

THE USE OF BIOLOG SYSTEM TO IDENTIFY SPECIES IN MINED-OUT SITES

Kateřina BOTUROVÁ, Hana VOJTKOVÁ

Department of Environmental Engineering, Faculty of Mining and Geology, VŠB – Technical University of Ostrava, Czech Republic

E-mail: katerina.boturova@vsb.cz

ABSTRACT

Poproč post-mining area in Slovakia is an old environmental burden which has been contaminated by toxic metals and semi-metals, predominantly arsenic and antimony, in connection with the extraction of antimony ores. Nowadays, revitalization work is undergoing in the closed mine with three adjacent sludge lagoons. The outcomes deal with anthropogenic manifestations of mining *in-situ* and their influences on microbes. Using the BIOLOG test system we identified representatives of indigenous microbes, whose adaptation to high levels of toxic metals and semi-metals are one of the main bioindicators of the state of the post-mining environment and a basic indicator of the evolution of effects on organisms of a higher ecological level. The analyses of microbial diversity in Poproč found the strain of *Firmicutes* to be the most abundant, with the highest diversity in the genus *Bacillus*. Based on the biochemical tests, the *Bacillus* genus was confirmed to be highly adaptable to the change in biogeochemical conditions in metal-contaminated substrates. The variable parameters of the identified representatives of the *Bacillus* genus correspond to its high diversity, which has been confirmed by a set of oxidative and assimilation tests.

Keywords: mined-out site, sludge lagoon, microbial diversity, BIOLOG system, *Bacillus*

1 INTRODUCTION

Microorganisms are a contact group of organisms, which are an important element for successful land reclamation because of their major role in nutrient cycling, plant establishment, biogeochemical transformations of present elements, and soils formation [1], [2], [3]. These are organisms that are highly adaptable to life under the conditions of heavily-burdened areas and extreme stress from the presence of toxic elements [4], [5], [6]. These organisms have an enormous biotechnological potential in the speciation of elements in post-mining substrates, thereby influencing the course of further revitalization of contaminated sites through the biotransformation processes of present toxic metals and semimetals [7], [8], [9], [10], [11]. Identification of these microorganisms after their isolation and cultivation enables the delimitation of taxa of present microorganisms within their diversity [12], [13]. The biochemical profiles of the representatives of the autochthonous microbiota provide valuable data on the enzyme production and substrate utilization, which can be used not only for assessing the microbial diversity, but also to predict its role in advancing biotransformation contaminants. This information also makes it possible to find differences between the microbial diversity of indigenous and environmentally-stressed areas in terms of its succession.

BIOLOG MicroStation system (MicroLog3, ver. 4.20.05) is a modern system through which a high amount of microbial metabolic information could be easily gained. The system is used for identification and phenotypic characterization of microorganisms based on highly accurate and patented biochemical tests [14]. Using 96-well GENIII microplates, a unique biochemical profile could be obtained for individual strains; the phenotypic fingerprinting of tested bacteria accurately reflects their catabolic potential and can be used to identify their bacteria using more phenotypic profiling.

2 MATERIAL AND METHODS

Site characteristics

The Poproč sludge lagoons are an old environmental burden. It is a site of already closed mine workings, which until 1965 belonged to very important sites of antimony ores in Slovakia. The mined ore had a polymetallic character and in addition to a significant proportion of antimony, it also contained other toxic and potentially toxic metals and half-metals (e.g. As, Pb, Cu, Fe, Zn, Co, Cd, Ni, and Au). The ore was further processed by the flotation method, which produced waste, which was deposited into three different sludge lagoons situated above the municipality of Poproč. The location of Poproč with adjacent extractive waste is located in the valley of Petrov, northeast of the village of Poproč in the eastern part of the Slovak Republic. Detailed description of the study area can be found in [15], Figure 1.



Figure 1. Beginning of the recultivation and revitalization of the territory Poproč (Google Maps and Kateřina Boturová, 2015)

Substrate sampling and isolation of bacterial strains

Samples of potable substrates Poproč have been taken systematically from north to south from different places to include all three of the sludge lagoons. Soils samples from a depth of 15 – 20 cm were taken using a sterile soil auger (in the amount of approx. 2 kg) and mechanically removed larger parts of plants (roots, twigs, etc.). The samples were subsequently enclosed into sterile plastic bags which were kept cool and immediately taken to the laboratory. After arriving in the laboratory, the samples were processed by the standard procedure: homogenizing, quarting and sieving to fraction <1 mm (according to norm ČSN ISO 3310-1). Part of the samples were used for basic physicochemical analyses and some samples were stored as appropriate to the biological nature of the samples until microbiological analyses were performed.

