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Nickel Biosorption by Free and Immobilized Cells of Marine *Bacillus subtilis* N10

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Abstract: The biosorption of nickel ions on marine *Bacillus subtilis* N10 free cells and immobilized cells on different porous solid supports has been studied in batch experiments to determine the effect of cell immobilization on metal accumulation. The influence of medium composition on the biosorption activities of these bacterial strains was investigated by the application of Plackett-Burman experimental design. Optimization results represents about 1.2 fold increase in Ni^{2+} uptake when compared with the control basal culture. Moreover, optimized cultures containing adsorbed cells on pumice particles showed the highest Ni^{2+} uptake.

Key words: Nickel, biosorption, marine, *Bacillus subtilis*, immobilization, Plackett-Burman

INTRODUCTION

The increasing amount of toxic heavy metals emitted into the biosphere as a result of rapid industrialization and urbanization indicate a potential hazard to the ecosystem (Vieira and Volesky, 2000). Unlike organic pollutants, which in most cases can eventually be destroyed, metallic species released into the environment tend to persist indefinitely, circulating and eventually accumulating throughout the food chain thus posing a serious threat to animals and man (Volesky, 1994). They also kill microorganisms during biological treatment of wastewater with a consequent delay of the process of water purification (Hussein *et al*., 2004).

Many industries such as electroplating and metal finishing discharge heavy metal-laden effluents into the environment, being one of the major contributors to heavy metal pollution in surface waters (Ajmal *et al*., 1996). In this type of effluent, there is usually a high concentration of nickel ion (Ni^{2+}) (Wong and Know, 1992).

With the increasing need for the safe removal of toxic heavy metals, the microbial processes play an important role in the future of waste management. Conventional techniques to remove toxic metals and radionuclides, e.g., ion exchange and precipitation, lack specificity and are ineffective, especially when the heavy metal ions are in solutions containing in the order of 1-100 mg dissolved heavy metal ions L^{-1} (Vieira and Volesky, 2000).

Biosorption of heavy metals by microbial cells has been recognized as a potential alternative to existing technologies for recovery of heavy metals from industrial waste streams (Hussein *et al*., 2004). Many aquatic microorganisms, such as bacteria, yeast and algae can take up dissolved metals from their surroundings onto their bodies and can be used for removing heavy metal ions successfully (Vieira and Volesky, 2000).

According to Beveridge (1989) bacteria make excellent biosorbents because of their high surface-to-volume ratios and a high content of potentially active chemosorption sites such as on teichoic acid in their cell walls. Bacterial cell walls are negatively charged under acidic pH conditions and the cell wall chemically functional groups display a high affinity for metal ions in solution (Collins and Stotzky, 1992). Fein *et al*. (1997) used *Bacillus subtilis* to examine the bacterium interaction with Cd, Cu, Pb and Al.

One of the main characteristics of the genus *Bacillus* is the ability of its members to produce heat-resistant-endospores (Jenkinson *et al*., 1980). Members of the genus *Bacillus* are generally easy to grow to high cell density and do not require expensive growth factors. Uptake of heavy metals, such as Cd, Cr, Fe, U and Zn, has been reported by *Bacillus* species (Brierly *et al*., 1989; EL-Helow *et al*., 2000).

For the industrial application of biosorption, the use of an immobilized biomass in a polymeric matrix or in other supports improves biomass performance and allows its use in many subsequent cycles in the usual unit processes characteristic of chemical engineering (Tsezos, 1988).

In the present study to determine the effect of cell immobilization on the Ni^{2+} accumulation properties of

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Bacillus subtilis N10, Ni^{2+} biosorption by free and immobilized cells in batch experiments has been studied. *Bacillus subtilis* N10 was isolated from a western harbor (Alexandria-Egypt) and selected for its high Ni^{2+} accumulation.

The influence of medium composition on the biocatalytic and biosorptive activities of these bacterial strains was investigated. Factors affecting the objectives were evaluated by application of two-level factorial experiments such as the Plackett-Burman design (Plackett and Burman, 1946).

MATERIALS AND METHODS

Microorganisms: Four marine *Bacillus* sp., *Bacillus pumilus* N1, *Bacillus thuringiensis* N9, *Bacillus subtilis* N10 and *Bacillus pumilus* N40, Were used in this study. They were isolated from a highly heavy metal-contaminated sea area in the East Port in Alexandria. During 2006, these strains were screened for their maximal Ni^{2+} uptake capability with different time of metal contacts. All strains were maintained as spore suspension.

