The Potential Role Of *Sphingomonas paucimobilis* In Bioremediation Of Soils Contaminated With Hydrocarbon And Heavy Metal

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ABSTRACT Aiming to find the solution to the problem of soil polluted by hydrocarbons and the associated heavy metals, the present study focused on the biodegradation and bioremediation capability of *Sphingomonas paucimobilis*. Morphological and biochemical tests have been used to identify bacterial isolates and to confirm that an automated instrument for bacterial identification (Vitek II) has been used. Based on the results, the bacteria were identified as *Sphingomonas paucimobilis*. The bioremediation capacity was monitored: the lowest inhibitory concentration (MIC) of lead (Pb) and cadmium (Cd) followed by an assay of the removal capacity of *S. paucimobilis* using the atomic absorption spectrometry (AAS) analyzer. The bacteria showed higher MIC value for Pb (2000 ppm) the Cd (500 ppm). The percentage of removal for Pb and Cd were 27.95% and 58.78%, and 22.37% and 48.21% for the concentration 25 ppm and 50 ppm, respectively. These findings showed the high aliphatic hydrocarbon biodegradation capacity of *S. paucimobilis*, with the percentage of degradation being 48.15% and 63.40% of the concentration of crude oil by 2% and 5%, respectively. *S. paucimobilis* can potentially be a safe biological treatment strategy to remediate soil polluted with hydrocarbons in crude oil extraction sites.

Key words: *Sphingomonas paucimobilis*, Bioremediation, Heavy Metal, Biodegradation, Hydrocarbons

1. INTRODUCTION

The decades-long extraction of oil from the south of Iraq, particularly in the Basra city led to significant damage to the environment, particularly the soil, as a result of the leakage of an enormous quantity of crude oil and associated pollutants, including heavy metals. The cumulative effect of organic and metallic pollutants on soil biology is more poisonous than that of hydrocarbons alone (Thavamani, 2012 ; Liu et al., 2013). The carcinogenic and mutagenic properties of both hydrocarbons and heavy metals have prompted researchers to study their combined effect on the environment (Lovelace et al., 2012).

Therefore, it is important to search for a solution to preserve the environment, taking into account that the required solution must be environmentally friendly. Bioremediation is a biological technique to recycle pollutants in a way that other

organisms can use and reuse. Recently, there has been great attention towards bioremediation by using of bacteria to treat and eliminate the pollutants from the environment. Bacteria are effective tools for decontamination of soil, water and sediments. Thus, the key for bioremediation is the selection of efficient and proliferative bacteria (Zheng, 2016). Past research has indicated that hydrocarbon-degrading bacteria mostly belong to the *Mycobacterium,Sphingomonas,Pseudomons, Bacillus*, and *Cycloclasticus clade* (Yao, 2015). Therefore, the objectives of this study were to isolate and identify *Sphingomonas* from soils contaminated with hydrocarbons and examine their capacity to treat soil contaminated with hydrocarbons and the associated heavy metal.

2. METHODS

2.1 Collection of Samples

A total of thirty soil samples was collected in up to 20 cm depth from two different selected stations in the North Rumaila oil field in the Basra city south of Iraq during January 2018. The samples were labeled and stored in plastic bags. Each sample was separately air-dried, milled using porcelain pestle and mortar, and then sieved using a 2 mm sieve. The fine soil fractions were collected in separate bags and stored in a dry place for further analysis.

2.2 Isolation and identification of bacteria

Mineral Salts (MSM) supplemented with different concentration of crude oil have been used to isolate bacteria from soil samples. Mineral salt media with oil as the sole carbon source has been used to maintain bacteria. The MSM composition was as follows: 0.3 g of KCl, 1.0 g of K2HPO4, 0.5 g of KH2PO4, 0.01 g of FeSO4.7H2O, 30.0 g of NaCl, 0.5 g of MnSO4.7H2O, 0.2 g of CaCl2 and 1000 ml DW (Fujisawa , 1980). The pH was adjusted to 7.0 - 7.8.

