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Bioaccumulation of Heavy Metals by Metal-Resistant Bacteria Isolated from *Tagetes minuta* Rhizosphere, Growing in Soil Adjoining Automobile Workshops

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Authors' Contribution
TG designed the study and KA did isolation, identification, biological activities and bioaccumulation studies. TG and SA supervised the study and wrote the article. SA analysed the data. FN and SE helped in experimental work. ZA and BAK helped in statistical analysis. MNA helped in concentration optimization and bioaccumulation studies.

Key words
***Tagetes minuta*, Biosoprtion, Heavy metals, Bioaccumulation capacity, Langmuir and Freundlich models.**

ABSTRACT

The present study deals with isolation, identification and characterization of some indigenous heavy metal resistant bacteria isolated from rhizosphere of *Tagetes minuta,* growing in soil adjoining automobile workshops to investigate their bioaccumulation capacity for three selected metals Cr(VI), Ni(II) and Cd(II). The plant was selected due to its natural abundance in the climate of Kashmir and also in the area of study. On the basis of morphological, biochemical, 16S rRNA gene sequencing, the isolates were identified as *B. cereus* BDBC01, *B. cereus* AVP12 and *B. cereus* NC7401. At maximum studied concentration of metal ions (250 mgl^{-1}), in above sequence, the strains accumulated 118.2, 121.87 and 90 mg/g Cr (VI), 135, 127.5 and 146.25 mg/g Ni (II) and 120, 129.37, 135 mg/g Cd(II). Langmuir and Freundlich models were applied and found suitable to describe biosorption of selected metals by the isolated strains.

INTRODUCTION

The advancement in industries has greatly increased the contamination of environment by the production of heavy metals (Bishop, 2000; Wang, 2002). Iron and steel, electroplating, leather, fertilizer, metallurgy, photography and a lot of other industries are continuously discharging their wastes containing heavy metals like Ni, Cu, Pb, Cr, Cd, *etc*. into the environment causing life threatening problems to human and other organisms on earth (Wang and Chen, 2006). In recent years, much attention has been given on the measurements to control this pollution of natural environment by such hazardous metals. In this respect, many techniques have been developed for the determination and removal of heavy metal ions. These methods include ion exchange, electro dialysis, evaporation, chemical precipitation and membrane separation (Gupta *et al.*, 2012). But all these techniques have many disadvantages like high energy consumption, low selectivity, high cost, incomplete removal and generation of toxic sludge (Celaya *et al*., 2000). Modern research focuses on efficient, ecofriendly and cost effective biological methods for heavy metal determination and their removal which may possess good potential to replace conventional methods (Malik, 2004). Many investigators have made developments to search for low cost adsorbents having biological origin like activated sludge, egg shells, rice husks *etc*. (Al-Qodah, 2006; Chuah *et al*., 2005; Vijayaraghavan and Yun, 2007). Microorganisms can be efficient adsorbents of metals and conventional techniques can be replaced by microbe related technologies for the determination and removal of potentially hazardous metals from the environment (Ozdemir *et al*., 2003, Rehman *et al*., 2007). This high efficiency of microbial biomass like

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bacteria, fungi and microalgae is due to the nature and composition of cell wall as it contains many functional groups capable of adsorbing metal ions. Carboxyl group a negatively charged, abundantly available functional group on microbial cell wall actively binds metal cations. Amine groups very effectively remove both cationic and anionic metal ions either by electrostatic interactions or hydrogen bonding (Kang *et al*., 2007; Tunali *et al*., 2006). Several other researchers have used different bacterial strains like *Bacillus* sp*., Bacillus licheniformis, Bacillus thuringiensis, Pseudomonas* sp*., Staphylococcus aureus* to for the biosorption as well as bioaccumulation of various metal ions like Cr(VI), Ni(II), Zn(II), Pd(II) *etc*. (Tunali *et al*., 2006; Şahin and Ozturk, 2005; Wang *et al*., 2010; Zhou *et al*., 2007; Ziagova *et al*., 2007).

