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Isolation and characterization of cadmium resistance *Enterobacter cloacae* strain BMSC1 from soil

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Abstract
A number of bacteria may be survive high cadmium exposure environment and appear to possess the genes for cadmium resistance. In the present study, a cadmium resistance bacterium was isolated from soil contaminated industrial area of Bilaspur district, Chhattisgarh, India. The isolate was selected based on high level of the cadmium in minimal broth. The minimal inhibitory concentration of strain BMSC1 was examined against the 10, 20, 30, 40 and 50mM of $CdCl_2$ in minimal broth. The isolate was also found to excellent growth at 7 pH. On the basis of morphological, biochemical characteristics and16S rRNA gene sequencing analysis revealed that the strain BMSC1 was authentically identified as the genus *Enterobacter cloacae*. For antibiotic sensitivity test of the isolated bacteria, different standard antibiotic discs such as, Streptomycin, Tetracycline, Chloromphenicol, Rifamycin, Ampicillin, Kanamycin, Gentamycin, and Nalidixic acid were used. This bacterium also shows the presence of *czcA* gene which is the confirmation of presence of heavy metal resistance in chromosomal gene. The results suggest that this identified cadmium resistant bacterium can be used for the bioremediation of cadmium contaminated area.

Keywords: cadmium, cadmium resistance bacteria, antibiotics, 16S rRNA gene, bio remediation

Introduction
Heavy metals contaminations have occurred as a result of both natural geologic processes and anthropogenic sources. The concentrations of metals in environment are increasing with contributions from a wide variety of industrial and domestic sources. Major Sources of the heavy metals are coal based thermal power plants and integrated iron and steel industries. Heavy metals such as lead, arsenic, cadmium, copper, zinc, nickel, and mercury are deposited in environment through geologic processes or discharged from industrial operations such as smelting, mining, metal forging, manufacturing of alkaline storage batteries, and combustion of fossil fuel (Bradl 2005; Naja and Volesky, 2009) [2, 13]. Cadmium is complex compounds which occurs in the earth's crust at a concentration of 0.1–0.5 ppm and is geologically associated with zinc, lead, and copper ores (Morrow, 2010) [12]. In nature, cadmium usually found as an oxidation state of +2, it also exists in the +1 state. The cadmium concentration of natural surface water and groundwater is usually <1 µg/L (Faroon, 2012) [6]. In atmospheric, cadmium is mainly in the forms of cadmium oxide and cadmium chloride.
An exposure very high concentration of cadmium can be cause irritatesthe stomach, leading to vomiting and diarrhea (Bernhoft, 2013) [1]. Plants can uptake cadmium from contaminated soil into their leaves, roots and tubers, and to a smaller degree their seeds, grains, and fruits. In plants, higher concentration of cadmium have present in cereals such as rice and wheat, green leafy vegetables, potato, carrot and celeriac than other (Clemens, *et al*. 2013; Chunhabundit, 2016) [4, 5]. Several techniques have been used to remediate of cadmium from contaminated environment. However, the major challenge of physical-chemical cadmium remediation technologies involves a huge cost for complete removal or containment. Bacteria that resisted high levels of cadmium can be adapted to tolerate the presence of cadmium on wastes (Nath *et al*. 2012) [14]. The application of heavy metal-tolerant bacteria may serve as a cost-effective tool for bioremediation of metal contaminated soil. The heavy metal-tolerant bacteria can be survived in heavy metal-contaminated sediments and removed high concentrations of Cd s from the contaminated soil and water. Therefore, the isolate may be useful as an indicator of potential toxicity of heavy metals in industrial effluents and play a role in bioremediation

Processes of heavy metal in polluted areas. Therefore, keeping in mind the core issues, with a view on current situation that can be carved out in future towards resolving this problem, we have decided to take up the present study.

