UNIVERSIDAD DE LOS ANDES
FACULTAD DE CIENCIAS
DEPARTAMENTO DE CIENCIAS BIOLÓGICAS

BIOSORPTION AND BIOACCUMULATION OF HEAVY METALS ON DEAD BIOMASS, LIVING BIOMASS AND S-LAYER OF *Bacillus sphaericus*

TESIS DE MAESTRÍA

LINA VELÁSQUEZ BRUN

DIRECTOR: JENNY DUSSAN GARZON Ph.D
DIRECTORA CENTRO DE INVESTIGACIONES MICROBIOLÓGICAS (CIMIC)
PROFESORA ASOCIADA, DEPARTAMENTO DE CIENCIAS BIOLÓGICAS
UNIVERSIDAD DE LOS ANDES

BOGOTÁ, MAYO DE 2009

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Abstract

The tolerance to As, Hg, Co, Fe and Cr was determined in different Colombian *Bacillus sphaericus* native strains, as well as the biosorption and bioaccumulation in living biomass. In addition, biosorption of Cr in dead cells was also determined. Living cells of the two most tolerant strains had the capacity to accumulate between 6 and 47% of Co, Hg, Fe and As. Living and dead cells of *B. Sphaericus* OT4b31 showed a biosorption of 25% and 44,5% of Cr respectively, while *B. Sphaericus* IV(4)10 showed a biosorption of 32% and 45%. These results are due to the absence of an active metabolism in dead cells and to the pH adjustment. [published in Journal of Hazardous Materials, (Velasquez and Dussán 2009)]. S-layer isolation from *Bacillus sphaericus* OT4b31, as well as the reassemble of the protein extracts was done. To prove if S-layer could be a mechanism of metal uptake in these cells, biosorption isotherms were made. Starting from the high initial Cr(VI) concentration (365,69 mg/l) the higher metal uptake was detected (230,52 mg of Cr(VI)/g of S-layer); which correspond to approximately 76% of metal uptake. This result shows that S-layer participate in metal sorption. The possibility to use this in a bioremediation processes depend of future experiments in adsorption improvement.

Key words: *Bacillus sphaericus*, biosorption, heavy metals, S-layer, living and dead cells.

1. Introduction

Several inorganic and organic compounds such as heavy metals, fuels and petroleum industry products cause soil and water contamination. For this reason, research focused toward the search of better decontamination methods and the development of new technologies has to be intensive.

Amongst the inorganic pollutants, remediation methods for heavy metals differ from those of organic compounds due to it being non-biodegradable (Gupta and Rastogi 2008). Therefore, immobilization and physical removal has to be made. Although some metals are necessary for biological processes, all of them are toxic at high concentrations. This is due to their oxidative capacity to form free radicals and their ability to replace essential metals in enzymes, interrupting their normal activity (Ghosh and Singh 2005). Other metals are not essential and accumulate in different organisms and because of this they are toxic even at low concentrations. Mercury, chromium, lead, arsenic, copper, cadmium, cobalt, zinc, nickel, beryllium, manganese and tin are the most toxic heavy metals according to the United States Environmental Protection Agency (EPA 2006).

Conventional physiochemical methods for metals remediation include precipitation, coagulation, ionic exchange, inverse osmosis and adsorption. Although these are efficient processes, they have disadvantages when used in industrial waste conformed by diluted metallic solutions. Furthermore, these processes are expensive in terms of energy and chemical products consumption (Atkinson, Bux et al. 1998). Bioremediation can be performed to remove metals in contaminated or waste water and to trap metals from soil and sediments.

Microorganisms are a feasible solution because they can achieve different transformation and immobilization processes. One of them is bioaccumulation, based on the incorporation of metals inside the living biomass. Another process is biosorption, in which metallic ions remain at the cellular surface by different mechanisms (Vijayaraghavan and Yun 2008).

Bacteria of the genus *Bacillus* are strictly aerobic or facultative anaerobic; form very resistant endospores and are broadly distributed in soil, water and air. Those characteristics have made this genus to be of great importance at the industrial and medical level (Gordon, Haynes et al. 1989). The formation of spores is a complex genetic mechanism of starvation that allows the ubiquity of these organisms and allows the survival under different conditions (Errington 1993). *Bacillus sphaericus* is very well-known specie for biological control of mosquito activity. Some strains synthesize a parasporal inclusion during sporulation, which contains two proteins of 51 and 42 kDa proteins. The mechanism of action of the binary toxin follows a series of steps that finish with the cell lysis (Baumann,

Clark et al. 1991). However, there are other characteristics of these bacteria, such as surface proteins that allow its presence in extreme environments as uranium mines. This make it very interesting not only for biological control but for other environmental processes.

Bacillus sphaericus JG-A12 is a strain isolated from an uranium mining waste pile in Germany capable of trapping different metals such as U, Cu, Pb, Al y Cd. Different studies have shown that this cells are covered by a proteinaceous surface called S-layer, which is able to bind high quantities of uranium (20 mg U g^{-1} protein) and others metallic ions (Pd(II), Pt(II) and Au(III)) in saline solutions (Sleytr, Györvary et al. 2003).

The S-layer is a surface and paracrystalline envelop present in several groups of bacteria and archaeas. This layer is formed by protein or glycoprotein monomers that can self assemble in two-dimensional structures (Sleytr, Györvary et al. 2003). It works as an interface between the cell and the environment. In Gram-positives it is attached to the Peptidoglycan in the cell wall and forms a porous protein compound of uniform size and morphology. The porosity is between 30 and 70% and the diameter of the pore between 2 and 8 nm (Sleytr and Beveridge 1999). This characteristic can be exploited for metal binding. In the organisms where it is present, this layer represents approximately 15% of the cell proteins, which is a high percentage (Pollmann, Raff et al. 2006). This is an important characteristic if large scale production is needed. In *Bacillus sphaericus* JG-A12 the S-layer represents 20% of the total proteins. The biological function is not clear; in different microorganisms this layer performs different functions. An important characteristic of this protein is its capacity to reassemble once isolated from the cell (Pollmann, Raff et al. 2006). S-layer subunits are bound to each other and with the cell wall through different non-covalent forces such as hydrogen or ionic bonds, hydrophobic or electrostatic interactions, which allow the disruption from the cell. What let the Reassamble is that bonds holding the subunits together are stronger than those between the layer and the cell envelope. Due to this, it can be used for bioremediation processes.

Approximately 60 percent of the amino acids that constitute S-layer are hydrophobic; 40% of them are structured in β-sheets and 10-20% as α-helices. The majority of S-layer proteins are weakly acidic, with an isoelectric point among 4-6. Few exceptions have been found in some archaea and *Lactobacilli* (Sleytr, Sára et al. 2001).

S-layer monomers can self assemble in different two-dimensional structures: oblique (p1, p2), square (p4) or hexagonal (p3, p6) symmetry. Depending on the lattice type, one morphological unit is composed of one, two, three, four or six identical subunits (p) with a center-to-center spacing of approximately 5–30 nm (Ilk, Egelseer et al. 2008).

Once isolated from the cell, there are different reassembly techniques. Reassembly in suspension generates self-assembly products of varying size and morphology. Additionally, the possibility to form mono or double layers exists. In double-layered structures, the two constituent monolayers are facing each other either with their inner or their outer face. Reassembly at the air–water interface generates coherent S-layer lattices at large scale. S-layers which have been recrystallized at liquid surface interfaces, including lipid films, can be transferred onto solid supports by standard Langmuir–Blodgett (LB) techniques (Sleytr, Györvary et al. 2003).

Tuzen et al. (Tuzen, Dogan et al. 2007) demonstrated metal bioadsorption processes by free and resin-immobilized inactive *B. sphaericus* cells. Srinath et al (2002) reported the biosorption of Cr(VI) by cells of *B. megaterium* from a treatment plant waste. They found that living and dead cells biosorbed 15.7 and 30.7 mg Cr/g dry weight respectively (Srinath, Verma et al. 2002). This results show that the bioadsorption process can be performed in both living and dead biomass.

The objective of the present study was to determine the tolerance of different Colombian *Bacillus sphaericus* native strains to different heavy metals, to evaluate the bioaccumulation and biosorption of the metals in living and dead biomass, to determine the presence of the S-layer and determine if S-layer could be a mechanism of Cr(VI) biosorption in the native *B. sphaericus* strain.

