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SCREENING AND ISOLATION OF BACTERIAL ISOLATES FOR HEAVY METAL BIOACCUMULATIONFROM CONTAMINATED INDUSTRIAL EFFLUENTS AROUND BANGALORE

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ABSTRACT

The increasing population along with urbanization and industrialization hasaugmentedwith a drastic accumulation of hazardous heavy metal wastes into the milieuposing potentialhazards to human health andthe environment. In comparison to chemical and physical remediation, microbial bioaccumulation is cost-effective and eco-friendly.Current investigation involved the screening and characterization of heavy metal-tolerantbacterial isolates fromindustrial-polluted soil samples around Bangalore.Of the 184bacterial colonies screened, 28isolates were characterized due to their significant growth in Luria Bertini (LB)containing100ppm of lead (Pb), cadmium (Cd), and chromium (Cr).The analysis exhibited isolates from all three soil sampleswithhighest resistance towards heavy metals as Pb, followed by Cr and Cd *i.e.*, Pb>Cr>Cd. Majority were observed to bespore forming Gram-positive bacillifollowed by Gram-negative bacteria.Subsequently, MALDI-TOF-MS identificationrevealedgenus *Bacillus* as predominant species, followed by *Staphylococcus sp*, *Pseudomonas, Acinetobacter, Enterococcus, Citrobacter,*and*Paenibacillus.*Heavy metal tolerance revealed, 3000ppm for Pb as highest, followed by 2200ppm for Cr and 700 for Cd. The highest metal tolerance was observed in*B. megaterium*with MIC values of 3000ppm, 1000ppm, and 1500ppm for Pb, Cd and Cr respectivelyindicating presence of multiple genes for remediation. However, significant results were revealed for Cr metal tolerance at 2200ppm by *S. xylosus,B. subtilis*and *B. cerues*showed Cd tolerance of 2200ppm. Among Gram-negative bacteria, *Pseudomonas aeruginosa* and *Citrobacter youngae* showed substantial Pb and Cr resistance at2500ppm and1000ppmrespectively and without much significance towards Cd. Current study highlights the potential soil bacteria withsignificant heavy metaltolerance that could be efficiently employed to develop sustainable bioremediationwithcompetentto restore ecological balance in heavy metal affected zones.

Keywords: Heavy metals, Industrial pollutants, Bioremediation, Soil bacteria, Heavy metal resistance, MALDI-TOF-MS, Bioaccumulation

Introduction

Heavy metal contaminationof theatmosphere isattributedtoequallynatural processes as well as anthropogenicactivities.However, contamination is further intensified by several human activities such as industrialization, expansion of urbanization, mining and refining activities, industrial waste disposal, and dumping of waste into the ecosystem [Kumar and Dwivedi, 2021; Briffa *et al.,* 2020]. Rapid development in industrialization and urbanizationled toheavy metal pollution as a global problem that has ill effects on humans, animals along with the surrounding ecosystem [Pande *et al.,* 2022].Although few heavy metals are required by living cells, most of them are not utilized by the cells as they are highly dense and extremely toxic at low concentrations. Heavy metals likeNickel(Ni), Copper (Cu), Mercury (Hg), Lead (Pb), Cadmium(Cd), Chromium(Cr), and Arsenic (Ar) are widespread in the ecosystem which is not a good insignia [Selvi *et al* ., 2019].Moreover, theyspread in the environmentthrough the disposal of industrial wastes posinga severe threat to living creatures**.** Hence,immediate measures need to be addressedto prevent the further spread of heavy metal pollution.

Traditionally, several physical methods include adsorption, soil washing, granular activated carbons, and electromagnetic techniques while, chemical methods used for removal of heavy metals from soil and water includes flocculation, coagulation, precipitation, ion exchange, and membrane filtration [Akhtar *et al.,* 2020].Although these methods are efficient ineliminating heavy metals,beinglowand cost-effectivethey are not eco-friendly as they generate sludge wastes and are hazardous chemicals that are difficult to treat and dispose of[Diep *et al.,* 2018; Akhtar *et al.,* 2020].In nature,microbes have the potential toutilize heavy metals through biosorption to drive their biochemical reactions and accumulate excess metals inside the cell referred to as bioaccumulation.Microbes detoxify heavy metals through redox transformation, leaching, chelation, and methylation making them less harmful. Microbial bioaccumulation is economical, versatile, more effective, andeco-friendly as it generates no hazardous waste [Nnaji *et al.,* 2023].

Bacterial microbes like, *Pseudomonas aeruginosa,* and *P. putida*have been reported to accumulate Cd and Pb inside the cells, respectively [Zolgharnein 2010, Rani 2009]. About 57% of Pb is accumulated by *Bacillus*, while, *Staphylococcus* sp., *Stenotrophomonas* sp., and *Klebsiella pneumonia* can accumulate and possess a capacity to tolerate about 1.0 to 1.6 mg ofPb, 0.5 to 1.0mg of Cr, and 0.7 to 1.0 mg of Ni,respectively [Slavin *et al.,* 2017; Hoque and Fritscher, 2019].A study on fungal species such as *Talaromyces helices*, *Trichoderma*

koningii, Gliocladiumroseum, and *Cladosporium cladosporioides*has been analyzedfor bioaccumulation of heavy metals. It has also been described that *Fusariumflocciferum* absorbs heavy metals like Ni, Cd, and Cu too[Mathew *et al.,* 2015]. Therefore, heavy metals bioremediation by using microbial system is a promising technique for treating contaminated ecosystems.

Materials and methods

Chemicals and media: Following chemicals and media were procured from Himedia, India such as Luria Bertani (LB) media, α-cyano-4-hydroxycinnamic acid, acetonitrile, water, lead acetate **(**$Pb(CH_3COO)_2$**),** Potassium dichromate ($K_2\ Cr_2\ O_7$), Cadmium chloride ($CdCl_2$),Gram's staining kit and Malachite green, ethanol etc., The analysis involving atomic absorption spectroscopy (AAS) (GBC Avanta, Victoria, Australia) was carried out at Biocentre, Bengaluru, and Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) at NIMHANS, Bengaluru.

Methods

Site description, soil collection,and preliminary screening of heavy metal-resistant bacterial isolates

The soil samples were collected from Bellandurriver bank (BRB), Peenya industrial area (PIA),and Byramangala agriculture land (BAL), Bengaluru, Karnataka, India corresponding to geographical coordinates 12º56'3'N 77º39'46E, 13.03ºN 77.514ºE, and 12º46'00.7"N 77º25'23.2"E respectively.The samples were collected using a sterilized container under possible aseptic conditions. The locationsofthe samples were cleaned from all debris and the places were dug to15cm depth to collect the samples using a sterile spatula. The samples were immediately processed after transferring to the laboratory by serialdilutiontechnique [Marzan*et al.,* 2017].

In 10ml of sterile saline, 1gm of soil sample was added and mixed thoroughly and serially diluted from 10^0 to 10^6 dilutions.Sterile LB agar plates were prepared using 100ppm of Pb, Cr, and Cd by adding lead acetate ($Pb(CH_3COO)_2$), Potassium dichromate ($K_2\ Cr_2\ O_7$), and cadmium chloride($CdCl_2$). Meanwhile, LB agar plates containing individual heavy metals at 100ppm were also prepared. Following this, the plates were allowed to solidify. From each dilution, 0.1ml of the sample wasadded on bothLB agar plates containing all three and individual heavy metal(s) and spread evenly. Plates were incubated at 37ºCfor 24-72hours in a bacteriological incubator. Followed by the incubation, plates were observed for bacterial colonymorphology and results were recorded.

SEM-EDX-based analysis of elemental contents of soil samples

The soil samples were characterised for morphological and elemental composition by SEM-EDX technique (Hitachi SU-8200 SEM and Oxford Instruments EDX). All the three samples were dried in a hot air over for 24hours at 40°C. Prior inserting into the SEM, on a stub, a small amount of soil sample measuring 1mm height and 1 mm width was placed.The stub was inserted and all images of the soil at defined places were captured and chemical compositions of selected area were studied by EDX [sun *et al.,* 2019; Akhtar *et al.,* 2023].

