

Original Research Article

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Resistance of Potential Plant Growth Promoting Rhizobacteria to Heavy Metals (Ni, Cd, Co, Pb) Isolated from Polluted Areas of Hyderabad

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ABSTRACT

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The toxicity and bioaccumulation tendency of heavy metals in the environment is a serious threat to the health of living organisms. Unlike organic contaminants, heavy metals cannot be broken down by chemical or biological processes. Hence, they can only be transformed into less toxic species. The present study was conducted to isolate promising heavy metal tolerant bacteria from various industrial polluted areas of Hyderabad, India and was tested for their plant growth promoting characteristics. Eleven strains isolated were identified as *Bacillus* (6) and *Rhizobium* (5) species and were tested for their maximum tolerance to heavy metals (Ni, Cd, Co, Pb) by supplementing its respective salts in different concentrations (0, 25, 50, 100, 150 and 200 mg/l) to Nutrient agar and Yeast extract mannitol agar. Among all strains AfSB-1, SfSB-5, AfSR-1 and SfSR-4 were able to tolerate upto 100 mg/l concentration. All the isolates were also screened for various biochemical traits like, Mineral solubilisation (P, K, Zn), HCN production, IAA production, Siderophore production, EPS production ACC deaminase activity. The best strains with both metal tolerance and multifunctional traits will enrich the studies on genetic diversity and could be exploited for bioremediation of heavy metal polluted environment.

Introduction

Heavy metal pollution is increases day by day due to industrialization in all over world. Pollution is generated due to metallic ferrous ores mining and smelting, fossil fuels, sewage, municipal wastes, pesticides and fertilizers. The occurrence of these heavy

metals in the environment has been a topic of great worry due to their toxicity, non-biodegradable nature and the long biological half-lives for their removal from biological tissues (Aiking *et al.*, 1984). Their high aquatic solubility triggers bioaccumulation and biomagnifications which eventually leads to insidious and irreversible health hazard

(including potential carcinogenicity) even in very minimal concentration range of about 1 mg/l. Gupta and Diwan (2017).

Bioremediation is defined as the process by which microorganisms are stimulated to rapidly degrade hazardous organic pollutants to environmentally safe levels in ground water, soil, substances, materials and sediments. Recently, biological remediation process have also been devised to either precipitate effectively immobilize inorganic pollutants such as heavy metals. Stimulation of microorganisms is achieved by the addition of growth substances, nutrients, terminal electron acceptor or donors or some combination thereby resulting in an increase in organic pollutant degradation and biotransformation. The energy and carbon are obtained through the metabolism of organic compounds by the microbes involved in bioremediation processes. (Fulekar, 2009; Singh *et al.*, 2014)

It is so necessary to separate bacterial strains with novel metabolic abilities and to start degradation pathways both biochemically and genetically. Screening out microbes from such an environment was done keeping in mind their multifunctional application especially for bioremediation. Among diverse soil microbes, plant-growth-promoting bacteria (PGPB) producing plant-growth regulators, mineral solubilizers, phytohormones, and various secondary metabolites have been reported to expedite the plant-growth and development and soothe plants against various environmental stresses including metal stress. Moreover, they have shown excellent results in reducing metal toxicity by promoting plant-growth when used as inoculants.

The aim of this study was to characterize metal tolerant microbial strains having plant growth promotion traits isolated from heavy

metal contaminated soil and to recommend them as potential bioinoculants.

Materials and Methods

Study area and collection of samples

Heavy metal polluted soil and water samples were collected from three sewage irrigated agricultural sites (Afzalgunj, Uppal, Student farm) in Hyderabad. Amount of heavy metals were also analyzed in water and soil samples by atomic absorption spectrophotometer (AAS) given by (Lindsay and Norway 1978).

Isolation, screening and characterisation of Heavy metal tolerant bacteria

Qualitative characterization of all the eleven isolates were characterized by following standard protocols.

Nitrogen fixing activity: Acetylene reduction assay

The nitrogen fixing capacity of the test organisms were evaluated by using acetylene reduction assay following the standard procedure (Bergersen, 1980).

Determination of Phosphate solubilization

For estimation of phosphate solubilization all isolates were inoculated on the Pikovskaya's agar medium. After 3 to 5 days of incubation at 28 ± 2 °C, when bacteria solubilised the phosphate clear zone appear around the spot inoculums. Halo zone around the growth was measured for the obtaining the phosphate solubilisation.

