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Cadmium Resistance and Accumulation Potential of *Klebsiella pneumoniae* Strain CBL-1 Isolated from Industrial Wastewater

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Abstract.- A bacterium, *Klebsiella pneumoniae* CBL-1, was isolated from heavy metal laden industrial wastewater and could tolerate Cd^{2+} (1500µg/mL), Cr^{6+} (800µg/mL), Cu^{2+} (600µg/mL), Pb^{2+} (700µg/mL), Zn^{2+} (800µg/mL) and Ni^{2+} (1000µg/mL). *K. pneumoniae* showed optimum growth at 30ºC and pH at 7. *K. pneumoniae* could decrease 46%, 68%, 83% and 93% of cadmium (100µg/mL) from the medium after 24, 48, 72 and 96 hours, respectively. *K. pneumoniae* was also capable to remove 54% and 82% cadmium from the industrial effluents after 4 and 8 days of incubation at room temperature. Multiple metal tolerance and high Cd accumulation by *K. pneumoniae* indicates its potential application in biological treatment of wastewaters contaminated with heavy metals.

Key Words: Metal resistant bacterium, bioremediation, Cd^{2+} accumulation, *Klebsiella pneumoniae*

INTRODUCTION

Heavy metal contamination due to natural and anthropogenic sources is a global environmental concern. The most abundant pollutants in the wastewater and in sewage are heavy metals. Human activities, such as mining operations and the discharge of industrial wastes, have resulted in the accumulation of metals in the environment and eventually are accumulated through the food chain, leading to serious ecological and health problems (Cheng, 2003; Chisti, 2004).

Cadmium (Cd) is a trace element, the presence of which in the environment is essentially due to human activities. It is a highly toxic non-biological heavy metal able to enter living cells via transporters usually used for the uptake of essential cations such as calcium, iron, zinc, and so forth (Clemens, 2006). It is extensively used in the industry for a number of applications, including electroplating, protection against corrosion and stabilizing plastic (Lebrun *et al.*, 1994). It is also obtained as a by-product of zinc production (Nies, 1999). Cd has been utilized eight times more during the last 40 years by mankind than in its entire history; the Cd^{2+} input into biosphere is estimated to be about 30,000 tons/year (Nriagu and Pacyna, 1988).

Cd is one of the most dangerous heavy metals both to human health and aquatic ecosystems. Company *et al*. (2004) studied that Cd had an inhibitory effect in the enzymatic defence system in hydrothermal vent mussel *Bathymodiolus azoricus.* Reported harmful effects of Cd on aquatic organisms include severe inhibition of such physiological processes as growth, photosynthesis, and nitrogen fixation (Trevors *et al*., 1986; Ganguly and Jana, 2002; Rangsayatorn *et al.*, 2002). The toxic dose, 10 or 15µg/mL produces great alterations due to high accumulation of Cd in the cell. Mitochondria are found to be degenerating, and alterations are mainly seen in the nucleus, which has an unusual shape (Piccinni and Albergoni, 1996).

Because traditional cleanup processes of heavy metal contaminated soils and waters are expensive and practical only in small areas, researchers have looked for new cost effective technologies than include the use of microorganisms, biomass, and live plants. Microbial metal removal has received much attention in the past years due to the potential use of microorganisms for cleaning metal-polluted water (Shakoori *et al.*, 2004; Rehman *et al*., 2007b; Maldonado *et al*., 2010; Resmi *et al.*, 2010). The present investigation deals with the isolation, growth and resistance to cadmium toxic ions of bacterium, isolated from industrial wastewater of Sheikhupura, Pakistan. Cadmium uptake ability of

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the bacterial isolate was assessed with a view to use it to detoxify industrial wastewaters contaminated with cadmium.

MATERIALS AND METHODS

Sample collection

Wastewater samples were collected in screw capped sterilized bottles from different industrial areas of Sheikhupura (Pakistan). Some physicochemical parameters of wastewater *viz.*, temperature (ºC), pH, dissolved oxygen and cadmium (µg/mL) were measured (APHA, 1989).

