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Acta Pharm. *55* (2005) 93–105

Original research paper

Uranium uptake by some locally isolated and some reference bacterial species

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In the present study, uranium absorption capacity of *Bacillus pantothenticus* and *Bacillus megaterium*, previously isolated from the environmental air surrounding the ^{60}Co gamma source, is reported. *Pseudomonas putida* and *Pseudomonas chlororaphis* were used as reference species. Concerning uranium uptake, the local species were more efficient than the reference ones. The maximum uptake of uranium was achieved by *B. megaterium* and *P. chlororaphis* at 20 μg U mL^{-1} and by *B. pantothenticus* at 30 μg U mL^{-1}. The transmission electron microscope examination indicated that uranium was absorbed onto the cell surface of the studied isolates.

Furthermore, the increase in biomass concentration has shown an increase in the total amount of uranium removed. Dead cells exhibited uranium uptake to the same or greater extent than living cells. *B. pantothenticus*, *P. putida*, and *P. chlororaphis* achieved maximum uptake at pH 4.0, whereas for *B. megaterium* it was at pH 6.0. Temperature had an important role in uranium absorption of all the studied species except *B. pantothenticus*. Metabolic inhibitors did not affect the uptake.

Received September 6, 2003
Accepted January 31, 2005

Keywords: uranium uptake, bacteria

Some of the aqueous discharges emanating from industrial processes, such as mining, smelting, ore processing, and energy production processes, contain dissolved heavy metals, which can generate significant environmental problems due to their chemical and radiological characteristics (1). Binding and removal of heavy metals and radionuclides using microorganisms has been recognized as a potential alternative to the existing technologies for recovery of heavy metals and radionuclides from polluted soil and industrial waste streams (2).

Both living and dead cells are capable of uptake and accumulation of uranium, and so are products produced or derived from microbial cells, such as excreted metabolites, polysaccharides and cell wall constituents (3, 4).

A variety of absorption mechanisms may be involved, ranging from physico-chemical interactions like adsorption and deposition to processes dependent on cell metabo-

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lism, such as transport, internal compartmentalization and precipitation by external metabolites (5–7).

Uranium uptake was investigated by several authors; they reported a rapid uptake of uranium from solution by resting cells of *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa* (1). Also, it was postulated that 99% of uranium (as uranium sulphate) was absorbed by *Saccharomyces cerevisiae* in one hour (8). Both living and dead cells exhibited a high metal uptake because yeast cells have reactive groups capable of chelating metal ions (5). A technique for continuous removal of uranyl ions from aqueous solution was reported previously, utilizing a biofilm of *Citrobacter* spp. (9). Preliminary studies have shown that living or dead cells of *Pseudomonas putida* EPS-5028, an exopolysaccharide--producing microorganism, can accumulate uranium from solution (10).

The aim of the present investigation was to evaluate the efficiency of some microbial isolates, previously isolated from the surrounding environment to absorb uranium from solutions. Also, the present work was aimed at using some local isolates, as an easy and inexpensive alternative method to the existing technologies, to remove and recover radionuclides from low-level activity wastes and to trap uranium from new promising areas. The choice of local isolates was based on their desirable characteristics, especially their high lipids and phospholipids content, heavy metal uptake and radiation resistance (11–13).

EXPERIMENTAL

Microorganisms

Bacillus pantothenticus and *Bacillus megaterium* (local species) were previously isolated from the environmental air surrounding the ^{60}C gamma-irradiation facility of the NCRRT (Cairo, Egypt). *Pseudomonas putida* 50198 DSM and *Pseudomonas chlororaphis* 50082 DSM were the reference species.

Growth medium

Bacterial species were maintained and cultivated on trypton, glucose, and yeast extract (TGY) medium containing in (g L^{-1} distilled water): trypton (5), glucose (2), yeast extract (3) and agar (15) (Oxoid, UK).

Preparation of the uranium solutions

All uranium solutions were prepared form uranyl nitrate (Merck, Germany). Uranium solutions of different concentrations (10–300 μg U mL^{-1}) were prepared. To study the effect of pH, uranyl nitrate solutions were prepared in the pH range of 2–10.

