

ACCUMULATION AND REDUCTION OF Cr (VI) IN INDUSTRIAL EFFLUENT BY *BACILLUS* SP. STRAIN CrM-1

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Abstract

A Gram-positive, spore forming *Bacillus* sp. strain CrM-1 was isolated from chromium-contaminated site that could resist up to $40 \mu\text{g mL}^{-1}$ of K_2CrO_4 on nutrient agar and $25 \mu\text{g mL}^{-1}$ in nutrient broth. The strain showed multiple heavy metals and antibiotics resistances and was able to accumulate chromate both by metabolic-dependent active uptake and energy independent passive uptake. Uptake of chromate was maximum in living cells as compared to heat-killed and dried cells. It reduced 92.25 %, 67.44 % and 25.27 % of Cr (VI) at an initial chromate concentration of 100, 500, and $1000 \mu\text{g mL}^{-1}$ after 24 hours with an inoculum size of 2.4^7 cells mL^{-1} . Different heavy metals at low concentrations did not affect the reduction potential of the strain. The strain was able to reduce 96.66 % and 79.23 % of Cr (VI) present in industrial effluent that contained initial Cr (VI) concentration of 150 and $300 \mu\text{g mL}^{-1}$, respectively.

Introduction

Industrialization has increased the harsh effects on the environment through the uneven disposal of heavy metals containing wastes as they are not easily biodegradable, hence, an efficient and cheap treatment process is required to selectively recover them for economic reasons. The removal of toxic metals from the environment by microorganisms has potential as an effective means of remediating heavy metals wastes. Microbe-based technologies can provide an alternative to conventional methods for metal removal.

Chromium, well recognized for its detrimental effects on the environment, where it accumulates throughout the food chain, is generated from leather tanning, metal finishing, wood preserving, ink and paint formation industries. Chromium is present as an element in several different oxidation forms. The most important are trivalent and hexavalent chromium. Trivalent chromium occurs naturally in the environment and an essential nutrient (Bahijri & Mufti, 2002). Hexavalent chromium is a well known carcinogen (Ortegel *et al.*, 2002). Besides this it can also cause skin ulcer, convulsions, kidney and liver damage. To avoid such toxic effects with the Cr (VI), it is exigent to convert this into Cr (III).

Microorganisms especially bacteria are common inhabitants of heavy metal contaminated soil, where they can accumulate and reduce toxic metals. Bacteria are capable of accumulating chromate ions due to metal-attracting compositions on their cell wall or via metabolism-dependent intracellular metal uptake mechanisms. Many bacterial genera such as *Bacillus* (Campos *et al.*, 1995), *Shewanella* (Guha *et al.*, 2001) *Desulfovibrio* (Mabbett *et al.*, 2002), *E. coli* (Puzon *et al.*, 2002), *Pseudomonas* (Park *et al.*, 2000; McLean & Beveridge, 2001) and *Alcaligenes* (Peitzsch *et al.*, 1998) successfully reduce Cr (VI) into Cr (III).

The objective of the present study was to measure the bioaccumulation-biosorption of Cr (VI) from aqueous solution by the bacterial strain in industrial wastewater and its reduction into less mobile Cr (III).

Materials and Methods

Bacterial strain and culture conditions: *Bacillus* sp. strain CrM-1 used in this study was routinely maintained at nutrient agar supplemented with 10 mg mL⁻¹ of K₂CrO₄ and stored at 4 °C.

Morphological, biochemical and physiological characteristics: Strain was characterized morphologically, biochemically and physiologically following Gerhardt *et al.* (1994). The resistance of the strain against different heavy metals (NiSO₄, 500 µg. mL⁻¹; ZnSO₄, 700 µg. mL⁻¹; MnSO₄, 1500 µg. mL⁻¹; CuSO₄, 1000 µg. mL⁻¹; CoCl₂, 500 µg. mL⁻¹; HgCl₂, 50 µg. mL⁻¹ and Pb (NO₃)₂ 1000 µg. mL⁻¹) and antibiotics (streptomycin, 500 µg mL⁻¹; ampicillin, 300 µg mL⁻¹; tetracycline, 30 µg mL⁻¹; kanamycin, 40 µg. mL⁻¹ and chloramphenicol, 5 µg. mL⁻¹) were tested on nutrient agar plates.

