

Cadmium Tolerance and Bioremediation Potential of Bacteria Isolated from Soils Irrigated with Untreated Industrial Effluent

Rizwan Ahmad*, Muhammad Mahmood-ul-Hassan, Muhammad Yasin and Vishandas Suthor
Land Resources Research Institute, National Agricultural Research Centre, Islamabad 45500, Pakistan

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Abstract. The present study was aimed to investigate the Cd tolerance of bacteria isolated from municipal effluent irrigated soils. Thirty bacterial strains were isolated and screened for their Cd⁺ tolerance by growing on nutrient agar plates amended with varying amount of Cd⁺. Out of them four bacteria (GS₂, GS₅, GS₁₀ and GS₂₀) were found highly Cd tolerant (600 ppm Cd). The minimum inhibitory concentration of Cd⁺ was found 200 ppm. The isolates showed optimum growth at 30 °C and pH 7.5-8.5. Growth curve study against different concentrations of Cd (0-600 ppm) revealed that GS₂ was more tolerant among selected strains showing only 33% reduction in growth compared to 64% by GS₅ and 77% by both GS₁₀ and GS₂₀ at 600 ppm Cd. Inoculation of maize seeds with Cd tolerant bacteria for root elongation demonstrated up to 1.7 fold increase in root elongation (in the absence of Cd) and up to 1.5 fold (in the presence of 50 ppm Cd) compared to the un-inoculated plants. The results of the study revealed that the bacterial isolates exhibiting great Cd tolerance and growth promoting activity can be potential candidates for bioremediation of metal contaminated soils and wastewaters.

Keywords: soil contamination, Cd tolerance, tolerance index, bioremediation

Introduction

The contamination of the environment with toxic heavy metals is a serious problem because it is associated with heavy metal accumulation in the food chain which later has an impact towards human health (Hamzah *et al.*, 2009). Municipal/industrial effluents contain considerable amounts of different metals as: chromium (Cr), cadmium (Cd), lead (Pb), nickel (Ni) and copper (Cu) in various combinations depending upon their source and nature (Khan *et al.*, 2013; Mahmood-ul-Hassan *et al.*, 2012). Release of untreated municipal/industrial effluents to agricultural lands and water bodies is a common practice in big cities of developing countries like Pakistan (Khan *et al.*, 2013; Mahmood-ul-Hassan *et al.*, 2012). Its long-term application can adversely affect soil and ecosystem health, ultimately human health (Singh and Bhati, 2005). Contamination of soil with heavy metals negatively affects biodiversity and the activity of soil microbial communities (McGrath *et al.*, 1995). Continuous application of untreated wastewater elevates the metal concentrations in surface soil to toxic levels. As soil is a rich habitat of all major groups of microorganisms (bacteria, actinomycetes, fungi and algae), long-term exposure of microorganism to high metal concentration develop the immunity in the

microorganisms (Akhtar *et al.*, 2013; Ezzouhri *et al.*, 2009).

Among metal pollutants of the surface soil, cadmium is one of the most toxic elements. Cadmium is used in industries like Ni-Cd battery manufacturing, electroplating, pigments manufacturing and stabilizers manufacturing. In plants, Cd affects nutrient uptake and homeostasis, inhibits root and shoot growth and frequently accumulated by agriculturally important crops (Sanita di Toppi and Gabrielli, 1999). Cadmium is the most dangerous heavy metal both to human and animal health as it is carcinogenic, embryo toxic, teratogenic and mutagenic (Hussain *et al.*, 2006). Excess Cd can damage kidney and lungs (Dhaliwal and Kukal, 2005). It may cause hyperglycemia, reduced immune potency and anemia, due to its interference with iron metabolism (Bueno *et al.*, 2008).

Several techniques (chemical and physical) are used for remediation of polluted soil and water. Chemical (precipitation and neutralisation) and physical (ion exchange, membrane separation and electro dialysis) techniques are applied to remove heavy metals from contaminated soils and waste water (Atkinson *et al.*, 1998). Such techniques have disadvantages like unpredictable metal ion removal, high reagent requirements, destruction of beneficial micro fauna and generation of toxic sludge (Ciba *et al.*, 1999).