Uranium uptake experiment

Bacterial cells were incubated at 30 °C in TGY broth medium and harvested after 24 h by centrifuging at 5000 rpm for 10 min. The cells were then washed three times with

sterile deionized-distilled water and re-suspended in 10 mL water. One mL of each cell suspension was dried in a hot air oven at 100 °C for 24 h to determine the dry mass. A known volume of each cell suspension, equivalent to 0.4 mg dry mass per mL of uranyl nitrate, was added, mixed well and incubated at 30 °C. After 24 h, the cells were removed from the mixture by centrifuging at 5000 rpm for 10 min; the remaining soluble uranium was measured with a UV-Vis Shimadzu (Japan) spectrophotometer UV-160A at 650 nm using Arsenazo III reagent (Aldrich, USA) (14).

To study the effect of biomass concentration, different cell concentrations ranging from 0.2–3 mg dry mass per mL were added to the solutions containing 20 and 30 μg U mL^{-1}.

To study the effect of metabolic inhibitors, the bacterial cells were treated chemically with $HgCl_2$ (1% solution) and formaldehyde (10% solution) (Adwic, El Nasser Pharmaceutical Chemicals Co., Egypt). Both were lethal to the bacterial cells; washed cells were exposed to the chemical agent at room temperature for 10 min under continuous stirring, the cells were then washed three times with water before contacting uranium solution (100 μg U mL^{-1}), or they were exposed to uranium in the presence of sodium azide (10^{-3} mol L^{-1}) (BHD, UK).

Mixtures were incubated at different temperatures (20, 30, 40, and 50 °C). To study the effect of the physiological state, the bacterial suspensions were heated at 100 °C for 15 min to kill the cells (no cells could be cultured after this treatment). The mean of three independent analyses is given in the histograms.

Electron microscopy

B. megaterium cells were treated with uranyl nitrate solution at a concentration of 100 μg U mL^{-1} and examined by the Transmission Electron Microscope (Model JEM-100 cx, JEOL, Japan) to assess the distribution of the metal on the cell. The cells were fixed, washed, dehydrated through an ethanol-propylene oxide series and processed into Epon 812 Premix kit (nonenyl succinic anhydride, vinyl cyclohexane dioxide and epoxy resin) (Electron Microscope Science Co., USA). Ultrathin sections (700 Å) were cut with ultratome (LKB ultratom III 8800, Austria) and mounted on copper grids (mesh 300). Unstained sections were examined without further processing (15).

RESULTS AND DISCUSSION

Uranium uptake by living cells

In the present study, the ability of the strains under investigation to absorb uranium was evaluated using the uranium concentration range from 10 to 300 μg U mL^{-1}. Results in Fig. 1 show that the highest total uranium uptake was achieved at low external concentrations, namely 10, 20 and 30 μg U mL^{-1} (0.4 mg dry mass per mL uranyl nitrate solution). The efficiency of uranium uptake decreased as uranium concentration increased and it reached the lowest value at uranium concentration of 300 μg U mL^{-1}. The maximum uptake by *P. putida* and *B. pantothenticus* was achieved at 30 μg U mL^{-1}. They removed 95 and 98.5% uranium while *P. chlororaphis* and *B. megaterium* exerted the maximum uptake at 20 μg U mL^{-1}, removing 87.6 and 98.9% uranium, respectively.

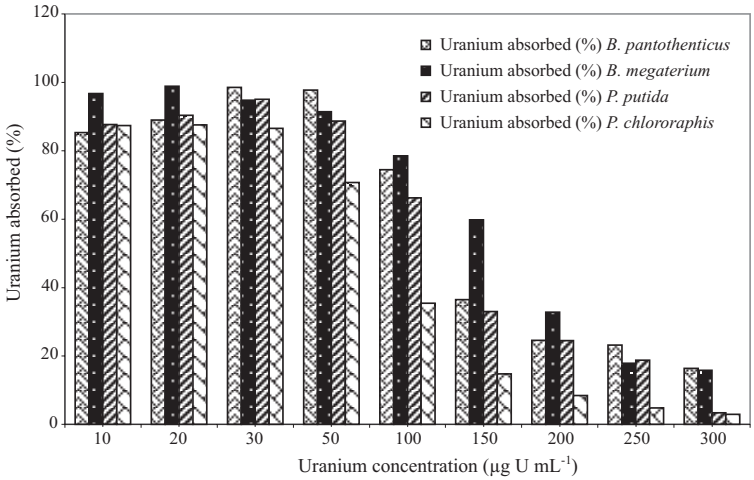

Fig. 1. Uranium uptake by the living cells.