Two concentrations of crude oil (2% and 5%) obtained from the Al - Shua'aba Refinery was added separately to the medium. The culture was cultivated at 30^{o}C for seven days. Nutrient agar (Hi media-India) was used to isolate *Sphingomonas paucimobilis.* It was identified by morphological and biochemical tests, and for better identification, the automated instrument for bacterial identification (Vitek II) (Biomerieux, USA) was used.

Heavy Metal Tolerance Assays

2.3 Preparation of the heavy metal concentrations

Stock solutions of the metal salts were made by dissolving the exact weight of Pb(NO3)2 and Cd(NO3)2.H2O in sterile deionized water. The working concentration of the Cd (II) and Pb (II) was prepared from the stock solution according to Etorki et al. (2014).

2.4 Tolerance determination

This test was carried out to assess the heavy metal tolerance of the isolated bacteria. The isolates were grown in nutrient, heavy metal-free broth (NB, Hi media) at 25°C for 24 hr. After incubation, a loopful was taken from the culture and then aseptically cultured on nutrient agar containing Cd and Pb, at different

concentrations (25, 50, 100, 250, 500, 1000, 1500, 1800 and 2000 ppm). The plates were incubated at 25°C for 48 hr. The minimal concentration of Cd and Pb that inhibited the growth was considered as the tolerance level. The test was repeated in triplicate and one control (Huët & Puchooa, 2017).

2.5 *Estimation of the bacterium's capability to remove heavy metal*

Bacteria were cultured in 10 ml of NB broth for one day at room temperature and 2 ml of the suspension was injected in NB broth containing 25 and 50 mg/l of Pb and Cd, respectively and incubated at 25°C for 24 hr. Then, the culture was centrifuged at 3000 rpm for 20 minutes and the supernatant was collected and examined to assess the Pb and Cd removal by using the flame atomic absorption spectrophotometer (AAS 6300, Shimadzu, Japan). The step was performed in triplicates. The equation below was used to calculate the percentage of removal:

% elimination = (reduction in heavy metal concentration ÷ Initial heavy metal concentration) × 100 (Huët & Puchooa, 2017).

2.6 Estimation of the oil degradation capability

Erlenmeyer flask containing 50 ml of MSM supplemented with 1 mL bacterial culture was incubated in an incubator shaker at 20˚C for 7 days at 120 rpm. The remaining crude oil was measured after one week and the test was carried out in duplicates (Alkanany et al., 2017).

2.7 Extraction of crude oil residues

The procedure outlined by Adebusoye et al. (2007) was used to extract the residual crude oil from the MSM medium. The extraction procedure included adding 50 ml of carbon tetrachloride (CCl4) solvent to the bacterial culture. This is commonly used to extract all hydrocarbons and inhibit bacterial growth and activity (Aizenman, 1989). The addition was performed during the step of continuous shaking. The culture was transferred to a separating funnel and allowed to settle. The suspension was removed, and the remaining oil was dried in an oven at 40˚C to get rid of CCl4.

Extraction of aliphatic fraction was done according to Farid (2006). The procedure included adding 35 ml of hexane to the remaining pre-extracted crude oil and pouring that entire solvent into a column closed with glass wool and topped with 8 g of silica gel. The remaining pre-extracted crude oil and solvent passed through the column after which Hexane aliphatic fraction was collected in 50 ml beaker and the remaining extract was left to dry completely; another 2mL of hexane was added to the beaker before measured using gas chromatography.

3. RESULTS AND DISCUSSION

3.1 Identification of Sphingomonas Paucimobilis

The isolated bacteria were identified based on their morphology and biochemical

test (Table1). For further identification, an automated instrument for bacterial identification (Vitek II, C8300 Biomerieux USA) was used. The result was within 95% confidence.

