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Evaluation of Cadmium Resistant Bacterium, *Klebsiella pneumoniae*, Isolated from Industrial Wastewater for its Potential Use to Bioremediate Environmental Cadmium

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Abstract.- A bacterium isolated from industrial wastewater was identified as *Klebsiella pneumoniae* on the basis of biochemical tests and 16S rRNA ribotyping. In addition to Cd^{2+} (13.3 mM), *K. pneumoniae* also showed high resistance against other metal ions *i.e.*, Zn^{+2} (17 mM), Pb^{+2} (13 mM), Cu^{+2} (2 mM), Cr^{+6} (2.6 mM), As^{+3} (1.7 mM) and Hg^{+2} (1.7 mM). *K. pneumoniae* showed optimum growth at 37°C and pH 7. Its growth pattern was significantly changed in Cd^{2+} stress. It could remove 40%, 50% and 57.4% Cd^{2+} from the medium after 2, 4 and 6 days of incubation. Change in temperature and pH also showed influence on Cd^{2+} processing ability of *K. pneumoniae.* Its maximum Cd^{2+} removing ability (40%) was determined at 37°C whereas it was least (2%) at 42°C. Similarly at pH 7, *K. pneumoniae* could remove 40.18% Cd^{2+} from the medium but at acidic (5) and basic pH (9), Cd^{2+} processing rate was decreased to 23.18% and 1.88%, respectively. An increase in glutathione and non-protein thiols levels was determined when *K. pneumoniae* was given Cd^{2+} stress (100mg/L). SDS-PAGE analysis showed that some protein bands expressed more and some less in metal treated *K. pneumoniae* as compared to the Cd^{2+}-untreated bacterial culture. To ascertain the metal resistance mechanism, FTIR, EDX and SEM were also performed. Significant Cd^{2+} uptake ability makes *K. pneumoniae* a potential candidate to remove Cd^{2+} from the environment.

Keywords: Cadmium resistant bacteria, *Klebsiella pneumoniae,* glutathione, bioremediation.

INTRODUCTION

Industrialization has raised our living standard very high but it has also destroyed our environment. Tons of industrial wastes are released in our environment every year. This industrial waste may contain very toxic and carcinogenic elements and cadmium is one of them (Fernández *et al.*, 2015). It is non-biodegradable and has no known role in cellular metabolism (Akosy *et al.*, 2014; Perez-chaca *et al*., 2014; Rahoui *et al*., 2014; Xu *et al*., 2014). It is accumulating in our environment as a result of its extensive release from different industrial processes such as mining and smelting of ores, electroplating, manufacturing plastics, color pigments and phosphate fertilizers (Ahmed *et al.*, 2015; Alidoust *et al.*, 2015). United States Environmental Protection Agency (EPA) has set maximum cadmium oral uptake at 0.5µg/Kg/Day in drinking water and 1 µg/Kg/Day in food (Zhai *et al.*, 2015b). In higher concentration it may cause some ill health effects in human including skeletal and cardiovascular dysfunctions, lungs, liver and kidney damage and reproductive problems (Ahmed *et al.*, 2015; Zhai *et al.*, 2015a). No treatment for cadmium toxicity has been approved so far (Zhai *et al.*, 2015b). There is an urgent need to protect and conserve our environment by reducing heavy metal pollution or else it would be soon out of our control to protect environment.

Several studies have reported the use of microorganisms to eliminate heavy metals from the environment as less expensive, cost effective and environmental friendly strategy (Yan and Viraraghavan, 2003; Feng and Aldrich, 2004; Vijayaraghavan and Yun, 2008; He *et al.*, 2011; Huang *et al.*, 2014). Some bacteria actively uptake heavy metal ions including Cd^{2+} along with essential metal ions, thus help removing heavy metal ions from aqueous environment (Rehman and Anjum,

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2009). The accumulated heavy metal ions are potent source of reactive oxygen species (ROS) production and thus interfere in bacterial metabolism by damaging DNA, RNA and proteins (Schirawski *et al.*, 2002; Teitzel *et al.*, 2006; Gibbons *et al.*, 2011; Sabdono, 2011). Bacteria have developed different defensive mechanisms to cope with the negative effects caused by heavy metal ions (Zahid *et al.*, 2012) including intracellular sequestration of metallothionein and other thiol containing compounds (Saluja and Sharma, 2014).

Glutathione is the most abundant low molecular weight thiol containing compound found in bacteria, plants and animals. It is a tripeptide consisting of three amino acids namely cysteine, glycine and glutamate. It is reported as free radical scavenger as it has high affinity for metal ions due to its cysteine residues. Thus it helps to protect the cell from the negative effects caused by heavy metal ions including Cd^{2+} (Meister and Anderson, 1983; Rehman and Anjum, 2011). It occurs in reduced (GSH) as well as in oxidized form (GSSG), but its reduced form is more important when cell combating with oxidative stress caused by heavy metal ions. GSH, as a strong reducing agent, reduces the H_2O_2 to H_2O and gets itself oxidized to GSSG (Penninckx, 2002; Galano and Alvarez-Idaboy, 2011).