The accredited laboratory of MORAVIA s. r. o. in Studénka, the Czech Republic, was used to carry out the chemical and physicochemical laboratory analyses for soil reaction. Samples of soil substrates of the sludge lagoon were determined by the SOP 44 method (ČSN EN 15933) for pH active and for pH_{KCl} was used SOP 06 method of the norm ČSN ISO 10390 (2005). For total organic carbon, the method according to SOP 56 (ČSN EN 15936) was used. The dry matter in the samples was determined using the Gravimetric method of the norm ČSN ISO 11465:1993. The contents of the elements were determined using an X-Ray Fluorescence Spectrometer (WD-XRF S8 Tiger, Bruker Co, USA) at the Centre of Advanced Innovative Technologies at VŠB – Technical University of Ostrava.

Bacteria were isolated by cultivation methods on nutrient media using the dilution plate method on trypton soya agar (M 290, HiMedia Laboratories Ltd., Mumbai, India) and cultivated at 30 °C for 48 h. All morphological varieties of colonies were selected and inoculated into the original nutrient medium to obtain pure bacterial strains. Pure cultures were identified morphologically and according to their characteristics. Biodiversity of microorganisms was determined according to the isolated and cultivable bacteria.

Identification and biochemical characterization of bacterial isolates

The isolated strains for identification were first cultivated on trypton soya agar medium (M290, HiMedia Laboratories, India) at 33 °C for 24h. The colonies were then inoculated into a special solution (IF, Biolog Inc.) and diluted to a recommended cell density of 98 %. This suspension was then inoculated according to the BIOLOG protocol to GENIII-MicroPlate plates in the amount of 100 µl per well and the microplates were incubated at 33 °C for 12 – 48 hours to allow the phenotypic fingerprint to form. During incubation, the bacterial cells react with

the test substance in the microplate wells, which the bacteria can use as a carbon source for growth. Positive metabolic reactions cause the reduction of the tetrazolium dye, resulting in varying purple colour intensity; negative wells remain colourless, as does negative control well (A1) with no carbon source. After incubation, the results of each test are evaluated and compared with the BIOLOG system database. If a match is found, the instrument reports successful species identification including probability and similarity levels with the pre-set database.

3 RESULTS AND DISCUSSIONS

Basic chemical and physicochemical analyses of the samples from the sludge removal sites were carried out to determine soil moisture, soil acidity (pH - H₂O and pH - KCl), the percentage of solids in the samples and the concentration of toxic and potentially-toxic metals and of the semi-metals.

Table 1. Physicochemical characteristics of substrates from the sludge lagoon Poproč (average values from 5 sampling points from POP 1, POP 2 and POP 3)

	POP 1	POP 2	POP 3
pH _{H2O}	5.8	5.1	4.5
pH _{KCl}	5.3	4.4	4.0
TOC	0.20	0.15	0.18
Dry matter (%)	87.13	88.08	80.05
As (mg/kg)	5266	3303	2562
Sb (mg/kg)	16455	7711	7401

Based on the phylogenetic analysis, the isolated bacterial strains (Figure 2) were composed of four phyla and represented by common strains in technogenic soils: *Firmicutes* (62.96 %), *Actinobacteria* (25.93 %), *Proteobacteria* (7.41 %), and *Bacteroidetes* (3.7 %). The strains *Proteobacteria* and *Firmicutes* were present in all studied substrates, the species of genus *Bacillus* accounted for the major part of cultivable bacteria in most of the samples (44.44 %). Smaller part of the cultivable bacterial community was represented by strains of *Actinobacteria* and *Bacteroidetes* (Figure 2).

