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Evaluation of the solubilization ability of two strains of *Bacillus megaterium* for heavy metals from residual phosphogypsum

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Abstract

One of the major problems of the phosphorus based fertilizers is the huge quantity of residual phosphogypsum resulted as by-product, about 5 tones of phosphogypsum per tone of produced phosphoric acid. Depending on the source of raw phosphate rock used in the phosphoric acid production, residual phosphogypsum contains among other compounds various quantities of heavy metals (chromium, copper, cobalt, zinc, cadmium, mercury, lead, uranium, radium, polonium etc.) that are a major threat for the environment. The aim of this paper is to evaluate the solubilization ability of some strains of Bacillus megaterium, a common soil bacterium, for copper, iron, manganese, cobalt, nickel and cadmium from residual phosphogypsum. A reference strain, BM_M, isolated from forest soil, and an adapted strain, BM_{30}, isolated from the vicinity of a phosphogypsum dump and adapted to high concentrations of phosphogypsum have been used in order to evaluate the solubilization capacity for heavy metals. Experiments showed that BM_{30} has a higher solubilization capacity than the witness strain BM_M. In culture media rich in phosphogypsum, the solubilization capacity of BM_{30} was directly proportional to the biomass quantity for iron and manganese and inversely proportional for copper. No detectable solubilization has been observed for cadmium and cobalt. At 2 - 6 g/L phosphogypsum added in the culture media, BM_{30} solubilizes large amounts of iron (42-72%), manganese (36-68%) and copper (12-30%). Larger concentrations of phosphogypsum in the culture media (10 g/L) lead to a considerable decrease both of the solubilized heavy metals and of the accumulated cellular biomass.

Keywords: biosolubilization, biosorption, *Bacillus megaterium,* heavy metals, phosphoric acid production, phosphogypsum.

Introduction

Residual phosphogypsum (PG) obtained in the technology of wet-process phosphoric acid production or fertilizer production from phosphate rock is still a problem to be solved. The wet process is economic, but generates a large amount of PG (5 tones of PG per tone of produced phosphoric acid) [1]. Large deposits of phosphate from igneous rock are found in USA, Canada, Russia, and Africa [2-4]. Depending on the phosphate rock source, the PG composition varies in impurities such as H_3PO_4, $Ca(H_2PO_4)_2 \cdot H_2O$, $CaHPO_4 \cdot 2H_2O$ and $Ca_3(PO_4)_2$, residual acids, fluorides (NaF, Na_2SiF_6, Na_3AlF_6, Na_3FeF_6 and CaF_2), sulfate ions and heavy metals (e.g. Cr^{3+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+} and Cd^{2+}) [5,6].

Heavy metals contamination has become a serious problem to the environment and human life. Because more than 8% of world production of PG is used as an amendment to alkaline soils, it is important to know the impact it has, under the action of soil's microorganism, in terms of heavy metal pollution. Toxicity of some metallic ions could be the result of competition with/or replacing a functional metal as well as causing conformational modification, denaturation and inactivation of enzymes and disruption of cellular and organelles integrity [7,8]. However, heavy metals may be mobilized by microorganism autotrophic and heterotrophic leaching, chelation by microbial and siderophores and

methylation, which can result in volatilization [9], or can be immobilized by sorption to cell components or exopolimers, transport into cell and intracellular sequestration or precipitation as insoluble organic and inorganic compounds, e.g. oxalates [9-11], sulfides or phosphates [12,13]. These mechanisms by which microorganism effect changes in metal speciation and mobility are fundamental components of biogeochemical application for metals [14]. Particularly, bacteria isolated from metal-contaminated soils are often more resistant to metals that those collected from uncontaminated environments [15], and may pose both positive and negative impacts on the bioavailability and mobility of heavy metals in soil, depending on the chemical nature of the element [16]. *Bacillus megaterium*, generally considered a soil microbe [17], is a Gram-positive bacterium and is considered a P-solubilizing bacterium [18,19] with a great potential for phytoremediation of metal polluted sites [17,20]. *Bacillus megaterium* was reported as a bacterium that reduces toxic and soluble Cr (VI) to non-toxic and insoluble Cr (III) [21] or that accumulates Cu (II) [22], Pb (II) [23] and other metals. However, the selective accumulation of several heavy metals (Mn, Co, Cd, Ni, Cu, Zn, Hg, Pb, U, Ra, Po) by *Bacillus megaterium* from a uranium waste pile was studied and has been shown that it possess accumulation capacity for heavy metals [24].