Isolation and screening of Cd-resistant bacteria

For isolation of cadmium resistant bacteria, 100µL of the wastewater sample was spread on Luria-Bertani (LB) agar plates containing 100µg of Cd^{2+}/mL of the medium. LB agar plates were prepared by dissolving 1 g NaCl, 1 g tryptone and 0.5 g yeast extract in 100mL distilled water, pH adjusted at 7.2 to 7.5 and then 1.5 g agar was added in the 250mL flasks. The medium was autoclaved at 121ºC and 15 Lb pressure for 15 minutes. The growth of the bacterial colonies was observed after 24 hours of incubation at 30ºC. Effect of Cd^{2+} on the growth of bacterial isolate was determined in acetate minimal medium (Pattanapipitpaisal *et al.*, 2001) which contained (g/L): NH_4Cl, 1.0; $CaCl_2.H_2O$, 0.001; $MgSO_4.7H_2O$, 0.2; $FeSO_4.7H_2O$, 0.001; sodium acetate, 5; yeast extract, 0.5; K_2HPO_4, 0.5 (pH 7) supplemented with Cd^{2+} (100µg/mL). Isolated colonies were picked up with sterilized wire loop and streaked on acetate minimal medium plate containing 100µg Cd^{2+}/mL. It was again incubated at 30ºC for 24 hours. This process was repeated with successively higher concentrations of Cd^{2+} until the minimum inhibitory concentration (MIC) of bacterial isolate was obtained. The MIC is defined as the lowest concentration of Cd^{2+} at which a single colony-derived streak could not grow.

Identification of the bacterial isolate

For further identification, genomic DNA was isolated and the 16S rRNA gene was amplified by PCR using two general bacterial 16S rRNA primers (RS-1; 5′-AAACTC-AAATGAATTGACGG-3′, RS-3; 5′-ACGGGCGGTGTGTAC-3′) (Rehman *et al.*, 2007a). PCR was performed by initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 2 minutes and a final extension at 72°C for 5 minutes. The PCR product of 0.5kb was removed from the gel and cloned in pTZ57R/T vector. The amplified 16S rRNA gene was purified with a Fermentas purification kit and the amplified products were electrophoresed on 1% agarose gel. Sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. FULLERTON, CA, USA. The 16S rRNA gene sequences were compared with known sequences in the GenBank database to identify the most similar sequence alignment.

Determination of optimum growth conditions

For optimum growth of the bacterial isolate, two parameters *i.e.* temperature and pH were considered. For determination of optimum temperature, 5mL LB broth was added in 4 sets, each of three test tubes, autoclaved and inoculated with 20 µL of freshly prepared culture of the isolate. The four sets of tubes were incubated at 25ºC, 30ºC, 37ºC and 42ºC. After an incubation of 12 hours, their absorbance was taken at 600 nm. For determination of optimum pH, test tubes having 5 mL LB broth were prepared in 9 sets, each containing 3 test tubes and their pH was adjusted at 5.0, 6.0, 7.0, 8.0, and 9.0 then autoclaved. These tubes were inoculated with 20 µL freshly prepared culture of the isolate. After incubation period of 12 hours at the optimum temperature, their absorbance was taken at 600 nm.

Effect of cadmium on bacterial growth

Growth curves of bacterial isolate were determined with (100µg Cd^{2+}/mL) and without cadmium (Control). For bacterial isolate 100mL acetate minimal broth was taken in one set consisting of 3 flasks, autoclaved and then inoculated with 20 µL of the freshly prepared inoculum. These cultures were incubated at 30ºC in a shaker at 80-100 rpm. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 0, 4, 8, 12, 16, 20 and 24 hours. Absorbance was taken at 600 nm wavelength.

Growth was plotted graphically.

