

ISOLATION AND MOLECULAR CHARACTERIZATION OF THREE *BACILLUS* STRAINS FOR THEIR TOLERANCE AGAINST VARIOUS HEAVY METALS

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ABSTRACT

Heavy metal contamination due to natural and anthropogenic sources is a global environmental threat which can produce harmful effects on human health when they are taken up in amounts that cannot be processed by the organism. Technologies involving microbial cells for metal removal and recovery may provide an alternative to conventional methods. In the present study, three cadmium resistant bacteria were isolated from soil collected from industrial area of Faridabad, Haryana, India. Screening of the bacterial isolates for metal resistance against Cd²⁺, Ni²⁺, Hg²⁺, Cu⁺² and Pb²⁺ was done by determining the minimal inhibitory concentration ranging from 10ppm to 250ppm. Moreover these isolates showed a significant ability to remove 70 to 78% of cadmium. These isolates were identified as *Bacillus sp.263ZY1 (MA5)*, *Bacterium YC-LK-LKJ45 (MB5)* and *Bacillus subtilis strain DHXJ07(MC5)* on the basis of 16S r-RNA gene sequencing. The ability of these microbes to tolerate high concentration of a range of heavy metals provides a scope of use of these bacterial strains for bioremediation of heavy metal from industrial effluent.

Keywords: Heavy metals, *Bacillus* strains, antibiotics sensitivity test, molecular characteristics, bioremediation

INTRODUCTION

The anthropogenic contamination of the environment with heavy metals is a global serious problem. Heavy metals like zinc, cadmium, copper, lead, nickel and mercury have been reported as the most toxic pollutants (Cameron, 1992). Although some heavy metals are essential trace elements, most can be at high concentration, toxic to all forms of life, including microbes, humans and animals. Among the list of heavy metals, the cadmium needs particular awareness because it is recognized as significant pollutant due to its high solubility and toxicity in the water. Cadmium gets released into the environment by various human activities such as mining, smelting, incineration of plastics and batteries, burning of fossil fuels etc (Tang et al., 2006). It is toxic at very low exposure levels and has acute and chronic effects on health and environment. Acute exposure to cadmium fumes may cause flu like symptoms including chills, fever, and muscle ache. The long term exposure of these metals result in physical, muscular, neurological degenerative processes that cause Alzheimer's disease (brain disorder), Parkinson's disease (degenerative disease of the brain), muscular dystrophy (progressive skeletal muscle weakness), multiple sclerosis (a nervous system disease that affects brains and spinal cord). Cadmium may also combat with other metals such as zinc and selenium for enclosure into metallo-enzymes and it may compete with calcium for binding sites on regulatory proteins such as calmodulin (Hu et al., 1998). Being non degradable in nature, it stays in circulation once released into the environment. Removal of cadmium from effluents before they are discharged into the environment can be accomplished by processes such as, chemical precipitation, cementation, solvent extraction, reverse osmosis and ion exchange (Meena and Rajagopal, 2003). The physical or chemical methods of heavy metals remediation are neither suitable nor widely accepted for practical applications. A lot of emphasis is being paid to the use of bacteria to remediate heavy metals and it has emerged as one of the alternatives to physicochemical methods. Their use offers economic and ecological benefits, not only as a scientific novelty but also for its potential application in industry (Chen et al., 2005 and De et al., 2008). Some microorganisms have the resistance against the heavy metals and they can grow in the heavy metal rich environment also. To avoid cellular damage caused by metal ions, bacteria evolved mechanisms of metal tolerance. Recent studies shows that the strains (bacteria, yeast and fungi) isolated from contaminated sites possess excellent capability of metal scavenging (Malik, 2003). In the present study the efforts are directed towards the isolation of cadmium resistant bacteria that also shows

resistance against other heavy metals and are potential candidates for the comprehensive treatment of metal-rich effluents.

MATERIAL AND METHODS

Sample collection

Soil samples were collected from five different sites of Faridabad industrial area, Haryana, India. All the samples were kept in clean sterile bags and stored at 4°C.

