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Isolation, Identification and Characterization of Cadmium Resistant Rhizobacterial Isolates from Long-Term Wastewater Irrigated Soils

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Abstract: **Long-term wastewater irrigation in the peri-urban areas of Varanasi, India has elevated the concentrations of heavy metals in soil, Cd in particular and may pose threat to food chain safety and human health. Cd is one of the highly mobile and toxic heavy metals. Therefore, the present study was carried out to isolate, identify, and characterize the Cd-resistant bacterial stains from the contaminated soils of the suburban area of Varanasi. Twenty four bacterial strains were isolated and were screened for Cd resistance. The tolerant strains, BHUJ Cd-1, BHUJ Cd-3, BHUJ Cd-10, BHUJ Cd-15, and BHUJ Cd-20 were grown on nutrient agar plates supplemented with Cd as $CdCl_2.H_2O$ ranging between 50 µg ml^{-1} to 500 µg ml^{-1}. The results showed that the strains BHUJ Cd-3, BHUJ Cd-10, and BHUJ Cd-15 are capable to grow *in-vitro* up to 300 µg Cd ml^{-1}, whereas BHUJ Cd-20 and BHUJ Cd 1can grow up to 350 µg Cd ml^{-1} and 450 µg Cd ml^{-1}, respectively. Based on morphological, biochemical, and molecular characteristics, the strains BHUJ Cd-1, BHUJ Cd-3, BHUJ Cd-10, BHUJ Cd-15, and BHUJ Cd-20 were identified as *Agrobacterium sp.* (MZ600148), *Microbacterium schleiferi* (MK 999915), *Agromyces sp.* (MK999982) *Stenotrophomonas sp.* (MK990331), and *Stenotrophomonas sp.* (MK962156), respectively. These strains also showed plant growth-promoting (PGP) characteristics such as the production of IAA, siderophore and ammonia, ACC deaminase activity, phosphate solubilization, etc. PGP traits of isolated Cd-resistant rhizobacterial strains indicated the strains could be useful as potential phytostimulators, biofertilizers, and stress ameliorators in achieving sustainable agriculture. The identified Cd-resistant bacterial strains may be used to develop bacterial consortia for the remediation of heavy metal contaminated soil.**

Index Terms: **Wastewater, Suburban, Cd-resistant rhizobacteria, PGP traits, Sustainable agriculture**

I. INTRODUCTION

Heavy metals such as Cd, Cu, Cr, Ni, Pb, etc., are the most abundant toxic contaminants present in different components of the environment (Kabata-pendias, 2010). Cadmium (Cd), a non-essential heavy metal is considered as one of the most toxic heavy metals because of its high mobility, bioaccumulation, persistent and non-biodegradable properties (Kumar et al., 2015). Cd is easily taken up by the crop plants growing on contaminated soil and pose risk to both food chain safety and human health. Daily consumption of heavy metal contaminated vegetables also poses health hazards to human beings (Gallego et al., 2012). When the Cd concentration exceeds a certain tolerance threshold, it can damage the cell membranes, change the specificity of enzymes, and damage the DNA. Cd toxicity is further induced by competing with macronutrients such as Zn for binding sites of transporters (Gratao et al., 2005) Microorganisms have developed mechanism such as bioaccumulation, biomineralization, biosorption and biotransformation for their adaptation in a heavy metal-rich environment (Ayansina et al., 2017). They have also several protection mechanisms to heavy metal resistance such as extracellular barrier, extracellular sequestration, active transport of metal ions (efflux) and intracellular sequestration (Bruins et al., 2000).

The conventional technique such as ion–exchange, adsorption, chemical precipitation, surface soil removal, etc., developed for the remediation of heavy metals contaminated soil are very expensive, less effective as well as non-eco-friendly (Danh et al., 2009). Whereas biological methods especially phytoremediation is a cost-effective, easy to achieve and an eco-friendly technology which involved plant species to remove the heavy metals from the contaminated soil either by accumulating or changing into less toxic complexes (Belimov et al., 2005). Metal

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resistant plant growth promoting rhizobacteria improved the efficiency of phtoremediation technique to fulfill of the nutrient and regulate the homeostasis of plant in metal contaminated soil. Plant growth-promoting rhizobacteria affect plants by improving growth and health, enhancing root development, nutrient uptake, and increasing their tolerance to various environmental stresses (Bhattacharyya and Jha, 2012).

Cd contaminated soil influenced the growth of many plants such as *Abelmoschus esculentum, Cucumis sativus L*., by inducing the physiological, biochemical and structural changes (Sharma et al., 2010; Feng et al., 2010). The adverse effects of Cd on the growth and metabolism of plants are associated with their interaction with heavy metal tolerant PGPR, which promotes their growth by different mechanisms such as rhizoremediation (Kuiper et al., 2004). In the rhizosphere of plants PGPR colonize and enhance the growth and development by indirect mechanisms such as induction of systemic resistance, competition for nutrients and niche with a pathogen, synthesis of antibiotics, or cell wall degrading enzymes (Ramette et al., 2006) and direct mechanisms. i.e. fixation of atmospheric nitrogen, soil mineral solubilization, siderophore synthesis and production of phytohormones and protect from the stress by synthesis of 1-aminocyclopropane1-carboxylate (ACC) deaminase (Kudoyarova et al., 2019).