Resistance to heavy metal ions

The cross heavy metal resistance of bacterial isolate was determined by using stock solutions of 10mg/mL of different metal salts (cadmium chloride, copper sulphate, lead nitrate, nickel chloride, potassium dichromate and zinc sulphate). Metals were added separately in the medium. The cross metal resistance was determined by increasing the concentration of respective metal in a stepwise manner with 100mg/L of metal checked resistance. This procedure was repeated with higher concentration of each metal ion in the medium. Every time, 100 mg/L of each metal ions were added more than that of the previous step, until the MIC of each metal was obtained. Inoculated cultures, containing metal ions, incubated at 30°C for a maximum period of four days.

Cadmium removal by bacterial isolate

The metal processing capability of bacterial isolate was checked by adding Cd^{2+} at a concentration of 100mg/L in the acetate minimal medium to minimize the complexation of the heavy metal ions. The control medium was also run for cadmium containing the same concentration as in treated one *i.e.*, 100mg/L but was without the bacterial isolate. The cultures were incubated at 30°C (pH of the treated culture was maintained at 7 after every 12 hours) for 96 hours and from each medium (control and treated) 5mL culture was taken out under sterilized conditions after 24, 48, 72 and 96 hours, respectively. The cultures were spun down at 3000 rpm for 5 minutes and the supernatants were used for the estimation of Cd by atomic absorption spectrophotometer (Varian, U.S.A) at wavelength 228.8 nm. In the present study metal uptake values were determined from the difference in final metal concentration between control flask without cells and test flask with cells at different time periods. The amount of metal in the supernatants was determined by using standard curve. The percentage decrease in the amount of Cd in the medium was calculated.

Cadmium removal from industrial wastewater

To check the efficacy of bacterial cells to remove cadmium from wastewater a lab-scale experiment was set up. Three plastic containers were taken. In the first container 10 L of tap water was taken along with 1.5 L of bacterial isolate grown to log phase. In the second container 10 L of industrial effluent (temperature, 31.5ºC; pH, 7.5; dissolved oxygen, 0.0153±0.03 g/L and Cd^{2+}, 1.43±0.04mg/L) was taken along with 1.5 L of 16 hours grown bacterial culture. In the third container only 10 L of industrial effluent was taken and 100mg/L of Cd stress was maintained in each container. Experiment was carried out at room temperature (30±2ºC). After 4 and 8 days of incubation samples were taken, centrifugated to separate the cells, and supernatants used to estimate the amount of Cd in wastewater and the quantity removed by the bacterial cells.

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken; their mean and standard error of the mean were calculated.

RESULTS AND DISCUSSION

Physicochemical characteristics of wastewater

Some physicochemical characteristics of industrial wastewater were ascertained, from where cadmium tolerant bacterium was isolated. The temperature of different samples ranged between 24.0ºC to 36.7ºC, pH ranged between 6.8 and 8.7, dissolved oxygen between 0.52±0.03 and 1.46±0.01mg/L and total Cd ranging between 0.76±0.04 and 1.60 ±0.03µg/mL.

Bacterial identification

The partially amplified (500bp) and sequenced 16S rRNA gene from local isolate was blast to check the maximum homology of this gene to confirm the species of this local isolate. The blast query revealed that this gene is 100% homologous to already reported gene of *Klebsiella pneumoniae*. The nucleotide sequences coding for 16S rRNA gene of *K. pneumoniae* have been submitted to the GenBank database under accession number EU924134.

Optimum growth conditions

The most suitable temperature for Cd-resistant bacterial isolate was found to be 30°C and *K. pneumoniae* showed maximum growth at pH 7. The growth curve pattern was studied by growing the organism in the presence of Cd^{2+} (100µg/mL) and comparing with the control culture in which no metal ions were added. Although the growth pattern of the isolate was not significantly different from those of control but the growth rate of the isolate was decreased in the presence of Cd^{2+}. The growth pattern has been shown in Figure 1.

