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Extracellular Polymeric Substances (EPS)-Producing by Bacteria for the Bioremediation of Heavy Metals

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SUMMARY

The city of Santo Amaro- (BAHIA-BRAZIL) gained visibility among the scientific community due to the contamination of the Subaé river by lead and cadmium from the PLUMBUM Mineração e MetalurgiaLtda industry, on the banks of the river in 1956, which produced lead ingots. Subaé river water was collected for physical chemical analysis and bacterial isolation. It was verified that all isolated bacteria produced expressive amounts of Exopolysaccharide (EPS). Thus, the optimization of this production in different sugars (sucrose, glucose and mannitol) and in three different pHs: 5.5; 6.5 and 7.5. All bacteria produced EPS in large quantities and the best sugar was sucrose at pH 7.5. In order to use the EPS for the bioremediation area, the adsorption test of lead and cadmium was carried out by the isolated EPS. 0.5 g of the EPS was dissolved in 50 ml of deionized water, then the solutions of metals, lead acetate and cadmium sulfate (procedure performed separately) were incubated at 28°C for 16 h after that period, were centrifuged. Samples were filtered to separate the insoluble EPS, and the filtrates obtained were used in the quantification of the metals by atomic absorption (FAAS). *Bacillus* spp., *Bacillus cereus, Staphylococcus* spp., and *Serratia marcescens*, all showed tolerance to the tested metals, due to the efficiency in the adsorption capacity of the EPS, and it was possible to distinguish seven genera, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Lysini bacillus* spp. to be used in the bioremediation of environments contaminated with heavy metals.

Keywords: Bioremediation; Heavy Metals; Exopolysaccharide; Adsorption; Bacteria; Subaé River

Abbreviations: EPS: Exopolysaccharide; pH: Potential Hydrogeologic; FAAS: Flame Atomic Absorption Spectrometry; TCA: Trichloroacetic Acid

Introduction

The contamination of natural waters by heavy metals has been one of the major problems of society due to the increase in industrial activities and great efforts are being devoted to the development of ecologically more appropriate, effective and low-cost technologies for the remediation of contaminated environments. The Subaé-BA river, whose sources are in Feira de Santana and mouth in Salvador, had its waters contaminated by heavy metals with the installation of PLUMBUM Mineração e Metalurgia LTDA in 1956, on the banks of the Subaé River and 10 km from its mouth at Todos os Santos Bay (BTS). During the production process of lead ingots at the factory, the slag generated was composed of heavy metals, such as arsenic, cadmium, bismuth and, mainly, lead, which were deposited in the environment where they were subject to chemical or biological weather. The activities of this company lasted until 1993, the year of its closure. Heavy metals have received special attention due to toxicity and the consequent accumulation in the ecosystem throughout the food chain, thus making the search for new technologies and techniques more efficient and economical that allow the removal of these chemical elements from the contaminated environment. Bioremediation emerges in this context as a viable alternative for the recovery of areas contaminated by heavy metals, since it is gaining more and more importance, due to the advantages it offers such as simplicity, efficiency and low cost. Since the bioremediation of degraded ecosystems is the first step in research aimed at using microbial activity to convert toxic substances into less harmful compounds [1].

In this context, some bacteria are promising candidates for having specialized systems for resistance to metals. Among the mechanisms of resistance to metals, we have bioaccumulation, biosorption, reduction, production of siderophores and formation of biofilms and biofilters (Figure 1), which can be explored for the development of clean technologies for the control of metal pollution in order to promote mitigation of environmental impacts [2]. Biofilms, for example, can be defined as a set of microorganisms involved in a matrix that consists of a mixture of polymeric compounds, mainly polysaccharides known as Exopolysaccharide (EPS) [3]. EPS have the function of protecting the bacterial cell against desiccation and phage attack, as well as antibiotics, toxic and protozoan compounds, sequester essential cations and involvement in the adhesion on solid surfaces, in addition to the formation of biofilms. Another possible function of EPS is that it can be excreted in response to environmental stressors, such as exposure to heavy metals, reducing its toxicity. They are also capable of acting on the colonization of surfaces and, therefore, they can have a surfactant action, which allows their use as surfactants or emulsifiers and confer potentials for industrial use and in bioremediation processes [4-5]. This research was conducted to assess the impact of heavy metals in Rio, to isolate bacteria tolerant to heavy metals and with a mechanism that could be applied to the decontamination of water impacted by heavy metals. Only 8 bacterial isolates were obtained, but all with Pb and Cd adsorption capacity in EPS. From these data, we sought to evaluate the resistance mechanisms of microorganisms and techniques in the bioremediation of surface waters contaminated with lead (Pb) and cadmium (Cd).