2. Materials and Methods

2.1. Microorganism and growth conditions

The *B. sphaericus* strains are shown in Table 1**.** All the strains were grown in nutrient broth agar for 24 h at 30°C. A colony of each strain was incubated in 3 ml of BHI broth (Brain

Heart Infusion) for 24 h at 30°C. 100 µl of each strain were transferred to fresh BHI broth and were incubated 24 h al 30°C. They were transferred to SPC agar (Standard Plate Count) and incubated 24 h at 30°C, for its storage.

Table 1 ***Bacillus sphaericus*** strains

Strain	Origin	Reference
2362	Reference strain	Donated by Delecluse, A.
OT4b25	Isolated from larvae,Beetles	(Dussán 2006)
OT4b48	Isolated from larvae,Beetles	(Dussán 2006)
OT4b51	Isolated from larvae,Beetles	(Dussán 2006)
OT4b56	Isolated from larvae,Beetles	(Dussán 2006)
OT4b31	Isolated from larvae,Beetles	(Dussán 2006)
III(2)3	Isolated from an oaks forest	(Lozano 1998)
IV(1)8	Isolated from an oaks forest	(Lozano 1998)
IV(4)10	Isolated from an oaks forest	(Lozano 1998)

2.2. Evaluation of metal tolerance

The evaluated metals were $Na_2HAsO_4.7H_2O$ (sodium arsenate), $HgCl_2$ (mercury chloride), $CoCl_2.6H_2O$ (cobalt chloride), $FeCl_3$ (iron chloride) and $K_2Cr_2O_7$ (potassium dichromate). A colony of each strain in SPC were transferred to 2 ml nutrient broth supplemented with 6240 and 9360 mg l^{-1} of As(V), 10 and 30 mg l^{-1} of Cr(VI), 5 and 10 mg l^{-1} of Hg, Co and Fe. Additionally, nutrient broth only with metals was prepared. They were incubated for 24 h at 30°C. Growth was taken as the indicative of tolerance. Strains that grew in most of the metals were selected.

2.3. Determination of the presence of S-layer

Presence of S-layer was determined for OT4b31 and IV(4)10 with or without metals. Cells from overnight grown culture were harvested by centrifugation at 10,000g for 1 min at 4°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell extracts were made in a 10% acrylamide separation gel and visualized by coomassie blue staining. The band of approximately 122 KDa, presumably S-layer protein, was blotted to polyvinylidene difluoride membranes. N-terminal sequencing of excised blot band was performed by Matrix-Assisted Laser Desorption/Ionization (MALDI) method. BLAST was carried out.

2.4. Metals biosorption and bioaccumulation quantification

The As, Co, Hg and Fe concentration was determined by atomic absorption spectroscopy (Perkin Elmer AAnalyst 300). Solutions of the highest concentration of each metal where each strain grew were prepared and the initial concentration was determined. For the biosorption and bioaccumulation quantification 0.5 ml of an overnight grown culture was inoculated into 35 ml of nutrient broth and incubated for 24 h at 30°C. Cells were centrifuged and the metal concentration in the supernatant was determined. The initial and final concentration of Cr(VI) was estimated by the 1, 5-diphenylcarbazide method (EPA 2006).

2.5. Metals biosorption in dead biomass

2.5.1. Dead biomass preparation

This experiment was carried out in triplicate with some modifications of the method described by T. Srinath et al. just for Cr (Srinath, Verma et al. 2002). Cells of OT4b31 and IV(4)10 were inoculated into 100 ml peptone water in a 500 ml flasks and incubated on a shaker at 150 rpm for 24 h at 30°C. The cells were grown to late exponential phase, harvested by centrifugation at 8,000 rpm for 30 min at 4C° and washed three times with deionized water. The harvested cells were conditioned to pH 2.5 by repeated washing with acidified deionized water (H_2SO_4). This pretreatment prevents changes in the solution pH after biomass addition. The pH-conditioned cells were then dried in an oven at 50°C for 12 h, crushed in a blender and sieved through a 24-mesh sieve.

2.5.2. Metal biosorption determination

The pH of the chromium solution (30 mg Cr(VI)/l) was adjusted to 4.0 with 1 M sodium hydroxide and 0.5 M acetic acid. The dead cells (around 10 mg dry weight) were added to 5 ml of chromium solution. The samples were shaken at 150 rpm. The Cr(VI) concentration before adding the biomass as well as at different times of incubation was determined by 1, 5-diphenylcarbazide method (EPA 2006). The experiments were carried out in triplicates.

2.6. Statistical analysis

Variance analysis for two factors with interaction was done to determine differences among strains in living cells, strains in dead cells and among living and dead cells at 24 h of incubation for the biosorption and bioaccumulation of Cr(VI) and among strains in living cells for the other metals.

2.7. Preparation of cell walls

This experiment was carried out with some modifications of the method described by Uwe B. Sleytr and Audrey M. Glautert (Sleytr and Glauert 1976). *B. sphaericus* OT4b31 was grown in batch culture on nutrient broth for 17-20 h at 30°C. Cells were harvested by centrifugation at 10,000 rpm for 15 min at 4C° and washed twice in cold 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.4), centrifuged, and suspended in 5 volumes of the same buffer. The cells were broken by sonification (Sonics VC 750). Four different sonication times were proved (3 pulses/50 s each, 2 pulses/60 s each, 7 pulses/50 s each and 5 pulses/50 s each). During sonication the cells were permanently maintained on ice to avoid proteolysis. The broken cells were centrifuged at 40,000 x *g*, 4°C, for 1 ½ h. The pellet was suspended in cold 50 mM Tris-hydrochloride buffer (pH 7.4) and washed three times in the same buffer. To decrease the amount of contamination with fragments of plasma membrane, the crude cell wall preparation was treated with 0.5% Triton X-100 for 10 min at 20°C with frequent shaking and then washed four more times in cold 50 mM Tris-hydrochloride buffer (pH 7.4). The purity of cell wall fragments was checked by SDS-polyacrylamide gel electrophoresis (PAGE).

2.8. S-layer purification

S-layer proteins were extracted from cell wall fragments with 5 M guanidine hydrochloride (GHCl) as described earlier (Egelseer, Schocher et al. 1996). Cell wall fragments were treated with GHCl (5 M GHCl in 50 mM Tris-HCl buffer [pH 7.2]) for 2 h at 20°C. After centrifugation at 40,000 x *g* for 20 min at 20°C, the supernatant containing the extracted S-

layer protein was dialyzed against distilled water at 4°C for 24 h. S-layer self-assembly products were sedimented by centrifugation of suspensions at 40,000 x *g*, for 20 min. Both sedimented S-layer self-assembly products and clear supernatants were checked by SDS-PAGE. The protein concentration was measured with Bradford protein assay (Bio-Rad). N-terminal sequencing of the clear supernatants was performed by Matrix-Assisted Laser Desorption/Ionization (MALDI) method.

2.9. Recrystallization at the air–water interface

For the recrystallization experiments, a volume of 100 µl of the clear supernatant of the S-layer solution was injected into the aqueous calcium-containing ($CaC1_2$ in 10 mM borate buffer, pH 8) subphase in mini petri dishes (30 mm diameter, 5 ml volume) (Pum and Sleytr 1995). Because the calcium concentration in the subphase is of significant importance, five different concentrations were used (1, 5, 10, 50 and 100mM $CaC1_2$). Electron micrographs were taken with a TECNAI G2 20 D345 (FEI) transmission electron microscope, operated at 200 kV. For electron microscopic examination, S-layers were transferred onto carbon-coated electron microscope grids. The grids were carefully placed horizontally onto the interface and removed after several seconds by hand with a forcep. After negative staining with 2.5% uranyl acetate, the protein monolayers could be imaged in the electron microscope. The stability of crystals on the electron microscope grids was increased by chemically crosslinking with 2.5% glutaraldehyde (in phosphate buffer, pH 7.4). This was done by placing the grids onto drops of glutaraldehyde for 15 min. All experiments were carried out at room temperature (22°C).

2.10. Biosorption isotherms

Experiments were carried out with 30 mg of protein. Different concentrations of potassium dichromate were prepared starting from a stock solution of 10000 mg l-1 and 1000 mg l-1. Solutions pH were between 5-7, being higher the solutions pH with less Cr(VI) concentration .Whole night recrystallized Proteins were added to the Cr(VI) solutions. They were incubated at 30°C and the concentration of Cr(VI) was estimated at different times of

incubation by the 1, 5-diphenylcarbazide method (EPA 2006)**.** To determine if the metal was bound to the protein Scanning Electron Microscopy and Energy Dispersive Spectroscopy (SEM/EDAX) was done.

