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Yeast Tolerance to Chromium Depends on Extracellular Chromate Reduction and Cr(III) Chelation

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Summary

The sensitivity of the yeast *Pichia guilliermondii* ATCC 201911 (L2) to Cr(VI) and Cr(III) has been investigated. This yeast was demonstrated to have much higher resistance to Cr(III), compared to Cr(VI). At chromate level of 2.0 mM, an expanded lag phase in growth was observed, followed by the active phase of cell proliferation. During the growth arrest, a residual external chromate concentration decreased gradually to the non-toxic level, and the duration of this period was directly dependent on the initial Cr(VI) concentration in the medium, *i.e.* the higher the Cr(VI) level, the longer the growth delay. Thus, the tolerance of *P. guilliermondii* to chromate depends on its capacity for extracellular reduction of Cr(VI). As a product of chromate reduction, Cr(III) forms complexes with the components of culture liquid, which are not effectively adsorbed by the cells and do not repress the growth of *P. guilliermondii*. When urea is used as a nitrogen source, not only chromate reduction, but also Cr(III) chelation were shown to decrease, compared to the medium supplemented with ammonium sulphate. The experimental data confirm this concept of extracellular reduction of Cr(VI) as an important mechanism, which provides the resistance of yeast cells to chromate.

Key words: yeast, *Pichia guilliermondii*, chromate tolerance, medium composition, chromate reduction, chromium(III) chelation

Introduction

Chromium compounds are among the most dangerous environmental pollutants due to their high toxicity and wide use in numerous industrial processes. It is assumed that chromium plays an important role in the metabolism of all living cells (*1*). However, at high concentrations it is toxic, mutagenic, and carcinogenic, especially in Cr(VI) form, which causes oxidative stress, DNA damage, and modulates the activity of regulatory apoptotic gene *p53* (*2–7*). In spite of a large number of works devoted to the study of biological role of chromium and its influence on various organisms, molecular mechanisms of chromium bioactivity remain vague, which prevents the development of an optimal strategy of its detoxification, bioremediation of industrial wastewaters, and treatment of diseases related to disturbed homeostasis of this element. Microorganisms, yeasts in particular,

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are the convenient objects for such studies, since some strains were found to be capable of growing at high concentrations of chromium compounds, and some of adsorbing or accumulating significant quantities of chromium in the cells and transforming them into chelated, less toxic, forms (*8–12*).

The dual role of chromium in cell metabolism foresees the presence of effective mechanisms of controlling its entry into the cells, accumulation, and detoxification. It has been suggested that the entrance of Cr(VI) into the yeast cells in an oxy-anionic form occurs *via* sulphate-specific transport systems (*13,14*). Genes involved in sulphate and chromate transport have been identified (*15,16*). The microbial cells are often impermeable for Cr(III), possibly because of the formation of its complexes with low solubility. They can be transferred through a cellular membrane, but the mechanism of such transport is still unknown (*14,17*). Up to date, it is not clear whether the known metal transporting systems are responsible for the accumulation of Cr(III) in the cells and whether there is a specific system to transport this cation in yeasts.

The consumption of chromium by yeast cells depends on its valence, chemical structure, and concentration of chromium species, method of cultivation, composition of the growth medium, energy source, and the presence of some modulators (sulphate, phosphate, iron ions, chelating agents) (*12,14,18*). It has been shown for different yeast cultures that chromate concentration determines the duration of the lag phase (*19,20*). The reasons for such atypical growth kinetics in the presence of chromate are not clear, but one can suppose that this phenomenon is related to the active detoxification of chromate to the less toxic species.

One of the most examined aspects of the metabolism of toxic chromate is its microbial reduction to Cr(III), which is one of the important mechanisms of chromate detoxification in bacteria. It has been suggested that the principal reason of the resistance to the chromium in yeasts is a decrease in its absorption (*14,21*). However, for the chromate-resistant strains of *Candida maltosa*, it was found that a NAD-dependent chromate-reducing activity took place mainly in the soluble protein fraction, and was less active in the membrane fraction (*22*). Recently, it has been discovered that the significant role in Cr(VI) detoxification belongs to extracellular reducing substances, which are secreted by the yeast cells (*12*) and chromate-resistant mutants of the yeast *Pichia guilliermondii* were selected with the ability to reduce 80–100 % of chromate to Cr(III) (*23*).

