

## HIGH CADMIUM UPTAKE ABILITY OF *BACILLUS CEREUS* STRAINS ISOLATED FROM RHIZOSPHERE OF *TARGETES MINUTA* L. GROWING IN CADMIUM-POLLUTED SOIL

KULSOOM AKHTER, TAHSEEN GHOUS<sup>1</sup>, MUHAMMAD SIDDIQUE AWAN<sup>2</sup>,  
ZAIN-UL-ABDIN\* AND BASHARAT HUSSAIN

Department of Chemistry, University of Azad Jammu and Kashmir,  
Muzaffarabad 13100, Pakistan

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### Abstract

Microbes resistant to heavy metals develop mechanisms to accumulate Cd(II) in their cells. Two bacterial strains, *Bacillus cereus* AVP12 and *B. cereus* NC7401 which grew at high Cd(II) concentration were isolated from roots of *Tagetes minuta* L. growing in Cd(II) contaminated and uncontaminated soil. Minimum inhibitory concentration (MIC) and percent removal capacity were determined as function of pH, contact time and initial Cd(II) concentration. Bioaccumulation capacity was determined to observe possible effect of two different rhizospheres on Cd(II) removal capacity of both strains. Both strains were resistant up to 300 mg/l Cd(II) concentration. The percent removal capacity of both strains was maximum at pH 7 and incubation time of 24 hrs. High bioaccumulation capacity was observed with increasing Cd(II) concentration. Both Langmuir and Freundlich models fitted well to data of Cd(II) bioaccumulation. Though, maximum adsorption capacity ( $Q^0$ ) was observed for strains isolated from both types of rhizospheres, however remarkable  $Q^0$  values of 434.0 and 212.7 mg/g were observed for *Bacillus cereus* AVP12 and NC7401, respectively isolated from polluted rhizosphere. *Bacillus cereus* strains growing in polluted rhizosphere can develop high Cd(II) uptake ability in comparison to non-polluted rhizosphere.

### Introduction

Cadmium is a toxic and non-biodegradable pollutant. It is highly water soluble metal with no physiological role (Pinto *et al.* 2003) and accumulates in the food chain. Cd(II) is used in metal plating, alloy preparation and electronic manufacturing industries (Volesky and Holan 1995). It is also used as plastic stabilizer especially for poly(vinyl chloride) products and for the production of colorant. Cd(II) compounds (chalcogens) are used in the photovoltaic cells, inks, quantum dots, enamels, paintings, plastics and display devices (Martelli *et al.* 2006). The improper disposal of waste of such industries by dumping and incineration leads to environmental contamination with Cd(II) which is mutagenic in nature and is classified as human carcinogen. Its exposure affects human immune system causing cancer of prostate, liver, stomach, hemopoietic system (Bouvard *et al.* 2009), urinary bladder (Kellen *et al.* 2007), endometrium (Åkesson *et al.* 2008) and pancreas (Kriegel *et al.* 2006). The maximum permissible level of Cd(II) recommended by World Health Organization is 0.003 mg/l. European chemical agency has considered Cd(II) as metal of high concern and its content in products is subjected to rigorous restrictions [Regulation (EC) No. 1907/2006 of European Parliament and of the Council, 2006]. The conventional methods involving physicochemical processes for Cd(II) removal are inefficient due to their high cost, high

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\* Author for correspondence: <zainulabdinchm@gmail.com>. <sup>1</sup>Department of Chemistry, Mirpur University of Science and Technology, Mirpur, Pakistan. <sup>2</sup>Department of Zoology, University of Azad Jammu and Kashmir, Muzaffarabad-13100, Pakistan.

