Progress in Biological Sciences
Vol. 2, No. 1, 1-11, Winter/Spring 2012

Lead and Cadmium Bioremoval by *Halomonas sp.*, an Exopolysaccharide-Producing Halophilic Bacterium

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Received: 12 September 2012; Revised: 30 October 2012; Accepted: 10 November 2012

Abstract

Toxic heavy metals, such as lead (Pb) and cadmium (Cd) are widely used in industry and their accumulation in the living tissues may cause serious health problems and ecological hazards. Twenty four moderately halophilic bacteria isolated from saline environments of Iran were used to study their ability to bioremediation of lead and cadmium. Amongst them, a Gram-negative rod shaped bacterium, designated as strain D showed remarkable ability for removal of Pb and Cd and could grow in media supplemented with 5 mM of these toxic heavy metals. Phenotypic characterization and phylogenetic analysis based on 16S rRNA gene sequence comparisons indicate this strain belongs to the genus *Halomonas.* Atomic absorption (AA) spectroscopy was used to estimate the removal rate of lead and cadmium by bacterial biomass, autoclaved biomass and exopolysaccharide (EPS) matrix under different conditions. The strain D could uptake more than 90% and 50% of lead and cadmium, respectively. Biomass showed the best lead removal at pH 3.0- 6.0, 35 °C and 5% NaCl (w/v), while the EPS showed maximum removal at pH 5.0, 35 °C and 10% NaCl (w/v). For cadmium removal by biomass, the best results were obtained at pH 3.0, 25 °C and 1% NaCl (w/v) while the EPS showed the optimal cadmium removal at pH 5.0, 45 °C and 1% NaCl (w/v). The results suggest that halophilic bacteria such as *Halomonas sp.* could be used for remediation of Pb and Cd in contaminated saline soils and wastes discharge sites.

Keywords: Atomic absorption, Bioremediation; Heavy metals; Moderate halophiles.

Introduction

Heavy metals can be produced through industrial processes such as mining, refining, electroplating, metallurgical and petrochemical processes, and manufacturing of plastics, fertilizers, and pigments (Malik, 2004; Tunali *et al.,* 2006; Esposito *et al.,* 2001). Heavy metals pollution represents an important problem due to their toxic effect and accumulation throughout the food chain, and leads to serious ecological hazards as a result of their solubility and mobility. Among the toxic heavy metals, Pb and Cd are widely produced in industry and their accumulation in theliving tissues may cause serious health problems (Volesky, 1995). The conventional techniques for metal remediation, such as chemical precipitation, electrochemical treatment, reverse osmosis and ion exchange, are very expensive and not environmentally acceptable. Hence, new bitechnological methods for remediation of these toxic metals which are more affordable and ecofriendly, are being considered recently (Chen *et al.,* 2005; Hawari and Mulligan, 2006; Iyer *et al.,* 2005).

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Furthermore, biosorption can overcome common problems associated with physicochemical processes such as high operational costs (Costly and Wallis, 2001). Various microorganisms including algae, bacteria, molds and yeasts have been shown capacities for metal removal from industrial and radioactive wastes through functional groups on their cell envelopes (Volesky, 1986; Gadd, 1988; Brierley, 1990). Exopolysacharides (ESP) produced by algae, bacteria, fungi and yeast has got a great deal of attention recently as a low-cost, non-hazardous and effective method for bioremoval of toxic metals from environment. Exopolysacharides are high molecular weight sugar polymers secreted by microorganisms into the surrounding environment. Heavy metal removal by EPS is a passive method in which the metal cations bind to the negative charges of acidic groups from exopolysaccharide. The structural properties of ESP affect their biosorption capacity (Iyer *et al.,* 2004; Salehizadeh and Shojaosadati, 2003). High concentration of salts in wastewater treatment systems have inhibitory effects on chemical reactions, and conventional biological treatments cannot function well at high salt concentrations. Halophilic and halotolerant microorganisms are suitable candidates for bioremediation processes, since they are able to grow on a wide range of salt concentrations (Margesin and Schinner, 2001; Mellado and Ventosa, 2003; Ventosa *et al.,* 1998). The purpose of this study was to isolate halophilic bacteria resistant to lead and cadmium and to study the possibility of using strains with high Minimum Inhibitory Concentration (MIC) values in bioremediation processes.