*Author for correspondence; E-mail: rizwan_narc@yahoo.co.in

Biological approaches have been considered as an alternative remediation for heavy metal contamination (Khan *et al.*, 2009). Recent advances have been made in understanding metal-microbe interaction and their application for bioremediation of metal contaminated soils (Chibuike and Obiora, 2014). Bioremediation is the use of microbes like fungi and bacteria for removal of heavy metals and have been successfully used (Congeevaram *et al.*, 2007). The metal tolerant microorganisms are helpful to alter the chemical status of the metal ions and in turn metal ion mobility. They act through processes such as reduction, bioaccumulation, mobilisation and biotransformation (Khan *et al.*, 2009). Bioremediation is an efficient strategy due to its low cost, high efficiency and eco-friendly nature. It can be applied without removing and transporting contaminated soils. As soil matrix is not disturbed, soil micro flora and fauna are preserved.

Bacterial surfaces have several types of functional groups that can react with dissolved metals. Bacteria are important microorganisms to be used for biosorption and bioaccumulation of metals and hence are an important factor in controlling the mobility and distribution of metals in contaminated soil and water (Burnett *et al.*, 2007). Biosorption is removal of heavy metals using passive binding process of living and dead biomass while bioaccumulation is referred as metal uptake into the cell across the membrane using active cell metabolism (Kotrba *et al.*, 2011). Considering the importance of bacteria in bioremediation, this study was designed to isolate indigenous bacteria from polluted sites and assess their cadmium tolerance potential to use in bioremediation.

Materials and Methods

Samples collection. Six composite surface soil samples (0-15 cm) were collected from heavy metal polluted peri-urban areas of Gujranwala and Sialkot, Pakistan for this research work. Sampling sites have been continuously (more than 10 years) irrigated with untreated industrial/municipal effluent with high metal contents. The surface soil samples were collected in sterilised plastic bottles and were transported to Soil Environment Laboratory, NARC, Islamabad, Pakistan in sealed containers. These containers were stored at 4 °C to ensure minimal biological activity till further process.

Isolation for cadmium tolerant bacteria. Dilution plate technique was used for isolation of microbes (Pepper and Gerba, 2004). Bacteria from soil were cultured with 10^{-4} - 10^{-7} dilutions on nutrient agar medium at 28 ± 2 °C for three days. Thirty prominent isolates

(twenty from Gujranwala and ten from Sialkot sites) with some distinguished morphological characters (colony colour, size, shape etc.,) were further cultured and purified through repeated streaking on the same medium. The cultured strains were preserved on slants for Cd tolerance test at 4 °C and refreshed within three months regularly.

Minimum inhibitory concentrations (MIC) of Cd. To determine MIC for Cd, the growth of isolated bacterial strains was tested on nutrient agar medium amended with ascending concentration of Cd starting from 50 ppm (Kalantri, 2008). Stock solution (1000 ppm) of Cd salt (CdCl_2) was prepared with sterile water and added to the nutrient agar in varying concentrations (50-600 ppm). The process was continued with 50 ppm interval till the growth was ceased. Highly tolerant strains (600 ppm Cd) were tested repeatedly for further confirmation.

Morphological and biochemical characterisation. For colony and cell morphology, bacterial strains were grown on nutrient agar medium at 28 ± 2 °C for 36-48 h. Each colony was characterised on the basis of colour, margin, elevation and cell shape with ocular and light microscopy. For gram staining, the slides of tolerant bacterial strains were prepared according to Benson (1994). A small loop of bacterial culture was taken and a thin smear on glass slides was prepared. The smear was air dried and heat fixed, stained with crystal violet stain for one minute and washed with water. Then the smear was flooded with iodine solution for 30 sec. After 30 sec it was washed with water and smear was decolourised with 75% ethanol for 30 sec. After washing, safranin was used for counter staining. The slide was rewashed with water, air dried and observed under light microscope.

Indole acetic acid (plant hormone) production of Cd tolerant bacterial strains was detected by using the method stated by Brick *et al.* (1991). Bacterial cultures were grown in 250 mL conical flasks containing 50 mL nutrient broth (Lab-lemco powder 1.0; Yeast extract 2.0; Peptone 5.0; Sodium chloride 5.0, each was on g/L basis) for 3 days at 28 ± 2 °C. Flasks were inoculated with different bacterial strains individually. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 mL) was mixed with two drops of orthophosphoric acid and 4 mL of the Salkowski reagent (50 mL, 35% of perchloric acid, 1mL 0.5M FeCl_3 solution). Development of pink colour indicates IAA production.