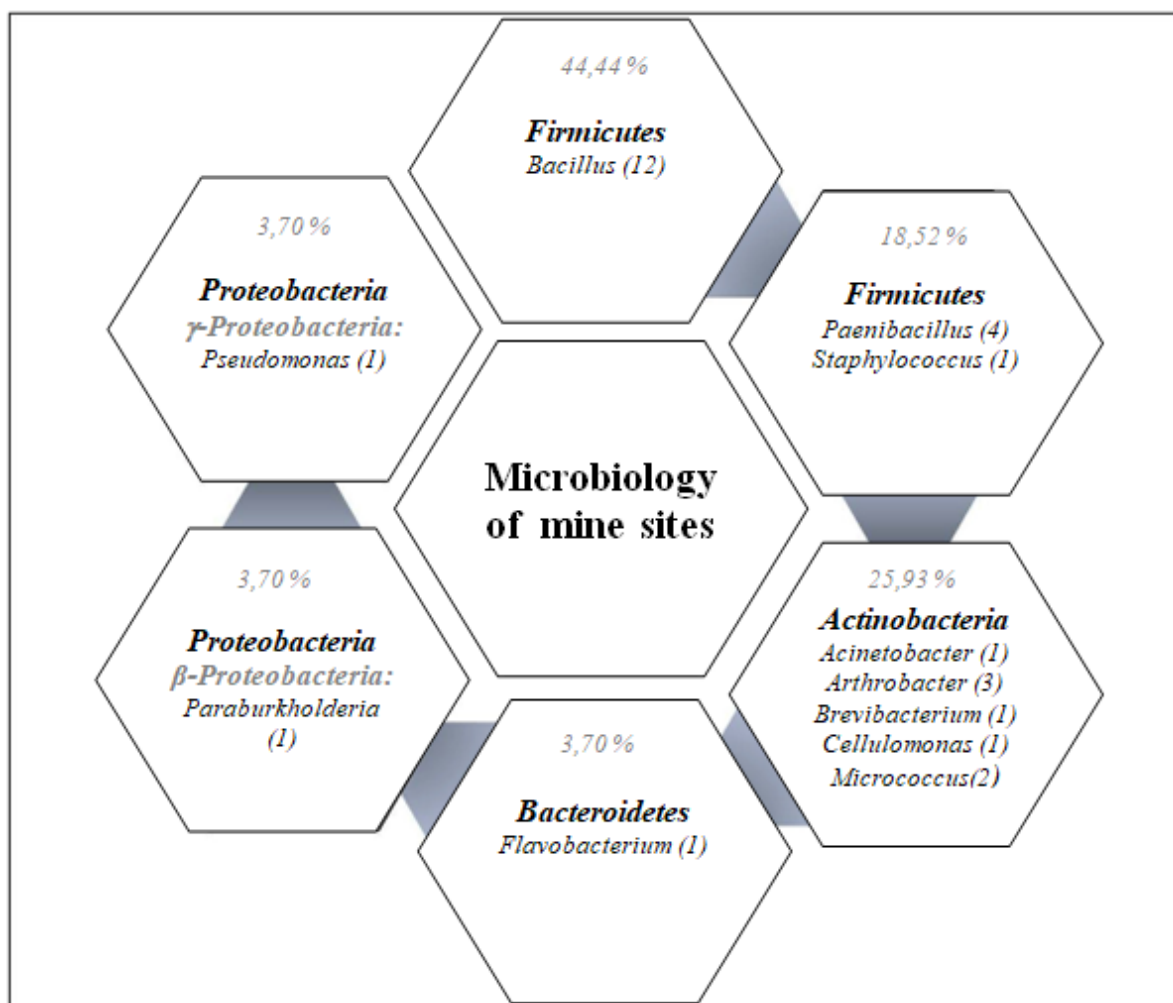


Figure 2. Abundance of isolated and identified bacterial phylum from mined-out site

In terms of diversity and highest abundance, we identified 17 bacteria of *Firmicutes* phylum of which 12 species belonged to the genus *Bacillus* in this technogenic soil (Figure 2). In addition, there were the genera of *Paenibacillus* (*P. illinoisensis*, *P. macerans*, *P. pabuli*, *P. polymyxa*), *Staphylococcus* (*S. warneri*), *Acinetobacter* (*A. radioresistens*), *Arthrobacter* (*A. histidinolovorans*, *A. ilicis*), *Brevibacterium*, *Cellulomonas*, *Micrococcus* (*M. luteus*, *M. yunnanensis*), *Flavobacterium*, *Paraburkholderia*, and *Pseudomonas* (*P. putida*).

