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Biosorption of copper by *Paenibacillus polymyxa* cells and their exopolysaccharide

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Summary

Biosorption of heavy metals by gram-positive, non-pathogenic and non-toxicogenic *Paenibacillus polymyxa* P13 was evaluated. Copper was chosen as a model element because it is a pollutant originated from several industries. An EPS (exopolysaccharide)-producing phenotype exhibited significant Cu(II) biosorption capacity. Under optimal assay conditions (pH 6 and 25 °C), the adsorption isotherm for Cu(II) in aqueous solutions obeyed the Langmuir model. A high q value (biosorption capacity) was observed with whole cells ($q_{max} = 112$ mgCu g^{-1}). EPS production was associated with hyperosmotic stress by high salt (1 M NaCl), which led to a significant increase in the biosorption capacity of whole cells ($q_{max} = 150$ mgCu g^{-1}). Biosorption capacity for Cu(II) of the purified EPS was investigated. The maximum biosorption value (q) of 1602 mg g^{-1} observed with purified EPS at 0.1 mg ml^{-1} was particularly promising for use in field applications.

Introduction

Aqueous waste released by a number of industries contains heavy metals that pollute the environment. This contamination poses serious health threats to humans and animals, as these heavy metals tend to persist in the environment indefinitely (Volesky & Holan 1995). Methods of treating heavy-metal contaminated effluents currently consist of chemical precipitation, solvent extraction, dialysis, electrolytic extraction, reverse osmosis, evaporative methods, treatment with ion-exchange resins, carbon adsorption and dilution. In recent years, there has been a significant effort to search for new methods of heavy metal removal from contaminated sites. Biological methods to remove metals from liquid effluents present many potential advantages. Heavy metal accumulation processes by biological cells are grouped together under the general term ''biosorption''. Biosorption is an important component in the integrated approach to the treatment of aqueous effluents. The mechanisms of biosorption may involve intracellular uptake and storage via active cationic transport systems, surface binding or some undefined mechanisms. The biological and chemical characteristics of these uptake processes are important not only as an aid in the understanding of the role of metallic ions in basic cellular functions but also for use in detoxification of metal-polluted industrial effluents by application of biomass. Emphasis has been placed on the use of microorganisms as biosorbents because they are propagated inexpensively (Volesky & Holan 1995; Salehizadeh & Shojaosadati 2003; Ince Yilmaz 2003). The use of bacteria and fungal biomass as biosorbents should be of special interest to industries in undeveloped countries where pollution generators cannot afford to install costly high-performance treatment facilities.

In this work we used copper as a model for the evaluation of biosorption capacity. Copper is found in effluents from various industries, including tanning, mining, metal-processing and -finishing, electroplating, the automobile industry, and the pharmaceutical industry. We have focused on the use of bacteria as agents of heavy-metal biosorption. In heavy metal pollution, bacterial exopolymers have become an alternative of interest as metal-binding agents in detoxification of contaminated waters (Gutnick & Bach 2000). Gram-positive bacteria that are designated GRAS (generally recognized as safe) organisms because they are non-pathogenic and non-toxicogenic are especially safe candidates for biosorption. Their cells are rigid and relatively insensitive to shear forces because of their thick cell wall. The exopolymers or exopolysaccharides (EPS) they form might play a crucial role in metal biosorption, as has been reported for other bacteria (Kim *et al.* 1996; Loaec *et al.* 1997).

We report here the purification and preliminary chemical analysis of the EPS produced by *Paenibacillus polymyxa* strain P13 isolated from a regional fermented sausage. As part of a survey of exopolymer production by bacteria, we examined osmotic stress condition using

cells from cultures grown in the absence and presence of high salt concentrations.

Materials and methods

Microorganisms and media

The *Paenibacillus polymyxa* strain P13 used in this study, which had been previously isolated by Piuri *et al.* (1998), was grown for 2 days in BHI broth (Merck) in shake culture (150 rev min^{-1}) at 32 °C. The effect of adding 2% glucose or 1 M NaCl was evaluated in the EPS production.