Metallothionein (MT) constitutes another intracellular defense system against metal stress. It is low molecular weight (6-7 kDa), thiol-containing, cysteine rich (20-30%), metal binding protein, induced by cadmium and other heavy metal ions and is involved in the sequestration of metal ions (Klaassen *et al.*, 2009; Chaturvedi *et al.*, 2012). Thiol groups of cysteine residues bind maximally seven cadmium ions per molecule of MT resulting in metal-thiolate complexes thus preventing interference of cadmium in cellular metabolism (Carpene *et al.*, 2007; Adam *et al.*, 2014). The bacterial MT locus is designated as smt locus which consists of *smtA* and *smtB* genes (Naz *et al.*, 2005).

This study was aimed at isolation and characterization of cadmium resistant bacterium from industrial wastewater, determining cadmium stress on bacterial growth and cell physiology (change in glutathione and non-protein thiol levels) and resistance to multiple heavy metal ions. To the metal resistance mechanism, some other experiments including amplification of *smtAB* genes, FTIR, EDX and SEM were also performed. A lab scale experiment was also designed to assess the cadmium processing ability of the bacterial isolate with a view to use it in removing cadmium from the environment.

MATERIALS AND METHODS

Sample collection

Industrial wastewater samples were collected in sterilized screw capped bottles from different industrial areas of Sheikhupura and Kot lakhpat, Lahore. Some physicochemical parameters of wastewater samples *viz.*, temperature (°C), pH and cadmium ion concentration (mg/L) were measured.

Isolation and purification of cadmium resistant bacteria

To isolate cadmium resistant bacteria, 100 µL of wastewater sample was spread on Luria-Bertani (LB) agar (1% NaCl, 1% tryptone, 0.5% yeast extract and 1.5% agar) plates supplemented with 1 mM Cd^{2+}. Bacterial growth was observed after incubation at 37°C for 24 h. Single colony was picked with sterilized wire loop and re-streaked on Cd^{2+} supplemented LB agar plates and again incubated at 37°C for 24 h. The process was repeated until the purified single colony obtained.

Determination of minimum inhibitory concentration (MIC)

The bacterial isolate was grown in minimal salt (MS) broth (1% $(NH_4)_2SO_4$, 0.086% $CaCl_2$, 0.15% K_2HPO_4, 0.1% KH_2PO_4, 0.1% $MgSO_4$, 0.02% $FeSO_4$ and 1% glucose) supplemented with 1 mM Cd^{2+} at 37°C for 24 h. Growth was observed by taking absorbance at 600nm. The process was repeated with high concentrations of Cd^{2+} until the growth of the isolate was inhibited. The minimum Cd^{2+} concentration at which bacterial isolate did not show growth was considered as its MIC.

Determination of optimum growth conditions

Optimum growth conditions of bacterial isolate were determined with respect to temperature and pH. To determine optimum temperature, isolate

was grown in LB broth at different incubating temperatures *viz.*, 20°C, 30°C, 37°C, 45°C and 55°C. After 24 h incubation, their absorbance was measured at 600 nm using spectrophotometer. To determine optimum pH, 250 mL flasks having 100 mL LB broth were prepared in 6 sets, each set containing 3 flasks, their pH were adjusted at 5, 6, 7, 8, 9 and 10 and autoclaved. These flasks were inoculated with 1% freshly prepared culture of the isolate. After 24 h incubation at optimum temperature, absorbance was taken at 600nm.

Effect of cadmium on bacterial growth

Effect of Cd^{2+} on the growth of bacterial strain was determined by growing in the presence (1mM Cd^{2+}) as well in the absence (control) of Cd^{2+}. MS broth (100 mL) was taken in a set of three 250 mL flasks, autoclaved, supplemented with 1 mM Cd^{2+}, inoculated with 1% of freshly prepared inoculum and incubated at 37°C in shaking incubator at 100 rpm. In one flask no Cd^{2+} was added which worked as a positive control. Growth in each culture was observed every 4 h by taking absorbance of an aliquot (1mL) at 600nm. Growth curves were plotted between time and absorbance.

Resistance to heavy metal ions

Resistance of bacterial isolate against heavy metal ions (zinc, lead, copper, chromium, arsenic and mercury) was determined by growing it in MS broth supplemented with respective metal ions. Stock solutions of 1 M concentration of heavy metal ions salts (zinc sulfate, lead nitrate, copper sulfate, potassium dichromate, sodium arsenate and mercuric chloride) were used. The concentration of metal ions was increased, 100 mg/L every time after 24 h of incubation, in a stepwise manner until the growth of the isolate was inhibited. The bacterial growth was determined by taking optical density (O.D) at 600nm after 24 h of incubation at 37°C.