In this article, the study on chromate reduction by *P. guilliermondii* and the influence of some factors on this process are presented, which confirms the extracellular pathway of Cr(VI) reduction as an important mechanism providing the resistance of the cells to chromate.

Materials and Methods

Strain and media

In this work, the strain of *Pichia guilliermondii* ATCC 201911 (L2) (*MAT-hisX-17*) was used (*MAT* is a mating type locus, *his* is a locus defining histidine biosynthesis).

The yeast cells were cultivated at 30 °C in the Erlenmeyer flasks on a circular shaker (200 rpm) in Burkholder's medium (*24,25*) with the addition of 0.1 % yeast extract (a semi-rich medium) or 0.2 % yeast extract+0.2 % peptone (a rich medium). Cr(VI) was added to the medium as potassium chromate. Liquid media were inoculated with *P. guilliermondii* cells from the early stationary growth phase in concentration of 5 mg/L. Incubation of the cells (0.3 g/L) with chromate was carried out from 2.5 hours to 6 days. Yeast biomass was determined turbidimetrically at 600 nm using gravimetrical calibration.

Analysis of chromate tolerance/sensitivity of the cells that survived in a medium containing 2 mM chromate was carried out using growth test on agar plates. With this aim, 3-day-old culture was diluted and spread on agar plates supplemented with standard growth components without chromate. A random set of 50 clones picked up from the separate colonies were tested on a selective agar medium supplemented with different concentrations of chromate (from 0.1 to 2 mM) by putting 5 µL of cell suspension at different dilutions (from 10 to 1000) on selective agar plates and checking for the appearance of growing colonies after 3 and 5 days of incubation.

Analytical methods

The determination of total chromium content in the cells was performed using atomic absorption spectrometer AAS-3 (Carl Zeiss, Germany) or photometric diphenylcarbazide method after oxidative conversion of different chromium forms to chromate by permanganate (*26*). Cells for analysis were prepared using acid-hydrogen peroxide mineralization as described earlier (*27*).

The assay of Cr(VI) concentration in a medium was carried out using diphenylcarbazide method (*28,29*). The concentration of the trivalent chromium was determined in the reaction with Chromazurol S (*27*).

Calculations

The presented quantitative data are average values resulting from 2–3 independent experiments. All analytical measurements were performed in 3 duplicates.

Results

Growth kinetics for the yeast *P. guilliermondii* in the presence of different concentrations of Cr(III) or Cr(VI) was investigated. The degree of growth inhibition by Cr(III) depends on its concentration in a medium. Upon incubation of the cells during 4 days in the rich medium supplemented with 4 mM Cr(III), the growth was repressed by 25 % when compared to the variant with 1 mM Cr(III) (Fig. 1a). At 12 mM Cr(III), the growth was observed only during the first day of cultivation, and biomass yield was 4-fold lower than in the presence of 1 mM Cr(III). Quite distinct kinetics of growth was observed during incubation of the cells with chromate (Fig. 1b). At Cr(VI) concentration of 1 mM, the growth was repressed, and at 2 mM a protracted delay of the growth (a 'plateau' in the interval between 1–4 days) was observed, after which the yeast began to grow in-

tensively accumulating finally the same biomass, as in a medium without chromate (Fig. 1b).

When yeast cultures supplemented with the lowest (1 mM) concentration of chromium were compared, the cells incubated in the presence of chromate were found to accumulate much more chromium (10-fold after incubation during 3.5 days) than those grown in the presence of Cr(III) (Figs. 1c and d). Similar to chromate-supplemented culture, a higher Cr(III) concentration in the medium resulted in an increased chromium content in the cells. In both cases, the major amount of chromium was retained in the medium. The difference in the kinetics of chromium accumulation in the cells was also revealed. In the presence of Cr(III), the cellular chromium increased gradually to the saturation level, contrary to chromate-supplemented cultures, where two-phase accumulation process was observed: the first with a positive slope in accumulation kinetics and the second one with a negative accumulation rate, which resulted in the decreased chromium content in the cells taken from the final (stationary) growth phase.