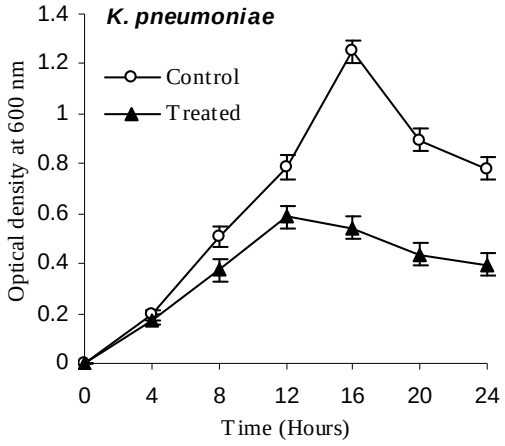

Fig. 1. Growth curves of Cd-resistant *K. pneumoniae* in acetate minimal medium containing (100µg Cd^{2+}/mL) and without cadmium after incubation at 30°C.

Multiple metal resistance

K. pneumoniae was found to be resistant to cadmium at a concentration of 1500µg/mL. The bacterial isolate was also checked for its resistance to various other heavy metals, *viz.*, chromium, copper, lead, zinc, mercury and nickel (Table I). *K. pneumoniae* showed maximum resistance against Ni^{2+} at a concentration of 1000µg/mL and the order of resistance regarding the metal concentration was $Ni^{2+}>Zn^{2+}=Cr^{6+}>Pb^{2+}>Cu^{2+}$. Cadmium tolerance has been studied in bacteria by many workers (Aiking *et al*., 1984; Holmes *et al.*, 1997; Shakoori and Qureshi, 2000; Sinha and Mukherjee, 2008; Hassan *et al.*, 2009). Cadmium-resistance in bacteria is mediated by several genetic systems (Silver and Phung, 1996; Nies, 1999).

Table I.- Cross metal resistance of Cd-resistant bacterial isolate from industrial wastewater against other heavy metals.

	Klebsiella pneumoniae
Cr^{6+} (µg/mL)	800
Cd^{2+} (µg/mL)	1500
Cu^{2+} (µg/mL)	600
Pb^{2+} (µg/mL)	700
Zn^{2+} (µg/mL)	800
Ni^{2+} (µg/mL)	1000

Cadmium uptake ability

Cadmium uptake capability of the bacterial isolate was checked by adding Cd^{2+} at 100µg/mL in the culture medium (Fig. 2). *K. pneumoniae* could decrease 93% of cadmium from the medium after 96 hours. The *K. pneumoniae* was also capable to remove Cd^{2+} (100µg/mL) 46%, 68% and 83% from the medium after 24, 48 and 72 hours, respectively. Pagnanelli *et al*. (2010) reported that Cd removal by bioprecipitation was 23% and Cd removal by biosorption of sulphate reducing bacteria was 77%. Thus a significant amount of Cd has been removed from the medium by the process of biosorption. The maximum adsorption capacity of *Geobacillus thermodenitrificans* for Cd was 42.9 mg/g (Chatterjee *et al*., 2010). Kao *et al.* (2009) reported that removal of Cd ions by *Escherichia coli* cells was up to 82.7% when the initial concentration of Cd was 10mg/L.

Cadmium uptake ability from industrial wastewater

In order to assess the ability of bacterial isolate to decrease Cd^{2+} in contaminated industrial effluents a mini large-scale experiment was done. Industrial wastewaters harbor a variety of microorganisms including bacteria, fungi, algae and ciliates. They were able to remove only 21 and 35% cadmium but in the presence of *K. pneumoniae* were able to remove 44 and 76% cadmium after 4 and 8 days of incubation at room temperature. This 54% greater removal of Cd^{2+} from the wastewater containing *K. pneumoniae* when compared with the original micro-flora present in the industrial effluent after 8 days indicates the ability of *K. pneumoniae* survival and removal of Cd^{2+} from the industrial wastewater. The *K. pneumoniae* was able to uptake 27 and 51mg/L of Cd^{2+} from distilled water after 4