2.11. Data evaluation

The amount of adsorbed Cr(VI) by the dead cells and S-layer proteins was calculated as follows:

$$q = \frac{v(C_i - C_f)}{m} \quad (1)$$

Where q is the metal uptake (mg metal/g of the biosorbent), v the liquid sample volume (l), C_i the initial concentration of the metal in the solution (mg/l), C_f the final concentration of the metal in the solution (mg/l), and m the amount of added biosorbent on the dry basis (g).

2.12. Adsorption models

Langmuir, Freundlich and Tempkin isotherms were used for the model of the experimental biosorption data.

3. Results and Discussion

3.1. Evaluation of metal tolerance

Table 2 shows the results of the growth of the 9 strains evaluated. The OT4b31 and IV(4)10 strains were selected for the quantification in living and dead cells because they showed tolerance to the majority of metals and they are native strains. Additionally they were isolated from different habitats. OT4b31 was tolerant to the two concentrations of As, Co, Fe and Cr. IV(4)10 was tolerant to the two concentrations of Hg, Co, Fe and Cr and to the lowest concentration of As.

Table 2 *Bacillus sphaericus* tolerance to different metal concentrations: As, Hg, Co, Fe and Cr. + growth - no growth

	As		Hg		Co		Fe		Cr	
Strain	20 mM	30 mM	5 mg/l	10 mg/l	5 mg/l	10 mg/l	5 mg/l	10 mg/l	10 mg/l	30 mg/l
2362	-	-	+	-	+	+	+	+	+	+
OT4b25	+	-	-	-	+	+	+	+	+	+
OT4b31	+	+	-	-	+	+	+	+	+	+
OT4b48	-	-	-	-	+	+	+	+	+	+
OT4b51	-	-	+	-	-	-	+	+	+	+
OT4b56	-	-	-	-	+	+	+	+	+	+
III (2) 3	-	-	+	+	+	+	+	+	+	+
IV (1) 8	-	-	+	+	+	+	+	+	+	-
IV (4) 10	+	-	+	+	+	+	+	+	+	+

3.2. Determination of the S-layer proteins presence

Figures 1a and 1b show the SDS-PAGE patterns of cell extracts for the chosen strains, with or without metals. Both figures showed an approximately 122 kDa band that evidence 100% homology with the S-layer (Table 3). All cases show high proteins concentration, because S-layer proteins represent between 15-20% of the total cells proteins

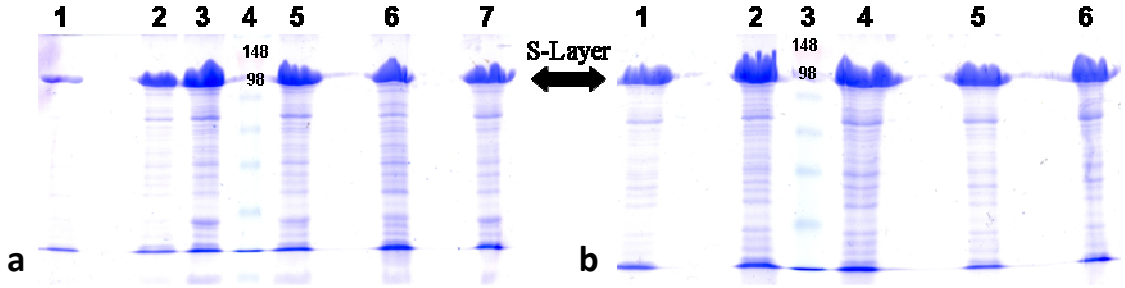

Figure 1 SDS-PAGE patterns of cell extracts of a) IV(4)10 (1) in presence of Hg (2)As (3) Fe (4) Molecular weight marker in kDa (5) Cr (6) Co (7) without metal, and b) OT4b31 (1)As (2) Fe (3) Molecular weight marker in kDa (4) Cr (5) Co (6) without metal.

Table 3 N-terminal sequencing

Peptide	AQLNDFNKISGY
Molecular weight	125 kDa
Identity	12/12 (100%)
Positives	12/12 (100%)
Gaps	0/12 (0%)
Proteins	Precursor S-layer
Bacteria	*B. sphaericus.*
GenBank access number	P38537

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3.3. Metals biosorption and bioaccumulation quantification

In living cells OT4b31 showed the highest percentage of biosorption for As, followed by Fe and Co. In IV(4)10 the highest percentage was for Hg followed by Fe, As and Co (figure 2). Data of the atomic absorption spectroscopy quantification is presented in table 4. There were no significant differences between strains in the Co and Fe concentrations after 24 hours ($P > 0.01$). Despite OT4b31 and IV(4)10 began with different As initial concentrations because of their tolerance level, 24 hours later they both reached approximately the same metal quantity. In the cobalt and iron case, they both began with the same concentrations ($P > 0.01$) and 24 hours later they also reached approximately the same quantity.

Living cells can make two processes, one of active metabolism in which metals accumulate inside the cell, and another of passive metabolism in which metals adhere to surface molecules such as the S-layer proteins. If an adsorption process is taking place, surface molecules as the S-layer proteins can have a saturation level in which no more metallic ions can adhere. If the process is metal accumulation, an efflux mechanism can be functioning at a certain metal concentration preventing more metal accumulation. These processes have been reported at *Pseudomonas aeruginosa* and *Cupriavidus metallidurans* (Ramírez, Díaz et al. 2008).

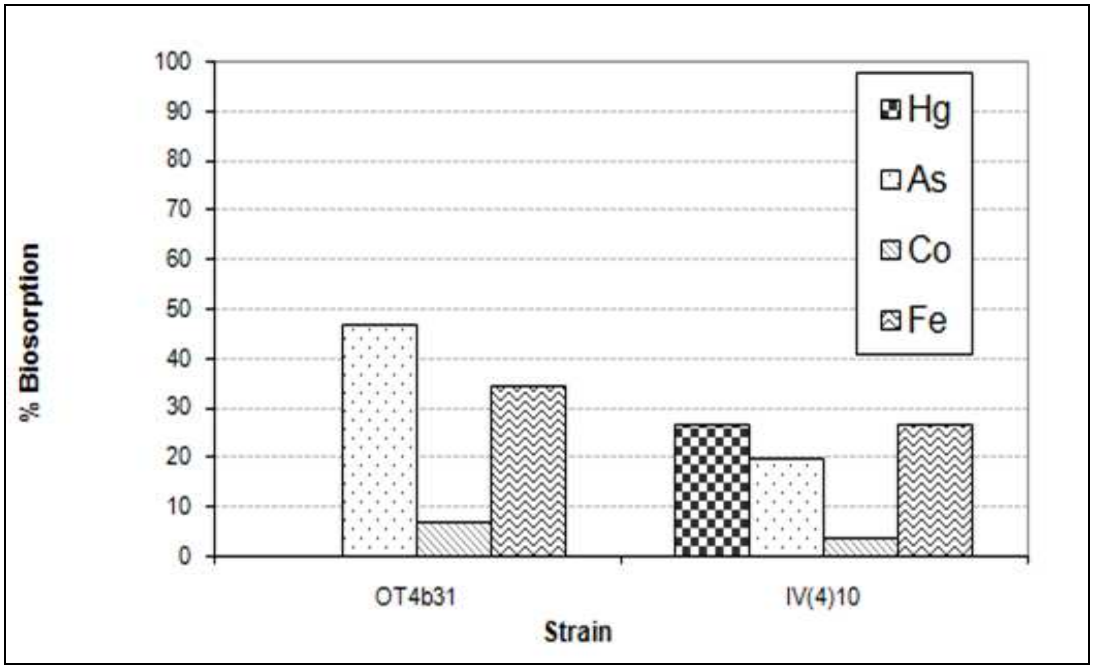

Figure 2 Heavy metals biosorption percentage in living cells. OT4b31 was tolerant to the two concentrations of As, Co, Fe and Cr. IV(4)10 was tolerant to the two concentrations of Hg, Co, Fe and Cr and to the lowest concentration of As.

Table 4 Heavy metals biosorption determination in living cells.

Strain	mg l-1	As	Co	Fe	Hg
OT4b31	Initial	2001,3	2,9	3,2	ND[a]
	24 hours	1069,7	2,7	2,1	ND
IV(4)10	Initial	1374,9	2,8	3,4	7,6
	24 hours	1105,8	2,7	2,5	5,6

Footnote: [a] Non determined.

