

## A COMBINED REMEDIATION TECHNOLOGY FOR THE REDUCTION AND BIOLEACHING OF HEXAVALENT CHROMIUM FROM SOILS USING ACIDITHIOBACILLUS THIOOXIDANS

Bruna FONSECA<sup>1</sup>, Joana RODRIGUES<sup>1</sup>, Tânia MENDES<sup>1</sup>, Ana QUEIROZ<sup>1</sup>,  
Teresa TAVARES<sup>1\*</sup>

**Abstract.** Contamination of soils due to the release of effluents or deposition of wastes containing hexavalent chromium has been arising serious environmental problems. Therefore, the development of cost effectiveness but also ecological cleaning techniques is a matter of great concern among the scientific community. Bioremediation is attracting more and more attention due to its efficiency, low impact in the ecosystems and low cost. In particular, this study approaches a bioleaching technique using an *Acidithiobacillus thiooxidans* DSM504 pure culture to clean a soil contaminated with hexavalent chromium. Eight batch tests were performed in order to evaluate the effect of combined parameters: operational temperature (26°C and Troom), hexavalent chromium concentration (50 mg kg<sup>-1</sup> and 100 mg kg<sup>-1</sup>) and pH of the contaminant solution (2 and pHfree). The bioleaching technique herein exposed presented removal values between 33.3% and 83.3%, undergoing higher deviations due to changes on the contamination pH. Generally, it was more efficient when applied to soils contaminated with acid solutions. The lowest and highest values were both observed for operational temperatures of 26°C and hexavalent chromium concentrations of 50 mg kg<sup>-1</sup>. Moreover, the highest value was observed for the soil contaminated with a hexavalent chromium solution of pH 2.

**Key-words:** Chromium; Bioleaching; *Acidithiobacillus thiooxidans*.

### Introduction

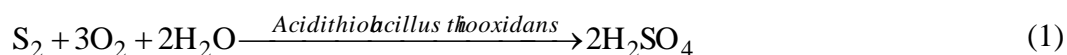
The release of hexavalent chromium, Cr(VI), into soils due to several anthropogenic activities, is more and more a matter of major concern, as this is a highly mobile, toxic and carcinogenic ion, also designated as a priority pollutant in various countries. Consequently, the development of cleaning technologies aiming its effects attenuation or its elimination has been a challenge for scientific researchers. Several physico-chemical techniques are already being used to decontaminate Cr(VI) polluted soils, but the “green” ones are an increasingly focus of attention, mostly because of their cost effectiveness. It is estimated that bioremediation using microorganisms can reduce total treatment costs in 28%, compared with conventional systems (Loukidou et al., 2004; Jeyasingh and Philip,

<sup>1</sup> Centre of Biological Engineering, University of Minho, 4710 - 057 Braga, Portugal

\* ttavares@deb.uminho.pt

2005; Ore and Grennberg, 2000; Mishra and Jena, 2009; Umrانيا, 2006; Fonseca et al., 2009; Krishna and Philip, 2005; Kumar and Nagendran, 2008).

Bioremediation is a bioremediation technique usually applied to heavy metals contaminated soils, sediments and sludge. Its application, using iron and sulfur oxidizing bacteria, either indigenous or laboratorial, has been fairly studied (Kumar and Nagendran, 2008; Gomez and Bosecker, 1999; Lombardi and Garcia, 2002; Kumar et al., 2008; Liu et al., 2007; Villar and Garcia, 2006; Kumar and Nagendran, 2009; Zagury et al., 2001; Park et al., 2007; Liu et al., 2003; Kumar and Nagendran, 2007; Ishigaki et al., 2005). However, there is a lack of specific studies on Cr(VI) bioremediation, as the metal is basically quantified concerning its total concentration. In 1922, Waksman (1922) isolated an acidophilic and chemoautotrophic bacteria - *Acidithiobacillus thiooxidans* - that can use elemental sulfur as their source of energy and consequently acidify the media through the production of sulfuric acid:



Later, Allegretti et al. (2006) proved that Cr(VI) can be reduced to a less toxic, less mobile and non-carcinogenic cation - trivalent chromium, Cr(III) - by the action of higher polythionates, which are intermediate compounds of the oxidation process of elemental sulfur, catalyzed by *Acidithiobacillus thiooxidans*. Furthermore, it is also known that, due to its high *redox* potential ( $E^\circ \geq 1.3\text{V}$ ), the Cr(VI) oxyanion can be easily reduced to Cr(III) by some biomaterials, especially in acidic media (Silva et al., 2009):



As a consequence of the above mentioned reasons, this study aims to be a contribution to the characterization and optimization of bioremediation operational parameters, using *Acidithiobacillus thiooxidans* DSM504, to clean soils contaminated with Cr(VI). With this purpose, and based on previous tests performed with a typical loamy sand soil from the North of Portugal (Fonseca et al., 2009a; Fonseca et al., 2009b) two values of the pH contaminant solution (pH 2 and pH<sub>free</sub>), of the Cr(VI) concentration in soil (50 mg kg<sup>-1</sup> and 100 mg kg<sup>-1</sup>) and of the operational temperature (26°C and  $T_{\text{room}}$ ) were evaluated by means of batch tests. Considering that each of the three parameters (n) could assume two values (L), eight experiments were performed,  $L^n$  (Plackett and Burman, 1946).