Determination of MIC (minimal inhibitory concentration) for copper ion

Analytical-grade $CuSO_4 \cdot 5H_2O$ (Merck) was used to prepare sterile 1000 mg l^{-1} stock solutions. BHI (Merck) agar plates were supplemented with different concentrations of heavy metal, adjusted to pH 7.0, and then inoculated. Analysis of metal resistance was performed by colony counts in duplicates of serial decimal dilutions of mid-log phase cultures on BHI agar plates containing different concentrations of metal salt. Results were obtained after 3 days of incubation at 32 °C. Linear regression was estimated from a plot of c.f.u. ml^{-1} against Cu concentration and MIC was obtained by interpolation.

Exopolysaccharide purification

In order to purify the exopolysaccharide (EPS) produced by *P. polymyxa*, an entire culture (cells plus broth) was boiled for 10 min to remove attached EPS and centrifuged at 20,000*g* for 20 min to remove cells. Trichloroacetic acid (TCA) was added to 10% final concentration to precipitate proteins and peptides. After centrifugation, the supernatant was filtered through a 0.45 μm filter membrane. One volume of cold ethanol (4 °C) was added to the supernatant and the crude EPS precipitated. After centrifugation, 1/10 original volume sterile distilled water was used to re-suspend it, and was dialysed overnight against sterile distilled water in cold room. The dialysed EPS was lyophilized overnight to remove the water, weighed and stored at 8 °C. For biosorption experiments, it was dissolved in sterile distilled water to give a stock solution with final concentration of 10 mg ml^{-1}, adjusting the pH to 6 with NaOH.

Thin-layer chromatography

Exopolymer samples (5 mg) were first hydrolysed in 8 M trifluoroacetic acid (TFA) at 110 °C for 2 h (Kachlany *et al.* 2001). The TFA was then removed by evaporation and the remaining residue was redissolved in 0.1 ml of sterile distilled water before thin-layer chromatography (TLC). A 10 μl volume of the hydrolysed or non-hydrolysed sample was spotted onto a silica gel TLC plate. The plate was developed in 9:1 (v/v) acetonitrile ± water for 1 h, dried and sprayed with a solution containing a mixture of 27 parts ethanol, 0.3 parts glacial acetic acid, 1.5 parts concentrated sulphuric acid and 1.5 parts *p*-anisaldehyde. The monosaccharides were visualized by charring at 110 °C until characteristic coloured spots were seen. Monosaccharide standards were run along with the samples.

FACE analysis

To further determine carbohydrate content of EPS, fluorescence-assisted carbohydrate electrophoresis (FACE) was used as described in Young (1996). Derivatization with 8-aminonaphthalene-3,6-trisulphonic acid (ANTS, Molecular Probes) was carried out according to Jackson (1994) in tubes containing dried hydrolysed EPS (5 mg in 8 M TFA 2 h 110 °C). ANTS was prepared in acetic acid/water (3/17, v/v) at 0.2 M as final concentration, $NaCNBH_3$ (1 M, made freshly and used immediately) was solubilized in DMSO for ANTS derivatization. Equal volumes of ANTS and $NaCNBH_3$ solutions (5 μl) were added to each dry sample. The reagents were mixed, centrifuged and incubated at 37 °C overnight. The solution was lyophilized. The derivatized sugars were re-suspended in 50 μl of glycerol 5% (w/v) and stored at −20 °C before use. Polyacrylamide gel electrophoresis was performed as follows: ANTS derivatized sugars were separated in 16 cm × 16 cm 30% (w/v) polyacrylamide gel and 0.8% (w/v) *N*,*N*-methylenebisacrylamide with a stacking gel (1.5 cm) of 8% (w/v) polyacrylamide and 0.2% (w/v) *N*,*N*-methylenebisacrylamide. The electrophoresis buffer system was 0.1 M Tris adjusted to pH 8.2 with boric acid (Tris–borate). Bromophenol-blue and monosaccharide sugars and hydrolysed dextran (2 M HCl 100 °C 2 h) ANTS-derivatized were used as markers. Fluorescence of Bromophenol-blue corresponds to the tetra-maltotetrose as described by Jackson (1994). The samples were submitted to electrophoresis, initially at 250 V for 30 min and then at 500 V for 60 min with cooling. To visualize the gels, they were scanned using Fuji CCD luminescent image analyser LAS1000 and software Image Gauge 3.122 (Fuji Film, Japan). The exposure time was optimized to increase sensitivity without saturating the intense bands. Fluorescence intensity was calculated for each lane with Image Gauge software 3.122, Fuji Film Japan and R_f calculated from plots with the same scale.

