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Biosorption of Cd and Ni by inactivated bacteria isolated from agricultural soil treated with sewage sludge

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Abstract

In this study, the capacities of the bacterial biomass in binding simultaneously two metals, Cd and Ni, isolated from soils subject to long-term sewage-sludge applications in Ahvaz, were investigated. Three strains of bacteria were isolated based on their resistance to these metals; namely, *Actinomycete* sp., *Streptomyces* sp. and *Bacillus* sp. The inactivated forms of these bacterial cells were investigated. *Actinomycete* sp. was the most tolerant isolate to Cd and Ni ions. Freundlich isotherms indicated that the sorption capacities on the biomass surfaces increased with increasing initial metals concentration of both metals. Generally, the biosorption of Ni was slightly higher than that of Cd. These results suggest that native bacteria in polluted area are competent candidates for bioremediation and improving soil quality for agricultural purposes.

Key words: Biosorption, metal resistant bacteria, MIC, Cd, Ni.

1. Introduction

Contamination by metals is a major global concern because of their toxicity and threat to human life and environment (Ceribasi, Yetis 2001). Metals are toxic and persistent pollutants released into the environment as a result of activities such as industrial, mining and agricultural activities and cannot be degraded or destroyed (Esposito *et al.* 2001; Coral *et al.* 2005). Cadmium and nickel are metals of major concern because of their widespread uses in developing countries and their non-degradable nature. Removal and recovery of metals are very important with respect to environmental and economic considerations (Nourbakhsh *et al.* 2002). Conventional physicochemical removal and recovery methods such as electrochemical, ion-exchange, precipitation, reverse osmosis, evaporation and oxidation/reduction treatments for metal removal from waste streams are expensive and not eco-friendly (Vijayaraghavan, Yun 2008). For this reason, there is a need to search for new techniques which have

an acceptable ability to remove pollutants to the levels required by law (Veglio, Beolchini 1997).

In recent years, the use of biological materials including living and non-living microorganisms to remove metal cations from industrial wastewaters has attracted much interest because of the performance, cost-effectiveness and eco-friendly nature of these sorbents (Kefala *et al.* 1999).

Both living (bioaccumulation) and non-living (biosorption) microbial biomass can act as effective metal scavengers. Bioaccumulation is based on the incorporation of metals inside the living biomass, while biosorption is a metabolism-independent process and metallic ions remain at the cellular surface. Biosorption results from different mechanisms such as complex formation, ion exchange, coordination, adsorption and chelation (Hu *et al*. 1996; Velasquez, Dussan 2009). Dead cells are more advantageous for use in industrial applications because non-living biomass usually exhibits higher metal uptake capacities. Additionally, non-living biomass can be applied more easily and sometimes with higher selectivity, using existing treatment technologies (Tobin *et al*. 1994). Moreover, this method is not affected by the toxicity of the metal ions or by adverse operating conditions (Chu, Hasim 2004). In addition, the use of dead biomass is easier to handle. Most microbial cells exhibit colloidal characteristics in the adsorption of metals or hydrolyzed metals similar to those of soil mineral oxides (Choi *et al.* 2009). Kureck *et al.* (1982) compared the Cd sorption by dead and active bacterial cells with abiotic soil components (clay and sand). They reported that when the same masses (on dry weight basis) of bacterial cells (*Serratia marcescens* and *Paracoccus* sp.), clay (montmorillonite) and sand were incubated with specified concentration of Cd, bacterial cells removed the largest quantity of Cd. Also, dead cells sorbed Cd much more readily than active cells in their study.

