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APPLICATION OF NAA METHOD TO STUDY CHROMIUM UPTAKE BY *ARTHROBACTER OXYDANS*

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INTRODUCTION

Environmental pollution by chromium is widespread in soils, sediments and groundwaters.[1] Being a transition element, it occurs in a number of oxidation states from Cr(II) to Cr(VI). However, only two stable, trivalent and hexavalent, species are significant in environmental systems. Hexavalent chromium compounds are highly soluble and toxic.[2] Epidemiological, animal and cellular studies have also established that Cr(VI) compounds are carcinogenic.[3] In contrast, most Cr(III) compounds are less soluble and less toxic. Moreover, trace amounts of Cr(III) appear to be essential for animal and human glucose and lipid metabolism.[2] The biological effect of Cr(VI) is generally attributed to cellular uptake, because Cr(VI), unlike Cr(III), is easily uptaken by cells via SO_4^{2-} and HPO_4^{2-} channels.[4] The toxicity of Cr(VI) compounds is ascribed to reactive intermediates (such as Cr(V), Cr(IV), radicals) generated during their reduction by living cells. Relatively, long-lived Cr(V) intermediates have been detected in the reduction of Cr(VI) both *in vitro* and *in vivo*.[4,5]

Toxic chromium compounds can be removed by chemical reduction, which is both difficult and expensive. So, the possibility of using indigenous microorganisms to transform the toxic heavy metal Cr(VI) into the Cr(III) state has stimulated intense interest in the study of metal-organic reactions.[6-8]

More recently, synchrotron radiation-based (SR) Fourier-transform infrared (FTIR) spectromicroscopy was applied to study chromate biotransformation on mineral surfaces.[9,10] *Arthrobacter oxydans*, the Cr(VI) reducing bacteria, isolated from Columbia basalt rocks, USA, was used as a model Cr(VI)-tolerant and reducing bacteria in these experiments. The study of the mechanisms of the Cr(VI) reducing ability of *A. oxydans* will continue in future experiments using biochemical and spectrometrical methods.[11,12]

The purpose of this study is (i) to study Cr(VI) and Cr(III) uptake by *A. oxydans* by means of instrumental epithermal neutron activation analysis (ENAA); (ii) to compare Cr(VI) accumulation and Cr(V) formation processes in *A. oxydans*; (iii) to determine the baseline chemical composition of *A. oxydans* using ENAA. ENAA was carried out at the IBR-2 pulsed fast reactor in FLNP, JINR, Dubna, which is characterised by a very high ratio of epithermal to thermal neutrons.

EXPERIMENTAL

Sample preparation

Chemicals. All experimental chemicals were ACS-reagent grade and purchased from Sigma (St. Louis, MO, USA).

Sample. Arthrobacter species are the member of the high mol % G+C actinomycete-coryneform bacteria.[13] *Arthrobacter oxydans*, isolated from Columbia basalt rocks collected from 75 meters below the ground surface of the Eastern Snake River Plain in USA were identified as gram- positive Cr(VI) reducer bacteria by Holman et al.[9] The life cycle of *A. oxydans* is characterized that their cells change from rod to cocci (almost spherical form), *i.e.*, in the exponential phase of growth *A. oxydans* cells are rods that change in size and shape.[13,14] In the course of exponential growth the rods get shorter and are eventually transformed into coccoid forms characteristic of a stationary phase structure.

The bacteria *A. oxydans* was kept in the nutrient medium recommended for *Arthrobacter* species[15]: 2 g of K_2HPO_4, 0.01 g of $FeSO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1 g of $C_6H_{11}O_7N$, 1 g of glucose, 1 g of yeast extract and 1 liter of distilled water. *A. oxydans* cells were grown in 250 ml Elenmayer flasks as a suspension. The medium was inoculated with 0.1 ml of overnight broth and incubated at 21 °C being shaked continuously.

To perform Cr(VI) and Cr(III) accumulation tests by ENAA, Cr(VI) [as K_2CrO_4] or Cr(III) [as $Cr(CH_3COOH)_3$] was added to the nutrient medium to provide the chromium concentration within a range of 10-200 mg/l. Both Cr(VI) and Cr(III) were added to the nutrient medium in the exponential phase of growth. After being cultivated for 5 days the cells were harvested by centrifugation (10,000 rpm, 15 min, 4 °C), rinsed twice in a 20 mM phosphate buffer. This wet biomass was then placed in an adsorption-condensation lyophilizer and dried following the procedure reported in.[16] The dry native biomass was finally pelletized to 5 mm pieces using a special titanium press form.

