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Bioremediation of Toxic Heavy Metals Pollutants By Bacillus spp. Isolated From Guilan Bay sediments, North of Iran

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Abstract. Conventional processes used for removal of heavy metals from industrial wastewaters include chemical precipitation, oxireduction, filtration, electrochemical techniques and sophisticated separation processes using membranes. These processes are usually expensive when heavy metals are present in moderate concentrations, such as 1 to 100 mg/L (1). This characteristic stimulates the use of alternative biotechnologies, due to their reduced cost and lower aggressiveness to the environment. This work presents some results on the use of microbes from the genus *Bacillus* for uptake of cadmium, zinc, copper and lead ions. Maximum copper bioaccumulations were 6.1 mol/g biomass for *B. cereus*, 5.8 mol/g biomass for *B. licheniformis* , 4.8 mol/g biomass for *B. amyloliquefaciens* and 5.9 mol/g biomass for *B. subtilis.* Maximum zinc bioaccumulations were 5 mol/g biomass for *B.subtilis*, 4.5 mol/g biomass for *B. licheniformis* and 4.1 mol/g biomass for *B. cereus* and *B. amyloliquefaciens* . Maximum cadmium bioaccumulations were 7.3 mol/g biomass for *B. licheniformis,* 10.7 mol/g biomass for *B. cereus*, 9.5 mol/g biomass for *B. cereus* and 7.2 mol/g biomass for *B. amyloliquefaciens* . Maximum lead biomaccumulations were 1.1 mol/g biomass for *B. amyloliquefaciens* and *B. licheniformis* and 1.8 mol/g biomass for *Bsubtilis*, and 0 .6 mol/g biomass for *Bcereus*. The different *Bacillus* strains tested presented distinct uptake capacities, and the best results were obtained for *B. subtilis* and *B. cereus*. The metal resistant isolates were identified using 16s rDNA sequencing .Thus the present work suggests that the brackish environment receiving a diverse anthropogenic input may provide a natural reservoir for the selection of heavy metal resistant bacterial strains.

Key words: Guilan Bay, heavy metals, bioaccumulation, *Bacillus*

1. INTRODUCTION

Regulation, handling and bioremediation of hazardous materials require an assessment of the risk to some living species other than human being, or assessment of hazard to the entire ecosystem. Assessment endpoints are values of the ecosystem that are to be protected and are identified early in the analysis. Such endpoints may include life cycle stages of a species and reproductive or growth patterns. Ecosystem risk assessment is at its dawn with this area of environment sciences still requiring extensive work in the industrialized nations of the world for sustainability of the global ecosystem.Heavy metals, such as cadmium, copper, lead, chromium and mercury, are important environmental pollutants, particularly in areas with high anthropogenic pressure. Their presence in the atmosphere, soil and water, even in traces, can cause serious problems to all organisms. Heavy metal accumulation in soils is of concern in agricultural production due to the adverse effects on food quality (safety and marketability), crop growth (due to phytotoxicity) and environmental health (1) . The mobilization of heavy metals into the biosphere by human activity has become an important process in the geochemical cycling of these metals. This is acutely evident in urban areas where various stationary and mobile sources release large quantities of heavy metals into the