Despite the reduced uranium uptake capacity, *B. pantothenticus* was the most efficient species, at uranium concentration of 50 μg U mL^{-1}, removing 97.8%, compared to 91.4, 88.7 and 70.8%, removed by *B. megaterium*, *P. putida* and *P. chlororaphis*, respectively. At higher concentrations (100, 150, 200 and 250 μg U mL^{-1}), there was a detectable reduction in the level of uranium uptake. As uranium concentration increased up to 300 μg U mL^{-1}, the local species were found more efficient than the reference ones, removing 16.4 and 15.9%, compared to 3.4 and 2.9%.

According to Beveridge and Koval (16), the total uptake of uranium by immobilized cells of *Escherichia coli* was the highest at uranium concentrations of 5, 10, and 21 μg U mL^{-1} and decreased at higher concentrations. This result agrees with that obtained in the present study.

These results could be attributed to the differences in cell wall composition between the Gram-negative reference species and Gram-positive local isolates. The cell wall of Gram-positive bacteria mainly consists of peptidoglycane, whereas that of Gram-negative bacteria consists of the outer and plasma membranes, which sandwich a thin peptidoglycane layer in the priplasmic space (17). For the most resistant species *P. chlororaphis*, the composition of cell wall may enable the bacterial cells to entrap the uranyl ions, preventing them from entering the cell cytoplasm and interfering with the metabolic processes concerning cell growth. The same may be suggested to explain the difference between the two local isolates, taking into account that the lipids and phospholipids content of *B. pantothenticus* is higher than that of *B. megaterium* (6.0 and 4.1 mg g^{-1} dry cell and (0.57 and 0.39 mg g^{-1} dry mass, respectively) (18). The obtained results could be discussed taking into consideration the fact that binding of metal ions takes place as a result of interactions between the positively charged metal ions and the negatively charged active sites on cell surfaces.

Other authors (19), assumed that at low uranium concentrations there was a small number of metal ions compared to the large cell surface area with active sites. Thereby, it

was easy for each metal ion to find its place on the cell surface. On the other hand, not all metal ions could be absorbed by increasing the metal concentration, leaving a residual amount of free metal ions.

Effect of the cell biomass

The effect of cell biomass of *P. chlororaphis*, *B. megaterium*, *P. putida* and *B. pantothenticus* on uranium uptake was tested using 0.2 to 3.0 mg dry mass per mL of uranyl nitrate solution containing 20 and 30 μg U mL^{-1}. As shown in Fig. 2a, the amount of uranium removed increased as the cell concentration increased; the maximum uptake was achieved at a cell concentration of 3.0 mg dry mass per mL. At 0.2 mg dry mass per mL, 88.0, 86.1, 91.9 and 86.0% were removed whilst at 3.0 mg dry mass per mL 96.7, 97.2, 98.8, and 99.0%, uranium were absorbed by *P. putida*, *P. chlororaphis*, *B. pantothenticus*, and *B. megaterium*, respectively, as shown in Fig. 2b.

The obtained results are found to be in agreement with those obtained by other authors (19), who mentioned that if the multiplicity of potential accumulation sites occurred in the cell wall, the accumulation of uranium should increase when the cell concentration increased.

On the other hand, by increasing the metal concentration, binding of metal ions at the same active sites takes place, leading to decreased percentage of the total amount removed. It is clear from the obtained results that there is a marked variation in the level of uptake between the studied species.

Anderson *et al.* (20) recommended the use of *Geobacter* species in bioremediation of uranium contaminated groundwater after optimization of the strategy of the long-term activity of the species.

Uranium uptake by dead cells

As shown in Fig. 3, the physiological state of the bacterial cells has an influence on uranium uptake at higher levels. The amount absorbed by dead cells was more or less the same as for living ones at lower uranium concentrations (10–50 μg U mL^{-1}). At a uranium concentration of 30 μg U mL^{-1}, both the living and dead cells of *P. putida* and *B. pantothenticus* removed 95.0, 94.6 and 98.5, 99.2%, respectively. For *B. megaterium*, this observation was found at the concentration of 10 μg U mL^{-1} L: 95.3 and 96.7% were removed, while for *P. chlororaphis* the percentage removed was 87.5, 88.8 and 86.6, 87.5%, at concentrations of 20 and 30 μg U mL^{-1}, respectively.