Materials and Methods

Chemicals

Lead nitrate, cadmium nitrate, nutrient broth and various salts were obtained from Merck (E. Merck, Darmstadt, Germany). The stock solutions were prepared in distilled water and filter-sterilized by microbial filter (0.22 μm). Working solutions were stored at 4 °C for approximately five days.

Isolation and cultivation

In total, twenty four moderately halophilic bacteria were isolated from hypersaline soils and waters located in Gheshm, Garmsar, Esfahan, Karaj, Qom, and Tehran in Iran. All strains were grown in saline medium with a final total salt concentration of 100 g l^{-1} supplemented with 5 g of yeast extract per liter. The salt solution composition which is also called SW-10 was prepared as follows (gl^{-1}): NaCl, 81; $MgCl_2$, 7; $MgSO_4$ 7 H_2O, 9.6; $CaCl_2$, 0.36; KCl, 2; $NaHCO_3$, 0.06; NaBr, 0.026 (Garabito *et al.,* 1997; Ventosa *et al.,* 1982). When necessary, the medium was solidified by adding 15 g agar. The cultures were incubated at 35 °C on an orbital shaking incubator (orbital incubator SI 50, Stuart Scientific) at 150 rpm for 48 h. The resistance of the strains to lead and cadmium was determined by growing the bacteria in nutrient agar containing different concentration of lead and cadmium and 10% NaCl (w/v) at 35 °C with the pH adjusted to 7.2-7.4 before autoclaving.

Identification of the isolates

Morphological and physiological features were determined in basal culture media containing 10% NaCl (w/v). Bacteria were grown either in saline nutrient broth or on agar medium. Gram reaction, motility, morphology and colony aspect, catalase and oxidase activities, nitrate reduction, hydrolysis of esculin, Tween 80 and Indole production were determined (Simbert and Krieg, 1994). Acid production from carbohydrates and utilization of carbon and nitrogen sources were evaluated as recommended by Ventosa *et al.,* (1982).

To determine the optimum temperature and pH for the growth of the strains, the cultures were incubated at a temperature range of 5-55 °C with increments of 5 °C and pH values of 5.0-11.0; pH values below and above 6 were adjusted with sodium acetate and Tris-HCl buffers, respectively.

For a more detailed characterization of a selected strain, a comparison of the 16S rRNA gene sequence was carried out. For this purpose, genomic DNA of the isolate was extracted, using a Genelute DNA extraction kit (Sigma) by following the manufacturer's recommended procedure. The 16S rRNA gene of the isolate was amplified using the universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG) and 1541R (5′-AAGGAGGTGATCCAGCCGC A-3′). The amplification was done by initial denaturation at 95 °C for 5 min followed by 10 cycles of 93 °C for 1 min, 63 °C for 1 min, 71 °C for 1.5 min, 20 cycles of 93 °C for 1 min, 67 °C for 1 min, 71 °C for 2 min and final extension at 71 °C for 5 min PCR product purification was conducted using a PCR purification kit (Bioneer, South Korea). The purified PCR product was sequenced in both directions using an automated sequencer by SeqLab laboratory (Germany). The phylogenetic relationship of the isolate was determined by comparing the sequencing data with sequences of the related type strains of species of the genus *Halomonas* (Gene Bank database of the National Center for Biotechnology Information). Phylogenetic analysis was performed using the neighbour-joining method with the software packages PHYLIP (Felsenstein, 1993) and MEGA version 3 (Kumar *et al.,* 2004) after multiple alignments of data available from public databases by CLUSTAL_X (Thompson *et al.,* 1997).

