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Uranium accumulation by a bacterium isolated from electroplating effluent*

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Summary

Pseudomonas MGF-48, a gram-negative, motile, oxidase-negative, catalase-positive, yellow-pigmented bacterium isolated from electroplating effluent, was found to accumulate uranium with high efficiency. Uptake of uranium was rapid and the amount increased in direct proportion to concentration, e.g., from 50 to 200 mg uranium per liter. The largest amount of uranium uptake was 174 mg per gram dry weight bacterial biomass and was observed to occur in stationary phase during incubation at 30 °C. Uptake was determined by flow injection analysis. Maximum uranium accumulation occurred at pH 6.5, with 86% of the uranium being taken up within 5 min of incubation. Release of uranium bound to the cells was accomplished by addition of sodium carbonate and EDTA solution (0.1 M), the cells were reusable, and served as a biosorbent. Cells immobilized in polyacrylamide gel took up 90% of the uranium. *Pseudomonas* MGF-48 showed excellent efficiency in biosorbing uranium, by both immobilized and free cells. The results of this study, compared with those of other reports of uranium accumulation by microorganisms, leads us to conclude that *Pseudomonas* MGF-48 shows excellent potential for bioremediating uranium-polluted aqueous effluents.

Introduction

Untreated effluents containing heavy metals from many industries have an adverse impact on the environment (Macaskie & Dean 1984; Norberg & Persson 1984; Nakajima & Sakaguchi 1986; Scott *et al.* 1986; Malone 1989). Generating large quantities of such aqueous effluents poses environmental disposal problems (Sanchez *et al.* 1999). A specific problem associated with heavy metals in the environment is accumulation in the food chain, as well as persistence.

Bioremediation of industrial wastes containing heavy metals has been demonstrated by several biotechnology companies employing bioaccumulation (Macaskie & Dean 1984; Lovely *et al.* 1991). Biosorption, bioprecipitation, and uptake by purified biopolymers derived from microbial cells provide alternative mechanisms, in addition to conventional physical and chemical methods (Higham *et al.* 1985; Figueira *et al.* 1997; Sar *et al.* 1998). Intact microbial cells, live or dead, and their products can be highly efficient bioaccumulators of both soluble and particulate forms of metals (Silver 1992; Lovely *et al.* 1993; Niu *et al.* 1993; Sar *et al.* 1998; Pieper & Reineke 2000). Various microbial species, mainly *Pseudomonas*, have been shown to be relatively efficient in the bioaccumulation of uranium, copper, lead, and other metal ions present in polluted effluents. Both as immobilized cells and in the mobilized state, bacteria are able to sequester metals (Lovely *et al.* 1993; Wong *et al.* 1993; Douglas & Beveridge 1998). We report here the bioaccumulation of uranium (UO_2^{2+} ions) by a bacterium isolated from the effluent of a metal smelting factory in the south of Tehran, Iran. The isolate, identified as a *Pseudomonas* sp., strain MGF-48, also accumulates lead, cadmium, and copper.

Materials and methods

Water, soil, and sludge samples were collected from the effluents of metal smelting and electroplating factories in the south of Tehran, Iran. Samples were diluted in sterile distilled water and plated on trypticase soy agar (Difco) plates containing 1 mM Cd ($Cd(NO_3)_2 \cdot 4H_2O$), 1 mM Cu ($CuSO_4 \cdot 5H_2O$), 1 mM Zn ($ZnSO_4 \cdot 7H_2O$), or 1 mM Pb ($Pb(NO_3)_2$). Plates were incubated at 30 °C for 72 h,

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after which colonies were randomly picked, isolated, and purified. In this preliminary screening, colonies showing resistance to Cd, Cu, Zn, and Pb were selected for further study.