Table 1. Morphological and biochemical characteristics of S. Paucimobilis

Colony Morphology	**Results**
Cell shape	Rod shape
Motility	Motile
Pigment	Yellow color colony
Gram reaction	-
Biochemical Tests	
H_2S formation	-
Catalase	+
Nitrate reduction	-
Urease	+
Oxidase	+
Glucose	-
Sucrose	-
Simon citrate	+

3.2 Metal Resistant Pattern

The MIC is defined as the lowest concentration of metals that inhibits bacterial growth (Yilmaz., 2003). The MIC is considered as an initial step to evaluate the susceptibility or tolerance of bacteria towards the remediation of heavy metal. Table 2 shows the MIC results; the bacterial isolates were resistant to high concentrations of Pb (2000 ppm) and Cd (500 ppm). The low MIC values showed the most toxic metals while the maximum MIC values indicated the least toxic ones (Mishra & Mishra, 2015).

Tangaromsuk et al. (2002) reported high tolerance of *S. paucimobilis* to the Cd. They found that the bacteria were capable of living in the concentration of cadmium higher than 200 mg/l. However, their resistance to Pb was higher than that to Cd. This is because, there is a high rate of lead in the environment compared to cadmium, leading to an increased resistance of the bacteria as a means of survival (Habi & Daba, 2009). A pollution with a particular metal can raise the level of tolerance of the bacterial community towards that metal (Mishra & Mishra, 2015). Other studies showed that the concentrations of metals as well as many physical and chemical factors played a prominent role in increasing the susceptibility of bacteria to different concentrations of metals (Afzal et al., 2017).

The results of this bioremediation study using two concentrations (25 and 50 ppm) from Pb and Cd are shown in Table 2 and Figure 1, indicating that *S. paucimobilis* was capable to eliminate Pb and Cd, with the percentage of Pb removal being 27.95 and 58.78% in the concentration of 25 and 50 ppm, respectively. The removal of Pb was concentration-dependent; as the concentration increased the percentage of

removal increased. The result of Cd removal was 22.37 and 48.21% for 25 and 50 ppm, respectively. The manner of removal was same to that for Pb. The result indicated that there was an increase in Pb and Cd removal as the concentration increased, indicating that the removal occurred in a diffuse manner. The higher the concentration, the greater the movement of molecules or ions (Ahemad & Malik, 2011).

There are also indications that the removal capacity of *S. paucimobilis* was higher for Pb than Cd. Allam (2017) reported that the removal ability of Pb was higher than Cd (57 and 53%).

Table 2. The minimum inhibitory concentration (ppm), and heavy metal removal.

	Heavy metals removal (%) /24h		
Heavy metals	MIC (ppm)	50 ppm	25 ppm
Pb	2000	58.78	27.95
Cd	500	48.21	22.37

