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RESEARCH ARTICLE

IDENTIFICATION OF POTENTIAL STRAIN- *BUTTIAUXELLA IZARDII DSM 9397* FOR REMEDIATION OF CADMIUM

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Abstract

The environmental disposal site in particular heavy metals contaminant has been studied in small scale industries located in Mira Bhayander road, Mumbai, India. The physico-chemical and microbial characterization of the waste has been carried out. Physico-chemical parameters studied found to provide favorable environmental conditions to the adapted microorganism. Microbial consortium assessed showed the presence of *Bacillus cereus, Bacillus licheniformis, Cloacibacterium normanense, Bacillus licheniformis, Klebsiela pneumoniae, Buttiauxella izardii, Citrobacter freundii.* Cadmium was exposed to microorganism consortium at concentrations such as 5 mg/l, 25 mg/l, 50 mg/l, 100 mg/l up to 800 mg/l to identify the organism which could survive at a higher concentration. Individual colony found was further identified by sequencing and developing phylogenetic tree based on bioinformatics tools – BLAST. Selected strain *Buttiauxella izardii* DSM 9397, was identified as potential microorganism for remediation of heavy metal- cadmium which will be applicable to decontaminate the environment.

Introduction

Heavy metals contaminants are found in the environment from the various sources like metal based industries, chemical industries, sewage and various processes as well as operations like electroplating, milling, cutting, rubbing, polishing etc. The small scale industries located in Mira Bhayander, Mumbai are carrying out such operations which generate wastes containing heavy metals. There is no proper treatment and disposal of the metal's waste.

The waste generated by each of the small scale industry are being swept, collected and dumped on the waste disposal site. The garbage, garlands and household wastes are also disposed on this site. The microorganisms are also finding a place to grow and multiply in the presence of contaminant due to the open air and nutrients. In the present study the waste disposal site has been characterized for physico-chemical and microbial characteristics. The microbial consortium was further assessed by exposing to cadmium at an increasing concentration to identify the potential organism.

The potential organism has been reported by (Shi *et al.* 2008) for remediation of cadmium. The cadmium remediation would be more effective using the selected identified potential *Buttiauxella izardii* DSM 9397 a selected strain (Sharma and Fulekar 2009).

Further, the potential of this strain was sequenced and identified based on phylogenetic tree and bioinformatics techniques such as BLAST (Sharma and Fulekar, 2008).

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Materials and methods

Soils physico-chemical and microbiological analyses

The environmental contaminated sites located at Mira road, Bhayander have been selected for sampling the soil, sediments for their physico-chemical and microbial characterization. Soil samples were collected from outside the metal industries (Site I, II) and dumping ground (Site III, IV). Samples were collected in sterilized sealed pack polythene bags. The physical and chemical parameters were analyzed as per described in standard methods for the examination of water and waste water, by using 17th edition of APHA methods (APHA, 1979). Soil was air-dried, ground and passed through a 2 mm pore size sieve and was stored in sealed containers at room temperature. Soil physico-chemical parameters organic carbon, total nitrogen, sulfate, phosphate, including biological characterization such as chemical oxygen demand, dissolved oxygen were analyzed (EPA, 1997).

Metals present in the soil were analyzed using nitric acid digestion method. Each soil sample was digested was digested with 10ml of a mixture perchloric acid: nitric acid ($HClO_4$:HNO_3-1:5 v/v) (Lone *et al.,* 2008). Acid digestion was carried out on a hot plate at 70-100˚C until yellow fumes of HNO_3 and white fumes of $HClO_4$ were observed. The digestion process was continued until a clear solution remained after volatilization of acids, and was stopped when the residue in the flask was clear and white. The digested sample was dissolved in distilled water, filtered through Whatman no.1 filter paper to remove impurities and made up to the desired volume (APHA, 1979).

