

Received June 1, 2013; reviewed; accepted July 3, 2013

EXTRACELLULAR ELEMENTS-MOBILIZING COMPOUNDS PRODUCED BY CONSORTIUM OF INDIGENOUS BACTERIA ISOLATED FROM KUPFERSCHIEFER BLACK SHALE – IMPLICATION FOR METALS BIORECOVERY FROM NEUTRAL AND ALKALINE POLYMETALLIC ORES

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Abstract: Extracellular compounds produced by a consortium of indigenous bacteria interact with the polymetallic Kupferschiefer black shale and implicate for elements mobilization and/or complexation. The extracellular compounds were identified by GC-MS as carboxylic acids, but also aromatic acids and alcohols. Due to their action in batch experiment 16 elements were mobilized from black shale. Among mobilized elements precious metals (Cu, Ni, Co, V) as well as toxic heavy metals (As) were determined. Extracellular metabolites produced by studied consortium may be utilized in non-contact biorecovery of precious metals from neutral or slightly alkaline ores, and in the bioremediation of heavy metal-contaminated sites.

Keywords: *microbial consortium, extracellular compounds, black shale, metals mobilization*

Introduction

Mineralytic effect of microorganisms based on the acidolysis and/or complexolysis is an integral process of rock bioweathering. Solubilization of mineral elements and the breakdown of minerals can be caused by a wide range of microbial extracellular metabolites. One group of such compounds responsible for bioweathering are by-products of microbial metabolisms. During degradation of organic matter heterotrophic microorganisms produce variety of low molecular weight organic acids such as carbonic, aliphatic, phenolic and amino acids. Beside the dissolution of minerals and elements mobilization some of them have also ability to complex

elements. The chemistry and role of such compounds have been reviewed by Birch and Bachofen (1990).

Besides the role of such extracellular metabolites in geochemical cycling of elements in natural environment they are also of potential use in biotechnologies of the recovery of metals from deposits and wastes. This phenomenon is of great importance especially in the case of neutral or slightly alkaline ores where acidic leaching is not applicable. Copper bearing alkaline ultrabasic and carbonatite deposits made up 4% of worldwide copper mining activity. This type of ore is located among other in the middle of Europe in a great area from Middle Germany (the eastern edge of the Harz Mountains and the Saale river) to Fore-Sudetic Monocline and North Sudetic Trough (SW Poland). These copper deposits called Kupferschiefer are ranked as one of the largest and the richest of Cu and Ag deposits in the world (Oszczepalski, 1999). The concentrations of copper and silver in the black shale profile range from a few to up to 10 wt. % and up to 100 ppm, respectively. Beside these two main elements Kupferschiefer contains also considerable reserves of other basic and highly valuable and rare elements (cobalt, nickel, vanadium) as well as toxic elements (arsenic, lead). The major difficulties restricting the exploitation of such abundant resources is the low efficiency of the conventional technical methods for recovering valuable metals, from mining extraction to metallurgical processing. Metal bearing minerals are mostly merged with sandstone (24-85 %) and dolomite (11-62 %). In this situation all known methods of acid metals bioleaching widely used throughout the world are not useful.

Bioshale project was one of first projects dedicated to development of biotechnology for safe, clean and viable beneficiation of black shale ores in Europe and to propose an innovative, environmentally and socially favourable model of mining activities and metals recovery (d'Hugues et al., 2008). In frame of Bioshale project the consortium of indigenous microorganisms was isolated from Kupferschiefer black shale (Matlakowska and Skłodowska, 2009). During our last studies we demonstrated that indigenous bacteria play a prominent role in the weathering of black shale and in the biogeochemical cycles of elements occurring in this rock (Matlakowska et al., 2010; Matlakowska et al., 2012). It was shown that bacteria directly interact with black shale organic matter and indirectly influence on ore minerals. As a result of bacterial activity the redistribution of P, Al, Si, Ca, Mg, K, S, Cu and Pb was confirmed. Continuing these studies we focused on the analysis of non-contact action of microbial extracellular products on the rock components and potential implication of these processes. The analysis of such phenomenon is important not only to understand the role of microorganisms in ancient black shale transformations but also to find the methods of precious elements biomining from neutral or slightly alkaline ores. In presented study we describe the impact of extracellular metabolites produced under laboratory conditions by a consortium of bacteria isolated from Kupferschiefer black shale on the biotransformation of elements present in this rock and we discuss the potential role of these processes in biomining.

