

NON-FERROUS METAL INDUSTRY WASTE DISPOSAL SITES AS A SOURCE OF POLY-EXTREMOTOLERANT BACTERIA

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Abstract: Waste disposal sites from non-ferrous metal industry constitute environments very hostile for life due to the presence of very specialized abiotic factors (pH, salt concentration, heavy metals content). In our experiments microflora of two waste disposal sites in Slovakia – brown mud disposal site from aluminium production near Ziar nad Hronom and nickel sludge disposal site near Sered - was analyzed for cultivable bacteria. Isolated bacteria were characterized by a combination of classical microbiological approaches and molecular methods and the most of isolated bacteria shown a poly-extremotolerant phenotype. The most frequently halotolerant (resistant to the high level of salt concentrations) and alkalitolerant (resistant to the high pH level) bacteria belonging to the Actinobacteria class were detected. The most of bacteria shown very high level of heavy metal resistance e.g. more than 500 µg/ml for Zn²⁺ or Cu²⁺. Based on our data, waste disposal sites thus on one side represents an important environmental burden but on other side they are a source of new poly-extremotolerant bacterial strains and species possibly used in many biotechnology and bioremediation applications.

Key words: Metal industry, waste, heavy metals, environment, extremotolerant bacteria, biotechnology

1. Introduction

During billions years of evolution bacteria have adapted to nearly all possible environments on Earth and evolutionary changes in the prokaryotes focused on metabolic diversity and the genetic capacities to explore and eventually colonize every conceivable environment on Earth, including extreme environments (HORIKOSHI and GRANT, 1998). Extremophile bacteria not only survive, but thrive under extreme conditions. This contrasts with an extremotolerant bacteria that may tolerate and

survive extreme conditions, but grows optimally under less extreme conditions. Extremotolerant bacteria can resist extreme temperatures, pH values, salinity, radioactivity, high concentration of heavy metals etc. which are detrimental to the majority of life on Earth. There are a variety of natural extreme environments on Earth that have very specialized abiotic factors. These habitats can be found in deep marine settings, earth crust interiors, highly mineralized soils, extreme radiation surroundings or extreme aquatic chemical environments e.g. hydrothermal deep-sea vents, thermal deep-sea black smokers, hot springs, soda lakes, salt pans etc. (PIKUTA *et al.*, 2007).

With the advent of industrial technologies a new type of extreme environments appeared with conditions unseen before. Especially waste disposal sites from non-ferrous metal industry constitute environments very hostile for life. Mining and ore processing consists of extracting minerals and metals from surrounding earth and ore. The extracted ore is then sent for crushing, washing, and various physical or chemical separation processes, like smelting, leaching, electrolyzing etc. Through the extraction and subsequent mineral processing, metals and metal compounds tend to become chemically more available, which can result in the generation of acid or alkaline drainage. In each stage of these operations the undesirable constituents are discarded as liquid or solid wastes. The residual waste frequently contains the toxic processing chemicals and/or elevated levels of metals (ADRIANO, 2001) and it is disposed by landfill which led to the pollution of environment especially groundwater.

In our experiments cultivable bacteria of two waste disposal sites in Slovakia – brown mud disposal site from aluminium production near Ziar nad Hronom (SCHWARZ and LALIK, 2012) and nickel sludge disposal site near Sered (MICHAELI *et al.*, 2012) - were analyzed for the resistance to the extreme conditions.

2. Materials and methods

2.1 Sample collection, isolation, growth, and identification of bacteria

Brown mud samples, samples of drainage water, and nickel sludge samples were collected from the Slovalco co. disposal site near Ziar nad Hronom (Slovakia) and nickel sludge disposal site of the Nickel smelter near Sered' (Slovakia), respectively. Solid samples were taken from at least two different locations and mixed together, the water sample was taken from a reservoir of drainage water. The samples were immediately brought to the laboratory and stored in the cold (4 °C) until microbiological analysis. pH of the brown mud sample was determined according to the ISO 10390:2005 standard.

To the 0.5 g of solid sample 10 ml of sterile phosphate-buffered saline solution were added and after 20 min of intensive mixing aliquots were spread on non-selective agar medium (TSA - Tryptone Soya Agar, Oxoid, USA). The samples of drainage water (50 µl per plate) were directly spreaded on TSA plates. Cultivation was conducted under aerobic conditions at 22 °C for 24 - 72 h. The individual isolates were selected on the basis of cell and colony morphology and used for further analyses. After repeated subculturing and control for the purity, the isolates were typed and identified by the MALDI TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry approach as specified recently (KOPCAKOVA *et al.*, 2014).