Cultural and Morphological identification of heavy metal resistant bacteria up to genus level

Following the growth on LB mediawith heavy metals, the resistant bacteria were selected for preliminary screening to be identified based on bacterial colony morphology such as colony size, shape, colour, and texture using standard microbiology manuals (Eklind and Lankford, 1967; Society of American bacteriologist, 1957). Further based on morphology, identical bacterial colonies were eliminated and non-identical bacterial colonies were sub-cultured and used for testing heavy metal tolerance by the minimum inhibitory concentration (MIC) method.

Evaluation of heavymetal tolerance of bacteria by minimum inhibitory concentration

The selected bacterial isolates from preliminary screening were subjected to MIC using heavy metals by agar dilution method [Wiegand *et al.,* 2008]. The stock solutions of each heavy metal*i.e.,* Pb, Cr, and Cd were prepared separately at 5000ppmconcentration and filter sterilized usinga 0.45 membrane filter. The LB plates containing various concentrations of heavy metals ranging from100 to 3000ppm were prepared from stock solutions,and bacterial inoculum was adjusted to McFarland standard corresponding to $03x10^{8}$cells/ml. On LB plates containing various heavy metals, 100µl of standard inoculum was added and spread uniformly, and the plates were allowed to stand for some time. Following this, plates were incubated at 37ºC for 24 to 72 hours. Post incubation, plates were observed for bacterial growth, and results were recorded. The minimum concentration of heavy metal(s) at which the complete growth of bacterial isolate was inhibited is recorded as MIC (Sanjay *et al.,* 2018; Mitra *et al.,* 2022).

MALDI-TOF-MS-based identification of heavy metal tolerant bacteria

The heavy metal tolerant soil bacteria with high concentrations were sub-cultured and identified by the MALDI-TOF-MS technique. A pure culture of metal-resistant bacterial isolate of 24 hours old was placed in wells of the steel target plate. To the wells containing pure bacterium 1µl of neat formic acid was added and dried. A matrix was prepared by dissolving 10mg/ml of α-cyano-4-hydroxycinnamic acid in a solution containing acetonitrile, water, and trifluoroacetic acid at 50%, 47.5%, and 2.5% respectively. One microliter of matrix was added to each well, dried, and analyzed using MALDI-TOF-MS [Alcolea-Medina *et al.,* 2019 ; Nazir *et al.,* 2020].

Study of bioaccumulation of heavy metal-resistant bacteria by AAS

The pure cultures of bacteria grown for 24 hours were inoculated in a sterile LB medium and incubated at 37ºC for 1 hour in a shaking condition at 150rpm until it reached O.D of 0.6 at $A_{600.}$ Followed by incubation,2ml of sterile heavy metal solution of 100ppm was added to respective flasks, and incubation was continued at the same conditions for 24 hours. The next day, the entire bacterial cells were recovered by centrifuging the solution for 15 minutes at 5000rpm. Supernatants thus obtained were mixed with double the volumes of 70% Conc. HNO_3. Further, acid-treated samples were boiled at 100ºC on a hot plate until the volume of the test sample was reduced to the initial volumes. The resultant extract was filtered in a filter paper using Whatman 42 and used for analysis by AAS to determine the heavy metal concentration accumulated in the metal-resistant isolates. The amount of heavy metals accumulation was calculated as shown below [Marzan *et al.,*2017 ;Aslam *et al.,* 2020].

$$\text{Heavy metal accumulation capacity,\%} = \frac{\text{Heavy metalutilized (ppm) by bacterium}}{\text{Heavy metaladded to the LB medium (ppm)}} \times 100$$

Heavy metalutilized (ppm) by bacterium =Heavy metaladded to the LB medium (ppm) – Heavy metalat the end of culture (ppm)

Results

Sample collection points and screening of heavy metal-resistant bacteria

The metal-accumulating bacteria from soil samples collected from three diverselocations of Bangalore regions were initially screened for metal resistance properties. Following theselection at the preliminary stage, these metal resistant bacteriawere evaluated for metal tolerance capacity. Further, bioaccumulation capacities of selected heavy metals by these bacteria were determined. The soil collection point *i.e.,*theBellandur river bank is surrounded by various metal industries and it is well known for industrial waste disposalas reported in daily news [DHNS, 2017]. Soil sample was collected from the Peenya industrial area which

is Asia's biggest industrial sector with approximately 8000 crores turnover and also consists of several metal industries[The Hindu, 2005;DHNS, 2015]. The effluents from the Peenya industrial area enter into the Vrushabhavati River which further flows into the Byramangala agricultural land which was chosen as the third soil sample collection point in the present study [**Figure 1.0**]. Since, all these succeeding locations are rich in metal industries and their waste disposals, it can be anticipated that bacteria habituating are heavy metal resistant and they accumulate excess heavy metals inside the cells.

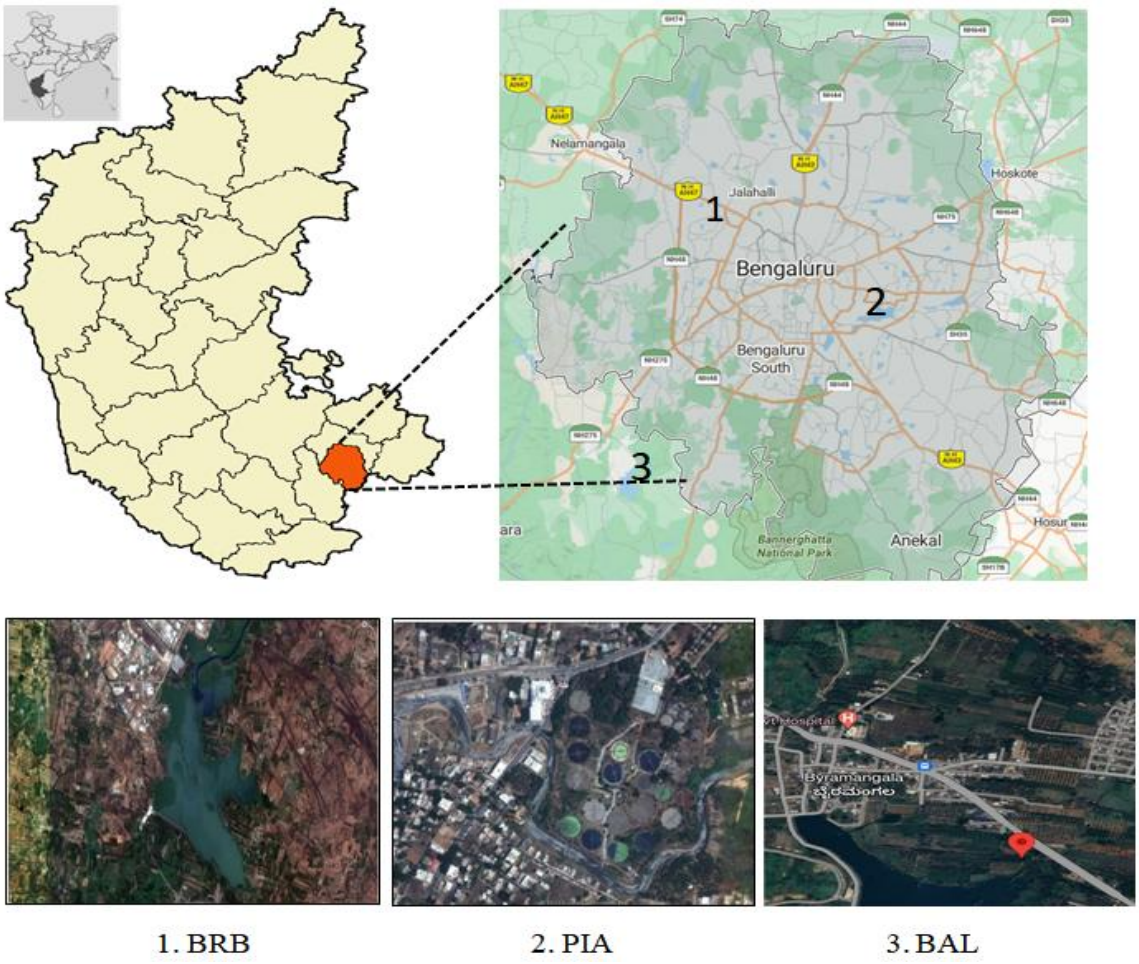

Figure 1.0 Geographical locations of soil collection for isolation of metal-resistant bacteria. Note: BRB - Bellandur River Bank, PIA - Peenya Industrial Area, BAL - Byramangala Agriculture Land.

