The kinetic reduction of Cr(VI) by yeast Saccharomyces cerevisiae, Phaffia rhodozyma and their protoplasts*

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Chromium in the sixth oxidation state may easily penetrate cellular membranes via non-specific sulfate transporters due to its tetrahedral symmetry (high similarity to SO$_4^{2-}$ and HPO$_4^{2-}$). This feature makes chromium a toxic and hazardous pollutant responsible for the deterioration of midland water quality. The aim of the study was to evaluate the capacity of two yeast species — *Saccharomyces cerevisiae* and *Phaffia rhodozyma* — and their protoplasts to reduce Cr(VI) to lower oxidation states. The study also deals with the behavior of the yeasts upon the presence of elevated sulfate ions as a competitive inhibitor of chromate transport by the sulfate transporters. The chrome-reducing activities were monitored by determination of Cr(V) free radical form with the use of L-band (1.2 GHz) EPR (electron paramagnetic resonance) spectroscopy. It was observed that both of the studied yeast strains exhibited the ability to reduce Cr(VI) applied at 4 mM. The cells of the studied yeast strains exhibited the ability to reduce resonant) spectroscopy. It was observed that both of the use of L-band (1.2 GHz) EPR (electron paramagnetic resonance) spectroscopy. It was observed that both of the studied yeast strains exhibited the ability to reduce Cr(VI) applied at 4 mM. The cells of *P. rhodozyma* showed about 3.5 times higher reduction than *S. cerevisiae*. The reduction efficiency was significantly improved when the protoplasts of both strains were used and reached 100% in the first 10 minutes of the reduction process which suggests that the cellular wall may have a notable influence on the uptake and/or inhibition of chromium reduction process. The reduction effect of *P. rhodozyma* cells and protoplasts may be associated with the more sufficient production of metabolites (such as glutathione and cysteine), which may also be responsible for the increased tolerance of the strain towards high concentrations of toxic chromium.

**Key words:** chromium, *Phaffia rhodozyma*, pollution, *Saccharomyces cerevisiae*

**Received:** 17 October, 2013; revised: 04 December, 2013; accepted: 04 December, 2013; available on-line: 29 December, 2013

**INTRODUCTION**

Contamination with heavy metals is a serious environmental problem. Pollution with chromium originating from several industrial processes such as leather tanning, electroplating, textile industries, nuclear power plant, water cooling, pulp production, ore and petroleum refining processes are the reason for deterioration of midland water quality. In trace amounts, being beneficial to living organisms, chromium serves as an essential element for glucose and fat metabolism as well as for the stabilization of the tertiary structure of proteins and nucleic acids (Pas et al., 2004). However, at higher concentrations it is extremely toxic, causing allergies, eczema, and respiratory tract disorders. It is also a strongly mutagenic and carcinogenic agent, especially in its oxidized form, Cr(VI) (Barceloux, 1999). Once inside the cell Cr(VI) is being reduced to the Cr(V) and can react with H$_2$O$_2$, which leads to the production of hydroxyl radicals (•OH) via the Fenton-like reaction. Cr(V) causes DNA breaks and various mutations in chromosomes.

Due to high toxicity, chromium is regarded as a priority pollutant by the US EPA. The adverse health effects and diverse cellular and molecular reactions make the studies on chromium toxicology and metabolism very crucial in terms of environmental protection and clinical medicine. Such studies are performed using eukaryotic organisms, mainly yeast, plants, mammalian cells and transgenic mice (Hedlam & Lay, 2001; Cervantes et al., 2001; Abbas et al., 2011). Among these, yeast has proved to be a very suitable model for the research of eukaryotic cell response to chromium stress and Cr bioremediation pathways (Kshepinska et al., 2005; 2010). Chromium, in the sixth oxidation state, can easily enter into the living cells because Cr(VI) ion has tetrahedral symmetry (same as SO$_4^{2-}$ and HPO$_4^{2-}$, unlike Cr(III) which has octahedral symmetry) and can get into the cell membrane through non-specific sulfate transporters by facilitated diffusion (Pereira et al., 2008). The competition between chromate and sulfate for the transporter has been reported by several authors, both in Procariota and Eucariota (Yoshimoto et al., 2002; Wysocki & Tamas, 2010). In *Saccharomyces cerevisiae* sulfate is transported into yeast cells via the system involving two permeases, one with a high and the other with a low sulfate affinity (Cherest, 1997).

