast or if it is peculiar to the other yeasts, incapable of fermentation, we have studied chromate reduction by some non-conventional yeasts. This includes the methylotroph *Hansenula polymorpha*, alkane-assimilating yeast *Yarrowia lipolytica*, the yeast *Rhodotorula pilimanae* which is able to synthesize the rhodothoric acid, as well as the flavinogenic yeast *Pichia guilliermondii* for which chromate is known to cause an

over-synthesis of riboflavin [29]. Different initial concentrations of Cr(VI) were chosen because of an essential difference in chromate tolerance of the tested yeast species, as shown by us previously [30].

As shown in Fig. 4, among studied yeasts, *P. guilliermondii* L2 can be regarded as the strain with the highest reducing activity. At the indicated conditions, 75 % of inoculated chromate is reduced extra-cellularly and the formed Cr(III) remained in extra-cellular liquid in a complex, "accessible" for assay only after its mineralization.

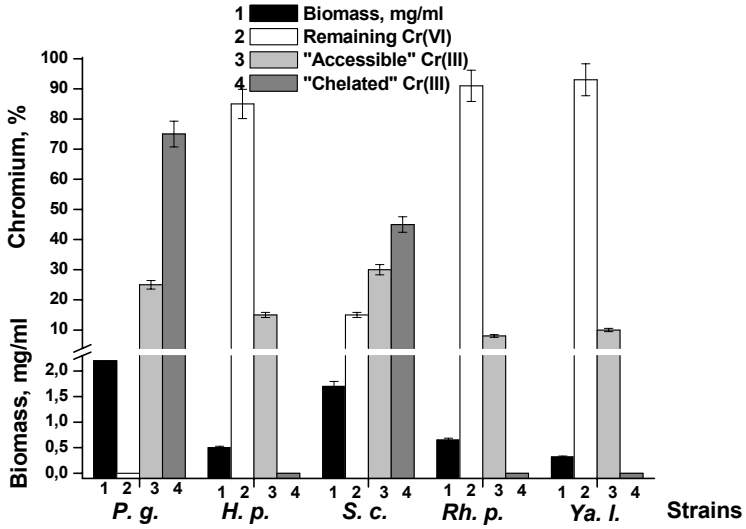

Fig. 4 Cell biomass and content of different chromium forms in cultural liquid of the yeast cultures grown in the presence of chromate (different concentrations of Cr(VI) were chosen because of a difference in chromate tolerance of the tested yeast species [30]). Abbreviations: *P.g.* - *Pichia guilliermondii* L2, *H.p.* - *Hansenula polymorpha* 356, *S.c.* – *Saccharomyces cerevisiae* "Enzym", *Rh.p.* - *Rhodotorula pilimanae* D-76, *Ya.l.* - *Yarrowia lipolytica* VKM Y-917. Conditions: yeasts *P.g. and H.p.* were incubated with 1.0 mM chromate; *S.c.*– 0.6 mM; *Rh.p.* and *Ya.l.* – 0.7 mM. The initial cell concentration for all yeasts – 0.3 mg/ml.

The optimal cultivation conditions for the yeast *P. guilliermondii* L2 resulting in the best yield of the "chelated" Cr(III)-form, localized in extra-cellular medium, were studied. A complete Cr(VI)-reduction and an effective chelation of Cr(III) were observed at chromate concentration 0.85 mM (0.3 mg/ml cells; incubation time 24 hours) and at 1 mM Cr(VI) (1 mg/ml cells; 48 hours). The increase in chromate concentration essentially repressed a reducing activity of the yeast cultures. At 2 mM initial concentration, a significant part of Cr(VI) remained in a cultural liquid in un-reduced form, and a portion of "chelated" Cr(III) decreased even at the prolonged incubation period. This influence could possibly be explained by a deteriorate effect of excess chromate on the cells.

To isolate a "chelated" Cr(III)-form, we used ion-exchange chromatography for fractionation of extra-cellular liquid taken from the chromate-supplemented culture *P. guilliermondii* L2. Previous analytical variants of chromatography carried out on different

ion-exchange resins, revealed a negative charge of the complex, so the preparative experiment was done using a column with the anionit Dowex 1x10 containing trimethylamine group. Acetic acid/sodium acetate system at pH 5.0 was used as a starting buffer. For elution of chromium-containing species absorbed on the column, a high concentration of sodium chloride in the initial buffer and a shift in pH of the buffer system were used. In all chromatographic fractions, chromium(III) content in an "accessible" form as well as a total amount after sample mineralization were assayed. The pool of the "chelated" Cr(III) has been determined as a difference between the total and "accessible" chromium contents.