Culture conditions and analysis of residual Ni^{2+}: Inocula from fresh slant were used to initiate preculture at 30°C. At late logarithmic phase of growth (A_{550} = 1), inocula of 1 mL were transferred and allowed to grow in 50 mL volume of the growth culture medium which has the following composition (g L^{-1}), Glucose, 3; yeast extract, 2; $(NH_4)_2SO_4$, 2; K_2HPO_4, 6; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.1; NaCl, 5; Fe SO_4, 0.001, $MnSO_4$, 0.01. in 250 mL conical flask at 30°C on a rotary shaker (250 rpm). At A_{550} =1.2, aliquots (0.2 g mL^{-1}) were harvested by centrifugation for 10 min at 5000 x g and washed with sterile glass distilled water. This biomass were suspended in 10 mL distilled water amended with 0.5 mM Ni^{2+} (29.2 μg mL^{-1}) at pH 7.4. This metal solution were incubated on a rotary shaker at 150 rpm for 2 h, after which the content were centrifuged and the supernatants were analyzed for residual Ni^{2+}. Residual Ni^{2+} was determined by atomic absorption spectrophotometer, as previously described (El-Sharowny, 2001). The same previous method of Residual Ni^{2+} determination was used to test the optimum pH for maximal biosorption.

Plackett-Burman design: To approach a near optimal response region of medium composition, a fractional factorial Plackett-Burman design (Plackett and Burman, 1946) was applied. The design included eleven variables and allowed each variable to be examined at low level (-1) and a high level (+1) (Table 1). Trials were performed in duplicates. The main effect of each variable was calculated as the difference between the average of measurements made at the high setting and the average of measurements observed at low setting of that factor. Accordingly, each of the calculated main effect figures reflected a comparison between two sets of data. For determining whether variations in the observed sets are the result of examined factors or experimental errors, statistical t-values for equal unpaired samples were calculated (Chatfield, 1975). Verification experiment was performed using medium component which has positive main effect in their high setting and medium component which has negative main effect in their low level, with the rest of the components as their basal level.

Table 1: Factors examined as independent variables affecting Ni^{2+} uptake (μg mL^{-1}) and their levels in the Plackett-Burman experiment

		Levels		
Factors	Symbol	-1	0	+1
Inoculum size (%)	IS	1.00	2.00	3.00
Peptone	P	0.50	1.00	1.50
Yeast extract (g L^{-1})	YE	0.50	1.00	1.50
Glucose (g L^{-1})	G	1.00	3.00	5.00
$(NH_4)_2SO_4$ (g L^{-1})	NH	0.50	1.00	1.50
K_2HPO_4 (g L^{-1})	K2	4.00	6.00	8.00
KH_2PO_4 (g L^{-1})	KH	0.00	1.00	2.00
$MgSO_4.7H_2O$ (g L^{-1})	Mg	0.05	0.10	0.15
NaCl (g L^{-1})	Na	2.50	5.00	7.50
$FeSO_4$ (g L^{-1})	Fe	0.00	0.001	0.002
$MnSO_4$	Mn	0.00	0.50	1.00

Immobilization of bacterial cells by adsorption: One milliter inoculum was added to Erlenmeyer flasks (250 mL capacity) containing 50 mL verified culture medium. From this flask, aliquots of 0.2 g were suspended in 50 mL sterile 0.5 mM Ni^{2+} solution containing sponge, Luffa, Pumice and clay separately. They were cut to small cubes (sponge) or pieces (luffa, pumice, clay) washed several times with water before use. Control flasks inoculum free were used. All flasks were shacked at 150 rpm for two hours after which 10 mL were centrifuged and the supernatants were analyzed for residual Ni^{2+}.

RESULTS

Screening for Ni^{2+} biosorption ability: From the four tested *Bacillus* strains *Bacillus pumilus* N1, *Bacillus thuringiensis* N9, *Bacillus subtilis* N10 and *Bacillus pumilus* N40 on the basal liquid medium amended with 29.3 μg mL^{-1} $NiSO_4$ (Table 2), it can be recognized that the highest uptake (17.6 μg mL^{-1}) was achieved in case of *Bacillus subtilis* N10. After 2 h of metal contact. Accordingly this strain was selected for its high capacity of Nickel removal.