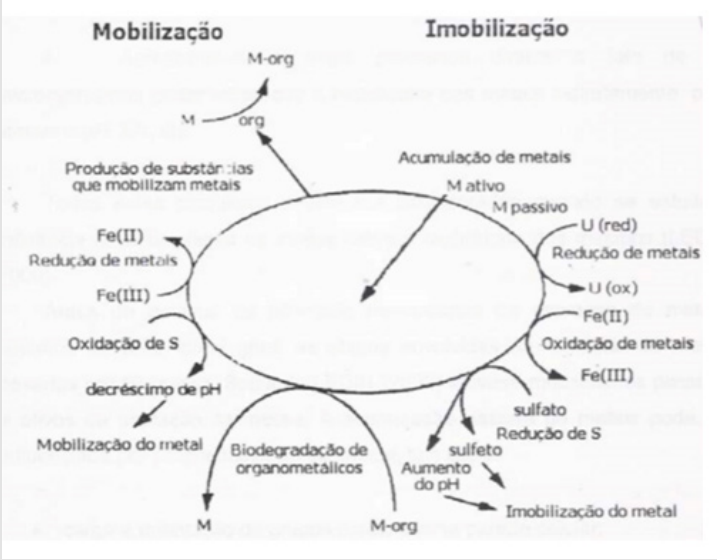

Figure 1: Interaction between metals and microorganisms. Source: Ledin (2000).

Materials and Methods

EPS Production

In May 2013, surface water samples from the Subaé River (Figure 1), in the Santo Amaro region, were collected approximately 500m from the PLUMBUM factory, to determine the physical-chemical and microbiological characteristics. The collection followed the standards described in the Standard Methods for Water and Wastewater Examination [6]. 900mL of surface water samples were collected at 6.1 m from the riverbank, in sterile glass bottles of 1 L capacity. From the water samples, eight bacteria were isolated and maintained in the laboratory, *Klebsiella pneumoniae* CCMB716, *Pseudomonas aeruginosa* CCMB717, *Lysinibacillus spp* CCMB718, *Bacillus spp* CCMB719, *Bacillus cereus* CCMB720, *Sthaphylococcus spp* CCMB721 and *Serratia marcescens* MBR7, and tested for the production of EPS in three sugars. EPS production was carried out in the base medium optimized by [7], with some adaptations, with sugars varying from 10% (sucrose, glucose and mannitol), pH (5.5, 6.5 and 7.5) and the standard incubation temperature of 28°C. The readings were performed with 24 hours of incubation. The method used was the one developed by [8]. This technique consists of inoculating 5µL of the active culture of each isolate [suspended in saline at an absorbance of 0.96 (+/- 600nm)] in sterile filter paper discs (5mm Ø), deposited on the solid culture medium. After the incubation period, a reading was made based on the formation of dense colonies on the discs. The level of biopolymer production was evaluated according to the diameter of the colony: from 0 to 6.9 mm considered without biopolymer production; from 7 to 14.9 mm, little production and above 15 mm, as great production.