Isolation and identification of cadmium resistant bacteria

The cadmium resistant bacteria were isolated by standard dilution method. The serially diluted soil samples were spread on the Luria-Bertani agar plates supplemented with 10mM of cadmium as cadmium nitrate. The plates were incubated at 30°C for 24 hours. The colonies that appeared on the medium supplemented with 10ppm cadmium were considered as cadmium resistant bacteria. Different colony characteristics such as colony color, elevation, shape, margin, texture were determined morphologically distinct colonies was picked and was purified by re-streaking on Luria Bertani medium plates. The isolates were stored on LB agar slants supplemented with 10ppm cadmium in a refrigerator and maintained by regular transfers.

Growth kinetics of bacterial isolates in the presence of cadmium

To study the growth kinetics of the isolates in the presence of heavy metals, LB broth and metal solution were separately autoclaved. The flasks were inoculated with 100µl of overnight grown bacterial culture with different concentration of cadmium (10ppm and 20ppm) and were incubated at 30°C in rotator shaker. LB broth (without cadmium nitrate) inoculated with the culture served as control. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 6, 12, 24, 48, 60, 72, 84, 96, 108 and 120 hours and growth was monitored at regular intervals by measuring the optical density (OD) at 620nm.

Determination of minimal inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of heavy metals was determined by the agar plate dilution method as described by Malik and Jaiswal (2000). Luria Bertani agar medium was prepared and amended with various amounts of heavy

metals (Cd, Pb, Ni, Cu and Hg) to achieve the desired concentration of 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, and 300ppm. Inoculum of bacterial strains was spread in the metal amended and control plates (without metal). The plates were incubated at 30°C for 48 hours. The MIC was defined as the lowest concentration of the metals that inhibits the visible growth (number of colonies) of the organisms.

Antibiotic susceptibility

Selected bacterial isolates were tested for susceptibility to 12 different antibiotics(Axiom Laboratories) by the disc diffusion method on Muller Hilton agar (2 g beef extract, 17.50 g Acid hydrolysate of casein, 1.5 g starch, 17 g Agar) plates. The antibiotics tested were: penicillin (20 µg), Co-trimoxazole (25 µg), cephalixin (30 µg), tetracycline (30 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), Levofloxacin (5 µg), linezolid (30 µg), cloxacillin (30 µg), roxithromycin (5 µg), Lincomycin (2 µg) and gentamicin (10 µg). The antibiotics discs were placed on the plates previously seeded with cell suspension. The plates were incubated at 30 OC for 72 h and observed for zones of inhibition.

Isolation of plasmid and electrophoresis

The bacterial isolates were screened for the presence of plasmid DNA using the alkaline lysis method (Sambrook et al., 1989). Plasmid profiles were determined on 0.8% agarose gels prepared in Tris–Borate–EDTA buffer, pH 8.0, stained with ethidium bromide and documented with an Imagemaster digital camera and associated annotation software (Syngene, USA).

Cadmium accumulation and removal assay by bacterial isolates

Selected isolates were grown in LB medium supplemented with 100µg/ml cadmium as cadmium nitrate. The flasks were incubated on a shaker for 120 hours at 30°C. Cells were harvested at 24 and 48h of incubation by centrifugation (1100xg for 10minutes at 4°C). Bacterial cell residue was dissolved in 1ml 95% nitric acid mixed well by vortexing and diluted to 10 ml with sterile DDW. Blanks were treated in the same way and analyzed by atomic absorption spectrometry. Cadmium was measured from the supernatant by the atomic absorption spectrometry. Percentage of Cd removal by the bacterial cells from the culture was calculated by taking difference between the initial metal content in the culture media and at the time of sampling (Sinha and Mukharjee, 2009).

