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Research Article

HEAVY METAL BIO-ACCUMULATING MICROBIAL ISOLATES FOR REMEDIATION OF METAL CONTAMINANTS FROM INDUSTRIAL EFFLUENTS

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Abstract- Textile and printing industries have tremendously increased the content of heavy metals in the soil and water ecosystem. Although the presence of other contaminants like xenobiotics, pesticides and organic matter cannot be ruled out, the extensively high levels of heavy metals in the environment pose a serious threat to the aquatic, microbial, plant and human systems creating imbalance in biogeochemical cycles. In the present study, microbes surviving in industrial effluents were isolated and characterized. These isolates were analyzed for their respective potential to tolerate and accumulate heavy metal contaminants. The remediation ability was studied against major life threatening heavy metal pollutants like Cu, Cr, Ni and Pb and their relative tolerance ability was estimated by calculating MIC. The isolates depicted significant potential to bio-accumulate heavy metal pollutants at an average concentration of 1mg/ml metal concentration. Different isolates were also analyzed for their rate of accumulation at different time intervals and most relevant bacteria were characterized for identification. Among these isolates *Bacillus subtilis*, *Salmonella* and *E.coli* were found to be most significant in reducing Pd, Ni and Cu heavy metal content respectively in the culture media. These isolates may be extensively used in the treatment of effluents from ink, printing and textile industries and preventing heavy metal intoxication in animals and humans.

Keywords- Bioremediation, Minimal inhibitory concentration, Effluents, Intoxication, Tolerance ability.

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Introduction

Heavy metals are the natural constituents found in the environment. But the excess human activities and commercialization has altered the geochemical cycle and balance of them. Although, the most significant natural sources are weathering of minerals, erosion, and volcanic activity, but the anthropogenic sources arising from human activities such as mining, smelting, electroplating, use of pesticides, and phosphate fertilizer discharge as well bio-solids (eg. livestock manures, composts, and municipal sewage sludge have led to a serious impact in the environmental levels of heavy metals.

As a result of industrial activities, excess of heavy metals such as cadmium, copper, lead, nickel, zinc etc. have been found to occur in the natural resources like the soil water and aquatic environments. Long time exposure and higher accumulation of such heavy metals can cause various life threatening diseases on human life and aquatic animals. Nowadays, Waste water released from various industries is the key concerns for environmental scientists and specialists. Industrial effluents contain a variety of toxic metals, harmful gases (carbon monoxide and nitrogen dioxide), and several other organic and inorganic compounds. Both the quality and quantity of waste water (effluent) result in various impacts on the availability of fine or good quality water as well as on aquatic environment.

Release of these contaminated discharges into the atmosphere creates various ecological, social and economic problems. Therefore long time contact of these toxic effluents results in numerous life threatening disease similar to Cancer, CNS depression, mutagenic changes, mental disorders etc.[1-2]. Industries conducting metal finishing, refining of petroleum, alloys industries like iron and nickel, manufacturing of textiles, electroplating etc. are examples, which releases such type of effluent [3-6].

Cadmium, arsenic, chromium, copper, lead, mercury, nickel, selenium, silver, zinc etc. are found to have not only cytotoxic but also carcinogenic and mutagenic property. For the proper development some metals are compulsory in very less amounts but the increasing concentration of several heavy metals in soil and water has generated a very harmful condition for the survival of human and aquatic life.

Several heavy metals like Cupper, chromium, nickel, lead, mercury and arsenic occur in extensive amounts causing environmental hazard to living beings. Large quantities of chromium are found in these industrial effluents in the form of Hexavalent chromium Cr (VI)) compounds which are regarded as very toxic causing several diseases like cancer and mutagenic diseases [7, 3] Arsenic is available as both metalloids and chemical compounds in the environment as a result of which it causes many pathogenic disorders [8]. Intakes of various types of insecticides, herbicides, and defoliants that are used for agricultural production are also a rich source of the heavy metals. Different type of inorganic salts and organic compounds of arsenite (As [III]) and arsenate (As [V]) are responsible for this situation. Lead originates from natural as well as synthetic sources. Manufacturing process of leaded pipes, solders, crystals and ceramics involve extensive use of lead. Some coloring quality is also found in lead and thus is also used in the coloring of ceramics [9]. Mercury is also released from different discharge and when it is dumped in the soil results in adverse effect on the