Keeping the above in view, the present study was aimed to isolate some native rhizobacterial species to determine their bioaccumulation capacity for three selected heavy metals, Cr(VI), Ni(II) and Cd(II). The plant *Tagetes minuta* was selected due to its natural abundance in the climate of Kashmir and also in the contaminated area of the study. Our main objective was to enhance a trend of making the use of rhizobacterial species in heavy metal removal technologies by exploring indigenous microbial isolates.

MATERIALS AND METHODS

Chemical and reagents

All chemicals and reagents used in this study were procured from Merck, Aldrich Chemicals Co., and BDH Chemicals. Sterilized nutrient broth medium was used to culture the strains. Sterilized distilled deionized water was used for solution preparation.

Plant collection

Whole plant of *Tagetes minuta* along with some soil adhered to its roots was picked up from an area where the waste water from automobile workshops was flowing. The sample was aseptically taken in sterile plastic bags and transported to the lab.

Isolation of microorganisms

Plant roots along with some soil were suspended in sterile ringer's solution and the suspension thus obtained was streaked on nutrient agar medium for bacterial growth. Distinct colonies obtained after 24 h were picked and inoculated in to nutrient broth to grow cultures at 37°C in a shaking incubator for 24 h. The whole process was repeated three times and pure colonies were obtained. Pure cultures of the isolates were maintained as their glycerol stocks in 50% sterilized glycerol and stored at -20°C.

Soil sample analysis

The rhizosphere soil adhering to the roots was gently shaken by hand and ground to a very small particle size. A nominal mass of 1 g was transferred in to sterile plastic tubes in which 25 ml deionized water was added. The mixture was periodically shaken for a minimum period of 24 h and the supernatant was collected and sent to Pakistan Institute of Nuclear Science and Technology, Islamabad for the determination of three dissolved metals Cr(VI), Ni(II) and Cd(II). The samples were analyzed by atomic absorption spectrophotometer.

Resistogram of bacterial isolates

The heavy metal resistance of nine distinct isolates KA17-KA25 was investigated using well diffusion method (Hassen *et al*., 1998) with increasing concentrations of Cr(VI), Ni(II) and Cd(II) ranging from 50-300 mg/l. Refreshed culture of each isolate (30 ml) in nutrient broth was mixed with 70 ml of nutrient agar and poured into plates. Wells 7 mm in diameter and 4 mm in depth were made in each plate and 100µl of appropriate metal salt solutions were added into the wells in triplicates. The plates were incubated at 37°C for 24 h and the zone of inhibition was measured.

Identification of microbial isolates

Three isolates showing maximum tolerance for heavy metals under study were identified presumably on the basis of following features: colony morphology, gram staining, acid and gas production, carbohydrate (glucose, lactose, sucrose) fermentation, urease, amylase, gelatinase production, and starch hydrolysis as described by Cappuccino and Sherman (2008). The isolates were further identified by 16S rRNA gene sequencing carried out by Macrogen Inc., Seoul, Korea.

Metal removal capacity study

The identified strains KA18, KA24 and KA25 were cultivated aerobically in properly labeled test tubes containing nutrient broth medium by incubating the test tubes at 37°C in shaking incubator. Bacterial suspension (5 ml) was mixed with 1ml metal solution in sterilized test tubes covered with aluminum foil and agitated at 150 rpm on a shaking incubator at 37°C. Effects of different parameters like pH, incubation time and initial metal ion concentration on percent metal removal capacity of each strain were studied. After 24 h incubation samples (1 mL) were centrifuged for 5 min at 13000 rpm in an eppendorf centrifuge (HERMLE Z230 HA) and the supernatant thus obtained was used for the estimation of metal ion concentration using double beam spectrophotometer (Shimadzu UV 1800). A control was also set containing

nutrient broth medium along with metal solution keeping all other conditions same except bacterial culture. Each test was performed in triplicates and average value was taken as a result. The percent metal removal capacity was calculated by equation:

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

Where, %R is the percent metal removal capacity, A_i is the absorbance at initial metal concentration and A_e is the absorbance after bioaccumulation.