One gram of each sample was immediately used for microbial count. The enumeration of bacteria and fungi was done according to a standard procedure (Kumar, 2004). Briefly, 1gm of soil was mixed with 10ml of sterile distilled water. An aliquot of 0.1 ml of dilutions for each soil samples was spread plated onto agar plates from the appropriate dilution tubes and incubated at room temperature. The bacterial colonies were counted after every 24 hrs of incubation at 37˚C, on minimal salt media. Only the plates showing between 25 to 300 colonies were tallied, and the results were averaged for each soil samples. The fungal colonies were counted after 48-72 h at 37˚C, on minimal salt media. Samples were preserved at 4°C for further microbial analysis (Collins, 1985). The isolates were then identified based on the morphological (cultural and microscopic) and biochemical characteristics following Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). The specialized agar medium was used for identifying bacterial strains as *E. coli, Salmonella* sp., *Shigella* sp., *Vibriocholerae* sp., *S. aureus, Clostridium* sp., *Pseudomonas* sp., *Streptococcus faecalis, Serratia* sp., and fungal strains (yeast and mould) (Klinge, 1960). Isolated colonies were further analyzed using specialized agar /16S rRNA sequencing. Other microorganisms present were identified using 16SrRNA sequencing were also studies.

Strains selection based on cadmium tolerance

Soil and sediment samples were further characterized for microbial analysis and to identify the potential microorganism responsible for cadmium bioaccumulation. Soil and sediments were serially diluted to 10,000 folds and plated on nutrient agar media.

A volume of 1ml bacterial culture was inoculated in nutrient broth and further, in 250 ml Erlenmeyer flasks containing 100 ml minimal media with a metal concentration of 5mg/l. Minimal media comprised (g/l): glucose 10; NH_4Cl 2.67; Na_2HPO_4, 5.35, amended with 6 ml mineral salts solution (CaCl2·2H20, 0.1 g; MgSO4·7H2O, 10 g; MnSO4·7H2O, 0.07 g; FeSO4·7H2O, 0.4 g; distilled water, 1000 ml). Cadmium was added from 1000 ppm stock solution of $CdNO_3{\cdot}4H_2O$ (2.74 mg/l). Flasks were kept in incubator shaker at 37°C, for 200 rpm. The pH was adjusted to 7.0 before inoculation. The pH and optical density at 600 nm were measured daily to analyze bacterial growth. Further, a volume of 1ml bacterial culture was transferred in solution containing cadmium concentration of 25 mg/l, and subsequently to 50 mg/l, 100 mg/l up to 800 mg/l to get potential microorganism.

A volume of 100 µl of samples of each concentration was plated on minimal media containing metal solutions of particular respective concentration. Single colony was isolated at higher

concentration and identified as potential microorganisms for cadmium tolerance ability.

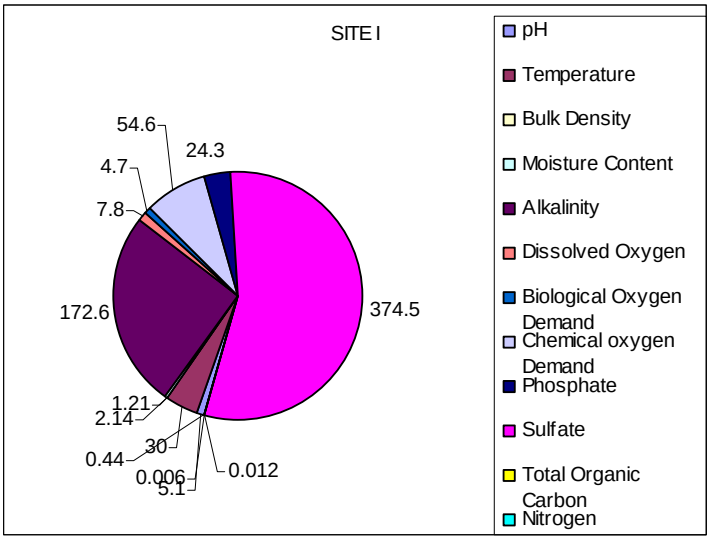
Bacterial strains characterization by genetic tools