Material and Methods

Collection of Soil and Isolation

These soil sample (250g) were collected in sterile plastic bags from industrial area of Bilaspur, Chhattisgarh. The soil sample was stored in refrigerator at 10 °C till further use. Serial dilution techniques were used for the isolation of arsenic and cadmium resistance bacteria. $A10^{-3}$ dilution of the sample was selected as inoculums for pour plate method to isolate cadmium resistance bacteria with 1mM concentrations of Cadmium chloride ($CdCl_2$). The plates were incubated at 37°C for 48 hrs to 72 hrs (till colonies developed). Individual colonies of bacteria with distinct shape and color were selected (Goswami *et al*. 2015). These isolated colonies were re-streaked and the process repeated to obtain pure culture and maintained as agar slants. Isolated colonies of cadmium tolerant bacteria were further screened out at different concentrations 5, 10, 20 30 and 40 mM of $CdCl_2$ in minimal broth.

Morphological and Biochemical characterization

Morphological and physiological characterization of the isolated bacterial colony was done following the standard methods of Bergey's manual of systematic bacteriology (2001) [15]. Different biochemical properties of the bacterial isolates such as enzyme activity (indole, urease and oxidase), coliform test, methyle red test, citrate utilization, ability to produce hydrogen sulphide, utilization of different carbon sources, utilization of gelatin and starch were tested by following the standard methods. To study the antibiotic sensitivity of the isolated bacteria, different standard antibiotic discs such as, Streptomycin, Tetracycline, Chloromphenicol, Rifamycin, Ampicillin, Kanamycin, Gentamycin, and Nalidixic acid were used (Brown 2007) [3].

Amplification 16S rRNA and *czcA* genes

From the pure culture, genomic DNA was isolated using Hi Media Bacterial DNA isolation kit (Hi Media Pvt Ltd, India). The polymerase chain reaction (PCR) the 16S rRNA was amplified by using 16S rRNA primers U1 (5′-CCAGCAGCCGCGGTAATACG-3′) and U2 (5′-ATCGGCTACCTTGTTACGACTTC-3′) (Lu *et al*., 2000) [10]. The PCR was completed with an initial denaturation step at 94 °C for 10 min, followed by 35 cycles with the denaturation at 94 °C, annealing at 55°C and extension temperature at 72 °C for 1 min, 1 min and 2 min respectively. Final extension was given at 72 °C for 10 min. Further, confirmation of cadmium resistance mechanism *czc*A gene was amplified with gene specific primers (Karelova *et al*. 2011) [8].

The 16S rRNA gene amplicons obtained from universal primers U1 and U2 were partially sequenced by using the Sanger dideoxy sequencing technique in order to ascertain the bacterial genus of unknown bacterial isolates. The sequences of the bacterial isolates were then converted to FASTA format and were deposited in the international gene bank repository of NCBI (National Centre for Biotechnology Information) getting an accession number for each isolates and the phylogenetic tree was prepared using Mega 6 software using "neighbor joining" method.