Bioaccumulation capacity of isolates

In this study, identified strains were cultivated in 500 mL conical flasks containing sterile nutrient broth in shaking incubator under aerobic conditions at 37°C and 150 rpm. The cells were harvested after 24 h incubation. The cells were then centrifuged and the pellets were dried at 65°C for 20 h and stored at -20°C. Dried cells (4 mg) were mixed with 1 mL of sterile metal ion solution in screw capped tubes which were then agitated on a shaking incubator at 150 rpm at 37°C for 1 h, centrifuged and supernatant was used for spectrophotometric determination of metal ions concentration before and after bioaccumulation. The amount of metal adsorbed on the bacterial biomass was calculated by the equation:

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

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

RESULTS AND DISCUSSION

Soil sample

Results of the study provided by the laboratory showed presence of Ni(II) and Cd(II) at concentrations of 107.7 and 8.34 ppb, respectively, while Cr(VI) was not detected.

Heavy metal tolerance and identification of bacterial isolates

Out of the studied isolates, KA24 exhibited complete resistance for all the three metals at all concentrations and no zone of inhibition was observed. Isolates KA18 and KA25 also tolerated Ni(II) and Cd(II) up to the maximum concentrations. However, these isolates showed sensitivity for Cr (VI) at 150 mg/L and above (Supplementary Table SI). These metal resistant isolates were characterized using standard physiological and biochemical tests (Supplementary Table SII) and were identified as *B. cereus*. All the three isolates were further subjected to 16S rRNA gene sequence analysis in order to check any possible difference at strain level. BLAST analysis showed genetic similarity of KA18, KA24 and KA25 with rRNA sequence of *B. cereus* BDBC01 (16S: 98% similarity with a reference strain JX276537.1), *B. cereus* AVP12 (16S: 99% similarity with reference strain KF527826.1) and *B. cereus* NC7401 (16S: 97% similarity with reference strain AB861980.1), respectively.

Metal removal capacity of isolates

Effect of pH

The pH of a solution is the most critical parameter for metal biosorption/bioaccumulation as it influences both the bacterial surface chemistry as well as solution chemistry of metal ions. In this study, pH values of metal ion solutions were adjusted in the range 5-9, before the addition of biosorbent. Figure 1 shows that all isolates exhibited maximum % removal capacity for Cr(VI) at pH5 and for Ni(II) and Cd(II) at pH7 with sharp decrease at pH9. This may be because in acidic medium functional groups on bacterial cell wall get protonated thus offering maximum binding capacity for negatively charged chromate ions compared to positively charged Ni(II) and Cd(II). As pH increases the functional groups become proton free favoring the greater bioaccumulation of Ni(II) and Cd(II)