Chromate uptake experiments: For experimental purpose strain was grown in nutrient broth at 37 °C with 150 rpm shaking for 24 hours. After 24 hours cultures were harvested and centrifuge at 13000 x g for 10 minutes. Supernatant was discarded and pellet were washed twice with autoclave-distilled water and stored at 4 °C. For uptake experiment three different initial K₂CrO₄ concentrations *i.e.* 100, 500, and 1000 µg. mL⁻¹ were used. The cell mass was treated in different ways as follows: i) One gram of fresh cell pellet was taken and dried at 60 °C for 48 hours (dried cells). ii) Same amount of pellet was taken and heat-killed at 121 °C for 20 minutes (heat killed) and iii) live cell mass. Aqueous solutions of K₂CrO₄ were made from analytical grade chemical in autoclaved distilled water. Known amount of cell mass was exposed to different concentrations of K₂CrO₄ at different time intervals. Samples were then harvested and centrifuged at 7000 x g for 10 minutes. Pellets obtained were washed twice in distilled water. The chromium contents in the pellet were determined by acid digestion method and optical density of the samples was monitored at 540 nm.

Cr (VI) Reduction experiments: To study reduction of hexavalent chromium to trivalent chromium by the strain, three initial K₂CrO₄ concentrations *i.e.* 100, 500, and 1000 µg. mL⁻¹ and two cell concentration *i.e.* 2.4⁷ cells. mL⁻¹ and 9.6⁷ cells mL⁻¹ were used. For reduction experiments, DeLeo & Ehrlich (1994) medium was used. Cultures were kept in an incubating shaker at 150 rpm at 37 °C. At regular time intervals *i.e.* after 24, 48, 72 and 96 hours, samples were taken aseptically and were analyzed for Cr (VI) reduction. Cr (VI) reduction was monitored with the diphenylcarbazide method (APHA, 1976). Optical density of the samples was taken at 540 nm.

Effects of heavy metals: To observe the effects of different metals on the reduction potential of the strain, cultures were also amended with Zr, 200 µg. mL⁻¹; Ni, 200 µg. mL⁻¹; Mn, 200 µg. mL⁻¹; Cu, 200 µg mL⁻¹; Co, 50 µg mL⁻¹ separately. After 24 hours, cultures were harvested and processed as above to check the amount of Cr (VI) reduced in to Cr (III).

Reduction in an industrial effluent: To check the reduction potential of the strain in industrial effluents, samples from metal finishing industry were collected in sterilized bottles. Physico-chemical parameters of the sample were also noted. Strain was exposed to the polluted sample and its subsequent dilutions for which initial Cr (VI) was known. Two different effluent samples were used *i.e.* sample I and II that contain 150 and 300 $\mu\text{g. mL}^{-1}$ of Cr (VI), respectively. Cultures were harvested after 40 hours and the amount of chromate reduced was measured as described above.

Results

Cells of the strain CrM-1 were Gram-positive, spore-forming rods, measuring 3 μm in length and 1 μm in width. The strain was motile, strictly aerobic, catalase and oxidase positive. Strain CrM-1 grew well up to 40 mg. mL^{-1} of K_2CrO_4 on nutrient agar and 25 mg. mL^{-1} in nutrient broth. The temperature preferences remained the same *i.e.* 37 °C both in the presence and absence of 1 mg. mL^{-1} of K_2CrO_4 . The optimum growth of the strain was observed at pH 7.0 but in the presence of K_2CrO_4 optimum growth was obtained at pH 8.0 (Fig. 1a). At low pH (5), growth of the strain was much less in the presence of K_2CrO_4 (Fig. 1a). The log phase in the growth curve of the strain started after 4 hours of incubation, but in K_2CrO_4 supplemented nutrient broth took more time *i.e.* after 8 hours to enter the log phase of the growth curve.

At low K_2CrO_4 concentration *i.e.* up to 2 mg. mL^{-1} in liquid medium, the growth of the strain was much better, but as chromate concentration increased, the growth decreased and at 4 mg. mL^{-1} growth decreased drastically. However, with further increase in chromate level reduction in growth was not that sharp. Strain showed multiple antibiotic and metal resistances. The strain was sensitive to some antibiotics such as streptomycin (500 $\mu\text{g. mL}^{-1}$), kanamycin (40 $\mu\text{g. mL}^{-1}$) and chloramphenicol (5 $\mu\text{g. mL}^{-1}$) but it showed resistance against tetracycline (30 $\mu\text{g. mL}^{-1}$) and ampicillin (300 $\mu\text{g. mL}^{-1}$). In the strain CrM-1, HgCl_2 and CoCl_2 showed more toxicity while Mn, Ni, Zn, Pb and Cu did not hinder the growth of the strain.