Soil analysis:

The elements found in the soil that are primarily found in alluvial formations are carbon (C), oxygen (O), aluminium (Al), silicon (Si), potassium (K), sodium (Na), iron (Fe), arsenic (As), and beryllium (Be), as described by the SEM-EDX analysis. The y-axis represents the overall concentrations of the soil sample, while the x-axis represents the elemental content (Fig. 2&3). The average percentage of components such as Cr, Fe, Pb, O, Na, Mg, Al, Si, Cd, K,

and Ca were also reported. Subsequently, heavy metal proportions analysed for BRBsample showed 4.97% of lead followed by 0.72% of chromium and 7.34% of cadmium, while, PIA soil showed Pb with concentration of 4.64%,0.47% of Cr and 0.25% of Cd. Our study results from BAL soil were found to be 0.87% for Pb, 0.34% for Cr and 0.50% of Cd. These components (as stated above) are found as natural deposits in soil samples as indicated by geological sources [Akhtar et. al., 2023].

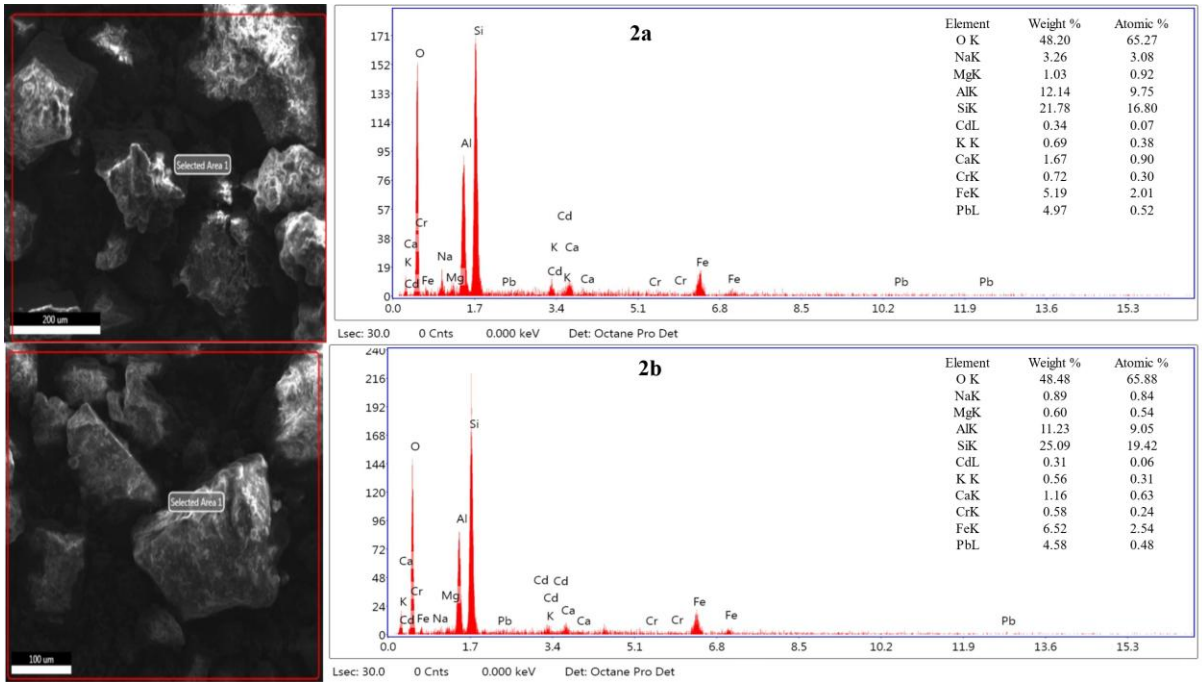

Figure 2a and 2b: SEM images and EDS of soil analysis from Site 1 - BRB - Bellandur River Bank

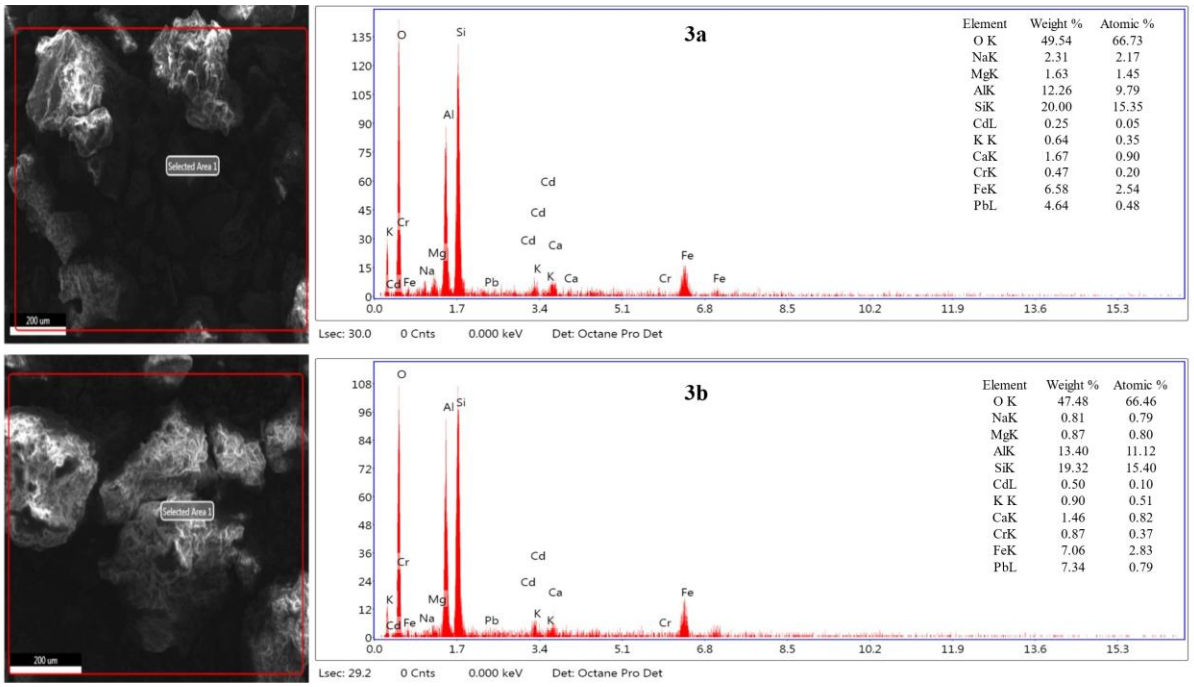

Figure 3: SEM images and EDS of soil analysis from Site 2 and Site 3 - PIA - Peenya Industrial Area (3a), BAL - Byramangala Agriculture Land (3b).

Soil from the above three locations was collected aseptically and processed by spread plate technique. Followed by the incubation, the average numbers of CFU/g of soil from each sample on respective heavy metal(s) containing LB plates were calculated(**Table 1.0)**. During the first 24 hours of incubation, slow growth of bacteria was noticed on the LB medium containing a single heavy metal and very less or no growth was seen in the LB plate containing two or three heavy metals. The growth on the LB plate containing 3 heavy metals was seen on the third day of incubation and followed by this no further increase in the bacterial colonies was observed.

