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Isolation, Identification and Cadmium Processing of *Pseudomonas aeruginosa* (EP-Cd1) Isolated from Soil Contaminated with Electroplating Industrial Wastewater

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Authors' Contribution
ARS conceived and designed the study. BM executed the experimental work and wrote the article. MJI designed the Primers and performed nucleotide sequencing. FRS analyzed the data.

Key words
Cadmium, czcA gene, *Pseudomonas aeruginosa*, Bioremediation

ABSTRACT

Cadmium resistant bacterial isolate EP-Cd1 on the base of 16SrRNA analysis identified as *Pseudomonas aeruginosa*. It showed high cadmium resistance in solid as well as in liquid media. It showed optimum growth at pH 6 and 35°C. Cadmium adversely affect the growth pattern of *P. aeruginosa*. Cadmium adsorption/efflux pumping gene *czc*A was detect in the isolated strain. SDS-PAGE analysis of EP-Cd1 revealed that cadmium induced some high molecular as well as low molecular proteins. It removed 80% of cadmium from medium as well as have capability to remove 95% of cadmium from the industrial wastewater after 72 h. of incubation. EP-Cd1 can be used as best candidate for bioremediation

INTRODUCTION

Heavy metals fall into three categories *viz.*, nontoxic essential (Mg^{2+} and Ca^{2+}); essential but toxic at high concentration (Fe^{2+}, Cu^{2+}, Mn^{2+}, Mo^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}), and toxic (As^{2+}, Pb^{2+}, Hg^{2+} and Cd^{2+}) (Valls and De Lorenzo, 2002). Cadmium is non-essential and is one of the most toxic heavy metals which entered into the environment from metal plating, smelting, cadmium-nickel containing batteries, phosphate fertilizers, industrial sludge, electroplating and mining industry. Due to the non-degradable nature it persist in environment and has high tendency to accumulate in living tissues. Cadmium exists in oxidation 2+ form in different salts like cadmium chloride, oxide and sulphide or sulphate (Valko *et al.*, 2005). Cadmium causes toxicity by mimicking other metals of 2+ oxidation state like calcium and zinc in biological systems.

Cadmium has severe consequences on human health and has ability to bind with respiratory enzymes and can create the oxidative stress. In human the high concentration of cadmium can induce different chronic diseases like hepatic, reproductive, cardiovascular edema, and is a potent human carcinogen (Zaki and Farag, 2010; Hong *et al.*, 2004; Koyu *et al.*, 2006; Tellez-Plaza *et al.*, 2008).

Physiochemical methods such as metal precipitation, flocculation, activated adsorption etc are expensive methods and at low concentration of metal become inefficient. So cost effective methods like bioremediation or biosorptions were adopted to remove the toxic metals even present in traces (He *et al.*, 2011). Microorganisms specifically bacteria have different mechanisms like efflux pumps, enzymatic detoxification, metal ion sequestration, metal accumulation and metal binding proteins to cope with metals present in polluted environment (Zhou *et al.*, 2013; Ozer *et al.*, 2013; Aktan *et al.*, 2013).

Pseudomonas is one of the most important bacteria present in almost all contaminated sites. Different species of *Pseudomonas* have ability to degrade pollutants like insecticides hydrocarbons fatty acids and heavy metals. *Pseudomonas* sp. have ability to resist the metals due to the presence of intracellular metal binding proteins and multiple efflux pumps (Chovanova *et al.*, 2004). Both Gram's positive and Gram's negative strains have different operons to cope with metals. In Gram's negative like *Pseudomonas aeruginosa* and *Cupriavidus metallidurans* CH34 *czc*ABC gene is responsible for (cobalt/zinc/cadmium) resistance (Perron *et al.*, 2004; Mergeay *et al.*, 2003). The czcA belongs to resistant nodulation cell division (RND) family, present in the periplasm and function as actual metal efflux protein (Raja and Selvam, 2012). In Gram-positive like *Bacillus* sp. and *Staphylococcus* sp. the cad operon has been well studied (Silver and Phung, 1996).