Results of the biosorption and bioaccumulation of Cr(VI) for living and dead cells are shown in table 5 and 6. There are no significant differences ($P > 0.01$) between living cells and dead cells strains. In living cells both strains must perform similar biosorption and bioaccumulation processes such as: adsorption in surface molecules as the S-layer proteins, accumulation through helper proteins that cells normally use for the incorporation of essential elements such as phosphorous and sulfur (Suárez and Reyes 2002) and/or reduction by enzymatic processes (Elangovan, Avispa et al. 2006). In dead cells, both strains must adsorb metals in the cellular surface or in S-layer. Nevertheless, there are little differences between living cells strains that cannot be watched between dead cells strains

(Data not shown). This happens because their metabolisms can be slightly different while in dead cells it is a passive process.

Table 5 Cr(VI) residual concentration for living cells (mg Cr(VI)/ l)

Strain	Initial	16 hours	24 hours
OT4b31	39,10 (1,29)[a]	32,48 (0,99)	29,28 (0,40)
IV(4)10	40,05 (0,47)	31,12 (1,15)	27,34 (1,02)

[a] Values in parenthesis are the standard deviation of the media (n = 3).

Table 6 Cr(VI) adsorb by dead cells (*q*= mg Cr(VI)/g dry mass). Cr(VI) concentration in the solutions before adding the cells were 33,38 mg/l for OT4b31 and 33,60 mg/l for IV(4)10

Strain	1 hour	3 hours	7 hours	24 hours
OT4b31	1,6[a]	2,28	3,68	7,44
IV(4)10	1,83	3,29	3,02	7,62

[a] Ci and Cf are a media (n=3)

Both, living and dead cells showed the capacity of Cr(VI) biosorption. In dead cells metals could have been adhered to surface molecules such as the S-layer which is a porous layer that can have a saturation velocity. Metallic ions must find a union target and pass through other cellular components before this happens. In living cells cellular density increases as the time passes, therefore there are more available binding sites for metals. Additionally, if the metal gets inside the cell it first needs to join surface molecules and then gets in through different mechanisms.

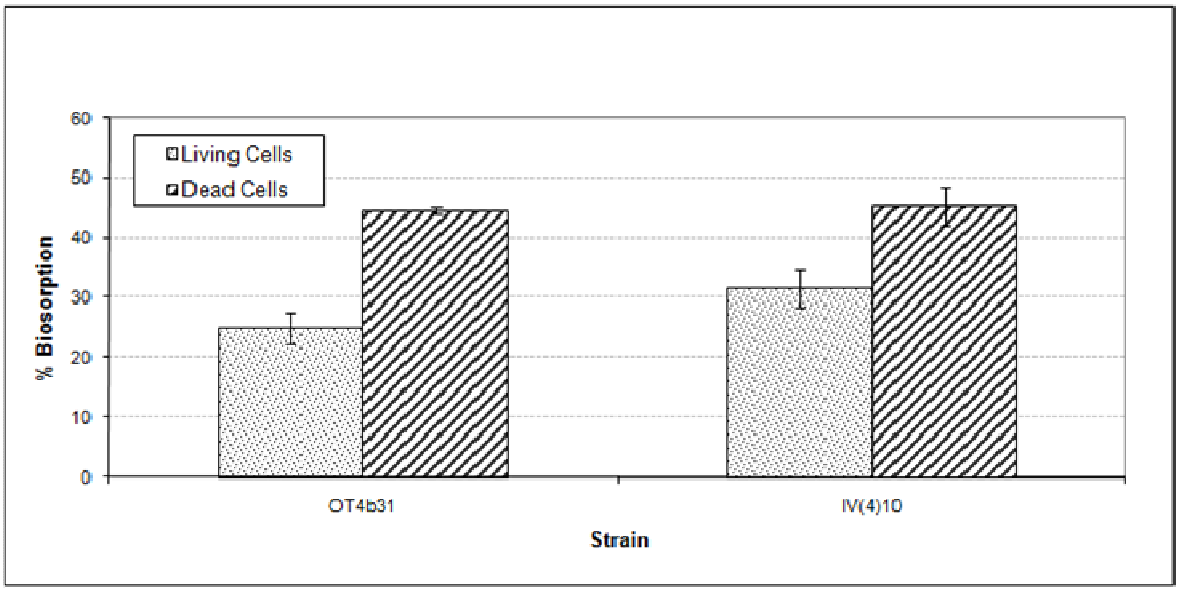

Figure 3 Cr(IV) biosorption percentage, 25% and 32% in living cells for OT4b31 strain and IV(4)10 strain respectively. 44.5% and 45% in dead cells for OT4b31 strain and IV(4)10 strain respectively.

There are differences in the concentration after 24 hours between living and dead cells (figure 3). In living cells metal accumulation could happen inside the cell but efflux mechanisms may predominate. Additionally, cellular growth decreases in presence of metal in comparison with free metals culture. This implies less available binding sites. In addition to the passive process, dead cells go through maceration process that can break the cellular wall, making even more available binding sites for metals if it binds to the S-layer.

3.4. S-layer purification

Considering that in the previous experiments dead cells had a greater metal uptake; the question if S-layer proteins could be one of the mechanisms of metals accumulation arose. It is known that biomass can carry out biosorption processes by different mechanism. One of them is cell wall surface biosorption, in which adsorption take place in polysaccharides, proteins and lipids; molecules that offer some functional groups, such as carboxylate, hydroxyl, sulphate, phosphate and amino groups. Physical adsorption take place by diverse mechanisms e.g., van der Waals' forces and ion exchange (Veglio and Beolchini 1997).

In order to evaluate if S-layer proteins may possibly have the ability to entrap metallic ions, either on living or dead cells protein biosorption isolation and regeneration had to be done. Three pulses of 50 s was the better condition of sonication. With this parameter the biggest protein concentration was obtained, consistent with the SDS-PAGE and Bradford measurement. 5 M GHCL, caused a disintegration and removal of the S-layers from the cell wall. When the GHCl was removed by dialysis, a white precipitate was formed containing the putative S-layer proteins as expected. SDS-PAGE shows the band that corresponds to the pure protein (figure 4). The sample was identified correctly. The identified protein is SLAP_BACSH with access code: P38537.

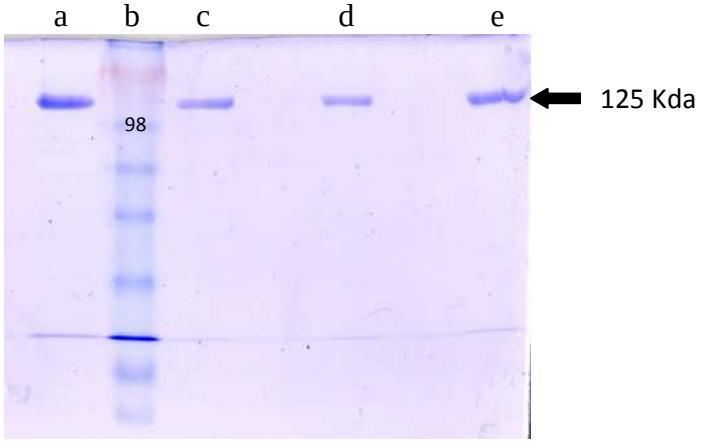

Figure 4 SDS-PAGE of dialyzed S-layer protein GHCL extracts from *B. sphaericus* OT4b31 at different sonication conditions. (a) 3 pulses of 50 s; (b) Molecular weight marker in KDa ; (c) 7 pulses of 50 s; (d) 5 pulses of 50 s; (e) 2 pulses of 60 s

3.5. Recrystallization experiments

The calcium concentration in the subphase is of great importance in the recrystallization of the S-layer at the air–water interface, like it has been reported in *Bacillus sphaericus* CCM 2177. Depending on this parameter many different morphologies can be found (Pum and Sleytr 1995). It is to be expected that divalent cations as calcium ions generate the necessary salt-bridges for the intermolecular binding among the S-layer subunits (Györvary, Stein et al. 2003). Fig. 5 shows electron micrograph of tenuous S-layer clusters obtained with 1 mM and 5mM $CaCl_2$. Larger and denser structures are obtained with a calcium concentration of 10 mM in the subphase. The larger structures were obtained with

a calcium concentration of 50 mM in the subphase. In 100 mM denser layers were obtained, in this concentration maybe bi or multi layers were formed. In double-layered structures, the two constituent monolayers can be facing each other either with their inner or their outer face (Sleytr, Györvary et al. 2003). 50 mM $CaCl_2$ was used for the following assays.