The present study is focused on a *Bacillus megaterium* strain isolated from the vicinity of the PG dump located in the neighborhood of a fertilizers plant from Bacău, Romania. The strain was adapted to the new and higher concentrations of PG and used to estimate changes in mobility of heavy metals in PG. The choice of the microorganism was based on the abundance of *Bacillus megaterium* in soil, occupying second place among the bacillus and on the capacity of living cells to remove metal ions from aqueous solution which is significantly influenced by biomass accumulated quantities during of life cycle. Our aim was also to evaluate the heavy metal solubilization capacity by *Bacillus megaterium*.

2. Material and Methods

2.1. Phosphogypsum samples

Phosphogypsum is a residue of the phosphoric acid production by extracting with sulfuric acid from apatites and phosphorites. About 6.3×10^9 kg of these solid wastes are stored in Bacău, Romania, on an area of 16 ha. PG samples (1 kg) were collected in plastic bags from twelve representative points, dried in oven at 80ºC for 24 h, homogenized, analyzed and used in bacterial growth experiments. The heavy metals content in studied samples compared with other PG samples previously reported are presented in Table 1.

Table 1: Content of selected metals in phosphogypsum samples collected from Bacău, Romania, compared to similar data previously reported in the literature

Additional information			Metals concentration (mg/kg)								Reference
Country	Phosphate rock source region	H_3PO_4 production place	Cd	Zn	Cu	Cr	Ni	Mn	Co	Fe	
Romania	Morocco	Bacău	5	212	6	17	N.D.	7.5	3	445	Present study
Tunisia	Morocco	–	5-20	50-315	5-18	10-30	3-5	-	8	-	[25,28]
Spain	Morocco	Huelva	5	2	6	-	5	-	-	-	[26]
Syria	Syria	Homs	0.8	37.2	51.7	-	-	-	-	-	[27]
Yugoslavia	Russia	Prahovo	7	45	17	-	20	8	10	785	[29]

N.D. - not detected

2.2. Isolation and screening

The *Bacillus megaterium* strains were isolated from two different soils: one from forest soil, noted BM_M – reference strain, and the other from polluted soil situated near the dump of PG, BM_{30}, in Bacău, Romania. The *Bacillus megaterium* strains were isolated on Standard Methods Agar plates in minimal medium agar [30,31] for 24 h at 30ºC. Isolates were

tested for catalase, anaerobic growth, hydrolysis of casein, gelatin, starch, nitrate reduced to nitrite, growth in 7% NaOH and growth at 50, 55, 65 ºC according to the standard protocol. The results were interpreted using Bergey's Manual of Systematic Bacteriology [32]. Microbial examinations of bacterial smears aimed to detect the presence of spores within cells, the size and shape of vegetative cells. The *Bacillus megaterium* strains isolated were maintained on extract-soil agar at 4ºC [31].

2.3. Media and culture growth conditions

In this step a mineral liquid medium (MLM = sucrose, 10 g/L; K_2HPO_4, 2.5 g/L; KH_2PO_4, 2.5 g/L; $(NH_4)_2HPO_4$, 1 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; Fe $SO_4 \cdot 7H_2O$, 0.001 g/L; $MnSO_4 \cdot 7H_2O$ 0.007 g/L dissolved in water) was prepared and autoclaved at 121ºC, 1.2 bar, for 15 minutes [33]. To prepare the inoculum from each strain of *Bacillus megaterium*, 100 mL MLM was inoculated with a single colony for 24 h at 30ºC and 119 rpm in a rotary shaker incubator. The optical density of inoculum was OD_{600} = 2.2848. MLM was inoculated with freshly grown bacteria in the ratio 1:100.

2.4. BM_{30} population adaptation protocol

The BM_{30} strain used in this work has previously grown on a high PG concentrate. The aim of this step was to adapt the BM_{30} strain to the new and higher concentrations of PG. First, the cells were adapted to the new substrate composition (sucrose-salts = sucrose, 10 g/L; K_2HPO_4, 0.8 g/L; KH_2PO_4, 0.2 g/L; $CaSO_4 \cdot 2H_2O$, 0.05 g/L; $FeSO_4 \cdot 7H_2O$, 0.001 g/L; KNO_3, 1 g/L; agar, 12 g/L dissolved in water) by successive subcultures at 1% PG. Successive subcultures were repeated until reproducible results were obtained. This adapted population grown at 1% PG was used as inoculum for new cultures of increasing PG concentrations. Again cultivations were carried on until adaptation was achieved using the same adaptation criterion. PG concentrations were in the range of 0.1 to 1% (w/v). The adaptation was validated by running parallel cultures with non-adapted cells at 1% PG [34-36].