At higher uranium concentrations (150–250 μg U mL^{-1}), dead cells showed a significant increase in the amount removed: 14.8 *vs.* 64.9%, 24.5 *vs.* 38.8%, 17.9 *vs.* 30.4%, 24.6 *vs.* 36%, for *P. chlororaphis P. putida*, *B. megaterium*, and *B. pantothenticus*, respectively. The percentage removed by the dead cells was 50.0, 14.3, 12.5, and 11.5% higher than that removed by the living cells, at uranium concentrations of 150, 200, 250, and 300 μg U mL^{-1}, respectively.

The uptake of metal ions by dead cells was a point of interest to several authors throughout many studies (14, 18, 21, 22). Besides, it was found that dead cells could accumulate heavy metals to the same or even greater extent than the living cells (23). This observation agrees with that obtained in our study for all four species. The percentages

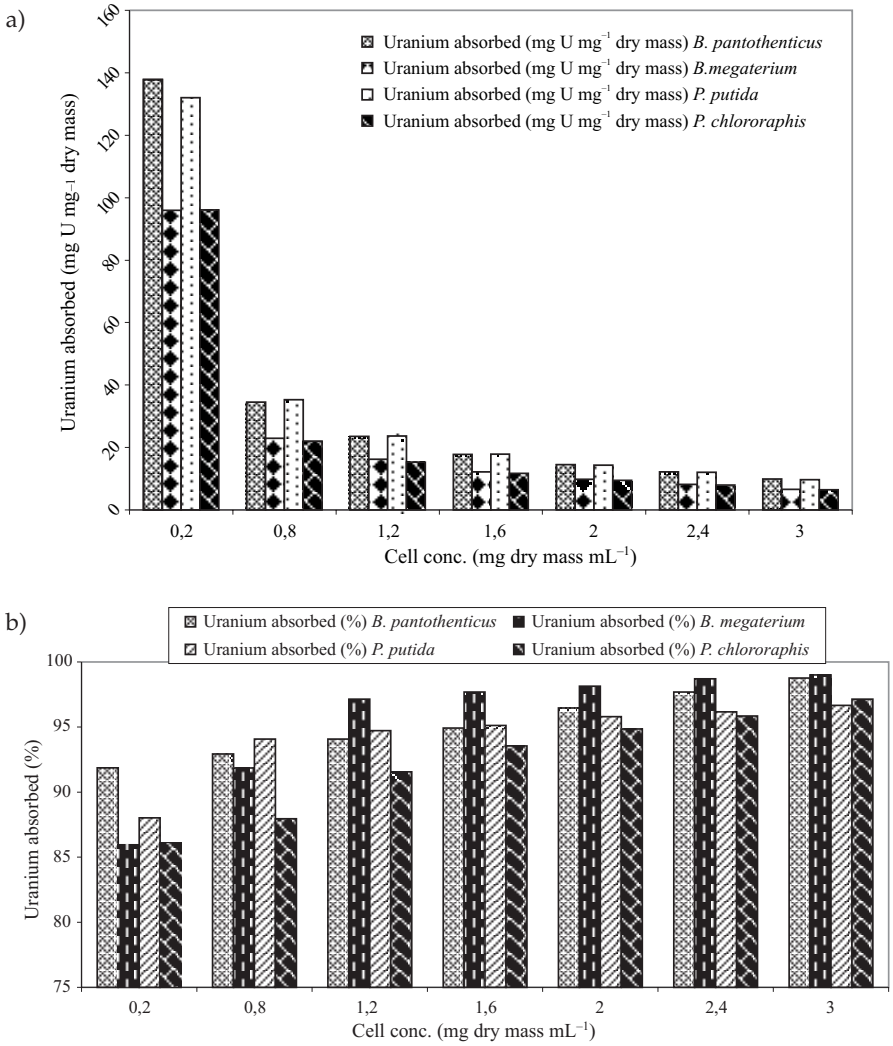

Fig. 2. Effect of cell concentration on the uranium uptake (20 μg U mL^{-1} for *B. megaterium* and *P. chlororaphis*; 30 μg U mL^{-1} for *B. pantothenticus* and *P. putida*).

removed by living and dead cells were comparable at lower uranium concentrations (10, 20 and 30 μg U mL^{-1}) but at higher concentrations (200 and 300 μg U mL^{-1}), the amount adsorbed by dead cells was larger than that removed by living cells.