environment. Cobalt is another heavy metal found in the effluent of various industries. These intoxicants are deposited into the ground and enter into the food and water supply. Most of the population is exposed to cobalt through food, water, and air and cobalt exposure is a well-known cause of cancer, DNA damage, and Heart and lung problems. With the increase in various industries the release of industrial effluent to the environment is increasing day by day with depletion occurring at a comparatively slower rate. The extra discharge of heavy metals into the soil and water is a global environmental and health associated issue. Because they cannot be broken down to non- toxic forms, they have a long lasting effect on the entire atmosphere. Many of the heavy metals are very toxic even when they are present in low concentrations.

For environmental conservation, it is necessary that the contaminated water and soil must be made free from such type of heavy metal pollutants and traces elements. Numerous techniques are used to eliminate these heavy metals like chemical precipitation, oxidation or reduction, filtration, ion exchange, reverse osmosis, membrane technology, evaporation and electrochemical treatment etc., most of them turning ineffective at low concentration of heavy metals (<100mg). Most heavy metal salts are water soluble and become dissolved in wastewater which means they cannot be separated by physical separation methods. Moreover, these methods are less economic and involve use of various chemicals. A more effective and environment friendly approach to overcome these limitations is the exploitation of biological sources for remediation. The diverse biochemical properties of microbes have widely been used for various purposes, bioaccumulation being one of the well studied characteristic of bacteria. In this study the bio-accumulative properties of microbes was characterized to allow the development of accost effective and simple approach to remediate heavy metal contaminated soil and water.

Moreover, biological methods like bio sorption and bioaccumulation for removal of heavy metal pollutants is a preferable approach as compared to physio-chemical methods since it involves the development of a sustainable remediation technology to rectify the natural condition of soil [10].

Therefore, in the present study microbial bioaccumulation properties were evaluated and characterized against some common heavy metal pollutants targeting the development of an effective strategy for treatment of industrial effluents and heavy metal bioremediation. The bacterial isolates with a significant heavy metal accumulative property were isolated and characterized with an ambition to use these isolates for soil and water remediation.

Materials and Methods

Collection of effluent sample

Effluent samples will be collected randomly from different textile and dye industries from across the regions of Dehradun, India. The samples were collected in polyethylene bottles washed with ethanol and distilled water. The total volume of the bottle was filled completely and a cap was locked enough, so that no air space could be remained inside the bottles.

Isolation and identification of bacteria

The effluent samples were diluted by serial dilution method in autoclaved distilled water. The diluted samples were then inoculated into 3 ml nutrient broth each. The inoculated media was further incubated a 37°C for 16-18 hrs. Pure bacterial isolates were isolated form mixed samples using agar plating and streaking methods. The pure cultures thus isolated were characterized according to colony morphology and simple staining methods and observed microscopically to determine cell shape.

Culture medium and heavy metal exposure

Cells were cultured in nutrient broth with the following composition-beef extract (3 gm), peptone (10gm), Di sodium phosphate (1gm), Sodium chloride (5gm) dissolved in one litre of distilled water. Final pH was around 7.4-7.6. The medium was autoclaved at 121°C for 20 minutes. Culture was maintained in agar slants (nutrient broth plus 30gm/liter agar). They were allowed to grow in the synthetic media having different heavy metals salts of Cu, Cr, Ni and Pb.

Estimation of MIC for different heavy metals

10 test tubes supplemented with increasing concentrations of heavy metal salts (ranging from 0.1- 2 mg/ml) were prepared, autoclaved and inoculated with different bacterial isolates. The inoculated culture tubes were incubated at 37°C for 16-18 hrs and turbidity was determined using spectrophotometer to detect Minimal inhibitory concentration.

Determination of λ max for heavy metal samples

The λ max of the sample was obtained by taking the nutrient broth solution as a reference and the heavy metal+ nutrient broth was taken as a sample. The optical density was measured at different wavelengths ranging from 200-800nm and a peak was derived to estimate the wavelength for maximum absorbance. The absorption studies were performed on Systronics UV-Vis double beam spectrophotometer.