To conduct a Cr(V) accumulation test by ESR method *A. oxydans* was cultivated in the same nutrient medium. In the exponential phase of growth two sets of experiments were performed: (1) *A. oxydans* cells were separated from the nutrient medium (by centrifugation), they were then added with chromate solution to have the Cr(VI) concentration within a range of 10 - 200 mg/l. ESR measurements were started in 30 min after the centrifugation; (2) chromate solution was added to the nutrient medium to provide a Cr(VI) concentration of 35, 70 or 140 mg/l. In 20 min or in 1 hour the cells were centrifugated and supernatants were subjected to the ESR measurements.

The elemental composition of the biomass of *A. oxydans* grown in the nutrient medium: 10 g of glucose, 10 g of peptone, 1 g of yeast extract, 2 g of caseic acid hydrolysate, 6 g of NaCl and 1 litre of distilled water – was determined by ENAA.

Analysis
ENAA

A. oxydans samples of about 0.5 g were packed in aluminum cups for long-term irradiation and were heat-sealed in polyethylene foil bags for short-term irradiation. The neutron flux density characteristics and the temperature in the irradiation channels equipped with a pneumatic system are described in.[16]

Long-lived isotopes were determined using the irradiation channel Chl. The samples were irradiated for five days, repacked and then counted twice after decays of 4 and 20 days. The counting time varied from 1.5 to 10 hours. To determine the short-lived isotopes of Mg, Al, Cl, Ca, V, Mn, and I, the irradiation channel Ch2 was used. The samples were irradiated for three minutes and measured twice after 3-5 and 20 min decay for 5-8 and 20 min, respectively.

Gamma-ray spectra were measured using a large-volume Ge(Li) detector with a resolution of 1.96 keV at the 1332.4 keV line of ^{60}Co with an efficiency of 30% relative to a 3'x3' NaI detector for the same line. The data processing and element concentration determination were performed on the basis of certified reference materials and comprators using software developed in FLNP JINR.[17]

Three certified reference materials (CRMs), namely, IAEA Lichen-336, IAEA Bottom Sediments SDM-2T and Nordic Moss DK-1 were used for quality assurance purposes.

Electron Spin Resonance (ESR) spectrometry

ESR investigations were carried out on the RE 1306 radiospectrometer with 100 kHz modulation at 9.3 GHz. A 0.5 ml bacterial sample was placed in a 4 mm diameter quartz tube. The samples were kept at room temperature for certain periods of time and then put into liquid nitrogen. ESR measurements were carried out at liquid nitrogen temperature (T=77 K).

RESULTS AND DISCUSSION

The cellular uptake of Cr depends on its chemical and physical state, the extracellular concentration and the exposure period. To determine the penetration ability of Cr(III) and Cr(VI) complexes into living systems,[4,8] a multitude of techniques, including radioactive tracer analysis, atomic absorption spectroscopy (AAS), gas-liquid chromatography (GLC) has been used.

We used the ENAA to study chromate uptake by *A.* oxydans cells. In Fig. 1 the level of Cr(VI) accumulation in *A. oxydans* at different Cr(VI) concentrations in the nutrient medium is presented.

As it follows from the data, in the discussed range of Cr(VI) concentrations the total content of intracellular chromium always increases, while the uptake of Cr is more intensive in the interval of Cr(VI) concentrations 10 -50 mg/l. At 50 mg/l the accumulated chromium is about 500 μg/g (after 5 days).

A non-monotonous behavior of Cr(VI) uptake is described by the function $Y = A_1(1-e^{-k_1 x})$, where A_1 = 405 ppm, k_1 = 0.0074 $(mg/l)^{-1}$. The solid line in Fig. 1 corresponds to calculation. The approximation was carried out by the nonlinear Levenberg-Marquardt method.