Materials and methods

Geological materials

Material for the study representing highly mineralized Kupferschiefer black shale was sampled at the underground Lubin mine below 600 m (Fore-Sudetic Monocline, SW, Poland). The crushed black shales with diameter fall in range: 0.25–0.5 mm; 1–2 mm and 3–4 mm were used. The whole rock analyses of the Kupferschiefer shale used in this study were performed by ACME Analytical Laboratories Ltd. Vancouver, Canada (<http://www.acmelab.com>) and its chemical composition is presented in Table 1.

Table 1. Chemical composition of Kupferschiefer black shale used in this study

Element (wt. %)		Element (mg kg ⁻¹)	
Na ₂ O	0.16	Cu	34958
K ₂ O	4.23	Co	1287
SiO ₂	34.50	Ni	336
Al ₂ O ₃	13.50	V	943
MgO	6.80	Mo	169
CaO	9.20	Mn	2224
Fe ₂ O ₃	3.50	Zn	26
S	1.89	As	1598
MnO	0.29	Fe	2450

Microorganisms

Bacterial strains were originally isolated from black shale (Lubin copper mine) and have been described previously (Matlakowska and Sklodowska, 2009). The mixed culture of microorganisms contained equal numbers of: *Microbacterium* sp. LM1 (EU821337), *Microbacterium* sp. LM2 (EU821338), *Acinetobacter* sp. LM3 (EU821339), *Bacillus* sp. LM4 (EU821340), *Pseudomonas* sp. LM5 (EU821341), *Pseudomonas* sp. LM6 (EU821342), *Pseudomonas* sp. LM7 (EU821343) and *Pseudomonas* sp. LM8 (EU821344).

Growth media

Luria-Bertani medium (LB) (Sambrook and Russell, 2001) was used for inoculum preparation. The mineral basal salts (MBS) medium containing glucose at concentration 20 mM (Hartmans et al., 1989) was used for production of extracellular metabolites.

Microorganisms cultivation

The mineral MBS medium was inoculated with an exponentially growing bacterial culture that had been cultivated in LB medium and then centrifuged (10 000 x g, at 4 °C, 10 min). Cultures were carried out in 500 millilitre flasks containing 250 ml of

mineral medium and were maintained in aerobic conditions, on a rotary shaker (150 rpm) at temperature 22 °C for 7 days. Sterile mineral medium with glucose was used as a control. Bacterial growth was monitored by plating diluted samples on solid LB medium to determine the number of CFU (colony forming units) and growth curves were plotted. The pH of bacterial culture and sterile control was measured at the end of experiment.

Dissolved organic carbon (DOC) analysis

The concentration of dissolved organic carbon (DOC) in the liquid phase of cultures was determined with Shimadzu TOC analyser TOC 5050A. A combustion/non-dispersive infrared gas analysis method was employed.

The analysis of extracellular compounds produces by bacterial consortium

Bacterial culture and sterile control were centrifuged (10 000 x g, at 4 °C, 10 min). 25 ml of samples of the cell-free supernatants was extracted with chloroform and then dried with anhydrous Na₂SO₄. Chloroform solutions were evaporated and then samples were prepared through the derivatization using BSFTA:TMCS 99:1 as silylating agent (Supelco, USA). GC-MS analyses were performed using an Agilent 7890A gas chromatograph associated to an electron impact ionization source 5975 series MSD (Agilent Technologies, USA). Identifications were carried out on a HP-5MS column (30 m long, 0.25 mm I.D., 0.25 µm film **thickness**, Agilent Technologies, USA) using He as carrier gas at 1 ml/min and an injection volume of 0.1 µl. The ion source was maintained at 230 °C; the GC oven was programmed with a temperature gradient starting at 100 °C for 3 min to 280 °C at 8 °C/min. MS analysis was carried out in the electron-impact mode at an ionizing potential of 70 eV. Compounds represented in the eluted peaks were identified by computer analysis using Wiley's mass spectra library (version 3.2, Copyright 1988–2000 by Palisade Corporation with The Wiley Registry of Mass Spectral Data, 8th Edition with Structures, Copyright 2000 by John Wiley and Sons, Inc.).

Elements mobilization from black shale by extracellular metabolites

10 g of crushed black shale was incubated with 100 ml of filter sterilized (pore size 0.22 µm) cell-free supernatant of bacterial culture in aerobic conditions on a rotary shaker (150 rpm) at temperature 22 °C for 24 hours. Sterile mineral medium supplemented with black shale were control for this experiment. All experiments were performed in triplicate. For chemical analysis the samples were centrifuged (10000 x g at 20 °C, 10 min), the supernatant was filtrated (pore size 0.22 µm) and mineralized with 65% nitric acid.