Cells of the strains were treated prior to the commencement of the experiment. Living cells showed maximum chromate uptake when compared with heat killed and dried cell mass (Fig. 2b). The amount of chromate uptake by the cells increased with the increase in initial K_2CrO_4 concentration in the medium. After two hours of contact time, the amount of chromium accumulated by the living cells was 76, 116 and 200 $\mu\text{g. g}^{-1}$ fresh weights at an initial chromate concentration of 100, 500 and 1000 $\mu\text{g. mL}^{-1}$, respectively; in heat-killed cells where chromate accumulated were 68, 100 and 172 $\mu\text{g. g}^{-1}$ fresh weights. The amount of chromium accumulated *i.e.* 16, 36 and 72 $\mu\text{g. g}^{-1}$ fresh weight by the dried cell mass was much less than the amount of chromium accumulated by the heat-killed and living cells. It was observed that after 8 hours, chromium accumulated by the living cells was much higher, specially at higher concentration relative to the other two categories *i.e.* heat-killed and dried cells. In all cell types the amount of chromium accumulation increased as contact time of cells with metal solution increased, but the accumulation in the heat killed and dried cells was not as high as was observed in the living cells after 8 hours. This effect was more pronounced at high chromate concentration *i.e.* at 1000 $\mu\text{g. mL}^{-1}$ where the difference in chromium accumulated in heat killed and living cells was much higher.

