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# Bioremediation of Cadmium by Mixed Indigenous Isolates *Serratia liquefaciens* BSWC3 and *Klebsiella Pneumoniae* RpSWC3 Isolated from Industrial and Mining Affected Water Samples

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**ABSTRACT:** A total of 58 Cadmium tolerant bacterial isolates were isolated from 26 samples collected from 20 villages/city of different contaminated water samples from industrial and mining affected areas of Chhattisgarh (India). Out of 58 bacterial isolates, 15 bacterial isolates were able to grow in presence of 40 mM cadmium chloride. These fifteen were further screened by biochemical characterization, antibiotic susceptibility and presence of *czcA* gene. However, finally five selected isolates (BSWC3, RgCWC2, RgUWC1, RpSWC3, KDWC1) were identified by 16S rRNA gene sequencing belonged to the genus *Serratia liquefaciens, Klebsiella quasipneumoniae subsp. similipneumoniae, Klebsiella pneumoniae, Pantoea dispersa* and *Enterobacter tabaci*, respectively. Among these two best culture *Serratia liquefaciens* BSWC3 and *Klebsiella pneumoniae* RpSWC3 were testes for their bioremediation efficiency individually as well as in mixed culture. Atomic Absorption spectrophotometer analysis of samples revealed that cadmium (Cd) tolerant bacterial isolates BSWC3, RpSWC3 and Combination of BSWC3 and RpSWC3 were significantly reduce of cadmium concentration i.e. 44.46%, 40% and 50.92%, respectively as compared to control. Therefore, the finding of the present study revealed the use of mixed culture or consortium of indigenous isolates is the better option for bioremediation of heavy metals.

**Keywords:** Cadmium Bacteria, Mixed Culture, Water, Bioremediation.

## INTRODUCTION

Cadmium is complex compounds which occurs in the earth's crust at a concentration of 0.1–0.5 ppm and geologically associated with zinc, lead, and copper ores (Morrow, 2010). Cadmium contamination in the water, soil and air has been occurring particularly in industrial and mining areas. In mining areas, coal contain with significant amounts of cadmium are mostly deposit in ends form of flue dust in Environment (Al-Kharabsheh & Taany, 2003; Hogervorst et al., 2007, Grant and Sheppard, 2008). Further, Cadmium is an extremely toxic element among the heavy metals for living organisms, with a wide range of organ toxicity and long elimination half-life (Jarup & Akesson, 2009). Cadmium (Cd) can be introduced into water sources in amounts harmful to human health by industrial effluents (Zheng et al., 2008). Acute exposure to cadmium may result in

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death from pulmonary edema. Its chronic exposure can cause kidney damage, osteoporosis, diabetes, cardiovascular disease and cancer. An exposure to very high concentration can irritates the stomach, leading to vomiting and diarrhea (Stuijfzand et al., 2000; Banjerdkji et al., 2005; Bernhoft, 2013). Microorganisms are an approach to bioremediation that ensures a more efficient clean-up of heavy metal polluted soils (Moghannem et al. 2015). The introduction of indigenous bacteria can provide a potential bioremediation process of contaminated soil without disturbing the target environment (Kermani et al., 2010; Kang et al., 2015). Some bacteria can be able to survive against high concentration of cadmium that leads to the evolution of different cadmium resistance systems such as efflux pumps, capable of binding cadmium and detoxifying (Prapagdee & Watcharamusik, 2009; Abbas et al., 2017).