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atmosphere and soil, exceeding the natural emission rates (2). Heavy metal bioaccumulation in the food chain can be especially highly dangerous to human health. These metals enter the human body mainly through two routes namely: inhalation and ingestion, and with ingestion being the main route of exposure to these elements in human population. Heavy metals intake by human populations through the food chain has been reported in many countries with this problem receiving increasing attention from the public as well as governmental agencies, particularly in developing countries (1). Industrialization is accelerating the deposition of heavy metals in soil and water bodies. In some ecosystems these metals can be easily incorporated by organic and inorganic fractions of the soil and by sediments. The extent of this incorporation depends on the concentration of metals and on characteristic biotic and abiotic factors. Nevertheless, in water bodies or soil, metals can be remobilized, acting as toxic elements. This way, it is essential to minimize deleterious effects of dispersion in natural waters, through the use of suitable technology-based techniques (3) . Beveridge focused his studies on the microbial morphology and incorporation of heavy metals; he concluded that the interaction between heavy metals and surface biological structures is inevitable (4). This surface accumulation occurs through chemical reactions such as complexation and ion-exchange with structural compounds present in the surface of microbes and other organisms (5). Incorporation is based on the polysaccharide composition of each particular organism, and is highly variable among distinct genera and even strains from the same species. Two particular groups of metals are of interest in this case: valuable metals, such as gold, platinum and silver, and toxic heavy metals, specially those from mining metallurgical activities (6,7,8). Thus, a detailed investigation of the chemical structures of bacterial cells and the understanding of the mechanism involved in the interaction is still missing in the study of the bioaccumulation process. The rationale for using *Bacillus* cells to study the uptake of heavy metal elements is the previous knowledge that Gram-positive cells accumulate a much higher amount of heavy metals than Gram-negative cells. Carboxyl groups are the main agents in the uptake of heavy metals. The sources of these carboxyl groups are the teichoic acids, associated to the peptidoglycan layers of the cell wall. In a broad review about the ultrastructure of the bacterial wall, surface structures were deeply detailed, providing a better understanding of the possible reaction sites (5, 6). The purpose of the present work was to investigate the ability of *Bacillus* species, isolated from sediments north of iran to accumulate copper, cadmium, zinc , lead and chromium . The objective is the selection of the best microbial species to be used in association with waste biomaterials to turn a batch process into a continuous process, with the advantage of suppressing costs of immobilization of the microbial cells. Some basic points about the surface structures of Gram-positive and Gram-negative bacteria should be briefly presented. A characteristic component of Gram-positive cells are teichoic acids and acids associated to the cell wall, whose phosphate groups are key components for the uptake of metals. The literature reports several studies on the interaction of heavy metals with bacterial surfaces, but just a few works consider these interactions at the molecular level (4,5). Thus, a detailed investigation of the chemical structures of bacterial cells and the understanding of the mechanism involved in the interaction is still missing in the study of the bioaccumulation process. The rationale for using *Bacillus* cells to study the uptake of heavy metal elements is the previous knowledge that Gram-positive cells accumulate a much higher amount of heavy metals than Gram-negative cells. Carboxyl groups are the main agents in the uptake of heavy metals. The sources of these carboxyl groups are the teichoic acids, associated to the peptidoglycan layers of the cell wall. In a broad review about the ultrastructure of the bacterial wall, surface structures were deeply detailed, providing a better understanding of the possible reaction sites (5, 6). Hazardous waste sites often contain complex mixtures of pollutants which include both organic contaminants and heavy metals .Microbial bioremediation of organic pollutants is a promising method of environmental cleanup. However, if the metals in soils are toxic to the microbes, removal of organic pollutants is slowed or prevented. Many reports have shown that (i) the short-term response to toxic metals is a large reduction in microbial activity and (ii) habitats that have had high levels of metal contamination for years still have microbial populations and activities that are smaller than the microbial populations and activities in uncontaminated habitats. Although these observations may suggest that metal contamination of soils retards bioremediation of organic pollutants, we take a different view, that metal contamination is an extreme environment (created by humans) to which microbes can respond (9).

2. MATERIALS AND METHODS

Sediments samples were collected using Peterson grab (July to September 2010) from sediments of Guilan province (anzali bay) in IRAN and transported on ice to the laboratory and processed within 10 h. Aerobic , cultivable bacteria were isolated by serially diluting 1 g of the sample in sterile distilled water. Gram-positive spore forming bacteria were isolated after heating the soil suspensions at 90 °C for 10 min in order to kill vegetative cells and 0.1 ml of the appropriate dilution were plated by spread plate technique on Luria Bertani (LB) Agar plate.Later,the plates were incubated at 25 °C for 24 h and observed for bacterial growth. Morphologically distinct colonies were picked, purified and stored at 4 °C for further analysis.The following characteristics were retained : Gram-positive rods, endospore producing, motile, catalase and oxidase positive.Clls were inoculated in Luria-Bertani broth (100 mL/flask) with the following composition : tryptone (10.0 g), yeast extract (5 g), sodium chloride (10 g), dissolved in one liter of distilled water. Final pH was around 7.4-7.6. The medium was autoclaved at 121ºC for 20 minutes.