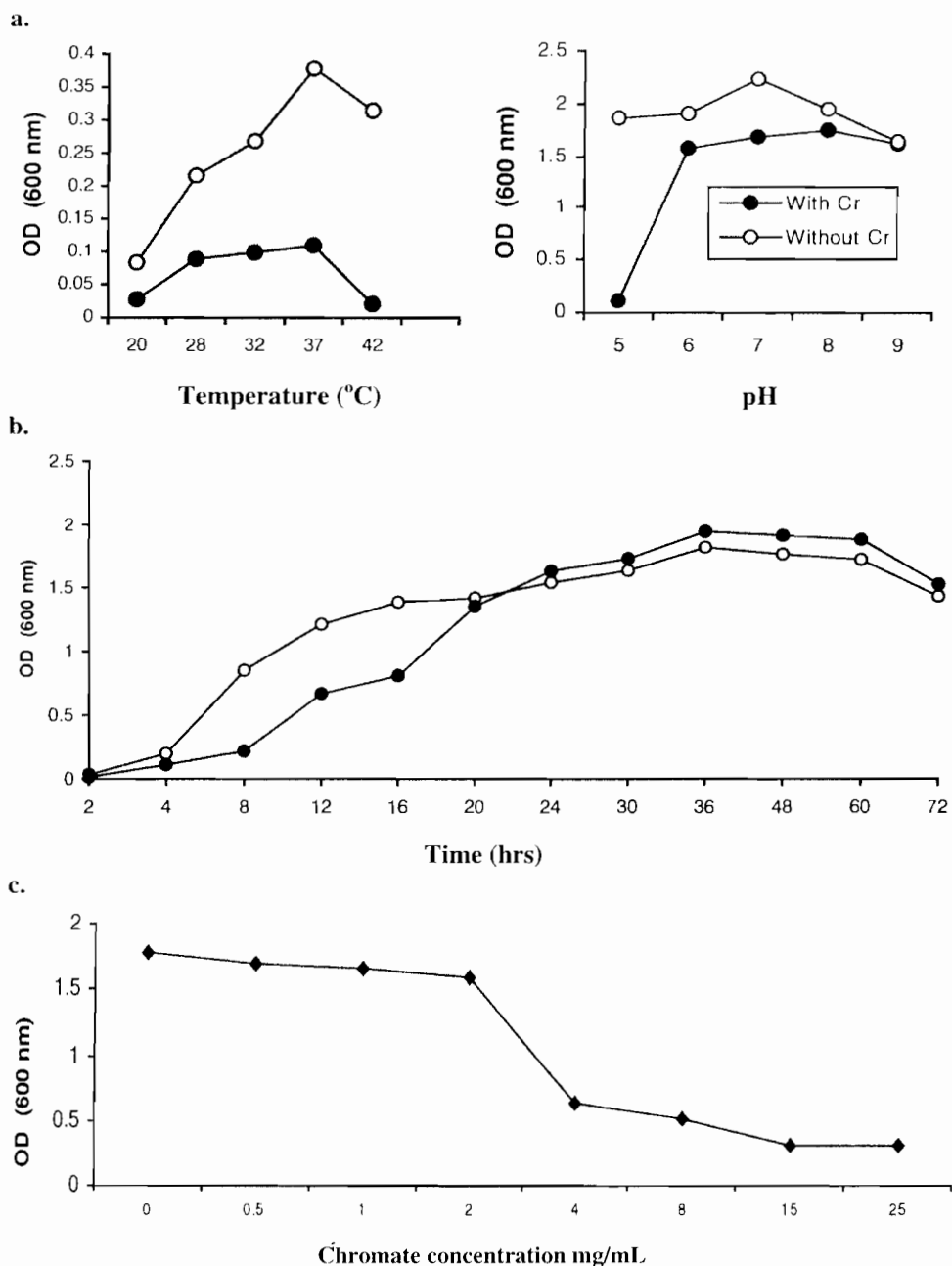


Fig. 1. **a)** Growth responses of bacterial strain in chromate supplemented (1 mg. mL^{-1}) and chromate free nutrient broth at different temperatures ($20\text{-}42 \text{ }^\circ\text{C}$) and growth pH ($5\text{-}9$). **b)** Growth responses of bacterial strain after different time of incubation in the presence and absence of chromate (1 mg. mL^{-1}). **c)** Growth responses of bacterial strain in nutrient broth at different concentrations of potassium chromate.

