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BIOSORPTION OF CADMIUM AND LEAD USING SUSPENDED AND IMMOBILIZED *Enterobacter cloacae* AT DIFFERENT PH

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Key words: metals, bacterial cultures, absorption, adsorption, biomass production

ABSTRACT

The aim of this work was to determine the best conditions for biomass production of the bacterium *Enterobacter cloacae* and for Cd and Pb retention by adsorption on the cell walls or by absorption and accumulation in the bacterial biomass. Suspended and immobilized cultures of this bacterium, acclimated to both metals, were grown for 24 h at pH 6, 7 and 9, in trypticase soy (TS) medium added with 2.73 mg/L of Cd or 16.8 mg/L of Pb. Immobilized cultures at pH 7 and 9 had the highest biomass production. Percentages of Cd retention by adsorption and absorption increased with increasing pH, but adsorption was higher in suspended while absorption was higher in immobilized cultures. Possibly because of different affinity of bacterial cells for these metals, the percentages of Pb retention were higher than those of Cd. In percentage, the highest Pb adsorption on cell walls was at pH 9, while retention by biomass decreased with increasing pH. The amount of metal retained by one g of bacterial biomass, efficiency was higher at pH 6, possibly because of the presence of dead or dying bacterial cells.

Palabras clave: metales, cultivo bacteriano, absorción, adsorción, producción de biomasa

RESUMEN

En este trabajo se determinaron las mejores condiciones para la producción de biomasa y para la retención de Cd y Pb por adsorción en las paredes celulares o por absorción y acumulación en la biomasa bacteriana de *Enterobacter cloacae*. Con esta finalidad, se mantuvieron durante 24 h a pH 6, 7 y 9 cultivos suspendidos e inmovilizados de esta bacteria aclimatada a los dos metales, en medio tripticasa de soya (TS) adicionado con 2.73 mg/L de Cd o 16.8 mg/L de Pb. La mayor producción de biomasa se obtuvo con los cultivos inmovilizados a pH 7 y 9. Los porcentajes de adsorción y absorción de Cd aumentaron con el incremento del pH, pero la adsorción resultó mayor en los cultivos libres, mientras que la absorción fue mayor en los inmovilizados. Los porcentajes de retención de Pb fueron mayores que los determinados para Cd, posiblemente debido a diferencias en la afinidad de estos metales para las células bacterianas. La mayor adsorción de Pb en las paredes celulares fue con pH 9, mientras que la retención en la biomasa disminuyó con el incremento del pH. En términos de cantidad de metal retenido por un gramo de biomasa bacteriana, la eficiencia de retención fue mayor a pH 6, posiblemente debido a la presencia de células muertas o poco viables.

INTRODUCTION

Industrial development produces environmental problems, and among these the most important is the presence of metals in the aquatic environment (Ribeiro et al. 2008). In the case of Cd and Pb, Mexico is in the fifth position worldwide regarding the production of both metals (Camimex 2017), which explains its presence in aquatic environments of the northwest of the country in relatively high concentrations that could exceed safe levels.

There are several techniques available for the bioremediation of aquatic ecosystems affected by metals. Among these, biosorption consists in the retention of metals dissolved in a liquid medium by substrates of different biological origin, including bacteria resistant or tolerant to metals (Vieira and Volesky 2000).

These microorganisms possess detoxification mechanisms based on the energy-dependent elimination of toxic metals (Silver 1996, Castrillón-Rivera and Palma-Ramos 2012), or they may tolerate the presence of several metals because of their immobilization within or outside their cell wall (Zur et al. 2016).

In previous experiments we showed that an adapted strain of *Enterobacter cloacae* may grow in cadmium (Cd) or lead (Pb) added TS medium and retain a sizeable fraction of these metals in their biomass (Bojórquez et al. 2016). In this work we evaluated the biosorption capacity of this strain grown at different pH in suspended and immobilized cultures.