Table 1.0 Heavy metal-resistant bacterial isolates from three selected locations in the Bangalore region

Soil location	The average number of heavy metal resistance bacteria isolated from soil (cfu/g)							Total cfu/g
	Pb	Cd	Cr	Pb+Cd	Pb+Cr	Cd+Cr	Pb+ Cd+ Cr	
BRB	33±2.5	06±1.5	15±2.0	01±0.5	10±1.5	00±0.0	01±0.5	$66x10^0$
PIA	35±2.0	12±2.0	18±2.5	01±0.5	11±2.5	00±0.0	01±0.5	$78x10^0$
BAL	18±2.6	06±2.0	10±2.0	00±0.0	06±2.0	00±0.0	00±0.0	$40x10^0$

Note: BRB; Bellandur river bank, PIA; Peenya industrial area, BAL; Byramangala agriculture land, Experiment conducted in triplicate, *n*=3.

In Total, 184 bacterial isolates were isolated from soils collected from three locations in which the highest heavy metal-resistant bacterial population *i.e.*, $78x10^0$CFU/g was noticed in PIA soil. Among these colonies, 35 colonies were resistant to Pb, 12 colonies were resistant to Cd, and 18colonies were resistant to Cr and one colony was resistant to both Pb and Cd,and 11colonieswere resistant to both Pb and Cr and no colonies were detected in the plate containing Cd and Cr. Only a single bacterial isolate was observed on the LB plate containingall three metals *i.e.*,Pb, Cd, and Cr.Similarly, soil collected from the BRB revealed almost equivalent bacterial population compared to PIA soil where majority of them were also resistant to Pb, followed by Cr and Cd. However, metal resistant bacteria isolated from BAL soilwerecomparatively the less in number.The total number of bacterial colonies and their heavy metal resistance obtained at preliminary screening of heavy metal resistanceis

shown detail in **Table 1.0.** Surprisingly, the trend ofheavy metal resistance among the bacterial population in all three locationswas found to be similar indicatingthe highest resistance to lead followed by chromium and cadmium *i.e.*,Pb>Cr>Cd as shown in **Figure 1.2**.

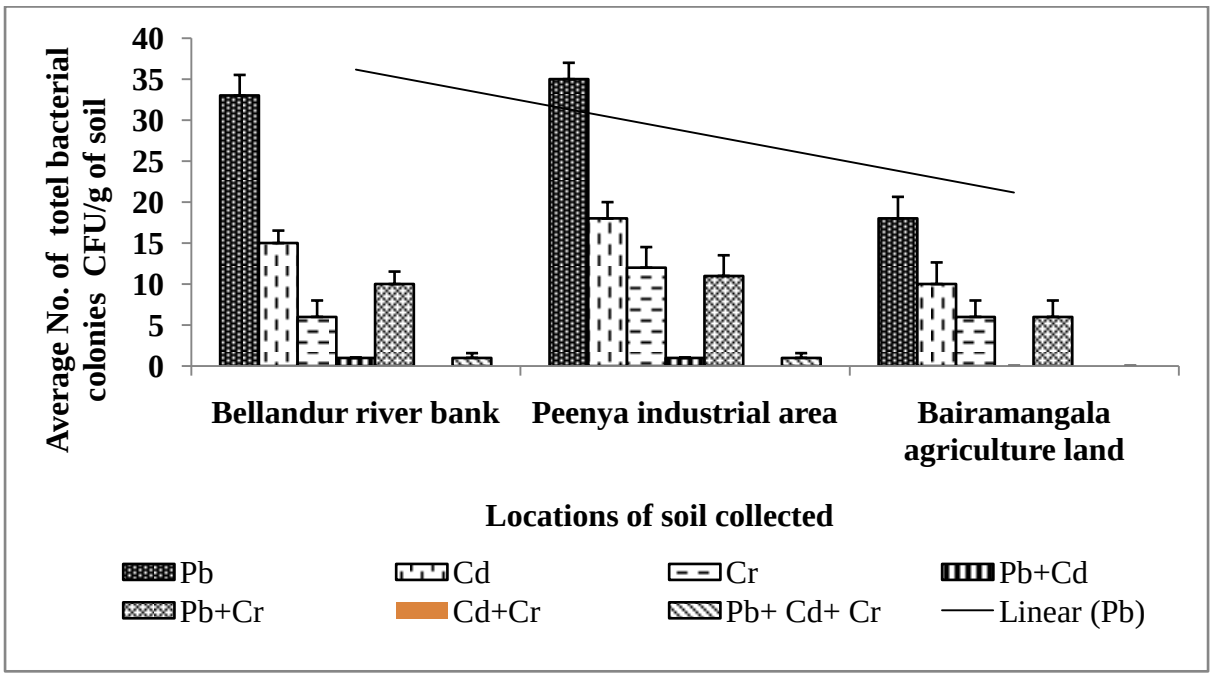

Figure 2.0Heavy metal resistance bacterial colonies isolated from three soil samples during preliminary screening.

Preliminary identification of heavy metal-resistant soil bacteria

The heavy metal-resistant bacteria strains were identified based on their colony morphology, Gram staining, and endospore staining. The bacterial isolates screened from different locations showed varying sizes of bacterial colonies ranging from 3mm to 6mm with various colony characteristics such as shape, texture, and colour. Majority of them were found to be Gram-positive while,Gram-negative bacteria were found to be exceptionally low. Most of the Gram-positive bacteria revealed endospore formation. The colonies with identical colony characteristics were considered as one group and a totally of 28 bacterial colonies with different colony characteristics were sub-cultured and coded with soil locations such as RBR 1 to 14, PIA 1 to 6, and BAL 1 to 8. The total number of positive bacterial isolates and their metal resistance to single, double, and triple metal are shown in **Figure 4.**

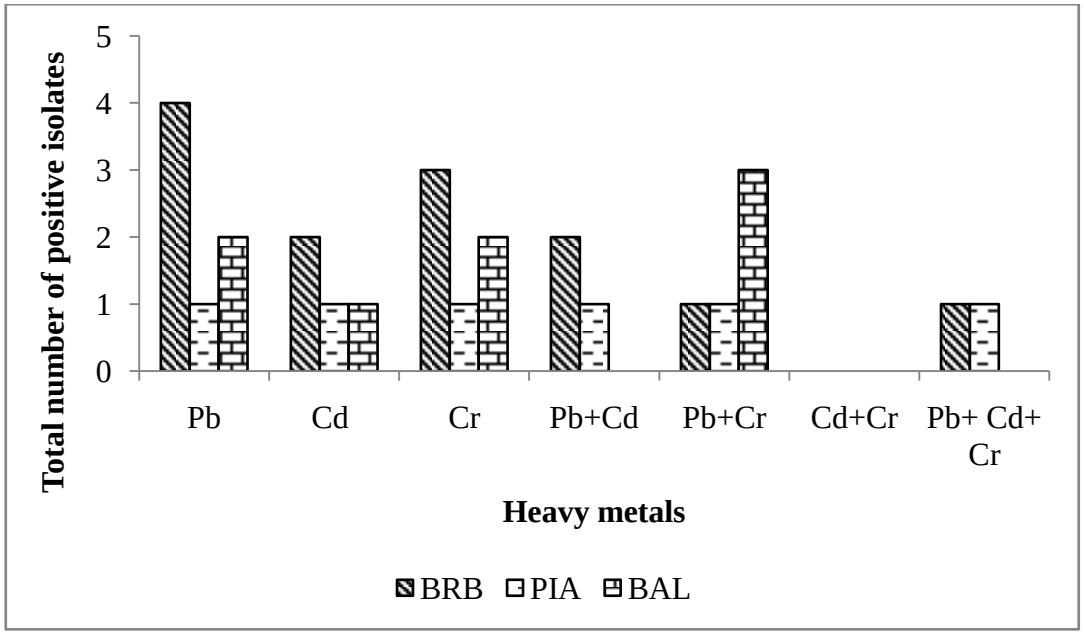

Figure 4.0 Total number of positive isolates resistance to single, double, and triple metals

Among 14 bacterial colonies isolated from BRB soil, 04 colonies were resistance to Pb, 02 colonies were resistance to Cd, 03 colonies were resistance to Cr, 02 colonies were resistance to both Pb and Cd, only single colony was resistance to Pb and Cr, and 01 colonies were resistant to Pb,Cd, and Cr. Similarly, among 06 colonies, each colony isolated from PIA soil was resistant to Pb, Cd, Cr, Pb+Cd, Pb+Cr and Pb+Cr+Cd. In eight colonies of BAL soil, 02 colonies were resistance to Pb, one colony was resistance to Cd, 02 colonies were resistance to Cr and 03 colonies were resistant to both Pb and Cr. The pure colonies were preserved in 30% glycerol stocks at -20ºC in a deep freezer. The bacterial colony morphology of all heavy metal-resistant isolates isolated from three soil locations was recorded in **Table 2.0**.