Physical, biochemical and molecular characterization

Bacterial isolate was identified on the basis of colony morphology and different biochemical tests such as Gram staining, catalase test, oxidase test, citrate utilization, fermentation of carbohydrates, H_2S production, nitrate reduction, indole and urease test. For molecular characterization 16SrRNA gene was amplified through polymerase chain reaction (PCR) by using general primers (RS-1; 5′-AAACTCAAATGAATTGACGG-3′, RS-3; 5′-ACGGGCGGTGTGTAC-3′) (Rehman *et al.*, 2007). Amplification of 16S rRNA gene was carried out by 35 thermal cycles each of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min followed by final extension at 72°C for 5 min. The PCR product (approximately 0.5kb) was purified through Fermentas Gene Jet Gel Extraction kit (#K0691) and sequenced from Macrogen, Korea. The sequence obtained was compared with known sequences in NCBI database using NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST) to identify the bacterial isolate. The accession number was obtained by submitting the sequence in the GenBank.

Cadmium uptake ability of bacterial isolate

To ascertain cadmium uptake ability, the bacterial isolate was grown in 100 mL LB broth supplemented with Cd^{2+} in the concentration of 100 mg/L in a set of three 250 mL flasks. In one flask Cd^{2+} was added in the same concentration but not inoculated with the organism, which worked as a control. Then 5 mL samples were taken out from each flask after 2, 4 and 6 days. Bacterial cells were harvested through centrifugation at 3,000 rpm for 5 min. The pellets [acid digested, H_2SO_4:HNO_3 (1:1)] and supernatants were used for the estimation of Cd^{2+} by atomic absorption spectrophotometer at a wavelength of 228.8 nm. Each pellet was divided into two portions by weighing (Electronic balance AUX220: Shimadzu Corporation, Japan), one portion was washed thrice with 0.5 M EDTA to collect Cd^{2+} adsorbed on the cell surface and second portion was acid digested to release absorbed Cd^{2+}. Total uptake values were calculated by substracting the metal concentration in treated flasks from the metal concentration in control flask. Standard curve was prepared to calculate amount of metal ions in pellets and supernatants. Percentage increase and decrease in the amount of metal ions were also calculated.

Effect of temperature and pH on Cd^{2+} uptake

To measure the effect of temperature and pH on Cd^{2+} uptake ability of the bacterial isolate, it was

grown in LB broth supplemented with 100 mg/mL Cd^{2+} at different incubating temperatures (25°C, 30°C, 37°C and 42°C) and pH (5,6,7,8 and 9). For each treatment three flasks were used. After 48 h of incubation, samples were collected and centrifuged at 3,000 rpm for 5 min. Pellets and supernatants were used to estimate the quantity of Cd^{2+} by tomic absorption spectrophotometer at 228.8nm.

Estimation of glutathione and other non-protein thiol contents

GSH, GSSG and total thiol contents were estimated according to Shamim and Rehman (2013). Briefly, bacterial isolate was grown in 100 mL of LB broth medium in a set of three 250 mL flasks. After 24 h of incubation at optimum temperature Cd^{2+} (100 mg/mL) was added in two flasks, whereas third flask worked as a control. All the flasks were again incubated for 24 h at optimum temperature. Bacterial pellets were collected through centrifugation of cultures at 6,000 rpm for 10 min. Each pellet was weighed, resuspended in 1 mL of 5% sulphosalicylic acid and sonicated two to three times for 15 sec with 60 sec interval. Tubes were then centrifuged at 14,000 rpm for 10 min and the supernatants were divided into two equal portions. One portion was used for the estimation of glutathione and other portion was used to determine non-protein thiols. In a micro-centrifuge tube 0.5 mL aliquot was taken and mixed with equal volume of reaction buffer (0.1 M phosphate buffer, pH 7.0, 0.5 mM EDTA) and 50 µL of 3 mM 5′-dithio-bis-2-nitrobenzoic acid. After 5 min of incubation at room temperature, absorbance was taken at 412nm for the estimation of GSH. In the same tube 100 µL of 0.4 mM NADPH and 2 µL glutathione reductase was added. After 20 min of incubation optical density was taken at 412nm for the determination of total glutathione (GSH+GSSG). The quantity of GSSG was calculated by subtracting the GSH from total glutathione concentration. A standard curve was prepared from different concentrations of reduced glutathione.

For the estimation of non-protein thiols, 100 µL aliquot was mixed with 0.5 mL of reaction buffer (0.1 M phosphate buffer, pH 7.0, 0.5 mM EDTA) and 0.5 mL of 1mM 5′-dithio-bis-2-nitrobenzoic acid. After 10 min of incubation absorbance was taken at 412nm. Non-protein thiol contents were calculated from standard curve prepared from varying concentrations of cysteine.

Protein extraction and SDS-PAGE

Bacterial isolate was grown in LB broth in the presence and absence of Cd^{2+}. Bacterial cells were harvested through centrifugation at 6,000 rpm for 10 min. Pellet was washed with deionized distilled water and resuspended in 150 µL of lysis buffer (1% SDS, 0.1% mercaptoethanol and 0.1% DTT). The cells were sonicated (Heilscher Ultrasonic Processors UP 400, S) 2-3 times at 4°C for 10 sec with 60 sec intervals, mixed with gel loading dye and incubated at 95°C in water bath (WB 10 Memmert GmbH Co KG Germany) for 10 min. Lysates were centrifuged at 14,000 rpm for 10 min to remove cell debris. The supernatant was collected carefully and used for SDS-PAGE. Proteins were resolved by SDS-PAGE on 12% acrylamide gel. Molecular weight standards were also run. Gels were stained with Coomassie Brilliant Blue and photographed after destaining.