The selected strains able to cadmium bioaccumulation at high potential were identified by 16SrDNA technique followed by sequencing and bioinformatics tools. The 16SrRNA analysis was done by using predetermined universal primers of 16SrRNA. DNA isolated from pure culture was used as template. PCR was performed with a 50µl reaction mixture containing primer 16S, DNA template buffer, MgCl2, dXTPs, Taq polymerase. PCR products were analyzed by electrophoresis in 1.8% agarose gel. PCR program was carried out in PTC-200 Peltier thermocycler which comprises of three steps; 1) Denaturation at 94ºC, for 1minute; 2) Annealing at 55ºC, for 1minute; 3) Extension at 72ºC, for 1minute (Bosshard *et al.,* 2003). The RNA sequences were compared with already submitted sequence in database using BLAST software. Further, most similar sequences were aligned by ClustalW and ClustalX software and phylogenetic tree was drawn using PHYLIP software to analyze evolutionary relationships among sequences of isolated microorganism and nearest neighbors (Fulekar, 2008).

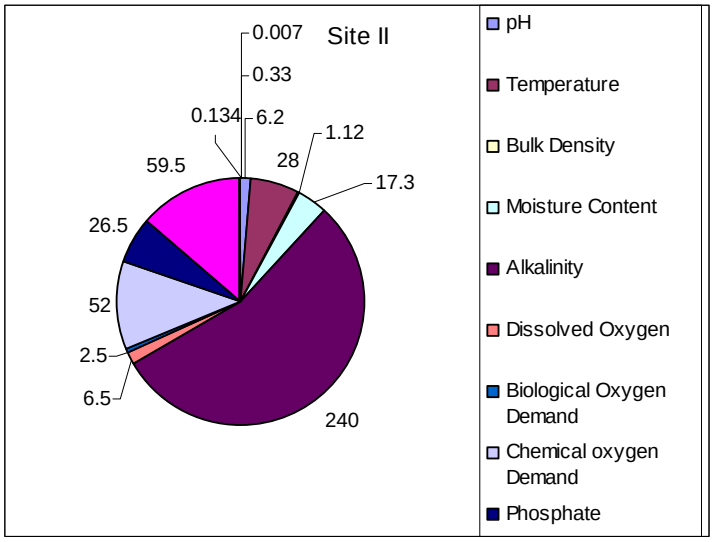
Results and discussion

Heavy metals contaminated soils has been assessed for characterization of physico-chemical properties and microbial consortium ability to cadmium bio accumulation. The microorganisms adapted in the contaminated environment change their proteomic and genomic characteristics and become versatile and develop potentiality for the survival in the contaminated site based on the nutrients available and bioaccumulation and biotransformation of heavy metal as contaminants. Heavy metals present in the contaminated soils are remediated by ability virtue of microbial consortium to action on the contaminants so as to obtain their food-nutrient (Sharma and Fulekar, 2009).

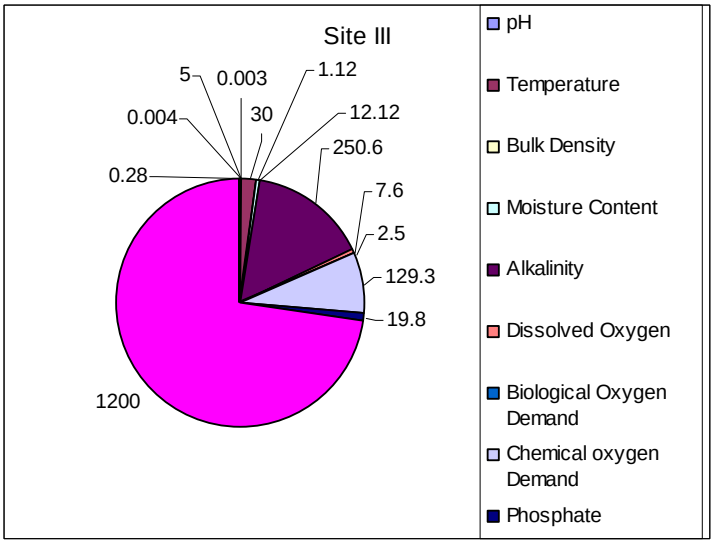
The physico-chemical parameters of soils studied are presented in figure 1 and table 1. The presence of phosphate (19.8-26.5mg/l), sulfate (59.5-1200 mg/l), nitrogen (0.003-0.4mg/l), BOD (2.5-5.5mg/l), COD (22-129.3mg/l) shows the favorable conditions i.e. supply of nutrients for the growth and multiplication of the microbial consortium. The microbial consortium assessed is presented in table 2. In the soils microbiota were found strains of species *Bacillus cereus, Bacillus licheniformis, Cloacibacterium normanense, Bacillus licheniformis, Klebsiela pneumoniae, Buttiauxella izardii, Citrobacter freundii.* The isolated strains were exposed to heavy metals with special reference to cadmium at increasing concentrations such as 5 mg/l, 25mg /l, 50 mg/l, and 100 mg/l up to 800 mg/l using scale up process technique by providing the minimal salt medium in controlled environment conditions of submerged cultivation.