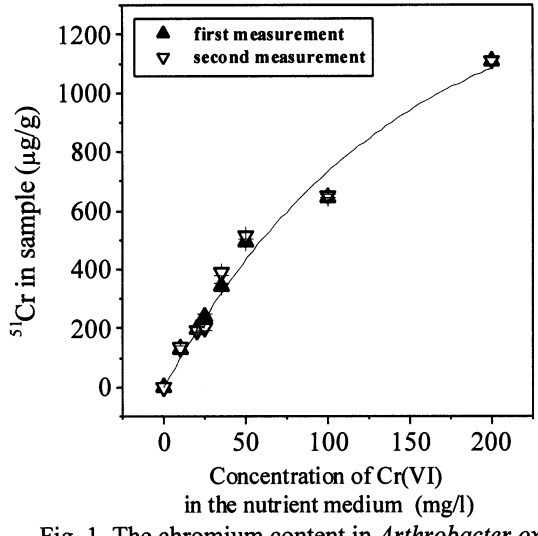


Fig. 1. The chromium content in *Arthrobacter oxydans* cells versus Cr(VI) content in the nutrient medium

It should be noted that when the Cr(VI) concentration in the nutrient medium exceeds 50 mg/l, the survival ability of *A. oxydans* cells decreases dramatically.[12] So, it can be assumed that the obtained result, a decrease in the accumulation rate at high concentrations of Cr(VI), is due to the effect of dose-dependent toxicity.

After Cr(VI) had been added to *A. oxydans* the ESR signal centered at g=1.980 with a peak-to-peak line width of 12 G appeared.[11] This signal is assigned to surface Cr(V)-diol complexes. Gram-positive bacteria contain different constituents in the cell wall, e.g. polycarbohydrates, teichoic acids *etc*, which, in turn, contain diol groups.[18] Cr(VI) as $(CrO_4)_2$ can be bound to diol moieties generating Cr(V) complexes.

In the second set of experiments, the dependence of Cr(V) formation on the Cr(VI) concentration was investigated (using ESR method). In Fig. 2 the relative intensity of the Cr(V) signal is plotted versus the concentration of Cr(VI). The chromate solution was added to the nutrient medium (insert) and directly to nutrient medium free *A. oxydans* cells.

The intensity of the ESR signal was measured after 30 min of chromate action (insert: after 20 min and after 1 hr). The time of Cr(V) measurements was

chosen on the basis of the data obtained from the study of the kinetics of Cr(V) formation.

It was observed that reduction from Cr(VI) to Cr(V) begins immediately and the Cr(V) signal intensity reaches its maximum in about 0.3-1 hr (the data are not shown) depending on the initial concentration of Cr(VI) in the nutrient medium.

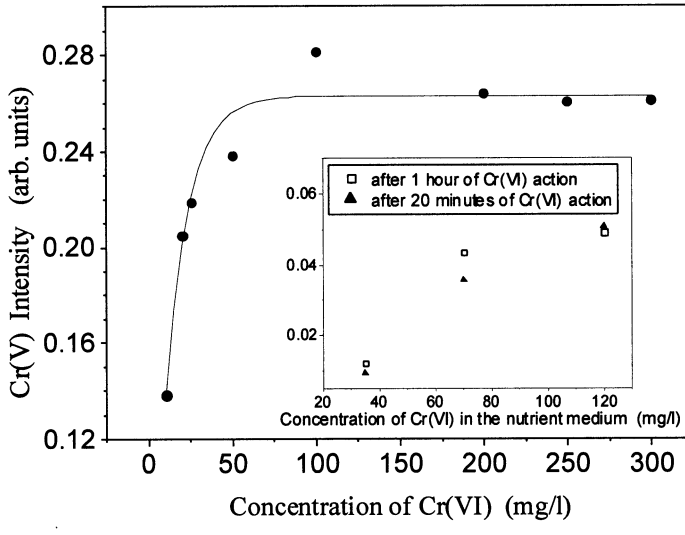


Fig. 2. Cr(V) formation by *Arthrobacter oxydans*

A chromate solution is added to bacterial cells separated from the nutrient medium. ESR measurements are carried out after 30 min. Insert: chromate solution is added to the nutrient medium.

Figure 2 shows that in both cases, the process of Cr(V) formation has a dose-dependent character. However, the intensity of the ESR signal is found to be about 5 times smaller when chromate solution is added to the nutrient medium (insert). A detail investigation of Cr(V) formation was performed when chromate solution was added directly to *A. oxydans* cells. At low concentrations of Cr(VI) (10 - 50 mg/l) the formation of Cr(V) goes fast but at higher concentrations the intensity of the ESR signal is practically constant. At a Cr(VI) concentration of 50 mg/l the maximal amount of Cr(V) is $\sim 10^{15}$ spin/sample (this value is observed after nearly half an hour of chromate solution action) i. e. about 25 ppm of Cr(V) (dry weight of sample is ~ 4 mg) have been formed in already half an hour of Cr(VI) action. So the formation of Cr(V) by *A.oxydans* is the process that goes at a high rate. Besides, Cr(V) is a highly reactive species and it is most likely easily transported to cells. So, it can be supposed that one part of chromium accumulated in bacterial cells is due to Cr(V).