Elements concentration analysis

The chemical composition of cell-free supernatants of bacterial culture after incubation with crushed shale was determined by inductively coupled plasma emission

spectrometry (ICP-ES) using an Optima 5300 DV spectrometer (Perkin-Elmer, USA). All analyses were performed in triplicate.

Results and discussion

One of the processes responsible for elements redistribution in environment is their mobilization and/or complexation by a variety of extracellular organic substances produced by microorganisms. Low molecular organic acids and alcohols are released as a by-product of microbial metabolism due to incomplete metabolic pathways (Birch and Bachofen, 1990). A majority of organic acids are generated by microorganisms as a byproduct of fermentation or aerobic respiration of glucose.

In this study the growth of consortium of indigenous microorganisms on liquid mineral medium with glucose under aerobic condition and the concentration of total dissolved carbon was monitored for 7 days (Fig. 1). A typical growth curve was observed. During bacterial growth the carbon concentration decreased during first 4 days and then it started to increase up to the end of experiment (Fig. 1).

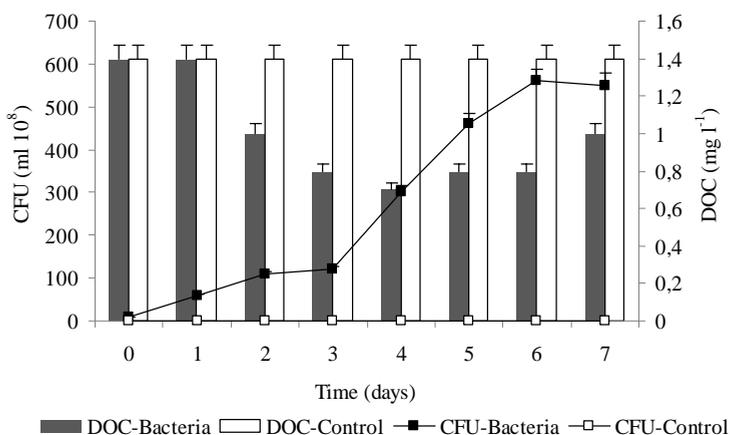


Fig. 1. The growth (CFU) of consortium of indigenous microorganisms on liquid mineral medium with glucose and the concentration of dissolved organic carbon (DOC)

The changes of the dissolved carbon concentration during bacterial growth suggest the utilization of glucose at the first days (decrease of the carbon concentration) and then the production of extracellular metabolites (increase of the carbon concentration). The pH of bacterial culture monitored during the bacterial growth decreased from 7.0 to 4.2 while the pH of sterile mineral medium (control) was stable (7.0).

The results of qualitative analysis of chemical compounds produced by consortium of indigenous microorganisms are presented in Table 2. Between compounds identified in culture sample 14 aliphatic (C7–C18) and 3 aromatic (C7–C8) carboxylic

acids were found (Table 2). In addition 5 alcohols were identified. All signals in the chromatogram of control sample (sterile mineral medium) originated from BSTFA:TMCS mixture.

The microbial extracellular metabolites such as organic acids increase mineral dissolution and mobilize elements by displacement of metal ions from the ore matrix by hydrogen ions or by the formation of metal complexes and chelates. The impact of organic acids and other metabolites on bioextraction of metal sulphide ore have been known for a long time. Parés (1964 a, b, c) described the solubilization of copper and other metals associated with laterite and clays using extracellular ligands formed by *Serratia marcescens*, *Bacillus subtilis*, *Bacillus sphaericus* and *Bacillus firmus*.

Table 2. The qualitative composition of extracellular compounds produced by mixture of microorganisms

Aliphatic carboxylic acids
heptanoic acid, octanoic acid, nonanoic acid, decanoic acid, undecanoic acid, 4-oxo-pentanoic acid, 5-dodecenoic acid, dodecanoic acid, 3-hydroxycapric acid, phenylpyruvic acid, 1,2-hydroxylauric acid, hexadecanoic acid, octadecanoic acid, 2,3-bishydroxypropyl laurate, 3-hydroxypalmitic acid
Aromatic carboxylic acids
benzoic acid, benzeneacetic acid, methyl benzoic acid
Alcohols
benzyl alcohol, glycerol, dodecanol, hexadecanol, octadecanol