Table 2.0 Colony morphology of heavy metal-resistant bacteria isolated fromthree soils

Bacteria colony	Colony characteristics of bacteria isolated from soil samples					
	Size (mm)	shape	Texture	Colour	Gram's stain	Endospore
BRB 1	04	Round	Smooth	Light green	-Bacilli	Absent
BRB 2	05	Round	Smooth	Pale Brown	+ Cocci	Absent
BRB 3	03	Lobate	Rhizoid	Cream	+ Bacilli	Absent
BRB 4	04	Round	Smooth	Cream	+Bacilli	Present
BRB 5	06	Lobate	Smooth	Cream	+ Bacilli	Absent
BRB 6	04	Round	Smooth	Pale Brown	+ Bacilli	Absent
BRB 7	04	Round	Smooth	Brown	- Bacilli	Present

BRB 8	03	Irregular	Lobate	Cream	+ Bacilli	Absent
BRB 9	06	Round	Smooth	Cream	+Bacilli	Absent
BRB 10	05	Round	Flat	Cream	+Cocci	Absent
BRB 11	05	Round	Smooth	Cream	+Bacilli	Present
BRB 12	03	Round	Craterifor m	Pale brown	-Bacilli	Absent
BRB 13	04	Round	Raised	Brown	+ Bacilli	Present
BRB 14	04	Irregular	Lobate	White	+ Bacilli	Present
PIA 1	06	Lobate	Rhizoid	Cream	+ Bacilli	Present
PIA 2	04	Hairy	Pigmented	Cream	+ Bacilli	Present
PIA 3	04	Round	Flat	Brown	+ Bacilli	Absent
PIA 4	06	Round	Rhizoid	Cream	+ Bacilli	Absent
PIA 5	04	Hairy	Pigmented	Cream	+ Bacilli	Absent
PIA 6	06	lobate	Rhizoid	white	+ Bacilli	Present
BAL 1	03	Round	Smooth	Cream	+ Bacilli	Present
BAL 2	04	Round	Smooth	Cream	+ Bacilli	Present
BAL 3	06	Round	Smooth	White	+ Bacilli	Present
BAL 4	06	Round	Smooth	Brown	+ Bacilli	Present
BAL 5	04	Round	Slimy	Cream	-Bacilli	Present
BAL 6	05	Lobate	Filamentou s	White	+ Bacilli	Present
BAL 7	03	Lobate	Rhizoid	white	+ Bacilli	Present
BAL 8	03	Round	Entire	white	+ Bacilli	Present

Further, all these heavy metal-resistant bacteria were selected aimed at evaluation for their metal tolerance potency against 100 to 3000ppm heavy metal concentration.

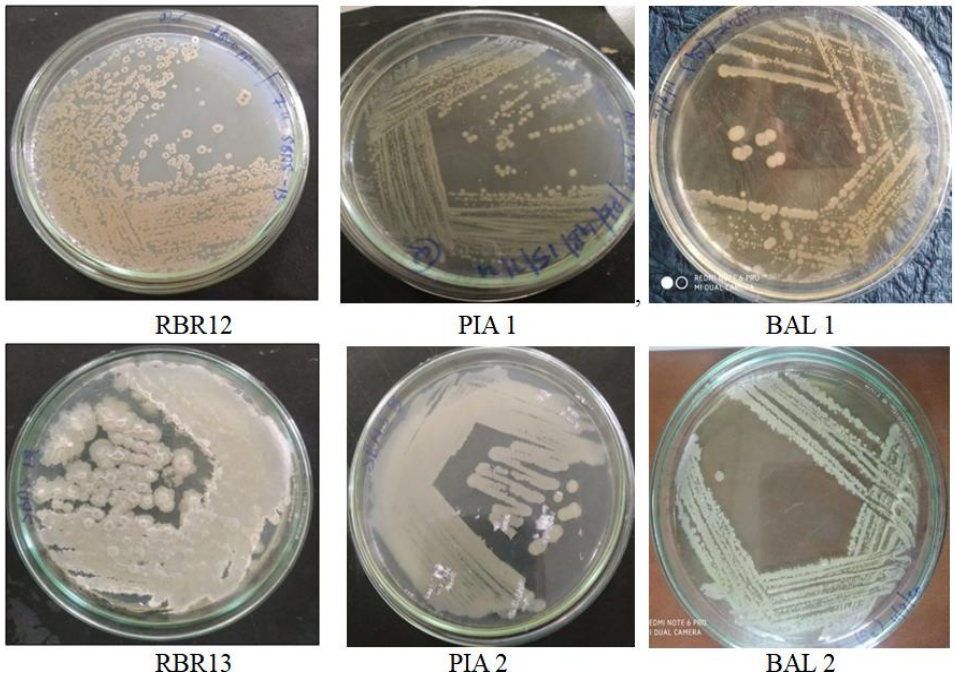

Figure 3.0 Preliminary screening of metal-resistant bacteria isolates from soil samples

Evaluation of soil bacteria for heavy metal tolerance by MIC techniques

Heavy metal-resistant bacterial isolates were evaluated for heavy metal tolerance potential against 100 to 3000ppm by MIC technique and results are indicated in **Figures 3.0 and 4.0**. The majority of bacteria population isolated from all three soils showed significant heavy metal tolerance towards Pb metal indicating the MIC value ranging from 1200 to 3000 ppm. However, the MIC value of heavy metal tolerance against Cd and Cr was observed in the range of 100 to 2200 ppm. Among bacterial colonies isolated from BRB soil, BRB3 showed significant metal tolerance against Pb and Cr with MIC values of 3000 and 1500 ppm respectively. Following this, colonies BRB1 and 3 were also resistant to 3000ppm of Pb, and isolates 4, and 6 to 11 showed Pb tolerance of 2500ppm.Isolates BRB 2, 3, 12, and 05 were shown the highest resistance Cr with MIC values of 2200ppm and 2000ppm respectively. All isolates of BRB soil indicated very little tolerance to Cd except BRB4, BRB1, and BRB5 which were resistant to 2200, 1200, and 500ppm of Cd respectively. In metal-resistant bacteria isolated from PIA and BAL soils, isolates PIA 1 to 5 revealed Pb tolerance of 2500 ppm and Cr tolerance of 700–1200ppm. Bacterial colonies BAL 1 and 3 showed potential tolerances to Pb with a MIC value of 3000ppm and additionally BAL 1 was also resistant to 1000ppm of Cd and 1500ppm of Cr. But BAL2 showed high resistance *i.e.*, 2000ppm to Cr, and low resistance to Cr *i.e.*, 300ppm. Isolate BAL 2, 3, and 5 indicated the maximum tolerance of Cr with a MIC value of 2000ppm. While, isolates such as BAL1 and 4 showed significant Cd heavy metal tolerance with MIC values of 1000 and 1500ppm. Hence,

bacterial isolates namely, BRB1 to 14, PIA1 to 6, and BAL1 to 6 were considered as potential metal-tolerance isolates concerning Pb, Cd, and Cr bioremediation. Significant heavy metal tolerance in all isolates of soil bacteria was noticed against Pb and Cd and the least tolerance was observed against Cr heavy metal. Potential heavy metal accumulating bacterial colonies were further selected for identification by the MALDI-TOF-MS technique.