The aim of the present study was to isolate the

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cadmium resistant bacteria from soil contaminated with industrial effluent and to determine the mechanism of cadmium resistance.

MATERIALS AND METHODS

Sampling and isolation of microorganisms

The soil sample was collected from Electroplating industrial area Ravi Road Lahore, Pakistan in sterile containers. pH of the soil was checked. For the isolation of cadmium resistant bacteria 1g of soil sample was added in 50 mL of LB (tryptone 1%, yeast extract 0.5% and NaCl 1%) sterilized liquid medium containing 50 µg/mL of cadmium and incubated at 100 rpm for 48 h at 37°C. The serial dilutions of enriched sample 10^{-1} to 10^{-10} were prepared, spread on LB agar plates containing 50 µg/mL of cadmium and incubated overnight at 37°C.

Determination of minimal inhibitory concentration (MIC)

For the determination of MIC of cadmium for the growth of bacterial isolates both liquid and solid media was used.

For solid medium, the isolated colonies were streaked on LB agar plates containing 100 µg/mL of cadmium and incubate overnight at 37°C. The process repeated till the MIC reached.

For liquid medium, ten sterlized100 mL conical flasks containing LB liquid media were prepared for each isolate. Different cadmium concentrations *i.e.*, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600 and 650 µg/mL were added in each flak respectively. Each flask was inoculated with isolated colony of bacterial isolate and incubated at 37°C in shaking incubator at 100 rpm till the growth appear.

16s rRNA gene sequencing

For the identification of bacterial isolates DNA was isolated (Sambrook *et al.*, 1989). The universal primers.

27F (AGAGTTTGATCCTGGCTCAG),

1492R (GGTTACCTTGTTACGACTT)

were used for the amplification of 16S rRNA gene (Weisburg *et al.*, 1991).

Amplification of the 16S rRNA gene was done by using the following conditions; 3 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 65°C and 1 min 30 s at 72°C, plus an additional 7 min cycle at 72°C. The PCR product was extracted by using Qiagen gel extraction kit. The sequencing was done using Genetic analysis system model CEQ-80 (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.

Growth conditions and effect of Cd on bacterial growth

Optimum pH, temperature and effect of cadmium on the growth of bacterial isolates was determine by the method describe earlier by Muneer *et al.* (2013).

To determine the optimum temperature the bacterial isolate was grown in LB broth at different temperatures *viz*., 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C and 55°C for 24 h. The absorbance was recorded at 600 nm using spectrophotometer. To determine optimum pH, 8 sets of 50 mL LB broth were prepared in 250 mL flasks. The pH of each set were adjusted at 3, 4, 5, 6, 7, 8, 9 and 10, sterilized and inoculated with 10^6 cells (freshly prepared). These flasks were incubated at optimum temperature for 24 h and absorbance was observed at 600 nm.

Effect of cadmium on the growth of bacterial isolate was determine by growing the bacterial isolate with (100 µg/mL) and without (control) cadmium stress. 100 mL LB broth was prepared in two flasks one was control other was experimental. The media was inoculated with 10^6 cells (freshly prepared) and incubated at optimum temperature. The sample was taken after regular interval of time of four hours and absorbance was recorded at 600 nm. Each experiment was performed in triplicate.

Detection of cadmium resistance genes

For the detection of cadmium resistant *czc*A gene genomic as well as plasmid DNA was isolated by the protocol described by Sambrook *et al.* (1989). The *czc*A gene was amplified by using a pair of

*czc*A-F5'-tcagtgaccgtgttccgccga-3' and
*czc*A-R5'-gctattagcagaagaacgggta-3'

primers by using genomic DNA and plasmid DNA separately as a template. PCR amplification was performed by using the following conditions: initial denaturation at 95°C for 5 min; then 35 cycles with 95°C for 60s, followed by 55°C for 60s and 72°C for 80s with an additional elongation at 72°C for 10 min. The reaction mixture (25 µL) contained 20 pmole of each primer, 0.2 mM dNTPs, 0.25 U *Taq* polymerase, 10 ng DNA, 50 mM KCl buffer and 1.5 mM $MgCl_2$.