16S rRNA sequence and phylogenetic analysis

The metal resistant isolates were identified using 16s rDNA sequencing. Genomic DNA was extracted from the isolates using CTAB method (Ausubel et al., 1987) and the quality of the product was checked on 0.8% TBE Agarose gel. PCR amplification of 16S rDNA with forward: 5'-AGHGTBTGHTCMTGNCTCAS-3' and reverse: 5'-TRCGGYTMCCTTGTWHCGACTH-3' primers was carried out using 10ng of genomic DNA. The amplification reactions were performed in a 25µl volume. The standardized conditions for PCR based amplification for each 100µl of reaction mixture were: PCR buffer containing 15mM MgCl₂ (10x); 100-200 ng of pure genomic DNA, 1000 pM of each primer, 25µM each of the dNTP's (100µM in a mixture of dNTPs) and 3 units of Taq polymerase. The volume was made up with milli Q water The reaction mixtures were incubated in a thermal cycler (ABI2720, Applied Biosystems) at 94°C for 5 min and then subjected to 35 cycles consisting of 94°C for 30s, 55°C for 30s and 72°C for 1 min15sec. Finally the mixtures were incubated at 72°C for 15 min. The amplified product was resolved on 2.0% agarose gel with a constant voltage of 20V for 1h followed by 50V for 3 h. The gels were stained with ethidium bromide and visualized under gel documentation. Sanger sequences were generated using an ABI 3130 Genetic Analyser (Applied biosystem). Phylogenetic analysis was carried out using distance-based phylogeny reconstruction method called "weighted neighbor joining," or "Weighbor" (Bruno et al., 2000). The trees were then validated by bootstrap.

RESULTS AND DISCUSSION

A total of 30 bacterial isolates were initially isolated from soil samples on LB agar plates. Out of thirty, three bacterial isolates were selected on the basis of cultural characteristics such as color, form, margin, and elevation and named as MA5, MB5 and MC5. A detailed result for colonial morphology, biochemical and physiology characteristics has been given in Table 1.

Table 1 Morphological, biochemical and physiological characteristics of selected isolates

Morphological observation	Bacterial isolates		
	MA5	MB5	MC5
Colony color	Off white	White	Milky white
Gram reaction	Positive	Positive	Positive
Cell shape	Rod	Rod	Rod
Spore formation	positive	Positive	Positive
Elevation	Flat	Flat	Flat
Margin	lobate	lobate	lobate
Biochemical tests			
Catalase test	Positive	Positive	Positive
Starch hydrolysis	Positive	Positive	Positive
H ₂ S production	Negative	Positive	Negative

Growth kinetics of Bacterial strains in the presence of different concentration of cadmium

Growth kinetics of each of the isolates was studied in the presence of cadmium. Results showed that growth was not considerably affected in the presence of cadmium indicating their resistance to cadmium. MB5 showed maximum growth after 120 hours in the presence of cadmium (Figure1).