2.2 Heavy metal resistance analysis

The minimal inhibitory concentrations (MIC) of the metals of isolated bacteria were determined by the plate dilution method as adopted by ALEEM *et al.* (2003). The lowest concentration that prevented bacterial growth was considered the MIC. Solid Nutrient medium 2 (Oxoid, USA) was used for heavy metal resistance determination in order to minimize complexation of the heavy metal ions tested.

2.3 NaCl and pH resistance analysis

For determining the salt tolerance of the isolated bacteria, they were streaked on TSA agar supplemented with increasing concentration of NaCl – 3 %, 5 %, and 10 % and cultivated for 24 hours at 22 °C. After the appearance of colonies the isolates were scored positive or negative for their ability to grow in different concentration of NaCl. Similar approach was used to test the pH tolerance of the isolated bacteria. The bacteria were streaked on buffered TSA plates (TSA medium with 100 mM TRIS-HCl, pH 7.5 or 10), and the growth was scored after 24 hours cultivation at 22 °C.

3. Results and discussion

The brown mud disposal site from aluminium production near Ziar nad Hronom and nickel sludge disposal site of the Nickel smelter near Sered belong to the most dangerous landfills in Slovakia. Brown mud is disposed as a slurry having a solid concentration in the range of 10-30 %, pH in the range of 13 and high ionic strength (SCHWARZ and LALIK, 2012). The nickel sludge contains of about 80 % of Fe and high concentrations of other metals e.g. 3.5 % of Cr₂O₃ and 0.17 % of Ni (MICHAELI *et al.*, 2012). Despite extremely harsh conditions (extreme pH values and heavy metal content in brown mud disposal site from aluminium production or high heavy metal content in nickel sludge) relatively high numbers of bacteria were recovered (Table 1) from these environments. The highest bacterial counts were detected in nickel sludge (32 000 cfu/g), the lowest in highly alkaline drainage water of brown mud landfill from aluminium production (80 cfu/ml). It seems there is a correlation between alkalinity and number of cultivable bacteria in studied ecosystems. In natural ecosystems such extreme pH values are very rare and for example in the Lonar lake in India, having pH 10.5, the total number of cultivated bacteria was found to be between 10²-10⁶ cfu/ml (JOSHI *et al.*, 2006).

Isolated bacteria were characterized by a combination of classical microbiological approaches (Gram reaction, cell and colony morphology, catalase activity, spore formation, H₂S production, and ability to grow on TSA plates at 22 and 37 °C) and MALDI TOF analysis. MALDI TOF analysis identified the highest variability of bacteria in the brown mud sludge (at least 16 taxa) followed by nickel sludge (at least 8 taxa) and the drainage water sample (at least 6 taxa). However, only limited number of isolates could be identified using MALDI TOF analysis (KOPCAKOVA *et al.*, 2014). Further microbiological analyses shown that in all tested samples Gram-

positive bacteria dominate (Table 1). While the nickel sludge and drainage water was dominated by *Actinobacteria*, the brown mud sample was dominated by *Firmicutes* (Low G+C content gram-positive bacteria). In both brown mud and nickel sludge actinomycetes were detected and identified as *Streptomyces variabilis* and *S. malaysiensis* for isolates from brown mud and as *Nocardioopsis alba* for isolate from nickel sludge respectively (data not shown). Similar halo-alkaliphilic actinomycetes such as *Nocardioopsis* sp. and *Streptomyces* sp. were collected e.g. from the mud soil of solar salt works in India (JOSE and JEBAKUMAR, 2012).

Table 1. Characteristics of studied environments and occurrence of poly-extremotolerant phenotypes in isolated bacteria.

Characteristics	Brown mud disposal site near Ziar nad Hronom		Nickel sludge disposal site near Sered
	Drainage water	Brown mud	
pH	13.1	11.6	8.1
Cultivable bacteria counts	80 cfu/ml	3 500 cfu/g	32 000 cfu/g
Total number of isolates	12	21	24
Gram-positive	10	18	24
<i>Actinobacteria</i>	7	8	21
<i>Actinomycetes</i>	0	2	1
Gram-negative	2	3	0
Number of species	6	16	8
Dominant genus	<i>Microbacterium</i>	<i>Bacillus</i>	<i>Arthrobacter</i>
Frequency of isolates growing at:			
pH=7.5	12/12	21/21	24/24
pH=10.0	12/12	19/21	22/24
3 % NaCl	12/12	21/21	24/24
5 % NaCl	11/12	8/21	23/23
10 % NaCl	5/12	2/21	0/23
500 µg/ml Zn ²⁺	0/12	19/21	24/24
500 µg/ml Ni ²⁺	0/12	14/21	3/24
200 µg/ml Co ²⁺	0/12	14/21	23/24
500 µg/ml Cu ²⁺	0/12	14/21	24/24

Microflora of metal industry waste disposal sites is not well understood and in the study of microflora of bauxite residue treated by using various added nutrients HAMDY and WILLIAMS (2001) reported the presence of multiple bacterial genera including *Firmicutes* and *Actinobacteria* (e.g. *Bacillus*, *Lactobacillus*, *Leuconostoc*, *Micrococcus*, *Staphylococcus*, *Pseudomonas*, *Flavobacterium*, and *Enterobacter*).

