J. Microbiol. Biotechnol. (2002), **12**(1), 137–141

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Bioaccumulation of Chromium Ions by Immobilized Cells of a Filamentous Cyanobacterium, *Anabaena variabilis*

KHATTAR, JASVIR I. S.*, TANGIRALA A. SARMA, DAVINDER P. SINGH, AND ANURADHA SHARMA

Department of Botany, Punjabi University, Patiala-147 002, India

Received: November 3, 2001
Accepted: December 24, 2001

Abstract *Anabaena variabilis* ATCC 29413 grew in chromium (Cr) containing Chu-10 (basal) and nitrate-supplemented media, and the growth of the organism in 100 μM chromium was found to be 50% of that in control medium. The growth in nitrate (NO_3^-) supplemented cultures was better as compared to cultures grown in basal medium. Free cells from basal and nitrate-supplemented media removed 5.2 and 7.4 nmol of chromium mg^{-1} protein in 8 h, respectively, from the medium containing 30 μM chromium. The efficiency of chromium removal increased 7-fold in imidazole buffer (0.2 M, pH 7.0). A cell density equivalent to 100 μg protein ml^{-1} was found to be optimum for maximum Cr removal. Entrapment of cells in calcium-alginate beads did not affect the rate of Cr uptake by the cells. The efficiency of the laboratory-scale continuous flow bioreactor (12.5×2 cm) loaded with alginate-immobilized cells (10 mg protein) and fed with 30 μM chromium solution was compared at different flow rates. The efficiency of the bioreactor varied with flow rates. In terms of percent removal of Cr from influent, a flow rate of 0.1 ml min^{-1} was found to be optimum for 6 h (54% Cr removal efficiency). Maximum amount of Cr (883 nmol) was removed by the cells in 3 h at a flow rate of 0.5 ml min^{-1}. The potential use of *A. variabilis* in removing Cr from industrial effluents is discussed.

Key words: Bioaccumulation, heavy metals, chromium, bioreactor, cyanobacteria, *Anabaena variabilis*

Contamination of water bodies by toxic heavy metals due to human activity and by industry is a world-wide environmental problem. Although, at low concentrations, some of these heavy metals stimulate biological processes, all become very toxic at threshold concentrations. Being nonbiodegradable, these metals act as persistent pollutants in the environment. Thus, heavy metal contamination of water poses a serious threat not only to the environment but also to human beings and animals, as these metals can enter the food chain at various trophic levels. Conventional precipitation methods or synthetic ion exchangers cannot alleviate the problem of water contamination, because of the high cost and low economic viability of these methods. Use of biomass is considered to be a viable alternative to conventional methods for metal recovery [24]. Several studies have been undertaken to evaluate the usefulness of microbes for the purification of wastewater containing heavy metals [2, 7, 22, 29]. Certain algae have been shown to accumulate heavy metal ions from aqueous solutions and to concentrate them in significant amounts [8, 17, 27]. Toxic effects of these metals and the mechanisms of their uptake have been well documented [10, 15, 18, 23]. The potential of living and nonliving microalgae to eliminate heavy metal ions from aqueous systems has been studied mainly for such cationic metal ions as Ni^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, and Pb^{2+} [1, 19, 22, 25, 28]. Not much information is available in the literature concerning the removal of Cr^{6+} ions by algae. Removal of Cr^{6+} ions by the cyanobacterium *Anacystis nidulans,* an isolate from polluted waters, has been demonstrated [11]. During the screening of various cyanobacteria for their ability to scavenge metal ions, *Anabaena variabilis*, a laboratory organism, was found to be tolerant to chromium. We have, therefore, attempted to determine the potential of immobilized cells of this cyanobacterium to remove Cr ions from water by characterizing its bioaccumulation.

MATERIAL AND METHODS

Anabaena variabilis Kuetz. ATCC 29413, a filamentous, diazotrophic cyanobacterium was obtained through the courtesy of Prof. P. Boeger of Konstanz, Germany. Clonal cultures of the organism were cultivated in 250-ml Erlenmeyer

*Corresponding author
Phone: 91-175-282461; Ext: 6282; Fax: 91-175-283073;
E-mail: jisk@pbi.ac.in

flasks containing Chu-10 medium devoid of combined inorganic nitrogen source (basal medium) as modified by Safferman and Morris [20] at 28±2°C in a culture room, and the cultures were illuminated with daylight fluorescent tubes (fluence 9.8 W/m^2 on the surface of culture vessels) for 14 h a day. The cultures were shaken by hand, twice daily. To obtain nitrate-rich medium, nitrate in the form of potassium nitrate (10 mM) was supplemented in the above medium.