reagent requirement and unpredictable removal (Chubar *et al.* 2003). Nowadays, use of microbiological methods involving bacteria (Vijayaraghavan and Yun 2008) to remove Cd(II) become popular due to their ability to work at relatively low concentration and environment friendly nature. Many investigators have successfully investigated different bacterial strains for Cd(II) bioaccumulation (Hryniewicz *et al.* 2014, Huang *et al.* 2014, Zouboulis *et al.* 2004, Ansari and Malik 2007). In present work, Cd(II) bioaccumulation studies of two *B. cereus* strains, *B. cereus* AVP12 and *B. cereus* NC7401 were carried out. Both strains were isolated from roots of *Tagetes minuta* growing at two different rhizospheres, i.e., metal polluted and non-polluted. Major objectives of the present investigation are: Selection of most Cd(II) resistant bacterial isolates from Cd(II) polluted and non-polluted rhizospheres of *Tagetes minuta* on the basis of MIC, evaluation of Cd(II) bioaccumulation potential of the selected rhizobacterial isolates using different initial Cd(II) concentrations and to finding interrelationship between metal pollution and bioaccumulation potential of bacterial isolates.

### Material and Methods

*Tagetes minuta* plants growing at heavy metal polluted and non-polluted rhizospheres were collected along with soil adhered to their roots. Identification was carried out by a taxonomist of Department of Botany, University of Azad Jammu and Kashmir, Muzaffarabad. The specimen was submitted in the herbarium of Botany department under the voucher number AKBH-000424-KA. Intact root systems were picked up and were aseptically taken in separately marked sterile plastic bags and transported to the laboratory.

Soil samples were taken at the time of plant collection from well-defined area around the roots of *Tagetes minuta* roots. The soil adhering to the roots was gently shaken off and the rhizospheric soil adhering to the roots was separated by hand, ground to very small particle size and 2.5 g nominal mass was transferred in to 25 ml deionized water containing plastic tubes. The mixture was shaken periodically for 24 hrs and supernatant was collected from each sample and sent to Pakistan Institute of Nuclear Science and Technology (PINSTECH), Islamabad for atomic absorption spectrophotometric determination of the dissolved Cd(II) concentration.

The plant roots were suspended in sterile ringer's solution and streaking of the suspension was performed on the sterile nutrient agar plates using quadrante method. The plates were incubated at 37°C for 24 hrs. Distinct colonies were picked and cultured in the sterile nutrient broth medium at 37°C and 150 rpm. The streaking was performed again on the agar plates and the whole procedure was repeated thrice to make sure the availability of the pure isolates. A total of ten isolates were obtained, five of them from polluted and five from non-polluted rhizospheres which were then stored in sterile liquid broth glycerol at -20°C.

Minimum inhibitory concentration determination of Cd(II) was performed by well diffusion method (Hassen *et al.* 1998) with increasing concentration of CdCl<sub>2</sub> ranging from 50-300 mg/l. Refreshed culture of each isolate 30 ml in nutrient broth after mixing with 70 ml of the nutrient agar was poured into plates and wells (7 mm in diameter and 4 mm in depth) were made in each plate. The 100 µl of metal salt solution was added into the wells in triplicates. The plates were incubated at 37°C for 24 hrs and zone of inhibition was measured. The isolates which showed maximum resistance for Cd(II) were selected for identification. All biochemical tests of Cd(II) tolerant isolates including gram staining, carbohydrate fermentation, catalase, amylase, urease, acid and gas production were performed as per standard procedures (Cappuccino *et al.* 1983). Further identification was performed at Macrogen Inc. Seoul, Korea by 16S rRNA gene sequencing.

Identified Cd(II) resistant isolates were cultivated in sterile nutrient broth medium at 37 °C for 24 hrs. In separate test tube, 1 ml of Cd(II) solution was added to 5 ml of bacterial culture, covered with aluminum foil and incubated at 37°C and 150 rpm for 24 hrs. Centrifugation was performed at 13000 rpm for 5 min and Cd(II) was estimated in supernatant using double beam spectrophotometer (Shimadzu UV 1800). The effects of three parameters including pH, contact time and Cd(II) initial concentrations on percent metal removal capacity were studied. A control containing nutrient broth was also set along with Cd(II) solution keeping all other conditions same except bacterial culture. All tests were performed in triplicates and mean value was taken. The following equation was used to calculate the percent metal removal capacity:

$$\% R = (A_i - A_e) / A_i \times 100$$

where %R represents per cent metal removal capacity,  $A_i$  and  $A_e$  are the absorbance at Cd(II) concentrations before and after bioaccumulation.