Long-term reuse of wastewater in agriculture, especially in the peri-urban areas of developing cities around the world has elevated the levels of heavy metals in soils and consequently in edible parts of the vegetable crops (Sharma et al., 2007; Singh et al., 2010). Heavy metal resistance PGPR have been widely explored for their potential to improve plant growth, alleviate metal toxicity, and mobilize/immobilize/transform heavy metals in soil, which may help to develop new microbe assisted phytoremediation and restoration strategies (Sarma and Prasad, 2019). Therefore, in the present study, an effort has been made to isolate Cd resistant rhizobacterial strains from the long-term wastewater irrigated agricultural soil of peri-urban areas of Varanasi, Uttar Pradesh, India. Further, the study aims to identify Cd resistant bacterial strains using morphological, biochemical, and molecular approaches.

II. MATERIALS AND METHODS

A. Soil sampling and analysis

Soil samples (500 g) were collected in triplicates from the rhizosphere of wheat, cabbage, tomato, green chili, brinjal, cauliflower, mustard, radish, and pea grown in wastewater irrigated areas of Lohata, located in suburban areas of Varanasi, Uttar Pradesh, India. The rhizospheric soil samples were collected from different fields under cultivation of various crops. The distance between two agricultural fields ranged from 100 to 200 meters. A monolith of 10 cm × 10 cm × 15 cm was dug out and placed in sterilized polyethylene bags. The soil samples were brought to the laboratory and separated into two parts. The first half part of each soil sample was used for the isolation of Cd resistance bacteria and microbial analysis, whereas the other half part of the soil sample was air-dried, crushed and passed through a 2 mm-mesh sieve and stored at room temperature for further analysis. The pH of the air-dried soil was measured in soil:water suspension (1: 5) using pH electrode (S/N 2517394, 2017). The electrical conductivity of the soil samples was also measured by using a conductivity meter (LMCM-20, 2017) in the same suspension. The organic carbon was determined using Walkley and black's Rapid Titration method of Allison et al. (1986). For the analysis of heavy metals in the soil samples, 0.25 g of dried soil was digested with 15 ml of HNO_3 and $HClO_4$ in 9:4 ratios at 80 °C until a transparent solution was obtained by Jackson (1973). Then, digested soil samples were filtered through the Whatman filter paper (No. 42) and the final volume was maintained to 25 ml using double distilled water (DDW). Concentrations of heavy metals in the filtrate were determined using an atomic absorption spectrophotometer (Perkin- Elmer model 2130, USA) fitted with a specific lamp of particular metal and appropriate drift blank. Quality control measures were used to assess the contamination and reliability of data. Both the blank and drift standards (Sisco Research Laboratories Pvt. Ltd. India) were run after the five determinations to calibrate the instrument. Precision and accuracy of analysis were also ensured through repeated analysis of samples against National Institute of Standards and Technology standard reference material (SRM 1570) for all the heavy metal and the results were found within 72% of the certified value.

B. Isolation of Cd resistant rhizobacteria

Half parts of the collected soil samples were preserved at 4 °C in the refrigerator for the isolation of Cd resistant rhizobacteria. The isolation of Cd resistant rhizobacteria was done by using the serial dilution and pour plate method (Aneja, 2003). Briefly, 10 g of soil was taken separately and transferred into a 250 ml Erlenmeyer flask containing 90 ml sterilized water and placed on a shaker at 120 rpm for 30 min and then serially diluted from 10^{-2} to 10^{-7}. One ml of the diluted sample was transferred into the respective plate and poured the nutrient agar medium (Peptone: 5 gL^{-1}, Beef extract: 3 gL^{-1}, NaCl 8 gL^{-1}, and Agar 15 gL^{-1}) amended with 50 µg Cd ml^{-1} as $CdCl_2.H_2O$. All the plates were incubated at 28±2 °C in an incubator shaker for 72 h. The morphologically different colonies were selected and transferred to another plate having the same medium composition for the purification. A single colony was transferred to the slant having the same growth medium without Cd salt and stored at 4 °C in a refrigerator for further studies.

2.3 Screening of Cd resistant rhizobacterial isolates

The isolated rhizobacteria were screened for Cd resistance using the minimum inhibitory concentration (MIC) approach. Freshly grown cultures were streaked on nutrient agar plates supplemented with Cd (0, 50, 100, 200, 300, 400, 500 mg ml^{-1}) as $CdCl_2.H_2O$ salts aseptically in the form of a single line by using an inoculation loop (having 10^{-8} cells ml^{-1} bacterial population). Inoculated plates were incubated at 28±2 ºC for 72 h for proper bacterial growth. A negative control plate i.e. without Cd was also incubated. All the experiments were conducted in triplicates.

C. Characterization and identification of bacterial isolates

1) Morphological characterization and identification

Gram staining of bacterial isolates was performed by using the method of Aneja (2003). A thin smear of pure culture was

prepared on clean grease-free slides and fixed by passing over a gentle flame. Heat fixed smear flooded with crystal violet solution for 60 s and then washed with DDW. The smear was again overwhelm with Gram's iodine for 30 s and rinsed with DDW then 70% ethanol used for decolorized for 30 s and finally washed with DDW. In the last steps safranine was used as a counter stain for 60 s rinsed with DDW and then allowed to air dry. The smears were seated on a microscope and observed under an objective lens. Gram-negative bacteria were stained with pink colour whereas gram-positive bacteria stained with purple in colour.