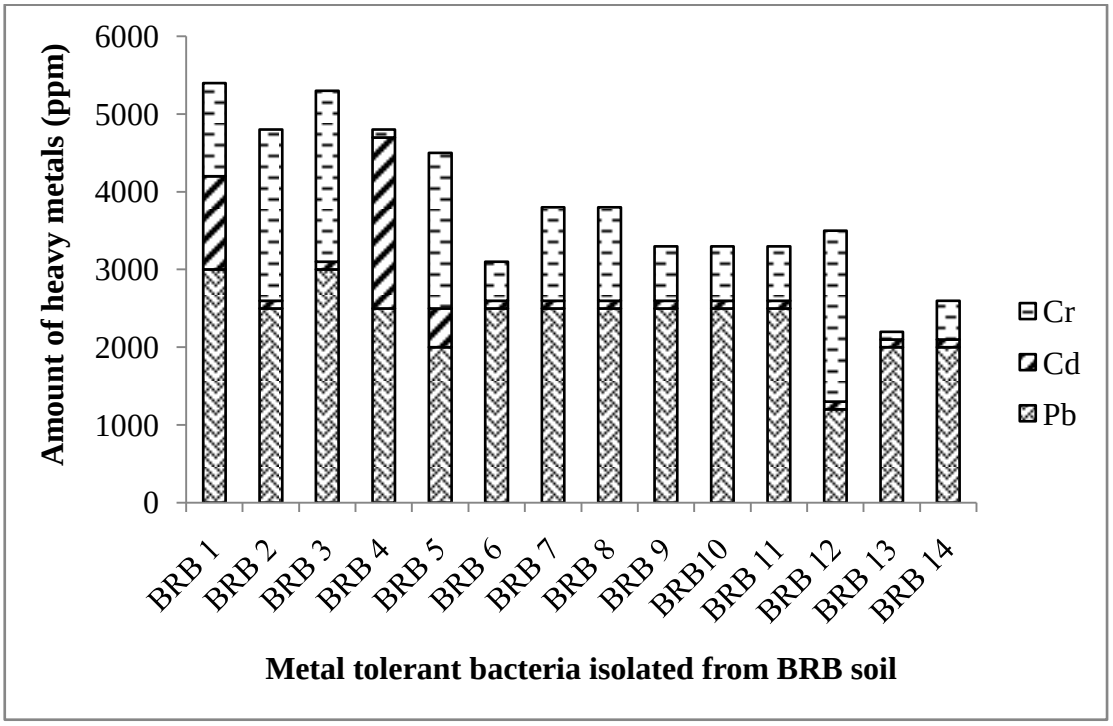

Figure 4.0 Heavy metal tolerant bacterial population isolated from BRB soil

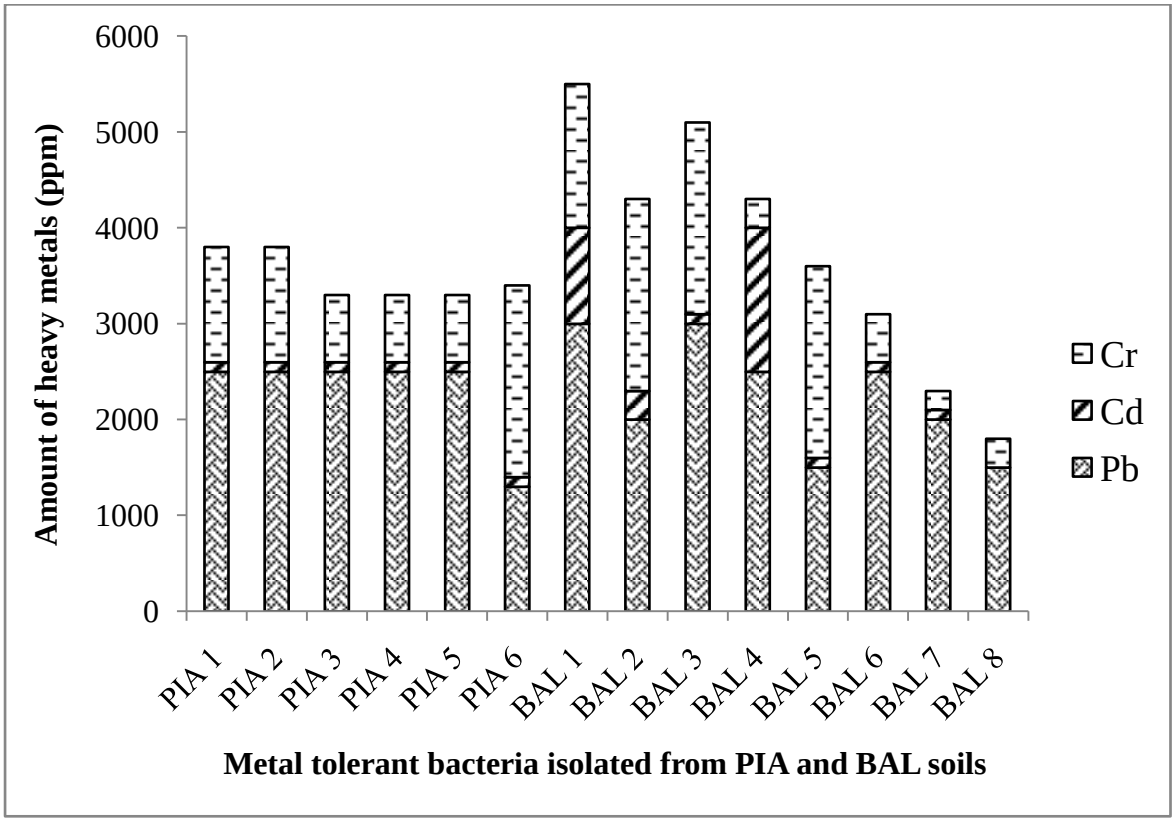

Figure 5.0 Heavy metal tolerant bacterial populations isolated from PIA and BAL soils

MALDI-TOF-MS andbiochemical-based identification of metal-resistant bacteria

Bacterial characterisation by MALDI-TOF-MS analysis was selected based on their MIC value *i.e.,*the growth of bacterial isolates at the highest MIC value or tolerant to high heavy metal(s) concentration that leads to accumulating heavy metals inside the cells. Potential heavy metal accumulating bacteria from BAL soil identified through MALDI-TOF-MS analysis were *B. megaterium*with highest metal tolerance to Pb (3000ppm), Cr (1500ppm), and Cd (1000ppm)While, *B. cereus group*was resistant to3000ppm of Pb, 2000ppm of Cr, 100 ppm of Cdand *S. epidermidis* showed resistance to 2500ppm of Pb, 1500ppm of Cd, 300ppm of Cr. Following this, bacterial isolates from PIA soil, *B. cereus group* revealed highest resistance to heavy metals (2500ppm of Pb, 100ppm of Cd, and 1200ppm of Cr). However,*Bacillus altitudinis/pumilus*showed resistance to Pb, Cd,and Cr with MIC values of 1200ppm,2500ppm, and 100ppm respectively.

