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Metal-Resistant Rhizobacteria Change Soluble-Exchangeable Fraction in Multi-Metal-Contaminated Soil Samples

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ABSTRACT: There is a complex interaction between various components of the soil ecosystem, including microbial biomass and soil chemical contaminants such as heavy metals and radionuclides, which may greatly affect the efficiency of bioremediation techniques. The aim of this study was to investigate microbial capacity to change pH, changes in the metal soluble-exchangeable fraction, and effects of initial heavy metal contents on soil samples in microbial solubilization/immobilization capacity. The soil samples used in this study were collected at a known metal-contaminated site. Three highly metal-resistant bacteria were isolated from rhizosphere soil samples collected on weed species identified as *Senecio brasiliensis*, *Senecio leptolobus*, and *Baccharis trimera*. A completely randomized experimental design in a factorial arrangement was used, with three replicates. In general, with an acid pH, the isolates neutralized the contaminated growth media. In a neutral or basic initial pH, increases in pH were observed in the media, so these bacteria have an alkalizing effect on the growth media. Soluble metal contents were quite different and depend on the microbial species and heavy metal contents in the soil samples. The soluble-exchangeable fraction of metal such as Cu, Zn, Ni, Cr, Cd, Pb, and Ba may be unavailable after inoculation with heavy metal-resistant rhizobacteria. A promising approach seems to be the application of inoculants with metal-resistant bacteria in bioremediation of multi-metal-polluted environments to improve the efficiency of this environmentally friendly technology.

Keywords: trace elements, bioremediation technology, rhizosphere, environmental contamination.

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INTRODUCTION

Heavy metals are derived from natural and anthropogenic sources. Anthropogenic contamination of the environment is the most widely distributed and most deleterious, probably as a result of its instability and solubility and, hence, bioavailability (Abdu et al., 2017). Around the world, many abandoned mining sites are highly contaminated with multiple heavy metals and, therefore, studies specifically focusing on isolating bacterial strains with resistance to heavy metals are necessary to find solutions for immobilizing or solubilizing these pollutants in the environment.

In some studies, microbes isolated from heavy metal-contaminated soil samples have been the focus of two lines of research. One applies these metal-resistant microorganisms to solubilization of heavy metals associated with plant species with a phytoaccumulation potential, enhancing metal extraction (phytoextraction), or applies them as plant growth-promoting or as plant protection-promoting microorganisms through the production of phytohormones, gel sequestration of metal, siderophores, atmospheric nitrogen biological fixation, phosphate solubilization, or biosurfactant production, among others. Another strategy has been to use these microorganisms to change the oxidation state of metal or to immobilize heavy metals by bioreduction or biosorption, decreasing metal-bioavailable forms (Baldi et al., 2001; Braud et al., 2006; Thavasi., 2011; Tak et al., 2013; Anaukwu et al., 2016; Giovanella et al., 2016; Abdu et al., 2017; Boechat et al., 2017; Chen et al., 2017; Giovanella et al., 2017).

There is complex interaction between various components of the soil ecosystem, including microbial biomass and soil chemical contaminants such as heavy metals and radionuclides, which may greatly affect the efficiency of bioremediation techniques. Furthermore, the bioavailability of heavy metals in soil to plants can be influenced by soil factors, including scaly cells and root exudates, microbial activities, and metal diversity and/or content (Kabata-Pendias, 2011; Ma et al., 2011; Ma et al., 2015; Boechat et al., 2016a; Chen et al., 2017).

We hypothesized that the metal in soil samples with different contents of heavy metal contamination may be immobilized by metal-resistant rhizobacteria, reducing the heavy metals available in the soil solution. Secondly, we hypothesized that the solubility of heavy metals may be changed by the levels of contamination in the soil samples.

The aim of this study was to investigate changes in pH and the metal soluble-exchangeable fraction in heavy metal-contaminated soil samples through the activity of metal-resistant rhizobacteria, analyzing their solubilization/immobilization capacity for bioremediation purposes.

