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Microbial biotechnology for remediation of aquatic habitats polluted with chromium

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Abstract. Chromium may occur in nine different forms of oxidation ranging from –II to +VI, with forms II, III and VI as the most commonly encountered. In Cluj county, chromium pollution dates well back in time and has caused important dysfunction to the mechanical-biological wastewater purification station of the city of Cluj (Coșier & Diță 1996). The purpose of this study was to develop one microbial method able to reduce hexavalent chromium (mobile, permeable to cell membrane, carcinogenic and mutagenic) (Ishikawa et al 1994) to the trivalent form (insoluble and an essential element for humans) (Song et al 2006). Different sources of chromium-reducing bacteria and many sources of carbon and energy added to the Kvasnikov mineral basal medium (Komori et al 1990) with increasing amount of chromate (200-1000 mg/l) were tested. Two bacterial strains, able to reduce even 1000 mg chromate/l, were isolated in pure culture. For one of these bacterial strains, we determined the optimum conditions for the reduction of Cr (VI).

Key Words: chromium-reducing bacteria, inocula, chromate, bacterial strain, molasses, sludge, mud, wastewater.

Résumé. Le chrome peut apparaître en neuf états d'oxydations différentes: de –II à +VI, les plus fréquents étant les états d'oxydation II, III, et VI. Dans le département de Cluj (Coșier & Diță 1996), la pollution avec chrome est signalée depuis longtemps, ceci étant la cause des disfonctionnements graves de la station d'épuration microbiologique du municipe où l'on décharge l'eau résiduelle dans la rivière Somes. Le but de notre étude a été le développement d'une méthode microbiologique afin de réduire le Cr(VI) (mobile, perméable pour les membranes cellulaires, carcinogène et mutagène) (Ishikawa et al 1994) au Cr(III) (insoluble, élément essentiel pour l'homme) (Song et al 2006). De différentes sources de bactéries réductrices de chrome et plusieurs sources de carbone et d'énergie ont été testées comme adjuvant au milieu minéral de base Kvasnikov (Komori et al 1990) qui contenait des quantités croissantes de chromât (200-1000mg/l). Deux souches bactériennes, capables de la réduction de 1000 mg chromât/l ont été isolées en culture pure, pour l'une d'elles on a déterminé aussi les conditions optimales de réduction de Cr(VI).

Mots clés: bactéries chrome réductrices, inoculum, chromât, souches bactériennes, mélasse, sédiment, eaux résiduelles.

Rezumat. Cromul poate apărea în 9 stări de oxidare diferite: de la - II la + VI, cele mai frecvent întâlnite fiind stările de oxidare II, III şi VI. In judeţul Cluj (Coșier & Diță 1996), poluarea cu crom este semnalată cu mult timp în urmă, cauzând disfuncţionalităţi grave staţiei de epurare mecano-biologice a municipiului care descarcă ape uzate în râul Someş. Scopul acestui studiu a fost dezvoltarea unei metode microbiologice de reducere a Cr(VI) (mobil, permeabil pentru membranele celulare, carcinogen şi mutagen) (Ishikawa et al 1994), la Cr(III) (insolubil, element esenţial pentru om) (Song et al 2006). Diferite surse de bacterii crom-reducătoare şi mai multe surse de carbon şi energie au fost testate ca adaos la mediul mineral bazal Kvasnikov (Komori et al 1990) ce conţinea cantităţi crescânde de cromat (200-1000 mg/l). Două tulpini bacteriene, capabile de reducerea a 1000 mg cromat/l, au fost izolate în cultură pură, pentru una din ele fiind determinte şi condiţiile optime de reducere a Cr(VI).

Cuvinte cheie: bacterii crom-reducătoare, inocul, cromat, tuplini bacteriene, melasă, sediment, nămol, ape uzate.