Subsequently, *P. aeruginosa*, *B. subtilis, S. xylosus,* showed significant metal tolerance to 3000ppm of Pb, 2200ppm of Cr, and 1200ppm of Cd respectively. *B cereus*isolated from BRB soil also showed metal resistance Pb,Cr, and Cd with MIC value of 2500ppm, 700ppm, and 100ppm respectively. A list of heavy metal-tolerant bacterial colonies identified through MALDI-TOF-MS from all three soil samples along with their MIC values of heavy metal is indicated in **Table 3.0.**

Table 3.0. Heavy metal tolerant soil bacteria identified through MALDI-TOF-MS

S. No.	Soil collection	Name of bacterial isolate	Heavy metal tolerance (ppm)		
			Pb	**Cd**	**Cr**
1	BAL1	*Bacillus megaterium*	3000	1000	1500
2	BAL2	*Bacillus subtilis/ amyloliquefaciers/ vallismortis*	2000	300	2000
3	BAL3	*Bacillus cereus group*	3000	100	2000
4	BAL4	*Staphylococcus epidermidis*	2500	1500	300
5	BAL5	*Klebsiella pneumoniae*	1500	100	2000
6	BAL6	*Bacillus subtilis/ amyloliquefaciers/ vallismortis*	2500	100	500
7	PIA1	*Bacillus altitudinis/ pumilus*	2500	100	1200
8	PIA2	*Bacillus cereus group*	2500	100	1200
9	PIA3	*Staphylococcus haemolyticus*	2500	100	700
10	PIA4	*Bacillus cereus group*	2500	100	700
11	PIA5	*Staphylococcus aureus*	2500	100	700
12	PIA6	*Paenibacillusthiaminolyticus*	1300	100	2000

13	BRB1	*Pseudomonas aeruginosa*	3000	1200	1200
14	BRB2	*Staphylococcus xylosus*	2500	100	2200
15	BRB3	*Bacillus subtilis/amyloliquefaciens/vallismortis*	3000	100	2200
16	BRB4	*Bacillus cerues group*	2500	2200	100
17	BRB5	*Bacillus subtilis/amyloliquefaciens/vallismortis*	2000	500	2000
18	BRB6	*Bacillus altitudinis/pumilus*	2500	100	500
19	BRB7	*Citrobacter youngae*	2500	100	1200
20	BRB8	*Bacillus licheniformis*	2500	100	1200
21	BRB9	*Bacillus altitudinus/pumilus*	2500	100	700
22	BRB10	*Enterococcus faecalis*	2500	100	700
23	BRB11	*Bacillus cereus group*	2500	100	700
24	BRB12	*Acinetobacter baumannii*	1200	100	2200
25	BRB13	*Staphylococcus aureus*	2000	100	100
26	BRB14	*Staphylococcus epidermidis*	2000	100	500

However, among the heavy metal tolerant bacteria that were screened and analyzed, few of them were found to be pathogens such as *S. epidermidis, S. haemolyticus, S. aureus, K. pneumonia, P. aeruginosa, Enterococcus faecalis*, and *A. baumannii*. Nevertheless,these pathogens are less in count in comparison to that of non-pathogenic isolates and might have acquired resistance by gene transfer that needs further studies that need to be explored for applications.

Bioaccumulation of heavy metals by bacterial isolates

Metal resistant bacterial isolates were further investigated for their bioaccumulation potential of heavy metals by AAS. Theseisolates were exposed to 100ppm of Pb, Cd, and Cr for 24 hours indicated various percentages of bioaccumulation of each heavy metal as shown in **Table 4**. All metal-tolerant bacteria accumulated the highest amount of Pb compared to the moderate amount of Cr accumulation. On the contrary all isolates showed very minimal absorption of Cd and the trend of bioaccumulation of heavy metals by all isolated were identical to metals tolerance *i.e.,* Pb>Cr>Cd. Isolate *Bacillus subtilis/amyloliquefaciers/ vallismortis* showed the highest amount of lead and cadmium absorption with 92% and 57% bioaccumulation respectively. While *Bacillus cereus group* revealed the highest amount of bioaccumulation of chromium *i.e.,* 87%. Among *Bacillus* species, *B.subtilis* showed significant bioaccumulation of Pb *i.e.,* 87% - 92% which is followed by the species *B.altitudinis/pumilus* with Pb bioaccumulation potential of 85%. Both *B. cereus* and *B. subtilis* revealed a substantial amount of bioaccumulation of Cr compared to considerable bioaccumulation of 40%-57% Cd by *B. subtilis*.

Table: Bioaccumulation of heavy metals and its triplicate measurements in comparison to control analyzed by Atomic Absorption Spectroscopy.

Sl.no.	Isolates	Sample ID	**% bioaccumulation by AAS**		
			Pb	Cr	Cd
1.	*Bacillus megaterium*	BAL1	78%	50%	40%
2.	*Bacillus subtilis/ amyloliquefaciers/ vallismortis*	BRB3	92%	80%	57%
3.	*Bacillus subtilis/ vallismortis*	BAL2	90s%	75%	50%
4.	*Bacillus subtilis/ amyloliquefaciers/*	BAL6	87%	70%	47%
5.	*Bacillus subtilis/ vallismortis*	BRB5	90%	73%	52%
6.	*Bacillus cereus group*	BRB4	80%	87%	20%
7.	*Bacillus cereus group*	BAL3	78%	80%	18%
8.	*Bacillus cereus group*	BRB11	72%	77%	21%
9.	*Bacillus altitudinis/ pumilus*	BRB6	85%	55%	24%
10.	*Bacillus altitudinis*	PIA1	80%	45%	24%
11.	*Bacillus cereus group*	PIA2	79%	64%	20%
12.	*Bacillus licheniformis*	BRB8	80%	60%	15%

Discussion

The toxicity of heavy metals such as Pb, Cd, and Cr on human health is a well-known concept and it is adversely affecting the development of the human healthcare system [Balali-Mood *et al.,* 2021]. The situation is more severe in urban and industrial areas where domestic and industrial wastes containing heavy metals are not treated properly and directly dumped into the water bodies connected to surrounding agricultural lands. Crops and vegetables grown in such a heavy metal-contaminated land absorb them and accumulate in high concentration. Consumption of these vegetables and crops contaminated with heavy metals presence shows a severe menace to human health. As a consequence, heavy metals disseminate from industrial effluents to the domestic areas through the consumption of contaminated crops and vegetables by heavy metals. The incidences are high in industrial areas associated with metal processing and manufacturing activities or wastes running out from these industries. Therefore, an immediate measure to be taken to prevent the spread of heavy metal contamination and posing human health hazardsrelated with their exposure [Mawari*et el.,* 2022; Rai *et al.,* 2019].

Bacterial populations thriving in metal-polluted areas are acclimatized to metal stress as they possess several mechanisms essential for bioremediation for heavy metals [Berman *et al.*, 2020]. For instance, Pb-resistant *Bacillus* species and Cd-resistant *Bacillus*sp, *S. aureus P. aeruginosa*, and *K. pnuemoniae*detoxify heavy metals through potential mechanisms such as active transport and *pbr* operon, and plasmid-based *cadA* operon respectively. While, Cr resistance employs one or several processes among biosorption, complexation, precipitation, cell surface binding, and exclusion. While, Cr-resistant Gram positive and negative bacteria, toxic Cr is neutralized through the reduction of Cr^{6+} (toxic) to Cr3+(non-toxic) by chromate reductase. But *Bacillus*sp, reduces chromate ions through aerobic process [Alotaibi *et al.*, 2021].Bioremediation by *Bacillus sps* have been well illustrated for their genes in several strategies involving biosorption, bioaccumulation and bioprecipitation (Wróbel et al.,2023). Our study also shows the relevance of the genus Bacillus as potential remediators.