Effect of pH: The effect of pH on the accumulation of Ni^{2+} by free cells, is shown in (Table 3). the pH of the solution had a marked effect on the accumulation of Ni^{2+} by free cells. After 2 h of contact at different pH, it can be

recognized that at pH 7.6, *Bacillus subtilis* N10 strain achieved a high Ni^{2+} accumulation (18.2 μg mL^{-1}).

Nutritional requirements for biosorption of Ni^{2+}: The design of the experiment, together with the observed Ni^{2+} uptake in μg $mL^{-1}$are given in (Table 4). The uptake obtained by experimental trials (in triplicates) were found to be in the range of 5-23 μg mL^{-1}. The mean values of the main effect together with the t-value of the examined factors of Ni^{2+} uptake were calculated and presented in Table 5. Based on these results, it is conceivable that the levels of K_2HPO_4 and NaCl, encouraged the accumulation of Ni^{2+} by *Bacillus subtilis* N10. Interaction between K_2HPO_4 and NaCl concentrations on Ni^{2+} accumulation Fig. 1 confirmed that low concentration of both factors promote Ni^{2+}uptake.

Table 2: Screening test of different marine *Bacillus* sp.

	Ni^{2+} uptake (μg mL^{-1})				
Organisms	1 h	2 h	4 h	8 h	12 h
Bacillus pumilus N1	13.5	10.5	10.9	13.2	11.2
Bacillus thuringiensis N9	16.1	17.1	17.3	14.0	13.6
Bacillus subtilis N10	15.4	17.6	17.0	15.1	16.8
Bacillus pumilus N40	15.2	16.2	18.4	17.4	16.5

Table 3: Effect of pH on Ni^{2+} uptake (μg mL^{-1}) by *Bacillus subtilis* N10

pH	Ni^{2+} uptake (μg mL^{-1})
5.8	16.1
6.2	14.3
6.6	12.7
7.0	11.6
7.4	16.6
7.6	18.2

According to data obtained, a near optimum culture was formulated as follows: (g L^{-1}) Glucose, 1 yeast extract, 1.5; Peptone 1.5, $(NH_4)_2$ SO_4, 1.5; K_2HPO_4, 8;

Table 4: Results of Ni^{2+} uptake (μg mL^{-1}) in Plackett-Burman experimental design for 11 factor

	Independent variables											Ni^{2+} uptake
Trial	G	NH	K2	KH	Mg	Na	Fe	Mn	IS	P	YE	μg mL^{-1}
1	+	-	+	-	-	-	+	+	+	-	+	12.7
2	+	+	-	+	-	-	-	+	+	+	-	4.9
3	-	+	+	-	+	-	-	-	+	+	+	18.5
4	+	-	+	+	-	+	-	-	-	+	+	4.6
5	+	+	-	+	+	-	+	-	-	-	+	10.0
6	+	+	+	-	+	+	-	+	-	-	-	8.4
7	-	+	+	+	-	+	+	-	+	-	-	3.1
8	-	-	+	+	+	-	+	+	-	+	-	11.7
9	-	-	-	+	+	+	-	+	+	-	+	1.3
10	+	-	-	-	+	+	+	-	+	+	-	5.5
11	-	+	-	-	-	+	+	+	-	+	+	16.5
12	-	-	-	-	-	-	-	-	-	-	-	13.6
13	0	0	0	0	0	0	0	0	0	0	0	23.4

+: Present, -: Absent

Table 5: Statistical analysis of the Plackett-Burman experiment

Factor	Symbol	Main effect	t-test
Inoculum size (%)	IS	-3.10	-0.95213
Peptone	P	2.30	0.657933
Yeast extract (g L^{-1})	YE	2.80	0.862858
Glucose (g L^{-1})	G	-3.15	-0.98615
$(NH_4)_2$ SO_4 (g L^{-1})	NH	2.10	0.626346
K_2HPO_4 (g L^{-1})	K2	1.30	0.379272
KH_2PO_4 (g L^{-1})	KH	-6.70	-2.54353
$MgSO_4.7H_2O$ (g L^{-1})	Mg	0.10	0.024757
NaCl (g L^{-1})	Na	-5.40	-1.86337
$FeSO_4$ (g L^{-1})	Fe	1.30	0.389403
$MnSO_4$	Mn	-0.05	-0.00991

t-value significant at the 1% level = 3.70, t-value significant at the 5% level = 2.446, t-value significant at the 10% level = 1.94, t-value significant at the 20% level =1.372, Standard t-values are obtained from Statistical Methods (Cochran and Snedecor, 1989)