For measurement of phosphate solubilising activity, a single colony of each strain culture was streaked on

Pikovskaya's medium containing tricalcium phosphate (EL-Komy, 2005) and incubated at 28 ± 2 °C for 3 days. Qualitative determination was done by optically observing clear P-zone (halo-zone) formation around the colonies.

Screening for acid producing ability was determined by using the bromothymol blue indicator along with nutrient broth. Each strain was grown in 250 mL conical flasks containing 50 mL nutrient broth for 3 days at 28 ± 2 °C. Traces of bromothymol blue indicator were added to each flask at the time of inoculation. Dark green colour appeared at neutral pH. Appearance of bluish green colour indicated the acid producing ability of the bacterial strain (Dupree and Wilcox, 1977).

Determination of optimum growth conditions. For optimum growth of bacterial isolates, two parameters i.e. pH and temperature were considered. To determine optimum pH, 30 mL test tubes having 10 mL nutrient broth were prepared in 5 sets for pH 6.0, 7.0, 8.0, 9.0 and 10 (each containing three test tubes) and autoclaved. These tubes were inoculated with freshly prepared culture of each isolate one by one. The tubes were incubated at 28 ± 2 °C and 70 rpm. After an incubation period of 24 h, their absorbance was taken at 600 nm wavelength on Spectronic Genesys 5 (Milton Roy Company, USA) and then a graph was plotted between pH (along x-axis) and absorbance (along y-axis).

For determination of optimum temperature, test tubes having 10 mL nutrient broth were prepared in 3 sets for 20, 30, and 40 °C. The pH of all the sets, each containing three test tubes was adjusted at 7 by using diluted HCl or NaOH solutions. Test tubes were autoclaved and then inoculated with freshly prepared cultures of different isolates individually. The tubes were incubated at 20, 30, and 40 °C, respectively, with 70 rpm. After an incubation period of 24 h, their absorbance was taken at 600 nm wavelength and then graph was plotted between temperature (along x-axis) and absorbance (along y-axis).

Growth curve and metal tolerance index. Nutrient broth with increasing concentrations of Cd i.e. 200, 400, 600 ppm was prepared and autoclaved. A control (0 ppm Cd) was also made. Growth of the selected heavy metal tolerant strains was studied at standard temperature (30 °C) and pH (7). Test tubes were inoculated and incubated for 24 h in water bath shaker at 70 rpm. After an incubation period of 24 h, their absorbance was taken at 600 nm wavelength and then a graph was plotted between Cd concentration (along x-axis) and absorbance (along y-axis).

Metal Tolerance Index (T_i) was calculated as the ratio of the optical density of the treated colony to that of the untreated colony.

$$T_i = \frac{OD_t}{OD_u}$$

Where:

OD_t = optical density of treated colony and

OD_u = optical density of the untreated colony.

Root elongation assay on filter paper culture. The plant root elongation promoting activity of the isolated bacteria was determined using the modified root elongation assay of Belimov *et al.* (2005). The seeds of maize variety ISD-gold were surface sterilised with a mixture of ethanol and 30% H_2O_2 (1:1) for 20 min, washed with sterile water and placed on wetted filter paper. Bacteria were grown in nutrient broth for 48 h at 28 ± 2 °C. Bacterial suspensions 5 mL or sterile water (un-inoculated control) were added to petri dishes containing filter papers, both in the presence and absence of 50 mg/L Cd. Root length of seedlings was measured after incubation of closed petri dishes for 7 days at 28 ± 2 °C in the dark. The assay was repeated twice with three dishes with 10 seeds per dish for each treatment.

