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PRELIMINARY SCREENING OF BACTERIAL ISOLATES FROM MINING WASTES

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ABSTRACT: Developing innovative biotechnology for obtaining new resources of high tech critical metals is strongly influenced by the need to reduce the potential risk of shortages, to support the development of industry at European level. To set up these new technologies is essential to isolate strains with high potential in bioleaching of ore, tailings and mine wastes and bioaccumulation of high tech critical metals. Microorganisms are capable of mediating metal and mineral bioprecipitation. In this paper are presented preliminary studies performed for the isolation of strains existing in mining residues containing high tech critical metals. Were used samples collected from various depths in an area of mining wastes containing high tech critical metals. The samples were fine grounded and the powder was washed with sterile saline water. Exact quantities of samples were dispersed in sterile saline water, shaken for a period of 60 minutes, diluted and plated in triplicate on selective agar. After several steps were isolated 3 strains of gram negative bacteria.

Keywords: critical metals, microbial population, biomining, mine waste, metal resistant bacteria

INTRODUCTION:

Taking into consideration the fact that the natural resources of raw materials are limited, and are rapidly diminishing due to intensive exploitation given by the increasing standard of living, urbanization and the world population explosion, the shortage of raw materials is expected in the near future. At worldwide level, the industrial demand for metals is high and the costs of extraction are rising, as a result of the fact that the sources of high quality ores is decreasing and so sources once considered as low quality must now be exploited (DEFRA, 2012).

Thinking of the lifetime of the planet, the exploitation of minerals can be considered a relatively recent activity that has grown exponentially during the last two hundred years (Arndt et al., 2012). Once the geological reservoirs of natural resources are consumed, they cannot be possibly replaced in a period of time significant to human beings, since geogenic mineral deposits are the end product of the prolonged formation of local environmental and geodynamic settings (Dill, 2010).

The availability of certain mineral resources is representing a common concern for governments and industries across Europe, because there is a great risk that some metals will become less available, thus negatively influencing the economy, industry and technology of the human society (Kaartinen *et al.*, 2013,; Kenward *et al*. 2011). Europe is confronted with an increasing supply risk of critical raw materials. These can be defined as materials of which the risks of supply shortage and their impacts on the economy are higher compared to most of other raw materials (Hennebel, 2015). Because other elements which have a low availability at this moment may become critical in the near future, it was taken the decision to update the list of critical raw materials every three years. Up to the present, were made public various reports (US Department of Energy, 2011; British Geological Survey, 2012; European Commission, 2014) which investigated the security of supply and scarcity of raw materials, the supply chain risks, trends and forecasts of the demand and supply, the political stability of the producing countries (being given the uneven geographical distribution of the supplies across the planet), the price volatility and the potential of substitution. The relative criticality of mineral supplies was evaluated using a wide range of parameters and in different contexts, for example, from the standpoint of their importance to national security, or to a specific industrial application. This is how is explained the multitude of classification schemes and differences in terminology regarding these supplies in the scientific studies, media and government reports. The core group of critical metals, listed in alphabetical order, includes: antimony (Sb), beryllium (Be), chromium (Cr), cobalt (Co), gallium (Ga), germanium (Ge), indium (In), lithium (Li), niobium (Nb), platinoids (PGM-platinum group metals), rare-earth elements (REE, including yttrium) and tungsten (W) (Chakhmouradian, *et al.* 2015).

Rare earth elements (REEs), Platinum group metals (PGMs), Lithium (Li), Indium (In), Cobalt (Co) and Antimony (Sb) were identified as presenting a high risk of supply shortage, thus having an increased impact on the global economy (Hislop *et al.*, 2011).