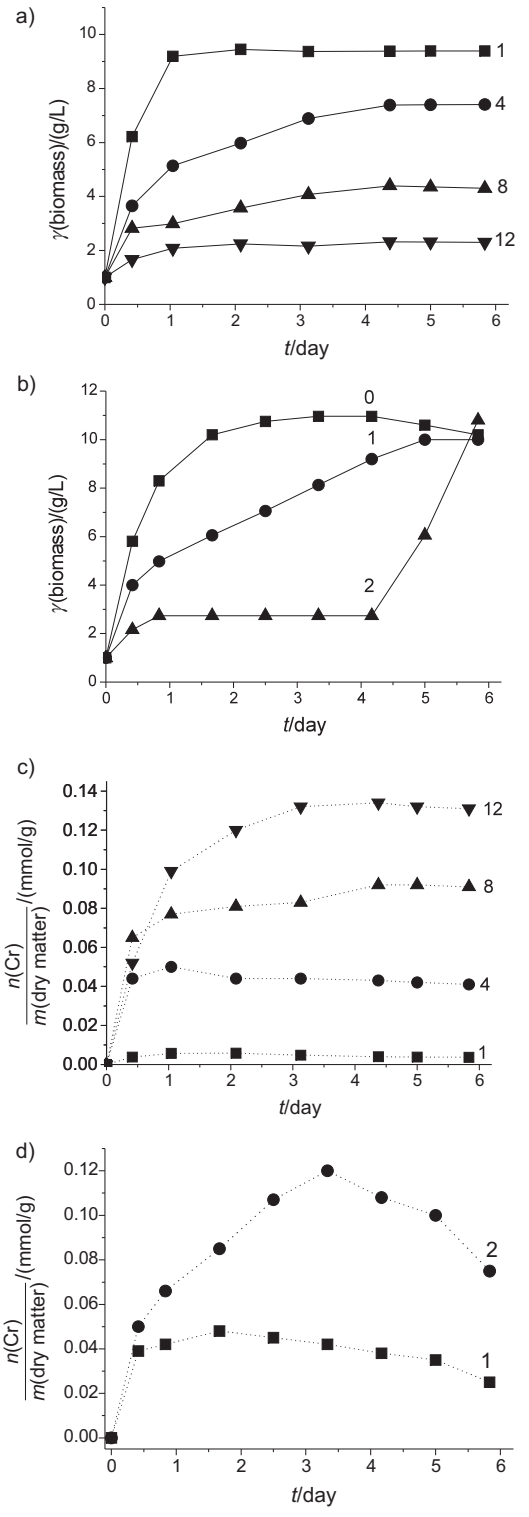

Fig. 1. The growth kinetics (a, b) and chromium accumulation (c, d) by *Pichia guilliermondii* L2 cells during incubation in sucrose-mineral medium supplemented with Cr(III) at 1, 4, 8 and 12 mM chromium (for a and c), and Cr(VI) at 0, 1 and 2 mM chromium (for b and d)

Restoration of the yeast growth after a protracted delay, observed for cultures with a higher chromate concentration, could suggest the appearance of the cells with a modified chromate resistance in the culture. Analysis of viability of the cells incubated for 3 days in the presence of 2 mM chromate showed that the number of living cells in the population decreased to 90 % of the initial count. However, the analysis of a random set of clones (50 representatives) which survived under the above mentioned cultivation conditions did not reveal any considerable change in sensitivity of the cells to chromate (the details of the test are described in section Materials and Methods).

The changes in chromium content in the medium and in the cells during incubation with different chromate concentrations were investigated. After incubation of the cells for 2 days in the presence of 0.5 mM chromate, no remaining Cr(VI) was detected in the culture liquid, and the cells accumulated only negligible quantities of chromium (Fig. 2). Moreover, only a very low concentration of the 'free' form of Cr(III) in extracellular medium was found, using the reaction with Chromazu-