2) Molecular characterization and identification

Out of 24 isolated pure bacterial cultures, only the selected isolates, resistant to Cd from 300 to 400 µg ml^{-1} were screened for molecular characterization. Bacterial cultures were grown on nutrient agar plates overnight for DNA isolation. The isolated DNA was used as a template in the colony PCR method and 16S rRNA was amplified by Thermocycler (Eppendorf) using the primers, 27 F' and 939 R' (Sigma-Aldrich Pvt. Ltd., India). Universal Primer 27 F' forward 5'AGAGTTTCCTGGCTCAG3' 939 R' reverse 5'CTTGTGCGGGCCCCCGTCAATTC3'. The PCR was performed in 50 µl reaction mixture containing 5 µl assay buffer (15X), 1 µl dNTP (10.0 mM) 1 µl each of forward and reverse primer, 0.5 µl of Taq polymerase (Takara, Clonotech, Japan), 2 µl of template DNA and 39.5 µl of HPLC grade water following amplification for 16s rRNA initial denaturation at 95 ºC for 5 min followed by 35 cycles of denaturation, annealing and extension (95 ºC for 1 min, 52 ºC for 1.30 min and 72 ºC for 1.30 min) and a final extension at 72 ºC for 10 min followed by a hold for infinity at 5 ºC. The amplified 16S rRNA PCR product was sequenced using an automated sequencer (SciGenom Labs Pvt. Ltd., Kochi, India.). The unknown micro-organisms were identified using the maximum aligned 16S rRNA sequences available in the GenBank, National Center for Biotechnology Information (NCBI), Building through BLAST search. The best sequence alignment results were noted. Phylogenetic analysis was performed using the MEGA 6.0 program and 16S rRNA sequence to test the evolutionary relationships of unknown micro-organisms.

3) **Plant growth-promoting traits of Cd resistant rhizobacteria**

Cd-resistant bacterial cultures were screened for PGP traits such as the production of indol-3-acetic acid (IAA), ammonia, siderophore, hydrogen cyanide (HCN), catalase, activities of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, phosphate solubilizing capacity, and antibiotics sensitivity. IAA production capacity of Cd-resistant bacterial culture was estimated in the broth medium supplemented with 150 and 300 µg ml^{-1} of tryptophane and was performed by using the method of Bric et al. (1991). Ammonia production was qualitatively and quantitatively estimated according to the method of Cappuccino and Sherman (1992). The modified method of (Penrose and Glick, 2003) was used to measure the amount of α-ketobutyrate produced when ACC deaminase cleaves ACC, a precursor molecule of ethylene as well as nitrogen source into CO_2 and ammonia. Phosphate solubilizing capacity was assessed quantitatively using the methods of Subba (1988). Siderophores producing bacteria can change the color of Chromo azurol S from blue to orange with a hollow zone around the colonies on the plate. Qualitative estimation of siderophore production was screened using blue agar plates containing chrome azurol S dye as described by Schwyn and Neilands (1987). Catalase, HCN production, and antibiotics tests for Cd-resistant isolates were performed by using the methods as described by Aneja (2003) and using ofloxacin, gentamicin ampicillin trimethoprim-sulfamethoxazole, and cefotaxime+ceftriaxone as described by Bauer et al. (1966), respectively.

D. Statistical analysis

The obtained data were analyzed by the Duncan Multiple Range Test (DMRT) to separate the treatment means and considered statistically significant at $P<0.05$ (n=3). All the statistical analyses were performed using SPSS software (SPSS, Inc., Version 16).

III. RESULTS

A. Characterization of wastewater irrigated soil

Wastewater irrigated soil was analyzed for different physicochemical properties such as pH, EC, organic carbon, and heavy metals (Table 1). The pH of soils was found slightly alkaline and electrical conductivity ranged between 109.1 µS cm^{-1} – 309 µS cm^{-1} (Table 1). The organic carbon and total phosphorus in the soil varied from a 3.7 % - 7.4% and 39.2 µg g^{-1} dw 87.3 µg g^{-1} dw, respectively. The concentrations of heavy metals such as Cd, Cu, Ni, and Co (µg g^{-1} dw) ranged between 1.3-8.7, 131-1246, 65-491, and 1.56, respectively.

Table 1. Physico-chemical properties and heavy metal concentrations in soil collected from the long-term wastewater irrigated areas of Varanasi