Heavy metal bioaccumulation studies

The salts used for bioaccumulation studies were copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$), potassium dichromate ($K_2Cr_2O_7$), Nickel Nitrate ($NiNO_3$), and lead nitrate ($PbNO_3)_2$. Stock salt solutions were prepared, filter sterilized and added in desired concentration into autoclaved Nutrient Broth according to the determined MIC. After inoculation, the culture vessels were kept under agitation in a rotary shaker, at 100 rpm for 48 hours at 35+_2 degree Celsius for absorbance determination.

Test tubes containing 4 ml broth (with metal salt solution in desired concentration) each were autoclaved and inoculated with different bacterial isolates. 100µl of fresh overnight grown culture of each isolate was inoculated into 8 test tubes containing nutrient broth along with heavy metal. The tubes were incubated at 37°C for a period of 24 hrs and samples were withdrawn at different time intervals. The culture withdrawn at different time intervals was centrifuged at 5000xg for 10 min to pellet out the bacterial cells. The supernatant was collected and its absorbance was estimated at λ max for each metal using UV- visible double beam spectrophotometer. Readings were taken at 1, 2, 4, 6, 12 and 24 hours respectively to detect the presence of heavy metal in the used culture medium. The absorbance at λ max depicts the presence of metal salt in the used medium which was later compared with the initial value.

Statistical analysis

Graphical representation of Bioaccumulation kinetics was used to determine the heavy metal accumulation properties of the respective isolates. The rate of removal for each metal salt was estimated from initial and final values.

$$\frac{(A_i - A_f)}{A_i} \times 100$$

Where Ai and Af are initial and final absorbance values respectively for each metal. Comparative studies of isolates against different metals were done by unpaired Student T test analysis.

Results and Discussion

The industrial effluent samples were collected from different ink and printing industries of Dehradun. Since, the effluents contain high levels of heavy metals, the indigenous microbial isolates derived from these effluents show heavy metal tolerance. Such microbes may be further used for evaluation of bioaccumulation activity. The microbial isolates were isolated using streaking and plating methods after serially diluting the samples. The isolates were morphologically differentiated according to their colony characteristics, size and morphology. The isolates derived from the industrial effluents are shown in [Table-1]. The morphological parameters were used to further purify these isolates and biochemical characterization of all the isolates was performed including MR test, VP test, Citrate Utilization, Gram's staining etc. The biochemical parameters of microbial isolates are given in [Table-2].

According to the biochemical characterization, it was observed that the isolates clearly resemble commonly found microbes like *E. coli*, *Salmonella*

Table-1 *Morphological Parameters of isolated bacterial strains*

Isolates	Gram's Staining	Cell shape	Culture characteristics
IE1	-	Rod shaped	Moist, white, glistening colonies
IE2	-	Rod shaped	Grey colored growth
IE3	-	Rod shaped	Thin green colonies
IE4	+	Rod shaped	Opaque, white colonies
IE5	+	Cocci	Yellow coloured smooth colonies

Pseudomonas, *Bacillus* and *Micrococcus* species [11]. According to the biochemical characterization, it was observed that the isolates clearly resemble commonly found microbes like *E. coli*, *Salmonella*, *Pseudomonas*, *Bacillus* and *Micrococcus* species [11]. The redundant isolates showing similar properties were removed during isolation and a single member was selected from every genus respectively to serve as a representative. From 24 isolates, 5 isolates with distinct biochemical characteristics were selected for bioaccumulation studies. Initially the metal tolerance in microbial isolates was evaluated by culturing them in Nutrient broth containing variable concentrations of heavy metal salts (colored salts were selected for visualization purpose and for measuring absorbance). To evaluate the Minimum inhibitory concentration for different isolates, nutrient broth with concentration gradient was prepared for every heavy metal salt. The results are shown in [Table-3].

Table-2 *Biochemical parameters of isolated bacterial strains*

Isolates	Starch hydrolysis	Lipid hydrolysis	Sucrose utilization	Lactose Utilization	H2S production	Indol production	Methyl red test	Citrate utililization	Nitrate reduction	Catalase activity	VP reaction	Urease activity
I.E-1	-	-	±	-	-	+	+	-	+	+	-	-
I.E-2	-	-	±	-	+	-	+	+	+	+	-	-
I.E-3	-	+	-	-	-	-	-	+	+	+	-	-
I.E-4	+	+	Acid production	-	-	-	-	-	+	-	±	-
I.E-5	-	-	-	-	-	-	-	+	±	-	-	+