Growth

Growth of the organism was monitored by taking absorbance of the cultures at 660 nm (Spectronic 20; Bausch & Lomb, U.S.A.) in medium with or without different concentrations (10–100 µM) of chromium. Stock solutions of chromium were prepared by dissolving $K_2Cr_2O_7$ (E. Merck, India) in water and were sterilized by autoclaving.

Immobilization

Cells were immobilized by entrapment in calcium-alginate. Thick suspension of exponentially growing cells was obtained by centrifugation and washed three times with distilled water. Protein content of the suspension was determined. Alginate solution was prepared by dissolving 5.5 g of sodium alginate in 150 ml of Chu-10 medium at 80°C. A concentrated suspension (50 ml) of cells with known protein content was added and mixed to sterilized and cooled alginate solution. By means of a syringe, the above mixture was added drop-wise into sterilized 0.1 M $CaCl_2$ solution at room temperature. Beads of uniform diameter of 4 mm were formed. These beads were hardened by suspending them in 0.1 M $CaCl_2$ solution for 24 h. From the total protein content of the suspension used and the number of beads so formed, the protein content of each bead was calculated. The beads were washed with sterilized distilled water and wiped with sterilized absorbent paper before use. When buffers were used for metal ion uptake studies, beads were prepared by dissolving sodium alginate in the corresponding buffer.

Estimation of Cr

In batch cultures, when free cells were used, suitable aliquots were withdrawn at regular intervals of 2 h and cell suspensions were quickly centrifuged. The amount of Cr ions left in medium/buffer was determined. In batch cultures using immobilized cells, suitable aliquots along with corresponding number of beads (so as to maintain constant amount of cell biomass per milliliter of incubation mixture throughout the experiment) were withdrawn and the concentration of Cr left in the aliquot was determined using a Cr electrode in a multichannel ion analyzer (Consort, Belgium). Control experiments with alginate beads without algal biomass revealed negligible amounts of Cr ion adsorption on beads.

Continuous Flow Bioreactor

For metal removal experiments in a continuous flow laboratory bioreactor, a glass bioreactor of 12.5 cm length and 2 cm internal diameter was used. Sterilized bioreactor was packed with biomass-loaded beads (10 mg protein). Cr (30 µM) in imidazole-HCl buffer (0.2 M; pH 7.0) was pumped with the help of a peristaltic pump connected to the reactor at the bottom of the vessel. The flow rates ranged from 0.05 ml to 0.5 ml min^{-1}. The eluants were collected at regular intervals and the amount of Cr removed was calculated by determining the amount of Cr left in the collected fractions. All metal uptake experiments were conducted in light at a radiant flux of 9.8 W/m^2 at 28°C in the culture room.

Protein content of the cells was determined after alkaline hydrolysis [9]. The data presented are averages of two independent experiments.

Results and Discussion

Metal-free nitrate-supplemented cultures exhibited 33% more growth of the organism when compared to basal medium (data not shown). When chromium (10–100 µM) was added to basal and nitrate-supplemented media, the latter again supported better growth. Growth of the organism in different concentrations of chromium in NO_3^--supplemented medium revealed that, although there was metal dependent decrease in the growth, the organism showed 50% growth in 100 µM chromium as compared to control (Fig. 1). Heavy metals are known to inhibit a number of physiological processes, which include respiration, photosynthesis, nitrogen

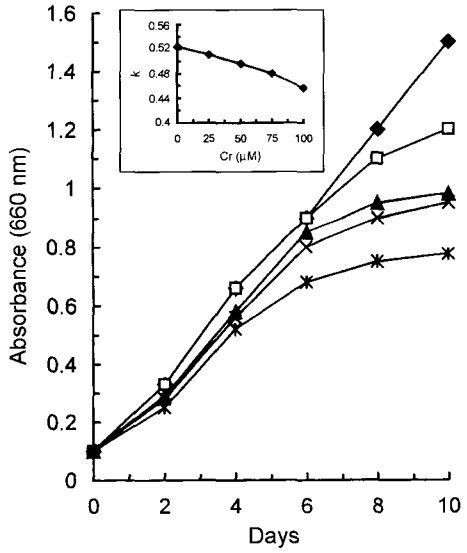

Fig. 1. Growth of *A. variabilis* in Chu-10 (◆) and Cr supplemented media (10 µM, □; 25 µM, ▲; 50 µM, ×; 100 µM, ✳). Inset: Effect of Cr on specific growth rate constant.