According to calculations, the best approximation (solid line in Fig. 2) again corresponds to the function $Y = A_2(1-e^{-k_2 x})$, where A_2 = 0.26 (arb. units) and k_2= 0.073 $(mg/l)^{-1}$ and so, $\frac{k_2}{k_1} \approx 10$. Thus the reduction from Cr(VI) to Cr(V) is a much faster process than the uptake of Cr(VI).

In Fig. 3 the results obtained in ENAA experiments with Cr(III) loading are presented.

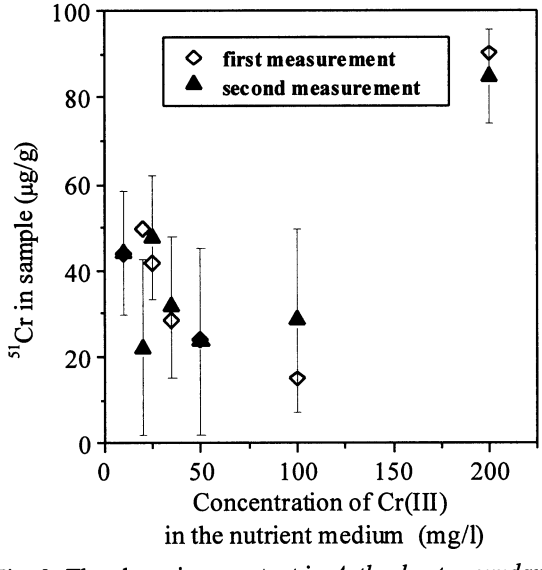
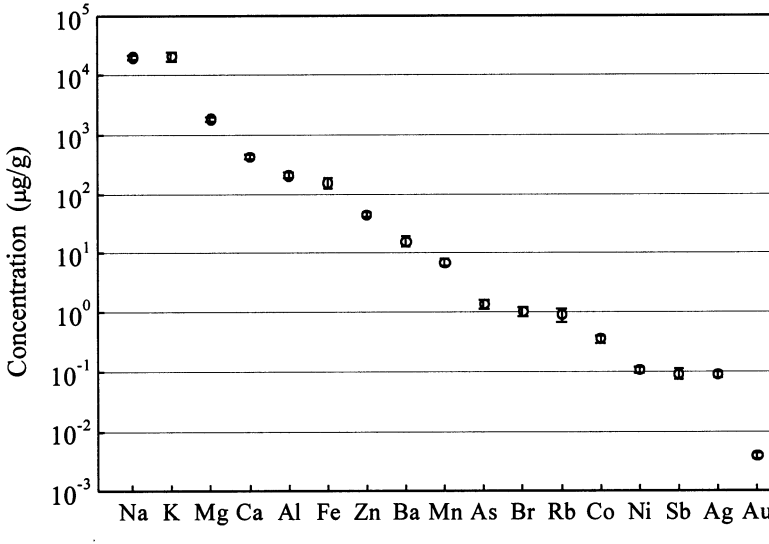


Fig. 3. The chromium content in *Arthrobacter oxydans* cells versus Cr(III) content in the nutrient medium

The figure shows, that in the investigated range of Cr(III) concentration in the nutrient medium, the accumulation of Cr(III) in bacterial cells does not practically take place. However, at a concentration of 200 mg/l Cr content in *A. oxydans* cells increases. Taking into account the fact that the rate and extent of chromium penetration into cells dependens on its extracellular concentration and exposure time, one can assume, that at the concentration 200 mg/l (after 5 days of Cr(III) action) the number of certain complexes of chromium (including Cr(III) complexes), that can be generated extracellularly and have high permeabilities, increases. This, however has to be studied more carefully in future experiments. It should be noted that Cr(III) inside the cells is one of the major reasons of Cr(VI) genotoxicity (Cr(VI) is not bound to DNA, but Cr(III) is).[4]

In the forth experiment, the ENAA was applied to determine the chemical composition of *A. oxydans*. The ENAA is found to be suitable for multi-element determination in biological samples by Frontasyeva and Steinnes.[19]

In Fig. 4 the background level of the chemical composition of *A. oxydans* is presented. As it follows from the figure, the concentration range is over 8 orders of magnitude, from major to ultra-trace elements. A total of 17 major, minor and trace elements were determined. The increased concentrations of Na and K in the samples



are due to that *A. oxydans* was freeze-dried in a Na-K phosphate buffer at pH 7.0.

