Cadmium, nickel and vanadium accumulation by three strains of marine bacteria

Ravanbakhsh Shirdam*[1], Anita Khanafari[2,3], Azam Tabatabaee[1,2]

[1]Department of Environment, Environmental Research Center, Laboratories Affairs Bureau, Pardisan Park, Hemmat highway, P.O. Box 14155-7383, Tehran, I.R. Iran, [2]Department of Marine Biology, Science and Research Branch, Islamic Azad University, Ponak Sq, Ashrafi Isfahani Highway, P.O. Box 14155-775, Tehran, I.R. Iran [3]Department of Microbiological Sciences, The North Tehran Branch, Islamic Azad University, Darband Str. Qods Sq., P.O. Box 19735-148, Tehran, I.R. Iran

Abstract
Three marine bacteria, *Pseudomonas putida* PTCC 1664, *Bacillus cereus* PTTC 1665 and *Pseudomonas pseudoalkaligenes* PTCC 1666 isolated from the East Anzali wetland sediments of the Caspian Sea, were resistant to heavy metals of Cadmium (Cd), Nickel (Ni) and Vanadium (V). Pseudomonas pseudoalkaligenes PTCC 1666 was found to be resistant to all 3 metals Ni, Cd, V. Heavy metal uptake was determined in both the biomass and supernatant by the Atomic Absorption Spectrophotometer (AAS). These bacteria showed enhanced absorption and growth in the presence of Cd and Ni at 80-100 mg/l and V at 40 mg/l concentrations. The high uptake of Cd, Ni and V was directly proportional to their respective concentrations, 5-100 mg/l for Cd and Ni and 5 - 40 mg/l for V. The maximum amount of heavy metal uptake occurred during stationary phase when cells were incubated at 30°C for 72h. The results revealed that these bacteria accumulated approximately 40-50% Cd, 5-6% Ni and 10-12% V. Bacterial cells Immobilized in alginate gel showed more efficiency in biosorbing heavy metals than free cells (80%). Scanning Electron Microscopy (SEM) results indicated that the marine bacteria were capable of accumulating several metals, showing that the isolated bacterial strains can be used as potential candidates for bioremediation, with respect to Cd, Ni and V removal from aqueous effluents.

Keywords: Bioremediation; Marine bacteria; Cadmium; Nickel; Vanadium.

INTRODUCTION

Heavy metals are released into the environment from a wide range of natural and anthropogenic sources. The rate of influx of these heavy metals into the environment far exceeds their removal by natural processes, thus leading to the accumulation of heavy metals in the environment, with aquatic ecosystems normally at the receiving end (Matagi *et al*., 1998).

The main problems caused by the release of toxic pollutants into receiving waters are toxicity to aquatic organisms and restrictions on the human use of these waters (Jung *et al*., 1996). As a result of natural and industrial processes heavy metals are increasingly found in microbial habitats. Thus, microbes have evolved several mechanisms to tolerate the presence of heavy metals (by efflux, complexation, or reduction of metal ions) and they also use them as terminal electron acceptors in anaerobic respiration. The intake and subsequent efflux of heavy metal ions by microbes usually include a redox reaction involving the metal, and some bacteria can use such reactions for energy and growth. Therefore, bacteria that are resistant to and grow on metals also play an important role in the biogeochemical cycling of those metal ions (Spain and Alm, 2003). As a result of these characteristics, microbes play an important role in cleaning up or remediating metal-contaminated environments (Spain and Alm, 2003). Environmental biotechnology refers to the utilization of microorganisms to improve environmental quality (Chen *et al*., 2005). Although conventional technologies such as precipitation, filtration, reverse osmosis, oxidation-reduction, and membrane separation are adequate to remove the bulk of the heavy metal contamination, they are often inadequate at reducing heavy metal concentrations to acceptable regulatory standards. It becomes clear that a cost-effective secondary polishing treatment process is needed for the removal of heavy metals at dilute concentrations (Zhaohui *et al*., 2002). Also conventional approaches (land-filling, recycling, pyrolysis and incineration) to the remediation of contaminated sites are inefficient and costly and can also lead to the for-

*Correspondence to: **Ravanbakhsh Shirdam, Ph.D.**
Tel: +98 21 88269921; Fax: +98 21 88268037
E-mail: shirdamr@yahoo.fr

mation of toxic intermediates. Thus, biological decontamination methods are preferable to conventional approaches because, in general, microorganisms degrade numerous environmental pollutants without producing toxic intermediates (Paul *et al*., 2005).