Furthermore, Chhattisgarh is one of the major coal producers state in India and the process of coal extraction, mostly opencast mining, and electrical generation by coal-fired power plants release a range of gaseous and solid chemicals and heavy metals like arsenic, cadmium, lead, nickel, manganese, silicon and aluminum into the atmosphere as a by-product of this process. This critical issue has been concealed for many years and only recently, there has been some noted scientific work carried out in the state. In a study, groundwater samples from 146 sites of Rajnandgaon district (C.G.) were analyzed. Arsenic concentration in tube well water was elevated above the WHO guideline by a factor of more than 10 with concentrations reaching 520 µg $l^{-1}$, whereas in the dug wells (general depth less than 50 m) As concentrations were as high as 880 µg $l^{-1}$ (Patel et al., 2017). Similarly, Cd present at concentration of 0.003 mg/L in river waster of Hasdeo (Chhattisgarh). The quality of river water were compared with national standards and revealed that the all samples are suitable for irrigation purpose but not for drinking (Bhaskar and Dixit, 2013). Korba city of Chhattisgarh, the nation's 'power hub' is also the fifth among its eighty-eight most critically polluted industrial hotspots (Das et al. 2018). Further, the metal concentration of groundwater in Bailadila iron ore mine area of this state follows the trend $Fe^{2+} > Zn^{2+} > Al+ > Cr^{6+} > Pb^{2+}$ in both pre and post monsoon seasons (Jareda et al., 2018). In another study, Pb, Ca in all the samples are exceeded WHO limits for drinking Water (Sharma et al., 2013). Most of them has been on its geochemical, mineralogical and physiochemical aspect leaving behind very few works on bioremediation and molecular aspect (Banerjee et al., 2015). Despite knowing the importance of bioremediation and numerous benefits it can deliver, especially by decontaminating heavy metals affected water through bacteria, no such significant work has been taken up by researchers in Chhattisgarh. 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

A total of 26 samples were collected from 20 villages/city of different district in Chhattisgarh (Fig. 1). They were transported immediately after collection and samples were stored at 4-10 $^{o}$C after taking to the laboratory for further use. $A10^{-3}$ 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 physiological characterization of the isolated bacterial colony was done following the standard methods of Bergey's manual of systematic bacteriology (2001). To study the antibiotic sensitivity of the isolated bacteria, different standard antibiotic discs such as, Streptomycin, Tetracyclin, Chloromphenicol, Rifamycin, Ampicillin, Kanamycin, Gentamycin, and Nalidixic acid were used (Brown, 2007). The inhibition zone of antibiotics obtained was measured by using Himedia (Hi Media, Pvt Ltd, India) antibiotic scale.

From the pure culture, genomic DNA was isolated using HiMedia Bacterial DNA isolation kit (HiMedia Pvt Ltd, India). Different sets of previously reported primers (table 1) were used to amplify 16S rRNA gene. Further, confirmation of cadmium resistance mechanism *czcA* gene was amplified with gene specific primers. The 16S rRNA gene amplicons obtained from universal primers U1 and U2 (table 2) 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.

Two best bacterial isolates were selected based on MIC Growth analysis and *czcA* gene amplification for bioremediation assay. The concentrations of metal in the form of Cadmium Chloride monohydrate (100ppm) were used for detail growth study. This concentration was previously used by several workers for isolating Cd resistant strains (Das et al., 2013; Kumar et al., 2017; Selvaraj et al., 2018) and concentration of metal salt in