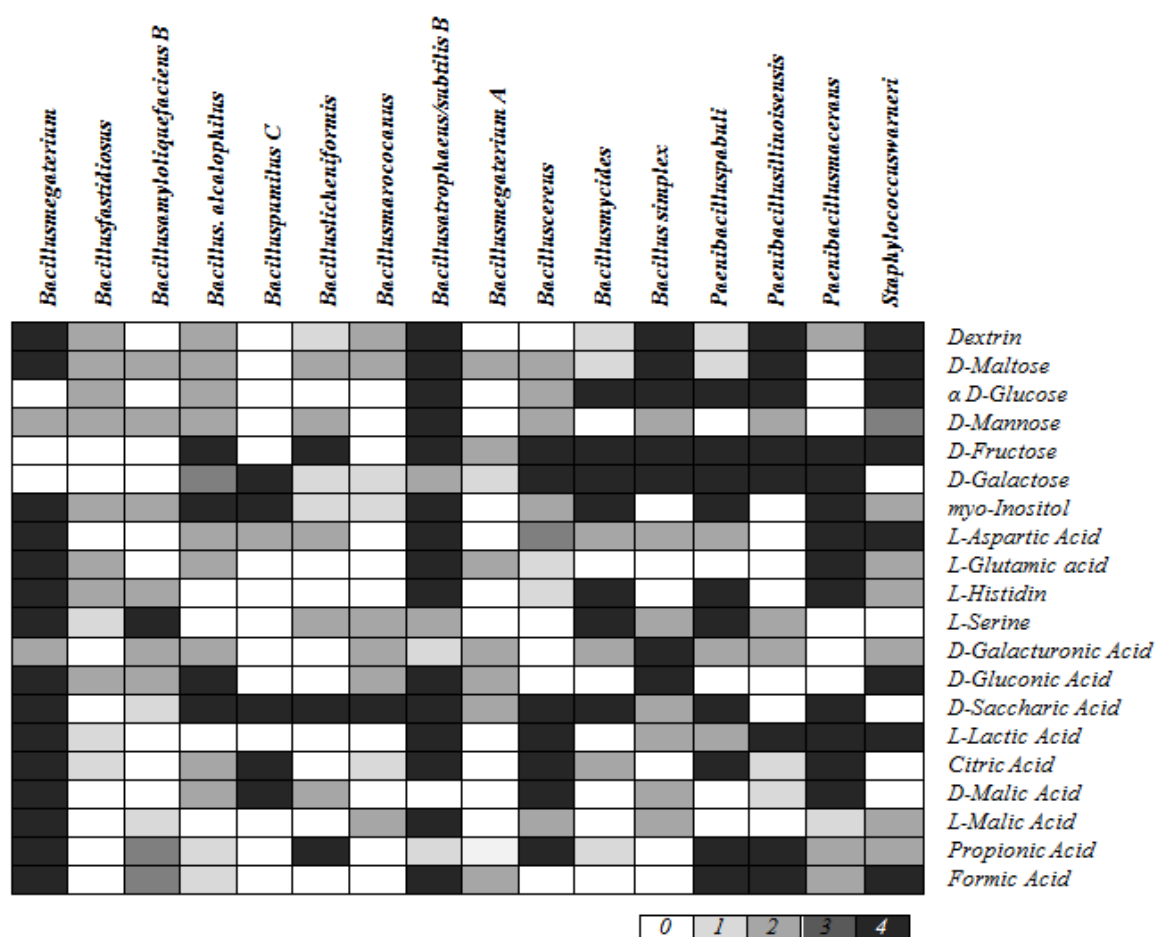


Figure 3. Biochemical identification of the *Firmicutes* phylum by BIOLOG and their variable parameters.

Bacteria are identified by oxidation and assimilation tests:

0 (white) - negative, 1 (light grey) - almost negative, 2 (medium grey) - border (+/-),
3 (dark grey) - almost positive, 4 (black) - positive

The results show a high variability of biochemical properties in all detected isolates, from which the utilization of sugars and organic acids appears in the biochemical profile as an essential characteristic. The taxonomic distance of the individual strains is well manifested in the identified representatives of the *Bacillus* genus in their biochemical profile (Figure 3). As part of the tests, the highest difference in utilization of dextrin and D-glucose was detected, from organic acids the greatest differences were found in the utilization of D-galacturonic acid, propionic acid and formic acid.