In recent years, several technologies have been developed with the aim of reducing or removing the presence of heavy metals in contaminated media. Among these technologies, those based on the use of microorganisms are of particular interest (Cabrera *et al*., 2005). The goal of the present investigation is to identify and isolate marine bacteria that selectively remove heavy metals such as Cd, Ni and V from waste water.

MATERIALS AND METHODS

Sampling: Sediment and water samples were collected from the East Anzali wetland of the Caspian Sea. Samples were plated onto BHI agar medium (Merck, Germany) containing 0.1M Cd (Cd $(NO_3)_2.4H_2O$), 0.1M Ni (Ni $(NO_3)_2.6H_2O$), 0.1M V (VCl_3) and a mixture of these metals. Plates were incubated at 30°C for 72 h and colonies were randomly picked, isolated and purified as described previously (Hussein *et al*., 2004; Malekzadeh *et al*., 2002; Lyer *et al*., 2004).

Preparation of heavy metal solutions: Different metal concentrations were prepared by diluting stock solutions (1000 ppm) in de-ionized water. The diluted solutions were sterilized by filtration through a flow pore filter with a 0.45 µm pore size and were used for the further preparation of different metal concentrations.

Determination of MIC and MBC: The metal-tolerance levels of the isolated bacterial strains were investigated by the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) tests (Piotrowska-Seget *et al*., 2005). Bacteria were grown for 72 h at 30°C in BHI agar medium containing heavy metals and used as inocula (approximately 3×10^8 cfu/ml). 1 ml of inocula was added to 10 ml of BHI broth medium (Merck, Germany) containing each of the three metals Cd, Ni, and V and a mixture of all of these, at the following concentrations of 10, 15, 20, 40, 60, 80, 100 ppm. Each of the sets was prepared in duplicates. One set of media without metals was also inoculated and kept as controls. After inoculation, the tubes were incubated at 30°C for 72 h. The MBCs of the isolates were determined by subculturing 0.1 ml of non-turbid samples onto agar medium without metals. Colony forming unit (cfu) in each subculture was determined by the colony count method (Lyer *et al*., 2004).

Growth measurements: 5 ml of the different metal-resistant precultures harvested from the exponential phase of growth were transferred to 100 ml of fresh BHI broth media supplemented with the different metal ions. The flasks were incubated at 30°C by shaking at 120 rpm using a rotating shaker (Hussein *et al*., 2005). Bacterial growth was monitored every 24 h for 14 days, by measuring the Optical Density (OD) of the cultures at 600 nm, using a UV/Vis spectrum spectrophotometer (Hussein *et al*., 2005; Malekzadeh *et al*., 2002).

Measurement of metal accumulation: The concentration of heavy metals in both harvested cells and culture supernatants were determined as follows: For determination of the cell dry weight, bacterial cells were pelleted out of the medium by centrifuging the culture broth at 605 g for 20 min at 20°C and the cell pellets were dried at 105°C for 24 h. 4 mg dry weight of harvested cells were then mixed with 0.5 ml of concentrated nitric acid (65% suprapur) and incubated in a water bath at 100°C for 1h, after which the mixture was cooled to 25°C and made up to a final volume of 5ml with distilled water. The concentration of heavy metals (Cd, Ni and V) in the resulting 5 ml solutions and culture supernatants were measured by the Thermo Elemental Solar AAS, using a specific lamp and wavelength for each metal (Cd in 228.8 nm, Ni in 232 nm, V in 318.5 nm) (Hussein *et al*., 2005; Malekzadeh *et al*., 2002).

Immobilization of bacterial cells: Alginate solutions with a concentration range of 0.5-10% can be used for cell immobilization. In the current study, a 4% alginate (Merck, Germany) concentration was studied. 4 g of alginate was dissolved in 100 ml of BHI broth and autoclaved at 121°C for 15 min. Cells were harvested during the mid-logarithmic phase of growth by centrifugation (192.5 g, 10 min), resuspended in 2 ml of phosphate buffer and added to 100 ml of sterilized alginate solution. This alginate/cell mixture (with stirring) was extruded drop by drop into a cold, sterile 0.2 M $CaCl_2$ solution through a sterile sampler. Gel beads, approximately 2 mm in diameter were obtained (Wang, 2004; Beshay, 2003). The beads were hardened by slowly resuspending them into fresh $CaCl_2$ solution with gentle agitation for 24h at 4°C. Finally these beads were washed with distilled water to remove excess calcium ions and free cells. The beads were

then transferred to 100 ml of BHI broth containing metals and were incubated at 30°C by shaking at 120 rpm (Wang, 2004; Beshay, 2003). Culture samples were harvested every 48h and the resulting supernatants were analyzed for the presence of heavy metals by AAS.