fixation and synthesis of pigments as a consequence of inactivation and denaturation of key enzymes of the metabolic pathways [4, 6, 23]. Inhibition of nitrogenase activity and nitrate reductase activity by chromium has been studied by Rai and Dubey [14] in *Anabaena doliolum.*

The growth of the organism in chromium-supplemented media may be due to the fact that the organism (i) shows resistance at the uptake level or (ii) is able to take up the metal and then detoxify it by some means. To test the ability of the organism to take up chromium ions, two types of uptake experiments were performed. In the first type, metal uptake was compared by free cells from basal medium (containing 30 μM chromium) in the presence and absence of nitrate. Cell mass equivalent to 200 μg protein ml^{-1} removed chromium ions at the rate of 5.2 and 7.4 nmol mg^{-1} protein in 8 h from basal and nitrate-supplemented media, respectively. Metal uptake by microorganisms has been shown to be pH dependent [12]. In the second type of uptake experiments, imidazole buffer with different pH values was used. The cell biomass and chromium concentration used were similar to those in the first type of experiments. A comparison of the metal uptake at different pH values (6.5 to 8.0) revealed that maximum uptake occurred at pH 7.0. Cells grown in basal and nitrate-supplemented media removed 38 and 49 nmol Cr, respectively, at this pH, which represents a 7-fold increase in uptake over the first type of experiments (Fig. 2). There are two mechanisms of heavy metal uptake by microbial cells: an initial uptake by adsorption on the cell surfaces, and a slower active intracellular accumulation [26]. Passive adsorption of metal cations on cell wall surfaces is due to the presence of negatively charged groups on cell walls or in the extracellular polymers of the mucilage [5, 13]. *A. variabilis* does not produce extracellular mucilage and the passive adsorption of chromate ions $(CrO_4)^{2-}$ on negatively charged cell walls in this organism is ruled out. This indicates that the cells actively take up Cr intracellularly. Cr uptake by free cells was also compared in buffer (pH 7.0; having 30 μM Cr) by taking different cell densities (50–300 μg protein ml^{-1}) to determine saturating cell concentrations. Increase in biomass of cells grown in basal medium from 50 to 100 μg protein ml^{-1} supported enhanced uptake from 30 nmol to 58.7 nmol of Cr taken up per mg protein in 8 h. With further increase in biomass to 200 and 300 μg protein ml^{-1} the uptake decreased to 38 and 25.6 nmol, respectively (Fig. 3). This may probably be due to decreased availability of Cr to cells. When nitrate grown cells were used, again 100 μg protein ml^{-1} was found to be the optimum cell concentration with 24% increase in uptake over that by cells grown in basal medium. Thus, for the 30 μM Cr concentration, cell biomass having 100 μg protein ml^{-1} was found to be the optimum cell density for maximum Cr removal efficiency. As nitrate grown cells performed better than those from basal medium, nitrate grown cells were used in subsequent experiments. The availability, uptake, and subsequent toxicity of a particular metal depends upon several factors including

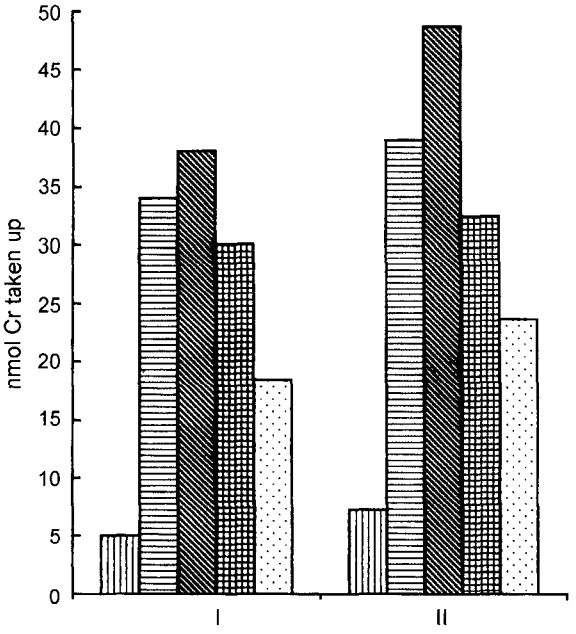

Fig. 2. Cr uptake (mg^{-1} protein) by free cells of *A. variabilis* grown in basal (I) and nitrate-supplemented (II) medium after 8 h of incubation from medium (▥) and imidazole-HCl buffer with different pH values (pH 6.5, ▤; pH 7.0, ▧; pH 7.5, ▦; pH 8.0, ▩).