Surface tension measurement and critical micellar concentration (CMC) determination

The surface tension of EPS samples was measured by the ring method using a tensiometer (Krüss, Optische-Mechanische Werkstatten, Germany). The instrument was calibrated with water to a reading of 70 mN m^{-1}.

The CMC was derived by dissolving known quantities ranging in concentration from 0.01 to 1.0 mg ml^{-1} of the dry EPS in water, by measuring the surface tension, and by plotting surface tension against EPS concentration. The CMC of the purified EPS was around 0.1 mg ml^{-1}.

Copper biosorption experiments

Whole cells or purified EPS were evaluated for biosorption capacity and were added to metal solutions at optimized condition of pH at 25 °C in duplicate. After biosorption had reached equilibrium, whole cells were directly removed by centrifugation. EPS was removed by adding one volume of cold ethanol to the solution and centrifuging at 20,000*g* for 20 min to remove insoluble EPS-metal complex. Residual, unsorbed metal in the supernatant was determined by the BCA method (Brenner & Harris 1995). In order to account for the effect of the ethanol precipitation on the metal solubility, a separate set of control experiments was run under the same conditions without adding EPS. All experiments were run in duplicate. The metal uptake (q) was determined as follows: $q = V(C_{\text{i-}C\_\text{f})/1000}\ W$ where V is the volume of solution in tube, W is the mass weight of adsorbent (EPS or cells) (g), C_i and C_f are the initial and equilibrium concentration of metal in solution (mg l^{-1}), respectively. The influence of other metal ions at different concentrations (25, 250 and 500 mg l^{-1}) was also evaluated on copper (50 mg l^{-1}) biosorption by EPS (0.25 mg ml^{-1}).

Results shown in the figures represented one of at least two or three independent experiments.

Calculation of adsorption isotherm parameters

The isotherm data were adjusted to the linear form of the Langmuir equation adsorption model ($C_\text{f}/q = (1/q_\text{max})\ b\ +\ C_\text{f}/q_\text{max}$), with regression features in Statistica 5.0, where q is the metal uptake by biosorbent (mg g^{-1}), C_f is the equilibrium concentration of metal in solution (mg l^{-1}), (b, q_max) are empirical constants of Langmuir isotherms representing affinity and maximum uptake capacity, respectively.

Results

Biosorption of Cu by whole cells

Paenibacillus polymyxa P13 produced exopolymers as observed by the mucous nature of the colonies on BHI agar and testing for ropiness with a sterile toothpick. The MIC for Cu metal was calculated as 3.5 ± 0.2 mM, involving a high resistance phenotype compared to that obtained for non-EPS-producing *Bacillus* cells and the one reported for *B. circulans* metal-resistant strain (Ince Yilmaz 2003). The presence of the EPS-producing phenotype has been associated with an increase in metal absorption (Loaec *et al.* 1997; Kachlany *et al.* 2001). Biosorption assays with stationary phase cells resulted in a higher biosorption capacity than those with vegetative cultures ($q_{stationary}$ was 2.5-fold × q_veg). This could be related to an increase in EPS synthesis during growth. The biomass needed for assays was 0.20 mg of whole cells ml^{-1} that adsorbed 30% of the metal in a 50 mg l^{-1} initial solution. When cells were heated (100 °C 10 min), the biosorption capacity decreased two-fold (data not shown). This may be due to a decreased EPS linked to the cells after heating.

As different carbon source (Ricciardi *et al.* 2002) and hyperosmotic stress conditions (Penaloza-Vazquez *et al.* 1997) affect EPS production, we evaluated the behaviour of glucose 2% (w/v) or NaCl (1 M)-grown cells. As observed in Figure 1, an increased biosorption was obtained in NaCl grown cells. While in 1 mg whole cells grown in BHI, q_max was 112 mg Cu g^{-1}, in NaCl grown cells, q_max was increased to 150 mg Cu g^{-1}.

We evaluated the yield of EPS from P13 in these growth media. We found that 100 ml of a stationary phase P13 culture formed 27 (± 4) mg (± SD) EPS in BHI medium containing 1 M NaCl compared to 15(± 4) mg (± SD) EPS in control BHI medium. The culture in high salt reached greater biomass yield ($DO_{600\text{nm}} = 3$) when compared with control ($DO_{600\text{nm}} = 2.4$). The addition of 2% glucose did not increase the EPS production.