Protein profiling

Bacterial cultural were grown in 50 mL LB broth with and without cadmium stress (300 µg/mL). Cells were collected by centrifugation at 10,000 rpm for 5 min at 4°C (Beckman JH-20 rotor). The supernatant was concentrated by freeze drying and stored at 4°C. The cells were resuspended in 50 mM Tris-HCl, pH 8.0 to concentrate these 10-fold. The cellular protein was extracted by sonification at a pulse rate of 30s with a rest of 1 min for 12-15 cycles until the samples were clear. Cell debris were

removed by centrifugation at 12,000 rpm for 15 min and the supernatant was collected and kept at -20°C until use. The soluble protein concentration was determined by the Lowry method (Lowry *et al.,* 1951) before performing gel electrophoresis.

Metal processing of bacterial isolates

Cadmium processing ability of bacterial isolates was checked in LB medium as well as in industrial effluent.

For LB medium, 100 mL of LB broth was prepared in 250 mL of conical flask containing 100 µg/mL of cadmium, 10^6 cells were added and incubated at their optimum pH and temperature. A control was also run containing 100 µg/mL of cadmium but without bacterial culture. The samples were drawn at regular intervals of time of four hours till 72 h. The samples were centrifuged at 6,000 rpm for 10 min at 4°C. The supernatant was collected and passed through 0.22 µm pore size filters with the help of 10 ml disposables syringe and stored at 4°C for further metal analysis by flame atomic absorption spectrophotometer at λ 228.8 nm.

For metal processing from industrial effluent cadmium content in the effluent sample was estimated by atomic absorption (AA). Treatment of effluent with metal-resistant bacteria was done in 5L flask containing 1000 ml of the effluent sample. After inoculation, the flask was kept under constant agitation in a rotator shaker at 150 r/min for 72 h. Control (without bacterial isolate) set was used to compare the uptake of metals with bacterial isolates. After 72 h, cells were separated by centrifugation and metal concentration in effluent sample was determined by AA.

RESULTS

Cd resistant isolates

A cadmium resistant bacterial strain EP-Cd1 was isolated from soil samples collected from electroplating industrial area Ravi Road Lahore, Pakistan. pH soil had 6.0 and cadmium concentration of 0.356 mg/L. On agar medium the minimum inhibitory concentration of cadmium was 650 µg/mL for the growth of EP-Cd1, while in liquid medium it could tolerate 500 µg/mL of cadmium. Comparison of 16S rRNA gene sequence with the one in the NCBI data base for comparative showed that the isolate belonged to the genus *Pseudomonas* and showed 99% homology with *P. aeruginosa*. The 16S rRNA sequence of EP-Cd1 was submitted in data base under the accession number LC102202. By neighbor joining tool the phylogenetic tree was constructed (Fig. 1) which shows the isolates relationship with their respective neighbors.

Growth conditions and effect of Cd on bacterial growth

Growth conditions *i.e.* pH and temperature and effect of cadmium on the growth of bacterial isolates was checked. It was observed that EP-Cd1 showed optimum growth at pH 6 while optimum temperature for growth was 35°C (Fig. 2).

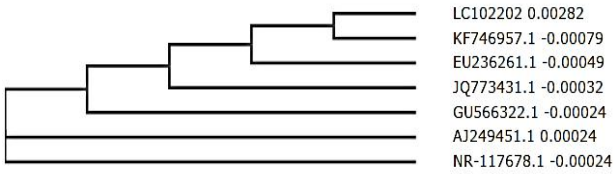

Fig. 1. Neighbor-joining tree is showing phylogeny of 16S rRNA (16S rDNA) gene sequences from heavy-metal-contaminated soil. Numbers indicate the GenBank accession number. LC102202 *P. aeruginosa* EP-Cd1; KF746957.1 *P. aeruginosa* ET6; NR-117678.1 *P. aeruginosa* DSM 50071; EU236261.1 Uncultured bacterium ZB1;AJ249451.1 *P. aeruginosa* AL98;JQ773431.1 *P. aeruginosa* RI-1;GU566322.1 *Pseudomonas* sp. NR2(2010).