Then, this medium was kept under agitation in a rotary shaker, at 80 rpm, for 48 hours at 27 ± 2ºC. Cells to be used in bioaccumulation experiments were separated by centrifugation. For quantification of the cell,they were quantified by direct weighing of the biomass, after drying at 105ºC for 24 hours. Solutions of copper, cadmium, zinc and lead sulphates were prepared in distilled water. Copper solutions presented the following concentrations: 1.7, 8.8, 17.6, 44.0 and 88.0 mg/L, namely Conc. 1, 2, 3, 4 and 5, respectively. Zinc solutions presented the following concentrations: 1.2, 5.7, 11.5, 28.7 and 57.5 mg/L, namely Conc. 1, 2, 3, 4 and 5, respectively. Cadmium solutions presented the following concentrations: 4.4, 22.0, 44.0, 110.0 and 220.0 mg/L, namely Conc. 1, 2, 3, 4 and 5, respectively. Finally, lead solutions presented the following concentrations: 1.2, 5.8, 11.7, 29.2 and 58.5 mg/L, namely Conc. 1, 2, 3, 4 and 5, respectively. All solutions were analyzed by atomic absorption spectrometry (Perkin-Elmer Analyst Model AA-300). Experiments of heavy metals bioaccumulation were done in Erlenmeyer flasks containing 100 mL of each heavy metal solution and 16.0 ± 1.0 mg of cells. To ensure equilibrium, cells and metal solution were maintained in contact for 24 hours, under constant agitation, at 27 ± 2ºC. In all experiments, cells were obtained from only one cultivation and collected from the same flask at the same growth stage. Microscopic observations revealed that cells did not grow or were lysed after incubation in the metal solutions. After 24 hours, cells were separated from the medium and residual metal concentrations were monitored by atomic absorption spectrometry. Experiments were done in triplicate.

The metal resistant isolates were identified using 16s rDNA sequencing .The chrosomal DNA was extracted according to standard procedure of Maniatis *et al*. and the quality of the product was checked on 0.8% TBE Agarose gel.The 16s rDNA was amplified using PCR with the universal primers 27f and 149r. The PCR products were cleaned using the QIAquick purification kit (Qiagen). The amplicons were sequenced in both forward and reverse direction by using an automated sequences were compared using BLAST program for identification of the isolates.

3. RESULTS AND DISCUSSION

Table 1. Presents the results of bioaccumulation of lead, zinc, cadmium and copper, by *Bacillus* spp.

Bioaccumulation by *B. licheniformis* ranged from 0 to 1.1 mol/g biomass for lead; from 0.3 to 7.3 mol/g biomass for cadmium; 0 to 4.5 mol/g biomass for zinc; and, from 0.1 to 5.8 mol/g biomass for copper Bioaccumulation by *B. cereus* and *B.amyloliquefaciens* ranged from 0 to 0.60 and 0 to 1.1 mol/g biomass for lead; from 0.1 to 10.7 and 0.1 to 7.2 mol/g for cadmium , 0 to 4.1 and 0 to 4.1 mol/g biomass for zinc; 0.1 to 6.1 and o.1 to 4.8 mol/g biomass for copper respectevily. Quantitative Bioaccumulation by *B. subtilis* ranged from 0.1 to 1.8 mol/g biomass for lead; from 0.2 to 9.5 mol/g biomass for cadmium; 0.1 to 5 mol/g biomass for zinc; and, from 0.2 to 5.9 mol/g biomass for copper. Results are expressed as mol/g in order to allow a direct comparison of results for the different metals.

