Journal of Environmental Science and Engineering A 13 (2024) 122-127
doi:10.17265/2162-5298/2024.04.002

Effects of Phytoremediation on Soil Contaminated with Pb and Cd in Tolerant Bacterial Populations

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Abstract: Bacterial populations isolated from treated soil, artificially contaminated with lead (Pb) and cadmium (Cd) and undergoing a phytoremediation process were studied to determine their potential application in soil remediation. The physicochemical parameters evaluated in the soil varied significantly. Ten bacterial strains were selected from each polluted soil to test tolerance and growth in contaminated media. The concentrations of heavy metals tested were 1,000 ppm for lead and 850 ppm for cadmium. These strains were morphologically identified through Gram staining. Four strains showing the most significant growth in both contaminants were then selected to verify their tolerance to different concentrations of heavy metals. The results demonstrated that the selected bacteria have high tolerance to Pb, resisting inhibition up to 2,000 ppm. In contrast, strains exposed to cadmium tended to slow their growth as the concentration increased.

Key words: Lead, cadmium, soil, bacteria, remediation.

1. Introduction

Heavy metals are naturally found in the Earth's crust as minerals or other substances; they are the products of volcanic eruptions and other sources. Furthermore, they are byproducts of human activities such as metal smelting, utensils production or industrial waste drainage [1, 2]. Some of these metals, such as zinc and iron, exhibit essential biological functions, while others as lead and cadmium, present high toxicity and are classified as environmental pollutants [3]. This has led scientists to seek for different alternatives to avoid pollution with these metals.

Incineration and excavation are the most commonly used techniques for heavy metals removal, however, these techniques increase the risk of exposure for populations close to the source of pollution. In contrast, biological remediation techniques are more environmentally friendly and are also used for this purpose.

Examples include bioadsorption, phytoremediation and remediation with microorganisms [4, 5].

The main microorganisms that have been used in heavy metal remediation are bacteria. Some genera that have proven their efficacy include *Anthrobacter*, *Bacillus* sp., *Citrobacter*, *Cupriavidus metallidurans*, *Cyanobacteria*, *Enterbacter cloacae*, *Pseudomonas aeruginosa*, *Streptomyces* sp., *Zoogloe aramigera*, *Alcaligenes*, *Sphinganonas*, *Rhdococcus*, *Mycobacterium* and *Arthrobacter* [6]. Bacteria are microorganisms with great metabolic diversity allowing them to use different chemical compounds as sources of energy and carbon. Species from *Pseudomonas* genus and *Rhizobiaceae* family, are usually found in places contaminated with heavy metals [7].

Research has reported that the genus *Bacillus*, specifically strains from *Bacillus* sp. and *Bacillus*

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cereus isolated from polluted environments show tolerance to heavy metals—an ability essential for bioremediation [8]. Furthermore, other species from *Bacillus* possess properties to detoxify polluted environments by the production of exopolysaccharides that chelate heavy metals [9]. Rhizobacteria *Bacillus* sp. were discovered to promote plant growth in seedlings from *Cyperus elegans* at different concentrations of cadmium. Results indicate that the rhizobacteria promoted growth 139% more than the control plant [10]. Some bacterial genera isolated from the rhizosphere of heavy metal-accumulator plants increase plant growth by producing IAA (Indoleacetic Acid). Other species such as *Burkholderia* sp., *Pseudomonas fluorescens* and *Microbacterium* sp., increased plant height in brown mustard, tomato and corn plants accumulating Cd and Pb [4].

2. Methodology

Black soil purchased from a greenhouse in the city of Veracruz was used. It contained stones and dry leaves to improve water drainage and prevent compaction. To achieve homogeneity in the soil composition, it was mixed and divided into portions of 1.5 kg each. The soil was then placed in polypropylene pots measuring 25 cm in diameter and 19.5 cm in height.