Isolates were aerobically cultivated in the sterilized nutrient broth medium at 37°C and 150 rpm for 24 hrs and then harvested by centrifugation. The obtained pellets were dried by keeping them at 65°C for 20 hrs and then stored at -20°C. In screw capped test tubes, 4 mg of the dried cells were mixed with 1 ml of Cd(II) solution. For bioaccumulation study, a range of Cd(II) solution was used from 50 - 250 mg/l. The test tubes were agitated at 150 rpm on a shaking incubator at 37°C for a period of 1 hr, centrifuged and Cd(II) concentration was determined spectrophotometrically before and after bioaccumulation by dithizone method (Di Nezio *et al.* 2005). The amount of Cd(II) adsorbed on the bacterial biomass was calculated by following equation:

$$q_e = (C_o - C_e)V/M$$

where,  $q_e$  is the amount of metal accumulated in mg/g of bacterial biomass at equilibrium,  $C_o$  is the initial metal ion concentration and  $C_e$  is the final metal ion concentration in mg/l, respectively.  $V$  is the solution volume taken in liters and  $M$  is the amount of the biosorbent used in g.

## Results and Discussion

The results showed the presence of Cd(II) at concentrations of 8.34 and 0.87 ppb in the soil samples of polluted and non-polluted rhizospheres, respectively.

Out of ten, four isolates were found to be tolerant for Cd(II) completely at all concentrations as no zone of inhibition was observed for them. These four Cd(II) resistant isolates when initially characterized by standard biochemical tests were found to be Gram positive, rod shaped with positive results for carbohydrate (glucose, lactose and fructose) fermentation and catalase production while no amylase, urease and gas production was observed. The BLAST analysis of the two isolates showed genetic similarity with rRNA sequence of *B. cereus* AVP12 (16S: 99% similarity with reference strain KF527826.1) and two with *B. cereus* NC7401 (16S: 97% similarity with reference strain AB861980.1). Each of them was found to be common in both polluted as well as non-polluted rhizosphere. The results of the per cent removal capacity of both types of *B. cereus* strains for Cd(II) from solution (100 mg/l) at three pH values 5, 7 and 9 is presented in Fig. 1.

Results show that Cd(II) per cent removal capacity was maximum at pH 7. The relation of per cent removal capacity of metal cations with pH can be explained on the basis of the presence of functional group on the bacterial cell wall as it primarily contains weak basic and acidic functional groups. At acidic pH, where the concentration of hydrogen ions is greater, the carboxyl

ions which could possibly be responsible for cationic binding are protonated because there is a competition between hydrogen ions and the cations for the binding sites resulting in poor binding of Cd(II). At pH 7, this competition becomes less resulting in maximum Cd(II) removal whereas at basic pH, the per cent removal capacity decreases due to precipitation.

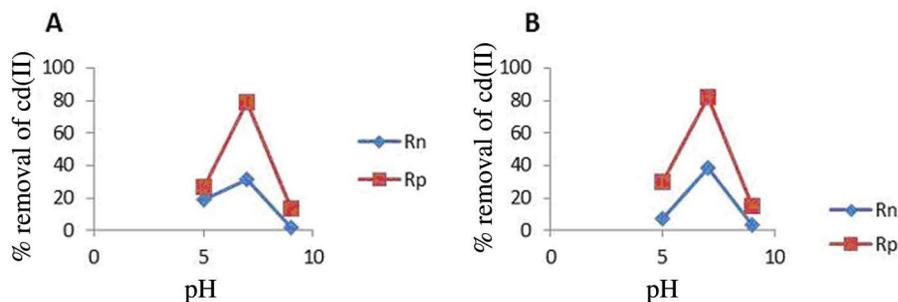


Fig. 1. Effects of pH on per cent Cd(II) removal efficiency by *B. cereus* strains: (A) *B. cereus* AVP12 and (B) *B. cereus* NC7401 (metal conc. 100 mg/l, contact time 24 hrs). Rn = Bacteria isolated from non-polluted rhizosphere, Rp = Bacteria isolated from polluted rhizosphere.