MATERIALS AND METHODS

Site and soil properties

The soil samples used in this study were collected at a known metal-contaminated site previously described in Boechat et al. (2016b). The soil was classified as an Entisol Orthent (Soil Survey Staff, 2014) or *Neossolo Litólico* (Santos et al., 2013). The contaminated soil samples were collected at previously determined points in order to form a systematic "square mesh" distribution to characterize initial heavy metal distribution at the site. Soil sample collections were collected in four points at 0.00-0.20 m layer. Soil samples were characterized chemically and physically by the methodology described in Tedesco et al. (1995). Soil sample fractions were air dried, passed through a 2 mm sieve, mechanically mixed to ensure homogeneity, and then stored prior to the experiment.

The soil samples were digested using the hot-block digestion procedure according to EPA 3050B (Usepa, 1996) for total metal content, which was analyzed in an inductively coupled plasma optical emission spectrophotometer (Perkin Elmer Optima 7000 DV, Perkin - Elmer Corporation, Norwalk, CT, USA). Selected properties of the soil samples collected for this study are shown in table 1.

Table 1. Chemical and physical properties of the soil samples from a multi-metal-contaminated site used in this study

Property	SS1	SS2	SS3	SS4
pH_{water}	6.0	6.0	5.3	5.1
P (mg dm^{-3})	30.0	7.9	7.0	3.4
K^+ ($cmol_c$ dm^{-3})	0.2	0.3	0.3	0.2
Ca+Mg ($cmol_c$ dm^{-3})	7.7	5.0	9.3	4.3
H+Al ($cmol_c$ dm^{-3})	2.8	2.2	1.0	0.7
CEC ($cmol_c$ dm^{-3})	10.7	7.5	12.9	10.7
V ($cmol_c$ dm^{-3})	74	71	73	42
SOM (%)	2.7	1.4	3.4	2.3
Clay (%)	14	12	15	15
Zn (mg kg^{-1})	167.0	42.0	170.0	9.4
Cu (mg kg^{-1})	61.0	4.7	9.5	0.8
Mn (mg kg^{-1})	13.0	4.0	30.0	37.0
Cd (mg kg^{-1})	2.0	0.4	2.2	<0.2
Ni (mg kg^{-1})	8.0	5.0	9.0	3.0
Cr (mg kg^{-1})	12.0	9.0	15.0	5.0
Pb (mg kg^{-1})	599.0	46.0	227.0	72.0
Ba (mg kg^{-1})	109.0	84.0	164.0	149.0
As (mg kg^{-1})	45.0	<2.0	7.0	<2.0

Clay: determined by the hydrometer method, using 0.1 mol L^{-1} NaOH; exchangeable Ca and Mg: extracted with 1 mol L^{-1} KCl; P and K^+: estimated by Mehlich^{-1}; H+Al: extracted with 0.5 mol L^{-1} calcium acetate solution, pH 7.0; SOM (soil organic matter): determined by the Walkley-Black combustion method (Tedesco et al., 1995). SB: sum of bases; CEC: cation-exchange capacity; V: base saturation.

Bacteria collection and identification

Three highly lead (Pb^{+2}) resistant bacteria [*Kluyvera intermedia* (Ki), *Klebsiella oxytoca* (Ko), and *Citrobacter murliniae* (Cm)] were isolated from rhizosphere soil samples collected on weed species identified as *Senecio brasiliensis* (Spreng.) Less., *Senecio leptolobus* DC., and *Baccharis trimera* (Less) DC., respectively, collected in a metal-contaminated area and used as a metal-tolerant strain in this study (unpublished data).

Bacterial isolates were incubated in Luria-Bertani culture (LB) for 24 hours in an orbital rotating incubator (140 rpm) at a temperature of 30 °C for subsequent extraction of chromosomal DNA. The DNA was extracted using the Wizard SV Genomic DNA Purification Kit System (Promega) according to the following protocol: 500 µL bacterial culture was centrifuged for 2 min and the supernatant discarded. Cells were suspended in 500 µL of lysis solution/RNase and incubated for 10 min in a water bath at 80 °C. Cell lysates were then transferred to a spin-column and centrifuged for 3 min, and the collection tube contents were discarded. After that, 650 µL of wash solution was added to the spin-column and then centrifuged for 1 min. This procedure was repeated three times. The quantity of 200 µL DNA rehydration solution was added to the spin-column, incubated for 2 min at room temperature, and centrifuged for 1 min. The extracted DNA was removed from the collection tube and stored in 2.0 mL micro centrifuge tubes at -5 °C.