a)



b)



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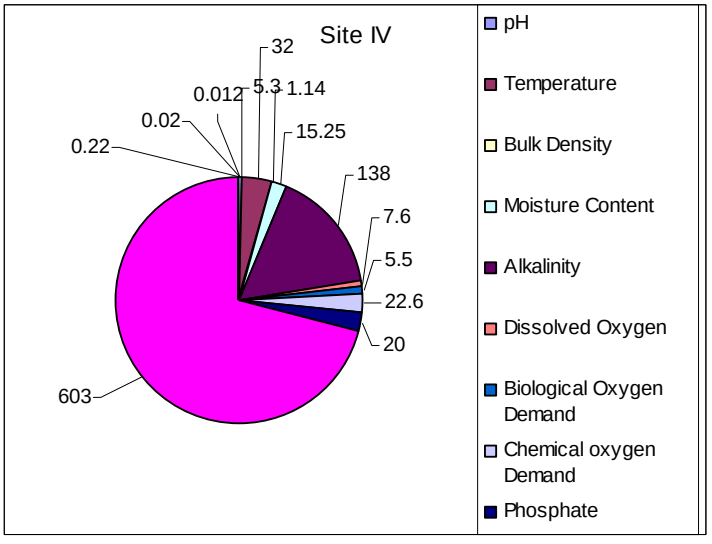

d)

Figure 1. *Physico-chemical parameters of studied soil sites: a) site I; b) site II, c) site III; d) site IV*

Table 1. *The concentration of metals in contaminated studied area*

Metals	Samples				
	I	II	III	IV	Avg.
Copper, mg/l	9.54	8.54	10.4	13.92	10.6
Iron, mg/l	10.6	7.6	9.9	15.8	10.98
Cadmium, mg/l	20.02	19.87	21.3	26.81	22.0

Table 2. *Quantitative characterization of soils microbiota*

Indicator number	Microbiological parameters	Specialized culture media	Level of cells in soils from:	
			Sample I	Sample II
1.	Total viable count, UFC*/g	Mac Conkey Broth	$4.2\ 10^4$	$6.9\ 10^4$
2.	Total coliform count, UFC/g	Plate Count Agar	$9.8\ 10^2$	$13.6\ 1^{03}$
3	Total yeast and mould count, UFC/g	Violet Red Bile Agar	$10.3\ 10^2$	$11.3\ 10^2$
4	*Pseudomonas* sp., UFC/g	Eosin Methylene Blue Agar	Present	Absent
5	*Escherichia coli* count, UFC/g	Sabourauds Chloramphenicol Agar	absent	Absent
6	*Clostridium* sp. count, UFC /25g	Baird Parker Agar	Absent	Absent
7	*Vibrio cholorae* count, UFC /25g	*Clostridium Botulinum* Isolation Agar	Absent	Absent
8	*Salmonella* sp., UFC /25g	TCBS Agar	Absent	Absent
9	*Staphylococcus aureus*, UFC /25g	Cetrimide Agar	Absent	Absent
10	*Shigella* sp., UFC /25g	Slanetz and Bartley Medium	Present	Present
11	*Streptococcus* sp., UFC /25g	Bismuth Sulphite Agar	Present	Present

*UFC -Units Forming Colony

The organism which has been found to survive at a higher concentration was further identified by sequencing, developing phylogenetic tree. The biochemical assays has also been done for identification of selected strains (table 3).

Based on sequencing, phylogenetic tree using bioinformatics tools such as BLAST as well as biochemical assays identified strain as *Buttiauxella izardii* (figure 2).