Introduction. The purpose of this study was to develop one microbial method able to reduce hexavalent chromium (mobile, permeable to cell membrane, carcinogenic and mutagenic) (Ishikawa et al 1994) to the trivalent form (insoluble and an essential element for humans) (Vitale et al 1994; Song et al 2006). This study was based on results of comparative analyses during a period of 15 months of observation, aimed to

demonstrate the usefulness of this work. The efficiency of microbial reduction of Cr(VI) was tested at the city's purification station. The activated sludge at the city's purification station retained chromium with improved efficiency so that upon discharge of the sewage the Cr concentration did not exceed maximal concentration admitted (MAC) during the 15 months of observation (Coșier & Diță 1996). Thermogravimetric (TG) and thermodifferential (DTA) analyses of the industrial sludge indicate the complete reduction of Cr(VI) to Cr(III) at 410^0C. The process requires high energy consumption, but it results in the least amount of waste.

In order to cultivate chromium-reducing bacteria in liquid media, we carried out four experiments, in which we used the Kvasnikov[1] medium (Komori et al 1990), but without addition of potassium acetate. As carbon and energy sources we used glucose, potassium acetate and molasses in different concentration. Hexavalent chromium was added in different quantities (0.2, 0.4, 0.6 and 1 g chromate/l). Different inocula as sources of chromium-reducing bacteria were tested, namely samples of sewage and sludge from "Clujana" tannery, samples of mud from the Tineretului Lakes 1 and 2 (Cluj-Napoca) and from the Ştiucii Lake (a lake located in the Natural Reservation Săcălaia, Cluj County) (Persecă & Coșier 1995), and the combinations of samples. The inoculated media were incubated under anaerobic conditions. The microscopic examination of the smears prepared from each culture and stained by the Gram procedure confirmed the macroscopic observation concerning the growth of chromium-reducing bacteria.

In order to obtain pure cultures of chromium reducing bacteria and to establish the range of chromate concentration at which bacterial growth takes place, we used Kvasnikov's mineral medium solidified with agar-agar. We operated transfers from the liquid cultures of the previous experiment, where reduction of Cr(VI) and bacterial growth were intense already on day 3 of the incubation (test tubes numbered 2, 18, 34 and S). The experiment was repeated by using colonies for inoculation of agar plates with increasing concentrations of chromate (200-1000mg/l). The colonies on plates number 2 and 18 originated from liquid cultures inoculated with a mixture of "Clujana" sewage and mud from the Tineretului Lake 1 grown under different conditions (Figures 1 and 2).