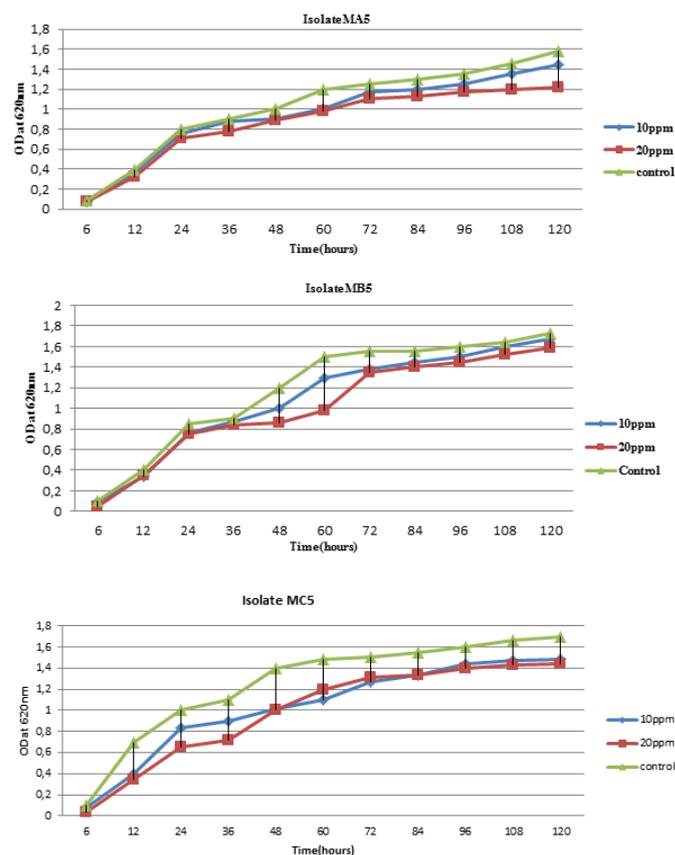


Figure 1 Growth curves of Cadmium resistance bacterial isolates (MA5, MB5 and MC5) in LB medium containing 10ppm and 20ppm concentration.

Minimal Inhibitory Concentration (MIC) of heavy metals

The isolates showed very high degree of resistance to all heavy metals. Minimum inhibitory concentration of Cd for all the isolates was 160ppm. MIC of Hg was 120ppm for MC5, while for the other strains it was 100ppm. MIC of Pb was 180 for all strains. Isolates MA5 and MB5 (100-200ppm) showed to same order of metals toxicity. Among all three bacterial isolates, MC5 showed a very high degree of resistance to heavy metals. The order of heavy metals toxicity towards MC5 was Hg>Pb=Cu>Cd>Ni

Table 2 Minimal inhibitory concentrations (MIC) of various heavy metals to the isolates

Bacterial isolates	Heavy metals(ppm)				
	Cadmium	Lead	Mercury	Copper	Nickel
MA5	160	180	100	180	200
MB5	160	180	100	180	200
MC5	160	180	120	180	200

Antimicrobial susceptibility and Plasmid profile

In order to determine the resistance to antibiotics, the reactions to 12 antibiotics were examined by the disc diffusion method. After incubating for 72 h, the appearance of colonies on plates with antibiotics disc was observed. Depending on the zone of inhibition results were interpreted as susceptible, intermediary susceptible and resistant, as recommended by CLSI. MA5 showed resistance against cefotaxime and levofloxacin. MB5 was resistant to ampicillin, cloxacillin, co-trimoxazole, cephalxin, and cefataxime and sensitive to other antibiotics. MC5 was found resistant to cefotaxime and sensitive to all the other 11 antibiotics.

Table 3 Antibiotic sensitivity for bacterial strains (MA5, MB5, MC5)

Antibiotics	Bacterial isolates		
	MA5	MB5	MC5
Ampicillin(20mcg)	S	R	I
Co-Trimoxazole(25mcg)	I	R	I
Cephalexin(30mcg)	I	R	I
Tetracycline(30mcg)	I	I	I
Cefotaxime(30mcg)	R	R	R
Lincomycin(2mcg)	R	I	I
Ciprofloxacin(5mcg)	S	S	I
Levofloxacin(5mcg)	S	S	S
Linezolid(30mcg)	S	S	S
Cloxacillin(5mcg)	I	R	S
Gentamycin(10mcg)	S	I	S
Roxithromycin(15mcg)	S	S	S

R-resistant, I-Intermediate resistance, S-sensitive

Isolation of plasmid and electrophoresis

All the three bacterial isolates were screened for the presence of plasmid. Plasmid profile (Figure 3) indicates the presence of a mega plasmid (more than 10,000 bp).

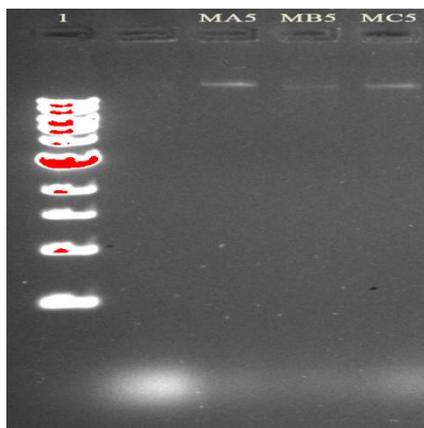


Figure 3 Plasmid DNA was extracted and separated by agarose gel electrophoresis (DNA ladder in lane 1); MA5 is loaded in lane3, MB5 in lane 4, MC5 in lane5.