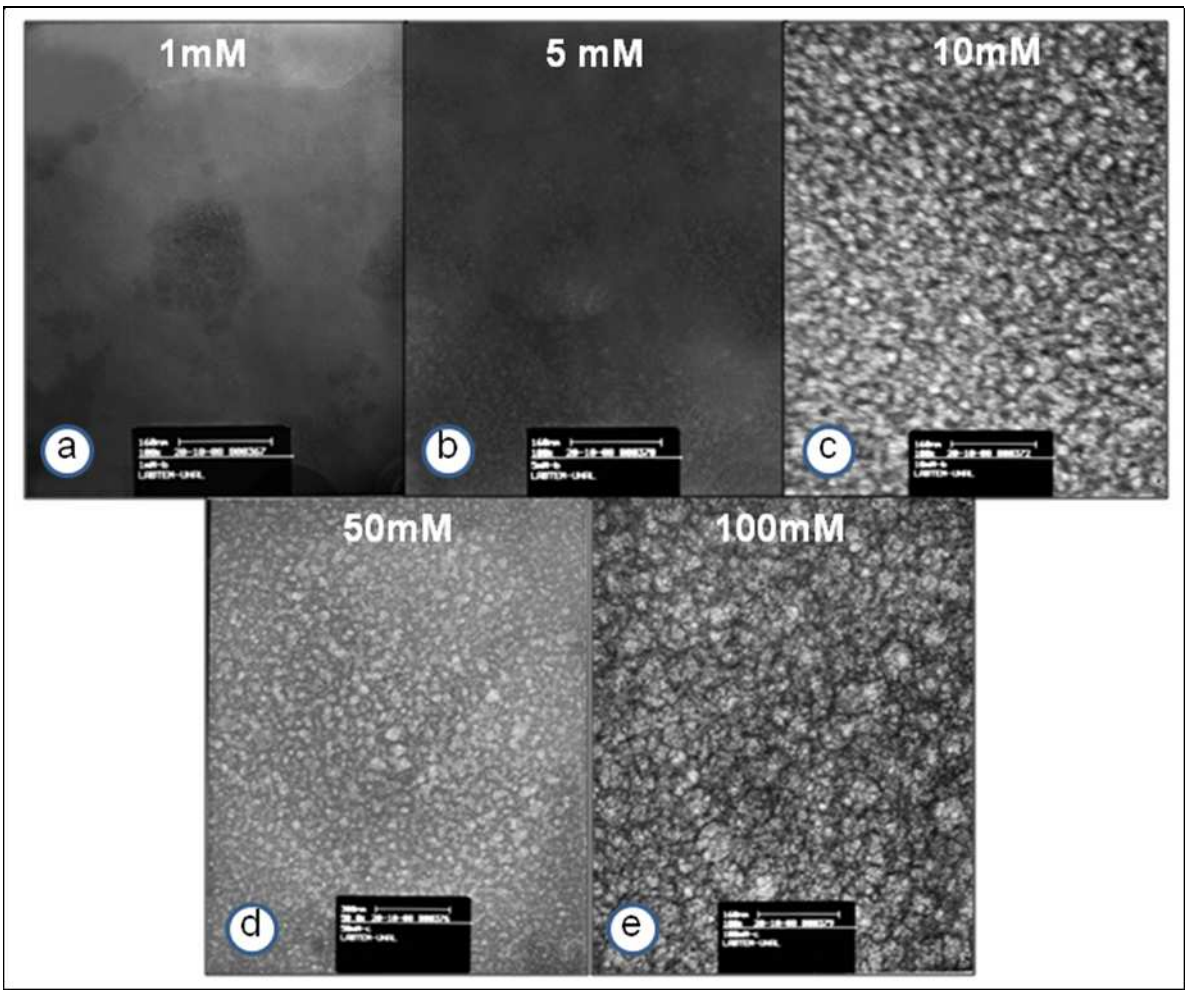

Figure 5 Electron micrograph (a) 1 mM $CaCl_2$ (b) 5 mM (c) 10 mM (d) 50 mM (e) 100 mM. Bar, 160 nm.

3.6. Biosorption isotherms

Figure 6 shows the evolution of the Cr(VI) concentration up to 72 h. Concentration at 72 h was used for *q* determination, because no increase in the metal bound occurred between 48 and 72 h (equilibrium time). Although shaking facilitates the system to reach the

equilibrium, the experiment was carried out in the lack of it, which resulted in a longer time to reach the equilibrium. The isotherms were done without shaking because the protein did not have a support. It is known that for the appropriately protein alignment, they need a support either a layer or an organized surface to join (Wetzer, Pfandler et al. 1998). For example, when the reassembling is done at the solid–liquid interface all S-layer protein subunits in the domains reveal the same orientation with respect to the substrate (Sleytr, Györvary et al. 2003). Silicon wafers is a solid support in which the proteins are bound as they are normally attached (N-terminal) to the cell wall. Nevertheless, the objective of the study was to establish if S-layer is one of the mechanism of biosorption of Cr(VI). To hand out the possibility of use the protein in a bioremediation process, the biosorption conditions have to be improved.

At low Cr(VI) initial concentrations, the residual concentration did not varied significantly through the time. As the initial concentration increases, Cr(VI) residual concentration decrease in a more significant way. These results are because at bigger metal concentration there is more chance that metal and biosorbent (S-layer) get in contact, increasing the adsorption kinetic. Metal uptake (*q*) was plotted against the final metal concentration in each solution (Figure 7), which represents the biosorption equilibrium isotherm. Table 7 shows Cr(VI) initial and final concentration (at equilibrium) and metal uptake (*q*). The percentage removal were 16%, 15%, 33%, 57%, 70% and 78% for 5.33; 32.91; 89.78; 159.80; 326.63; 365.69 mg Cr(VI)/l respectively. Under these conditions the process is efficient when the Cr initial concentration is high, but would not be a good process for diluted concentrations.

As it had been said previously, pH is a key factor in adsorption processes. The adsorption, understood like a physical phenomena, can occur by different mechanisms involving electrostatic interactions as Van der Waals forces. In aqueous solution, Cr(VI) is present as either dichromate ($Cr_2O_7^{2-}$) in acidic environments or as chromate (CrO_4^-) in alkaline environments, which could favor the binding to positive charges of the whole ion. This isotherm shows that when pH is lower, adsorption is higher. This could happened because at low pH there are more protons (H^+) that does not compete with chromate for positive binding sites. In the opposite, others metal e.g. Pb, Cd, Cu are present as positive ions in

solutions, in this case a basic solution favors the uptake, because no H^+ compete for binding sites. In addition S-layer is an acid protein with pI between 4-6, pH values above the pI can cause a negative net charge, which decrease the chromate binding site. Although, it is also necessary to take into account that the solutions with lowest pH were those that had greater Cr(VI) concentration, which could have favored the process.

Protein surface was analyzed by SEM/EDX after adsorption studies. Figures. 8 and 9 show the sample composition profile and cell map containing adsorbed Cr(VI) respectively. As the sample is a protein, carbon, nitrogen and oxygen were detected. Ca and Cl correspond to the $CaCl_2$ recrystallization solution. Al and La are the constituent elements of the stib-surface. The adsorbed Cr(VI) is represented by the white points in figure 9. It is important to say that the profile in figure 8 is only for one of the white points in figure 9.