Precisely 20 µL extracted DNA were used to verify that DNA extraction was efficient. The reaction was evaluated by agarose gel electrophoresis (1.0 % w/v) in TAE 1× buffer (Tris-acetate 40 mmol L^{-1}, EDTA 1 mmol L^{-1}) and stained with blue green loading dye according to the manufacturer's specifications. Observation was performed under ultraviolet light in a horizontal transilluminator.

Isolates were identified based on partial sequencing of the 16S rRNA region. The universal primers used in bacteria were 27F (5'-AGATTTGATCMTGGCTCAG-3') and 1492R (TACGGYTACCTTGTTACGACTT 5'-3') for amplification of 16S ribosomal RNA in PCR. The PCR reaction mixture consisted of 12.5 µL of PCR Master Mix (Promega, Madison, WI, USA), 0.5 µL of genomic DNA sample, 2.5 µL (12.5 umol) of 27F primer oligonucleotide, and 2.5 µL (12.5 umol) primer oligonucleotide 1492R, and a final volume of 25 µL was completed with nuclease-free water.

Amplification was performed in a thermocycler (TECHNE CT - 512) using a basic program consisting of 35 cycles (initial denaturation at 95 °C for 5 min, subsequent denaturation at 95 °C for 30 s, annealing at 50 °C for 1 min, extension at 72 °C for 1 min, and final extension for 5 min). Millipore Montage PCR Filter Units (Millipore, Billerica, MA, USA) was used to remove oligonucleotide primers salts, and unincorporated dNTPs according to the manufacturer's instructions.

A BigDye terminator kit (Applied Biosystems, Foster City, CA, USA) was used for the DNA sequencing cycle with the primers 27F (5'-AGATTTGATCMTGGCTCAG-3') and 1492R (TACGGYTACCTTGTTACGACTT 5'-3'). The reactions were read in an ABI-PRISM 3100 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA, USA). All sequences generated were analyzed using the Genbank database at the National Center for Biotechnology Information (NCBI, USA).

Bacterial isolates were identified based on partial sequencing of the 16S rRNA region deposited in the Genbank database under accession numbers NR028803.1, NR028802.1, and NR028688.1. The similarity of gene sequencing of 16S rRNA was 96 % with *K. intermedia*, 98 % with *K. oxytoca*, and 97 % with *C. murliniae*.

Inoculum preparation

Bacteria were grown and maintained in Luria-Bertani liquid medium, which contained 5.0 g yeast extract, 10.0 g tryptone, 10.0 g sodium chloride, and 300 mg L^{-1} Pb^{2+}, with an initial pH of 6.5. Metal-tolerant bacterial cells were inoculated into 250 mL Erlenmeyer flasks containing 150 mL of sterile medium, which were cultivated aerobically in an orbital/rotary shaker (140 rpm) at 30 °C for 24 h. The non-bacterial (control) inoculum was prepared with the same sterilized medium under the same conditions.

The liquid media in which inoculums were grown were centrifuged at 10,000 rpm at a temperature of 6 °C for 6 min. The supernatant was discarded and the pellet suspended with saline solution (0.8 % NaCl). This procedure was performed three times. The pellet was then suspended and adjusted to a concentration of approximately 2.0×10^8 colony forming units (CFU) per gram of soil based on optical density (OD; λ600) using a spectrophotometer (Spectrumlab 22PC).

pH medium changes

Luria-Bertani culture medium (LB) supplemented with 300 mg kg^{-1} Pb^{2+} was used to evaluate the effect of isolated changes in pH. Sterilized LB-Pb^{2+} was adjusted to pH 3.5, 4.0, 5.0, 6.0, 6.5, and 7.0 with predetermined amounts of sterilized HCl 1 mol L^{-1} or NaOH 0.1 mol L^{-1}. The media were inoculated with 100 uL of each isolate (10× dilution of inoculum) and incubated under orbital shaking (140 rpm) at 30 °C for 24 h, with three replications. The media containing the inoculums were centrifuged at 1,225 *g* for 6 min at a temperature of 4 °C. After centrifugation, the culture supernatants were diluted five times to directly read the final pH in a benchtop pH meter (Digimed - DM22).