Table-3 *Minimal inhibitory concentration for respective isolates against heavy metals. *Strains with potential metal bio-accumulative ability*

Isolates	Cu (mg/ml)	Cr (mg/ml)	Ni (mg/ml)	Pb (mg/ml)	Mean
IE1 *E. coli*	0.8	0.5	0.4	0.2	0.475
IE2 *Salmonella spp.*	0.8	0.7	0.2	0.1	0.45
IE3 *Pseudomonas spp.*	0.5	0.3	0.6	1.5	0.725
IE4 *Bacillus spp.*	0.8*	0.3	0.2	1.8	0.775
IE5 *Micrococcus spp.*	0.5*	0.4*	0.5*	0.5*	0.475
Mean	0.68	0.44	0.38	0.82	

Table-4 *Absorbance values at 525nm for Copper at different time intervals*

Copper (Cu)	1hr	2hr	4hr	6hr	12hr	24hr
IE1	1.4	1.35	0.6	0.2	0.01	0.01
IE2	0.9	0.73	0.624	0.521	0.49	0.38
IE3	0.96	0.87	0.82	0.79	0.71	0.67
IE4	1.35	1.15	0.84	0.56	0.22	0.1
IE5	0.7	0.62	0.54	0.55	0.48	0.42

The Minimal Inhibitory concentration and metal tolerance observed in the isolates was in correlation with the other studies in the field. MIC refers to the minimum concentration of the metal, which inhibits the growth of bacterial isolate. In the present study, it was observed that *Bacillus, Salmonella* and *E. coli* show maximum resistance against Cu upto 0.8mg/ml. *Salmonella spp.* was found to have maximum tolerance against Chromium, *Pseudomonas* against Nickel and *Bacillus spp.* against Lead. Micrococcus has been observed to show maximum tolerance against copper and lead as depicted by earlier studies [12]. The increased metal tolerance developed in the bacterial strains may be due to the irrational drainage of industrial effluents in surrounding soil and water bodies. The use of effluents for agricultural practices and presence of high heavy metal levels in the soil leads to increased microbial adaptability and development of metal resistant strains. From these resistance studies the pattern of heavy metal toxicity was determined. Among these heavy metals chromium and nickel were found to be more toxic to bacterial isolates as compared to lead and copper. The order of metal toxicity is Ni>Cr>Cu>Pb with most microbial isolates showing maximum resistance against Pb. The changes in the resistant abilities of isolates may be due to their increased ability for environmental adaptation. Moreover, it was observed that *Pseudomonas, Bacillus* and *Micrococcus spp.* were more resistant to heavy metal intoxication as compared to *E. coli* and *Salmonella.*

Table-5 *Absorbance values at 435 nm for Chromium at different time intervals*

Chromium (Cr)	1hr	2hr	4hr	6hr	12hr	24hr
IE1	0.8	0.72	0.705	0.69	0.52	0.45
IE2	0.9	0.65	0.46	0.2	0.17	0.2
IE3	0.9	0.87	0.54	0.51	0.48	0.32
IE4	0.85	0.79	0.73	0.65	0.545	0.5
IE5	0.98	0.43	0.34	0.21	0.05	0.1

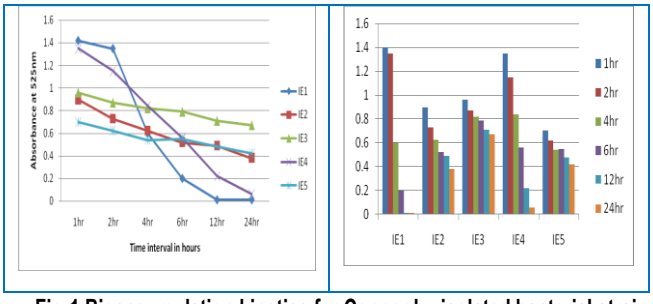

Fig-1 Bioaccumulation kinetics for Copper by isolated bacterial strains

λ max of different heavy metals salts was estimated by measuring the optical density of the samples in a range of 200- 800 nm wavelength. The λ max of Cu, Cr, Ni and Pb was found to be 525nm, 435nm, 415nm and 512 nm respectively. The culture conditions were optimized for standardization of bio-accumulative properties. The isolates were allowed to grow at different pH and temperature ranges along with the heavy metal salts present in the nutrient media. It was observed that maximum decrease in the absorbance occurred at a temperature of 37°C and at pH ranging from 6-7.