Production of Indole acetic acid

Indole acetic acid production was quantitatively measured by the method given by Gordon and Weber (1951). Bacterial

cultures were grown in a Luria-Bertani broth amended with tryptophan (5 mM) for 3 - 4 days. Cultures were centrifuged at 10,000 rpm for 20 min. Two ml of supernatant was mixed with two drops of orthophosphoric acid and 4 ml of salkowski reagent. Tubes were incubated at room temperature for 25 min. The intensity of pink color was read at 530 nm spectrophotometrically and the amount of IAA produced was extrapolated from the standard curve.

Production of HCN

All the isolates were tested for the HCN production with the help of methodology of Castric and Castric (1983). A Whatman no.1 filter paper placed on lid of Petri plates and then autoclaved. Selected isolates were streaked on nutrient agar yeast extract mannitol agar plates amended with 4.4 g/l of glycine.

Soaked the filter paper in 2% sodium bicarbonate and 0.5% picric acid solution and put inside the lid of the plates and sealed properly with parafilm and incubated for 3-4 days at $\pm 30^{\circ}\text{C}$. HCN production is indicated by the appearance of color of filter paper from light brown to dark brown.

Siderophore production

Siderophore production was estimated qualitatively. By taking 0.5% of cell free culture supernatant and added to 0.5 ml of 0.2% aqueous Ferric chloride solution. Appearance of orange or reddish brown colour indicated the presence of Siderophore (Yeole and Dube, 2000).

EPS extraction

The culture broth of all the isolates was centrifuged at 6000 rpm for 10 min to remove cells. Two volumes of cold ethanol (4°C)

were added to the supernatant and the crude EPS precipitate was dried in a dessicator overnight (Rasulov *et al.*, 2013).

Screening for ACC deaminase activity

All the heavy metal tolerant isolates were grown in 5 ml of trypticase soy broth medium incubated at 28°C at 120 rpm for 24 h.

The cells were harvested by centrifugation at 3000 g for 5 min and washed twice with sterile 0.1 M Tris-HCl (pH 7.5) and spot inoculated on petri plates containing modified Dworkin and Foster salts minimal medium (Dworkin and Foster, 1958) supplemented with 3 mM ACC as sole nitrogen source. Plates containing only DF salts minimal medium without ACC as negative control and with $(\text{NH}_4)_2\text{SO}_4$ (0.2% w/v) as positive control. The plates were incubated at 28°C for 72 h.

Growth of isolates on ACC supplemented plates was compared to negative and positive controls and was selected based on growth by utilizing ACC as nitrogen source.

Metal tolerance test

Heavy metals tolerant bacteria were isolated on nutrient agar and yeast extract mannitol agar supplemented with various concentration (0, 50, 100, 150 and 200 mg/l) of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, CdSO_4 , CoCl_2 and PbCl_2 .

The agar amended with heavy metal salts was sterilized at 121°C for 15 min and allowed to cool $40-45^{\circ}\text{C}$ and transfer into petri plates. The pure cultures were streaked on heavy metal enriched medium and resistance was determined by the appearance of growth of bacteria after the 3 to 4 days of incubation.

The minimal inhibitory concentration (MIC) was determined as the lowest concentration of metal ion that completely inhibited growth.

Results and Discussion

Initial heavy metal concentration of various polluted and unpolluted samples

The collected soil samples were prepared by drying and ground to pass through a 0.5 mm sieve. The heavy metals like Nickel (Ni), Cadmium (Cd) Cobalt (Co) and Lead (Pb) in soil determined using DTPA method by Atomic absorption spectrophotometer given by (Lindsay and Norvell1978). Results showed that among the four heavy metals Pb was more ranging from 5.90- 14.58 mg/kg. The details of the Heavy metal concentrations were given in table 1.

Isolation and cultural characterization of purified bacterial strains

A total of 28 bacterial isolates were isolated from soil and water by serial dilution and plate count method. These pure cultures of different bacterial isolates were preserved and carried out further analysis. The details of soil samples and bacterial codes were presented in table 2.

The details of cultural and morphological characteristics of all the bacterial isolates were given in table 3. On Nutrient agar medium plates, a total of six *Bacillus* isolates showed dull to off white, irregular, non -spreading smooth, flat, opaque, viscid colony characteristics. All the 6 isolates showed Gram positive reaction, rods with endospore formation, when observed under microscope

The colony morphology of isolates was examined on YEM agar plates. About five *Rhizobium* isolates showed small to medium, milky translucent, raised, mucoid colonies and formed non-spreading type of colonies. These pure cultures were checked for purity by streaking on different media like Hofer's alkaline agar, glucose peptone agar and

lactose agar plates. None of the isolates showed any growth on these media plates.