Scanning Electron Microscopy (SEM) examinations: For morphological studies of bacterial cells immobilized in alginate beads, investigation of the internal structure of alginate beads and the search for presence of metals, the technique of SEM was used (Beshay, 2003). The alginate beads were fixed with 2% glutaraldehyde (Merck, Germany) at 4°C for 24h and then rinsed with 0.2 M phosphate buffer ($NaH_2PO_4.2H_2O$ and Na_2HPO_4), pH 7.2 and fixed for another 2h in 2% osmium tetraoxide (Merck, Germany). After being washed three times in phosphate buffer, beads were dehydrated by being passed through a graded series of alcohol (30-100%) and frozen rapidly in liquid nitrogen prior to freeze-drying. Dried beads were coated with gold and observed by the LEO 440i Scanning Electron Microscope (Beshay, 2003; Lopez-Jimenez and leborgne, 2003).

Semi-continuous cultures: The semi-continuous culture systems with its consistent medium delivery was used in order to investigate the stability of the alginate beads and its ability to take up metals under repeated batch cultivations. This process was carried out by decanting the spent medium every 48h and replacing it with fresh medium containing metals, in 5 cycles (Beshay, 2003), The resulting culture supernatants were analyzed by AAS.

Effect of the ASW4 strain on wastewater containing metals: Contaminated waste that contained Cd, Ni, and V was used for metal analyses. Initially 100 ml of waste was autoclaved at 121°C for 15 min, to which the ASW4 beads were added and incubated at 30°C by shaking at 120 rpm. Culture samples were taken every 48h and their metal concentrations were measured by AAS (Wang, 2004).

RESULTS

Bacterial growth was observed in plates containing metals after 72h of incubation at 30°C (Malekzadeh *et al*., 2002).

Isolation of heavy metal resistant bacteria: In this preliminary screening, in total, 22 bacterial strains resistant to heavy metals were isolated from sediment samples of the East Anzali wetland of the Caspian Sea. Isolates were maintained on BHI agar slants containing metals at 4°C and were used for subsequent studies. 5 strains were tolerant to Ni, 6 strains to Cd, 6 strains to V and 5 strains were capable of accumulating all 3 metals tested in this study.

Bacterial tolerance to metals (MIC & MBC): All isolates were examined for metal tolerance levels. 7 of the strains including ASW1, ASW1-1, ASW2-1, ASW3, ASW3-1, ASW4 and ASW4-1, showed a high tolerance to Cd, Ni and V. Characteristically, every strain isolated on medium supplemented with the specific metal showed the highest MIC for this metal, among all bacteria tested. Table 1 shows MIC and MBC values for different isolates.

Table 1. Growth response of bacteria isolated from sediments in media containing different concentrations of metals.

Isolates	(After 72 h at 30°C)								(ppm) MIC	MBC
	5	10	15	20	40	60	80	100	After 48 h	After 48 h
AWS1	+	+	+	+	+	+	+	After 5 days +	100	120
AWS1-1	+	+	+	+	+	+	After 5 days +	-	80	100
AWS2-1	+	+	+	+	+	+	After 10 days +	-	80, 100	120
AWS3	+	+	+	+	+	-	-	-	60	80
AWS3-1	+	+	+	-	-	-	-	-	20	60
AWS4	+	+	+	+	+	+	+	+	120	140
AWS4-1	+	+	+	+	+	+	+	-	100	120

+ : The ability of an organism to grow when subjected to Cd, Ni and V, in a nutrient medium. MIC: represents the Minimum inhibitory concentration; MBC: represents the Minimum Bactericidal concentration.