S. No.	pH	EC (µS cm^{-1})	OC (%)	P(µg g^{-1})	Heavy metal (mg kg^{-1}dw)			
					Cd	Cu	Ni	Co
1	7.7^{ab}	309.0^{b}	3.7^{f}	47.5^{d}	6.2^{b}	499^{c}	335^{d}	1.4^{g}
2	7.6^{ab}	246.2^{d}	7.1^{b}	85.0^{a}	5.7^{b}	999^{b}	488^{a}	16.9^{a}
3	7.7^{ab}	210.4^{f}	7.4^{a}	39.2^{e}	1.3^{c}	430^{d}	457^{d}	14.9^{ab}
4	7.8^{ab}	351.3^{a}	6.9^{b}	75.4^{b}	8.7^{a}	124^{a}	477^{a}	16.1^{a}
5	7.7^{ab}	277.3^{c}	7.0^{b}	57.9^{c}	8.4^{a}	432^{d}	128^{d}	3.1^{ef}
6	7.7^{ab}	229.0^{e}	6.7^{c}	73.3^{b}	8.4^{a}	131^{g}	116^{d}	3.7^{e}
7	7.9^{a}	109.1^{i}	6.6^{c}	68.0^{b}	6.5^{b}	141^{ef}	85^{e}	4.0^{e}
8	7.6^{b}	150.1^{h}	6.1^{d}	87.3^{a}	5.9^{b}	140^{ef}	69^{f}	3.9^{e}
9	7.7^{ab}	155.7^{g}	4.8^{e}	74.2^{b}	6.5^{b}	160^{e}	65^{f}	6.0^{d}

Values are mean ± SE (not given) of three replicates.
Values within a column followed by different letter are statistically different at $p<0.05$ (Duncan Multiple Range Test).

B. Characterization of Cd-resistant rhizobacteria

In the present study, twenty-four Cd resistant bacterial isolates were isolated using nutrient agar plate amended with 50 µg Cd ml^{-1}. All these isolates were screened using the MIC approach to identify their best resistant capacity. Out of 24 isolates, five isolates, BHUJ Cd-1, BHUJ Cd-3, BHUJ Cd-10, BHUJ Cd-15, and BHUJ Cd-20 had the potential to tolerate Cd at 300 µg ml^{-1} and 450 µg ml^{-1} (Table 2 and Fig1).

C. Morphological and Biochemical characterization of Cd-resistant rhizobacteria

The morphological characteristics of Cd-RPGPR such as BHUJ Cd-1, BHUJ Cd-3, BHUJ Cd-10, BHUJ Cd-15, and BHUJ Cd-20 are shown in Table 3. The efficient isolates BHUJ Cd-1, BHUJ Cd-10, BHUJ Cd-15, and BHUJ Cd-20 were rod-shaped and Gram-negative rhizobacteria, While BHUJ Cd-3 was a rod-shaped and Gram-positive. All bacterial isolates colonies showed smooth edges on the plates. BHUJ Cd-1 and BHUJ Cd-10 appeared as whitish colonies, while BHUJ Cd-3 as yellowish green and BHUJ Cd-15 BHUJ Cd-20 appeared as brownish black colonies on the nutrient agar plates. The results further indicate that BHUJ Cd-1, BHUJ Cd-15 and BHUJ Cd-20 were as fast growing as compared to the BHUJ Cd-3 and BHUJ Cd-10 on the nutrient agar plate and in broth medium.

D. Molecular characterization of Cd-resistant rhizobacteria

A comparison with the 16S rDNA sequences available in the GenBank database indicated that the BHUJ Cd-1, had 98% similarity with *Agrobacterium sp.* (Accession No. MZ600148) BHUJ Cd-3 strain had 98% similarity with *Microbacterium schleferi* (Accession No. MK999915). The strains, BHUJ Cd-10, BHUJ Cd-15, and BHUJ Cd-20 showed 98% similarity with *Agromyces sp*. (Accession no KM999982), 97% with *Stenotrophomonas sp*. (Accession no MK 990331), and 98% with *Stenotrophomonas sp*. (Accession No MK962156), respectively. Similarity index of the partial gene sequence confirmed strains as *Agrobacterium sp.*, *Microbacrium schleferi*, *Agromyces sp*., *Stenotrophomonas sp*., and *Stenotrophomonas sp.* respectively. The sequences of the BHUJ Cd-1, BHUJ Cd-3, BHUJ Cd-10, BHUJ Cd-15, and BHUJ Cd-20 strains were deposited in the NCBI Gene Bank database with accession number MZ600148, MK999915, MK999982, MK990331, and MK962156, respectively (Fig 2).

Table 2. Screening of Cd resistant isolates from wastewater irrigated areas of Varanasi for Cd concentration ranging from 0-500 µg ml^{-1} using MIC approach

Bacterial strains	Cd concentration (µg ml^{-1})										
	0	50	100	150	200	250	300	350	400	450	500
BHUJ Cd-1	++	++	++	++	++	++	+	++	+	+	-
BHUJ Cd-3	++	++	++	++	+	+		-	-	-	-
BHUJ Cd-10	++	++	++	++	++	++	-	-	-	-	-
BHUJ Cd-15	++	++	++	++	++	++	++	-	-	-	-
BHUJ Cd-20	++	++	++	++	++	++	+ +	+	-	-	-

++ (Prominent growth); + (Growth); - (No growth)

E. Plant growth-promoting traits of Cd-resistant rhizobacteria

To estimate IAA production, Cd resistant bacterial strains were grown on media containing 150 and 300 µg tryptophan ml^{-1}, as a precursor molecule in the broth for IAA synthesis for 48 to 72 h. The bacterial strains, BHUJ Cd-1 and BHUJ Cd-20 produced significantly higher amounts of IAA than BHUJ Cd-3, BHUJ Cd-10 and BHUJ Cd-15 amended with 150 and 300 µg tryptophan ml^{-1} for 48 and 72 h, respectively (Table 4). BHUJ Cd-1 have higher IAA production at 300 µg tryptophan ml^{-1} as compared to BHUJ Cd-20, BHUJ Cd-15, BHUJ Cd-10 and BHUJ Cd-3. The highest IAA production by tested Cd resistant strains ranged between from 5 to 32 µg tryptophan ml^{-1}.