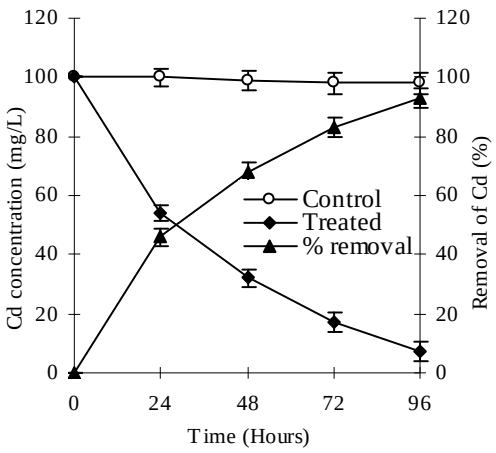

Fig. 2. Cadmium uptake by *K. pneumoniae* from the medium containing (100µg Cd^{2+}/mL). Estimations were done at different time periods.

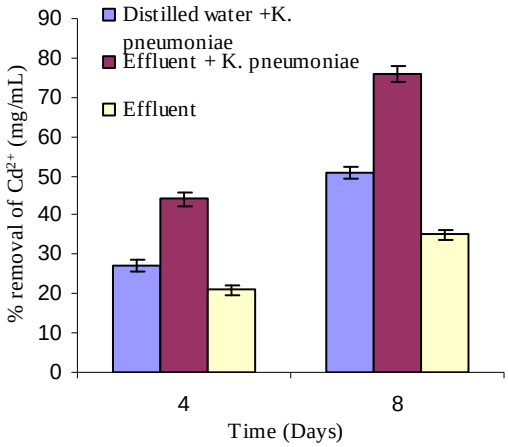

Fig. 3. Percentage removal of Cd^{2+} by *K. pneumoniae* from 10 L aqueous solutions (distilled water and industrial effluent) with initial concentration of 100µg Cd^{2+}/mL after 4 and 8 days of incubation at room temperature.

and 8 days, respectively. The amount of Cd-removal from effluent + *K. pneumoniae* was higher than that from distilled water + *K. pneumoniae* due to the presence of microorganisms in the wastewater. These microorganisms also contribute in removing cadmium ions from the wastewater but these microorganisms were not present in distilled water and the total Cd-removal was done only by *K. pneumoniae* (51mg Cd/L after 8 days) but microorganisms along with *K. pneumoniae* were able to remove 76 mg Cd/L after 8 days from the industrial effluent (Fig. 3).

Hassan *et al*. (2009) reported that *Pseudomonas stutzeri* KCCM 34719 adsorbed 43.5mg/g of Cd from the medium while Lu *et al.* (2006) reported that *Enterobacter* sp. J1 was able to adsorb 46.2mg/g Cd. Maximum specific biosorption of Cd by *Streptomyces* sp F4 was 42.7mg/g dry weight after 7 days of growth (Sineriz *et al*., 2009). In another study, *Pseudomonas aeruginosa* BC15 was capable of absorbing 50% Cd within 48 hours from the medium containing 100 mg of cadmium per liter (Raja *et al*., 2006). Aiking *et al*. (1984) reported that *K. aerogenes* NCTC 418, growing in the presence of cadmium under exhibits two different cadmium detoxifying mechanisms. Besides sulfide formation, increased accumulation of Pi is demonstrated as a novel detoxification mechanism. Intracellular cadmium is always quantitatively counterbalanced by a concerted increase in both inorganic sulfide and Pi contents of the cells. Cd toxicity has been overcome by biotransformation of cadmium ions into photoactive, nanometer-sized CdS particle in *K. pneumoniae* (Holmes *et al*., 1997).

CONCLUSIONS

During the present investigation *K. pneumoniae* showed excellent ability to accumulate cadmium *i.e.,* 93%. The metal resistant *K. pneumoniae* showed high level of cadmium tolerance (1500µg/mL) and decreased substantial amount of Cd^{2+} from the medium and therefore may be employed for the treatment of Cd^{2+} contaminated wastewater.

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