The effect of contact time on per cent removal capacity of the metal ions of two *B. cereus* strains was investigated by contacting 100 mg/l Cd(II) at pH 7 for a period of 4 - 96 hrs. Results are presented in Fig. 2, which illustrate that by incubating the bacterial strains for 4 hrs almost 20 and 47% uptake was observed in case of *B. cereus* AVP12 and *B. cereus* NC7401, respectively isolated from polluted rhizosphere which increased up to 77 and 92% after 24 hrs incubation period.

In case of the strains isolated from non-polluted rhizosphere, no significant uptake was observed after 4 hrs incubation which reached to a maximum value of 29 and 38% Cd(II) removal. The Cd(II) bioaccumulation rate was high up to a period of 24 hrs because the bioaccumulation sites were open and their availability was greater. After 24 hrs, an equilibrium was established which might be due to the reason that a large amount of Cd(II) get attached and no more binding sites were available. As a compromise, 24 hrs incubation time was selected for further study.

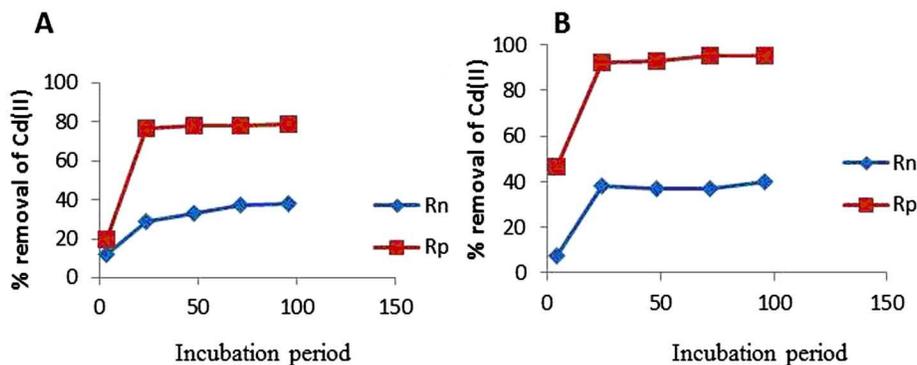


Fig. 2. Effect of contact time on per cent Cd(II) removal efficiency by *B. cereus* strains: (A) *B. cereus* AVP12 and (B) *B. cereus* NC7401 (metal concentration 100 mg/l, pH 7). Rn = Bacteria isolated from non-polluted rhizosphere, Rp = Bacteria isolated from polluted rhizosphere.

Effect of initial concentration of metal ion: Fig. 3 shows the effect of different initial concentrations of Cd(II) ions in the range of 50-250 mg/l on per cent removal capacity under the optimized conditions of pH 7 and incubation period of 24 hrs at 25°C. It can be seen from Fig. 3 that, as the concentration of Cd(II) was increased, the per cent removal capacity was decreased.

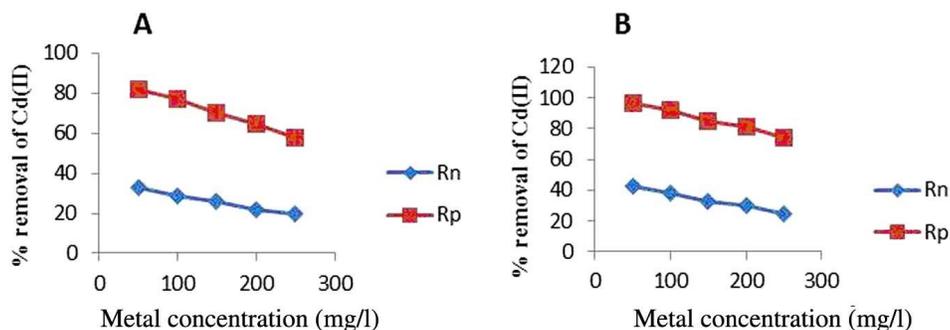


Fig. 3. Effect of initial concentration on per cent Cd(II) removal efficiency by *B. cereus* strains: (A) *B. cereus* AVP12 and (B) *B. cereus* NC7401 (contact time 24 hrs, pH 7). Rn = Bacteria isolated from non-polluted rhizosphere, Rp = Bacteria isolated from polluted rhizosphere.