Determination of lead and cadmium tolerance

The agar dilution method was used to determine the tolerance of strains to lead and cadmium (Washington and Sutter, 1980). Volumes of 20 ml of melted nutrient agar containing 10% NaCl (w/v) plus various concentrations of lead and cadmium (0.3, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 and 30 mM) were poured into 8 cm plates. Then 10 µl of the bacterial suspension (1.5×10^8 cfu ml^{-1}) was inoculated on each plate using a sampler followed by incubation at 35 °C for 7 days. Minimum inhibitory concentration (MIC) for lead and cadmium was determined. Each type of plate was prepared in triplicate (Nieto *et al.,* 1989).

Lead and cadmium removal experiments

The 100 ml Erlenmeyer flasks containing 20 ml of saline medium supplemented with 5% (w/v) NaCl and 0.5 mM lead or cadmium nitrate were prepared for the removal experiments. The bacterial suspensions with the final concentration of 1.5×10^6 $CFUml^{-1}$ were prepared and used as inoculums. The medium was inoculated with 0.2 ml of the bacterial suspensions and incubated aerobically at 35 °C on a rotary shaker (150 rpm) for 2 days. The cells were pelleted out by centrifuging at 3000 ×g for 15 min and the supernatants were used to determine the residual lead and cadmium, by atomic spectroscopy. Based on results of MIC tests and also removal experiments, strain D was selected for further studies.

Evaluation of lead and cadmium removal by biomass, autoclaved biomass, and exopolymeric substances of the selected strain

Removal of lead and cadmium by biomass, autoclaved biomass, and exopolymeric substances of the selected strain was evaluated for better understanding the biosorption process. Bacterial cultures in

saline nutrient broth were centrifuged at 4000 ×g for 15 min and cell pellets were washed with distilled water three times and centrifuged again under the same conditions. Cell pellets were then ready to be added as biomass to stock solutions of lead and cadmium at a concentration of 100 ppm for evaluating the removal rate. In order to evaluate the removal rate by autoclaved biomass, Erlenmeyer flasks containing bacterial cultures were autoclaved and then centrifuged at 4000 ×g for 15 min and cell pellets were washed with distilled water three times and centrifuged again under the same conditions. Cell pellets were then ready to be added as dead biomass to stock solutions of lead and cadmium at a concentration of 100 ppm for evaluating the removal rate.

To study the removal rate by exopolymeric substance, after three days of aerobic incubation, bacterial suspension was centrifuged at 60, 000 × g for 80 min at 4 °C. The cell pellet was washed with saline for 2-3 times, and its saline supernatant containing bound EPS was saved for the EPS to be recovered, while the saline-washed cell pellet containing no EPS was used as cell biomass. The exopolymeric substance was then recovered by alcohol (ethanol 96%) precipitation of the supernatant (volume ratio of alcohol to the supernatant: 3:1) at 4 °C for one night. (Quesada *et al.,* 2004). EPS was then ready to be added to stock solutions of lead and cadmium at a concentration of 100 ppm for evaluating the removal rate. One g of biomass, autoclaved biomass and EPS were brought into contact with 50 ml of pure lead and cadmium solutions. After 1 h of exposure at room temperature on a rotary shaker (150 rpm) contents were centrifuged at 3000 ×g for 15 min and the supernatants were used to determine the residual lead and cadmium by atomic spectroscopy (Model: Varian Spectr AA 200).