In the case of critical metals, the scarcity is perceived as an increased risk faced by the industry and characterized by the price volatility. To avoid the risk of price volatility and to stockpile raw materials for future generations, there is a need to identify secondary sources and to develop suitable technologies for their recovery (Nancharaiah *et al*., 2015). Developing innovative biotechnology for obtaining new resources of high tech critical metals is strongly influenced by the need to reduce the potential risk of shortages, to support the development of industry at European level. To set up these new technologies is

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essential to isolate strains with high potential in bioleaching of ore, tailings and mine wastes and bioaccumulation of high tech critical metals. Microorganisms are capable of mediating metal and mineral bioprecipitation (Gadd, 2010).

Therefore, there is a great need to research for the isolation and characterization of novel microorganisms which can thrive in complex physicochemical conditions of waste streams and concentrate diffuse critical metals in a recoverable form. The major challenges for the critical metals recovery include their low concentration, low pH, co-existing metals and salts (Nancharaiah, *et al.*, 2016).

The aim of the present research was to perform preliminary studies for the screening and isolation of bacterial strains existing in mining residues containing high tech critical metals.

MATERIALS AND METHODS:

Sample collection

A number of nine samples were collected from mining wastes containing high tech critical metals, from various depths of the same situs. Samples were aseptically collected from mine tailings at a depth of approximately maximum 50 cm below surface, after which they were placed clean sterile bags, labeled accordingly and stored at 4 ºC until further analysis (Table 1). In order to be used for the microbiological studies, the samples (named P1 to P9) were grounded to obtain a coarse powder.

Table 1.
Distribution of the samples collected

Label	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**
Depth of prelevation (cm from surface)	0	0	0	10±5	10±5	10±5	50±5	50±5	50±5

Chemicals and media

Stock solutions of critical metals (1000 mg L^{-1}) prepared in distilled water were sterilized by filtration through 0.22 μm Merck Millipore filters. The tested metals employed were Magnesium nitrate hexahydrate $Mg(NO_3)_2 \cdot 6H_2O$; Gallium nitrate $Ga(NO_3)_3 \cdot H_2O$; Sodium tungstate dihydrate, $Na_2WO_4 \cdot 2H_2O$; Palladium nitrate dihydrate $Pd(NO_3)_2 \cdot 2H_2O$; Platinum cyanide, $Pt(CN)_2$.

Nutrient broth, nutrient agar and soil extract medium was used for isolation of the bacterial strains. The soil extract medium contained 500 mL / L soil extract and 15g / L agar. The soil extract was prepared after a recipe described earlier by Karelova (Karelova *et al.*, 2011) by mixing 1000 g of soil with 2 L of double distilled water and incubating overnight at room temperature. The mixture was filtrated and centrifuged at 15000 rpm. The supernatant was sterilized three times by autoclaving.

Isolation of bacterial strains

Two different strategies (S1 and S2) were employed for the isolation of bacteria from the mine wastes samples.

S1: Ten grams of waste powder were suspended in sterile saline water and shaken for a period of 60 minutes. The solution obtained was kept for 24 hours in sterile condition, at room temperature, with occasional shaking. After 24 hours, the suspension was filtered through Whatman 1 filter paper, under sterile conditions. These bacterial suspensions were serially diluted and aliquots (0.1 mL) were used as inoculum on soil extract agar prepared as mentioned above. The plates were incubated at 37 ° C, for bacterial growth.

S2: Two grams of powder were added to 250 ml Erlenmeyer flasks containing nutrient broth. The flask were left to incubate at variable temperatures, between 10 - 50 ° C. When the bacterial growth became visible, the strains were streaked on nutrient agar to ensure their purity.

Independently growing colonies were randomly selected (on the basis of morphology) subculture continuously and purified by repeated striking and dilution plate methods (Choudhary *et al*., 2009). After purification Gram status was determined. The bacterial isolates obtained were maintained on nutrient agar at 4 ° C. Afterwards the strains were evaluated for the ability to grow on minimal agar in the presence of different critical metal concentrations.