2.5. Metal biosolubilization quantification

The Cu, Mn, Fe, Co, Ni and Cd concentrations were measured by atomic absorbtion spectroscopy (Perkin Elmer 3300). For the biosolubilization quantification produced by BM_M and BM_{30} strains, the cell was inoculated in three experimental treatments respectively with 2, 6 and 10 g/L PG, and incubated on a rotary shaker incubator (GPL 3033) at 30ºC and 190 rpm. The biosolubilization of heavy metals was observed during the life cycle at 18, 24 and 30 hours. The cells were grown to each hour, harvested by centrifugation at 9,000 rpm for 30 min at 4ºC. For biosolubilization quantification, the metal concentration was determined from the supernatant. The experiment was carried out in triplicate for each concentration of PG added and every hour.

2.6. Quality assurance

The method was calibrated with five aqueous standards of the analyzed metals. The regression coefficients (r^2) obtained were all greater that 0.995. The method precision was determined as the relative standard deviation (RSD) of ten independent analyses for a solution containing 1 ppm for cadmium, cobalt, copper, nickel and one of 3 ppm for iron and manganese. The limit of detection (LOD), the lowest analyte concentration that produces a response detectable above the noise level was calculated as the blank (n = 10) signal plus three standard deviation. In this study, the blank reagent was MLM, the matrix in which heavy metals were biosolubilized. The limit of quantification (LOQ) is the lowest level above which quantitative results may be obtained with a specified degree of confidence. The LOQ was calculated as the blank (n = 10) signal plus ten standard deviation (Table 2).

Table 2. Analytical performances of the proposed method for the determination of heavy metals in supernatant

Metal	r^2	LR (ppm)	LOD (ppm)	LOQ (ppm)	RSD (%)
Cu	0.999	0.2 - 1.2	0.021	0.049	3.41
Fe	0.995	0.5 - 2.5	0.079	0.167	2.43
Co	0.996	0.5 - 2.5	0.034	0.062	3.63
Cd	0.995	0.1 - 0.5	0.14	0.043	2.47
Ni	0.997	0.2 - 1.2	0.26	0.057	2.68
Mn	0.999	0.25 - 1.5	0.095	0.173	2.96

Notations: r^2 - regression coefficient; LR - linear range; LOD - limit of detection; LOQ - limit of quantification; RSD - relative standard deviation (n = 10).

3. Results and Discussion

3.1. Isolation and screening

The isolated BM_M and BM_{30} strains were rod shaped, endospore forming, Gram-positive and identified as aerobic bacteria. Morphological and physiological features revealed that the colonies grown on nutrient agar are large, smooth, convex, creamy white, glistening, and with entire margins. The biochemical profiles of the BM strains are presented in Table 3. In addition, a *Bacillus megaterium* reference strain (ATCC 14581) was included in this study.

Table 3. Biochemical properties and morphological characteristics for *Bacillus megaterium* isolates from soil samples (n = 10)

Isolates	ATCC 14581	BM_M	BM_{30}
V-P test	+	+	+
Catalase test	+	+	+
Mannitol utilization	+	+	+
Arabinose utilization	+	+	+
Casein hydrolysis	+	+	+
Starch hydrolysis	+	+	+
Reduction of nitrate to nitrite	+	+	+
Growth in NaOH 7%	+	+	+
Growth at 50ºC	-	-	-
Growth at 65ºC	-	-	-
Anaerobic growth	-	-	-
Gram strain	+ ve	+ ve	+ ve
Cell size, μm	2.82 – 3.17	2.67-3.05	2.34-2.97
Spore	present	present	present

3.2. Metal biosolubilization quantification

Because PG is insoluble in MLM at pH = 7, FAAS analysis of heavy metal in non-mineralized samples is irrelevant. The mineralization of PG samples was performed with *aqua regis* (HCl + HNO_3) but, even in these conditions, a fraction of the sample remains non-mineralized, that provides the existence of very stable compounds. In this study, the heavy metals monitoring was performed on mineralized samples. Details on the amount of heavy metals dissolved in MLM without *Bacillus megaterium* inoculum, in non-mineralized samples, are presented in Tables 4-6. The presence of Mn in the non-mineralized sample is due to the contribution of MLM. The amount of analyte that was dissolved in the MLM, in which PG was added, remained constant over the entire established schedule of the experimental protocol.

These parameters were monitored for 18, 24 and 30 h, the pre-established hours of the experimental protocol. In the case of the samples for which there was recorded a lower signal than LOD, the reporting was done for the total amount of analyte initially found in the mineralized sample, and for the samples in which was found a lower signal than LOQ, half of the amount of analyte corresponding to the LOQ was considered.