The cultures were withdrawn at different time intervals and centrifuged so as to sediment the bacterial cells. The supernatant was collected at each step and was spectrophotometrically analyzed to determine the presence of heavy metal. The optical density to detect the heavy metal was takes at it specific λ max. The absorbance values thus obtained at different incubation time interval for copper are depicted in [Table-5]. The results indicated that the accumulation of heavy

metal increased with the growth of the bacteria, with correlation to growth kinetics. IE1 was found to have high ability to bioaccumulate copper as compared to the other microbes. The maximum copper accumulation was thus observed in IE1 and minimum in IE5 [Fig-1]. On the contrary, Chromium accumulation was found to be most efficient in IE5 and minimum in IE1 as depicted in [Fig-2].

For the accumulation of nickel was observed in *Pseudomonas* then in the other isolates. The comparative analysis among the isolates shows that *Pseudomonas* species are highly efficient, but other isolates like *Salmonella* and *Micrococcus* species also show significant bioaccumulation and can be further explored for reclamation studies [Fig-3], [Table-6]. Moreover, the removal of lead was also estimated by absorbance studies. It was clearly observed through absorbance values and graphical representation that *Bacillus spp.* showed a considerable decrease in the levels of lead in the media during incubation. The reduction on lead levels is significantly high in case of *Bacillus spp.* as compared to *E. coli*. Although *Salmonella* also shows some reduction in lead levels in the culture medium but the reduction is significantly low. [Fig-4] [Table-7]. The comparative analysis reveals that the removal of copper was best exhibited by *E. coli* whereas Cr by *Micrococcus spp.*, Ni by *Pseudomonas spp.* and Pb by *Bacillus spp.* The % removal was estimated using absorbance values form initial and final samples [Table-8].

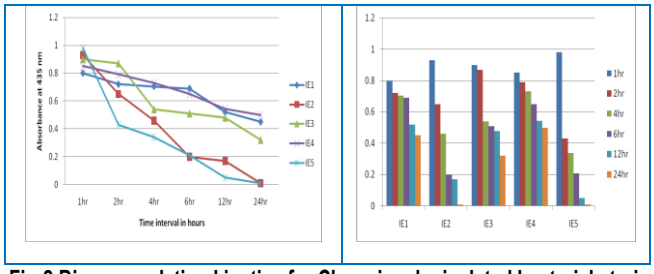

Fig-2 Bioaccumulation kinetics for Chromium by isolated bacterial strains.

Table-6 *Absorbance values at 415 nm for Nickel at different time intervals.*

Nickel (Ni)	1hr	2hr	4hr	6hr	12hr	24hr
IE1	1.14	0.95	0.91	0.85	0.78	0.73
IE2	0.98	0.87	0.65	0.54	0.34	0.2
IE3	0.8	0.47	0.21	0.12	0.04	0.1
IE4	1.22	1.12	1.04	0.92	0.87	0.82
IE5	0.87	0.81	0.65	0.53	0.51	0.4

Table-7 *Absorbance values at 512 nm for Lead at different time intervals*

Lead (Pb)	1hr	2hr	4hr	6hr	12hr	24hr
IE1	0.85	0.83	0.76	0.72	0.7	0.71
IE2	0.94	0.93	0.76	0.65	0.51	0.49
IE3	1.16	0.76	0.72	0.67	0.59	0.43
IE4	0.89	0.42	0.15	0.02	0.01	0.1
IE5	0.99	0.89	0.81	0.72	0.7	0.56

Table-8 *Percentage of removal of heavy metals in 24 hours*

Isolates	Cu removal (%/24 hrs)	Cr removal (%/24 hrs)	Ni removal (%/24 hrs)	Pb removal (%/24 hrs)
IE1	92	43	35	16
IE2	54	77	79	47
IE3	44	64	87	62
IE4	90	26	32	88
IE5	40	87	54	43

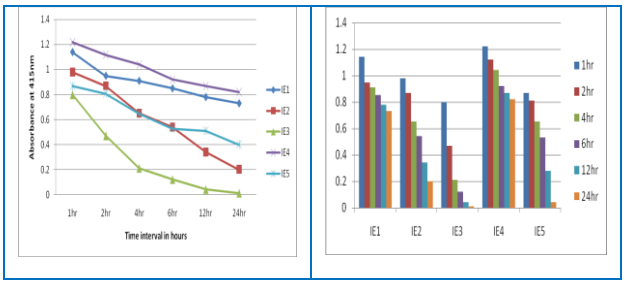

Fig-3 Bioaccumulation kinetics for Nickel by isolated bacterial strains.