Characterization of EPS

As bacterial EPS is known to interact with toxic metals by various mechanisms including van der Waals' forces, electrostatic attraction and surface complexation (Gutnick & Bach 2000), it is helpful to characterize the chemical properties of the purified EPS in order to better understand the metal-polymer interactions.

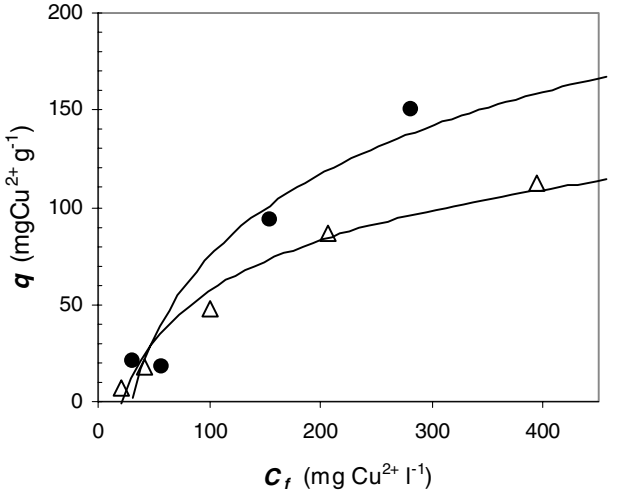

Figure 1. Biosorption of Cu^{2+} by whole cells: Growth was obtained in BHI (Δ) and BHI + NaCl (●). Harvested cells were washed and used for biosorption assays as described in Materials and methods.

In purified EPS, proteins were not detected. The carbohydrate content of EPS was evaluated by monosaccharide composition of purified EPS preparation by TLC. TLC allowed the identification by comparison with authentic monosaccharides standards of the predominant sugar present in the acid-hydrolysate of partially pure EPS preparations, which was mannose (Figure 2a). Many sugars may go undetected by this technique. Therefore, a more sensitive analysis was performed: fluorescence-assisted carbohydrate electrophoresis (FACE) (Figure 2b). FACE confirmed that the polysaccharide produced by P13 was a mannose polymer.

Table 1. Biosorption kinetics and effect of pH.

hs	pH		
	4	6	10
	mg Cu^{2+} l^{-1} retained		
2	10 ± 1	49 ± 2	0.2 ± 1
5	9 ± 1	47 ± 2	0.2 ± 1
16	3 ± 1	47 ± 2	0.9 ± 1

Time (h) of incubation at 25°C and pH are indicated using 100 mg Cu^{2+} l^{-1} initial metal concentration and 0.5 mg EPS ml^{-1}.

Biosorption kinetics and effect of pH

The activity of the functional groups of the EPS was affected by pH, the optimum pH being 6 (Table 1). Biosorption investigations on bacteria in the acidic pH range have demonstrated a reduction in the available metal-binding sites because of protonation (Savvaidis *et al.* 2003) or interaction between cations and negative charges of acidic functional groups of polysaccharide (Salehizadeh & Shojaosadati 2003). This behaviour can also explain the partial insolubilization of EPS observed in the present work, decreasing the specific biosorption capacity. At basic pH, the adsorption capacity of polysaccharide was absent due to insolubilization of the Cu^{2+} ions with the dominance of polyhydroxylated metal species (Savvaidis *et al.* 2003) and probably alkaline inactivation of EPS.

The time course of the metal adsorption was almost complete after 2 h incubation at 25 °C when compared to equilibrium time of 16 h at optimum pH as shown in Table 1. Moreover, when analysing the first 2–30 min of incubation (data not shown), about 99% of the Cu^{2+} was already retained in the EPS. Comparable adsorption kinetics has been obtained by other researchers working with *Bacillus firmus* (Salehizadeh & Shojaosadati 2003) and *Ochrobactrum anthropi* (Ozdemir *et al.* 2003).