Heavy metal uptake

Bacterial isolate MGF-48 was grown in 500 ml flasks containing a glucose and mineral salts medium (GMS) (glucose, 10 g; NH_4Cl, 2.67 g; Na_2HPO_4, 5.35 g; distilled water, 1000 ml) amended with 6 ml mineral salts solution ($CaCl_2 \cdot 2H_2O$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 10 g; $MnSO_4 \cdot 7H_2O$, 0.07 g; $FeSO_4 \cdot 7H_2O$, 0.4 g; distilled water, 1000 ml). The glucose was autoclaved separately as a solution (1%), with the pH adjusted to 7.0 before autoclaving. Insulated flasks were incubated at 30 °C for 72 h, with shaking (200 rev/min). Bacterial cells were harvested by centrifugation at $9000 \times g$ for 20 min at 4 °C and washed twice with distilled water. Freshly harvested bacterial cells were suspended in deionized water to a final concentration of 2.5 mg dry weight per millilitre. About 10 ml of the suspension was added to 40 ml of selected concentrations of uranyl nitrate ($UO_2(NO_3)_2 \cdot 6H_2O$). Suspensions were centrifuged at $9000 \times g$ for 20 min, after shaking at 100 rev/min for 1 h at room temperature. The bacterial cells were heated at 105 °C overnight after which the dry weight was measured. The harvested cells, 4 mg dry weight, were mixed with 0.5 ml concentrated nitric acid and incubated in a water bath (100 °C) for 1 h, after which the mixture was cooled to 25 °C, the volume brought to 5 ml with distilled water, and the concentration of uranium measured by flow injection (model FIA-1 Autoanalyzer, Beijing, China). This procedure was used to minimize aerosol hazard from the uranium. Uptake of other metals was measured by atomic adsorption spectrophotometry (Perkin-Elmer, Norwalk, CT, USA, Model No. 300 S). The identical protocol was followed using lead ($Pb(NO_3)_2$), cadmium ($Cd(NO_3)_2 \cdot 4H_2O$), copper ($CuSO_4 \cdot 5H_2O$), nickel ($NiCl_2 \cdot 6H_2O$), zinc ($ZnSO_4 \cdot 7H_2O$), silver ($Ag(NO_3)$), cobalt ($CoCl_2 \cdot 6H_2O$), and chromium ($K_2Cr_2O_7$) at concentrations of 50–100 mg/l. Results were expressed as mean of experiments carried out in triplicate and the data compared with those for loss of uranium from uninoculated controls.

Recovery of uranium from cells

To measure uranium bioaccumulation, i.e., the amount of uranium taken up by strain MGF-48, the cells were washed with distilled water for 15 min and mixed with 0.1 M sodium citrate, EDTA, sodium carbonate, or nitric acid. The concentration of uranium released was measured and cells were reused, serving as a biosorbent.

Transmission electron microscopy

To determine the location of accumulated metals in the cells, the bacteria were collected by centrifugation and fixed for transmission electron microscopy, using 3% glutaraldehyde in phosphate buffer (pH 7.2), for 3 h at 4 °C. The preparations were centrifuged at $4000 \times g$ for 10 min, embedded in agar, and divided into 1.1 mm segments. The segments were fixed in 2% osmium tetroxide for 2 h at 4 °C, after which specimens were dehydrated with ethanol at selected concentrations and embedded in epoxy resin. Ultrathin sections were prepared, stained with 2% uranyl acetate, and examined by transmission electron microscopy (Phillips Model EM 400, Netherlands).

Immobilization of bacterial cells

The bacterial isolate MGF-48 was grown in 100 ml GMS medium in 500 ml flasks and incubated at 37 °C, with shaking at 200 rev/min. Cells were harvested after incubation for 36 h at 37 °C by centrifugation at $9000 \times g$ for 10 min at 4 °C, and washed three times with MES (2-*N*-morpholino-ethanesulphonic acid) buffer. About 50 mg (dry weight) of freshly harvested bacterial cells were suspended in 9.0 ml MES buffer and mixed with 6.0 ml of 20% polyacrylamide stock solution (18.2 g acrylamide and 1.8 g *N*,*N*-methylene-bis-acrylamide dissolved in 50 ml distilled water and diluted with distilled water to a final volume of 100 ml) and 100 ml potassium persulphate (10%). About 10 ml of TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine) was added to the mixture, with polymerization at 25 °C for 1 h. The gel was ground into small pieces (20 mesh), washed thoroughly with MES buffer, and resuspended in MES buffer for heavy metal ion uptake measurement.

In batch ion uptake experiments, immobilized bacterial cells (approximately 50 mg dry weight in 15 ml of gel) were suspended in 20 ml MES buffer amended with 100 mg/l of uranium, cadmium, or copper, and packed into a column (1.5×15 cm^2). After exposure for 60 min, the buffer was drained off and the concentration of heavy metal ion in the buffer determined by atomic absorption spectrophotometry. Experiments were carried out in triplicate and controls (gels without bacteria) were included. Removal efficiency of the immobilized bacterial cells is defined as the percentage of added heavy metal ion removed, including the amount absorbed by the bacterial cells.