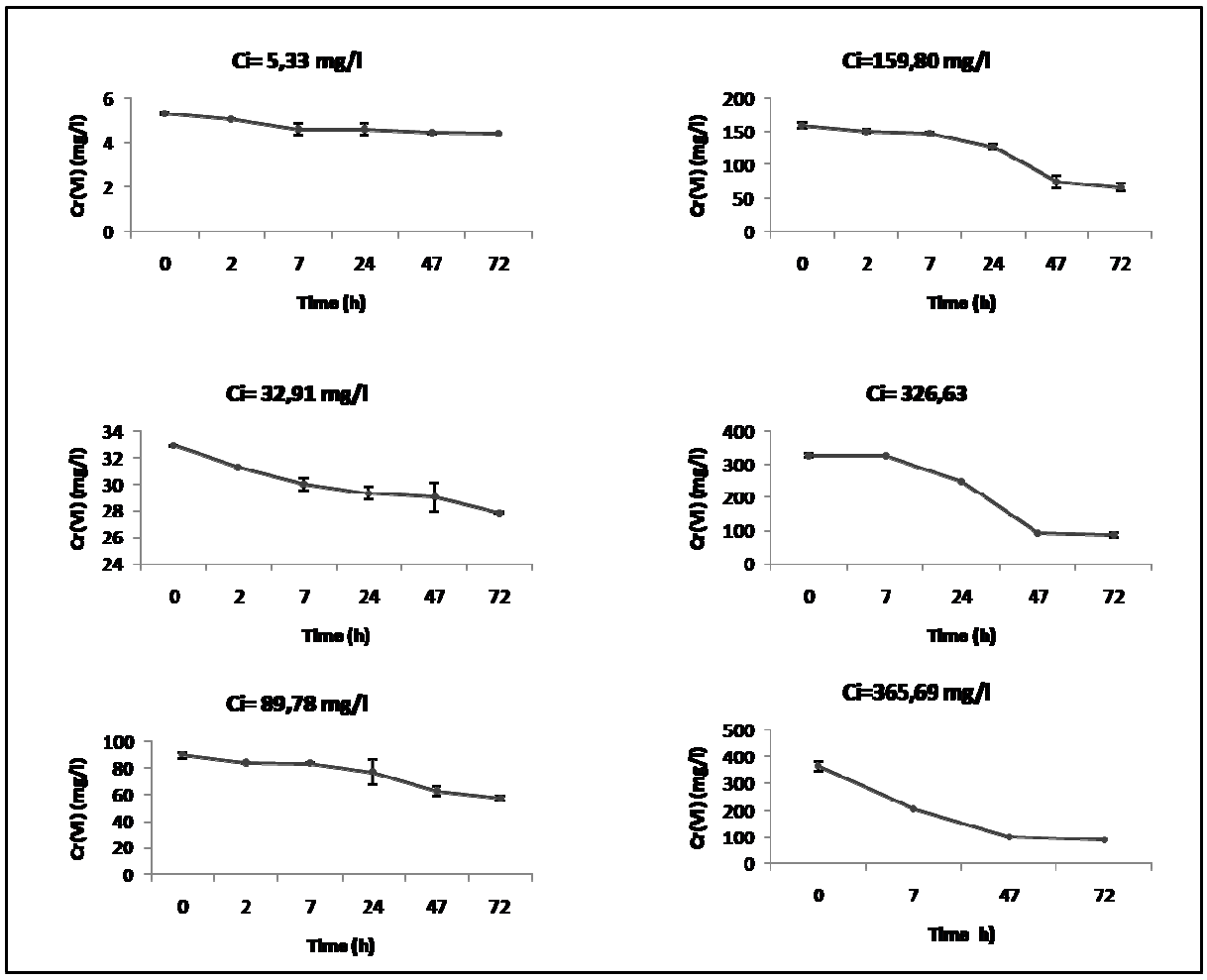

Figure 6 Kinetic of Cr(VI) binding to the S-layer. Data correspond to a media (*n*=2)

It was attempted to make biosorption isotherms with a larger quantity of protein, to test whether a larger quantity of biosorbent would influence metal uptake. However, after purify the protein again and make SDS-PAGE degradation was evidenced. An additional band of approximately 70 kDa or many of different molecular weight were detected. It has been shown that *B. sphaericus* produce proteases as serine proteinases capable of cause proteolysis of a high molecular weight protein (100 kDa) (Thanabalu and Porter 1995; Yang, Wang et al. 2007). Some serine proteinases cleaves peptide bonds selectively on the large hydrophobic amino acids such as tryptophan, tyrosine, phenylalanine, and methionine (Berg, Tymoczko et al. 2002). S-layer amino acids are mainly hydrophobic, which makes it vulnerable to this kind serine proteases attack.