The database sequence available for known organisms compared with this identified organism also confirmed as *Buttiauxella izardii*. Therefore the identified organism has been coded as *Buttiauxella izardii* DSM 9397 (figure 3).

This organism has been reported as a potential for bioaccumulation and remediation of cadmium (Shi *et al*., 2008) the research findings has shown that microorganism adapted in the contaminated site if identified, cultured and characterized will become a significant source to use as a biomass for bioremediation of heavy metals.

The microbial remediation of heavy metals using the biomass metal accumulation potential will contribute to decontamination of the metal polluted environments.

Table 3. *Biochemical properties of the selected strain Buttiauxella izardii DSM 9397*

Characteristics	Result	Characteristics	Result
ONPG(β galactosidase)	+	Adonitol production	-
Lysine metabolism	-	Rhamnose metabolism	+
Ornithine metabolism	-	Voges-Proskauer	-
Urease	V*	Cellobiose metabolism	-
TDA (Target detection Assay)	-	Melibiose metabolism	-
Nitrate metabolism	+	Rhaffinos metabolism	-
H_2S production	V	Trehalose metabolism	-
Citrate utilization	-	Glucose metabolism	+
Methyl red	+	Lactose metabolism	V
Malonate metabolism	-	Rhaffinos metabolism	-
Arabinose metabolism	V	Trehalose metabolism	-
Xylose metabolism	+		