Results and discussion

Heavy metal resistant isolates

A total of 50 bacterial strains resistant to heavy metals were isolated from samples of metal smelting and electroplating effluent. Some of the strains were resistant to all four metals tested in this study (Cu, Pb, Cd, Zn). Only one strain was capable of accumulating uranium, when transferred to GMS broth containing uranium nitrate. This isolate was found to be a gram-negative,

motile, yellow-pigmented, oxidase-negative, catalase-positive, aerobic, rod-shaped bacterium.

Identification was carried out following the criteria of Bergey's Manual of Determinative Bacteriology (Krieg & Holt 1984) and employing the Biolog system for gram-negative bacteria (microplate Pl 001, Biolog, Inc, Hayward, CA, USA). Biochemical characteristics and enzymatic activities of the strain were studied (data are not shown). The isolate was identified as either *Pseudomonas* or *Chryseomonas* and is designated as *Pseudomonas* strain MGF-48. Confirmatory identification employing molecular methods is in progress.

Effect of growth phase and uranium concentration on uranium accumulation

When bacterial suspensions were prepared from 24, 48, and 72 h cultures incubated at 30 °C, with shaking (200 rev/min), and uranium uptake measured, employing a 50 ml uranium nitrate solution (50 mg/l), the amount of uranium accumulated was 163.5, 155.5 and 162.5 mg/g dry weight of cells, respectively. No significant difference relative to the stage of growth was evident. Thus, in subsequent experiments, cultures incubated for 3 days at 30 °C were used. Uranium uptake by the cells was measured after incubation in media amended with concentrations of uranium ranging from 50 to 500 mg/l. It was found that the amount of uranium taken up by the cells increased with increase in concentration of uranium in the range of 50–200 mg/l (Figure 1). No increase in uptake of uranium was observed at concentrations greater than 200 mg/l. The highest concentration of uranium taken up by *Pseudomonas* MGF-48 was 174 mg/g dry weight of bacterial biomass.

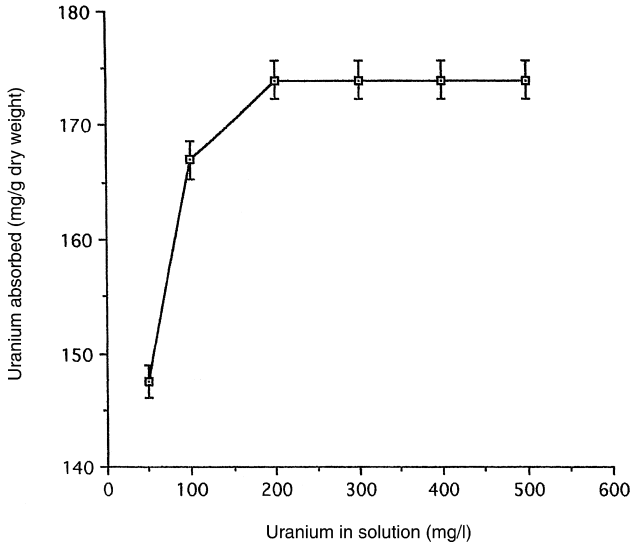

Figure 1. Effect of uranium concentration (mg/l) in solution on uptake of the metal by *Pseudomonas* MGF-48 after incubation in uranium solution for 1 h.

Effect of pH on uranium accumulation

As shown in Figure 2, the initial pH of the solution had a significant effect on uranium accumulation. At pH 6.5, maximum accumulation of uranium was obtained (86% within the first 5 min). In this study, *Pseudomonas* MGF-48 was found to be highly efficient in accumulating uranium, up to 174 mg/g dry weight bacterial biomass, when grown in GMS broth. The maximum accumulation of uranium was achieved when the bacterium was in stationary phase of growth at pH 6.5 and incubated at 30 °C. Uptake was relatively rapid under these conditions. At extremely high or low pH, the amount of uranium accumulation was significantly reduced (Figure 2).

Recovery of uranium

Uranium was released from cells by addition of sodium carbonate, sodium citrate, EDTA, and nitric acid (Figure 3). These reagents were tested to determine the efficacy of the reagents for recovery of uranium from treated cells. Because of the formation of a very stable uranyl–carbonate-complex (U–EDTA), washing the cells three times with the reagents was required to recover the maximum amount of accumulated uranium. The amount of uranium recovered in the first wash with sodium carbonate and EDTA was 95%, and the two additional washings yielded 94.4% of the remaining uranium. Recovery was significantly reduced when the cells were washed with sodium citrate or nitric acid (77.9 and 78.1%, respectively), compared to EDTA and sodium carbonate.