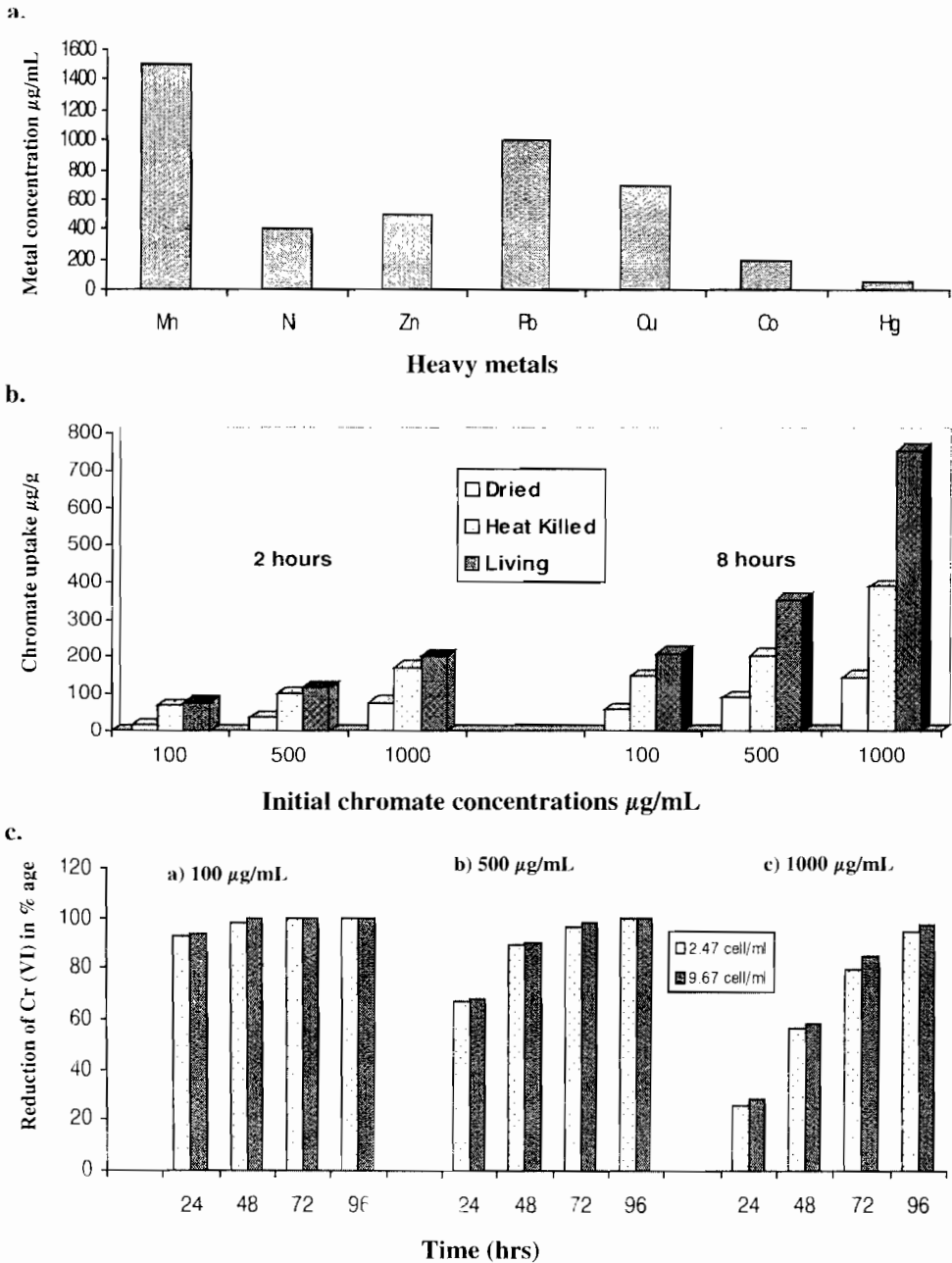


Fig. 2. **a)** Heavy metal resistance profile of chromium resistant bacterial isolates. **b)** Uptake of potassium chromate at three chromate concentrations. Cells were used as dried, heat killed and as live. Contact time A) after 2 hours B) after 8 hours. **c)** Reduction of hexavalent chromium (in %) in the presence of two inocula sizes ($2.47 \text{ cells. mL}^{-1}$ and $9.67 \text{ cells. mL}^{-1}$) at different time intervals: a) $100 \text{ µg. mL}^{-1} \text{ K}_2\text{CrO}_4$, b) $500 \text{ µg mL}^{-1} \text{ K}_2\text{CrO}_4$, c) $1000 \text{ µg. mL}^{-1} \text{ K}_2\text{CrO}_4$.

Table. 1A. Effects of different heavy metals on Cr (VI) reduction potential of the strain (Initial chromate concentration 500 $\mu\text{g. mL}^{-1}$). B. Chromate reduction in an industrial effluent. (a*= Initial Cr (VI) concentration 150 $\mu\text{g. mL}^{-1}$, b= Initial Cr (IV) concentration 300 $\mu\text{g. mL}^{-1}$).**

Source	% Reduction
A. Metals ($\mu\text{g. mL}^{-1}$)	
Control	86.2
Ni (200)	86.4
Mn (200)	87.4
Zn (200)	86.6
Cu (200)	85.4
Co (50)	85.2
B. Industrial effluents	
a*	96.66
b**	79.23

Fig. 2c shows hexavalent chromium reduction percentage by the strain CrM-1 at three different initial chromate concentrations *i.e.* 100, 500 and 1000 $\mu\text{g. mL}^{-1}$ by two different inocula sizes *i.e.* 2.4⁷ cells. mL⁻¹ and 9.6⁷ cells. mL⁻¹. At an initial chromate concentration of 100 $\mu\text{g. mL}^{-1}$, strains completely reduced entire chromate present in the medium within 72 hours with both cell concentrations. At a high chromate concentration especially at 1000 $\mu\text{g. mL}^{-1}$, the percentage of hexavalent chromium reduction decreased, but overall more amount of chromate was reduced with the same time period. No major difference in reduction potential of the strain was observed under both inocula sizes, but with more inoculum size chromate reduction was slightly more.

At initial concentration of 1000 $\mu\text{g. mL}^{-1}$, the strain reduced 25.27 %, 56.78 %, 79.04 % and 94.62 % of chromium (VI) after 24, 48, 72 and 96 hours, respectively, in comparison with a high inoculum size where these reduction percentages were 28.64 %, 58.44 %, 84.32 % and 96.63 %. Different metals such as Ni, Zn, Cu, Mn and Co at low concentrations did not affect the reduction potential of the strains as compared to the control (Table 1). Strains reduced 84.4 %, 87.4 %, 86.6 %, 85.4 % and 85.2 % in the presence of Ni, Mn, Zn, Cu and Co, respectively, in comparison to control where it reduced 86.2 % of chromium (VI) within 24 hours. In another experiment where the industrial effluent was used directly, strain reduced 96.66 % and 79.23 % of chromium (VI) in sample-1 and sample-2, respectively after 40 hours of incubation.