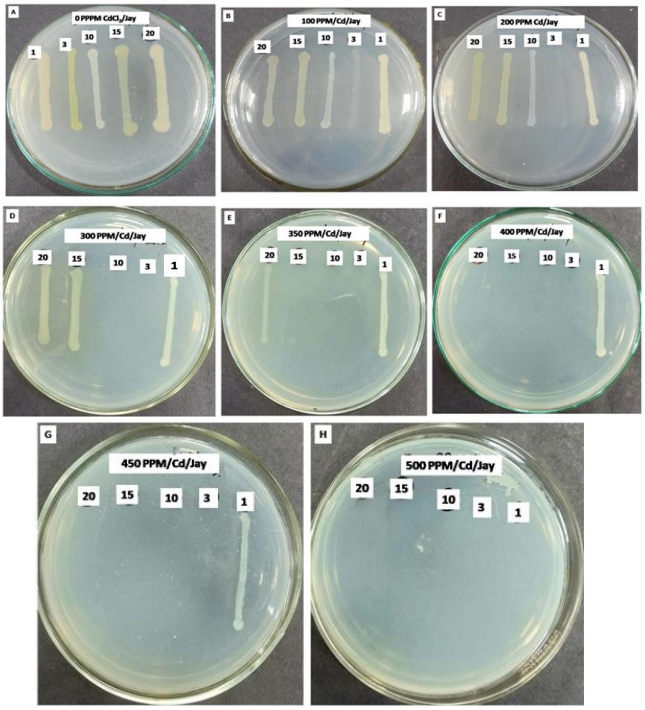

Fig 1: Plates (A-H) showing Cd tolerance potential of bacterial strains isolated from wastewater irrigates areas of Varanasi

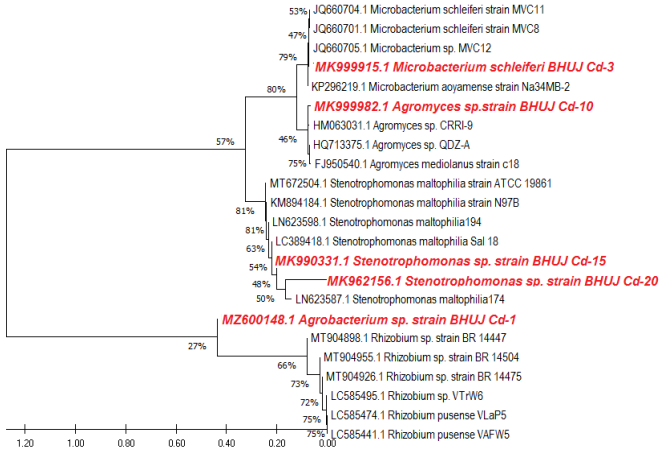

Fig. 2. Phylogenetic tree showing the relationship between the strains BHUJ Cd-1, BHUJ Cd-3, BHUJ Cd-10, BHUJ Cd-15 and BHUJ Cd-20 strain. In phylogenetic tree analysis, the strain was in the same cluster with different strains of *Agrobacterium sp*., *Microbacterium schleiferi*, *Agromyces sp*., *Stenotrophomonas sp*. and *Stenotrophomonas sp*. strains had 99.8% homology. The phylogenetic tree was drawn by MEGA 6 software using Neighbour-joining method and the significance of junctions was established using bootstrap method.

Selected Cd resistant bacterial strains were also screened for qualitative and quantitative estimation of ammonia production and results are shown in Table 4. All the selected bacterial strains showed a positive test by changing the broth medium color from yellow to orange or pink as compared to the control (without inoculation). Strains BHUJ Cd-20 and BHUJ Cd-15

produce higher ammonia production followed BHUJ Cd-1, BHUJ Cd10 and BHUJ Cd-3 and least in control at 24 and 48 h incubation, respectively (Table 4). Table 4 further showed that BHUJ Cd-15 and BHUJ Cd-20 showed higher ACC deaminase activity (29.9 ± 0.02 and 32.6 ± 1.5 µmol mg^{-1} h^{-1}, respectively) over BHUJ Cd-3,BHUJ Cd-10 and BHUJ Cd-1 (19.1 ± 0.29, 26.5 ± 0.27 and 25.0 ± 0.35 [d] µmol mg^{-1} h^{-1}, respectively).

