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Reduction of chromate and carotene-synthesizing activity of selenite-resistant mutants of the yeast *Xanthophyllomyces dendrorhous (Phaffia rhodozyma)*

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Aim. *The yeast P. rhodozyma is a perspective microbial producer of carotenoid pigment astaxanthin with a high antioxidant power. The aim of the work was to study the ability of the selenite-resistant strains of this yeast to reduce chrome(VI) compounds, as well as to analyze the relations between synthesis of carotenoids, resistance to selenite and chromate-reducing activity of P. rhodozyma.* ***Methods***. *The yeast cells were grown at standard conditions for this species. The residual chromate content in cultural liquid was determined colorimetrically using diphenylcarbazide. The carotenoid content was determined after extraction of the pigments from the previously permeabilized cells by organic solvents.* ***Results***. *The selected selenite-resistant mutants of the yeast P. rhodozyma revealed the different combinations of the phenotypes related with tolerance/sensitivity to chromate and selenite, as well as ability to reduce chromate.* ***Conclusions***. *The obtained results give reasons for suggesting that pathways of detoxification of chromate and selenite by the yeast P. rhodozyma are different, although run through a common reductive type. The isolated mutant strains would be served as the useful models to study relations between homeostasis of Se and Cr oxyanions and biosynthesis of carotenes.*

Keywords: chromate, reduction, selenite, carotenes, Xantho- phyllomyces dendrorhous (Phaffia rhodozyma).

Introduction. Due to wide industrial application and distinguishing oxidizing abilities, chromium(VI) compounds are considered to be dangerous environment pollutants. Chromates (CrO_4^{2-}) and bichromates ($Cr_2O_7^{2-}$) are water soluble and capable of penetrating biological membranes. In microorganisms, in the yeasts in particular, this process is mediated by sulphate transporters, the products of *SUL1* and *SUL2* genes [1], which is the basis for selection of mutants

impaired in sulphate transportation related to chromate resistance [2]. After penetrating a cell, Cr(VI) compounds react with intercellular proteins, nucleic acids, and other cell components, causing mutagenic and carcinogenic effects [3, 4].

Living cells are capable of intracellular reduction of Cr(VI) to Cr(III). This process is related to detoxification, as it was shown that Cr(III) compounds are 100-fold less toxic for bacteria and fungi compared to Cr(VI) compounds. Intracellular reduction may take place via non-enzymatic and enzymatic pathways. Ascorbic acid, glutathione, and cysteine are efficient reducers of Cr(VI) to Cr(III) at physiological conditions. The mechanisms of enzymatic reduction of chromates are well studied in bacteria and may function both at aerobic and anaerobic conditions. For instance, Cr(VI)-resistant strains of *Enterobacter cloacae* reduce chromates in anaerobic conditions, using Cr(VI) as electron acceptors. In bacteria, causing aerobic chromate reduction (*Pseudomonas*, *Aeromonas, etc.*), this process is catalyzed by NADH-and NADP(H)-dependent reductases [5, 6].

As for eukaryotic microorganisms, yeasts in particular, it is yet to be determined which reduction system – either enzymatic/non-enzymatic one or intra-/extracellular one – plays a decisive role in chromate-detoxification processes. Besides, there are no data on the ability of biotechnologycally promising yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) to detoxicate and remediate chromates. Astaxanthin, the main carotenoid pigment, synthesized by the mentioned yeast, is notable for its powerful antioxidant activity that is 500 times higher than that of -tocopherol, which is the highest among all the known carotenoids [7–9]. Due to this fact the yeast *X. dendrorhous* is considered to be a promising source in production of pharmacological preparations for cancer prevention, improvement of immune response, and protection from the activity of free radicals.

At the same time, considerable differences in redox-potentials of astaxanthin (E^0 = 0.75 V) and chromate (E^0 = 1.33 V) [10, 11] allow the assumption on increased activity of carotene-synthesizing yeast *X. dendrorhous* related to chromate reduction. In order to increase reduction detoxification potential of astaxanthin-synthesizing yeast we performed a selection of mutants of the yeast *P. rhodozyma*, resistant to selenite [12].

The aim of this work was the investigation on reduction features of selenite-resistant strains of carotene-synthesizing yeast *P. rhodozyma* related to chromium(VI) compounds and the analysis of the interrelation between the level of carotenoid synthesis, resistance to selenite, and ability of chromium reduction.