Results & Discussion

Bacterial isolate BMSC1 was isolated successfully as cadmium tolerant strain. The isolate was found to excellent growth at 7 pH. However, BMSC1 was also able to grow on low pH (4 pH) and high pH (pH 9). It was also reported that the bio sorption ability cadmium resistant bacteria was also found changing with varying pH. Neutral (pH 7) or nearly neutral pH (pH 6) was found most suitable (Khan *et al*. 2016) [9]. Similarly, Worden *et al*. (2009) [17] demonstrated that transcriptional responses of Escherichia coli to cadmium are affected by pH and suggested that numerous stress responses, transport, and hypothetical genes play roles in the mechanism by which pH mediates cadmium toxicity. Basic (pH 8) and strongly basic pH (pH 9) decreased Cd2+ removal to 2.9 and 0.53 mM/g, respectively. After incubation of 24h at 37 ^{0}C these isolate changed their colony colour (violet) which indicated a positive reaction for the coliform. Coliform bacteria generally belong to Enterobacteriaceae family; *Citrobacter, Enterobacter, Esherichia and Klebsiella*. Not all strains of these four genera meet the coliform definition, while a few bacterial strains outside these genera will (e.g., *Aeromonas* spp). *Escherichia coli* are the most well-known coliform. This isolate was negative for starch and gelatin hydrolysis and also showed only fermentation of glucose and could not able to ferment lactose and sucrose but positive for gas production and citrate utilization. The amplified products were partially sequenced by Sanger sequencing technique and the gene sequences were deposited in NCBI gene bank retrieving the following accession number MH915558. This isolate belonged to the genus *Enterobacter cloacae* BMSC1 after sequencing of 16S rRNA gene sequencing. Earlier reports have shown the Cd biosorption capacity of the *Enterobacter* strain J1 isolated from a local industry wastewater treatment plant (Lu *et al*. 2006) [11]. There are likewise other research confirming cadmium uptake by *Enterobacter* species as the ones published by Pishchik *et al*. (2002) [16] and Jha *et al*. (2011) [7]. Then, it can be stated that *Enterobacter* sp. has shown cadmium uptake capacity and high resistance to various heavy metals, including cadmium at the rhizosphere and rhizoplane levels (Pishchik *et al*. 2002) [16]. This bacterium also shows the presence of *czcA* gene which is the confirmation of presence of heavy metal resistance in chromosomal gene. Generally, the *czc*A gene system detoxifies the cell by efflux, the three metal, viz. cobalt, zinc and cadmium, which are taken up into the cell by fast and unspecific transport system for Mg2+ are actively extruded from cell by products of *czcA* resistance determinants (Nies *et al*. 1989) [15].

Conclusions

The Cadmium resistant bacterium isolated in this study was *Enterobacter cloacae* based on phylogenetic analysis of 16S rRNA sequence. *Enterobacter cloacae* are highly resistant against cadmium metal and survive in the presence of high concentration of cadmium (40mM). This bacterial strain has evolved mechanisms to regulate cadmium resistant genes (*czc*A gene). The results concluded that strain BMSC1 can be used for the bioremediation of cadmium contaminated area.

Table 1: Morphological and Biochemical Characterization of *Enterobacter cloacae* strain BMSC1

Morphological Characterization	
Gram stain +/-ve	-
Shape	Rod
Colony Shape	Circular
Margins	Entire
Elevation	Convex
Color	Yellowish
Texture	Smooth
Biochemical Characterization	
Coli form	+
Catalase test	+
Starch Hydrolyze	-
Citrate Utilization	+
Gelatin Iron	-
Indole test	-
Methyl Red Test	-
Urease Test	-
Glucose	+
Lactose	-
Sucrose	-
H_2S	+
Gas	+

(+) =Positive, (-) =Negative

Table 2: Antibiotic susceptibility test of *Enterobacter cloacae* strain BMSC1

Antibiotic discs	**Zone of inhibition (mm)**
Streptomycin	14
Tetracycline	21
Chloromphenicol	25
Rifamycin	16
Ampicillin	-
Kanamycin	14
Gentamycin	17
Nalidixic acid	24