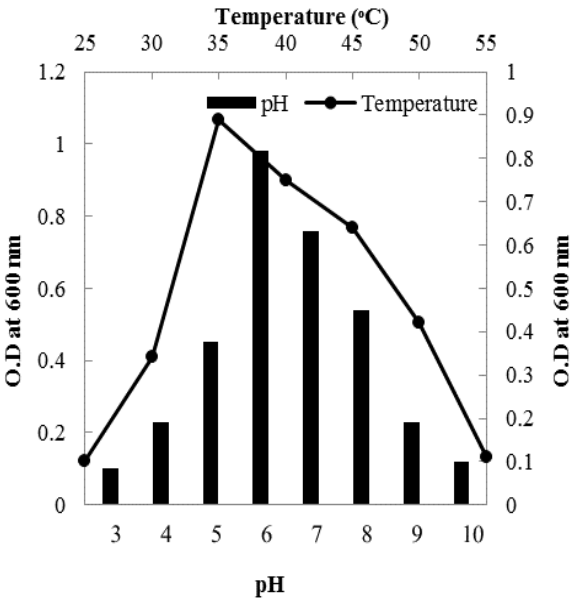

Fig. 2. Growth conditions *i.e.* pH and temperature of bacterial strain EP-Cd1.

When bacterial strain was grown in the presence of cadmium (100 µg/mL) it was observed that cadmium adversely affected the growth of bacterial isolate. *P. aeruginosa* **(**EP-Cd) showed a typical growth pattern

(Fig. 3). They showed prolonged lag phase of about 4 h in the presence of cadmium stress while it was about 1-2 h in the absence of cadmium stress.

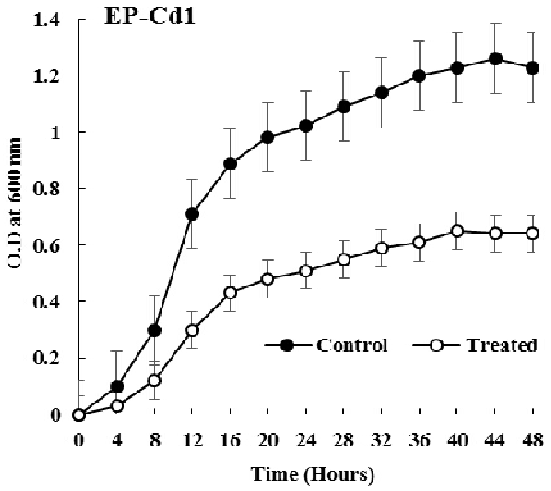

Fig. 3. Effect of Cd^{2+} on the growth of EP-Cd1, isolated from the wastewater contaminated soil, represented by the O.D at 600 nm, after 48 h of inoculation at 35°C in LB medium with and without 100 µg/mL of Cd^{2+}.

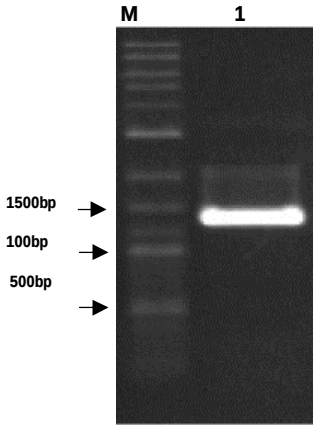

Fig. 4. PCR amplification of *czc*A gene from the chromosomal DNA of EP-Cd1. M:DNA marker.

Cadmium resistance genes

Approximately 1.5 kb fragment of *czc*A gene was amplified by using the genomic DNA of EP-Cd1 (Fig. 4), no amplification was observed with plasmid DNA. After sequencing it was confirmed that bacterial strain has metal transporter gene on chromosome responsible for cadmium resistance in different *Pseudomonas* spp. The sequence obtained has been deposited in the Genbank under the nucleotide sequence accession number LC102806.