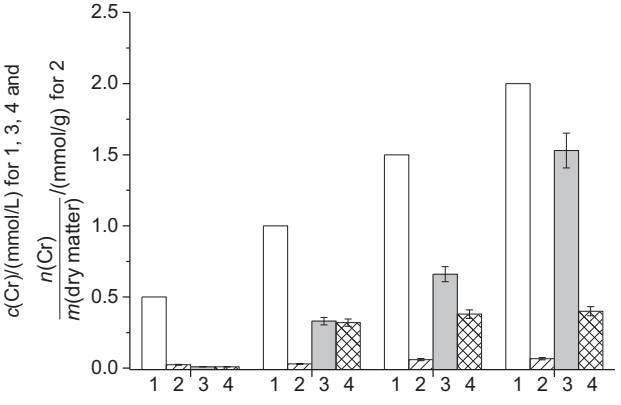

Fig. 2. Chromium content in the medium and in *P. guilliermondii* L2 cells after incubation of the cells (0.3 g/L) for 2 days in sucrose-mineral medium supplemented with different chromate concentrations (0.5, 1.0, 1.5 and 2.0 mM): 1 – initial chromate level in the medium; 2 – chromium content in the cells; 3 – residual chromate concentration in a medium; 4 – 'free' form of Cr(III) in the medium (measured without mineralization of the sample)

rol S without mineralization of the sample. At higher concentrations of the added chromate (1–2 mM), Cr(VI) still remained in the culture; however, the total content of chromium in the culture liquid (without mineralization) and in the mineralized cells at all tested chromate concentrations was lower compared to the initial chromate level in the medium: the lower the concentration of chromate, the more expressed negative balance in chromium content in the cultures was observed.

Since the decreased levels of Cr(III) compared to the expected ones without mineralization of an extracellular medium were found, it is important to check a possible negative effect of chromate on Cr(III) assay. As shown earlier (*30*), chromate anions do not interfere with the determination of Cr(III) using the reaction with Chromazurol S in molar ratio of Cr(VI)/Cr(III) at least 14:1. The modified method for Cr(III) assay, converting chromium into triple complexes with Chromazurol S and detergents (SDS and CTMAB) (*27*) was used, and in this case any negative effects of chromate on Cr(III) assay in the similar ratio of Cr(VI)/ Cr(III) were not found. On the contrary, an insignificant over-estimating effect of chromate on Cr(III) assay (less than 7–8 %) was observed, probably due to a slight reduction of chromate to Cr(III) during the heating procedure for conversion of Cr(III) into the coloured complex with Chromazurol reagent (data not shown). Therefore, the presence of chromate in the analyzed samples could not explain an apparent 'decreased' Cr(III) content in the culture during chromate reduction. Moreover, at zero concentration of the remaining chromate in the culture (see variant with 0.5 mM chromate in Fig. 2), the pool of Cr(III), available for reaction with Chromazurol S, is the lowest.

It could be speculated that Cr(III) generated in an extracellular medium can exist in two forms: chelated species (unavailable for reaction with Chromazurol S) and 'free' species, and both are much less toxic than initial chromate. This could result in the restoration of the cell growth following a period of its repression. It was also supposed that an observed difference in chromium accumulation by the cells at different chromate level could be related to the difference in the ratio of different Cr(III) species formed in the extracellular medium. Moreover, stable chelated Cr(III) species, found recently in the yeast cultures (*12*), could render chromium unavailable for the yeast cells.

To find out the reasons for atypical growth kinetics in the presence of chromate and a 'negative' balance between the added chromate and the chromium detected in the culture liquid and in the cells, the changes in Cr(VI) and Cr(III) concentration were analysed during incubation of *P. guilliermondii* cells at different concentrations (1 and 2 mM) of chromate (Figs. 3a and b). It was revealed that in the presence of 1 mM chromate in the medium, the decrease of the Cr(VI) concentration, due to its active reduction, coincided with the phase of the growth delay and resulted in a gradual accumulation of Cr(III) in the culture liquid. With increasing duration of incubation, a substantial drop in both Cr(VI) and 'free' Cr(III) concentration was observed, followed by the restoration of the growth when neither Cr(VI) nor 'free' Cr(III) were found in the medium. Taking into consideration that the amount of chromium in the cells was usually only 4–6 % of the initial chromate content, it can be concluded that a great part of extracellular Cr(III) can exist in strongly chelated form, unavailable for reaction with Chromazurol S (*12*). In the presence of 2 mM chromate, the growth of the yeast cells and the reduction of Cr(VI) were strongly decreased, obviously as a result of repression of metabolic processes by a higher level of toxic chromate.