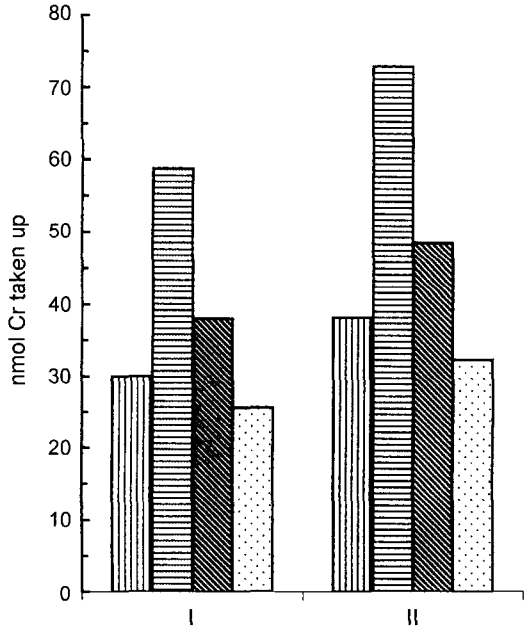

Fig. 3. Cr uptake (mg^{-1} protein) by free cells of *A. variabilis* at varying cell densities (μg protein/ml) from imidazole-HCl buffer (0.2 M, pH 7.0) after 8 h of incubation.
Experimental conditions were similar to that described in Fig. 2 (50, ▥; 100, ▤; 200, ▧; 300, ▦).

pH, amount of biomass present in the system, and the metal species and its concentration [14, 16, 30].

Alginate-immobilized cells in batch cultures, like free cells, exhibited maximum Cr removal efficiency at a cell density of 100 μg protein ml^{-1} and a pH of 7.0. This showed that the rate of uptake of Cr by the organism was not altered when immobilized in alginate. Asthana *et al.* [1], while studying metal removal by *Nostoc muscorum* cells, showed that alginate beads alone contributed towards 43% of Ni adsorption in 2 h. In the present study, alginate beads without biomass did not adsorb Cr ions.

In continuous flow bioreactor experiments, chromium solution (30 μM) was pumped into the reactor at different flow rates. Thus, fractions with different volumes were available at regular intervals of time. The rates of metal bioaccumulation have been calculated on the basis of each milliliter of metal solution passed through it. At a flow rate of 0.05 ml min^{-1}, the maximum uptake rate was during the first half hour, after which it leveled off at 1.0 nmol Cr being taken up mg^{-1} protein h^{-1} from each ml of solution passed through it. When the flow rate was fixed at 0.1 ml min^{-1}, metal uptake rate increased up to 45 min and then leveled off at 1.66 nmol Cr removed (Fig. 4). At a flow rate of 0.2 ml min^{-1}, there was an increase in uptake rate during the first half an hour, whereafter it leveled off at 1.5 nmol of Cr removed for up to 2 h, and then it gradually decreased. The bioreactor worked for 3 h only at the flow rate of 0.5 ml min^{-1} (Fig. 4). The efficiency of the bioreactor for metal removal at different flow rates is given in Table 1. If overall efficiency of the bioreactor for 6 h is calculated, then the flow rate of 0.1 ml min^{-1} turns out to be optimum with 54% metal removal efficiency. With increase in flow rate up to 0.5 ml min^{-1}, the efficiency of the reactor decreased to 16.35% and 32.7% when calculated for 6 h and for 3 h, respectively. On the other hand, if we calculate the total amount of Cr accumulated, then the flow rate of 0.5 ml min^{-1} seems to be optimum with 883 nmol of Cr being removed. The reactor worked for 3 h only at this flow rate. It seems that cells became saturated with this amount of Cr and were then no longer able to accumulate more metal ions. Although the use of isolated mother cell walls or nonviable biomass has been suggested for removal of metal ions from aqueous solutions [3, 21, 25, 26], whole cells are required for continuous uptake of metal ions. Our results show that cells of *A. variabilis* can efficiently remove Cr ions from the influent in a laboratory-scale continuous flow bioreactor. This organism thus has a good potential to be employed for removal of Cr ions from industrial effluents.