## Material and Methods

### Material

*Soil:* Various soil samples of a loamy sand soil were collected in Póvoa de Varzim, Oporto, Portugal (41°25'15.58''N and 8°45'58.27''O), homogenized, and characterized over again as described elsewhere (Fonseca et al., 2009a; Krishna and Philip, 2005; Fonseca et al., 2009b). Table 1 resumes the main characteristics of this soil sample.

*Contaminant:* The potassium dichromate ( $K_2Cr_2O_7$ ) used to contaminate the soil was 99.5% pure and was purchased from Panreac.

*Inoculum:* The bacteria used for leaching experiments were the *Acidithiobacillus thiooxidans* DSM 504, obtained from the German Collection of Microorganisms and Cell Cultures. The bacteria was revived from frozen cultures (-80 C in 20% glycerol) by growing cells in DSMZ 35 medium: 0.10 g of  $NH_4Cl$ , 3.00 g of  $KH_2PO_4$ , 0.10 g of  $MgCl_2 \cdot 6H_2O$ , 0.14 g of  $CaCl_2 \cdot 2H_2O$  and 10 g of elemental sulfur, per liter of distilled water. The inoculum for the bioleaching assays resulted from a six day incubation of 500 mL of culture media in a rotary shaker at 150 rpm and 26°C. The absorbance of the inoculum at 620 nm was averagely 0.034, corresponding to the beginning of the exponential phase of the growth curve.

**Table 1.** Chemical and physical properties of the original soil sample (before contamination)

| Parameters  |            |
|---|------------|
| Texture (%)   |            |
| Clay  | 1.43       |
| Silt  | 19.29      |
| Sand  | 78.96      |
| pH (H <sub>2</sub> O)                                   | 6.3 ± 0.1  |
| Organic Matter Content (%)                              | 4.3 ± 0.2  |
| Metal oxides  |            |
| [Feox]/(mg kg <sup>-1</sup> )                           | 3800 ± 100 |
| [Mnox]/(mg kg <sup>-1</sup> )                           | 100 ± 0    |
| [PO <sub>4</sub> <sup>3-</sup> ]/(mg kg <sup>-1</sup> ) | 7.2 ± 0.8  |
| [Cr]/ (mg kg <sup>-1</sup> )                            | 8.7 ± 0.0  |

### ***Soil preparation and analysis***

Four sub-samples of the collected soil were spiked with solutions of  $K_2Cr_2O_7$ , in order to obtain two contamination levels of Cr(VI):  $50 \text{ mg kg}^{-1}$  and  $100 \text{ mg kg}^{-1}$ . The pH of two contaminant solutions, with different concentrations, was adjusted to 2 using concentrated  $HNO_3$  (65%). The pH of the two remaining solutions was kept unadjusted ( $pH \approx 7.7$ ). The mixtures were stirred frequently till they were dry (2/3 days). For each sample, including the original soil, the real concentration of Cr(VI) was determined by Flame Atomic Absorption (FAA; Varian SpectrAA-250 Plus), after an acid digestion (CEM MDS-2000) of the soil, according to the US EPA method 3051 (USEPA, 2007). The concentration of phosphates,  $[PO_4^{3-}]/(\text{mg kg}^{-1})$ , was determined using the stannous chloride method, described in standard methods (APHA, 1998), after the extraction with sodium bicarbonate as described by Olsen et al. (1954). The pH was determined according to the US EPA method 9045D (USEPA, 2004). The analyses were made in triplicate. Table 2 compiles the main characteristics of the four contaminated samples.