The biosorption of metals is affected by many factors such as the pH, temperature, biomass concentration and type, presence of different metallic ions in solution, contact time, etc. (Özdemir *et al.* 2009). A large number of microorganisms such as bacteria, fungi, yeasts, and algae have been reported to bind a variety of metals to different extents in solutions (Volesky, Holan 1995). Bacteria are preferred as biosorbents more than other microorganisms because of (a) their high surface-to-volume ratios, (b) a high content of potentially active chemosorption sites such as on teichoic acid in their cell walls (Beveridge 1989) and (c) their abundance in all environments such as water, soil and air. They also can be easily propagated under laboratory conditions. The metal ions in solution are adsorbed on the bacterial surface through interactions with chemical functional groups such as carboxylate, amine, amide, imidazole, phosphate, thioether, hydroxyl and other functional groups found in the cell wall biopolymers (Crist *et al.* 1981; Madrid *et al.* 1997; Gupta *et al.* 2000; Vijayaraghavan, Yun 2008).The sites for metal binding are different according to bacterium species and metals. On the other hand, biosorpton studies have focused mostly on the selection of metal-resistant microorganisms from polluted environments. De Vicente *et al.* (1990) found a strong correlation between the extent of metal pollution and bacterial resistance to metals in aqueous environments. It could be hypothesized that microorganisms of metal-polluted soils have developed tolerances to the metals and probably increased metal biosorption capacities (Iqbal *et al.* 2005). Long-term application of wastewater and sewage sludge may result in the accumulation of metals such as Cd^{+2} and Ni^{2+} in soils and exert a selection pressure on soil microorganisms (Ansari, Malik 2007). The pollution of the environment with metals has led to the appearance of metal-resistant microorganisms in the soil and water of industrial regions (Aleem *et al.* 2003). Therefore, it is important to explore microbes from such environments for use in metal biosorption. Most of the studies involve the removal from aqueous solutions of only one kind of metal ion by microorganisms. However, multi-metal biosorption systems more closely would represent the composition of industrial effluents, as these effluents generally include more than one metal. The specific metal sorption is dependent on both the metal ion concentrations and metal species. The presence of different metal species in different concentrations in a solution substantially affects the adsorption capacity of the dead microorganisms (Dias *et al.* 2002).

Therefore, the objectives of the present study are the isolation and identification of metal resistant bacteria from agricultural soils treated with wastewater, the evaluation of their Cd and Ni biosorption potentials in a competitive system and the investigation of their Freundlich isotherms. The Freundlich model provides a physically more realistic description of metal adsorption by organic matter due to its inclusion of different binding sites (Aksu, Dönmez 2006).

2. Materials and methods

2.1. Sample collection, isolation and characterization of bacteria

Soil samples were collected in sterile plastic bags from the surface layer (0-30 cm) of an agricultural field in the west of Ahvaz, Iran. The soils had been treated for more than one decade with sewage sludge. The soil samples were transported

to the laboratory to isolate bacterial cells. 10 g of soil sample was suspended in 90 ml of sterile saline solution (8 g NaCl in 1000 ml distilled water) for 2 hours, under shaken (150 rpm), and serially diluted to 10^{-6} with saline solution. Then, 0.1 ml of diluted suspension was placed on nutrient agar plates amended with 100 ppm of Cd and Ni (as $CdCl_2$ and $NiCl_2$). These Plates were incubated at 30°C for 48-72 h. The most frequent strains of the bacteria isolated were chosen for next experiments, encoded as I, II**,** III and stored onto nutrient agar at 4°C.

A subsample of soil was air-dried and sieved through a 2-mm plastic sieve and used to determine the extractable concentration of metals using DTPA solution and an atomic adsorption spectrophotometer (AAS, Model: Unicam 939). The sampled soils showed high contents of Zn (29.04 mg kg^{-1}), Fe (24.48 mg kg^{-1}), Mn (11.59 mg kg^{-1}), Cu (4.18 mg kg^{-1}), Pb (1.34 mg kg^{-1}), Ni (0.88 mg kg^{-1}) and Cd (0.187 mg kg^{-1}).

2.2. Determination of minimum inhibitory concentrations (MIC) for Cd and Ni

The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of a metal that inhibits the visible growth of microorganisms. The MIC of Cd- and Ni-resistant bacteria isolates were determined by the nutrient agar dilution method (Aleem *et al.* 2003). The salts of $CdCl_2$ and $NiCl_2$ were used to prepare 1000 mg ml^{-1} stock solutions in sterile deionized water and were added to the nutrient agar in various concentrations (from 50 to 850 mg ml^{-1}) and then the isolated bacteria were inoculated individually into plates. The bacteria were incubated at 30°C for 72 h. The lowest concentrations of Cd and Ni that completely prevented growth of each bacterium were considered as MIC.