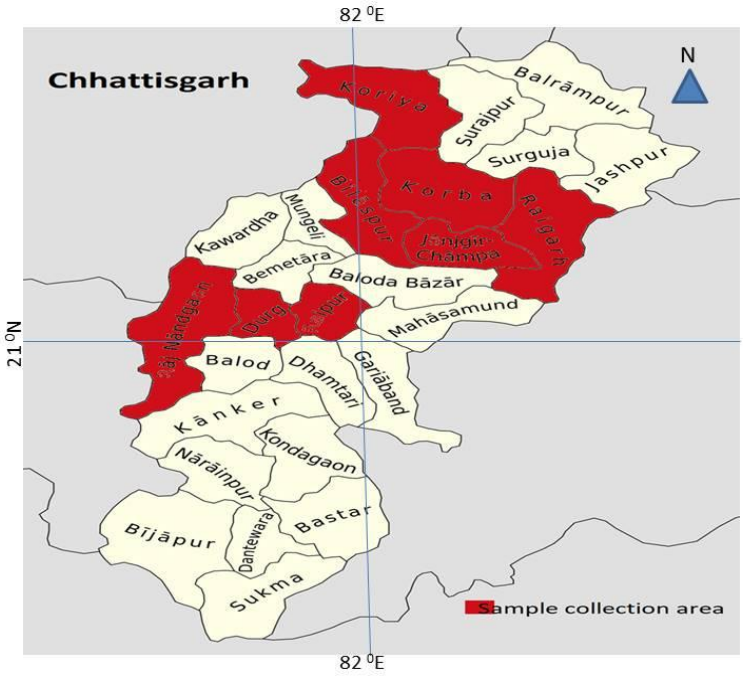


**Fig. 1. Pictorial view of sample collection sites in Industrial and Mining affected area of Chhattigarh (India).**

ppm was also helpful for AAS analysis. In each treatment having respective isolates with/ without cadmium salt were taken in 100ml minimal broth along with uninoculated control. All flasks were incubated at 37 $^{o}$C with continuous shaking at 100rpm. Culture (0.6 OD) without heavy metals was treated as positive control. Growth was monitored as a function of biomass by measuring absorbance at 600 nm using visible spectrophotometer. Growth of the isolates on minimal broth with or without metal supplementation were determined and compared by plotting the optical density at 600 nm (O.D. at 600nm) to time in days. Estimation of Cd concentration was done Atomic absorption spectroscopy (AAS) as per the procedure followed by Baghel (2016).

## RESULTS AND DISCUSSION

A total of 58 Cd tolerant bacterial strains were isolated from contaminated water samples collected from industrial and mining affected areas of Chhattisgarh (India). All bacterial isolates were obtained in minimal agar medium containing 1mM cadmium chloride ($CdCl_2$). The isolation of bacterial strains was possible on the basis of their ability to grow in the presence of added at a concentration of cadmium chloride. These isolates were selected on the basis of color, size and morphology. Further screening was done at minimal ager plats on the basis of maximum metal tolerance for Cadmium. Out of 58 bacterial isolates, only 15 bacterial isolates were able to grow in the presence of 40 mM cadmium chloride whereas 21 isolates were able to grow in 30 mM cadmium chloride. The minimum inhibitory concentration of all selected cadmium tolerant isolates were determined by measuring the O.D. at 600 nm after 48h growth in Minimal broth supplemented with different concentrations of cadmium chloride (10, 20, 30, 40mM and 50mM). All selected bacterial isolates were able to tolerate at 40mM cadmium chloride and no growth found in the presence of 50mM cadmium chloride. The cadmium tolerant bacterial isolates BSWC1 was found to highest resistance cadmium followed by RpSWC3 bacterial isolate. Similarly, Mathivanan and Rajaram (2014) studied that some cadmium tolerant bacteria able to tolerance up to 400 mg $L^{-1}$ of $Cd^{2+}$ which were isolated from the polluted coastal.

**Table 1. Gene specific PCR primers set used in the study.**

| Gene | Primer | Primer sequence | References |
|---|---|---|---|
| 16S rRNA gene | U1 | 5′-CCAGCAGCCGCGGTAATACG-3′ | Lu et al., 2000 |
| | U2 | 5′-ATCGGCTACCTTGTTACGACTTC-3′ | |
| czcA | czcAF | 5′-GAC TTC GGC ATCATCRTCGAYGG-3 | Karelova et al. 2011 |
| | czcAR | 5′-CGTTGAASCGCARCTGGATCGG -3′ | |

**Tabel 2. Biochemical Characterization of selected Cd tolerant bacterial isolates**

| S.N. | Name of Isolates | Coli form | Catalase test | Starch Hydrolyze | Citrate Utilization | Gelatin Iron | Indole test | Methyl Red Test | Urease Test | Glucose | Lactose | Sucrose | H2S | Gas |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | BSWC3 | - | + | + | + | - | - | - | + | + | - | - | + | + |
| 2 | KDWC1 | + | + | + | + | + | - | + | + | + | - | - | - | - |
| 3 | RgUWC1 | + | + | + | + | - | - | + | - | + | - | - | + | + |
| 4 | RpSWC3 | - | + | - | + | - | - | - | + | + | + | + | - | - |
| 5 | RgCWC2 | - | + | + | + | + | - | - | + | + | + | + | - | + |