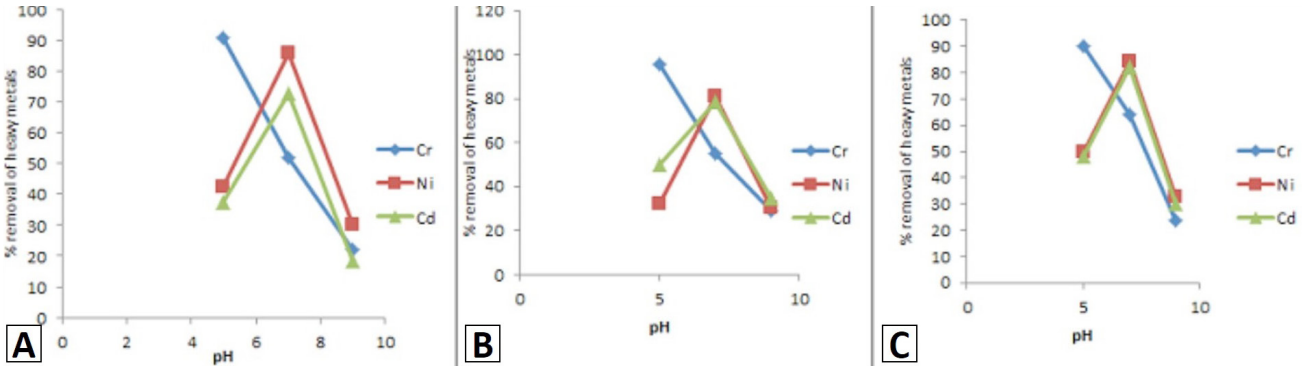

Fig. 1. Effect of H on percent metal removal efficiency by *B. cereus* strains KA18 (A), KA24 (B) and KA25 (C) (metal conc. 100 mg/l, incubation time 24 h).

to the cell surface. The low percentage removal at pH 9 may be attributed to decrease in solubility of Ni(II) and Cd(II) as a result of precipitation decreasing the free metal ions availability to bind with cell wall (Lopez *et al*., 2000).

Effect of incubation time

Incubation time is one of the most effective factors in batch biosorption studies to determine equilibrium stage of the process. Percent removal capacity of metal ions was investigated for period of 4-96 h. Figure 2 shows maximum uptake of Cr (VI) at 4 h and reached at equilibrium after 24 h. Whereas percent removal capacity of Ni(II) and Cd(II) onto KA18 and KA24 was significant at 24 h and 48 h on to KA25. As a compromise 24 h incubation time was selected for further study.

Effect of initial concentrations of metal ions

Effect of metal ions concentration on percent removal capacities by the strains was studied in the range of 50-250 mgL^{-1}. As is shown in Figure 3, as the concentration of Cr(VI) increased there was a decrease in percent removal capacity. At 50 mgL^{-1} removal was 100% by all three strains while at 250 mgL^{-1}, 63% and 65% removal was shown by KA18 and KA24 and 42% by KA25. For Ni(II) and Cd(II), all three strains have shown similar trend and percent removal capacity of the metals remained between 85-65% in the studied concentration range. The decrease in metal removal capacities for Cr(VI) with increase in concentration may be due to the to the absence of the metal ion in the environment of the study. The bacterial strains exposed to high levels of Ni(II) and Cd(II) may have gained biological resistance against their adverse effects by developing various resistance mechanism like; plasmid-encoding or enzyme activity at cell surface. This may also be linked with functional groups like; amino, carboxylic, sulfhydryl on bacterial cell surface which may have different metal binding capacity for negatively charged chromate ions and positively charged Ni(II) and Cd(II).

Bioaccumulation capacity study

Results of the study (Fig. 4) reflects the trends of bioaccumulation capacity of each strain for the studied metal ions. Bioaccumulation capacity for Cd(II) and Ni(II)