### ***Bioleaching experiments***

The batch bioleaching tests were performed in 250 mL Erlenmeyer flasks. The working volume of 150 mL was constituted by 10 % (v/v) of inoculum, 1% (w/v) of elemental sulfur, 3% (w/v) of contaminated soil and 90% of DSMZ 35 medium. The absorbance of the inoculum was  $0.034 \pm 0.022$ . For each set of experiments, eighteen flasks, sealed with solid silicone caps, were incubated in a rotary shaker, at 150 rpm, for seventy days. A pair of flasks, duplicates, was removed, each five days during the first twenty days, and each ten days for the remaining period, in order to keep the chemical equilibrium undisturbed. The liquid and solid phases were separated by centrifugation (5000 rpm, 10 min) and properly stored for future analyses. Eight sets of experiments were prepared, by varying the contamination level -  $50 \text{ mg kg}^{-1}$  and  $100 \text{ mg kg}^{-1}$  - the pH of contaminant solution - pH 2 and  $pH_{\text{free}}$  - and the operation temperature -  $26^\circ\text{C}$  and  $T_{\text{room}}$ . A different code was ascribed to each experimental set, according to the studied variable (Table 2). Three sets of blank experiments were undertaken. In Set I (chemical control) the soil was autoclaved, in Set II (biological control) the elemental sulfur was annulled, and in Set III (biological control) the flasks were not inoculated with *Acidithiobacillus thiooxidans*.

**Table 2.** References ascribed to each assay, according to the variable parameters. Chemical properties of the soil samples contaminated with hexavalent chromium

| Code                     |                       | Characteristics of the soil samples |                       |   |
|--------------------------|-----------------------|-------------------------------------|-----------------------|---|
| $T = 26^{\circ}\text{C}$ | $T = T_{\text{room}}$ | [Cr]/(mg kg <sup>-1</sup> )         | pH (H <sub>2</sub> O) | [PO <sub>4</sub> <sup>3-</sup> ]/(mg kg <sup>-1</sup> ) |
| C50pH2T26                | C50pH2T               | 46 ± 0                              | 5.9 ± 0.0             | 7.8 ± 1.2   |
| C50pHT26                 | C50pHT                | 51 ± 2                              | 6.7 ± 0.1             | 10.8 ± 0.7  |
| C100pH2T26               | C100pH2T              | 120 ± 2                             | 6.1 ± 0.1             | 5.5 ± 0.3   |
| C100pHT26                | C100pHT               | 118 ± 0                             | 7.0 ± 0.0             | 16.9 ± 2.0  |

### Chemical analyses

The liquid phase was analyzed for total chromium by FAA and for Cr(VI) by US EPA method 7196A (USEPA, 1992). The concentration of the Cr(III) in solution, C<sub>Cr(III)</sub>/(mg L<sup>-1</sup>), and the mass of total chromium desorbed per mass of soil, S<sub>Cr</sub>/(mg kg<sup>-1</sup>), were determined by the expressions:

$$C_{Cr(III)} = C_{Cr} - C_{Cr(VI)} \quad (6)$$

$$S_{Cr} = (C_{Cr}V)/w_{soil} \quad (7)$$

Here C<sub>Cr</sub> and C<sub>Cr(VI)</sub> are, respectively, the concentrations of total and hexavalent chromium in solution (mg L<sup>-1</sup>), V is the batch working volume (L) and w<sub>soil</sub> is the mass of soil sample (kg). In order to follow the production of sulfuric acid, the sulfates - [SO<sub>4</sub><sup>2-</sup>]/(mg L<sup>-1</sup>) - were quantified in the liquid phase according to the modified turbidimetric method described by Kolmert et al. (2000). The variation of phosphates - [PO<sub>4</sub><sup>3-</sup>]/(mg L<sup>-1</sup>) - in solution was also evaluated applying the stannous chloride method to the liquid samples.