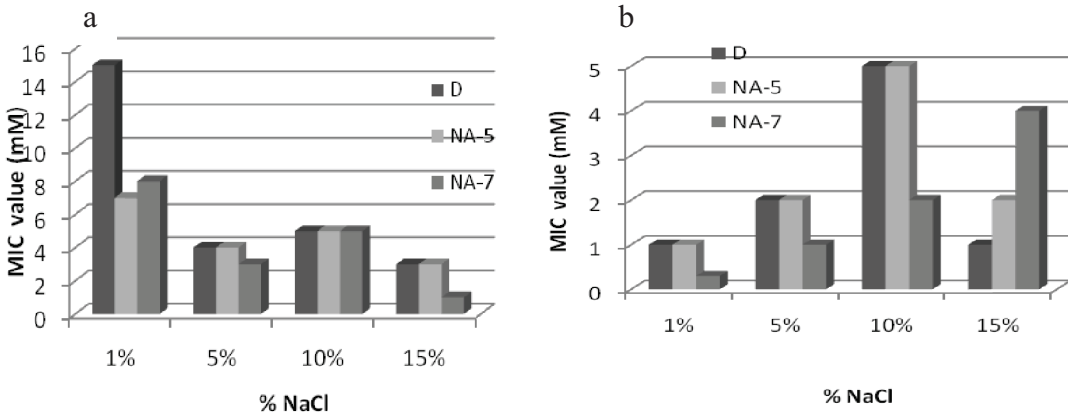

Figure 1. The effect of salinity on tolerance to lead (a) and cadmium (b) in three moderately halophilic strains: D, NA-5 and NA-7 in saline nutrient broth.

Effect of various factors on lead and cadmium removal

To study the effect of different factors on removal rate of toxic heavy metal, one g of biomass and exopolymeric substances of the selected strain was added to 50 ml of pure lead and cadmium solutions at a concentration of 100 ppm. Exposure was performed at different pH values (3, 4, 5 and 6), temperatures (25, 35, 45 and 55 °C) and NaCl concentrations (1%, 5% and 10% w/v), on a rotary shaker (150 rpm) for 1 h. Contents were then centrifuged at 3000 × g for 15 min and the supernatants were used to determine the residual lead and cadmium by atomic spectroscopy.

Results

Screening of moderately halophilic bacteria tolerant to lead and cadmium

Among many moderately halophilic bacteria isolated from various environments in Iran, twenty four strains were studied for their resistance to lead and cadmium. All strains tolerate a concentration of 0.5 and 0.1 M of lead and cadmium, respectively. However, three strains (D, NA-5 and NA-7) showed a higher tolerance to these metals and they were selected for further studies (Table 1). Strain D showed the maximum MIC which was higher than previous reports for other moderate halophiles such as *Marinococcus halophilus* (ATCC 27964) which grew at concentrations of lead up to 2.5 mM lead and at concentrations of cadmium up to 0.5 mM (Nieto *et al.,* 1989).

Effects of different NaCl concentrations on tolerance to lead and cadmium were determined (Fig. 1 a and b). According to the results, at increasing concentrations of NaCl from 1% to 15%, decreased tolerance to lead was observed. However, with respect to cadmium, decreasing NaCl concentration from 10% to 1%, resulted in decreased tolerance to this metal. At 15% NaCl (w/v), tolerance to cadmium was also very low.

Estimation of metal removal in three selected strains

All three toxic metal tolerant isolates were able to remove these compounds, but strain D exhibited slightly better removal of metals overall. It could uptake after two days of growth more than 90% of lead when grown in 5% NaCl and 0.5 mM lead nitrate and 50% of cadmium when grown in 5% NaCl and 0.5 mM of this metal (Fig. 2). There was no further removal after growth for 96 hours. On the basis of the MIC results and toxic metal removal experiments, strain D was selected for further studies.

Strain D was shown to be a Gram-negative, non-spore forming, motile, facultative aerobic rod and produced catalase and oxidase. The colonies appeared round, smooth, formed a creamy pigment and were 2 mm of diameter after 48 h. The phenotypic features of this strain in comparison with other studies, lead us to place it on the genus *Halomonas*. To confirm the identity of the isolate, PCR amplification and sequencing of the 16S rRNA gene was performed. 1452 bp of 16S rDNA gene of the D2 strain (GenBank accession no. DQ767691) was determined. The phylogenetic tree constructed by the neighbour-joining method indicated that the isolate D was part of the cluster within the genus *Halomonas* (Fig. 3). Among the described species, the closest relative of strain D was *Halomonas elongata* with 99% similarity.