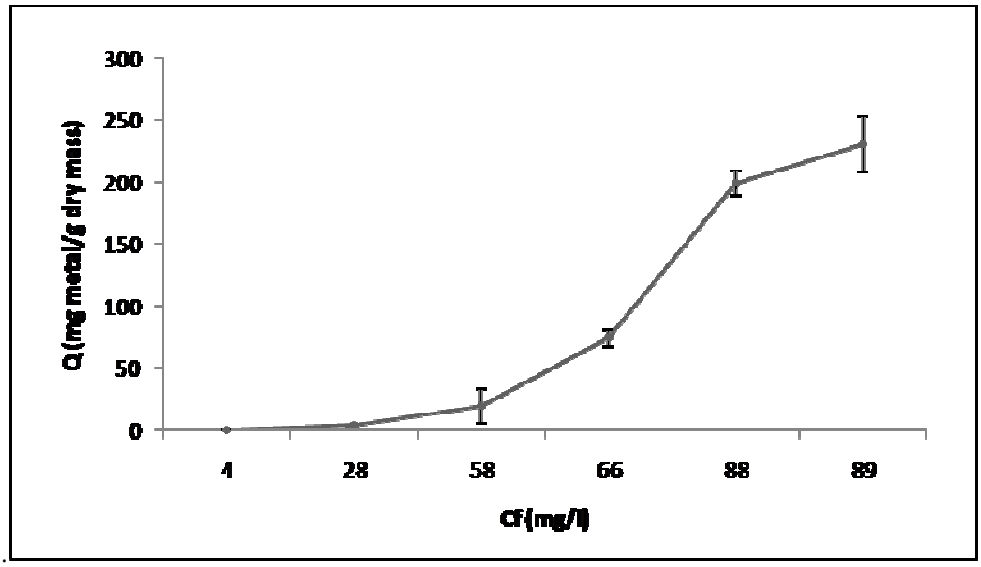

Figure 7 Biosorption equilibrium isotherm

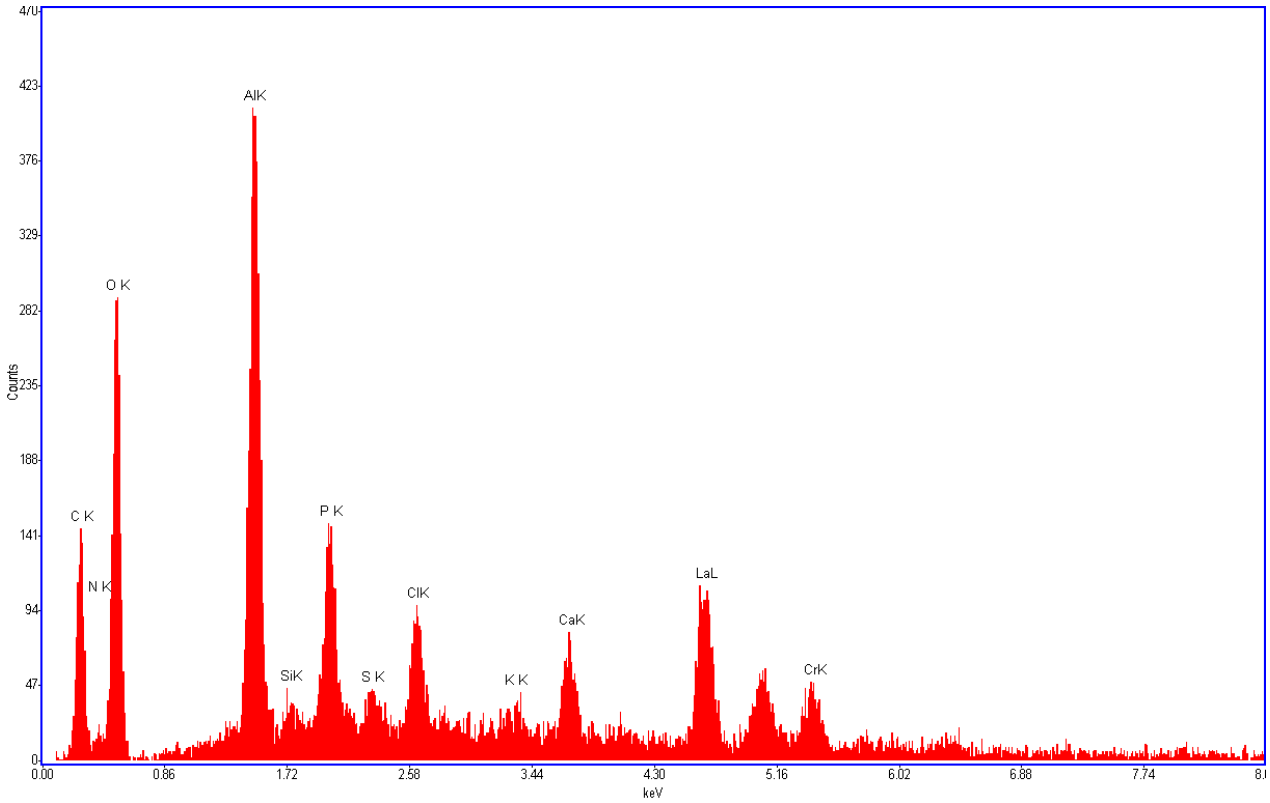

Figure 8 SEM/EDAX of Cr(VI) adsorption

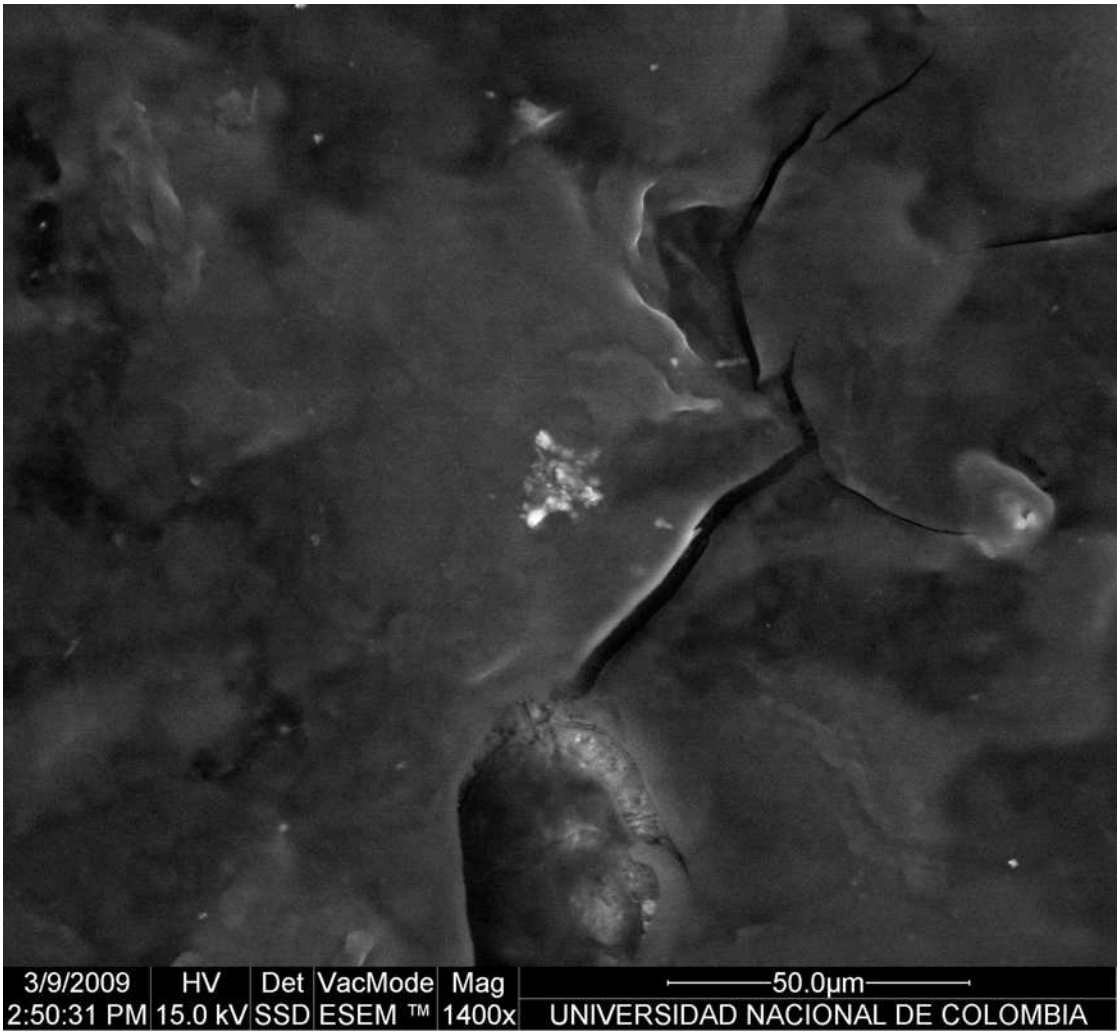

Figure 9 SEM/EDAX image of Cr(VI) adsorption.

Table 7 Cr(VI) initial and final concentration (at equilibrium) in mg/l and metal uptake (q [mg metal/g biosorbent])

Initial	5,33 (0,05)	32,91 (0,08)	89,78 (2,50)	159,8 (4,24)	326,63 (5,66)	365,69 (17,47)
Final	4,43[a]	27,84 (0,08)	57,71 (1,66)	66,27 (5,56)	87,89 (17,47)	89,07 (9,15)
q	0,40 (0,55)[b]	4,23 (0,14)	19,40 (13,82)	74,07 (6,57)	198,94 (9,85)	250,21 (22,19)

[a] n=1

[b] Values in parenthesis are the standard deviation of the media (n = 2)

3.7. Adsorption models

Langmuir isotherm (Langmuir 1918), Freundlich (Freundlich 1906) and Tempkin(Tempkin and Pyzhev 1940) are common adsorption isotherms models capable of describing the concentration of the metal in the liquid phase when reaching the thermodiynamics equilibrium with the metal adsorbed, important in optimizing the use of the latter.

The Cr(VI) adsorption isotherm followed the linearized Freundlich model (Figure 10). Freundlich isotherm model the relation between the metal uptake capacity q (mg/g) of adsorbent and the residual metal ion concentration C_f (mg/l). It is presented by the following equation and its linearized form:

$$q = k \cdot C_f^{1/n}, \quad (2)$$

$$\log q = \frac{1}{n} \log C_f + \log k \quad (3)$$

where the intercept ln k is a measure of adsorbent capacity, and the slope $1/n$ is the adsorption intensity. The values of the constants k and 1/n, calculated from the plot of the linearized model (Log q against Log $C_{f)}$ were $2{,}56 \times 10^{-5}$ and 3,55 respectively. Since the value of 1/n is more than 1 and K is small, it indicates that adsorption is not very favorable.

Figure 11 represent the linearized Langmuir model. Langmuir isotherm model relates solid phase adsorbate concentration q to the equilibrium liquid concentration C_f. It is presented by the following equation and its linearized form:

$$q = \frac{q_{\max} \cdot b \cdot C_f}{1 + b \cdot C_f}, \quad (4)$$

$$\frac{1}{q} = \frac{1}{q_{\max} \cdot b} \frac{1}{C_f} + \frac{1}{q_{\max}}, \quad (5)$$

where q_{max} and b are the Langmuir constants, representing the maximum adsorption capacity for the solid phase loading and the energy constant related to the heat of adsorption, respectively. Where b was obtained from the linear regression and q_{max} is the metal uptake at the equilibrium residual concentration of 365,69mg Cr(VI)/l

Figure 12 correspond to the Tempkin model. Tempkin and Pyzhev considered the effect of some indirect sorbate/adsorbate interactions on the adsorption isotherm. This isotherm assumes that; the heat of adsorption of all the molecules in a layer decreases linearly with surface coverage of adsorbent due to sorbate–adsorbate interactions. This adsorption is

characterized by a uniform distribution of binding energies (Colak, Atar et al. 2009). It is presented by the following equation and its linearized form:

$$q = B\ln(A \cdot C_f) \quad \textbf{(6)}$$

$$q = B\ln A + B\ln C_{f,} \quad \textbf{(7)}$$

Where A is the equilibrium binding constant and the constant B is related to the heat of adsorption, calculated from the plot of q against ln C_f.