## **Results and Discussion**

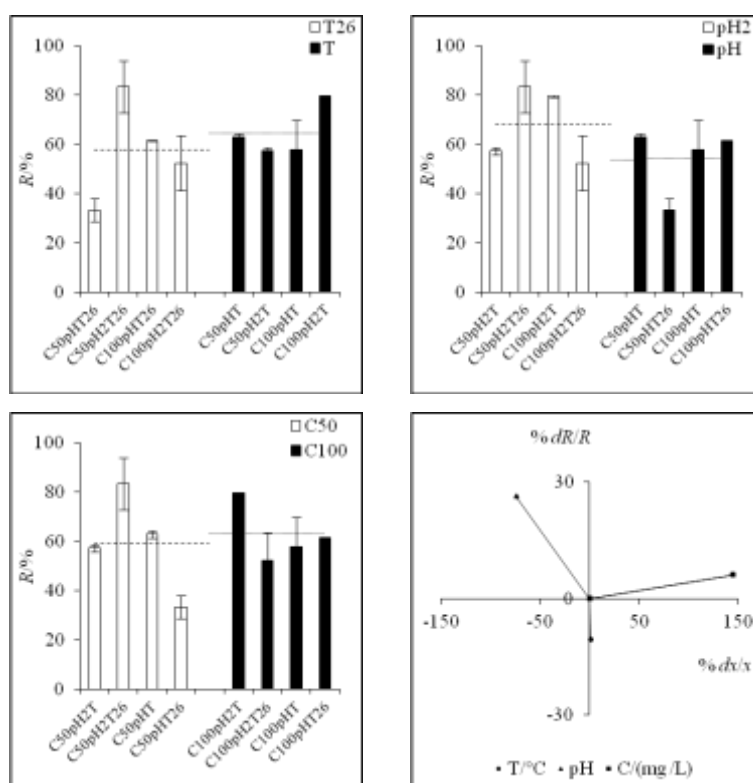
### ***Soil characterization***

As mentioned before, the soil used to perform the tests herein described was previously characterized (Fonseca et al., 2009a; Fonseca et al., 2009b). However, to ensure the soil steadiness, some of its characteristics were reevaluated. As expected, physical characteristics like the particle size distribution did not reveal significant discrepancy and the loamy sand soil designation still fits. Values concerning the pH, organic matter content and metal oxides concentration were kept almost equal. However, the total chromium concentration did decrease in the meanwhile. This can be explained by the time interval between sampling: the atmospheric conditions, especially the rain, may have drained the chromium sorbed at the superficial layers, A-horizon and O-horizon. This phenomenon greatly justifies the need of this study. Finally, the phosphates concentration was also determined and these compounds were followed during the bioleaching assays in order to evaluate the dissolution of rock phosphates (Waksman and Joffe, 1922a). However, the concentration found was very low.

### ***Total chromium removal: influence of concentration, pH and temperature***

First and foremost, it is essential to refer that sulfur oxidation to sulfuric acid was recorded for some of the blank tests. Concretely, either for the sterilized and unsterilized soil - Set I and III - the oxidation was not very extent, but sporadic values of 30% were achieved for the Cr(VI) removal. This fact is in accordance with the statement of Waksman and Joffe (1922a): when sulfur is added to soil, it is slowly oxidized to sulfuric acid, depending on the substances present in the sample. However, for the Set II, with no sulfur added, maximum values of 2% were achieved for the removal of Cr(VI). This may correspond to the fraction that was weakly sorbed to the soil matrix through van der Waals forces (Krishna and Philip, 2005).

Even so, the main purpose of this study was to test the ability of the *Acidithiobacillus thiooxidans* DSM504 bacterium to catalyze and consequently accelerate the sulfur oxidation, while it is reducing and leaching Cr(VI) from a contaminated soil, in different scenarios. Figure 1 resumes the main results obtained. The removal values, concerning total chromium, were represented according to the tested parameter, for the eight experimental sets.



**Figure 1.** Cr(VI) removal obtained for the variation of each parameter. Dot lines represent the removal mean values, concerning each parameter variation.

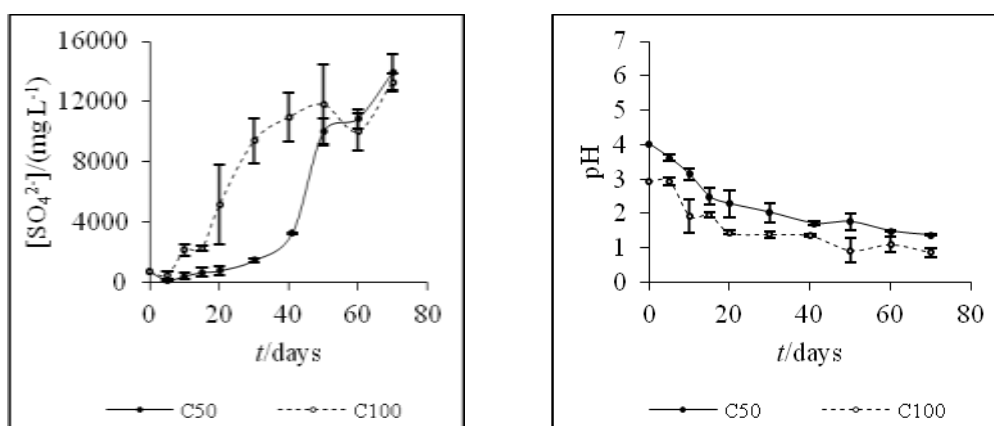
The lowest removal values, 33.3% and 53.4%, were obtained for the tests at 26 °C, using the soil contaminated with 50 mg kg<sup>-1</sup> and 100 mg kg<sup>-1</sup>, and with solutions of free pH and pH 2, respectively. The higher removal values, 83.3% and 79.5%, were obtained for the tests performed with contaminant solutions at pH 2, performed at 26 °C and at room temperature, with either 50 mg kg<sup>-1</sup> or 100 mg kg<sup>-1</sup> of Cr(VI). Herein appears the first evidence of the notorious influence of soil pH in the efficiency of the chromium dissolution process. It should be noted that the contamination using solutions with pH 2 resulted in the acidification of the soil samples, Table 2. According to the adopted sensitive analysis method, described by Hatzikioseyan et al. (2001) and presented in Figure 1, the relative change of the contamination pH resulted in a higher relative change of the Cr(VI) removal, compared with the other two parameters, T/°C and S<sub>Cr</sub>/(mg kg<sup>-1</sup>). This method was applied considering the mean values of free contamination pH, room temperature and contamination level of 50 mg kg<sup>-1</sup>, as the base point. Still focusing on this graphic, a relative lower interference of the operation temperature and of the contaminant concentration was observed. These observations are strengthened by the removal mean values, Figure 1, concerning the variation of temperature and concentration parameters, that dist very