In order to see in which growth phase of the microorganism the maximum biosolubilization occurs, each analyte was monitored during the life cycle of *Bacillus megaterium*. The highest solubilization degree in the BM_{30} supernatant was observed for iron, followed by manganese and copper. The levels for cobalt and cadmium were recorded below LOD in all variants and at all the ages of the microorganism (Figs. 1-3). Data of the atomic absorption spectroscopy quantification, for each strain, are presented in Tables 4-6. The BM_{30} biosolubilization capacity is from 2 up to 7 times higher then for the BM_M.

Table 4: Evolution of heavy metals biosolubilization by *Bacillus megaterium* strains at 2 g/L PG added in the culture media

Sample	Biosolubilized heavy metals (mg/L)					
	Time (h)	Cu	Fe	Co	Cd	Mn
Non-mineralized sample	0	N.D.	N.D.	N.D.	N.D.	1.2
	30	N.D.	N.D.	N.D.	N.D.	1.2
Mineralized sample	-	0.1578	3.5950	0.1350	N.D.	1.5520
Strain BM_{30} supernatant	0	N.D.	0.0328	N.D.	N.D.	< LOQ
	18	N.D.	2.0197	N.D.	N.D.	0.7230
	24	N.D.	2.6890	N.D.	N.D.	1.1698
	30	0.0465	2.4528	N.D.	N.D	1.0357
Strain BM_M supernatant	0	N.D.	0.3150	N.D.	N.D.	N.D.
	18	N.D.	1.4667	N.D.	N.D.	0.4549
	24	N.D.	1.9000	N.D.	N.D.	0.7595
	30	0.0066	2.0000	N.D.	N.D.	0.7839

N.D. – not detected; < LOQ – below the limit of quantification

Table 5: Evolution of heavy metals biosolubilization by *Bacillus megaterium* strains at 6 g/L PG added in the culture media

Sample	Biosolubilized heavy metals (mg/L)					
	Time (h)	Cu	Fe	Co	Cd	Mn
Non-mineralized sample	0	N.D.	N.D.	N.D.	N.D.	1.2
	30	N.D.	N.D.	N.D.	N.D.	1.2
Mineralized sample	-	0.2542	6.512	0.2182	< LOQ	2.2545
Strain BM_{30} supernatant	0	0.0199	0.0630	N.D	N.D.	0.0510
	18	0.0066	2.6890	N.D	N.D.	0.9626
	24	0.0730	1.3898	N.D	N.D.	0.7433
	30	0.0332	2.7677	N.D	N.D.	0.8123
Strain BM_M supernatant	0	N.D.	0.0630	N.D	N.D.	< LOQ
	18	N.D.	0.8000	N.D	N.D.	0.3615
	24	0.0066	0.8667	N.D	N.D.	0.4184
	30	0.0199	1.1333	N.D	N.D.	0.4265

N.D. – not detected; < LOQ – below the limit of quantification

Table 6: Evolution of heavy metals biosolubilization by *Bacillus megaterium* strains at 10 g/L PG added in the culture media

Sample	Biosolubilized heavy metals (mg/L)					
	Time (h)	Cu	Fe	Co	Cd	Mn
Non-mineralized sample	0	N.D.	< LOQ	N.D.	N.D.	1.2
	30	N.D.	< LOQ	N.D.	N.D.	1.2
Mineralized sample	-	0.322	7.415	0.242	< LOQ	2.863
Strain BM_{30} supernatant	0	0.0199	0.0860	N.D.	N.D.	0.0780
	18	0.0332	0.4449	N.D.	N.D.	0.2140
	24	0.0730	0.6811	< LOQ	N.D.	0.4020
	30	0.0345	1.5472	< LOQ	< LOQ	0.3510
Strain BM_M supernatant	0	N.D.	0.0860	N.D.	N.D.	0.0780
	18	N.D.	0.4667	N.D.	N.D.	0.1300
	24	0.0199	0.5667	N.D.	N.D.	0.1625
	30	0.0066	1.0333	N.D.	N.D.	0.1909

N.D. – not detected; < LOQ – below the limit of quantification

When 2 g/L PG are added to the culture media, iron and manganese are solubilized in similar proportions, the solubilization significantly increasing during the life cycle for both BM_{30} (Pearson correlation, $p = 0.044$, respectively $p = 0.043$) and BM_M ($p = 0.010$, respectively $p = 0.017$). For copper, it was noticed that it is solubilized in a proportion 7 times higher for BM_{30} than for BM_M ($p = 0.001$) after 30 hours (Fig.1).