MATERIALS AND METHODS

The bacterial strain was isolated with standard techniques from a sample of domestic wastewater obtained from the water discharges of the Universidad Politécnica de Sinaloa (Mazatlán, Sinaloa, Mexico). After its identification with molecular techniques, it was acclimated and maintained in trypticase soy (TS) growth medium with close to 2.8 and 17 mg/L of Cd and Pb, respectively (Bojórquez et al. 2016).

Optical density readings obtained in preliminary experiments showed that *E. cloacae* grew well between pH 10 and 6, but performed poorly outside this range. Therefore, biomass production in metal-free growth medium and the following retention experiments were performed with suspended and immobilized cultures at pH values of 6, 7 and 9, in TS medium with the programmed amounts of Cd or Pb, added as chlorides. The final concentrations, determined by atomic absorption spectrophotometry (Varian SpectrAA-220), were 2.73 mg/L of Cd and 16.8 mg/L of Pb, respectively.

Cultures (20 suspended and 20 immobilized) were started with 0.1 mL of an actively growing culture in Cd- or Pb-added medium and grown for 24 h in 20-mL test tubes with 15 mL of TS medium added with the respective metal. Immobilized cultures had a 4 × 6 cm (48 cm^2, considering both sides) of acid-treated grey polypropylene netting of known weight for biofilm support.

The initial pH of the medium was adjusted to 6, 7 and 9 using HCl or NaOH. After 24 h, cultures were filtered through 4.7 cm Whatman GF/C glass fiber filters of known weight. Suspended and attached bacterial biomass was determined with a semi-micro analytical balance (0.01 mg) after drying separately the filters and the polypropylene support with attached bacteria at 60 ºC for at least 24 h or until constant weight.

Metal retention was determined in two separate experiments with triplicate cultures. After 24 h of growth in the respective medium, substrates were removed from the immobilized cultures, gently rinsed with a known volume of metal-free fresh medium, which was added to the original cultures. After this, all cultures were centrifuged at 5000 rpm (340 × g) in a refrigerated centrifuge (Sorvall Legend T, 4 ºC) for 30 min and the supernatant was decanted and stored for metal analysis.

The metal adsorbed was separated from the bacterial cell surface vortexing pellets and substrates in 10 mL of 10 mM ethylenediaminetetraacetic acid (EDTA) (Bashkar and Bhosle 2006). After centrifugation for 15 min, the EDTA was recovered and stored for metal analysis. The resulting pellet was digested in 3 mL of concentrated nitric acid (Fluka) in a Teflon vessel at 120 ºC for 4 h. Substrates and adhered bacteria were acid-digested with the same technique.

Samples of the acid-digested bacterial biomass diluted with 7 mL of Milli-Q water, as well as samples of the spent medium and of the EDTA with adsorbed metal, were stored until analysis at 4 ºC in polypropylene vials (Frías-Espericueta et al. 2009). Metal concentrations were determined by atomic absorption spectrophotometry. Data for the spent medium of immobilized cultures were corrected for dilution with the volume of metal-free medium used to rinse the substrate.

For QA/QC blanks were used every 25 samples and the accuracy of the analytical method was evaluated using two certified standard reference materials by the International Atomic Energy Agency, IAEA-392 (algae) and IAEA-331 (spinach) (IAEA 2009). Recoveries for Cd in algae and spinach were 107.02 and 97.31 %, respectively. For Pb in algae, recovery was 90.42 % (Bojórquez et al. 2016).

Mean biomass and mean percentages of metal retained on the surface or within bacterial cells were compared with two-ways ANOVA tests after R1 rank transformation and multiple comparisons Holm-Sidak tests, with $\alpha = 0.05$ (Zar 1996, Conover 2012).

RESULTS

Biomass production in metal-free growth media was higher for immobilized than for suspended cultures. In both cases, it was similar at pH 7 and 9 (0.600-0.605 and 0.810-0.815 g/L for suspended and immobilized cultures, respectively) and higher than at pH 6 (0.350 and 0.450 g/L) (**Table I**).

Cadmium retention by extracellular products increased progressively with increasing pH values and was higher in suspended cultures. Percentages of retention ranged from 3.5 to 8.6 % in suspended and from 1.8 to 6.5 % in immobilized cultures. Absorption in mg/g had the same trend, with values ranging from 0.090 to 0.393 mg/g of bacterial biomass.