little from each other, compared with the results obtained due to pH variation. However, it is important to refer that, averagely, an increasing removal was observed for the increase in the contaminant loading, as reported by Jeyasingh and Philip (2005) for indigenous microorganisms. Also, the expected enhancement of the contaminant removal for increasing temperatures, frequently associated to biological treatments, was not observed at such minor variation (Umrana, 2006).

During similar studies with contaminated sludge, Villar and Garcia (2006) did not notice a significant discrepancy between removals for an initial pH of 4 and 7. On the other hand, but in longer assays, Kumar and Nagendran (2007) observed a difference of 18% between bioleaching efficiencies, on soil systems at initial pH of 3 and 7, but with opposite trend, and registered a maximum removal, 90%, for the assay with an initial pH of 5. It should be noticed that, besides the shorter contact time and the lower variation on pH in the first case, only total chromium was determined in both studies and, as it is well known, trivalent chromium cations and hexavalent oxyanions have different mobility in acid/basic media (Fonseca et al., 2009a). Therefore, these removal values may be essentially related with one of the forms present in the contaminated sludge or soil, but also with the ideal pH for the bacteria growth. In order to clarify this, a closer approach was made through the evaluation of important parameters like media pH, Cr(VI) dissolution and sulfate production, along the most efficient assays for each contaminant concentration - C50pH2T26 and C100pH2T.

### ***Sulfur oxidation, pH variation and Cr(VI) dissolution***

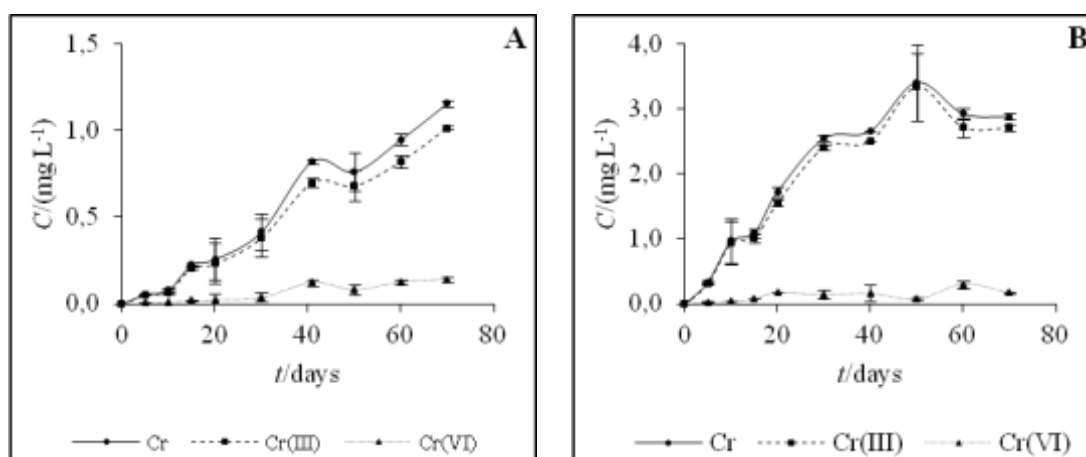
As referred in Introduction, the *Acidithiobacillus thiooxidans* bacteria have the ability to oxidize elemental sulfur and produce sulfuric acid. Therefore, the sulfates production was followed, in all assays, and a representative result is shown on Figure 2, for the assays C50pH2T26 and C100pH2T.



**Figure 2.** Sulfates concentration and pH in the liquid phase, registered along the most successful assays, performed with the soil samples contaminated at  $50 \text{ mg kg}^{-1}$  (C50) and  $100 \text{ mg kg}^{-1}$  (C100).