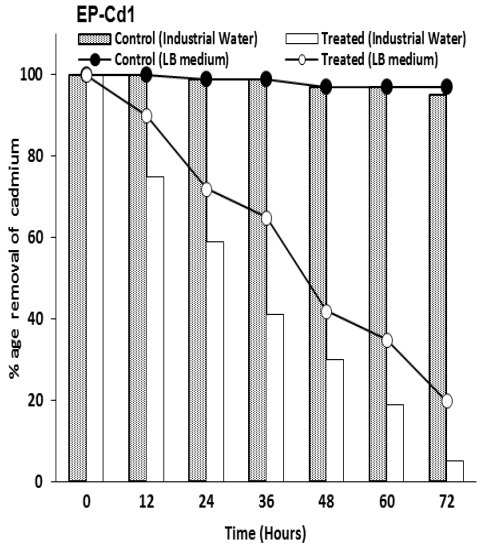

Fig. 5. Cadmium processing ability of EP-Cd1 after 72 hours of incubation in LB medium as well as in industrial wastewater.

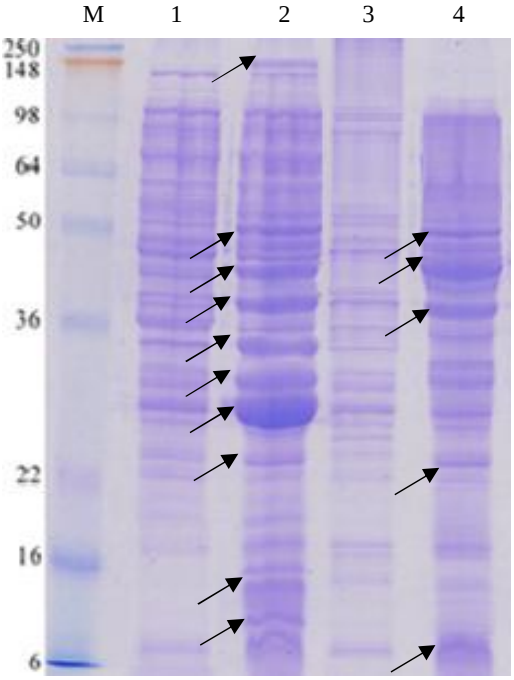

Fig. 6. The protein profile of EP-Cd3 treated (300 µg/mL) and non-treated (Control) cells. M: Protein marker, 1 Cell lysate non treated, 2: Cell lysate treated (300 µg/mL), 3: Supernatant non treated, 4: Supernatant treated (300 µg/mL).

Metal processing of bacterial isolate

Figure 5 shows the metal processing ability of bacterial isolate. EP-Cd1 removed high concentration of cadmium during first 48 h. It was observed that EP-Cd1 have ability to remove 80% of cadmium from the medium after 72 h of incubation. EP-Cd1 removed 95% of cadmium from the industrial effluent.

Protein profiling

Figure 6 shows the protein profile of EP-Cd1 treated (300 µg/mL) and non-treated (Control) cells. In the presence of cadmium stress proteins of different molecular weight were detected in supernatant *i.e.* 50, 46, 40, 24 and 8 kDa as well as in cell lysate *i.e.* 148, 50, 46, 40, 34, 32, 30, 24, 14 and 10 kDa which were absent in the control (non treated) cells. These high and low molecular proteins induced in the presence of cadmium stress can be cadmium binding protein like metal-transporters, metal-detoxifying enzymes, and metallo-thioneins, to protect the bacteria.

DISCUSSION

Heavy metal contaminated sites contain different types of indigenous microorganisms which have ability to tolerate different metal concentrations (Muneer *et al.*, 2013; Xiao *et al.*, 2013; Xie *et al.*, 2010, Muneer *et al.*, 2007). The present study focus on the isolation of cadmium resistant bacteria EP-Cd1 from soil contaminated with industrial effluent. The EP-Cd1 on the base of 16SrRNA gene identified as *P. aeruginosa* showed high cadmium tolerance in solid (650 µg/mL) as well as in liquid media (500 µg/mL).