Screening of bacterial isolates with critical metal tolerance

The isolated strains were tested for their metal tolerance using the agar diffusion method. The agar was supplemented with successively higher concentrations (0, 5, 10, 25, 50, and 100 mg /L) of the critical metals mentioned above. The plates were inoculated with the selected bacterial isolates and incubated for 48 h. The growth of bacteria on the plates containing culture media with no metals was considered as control.

RESULTS AND DISCUSSION:

The experiments performed aimed a preliminary screening of bacterial isolates from mining wastes followed by the isolation of bacterial isolates in order to identify potential candidates for recovery of high tech critical metals from mine wastes.

A total of nine samples were collected from the mine wastes as shown in Table 1. After applying two different strategies (S1 and S2) for isolation of bacterial strains, resulted one bacterial strain labelled B1, from S1, and two strains labelled B2 and B3 from the second protocol performed. All of the strains resulted from the samples collected from 10 cm below the surface of the tailing. The strain B2 was isolated from the plates incubated at ambient temperature (23 -

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24 ° C) and the strain B3 was isolated from the plates incubated at temperatures above (50 ° C). The bacterial colonies were studied with respect to size, color, opacity, and form, (data not shown). All bacterial strains proved to be Gram-negative.

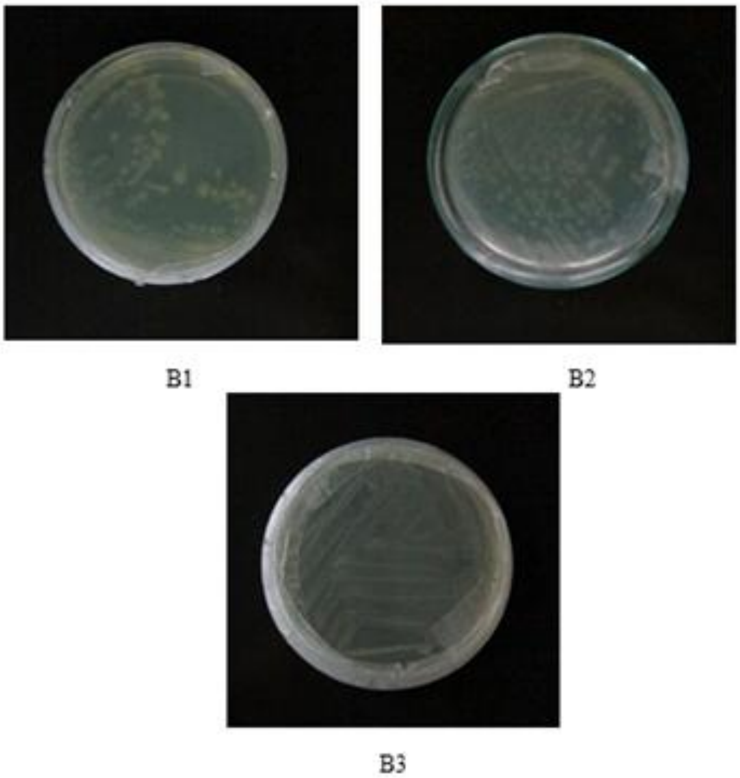

Fig. 1. The aspect of purified bacterial isolates on nutrient agar

Up to the present, the research studies performed, in terms of the ability of microorganisms to accumulate certain metals were focusing on the use of microorganisms (bacteria or fungi) in bioremediation through bioaccumulation of toxic metals, heavy metals and residual dyes. The biological approaches based on metal-resistant microorganisms have received a great deal of attention as alternative remediation processes of polluted soils (Gadd, 2010). The study on bioleaching of high tech critical metals is the research is at a very starting point. It must be taken into consideration that bioleaching process is influenced by a wide range of parameters including physicochemical and microbiological factors, which can affect both the growth of the microorganisms and their leaching behavior. For bioleaching to be successful, it is obvious that optimal growth conditions must be maintained and the microorganism must be able to leach the material. In addition, and most importantly, the microorganism must be resistant to the metals that are leached out (Monballiu et *al*., 2015).