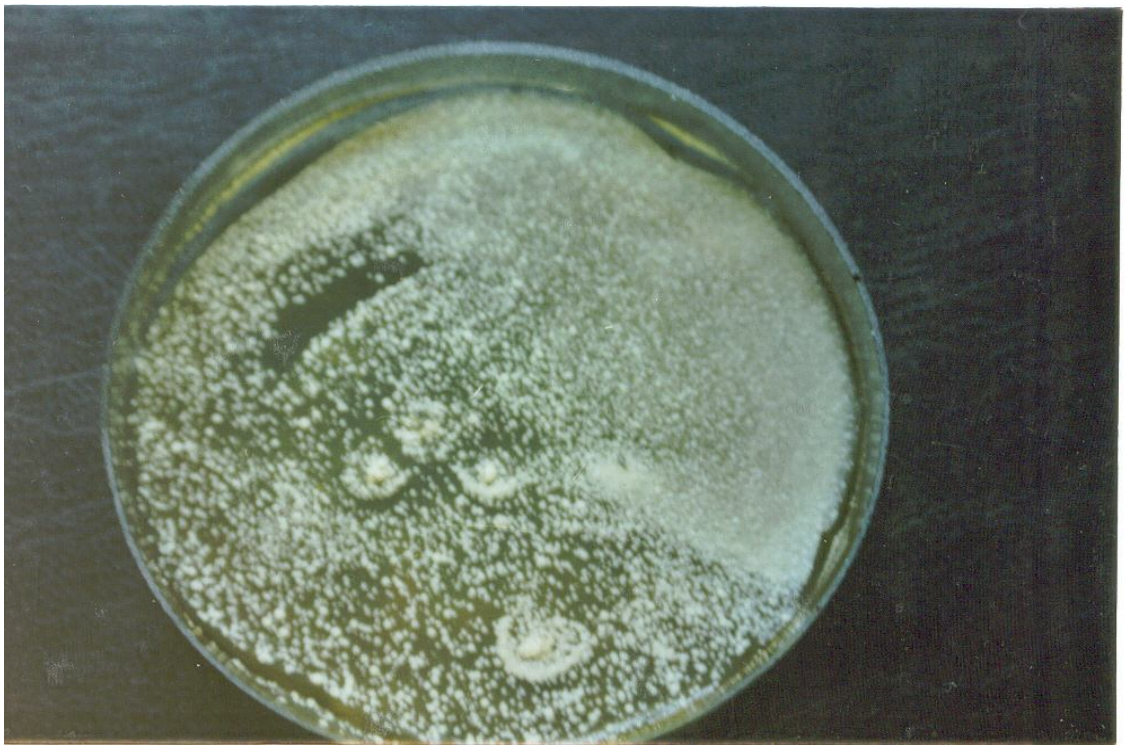

Figure 1. The bacterial culture with tube no. 18 inocula (mud of the Tineretului Lake 1 and the sewage from "Clujana"); medium with 0.6 g chromate/l.

[1] Kvasnikov mineral basal medium (in g/l): NH_4Cl – 0.03; K_2HPO_4-0.03; KH_2PO_4- 0,05; NaCl-0.01; $MgSO_4 \bullet 7H_2O$-0.01; $CaCO_3$-0.005; $FeCl_3 \bullet 7H_2O$

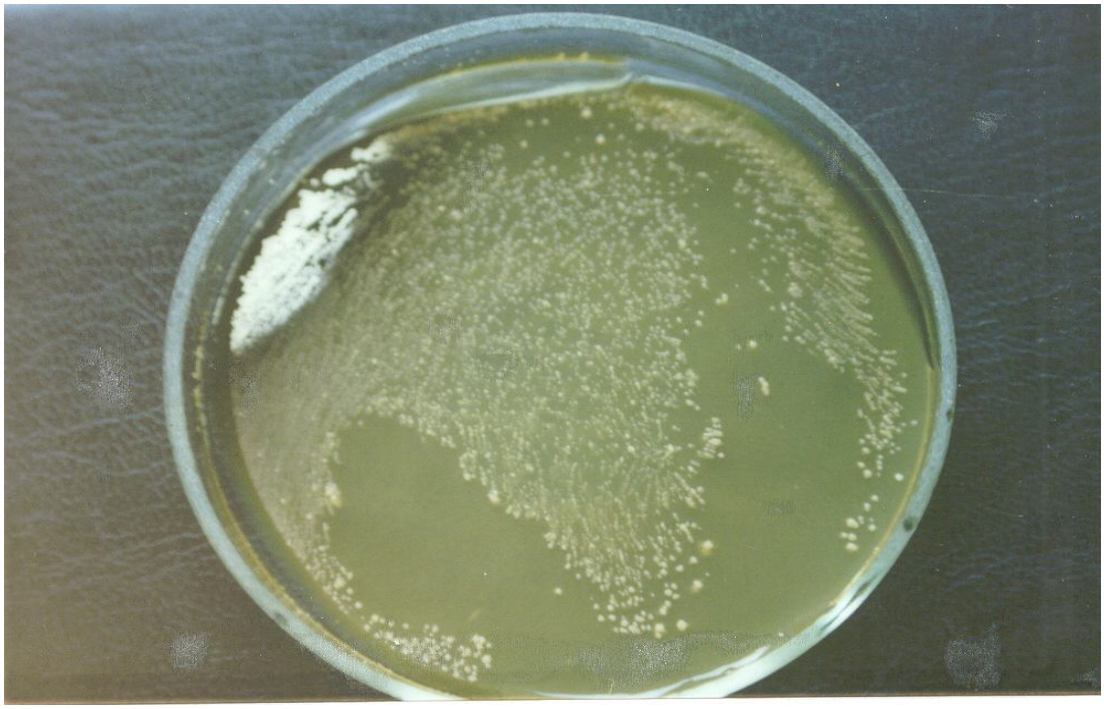

Figure 2. The bacterial culture with tube no. 2 inocula (mud of the Tineretului lake 1 and the sewage from "Clujana"); medium with 1 g chromate/l.