Metal accumulation

Pseudomonas MGF-48 was also capable of accumulating several other metals (Pb, Cd, Cu, Ag, Ni), in the following order: U > Pb > Cd > Cu > Ag > Ni (Figure 4). Affinity for Cr and Co was much lower and the amount of biosorption of Pb, Cd, Cu, Ag, and Ni was 57.5, 54, 26, 18, and 6 mg/g dry weight bacterial biomass, respectively (at an initial concentration of 100 mg/l). Bacterial cells grown in a sulphate-limiting medium did not show significantly increased copper removal (20.5 mg/g dry weight bacterial biomass at 50 mg/l and 27.5 mg/g at 100 mg/l).

Uranium uptake was much greater than that observed for the other metals included in this study. Furthermore, *Pseudomonas* MGF-48 exhibited a specificity for uranium, as well as accumulating Pb, Cd, and Cu. Specificity for a given metal ion by a bacterial species has been reported by other investigators (Nakajima & Sakaguchi 1986; Lovely *et al.* 1993; Suttleworth & Unz 1993; Wong & So 1993). For example, Lovely & Phillips (1992) and Phillips *et al.* (1995) showed uranium reduction by *Desulfovibrio desulfuricans*. Simmons *et al.* (1995) used *Saccharomyces* and *Candida* spp. to remove Ag and Cu from effluents.