Higher MIC is reported in solid media as compared to the liquid media due to the difference in metals availability, diffusion capacity and metal binding ability in both states (Kumar *et al.*, 2013). The level of tolerance of heavy metals among microorganisms depend upon the concentration of metals at the contaminated site (Abou Shanab *et al.*, 2007). Zeng *et al.* (2009) isolated cadmium resistant *P. aeruginosa* E1 from metal contaminated site tolerate 360 ppm of cadmium.

Growth conditions like pH and temperature adversely effect on the growth rate of bacteria (Chakravarty and Banerjee, 2012). *Bacillus* and *Pseudomonas* sp. have ability to remove maximum concentration of different metals like Cd, Cu, and Pb at pH 7 and 6 respectively (Rani *et al.*, 2010). Metal up take by binding the metal at the surface of bacterial cell is pH dependent (Wang and Chen, 2006). In *Pseudomonas* sp. the metal removal greatly enhance above pH 5 (Pandit *et al.*, 2013). Metal removal by bacteria is an energy dependent mechanism and depend upon the temperature. The temperature effect on stability, configuration as well as the state of ionization of the cell wall. These elements effect on the binding sites of metals on the surface of microorganisms. Energy-independent mechanisms for metal removal are physiochemical in nature so they are less likely to be affected by temperature (Gulay *et al.*, 2003). Maximum removal of cadmium by *P. aeruginosa* JN102340 was observed at 35ºC (Hussein *et al*., 2004).

In bacteria heavy metal as well as antibiotic resistant gene can be located on plasmid or on chromosomal DNA. EP-Cd1 harbor 23kb plasmids. The same size plasmid DNA was detected in lead, chromium cadmium and titanium resistant *P. aeurignosa* (Kassab and Roane, 2006; Park *et al*., 2006). The amplification of the gene by using the chromosomal DNA indicate the presence of the cadmium resistant gene on the chromosomal DNA and sequencing of the fragment confirmed the presence of czcA gene responsible for cadmium resistance (efflux pump) in EP-Cd1. Raja and Selvam (2009) determine the cadmium resistance gene on chromosomal DNA in *P. aeurignosa*.

In the present study induction of different low molecular weight as well as high molecular weight proteins was observed in the presence of cadmium stress. In *P. aeurignosa* the induction of different molecular weight proteins under cadmium stress was observed by Soltan *et al*. (2008). Different proteins like ATPases, metallothioneines, oxidoreductases, and transport proteins induced in the presence of cadmium stress (Waldron and Robinson, 2009).

EP-Cd1 removed the high concentration of cadmium from medium as well as from industrial effluent. Sankarammal *et al.* (2014) reported that the *Pseudomonas* sp have ability to remove the high concentration of cadmium. In the present study it was observed that the percentage of metal removal increase with the increase in the time of incubation. The biomass increases with the increase of time of incubation which enhance the surface area for metal adsorption or it increase the active binding sites for metal on the cell surface (Bai *et al.*, 2002; Veni and Ravindhranath, 2012). It was observed that *P. aeurignosa* removed 90% of cadmium from effluent as compared to the one with nutrients *i.e.* 67% (Olawale, 2014).

CONCLUSIONS

In the present study the bacterial strain EP-Cd1 was isolated from the soil sample contaminated with industrial effluent of electroplating industry and identified as *P. aeurignosa* (LC102202). It showed high cadmium tolerance in both liquid as well as in solid media *i.e.* 500

µg/mL and 650 µg/mL. The bacterium showed maximum growth at pH 6 and 35°C. The *czc*A gene responsible for cadmium binding/cadmium efflux was detected and submitted in NCBI data base under the accession number LC102806. When the bacterial strain was grown in the presence of cadmium stress it was observed it removed about 80% and 95% of cadmium from medium and industrial wastewater, respectively. The results of the present study suggest the potential use of the isolated strain EP-Cd1 as a potent candidate of bioremediation.

Statement of conflict of interest

Authors have declared no conflict of interest.

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