2.3. Biosorbents preparation

Resistant bacteria were grown in TSB (Tryptic Soy Broth, Merck, Germany) medium using the shaken flask (50 ml/250 ml Erlenmeyer flask) method. The flasks were aitated on a rotary shaker (150 rpm) for 48 h at 30°C. The resultant biomass was pelletized by centrifugation at 7500 rpm for 15 min, washed five times with 0.1 M NaCl, autoclaved at 121°C for 15 min and re-centrifuged at 7500 rpm for 15 min. After that, inactive cells were washed with 0.1 M NaCl. The washed cells were dried at 80°C for 24 h in an oven, ground in a blender and stored at 4°C to be used for the biosorption experiments.

2.4. Preparation of Cd and Ni solutions

Standard solutions were prepared by dissolving the chloride salts in distilled water. Cd and Ni solutions of 1000 mg l^{-1} were prepared with deionised water. Solutions of varying concentrations (50, 100, 200 and 400) were prepared by diluting the stock solution with deionised water. These solutions subsequently were sterilized at 121°C for 15 min. Competitive adsorption isotherms were determined by adding Cd and Ni at 1:1 concentration ratios.

2.5. Biosorption experiments and kinetics of sorption

The batch biosorption experiments using the three bacterial isolates were performed in 40 ml tubes. 0.1 g (dry weight) of each type of dead cells was added individually to 20 ml of metal solution (representing 5 g l^{-1} biomass content). The tubes were placed on a shaker at 150 rpm for 24 h. Then, the samples were centrifuged at 7500 rpm for 5 min to separate the solid phase from the liquid phase. The liquid phase suspensions were analyzed for residual metal concentrations using AAS (Unicam 939) after dilution of the samples.

The effect of biosorbent content on the sorption value was studied by varying the dosages (1, 2 and 5 g l^{-1}) of each inactive bacterial cell. All experiments were carried out in triplicate and the reported data were given as a mean. All materials used in the growth and biosorption of the microorganisms were previously rinsed with acid, and then several times with deionized water. The amount of biosorbed element per gram of each dried cell was calculated using the following equation:

$$q = (C_o - C_e)\ V/M \qquad (1)$$

Where q is the amount of biosorbed metal (mg of metal g^{-1} of biomass), C_o is the initial concentration of the metal in solution (mg ml^{-1}), C_e is the equilibrium concentration of metal in solution (mg ml^{-1}), V is the volume of the reaction mixture (ml), and M is the weight of dried cells (g).

In order to analyze the obtained data from adsorbing Cd and Ni in different pollution levels by means of all the three bacterial biomass, SPSS software was used and the results related to adsorbed Cd and Ni were calculated and the average of data sets (groups) were evaluated through Two-Way ANOVA and Duncan's test. To determine the existence or non-existence of significant statistical variation among samples, the significant level of $P < 0.05$ was considered.

The relationship between the concentration of dissolved and adsorbed metal was expressed by the Freundlich isotherm. The linear form of the Freundlich isotherm is given by:

$$\log q = \log K + 1/n \log C \qquad (2)$$

Where q (or x/m) is the amount of adsorbed metal per gram of sorbent (mg g^{-1}), C is the equilibrium

concentration of the adsorbate (mg ml^{-1}), and *K* and n are Freundlich constants related to adsorption capacity and adsorption intensity, respectively. Freundlich parameters can be obtained by plotting *log Q* vs. *log C*, with *1/n* being the slope and *log K* being the intercept of the line.

3. Results and discussion

3.1. Characteristics of resistant isolates

Those bacteria that could tolerate 100 Cd and Ni were selected, purified and identified based on morphological and biochemical features following Bergey's Manual of Determinative Bacteriology (Bergey, Holt 1994). I, II, III isolates which showed a tolerance to high concentrations of cadmium and nickel were identified as *Bacillus* sp., *Actinomyces* sp. and *Streptomyces* sp., respectively. Table I gives the morphological and biochemical characteristics of the identified bacteria. These bacteria were selected for determination of MIC and biosorption values because of their resistance to both metals.

Minimum Inhibitory Concentration

The MIC of Cd^{2+} for I, II and III isolates were 350, 400 and 400 µg ml^{-1}, respectively, and in the case of Ni^{2+},were 450, 500 and 350 µg ml^{-1} (Table II). *Actinomycete* sp. was the most resistant isolated bacteria. The occurrence and abundance of metal-resistant microbes in metal polluted sites are well-documented in earlier studies (Srinath *et al.* 2002; Jankowska *et al.* 2006; Chen *et al.* 2006). It seems that long-term use of sewage sludge might have exerted selection pressure on soil microbial populations and developed their resistance to metals (Malik, Jaiswal 2000). This is consistent with the results of Zafar et al (2007), who have isolated various filamentous fungi from agricultural soils receiving long-term (> 20 years) applications of municipal wastewaters, and who observed fungi growing in the presence of high concentrations of metal ions.