Table 3. Selected morphological characteristics of Cd resistance strains isolated from wastewater irrigated areas of Varanasi

Bacterial strains	Cell morphology		Colony morphology			
	Gram staining	Shape	Form	Elevation	Margin	Color
BHUJ Cd-1	Negative	Rod	Circular	Flat	Undulate	Milky whitish
BHUJ Cd-3	Positive	Rod	Spindle	Flat	Undulate	Greenish
BHUJ Cd-10	Negative	Rod	Filamentous	Oval	Curried	Whitish
BHUJ Cd-15	Negative	Rod	Circular	Flat	Curried	Blackish brown
BHUJ Cd-20	Negative	Rod	Circular	Flat	Undulate	Brown

Table 4. Selected biochemical characteristics of Cd resistance bacterial strains isolated from waste water irrigated areas of Varanasi

Bacterial strains	IAA production (µg tryptophan mL^{-1})				Ammonia production (µg mL^{-1})		ACC deaminase activity (µmol mg^{-1} h^{-1})
	48h		72h				
	150	300	150	300	24 h	48 h	
Control	1.6[e]	2.8[e]	1.7[e]	2.7[e]	6.4[d]	8.7[e]	
BHUJ Cd -1	14.7[d]	26.8[d]	17.1[c]	32.0[c]	26.5[d]	31.3[d]	25.0[d]
BHUJ Cd -3	5.4[c]	4.9[c]	7.5[d]	7.7[c]	6.7[c]	12.9[c]	19.1[c]
BHUJ Cd -10	5.6[c]	6.4[c]	5.1[d]	7.2[c]	12.0[b]	26.7[b]	26.5[d]
BHUJ Cd -15	13.6[b]	17.7[b]	19.8[b]	24.9[b]	23.9[b]	41.9[b]	29.9[b]
BHUJ Cd -20	19.0[a]	24.5[a]	23.0[a]	32.0[a]	33.3[a]	54.0[a]	32.6[a]

Values are mean ± SE (not given) of three replicates.
Values within a column followed by different letter are statistically different at p<0.05 (Duncan Multiple Range Test).

Table 5. Phosphate solubilization, siderophore catalase test and HCN production, by isolated Cd resistant bacterial strains from waste water irrigated areas of Varanasi.

Bacterial strains	Phosphate solubilization (µg mL^{-1})			Siderophores	Catalase	HCN production
	4 days	8 days	12 days			
Control	0.8[d]	1.3[c]	2.7[e]	-	-	-
BHUJ Cd -1	2.8[b]	4.0[b]	4.5[d]	+	+	+
BHUJ Cd -3	4.1[c]	5.0[b]	4.9[d]	+	-	-
BHUJ Cd -10	2.9[b]	4.6[b]	3.7[b]	-	+	+
BHUJ Cd -15	4.3[b]	6.0[a]	5.5[b]	+	+	+
BHUJ Cd -20	5.1[a]	5.8[a]	5.8[a]	+	+	+

Values are mean ± SE (not given) of three replicates.
Values within a column followed by different letter are statistically different at p<0.05 (Duncan Multiple Range Test).
+ : Presence; - : absence

Cd-resistant bacterial strains also showed phosphate solubilization ability at different incubation times i.e. 4, 8, and 12 d (Table 5). The BHUJ Cd-20 has higher and significant phosphate solubilization capacity (5.8 ± 0.08 µg ml^{-1}) followed by BHUJ Cd-15 (4.3 ± 0.82) when compared to control at 12 d of incubation. BHUJ Cd-20 also showed better phosphate solubilization activity (5.1 ± 0.1 µg ml^{-1}) followed by BHUJ Cd-15, BHUJ cd-3 , BHUJ Cd-10 and BHUJ Cd-1 (4.31 ± 0.82, 4.1 ± 0.40, 4.1 ± 0.40, 2.9 ± 0.43 and 2.8 ± 0.11 [c]) respectively when compared with control (without inoculation) at 4 d of incubation (Table 5). BHUJ Cd-15 showed optimum phosphate solubilization activity (6.0 ± 0.06 µg ml^{-1}) followed by BHUJ Cd-20,BHUJ Cd-3, BHUJ Cd-10, and least in BHUJ Cd-1 (5.8 ± 0.07, 5.0 ± 0.18, 4.6 ± 0.4 and 4.0 ± 0.36 µg ml^{-1}) respectively when compared with control at 8 d of incubation. The phosphate solubilization activity of BHUJ Cd-20 was higher followed by BHUJ Cd-15 BHUJ Cd-3 and BHUJ Cd-1(5.8 ± 0.08, 5.5 ± 0.14, and 4.9 ± 0.05 µg ml^{-1}, 4.5 ± 0.28) respectively at 12 d of incubation. BHUJ Cd-1, BHUJ Cd-3, BHUJ Cd-15, and Cd-20 showed a positive test for siderophore production, whereas BHUJ Cd-10 gave negative results (Table 5).

The production of siderophores, low molecular weight metal iron chelators, was detected in the isolates conferring them a competitive advantage to bio-control agents and contributes to disease suppression due to the limited supply of essential trace minerals in natural habitats. In this present study, strain BHUJ Cd-1, BHUJ Cd-3, BHUJ Cd-10, BHUJ Cd-15, and BHUJ Cd-20 showed t catalase activity except for BHUJ Cd-3 (Table 5). These strains were screened for HCN production and results are presented in (Table 5). The yellow colour filter paper changed into the brown colour as compared to control which indicates that all the strain, except BHUJ Cd-3, produced HCN for 4 d of incubation.