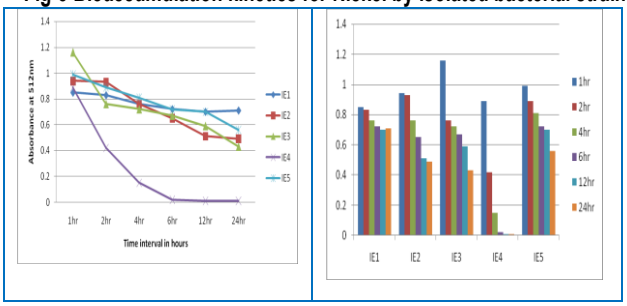

Fig-4 Bioaccumulation kinetics for Lead by isolated bacterial strains

In contrast to the other degradable pollutant, heavy metals are extremely persistent in polluted sites. The difficult removal of heavy metal from the environment leads to the development of diverse strategies for reclamation. The methods like chemical precipitation and solvent extraction have been used for remediation purpose earlier but these methods suffer from certain limitations like application on large areas, economically inefficient and environmentally inadaptable [13]. Biological approaches are currently being evaluated for the reclamation of polluted areas due to their easy availability and high efficiency without adversely affecting the environmental niches. The uptake of metal in microbes occurs either actively or passively through bioaccumulation and biosorption respectively [14-15]. In earlier reports, *Bacillus* sp., *Pseudomonas* sp. and *Micrococcus* species have been identified to be resistant against Cu, Cd and Pb in the earlier reports. Pardo *et al.* (2003) have reported that *Pseudomonas sps.* have an explicit quality to remove cadmium for polluted industrial samples. They estimated 80% removal for cadmium, lead, zinc and copper, and concluded that it can be further utilized for the quick reclamation of heavy metal polluted regions. Similarly, Alam *et. al.* (2011) have reported the removal of chromium by bacterial isolates for treatment of tannery effluents [2].

Srinath *et al.* (2002) also studied the bioaccumulative activity of *Bacillus* sps and concluded that they can be used for reclamation of Cr intoxicated regions [20]. Similarly, Issazadeh *et al.* (2011) concluded that *Bacillus* sps show effective lead bioaccumulation [21]. Blackwell *et al*., (1995) suggested that the bioaccumulative properties of microbial isolates depend on the biochemical, physiological and structural properties of the bacterial cell membrane as well as genetic adaptability [22].

The bioaccumulation of the microbe is growth dependant [17] and is exhibited by both living as well as dead biomass [18]. The remediation of heavy metal contaminated ecosystems using microbes is of considerable interest because of being ecofriendly and cost effective. The bacterial isolates identified in this study can be efficiently used for the reclamation of heavy metal contaminated regions and also for the pre-release treatment of industrial effluents from ink and printing industries [19]. The increased microbial resistance to heavy metals present in the environment is hazardous to the ecosystem but on the contrary these bacterial isolates may also be utilized for the reclamation of heavy metal polluted environmental regions. Therefore the present study highlights the role and importance of the biological methods and the applications of bacterial isolates in remediation of polluted areas as well as treatment of industrial effluents prior to release. This method may be useful for wide application in industrial and agricultural purpose.

Conclusion

The heavy metal contaminants in the ecological niche create a crucial environmental hazard. The microbial systems, with the ability to metabolize and bio-accumulate these heavy metal contaminants, are thus a substantial source of remediation. Hence, the present study is a move in the microbial based bioremediation approaches for the development of microbial isolates for heavy metal contaminants. The microbial strains isolated from effluents with specific high metal resistance are hypothesized to be a significant source for ground based bioremediation techniques. Further studies are yet required to understand the biochemical mechanisms and genes behind such metal resistant and

accumulative properties.

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Conflict of Interest
The authors declare that they have no conflict of interest.

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