The bacterial strain on plate number 2 was tested in order to establish the optimum conditions for the reduction of Cr(VI). The effects of temperature, pH, amount of chromate and oxygen upon the reduction of Cr(VI) were tested. The test was carried out in Kvasnikov's liquid media with molasses (20 g/l), at a set cell density and a known chromate concentration. The results are shown in the following figures (3, 4 and 5).

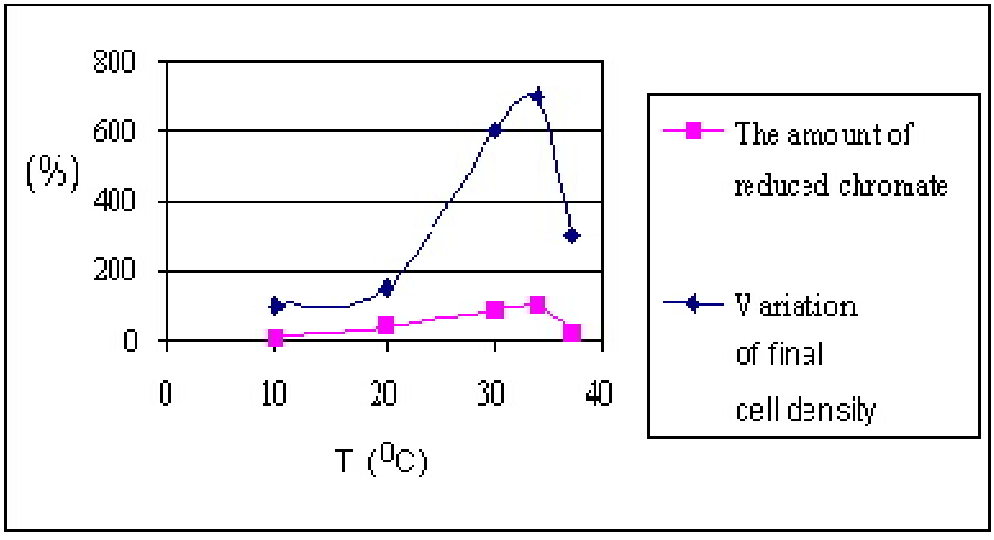

Figure 3. Relation between the amount of reduced chromate and the variation of cell density depending on the temperature.

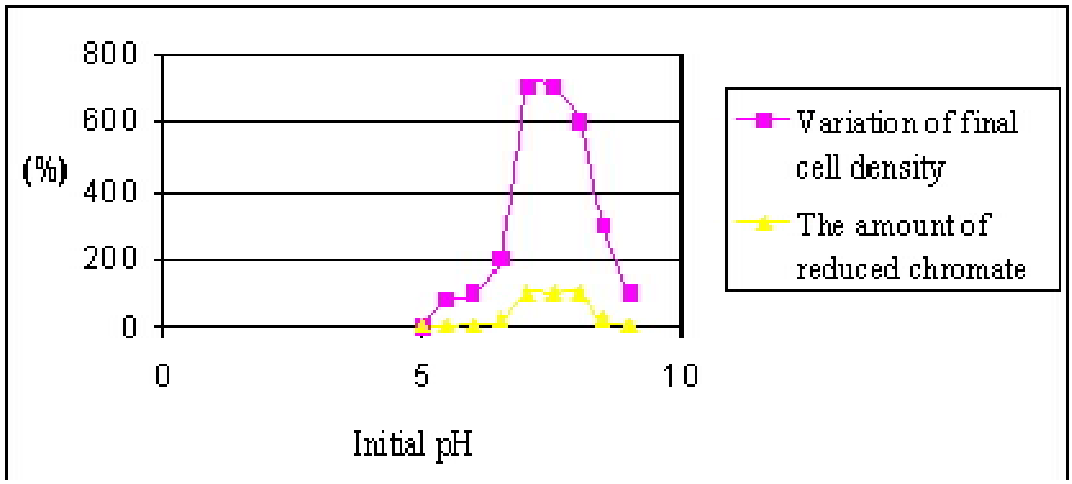

Figure 4. Relation between the amount of reduced chromate and the variation of cell density depending on the initial pH.