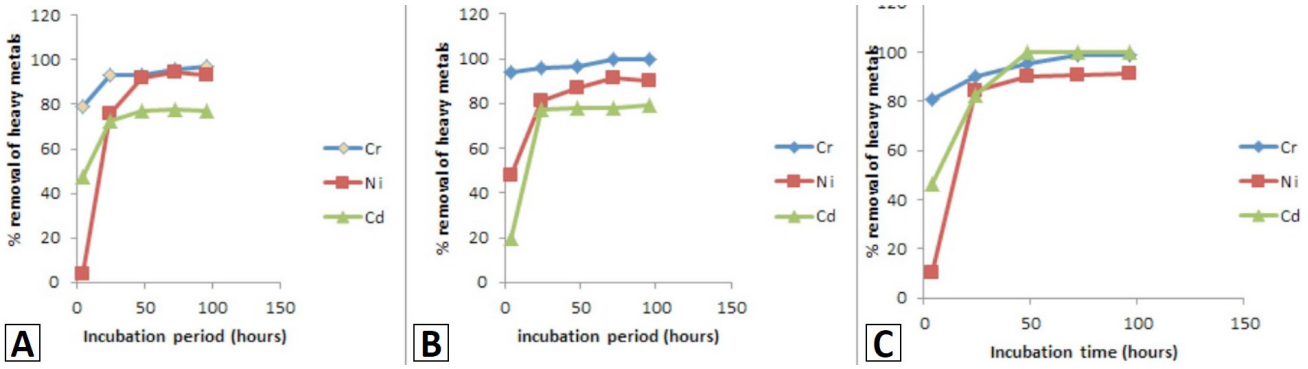

Fig. 2. Effect of incubation time on percent metal removal efficiency by *B. cereus* strains KA18 (A), KA24 (B) and KA25 (C) (metal conc. 100 mg/l).

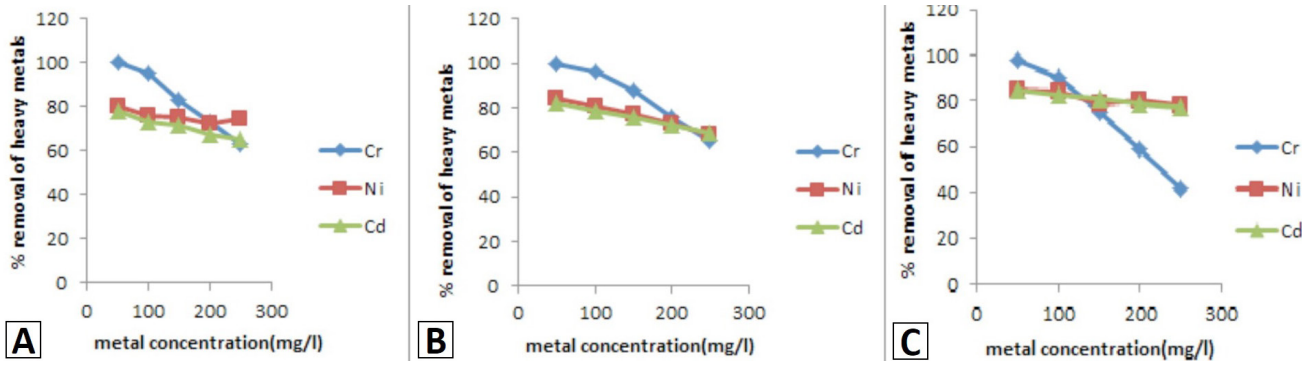

Fig. 3. Effect of metal concentration on percent metal removal efficiency by *B. cereus* strains KA18 (A), KA24 (B) and KA25 (C) (incubation time 24 h).

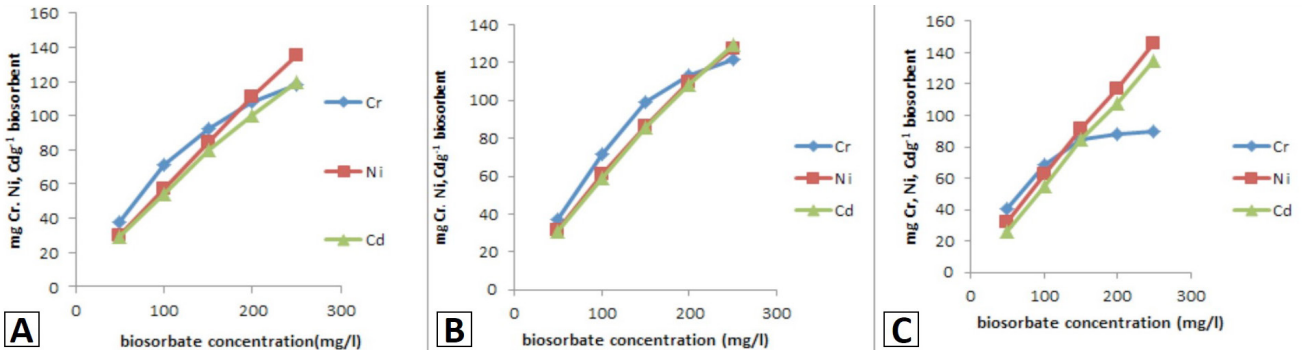

Fig. 4. Bioaccumulation capacities shown by *B. cereus* strains KA18 (A), KA24 (B) and KA25 (C) (incubation time 24 h).