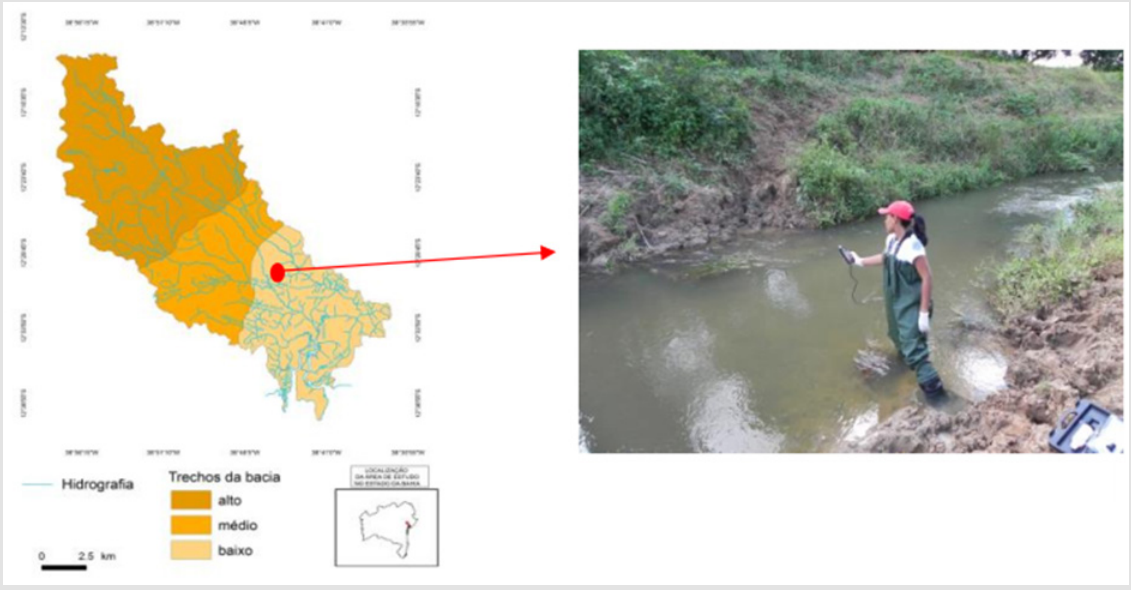

Figure 2: Map of the location of water collections in the lower course of the Hydrographic Basin of the Subaé River in the region of Santo Amaro-BA.

A rapid test was carried out to confirm the production of EPS, mixing a range of the potentially biopolymer-producing colony in 2mL of absolute ethanol. Half the ethanol capacity was placed in a glass test tube and with the platinum handle it took the contents around the disc (the viscous material that formed on the discs inserted in the EPS medium) and transferred it to the tube with ethanol so that this content comes off the platinum handle. The formation of a precipitate in the medium was indicative of the production of polysaccharide-type biopolymers. So, if there was precipitation without turbidity, it is because the content that grew at the bottom of the tube is EPS. If there was only turbidity, it is because the contents of the disc are bacterial colony mass. When testing the production of EPS with absolute ethanol, the result was positive for all eight bacteria isolated. Thus, after confirmation, isolates were selected that had the highest amount of mucoid substance for testing under different conditions. For this analysis, the pHs (5.5, 6.5 and 7.5), incubation temperature (28º, 35º and 42°C) and the carbon sources (sucrose, glucose and mannitol) at a concentration of 10% were varied, in order to verify which of these conditions would favor greater EPS production. The culture medium used was the same used for the initial selection of EPS production, with variation of the conditions mentioned. For each isolate, the experiment was carried out in triplicate. The experimental analysis consisted of measuring, with a caliper, the diameter of the mucoid layer around the filter discs and to optimize the tested conditions, the DOE-HLERT model was used [9].

Biochemical Characterization of EPS

The biochemical characterization of EPS was performed, its biochemical groups were analyzed and its potential for adsorption of heavy metals was tested, since the biosorption process occurs as a result of the interactions of metals and/or organic pollutants with the functional groups present in the constituent organic polymers cell surface or as a result of metabolic processes. Dry EPS analysis was performed by Fourier transform Infrared Spectroscopy (FTIV OR FTIR) with attenuated total reflectance (ATR-FTIR) in Shimadzu's IRAffinity-1 FTIR equipment, at Unifesp São José dos Campos, Instituto de Ciência e Environmental Technology/Biotechnology. Based on [10], the EPS spectra were obtained as follows: first 5 mg of the sample was spread over the surface of the ATR crystal, then the spectrum was obtained. A simple and quick instrumental technique that can show different functional groups of a molecule, as it depends on the interaction of the molecule with electromagnetic radiation in the Infrared (IV) region, and the absorption of electromagnetic radiation in the IV region interferes with the vibrations of the connections covalent, increasing their amplitude.