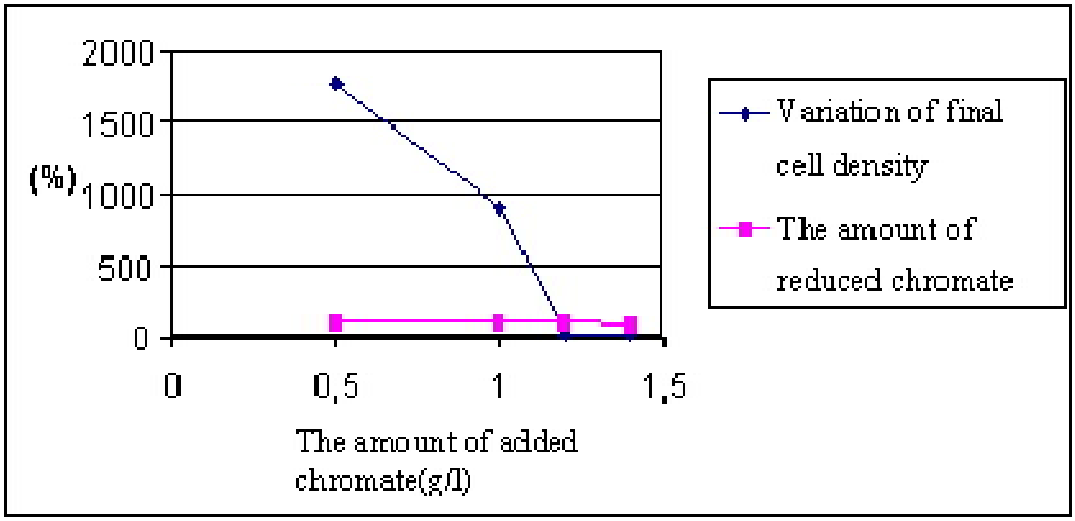

Figure 5. Relation between the amount of reduced chromate and variation of cell density depending on the amount of added chromate.

Materials, Method and Results. The experiment included three stages: 1. Cultivation of chromium reducing bacteria in liquid media; 2. Cultivation of chromium-reducing bacteria in solid media; 3. Testing of the isolated bacterial strain. Determination of optimum conditions for the reduction of Cr(VI) by the bacterial strain isolated in pure culture (Figures 3, 4 and 5).

1. Cultivation of chromium reducing bacteria in liquid media.

We used Kvasnikov's mineral basal medium (Komori et al 1990) with the composition (in g/l): NH_4Cl – 0.03; K_2HPO_4-0.03; KH_2PO_4- 0.05; NaCl-0.01; $MgSO_4 \bullet 7H_2O$-0.01; $CaCO_3$-0.005; $FeCl_3 \bullet 7H_2O$ in which we added glucose, potassium acetate or molasses in different concentration to serve as a carbon and energy sources. Hexavalent chromium, in the form of potassium chromate, was added to the culture media in different quantities (0.2, 0.4, 0.6 and 1 g/l). The chromate rendered the media in yellow. The inoculated media were incubated at 34^0C under anaerobic conditions for 10 days. Observation were made regarding a) decolouration of the initially yellow media, which indicates that the reduction of Cr(VI) took place; b) appearance of opalescence, due to the growth of chromium-reducing bacteria and c) formation of a sediment containing the reduced Cr(III) removed from the liquid phase. The microscopic examination of the smears

prepared from each culture and stained by the gram procedure confirmed the macroscopic observations concerning the growth of chromium-reducing bacteria. Based on the results obtained, we could establish the optimal combinations of three factors: sources of chromium-reducing bacteria (type of inoculum), kind of the carbon and energy sources and amount of the reduced chromium.