Fig. 4. The elemental distribution in lyophilized samples of *Athrobacter oxydans*

As it follows from the same figure high concentrations of the following components – Mg, Al, Ca and Fe were observed. The concentration of Mg was found to be especially high, 1880 g/g. The Columbia basalt samples from the studied site are fine-grained silicate rocks with calciplagioclase feldspar [$(Na,Ca)(AlSi)_4O_4$], pyroxene [$(Ca,Na,Mg,Fe)(Al,Si)O_3$], and olivine ($Mg_{1.8}Fe_{0.2}SiO_4$) being essential minerals. Magnetite ($Fe^{2+}Fe^{3+}{}_2)O_4$ is also often present.[10] So, it can be supposed that the element composition of *A. oxydans* reflects the chemical composition of the environment to which it was confined. In future obtained results will be compared as to the chemical composition of other Cr(VI) reducer endolithic bacteria.

CONCLUSIONS

Using instrumental neutron activation analysis the uptake of chromate by *A. oxydans* was investigated. It was established that chromate accumulation is dose-dependent and it is more intensive in the interval of Cr(VI) concentrations (10-50) mg/l. The most intensive formation of Cr(V) was also found at low concentrations of Cr(VI) (up to 50 mg/l) (using the ESR method). Besides, it was calculated that both Cr(VI) uptake and Cr(V) formation are described by the function $Y = A(1-e^{-kx})$, while the reduction from Cr(VI) to Cr(V) was found to be a faster process than the uptake of Cr(VI).

According to ENAA measurements, Cr(III) in contrast to Cr(VI), is not accumulated in *Arthrobacter oxydans* cells up to a concentration of 200 mg/l.

Also, the chemical composition of *A. oxydans* was studied using the ENAA. The background levels of 17 major, minor and trace elements were determined in the bacteria. In future the obtained results will be compared with the chemical composition of other Cr(VI)-reducing endolithic bacteria.

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REFERENCES

1. E.E. Cary. Chromium in air, soils and natural waters, in: S. Langard, ed. Biological and environmental aspects of chromium. Elsevier, Amsterdam, the Netherlands, (1989), p. 49.
2. S. Langard, The carcinogeneicity of chromium compound in men and animals, in: S. Borrow, ed. Chromium - metabolism and toxicity, CRS Press, (1983), p. 13.
3. S. Deflora, K.E. Wetterhahn, Life Chem. Rep., 7 (1989)169.
4. R. Codd, C.T. Dillon, A. Levina, P.A. Lay, Coord. Chem. Rev., 216-217 (2001) 537.
5. K.Y. Appenroth, M. Bischoff, H. Gabrys, J. Stoeckel, H.M. Swartz, T. Walczak, K. Winnefeld, J. Inorg. Biochem., 78 (2000) 235.
6. Y. Ishibashi, C. Cervants and S. Silver, Appl. Environ. Microbiol., 56 (1990) 2268.
7. J. McLean and T.J. Beveridge, Appl. Env. Microbiology, 67 (2001) 1076.
8. H.H. Popper, E. Grygar, E. Ingolic, O. Washinek, Inhal. Toxicol. 5 (1993) 345.
9. H.-Y. Holman., D. Perry and J. Hunter-Cevera, J. Microbiol. Methods, 34 (1998) 59.