Multifunctional traits *Bacillus* and *Rhizobium* isolates for their PGPR characterization

All the isolates show three or four traits of PGPR shown in the Table 4. Among all the eleven isolates 5 *Bacillus* and 1 *Rhizobium* isolates were able to form clear zone of phosphate solubilisation on Pikovyskaya's agar plate ranged from 5-13 mm. Among them SfS-4 recorded the highest solubilisation zone (12.76 mm) followed by AfS-2 (12.1 mm).

Except one *Bacillus* strain (SfS-17) all bacterial strains were found to be positive for IAA production ranging in concentration from 1.50 to 21 µg/ml as determined by the development of pink color after reaction with Salkowski reagent. *Rhizobium* strain UpS-16 from YEMA plate and *Bacillus* strain SfSB-5 from nutrient agar plate showed highest IAA production (21.51 µg ml⁻¹) and (19.16 µg ml⁻¹) respectively with spectrophotometer readings.

The development of a mucoid aspect, indicating a possible EPS development by the strains; and more evident was by adding two volumes of cold ethanol (4°C) to the supernatant. EPS production was shown by only three *Rhizobium* isolates AfS-15, UpS-16, and SfS-17 and only by two *Bacillus* AfSB-1, SfSB-5 isolates.

All the bacterial isolates were grown on DF salts minimal medium plus either ACC or ammonium sulphate and were assayed for ACC deaminase activity by incubating the extract with ACC and observing the growth. Four out of six *Bacillus* strains were positive for ACC deaminase activity; two isolates showed moderate (++) ACCd production

(AfSB-2 and SfSB-5), remaining two isolates showed weak (+) ACCd production. Three out of five *Rhizobium* strains were grown on the enriched medium and utilised 1-aminocyclopropane- 1-carboxylase as a sole nitrogen source, AfSR-15 and SfS-18 were moderate (++) for ACCd, while UpS-17 and AfW-19 were weak (+) producers.

Isolated bacteria strains were examined for Hydrogen cyanide (HCN) and Siderophore production. Nine among eleven were positive for HCN and their level of production was based on their change of filter paper from yellow to orange. AfS-1, SfS-5, SfS-18 were strong producers.

Siderophore production was shown by all the isolates and among them *Rhizobium* strain AfS- 15 is the strong producer (+++); showed

intense colour at the end. And most of them were moderate producers (++) AfS-1, UpS-3, SfS-4, SfS-17, SfS-18 and AfW-19.

Assessment of MIC against each heavy metal

Minimum inhibitory concentration (MIC) for each heavy metal was examined ranging from 25 to 200 mg/L given in Table 5. Only four bacterial strains (AfS-1, SfS-5, AfS-15 and SfS-18) were resistant to higher concentration of Heavy metal salt (upto 100 mg/l). Among 6 *Bacillus* isolates SfS-5 shown resistance to higher concentration (*ie* upto 100 mg/l of Ni, Co; upto 150mg/l Cd and upto 200 mg/l of Pb) and SfS-4 of *Rhizobium* isolates showed resistance (upto 100 mg/l of Ni, Cd and Co salts and upto 150 mg/l of Pb salt).

Table.1 Heavy metal concentration of various polluted samples (ppm)

Samples	Nickel (Ni)	Cadmium (Cd)	Cobalt (Co)	Lead (Pb)
Afzulung soil	3.16	1.01	2.55	8.56
Uppal soil	2.98	1.98	1.59	12.61
Student farm soil	3.11	1.23	1.62	14.58
Afzulung water	2.61	0.54	0.86	5.90
Uppal water	2.35	0.95	0.42	6.95
Student farm water	2.54	0.87	0.53	9.32

Table.2 Isolated and purified bacterial strains from different sources

Sampling source	Number of bacterial isolates	Codes of Isolates
Afzalung soil	3	AfS-1, AfS-2, AfS-15
Uppal soil	2	UpS-3, UpS-16
Student farm soil	4	SfS-4, SfS-5, SfS-17, SfS-18
Afzalung water	1	AfW-19
Student farm water	1	SfW-6

Table.3 Morphological and cultural characteristics of *Bacillus* and *Rhizobium* isolates