Each portion of soil was irrigated with 250 mL of contaminant solution (1,000 ppm of lead nitrate ($Pb(NO_3)_2$) and 850 ppm of cadmium nitrate tetrahydrate ($Cd(NO_3)_2 \cdot 4H_2O$), with a total of three pots per treatment and three control pots irrigated with deionized water. All pots were mixed manually to distribute the solution evenly.

Finally, one sunflower seedling was planted in each pot and watered with 200 mL of potable water every other day. Biodegradable disposable plates were placed at the bottom of the pots to capture the leachate and return it to the soil to avoid heavy metal loss.

The phytoremediation experiment was carried out for 30 days. Sample soils for analysis were collected into sterile 100-mL clinical cups and stored at 4 ℃ until processing. The physicochemical tests were performed following the methods established by the LaMotte soil handbook, which is based on colorimetric techniques to estimate the concentration of various soil components.

For the quantification of microbial colonies, the method of serial dilutions was applied under sterile conditions. For the initial flask, 10 g of soil was added to a flask with 90 mL of sterile water. Subsequently, ten-fold dilutions in sterile 15-mL plastic tubes with 10 mL of working volume were made up to 10^{-10}. These dilutions were spread on NA (Nutrient Agar) using a Drigalski spatula. The agar plates were incubated at 37 ℃ until colony growth was observed. Then, their morphology was analyzed, and CFUs (Colony-Formation Units) were counted by direct-count.

Colonies of bacteria with different morphologies were selected from agar plates containing lead and cadmium for further isolation using the streaking method on LB (Luria-Bertani) agar medium. First, the colonies were inoculated in separate flasks with LB broth containing 1,000 ppm of lead and 850 ppm of Cd, respectively, to observe tolerance to these contaminants. Then, the bacterial suspension from the last experiment was inoculated in other flasks with the same medium and incubated at 30 ℃ for 48 h. The samples were centrifuged and quantified under sterile conditions to achieve an inoculum of 1 mg/mL of each isolated strain. Two flasks with treatment and two control flasks were analyzed to determine the amount of biomass. Bacterial samples with the best results were then used for a second trial with concentrations of 1,200, 1,400, 1,600, 1,800 and 2,000 ppm for Pb and 400, 550, 700, 850 and 1,000 ppm for Cd.

3. Results

The first batch of soil samples, before contamination with heavy metals, was medium brown with lumps and variations in texture (Fig. 1).

The characteristics of soil before and after the phytoremediation process are shown in Table 1.

Fig. 1 Batch of soil before the experiments.

Table 1 Characteristics of soil before and after the phytoremediation process.

Component	Control	Pb	Cd
Nitrate (ppm)	15	55	95
Nitrite (ppm)	1	3	4
Phosphorus (ppm)	2.5	2	2
Magnesium (ppm)	5	3	1
Calcium (ppm)	6,000	1,700	3,000
Sulfate (ppm)	100	20	10
Humus (%)	20	25	10
Ammoniacal nitrogen (ppm)	3	5	5
Manganese (ppm)	5	1.5	2
pH	6.8	7.1	5.8

The concentration of nitrogen compounds (nitrate, nitrite and ammoniacal nitrogen) increased in contaminated soil. The levels of Mg, Mn, Ca, and sulfate significantly decreased in soils contaminated with lead and cadmium. The humus content increased in polluted soils; this value is directly related to the number of microorganisms that inhabit the soil and facilitate organic matter decomposition [11, 12].. The content of microorganisms present in soil is shown in Table 2.

There was no significant variation among the populations of control soil at the beginning and after 30 days of experimentation with phytoremediation. However, contrary to what was expected, an increase in the number of bacterial CFU in the presence of Pb and Cd was noted. Parallel experiments in which this effect was studied indicate the nitrate present in the salt of the contaminants used caused this phenomenon.

A total of 20 bacterial strains were isolated, 10 from each contaminated soil, and were studied in liquid medium. The growth percentages in relation to each control are shown in Table 3.

The four strains with the best growth results in both pollutants were selected and because the growth of the selected strains in lead, on average, exceeded 50% in relation to the control, it was decided to test with higher concentrations of contaminants. The growth results are shown in Fig. 2.