EPS Production for Metal Adsorption (Lead and Cadmium)

The bacteria were activated in Broth Müeller Hinton (CMH) medium at 37°C for 24 h. After that period, the bacteria were suspended in 0.85% saline until absorbance of 0.96 (+/- 600nm) was obtained. Then, 1% of the bacterial solution was inoculated in 100 mL of EPS medium, pH 7.0 and incubated at 28°C for 24 h, with and without shaking at 150 rpm. All tests were done in triplicate. The extraction of the EPS produced occurred in 02 ways: one with the use of 10% trichloroacetic acid (TCA), CCl_3COOH, and the other without TCA. TCA is widely used in biochemistry for the precipitation of macromolecules such as proteins, DNA and RNA. Thus, EPS WITHOUT TCA contains proteins and COM TCA does not, to analyze whether protein groups interfere or not with metal adsorption, since TCA has a fragmenting effect on the protein chains present in the produced polymers. For EPS extracted with 10% TCA, 100 ml of 10% TCA was added to each 100 ml of the medium with the inoculated culture, maintaining the ratio (1:1), stirred in the shaker at 90 rpm, 25°C +/- 2 for 30 minutes.

Discussion

EPS Production

All bacteria produced EPS in large quantities and the best sugar was sucrose with pH 7.5, a condition in which the EPS production diameter exceeded 18 mm (Table 1). Among the tested carbon sources, sucrose provided a more expressive production of EPS. This result corroborates with data found in the literature, where it is observed that glucose and sucrose are the most used carbon sources for the production of EPS [11-16]. However, there is a cultivation time and an optimal concentration of carbon source for the production of EPS for each microorganism [17]. The pH variation did not differ, with pH 7.5 being defined for EPS production. Of all the bacteria, *Pseudomonas aeruginosa* was considered the best isolate for the production of EPS (Sucrose 10% at 28ºC) with TCA 10%, as it presented greater dry weight under the conditions tested. For this reason, the *P. aeruginosa* bacterium was selected to produce EPS to perform the metal retention test to elaborate the Biofilter. The yields obtained are compatible with other findings in the literature, where the constant dry weight varies from 0.2512 to 2, 2130 g EPS. [18] obtained 2.2000 g in the production of EPS, [19] found 1.5750 g in strains of *Pseudomonas ssp*., Demonstrating that the methodology of the experiments could verify the interaction of the parameters to obtain a better response, which minimizes analysis time and improves conditions for a larger experiment.

Table 1: EPS production in different carbohydrates and pH of bacterial isolates: *Klebsiella pneumoniae, Pseudomonas aeruginosa, Lysin bacillus spp, Bacillus spp, Bacillus cereus, Staphylococcus spp* and *Serratia marcescens* from samples collected from the banks of the Subaé river in the municipality of Santo Amaro / BA.

Carbon Source	pH	Isolates producing the EPS/Ø
Glucose	5,5	All (above 15mm)
	6,5	All (above 15mm))
	7,5	None produced
Sucrose	5,5	All (above 15mm)
	6,5	All (above 15mm)
	7,5	All (above 15mm)
Mannitol	5,5	All (above 15mm)
	6,5	All (above 15mm)
	7,5	All (above 15mm)

Biochemical Characterization of EPS

The biochemical characterization of EPS made it possible to analyze some chemical groups that may be interacting with the heavy metals present in the waters from which these bacteria were isolated, facilitating the understanding of their resistance mechanisms in these environments considered extremophiles due to the high degree of environmental contamination. The EPS samples produced by the bacteria showed similar spectra (Figure 3). Correlation tables in IV were used to extract structural information from the spectrum. All have peaks between 3600 and 3200 cm^{-1} correspond to bands of hydroxyl groups (OH). The second peak is located around 1660 cm^{-1}, indicating that there is a stretch of C = O and C–N (amines and amides in amino acids), which indicates the presence of proteins in the sample. The peak at about 1060 cm^{-1} again indicates the presence of stretching of the OH group. The carbonyl elongation of carboxylic acids appears close to 1750 cm^{-1}. A band at 1420 cm^{-1} may be due to the C-OH deformation vibration with the contribution of the O-C-O symmetrical

vibrational stretching of the carboxylate group. The bands 1260 cm^{-1} and 1160 cm^{-1} can be attributed to the presence of sulfate ester groups (S = O). The peak around 830 cm^{-1}, present in the samples of the bacteria *Bacillus spp*, *Bacillus cereus* and *Sthaphylococcus spp* indica the presence of a fold in a substituted trialcene. There are no studies on the characterization of EPS produced by these microorganisms aimed at environmental contamination areas, and the process of biofilm formation, both *P. aeruginosa* by *Staphylococcus aureus* or by *Serratia marcescens*, is further investigated, focused on their impact on the development of resistance to antimicrobials, evidencing the current pioneering research in the production of EPS aimed at bioremediation of environments with heavy metals with these microorganisms.