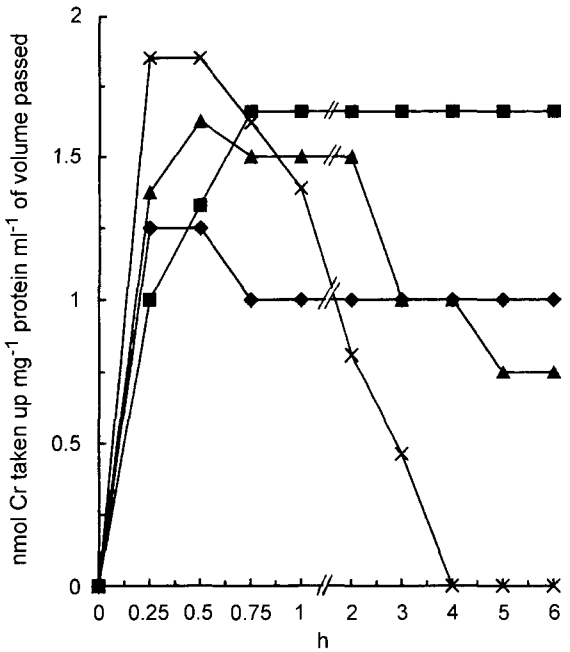

Fig. 4. Cr removal by immobilized cells of *A. variabilis* (10 mg protein) in a continuous flow bioreactor at different flow rates (ml min^{-1}).
(0.05, ◆; 0.1, ■; 0.2, ▲; 0.5, ✳.) Imidazole-HCl buffer (0.2 M; pH 7.0) containing 30 μM Cr was pumped into the bioreactor.

Table 1. Efficiency of the bioreactor for Cr removal at different flow rates for 6 h.

Flow rate (ml min^{-1})	Total volume passed (ml)	Total nmol passed	Total nmol removed	Efficiency (%)
0.05	18	540	182	33.70
0.1	36	1,080	585	54.10
0.2	72	2,160	780	36.1
0.5	180	5,400	883	16.35
	(90)	(2,700)	(883)	(32.7)*

*The values in parentheses refer to those when running time for the bioreactor is considered to be 3 h.

Acknowledgments

The authors are thankful to Head, Department of Botany, Punjabi University, Patiala for providing necessary laboratory facilities. Financial assistance from Council of Scientific & Industrial Research, New Delhi in the form of a research Scheme No. 38(888)-95-EMR-II and University Grants Commission, New Delhi in the form of Departmental Research Support under Special Assistance Program is gratefully acknowledged.

References

1. Asthana, R. K., S. Chatterjee, and S. P. Singh. 1995. Investigations on nickel biosorption and its remobilization. *Process Biochem.* **30:** 729–734.
2. Bender, J., F. L. Richard, and P. Phillips. 1995. Uptake and transformation of metals and metalloids by microalgal mats