Table 2 Content of microorganisms in soil.

Soil sample	Total CFU (organisms $\times 10^6$/g soil)	Total bacteria (bacteria $\times 10^6$/g soil)
Control T_0	3.12	2.91
Control T_1	3.35	3.01
Pb 1,000	5.63	4.82
Cd 850	4.83	3.96

Table 3 Results of the tolerance to Pb and Cd.

Strain	Pb (%)	Cd (%)
Cd850-1	4.477	3.379
Cd850-2	21.895	10.599
Cd850-3	50.921	9.042
Cd850-4	71.977	24.789
Cd850-5	86.236	40.257
Cd850-6	36.902	21.500
Cd850-7	33.889	21.111
Cd850-8	30.300	42.913
Cd850-9	67.177	40.600
Cd850-10	12.895	15.599
Pb1000-1	41.430	33.151
Pb1000-2	24.190	41.538
Pb1000-3	32.459	19.300
Pb1000-4	52.257	20.942
Pb1000-5	37.391	7.561
Pb1000-6	80.820	29.665
Pb1000-7	45.120	50.153
Pb1000-8	82.399	48.007
Pb1000-9	94.280	34.064
Pb1000-10	15.788	13.509

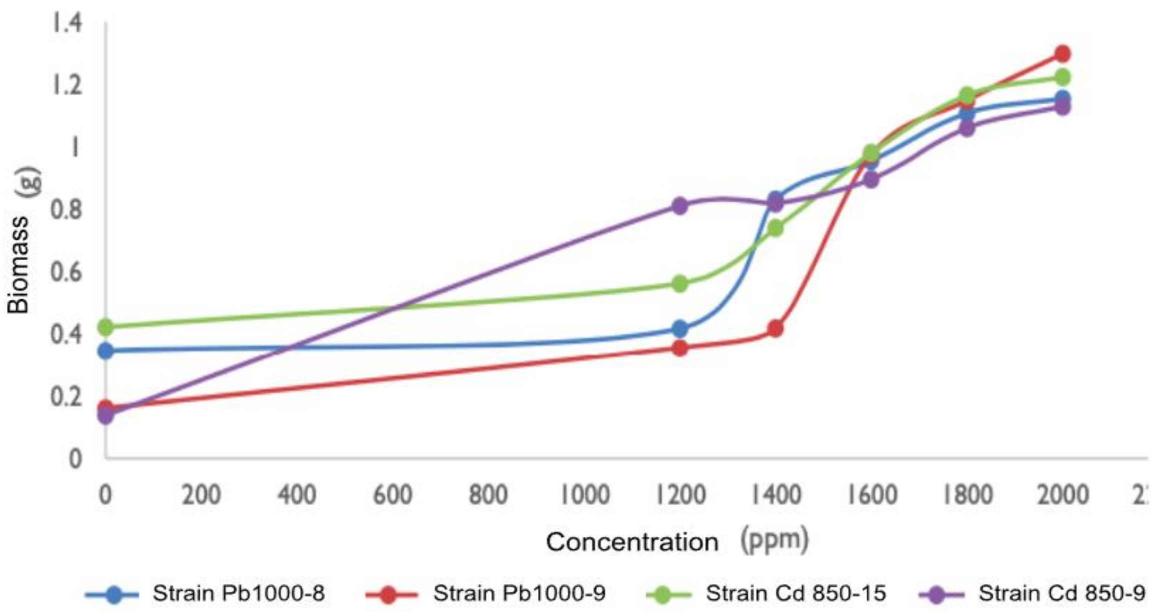

Fig. 2 Tolerance to lead test.