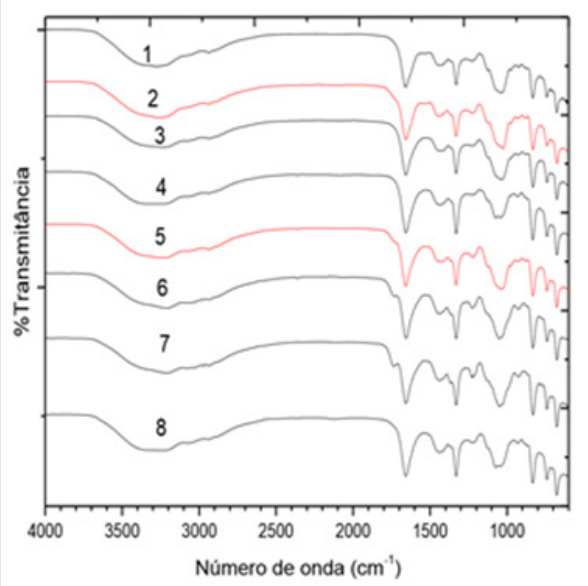

Figure 3: Set of EPS infrared spectra of all bactéria.

EPS Production for Metal Adsorption (Lead and Cadmium)

The EPS produced without the addition of 10% TCA by all the tested bacteria were firmer and more compacted in the bottom of the containers (showing crystalline visual aspects). One of the vials with the centrifuged content of the colony K. pneumoniae, two of the colony B. cereus and two of the vials of the colony Serratia marcescens (without the addition of 10% TCA) showed a biphasic visual aspect and during the addition of ethanol (92, 8°GL) were softer, more flexible and bulky. For Bacillus cereus bacteria, prior to centrifugation, the Erlenmeyer contents were viscous (with a honey consistency of around 59.60 mPa.s) and with EPS precipitates at the bottom of the containers. And for the Serratia marcescens bacteria, before the centrifugation, the the colony Sthaphylococcus spp. For all eight bacteria, the EPS produced with the addition of 10% TCA was presented visually in the form of a semi-granulated solid material, and their mechanical separation with the liquid phase became difficult, requiring the centrifugation to provide this separation. This probably occurred due to the proteolysis that EPS underwent, precipitating low molecular weight peptides and free amino acids, due to the action of TCA 10%, which was enhanced with the use of chilled ethanol.

Adsorption of Pb by EPS Produced by Microorganisms

In all EPS of the tested bacteria, it was found that the removal efficiency was greater for Cd than for Pb, with adsorption of both Cd and Pb being more efficient by bacterial EPS without TCA (Table 2). The best adsorption performance of metals was from EPS without ATA extracted from *Klebsiella pneumoniae*, the maximum biosorption capacity for Cd and Pb was 0.32666 mg L^{-1} and 0.10339 mg L^{-1}, respectively. This good performance was also verified in the work of [20] whose Pb adsorption was 99.5 mg g^{-1} by the EPS produced by the genus Klebsiella sp. EPS ATA produced with *Bacillus spp*, *Bacillus cereus* and *Lysinibacillus spp* did not adsorb Cd. The lowest efficiency for Cd removal was from EPS ATA without *Bacillus spp*, 0.00001 mg L^{-1} and the lowest efficiency for adsorbing Pb it was from EPS with ATA produced by Pseudomonas aeruginosa and *Sthaphylococcus spp*, 0.00012 mg L^{-1}. This fact can be justified by a greater metallic affinity to EPS than to the cell surface *in vivo* systems, since EPS contain ionizable functional groups such as carboxyl, phosphate, amine and hydroxyl, based on that described in IV, which enable them to kidnap metal ions. Metals such as Cu^{+2}, Cd^{+2}, Ni^{+2}, Pb^{+2}, Zn^{+2}, Co^{+2} and Cr^{+3}, due to their similar chemical coordination characteristics, end up competing substantially with each other for biosorbent binding sites [21].