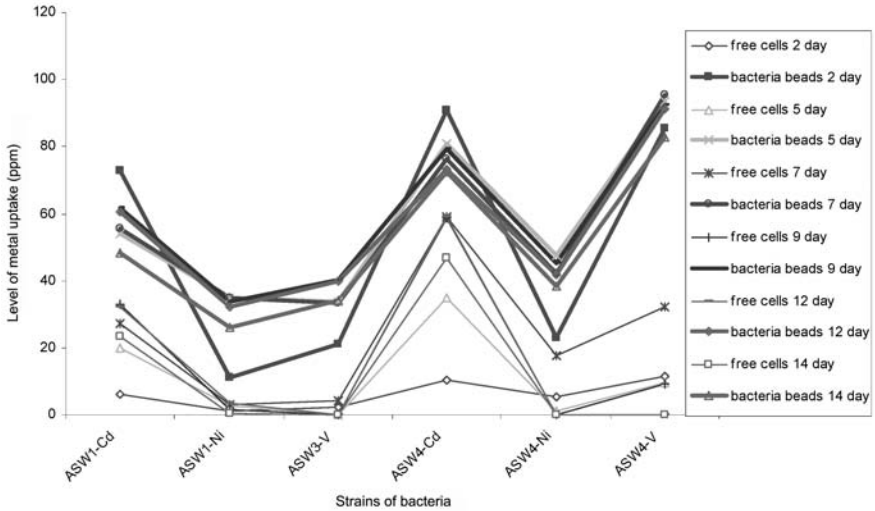

Figure 1. Comparison of metal uptake by bacterial strains as free and immobilized cells (2-14 days). -ASW1: resistant to Cd, Ni; ASW3: resistant to V; ASW4: resistant to Cd, Ni,V.

Effect of growth stage and metal concentration on metal accumulation: From the 7 bacterial strains grown in BHI broth supplemented with various metals, only 3 strains grew strongly and displayed high levels of heavy metal uptake. The amount of uptake of Cd by free ASW1 bacterial cells was 45%, Ni by free ASW1 bacterial cells was 6.5%, and V by free ASW3 bacterial cells was 10%. The amount of uptake of Cd, Ni and V were 60%, 17%, 32% respectively, by free ASW4 bacterial cells after 7 days. The highest concentration of metals taken up by bacterial strains was during the stationary phase of growth.

Heavy metal uptake by immobilized cells: The efficiency of Cd removal by ASW1 immobilized cells was 90%; Ni removal by ASW1 cells was 45%; V removal by ASW3 cells was 100% and Cd, Ni, V removal by ASW4 immobilized cells were 90%, 50% and 95%, respectively.

Comparison of metal uptake by bacterial strains as free and immobilized cells: Figure 1 shows metal uptake by the alginate immobilized cells as compared to free cells. The results show that Cd metal uptake by ASW1 immobilized cells approached 72.7 ppm after 2 days, Ni uptake by ASW1 immobilized cells approached 35.1 ppm after 5-7 days, V uptake by ASW3 immobilized cells approached 40 ppm after 5-9 days and Cd uptake approached 90.8 ppm after 2 days. In ASW4 immobilized cells, Ni uptake approached 47.5 ppm after 5 days, V uptake approached 95.6 ppm after 7 days. This activity continued to increase and reached a maximum value, at which it remained approximately constant. Thus, metal uptake by the alginate immobilized cells was 80% higher than that obtained by free cells used as the control.

Scanning electron microscopy studies: The internal and external structure of alginate beads in the presence of immobilized bacterial cells were studied by SEM. The SEM photographic plates showed that the cells were randomly distributed in alginate beads (Fig. 3). The highest Cd, Ni and V metal concentrations were observed in regions of beads that included bacteria.

Semi-continuous culture: In the semi-continuous culture studies the operational stability of the alginate beads obtained under optimal immobilized conditions was followed during 5 cycles for the ASW4 strain (Fig. 2).

The results, obtained after the transfer of fresh medium into previous medium at the end of the third cycle of cultivation (144h), showed a significant increase (50-90%) in Cd and Ni metal removal by entrapped cells, compared to those by free cells.

The highest activity and uptake of metals obtained during the semi-continuous culture experiment was obtained in the third batch approximately 6 days after the start of the repeated batch cultivation. Further replacements with fresh medium did not improve levels of Cd and Ni uptake, which remained between 50-90% during the fifth cycle, after 10 days (Fig. 2). Replacement with fresh medium containing the heavy metal V, did not enhance the uptake of the V metal during the 5 cycles (Fig. 2).