3.2. Biosorption of Cd and Ni by dead bacterial cells and Fruendlich constants

As shown in Fig. 1a (Cd biosorption) and b (Ni biosorption), all three types of inactivated cells indicated similar behaviors to be different concentrations of Cd and Ni. In general, it can be stated that Ni biosorption decreased in the following sequence: *Actinomyces* sp. > *Streptomyces* sp. > *Bacillus* sp., but Cd biosorption was less regular. Although the initial concentrations of Cd and Ni were equal in all of the samples, the biosorption of Ni was higher than that of Cd, excluding the results for *Bacillus* sp. At the treatment levels of 100 and 400 µg ml^{-1}, in which Cd biosorption was higher than Ni. Overall, there was a lower affinity of all biosorbents for Cd comparing to Ni. Biosorption of Ni ions by *Actinomyces* sp. and *Streptomyces* sp. was preferential to that of Cd ions but *Bacillus* sp. preferred Cd more than Ni. The results showed that *Actinomyces* sp. was a more effective biosorbent than the two other bacterial cells.

Table I. Morphological and biochemical characteristics of metal resistant soil bacterial isolates.

Morphological and biochemical tests	Bacterial species		
	I	**II**	**III**
Cell shape	Rod	Rod	Rod
Gram test	+	+	+
Catalase test	+	-	-
Oxidase test	-	-	-
Methyl red test	-	-	-
Voges-Proskauer test	-	-	-
Oxidation/fermentation (O/F)	F	F	F
Growth on McConkey Agar	-	-	-
Growth on NaCl (6.5%)	+	-	+
Motility	+	+	+
Indole production	-	-	-
Gelatine hydrolysis	-	-	-
H_2S production in TSI	-	-	+
Citrate utilization	-	-	+
TSI	Alk/Acid	Acid/Acid	Alk/Acid
Sugar fermentation			
Glocose utilization	+	+	+
Sacarose utilization	-	+	-
Lactose utilization	-	+	-
Type of specie	*Bacillus* sp.	*Actinomyces* sp.	*Streptomyces* sp.

(+) and (-) refers to positive and negative test performance, respectively.

Table II. Minimum Inhibitory Concentration (MIC) of metals for resistant bacteria

metals	MIC		
	Bacillus sp.	*Actinomyces* sp.	*Streptomyces* sp.
Cd	350	400	400
Ni	450	500	350

Biosorption rates of Cd and Ni ions on all of the inactivated bacterial cells increased with increasing initial concentrations of these metals, so the minimum and maximum metal sorptions occurred at the pollution levels of 50 and 400 μg ml^{-1}, respectively (Fig. 1a and b). As an example, when initial metal concentrations increased from 50 mg l^{-1} to 400 mg l^{-1}, Cd biosorption increased from 4.11 to 32.63 mg g^{-1} for *Actinomyces* sp., 3.39 to 33.18 mg g^{-1} for *Streptomyces sp.* and 3.96 to 38.71 mg g^{-1} for *Bacillus* sp., respectively and Ni biosorption increased from 7.1 to 36.55 mg g^{-1} for *Actinomyces* sp., 5.47 to 34.82 mg g^{-1} for *Streptomyces* sp. and 4.08 to 32.8 mg g^{-1} for *Bacillus* sp.. This increase in sorption capacity of the biosorbents with the increase in initial metal concentrations could be due the greater availability of metal ions for sorption (Vimala, Das 2009), or to an increase in electrostatic interactions involving sites of progressively lower affinity for metal ions (Al-Garnı 2005).