The tolerance of the bacterial isolates (B1 to B3) to different critical metals is presented in Table 1. Some of the bacterial isolates presented a good growth in the presence of low concentrations of some of the critical metals, while only B1 could grow at higher concentrations (100 mg / L). However, no isolates could tolerate wolfram/tungsten and gallium at any of the concentrations tested. Furthermore, the strain B2 did not tolerate palladium either, and the strain B3 did not tolerate magnesium at any of the concentrations tested. Among the bacterial isolates, B1 showed multiple-metal resistance, at all concentrations to Magnesium, a medium growth for Platinum (5 mg / L) and low growth to Platinum (10 mg / L) and Palladium (5 mg / L).

The difference in the tolerance toward the bacterial isolates could be explained by the conditions of bacterial isolation and the nature and physiological characteristics of each bacterial isolate.

Table 2.
Resistance of bacterial isolates to critical metals and concentrations

Critical metal	Concentration (mg L^{-1})	Bacterial isolate		
		B1	***B2***	***B3***
Magnesium	0	+++	+++	+++
	5	+++	++	X
	10	+++	++	X
	25	+++	X	X
	50	+++	X	X
	100	+++	X	X
Palladium	0	+++	+++	+++
	5	±	X	+++
	10	X	X	±
	25	X	X	±
	50	X	X	X
	100	X	X	X
Platinum	0	+++	+++	+++
	5	++	±	+++
	10	±	X	+++
	25	X	X	X
	50	X	X	X
	100	X	X	X
Gallium	0	+++	+++	+++
	5	X	X	X
	10	X	X	X
	25	X	X	X
	50	X	X	X
	100	X	X	X
Wolfram/tungsten	0	+++	+++	+++
	5	X	X	X
	10	X	X	X
	25	X	X	X
	50	X	X	X
	100	X	X	X

X=Negative; +++ Positive; ++ Medium growth ± Low growth

Recovery of critical metals from several sources such as contaminated waters, sites, biomining leachates, and solid wastes, is of great interest for the European market. Some authors consider that naturally occurring microorganisms are a cost-effective method for concentrating diffuse elements from effluents and leachates prior to recovery (Nancharaiah, *et al.*, 2016).

Other authors, such as Van Passel *et al.* (2013) have a less optimistic point of view. The author considered that a substantial economic potential exists for mine waste projects and describes several economic methodologies for exploring the economic potential of landfill mining (Van Passel *et al.*, 2013), including private and social costs and benefits, where the inclusion of recovered critical metals definitely increases the profits. Unfortunately, the technology performance from efficiency and profitability point of view is an uncertainty that keeps the investors out of these projects (Krook *et al.*, 2012).

Temperature, pH, oxygen, salinity and the availability of nutrients are the most important parameters that must be taken into account for the growth and maintenance of the microorganisms tolerant to critical metals. These parameters are species dependent and therefore it is good to determine to which classification the microorganism belongs.

CONCLUSIONS:

Critical metals are defined as elements which are essential for economic development but are associated with limited availability and a supply security risk. The scarcity of the high tech critical metals is seen as an increased supply risk and is resulting in a great price volatility. High tech critical metals are essential in electronic and green energy technologies, but are only available at low abundance or can be found in only a few geographical areas.

Most of the critical metals are currently obtained from primary sources which are limited, present unequal geographical distributed, and are rapidly diminishing as a result of urbanization, increasing standards of living, and the population explosion. There is thus an urgent need to find new and innovative solutions to source these critical elements from alternative sources using sustainable technologies.

The studies undergone so far regarding metal-microorganisms interactions were focused on bioremediation, meaning the use of naturally occurring microorganisms to break down hazardous substances into less toxic or nontoxic substances by precipitation, sorption, and accumulation. It would be a great achievement if the research activities should stronger focus on technologies that unite bioremediation with element recovery and reuse for the recuperation of critical metals.

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