Since the same concentration of living and dead cells was used, the reason for such increase in the percentage removed by dead cells may be due to the death process itself, which denaturated the cell wall leaving the uranium binding sites, which may be constrained in the cell membrane, much more exposed to metal ions (16). In addition, killed cells became immune to metal toxicity and other adverse operation conditions (5).

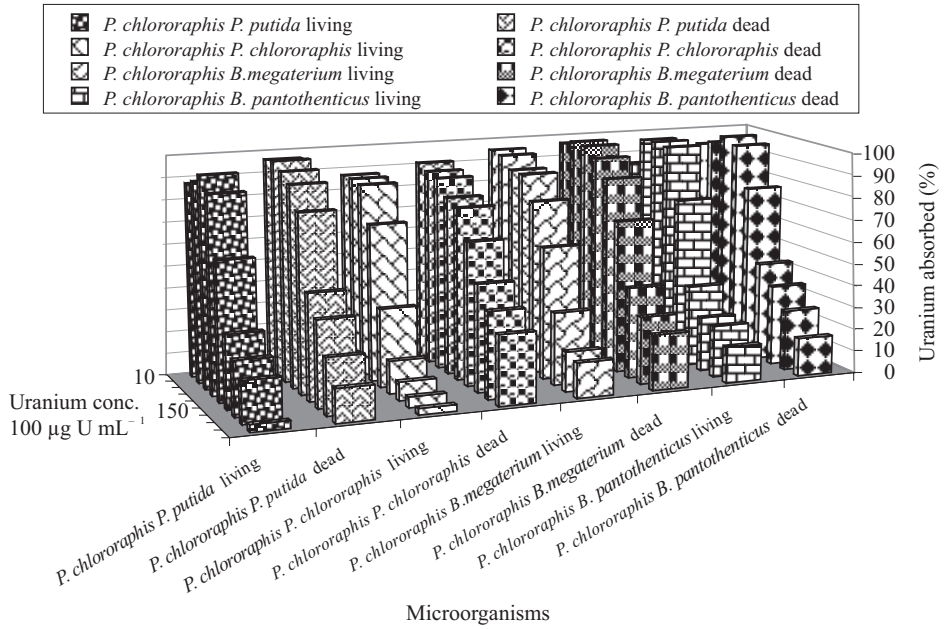

Fig. 3. Effect of the physiological state of the bacterial species on uranium uptake.

It was already reported that uranium accumulation by killed cells of *Streptomyces* spp. was slightly higher than that of living ones (18). Also, both living and dead cells of *Pseudomonas* sp. EPS-5028 removed all the uranium (50 μg U mL^{-1}) within one hour contact; at higher concentrations (200 and 500 μg U mL^{-1}), the dead cells took up 78 mg U g^{-1} dry cell, compared to 50 mg U g^{-1} dry cell removed by the living cells within the same time (10).

Effect of temperature and pH

As shown in Fig. 4, there is a variation in the response of the bacterial species due to the change of temperature. There was no change in the uptake capacity of *B. pantothenticus*, over the temperature range 20–50 °C; the percentage removed was 80.3, 81.2, 80.0 and 82.1% at 20, 30, 40, and 50 °C, respectively. The capacity of *B. megaterium*, *P. chlororaphis*, and *P. putida* increased the percentage removed being 55.1, 50.1, and 54.4% at 20 °C, while at 50 °C it was 67.0, 68.1, and 75.5%, respectively.

As regards the bacterial species under investigation, increasing the temperature from 20 to 50 °C did not influence the uptake capacity of B. *pantothenticus.* This result was in agreement with the amount of uranium removed by cells of *Pseudomonas* EPS-5028, which was almost constant over a temperature range of 20 to 50 °C with removal of about 23 mg U g^{-1} dry mass (10). In our study, increasing the temperature promoted the uptake of uranium by *B. megaterium*, *P. putida*, and *P. chlororaphis*. Such phenomena were already discussed by other authors, who mentioned that the rate of ura- nium uptake by *Saccha-*