Amplification of smtAB

An internal fragment of 480 bp of known *smtAB* gene was amplified from both plasmid and genomic DNA through PCR using known primers (smtAB-F; 5′- GAT CGACGTTGCAGA GACAG-3′, smtAB-R; 5′- GATCGAGGGCGTTTTGATAA-3′) reported by Naz *et al.* (2005). The reaction mixture (20 µL) contained 0.2 mM dNTPs, 20 pmole each primer, 10 ng DNA, 0.25 U taq polymerase, 50 mM KCl buffer and 1.5 mM $MgCl_2$. Amplification was carried out by 35 thermal cycles each of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 2 min followed by final extension at 72°C for 5 min.

FTIR, EDX and SEM analysis

Infrared spectra of the *K. pneumoniae* with and without Cd^{2+} treatment were recorded on a FTIR spectrometer (Bruker, alpha) in the region 4000 to 400 cm^{-1}. A drop of bacterial suspension (both untreated and treated with 1mM Cd^{2+}) was mounted and dried on aluminum stub and furthermore coated with gold for better contrast. Then these drops were analyzed by scanning

electron microscope (Nova NanoSEM 450) equipped with Oxford Energy Dispersive X-ray (EDX) microanalysis system.

RESULTS

Physicochemical characteristics of wastewater

Some physicochemical parameters of wastewater were measured at the time of sampling. Temperature of different samples ranged between 25°C to 40°C, pH was between 6.2 to 9.0, dissolved oxygen ranged between 0.69±0.01 and 1.39±0.01 mg/L and Cd^{2+} ranged between 0.42±0.01 and 1.39±0.01 mg/L.

Cd^{2+} resistant bacteria

The bacterium which showed highest resistance against Cd^{2+} (13.3mM) was selected for further studies and dubbed as C2. It was also found to be resistant against other heavy metal ions, *viz*., Zn^{+2} (17mM), Pb^{+2} (13mM), Cu^{+2} (2mM), Cr^{+6} (2.6mM), As^{+3} (4.4mM) and Hg^{+2} (1.7mM). The order of resistance regarding metal ions concentration was $Zn^{+2} > Pb^{+2} > As^{+3} > Cr^{+6} > Cu^{+2} > Hg^{+2}$.

Identification and growth characteristics of bacterial isolate

Bacterial isolate was identified on the basis of morphological, biochemical characteristics and 16S rRNA ribotyping. Morphologically the bacterial colonies were found to be irregular in shape, glistening surface, creamy colour, raised elevation and opaque in nature. The Gram negative bacterium showed positive results for catalase, citrate, lactose fermentation, nitrate and urease test but was negative for oxidase, H_2S production and indole test. The partial sequence of 16S rRNA gene showed 99% homology with 16S rRNA sequence of *Klebsiella pneumoniae* already submitted to NCBI database. Then the sequence was submitted to Genbank under the accession number JQ423115.

The most suitable temperature for *K. pneumoniae* was found to be 37°C and it showed maximum growth at pH 7. The growth of bacterium was significantly decreased in the presence of Cd^{2+} (Fig. 1).

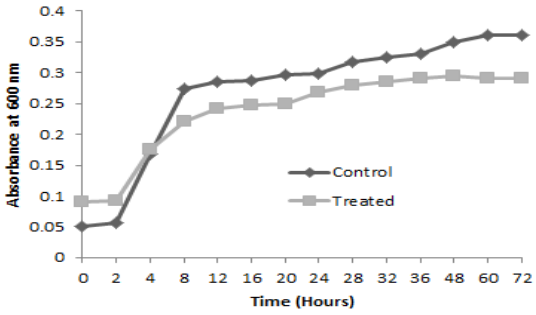

Fig. 1. Growth curves of *K. pneumoniae* in MS culturing medium with (1 mM Cd^{2+}) and without Cd^{2+}.

Biosorption of Cd^{2+}

Biosorption capability of *K. pneumoniae* was assessed by growing it in culture medium supplemented with 100 mg/mL of Cd^{2+} (Fig. 2). *K. pneumoniae* removed 40%, 50% and 57.4% from the medium after 2, 4 and 6 days, respectively. The uptake of Cd^{2+} by *K. pneumoniae* after 2, 4 and 6 days was 33.5%, 36.8% and 38.9%, respectively. The bacterium was able to adsorb 6.5%, 13.2% and 18.5% of Cd^{2+} on its surface after 2, 4 and 6 days, respectively (Fig. 2).