The presented results suggest that the formed Cr(III) binds into complexes with extracellular metabolite(s) and becomes unavailable for the reaction with Chromazurol S. After acidic-oxidative mineralization of the culture liquid, taken on the third day of cultivation, 89–100 % of the initial amount of chromium, added to the medium as chromate, were detected.

It can be suggested that the formation of organo--chromium(III) complexes makes the chromium less available to the cells, which could result in the restoration of yeast growth. To verify this hypothesis, the influence of naturally chelated Cr(III) on the growth of *P. guilliermondii* cells (Fig. 4) was studied. A culture liquid was used as a source of chelated Cr(III) obtained from a 3-day-old

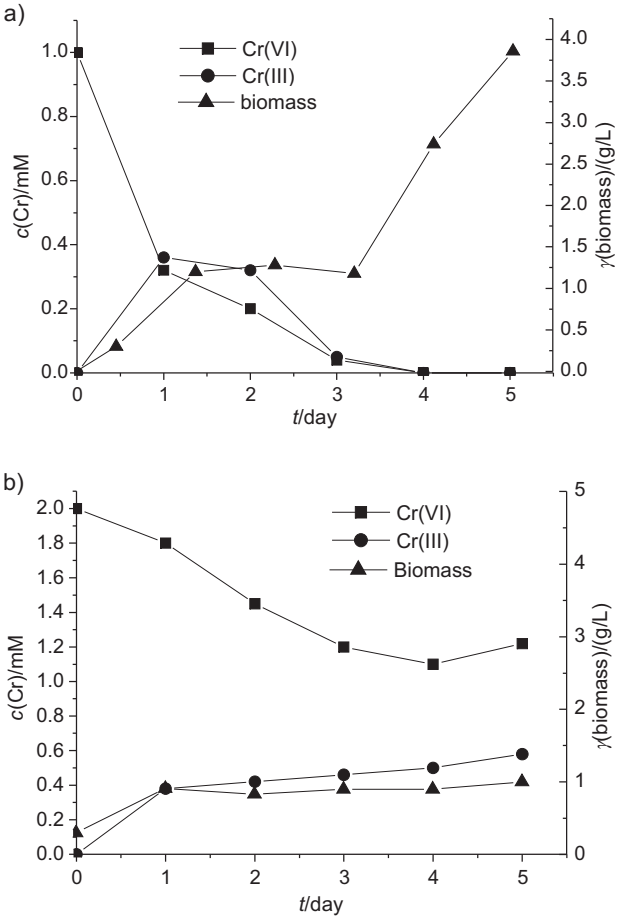

Fig. 3. Dynamics of the yeast growth and chromate reduction by *P. guilliermondii* L2 cells incubated with (a) 1 mM and (b) 2 mM chromate. Chromate reduction was characterized by measuring in the extracellular medium the residual concentration of chromate and the concentration of the 'free' form of Cr(III) available in the reaction with Chromazurol S

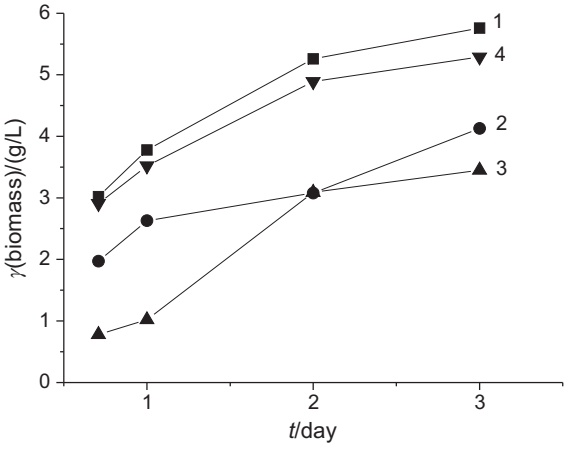

Fig. 4. Influence of different chromium species (0.5 mM) on the growth of *P. guilliermondii* L2 cells: 1 – control (without chromium), 2 – chromate, 3 – Cr(III) sulphate, 4 – culture liquid taken from 3-day-old culture with completely reduced 0.5 mM chromate (a source of Cr(III) biocomplexes)