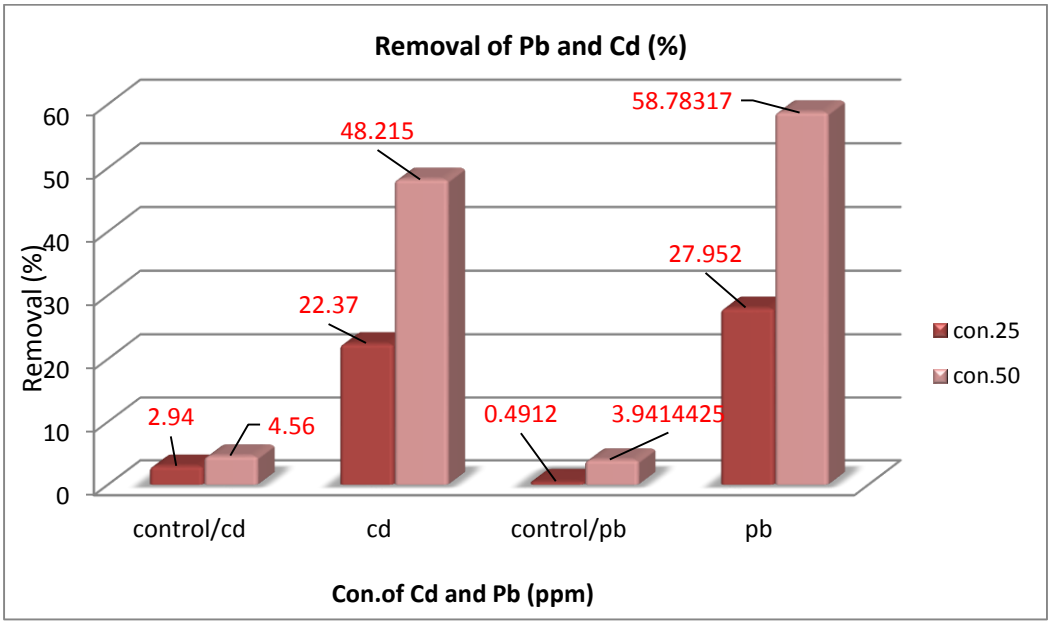

Figure 1. Removal of Pb and Cd (%) by S. Paucimobilis

3.3 Degradation study

To examine the ability of *S. paucimobilis* to degrade hydrocarbons (aliphatic part), GC-MS analysis of the control (only crude oil and without microorganisms) showed that it was a mixture of different aliphatic hydrocarbons at the different concentration of crude oil (5% and 2%) (Fig.2.A and Fig.3.A). *S. paucimobilis* was capable of actively degrading the total mixture of hydrocarbons present in the crude oil of two concentrations (5% and 2%) during the

seven days of incubation. The result was confirmed by an almost total reduction of the amount of each compound peak (Fig.2.B and Fig.3.B).

Table 3 shows the percentage of oil degradation by *S. paucimobilis*. S. *paucimobilis* was more effective to degrade oil in 5% (63.40%) than 2% concentration (48.15). The ability of *S. paucimobilis* to degrade the aliphatic compound was also reported in previous work by Al-Taee et al. (2017) where they showed the percentage of removal of 67% for the 2% concentration of crude oil. Barth (2003) reported that *Sphingomonas* bacteria was typical in hydrocarbon degradation. Zhuang et al. (2003) referred to *Sphingomonas* as one of hydrocarbon degradation bacteria.

One of the factors that can play crucial roles in the ability of a bacterium to degrade hydrocarbons include the available concentration of hydrocarbons to the bacteria. The higher the available concentration of hydrocarbons, the higher the percentage of removal and the degree of interactions between bacteria and hydrocarbons required for the oxygenation process (Hua & Wang, 2014).

Therefore, bacteria with the ability to remove high amounts of hydrocarbons seem to develop an adhesive ability through changing their surface component (Krasowska & Sigler, 2014). The composition of hydrocarbons is considered as a vital factor as reported by Varjani (2017). Hydrocarbons can be arranged according to the degradation as follows: linear alkanes > branched alkanes > low molecular weight alkyl aromatics > monoaromatics > cyclic alkanes > polyaromatics > asphaltenes.

The chemical and physical properties of substrate materials as well as their bioavailability affect the ability of bacteria to contact, transport, and convert hydrocarbons (Varjani & Upasani, 2017).