Table 6. Antibiotic sensitivity of Cd resistant strains isolated from wastewater irrigated areas of Varanasi

Bacterial strains	Inhibition zone diameter (mm)				
	Ofloxacin	Gentamicin	Ampicillin	Trimethoprim + sulfamethoxazole	Cefotaxime + ceftriaxone
BHUJ Cd -1	15	11	10	NZ	9
BHUJ Cd-3	30	12	30	35	9
BHUJ Cd-10	25	23	27	30	24
BHUJ Cd-15	33	9	NZ	24	NZ
BHUJ Cd-20	19	20	14	NZ	NZ

F. Antibiotics sensitivity of Cd-RPGPR

Cd resistant strains also can tolerate antibiotics such as ofloxacin, gentamycin, ampicillin, trimethoprim-sulfamethoxazole, and a combination of cefotaxime and ceftriaxone (Table 6). Cd resistant strain used for screening is based on MIC against five antibiotics on the nutrient agar plate. The results showed that Cd-1senstive to ofloxacin, and sensitive to gentamicin, ampicillin,trimethoprim + sulfamethoxazole, cefotaxime + ceftriaxone, Cd-3 was sensitive to ofloxacin, ampicillin, and trimethoprim-sulfamethoxazole, and resistant to gentamicin and cefotaxime+ceftriaxone, whereas BHUJ Cd-10 was found sensitive to all the tested antibiotics. BHUJ Cd-15 and BHUJ Cd-20 showed resistance to these five antibiotics. BHUJ Cd-15 was found sensitive to ofloxacin only (Table 6). The

multiple antibiotic resistances of the Cd resistant strains might be associated with a high degree of resistance to heavy metals.

IV.DISCUSSION

Wastewater has been used for irrigation of agricultural soils since long-time, therefore, the concentration of heavy metal has been elevated in the long-term wastewater irrigated soil as compared to soil under immediate use of wastewater. Due to toxic in nature heavy metal harms soil microflora and microbial biodiversity (Ahmad et al., 2016). But the previous study has reported that microbes from the plants' rhizosphere enriched in heavy metal have PGP activity (Ahmad et al., 2016), So the site selection is one of the best criteria for the isolation of Cd-resistant PGPR. In the present study, soil pH was slightly alkali (above the 7.5) which may be ascribed to reduce acid production by the decreased population of phosphate solubilizing bacteria in the summer season. Both the soil pH and EC are strongly correlated. Proton deficient clay particles release the ions to the soil solution which increases EC (Thomas, 1996). The concentrations of tested heavy metals in wastewater irrigated soil were found higher than the previously reported by (Sharma et al., 2007) for the same study area. The permissible limits of Cd in soil ranged between 3–6 µg g^{-1} dw (Sharma and Agrawal, 2005). The concentrations of Cd and Cu in the soil are many-fold higher than the range in uncontaminated soil (0.01- 0.7 µg g^{-1} dw and 100 µg g^{-1} dw, respectively) (Sharma and Agrawal, 2005). In the present study, the concentration of Ni in the soil was within the range reported for the uncontaminated soil i.e.10-100 µg g^{-1} dw (Sharma and Agrawal, 2005). The range of Cd, Cu and Ni concentrations in the soil were also found above the permissible range of Indian standards i.e. 3-6 µg g^{-1} dw, 135 - 270 µg g^{-1} dw and 75-150 µg g^{-1} dw (Sharma and Agrawal, 2005). The higher concentrations of tested heavy metals in the soil may be ascribed to repeated use of wastewater, a mixture of domestic and industrial effluents from Varanasi city for the last four decades. Thus, the present study strongly supports earlier studies that concluded that wastewater reuse in agriculture especially in peri-urban areas of developing cities in the world has elevated the heavy metal concentration in soil (Sharma et al., 2007; Singh et al., 2010).

Bacteria residing in the extreme rhizospheric environment e. g. in metal contaminated soils have developed tolerance to stress as compared to non-contaminated soils (Ahmad et al., 2014). Therefore, isolates were further screened based on MIC for Cd. Only four highest resistant isolates were found (Fig.1). Several Cd-RPGPR such as *Ochrobactrum*, *Stenotrophomonas*, *Serratia*, *Bacillus*, *Bradyrhizobium*, and *Klebsiella* have reported form different parts of the globe (Ahmad et al., 2014; Pramanik et al., 2018). 24 bacterial strains were isolated from the wastewater irrigated areas of Varanasi and screened for Cd resistance. The results of the present study showed that strains, BHUJ Cd-1, BHUJ Cd-3, BHUJ Cd-10, BHUJ Cd-15 and BHUJ Cd-20 were able to resist the high concentration of Cd (300-400 µg ml^{-1}). Khatri et al. 2020, have also reported that *Bacillus subtilis* and *Pseudomonas putida* from a colder region of Indian Himalaya have tolerated Cd up to 18 µg ml^{-1} and 20 µg ml^{-1} respectively. The molecular characterization of Cd tolerant bacterial isolates based on partial 16S rDNA gene sequencing, Cd resistant PGPR strains BHUJ Cd-1, BHUJ Cd-3, BHUJ Cd-10, BHUJ Cd-15, and BHUJ Cd-20 were identified as *Agrobacterium sp.*, *Microbacrium schleferi*, *Agromyces sp*., *Stenotrophomonas sp*., and *Stenotrophomonas sp*. A number of bacterial species were isolated from the heavy metal contaminated soil e.g. *Enterobacter sp.* (Pramanik et al., 2018). IAA, a plant hormone is involved in cell division, cell enlargement tissue differentiation, lateral as well as adventurous root initiation, and pigment formation which help in maintaining the fitness of plants under Cd stress (Verma et al., 2010). Plants growing in a Cd contaminated soil produce reactive oxygen species (ROS) which disrupt the metabolic activities as well as block the hormonal pathway and induce senescence (Tran and Papova, 2013). IAA and other phytohormones produced by Cd-RPGPR reduced the toxicity of ROS and promote the seedling growth of *Oryza sativa* L. plants (Bhattacharyya and Jha, 2012). IAA producing Cd RPGPR increases roots biomass by enrichment of rhizosphere with nutrients (Chmielewska et al., 2014). Similarly, IAA produced by Cd resistant PGPRs in a Cd contaminated soil act as a phytostimulator and increased the plant growth (Ahmad et al., 2016; Pramanik et al., 2018).