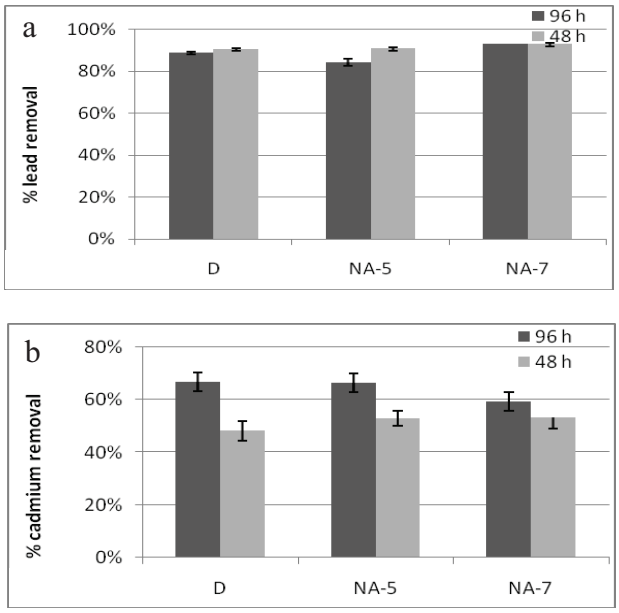

Figure 2. Lead (a) and cadmium (b) removal by three moderately halophilic strains at 48 and 96 h. Results represent the means of three separate experiments, and standard deviation bars are indicated.

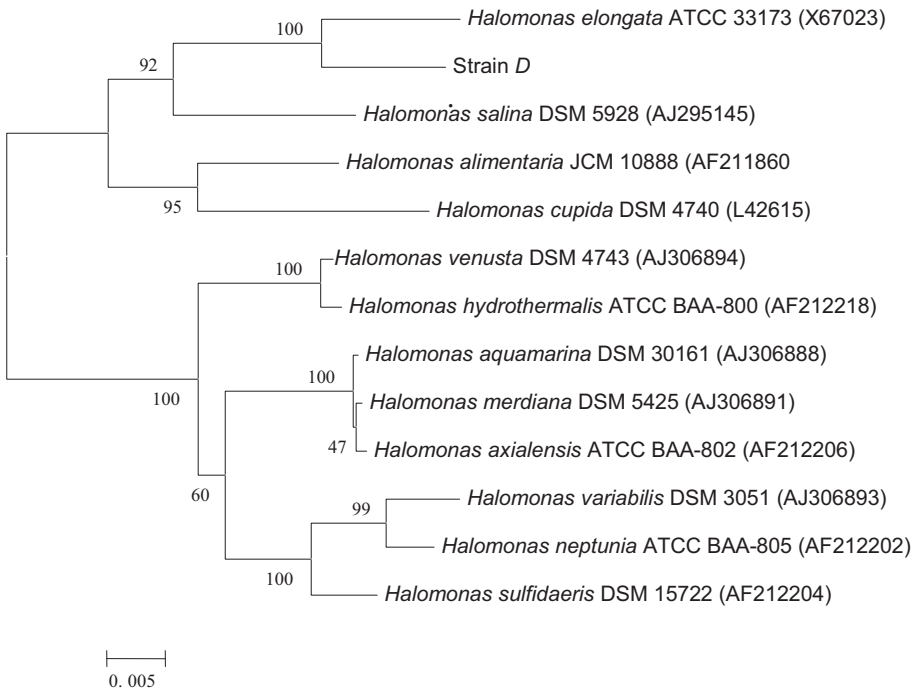

Figure 3. Phylogenetic tree showing the position of the halophilic isolate s train D, based on 16S rRNA gene sequence comparison and the neighbour –joining method. The accession numbers for the reference strains are included in brackets. Bootstrap values are indicated on the branches.