The maximum sulfate production is in accordance with the concentration range reported by other authors in similar studies (Kumar et al., 2008; Liu et al., 2003; Kumar and Nagendran, 2007). An exponential increasing of the sulfate concentration is clear between the days 6 and 60. According to Liu et al. (2003), this is an evidence of positive correlation between the exponential growth phase of the bacteria and the oxidation of elemental sulfur. However, the production of sulfuric acid kept growing after 60 days, meaning that other mechanisms than the bacteria metabolism may be involved in its production (Liu et al., 2003). Therefore, a decreasing pH was noted during the whole experiment due to the constant production of sulfuric acid, as it can be seen in Figure 2. As expected, a higher total production of sulfuric acid, during the test with the soil contaminated with  $100 \text{ mg kg}^{-1}$  of Cr(VI), resulted in lower pH values, and consequently in a highly oxidizing environment (Kumar and Nagendran, 2008). Most of the heavy metal cations are then easily removed from soil matrices, except Cr(VI) that exists mainly as oxyanions in natural environments, being less mobile in such acidic media (Fonseca et al., 2009a; Fonseca et al., 2009b). Even though, dissolution of total chromium was recorded along the bioleaching batch tests, Figure 3.



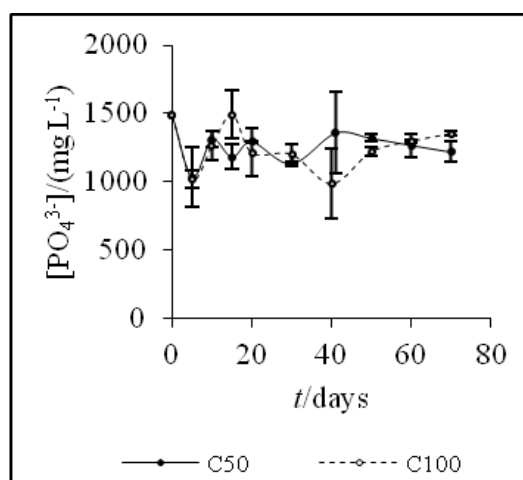
**Figure 3.** Cr, Cr(III) and Cr(VI) concentrations in the liquid phase, along the batch tests.  
 A) Test undertaken at  $26^{\circ}\text{C}$  with the soil contaminated with  $50 \text{ mg kg}^{-1}$  of Cr(VI), at pH2 (C50pH2T26).  
 B) Test undertaken at room temperature with the soil contaminated with  $100 \text{ mg kg}^{-1}$  of Cr(VI), at pH2 (C100pH2T).

A closer analysis of Figure 3 reveals that almost all the chromium in solution is in the trivalent form, which means that the Cr(VI) was reduced and then leached. As mentioned previously, Allegretti et al. (2006) proved that the oxidation of elemental sulfur generates a few sulfur compounds - polythionates,  $\text{S}_x(\text{SO}_3)_2\text{H}_2 \cdot w\text{H}_2\text{O}$  – that may be responsible for Cr(VI) reduction. Actually, these polythionates are known to be oxidized by substances with less reduction

potential than Cr(VI) oxyanions. Steudel (1989) proposed the spherical unilamellar and multilamellar vesicles as the model action: elemental sulfur is dissolved in a hydrophobic membrane, polythionates, that constrains an aqueous internal cavity. When the environment is acid these vesicles lose stability and the polythionates are slowly oxidized and released to the media. Therefore, given the high oxidizing environment during the bioleaching tests, the polythionates were responsible for the Cr(VI) reduction, and consequently for the bioleaching of Cr(III) cations, also favored by the acidic conditions. It is evident that higher levels of sulfur oxidation, expressed by the high concentration of sulfuric acid, in Figure 2, resulted in higher Cr(VI) reduction, explaining the elevated removal values, Figure 1. The importance of pH in the all process may also explain the lower removal values, obtained for the scenarios where a contaminant solution with non-adjusted pH was used, Figure 1. The low pH registered for these soils, Table 2, resulted in a faster decrease of the media pH, due to the soil buffering capacity, favoring the Cr(VI) removal. Some authors reported the remnant chromium as bounded to the organic and residual fractions, and also noted an increasing on bioleaching efficiency with contact time. From those authors' perspective, and due to the fact that only total chromium was determined, it is plausible to attribute the non-removed chromium to the hexavalent fraction. On the other hand, the same authors refer consistently the exchangeable and Fe/Mn oxide-bound fractions as the most mobile and bioavailable, which may justify the presence of little dissolved Cr(VI), Figure 2 (Kumar et al., 2008; Liu et al., 2007; Kumar and Nagendran, 2007).

### ***Phosphates dissolution***

The digestion of soil organic matter and erosion of the original rock, and the consequent release of bounded nutrients are often attributed to the highly oxidizing environment and to the low pH, registered during the bioleaching experiments (Kumar and Nagendran, 2008; Waksman and Joffe, 1922b) . Therefore, this may represent a clear disadvantage on the application of this technique, as nutrients such phosphorus and nitrogen may be lost during the process. An evaluation of the flotation of the low phosphate concentration found in the soil in the liquid phase was performed and a relatively constant behavior was observed, Figure 4. The slight decrease at the beginning was clearly related with phosphorus consumption during bacteria growth. Therefore, should the soil be poor or not in phosphorus, the adding of this nutrient is mandatory for the success of the operation.