Materials and Methods. The objects of our investigation were wild-type strains of *X. dendrorhous* (*P. rhodozyma*) NRRL Y-10921 from the collection of microorganisms of the Institute of Cell Biology, NAS of Ukraine, and mutants of *P. rhodozyma,* obtained by us, which demonstrated resistance to sodium selenite (*sit* strains).

The yeast was grown on rotor shaker at 250 rpm, t = 22°C, in 100 ml Erlenmeyer flasks, filled with 10 ml of the medium with the following composition (g/l): KH_2PO_4 – 1; $MgSO_4 \cdot 7H_2O$ – 0.5; $(NH_4)_2SO_4$ – 2; $CaCl_2$ x $2H_2O$ – 0.1; yeast extract– 2; sucrose – 20; biotin – $1 \cdot 10^{-6}$.

The selection of spontaneous *sit*-mutants of *X. dendrorhous* was performed collecting the yeast colonies, grown on agarose medium in the presence of 7.5 mM Na_2SeO_3.

The exponential growth cells (the first 24 hours) were used in experiments. A sterile solution of potassium chromate was added to cells' suspension with the concentration of 0.5 mg/ml with subsequent incubation at t = 22°C and aeration. The biomass was determined using optic density of cells' suspension at the wavelength of 540 nm with subsequent calculation into absolute dry biomass of cells in accordance to the calibration curve.

The resistance of mutant *P. rhodozyma* strains to sodium selenite was analyzed by applying cells' suspension (0.2 mg/ml) on agarose medium with the range of selenite concentration from 1 to 30 mM with the subsequent study of growth kinetics in 2 days growing at t = 22°C.

The content of carotenoids was determined after permeabilization of yeast cells by dimethyl sulphoxide with subsequent extraction of carotenoids into hexane-ethyl acetate mixtures [13]. During the incubation of yeast cells with chromate, the concentration of residual chromate in the cultural liquid was determined by

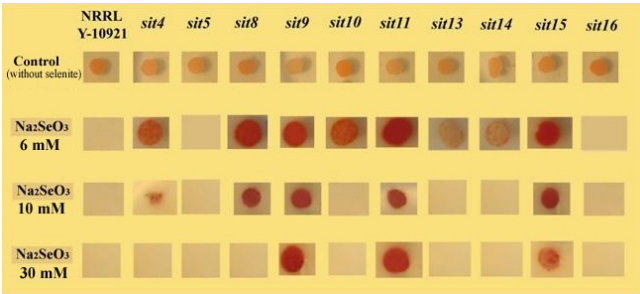

Fig. 1 Test for resistance of the cells of wild-type strain of *P. rhodozyma* NRRL Y-10921 and mutant *sit*-strains to sodium selenite

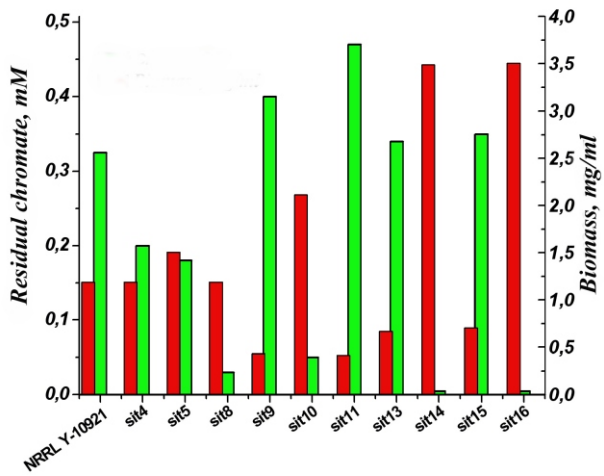

Fig. 2 Growth and residual content of chromate in extracellular liquid of the yeast *P. rhodozyma* on the 4th day of incubation of the cells with 0.9 mM chromate: redcolumns – biomass; green columns – Cr(VI).

means of colorimetric diphenylcarbaside method [6]. All the experiments were repeated three times.

Results and Discussion. There are numerous literature data on the ability of sulphate-reducing cells of microorganisms to reduce Cr(VI) [14]. As selenate and sulphate are of the same reduction system, characterized by mutual competitiveness [15, 16], one may extend this analogy and assume that compounds of chromium and selenium may also have common enzymatic mechanisms of reduction. Fig.1 demonstrates the results of testing initial and derivative *sit*-mutants of *X. dendrorhous*, selected via positive selection in the medium with 7.5 mM of potassium selenite, concerning their resistance to selenite.