A. In the first experiment, different sources of chromium reducing-bacteria (different types of inocula) were tested. Molasses (10g/l) served as carbon and energy sources. Potassium chromate was added to the medium in an amount of 0.2 g/l. The medium was inoculated with decimal dilution (10^{-1}-10^{-6}) of samples taken from mud from the Tineretului Lake 1; mud from the Tineretului Lake 2; sludge from "Clujana"; sewage from "Clujana"; sludge plus sewage from "Clujana" and mud from the Ştiucii Lake. All types of inocula tested were active in the reduction of Cr(VI) to Cr(III), some of them showing a better start. Upon microscopic examination, gram-positive cocci and Gram-negative rods occurring as single cells or pairs were found.

B. Further on, we tested the sensitivity of the chromium-reducing bacteria from different sources (types of inocula) to different amounts of chromate added to the medium. As compared to the previous experiment, other two combinations of inocula were tested, namely the combination of sewage from "Clujana" and the muds from the Tineretului Lakes 1 and 2, respectively. The results have shown that, at the chromate concentration of 0.2 g/l, the most active inocula were samples of sludge from "Clujana" used alone or in combination with muds from the Tineretului Lakes 1 and 2, whereas, at the chromate concentration of 0.5 g/l, the sludge from "Clujana" was the most active. Upon the microscopic examination of the culture, we noticed the presence of Gram-positive cocci in association with Gram-positive rods as single cells or grouped in chains.

C. Further on, we tested the influence of the carbon and energy sources as well as the amount of the added chromate upon the reduction of Cr(VI) using the types of inocula that proved very active in the previous experiment. The sources of carbon and energy employed were potassium acetate (0.2 g/l), glucose (1 g/l), and molasses (10 and 20 g/l). The number of cultures that we operated with was 72 including the controls.

The results recorded on macroscopic and microscopic examination were very useful in establish the optimal combinations among the type of inoculum, the source of carbon and energy and the amount of reduced chromium. Molasses at the 20 g/l concentration prove to be the best sources of carbon and energy for chromium-reducing bacteria, because in these cultures reduction was also noticed at the chromate concentration of 1000 mg/l. For this reason, in the following experiments we only used this source of carbon and energy, which, moreover, is also very cheap. Upon the microscopic examination of the smears prepared from these cultures and submitted to Gram staining, we found two preparations, which contained only Gram-positive cocci.

D. The last experiment in liquid culture media aimed at testing those types of inocula, which in the previous experiment were found to be the most active in the reduction of chromate. In this experiment, the concentration of added chromate was 1000 mg/l. Again three types of inocula, namely the sewage from "Clujana" in combination with mud from the Tineretului Lakes 1 and 2, as well as the sludge from "Clujana" proved to be the best sources of very active chromium-reducing bacteria. The microscopic examination of the culture of these bacteria showed the presence of Gram-positive cocci not associated or associated to Gram-negative rods.

2. Cultivation of chromium-reducing bacteria on solid media.

In order to obtain pure culture of chromium-reducing bacteria, we used Kvasnikov's mineral medium solidified with agar-agar. The medium contained chromate at different concentrations (0.2, 0.4, 0.6 and 1 g/l). We used the same carbon and energy source – molasses (20 g/l) in the experiments. We operated transfers from the liquid cultures of the previous experiment, where reduction of Cr(VI) and bacterial growth were intense already on the day 3 of the incubation (test tubes numbered 2, 18, 19, 34 and S). The inoculated solid media (agar plates) were incubated at 34^0C for 72 hours. The cultures were examined and photographs were taken. Bacterial colonies appeared on the agar plates containing 0.2 and 0.4 g chromate/l. Recordings were made of the number and morphology of the colonies. The plates maintained the numbers of the liquid cultures

used for inoculation. No growth was observed on agar plates with 0.6 and 1 g chromate/l. Therefore, the experiment was repeated by using colonies for inoculation on agar plates with 0.6 and 1 g chromate/l. Only plates number 2 and 18 with 1 g chromate/l made possible the bacterial growth. It should be mentioned that the colonies on plates number 2 and 18 originated from liquid cultures inoculated with a mixture of "Clujana" sewage and mud from Tineretului Lakes 1 and 2, grown under different conditions. Microscopic examination shows that the colonies, which developed on plate number 2, form a pure culture consisting only of Gram-positive cocci; and the plate number 18, Gram-positive cocci, grouped in chains. It should be added that the colonies on plate no. 2 grew more abundantly than those on plate no. 18 (Figures 1 and 2).