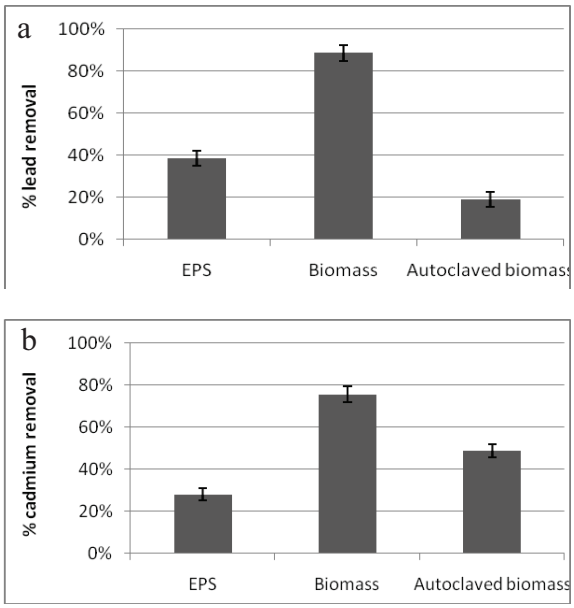

Figure 4. (a) Lead removal by biomass, autoclaved biomass and EPS of strain D. (b) Cadmium removal by biomass, autoclaved biomass and EPS of strain D. Results represent the means of three separate experiments, and standard deviation bars are indicated.

Strain D could grow in a wide range of temperature (15-45 °C), pH conditions (5.0-10.0) and salt range (1-27%). However, optimum growth was observed at 42 °C, pH 7.4, and 7.5% (w/v) NaCl. This bacterium can be considered as a moderate halophile.

Determination of toxic metal removal by biomass, autoclaved biomass, and exopolysaccharide of the selected strain

Lead and cadmium removal by biomass, autoclaved biomass, and exopolymeric substances produced by strain D was measured and maximum removal obtained when using biomass which was 91.3% for lead and 78.4% for cadmium, after 1 h of exposure (Fig. 4).

As can be observed in Fig. 4, maximum lead absorption was achieved by biomass, then exopolymeric substances and finally by autoclaved biomass, while the maximum cadmium absorption was observed by biomass, then autoclaved biomass and finally EPS.

Effects of pH, temperature, and NaCl concentration on removal rate

As shown in Fig. 5 a and b, the pH had no significant effect on lead removal by biomass. In cadmium removal, the best removal rate was achieved in pH 3 and pH 5 when using biomass and EPS, respectively. In our study, lead nitrate and cadmium nitrate solutions prepared for exposure to biomass, had high metal concentrations. Therefore, performing experiments at room temperature and by adding NaOH for adjusting the pH of the medium resulted in white precipitation of lead and cadmium hydroxide.

Lead and cadmium removal were studied at various temperatures ranging from 25 to 55 °C (Figs. 5 c and d). Temperature has no significant effect on removal rate of either of the metals.

To determine the effects of different concentrations of salt on the toxic metal removal capacity of the strain, sodium chloride was added at concentrations of 1% to 10% (w/v). Maximum lead removal was observed at 5% NaCl (w/v) and 10% NaCl (w/v) by biomass and exopolysaccharide, respectively (Fig. 5 e). Also, Maximum cadmium removal was observed in the presence of 5% NaCl (w/v) and 1% NaCl (w/v) by biomass and exopolysaccharide, respectively (Fig. 5 f).

Discussion

Moderately halophilic bacteria comprise a diverse group that grows from low-salt media to high salt concentrations showing optimal growth at 5 to 15% salts (Ventosa *et a l.,* 1998). These bacteria can be used in treatment processes of toxic waste waters and polluted environments (Margesin and Schinner, 2001; Mellado and Ventosa, 2003; Ventosa *et al.,* 1998; Woolard and Irvine, 1992). Identification of metal resistant strains is the first step in applying them in bioremediation processes (Trevors *et al.,* 1985).

For strain D, a decreased tolerance to cadmium along with decreasing NaCl concentration from 10% to 1% was observed. Nevertheless, in 15% NaCl (w/v) the tolerance was also very low. The same results were reported by Nieto *et al.,* (1989) for some moderately halophilic bacteria, which decreasing NaCl concentration in their growth medium led to increasing cadmium toxicity. However, our data showed that decreasing NaCl concentration in the growth medium led to higher tolerance to lead. Moderate halophiles are good candidates because they naturally need high anion and cation concentrations for their growth while increasing salt concentrations inhibits growth of other microorganisms (Ventosa *et al.,* 1998).