All the selected isolates were further characterized on the basis of gram staining and colony morphology. All 15 selected Cd bacterial cultures were spread plate on metal agar plates having 1mM $CdCl_2$ in presence of 8 antibiotic discs and were inoculated at $37^0$C for 24-48 hours to get an even zone of bacterial growth. The results revealed that the maximum resistance was showed by all the isolates towards antibiotic *viz* Streptomycin, ampiciline and rifamycin, whereas highly susceptible was showed to against *viz* antibiotic tetracycline and chloramphenicol. Bacteria isolate RgCWC3 was complete resistance against two antibiotics chloramphenicol and ampicillin. Chloramphenicol and tetracycline are considered as macrolides and aminoglycosides, respectively. These molecules mainly bind to ribosomes and alter their function. Tetracycline had the highest inhibitory effect on all the isolates (table 2 &3). These days with increase in environmental contaminants, microbes are known to be showing more antibiotic resistance (Samanta et al. 2012). Antibiotics are being extensively used in agriculture and aquaculture (Seiler and Berendonk 2012). Some reports suggest that presence of heavy metals often acts as a selective agent for antibiotic resistance in microbes which exerts a selection pressure in environment resulting in co-selection of heavy metal and antibiotic resistance (Baker-Austin et al. 2006). Several antibiotic resistance mechanisms are markedly influenced by the presence of metals showing a positive co-relation between antibiotic- metal resistances. This mechanism for co-selection is based on cross resistance and co-resistance. Cross resistance contains genes encoding non-specific mechanisms showing resistance to both antibiotics and metals while co-resistance genes are present separately integrated in the same genetic material like plasmid or chromosome (Knapp et al. 2011). In this way the microbes showing resistance for heavy metals may also show resistance for antibiotics (Sinegani and Younessi 2017).

**Table 3. Antibiotic susceptibility test of Cd tolerant bacterial isolates**

| S.N. | Name of Isolates | Zone of inhibition (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Str | Tet | Chl | Rif | Amp | Kan | Gen | Nal |
| 1 | BSWC3 | 17 | 22 | 16 | 14 | - | 16 | 14 | 15 |
| 2 | KDWC1 | 26 | 27 | 14 | 13 | 14 | 12 | 14 | 15 |
| 3 | RgUWC1 | 13 | 23 | 24 | 10 | - | 14 | 11 | 14 |
| 4 | RpSWC3 | 16 | 21 | 16 | 14 | - | 12 | 13 | 15 |
| 5 | RgCWC2 | 18 | 23 | 17 | 11 | - | 17 | 16 | 25 |

Str = Streptomycin, Tet = Tetracycline Chl = Chloramphenicol, Rif = Rifamysin, Amp = Ampicillin, Kan = Kanamycin, Gen = Gentamycin, Nal = Nalidixic acid

Five best cadmium tolerant isolates (BSWC3, RgCWC2, RgUWC1, RpSWC3, and KDWC1) were identified by sanger sequencing which belong to four genera. However, five isolates (BSWC3, RgCWC2, RgUWC1, RpSWC3, KDWC1) were belonged to the genus *Serratia liquefaciens, Klebsiella quasipneumoniae subsp. similipneumoniae, Klebsiella pneumoniae, Pantoea dispersa* and *Enterobacter tabaci*, respectively (Fig 2). However, previously bacterial genera *Alcaligenes xylosoxidans, Comamonas testosteroni, Klebsiella planticola, Pseudomonas putida, ciens* (Kanazawa and Mori, 1996; Barberio & Fani, 1998; Chovanová et al., 2004), *Stenotrophomonas acidaminiphila, Pseudomonas aeruginosa and Delftia tsuruhatensis* (Lin et al., 2016) also showed tolerance to metals, especially Cd , Zn and Pb. Interestingly cadmium

resistance in *P. dispersa* and *E. tabaci* was not much reported especially *E. tabaci* which is a newly identified species (Duan et al., 2015). Furthermore, gram-negative bacterial species such as *E. coli* (Cohen et al., 1991), *P. putida* (Higham et al., 1984), *P. syringae* (Cabral, 1992), *P. aeruginosa* (Hassen et al., 1998) was recognized on production of intracellular cadmium-binding proteins. The amplified products were partially sequenced by Sanger sequencing technique and the gene sequences were deposited in NCBI gene bank retrieving the following accession numbers from MH915559- MH915563.