*Variance

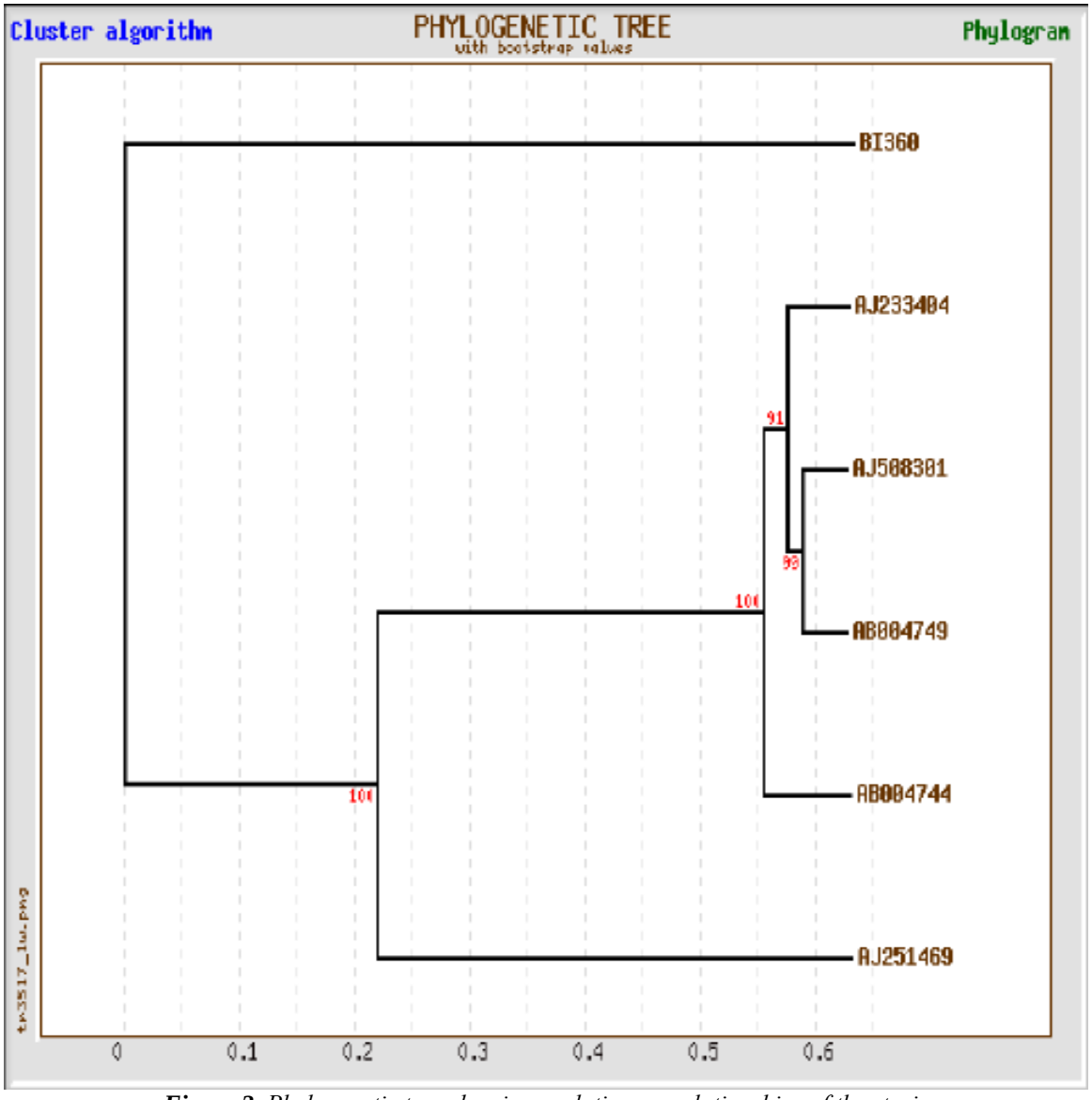

Figure 2. *Phylogenetic tree showing evolutionary relationships of the strain Buttiauxella izardii*

GCTTGCGGGGTTCCTACATTGGAAGTCGAGCGGTAGCACAGAGAGCTTGCTC
TCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGG
GATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGG
ACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTA
ACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGA
ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG
GCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCA
CTTTCAGCGGGGAGGAA
GGCGTTAAGGTTAATAACCTTGGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAA
CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGG
CGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTG
GGAACTGCATTCGAAACTGGCAGGCTAGAGTCTT
GTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGACCTGAGGAATA
CCGGTGGCGAAGCGGCCCCTGGACAAAGACTGACGCTCAGGTCGAAACCTGGGGAGC
AAACAGGATAATACCCGGTAGCCCACCC

Figure 3. *The 16SrDNA sequence of Buttiauxella izardii, strain coded DSM 9397 selected based on their potential of cadmium tolerance and bioremediation*

Table 4. *Hit list and classification of the nearest neighbors of the bacterial strains by using genetic tools*

Sample	Gene bank entry	Domain	Phylum	Class	Order	Family	Genus	Species
BI 360	AJ508301	*Bacteria*	*Proteo-bacteria*	*Gammaproteo-bacteria*	*Entero-bacteriales*	*Entero-bacteriaceae*	*Buttiauxella*	*izardii*
	AJ251469	*Bacteria*	*Proteo-bacteria*	*Gammaproteo-bacteria*	*Entero-bacteriales*	*Entero-bacteriaceae*	*Enterobacter*	*cloacae*
	AB004744	*Bacteria*	*Proteo-bacteria*	*Gammaproteo-bacteria*	*Entero-bacteriales*	*Entero-bacteriaceae*	*Enterobacter*	*kobei*
	AB004749	*Bacteria*	*Proteo-bacteria*	*Gammaproteo-bacteria*	*Entero-bacteriales*	*Entero-bacteriaceae*	*Enterobacter*	*amnigenus*
	AJ233404	*Bacteria*	*Proteo-bacteria*	*Gammaproteo-bacteria*	*Entero-bacteriales*	*Entero-bacteriaceae*	*Enterobacter*	*asburiae*

Conclusions

The presence of physical parameters pH, mesophilic temperature as well as the nutrients such as phosphorus, sulfate, and nitrogen proliferated growth of the microbial consortium present in the metal contaminated waste disposal site. The identified potential organism for bioaccumulation/biotransformation/bioadsorption has significance for remediation of the metals from the contaminated environment.

The present study deals with the evaluation of the potential of selected bacterial strain *Buttiauxella izardii* DSM 9397 to cadmium bioremediation from metal contaminated environment. The strain *Buttiauxella izardii* DSM 9397 has been identified by biochemical assays, sequencing and developing phylogenetic tree based on bioinformatics techniques such as BLAST, ClustalW and FASTA. Strain *Buttiauxella izardii* DSM 9397 would serve as a significant role for bioaccumulation and remediation of heavy metals for environmental cleanup.

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