Soluble-exchangeable heavy metals

The experiment was carried out in 50 mL autoclaved conical tubes under laboratory conditions in a 4 × 4 factorial arrangement of four contaminated soil samples and three bacterial inoculums plus a control treatment without inoculation.

In the first step, 10 g of each autoclaved soil sample were transferred to autoclaved conical tubes. Soil samples were autoclaved for 40 min at a temperature of 121 °C under a pressure of 1 atm in order to eliminate all microorganisms. Soil samples were irrigated with ultrapure water at 60 % of water-holding capacity. Treatments were added to each tube and then all the conical tubes were incubated at 30 °C for 20 days.

In the second step, extraction was carried out in triplicate, using 1 g of air-dried soils. Soil samples were placed in 50 mL conical centrifuge tubes and 15 mL of $CaCl_2$ 0.1 mol L^{-1} was added to the tubes. All tubes were placed in a rotary shaker (120 rpm) at room temperature for 2 h. After equilibration, the solution and solid phases were separated by centrifugation at 1,225 *g* for 10 min. The supernatants were filtered through a 0.45 µm membrane (Silveira et al., 2006; He et al., 2013) and then analyzed for Cu, Zn, Ni, Cr, Cd, Pb, As, and Ba in an inductively coupled plasma-optical emission spectrometer (ICP-OES, Perkin Elmer Optima 7000 DV, Perkin - Elmer Corporation, Norwalk, CT, USA) in triplicate.

Experimental setup and statistical analyses

A completely randomized experimental design in a factorial arrangement was used, with three replicates. The data obtained from the experiment were subjected to analysis of variance and means were compared by Tukey's test at 1 %. The Sisvar statistical program (Ferreira, 2011) was used for statistical analyses of data.

RESULTS AND DISCUSSION

Changes in pH of the medium

Isolated bacteria resisted media with pH >4.0 but not media with pH ranging from 3.5 to 4.0, as shown in table 2. In general, in an acidic pH (5.0), isolates neutralize the growth media to a pH between 6.1 and 6.4. In an initial neutral or basic pH (6.0 and 6.5), increases in the pH of the media up to 7.3 were observed; thus, these bacteria have an alkalizing effect on the growth media (Table 3). However, at an initial pH of 7.0, the isolates under study did not change the pH of the media (Table 2). Sabullah et al. (2016) observed that activity by inhibition of molybdenum reduction was dramatic at pH lower than 5.0 by the *Klebsiella oxytoca* strain Saw-5. In contrast, Liu et al. (2016), studying 16 different *Klebsiella* species, observed a pH tolerance level from 3.5-10.5 and optimum pH for growth from 4.0-10.0, which is very common in these groups of bacteria.

Soluble-exchangeable heavy metals

Soluble heavy metal contents were quite different and depended on the microbial species and heavy metal concentrations in the soil samples (Tables 3 and 4). The concentrations

Table 2. Changes of pH in Luria-Bertani culture medium (LB) supplemented with 300 mg kg^{-1} Pb^{2+} by metal-resistant rhizobacteria

pH of liquid medium				
Initial	Final*			
	Ki	Ko	Cm	Control
3.5	ng	ng	ng	3.5
4.0	ng	ng	ng	3.9
5.0	6.1	6.3	6.4	5.1
6.0	6.7	6.8	6.7	6.2
6.5	6.6	7.1	7.3	6.5
7.0	7.0	7.0	6.9	7.2

* Mean values of three replicates; Ki: *Kluyvera intermedia*; Ko: *Klebsiella oxytoca*; Cm: *Citrobacter murliniae*; Control treatment: liquid medium without inoculum; ng: no growth.

of available Cu decreased with the *K. intermedia* (Ki), *K. oxytoca* (Ko), and *C. murliniae* (Cm) treatments in SS1 by 34.8, 38.5, and 38.0 %, respectively, compared to the control treatment (no inoculum). No significant differences in available Cu were found in soil samples SS2, SS3, and SS4 for any treatments. Microorganisms take up metals, several of which play important cell metabolic functions. However, it has been shown that cells of microorganisms may have quite variable affinities for radionuclides that are necessarily related to their biological function (Kokke, 1972; Arigony et al., 2013).