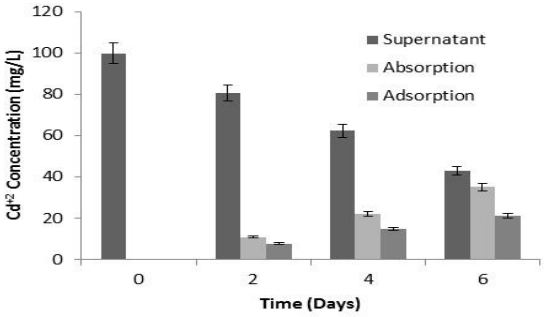

Fig. 2. Biosorption capability of *K. pneumoniae* growing in culture medium supplemented with 100 mg/mL of Cd^{2+}.

Effect of temperature and pH on Cd^{2+} uptake ability

Effect of temperature and pH on Cd^{2+} uptake ability of *K. pneumoniae* was determined by changing temperature and pH of the culturing medium. *K. pneumoniae* removed 26.2% and 34.2% Cd^{2+} from the culturing medium at 25°C and 30°C, respectively. The maximum Cd^{2+} removing ability (40%) of *K. pneumoniae* could uptake 40% Cd^{2+} at

37°C whereas at 42°C it was able to remove only 2% Cd^{2+} (Fig. 3A). *K. pneumoniae* removed 23.81% Cd^{2+} at pH 5. It removed 35.79%, 40.18%, 10% and 1.88% Cd^{2+} at pH 6, 7, 8 and 9, respectively (Fig. 3B).

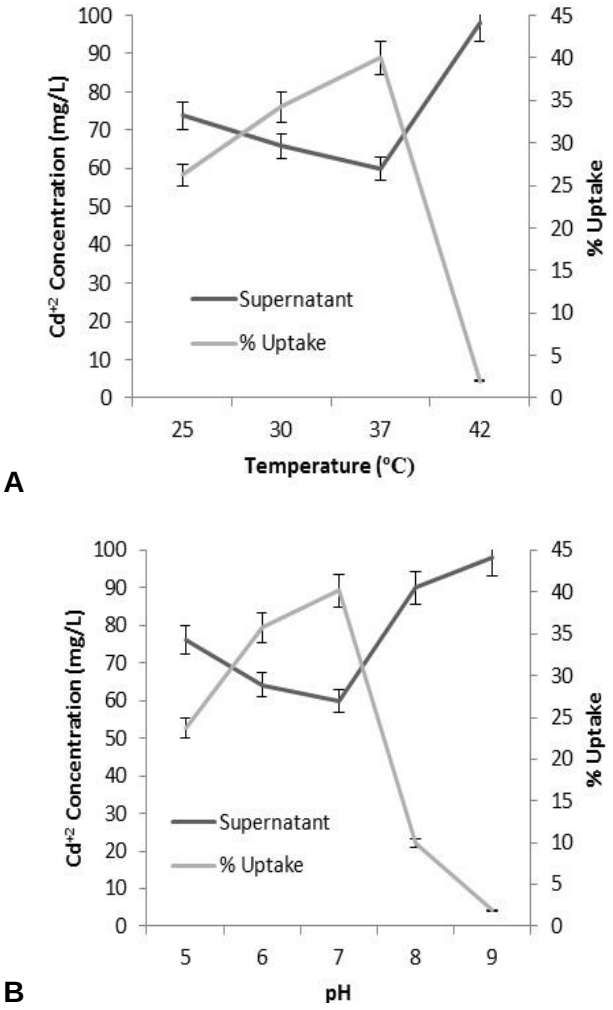

Fig. 3. Effect of temperature (A) and pH (B) on Cd^{2+} uptake ability of *K. pneumonia.*

Cadmium induced levels of glutathione and non-protein thiols

The levels of GSH and GSSG were altered in the presence of Cd^{2+} (100 mg/L). There was 94% and 175% increase in GSH level and GSH/GSSG ratio, respectively. Whereas 41.37% increase in non-protein thiols level was recorded in bacterial cell in the presence of Cd^{2+} (Table II).

Table II.- Levels of reduced (GSH) and oxidized glutathione (GSSG), total glutathione, reduced and oxidized glutathione ratio, and non-protein thiols in *K. pneumoniae* exposed to 100mg/L of Cd^{2+}.

	Cd^{2+} Concentration (µg/mL)	
	0	100
GSH (mM g^{-1} FW)	9.4±0.17[a]	18.33±1.14
GSSG (mM g^{-1} FW)	10.16±0.06	6.29±0.21
GSH+GSSG (mM g^{-1} FW)	19.56±0.06	24.62±0.8
GSH/GSSG	0.92±0.08	2.91±0.17
Non-protein thiols	2.9±0.9	4.1±0.6

[a]±SE (n=3)

smtAB *genes of bacterial isolate*

The amplification of *smtAB* genes was visualized with genomic DNA but no amplification was detected when plasmid DNA was used as a template (Fig. 4).