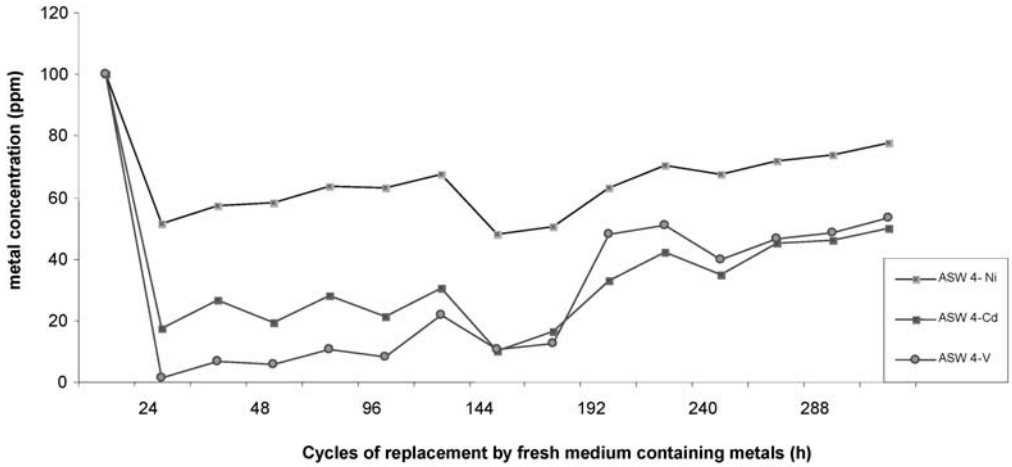

Figure 2. Time- course of semi-continuous cultures with ASW4 immobilized cells.

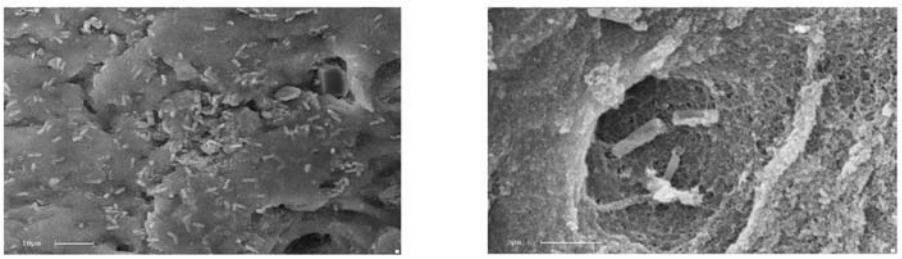

Figure 3. Scanning electron microscopic observations of strains entrapped in alginate beads.

Identification of bacterial strains: Table 2 describes the identification of bacterial strains used in this study by the Persian Type Culture Collection (PTCC). The PTCC Identified the bacterial strains used in this study according to Bergey,s Manual of Determinative Bacteriology. The strains Identified were *Pseudomonas putida*, *Pseudomonas pseudoalkaligenes* and *Bacillus cereus*.

Efficiency of removal of heavy metals from wastewater by ASW4 strain: The amount of the uptake of Cd, Ni and V metals in wastewater containing metals (initial concentration of 100 mg/l) was investigated. The results indicated a removal efficiency of 90% for Cd; 50% for Ni and 95% for V by the ASW4 strain.

DISCUSSION

The good metal removal properties of bacterial beads in waste containing Cd, Ni and V, proved the effectiveness of bacterial beads in the widely used method of bioremediation of effluents contaminated with metals. Microorganisms, collectively, are highly diverse in

Table 2. Identification of bacterial strains by the PTCC.

Isolates	Isolated place	Final identification		PTCC
AWS1	East Anzali wetland sediments of Caspian Sea	Gram negative Bacilli	*Pseudomonas putida*	*1664*
AWS3	East Anzali wetland sediments of Caspian Sea	Gram positive Bacilli	*Bacillus cereus*	*1665*
AWS4	East Anzali wetland sediments of Caspian Sea	Gram negative Cocco Bacilli	*Pseudomonas pseudoalkaligenes*	*1666*

metabolism, so that (theoretically) any of a wide range of pollutants can be degraded, decreased and taken up given a suitable choice of microorganisms (Hong and Shan-shan, 2005; Chen *et al*., 2005). The capacities of various microbes to accumulate an ample amount of metal species have been described by a number of researchers (Table 3). Microorganisms have a high surface area-to-volume ratio because of their small size and therefore, they can provide a large contact interface, which can interact with metals from the surrounding environment (Zouboalis, 2004).Thus biological decontamination methods are preferable to common physico-chemical techniques that are expensive, and inefficient. Furthermore, microorganisms generally degrade numerous environmental pollutants without producing toxic intermediates (Lyer *et al*., 2004; Paul *et al*., 2005).