Generally, this characteristic is related to metal resistance of the microorganism through various mechanisms developed to adapt to these environments. All mechanisms are found either to prevent entry of metal ions into the cell or to actively pump the metal ions out of the cell (Hughes and Poole, 1989; Silver, 1996) and, these mechanisms appear to be related to a higher concentration of the metal ion in the medium, e.g., the Cu concentration in SS1 was 76 times higher than the concentration in SS4 (Tables 1 and 3).

For Zn in soil sample SS3, lower amounts of the element in solution were observed in the Ki, Ko, and Cm treatments, with a decrease in the soluble Zn concentration of 41.2, 9.8, and 54.9 %, respectively, compared to the control; the lowest concentration (36.06 mg L^{-1}) was observed in the Cm treatment (Table 3). In the SS1 soil sample, treatments decreased Zn concentrations in the soil solution an average of 45.9 %. The highest available Zn concentrations were found in SS3, followed by SS1, and lower concentrations were found in SS2 and SS4 (Table 3).

Table 3. Metal concentration available in soil samples after application of bacterial inoculum

Treatment	Cu	Zn	Ni	Cr
	mg L^{-1}			
	Soil sample SS1			
Ki	1.44±0.12 bA	22.72±1.14 bB	0.06±0.017 abB	0.07±0.007 bB
Ko	1.36±0.04 bA	21.83±0.82 bB	0.05±0.013 bB	0.07±0.002 bC
Cm	1.37±0.06 bA	21.38±1.19 bB	0.06±0.01 abB	0.07±0.003 bBC
Control	2.21±0.12 aA	40.64±0.96 aB	0.09±0.009 aB	0.09±0.002 aB
	Soil sample SS2			
Ki	0.11±0.07 aC	6.30±0.38 aC	0.02±0.009 aC	0.06±0.001 aC
Ko	0.10±0.01 aC	5.27±0.23 aC	0.02±0.007 aB	0.06±0.002 aC
Cm	0.09±0.006 aC	5.09±0.11 aC	0.02±0.007 aC	0.06±0.001 aC
Control	0.12±0.02 aC	7.58±0.21 aC	0.03±0.006 aC	0.07±0.004 aC
	Soil sample SS3			
Ki	0.67±0.06 aB	46.88±8.45 cA	0.17±0.03 bA	0.07±0.003 bBC
Ko	0.79±0.12 aB	72.00±2.17 bA	0.29±0.003 aA	0.09±0.002 aB
Cm	0.73±0.06 aB	36.06±1.64 dA	0.13±0.01 cA	0.07±0.001 bB
Control	0.79±0.03 aB	79.85±4.89 aA	0.32±0.003 aA	0.09±0.004 aB
	Soil sample SS4			
Ki	0.009±0.008 aC	2.48±0.24 aC	0.009±0.004 aC	0.13±0.003 bA
Ko	0.001±0.0000 aC	1.80±0.13 aC	0.02±0.001 aB	0.14±0.001 abA
Cm	0.000±0.001 aD	1.67±0.10 aD	0.006±0.003 aC	0.13±0.005 bA
Control	0.001±0.002 aC	1.06±0.12 aC	0.01±0.004 aC	0.15±0.004aA
CV (%)	9.97	11.12	13.72	3.55

Data were displayed as mean ± standard deviation. Means followed by the same lowercase letter do not differ among treatments in each soil, and means followed by the same uppercase letter do not differ among soils in each treatment by Tukey's test ($p<0.01$); Ki: *Kluyvera intermedia*; Ko: *Klebsiella oxytoca*; Cm: *Citrobacter murliniae*; control treatment: liquid medium without inoculum; ng: no growth; CV: coefficient of variation.

For the element Ni, soil samples SS1 and SS3 have higher available Ni concentrations, followed by SS2 and SS4 (Table 3). These results were according to the total Ni concentrations presented in table 1 using the acid digestion method for soil sample analysis. In SS1 for the treatment with *K. oxytoca* and in SS3 with *K. intermedia* and *C. murliniae*, a decrease in available Ni concentration was observed. This occurs because bacteria immobilize metals present in the soil. Since most heavy metal-microbe interactions are initiated at the level of uptake, the uptake mechanism is likely to be closely linked to the mechanism of heavy metal resistance in the microorganism (Kang and So, 2016).