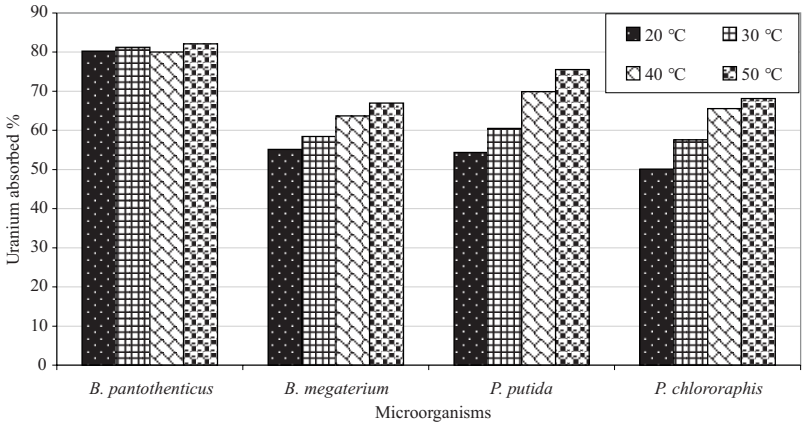

Fig 4. Effect of temperature on the uptake of uranium by the bacterial species treated with 100 μg U mL^{-1}.

romyces cerevisiae increased with increasing temperature between 20° and 50 °C, and that uranium biosorption by *Rhizopus arrhizus* was higher at 40 °C compared to that at 23° and 5 °C (24, 25). The authors considered that the uptake process looks like the ion-exchange-type mechanism and the processes of adsorption and ion-exchange are of endothermic type. Accordingly, the previously mentioned results in our study were in agreement with those obtained by other authors.

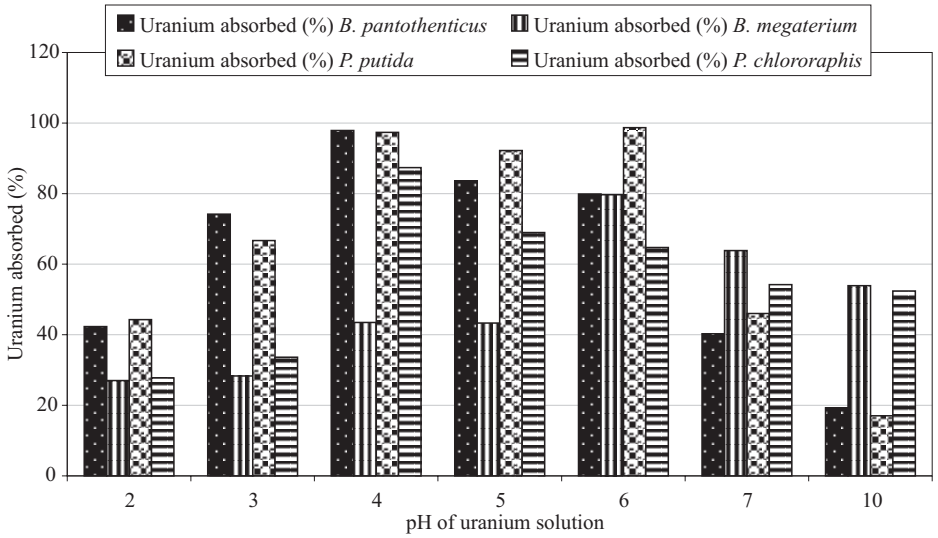

Fig. 5. Effect of pH of uranium solution on the uptake of uranium by the bacterial species (100 μg U mL^{-1}).

The uptake of uranium increased as pH increased (Fig. 5) for *P. chlororaphis*, *B. pantothenticus*, and *P. putida*. It achieved the maximum value at pH 4.0, 87.4, 97.9, and 97.4% uranium being removed, respectively. For *B. megaterium*, the maximum uptake was achieved at pH 6.0, removing 79.7% uranium. In both cases, further alkalization decreased the efficiency of metal uptake. It reached the lowest value of 19.3, 53.9, 17.0, and 52.4% at pH 10.0 for *B. pantothenticus*, *B. megaterium*, *P. putida*, and *P. chlororaphis*, respectively.

The pH of the solution plays a major role for the extent of metals binding to microorganisms (26). The acidic conditions can repress both biosorption and intracellular uptake, and an acid can act as an effective desorption agent (5). Our results could be discussed taking into account the suggestion of other investigators (24) that UO_2^{2+} may be the first species biosorbed and it is the only species present at pH 2.0. Due to the hydrolysis of uranyl ion, about 80% of uranium is in the form of UO_2^{2+} at pH 4.0, while at pH 5.0 only 9% is found in this form. Moreover, uranium occurring in the form of uranyl nitrate is best accumulated by the biomass of various microorganisms in the pH range of 4.5 to 6.5 (27). Also, at pH above 5.0 precipitation of uranium oxides in metal solution occurs (24).