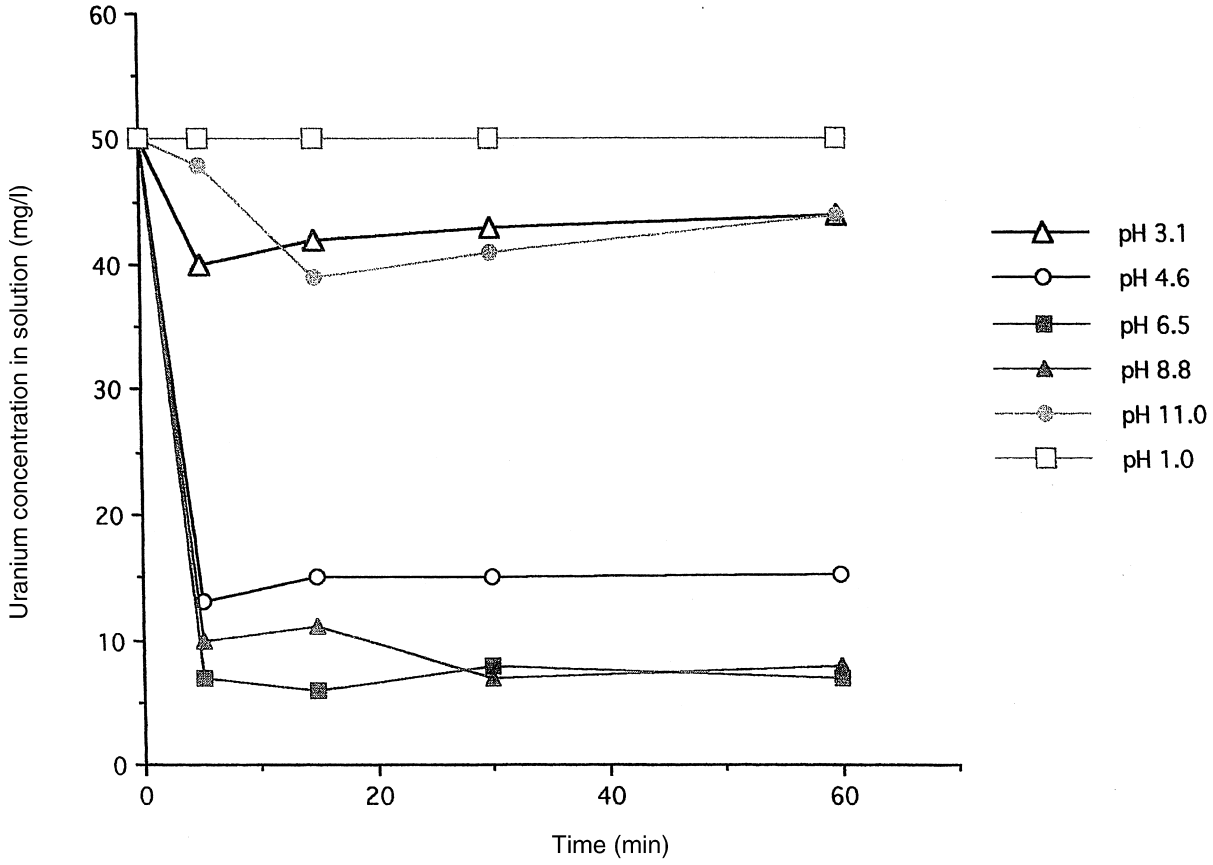

Figure 2. Effect of pH on uranium uptake by *Pseudomonas* MGF-48. At pH 1 (□) no change was observed in uranium concentration in solution. At pH 3.1 (△) ca. 20% of uranium was removed from the solution. At pH 4.6 (○) ca. 74%, at pH 6.5 (■) ca. 88%, at pH 8.8 (▲) ca. 80%, and at pH 11 (●) ca. 22% of the uranium was taken up by the bacterial cells. All assays were conducted after incubation for 15 min. Controls comprised uninoculated media at the respective pH and incubation for 15 min.

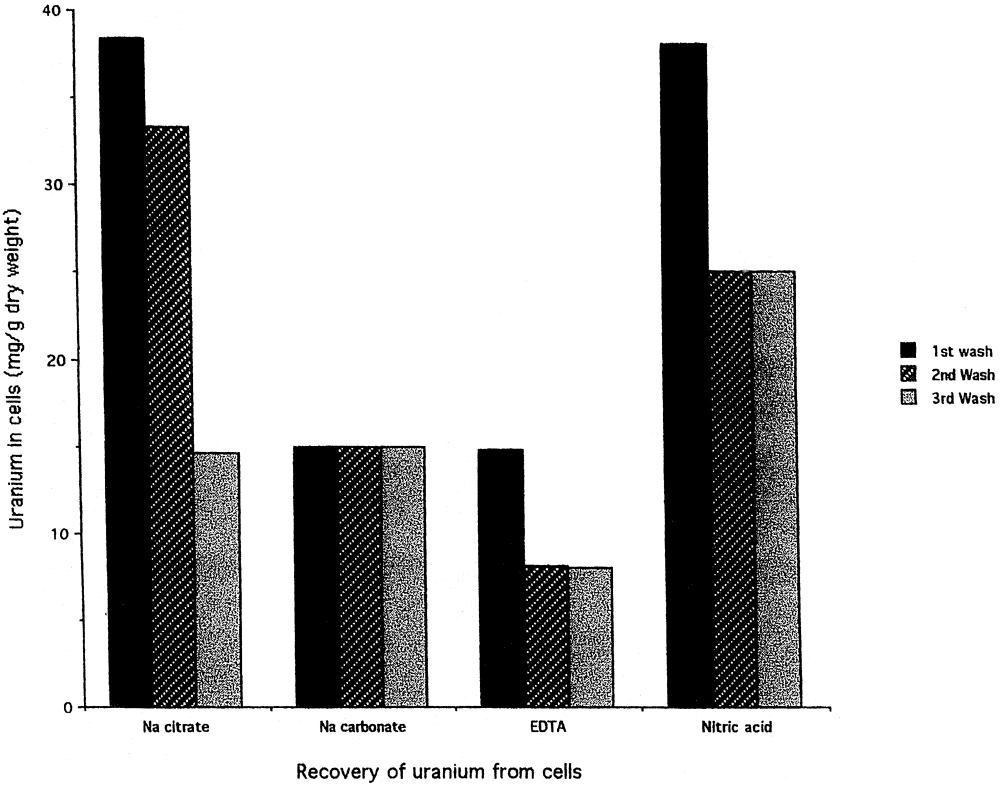

Figure 3. Recovery of uranium from *Pseudomonas* MGF-48 cells. Each of the three bars indicates efficacy of the specific chemical reagent in releasing uranium from the cells at each of three successive washes.