Cadmium removal assay

The isolates were examined for their ability to remediate the cadmium from aqueous solution. It was observed that the isolates could remove upto 78% of the Cd from medium amended with 100ppm cadmium (Tab 4). Isolate MA5 showed maximum removal of Cd (74 %and 78%), after 24 and 48hours of incubation respectively and isolate MB5 could efficiently remove 70% and 72% of Cd after 24 and 48hours of incubation respectively. In case of MC5, the concentration of Cd was reduced to 74% from an initial concentration of 100ppm in 48 hours.

Table 4 Percentage removal of cadmium by bacterial isolates from medium with initial concentration of 100 ppm cadmium after 24 and 48hours of incubation at 30°C.

Bacterial isolates	Cadmium removal (%)	
	After 24h	After 48h
MA5	74	78
MB5	70	72
MC5	72	74

16S r-RNA sequence and phylogenetic analysis

The genomic DNA (Figure 4) was isolated and amplified with 16S rRNA specific primers. The length of the amplified DNA fragments is about 1.5bp (Figure 5). Sequence analysis of the DNA fragments was performed and compared against the GenBank database using the NCBI Blast program. Selected sequences of other microorganisms with greatest similarity to the 16SrRNA sequences of bacterial isolates were extracted from the nucleotide sequence databases and deposited in GenBank under accession number of KU513823, KU513824, KU513825 for MA5, MB5and MC5 respectively (Tab 5). These 16S rRNA sequences were aligned by MUSLE for construction of phylogenetic tree using neighbor joining method (Saitou and Nei 1987). Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2015).

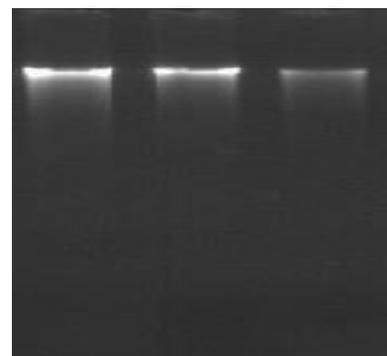


Figure 4 Extraction of Genomic DNA from Bacterial sample using the Bacterial Genomic DNA Isolation Kit (RKN15).

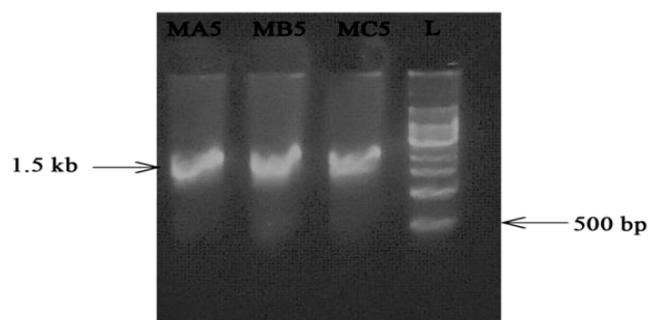


Figure 5 PCR amplification of 16s rDNA fragment from Bacterial sample. The size of PCR amplified product is ~1.5kb.

Table 5 Molecular identification of the isolates based on partial 16S rDNA analysis

Bacterial isolates	Bacterial strain showing maximum homology	Identity (%)	GenBank accession No.
MA5	<i>Bacillus sp. 263ZY1</i>	99%	KU513823
MB5	<i>Bacterium YC-LK-LKJ45</i>	99%	KU513824
MC5	<i>Bacillus subtilis strain DHXJ07</i>	99%	KU513825