**Figure 4.** Phosphates concentration in the liquid phase, along the batch tests with soil contaminated at 50 mg kg<sup>-1</sup> (C50) and 100 mg kg<sup>-1</sup> (C100), concerning the higher removal values.

## Conclusions

This study investigated the applicability of a bioleaching technique using *Acidithiobacillus thiooxidans* DSM 504, on the decontamination of soils polluted with Cr(VI). Parameters such operation temperature, contamination pH and contaminant concentration were evaluated and a maximum removal of 83% was obtained for the bioleaching test concerning the soil contaminated with 50 mg kg<sup>-1</sup> of Cr(VI), at pH 2, and at 26 °C. The parameter that influenced the most the efficiency of the process was the contamination pH, which decrease improved the removal of Cr(VI). On the other hand, at the temperature range tested no significant changes were observed in the yield of the method. Finally, the increased contaminant concentration affected positively the same factor. It was also proved that, during the oxidation of sulfur, Cr(VI) was reduced to Cr(III) that was easily leached in the acidic environment.

In conclusion, this laboratory scale study proofs that bioleaching of Cr(VI) contaminated soils can be economically performed, using *Acidithiobacillus thiooxidans*: no temperature control is needed in temperate climate; high Cr(VI) charges favor its reduction rate to Cr(III); stream treatments can be eliminated as Cr(III) is less toxic and less mobile and, finally, the most acidic character of Cr(VI) contamination proved to enhance the efficiency of the bioleaching process. Even though, larger scale work is needed to fully assess this technology.

**Acknowledgments**

Financial support was provided by the Fundação para a Ciência e Tecnologia, Ministério da Ciência e Tecnologia, Portugal and Fundo Social Europeu (FSE) through the PhD grant of Bruna Fonseca (SFRH/BD/27780/2006).

**References**

- Loukidou, M.X., Zouboulis, A.I., Karapantsios, T.D., Matis, K.A., (2004) - Equilibrium and kinetic modeling of chromium(VI) biosorption by *Aeromonas caviae*, *Colloids Surf., A* 242, 93-104.
- Jeyasingh, J., Philip, L., (2005) - Bioremediation of chromium contaminated soil: optimization of operating parameters under laboratory conditions, *J. Hazard. Mater.*, 118, 113-120.
- Ore, S., Grennberg, K., (2000) - The effect of metal pollutants leaching from the road constructed with bottom ash, *Proceedings from: 7th International Conference on the Biogeochemistry of Trace Elements*, Uppsala, Sweden.
- Mishra, S., Jena, S., (2009) - Bioreduction based bioremediation of hexavalent chromium Cr (VI) through potential indigenous microbes, Department of Chemical Engineering, National Institute of Technology, Rourkela, Orissa, India.
- Umrana, V., (2006) - Bioremediation of toxic heavy metals using acidothermophilic autotrophes, *Bioresour. Technol.*, 97, 1237-1242.
- Fonseca, B., Teixeira, A., Figueiredo, H., Tavares, T., (2009a) - Modelling of the Cr(VI) transport in typical soils of the North of Portugal, *J. Hazard. Mater.*, 167, 756-762.
- Krishna, K.R., Philip, L., (2005) - Bioremediation of Cr(VI) in contaminated soils, *J. Hazard. Mater.*, 121, 109-117.
- Kumar, R.N., Nagendran, R., (2008) - Changes in nutrient profile of soil subjected to bioleaching for removal of heavy metals using *Acidithiobacillus thiooxidans*, *J. Hazard. Mater.*, 156, 102-107.
- Gomez, C., Bosecker, K., (1999) - Leaching Heavy Metals from Contaminated Soil by Using *Thiobacillus ferrooxidans* or *Thiobacillus thiooxidans*, *Geomicrobiol. J.*, 16, 233-244.
- Lombardi, A.T., Garcia, O., (2002) - Biological leaching of Mn, Al, Zn, Cu and Ti in an anaerobic sewage sludge effectuated by *Thiobacillus ferrooxidans* and its effect on metal partitioning, *Water Res.*, 36, 3193-3202.
- Kumar, R.N., Nagendran, R., Parvathi, K. (2008) - Bioleaching of heavy metals from contaminated soil using *Acidithiobacillus thiooxidans*: effect of sulfur/soil ratio, *World J. Microb. Biot.*, 24, 1539-1546.
- Liu, Y.G., Zhou, M., Zeng, G.M., Li, X., Xu, W.H., Fan, T., (2007) - Effect of solids concentration on removal of heavy metals from mine tailings via bioleaching, *J. Hazard. Mater.*, 141, 202-208.