At an addition of 6 g/L PG to the culture media, the solubilization differences of the two strains are significant; the BM_{30} strain dissolves a quantity up to 3 times higher from each analyte than the reference strain. Although the amount of PG added is higher, iron and manganese remain solubilized in equal proportions but decreased, compared to the first treatment (2 g/L PG added). A variation of the solubilized metal quantity can be observed, during the growth phases for iron and manganese. The BM_{30} life cycle is 30 hours: logarithmic phase growth between 6-24 hours, stationary phase between 24-30 hours and the declining phase after 30 hours. In the exponential growth phase, both for iron and manganese, an increase of the biosolubilized analyte quantity until the age of 18 hours can be observed, followed by a decrease of their concentration in supernatant, then the concentration of the analyte returns to the initial threshold in the stationary phase after 30 hours. This means that in the microorganism's logarithmic growth phase a solubilization of the iron and manganese ions takes place, followed by an accumulation of their biomass. According to VELASQUES & al. [37], this action is based either on the active metabolism in which metals accumulate inside the cell, either on passive metabolism in which metals adhere to surface molecules. If the process in the 18-24 h interval is metal accumulation, in the stationary phase the process that occurs is the efflux mechanism, which can prevent more metal accumulation [38].

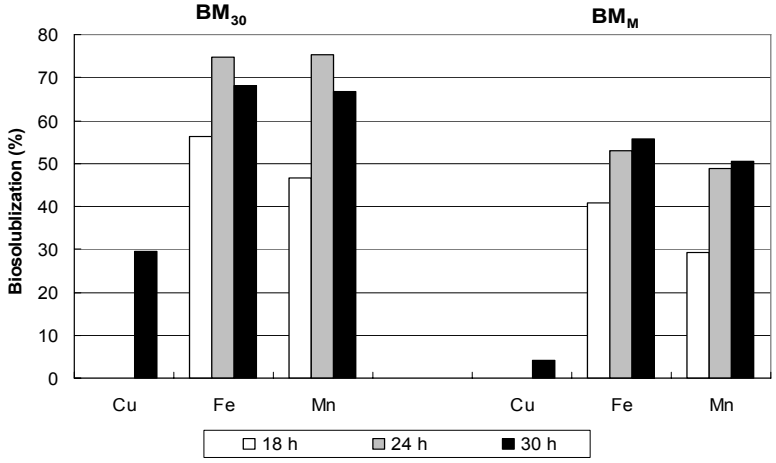

Fig. 1. Metals biosolubilized by *Bacillus megaterium* strains in culture media with 2 g/L phosphogypsum added.

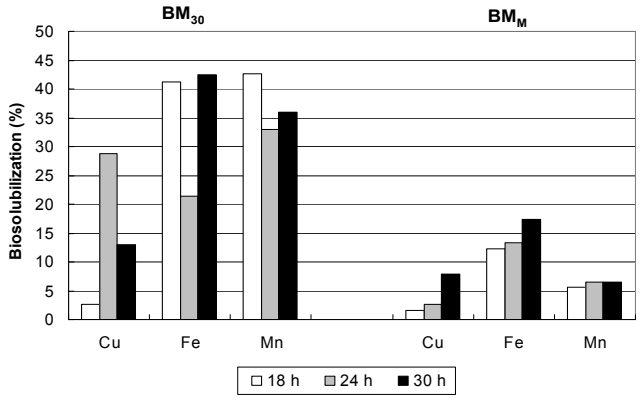

Fig. 2. Metals biosolubilized by *Bacillus megaterium* strains in culture media with 6 g/L phosphogypsum added.

Noteworthy is the fact that copper is solubilized in the same proportion as in the treatment with 2 g/L PG. That can be explained by chelating of the insoluble form of cations and their conversion to soluble forms through a chelation-mediated mechanism. The biosolubilization of the analytes for BM_{30} is 30-42% and for the control strain is between 2-17% (Fig. 2).

In the case of 10 g/L PG added to the culture media, both strains manifest a low capacity of biosolubilization, up to 15% (Fig. 3). There is a decrease of the metabolic activity of the BM_{30} strain, the amount of PG reaching the toxic threshold of the microorganism's growth. This effect is also underlined by the microscopic observation of the BM_{30} cells. Also, there were observed quantitative changes of color and consistency of the biomass.

In support of the above assertion, Figure 4 shows the accumulation of cell biomass during of the life cycle in presence of different PG additions.