TABLE I. MEAN BIOMASS (g/L) (± standard deviation) OF *E. cloacae* IN SUSPENDED (SC) AND IMMOBILIZED (IC) IN TS MEDIUM WITH INITIAL pH 6, 7 AND 9

pH	SC	IC
6	0.350 ± 0.003a	0.450 ± 0.002b
7	0.605 ± 0.015c	0.815 ± 0.003d
9	0.600 ± 0.002c	0.810 ± 0.003d

Different letters indicate significant differences (two-ways ANOVAs on R1 rank-transformed data, $\alpha = 0.05$, a < b < c < d)

Cd contained (absorbed) in the microbial biomass was higher than in extracellular products. In all cases values were higher in immobilized cultures, which retained from 17.8 to 52.7 % of the initial value and from 0.598 to 1.779 mg/g (**Table II**).

There were no differences in the percentages of Pb retention by extracellular products, with values ranging from 22.4 to 31.8 % in the suspended cultures at pH 6 and 9, respectively. In terms of mass (mg/g), retention by immobilized cultures was significantly lower, and the lowest was at pH 7. However, retention by absorption in the microbial biomass was consistently higher in the immobilized cultures, with values decreasing with increasing pH: retentions at pH 6 were 63.7 and 75.1 %, and decreased at pH 9 to 8.2 and 41.9 % in suspended and immobilized cultures, respectively.

Retention in mass units had the same pH-dependent trend, but there were no differences in the amounts of Pb retained by suspended and immobilized cultures at pH 6 (30.30 and 28.06 mg/g) and 7 (9.59 and 10.58 mg/g), while at pH 9 retention was lower in suspended cultures (2.29 and 8.68 mg/g) (**Table II**).

DISCUSSION

The higher biomass production in the immobilized cultures observed in the growth experiments coincides with the results obtained by several authors with different bacterial cultures (Garzón-Jiménez and Barragán-Huerta 2008, Martins et al. 2012, 2013, Zur et al. 2016), and is probably explained by the higher availability of dissolved and particulate nutrients adsorbed on the film of extracellular organic compounds used for adhesion by bacterial cells (Cohen 2001, Garrett et al. 2008). This organic film might also participate in metal resistance and retention, through immobilization on its active sites (Lau et al. 2005).

TABLE II. MEAN RETENTION OF Cd AND Pb IN EXTRACELLULAR PRODUCTS AND BIOMASS OF *E. cloacae* GROWN IN SUSPENDED (S) AND IMMOBILIZED (I) CULTURES IN TS MEDIUM AT pH 6, 7 AND 9, WITH 2.73 mg/L OF Cd OR 16.8 mg/L OF Pb*

pH	Culture	Extracellular (%)	Biomass (%)	Extracellular (mg/g)	Biomass (mg/g)
			Cd		
6	S	3.5 ± 1.3b	6.5 ± 2.4b	0.28 ± 0.10bc	0.51 ± 0.11b
	I	1.8 ± 0.4a	23.8 ± 2.2d	0.11 ± 0.02a	1.45 ± 0.10c
7	S	4.5 ± 2.2b	2.7 ± 1.3a	0.20 ± 0.10b	0.12 ± 0.06a
	I	2.7 ± 1.1a	17.8 ± 2.9c	0.09 ± 0.03a	0.60 ± 0.09b
9	S	8.6 ± 3.3c	14.3 ± 1.5c	0.39 ± 0.10c	0.65 ± 0.06b
	I	6.5 ± 2.8c	52.7 ± 3.2e	0.22 ± 0.09b	1.78 ± 0.11d
			Pb		
6	S	22.4 ± 3.5a	63.7 ± 4.3d	10.78 ± 1.72e	30.60 ± 2.01d
	I	22.8 ± 4.0a	75.1 ± 6.4e	8.52 ± 1.50d	28.06 ± 2.42d
7	S	25.5 ± 2.8a	34.5 ± 3.1b	7.07 ± 0.80c	9.59 ± 0.80c
	I	24.9 ± 1.6a	52.8 ± 7.0d	5.14 ± 0.29a	10.88 ± 1.38c
9	S	31.8 ± 3.0b	8.2 ± 1.1a	8.89 ± 0.83e	2.29 ± 0.30a
	I	31.0 ± 3.8b	41.9 ± 6.1c	6.44 ± 0.71b	8.68 ± 1.19b