The concentrations of available Cr decrease for all bacterial treatments in the SS1 soil sample and for *K. intermedia* and *C. murliniae* treatments in SS3 and SS4. Comparing the concentration of available Cr among soil samples, SS4 exhibited higher values, but for total concentration, SS4 had a lower concentration (Tables 1 and 3).

In table 4, in soil sample SS1, average decreases of 34.6, 34.1, and 15.72 % were observed in the available Cd, Pb, and Ba concentrations, respectively, compared to the control treatment. In the SS2 and SS4 soil samples, no significant differences were observed among inoculum treatments for Cd, Pb, and As. In the SS3 soil sample, there were no significant differences for As and Pb. However, available Cd concentrations decreased in the bacterial treatments compared to the control treatment. In all soil sample bacterial treatments, there was a decrease in the available concentration of Ba compared to the control treatment (Table 4).

Comparing available heavy metal concentrations among soil samples, higher concentrations of Cd in Ki, and Cm were observed in SS1 and in SS3 for all treatments. For Pb, a higher concentration was observed in SS1. For As, no significant differences were observed among soil samples, and for Ba, significant differences were found in the SS4 soil sample (Table 4).

Table 4. Available heavy metal contents in soil samples after application of bacterial inoculum

Sample	Cd	Pb	As	Ba
	mg L^{-1}			
	Soil sample SS1			
Ki	0.17±0.009 bA	0.33±0.06 bA	0.06±0.05 aA	27.0±0.47 bC
Ko	0.17±0.002 bB	0.31±0.03 bA	0.09±0.01 aA	26.8±0.68 bC
Cm	0.17±0.007 bA	0.33±0.05 bA	0.03±0.03 aA	26.8±0.49 bC
Control	0.26±0.007 aB	0.47±0.06 aA	0.02±0.03 aA	31.8±0.39 aC
	Soil sample SS2			
Ki	0.05±0.003 aB	0.14±0.01 aB	0.06±0.02 aA	31.1±0.64 bB
Ko	0.05±0.004 aC	0.11+0.01 aB	0.09±0.06 aAB	31.6±0.34 bB
Cm	0.04±0.003 aB	0.10±0.01 aB	0.03±0.03 aA	31.2±0.46 bB
Control	0.07±0.002 aC	0.10±0.01 aB	0.02±0.03 aA	35.4±1.3 aB
	Soil sample SS3			
Ki	0.20±0.03 cA	0.08±0.019 aBC	0.05±0.04 aA	25.2±0.62 bC
Ko	0.27±0.01 bA	0.06±0.008 aB	0.03±0.03 aAB	28.9±0.76 aBC
Cm	0.16±0.01 dA	0.10±0.039 aB	0.07±0.06 aA	23.5±0.28 bD
Control	0.30±0.01 aA	0.08±0.012 aB	0.03±0.03 aA	29.7±1.0 aC
	Soil sample SS4			
Ki	0.00±0.000 aC	0.03±0.007 aC	0.0±0.00 aA	60.2±1.22 bA
Ko	0.00±0.000 aD	0.02±0.029 aB	0.0±0.00 aB	59.0±2.32 bA
Cm	0.00±0.000 aC	0.03±0.034 aB	0.0±0.00 aA	58.3±1.17 bA
Control	0.00±0.000 aD	0.05±0.071 aB	0.02±0.03 aA	67.6±1.02 aA
CV (%)	7.91	22.49	79.85	2.6

Data were displayed as mean ± standard deviation. Means followed by the same lowercase letter do not differ among treatments in each soil, and means followed by the same uppercase letter do not differ among soils in each treatment by Tukey's test ($p<0.01$). Ki: *Kluyvera intermedia*; Ko: *Klebsiella oxytoca*; Cm: *Citrobacter murliniae*; control treatment: liquid medium without inoculum; ng: no growth; CV: coefficient of variation.

These results indicate that solubilization and/or immobilization of heavy metals in the soil is affected not only by the microbial species, but also by the type and concentration of the chemical element, the pH of the medium, and the presence of other metallic ions. The understanding that the soluble-available metal content is not only dependent on the total concentration of the element in the soil, but on local environmental characteristics, soil parent material, mineralogical and chemical properties of soil, and biological activity (Kabata-Pendias, 2011), the contents of elements and dynamics among elements become important from the environmental and food security perspective.