Isolates		Gram reaction	Cell Shape	Colony characteristics
<i>Bacillus</i>	AfSB-1	+ve	Rod	Dull white, irregular, spreading, smooth, flat, opaque, undulate margin
	AfSB-2	+ve	Rod	Off white, irregular, non-spreading, smooth, flat, opaque, undulate margin, viscid colony
	UpSB-3	+ve	Rod	Dull white, irregular, spreading, smooth, flat, opaque, undulate margin
	SfSB-4	+ve	Rod	Dull white, irregular, large, smooth, flat, opaque, undulate margin, viscid colony
	SfSB-5	+ve	Rod	Off white, irregular, non-spreading, smooth, flat, opaque, undulate margin, viscid colony
	SfWB-6	+ve	Rod	Dull white, irregular, spreading, smooth, flat, opaque, undulate margin
<i>Rhizobium</i>	AfSR-1	-ve	Rod	Gummy white, round, non-spreading, smooth, raised, translucent, mucoid colony
	UpSR-2	-ve	Rod	Gummy white, round, non-spreading, smooth, raised, translucent, mucoid colony
	SfSR-3	-ve	Rod	Gummy white, round, non-spreading, smooth, raised, translucent, mucoid colony
	SfSR-4	-ve	Rod	Gummy white, flat, entire, wrinkled edge
	AfWR-5	-ve	Rod	Flat, entire, wrinkled edge

Table.4 Multiple plant growth promoting activities of *Bacillus* and *Rhizobium* isolates from the Heavy metal polluted soil

Isolates	PO ₄ Solubilisation(mm)	Nitrogenase activity-ARA (nmol C ₂ H ₄ mg protein ⁻¹ h ⁻¹)	IAA Production (µg ml ⁻¹)	EPS Production	ACC deaminase	HCN Production	Siderophore Production
AfS-1	10.3	ND	16.00	++	+	+++	++
AfS-2	12.1	ND	9.38	-	++	++	+
UpS-3	-	ND	18.21	-	-	-	++
SfS-4	12.76	0.18	1.51	-	-	++	++
SfS-5	09.33	ND	19.16	+	++	+++	+
SfW-6	08.00	ND	5.81	+	+	-	+
AfS-15	4.11	2.20	19.03	+	++	+	+++
UpS-16	-	2.16	21.51	++	+	+	+
SfS-17	-	4.23	-	++	-	+	++
SfS-18	8.78	4.54	16.34	++	++	+++	++
AfW-19	-	2.66	12.44	-	-	+	++

Table.5 Heavy metal tolerance of *Bacillus* and *Rhizobium* spp

HEAVY METAL TOLERANCE (mg/l)				
Isolates	Ni	Cd	Co	Pb
AfS-1	100	100	100	150
AfS-2	50	50	100	150
UpS-3	25	50	25	100
SfS-4	50	100	50	150
SfS-5	100	150	100	200
SfW-6	100	50	50	100
AfS-15	100	100	100	100
UpS-16	50	150	50	100
SfS-17	25	100	25	100
SfS-18	100	100	100	150
AfW-19	100	50	50	100

The *Bacillus* and *Rhizobium* strains isolated from the heavy metal polluted soils showed resistance to 25 mg/l concentration of heavy metals with at least three PGPR activities. Research reports suggested that diverse group of free-living soil bacteria can improve host plant growth and alleviate toxic effects of heavy metals on the plants (Belimov *et al.*, 2004; Wani *et al.*, 2008). Pandey *et al.*, (2011) isolated and characterized two Pb and arsenate tolerant *Bacillus* sp. from slag disposal site. Similar type of results reported by Singh *et al.*, (2015) isolated *Bacillus safensis*KM39 (LT) and evaluated for plant growth promoting traits, Heavy metal (Pb and Cd) resistance and bioaccumulation of heavy metals and showed positive results for qualitative parameters viz. inorganic Phosphate solubilization, production of siderophores, Indole acetic acid, HCN and ammonia production.

Wani and Khan (2012) isolated *Rhizobium* strain RL9 from the nodules of lentil grown in metal contaminated soils. The strain tolerated Pb upto a concentration of 1600 µg/ml. It also

produced a good amount of IAA which found to produce a maximum amount of 33 µg/ml of IAA at 100 µg/ml of tryptophan. Similar type of results was reported by Wani *et al.*, (2008), they isolated nickel and zinc-tolerant plant growth-promoting (PGP) *Rhizobium* sp. RP5 from nodules of pea, grown in metal-contaminated Indian soils. Strain RP5 displayed a high level of tolerance to nickel (350 µg ml⁻¹) and zinc (1500 µg ml⁻¹) and showed PGP activity like nitrogen fixation, growth promotion, and the ability to reduce the toxicity of nickel and zinc under *in vitro* conditions and reported their importance in augmenting the growth and yield of pea, in nickel- and zinc-polluted soils.

In conclusion, the study has been done is demonstrated that microorganism are helpful for reduction of leaching of heavy metals in environment. Hence, we can use these isolates for minimization of toxicity and enhancement of plant growth by its PGPR activity even under metal stress condition. This study is also helpful for bioremediation because of high level of MIC of isolates.

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