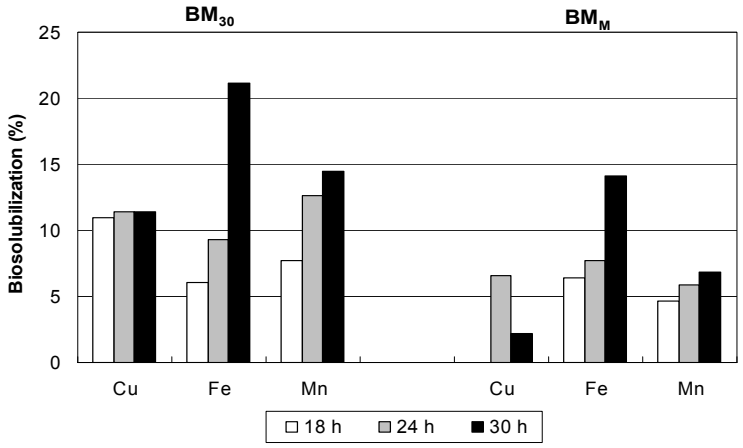

Fig. 3. Metals biosolubilized by *Bacillus megaterium* strains in culture media with 10 g/L phosphogypsum added.

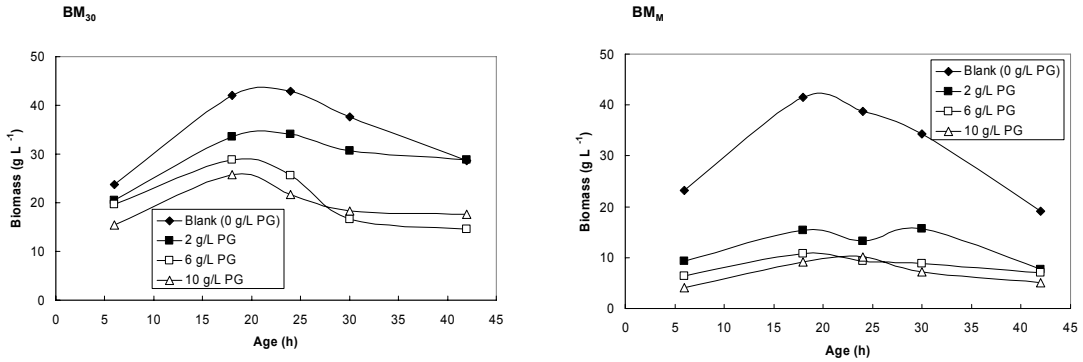

Fig. 4. Biomass accumulation of BM_{30} (A) and BM_M (B) at different PG additions.

Comparing the results obtained from all the three treatments (2, 6 and respectively 10 g/L PG added in the culture media), it can be observed a decreasing in the solubilized analyte quantity as quantity of PG added in MLM was increased. This finding may be explained considering that *Bacillus megaterium* posses a higher ability to bind heavy metals [24], and a higher capacity to accumulate heavy metals to the cellular biomass.

The much lower quantity of solubilized copper occurs because *Bacillus megaterium* is known as having an increased capacity to accumulate copper, cobalt and cadmium ions [24,39]. This could explain the cobalt and cadmium concentrations detected below LOD, also taking into account the fact that these three metals are found in low concentrations in the PG sample (Table 1).

Conclusions

In this study the growth and metal solubilization capacity of BM_{30} were significantly correlated. For iron and manganese the solubilizing capacity was directly proportional to the biomass quantity and for copper it was inversely proportional to the biomass quantity.

Cadmium and cobalt, metals known as having toxic potential, were not detected in the supernatant, indicating, that from this point of view, *Bacillus megaterium* does not bring an

important contribution to their accumulation in plants, especially if it has a high abundance in soil.

On the other hand, the solubilizing order of the PG contained metals in aqueous environments is Fe > Cu > Mn > Cd > Co [26], and in the presence of the BM_{30} strain the solubilizing order is Fe > Mn > Cu; Cd, and Co not being detected in the supernatant. The small amount of solubilized copper and the lack of the other metals (cadmium and cobalt) in the supernatant lead to the assumption that these ions are accumulated in the cell, similar behavior being previously reported by PEREZ-LOPEZ & al. [40]. BM_{30} can solubilize high concentrations of iron, manganese and copper in a 2-6 g/L PG range. The presence of 10 g/L PG in the microorganism's growth medium leads to a considerable decrease both of the solubilized heavy metals concentration and of the accumulated cellular biomass, similar results being highlighted also for *Desulfomicrobium sp.* by AZABOU & al. [25]. The BM_{30} solubilized ions are basic micronutrients for plant growth and development and do not exceed the maximum limit of the applicable laws. Also, these metals play a cofactor role in the enzymatic metabolism for both microorganisms and plants.