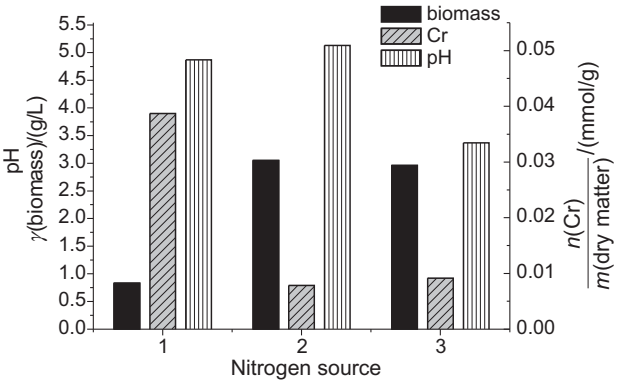

Fig. 5. Cell biomass, medium pH, and cellular chromium content after 1-day incubation of the yeast cells in sucrose medium supplemented with 0.5 mM chromate in the presence of urea or ammonium sulphate as nitrogen sources: 1 – urea; 2 – ammonium sulphate+urea; 3 – ammonium sulphate. Biomass in controls (without chromate; in g/L): 1 – 7.51; 2 – 6.82; 3 – 5.40

culture after complete reduction of 0.5 mM chromate. In control, the incubation of the cells was carried out in the presence of 0.5 mM Cr(III) or Cr(VI) added in the form of inorganic compounds (sulphate or potassium salts, respectively). It was observed that natural Cr(III) chelate(s) did not repress the growth of the yeast, and after 3 days of incubation the cells contained only a negligible amount of chromium (0.005 mmol/g), suggesting that the cells took up the formed Cr(III) biocomplexes very inefficiently. Thus, the extracellular reduction of chromate to a less toxic Cr(III), which is not able to enter the cell in the form of organo-complexes, can predetermine the resistance of the yeast cells to chromate and restoration of the growth after the lag phase. As it has been shown earlier (*12*), such complexes have a negative charge (they bind strongly with anion exchange resin), and this characteristic can explain the inability of the formed Cr(III) biocomplexes to be adsorbed by negatively charged cellular barrier of the yeast cell.

In a previous study (*31*) it has been demonstrated that sensitivity of yeast cells to chromate during incubation in a medium with urea is substantially higher than when using ammonium sulphate as a nitrogen source. Fig. 5 shows growth yield, medium pH value, and cellular chromium content during incubation of the yeast cells with 0.5 mM chromate in the medium, supplemented with urea or ammonium sulphate as the nitrogen source. In the presence of ammonium sulphate, the yeast growth was fully recommenced after 1 day of incubation, and neither Cr(VI) nor 'free' Cr(III) was found in the medium. It is not clear why the yeast cells cultivated on ammonium sulphate as a source of nitrogen are more resistant to chromate. Three factors could be considered for explanation of this phenomenon: (*i*) as a structural analogue of chromate, sulphate ion can competitively repress chromate transport into the cells, preventing its toxic effect on the cellular metabolism; (*ii*) different chemical composition of the medium can affect distribution of Cr(III) species formed in the extracellular environment; (*iii*) due to a strong acidification of the medium when ammonium is used as a source of nitrogen, more optimal conditions for chromate reduction and, possibly, for Cr(III) chelating are created, contrary to the urea medium. The first suggestion is based on the well known fact that sulphate and chromate are competitors for the same transport system. Based on this phenomenon, the positive selection procedure was developed for many microorganisms to isolate mutants with impaired sulphate transporters *(e.g. sul1*, *sul2*, and *sul3* mutants in the baker's yeast), collecting colonies resistant to chromate (or selenate) (*13*,*14*).

Dynamics of the yeast growth and chromate reduction by *P. guilliermondii* cells incubated with 0.5 mM chromate depending on a nitrogen source are presented in Figs. 6a and b, respectively. Chromate reduction was characterized by measuring the residual concentration of chromate in extracellular medium and the level of a 'free' Cr(III) available in the reaction with Chromazurol S. It is clearly seen that both chromate reduction and Cr(III) formation run more quickly on ammonium sulphate medium than in the presence of urea.