Then water-soluble Cr(VI) compounds are reduced to various unstable reactive intermediates, like Cr(V) and/or Cr(IV) and reach the stable and less toxic Cr (III), which can be easily removed by precipitation (Cieslak-Golonka, 1996). The gradient of Cr(VI) between the two sides of the cell membrane is maintained metabolically and it is continuously reduced by both enzymatic and non-enzymatic pathways (Jannik & Raspor, 2003). It is unclear whether enzymatic/non-enzymatic and both intra — and extracellular reductions of Cr(VI) play a decisive role of Cr(VI) detoxification in eukaryotic microorganisms. Ascorbic acid, glutathione, cysteine, methionine are efficient non-enzymatic reducers of Cr(VI) to Cr(III) at physiological conditions of living cells (Smutok et al., 2011). The enzymatic mechanism of the Cr(VI) reduction is more complicated as the chromate causes a strong decrease in sulfur assimilation and induction of genes and enzymes of the sulfur amino acid pathways (Pereira, 2008) as well as stress induced proteins.

**Abbreviations:** Cr, Chromium; *P. rhodozyma*, *Phaffia rhodozyma*; *S. cerevisiae*, *Saccharomyces cerevisiae*
The viability study of *S. cerevisiae* and *P. rhodozyma* were collected in the lag phase of growth, centrifuged and then the supernatant, free of yeast cells was taken to the reduction process measurements.

**Determination of the minimum content of sulfate ions necessary for the growth of the yeast.** Free of sulfate medium (modified composition of Bushnell-Haas medium) was tested in the yeast viability experiments. In order to find minimal concentration of sulfate necessary for cell growth the Bushnell-Haas mediums with varying sulfate concentrations were tested. It was found out that the minimal sulfate concentrations in which yeast cells avoid sulfur starvation was approximately 100 μM, which corresponds to the literature (Pereira *et al.*, 2008).

**Protoplast isolation procedure.** The protoplasts were isolated using the method developed and optimized by the authors with the use of the Zymolyase enzyme produced by a culture of *Arthrobacter lutescens*.

Yeast cells were incubated until the log phase of growth was reached (24 h for *S. cerevisiae* at 30°C and 48 h, 25°C for *P. rhodozyma*, respectively). Next the cells were washed 3 times with distilled water and the sediment was suspended in the 4 ml of buffer pH 7.5 with addition of 0.4 mg Zymolyase 20T and 50 mM DTT (dithiothreitol). Cells were incubated for 1h in 30°C temperature on a rotary shaker. After the incubation, 6 ml of buffer (pH 7.5) was added to the mixture of protoplasts and fragments of the cell wall. Then the mixture was gently centrifuged for 5 min, 400 G. Proto protoplasts were irrigated twice in 10ml of buffer (pH 7.5) by gentle centrifugation (400G, 5 min).

**EPR — L band chromium reduction measurements.** Reduction process was carried out in 120 minutes with the final concentration of Cr(VI) equal to

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The kinetic reduction of Cr(VI) by yeast Saccharomyces cerevisiae and Phaffia rhodozyma

4 mM in each probe. The scan of the spectra was recorded 5 times for each measurement.

The kinetics of chromate reduction were monitored by means of Cr(V) free radical form determination using the L-band (1.2 GHz) EPR (electron paramagnetic resonance) spectrometer. The spectrometer was equipped with a microwave bridge with the operating frequency of 1.2 GHz and an extended surface coil-type high frequency resonator having an internal diameter of 16 mm. The following settings of the spectrometer were typically used: 40 mW maximum microwave power, 27 kHz field modulation frequency, 40 G magnetic field scan. The 5 scans of EPR signal were averaged. The typical EPR-L band spectra of Cr (V) is shown in Fig 1. Every EPR signal was standardized to free-radical probe (TEMPO) at constant concentration. The spectrum of the probe has been taken at the beginning of each measurement. The procedure allows to compare amplitudes of all samples signals.

RESULTS

The reduction of Cr(VI) by the cellular reducing system of living organisms generates paramagnetic long-lived reactive intermediate, Cr(V). The data given in Fig. 2 show the formation of Cr(V) during the reduction process conducted by S. cerevisiae and P. rhodozyma which is much faster in the case of Phaffia rhodozyma. The initial slopes of the curves illustrating Cr(V) formation reaction in the time-depending mode suggest that the reduction process is 2.5 times faster in the case of Phaffia rhodozyma compared to the Saccharomyces cerevisiae cells.

This result was expected because Phaffia rhodozyma is far more resistant to Cr(VI) than Saccharomyces cerevisiae. The conclusion also can be drawn out from the viability experiments — data shown in Table 1 and other literature (Horvath et al., 2011; Smutok et al., 2008). It was interesting to verify whether the faster reduction of Cr(VI) by Phaffia rhodozyma is connected with chemical groups of cell wall or and low molecular mass reductants secreted to the extra-cellular space by the yeast cells. The other explanation is the effective penetration of chromate into the cells and subsequently enzymatic and non-enzymatic reduction. In order to check occurrence of above mentioned phenomena the reduction of Cr(VI) was done with protoplasts (cells without the cell wall) of both strains. The data is shown in Fig. 3.