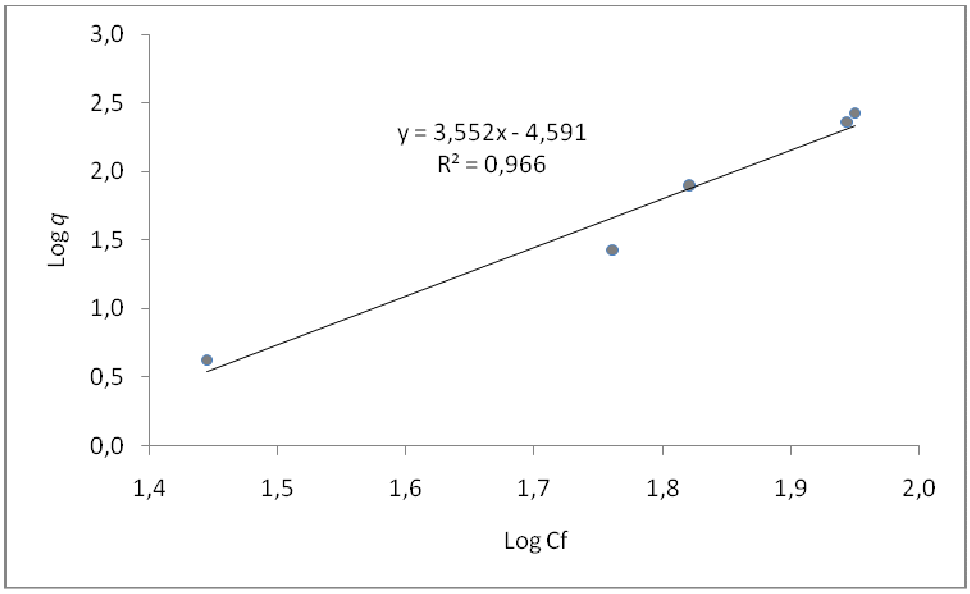

Figure 10 Linearized Freundlich model: Log q= Log K+ 1/n Log $C_{f.}$

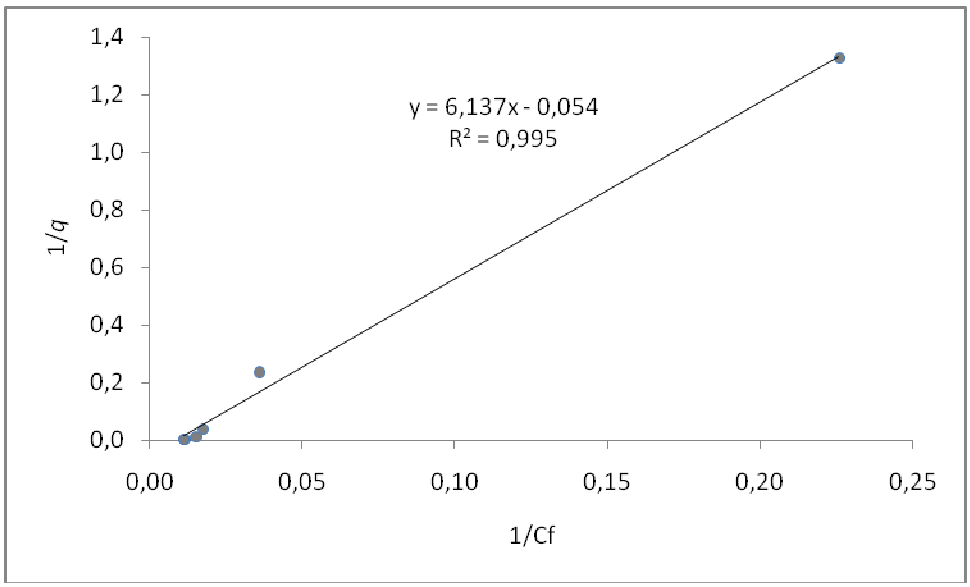

Figure 11 Linearized Langmuir model: $1/q = 1/q_{max}\,(1/bC_f + 1)$

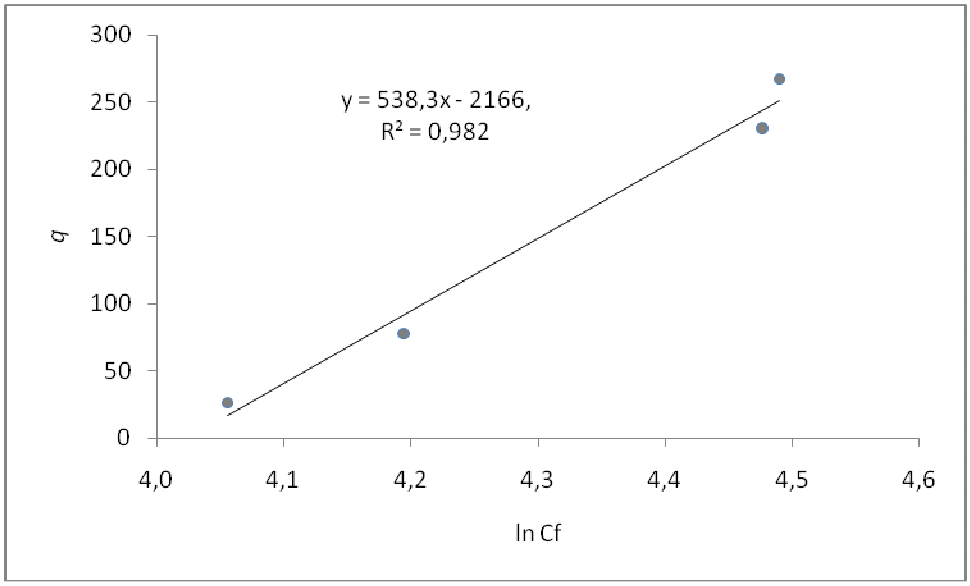

Figure 12 Linearized Tempkin model: q = B ln A + B ln C_f

Although the R^2 was high for the three linearized models, when calculating q from the parameters of each model the model that better adjusts to the data in its totality is Freundlich. Nevertheless, Tempkin fit the data at high initial of Cr(VI) concentrations very well. Langmuir is not a good model for this adsorption isotherm, in spite of having the best R2. This can be due to the wide range between the 1/Cf data, which makes linear regression good (figure 13). Table 8 show the estimated parameters values for each model.

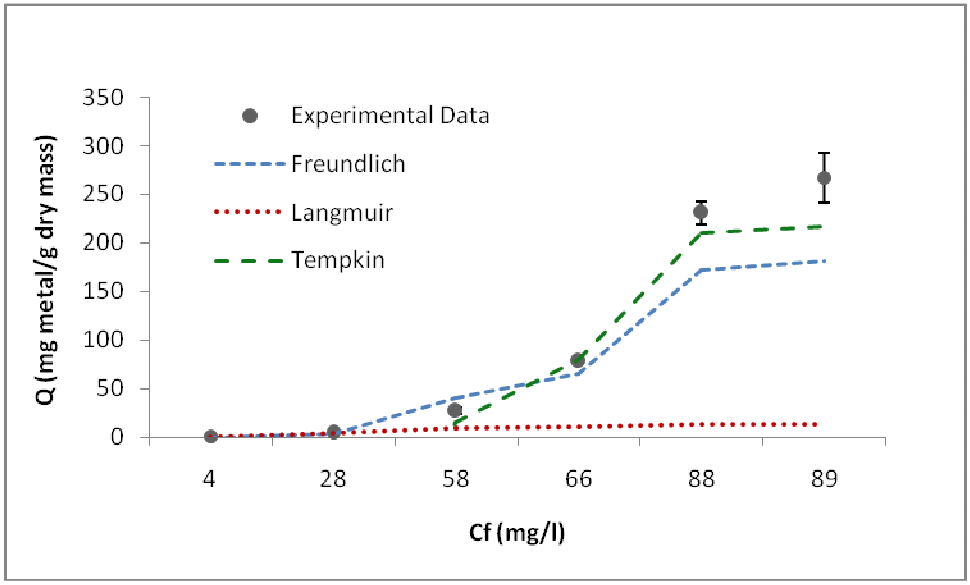

Figure 13 Adsorption isotherms based on calculated parameters.

Table 8. Model parameters calculated from the linearized model.

Freundlich		Lagmuir		Tempkin	
k (l/g)	$1/n^a$	q_{max} (mg/g)	b (l/mg)	A(l/mg)	B^a
$2{,}56 \times 10^{-5}$	3,55	290	$5{,}62 \times 10^{-4}$	0,0179	538,4

[a]dimensionless

4. Conclusions

Both, dead and living cells showed the capacity of metal biosorption. Dead cells can be stored easily since they are powdered. Enriched mediums are not needed since their growth occurs before supplementing the metal, contrary to living cells that can not grow in minimum mediums because they must tolerate higher pressure in presence of metals. Both strains grew in peptone water in absence of Cr(VI) and maintained the cell density when the metal was added.

The pH is an important factor that influences the biosorption phenomenon. In metallic ions such as Cr(VI) this process occurs by acidic adsorption, mechanism in which a liquid must have enough protons for an ionic exchange to occur. (Srinath et al., 2002) Adjusting cells pH to 2.5 and the Cr solution pH to 4.0, cells remain acid or positive while Cr ions remain more basic or negative. This pre-treatment cannot be applied to living cells because of their intolerance to low pH levels. Additionally, metabolically active cells produce redox reactions between the cells and the aqueous medium generating pH increases.

Although, it was proved that S-layer proteins execute a trapping role of Cr(VI) in *B. sphaericus* cells, it is necessary to consider that dead cells are more stable than proteins. The last are extremely sensible to pH and temperatures changes. In addition, they need a proper structure assemble, in this case S-layer may need a support surface to play the same role as in living or dead biomass. Nevertheless, using the purified protein reduces the risk of introducing living microorganisms to the environment. Making this process a nature friendly non chemical method. Adsorption conditions have to be improved to handout this possibility.

Acknowledgements

This work was funded by the Research Fund from de Science Faculty of Universidad de los Andes, the Microbiological Research Center (CIMIC) and Colciencias. The authors acknowledge the contribution of the Electron Microscopy laboratory of Universidad Nacional de Colombia and of Centro de Biología Molecular Severo Ochoa (CBMSO), Universidad Autonoma de Madrid España for protein sequencing.

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