Effect of initial polysaccharide concentration on copper removal

The initial polysaccharide concentration in solution affected the metal adsorption capacity (Figure 3). An increase in polysaccharide concentration led to interference between binding sites. The decrease of specific uptake might be attributed to metal concentration shortage in solution (Valdman & Leite 2000). This behaviour may also be explained by the EPS aggregation at higher concentrations suggesting interference between possible binding sites (Salehizadeh & Shojaosadati 2003). Similar drop profiles for biosorption capacity (q) and surface tension (ST) were also verified with increasing EPS concentrations (Figure 3). This behaviour validated the hypothesis that the formation of micelles decreases copper biosorption capacity as observed for biosurfactants at the critical micellar concentration (CMC) (Singh & Cameotra 2004).

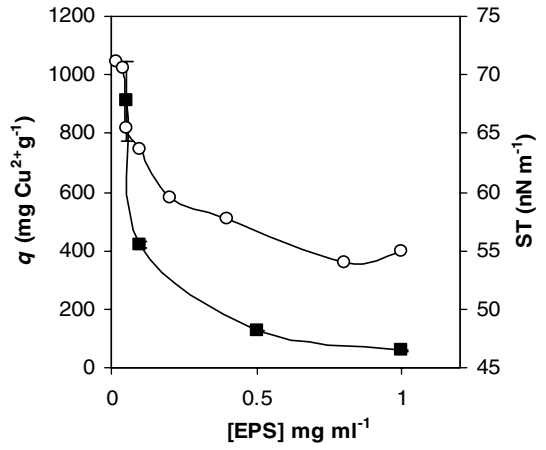

Figure 3. Effect of polysaccharide concentration: Copper biosorption (q) (■) and surface tension (ST) (○) at different EPS concentrations (pH 6).

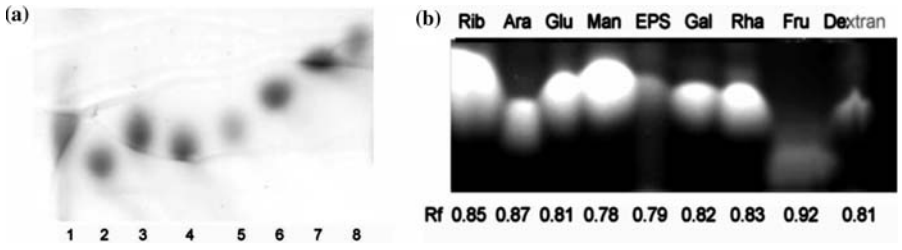

Figure 2. Thin-layer chromatography (TLC) and FACE analysis of partially purified EPS: (a) TLC, Lane 1, hydrolysed EPS; Lanes 2 to 8 monosaccharide standards: 2, galactose, 3, mannose, 4, glucose; 5, fructose, 6, arabinose, 7, ribose; and 8, rhamnose. (b) FACE analysis: Monosaccharide standards ANTS-derivatized: Rib (ribose), Ara (arabinose), Glu (glucose), Man (mannose), Gal (galactose), Rha (rhamnose), Fru (fructose), Samples ANTS-derivatized: EPS (hydrolysed exopolysaccharide) and Dextran (hydrolysed dextran).

Biosorption isotherm of Cu by EPS

We investigated the adsorption capacity of the purified EPS from P13. The metal uptake isotherms for Cu plotted against final metal concentration C_f in aqueous solutions are shown in Figure 4. The results shown demonstrate that copper was taken up more effectively by 0.1 mg EPS ml^{-1} than by 1.0 mg EPS ml^{-1}. It can also be observed that the Langmuir model was able to describe the passive biosorption equilibrium between Cu(II) ions and the biopolymer. To determine the maximum uptake capacity (q_{max}) of Cu(II) and affinity (b) constants of this adsorption model, regression features in Statistica 5.0 were used (Table 2). While the affinity constants (b) for both concentrations were of the same order of magnitude (10^{-3}), the maximum uptake capacity ($q_{max} = 1602$ mgCu g^{-1}) obtained for 0.1 mg EPS ml^{-1} was almost 3-fold higher than that observed for 1.0 mg EPS ml^{-1} (583 mgCu g^{-1}). Almost no difference with the control condition was observed for the isotherm obtained with EPS from NaCl culture (Figure 4).