Results and Discussion

Total heavy metals in Gujranwala and Sialkot soils. Surface soil samples used in this study were collected from peri-urban area of Gujranwala and Sialkot being irrigated with untreated wastewater (Table 1). The wastewater was a mixture of cottage industries and domestic effluent. Soil organic matter content was 0.7-2.0 %. The soils were alkaline in reaction (pH from 7.0 to 8.5), non-saline (electrical conductivity from 0.3 to 1.2 dS/m) and calcareous in nature (lime contents from 1.5 to 15.2%). The concentrations of different metals in soil of the study areas were; Cd ranged from 2 to 8.4 mg/kg; Cu 60 to 380 mg/kg; Pb 205 to 250 mg/kg; Cr 80-330 mg/kg and Ni from 90 to 130 mg/kg. The total soil Cd, Cu, Cr, Pb and Ni content in almost all the soil samples were higher than the permissible limits, i.e., 3, 100, 100, 100 and 50 mg/kg, respectively, as proposed by FAO/WHO (2001). Heavy accumulation of these metals in the soils are results of their use in different industries like ceramics, sanitary fittings, electrical and gas appliances, detergent manufacturing, dry batteries, plastic-ware, kitchen-ware and tanneries. The elevated concentrations of heavy metals in the soils are most likely due to long-term continuous application of

Table 1. Geographical position of peri-urban sites of sample collection

Site	City	North	East
1	Gujranwala	32° 06'	74° 10'
2	Gujranwala	32° 07'	74° 10'
3	Gujranwala	32° 07'	74° 11'
4	Gujranwala	32° 09'	74° 11'
5	Sialkot	32° 28'	74° 30'
6	Sialkot	32° 29'	74° 32'

untreated municipal/ industrial effluent containing these heavy metals.

Screening and characterisation of Cd tolerant bacterial strains. In this study, a total of 30 soil bacteria were isolated from the heavy metal contaminated soils. Minimum inhibitory concentration of Cd was found 200 ppm. Similar results were also reported by Ansari and Malik (2007), who reported MIC of 200 ppm for Cd. Out of 30, 21 bacterial strains tolerated cadmium (Cd) up to 200 ppm, thirteen strains were moderately tolerant (400 ppm Cd) and only 4 strains (GS₂, GS₅, GS₁₀ and GS₂₀) were found highly tolerant (600 ppm Cd).

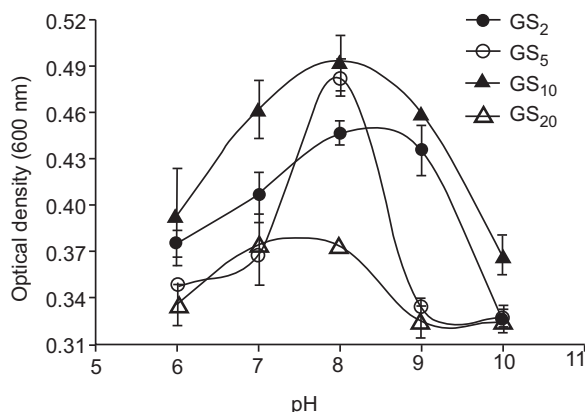
The selected 4 bacteria were characterised morphologically and bacteria were also observed under microscope for cell shape (Table 2). Most of the strains had phosphorus solubilisation and acid producing activity with bacillus cell shape and gram negative staining. None of the tolerant strain had IAA producing ability in the absence of L-tryptophan. IAA producing ability in the presence of L-tryptophan was not studied.

Results have shown that microorganisms in a contaminated environment could have adapted to that environment over a period of time. Piotrowska-Seget *et al.* (2005) also found in his study that, prolonged exposure of soil bacteria to Cd can develop resistance to its toxicity by activating the

tolerance mechanism towards Cd. Most of the bacteria they studied were gram negative.

Optimum growth conditions, growth curve and tolerance index. The effect of pH on the growth of selected 4 bacterial isolates is shown in Fig. 1 and the effect of temperature is shown in Fig. 2. Optimum pH varied from 7.5 to 8.5 which was in accordance with the pH of the soils from which the strains were isolated (Mahmood-ul-Hassan *et al.*, 2012). Regarding temperature, optimum growth of bacterial isolates was found at 30 °C ± 2. It shows that the selected bacteria are well adapted to soil as well as the climatic conditions of the regions from where they were isolated and can be reused in field conditions of the same ecology.

Growth curves of 4 highly Cd tolerant bacterial strains were made against different concentrations of Cd ranging from 0 to 600 ppm (Fig. 3). It is obvious from the result that at low concentration of Cd (200 ppm) the bacterial growth was high as compared to control; however, at 400 and 600 ppm Cd, the growth of all bacterial strains was also suppressed.