The metabolic pathway of D-glucuronic acid and that of D-glucose have much in common with each other – part of the enzyme system which is capable of oxidizing D-glucose can be stimulated by the adaptation of bacteria to D-glucuronic acid [16].

Many scientific studies show that some microorganisms are able to survive in soils and technogenic sediments contaminated by high concentrations of toxic metals and semi-metals [17], [18], [19]. An example is the *Firmicutes* phylum with the identified genus *Bacillus*, which occurred in the sludge substrates with the highest observed abundances (62,96%). Bacteria of the genus *Bacillus* are known for their secondary metabolism and the ability to produce a wide range of metabolites-enzymes, organic acids, vitamins and toxins [20], [21]. In addition, many of the metabolites production processes have now been improved and supported by molecular genetic interventions in the bacterial genome, including areas that provide genetically conditioned adaptation to increased arsenic and antimony in the environment [21].

Ars-operon is now a well-known carrier of bacterial resistance to arsenic, which has been subsequently detected by a number of Gram-negative and Gram-positive bacteria, including the archaea (e.g. *Escherichia coli*, *Pseudomonas aeruginosa* and many others) [22], [23]. The analogous genetic system (*ARR*, *ACR*) has also been previously described for other microorganisms, for example, in *Saccharomyces cerevisiae* yeast [24] and similar systems can therefore also be expected in the case of microscopic fungi, where the published results are currently limited. Also in the case of observed bacterial isolates from the site of the Poproč was confirmed in several

representatives of autochthonous microbiota that *Ars*-operon and other systems of resistance to As and Sb are in the genome of isolated bacteria present and widespread [25]. In view of the fact that these genes may be subject to horizontal transmission, it can be assumed that other isolated bacterial autochthonous microbes *in situ* are also part of the genome.

The ability to withstand long-term exposures to increased concentrations of toxic metals and half-metals has been previously supported in bacteria *Bacillus* by a number of studies [26], [27], [28]. In the Poproč sludge lagoons, a very high abundance of arsenic (≥ 5266 mg/kg) and antimony (≥ 16455 mg/kg) has been observed, leading to the selection of specifically resistant microorganisms for which interesting phenotypic properties have been confirmed by the BIOLOG system using the whole range of oxidative and assimilation tests. Also due to the physiological capacities of the sporulation, the *Bacillus* genus can well survive the strong selection pressure of the metal-contaminated substrates and can thus be considered as a representative of the indicator group of highly adaptable microorganisms to metallic stress [12], [29], [30], [31]. The fact that the *Bacillus* genus has been confirmed to be the predominant cultivable part of the autochthonous microbes detected is evident in the diversity of its metabolic pathways, and thus seems to have a key role in the biological biotransformation of metals in a gradual change environmental conditions after mining and industrial activities.

One of the most used representatives of the genus *Bacillus* in biotechnological production is *B. subtilis*, whose genome is in part reserved for the synthesis of secondary metabolites [20]. This species produces a wide range of substances such as riboflavins, D-ribose, glutamic acid, or α -amylase (*B. circulans*) and β -amyl (*B. cereus*). These substances have wide applications in the pharmaceutical, cosmetic, textile, paper and food industries [21], [32].

4 CONCLUSION

The biotransformative role of the autochthonous microflora in the metal-contaminated area is closely related to the course of the geochemical processes that affect the toxicity of the environment, especially the release of toxic metals and metal to the surrounding environment. Microorganisms as the first contact biota, which interacts with changes in environmental conditions, are bioindicators of the state of the soil environment, and the analysis of its diversity is thus an indicator of negative influences also on the organisms of higher orders.

From analyses of the As, Sb-highly contaminated area Poproč (Slovakia) with secondary wastes shown that the most typical phylum correspond to *Firmicutes*, whose biochemical properties have been verified by the BIOLOG system. Variable parameters in the identified representatives of the *Bacillus* genus correspond to high diversity, which was confirmed by a set of oxidative and assimilation tests in the BIOLOG system. It has been confirmed that the *Bacillus* genus is very variable and adaptable, therefore it can survive well in metal-contaminated substrates and its specific physiological profiles of identified species illustrate the possibility of rapid physiological adaptation to negative physical, chemical and biological environmental conditions. The confirmation of the presence of predominant representatives of the genus *Bacillus* in the sludge substrates after the extraction of arsenic and antimony implies a direct link to the limit of revitalization of the landscape affected by mining and restoration of the original biological function of the environment.

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