Discussion

The fate of chromium in the environment strongly depends on its valence state. Hexavalent chromium is readily bio-available due to high solubility and mobility as compared to trivalent chromium. To restrict the mobility and bioavailability of Cr (VI) in the environment it is necessary to convert this carcinogenic oxidation state of chromium into Cr (III). Currently, different biological and chemical methods are in way to treat Cr (VI). Chemical methods are much more expensive and the production of by-products during chemical reaction also have side effects. Biological methods of Cr (VI) reduction are less expensive and free from any side effect. Microorganisms such as bacteria and fungi play a suitable role in the decomposition and detoxification of heavy metals. Many genera of bacteria are reported to successfully convert toxic Cr (VI) into Cr (III) both

aerobically as well as anaerobically. The present study focused on the uptake and reduction of toxic hexavalent chromium into trivalent chromium by the bacterial strain CrM-1 which showed very high-level resistance to chromate, up to 40 mg. mL⁻¹ of K₂CrO₄ on nutrient agar and 20 mg. mL⁻¹ in nutrient broth. Strains used by Ganguli & Tripathi (2002) could resist up to 50 µg. mL⁻¹ of chromate while *Bacillus subtilis* used by Garbisu *et al.* (1998) was resistant to 1 µM K₂CrO₄. Hence the resistance level of CrM-1 is very high as compared to strains reported in literature. Due to the presence of chromate, the log phase was delayed almost 8 hours in comparison to simple medium where log phase started immediately after 4 hours because under chromate stress, the cells take more time to divide. Ganguli & Tripathi (2002) found that in the presence of 100 µg. mL⁻¹, the generation time increased to 57 minutes as compared to normal 42 minutes.

In the Cr (VI) reduction experiments the concentration of hexavalent chromium in all the three initial chromate concentration decreased with increasing incubation time. At low initial chromate concentration, the strain was able to remove all the Cr (VI) present in the solution with 72 hours of incubation but at higher concentration *i.e.* 1000 µg. mL⁻¹ was unable to remove all the Cr (VI) present in the solution. We used high initial Cr (VI) concentration *i.e.* 100, 500 and 1000 µg. mL⁻¹ compared with other workers. Quilntana *et al.* (2001) used an initial chromate concentration of 10 µg. mL⁻¹ for reduction experiments, while Ganguli & Tripathi (2002) used an initial Cr (VI) concentration of 10, 25, 50, and 100 µg. mL⁻¹.

Different heavy metals did not appear to have any significant effect on the chromium reduction potential of the strain. A slight decrease in Cr (VI) reduction *i.e.* 0.92 % and 1.16 % was noted with Cu (200 µg. mL⁻¹) and Co (50 µg. mL⁻¹), respectively, after 24 hours' incubation period when compared with control (Table-1). McLean & Beveridge (2001) observed that at an initial Cr (VI) concentration of 20 µg. mL⁻¹ heavy metals such as Ar (60 µg. mL⁻¹) and Cu (20 and 40 µg. mL⁻¹) did not affect the reduction potential of *Pseudomonad*. The successfulness of any bacterial strain is possible if it gave same results in the field where a mixture of pollutants is gathered. Keeping in view this factor, the effluent sample was collected from chromium-polluted areas and the strain was exposed directly to this effluent. Interestingly, the strain CrM-1 reduced a considerable amount of Cr (VI) present in the effluent sample within 40 hours of incubation.