**Fig. 1.** Growth of Cd-tolerant bacteria as affected by different pH at 30 °C (N=3).**Table 2.** Morphological, biochemical and microscopic characteristics of Cd-resistant bacterial strains

Characteristics	Bacterial strain			
	GS ₂	GS ₅	GS ₁₀	GS ₂₀
Colony shape	Filamentous	Filamentous	Filamentous	Irregular
Colony colour	Yellowish white	White	Yellowish white	Creamy
Elevation	Umbonate	Concave at centre	Concave at centre	Umbonate
Margin	Undulate	Lobate	Erose	Undulate
Cell shape	Bacillus	Bacillus	Coccus	Bacillus
Gram staining	-	-	-	-
P-solubilising	+	-	+	+
Acid production	+	-	+	+
IAA-production	-	-	-	-

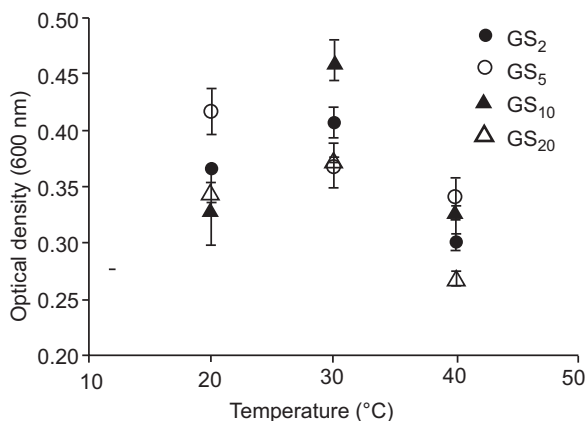


Fig. 2. Growth of Cd-tolerant bacteria as affected by different temperatures at pH 7 (N=3).

At 200 ppm Cd, optical density of GS₂, GS₁₀ and GS₂₀ showed an increase of 30, 12 and 13%, respectively, over control (0 ppm Cd). However, there was 7% reduction in case of GS₅. The results show that except GS₅ all other bacterial strains have so much adapted to Cd that they have bio-accumulated it to some extent. At 400 ppm Cd, growth of all the bacterial strains was reduced. GS₂ was found more tolerant with 10% reduction in growth and GS₅ least tolerant with 64% reduction. Reduction of growth in case of GS₁₀ and GS₂₀ was 35 and 55%, respectively, (Fig. 3).

Contrary to other bacterial strains, GS₂ again was found more tolerant at 600 ppm Cd with 33% reduction in growth. The growth of GS₅ at 400 and 600 ppm Cd was almost similar (64% reduction); however there was a sharper decrease in the tolerance indices of GS₁₀ and GS₂₀ at 600 ppm than at 400 ppm. There was up to 77% decrease in the growth of GS₁₀ and GS₂₀ at 600 ppm Cd (Fig. 3).

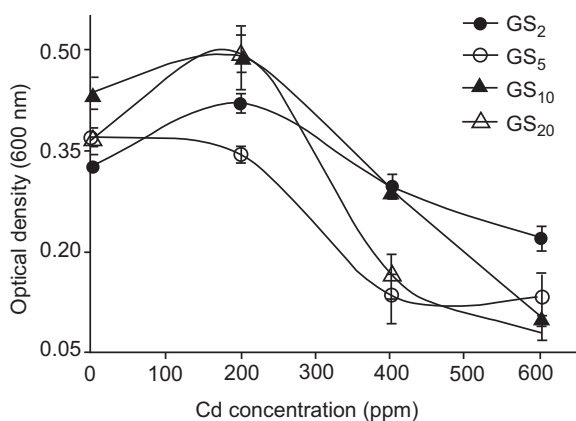


Fig. 3. Growth curve of Cd-tolerant bacteria as affected by different concentration of Cd at pH and temperature 30 °C (N=3).

Cadmium tolerance indices of all tested bacterial strains at different Cd concentrations are presented in Fig. 4. GS₂ being more tolerant among all strains showed highest tolerant index at highest concentration of 600 Cd. Tolerant indices of other bacterial strains revealed the order of tolerance as; GS₅ > GS₁₀ > GS₂₀.