The strains isolated from polluted rhizosphere showed highest percentage removal at 50 mg/l where it was 82 and 96 for *B. cereus* AVP12 and *B. cereus* NC7401, respectively while at 250 mg/l, 58 and 74 removal was shown by both the strains. In contrast, strains isolated from non-polluted rhizosphere have shown low per cent removal capacity values between 33 - 20 and 43-25 in the studied concentration range.

Trend of bioaccumulation capacity reflected from Fig. 4 that both the strains isolated from two different rhizospheres showed increase in the amount of Cd(II) adsorbed per unit weight of the adsorbent with the increase (50 - 250 mg/l in the concentration of metal) (Fig. 4).

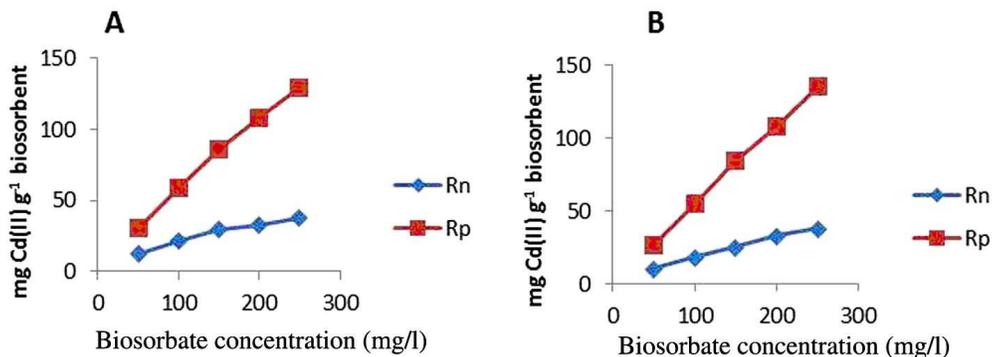


Fig. 4. Bioaccumulation capacity shown by *B. cereus* strains: (A) *B. cereus* AVP12 and (B) *B. cereus* NC7401 (metal conc. 100 mg/l, contact time 24 hrs, pH 7). Rn = Bacteria isolated from non-polluted rhizosphere, Rp = Bacteria isolated from polluted rhizosphere.

This increase in the adsorption could be due to electrostatic interactions of cationic Cd(II) with the negatively charged functional groups on bacterial cell surface and possibility of bridging between negatively charged functional groups of the biomolecules of bacterial cell wall including; nucleic acids, carbohydrates, proteins or lipids. At an initial Cd(II) concentration of 50 mg/l,

bioaccumulation capacity values shown by *B. cereus* AVP12 and *B. cereus* NC7401 isolated from polluted rhizosphere were 30.9 and 26.25 mg/g which reached a maximum value of 129.37 and 135 mg/g biosorbent, respectively at 250 mg/l Cd(II) concentration. These values for the same strains isolated from non-polluted rhizosphere with the same sequence were found to be 12.37, 10.75 and 37.5 mg/g at initial and final concentrations, respectively.

Equilibrium adsorption isotherms proved to be very helpful in understanding the adsorption mechanism. Langmuir and Freundlich models are widely used for this purpose. Langmuir isotherm model was found to be successfully applicable for monolayer adsorption and it showed the adsorption on homogenous sites. The constants evaluated from both the isotherm models are presented in Table 1.