The chromatographic profile and characteristics of the chromium(III)-containing fractions are shown in Fig. 5 and Table 1. The presented data allow the conclusion that during chromate reduction by yeast culture at least two Cr(III)-containing complexes are formed in extra-cellular liquid. Both complexes have a total negative charge and can be easily separated by ion-exchange chromatography. A total yield of the two isolated species is approximately 65 % (from the initial level of chromate) with a relative molar ratio 8:5. The primary characterization of the isolated complexes by UV- and EPR-spectroscopy approved 3-valent state of their chromium: absorption peaks were observed at 563-568 mm (versus 379 mm for chromate) and g-factor was 3.76 (spectra are not shown). The obtained results will serve as a basis for preparative isolation of the discovered complexes, their structural characterization, as well as for studying their potential importance for pharmacology as chromium-containing bio-complexes.

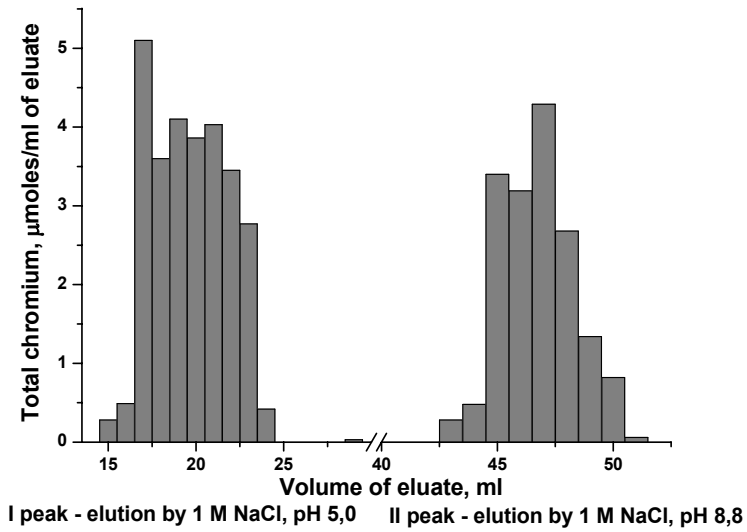

Fig. 5 Chromatographic purification of Cr(III)-complexes from a cell-free supernatant of the yeast *P. guilliermondii* L2 culture after cultivation of the cells in the presence of 1.0 mM chromate during 2 days.

Put on a column	Cr(III), micromoles Determined after chromatography		Yield, %
139 (=100 %)	Non-sorbted fraction and washings by starting buffer	19	14
	I eluate (1 M NaCl, acetate buffer, pH 5.0)	56	40
	II eluate (1 M NaCl, Tris-HCl buffer, pH 8.8)	35	25

Table 1 Characteristics of the fractions isolated during ion-exchange chromatography of 160 ml cultural liquid of the yeast P. *guilliermondii* L2 culture after incubation of the cells with the 1.0 mM chromate.

4 Conclusions

The experimental data affirm an essential role of extra-cellular medium in chromate reduction by yeast cultures. This process is tightly related with a formation of the stable Cr(III)-complexes which are accumulated out of the cells and are "inaccessible" in the reaction with chromazurol S without mineralization. Chromate reduction and formation of Cr(III)-biocomplexes are dependent on the energetic status of the cells. This was shown by the activation of these processes from increased sucrose concentrations and their inhibition by sodium azide and rotenone. The revealed extra-cellular chromate reduction can be considered as an important part of an universal chromate detoxification system, common for baker's and non-conventional yeasts, because of the lower toxicity of Cr(III) species compared to Cr(VI).

Cr(III)-biocomplexes, produced in extra-cellular medium, are of a different chemical nature and can be separated into at least two components by ion-exchange chromatography on anionit Dowex 1x10. These results will be used for preparative isolation of the discovered Cr(III)-biospecies, their structural characterization, as well as for studying their potential importance for pharmacology as chromium-containing bio-complexes.

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