Findings of other researchers also revealed that, Cd has inhibitory effect on bacterial growth (Kalantri, 2008; Laddaga and Silver, 1985). Higher Cd concentration can reduce the activities of essential enzymes such as protease, urease, arylsulphatase and alkaline phosphatase (Lorenz *et al.*, 2006).

Root length promotion. The effects of 4 Cd-resistant bacterial strains on root elongation of maize variety ISD-gold in the absence of Cd is shown in Table 3. Addition of 50 mg/L Cd to the filter paper culture inhibited root elongation of un-inoculated seedlings by 33%. Inoculations with Cd-resistant bacteria in the absence and presence of Cd significantly increased the root length of maize seedlings over un-inoculated seedlings. The maximum root length promoting effect on Cd-treated plants was observed after inoculation with strains GS₂ (150% over control). It was followed by GS₁₀ and GS₂₀; both produced 130% increase over control. The minimum increase in root length where seeds were treated with Cd was observed after inoculation with strains GS₅ (78%). Statistically similar trend was observed where seeds were inoculated in the absence of Cd.

Sheng and Xia (2006) and Belimove *et al.* (2005) also observed root growth promotion of Indian mustard inoculated with Cd resistant bacteria over un-inoculated seedlings in the presence of Cd. Rhizobacteria belonging

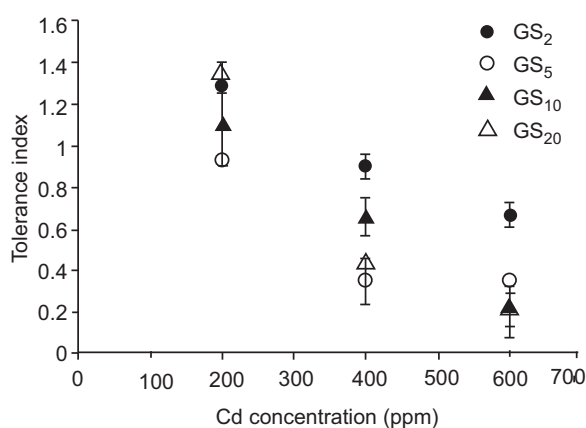


Fig. 4. Tolerance index of Cd-tolerant bacteria as affected by different concentration of Cd (N=3).

Table 3. Root length of maize seedlings inoculated with Cd-resistant bacterial strains grown in absence or presence of Cd in nutrient solution (N=3)

Bacterial strains	Untreated seedlings		Treated with 50 mg/L Cd	
	Root length (mm)	Bacterial effect (%)	Root length (mm)	Bacterial effect (%)
Uninoculated control	33.0 ^c	-	22.4 ^d	-
GS ₂	92.1 ^a	+176.6	56.0 ^a	+150.0
GS ₅	54.5 ^d	+ 63.7	40.0 ^c	+78.6
GS ₁₀	63.0 ^b	+ 89.2	51.5 ^b	+129.9
GS ₂₀	59.8 ^c	+ 79.6	51.5 ^b	+129.9
LSD	3.3	-	2.7	-

to different genera such as *Pseudomonas*, *Mycobacterium*, *Agro-bacterium* and *Arthrobacter* were found to have plant growth-promoting characteristics that can potentially support heavy metal uptake and reduce stress symptoms in plants (Dell'Amico *et al.*, 2005). He *et al.*, (2009) observed an increase in root growth and Cd contents in above ground tissues of hyperaccumulator tomato grown in Cd contaminated soil when inoculated with two metal-resistant bacteria; *Pseudomonas* sp. and *Bacillus* sp. Both the bacteria were indole acetic acid and aminocyclopropane-1-carboxylate deaminase producers. Thus proliferation of root growth in metal contaminated soil either by presence of indole acetic acid or aminocyclopropane-1-carboxylate deaminase enzyme could lead to enhanced uptake of heavy metals in hyperaccumulator plants which could help in bioremediation.

Conclusion

Bacteria isolated from heavy metal-contaminated peri urban areas of Gujranwala have the ability to tolerate higher concentrations of Cd. Cadmium resistance potential and root growth promoting activity of these isolates demonstrated that, these bacteria could be used as a potential candidate in the bioremediation of Cd contaminated wastewater and soil.

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