Isolated mutant strains of *P. rhodozyma* showed different degrees of resistance to this toxic factor. Wild-type strain of *P. rhodozyma* NRRL Y-10921 did not grow at 6 mM Na_2SeO_3, while six *sit*-strains out of ten were actively growing at this concentration of sodium selenite. Even in case of five-fold increase in Na_2SeO_3 concentration in agarose medium (30 mM), there still were three strains – *sit9*, *sit11*, and *sit15*, the growth of which was steady; *sit9* and *sit11* showed the highest resistance to selenite.

The ability of selenite-resistant strains of carotene-synthesizing yeast *X. dendrorhous* to reduce chromium(VI) compounds was studied via monitoring of growth and content of residual chromate in the cultural liquid for 6 days of yeast incubation (initial concentration – 0.5 mg/ml) with 0.9 mM of chromate. Fig.2 demonstrates the results of the experiment on the 4th day of yeast incubation.

It was revealed that isolated mutants with different level of resistance to selenite demonstrate different tolerance to chromate, both in the ability to grow in the presence of chromate and in the level of its reduction. For instance, two selenite resistant strains *sit14* and *sit16* reduced all the chromate and had the highest biomass increase – 5.40 and 5.36 mg/ml, respectively. The strains with remaining 3-22% chromate in their culture (*sit4*, *sit5*, *sit8*) were characterized by more intensive growth compared to strains, reducing about a half of added chromate (*sit9*, *sit11*, *sit13*, *sit15*). The correlation analysis of growth activity data and values of residual content of chromate for tested strains revealed clear negative correlation between these parameters (R = –0.825). Thus, the strains, reducing chromate the most efficiently (lower level of residual Cr(VI) in the cultural liquid) usually grow better in the presence of Cr(VI).

The study on carotene-synthesizing activity of investigated *P. rhodozyma* strains revealed inhibition of this process in the presence of high concentrations of chromate in the incubation mixture. The cells did not have any colour until chromate concentration in the medium decreased to app. 0.2 mM, which caused active growth of yeast and synthesis of carotenoids. Therefore, chromate inhibits the process of carotenoid synthesis along with growth inhibition.

Fig.3 demonstrates the growth kinetics and process of chromate reduction for some *sit*-mutants of the yeast. Strains *sit15* and *sit16* showed the highest activity in their growth and chromate reduction, besides, chromate disappeared from the cultural liquid sooner – in 4 days of incubation, while concentration of chromate on the

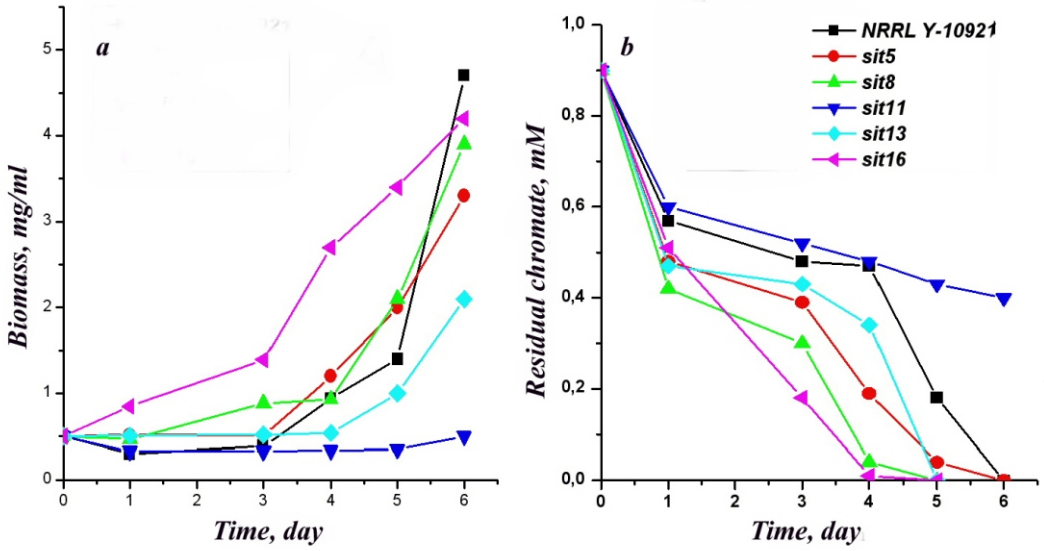

Fig. 3 Growth kinetics (*a*) and chromium reduction (*b*) for wild-type strain of the yeast *P. rhodozyma* NRRL Y-10921 and mutants, resistant to selenite.