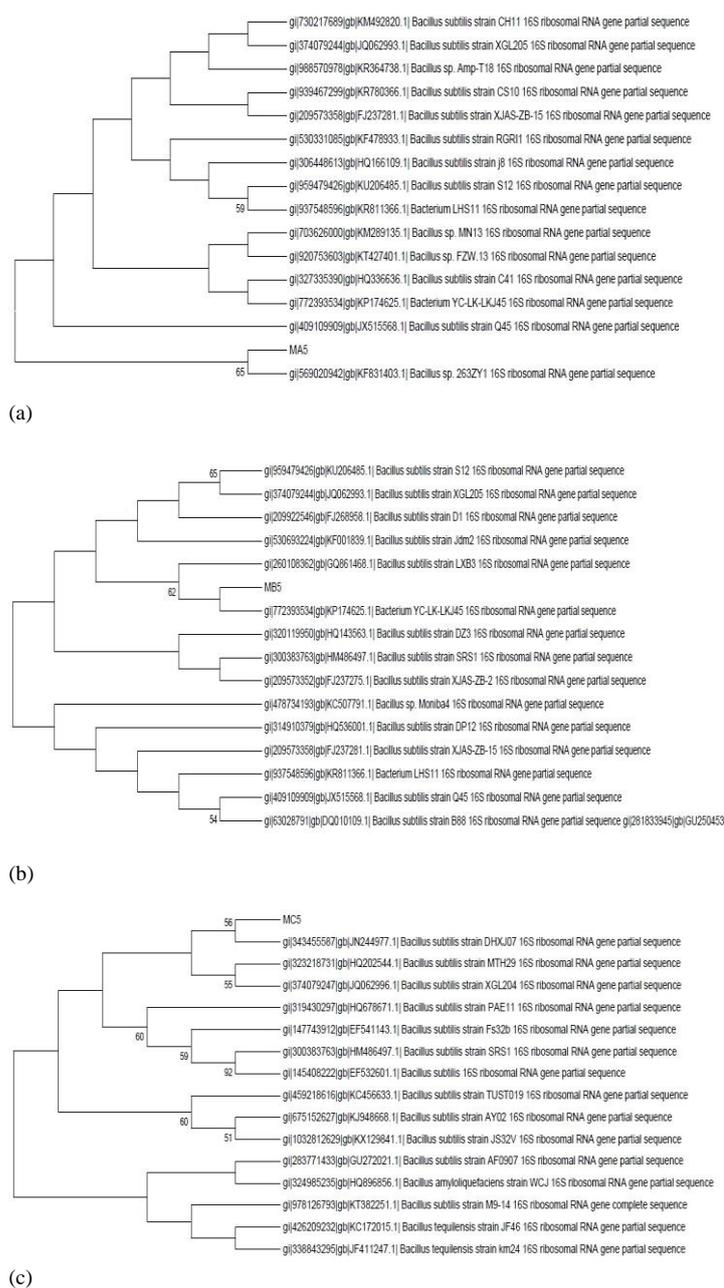


Figure 6 Phylogenetic analysis of the isolates based on 16S rDNA sequence analysis. (a) Phylogenetic tree of isolate MA5, (b) phylogenetic tree of isolate MB5, and (c) phylogenetic tree of isolate MC5.

DISCUSSION

Metal polluted environment contains microorganisms which have adapted to toxic concentrations of heavy metal. Such a microbial population is resilient to metal toxicity by various mechanisms involving bioaccumulation and bioadsorption, oxidation and reduction, methylation and demethylation and extrusion of toxic ions by efflux systems (Bolton and Gorby, 1995). These microorganisms can potentially remove heavy metals from the polluted environment. Many bacterial species, both gram positive and gram negative, have been implicated in bioremediation studies. In the present study, three cadmium resistant bacteria were isolated from industrial soil samples which tolerated high concentrations of Cd²⁺, Pb²⁺, Ni²⁺, Cu²⁺ and Hg²⁺. These isolates were also found to be resistant to many antibiotics. Since there is a documented association between metal resistance and antibiotic resistance and the genes conferring the same are thought to reside on a plasmid, all the three bacterial isolates were screened for the presence of plasmids and it was found that all the isolates harboured the plasmid. Many studies indicate a close association between the types and levels of metal contamination and specific patterns of antibiotic resistance. The mechanisms that underlie this co-selection process include co-resistance and cross-resistance (Baker-Austin et al., 2006). The selected isolates were found to be resistant to a range of heavy metals demonstrating that environmental bacteria may adapt to their ecological conditions and may have