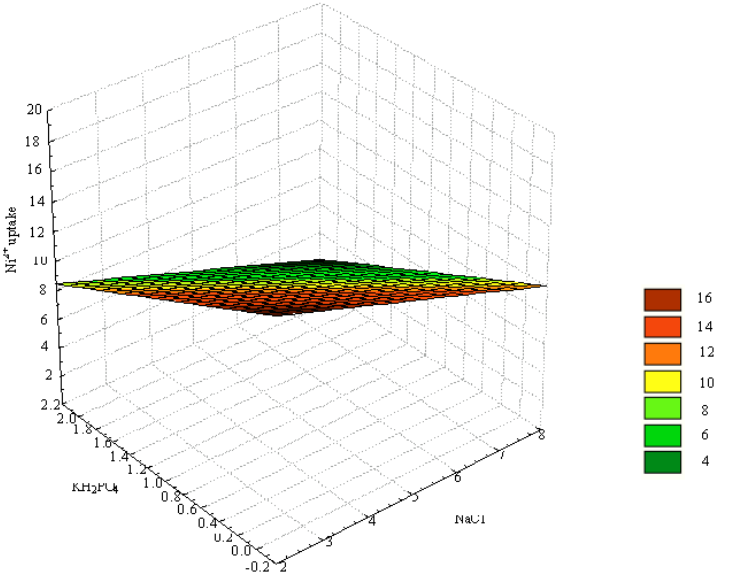

Fig. 1: Effect of interaction between NaCl and KH_2PO_4 on Ni^{2+} uptake (μg mL^{-1}) by *Bacillus subtilis* N10 free cells

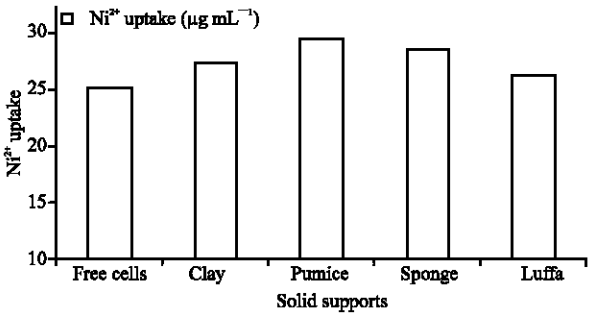

Fig. 2: Ni^{2+} uptake (µg mL^{-1}) by *Bacillus subtilis* N10 adsorbed on different solid porous supports

$MgSO_4$. $7H_2O$, 0.15 NaCl, 2.5; $FeSO_4$, 0.002, with inoculum size of 0.5 mL. This approach verified the validity of the applied design. A verification experiment was applied to evaluate the basal versus the optimized medium. Verification results showed 25 µg mL^{-1}which represents about 1.2 fold increase in Ni^{2+} uptake when compared with the control basal culture.

Effect of *Bacillus subtilis* N10 adsorption on Ni^{2+} uptake: The results recorded in Fig. 2 showed that a high cell adsorption occurred on all supports. However, Ni^{2+} uptake of adsorbed cultures was higher than that of cell free culture. Moreover, cultures containing adsorbed cells on pumice particles showed the highest Ni^{2+} uptake (29.2 µg mL^{-1}).

DISCUSSION

The high incidence of heavy metal-contamination in aquatic environments is known to cause severe damage to aquatic life. In addition, these metals kill microorganisms during biological treatment of wastewater with a consequent delay of the process of water purification (Hussein *et al*., 2004). Most of the heavy metal salts are soluble in water and form aqueous solutions and consequently cannot be separated by ordinary physical-chemical means of separation. Biological methods such as biosorption/bioaccumulation for the removal of heavy metal ions may provide an attractive alternative to physico-chemical methods (Kapoor and Viraraghavan, 1997).

Bacteria has been proved to act as a very good metal sequester like the bacterial strains; *Pseudomonas ambigua*, *Desulfovibrio vulgaris*, *Enterobacter cloacae* Ho-1, *Alcaligenes eutrophus*, *Dinococcus radiodurans* R1 (Igwe and Abia, 2006).