Two best Cd tolerant bacterial isolates *Serratia liquefaciens* BSWC3 and *Klebsiella pneumoniae* RpSWC3 positive for *czcA* gene PCR were selected for bioremediation assay. Actually, the a *CzcCBA* efflux system (*CzcC*, *CzcB* and *CzcA*) encode a membrane-bound protein complex that achieves metal resistance by active cation efflux driven by a cation-proton antiporter (Nies, 1995; Kim et.al. 2011) and the loss of *CzcA* increases sensitivity to Cd (Rensing et al., 1997;). Hence, the presence of this *czcA* gene in the bacterial genome confirms its metal resistance ability. A growth curve was prepared by periodically taking the optical density of each bacterial isolates by spectrophotometer at 600nm for 10 days. The growth curve for cadmium tolerant bacteria isolate *Serratia liquefaciens* BSWC3 and *Klebsiella pneumoniae* RpSWC3 were showed that in absence of cadmium, the growth of bacteria enter into the log phase and increased their growth up to 3rd days after than it entered into death or declining phases. However, in presence of Cd, it was observed that a prolonged log phase of 1 to 4 days with a brief stationary phase after which it enters into declining phase from 5$^{th}$ day onwards. Prolonged log phase in presence of cadmium as compared to shorter one in its absence shows that accelerated growth was achieved when the bacteria was grown under presence of cadmium. The bacterial isolates *Serratia liquefaciens* BSWC3 supplement with Cadmium chloride was showed maximum growth compare to RpSWC3 and combination of *Serratia liquefaciens* BSWC3 and *Klebsiella pneumoniae* RpSWC3 (fig 3). Recently, *Serratia liquefaciens* was also from industrial effluents polluted with heavy metals (Ramya & Boominathan, 2017).