Several studies have reported metal removal by bacteria in culture media (Iyer *et al.,* 2004; Iyer *et al.,* 2005; Zouboulis *et al.,* 2004). Data for an unknown halophilic bacterium showed 70.5% and 44.9% removal capacity for lead and cadmium, respectively, after two weeks of exposure (Massadeh *et al.,* 2005), which was significantly lower than removal rate carried out in this study by strain D after only two days.

In this study we observed that the removal rate was much higher when using biomass of strain D. Similar results were reported for lead removal by *Saccharomyces cerevisiae* in which decreasing the lead concentration was 10 times greater when using biomass in comparison to using dead biomass (Zouboulis *et al.,* 2004).

Heavy metal cations get trapped in surface structures of bacterial cell which will lead to their attachment to binding sites present in the cellular surfaces and subsequently, biosorption takes place. This process is non-metabolic and energy-independent and is called passive uptake. Also, the heavy metals can enter the cell through metabolic cycle which is energy dpendent and is called active uptake. Both active and passive modes of metal uptake can be called bioaccumulation (Iyer *et al.,* 2004).

Table 1. The MIC values in 24 moderately halophilic strains in nutrient agar containing 10 % NaCl (w/v).

Strain	$PbNO_3$	MIC (mM) $CdNO_3$
NA-5	5	5
D	5	5
HS-2	5	4
X159	2	0. 3
K-1	4	4
E	4	3
H	4	4
T142	0. 5	0. 3
A73	2	0. 3
Y105	2	0. 3
F	4	3
J	3	3
F – 2	2	2
Z	3	2
L1	2	2
D2	1	0. 1
ZG13	3	2
NA-7	5	2
QW-6	3	1
ViGi	4	2
Halomonas maura (DSM 13445)	1	1
Halomonas halophila (DSM 4770)	2	2
Halomonas elongate (DSM 2581)	5	3
Halobacillus karajensis (DSM 14948)	2	1