Microbes have been used as potential agents to clean up the contaminated areas and effluents by different mechanism such as biosorption, bioaccumulation, bioleaching etc to detoxify the pollutants. Earlier reports have also shown the role of Bacillus*drentensis* (MK217088), *Bacillus safensis* (MK774729), *Bacillus haynesii* (MK192808), *Bacillus subtilis* (MK217089), and *Bacillus cereus* (MK801278) as effective chromium degradation being an alternative biological method for treating heavy metal degradation (Kalaimurugan et al. 2020; Singh et. al. 2021). Correspondingly, our study also highlights the Bacillus genus to be the potential bioremediating microbes. Their study also analysed few pathogens *Citrobacter* sp. and *Enterobacter* sp. removed 87%, 79% and 43% and 86%, 78% and 51% of Ni, Cd and Pb, respectively. Similarly, the current study also emphasise the role of different pathogens as shown in Table 3 that were remediating the heavy metals. These pathogens like others might have gained the microbial gene sequence responsible for metal oxidation, reduction and remediation have made these heavy metal resistant microbes for remediation (Njoku et. al., 2020). The mechanism of transformation or accumulation of heavy metal by genes responsible in bacteria has also been significant in promoting plant growth traits (Vezza et. al., 2020; Abdollahi et.al.,2020).

Therefore, bioremediation is an eco-friendly technique that can be explored as a control measure in metal-polluted areas.

In the current work heavy metal accumulating bacteria were isolated from three metal waste-polluted industrial areas of Bangalore city known for the inflow and dumping of industrial effluents.The majority of the bacterial populations isolated from all locations showed high

resistance to Pb compared to Cr and Cd when screened at 100 ppm. Only a few bacterial colonies were resistant to double and triple heavy metals *i.e.*, 100ppm of Pb+Cd, Pb+Cr, and all three heavy metals.Resistant bacterial isolates further indicated significant metal tolerance against Pb and Crcorresponding to the MIC value of 1200 to 3000ppm and 100 to 2200ppm respectively**.** However, heavy metal tolerance against Cd was not much significant except few isolates such as *B. megaterium, S. epidermidis, P. aeruginosa,* and *B. cerues* that showed Cd resistance of 1000ppm, 1500ppm, 1200ppm, and 2200ppm respectively. **A study on** soil samples collected from chromium mining area of Sukinda of Odisha indicated 42 heavy metal resistant bacteria, of which 11, 09, 14, and 08 isolates were resistant to Pb, Cd, Cr, and As metals. Among them, four potential bacteria *viz.,B.fungorum*, *B. thuringiensis, B. subtilis,* and *Pseudomonas argentinensis* showed heavy metal-tolerance between 50-1000ppm and *P. argentinensis*revealed highest Cr metal absorption capacity *i.e.,* 73.4% [Acharya *et al.,* 2024].

All potential metal-resistant bacteria were identified through MALDI-TOF-MS and isolates were belonged to *Bacillus, Staphylococcus, Klebsiella, Pseudomonas, Paenibacillus, Citrobacter, Enterococcus,* and *Acinetobacter* species in which *Bacillus* species were dominant in both number and metal resistance.Comparatively, a study by Nazir et al., 2020 showed the presence of *Staphylococcus sps* such as *S. equrum, S. warneri* and *Bacillus sps* such as *B. safensis* and *B thuringenisis*while, our study showed *S haemolyticus, S. aureus, S. xylosus* and *S. epidermidis*with different Bacillus species.

Among these isolates, the substantial heavy metal accumulating capacity was shown by *B. cereus* with Pb, and Cr tolerance capacity of 3000 and 2000ppm compared to the reported tolerance value of 1000 and 200 mg/literby*B. cereus* NWUAB01**[**Ayangbenro*et al.,* 2020]. A study revealed about 88% of the bacterial population is dominated by *Bacillus* and *Pseudomonas* species with heavy metal tolerant of about 300ppm to cadmium isolated from Rhizosphere soil [Arce-Inga *et al.,* 2022].While in the present study, *Bacillus* species were dominant and showed significant metal tolerance particularly species like *B. altitudinis/pumilus, B. licheniformis, Bacillus subtilis/amyloliquefaciens/vallismortis* showed substantial Pb tolerance of 2500ppm and with Cr-resistance of up to 1200 - 2200ppm. On the other hand, *P. aeruginosa* revealed a Pb tolerance of 3000ppm and Cr and Cd-tolerance of 1200ppm.

The lead-resistant *B. megaterium* and *P. marginalis* isolated from heavy metal polluted areas revealed resistance to 0.6 mM and 2.5 mM of Pb [Roane, 1999]. *B. megaterium* is also capable of detoxifying Cr(VI) to Cr(III), a non-toxic form, and the isolate recovered from tannery effluent treatment has a Cr bioaccumulation capacity of 32mg/g dry weight **[**Srinath

et al., 2002]. However, in contrast to the reported value of metal tolerance, *B. megaterium* isolated in the current study showed significant tolerance towards metals such as Pb, Cd, and Cr with MIC values of 3000, 1000, and 1500ppm respectively. Following this, *B. cereus* showed the second highest metal tolerance to Pb of 3000ppm and Cr 2000ppm. In addition to *Bacillus*sp, *Staphylococcus* species *viz.,S. epidermidis, S.haemolyticus,* and*S. aureus*indicated substantial Pb resistance of 2500ppm and *S. xylosus* showed Pb tolerance of 2000ppm and only *S. epidermidis* showed Cd resistance of 1500ppm. However, It has been reported that *S. xylosus* isolated from the mining area showed metal tolerance capacity of 1300 mg/L Pb, 1000 mg/L Cd, and 50 mg/L Cdcompared to 2500ppm Pb, 100ppm Cd, and 2200ppm Crrevealed in the present study [Rahal et al., 2024]. *Staphylococcus* sp. MB371 and *Klebsiella pneumoniae* MB361 isolated from industrial effluents showed Pb and Cd accumulation potential of 1000-1600µg/ml and 500-1000 µg/ml respectively [Aslam *et al.,* 2020]. The present study also proposes some other bacteria namely *Paenibacillusthiaminolyticus, Citrobacter youngae, Enterococcus faecalis,* and *Acinetobacter baumannii* with significant heavy metal-resistance potential.

Non-pathogenic and potential metal tolerance bacterial species particularly *Bacillus* species evaluated for bioaccumulation of heavy metals in which the highest Pb bioaccumulation *i.e.,* 92% were revealed by *Bacillus subtilis/ amyloliquefaciers/ vallismortis* followed by *Bacillus altitudinis/ pumilus Bacillus altitudinis/ pumilus,*and*Bacillus licheniformis. Bacillus cereus* and *Bacillus subtilis* indicated 87% and 80% bioaccumulation ofCr, respectively. Whereas, the highest bioaccumulation was indicated by *Bacillus subtilis/ amyloliquefaciers/ vallismortis* followed by *Bacillus subtilis/ vallismortis.* The substantial metal-tolerance property of proposed isolates in the current study can be explored for various industrial applications including heavy metal detoxification during industrial effluent treatment, as a bioremediation method for environment cleaning in metal-polluted areas, and as a heavy metal supplier to plants in form of biofertilizers [Oziegbe*et al.,* 2021; Saha *et al.,* 2022; Haroun *et al.,* 2023].A study by Green et al., 2003, has highlighted the role of soil microbes can help in reducing the metal toxicity by absorption and support in plant growth by means of soil phytoremediation.

However, effective prevention of metal pollution and environmental cleaning is only achieved with synergetic efforts made by metal industries for proper disposal of wastes, local government that implements strict laws against metal waste disposal, and creating awareness methods about adverse effects of waste disposal and heavy metal toxicity [Chen and Ding, 2023].

Conclusion

The role of microbes for heavy metal detoxification has evolved over the period of times using different strategies. The present study highlights the role of high resistant bacterial isolates towards heavy metals like Pb, Cr and Cd that can cause delirious effect on health and environment in higher concentrations. The study proposes important bioremediating microbes by biochemical and MALDI-TOF analysis. We also analysed some pathogens in the process necessitating the transfer of resistance plasmids in them. Following study highlights the role of*Bacillus sps* as potential candidates of heavy metal bioremediation that can be formulated as a means of detoxifying industrial effluents. Though, additionalwork needs to be carried out to elucidate the molecular characterisation and elucidate molecular mechanism to help in applications.

Conflict of interest

The authors has no conflict of interest

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