Table 2. Degradation percentage of crude oil

S. Paucimobilis	
Concentration/ 2%	Concentration/ 5%
48.15%	63.40%

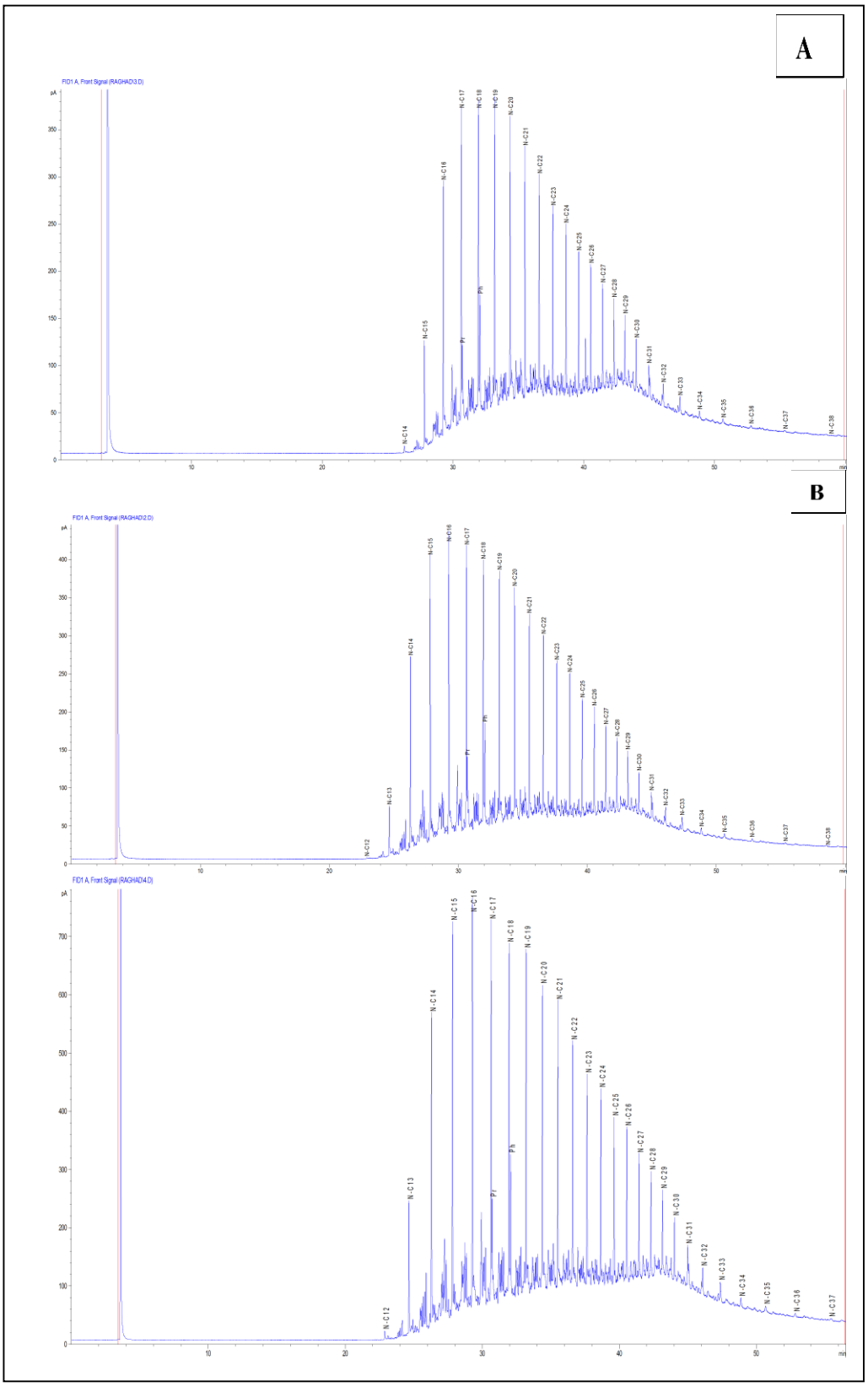

Figure 2. A: Crude oil (aliphatic fraction, 5%) without; bacteria and B: Crude oil (5%) with *S. Paucimobilis*