Bacterial population in alkaline Lonar lake was dominated by *Firmicutes*, followed by γ -*Proteobacteria*, and α -*Proteobacteria*. *Actinobacteria* were found to be the least

represented class of bacteria (JOSHI *et al.*, 2006). In extremely alkaline (pH > 12) ground water of the Lake Calumet area of Chicago, Illinois, where historic dumping of steel slag has filled in a wetland, the presence and growth of a variety of alkaliphilic β -Proteobacteria, *Bacillus*, and *Clostridium* species was detected (ROADCAP *et al.*, 2006). In this report the non-cultivation based approach was used however.

Most of the isolated bacteria have shown poly-extremotolerant phenotype. The most frequently halotolerant (resistant to the high level of salt concentrations) and alkalitolerant (resistant to the high pH level) bacteria belonging to the Actinobacteria class were detected (Table 1). The highest level of halo- and alkalitolerance was observed among isolates from the drainage water. The isolates from solid wastes have shown similar degree of alkalitolerance despite dramatically different pH in these environments (11.6 in brown mud versus 8.1 in nickel sludge). Surprisingly, two isolates from the brown mud (both Gram-negative) were unable to grow at pH 10 under *in vitro* conditions. The lowest salt tolerance was observed in isolates from nickel sludge.

In both waste disposal sites, relatively high concentrations of heavy metals were detected. Brown mud heavy metal content is 10 ppm for Hg, 220 ppm for Cu, 400 ppm for Cr, 700 ppm for V, 150 ppm for Pb and 800 ppm for As (KOPCAKOVA *et al.*, 2014). The average heavy metal content of nickel sludge is Fe (45.9 %), Cr₂O₃ (2.1 %), SiO₂ (15.0 %), Al₂O₃ (4.1 %), CaO (3.5 %), NiO (0.2 %), MgO (2.3 %) (MICHAELI *et al.*, 2012). Heavy metals may exert an inhibitory action on microorganisms by blocking essential functional groups, displacing essential metal ions, or modifying the active sites of biological molecules (MOFFETT *et al.*, 2002). Numerous studies have examined the heavy metal sensitivity or resistance of bacteria isolated from different habitats and many microorganisms shown adaptation to the toxic metals to which they are exposed (MÜLLER *et al.*, 2001). Accordingly, most of isolates from brown mud and nickel sludge shown very high levels of resistance against heavy metal present in disposal sites (e.g. more than 500 $\mu\text{g/ml}$ for Cu²⁺ or Co²⁺) as well as to Zn²⁺ used as a control. Surprisingly, the nickel resistance was more frequently encountered in the brown mud compared to the nickel sludge isolates. All three actinomycetes belonged among the most heavy metal resistant isolates. Heavy metal resistance is frequently occurred in actinomycetes (ALVAREZ *et al.*, 2013). On other hand, the isolates from the drainage water were much more sensitive to the heavy metals, probably as a consequence of extreme pH in drainage water at which most heavy metals form insoluble hydroxides. Thus heavy metal concentrations in drainage water are much lower compared to the brown mud (e.g. 0.3 ppm of Cu in drainage water compared to 220 ppm of Cu in brown mud (unpublished data).

In the last years an interest in extremotolerant bacteria increased dramatically either in biotechnology or bioremediation applications. The use of metabolic abilities of extremotolerant bacteria for degradation/removal of environmental pollutants provides an economic and safe alternative compared to other methodologies (PERPETUO *et al.*, 2011). Based on our data, metal industry waste disposal sites on one side represents an important environmental burden but on other side they are a source of new poly-extremotolerant bacterial strains and species possibly used in many biotechnology and bioremediation applications.

4. Conclusions

Despite extremely harsh conditions (extreme pH values and heavy metal content in brown mud disposal site from aluminium production or high heavy metal content in nickel sludge) relatively high numbers of bacteria were recovered from both metal industry disposal sites. At least some of isolated bacteria are poly-extremotolerant. The most frequently halotolerant (resistant to the high level of salt concentrations) and alkalitolerant (resistant to the high pH level) bacteria belonging to the *Actinobacteria* class were detected. The most of bacteria showed very high heavy metal resistance levels. Waste disposal sites are a source of new poly-extremotolerant bacterial strains and species possibly used in many biotechnology and bioremediation applications.

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