As can be seen in Fig. 3 the rate of chromium reduction is very fast and there is almost no difference between the protoplast of both strains. It means that the lack of significant differences in fast reduction of the metal by the P. rhodozyma and S. cerevisiae cells is not related with intra-cellular both enzymatic and non-enzymatic processes. The differences is explained by the data given in Fig. 4, where the reduction Cr(VI) by the free-cell medium is shown.

In the case of P. rhodozyma free-cell medium treated with 4 mM of Cr(VI) the reaction of Cr(V) formation reaches the maximum after 30 min while in the medium of S. cerevisiae, the reduction process is running during whole time of the experiment. The gradual decrease in the amplitude signals was observed after 180 min in the case of S. cerevisiae (data not shown). These results suggest that the P. rhodozyma medium culture, after rejection of the intact cells, was more active in terms of Cr(VI) reduction. The difference in such activity is about 70 times bigger in the case of free-medium of P. rhodozyma than S. cerevisiae, which can be judged from the initial slope of the curves presented in Fig. 4.

Such difference can be caused by metabolic mechanisms responsible for secretion of the reductants to the extra-cellular space in P. rhodozyma cells. Some metabolic pathways of the reductant synthesis can be triggered by
chromium (Jamnik et al., 2003). If so, it can be verified with the use of sulfate (added before chromium) which blocks the Cr(VI) transporters. The 30 mM and 100 mM sulfate concentrations were chosen based on the experiments of (Pepi, Baldi 1992). The data of the Cr reduction in the presence of sulfate in the media were shown in Fig. 5 and 6.

The presented data was normalized to the signal of paramagnetic spin probe, and it is possible to compare sample values of EPR signals coming from different set of measurements. As can be seen in Fig. 5 the formation of free-radical Cr(V) by the S. cerevisiae cells in the presence of 30 mM sulfate ions is enhanced about 2 times, compared to the sample in the absence of sulfate (Fig. 2). Analyzing the time-dependent formation of Cr(V) with addition of sulfate and without sulfate (Fig. 2) one can observe that after 30 min the reduction of Cr(VI) by S. cerevisiae cells achieve a plateau. While in the presence of 30 mM sulfate after 35 min the formation of Cr(V) reaches the maximum and after that the signal decreases (Fig. 5). It is worth noticing that in the presence of 30 mM of sulfate the EPR amplitude is significantly higher than in the case of sulfate absence and it can suggest that sulfate induces the production of chromium reductants. The 100 mM concentration of the sulfate inhibits the reduction process about 2 times in the case of Saccharomyces cerevisiae (Fig. 2, 5).

DISCUSSION

The reduction of chromate by living organisms has been demonstrated by various methods in animal system (Appenroth et al., 1996), bacteria (Shi et al., 1994) and plant (Kaszycki et al., 2005). The EPR-L band method was used successfully to monitor the Cr (VI) reduction processes. The usage of the L-band techniques has several advantages, mainly the formation of paramagnetic species can be measured continuously in vivo with minimal disturbance of the living systems. Samples placed into surface coil resonator are not disturbed and there is no need to cut or disintegrate them as required in other method, e.g X-band spectroscopy. With the EPR L-band spectroscopy direct kinetic studies can be carried out in whole cells under physiological condition. Using this method the chromate reduction by S. cerevisiae and P. rhodozyma was undertaken.

In the study those yeast strains were chosen because one of them, P. rhodozyma is highly resistant to the toxic chromate, much more resistant than moderate S. cerevisiae. Their response to sub-lethal concentration of the metal might reveal some differences in the reduction mechanism.

The data presented in Fig. 2, Tables 1 and 2 confirm that the P. rhodozyma is much more resistant to chromium than S. cerevisiae (Nechay et al., 2009). Based on the results shown in Figs. 3 and 4 it can be suggested that the higher resistance of P. rhodozyma to toxic chromate is related to the extra-cellular reductants. The dominant role of the extra-cellular reductants of Cr(VI) was suggested by the Gonchar group (Ksheimenska et al., 2006) and the suggestion was based on “disappearance” of Cr(VI) which resulted in the detection of Cr(III) complexes in extra-cellular space of some yeast species. The data shown in Fig. 4 for the first time illustrates direct role of extra-cellular compounds secreted by the yeast strains in terms of free-radical scavenging. The reduction capacity of
**CONCLUSION**

Protoplasts of both strains showed coupled times faster reduction of chromium compared to intact cells which suggests the high influence of the cell wall on uptake into the cell and the reduction of Cr(VI). What is more **P. rhodozyma** has demonstrated greater Cr(VI) reduction than *S. cerevisiae* both in intact cells and its isolated protoplasts what could be connected with more sufficient production of metabolites like glutathione and cysteine which also can be a factor responsible for its resistance for high concentrations of toxic chromium.

**Acknowledgements**

This work was supported by grant Ministry of Science and Higher Education, Poland, No. NN304326136.

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