References

1. US EPA, U.S. Environmental Protection Agency, 2002. National Emission Standards for Hazardous Air Pollutants, Subpart R (2002).
2. Mineral Information Institute, Environmental chemistry, Geology, Agriculture and Food, Human health and Minerals and mining (2008).
3. A.C. KNUDSEN, M.E. GUNTER, Sedimentary phosphorites—an example: Phosphoria formation, southeastern Idaho, USA. In: M.J. Kohn, J. Rakovan and J.M. Hughes, Editors, *Phosphates — geochemical, geobiological, and materials importance*, *Reviews in mineralogy and geochemistry*, Mineralogical Society of America, Washington, 48, 363–389 (2002).
4. U.S. EPA, Identification and listing of hazardous waste, toxicity characteristics. 40 CFR 261.24, Federal Register, 18 (261), 55–56, (1999).
5. P.M. RUTHERFORD, M.J. DUDAS, R.A. SAMEK, Environmental impacts of phosphogypsum. *The Science of the Total Environment* 149 (1–2), 1–38 (1994).
6. H. TAYIBI, M. CHOURA, F.A. LOPEZ, F.J. ALGUACIL, A. LOPEZ-DELGADO, Environmental impact and management of phosphogypsum. *Journal of Environmental Management*, 90, 2377–2386 (2009).
7. A.A. ABOU ZEID, W.A. HASSANEIN, H.M. SALAMA, G.A.A. FAHD, Biosorption of some heavy metal ions using bacterial species isolated from agriculture waste water drains in Egypt. *J. of Applied Sciences Research*, 5(4), 372-383 (2009).
8. K.J. BLACKWELL, I. SINGLETON, J.M. TOBIN, Metal cation uptake by yeast: A review. *Appl. Microbial., Biotechnol*., 43, 571-584 (1995).
9. G.M. GADD, Microbial metal transformations: Minireview. *The Journal of Microbiology*, 39(2), 83-88 (2001).
10. M.M. GHARIEB, J.A. SAYER, G.M. GADD, Solubilization of natural gypsum ($CaSO_4 \cdot 2H_2O$) and the formation of calcium oxalate by *Aspergillus niger* and *Serpula himantioides*. *Mycol. Res.*, 102, 825-830 (1998).
11. J.A. SAYER, G.H. GADD, Solubilization and transformation of insoluble inorganic metal compounds to insoluble metal oxalates by *Aspergillus niger*. *Mycol. Res*., 101, 653-661 (1997).
12. C. WHITE, G.M. GADD, Mixed sulphate-reducing bacterial cultures for bioprecipitation of toxic metals: factorial and response-surface analysis of the effects of dilution rate, sulphate and substrate concentration. *Microbiol.*, 142, 2197-2205 (1996).
13. K.CHOJNACKA, Biosorption and bioaccumulation – the prospects for practical applications: A Review. *Environment International*, 36, 299–307 (2010).
14. G.M. GADD, Microbial influence on metal mobility and application for bioremediation. *Geoderma*, 122, 109-119 (2004).
15. A.M. CHAUDRI, S.P. MCGRATH, K.E. GILLER, Metal tolerance of isolates of *Rhizobium leguminosarum* Biovar *Trifolii* from soil contaminated by past applications of sewage-sludge. *Soil Biol. Biochem.,* 24, 83–88 (1992).