Statistical analysis results indicated that the adsorption rate has increased significantly, as the pollution level increased. According to obtained results, the highest adsorption took place in the pollution level 400 ppm for all the three bacteria. Results also showed that the sort of bacteria and elements did not have significant variation in the adsorption rates. In other words, all the three bacteria had similar efficiency through the adsorption and accumulation of Ni and Cd elements in the same pollution level. Summarized results from comparison average table also confirmed that adsorption rates increase through increasing pollution levels (Tables III and IV). Columns and rows with uncommon letters have a significant statistical difference at the level of 5%

To compare the biosorption performances of the bacterial cells on metals, Freundlich competitive sorption isotherms were described and the Freundlich constants (K and n) along with correlation coefficients (R^2) were calculated from the Freundlich adsorption isotherms for the biosorption of nickel and cadmium. The adsorption constants for the competitive Freundlich model are given in Table V. K, n and r are Freundlich parameters and are indicative of sorption capacity, intensity and

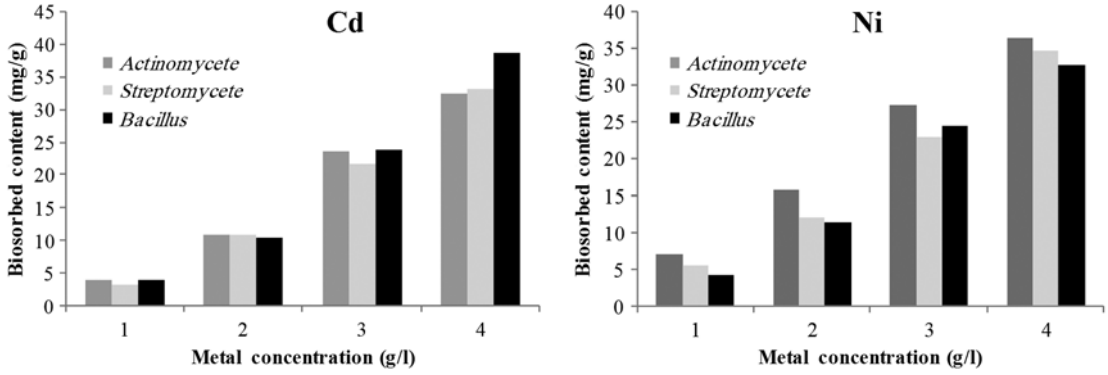

Fig. 1. Biosorption rates of Cd and Ni at different concentrations of the metals by inactivated isolates.

Table III. Variance analysis of biomass type effectiveness, pollution level and the type of metal on biosorption rate.

The variation resources	Degree of freedom	Mean squares	Value F
Pollution level	3	4357.38	3.41*
Bacteria species	2	1606.37	1.26ns
Metal	1	1534.32	1.2ns
Experimental error	42	1275.3	

Table IV. The comparison of the mean effectiveness of biomass type, pollution level and the type of metal on biosorption rate.

***Bacillus* sp.**		***Streptomyces* sp.**		***Actinomyces* sp.**		**Pollution level**
Ni	**Cd**	**Ni**	**Cd**	**Ni**	**Cd**	
4.08e	3.96e	5.47e	3.39e	7.1de	4.11e	50 ppm
11.28c	10.47cd	12.03c	10.92cd	15.78c	11.05c	100 ppm
24.46b	24.03b	23.01b	21.76b	27.3b	23.73b	200 ppm
32.8a	38.71a	34.82a	33.18a	36.55a	32.63a	400 ppm

correlation coefficient, respectively. The correlation coefficients for both Cd and Ni at all biomasses were found to be > 0.94, which indicates that metal biosorption by the dead bacterial cells favorably fits the Freundlich isotherm model.

The adsorption capacities (*K*) of Cd for *Actinomyces* sp., *Streptomyces* sp. and *Bacillus* sp. were 2×10^{-2}, 1.24×10^{-2} and 1.22×10^{-2}, respectively, and 8.5×10^{-2}, 4.1×10^{-2} and 1.95×10^{-2} for Ni. As mentioned, the biomass of *Actinomycs* sp. had a higher sorption capacity compared with those of the dead cells of *Streptomyces* sp. and *Bacillus* sp. The greater the *K* value, the higher the affinity between bacterial cells and metal ions. Our results suggested that *Actinomyces* sp. is a better biosorbent for Cd and Ni.