Effect of metabolic inhibitors

To find whether the process of uranium uptake by *B. pantothenticus*, *B. megaterium*, *P. putida*, and *P. chlororaphis* involved any metabolic activities or not, the bacterial cells were exposed to uranium in the presence of metabolic inhibitors.

As shown in Fig. 6, the treatment of *P. chlororaphis*, *P. putida*, *B. pantothenticus*, and *B. megaterium* with sodium azide solution (10^{-3} mol L^{-1}) did not disturb the uranium uptake by the treated cells. On the contrary, the percentage removed increased in the presence of sodium azide. It was 75.5, 73.7, 89.6, and 82.1% compared to 44.0, 56.5, 63.6, and 58.2% for the control (no azide). This result is in agreement with that described by Marques *et al.* (10), who found that treatment of cells of *Pseudomonas sp.* EPS-5028 with sodium azide (10^{-3} mol L^{-1}) or 2,4-dinitrophenol (5×10^{-3} mol L^{-1}) did not affect uranium uptake. Similarly, the cells of *S. cerevisiae* and *P. aeruginosa* were not affected by the treatment with either metabolic inhibitor (28). On the other hand, pre-treatment with mercuric chloride and formaldehyde decreased the percentage removed. Formaldehyde-pretreated cells exceeded the $HgCl_2$-pretreated ones with 38.6, 52.5, 43.6, and 40.0% *vs.* 30.0, 44.0, 37.2, and 31.0% for *P. chlororaphis*, *P. putida*, *B. pantothenticus*, and *B. megaterium*, respectively. This reduction could be understood if we consider that both treatments could modify some structures involved in uranium absorption (10). Besides, Hg^{2+} as a divalent ion has a complete chance to bind with the active groups that reside on cell surfaces during the pre-treatment. If this happens the available active groups participating in uranium binding will be reduced compared to the control (no $HgCl_2$). In comparison, heat killed cells were more efficient; they removed 78.4, 86.5, 87.3, and 83.5% of uranium.

To verify the location of heavy metals and radionuclides accumulated by different microorganisms, thin sections of the microbial cells, which had taken up metal ions from solution, along with thin sections of metal-free cells (control), were examined with a transmission electron microscope. All microphotographs have shown that uranium was adsorbed onto cell surfaces, while intracellular deposition was not clear (Figs. 7a, b). Uranium deposits appeared as electron-dense particles. This surface binding may be at-

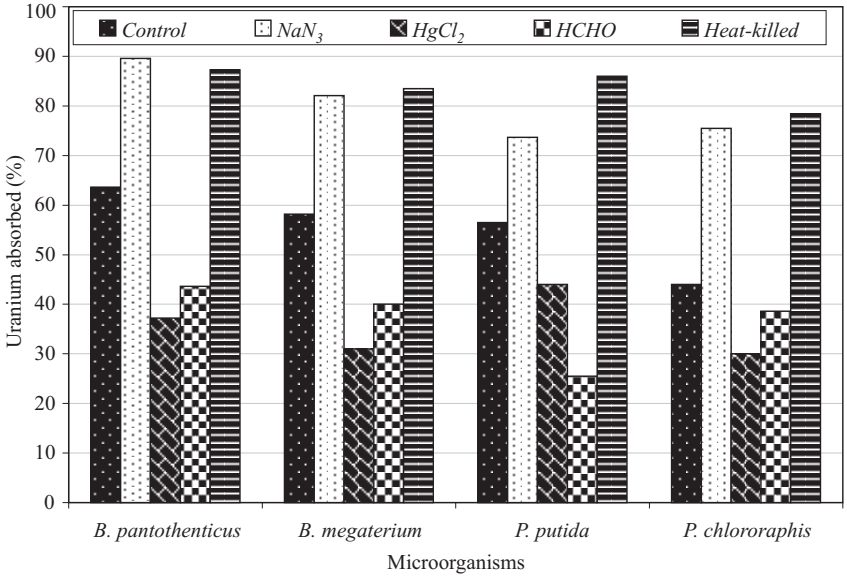

Fig. 6. Effect of metabolic inhibitors on the uptake of uranium by the bacterial species (100 μg U mL^{-1}).