Table1.Range Uptake (mol metal/g biomass) lead, cadmium, zinc and copper by *Bacillus licheniformisB. cereus, B. amyloliquefaciens and B. subtilis* isolated from sediments of Guilan bay(IRAN),

Bacteria	lead	cadmium	zinc	Copper
B. licheniformis	0-1.1	0.3-7.3	0-4.5	0.1-5.8
B. cereus	0-0.60	0.1-10.7	0-4.1	0.1-6.1
B.amyloliquefaciens	0-1.1	0.1-7.2	0-4.1	0.1-4.8
B. subtilis	0.1-1.8	0.2-9.5	0.1-5	0.2-5.9

An increasing uptake pattern can be observed for all the metals (Table.1). Saturation of biomass by metals was not observed, indicating that available sites probably exist. More concentrated metal solutions should be used to reach saturation. However, determination of saturation levels was not the purpose of the present investigation, but the determination of the potential ability of the cells to accumulate heavy metals to be used as metal concentrators in wastewater treatment, immobilized on the surface of waste biomaterials. Classical adsorption equations were not here used, because probably uptake was not restricted to surface phenomena, once viable cells were here used. Any metabolic activity could be in action during the uptake.

Table.1 also shows a selective uptake: cadmium > copper > zinc > lead.

In order to select a suitable *Bacillus* strain for further studies, a simple mathematical analysis was performed with the overall results obtained in the four groups of experiments. The first set of four bars from were compared and the most suitable strain to accumulate the metals at each concentration level was detected. The same comparison was done with the other sets of bars. For bioaccumulation of copper *Bacillus licheniformis* produced two results that were statistically distinct from the average values for all microbial cells; *B. cereus* presented four values that differed significantly from the average; *B. amyloliquefaciens* and *B. subtilis* presented two of these values. Based on these results, *B. cereus* was selected as the best copper biosorber. *B. subtilis* was the best zinc accumulator, *B. cereus* the best cadmium accumulator and *B. subtilis*, again, the best lead biosorber. The selected *Bacillus* strains can be used, in the future, for heavy metals removal, immobilized on waste biomaterials. Input of heavy metals imposes a selective pressure that may favor the growth and activity of resistant/tolerant microbes. The development of a metal-resistant population in a contaminated soil can result from: (i) vertical gene transfer (reproduction), (ii) horizontal gene transfer (including transposons and broad host range plasmids), and (iii) selection pressures on spontaneous mutants (due to the presence of metals). Transposable elements carrying mercury resistance genes have been linked to the distribution of this trait in nature. There are six or more systems for bacterial cadmium resistance known today. However, little physiological and biochemical work has been done. Only one of these systems has been cloned, and DNA sequencing has just been completed in our laboratory. Therefore, our understanding of bacterial cadmium resistance is preliminary and tentative. Cadmium ions are taken into sensitive bacterial cells by the energy dependent manganese transport system, where they cause rapid cessation of respiration by binding to sulfahydryl group in protein. Resistance to cadmium is a common plasmid specified function in *S. Aureus* (11). Thus the proposed cellular copper sequestration might be the basic mechanism of copper resistance. MIC some of heavy metals were showed in Table. 2

Table 2. Minimum Inhibitory Concentration (MIC) of Cr, Pb, Cu, Cd and Ni to bacteria isolated from heavy matals contaminated soils from an old tannery site in Michigan

Strain Name	K_2CrO_4 (mM)	$Pb(NO_3)_2$ (µM)	$CuSO_4$ (µM)	$CdSO_4$ (µM)	$NiCl_2$ (µM)
Cr2	0.1	50.0	50.0	10.0	25.0
Cr3	10.0	50.0	100.0	10.0	25.0
Cr4	0.1	50.0	100.0	25.0	25.0
Cr7	5.0	50.0	100.0	10.0	25.0
Cr8	0.2	50.0	50.0	10.0	10.0
Cr9	2.0	0.0	100.0	25.0	50.0
Cr10	>20.0	50.0	100.0	50.0	50.0
Cr11	0.0	0.0	25.0	25.0	na
Cr12	1.0	50.0	100.0	10.0	50.0
Cr13	>20.0	50.0	100.0	10.0	50.0

Cr14	0.2	50.0	100.0	25.0	25.0
Cr15	>20.0	50.0	100.0	10.0	50.0
Cr18	10.0	0.0	25.0	10.0	100.0
Cr19	1.0	25.0	50.0	25.0	50.0
Cr20	2.0	0.0	50.0	10.0	100.0

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