More detailed analysis of the influence of the source of nitrogen on chromate metabolism is presented in Table 1 and Fig. 5. Yeast cells were incubated for 1 day in the presence of 0.5 mM chromate in three types of medium containing: (*i*) urea, (*ii*) a mixture of urea and ammonium sulphate, and (*iii*) ammonium sulphate. In the presence of ammonium sulphate, cells were more resistant to chromate than in the presence of urea, and accumulated 3.6-fold less chromium in the cells. After 1 day of incubation in the presence of ammonium sulphate, chromate was completely reduced, while in the presence of urea it remained at 50 % of the initial level. In the presence of urea, 6–10 times higher levels of free Cr(III) were found, compared to ammonium sulphate medium. The assay of the balance of different chromium forms in the yeast culture allowed drawing the conclusion that in the presence of urea as a source of nitrogen not only the reduction of chromate, but also Cr(III) chelation is substantially lower, compared to ammonium sulphate medium.

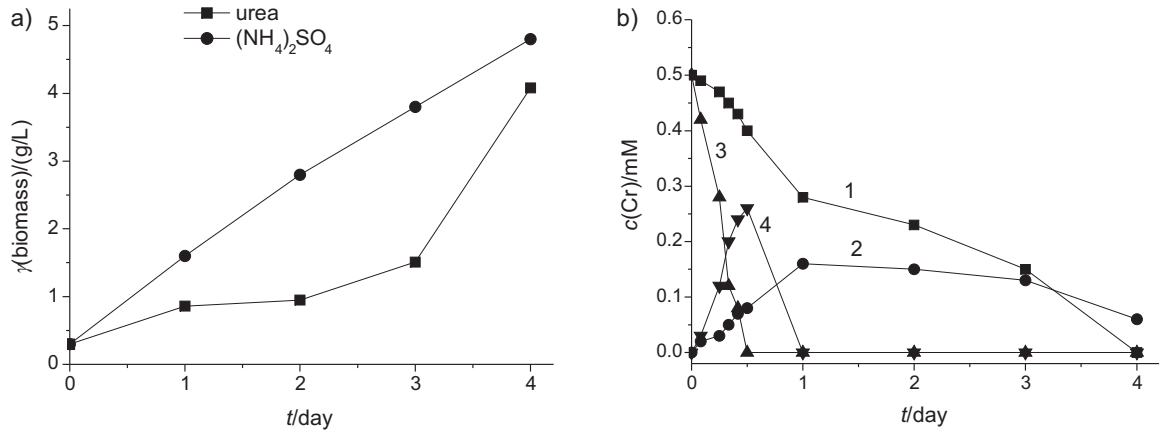

Fig. 6. Dynamics of (a) the yeast growth and (b) chromate reduction by *P. guilliermondii* L2 cells incubated with 0.5 mM chromate depending on a nitrogen source: 1 – residual Cr(VI), urea; 2 – formed 'free' Cr(III), urea; 3 – residual Cr(VI), ammonium sulphate; 4 – formed 'free' Cr(III), ammonium sulphate. Chromate reduction was characterized by measuring in the extracellular medium the residual concentration of chromate and the level of formed Cr(III) available in the reaction with Chromazurol S ('free' form)

Table 1. The reduction of chromate (0.5 mM) and Cr(III) chelation by the yeast *P. guilliermondii* grown for 1 day in the media supplemented with different nitrogen sources

Nitrogen source	*c*(Cr)/(mmol/L)				
	Measured chromium				Chelated Cr(III) in culture liquid***
	'Free' Cr(III)*	Remained Cr(VI)	Chromium accumulated by the cells**	After mineralization of the culture	
1	2	3	4	5	6
U	0.120±0.010	0.250±0.020	0.032±0.003	0.476±0.040	0.074
U+AS	0.201±0.015	0	0.024±0.002	0.501±0.041	0.276
AS	0.020±0.002	0	0.009±0.001	0.491±0.039	0.462