Such species as *Bacillus circulans* and *Bacillus mucilaginosus* were described as able to leach manganese from ores using different organic compounds as reducing agents (Groudev, 1987). The most effective in metal solubilization are fungi: *Aspergillus* and *Penicillium* (Burgestaller and Schinner, 1993; Bosecker, 1997). Wenberg et al. (1971) reported the isolation of the fungus *Penicillium* sp. from a mine-tailing pond which produced unidentified metabolites that could mobilize copper from sedimentary ores of the White Pine deposit (Michigan, USA). Anjum et al. (2010, 2012) described the role of organic acids produced by *A. niger* in mobilization of copper, cobalt and zinc from black shale located in Pakistan. Also our studies of yeast *Rhodotorula mucilaginosa* sp. LM9 isolated from Kupferschiefer black shale showed that organic acids (malic and oxalic) produced by this strain can effectively mobilize copper from this sedimentary rock (Rajpert et al., 2013).

Despite extensive information on this topic in the literature, up to our knowledge this paper for the first time presents the simultaneous mobilization of dozen of elements from multielements sedimentary rock by the action of compounds of bacterial origin. Figure 2 shows the concentrations of nine elements after 24 hours of black shale incubation with cell-free supernatant containing bacterial metabolites and sterile mineral medium (control) and Table 3 presents the efficiency of mobilization of all studied elements.

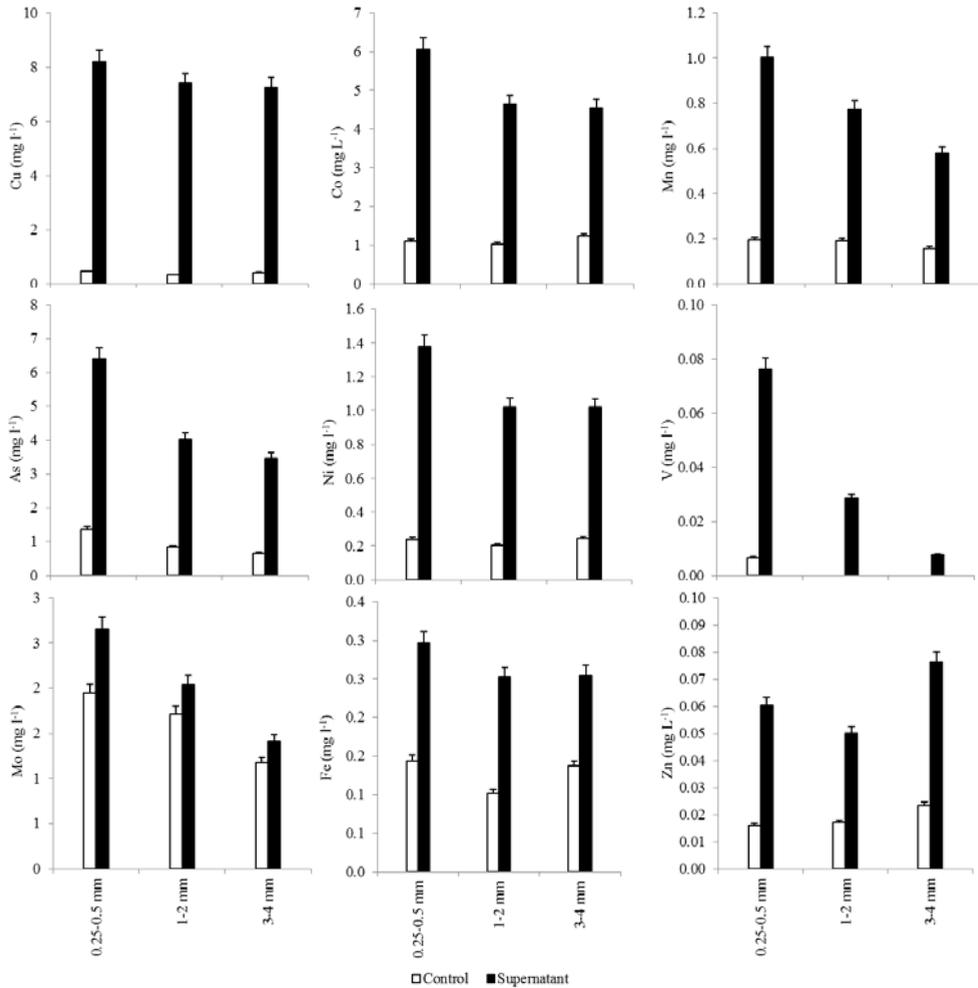


Fig. 2. Concentrations of elements mobilized from black shale by cell-free supernatant of bacterial cultures and sterile mineral medium (control) after 24 hours of incubation. Bars represent the standard deviation of measurements

The role of bacterial extracellular metabolites in mobilization of Ca, Na, Mg, Si, Al, Cu, Co, As, V, Zn, Ni, Mo, Fe, and Mn was clearly evident. The concentrations of K and S were lower in studied sample than in control where only chemical weathering was observed. The mobilization of elements was depended on the diameter of crushed shale and it was the highest in the case of fine crushed shale (0.25–0.5 mm) that could be simply explained by the differences in active surface area of different fractions used in this study.