The formation of the weakest links occurs as the strongest sites become saturated. Most studies carried out emphasize the formation of chelating rings, but this mechanism cannot be considered alone, the characterization of EPS in relation to the chemical groups responsible for the adsorption of metals, corroborates in a better understanding of the Bacterial-metal EPS interaction process [22].

Table 2: Results of the Pb and Cd adsorption test in EPS of bacteria isolated from the Subaé-BA river.

BACTÉRIA	TIPO DE ADSORÇÃO	Média Adsorção ao Pb Conc. (mg L^{-1})	Média Adsorção ao Cd Conc. (mg L^{-1})	Desvio Padrão
*Klebsiellapneumoni*ae	SEM ATA	0,10339	0,32666	0,157876
	COM ATA	0,050133	0,19333	0,101256
Pseudomonasaeruginosa	SEM ATA	0,0005	0,02323	0,016073
	COM ATA	0,00012	0,00003	$6,36x10^{-5}$
Klebsiellapneumoniae	SEM ATA	0,000597	0,140007	0,098578
	COM ATA	0,000140	0,00016	$1,41\ x10^{-5}$
Lysinibacillusspp	SEM ATA	0,000477	0,23319	0,164553
	COM ATA	0,00025	Não adsorveu	0,000177
Bacillusssp	SEM ATA	0,000163	0,00001	0,000108
	COM ATA	0,000150	Não adsorveu	0,000106
Bacilluscereus	SEM ATA	0,00135	0,258567	0,18188
	COM ATA	0,0007	Não adsorveu	0,000495
Sthaphylococcusspp	SEM ATA	0,00023	0,193333	0,136544
	COM ATA	0,00012	0,00008	$2,83x10^{-5}$
Serratiamarcescens	SEM ATA	0,00038	0,129973	0,091636
	COM ATA	0,00022	0,00008	$9,9x10^{-5}$

However, in this experiment there was no competitive adsorption of Pb and Cd as the tests were performed separately for each metal. Possibly, the adsorptive behavior of Cd in relation to Pb may be due to its greater metallic affinity and attraction of electrostatic charges to the chemical groups present in EPS, which are influenced by the reactions of oxide-reduction and kinetics of the reactions. The results demonstrate the efficiency of bacterial EPS isolated in the adsorption of different metallic ionic species from aqueous solutions. In this context, it is worth noting that the existing methods for reducing the concentration of heavy metals in water and effluents present difficulties in application and high cost, in addition to their efficiency being relatively low. In industry, there are few cases in which alternative techniques are used to reduce the levels of these metals in their effluents. In the vast majority of cases, chemical precipitation is the technique used. More efficient processes, such as reverse osmosis, ultrafiltration and ion exchange, still do not have a relevant practical application in the field of industrial effluent treatment because they are expensive and difficult to operate [23-31].

Conclusion

Cultivable bacteria identified in the surface waters of the Subaé river in the Santo Amaro-BA stretch, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Klebsiella pneumoniae, Lysinibacillus spp, Bacillus sp, Bacillus cereus, Sthaphylococcus spp,* Serratia marcescens have a tolerance to heavy metals and capacity EPS production line.

The EPS of each bacterial strain, tested in isolation, showed high affinity and adsorption capacity, mainly for Cd.

The results showed that the EPS produced by all eight bacteria represent one of the biochemical mechanisms they developed to resist these metals, presenting the potential for bioremediation as a technologically efficient alternative to control the migration of heavy metals along the Subaé River. In this sense, this work expands the strategies in the control of pollutants and creates tools for the development of new technologies for environmental repairs and recoveries, making it necessary a more detailed study about the characteristics of this material, as well as the variables involved in its behavior during the metal adsorption process.

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Highlights

1. This work made it possible to discover the bacterial biodiversity of the Subaé River (Santo Amaro-BA)

Bacteria present in the surface waters of the Subaé River in the Santo Amaro-BA section showed tolerance to heavy metals.

2. The adsorption capacity of lead and cadmium by the Exopolysaccharide (EPS) produced by all bacterial isolates was verified.

3. The great potential of bacterial isolates to improve the bioremediation of Pb and Cd in a contaminated system was identified.

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