3. Testing of the isolated bacterial strain. Determination of optimum conditions for the reduction of Cr(VI) by the bacterial strain isolated in pure culture (plate no. 2).

The bacterial strain on plate no. 2 was tested in order to establish the optimum conditions for the reduction of Cr(VI). The effects of temperature, pH, amount of chromate and oxygen upon the reduction of Cr(VI) were tested. The test was carried out in Kvasnikov's liquid media with molasses (20 g/l), at the determined cell density and the known chromate concentration. At the end of the experiment, cell density and the amount of reduced chromium were determined. Cell density was determined by methods of plate cultures. Chromium was measured colorimetrically after treatment with diphenylcarbazide and recording of the extinction on an FEK-M photocolorimeter.

a) The effect of temperature on the reduction of chromate and cell density.

The tested incubation temperatures were 10, 20, 30, 34 and 37^0C. Initial cell density was found to be 10^6 cells/ml. The effect of temperature on the reduction of Cr(VI) and cell density is shown in Figure 3, which presents the amount of reduced chromate (the difference between the initial and the final concentrations), expressed in percentage (as related to the value of the initial concentration) and the variation of cell density, also expressed in percentage. The optimum temperature for the reduction of chromate with this strain is situated in the interval of 30-34^0C, the removal percentage in this interval being of 86.9 and 99.21, respectively. At these temperatures, cell density ranged from 7 x 10^6 to 8 x 10^6 cells/ml.

b) The effect of pH on the reduction of Cr(VI).

During this experiment, we worked with pHs ranging from 5 to 9. The initial cell density was again 10^6 cells/ml and the initial chromate concentration was 1000 mg/l. The relation between the amount of reduced chromate, cell density and pH is shown in Fig. 4. The initial optimum pH for the reduction of Cr(VI) was 7.0 – 7.5 with a Cr(VI) removal percentage of 100% at pH 7.0. The final cell density of 8 x 10^8 recorded at the initial pH of 7.0 and 7.5 began to drop at ph 8.0, where the Cr(VI) removal percentage was, however, 99%.

c) Dependence of the reduction of chromate on the amount of chromate added to the medium.

We worked with chromate concentrations of 0.5, 1, 1.2 and 1.4 g/l. Initial cell density was 8 x 10^8 cells/ml. Subsequent of this experiment, the results of which are shown in Figure 5, we noticed that the amounts of chromate added to the medium led to the increase of the reduction of Cr(VI) up to the certain concentration. At 1.2 and 1.4 g chromate/l, the percentage of chromium removal decreased either because of insufficient source of carbon and energy for maintaining the bacterial growth, or due to the toxic effect of the chromate in the medium.

d) The effect of oxygen on the growth of the bacterial strain in a medium containing 1 g chromate/l.

The incubation conditions were modified for a sample and its controls by blowing air. The final cell density was not determined because, subsequent to the measurements of the amount of reduced chromium in the aerobically incubated sample, it was noticed that the oxygen completely inhibited the reduction of chromate by the strain.

Conclusions. The wastewater from "Clujana" and the muds from Tineretului Lakes 1 and 2 proved to be the best sources of chromium-reducing bacteria. From these sources, we isolated the bacterial strain (plate no. 2). It was established that the bacterial strain is

capable of completely reducing the chromate at concentrations up to 1000 mg chromate/l under anaerobic conditions. The optimum incubation temperature is 34^0C, and the optimum pH is 7.0. At higher chromate concentrations (1.4 g/l), the percentage of removal was 75%, at the initial cell density of 8 x 10^8 cells/ml. For these reasons, subsequent research was focused on the development of an application on zeolitic volcanic tuffs, which – in combination with the microbial method – can remove from wastewater over 1000 mg chromate/l.

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