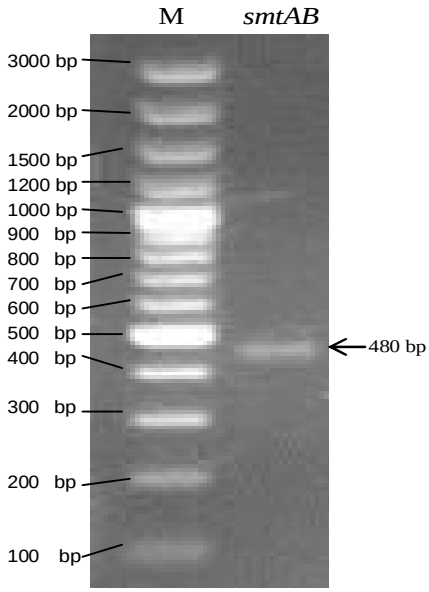

Fig. 4. Amplification of *smtAB* gene through PCR. M represents DNA ladder.

Cd induced proteins

Total protein profiles of *K. pneumoniae* in the absence and presence of Cd^{2+} were compared by polyacrylamide gel electrophoresis (Fig. 5). This comparison clearly indicated the induction as well as suppression of some proteins due to Cd^{2+} stress. A band of 36 kDa disappeared whereas a band of 40 kDa was found to be more prominent in the presence of Cd^{2+}.

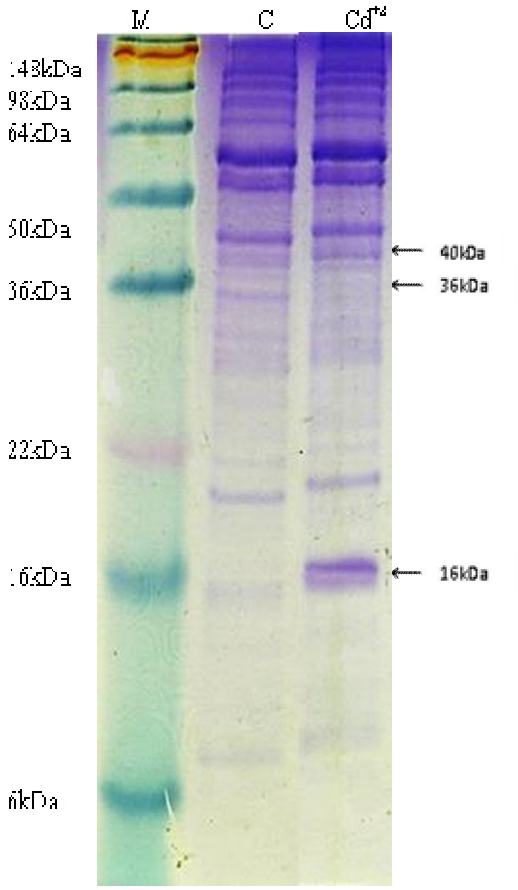

Fig. 5. Total protein profile of *K. pneumoniae* in the absence and presence (100 mg/L) of Cd^{2+}. M: protein marker; C, control (without Cd^{2+}); Cd^{2+}, with Cd^{2+}.

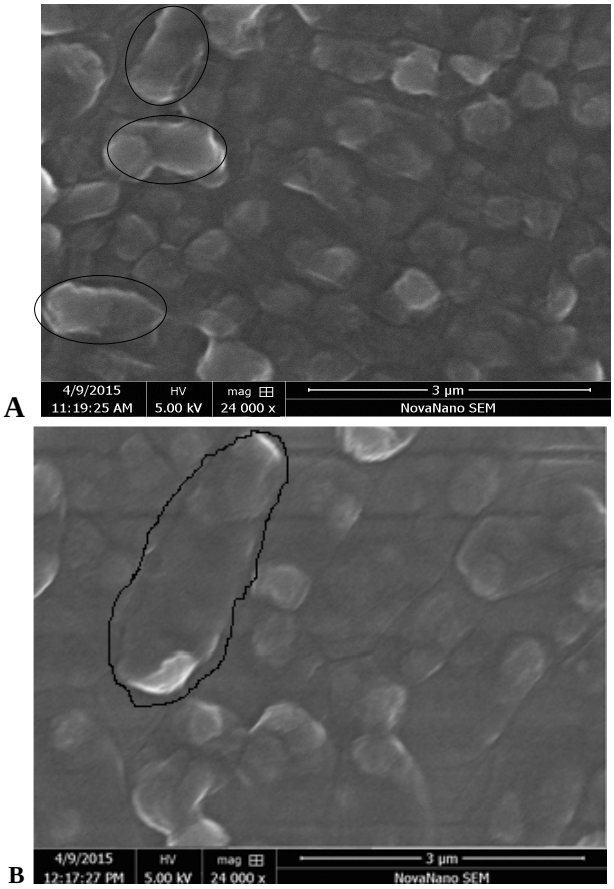

Fig. 6. Images of scanning electron microscopy of *K. pneumoniae* in the absence (A) and presence (B) of Cd^{2+} (100 mg/L).

SEM, FTIR and EDX

The size of bacterium increased 2.2 folds due to accumulation of cadmium, which is evident from the difference in size shown in SEM images (Fig.6). The accumulation of cadmium was confirmed by the Energy Dispersive X-ray (EDX) analysis (point and ID scan) of SEM images (Fig. 7). The Fourier Transform Infrared spectroscopy (FTIR) analysis confirmed the presence of carboxyl, amino and phosphate moieties in bacterium and this analysis was also used to observe the binding of Cd^{2+} with bacterium. It is clear that the peaks attributed to amide linkage, appearing at 1634 and 1538 cm^{-1} (control) are shifted to 1655 and 1543 cm^{-1} respectively, in the presence of Cd^{2+} (Fig.8).