Soil bioaugmentation with the metal-resistant bacteria enhanced Cd and Pb uptake by the plant species *Mucuna deeringiana* grown in soil samples with high heavy metals contents. In general, these bacteria had either no significant effect in a soil sample with a lower metal concentration, except to Cr in SS4 soil sample (Table 3). The authors concluded that the fate of the soil heavy metals depended on two opposite phenomena which acted simultaneously, the first being metal immobilization by microorganisms through their passive adsorption onto the cells and/or to their active bioaccumulation into the cells, and the second being increase in metal bioavailability through the extracellular synthesis of biosurfactants, siderophores, and/or organic acids. As a consequence, according to biotic and abiotic conditions in the soil, there was an increase or a decrease in metal bioavailability (Braud et al., 2006; Boechat et al., 2017).

Therefore, changes in metal bioavailability in the soil samples studied may be caused by products of microbial origin, such as biosurfactants, siderophores, and organic acids. In general, resistance mechanisms are associated with bacterial adaptation to metal stress environments (Tak et al., 2013). These mechanisms may be utilized in detoxification and removal of heavy metals from contaminated soils (Ahmed et al., 2005), e.g., biosurfactants are a structurally diverse group of surface-active substances produced by microorganisms. All biosurfactants are amphiphiles, they consist of two parts - a polar (hydrophilic) moiety and non-polar (hydrophobic) group. A hydrophilic group consists of mono-, oligo-, or polysaccharides, peptides, or proteins, and a hydrophobic moiety usually contains saturated, unsaturated, and hydroxylated fatty acids or fatty alcohols (Lang, 2002). A characteristic feature of biosurfactants is a hydrophilic-lipophilic balance (HLB), which specifies the portion of hydrophilic and hydrophobic constituents in surface-active substances (Pacwa-Płociniczak et al., 2011).

Anaukwu et al. (2016) observed biosurfactant production by the bacterial species *Citrobacter murliniae* AF025369. The authors said that the biosurfactant produced performed better than the synthetic surfactant in crude oil biodegradation. Biosurfactants increase the bioavailability of hydrocarbon, resulting in enhanced growth and degradation of contaminants by hydrocarbon-degrading bacteria present in polluted soil. In heavy metal-polluted soils, biosurfactants form stronger ionic bonds with metals at the soil interface, which is followed by desorption of the metal and removal from the soil surface, leading to increased metal ion concentrations and bioavailability of these metal ions in the soil solution (Pacwa-Płociniczak et al., 2011; Thavasi, 2011).

Furthermore, the siderophore-producing bacteria *Kluyvera ascorbate* SUD165 was able to provide sufficient ions for the growth of *Brassica juncea* and *Lycopersicun esculentum* in the presence of high contents of Ni, Pb, and Zn (Burd et al., 1998). Baldi et al. (2001) cultivated the bacteria *Klebsiella oxytoca* strain BAS-10 anaerobically in a 50 mL medium previously boiled for 5 min to degas O_2. The authors observed, under laboratory conditions, that polymer secreted by BAS-10 binds Pb(II) efficiently and, to a lesser degree, Cd(II) and Zn(II), but not Fe(II). Metal co-precipitation occurring in the iron gel indicates that BAS-10 reduces metal mobility.

CONCLUSIONS

Application of metal-resistant bacteria in environmental technologies (bioremediation and phytoremediation) was studied. The isolated bacteria *Kluyvera intermedia*, *Klebsiella oxytoca*, and *Citrobacter murliniae* do not grow in an acidic pH medium (<5.0) and change the pH of the medium to a range of 6.1 to 7.3. The soluble-exchangeable fraction of metals such Cu, Zn, Ni, Cr, Cd, Pb, and Ba can be unavailable in contaminated soil samples by the isolated bacteria *Kluyvera intermedia*, *Klebsiella oxytoca*, and *Citrobacter murliniae*. Metal-resistant rhizobacteria does not have significant effect in metal availability in a soil sample with a lower concentration with exception of chromium. A promising approach seems to be the application of inoculants of metal-resistant bacteria in the bioremediation of environments polluted by multi-metals to improve the efficiency of this environmentally friendly technology. The initial soil heavy metal concentration affects the heavy metals available in the soil samples studied. Additionally, we demonstrated that the heavy metal-resistant microorganisms may be potential agents for remediation programs.

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