Metal ion homeostasis is regulated principally by metalloregulatory proteins that control metal ion uptake, storage and efflux genes. Moore *et al*. (2005) have used transcriptional profiling to survey *Bacillus subtilis* for genes that are rapidly induced by exposure to high levels of metal ions including Ag(I), Cd(II), Cu(II), Ni(II) and Zn(II) and the metalloid As(V). Many of the genes affected by metal stress were controlled by known metalloregulatory proteins

Hence, four marine *Bacillus* strains isolated from a highly heavy metal-contaminated sea area in the East Port in Alexandria were examined for their ability for sequestering Ni^{2+} from effluents. These four strains were proved to have a very high affinity for azodyes sequestering (El-Sersy, 2001). The obtained results showed that the strain *Bacillus subtilis* N10 achieved the greatest rate of Ni^{2+} uptake (60.1%) in only 2 h as contact time. The other three, showed also a high efficiency for Ni^{2+} and these results were predictable as these strains were initially isolated from a highly heavy metal contaminated sea area. These results are also in good agreement with those claimed by Fein *et al.* (1997) who used *Bacillus subtilis* to examine the bacterium interaction with Cd, Cu, Pb and Al. There is also López *et al*. (2002), who used the strain *Pseudomonas fluorescens* 4F39, which was isolated from a sludge of an electroplating effluent in Barcelona (Spain) and selected for its high Ni^{2+} accumulation, 110 mg Ni^{2+}/g dry cells (11% of its dry weight) (López *et al*., 2000).

The physiological state of the organism, the availability of micronutrients during their growth and the environmental conditions during the biosorption process (such as pH, temperature and the presence of certain co-ions) are important parameters that affect the performance of a living biosorbent. Therefore, on applying different pH values on the reaction solution, the maximal uptake value was seen at pH 7.6, where up to 62.1% of the total dissolved Ni^{2+} was sequestered. According to López *et al*. (2002), they found that the pH of the solution had a marked effect on the accumulation of Ni2+ by free cells. After 1 h contact at pH 6.5, *P. fluorescens* 4F39 cells accumulated 8 mg Ni^{2+}/g dry cells. At pH 8, Ni^{2+} accumulation increased markedly to 110 mg Ni^{2+}/g dry cells. According to the distribution of ionic species of nickel by the computer program SOL 1 (Izquierdo and Beltran, 1988), in the range of pH 1 to 7, one major hydrolyzed Ni^{2+} (90%) exists in the solution. The increase of Ni^{2+} accumulation as the pH increased was a consequence of the increase of the negative charge at the surface of the cells and a more efficient competition of Ni^{2+} with H^+ for binding sites. The drastic increase at pH 8 corresponded to the appearance of hydroxylated species. Collins and Stotzky (1992) considered the ability of some species, especially if hydroxylated, to be well adsorbed on the cell surface. Ni^{2+} accumulation by beads decreased as the pH of the Ni^{2+} solution increased.

Plackett-Burman design (Plackett and Burman, 1946) was constructed to determine the nutritional requirements

for biosorption of Ni^{2+}. Optimized medium, results in cells having a high efficiency for Ni^{2+} accumulation and achieving a level of 25 μg mL^{-1} which represents about 1.2 fold increase in Ni^{2+} uptake when compared with the control basal culture. The selective sequestering of metal ions from aquatic solutions by binding to the extracellular surface of the microbial cell is a well known phenomenon (Volesky, 1994). Several mechanisms by which metals interact with microbial cell walls and envelopes are established, including adsorption type reactions, metal reduction, complexion reactions and precipitation type reactions (Brierley, 1990). Microbial cell walls are chemically complex and each group of microorganisms (bacteria, fungi, yeasts and algae) has differing cell wall structures. However, in terms of metal binding, the interaction between the cell wall and a metal is quite similar and the cell walls can be considered to have polyfunctional metal-binding capabilities (Gazsó, 2001). Medium composition has a very remarkable effect on cell wall composition and consequently the cell affinity to specific metal cation (El-Helow *et al*., 2000).

On adsorbing bacterial cells on different supports showed that a high cell adsorption occurred on all of them. However, Ni^{2+} uptake of adsorbed cultures was higher than that of cell free culture. Moreover, cultures containing adsorbed cells on pumice particles showed the highest Ni^{2+} uptake**.** According to Ghanem and Abou-Elela (2006)**,** They reported that the immobilized mycelia of marine *Nocardiopsis aegptia* sp. on clay particles showed higher Cu^{+} uptake than free cells.

For future studies, An economical biological process of bioaccumulation or biosorption of toxic metal contaminants, will be considered the following features: inexpensive biomass, high metal binding capacity, selective metal binding, effective desorption methods, recycling of desorbents and repeated use of biomass (Gazsó, 2001).

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