DISCUSSION

Industrialization is responsible for increasing heavy metal pollution in our environment. Cadmium is the most toxic heavy metal which has been reported to cause oxidative damage and alteration in glutathione and non-protein thiol levels in cell (Dorta *et al.*, 2003). The main objective of this study was to screen and characterize Cd^{2+}-resistant bacterium from the environment which has potential to decrease Cd^{2+} pollution. In the present study a bacterium, *K. pneumoniae*, was isolated from the environmental samples could resist Cd^{2+} upto 13.3 mM. Many researchers have reported that a strain can be declared as Cd^{2+} resistant if its MIC exceeds 100 mg/L (Akinbowale *et al.*, 2007) or 112.4 mg/L of Cd^{2+} (Abou-Shanab *et al.*, 2007).

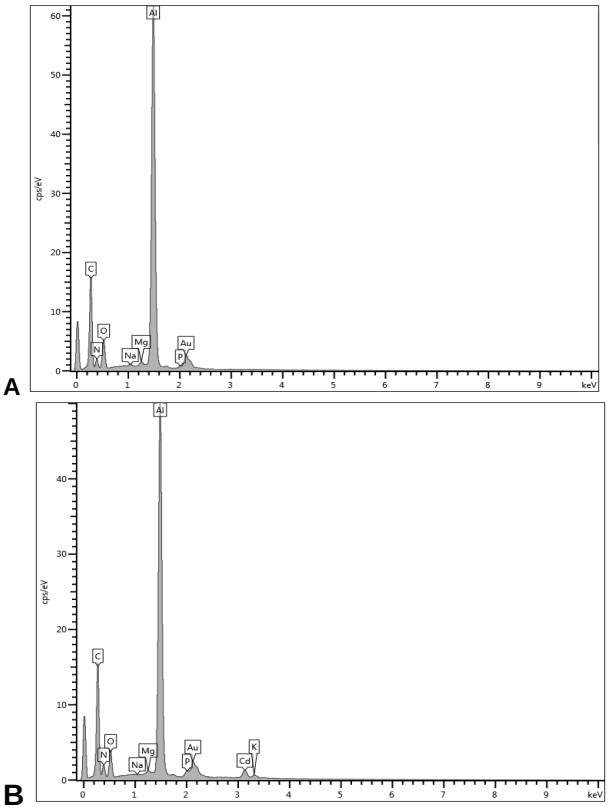

Fig. 7. Energy dispersive X-ray spectroscopy through SEM of *K. pneumoniae* (A) control and (B) Cd^{2+}-treated.

Resistance in bacteria against Cd^{2+} has reported in several investigations (Sinha and Mukherjee, 2008; Hassan *et al.*, 2009). Many researchers have reported the use of microorganisms (bacteria, yeasts and algae) as a potent source to decrease heavy metal pollution (Yan and Viraraghavan, 2003; Feng and Aldrich, 2004; Vijayaraghavan and Yun, 2008).

Bacteria have great potential to remove Cd^{2+} contaminants from the environment either through metabolism independent extracellular adsorption or metabolism dependent intracellular accumulation of Cd^{2+}, thus reducing environmental heavy metal pollution (Kuroda and Ueda, 2003; Zouboulis *et al.*, 2004; Vargas-García *et al.*, 2012; Huang *et al.*, 2013). Present study investigated the adsorption and intracellular accumulation of Cd^{2+} by *K. pneumoniae* after different time intervals. *K. pneumoniae* could adsorb 6.5%, 13.8% and 18.5%

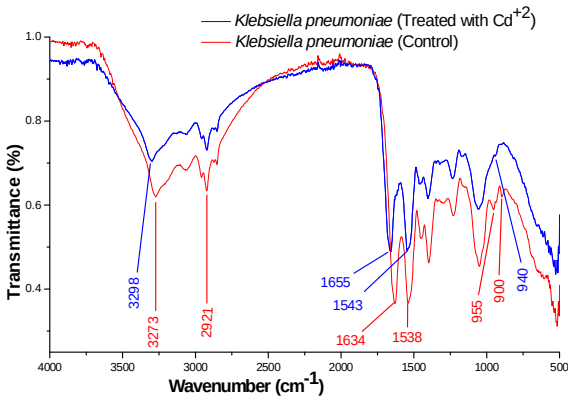

Fig. 8. FTIR of *K. pneumoniae* in the absence and presence of Cd^{2+}.

Cd^{2+} after 2, 4, and 6 days, respectively. According to Hassan *et al.* (2009) *Pseudomonas stutzeri* could remove 43.5 mg/g Cd^{2+} by adsorption while Lu *et al.* (2006) reported 46.2 mg/g adsorption of Cd^{2+} by *Eterobacter* sp. The functional groups like carboxyl (COOH-), hydroxyl (-OH), amine ($-NH_2$) and phosphate ($-PO_4$) are present on bacterial surface and help adsorb Cd^{2+} (Das *et al.*, 2007).