Where “ $C_{eq}$ ” represents equilibrium concentration (mg/l) and “ $q_{eq}$ ” represents the amount of metal ions adsorbed by one gram of the dried biomass at equilibrium (mg/g). “ $Q^0$ ” represents the maximum adsorption capacity (the maximum amount of the metal ion adsorbed per unit weight of biomass to form a complete monolayer on bacterial surface bound at high  $C_{eq}$  (mg/l)). ‘ $b$ ’ is the Langmuir constant which is related to the binding affinity of the binding sites. The values of “ $Q^0$  and  $b$ ” can be determined from the linear plot of  $C_{eq}/q_{eq}$  versus  $C_{eq}$ .

**Table 1. Isotherm model constants for bioaccumulation of Cd(II) on *Bacillus* strains.**

Strain	Rhizosphere	Langmuir isotherm parameters			Freundlich isotherm parameters		
		b	$Q^0$	$R^2$	$K_F$	n	$R^2$
<i>B. cereus</i> AVP12	Polluted	1.13	434.0	0.994	16.82	5.8	0.988
	Non-polluted	5.95	68.02	0.992	1.11	1.52	0.986
<i>B. cereus</i> NC7401	Polluted	0.93	212.7	0.974	1.67	0.93	0.987
	Non-polluted	12.77	89.28	0.990	1.42	1.33	0.998

**Table 2. Comparison of maximum adsorption capacity ( $Q^0$ ) values for Cd(II) with various adsorbents.**

Adsorbents	$Q^0$	$K_F$	References
Wheat straw	40.48	5.41	(Dhir and Kumar 2010)
Rice straw	39.68	8.45	(Dhir and Kumar 2010)
Activated carbon	0.53	0.40	(Bohli <i>et al.</i> 2013)
Multi walled carbon nanotubes	25.7	-	(Vuković <i>et al.</i> 2010)
Perlite	1.79	0.79	(Torab-Mostaedi <i>et al.</i> 2010)
<i>Salvinia</i> biomass	39.06	8.19	(Dhir and Kumar 2010)
<i>Pantoea</i> sp. TEM18	58.1	10.34	(Ozdemir <i>et al.</i> 2004)
<i>Pseudomonas florescens</i>	500	41.2	(Hussein <i>et al.</i> 2004)
<i>E. Coli</i>	2.24	0.79	(Adedirin <i>et al.</i> 2011)
<i>Bacillus subtilis</i>	2.65	0.86	(Adedirin <i>et al.</i> 2011)
<i>Bacillus thuringiensis</i> OSM29	59.17	2.57	(Adedirin <i>et al.</i> 2011)
<i>Bacillus thuringiensis</i> OSM29	434.0	16.82	(Oves <i>et al.</i> 2013)
<i>B. cereus</i> AVP12 (polluted)	68.02	1.11	Self-devised
<i>B. cereus</i> AVP12 (non-polluted)	212.7	1.67	Self-devised
<i>B. cereus</i> NC7401 (polluted)	89.28	1.42	Self-devised
<i>B. cereus</i> NC7401 (non-polluted)			Self-devised

The value of  $Q^0$  is helpful in comparing the performance of all the strains with each other to identify which strain has the highest bioaccumulation capacity. Langmuir parameters showed high  $Q^0$  values of 434 and 212.7 mg/g for *B. cereus* AVP12 and *B. cereus* NC7401 isolated from polluted rhizosphere, whereas in case of both these strains isolated from non-polluted rhizosphere, these values were found to be 68.02 and 89.28 mg/g, respectively. A comparison is shown in Table 2 for the Cd(II) adsorption capacity values reported in literature with various other adsorbents and *B. cereus* strains used in the present study. Though, it is not so easy to have a direct comparison, as the experimental conditions vary in case of different studies. However, it is seen that *B. cereus* strains in this study have great adsorption capacity as compared to other adsorbents.

All studied strains illustrated strong resistance against Cd(II) and showed potential bioaccumulation capacity especially those isolated from polluted rhizosphere. These strains could be promising candidates for the removal of heavy metals from polluted agricultural, sewage and industrial effluents. Plants growth on the polluted sites could be excellent ecosystems to isolate bacterial genes involved in metal resistance and/or plant growth promotion.

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