and their use in bioremediations. *J. Indus. Microbiol.* **14:** 113–118.
3. Butter, T. J., L. M. Evison, I. C. Hancock, F. S. Holland, K. A Matis, A. Philipson, A. I. Sheikh, and A. I. Zouboulis. 1998. The removal and recovery of cadmium from dilute aqueous solutions by biosorption and electrolysis at laboratory scale. *Wat. Res.* **32:** 400–406.
4. De Filippis, L. F. and C. K. Pallaghy. 1976. The effect of sublethal concentrations of mercury and zinc on *Chlorella*. II. Photosynthesis and pigment composition. *Z. Pflanzen. Physiol.* **78:** 314–322.
5. Doers, M. and D. L. Parker. 1988. Properties of *Microcystis aeruginosa* and *M. flos-aquae* (Cyanophyta) in culture. *J. Phycol.* **24:** 502–508.
6. Fernandez-Pinas, F., P. Mateo, and I. Bonilla. 1991. Physiological and structural responses of the cyanobacterium *Nostoc* UAM 208 to free Cd ions. *Plant Physiol. (Life Sci. Adv.)* **10:** 173–178.
7. Gadd, G. M. 1990. Biosorption. *Chem. Ind.* **13:** 421–426.
8. Gadd, G. M. 1990. Accumulation of metals by microorganisms and algae. pp. 401–433. *In* K. J. Rehm (ed.), *Biotechnology Handbook, 6B: Special Microbial Processes.* VCH Verlagsgesellschaft, Weinheim.
9. Hewitt, B. R. 1958. Spectrophotometric determination of proteins in alkaline solutions. *Nature (Lond.)* **182:** 246.
10. Jin, X., C. Nalewajko, and D. J. Kushner. 1996. Comparative study of nickel toxicity to growth and photosynthesis in nickel resistant and sensitive strains of *Scenedesmus acutus f. alternans* (Chlorophyceae). *Microbial. Ecol.* **31:** 103–114.
11. Khattar, J. I. S., T. A. Sarma, and D. P. Singh. 1999. Removal of chromium ions by agar immobilized cells of the cyanobacterium *Anacystis nidulans* in a continuous flow bioreactor. *Enzyme Microbial Technol.* **25:** 564–568.
12. Peterson, H. G., F. P. Healey, and R. Wagemann. 1984. Metal toxicity to algae: a highly pH dependent phenomenon. *Can. J. Fish Aquat. Sci.* **41:** 974–979.
13. Plude, J. L., D. L. Parker, O. J. Schommer, R. J. Timmerman, S. A. Hagstrom, J. M. Joers, and R. Hnasko. 1991. Chemical characterization of polysaccharides from the slime layer of cyanobacterium *M. flos-aquae* C3-40. *Appl. Environ. Microbiol.* **57:** 1696–1700.
14. Rai, L. C. and S. K. Dubey. 1988. Chromium toxicity to a cyanobacterium: Possible role of carbon sources in toxicity amelioration. *Microbios* **55:** 193–203.
15. Rai, L. C., J. P. Gaur, and H. D. Kumar. 1981. Phycology and heavy metal pollution. *Biol. Rev.* (Cambridge) **56:** 99–151.
16. Rai, L. C., Y. Husaini, and N. Mallick. 1998. pH altered interaction of aluminium and fluoride on nutrient uptake, photosynthesis and other variables of *Chlorella vulgaris*. *Aquatic Toxicol.* **42:** 67–84.
17. Rai, L. C., H. D. Kumar, F. H. Mohn, and C. J. Soeder. 2000. Services of algae to environment. *J. Microbiol. Biotechnol.* **10:** 119–136.
18. Raizada, M. and L. C. Rai. 1985. Metal induced inhibition of growth, heterocyst differentiation, carbon fixation and nitrogenase activity of *Nostoc muscorum:* Interaction with EDTA and calcium. *Microbios Lett.* **30:** 153–161.
19. Robinson, P. K. and S. C. Wilkinson. 1994. Removal of aqueous mercury and phosphate by gel entrapped *Chlorella* in packed bed reactors. *Enzyme Microbial Technol.* **16:** 802–807.
20. Safferman, R. S. and M. E. Morris. 1964. Growth characteristics of the blue-green algal virus LPP-I. *J. Bacteriol.* **88:** 771–775.
21. Sandau, E., P. Sandau, and O. Pulz. 1996. Heavy metal sorption by algae. *Acta Biotechnol.* **16:** 227–235.
22. Singh, A. L., R. K. Asthana, S. C. Srivastava, and S. P. Singh. 1992. Nickel uptake and its localization in a cyanobacterium. *FEMS Microbiol. Lett.* **99:** 165–168.
23. Stratton, G. W. and C. T. Corke. 1979. The effect of cadmium ion on the growth, photosynthesis and nitrogenase activity of *Anabaena inaequalis*. *Chemosphere* **10:** 731–740.
24. Volesky, B. and Z. R. Holan. 1995. Biosorption of heavy metals. *Biotechnol. Prog.* **11:** 235–250.
25. Wehreim, B. and M. Wettern. 1994. Biosorption of cadmium, copper and lead by isolated mother cell walls and whole cells of *Chlorella fusca*. *Appl. Microbiol. Biotechnol.* **41:** 725–728.
26. Wehreim, B. and M. Wettern. 1994. Comparative studies of heavy metal uptake by whole cells and different types of cell walls from *Chlorella fusca*. *Biotechnol. Tech.* **8:** 227–232.
27. Wilde, E. W. and J. R. Benemann. 1993. Bioremoval of heavy metals by use of micro-algae. *Biotechnol. Advances* **11:** 781–812.
28. Wilkinson, S. C., K. H. Goulding, and P. K. Robinson. 1990. Mercury removal by immobilized algae in batch culture systems. *J. Appl. Phycol.* **2:** 223–230.
29. Wong, P. K. and S. C. Kwok. 1992. Accumulation of nickel ion (Ni^{+2}) by immobilized cells of *Enterobacter* species. *Biotechnol. Lett.* **14:** 629–634.
30. Yetis, V., G. N. Demirer, and C. F. Goekcay. 1999. Effect of chromium (VI) on the biomass yield of activated sludge. *Enzyme Microbial Technol.* **25:** 48–54.