The adsorption of Cd^{2+} on bacterial surface is greatly influenced by change in pH and temperature (Chakravarty and Banerjee, 2012). At low pH, negatively charged sites on bacterial surface become positive and repel Cd^{2+} and at high pH (above pH 7), Cd^{2+} exist either in the form of $[Cd(OH)_3]^-$ or $[Cd(OH)_4]^-$ and thus repelled by the negatively charged bacterial surface (Panda *et al.*, 2006), thus lowering the adsorption capability of bacteria. At neutral pH, adsorption of Cd^{2+} is maximum due to the presence of free negatively charged functional groups. Present study clearly revealed the decrease in adsorption and overall metal removing capability of *K. pneumoniae* at low pH. At pH 5 and 6, *K. pneumoniae* removed 23.18% and 35.7% Cd^{2+}, respectively while at pH 7 it showed maximum removal *i.e.*, 40.18%. Chakravarty and Banerjee (2012) also reported decreased Cd^{2+} adsorption at low pH in acidophilic bacteria.

High temperature increases the kinetic energy of reacting molecules as well as vibrational energy of constituent elements of bacterial envelop. It breaks the bonds between Cd^{2+} and functional groups on bacterial surface thus decreasing

adsorption and metal bioaccumulation in bacteria. In this study an experiment was carried out to observe the effect of temperature on the biosorption capability of *K. pneumoniae* which indicated that high temperature decreases the biosorption rate of Cd^{2+} as described by Özdemir *et al.* (2009). Cd^{2+} removal by *K. pneumoniae* was 26% at 25ºC which increased up to 40% at 37ºC. Whereas at 42ºC only 2% removal was observed. *K. pneumoniae* showed 33.5%, 36.2% and 38.9% intracellular accumulation after 2, 4 and 6 days, respectively. An increase in the size of *K. pneumoniae* was observed due to Cd^{2+} accumulation under scanning electron microscope as described by Chakravarty and Banerjee (2008).

In the present study proteins banding pattern in *K. pneumoniae* was analyzed by SDS-PAGE in the absence and presence of Cd^{2+}. A 36 kDa protein band was disappeared whereas 40 kDa and 16 kDa bands were over-expressed due to Cd^{2+} presence. Durve *et al.* (2013) also reported disappearance and over-expression of some protein bands in the presence of heavy metal ions. The size of bacterium (*K. pneumoniae*) was increased many folds due to Cd^{2+}-accumulation. This marked difference in size is due to internalization of metal ions in Cd^{2+} treated *K. pneumoniae* culture as compared to culture grown without the metal ions.

Energy Dispersive X-ray analysis also confirmed the accumulation of Cd^{2+}. A cadmium peak was detected when *K. pneumoniae* was grown in the presence of Cd^{2+}. The FTIR analysis confirmed the presence of carboxyl, amino and phosphate moieties in bacterium and Figure 8 clearly indicates the peaks attributed to amide linkage, appearing at 1634 and 1538cm^{-1} (control) are shifted to 1655 and 1543 cm^{-1} respectively, in the presence of Cd^{2+}. This suggests that cadmium mainly adsorbed on to nitrogen atom of amide group. The stretching and broadening of peaks appearing in range of 1000-1320 cm^{-1}, represents the presence of C=O and indicates the metal to C=O interaction. The shifting of characteristic peak of phosphate group, from 955 to 943 cm^{-1}, indicates its chemical link with cadmium on the surface of bacteria. Addition to this, the percentage transmittance of peaks in bacterium treated with cadmium is considerably lesser than those of control bacterium. This shows that stretching of bonds occurs to lesser degree due to presence of metal atoms and results in reduce transmittance. These results are comparable to the results reported byChakravarty and Banerjee (2012).

CONCLUSIONS

In the present investigation *Klebsiella pneumoniae*, isolated from industrial wastewater, was able to resist Cd^{2+} (13.3 mM). *K. pneumoniae* showed optimum growth at 37°C and pH 7. The bacterium was able to remove 40%, 50% and 57.4% Cd^{2+} from the medium after 2, 4 and 6 days. Cd^{2+} processing ability of *K. pneumoniae* was also influenced by the change in temperature and pH. The maximum Cd^{2+} removing ability (40%) was observed at 37°C whereas minimum was (2%) at 42°C. Likewise at pH 7, *K. pneumoniae* could remove 40.18% Cd^{2+} from the medium but at acidic (5) and basic pH (9), metal removing rate was decreased to 23.18% and 1.88%, respectively. Glutathione and non-protein thiols levels were increased when *K. pneumoniae* was given Cd^{2+} stress (100mg/L). FTIR, EDX and SEM all showed that when the bacterium was grown in the medium containing metal, the metal has been accumulated by the bacterial cells. Significant Cd^{2+} resistance and uptake capability of *K. pneumoniae* makes it a potential candidate to detoxicate the environment.

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Conflict of interest

The authors have declared that no competing interests exist.

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