The maximum accumulation of the 3 metals was achieved when bacterial strains were in the stationary phase of growth. This has also been reported by other investigators (Pongratz and Klaus, 1999; Malekzadeh *et al*., 2002). In this study the highest amount of metal uptake (Cd, Ni and V) was by cells immobilized in alginate, such as the ASW4 strain (Cd 90%, Ni 50%, V 95% at an initial concentration of 100 ppm). Similar findings have also been reported by other investigators (Table 4).

Beshay (2003) has also shown that metal uptake by immobilized cells is superior to that of free cells because it leads to higher volumetric activities within the same time of intake. Semi-continuous cultivation also showed the capacity of metal uptake by immobilized cells are 2-3 times higher than that obtained by free cells (Table 5) (Beshay, 2003).

With the aid of SEM, it was possible to observe the random distribution of bacteria in alginate and the amount of metals (Cd, Ni, and V) present in the bacteria. These studies are in agreement with Beshay (2003) who found that the immobilized *Teredinobacter turnirae* cells were randomly distributed in alginate beads.

In conclusion, the uptake of metal ions can be divided into two stages: rapid and slow stage. In the "rapid" stage, the metal ions are absorbed onto the surface of the microorganism. In the "slow" stage the metal ions

Table 3. Results of past and current research regarding the accumulation of metals.

NO.	Reference	Bactrial isolate	Heavy metal	Location
1	Costa *et al*. (2001)	*Bacillus subtilis*	Pb, Zn, Cu	-
2	Wong *et al*. (1993)	*Pseudomonas putida*	Cu up to 6.5% dry weight	-
3	Malekzadeh *et al*. (2002)	*Pseudomonas MGF-48*	uranium up to 174 mg/g dry weight	-
4	Anita lyer *et al*. (2004)	*Enterobacter cloacae*	Cd (65%), Cu (20%), Co (8%) at 100 mg/l heavy metal concentration	the west coast of India
5	Hussein *et al*. (2005)	a group of *Pseudomonas* sp	Cr and Cu up to a concentration of 3 Mm/l, Ni up to 5 Mm /l and also Cd up to 10 Mm /l	wastewater
6	Rang Sayatorn *et al*. (2004) and Horikoshiet *et al*. (1979)	*Spirullina platensis*	Cd rapidly adsorbed during the first 5 min	
		C. regularis	Cd rapidly adsorbed within 6 min	-
7	Present study	*Pseudomonas putida*	Cd (40-50%) (Concentration 80 ppm Cd) and Ni (5-6%) (concentration 80 ppm Ni)	East Anzali wetland sediments of Caspian Sea
		Bacillus cereus	V (10-12%) (concentration 40 ppm V)	
		Pseudomonas pseudoalkaligenes	3 metals up (Cd 60%, Ni 17%, and V 32% in concentration 100 ppm).	

Table 4. Results of past and current research regarding the accumulation of metals by immobilized cells.

NO.	Researchers	Immobilized cells	Accumulation of
1	Macaskie and Dean (1984), Malone (1989), Marques *et al*. (1991), Wong (1993)	Incorporating pre-grown cells in polyacrylamide gels.	A wide range of heavy metals, including some in the uranium family and other metals such as Cd, Pb and Ag
2	Wong *et al*. (1993)	Cells immobilized in polyacrylamide gel	More than 90% of the uranium
3	Present study	Cells immobilized in sodium alginate	Cd 90%, Ni 50%, V 95% in initial concentration 100 ppm

Table 5. Results of past and current research involving semi- continuous cultivation.

NO.	Researchers	Immobilized cells	Performance
1	Beshay (2003)	Protease production by *Teredinobacter turnirae* immobilization	2-3 times higher than that obtained with free cells
2	Present study	Capacity of metals uptake by immobilized cells	2-3 times higher than that obtained with free cells

are transported across the cell membrane into the cytoplasm (Hong and Shan-shan, 2005) (Table 3).

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