- Villar, L.D., Garcia, J., (2006) - Effect of Anaerobic Digestion and Initial pH on Metal Bioleaching from Sewage Sludge, *J. Environ. Sci Health, Part A: Toxic/Hazard. Subst. Environ. Eng.* 41, 211-222.
- Kumar, R.N., Nagendran, R., (2009) - Fractionation behavior of heavy metals in soil during bioleaching with *Acidithiobacillus thiooxidans*, *J. Hazard. Mater.*, 169, 1119-1126.
- Zagury, G.J., Narasiah, K.S., Tyagi, R.D., (2001) - Bioleaching of metal-contaminated soil in semicontinuous reactor, *J. Environ. Eng.*, 127, 812.
- Park, H.S., Lee, J.U., Ahn, J.W., (2007) - The effects of *Acidithiobacillus ferrooxidans* on the leaching of cobalt and strontium adsorbed onto soil particles, *Environ. Geochem. Health*, 29, 303-312.
- Liu, H.L., Chiu, C.W., Cheng, Y.C., (2003) - The effects of metabolites from the indigenous *Acidithiobacillus thiooxidans* and temperature on the bioleaching of cadmium from soil, *Biotechnol. Bioeng.*, 83, 638-645.
- Kumar, R.N., Nagendran, R., (2007) - Influence of initial pH on bioleaching of heavy metals from contaminated soil employing indigenous *Acidithiobacillus thiooxidans*, *Chemosphere*, 66, 1775-1781.
- Ishigaki, T., Nakanishi, A., Tateda, M., Ike, M., Fujita, M., (2005) - Bioleaching of metal from municipal waste incineration fly ash using a mixed culture of sulfur-oxidizing and iron-oxidizing bacteria, *Chemosphere*, 60, 1087-1094.
- Waksman, S.A., (1922) - Microorganisms concerned in the oxidation of sulfur in the soil V. Bacteria oxidizing sulfur under acid and alkaline conditions, *J. Bacteriol.*, 7, 609-616.
- Allegretti, P., Furlong, J., Donati, E., (2006) - The role of higher polythionates in the reduction of chromium(VI) by *Acidithiobacillus* and *Thiobacillus* cultures, *J. Biotechnol.*, 122, 55-61.
- Silva, B., Figueiredo, H., Neves, I.C., Tavares, T., (2009) - The role of pH on Cr(VI) reduction and removal by *Arthrobacter viscosus*, *Int. J. Chem. Biol. Eng.*, 2, 100-103.
- Fonseca, B., Maio, H., Quintelas, C., Teixeira, A., Tavares, T., (2009b) - Retention of Cr(VI) and Pb(II) on a loamy sand soil: Kinetics, equilibria and breakthrough, *Chem. Eng. J.*, 152, 212-219.
- Plackett, R.L., Burman, J.P., (1946) - The design of optimum multifactorial experiments, *Biometrika*, 33, 305-325.
- USEPA, (2007) - Microwave assisted acid digestion of sediments, sludges, soils, and oils, 3051 United States of America, Environmental Protection Agency.
- American Public Health Association (APHA), (1998) - Standard methods for the examination of water and wastewater, American Public Health Association Publications, Washington.

- Olsen, S.R., Cole, C.V., Watanabe, F.S., Dean, L.A., (1954) - Estimation of available phosphorus in soils by extraction with sodium bicarbonate, Circ. 939, U. S. Department of Agricultural.
- USEPA, (2004) - Soil and waste pH, Method 9045D United States of America, Environmental Protection Agency.
- USEPA, (1992) - Chromium, hexavalent (colorimetric), 7196A United States of America, Environmental Protection Agency.
- Kolmert, A., Wikstrom, P., Hallberg, K.B., (2000) - A fast and simple turbidimetric method for the determination of sulfate in sulfate-reducing bacterial cultures, *J. Microbiol. Methods*, 41, 179-184.
- Waksman, S.A., Joffe, J.S., (1922a) - The chemistry of the oxidation of sulfur by microorganisms to sulfuric acid and transformation of insoluble phosphates into soluble forms, *J. Biol. Chem.*, 50, 35-45.
- Hatzikioseyan, A., Tsezos, M., Mavituna, F., (2001) - Application of simplified rapid equilibrium models in simulating experimental breakthrough curves from fixed bed biosorption reactors, *Hydrometallurgy*, 59, 395-406.
- Steudel, R., (1989), - On the Nature of the Elemental Sulfur Produced by Sulfur-oxidizing Bacteria, in: Schlegel H.G., B. Bowien (Eds.), *Biology of Autotrophic Bacteria*, Science Tech. Publ., Madison, WI. 289-303.
- Waksman, S.A., Joffe, J.S., (1922b) - Microorganisms concerned in the oxidation of sulfur in the soil II. *Thiobacillus thiooxidans*, a new sulfur-oxidizing organism isolated from the soil, *J. Bacteriol.*, 7, 239-256.