(-) = High Resistance

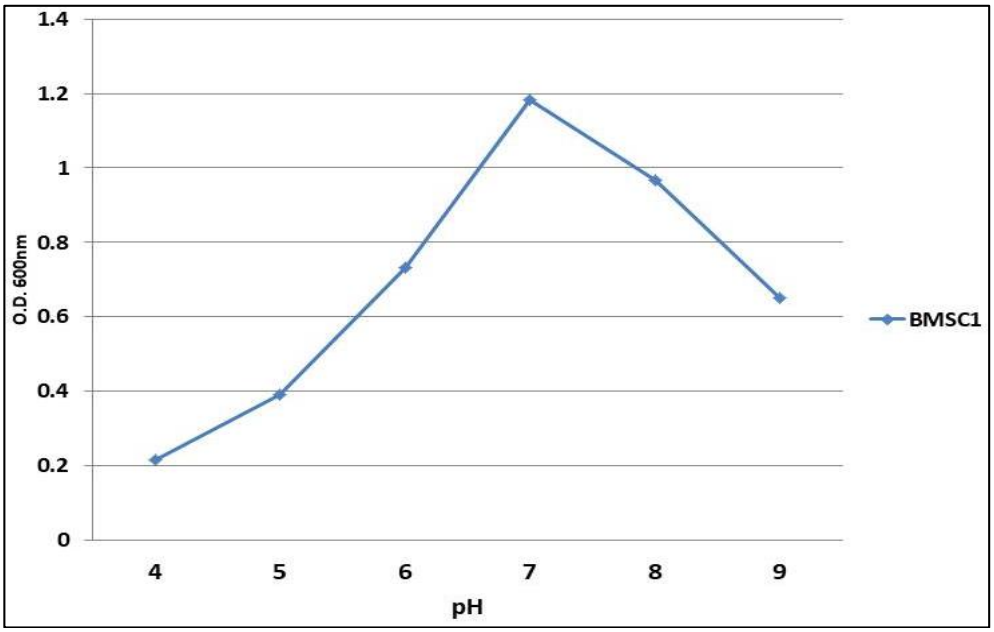

Fig 1: Effect of pH on the growth of *Enterobacter cloacae* strain BMSC1

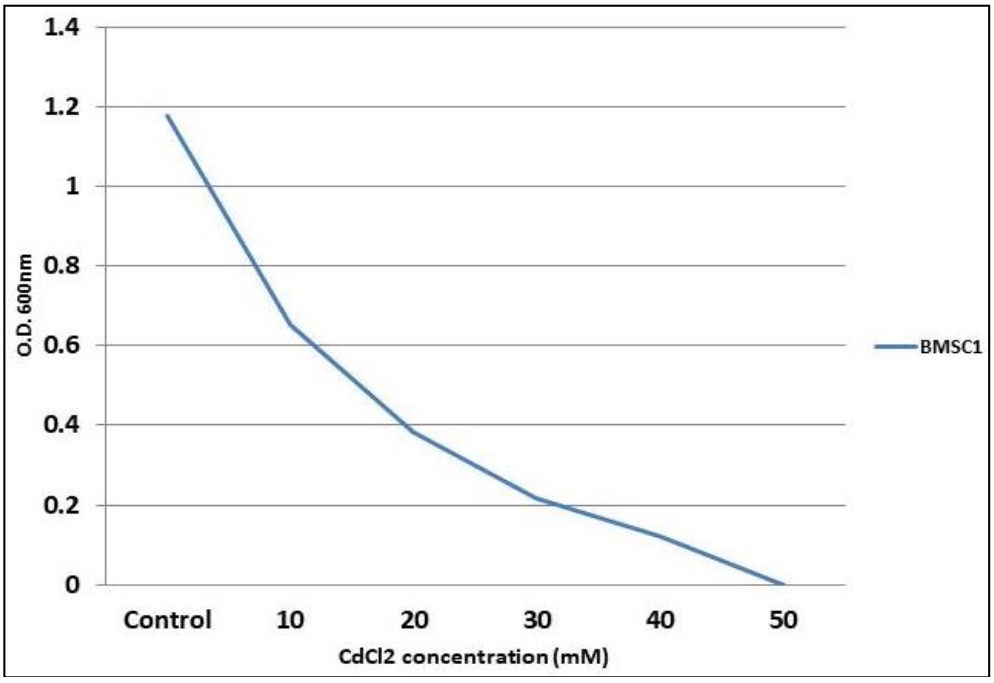

Fig 2: Effect of different concentrations of cadmium on the growth of *Enterobacter cloacae* strain BMSC1

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