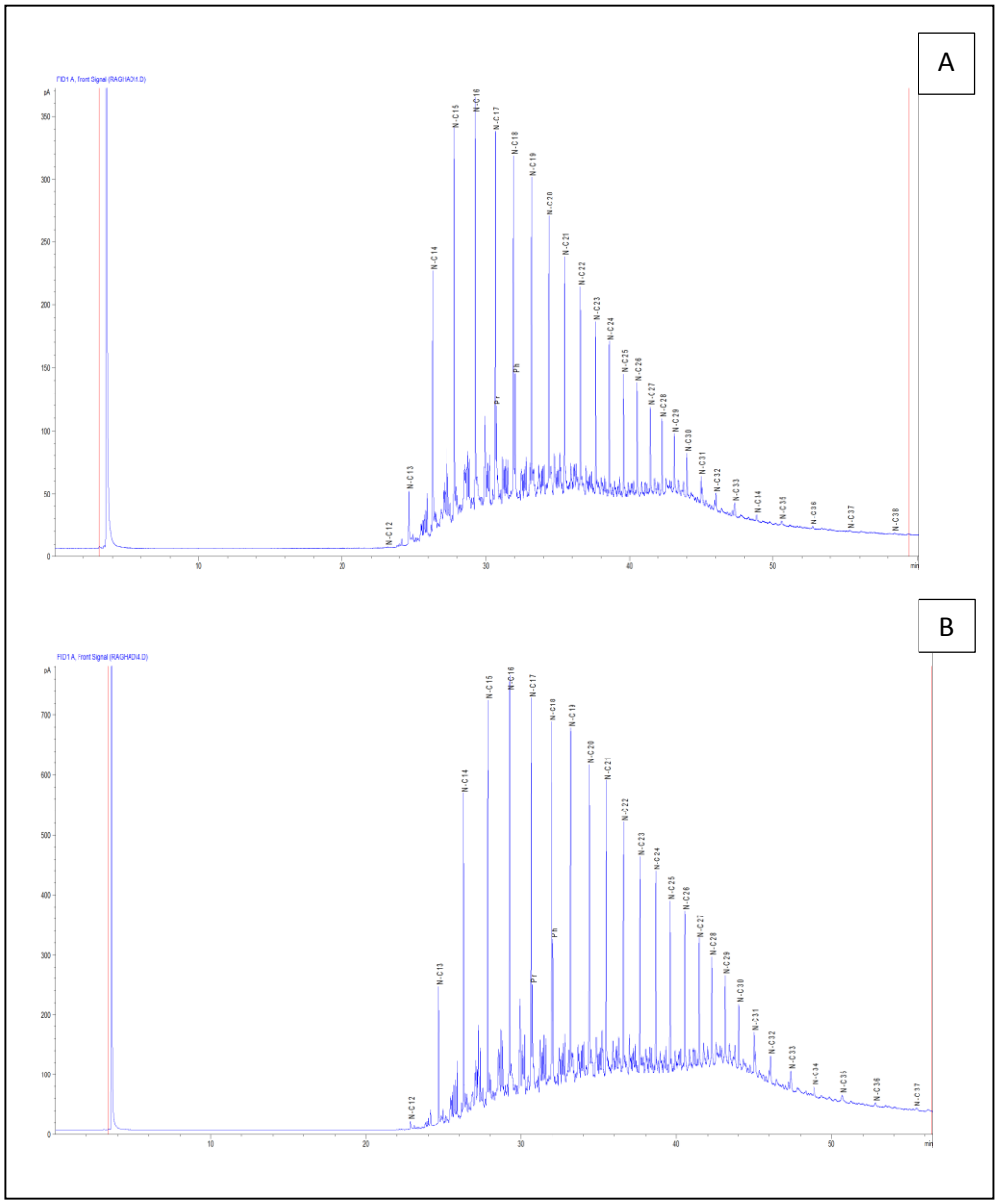

Figure 3. A: Crude oil (aliphatic fraction, 2%) without; bacteria and B: Crude oil (2%) with *S. Paucimobilis*

4. CONCLUSIONS

Based on these findings, *S. paucimobilis* is considered as an excellent agent in bioremediating soil polluted with both hydrocarbons and associated heavy metal since the isolated organism showed perfect tolerance and removal capacity. The possibility of isolating these bacteria from the soil is a good indicator for the healthy state of the soil, meaning that the soil is capable of self-treatment. Therefore, in order to improve this possibility, reviving these bacteria is important to increase their

efficiency and potential in soil bioremediation as a safe and environmentally friendly treatment.

5. FUTURE RECOMMENDATION

These bacteria can be used in-situ bioremediation after increasing its biomass construction in a large-scale bioreactor. Further research is needed to uncover the bacterial capacity to perform bioremediation of other heavy metals that cause environmental pollution. Finally, it is important to search for the genes responsible for the bioremediation to develop recombinant strains.

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