Effect of other ions

Metallic ions in nature are rarely represented by a single kind of metal. Na, Ca, Mg and K are elements frequently found in nature and of great solubility. The competitive effect of different metal ions on Cu^{2+} adsorption to EPS was studied by pairing Cu^{2+} with each of the other ions at three different concentrations (50, 250 and 500 mg l^{-1}). The process of Cu^{2+} removal was inhibited in the presence of other ions (Figure 5). Particularly, Ca^{2+} was the greater inhibitor although high concentrations are required to observe the complete displacement of Cu^{2+} by Ca^{2+}. In Figure 5 it can be seen that Ca^{2+} and Zn^{2+} produced an important inhibition of Cu^{2+}.

Table 2. Maximum uptake capacities (q_{max}) and affinity constants (b) for copper adsorption by purified EPS from P13 grown in BHI (Control) and BHI + 1 M NaCl (NaCl) according to Langmuir equation $q = bC_f q_{max}/(1 + bC_f)$. R^2 is the correlation coefficient. Mean and standard deviation (SD) are shown for q_{max} in each condition and EPS mass. No statistical significance was obtained at the $P < 0.05$ level of probability between q_{max} values for each EPS mass.

Langmuir parameters EPS (g l^{-1})	q_{max}(SD) (mg g^{-1})	b (l mg^{-1})	R^2
Control			
0.1	1602 (558)	0.0065	0.972
1.0	583 (146)	0.0024	0.98
NaCl			
0.1	2066 (383)	0.0021	0.977
1.0	469 (133)	0.0047	0.964

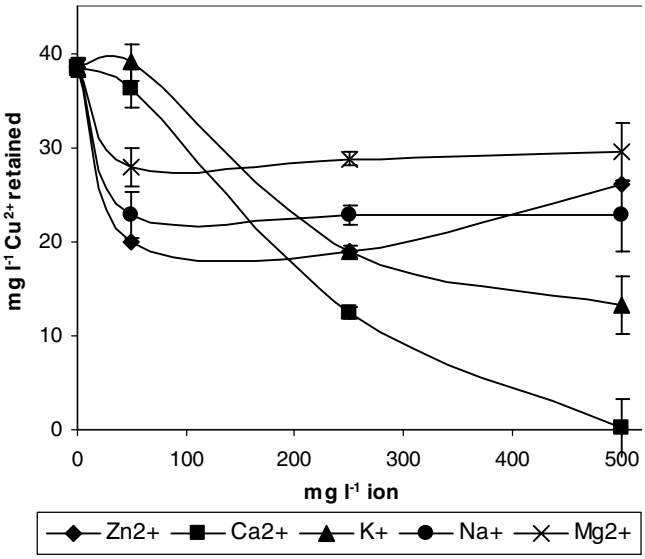

Figure 5. Effect of other ions: The competitive effect was tested by pairing Cu^{2+} with each of the other ions. The presence of increasing ion concentration was evaluated using 50 mg l^{-1} initial Cu^{2+} concentration and 0.25 mg EPS ml^{-1}.

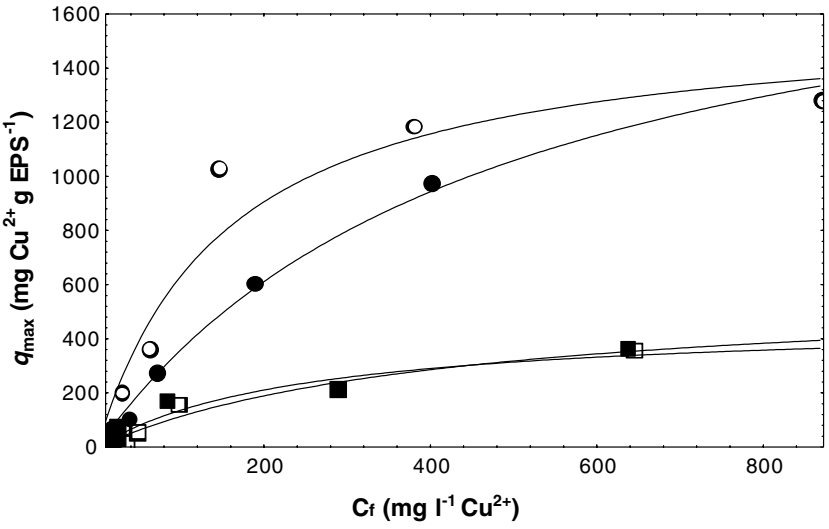

Figure 4. Isotherms of Cu^{2+} biosorption at two EPS concentrations: 0.1 mg ml^{-1} (circles) and 1.0 mg ml^{-1} (squares). Filled symbols represent biosorption capacity of EPS obtained from NaCl culture conditions. Isotherms are consistent with the Langmuir model; see constants in Table 1. No difference was observed for the isotherms when compared to the control condition (open symbols).