Results obtained from this study show that the living cells had a high accumulation of K₂CrO₄ *i.e.* 208, 352 and 756 µg. g⁻¹ at an initial chromate concentration of 100, 500 and 1000 µg. mL⁻¹ after 8 hours of incubation in comparison with the accumulation amount of 76, 116 and 200 µg. g⁻¹ after 2 hours. While in heat-killed cells, the amount of chromium accumulated after 2 hours of incubation was nearly same as in living cell but this difference was much increased after 8 hours (Fig. 2b). This might be due to the initial passive uptake (transport) of K₂CrO₄ along with water for some time, but with the passage of time the cells attained a maximum level of K₂CrO₄ and further uptake stopped while in the living cells through active transport metabolic dependent chromium uptake continued. This effect was clearly observed in living cells after 8 hours. The reduction in K₂CrO₄ accumulation capacity of dried cell mass in comparison with heat-killed and living cells may be attributed to the loss of intracellular uptake, as the cell organelles may be damage or deformed. It shows, that in this strain two types of chromate uptake mechanisms are involved *i.e.* metabolism-dependent active transport and passive uptake.

Due to strong affinity for accumulating and reducing Cr (VI) in solution, strain CrM-1 can be considered as a good object for application at an industrial scale for removing toxic Cr (VI).

References

- APHA, 1976. *Standard Methods for the Examination of Water and Waste Water*. 16th Ed., Amer. Public Health Assoc., Washington, D. C.
- Bahijri, S.M.A and A.M.B. Mufti. 2002. Beneficial effects of chromium in people with type 2 diabetes. and urinary chromium response to glucose load as a possible indicator of status. *Biol. Tr. Elem. Res.*, 85: 97-110.
- Campos, J., Martinez-Pacheco and C. Cervantes. 1995. Hexavalent chromium reduction by a chromate-resistant *Bacillus* sp. Strain. *Anton. van Leeuwenh.*, 9: 133-141.
- DeLeo, P.C. and H.L. Ehrlich. 1994. Reduction of hexavalent chromium by *Pseudomonas fluorescens* LB 300 in batch and continuous cultures. *Appl. Microb. Biotech.*, 40: 756-759.
- Ganguli, A. and A.K. Tripathi. 2002. Bioremediation of toxic chromium from electroplating effluents by chromate-reducing *Pseudomonas aeruginosa* A2Chr in two bioreactors. *Appl. Microb. Biotech.*, 58: 416-420.
- Garbisu, C., I. Alkorta, M.J. Llama and J.L. Serra. 1998. Aerobic chromate reduction by *Bacillus subtilis*. *Biodegradation*, 9: 133-141.
- Gerhardt, P., R.G.E. Murry, W.A. Wood and N.R. Kreig. 1994. *Methods for General and molecular Bacteriology*. Amer. Soc. for Microb., Washington, D.C.
- Guha, H., K. Jayachandran and F. Maurrasse. 2001. Kinetics of chromium (VI) reduction by a type strain *Shewanella* alga under different growth conditions. *Environ. Pollut.*, 115: 209-218.
- Mabbett, A.N., J.R. Lloyd and L.E. Macaskie. 2002. Effect of complexing agents on reduction of Cr (VI) by *Desulfovibrio vulgaris* ATCC 29579. *Biotech. Bioeng.*, 79: 389-397.
- McLean, J. and T.J. Beveridge. 2001. Chromate reduction by a Pseudomonad isolated from a site contaminated with chromated copper arsenate. *Appl. Environ. Microb.*, 67: 1076-1084.
- Ortegel, J.W., E.D. Staren, L.P. Faber, W.H. Warren and D.P. Braun. 2002. Modulation of tumor-infiltrating lymphocyte cytolytic activity against human non-small cell lung cancer. *Lung Cancer*, 36: 17-25.
- Park, C.H., M. Keyhan, B. Wielnga, S. Fendorf and A. Matin. 2000. Purification to Homogeneity and Characterization of a novel *Pseudomonas putida* Chromate Reductase. *Appl. Environ. Microb.*, 66: 1788-1795.
- Peitzsch, N., G. Eberz and D.H. Nies. 1998. *Alcaligenes eutrophus* as a bacterial chromate sensor. *Appl. Environ. Microb.*, 64: 453-458.
- Puzon, G.J., J.N. Petersen, A.G. Roberts., D.M. Kramer and L. Xun. 2002. A bacterial flavin reductase system reduces chromate to a soluble chromium (III)-NAD⁺ complex. *Biochem. Biophys. Res.*, 294: 76-81.
- Quilntana, M., G. Curutchet and E. Donati. 2001. Factors affecting Cr (VI) reduction by *Thiobacillus ferrooxidans*. *Biochem. Eng. J.*, 3519: 1-5.