The extracellular metabolites can be beneficial for microorganisms – they can be utilized as a means of sequestering of some metals from external environment to

supplement their nutritional needs. They can be also used as a way of detoxication against toxic elements. Black shale represents a type of extreme environment characterized by high concentrations of heavy metals and limited access to sources of carbon and energy. According to detailed petrographic and geochemical characterization of Kupferschiefer black shale performed by Speczik (1994) and Oszczepalski (1999) about 60 different major and trace elements in more than 100 different minerals occur in this ore. Rock-forming minerals such clay minerals, feldspars, dolomites and calcites are potential sources of Na, K, Al, Si, Ca and Mg. Such elements as Cu, Zn, Fe, Mo, S are mobilized from ore minerals - Cu-S, Cu-Fe-S, Cu-As-Sb-S, Pb-S and Zn-S Cu-Mo-S Mo-S. A part of mobilized elements can originated from metalloorganic compounds and geoporhyrins. Among them is first of all vanadium, but also it can be nickel, cobalt and molybdenum (Matlakowska et al., 2013).

Table 3. The efficiency (%) of elements mobilization from black shale by a microbial extracellular compounds

Element	Fraction size					
	0.25–0.5 mm		1–2 mm		3–4 mm	
	Supernatant	Control	Supernatant	Control	Supernatant	Control
Na	3.56	0	2.94	0	2.61	0
K	0.79	0.94	0.48	0.74	0.42	0.60
Al	0.011	0.0023	0.0098	0.00054	0.0069	0.00068
Si	0.035	0.025	0.025	0.022	0.023	0.021
Mg	0.31	0.31	0.29	0.24	0.27	0.19
Ca	1.3	0.76	0.83	0.52	0.73	0.38
Fe	0.012	0.0058	0.010	0.0042	0.010	0.0056
Mn	0.45	0.087	0.34	0.084	0.26	0.07
S	3.54	5.32	2.17	3.49	2.17	3.33
Cu	0.23	0.013	0.22	0.0092	0.21	0.012
Co	4.69	0.85	3.59	0.79	3.52	0.94
Ni	4.08	0.71	3.04	0.61	3.03	0.73
V	0.08	0.0074	0.03	0	0.0085	0
Mo	15.68	11.48	12.01	10.11	8.34	6.92
Zn	2.3	0.62	1.92	0.65	2.92	0.88
As	4.02	0.85	2.52	0.53	2.18	0.41

The efficiency of elements mobilization was the highest for cobalt (4.69%), nickel (4.08%) and arsenic (4.02%). The distribution of arsenic in black shale is very similar

to that of cobalt and nickel. Biorecovery of nickel and cobalt by mean of extracellular metabolites is very promising solution but also the mobilization of toxic arsenic is of great importance. Arsenic is an element that is very often associated in copper minerals. Contamination of copper ore with this metalloid is troublesome for hydrometallurgical processes. During smelting processes the volatile arsenic compounds are released into the atmosphere, which due to the toxicity of these compounds is a significant environmental hazard. Besides volatile arsenic compounds, high concentrations of arsenic are found also in the dust from tailings ponds. For this reason, very important, both from an economic and environmental point of view, is to develop effective methods for controlled removal of arsenic from copper deposits.

Conclusion

Taken together, the results of present study increase our knowledge about black shale bioweathering and confirm that bacteria may indirectly influence on the simultaneous mobilization of elements from sedimentary rock. This phenomenon has the potential application in biorecovery of metals from neutral or alkaline ores and wastes containing such ores and it could be used as the non-contact and environmentally friendly method of metal recovery.

The application of this biotechnology requires further research focused on finding a cheap source of extracellular compounds and process optimization. Another aspect requiring further analysis is the possibility of including these microbial metabolites into hydrometallurgical processes and recovering the mobilized metals as well as re-using the solutions.

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