6th days of incubation for strains *sit9* and *sit11* was decreased only by half and amounted to 0.4–0.5 mM at the end of experiment. Strains *sit9* and *sit11* were also notable for the slowest growth rate. Growth intensification of the rest of mutants coincided with chromate decrease in the cultural liquid; strains *sit8, sit13* and *sit5,* wild-type strain reduced all the chromate on the 5th and 6th days of experiment, respectively.

Therefore, the analysis of ability to reduce chromate of mutant strains with the highest resistance to selenite (*sit9*, *sit11*, *sit15*) showed that chromate in the concentration of 0.9 mM inhibits their growth considerably; at the same time they reduce it much worse compared to strains *sit14* and *sit16*, resistance to selenite of which is less expressed. It may testify to a difference in reduction pathways of these two anions. Our previous works on the model of flavinogenous yeast *Pichia guilliermondii* [17] demonstrated that mutants, resistant to selenite, are characterized by the ability of accumulating elementary selenium (Se^0) in cells, while reduction detoxification of chromate in this yeast results in accumulation of Cr(III) biocomplexes in the cultural liquid [6, 18].

Thus, different nature of end products of reducing toxic oxianions of chromium and selenium and differences in their localization may result in ambiguous interrelation between resistance phenotypes to selenite and chromate. It is also possible that increased ability to reductive detoxification of selenite in selenite-resistant strains of *X. dendrorhous* results in the increase in the pool of a very toxic radical Cr(V) – the intermediate of Cr(VI) reduction. We intend to verify this hypothesis via monitoring of Cr(V) generation in the cultures of *sit*-mutants using EPR-spectroscopy, as we have done before for chromate-resistant strains of flavinogenous yeast *P. guilliermondii* [20, 21].

We cultivated yeast cells for 4 days and on obtaining the maximal level of carotenoid synthesis [19] we determined total content of carotenoids and biomass increase for wild-type strains and mutants (Fig.4).

The majority of selenite-resistant strains synthesized less carotenoids than the initial strain, which produced 327 μg/g of dry biomass, and the coefficient of decrease in carotene-synthesizing activity for mutants was fluctuating from 1.1 to 2.5. Only strain *sit16* produced carotenoids on the level, similar to that of wild-type strain. The analysis of interrelation between resistance to sodium selenite and the content of carotenoids, produced by the tested strains, revealed that mutants *sit9*, *sit11*, *sit15* are the most resistant to sodium selenite and they synthesize approximately twice less carotenoids compared to the wild-type strain. This fact may testify to the absence of direct correlation between the level of carotenogenesis and ability to selenite detoxification.

We have analyzed the resistance of obtained *sit*-mutants of the yeast *P. rhodozyma* to selenite (Fig.1), compared obtained results to the data on their

Phenotype parameters of resistance of P. rhodozyma sit-mutants to selenite and chromate

Phenotype group	Chromate	Selenite	*P. rhodozyma* strains
I	Resistant	Resistant	*sit8, sit10*
II	Resistant	Level of control	*sit14, sit16*
III	Sensitive	Over-resistant	*sit9, sit11, sit15*
IV	Sensitive	Level of control	*sit4, sit5, sit13*

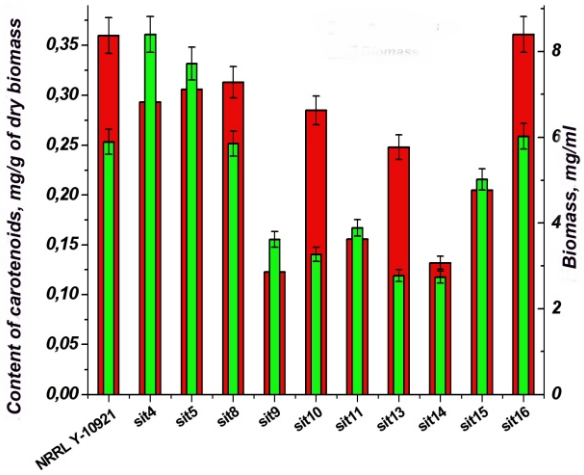

Fig. 4 Growth and synthesis of carotenoids for wild-type strain of *P. rhodozyma* NRRL Y-10921 and mutants, resistant to sodium selenite, in the medium without the addition of the latter: red columns – carotenoids; green columns – biomass

ability to reduce chromate (Fig.2), and divided all the investigated strains into three groups according to phenotype characteristics (Table).