10. H.-Y. Holman, D. Perry, M. Martin, G. Gamble, W. McKinney and J. Hunter-Cevera, Geomicrobiology J., 16 (1999) 307.
11. M. Abuladze, N. Asatiani, N. Bakradze, H.-Y. Holman, T. Kartvelishvili, T. Kalabegishvili, L. Mosulishvili, A. Rcheulishvili, N. Sapojnikova, N. Tsibakhashvili, Fresenius Environmental Bulletin, 11(2002)N 7.
12. N. Tsibakhashvili, M. Abuladze, N. Asatiani, N. Bakradze, H.-Y. Holman, T. Kartvelishvili, T. Kalabegishvili, A. Rcheulishvili, N. Sapojnikova, L. Mosulishvili, Proc. Intern. Confer. on Protection and Protection and Restoration of the Environment, Skiathos Island, Greece, 2002 (in press).
13. Loveland-Curtze J., Sheriden P., Gutshall K and Brenchley J. Arch. Microbiol. 171 (1999) 355.
14. N. Tsibakhashvili, N. Asatiani, M. Abuladaze, B. Birkaya, N. Sapojnikova, L. Mosulishvili, H.-Y. Holman. Biomed. Chrom., 16 (2002) N5, 327.
15. B. Birard, and E. Snell (1983), Biochemical; factors, in: Manual Methods for General Bacteriology (ed. P. Gerhardt), Mir, Moscow.
16. L.M. Mosulishvili, Ye.I. Kirkesali, A.I. Belokobilsky, A.I. Khizanishvili, M.V. Frontasyeva, S.F. Gundorina, C.D. Oprea, J. Radioanal. Nucl. Chemistry, 252(2002) № 1, 15.
17. T.M. Ostrovnaya, L.S. Nefedyeva, V.M. Nazarov, S.B. Borzakov, L.P. Strelkova, Proc. Analysis in Environment Protection, D-14-93-325, Dubna, 1993, p. 319.
18. The Biology of Actinomycetes . Edited by M. Goodfellow, M. Mordarski and Williams S.T. Academic Press, 1984, London, p. 226.
19. M.V. Frontasyeva, E. Steinnes, Proc. Intern. Symp. on Harmonization of Health Related Environmental Measurements Using Nuclear and Isotopic Techniques, Hyderabad, India, 4 -7 November, 1996, IAEA 1997, p. 301.

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Цибахашвили Н. Я. и др. E18-2002-129
Применение метода нейтронного активационного анализа
для изучения хрома, поглощенного *Arthrobacter oxydans*

Для изучения поглощения хрома *Arthrobacter oxydans* (Cr(VI)-редуцирующая бактерия, выделенная из колумбийских базальтовых пород, США) использовался метод инструментального активационного анализа. Установлено, что аккумуляция хрома зависит от дозы и наиболее интенсивна в интервале концентраций Cr(VI) от 10 до 50 мг/л. На основе метода электронно-парамагнитного (спинового) резонанса было также определено, что наиболее интенсивное образование Cr(V) происходит при низких концентрациях Cr(VI) (до 50 мг/л). Кроме того, было установлено, что процесс восстановления Cr(VI) в Cr(V) происходит быстрее, чем поглощение Cr(VI). По результатам измерений ЭНАА Cr(III), в отличие от Cr(VI), не аккумулируется клетками *Arthrobacter oxydans* вплоть до концентраций 200 мг/л. С помощью эпитеплового нейтронного активационного анализа в *Arthrobacter oxydans* был определен фоновый уровень 17 макро- и микроэлементов.

Работа выполнена в Лаборатории нейтронной физики им. И. М. Франка ОИЯИ и в Институте физики им. Э. Л. Андроникашвили АН Грузии.

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Tsibakhashvili N. Ya. et al. E18-2002-129
Application of NAA Method to Study Chromium Uptake
by *Arthrobacter oxydans*

To study chromium uptake by *Arthrobacter oxydans* (Cr(VI)-reducer bacteria isolated from Columbia basalt rocks, USA) instrumental neutron activation analysis method was applied. It was established that chromate accumulation is dose-dependent and it is more intesive in the interval of concentrations of Cr(VI) (10–50 mg/l). At low concentrations of Cr(VI) (up to 50 mg/l) the most intensive formation of Cr(V) was also found (using ESR method). Besides, it was estimated that reduction from Cr(VI) to Cr(V) is faster process than the uptake of Cr(VI). According to ENAA measurements Cr(III), in constant to Cr(VI), is not accumulated in *Arthrobacter oxydans* cells up to concentration of 200 mg/l. Using epithermal neutron activation analysis the background levels of 17 major, minor and trace elements were determined in *Arthrobacter oxydans*.

The investigation has been performed at the Frank Laboratory of Neutron Physics, JINR and at the E. L. Andronikashvili Institute of Physics of the Georgian Academy of Sciences.

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