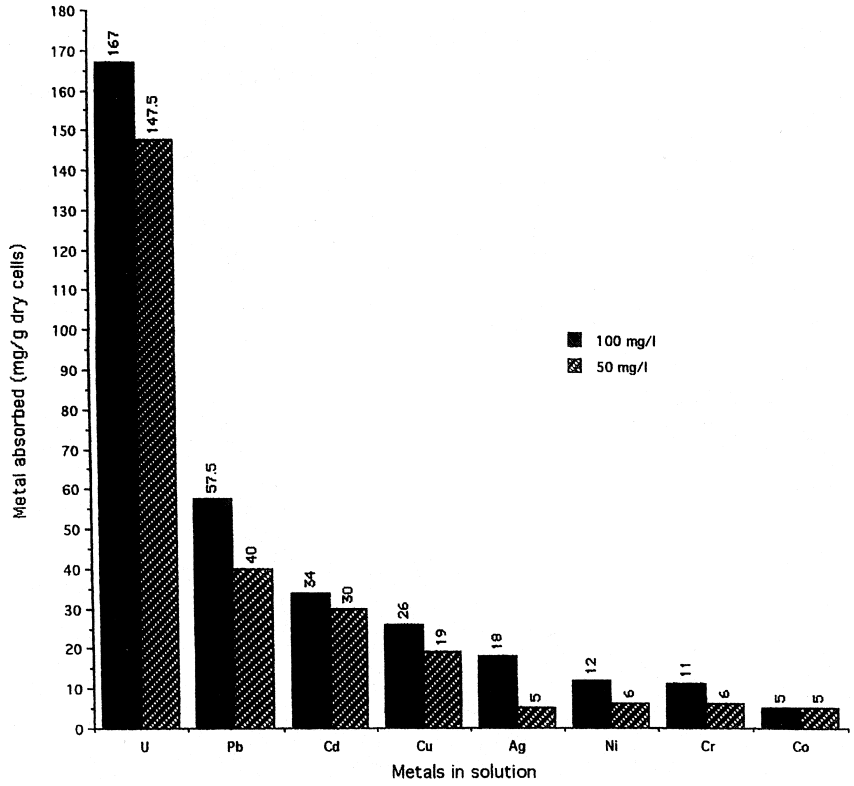

Figure 4. Comparison of metal uptake by *Pseudomonas* MGF-48 at 50 and 100 mg/l concentrations of each metal.

Electron microscopy

To locate sites of uranium accumulation within the cells, transmission electron microscopy was employed. Cells were stained with uranyl acetate. Untreated cells were used as a control. It was observed that uranium accumulation in the cells grown in the presence of 50 mg uranium per litre could be detected as electron dense areas in thin sections, compared with cells grown in the absence of uranium (pictures are not shown).

Heavy metal uptake by immobilized cells

Immobilized cells of the bacterium were prepared and used to determine the efficiency of removal of uranium, cadmium, and copper in concentrations of 100 mg/l. Uranium was efficiently (more than 90%) absorbed by *Pseudomonas* MGF-48 and the bioadsorbed uranium could be recovered by elution with 0.1 M Na_2CO_3. The immobilized cells also absorbed 28% of the cadmium and 21% of the copper. No significant difference was observed in the removal efficiency of immobilized vs. free cells, with a maximum removal obtained at a flow rate of 44 ml/h at 25 °C.

Several principal sites of uranium complex formation in biological systems have been proposed, including accumulation in the cell wall, carbohydrate or protein polyphosphate–uranium complexation, complexing with the carboxyl group of the peptidoglycans in the cell wall, or entry into cells via an energy-dependent mechanism (McLean *et al.* 1996). Strandberg *et al.* (1981) and Macaskie (1991) provide an excellent overview of these alternatives, giving detailed descriptions of mechanisms involved in metal–microbe interactions. Electron microscopy study showed that the bacteria accumulate uranium in the cell wall and along the external cell surfaces, as well as internally with formation of electron-dense areas along the cell envelope and inside the cells (micrographs are not shown). In this study, uptake of uranium was found to be rapid, i.e., occurring within the first 5 min of incubation. Most of the uranium adsorbed could be removed when cells were washed with Na_2CO_3 and EDTA. These findings suggest that uranium uptake involves both surface phenomena and diffusion, the latter most likely a result of increased membrane permeability. Since surface adsorption (metabolism-independent biosorption) is frequently reversible, without causing damage to the biomass or impairment for subsequent use, cells of *Pseudomonas* MGF-48 offer an efficient, inexpensive, and feasible method for removal of metals when applied in aqueous systems (Mullen *et al.* 1989; Marques *et al.* 1991).

Uranium and other metals, such as cadmium, lead and silver, can be removed actively by incorporating pre-grown cells in polyacrylamide gels. From results of studies reported here, more than 90% of the uranium was removed by cells immobilized in polyacrylamide gel. In conclusion, *Pseudomonas* MGF-48 demonstrates a significantly high efficiency of uranium bioaccumulation and, therefore, is an excellent candidate for use in

bioreactors to remove uranium from polluted aqueous effluents.

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