Ammonia plays a signaling role when the PGPRs and plants interact with each other (Becker et al., 2002). Besides, ammonium transporters found in several PGPRs are thought to be involved in the reabsorption of $NH4^{+}$ released through diffusion from the bacterial membrane (Van Dommelen, 1997). Most of the PGPR have the capacity to fix atmospheric nitrogen and fulfill nitrogen deficiency of plants, also replacing the use of chemical nitrogen fertilizer [9]. Similarly, Cd-RPGPR strains fixed atmospheric nitrogen in a heavy metal stress environment (Bhattacharyya and Jha, 2012; Pramanik et al., 2018). It is now widely accepted that Cd-RPGPR isolates having ACC deaminase activity under Cd stress therefore it cleaves the ACC and lowering the ethylene level and improves the plant growth in the stress condition. The ACC deaminase producing ability in *Klebsiella michiganensis* MCC3089 was increased under Cd stress (Mitra et al., 2018). Various microorganisms with a wide range of ACC deaminase activity (approximately ≥20 nmol α-ketobutyrate mg^{-1} h^{-1}) can act as PGPR (Penrose and Glick, 2003). Isolates with higher levels of ACC deaminase activity ranges between 0.003 to 0.004 mmol α-ketobutyrate mg^{-1} h^{-1} are very effective for the promotion of plant growth. In the present study, all the four Cd resistant isolates had very high ACC deaminase. These rhizobacteria may be used to promote the growth of plants, particularly under stressful conditions such as heavy metals and delay senescence (Burd et al., 1998; Ali et al., 2012). This activity could be utilized for the production of a wide range of agricultural as well as horticultural crops by providing extra nutrients from the degradation of ACC and phytoremediation (Nascimento et al., 2014).

Despite the abundance of phosphorous in the soil, plants are unable to take phosphorous from the soil due to its insoluble form (Glick, 2012). Several PSB has the capacity to convert the insoluble form of phosphate into its soluble form by secreting organic acid or proton (Bhattacharyya and Jha, 2012). PSB increased the available phosphorus content in soil contaminated with heavy metal and also had the potential to immobilize heavy metals (Park et al., 2011). Under heavy metal stress, Cd resistant

PGPR produce siderophore and reduce their toxicity by immobilization of heavy metals in soil and pathogen infection by nutrients competition (Rajkumar et al., 2010). The assayed bacteria able to produce siderophores may secrete directly antimicrobial compounds caused by a stimulated biosynthesis. In the development of the antagonism, the siderophore production of the bacteria has an important role; such bacteria functioned as stress factors including local and systematic host resistance (Beneduzi et al., 2012). HCN, a volatile secondary metabolite is produced by microbes that are responsible for reducing the growth of pathogens by inhibiting metal enzymes, especially cytochrome C oxidizes in an electron transport system (Siddiqui and Ahmed, 2006). HCN production by PGPR indirectly helps in plant growth by suppressing the growth of soil-borne phytopathogens and it blocked the electron chain in pathogens for decreasing their population. PGP traits shown by the tested Cd-resistant PGPR strains indicated that these strains are phytostimulator, biofertilizer, and stress ameliorator. Therefore, these strains might be very useful in agricultural applications acting as potential PGPR in Cd contaminated areas to achieve sustainable agriculture.

The present study further showed that isolated bacterial strains were resistant to antibiotics which represent the plasmid associated R factor is responsible for heavy metal resistance. Many studies have reported the metal and antibiotics resistance of rhizobacteria (Wani et al., 2009). It has been suggested that antibiotic-resistant microorganisms will adapt faster under metal stress by the spread of R-factor instead of mutation and natural selection (Silver and Misra, 1988). Similar observation on antibiotics resistance by PGPR strain has also been reported by (Thacker et al., 2007). The variations in the resistance of rhizobacteria to different antibacterial drugs in the present study may be possible due to the differences in growth condition as well as the presence or absence of resistance mechanisms that could be encoded either by chromosomes and r-plasmid (Spain and Alm, 2003).

The identified Cd resistant bacterial strains possess the plant growth promoting traits. Thus, these bacterial strains can be used to develop a bacterial consortium for the remediation of heavy metal contaminated soils. The present study suggests to explore the biotechnological applications of such bacterial strains.

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Conflict of Interest

The authors declare that there is no conflict of interest for this publication.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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