Table I.- Isotherm model constants for adsorption of metal ions on to *B. cereus* strains.

Strain	Metal ion	Freundlich			Langmuir		
		K_F	n	R^2	B	Q^o	R^2
KA18	Cr(VI)	14.62	2.45	0.995	2.24	192.30	0.956
	Ni(II)	2.16	1.15	0.992	1.01	769.23	0.943
	Cd(II)	1.93	1.42	0.995	1.00	416.66	0.981
KA24	Cr(VI)	39.17	8.0	0.992	2.37	181.0	0.990
	Ni(II)	29.71	7.4	0.947	1.22	370.0	0.975
	Cd(II)	16.82	5.8	0.988	1.13	434.0	0.994
KA25	Cr(VI)	12.95	2.5	0.912	3.39	107.5	0.994
	Ni(II)	1.60	1.11	0.999	1.16	769.0	0.940
	Cd(II)	1.67	0.93	0.987	0.93	212.7	0.974

KA18, *B. cereus* BDBC01; KA24, *B. cereus* AVP12; KA25, *B. cereus* NC7401.

increased with increase in initial metal ions concentration (50-250 mgL^{-1}) which may be due to electrostatic interactions of these cationic species with the negative charged density on bacterial cell surface with added possibility of their bridging between negatively charged functional groups of biomolecules like; nucleic acids, proteins, carbohydrates or lipids of bacterial cell wall. For Cr(VI) all three strains KA18, KA24 and KA25 showed maximum bioaccumulation capacity (92.25, 99 and 84.37 mg/g, respectively) at150 mgL^{-1} and reached at saturation value at 200 mgL^{-1}. This may be explained as, at acidic pH Cr(VI) exists as different anionic species like CrO^{4-}, $HCrO^{4-}$ and CrO_7^{2-} which might have different affinity for positively charged functional groups and availability of such functional groups on bacterial cell surface. KA18 accumulated 135 and 120 mg/g of Ni(II) and Cd(II) while KA24 and KA25 showed bioaccumulation capacity values of 127.5 and 146.25 mg/g for Ni(II) and 129.37 and 135mg/g for Cd(II), respectively. Isotherm model constants for adsorption of Cr(VI), Ni(II) and Cd(II) on *Bacillus* strains are given in Table I. Where Ceq is equilibrium concentration (mgL^{-1}) and qeq is the amount of metal ions adsorbed by one gram of the dried biomass at equilibrium (mg/g) .Q^o the maximum adsorption capacity is the maximum amount of metal ion adsorbed per unit weight of biomass to form a complete monolayer on bacterial surface bound at high Ceq(mg/L). 'b' is the Langmuir constant which is related to the binding affinity of the binding sites. The values of Q^o and 'b' can be determined from the linear plot of Ceq/qeq versus Ceq. Q^o helps to compare the performance of all the three strains with each other to identify which strain has the highest adsorption capacity. Langmuir parameters also show maximum adsorption capacity (Q^o) observed for Cr(VI) 192.0, 181.0 and 107.5mg/g, Ni(II) 769.23, 370.0 and 769.0 mg/g and Cd(II) 416.0, 434.0 and 212.by KA18, KA24 and KA25, respectively.

CONCLUSION

To conclude, our results revealed that all studied strains illustrated remarkable tolerance against heavy metals and tremendous bioaccumulation capacity. The strains could be potential agents for the bioremediation of heavy metals polluted agricultural, swage and industrial waters. Plants growing on contaminated sites could be excellent ecosystems to isolate bacterial genes involved in metal resistance and/or plant growth promotion.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2017.49.s5.1841.1846

Statement of conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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