Discussion

The results presented in this study show that the exopolysaccharide produced from *Paenibacillus polymyxa* was a powerful copper adsorbent.

Gram-positive bacteria have the advantage of having rigid cell walls and thus are less sensitive to shear forces due to the thick cell wall surrounding the cells, and thus potentially more suitable for field applications such as biosorption. Furthermore, we found an increased biosorption capacity in cells grown in NaCl. In this case cultures reached higher OD_{600} nm since sporulation fails in this condition as previously reported (Ruzal *et al.* 1998) and favours the formation of biomass, with the consequent 2-fold increase in the recovery of EPS. These results explain the best biosorption observed with whole cells grown in high salt. However, we did not observe a significant increase in biosorption capacity for EPS from high salt culture when compared to EPS obtained from non-salt medium (Table 2 and Figure 4). FACE analysis of purified EPS from NaCl medium showed the same results as the control (data not shown). A difference in affinity at low EPS concentrations (b value 0.0065 and 0.0021 for control and NaCl respectively) was observed. However, Langmuir parameter q_{max} involving maximum biosorption capacity for the purified EPS of both cultural conditions showed no significant difference. Therefore, although we do not know the exact chemical structure of the EPS we postulated that it would probably be the same in both cultural conditions.

Various polysaccharides and other biopolymers exhibit metal-binding properties (Kim *et al.* 1996; Loaec *et al.* 1997; Salehizadeh & Shojaosadati 2003). For electrostatic interactions, the binding of cations to bacterial biopolymers generally occurs through interaction with negatively charged functional groups. In addition, there may also be cation-binding by positively charged polymers or coordination with hydroxyl groups (Gutnick & Bach 2000). So, an increase on the availability of possible cation-binding groups would be expected as the biopolymer is disperse in the contaminated solution. This could explain the higher copper uptake obtained at EPS concentrations below the CMC value (Fig. 3) where micelle formation and electrostatic impediments are not yet verified. Surface tension (ST) of the solution also decreased with increment of EPS concentration until micelles were formed.

The bacterial exopolymer from *Paenibacillus polymyxa* (P13), a strain isolated from Argentinian regional fermented sausages, showed a strong ability to bind heavy metals under the conditions of batch isotherms. Maximum binding capacities were four-fold higher for whole cells and double for purified EPS than those reported for other members of the *Bacillaceae* (Salehizadeh & Shojaosadati 2003; Ince Yilmaz 2003). The use of the inhibition assay was the approach used to determine that EPS was able to adsorb other ions. Other heavy metals, like zinc, inhibited biosorption, so they would therefore interact with EPS. This is probably due to the very close characteristics of Ca^{2+} and Zn^{2+} ions to Cu^{2+}, the same charge and practically the same hydrated ionic radii, approximately 600 pm (Kielland 1937). K^{+} (hydrated ionic radius: 300 pm), presents a ratio radius:charge similar to Cu^{2+}, suggesting that the charge density plays an important role in the adsorption process. Mg^{2+} has a bigger radius (800 pm) and Na^{+} (450 pm) a smaller charge density. The four elements tested belong to the same period of the periodic table, Cu and Zn are neighbours and both are generally present in polluted wastes.

The different metal uptake capacities of EPS from P13 can also be attributed to types of conformation of polymer with adsorbed ions where a strong interaction and flat conformation or a weak interaction and looped conformation can be verified as described by Salehizadeh & Shojaosadati (2003). A study on the combined effect of two or more metals will be conducted.

We believe that the finding that the maximum value uptake was of 1,602 mg Cu g^{-1} EPS is particularly interesting since this value indicates that it may have potential for use in applications in water treatment and biodetoxification of metal-polluted waters. In addition, EPS immobilization techniques are also under investigation to continuously treat metal-bearing industrial effluents in large-scale columns.

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