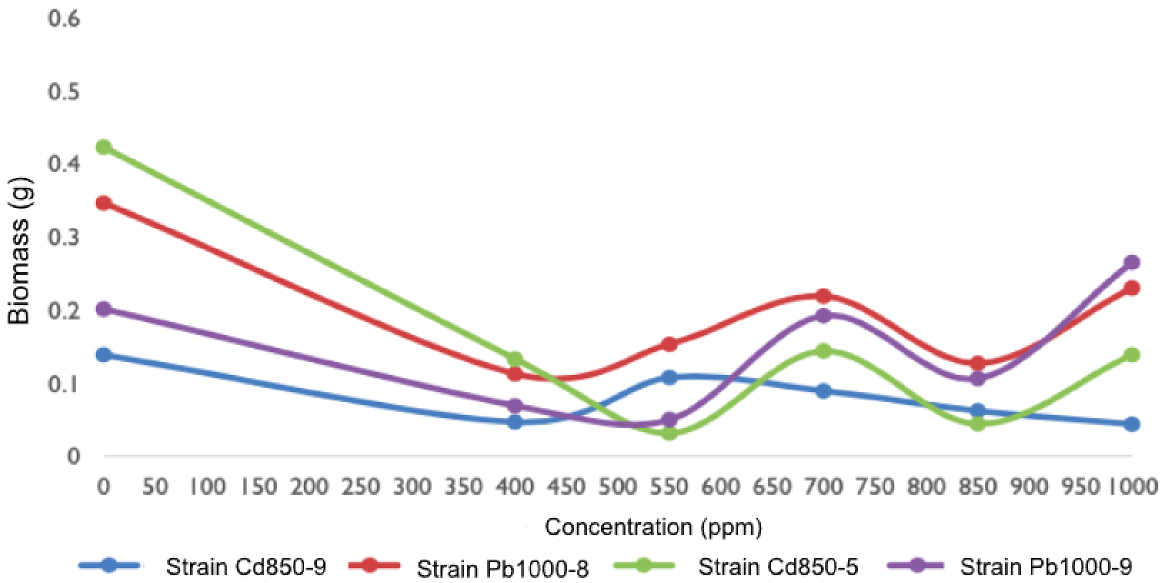

Fig. 3 Tolerance to Cd test.

As can be seen in the previous graph, the growth of the strains in general in the contaminant showed unexpected results, since as the concentration increased, the strains tended to increase in growth, so it was not possible to obtain a specific value of concentration of inhibition in this contaminant. There are studies that have investigated the ability of *Pseudomonas aeruginosa* to adsorb lead and nickel, demonstrating that this genus of bacteria was able to adsorb up to 79 mg of lead per gram of living cells. Other bacterial species related to this are *Acinetobacter junii* and *Azotobacter chroococcum* [13].

In the case of the experiments with cadmium, the growth of the selected bacteria, on average, did not exceed 50% in relation to the control, it was determined to reduce the concentration under 850 ppm and test with 400, 550 and 700 ppm; and at the same time try with 850 and 1,000 ppm. The growth results are shown in Fig. 3.

The graph shows that the behavior of the strains in the presence of Cd tends to decrease as the concentration of the contaminant increases, indicating that Cd is causing an inhibitory effect on the strains. However, it was not possible to determine a concentration at which the contaminant completely inhibited the growth of the strains.

Gram tests determined that strain Pb1000-8 is a Gram-negative bacillus, strain Pb1000-9 is a Gram-negative coccus, strain Cd850-5 is a Gram-negative coccus, and strain Cd850-9 is a Gram-negative coccus.

4. Conclusion

The soils underwent significant changes at the end of the phytoremediation treatments, including physicochemical parameters relevant to the behavior of the microorganisms. The content of nitrogen compounds seems to influence bacterial growth, and humus reflects the activity of microorganisms in soils.

Various concentrations of lead were tested, and the growth kinetics showed that as the concentration increased, the strains tended to increase their biomass. Thus, it was not possible to obtain a specific concentration value of the contaminant inhibition. The increase in biomass in the presence of lead could be attributed to processes of intracellular or extracellular bioaccumulation of metals by bacteria, which have been previously reported.

For cadmium concentrations, the growth kinetics show an inhibitory effect on all strains. It was not possible to determine a concentration at which the contaminant completely inhibited the growth of the strains.

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