U – 17 mM urea; AS – 23 mM ammonium sulphate
*available in the reaction with Chromazurol S
**recalculated per cells in 1 L (c/(mmol/L) = $\frac{n(\mathrm{Cr})}{m(\text{dry matter})}$/(mmol/g)·$\gamma$(biomass)/(g/L))
***calculated as a difference between column 5 and the sum of columns 2, 3 and 4

Discussion

Depending on a dose, chromium is a useful microelement, but it can be toxic at higher concentrations. That is why there should be effective mechanisms controlling its entry into the cells, assimilation and storage, and preventing its hyperaccumulation. Identification of the molecular components of these mechanisms is necessary for understanding not only the physiological need for this element, but also for estimating its potential toxicity. Such information is also important for genetic engineering of organisms, capable of removing this toxic element from polluted environment or synthesizing Cr(III) biocomplexes with potential pharmacological activity.

The obtained results suggest that the extracellular reduction of chromate to Cr(III) is an important mechanism which restores the yeast growth after a prolonged lag phase. During this period, chromium assimilation processes are switched on to transform toxic chromium species into less dangerous ones. The reduction of Cr(VI) to the less toxic Cr(III) is the basic mechanism of the resistance to chromate in bacteria (*13*). The resistance to chromate in yeasts can be caused by deficient sulphate transport (*16*,*32*) and by impaired chromium uptake by the cells (*21*,*33*). The role of reduction in the resistance of the yeast cells to chromate is yet to be studied. However, the yeast strain of *Candida* sp. RR1, capable of growing at high concentrations of chromate (2 mM), was described. The chromate resistance of this strain was correlated with the ability to reduce Cr(VI) (*10*). It was shown that NAD-dependent chromate-reductase activity of chromate-resistant *Candida maltosa* strains was associated with the soluble protein fraction and to a lesser extent with the membrane fraction (*22*). Recently, the extracellular chromate reduction has been shown to be characteristic not only for *P. guilliermondii*, but also for *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (*12*). At the middle content of chromate in the medium, restoration of the growth after the protracted delay phase does not occur, and only a small part of chromium enters the cells (less than 7 %). At higher chromate concentrations, the lethal effect of chromate coincides with an inhibition of the cell redox activity as a result of cellular metabolism inhibition, in particular of energy supply. In favour

of this hypothesis, a substantial inhibition of chromate--reducing ability of the cells by sodium azide (a respiration inhibitor and a protonophore) was revealed (*12*).

A decline in Cr(VI) reduction as a result of Cr(III) accumulation in *Shewanella* cells was also described (*34*). However, in the presence of chelating agents, the level of reduction grew in proportion with the complexed Cr(III). It was discovered that Cr(III), generated during chromate reduction by *P. guilliermondii*, forms complexes with the extracellular metabolite(s). The chelation of Cr(III) increases the survival of the cells and strengthens the reduction of chromate. The formation of organo--Cr(III) complexes during chromate reduction was studied in detail during the investigation of the effect of chromium on human organism (*35*). The formation of soluble organo-Cr(III) complexes during the reduction of chromate by bacteria was also demonstrated, and it was shown that some microorganisms were able to destroy such complexes, which resulted in absorption of Cr(III) on the cell wall (*36*). Chelated Cr(III) species discovered in *P. guilliermondii* are not absorbed by the cells, which results in the restoration of growth after transforming toxic chromate into less toxic Cr(III) complexes. As shown previously (*12*), such complexes have a negative charge (they bind with anionites) and this finding explains the inability to facilitate an uptake of Cr(III) biocomplexes by negatively charged cellular barrier of the yeast.

Conclusions

Chromate-reducing capacity of the yeast cells depends on many factors, in particular on the source of nitrogen. The obtained results suggest that not only the chromate reduction, but also the chelation of the formed Cr(III) are inhibited in the medium with urea, compared to ammonium sulphate culture. The process of extracellular chromate reduction by the yeast cells and the nature of the formed organo-complexes require a more detailed study. This phenomenon could be useful for the development of microbial chromate reduction technologies and bioremediation processes. Evaluation of this problem would promote the application of the yeast cells for chromate bioremediation and its transformation to Cr(III) biocomplexes with potential pharmaceutical and nutritional importance.

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