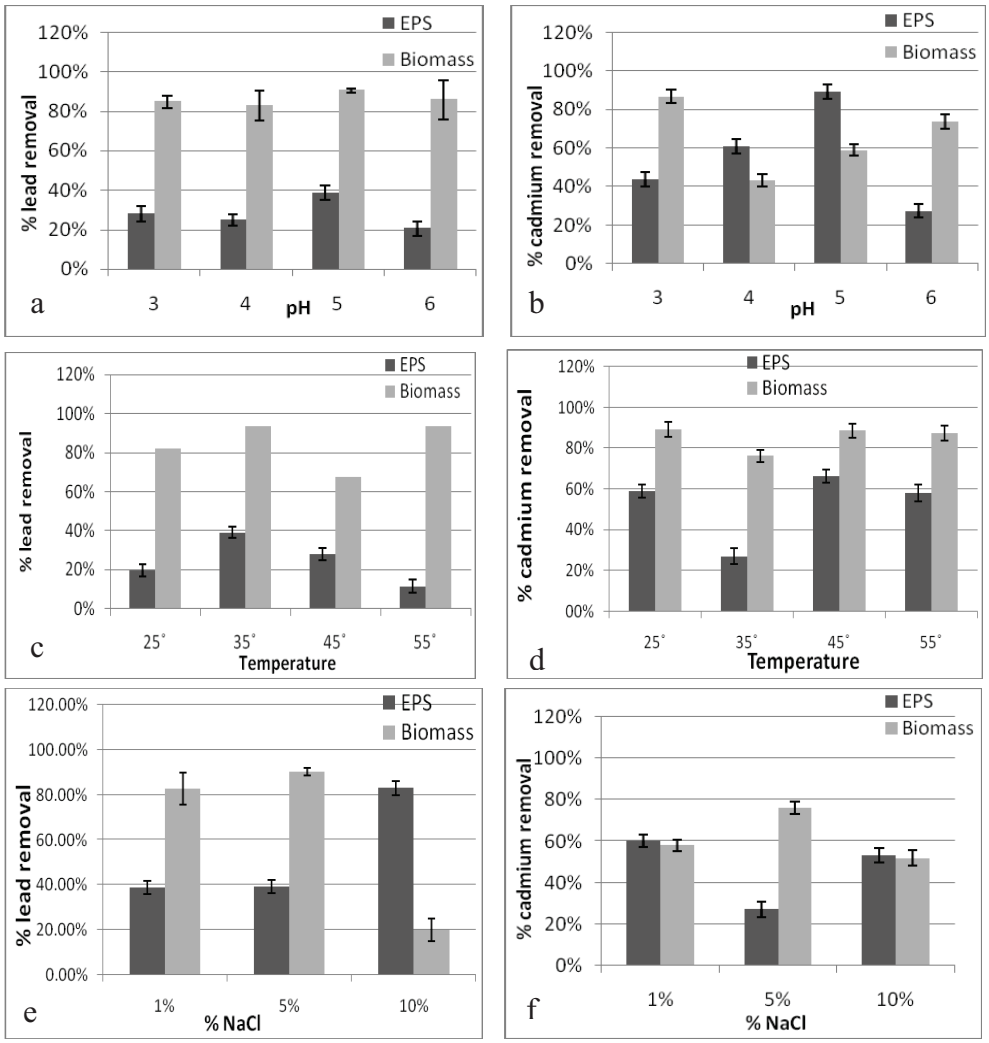

Figure 5. The effect of pH (a, b), temperature (c, d) and NaCl concentrations (e, f) on lead (a, c, e) and cadmium (b, d, f) removal by biomass and EPS of strain D. Results represent the means of three separate experiments, and standard deviation bars are indicated.

Decreased removal rate by autoclaved biomass can be caused for two reasons: i) the heavy metal attaches onto the surface of the cell wall and membrane and it has no penetration into the cell; ii) Some binding sites of the cell surface for the metal ions may be damaged or transformed as the result of autoclaving at high teperature (Suh *et al.,* 1998).

Effect of different pH values in cadmium asorption was in consistency with pervious rsults, indicating that metal ion adsorption is generally pH dependent, as the pH affects the availability of metal ions in solution as well as the metal binding site onto cell surface (Zouboulis *et al.,* 2004).

In our experience, temperature had no signifcant influence on the metal removal by exopolymeric substances, while in meta removal by biomass, best results were obtained at temperatures near to strain′s optimum growth temperature. Likewise, Previous reports showed that the uptake of cadmium ions into *Bacillus licheniformis* biomass was not significantly influenced by temperature. The temperature of the adsorption medium is important only in the case of active uptake which is energy

dependent. Otherwise, temperature does not seem to affect the process and biosorption is more dependent on physico-chemical (electrostatic forces) bonds (Zouboulis *et al.,* 2004).

About the effect of NaCl concentration, the best metal removal results by biomass were obtained at NaCl concentrations near to optimum NaCl concentration for the growth of D strain. Whereas, in the case of metal removal by exopolymeric substances, increasing NaCl concentration led to best Pb removal rate and decreasing NaCl concentration led to best Cd removal rate. It should also be considered that there are reports stating the biosorption of heavy metals can be influenced by the composition of exopolysaccharide, especially by the presence of Uronic acid (Iyer *et al.,* 2005).

The increase of industrial activities has intensified environmental pollution problems. These polluted environments often contain different salts. Although bioremediation is usually one of the best processes for removing heavy metal pollutants, in salty conditions it seems to be hindered and active sludge's activity is clearly reduced. Therefore, the isolation of halophilic microbial strains such as strain D, which is able to tolerate many toxic metal ions, can be promising for bioremediation of contaminated saline soils and waste discharge sites.

Acknowledgement

The study was supported by Iran National Science Foundation (No. 85043/13).

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