been selected for certain metal tolerance mechanisms. Such bacterial strains may be potential candidates for their simultaneous removal from wastes. Several studies on application of growing microbial cells for metal scavenging have been reported (Malik, 2004). Under lab conditions, these isolates were able to remove upto 70-78% cadmium. A slight difference in phenotypic characteristics was observed among three bacterial isolates, suggesting the presence of different species. The morphological characters and biochemical attributes of isolates MA5, MB5 and MC5 provide evidence to their resemblance with *Bacillus* genera. Further molecular characterization was done to confirm identification. The 16S-rDNA sequencing confirmed identification of MA5, MB5 and MC5 as *Bacillus sp.263ZY1*, *Bacterium YC-LK-LKJ45* and *Bacillus subtilis strain DHXJO*, respectively.

Many studies have implicated *Bacillus spp.* in bioremediation of heavy metals. *Bacillus safensis* strain was found to tolerate cadmium up to the concentration of 80 ppm and the reduction and absorption of cadmium was 83.5, 39% and 98.10, 92% at 40 and 60 ppm of cadmium, respectively at pH 7. Khosro et al. (2011) used different *Bacillus* strains for the removal of heavy metals. The results revealed that maximum cadmium accumulation were 7.3mol/g biomass for *B.licheniformis*, 10.7 mol/g biomass for *B.cereus*, 9.5 mol/g biomass for *B. subtilis* and 7.2 mol/g biomass for *B. amyloliquefaciens*. In another study, *Bacillus subtilis* isolated from industrial effluent soil showed maximum accumulation at 200µg/ml and showed saturation above that concentration (250 µg/ml) (Gayathamma et al., 2013). Significant reduction of soluble Cd was observed during growth of plasmid-bearing *Bacillus* strain H9 and *Pseudomonas* strain H1 (Roane and Pepper, 2000).

The environmental contamination arising primarily from agricultural and industrial sources, as a result of the increase in population, industrial activities, and the modernization of agricultural practices, such as the increasingly widespread use of pesticides, resulting in an increase of the amount of effluents thrown into the air, water, and soil, and have consequences on food quality and human health which are very serious worldwide problems. Bacteria have been so continuously exposed to heavy metal contaminants of the environment that they have developed genetically determined resistance systems against heavy metal toxicity. Contaminated environments like those in the vicinity of industries or industrial dump grounds accumulate a heavy burden of toxic metal ions, organic wastes and antibiotics. A large number of bacterial isolates from these areas have depicted a pronounced capability of processing and resisting toxic industrial wastes. All the three bacteria isolated in this study show a high efficiency in removal of Cd from the medium. They accumulated a significant amount of Cd within 24h. These three strains were belong to *Bacillus spp.* Cadmium uptake is reported to be through an ATPase pump in Gram positive bacteria and by the action of proton-cation anti-porters in Gram negative bacteria (Nies and Silver1995). These capabilities of the selected strains make them suitable for bioremediation of contaminated environment.

CONCLUSION

The present investigation was designed to isolate cadmium resistant bacteria which possessed multiple resistances to various heavy metals and consequently could be used for the simultaneous removal of more than one heavy metal from a contaminated environment. The capability of microbial strains to grow in the presence of heavy metals would be helpful in the waste water treatment. In the present study, bacterial isolates belonging to genera *Bacillus*, showing high degree of resistance to various heavy metals was isolated from soil. These strains were capable of removing upto 78 % of cadmium, signifying that these *Bacillus* strains can be efficiently used for bioremediation and removal of cadmium present in polluted water with minimum cost and high efficiency.

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