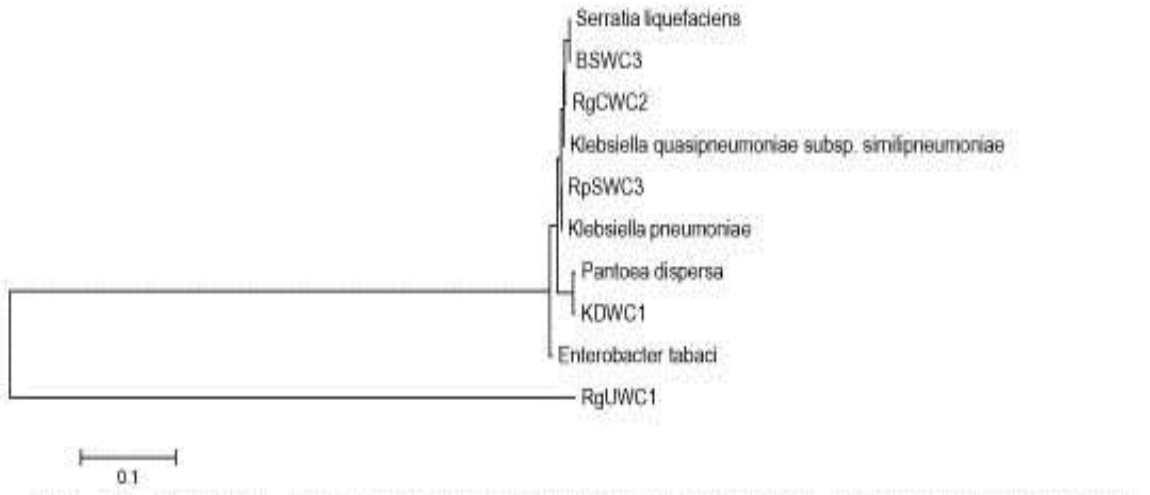


**Fig. 2. 16s rRNA sequencing based Phylogeny tree of selected isolates.**

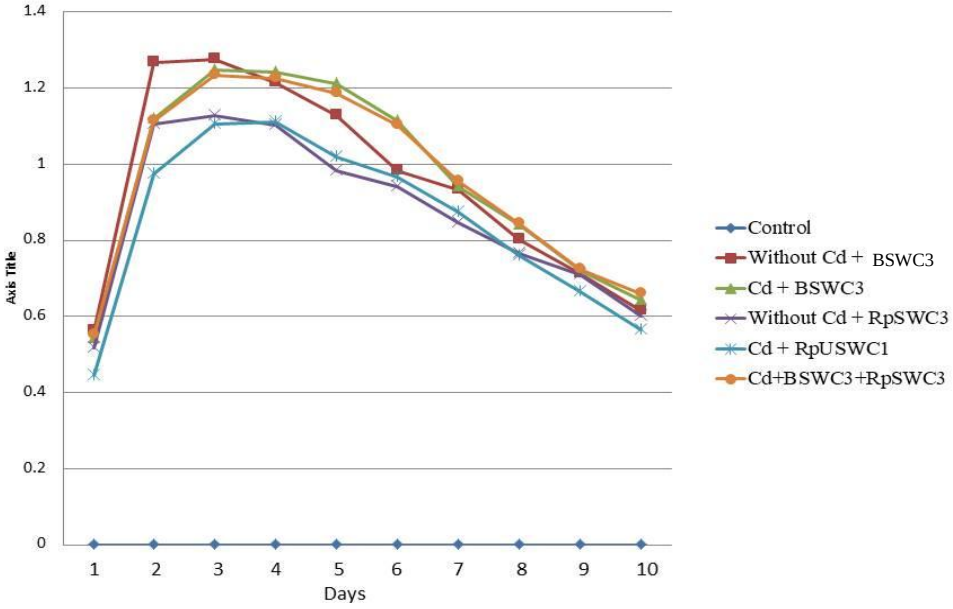


**Fig. 3. Growth curve of Serratia liquefaciens BSWC3, Klebsiella pneumoniae RpSWC3 and mixed culture.**

Absorption or remediation of cadmium by these bacteria isolates were estimated through Atomic Absorption Spectroscopy (AAS). The cadmium reduction capability of bacterial isolate was checked by adding cadmium chloride at a concentration of 100ppm in the minimal broth. The control medium was also run for cadmium containing the same concentration as in treated one i.e., 100ppm but was without the bacterial isolate. The Cd tolerant bacterial isolates BSWC3, RpSWC3 and Combination of BSWC3 and RpSWC3 were significantly reduction of cadmium i.e. 44.46%, 40% and 50.92%, respectively as compared to control. The bacterial combination isolate BSWC3 and RpSWC3 was found to highest reduction (50.92%) of cadmium followed by single bacterial isolate BSWC3. Results of AAS for Cd content clearly indicate the differential uptake depending upon the bacterial species. Laboratory scale bioremediation study indicated effective cadmium removal by mixed culture as well as individual strains. This might be because of synergistic effect of the isolates. It was also reported in previous studies that a consortium works better than individual strains (Kozyrovska et al., 2004; Chowdhury et al., 2011; Li et al., 2017). As reported by Wong et al. (2013), consortium exposed to Cd observed that a dense area around the bacteria compared with control. The dense area showed cadmium adsorption on the surface of cells, possibly coated with exopolysaccharide, suggesting this to be the mechanism employed by consortium to remove Cd from broth (Wong et al. 2013). It has been shown that microbes have the capability to reduce heavy metals, but whether they reduce it for detoxification or for its growth is a matter of concern. Further, Lee et al., (2008) reported that a consortium isolated from lake sediments was able to remove 99-100% of different heavy metals including copper, chromium, nickel, lead and zinc from heavy metal contaminated water. However, reports on cadmium bioremediation by bacterial consortia are very limited (Malekzadeh et al. 2012, Sen et al. 2014). In one more research, cadmium resistant bacteria were also isolated from Cd-contaminated soils by Prapagdee and Watcharamusik (2009).

## CONCLUSION

In the present study, the cadmium resistant bacterial isolates were isolated from the water samples collected from industrial and mining affected area of Chhattisgarh state. These isolates were found to be resistant to cadmium. The mechanism involved in conferring resistance to cadmium was found to be chromosomal. It is clear from the above results that resistance to Cd in both the diversified isolates *Serratia liquefaciens* BSWC3 and *Klebsiella pneumoniae* RpSWC3 is due to the *czc* gene present on the chromosomal DNA and involves metal binding and/or an efflux mechanism of resistance. Furthermore, the results of in vitro, AAS analysis suggest that the strains may have considerable potential as an agent for bioremediation with reference to Cd. The detoxification efficiency of these two isolates *S. liquefaciens* BSWC3 and *K. pneumoniae* RpSWC3 in combination indicates good potential for application in bioremediation of cadmium from polluted sites.

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