It turned out that there are practically all the combinations of phenotypes related to resistance/sensitivity to chromate and selenite which testifies to the absence of definite dependence between resistance to oxianions of chromium and selenium.

Conclusions. The results obtained allow the assumption that the pathways of chromate and selenite detoxification in the yeast *P. rhodozyma* are different though these processes take place according to a common reductive type. The absence of definite dependence between the resistance to chromate and selenite and the ability of the yeast to synthesize carotenoids testifies to the necessity of searching for biochemical and genetic mechanisms, responsible for these processes. Obtained mutants with different combination of phenotypes of resistance to selenium and chromium oxianions will serve a convenient model in the study on interrelation between homeostasis of the mentioned elements and biosynthesis of carotenoids with a high antioxidant activity.

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Редукція хромату та каротиносинтезувальна активність резистентних до селеніту мутантів дріжджів *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*)

Резюме

Мета. *Дріжджі P. rhodozyma є перспективними біопродуцентами, оскільки синтезують каротиноїдний пігмент астаксантин з високою антиоксидантною активністю. Мета роботи полягала у вивченні здатності резистентних до селеніту штамів редукувати сполуки хрому(VI) і в аналізі взаємозв'язку між рівнем синтезу каротиноїдів, стійкістю до селеніту та редукуючими властивостями щодо хромату.* ***Методи****. Дріжджі вирощували за стандартних для даного виду умов. Вміст залишкового хромату в культуральній рідині визначали колориметрично дифенілкарбазидним методом. Кількість каротиноїдів виявляли екстракцією пігментів органічними розчинниками із попередньо пермеабілізованих клітин.* ***Результати****. Виділені селеніт-резистентні мутанти дріжджів P. rhodozyma проявили різні комбінації фенотипів за стійкістю/чутливістю до хромату і селеніту та здатністю до редукції хромату.* ***Висновки****. Одержані результати дають підставу припустити, що шляхи детоксикації хромату і селеніту у дріжджів P. rhodozyma є різними, хоча і здійснюються за однаковим редукційним типом. Мутантні штами можуть стати зручною моделлю для вивчення взаємозв'язку між гомеостазом оксіанінів селену і хрому та біосинтезом каротиноїдів.*

Ключові слова: хромат, редукція, селеніт, каротиноїди, Xanthophyllomyces dendrorhous (Phaffia rhodozyma).

Г. И. Нечай, Г. П. Кшеминская, А. В. Колесник, М. Гжондка, М. В. Гончар

Редукция хромата и каротинсинтезирующая активность резистентных к селениту мутантов дрожжей *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*)

Резюме

Введение. *Дрожжи P. rhodozyma – перспективный биотехнологический продуцент, синтезирующий каротиноидный пигмент астаксантин с высоким антиоксидантным потенциалом. Цель работы состояла в изучении способности резистентных к селениту штаммов редуцировать соединения хрома (VI) и в анализе взаимосвязи между уровнем синтеза каротиноидов, стойкостью к селениту и редуцирующими свойствами по отношению к хромату.* ***Методы.*** *Клетки дрожжей выращивали в стандартних для этого вида условиях. Остаточное содержание хромата в культуральной жидкости определяли колориметрически, используя дифенилкарбазид. Содержание каротиноидов определяли экстракцией пигментов смесью органических растворителей из предварительно пермеабилизованных клеток.* ***Результаты.*** *Выделенные селенит-резистентные мутанты дрожжей P. rhodozyma проявили различные комбинации фенотипов стойкости/чувствительности к хромату и селениту, а также способности к редукции хромата.* ***Выводы.*** *Полученные результаты дают основание полагать, что пути детоксикации хромата и селенита у дрожжей P. rhodozyma разные, хотя осуществляются по общему редукционному типу. Мутантне штаммы могут стать удобной моделью для изучения взаимосвязи между гомеостазом оксианионов селена и хрома и биосинтезом каротиноидов.*

Ключевые слова: хромат, редукция, селенит, каротиноиды, Xanthophyllomyces dendrorhous (Phaffia rhodozyma).

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