16. S.C. WU, Y.M. LUO, K.C. CHEUNG, M.H. WONG, Influence of bacteria on Pb and Zn speciation, mobility and bioavailability in soil: A laboratory study. *Environmental Pollution,* 144(3), 765-773 (2006).
17. T.M. ROANE, Lead Resistance in Two Bacterial Isolates from Heavy Metal–Contaminated Soils. *Microb. Ecol.,* 37, 218–224 (1999).
18. S.C. WU, Z.H. CAO, Z.G. LI, K.C. CHEUNG, M.H. WONG, Effects of biofertilizer containing N-fixer, P and K solubilizers and A.M. fungi on maize growth: a greenhouse trial. *Geoderma,* 125, 155-166 (2005).
19. K.H. CHEUNG, JI-DONG GU, Chromate reduction by *Bacillus megaterium TKW3* isolated from marine sediments. *World Journal of Microbiology & Biotechnology,* 21, 213–219 (2005).
20. S.P. MOHAPATRA, M.A. SIEBEL, G.J. ALAERTS, Effect of *Bacillus megaterium* on removal of copper from aqueous solutions by activated carbon. *Journal of Environmental Science and Health*, A28, 615-629, (1993).
21. P.S. VARY, Prime time for *Bacillus megaterium*. *Microbiology*, 140, 1001–1113 (1994).
22. S.C. WU, K.C. CHEUNG, Y.M. LUO, M.H. WONG, Effects of inoculation of plant growth promoting rhizobacteria on metal uptake by *Brassica juncea*. *Environ. Pollut*., 140, 124-135 (2006).
23. L.H. WU, Y.M. LUO, Rhizon-soil moisture sampler - an introduction to an innovative *in situ* soil solution sampler. *Soils*, 31, 54-56 (1999).
24. S. POBELL-SELENSKA, P. PANAK, V. MITEVA, I. BOUDAKOV, G. BERNHARD, H. NITSCHE, Selective accumulation of heavy metals by three indigenous Bacillus strains, *B. cereus, B. megaterium* and *B. sphaericus*, from drain waters of a uranium waste pile. *FEMS Microbiology Ecology*, 29, 59-67 (1999).
25. S. AZABOU, T. MECHICHI, B. K.C. PATEL, S.SAYADI, Isolation and characterization of a mesophilic heavy-metals-tolerant sulfate-reducing bacterium *Desulfomicrobium* sp. from an enrichment culture using phosphogypsum as a sulfate source. *Journal of Hazardous Materials,* 140, 264–270 (2007).
26. R. PEREZ-LOPEZ, A.M. ALVAREZ-VALERO, J. MIGUEL NIETO, Changes in mobility of toxic elements during the production of phosphoric acid in the fertilizer industry of Huelva (SW Spain) and environmental impact of phosphogypsum wastes. *Journal of Hazardous Materials*, 148, 745–750 (2007).
27. M.S. AL-MASRI, Y. AMIN, S. IBRAHIM, F. AL-BICH, Distribution of some trace metals in Syrian phosphogypsum. *Applied Geochemistry*, 19, 747–753 (2004).
28. H. TAYIBI, M. CHOURA, F.A. LOPEZ, F.J. ALGUACIL, A. LOPEZ-DELGADO, Environmental impact and management of phosphogypsum: A Review. *Journal of Environmental Management*, 90, 2377–2386 (2009).
29. M.B. RAJKOVIC, D.V. TOSCOVIC, Investigation of the possibilities of phosphogypsum application for building partitioning walls – elements of a prefabricated house. *APTEFF*, 33, 71-92 (2002).
30. R.M. RONALD, Handbook of microbiological media for the examination of food – 2nd ed., CRC Press Taylor & Francis Group, 2006.
31. P.H.A. SNEATH, Endospore-forming Gram-Positive Rods and Cocci, in: Bergey's Manual of Systematic Bacteriology, vol 2, Staley, J.T., Bryant, M.P., Pfenning, N., Holt, J.G., 1989. Bergey's Manual of Systematic Bacteriology, Williams and Wilkins Co., Baltimore, 1986, 1104-1139.
32. J.T. STALEY, M.P. BRYANT, N. PFENNING, J.G. HOLT, Bergey's Manual of Systematic Bacteriology, vol. 3, Williams and Wilkins Co., Baltimore, 1989, 1582–2298.
33. R.M. ATLAS In: C.L. Parks: Handbook of Microbiological Media, CRC Press, Boca Raton, 1993.
34. E. DONATI, G. CURUCHET, S. PORRO, P. TEDESCO, Bioleaching of metallic sulphides with *Thiobacillus ferrooxidans* in the absence of iron (II). *World J. Microbiol. Biotechnol*., 8, 305–308 (1992).
35. C. ASTUDILLO, F. ACEVEDO, Adaptation of *Sulfolobus metallicus* to high pulp densities in the biooxidation of a flotation gold concentrate. *Hydrometallurgy*, 92, 11–15 (2008).
36. D.F. HAGHSHENAS, E.K. ALAMDARI, M.A. TORKMAHALLEH, B. BONAKDARPOUR, B. NASERNEJAD, Adaptation of *Acidithiobacillus ferrooxidans* to high grade sphalerite concentrate. *Minerals Engineering*, 22, 1299–1306 (2009).
37. L. VELASQUES, J. DUSSAN, Biosorption and bioaccumulation of heavy metals on dead and living biomass of *Bacillus sphaericus*. *Journal of Hazardous Materials*, 167, 713-716 (2009).
38. M. RAMÍREZ, C. DÍAZ, E. VARGAS, H. RIVEROS, J. CAMPOS, C. CERVANTES, Mechanisms of bacterial resistance to chromium compounds. *Biometals*, 21, 321–332 (2008).
39. M.D. SCHOLLE, C.A. WHITE, M. KUNNIMALAIYAAN, P.S. VARY, Sequencing and Characterization of pBM400 from *Bacillus megaterium* QM B1551. *Applied and Environmental Microbiology*, 69(11), 6888–6898 (2003).
40. A. CHOI, S. WANG, M. LEE, Biosorption of cadmium, copper, and lead ions from aqueous solutions by *Ralstonia sp*. and *Bacillus sp*. isolated from diesel and heavy metal contaminated soil. *Geosciences Journal*, 13(4), 331 − 341 (2009).