tributed to the cell wall structure. In *B. subtilis* and other Gram-positive microorganisms, the carboxyl groups of D-glutamic acid residues of the peptidoglycane are the most potent metal scavengers and play a meaningful role in the biosorption (29). In Gram-negative bacteria, the metal ions may interact with the polar head groups of the phospholipids and the available anionic sites of lipopolysaccharide (7). The acidic groups of the exposed peptides may be involved in metal binding, as suggested for *E. coli* k-12 (17). Uranium was deposited in the cell walls of *Rhizopus arrhizus*, while electron microscopy of the cell interior did not indicate any concentration of electron-dense material (26). It was also observed that not all the cells have electron-dense deposits, *i.e.*, although many

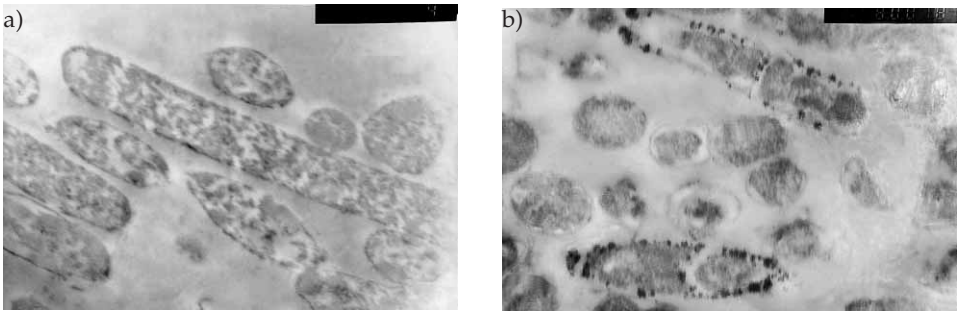

Fig. 7. Electron micrograph of living *B. megaterum* (80,000 x): a) untreated, b) treated with 100 μg U mL^{-1}.

bacteria possess electron-dense uranium deposits, others appear unloaded. Strandberg *et al.* (24) found that 32% of *S. cerevisiae* and 44% of *P. aeruginosa* cells had uranium deposits. Similarly, other authors (30) described uneven distribution of silver deposits within a population of bacteria. Two possibilities were suggested: either only a proportion of the bacterial cells was responsible for metal accumulation, or metal deposits were removed by attrition, a well known phenomenon in bioreactors.

CONCLUSIONS

The cell surface structure and composition of bacterial species played the major role in the uptake of uranium. The Gram-positive local isolates bound larger quantities of uranium compared to the Gram-negative reference species. Dead cells (heat-killed) showed a higher uptake of uranium than the living cells, especially at higher uranium concentrations. The uptake of uranium by *B. pantothenticus* was temperature-independent while it was temperature-dependent for *B. megaterium*, *P. putida*, and *P. chlororaphisi*. The absorption of uranium by these bacterial species was presumably not mediated by any metabolic activities. For all bacterial species, the uptake of uranium was pH-dependent. Transmission electron microscope examination revealed that uranium was absorbed onto cell surfaces rather than intracellular accumulation.

Acknowledgement. – We thank the electron microscope stuff in the central laboratory of the NCRRT for their cooperation.

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S A Ž E T A K

Apsorpcija urana u izoliranim i referentnim bakterijskim vrstama

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U radu je proučavan kapacitet apsorpcije urana bakterija *Bacillus pantothenticus* i *Bacillus megaterium* izoliranih iz zraka izloženog izvoru gama-zračenja iz ^{60}Co. Te bakterije su se pokazale učinkovitije u usporedbi s referentnim vrstama *Pseudomonas putida* i *Pseudomonas chlororaphis*. Maksimalna postignuta koncentracija bila je 10, 20, odnosno 30 μg U mL^{-1}. Nadalje, povećanje koncentracije u biomasi pratilo je povećanje ukupne količine uklonjenog urana. Mrtve stanice su apsorbirale uran u istoj ili većoj mjeri nego žive stanice. Maksimum apsorpcije *B. pantothenticus*, *P. putida* i *P. chlororaphis* postignut je pri pH 4,0, a *B. megaterium* pri pH 6,0. Kod svih ispitivanih vrsta osim *B. pantothenticus*, temperatura je značajno utjecala na apsorpciju dok inhibitori metaboličkih reakcija nisu utjecali. Pretraživanje transmisijskim elektronskim mikroskopom ukazalo je da se uran apsorbirao na površinu stanice.

Ključne riječi: apsorpcija urana i bakterije

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