On the other hand, the comparison of metal sorption capacities showed that the Ni adsorption capacities of *Actinomyces* sp., *Streptomyces* sp. and *Bacillus* sp. were more than their Cd adsorption capacity. The reason for this difference could be the smaller ionic radius of Ni (0.69 Å) compared with Cd (0.96 Å). The binding strength of a metal ion to biomass is dependent upon factors such as hydration, hydrolysis and covalent binding of a metal ion (Hawari, Mulligan 2007). Tobin *et al.* (1984) demonstrated that ions having a smaller ionic radius could be more quickly adsorbed onto a fixed adsorption area. The results obtained by Tobin *et al.* (1984) revealed that the available binding sites of the biomass may have different affinities towards specific metal ions. The Pearson classification categorized metals into three broad categories: those that are polarizable or "soft", those that are non-polarizable or "hard" and those that are borderline (Williams *et al.* 1998). Nickel is classified in the borderline category according to this classification, while Cd ions fall into the soft category (Sen Gupta 2002). Soft cations form more stable complexes with soft donors, while hard cations prefer hard donors (Buffle 1988). Examples of hard ligands are carbonate, phosphate, sulfate, carboxylate and hydroxyl groups, while soft ligands include sulfhydryl and amino groups (Bell 1977; Nieboer, Richardson 1980). As a fact, these biomasses had a lower affinity for cadmium ions. Therefore, it could be deduced that the main functional groups involved in metal sequestration are likely to be hard ligands (i.e. carbonate, phosphate, sulfate, carboxylate and hydroxyl groups).

3.3. The relation between MIC and biosorption

In the present study, bimetal-tolerant *Actinomyces* sp. and it's less tolerant counterparts including *Bacillus* sp. and *Streptomyces* sp. were evaluated for their biosorption potential for Cd and Ni. The differences in biosorption content by different microbial cells may be attributed to the wide variations in the characteristics and chemical composition of their cell walls and, consequently, to their tolerance for metal ions (Gadd 1990).

These findings indicate a close relationship between the level of metal resistance and biosorption capacity in bacterial isolates. Using the results obtained in this study, it was observed that bacteria having a higher MIC, can retain more metals ions than their cell surfaces. Therefore, bacteria showing a high tolerance to toxic metals may be more useful in metal recovery systems. Their resistance mechanisms maybe based onto metal sorption onto the surface of a cell, the intracellular transformation of the metal into less toxic forms, the release of metal from a cell with the help of polymers, and reduced permeability of their cell membranes (Macaskie, Dean 1989; Tobin *et al.* 1990; Nies 2003). Rho and Kim (2002) compared the metal biosorption characteristics of four streptomycetes and observed their different sorption behaviors according to bacterium species and metal. They stated that these differential metal binding capacities of the cell walls might be due to the differences in binding strength or to the binding selectivity of cell wall components, while metal biosorption is not the basis of the metal tolerance.

Conclusion

The findings of this study suggest that soil treated with sewage sludge containing high levels of several types of toxic metals may have led to the soil microbial population developing resistance strategies against metal toxicity. Biosorption of Cd and Ni was studied at initial metal concentrations of 50, 100, 200 and 400 μg ml^{-1}. According to the results, the dead biomasses of *Actinomyces* sp., *Streptomyces* sp. and *Bacillus* sp. can be used as potential biosorbents for the removal of Cd and Ni ions from aqueous solutions. The results of this study indicate that biomass type and initial concentration

Table V. Freundlich isotherm parameters for sorption of Cd and Ni by three bacterial cells.

Bactrial cell	Cadmium			Nickel		
	K	*n*	*r*	*K*	*n*	*r*
Bacillus sp.	1.22×10^{-2}	0.892	0.972	1.95×10^{-2}	0.98	0.942
Actinomyces sp.	2×10^{-2}	0.982	0.952	8.5×10^{-2}	1.282	0.952
Streptomyces sp.	1.24×10^{-2}	0.916	0.954	4.1×10^{-2}	1.128	0.979

of metal, partly affect biosorption performance, but the sorption capacity of the biomass surfaces increased strongly with increasing initial metal concentrations for both metals. Maximum biosorption of metals from solution by *Actinomyces* sp. isolate occurred at 400 μg ml^{-1} of initial concentration and *Actinomyces* sp. dead cells showed more sorption of both metals than other biosorbents.

The metal adsorption isotherms for the trace elements were adequately described by the Freundlich adsorption model. The adsorption affinity sequences for three dead cells were found to be: *Actinomyces* sp. >> *Streptomyces* sp. >> *Bacillus* sp. for Ni and *Actinomyces* sp. > *Streptomyces* sp. ≥ *Bacillus* sp. for Cd. These sequences were almost consistent between resistance sequences. Also, the sorption capacity of Ni by the bacterial cells was more than that of Cd.

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