*Values in percentages (± standard deviation) of the initial concentration (%) and in mg of metal/g of microbial biomass (mg/g). Different letters indicate significant differences between values in the same column (two-ways ANOVA, and Holm-Sidak tests using R1 rank-transformed data, $\alpha = 0.05$, a < b < c < d < e)

Metal retention by bacterial biomass is highly dependent on environmental conditions. Among these, pH is of paramount importance for the adsorption/absorption process (Kelly et al. 2003, Ahluwalia and Goyal 2007). This is demonstrated by the significant differences in Cd and Pb retention at pH 9 in comparison to the remaining pH conditions.

The only data concerning Cd and Pb retention by live cultures of *E. cloacae* refer to experiments performed by Banerjee et al. (2015). In these, Cd retention after one day was equivalent to 22.75 % of the initial concentration, while cultures retained approximately 64 % of the initial Pb. Both values are similar to those obtained at pH 7 in this study, which was the only pH value used in that experiment.

We could not trace additional examples of studies dealing with metal retention by *E. cloacae*, but pH-dependent variations of metal retention similar to those of this study were observed in *Enterobacter* sp. cultures by El-Shanshoury et al. (2013), who found that Cd retention increased with increasing pH between 5 and 8, while in the case of Pb it decreased significantly in the 8 to 9 pH range.

The importance of pH for metal biosorption has been stressed by several authors, either because it modifies the uptake process of metals at the membrane level, or due to its effect on metal speciation (surface charges of organic carbon), since both modify the amount of metal available for direct uptake by bacterial cells (Kelly et al. 2003). Besides, acid pH values used in the present study are similar to those reported by Ruiz-López et al. (2010) in mining waste effluents.

The lower percentages of extra- and endo-cellular Cd, in comparison to those determined for Pb, might be due to the greater affinity for Pb than for Cd, which was determined experimentally by several authors (Fowle and Fein 1999, Borrock and Fein 2005), or to different mechanisms of detoxification and active expulsion, such as those described by Leedjärv et al. (2008) for both metals and by Hynninen et al. (2009) as an exclusive bacterial detoxification mechanism for Pb.

Generally, high metal retention in immobilized cultures confirms the protective role of the bacterial biofilm. This apparently confers resistance providing binding sites which sequester metals, as was shown for Pb in several bacterial species which use intra- and extra-cellular binding factors to avoid toxicity of water-borne Pb (Levinson et al. 1996, Roane 1999, Mire et al. 2004).

In spite of the significantly lower biomass at pH 6, removal efficiency measured in units of mass of Cd removed (including mass determined in extracellular products) by one gram of bacterial biomass, was more than twice as high than at pH 7, and only slightly lower than at pH 9. Since the biosorption capacity of viable cells is considerably lower than that of their dead biomass (Kurek et al. 1982, Gabr et al. 2008) these differences are probably due to the poor metal exclusion mechanisms of dead or dying bacterial cells, as observed in suboptimal culture conditions by El-Shanshoury et al. (2013).

CONCLUSIONS

Biomass production and retention of Cd and Pb by *E. cloacae* are dependent on the pH of the growth medium: at pH 6, biomass is significant lower than at pH 7 and 9, both in immobilized and suspended cultures. Retention of Cd by adsorption on microbial cell walls, as well as active absorption within bacterial biomass were higher at pH 9 and significantly better in immobilized cultures.

In the case of Pb, adsorption showed an increasing trend with increasing pH values, and there were no significant differences between suspended and immobilized cultures. Absorption by biomass was consistently better for immobilized cultures and values were significantly higher at pH 6 (as reported in some mining effluents) than at pH 7 and 9.

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