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Potential of *Citrobacter freundii* for bioaccumulation of heavy metal - copper

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Abstract
The small scale industries located at Mira Road, Mumbai, are engaged in various processes/operations which generate the wastes. The waste generated are collected and disposed of at the dumping ground near the industrial estate. In the present study, the physico-chemical and microbial status of the contaminated sites has been carried out. The heavy metal with special reference to copper was exposed to microbial consortium at increasing concentrations viz 5, 25, 50, 75, 100 up to 800ppm to isolate potential microorganism for bioremediation. *Citrobacter freundii* has been identified by 16SrDNA technique as potential microorganism for bioaccumulation/bioremediation of copper. This organism can be used for remediation of copper from contaminated environment.

Keywords: Bioremediation, bioaccumulation, heavy metals.

Introduction

The metals found in the environment come from the natural weathering processes of earth's crust, soil erosion, mining ,industrial discharge, urban runoff, sewage effluents, air pollution fallout ,pest or disease control agents applied to plants and other sources. Since the industrial revolution the use of metals is a mainstay of the economy of many developed countries, including the United States (Sharma et al. 2000). However, with the increasing of mining for metal ores, health and exposure risks to workers and the general public has become an increasing concern. The improvement has been made by various techniques such as exacavation and landfill, In situ vitrification, solidification & stabilization, In situ redox manipulation, soil washing , soil flushing etc. in reducing the level of general environmental pollution problems. In spite of the present treatment technology, several heavy metals, such as lead, cadmium, copper and mercury are found persisting in the environment (Fulekar, 2005a).

The small scale industries located at Mira Road, Bhayander (Mumbai) involves various processes and operations such as grinding, mixing, plating, washing etc. The wastes generated from these processes/operations are disposed of at the disposal ground located near to these industries. The characterization of the wastes including the microbial studies is important to identify the potential microorganism to develop a strategy for the decontamination of the environment. In the present study, besides physico-chemical status of the contaminant, 16SrDNA technique has been used to develop phylogenetic tree for identification of microorganism responsible for bioaccumulation of heavy metals with special reference to copper.

Material and Methods

The environmental contaminated sites located at Mira Road, Bhayander (Mumbai) have been selected for sampling the soil, sediments for their physico-chemical and microbial characterization. Samples were collected in sterilized sealed pack polythene bags. The physical and chemical parameters were analyzed as per Standard Methods for the Examination of Water and Waste Water, 17th edition, APHA (1989).

Soil analysis

Soil was air-dried, ground and passed through a 2mm pore size sieve and was stored in sealed containers at room temperature. Soil physico-chemical parameters, biological characterizations were analyzed (EPA 1997).

Microbial analysis

One gram of each sample was immediately used for microbial enumerations. 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 on to agar from the appropriate dilution tubes and incubated at room temperature (Collins, 1985). The bacterial colonies were counted after every 24 hrs. 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. Samples were preserved at 4°C for further microbial analysis (Chao et al. 2003). Isolated colonies were further analyzed using specialized agar / 16SrDNA sequencing. The isolates were then identified based on the morphological, cultural and biochemical characteristics following Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). The specialized agar (from Hi media) was used for identifying *E. coli, Salmonella, Shigella, Vibriocholerae , Yeast and Mould, S. aureus, Clostridium, Pseudomonas , Streptococcus faecalis, Serratia* (Klinge 1960). Other microorganisms present were identified using 16SrDNA sequencing.

Metal analysis

Each soil sample 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 70-100C 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).

Isolation of potential microorganism

Soil and sediments were serially diluted to 10,000 folds and plated on nutrient agar. 1ml bacterial culture was inoculated in nutrient broth and further, in 250ml erlenmeyer flasks containing 100ml minimal media with a metal concentration of 5mg/l. Minimal media comprised of Na_2HPO_4, NH_4Cl, glucose blended with 0.6ml of trace elements solution. Trace elements solution contains $CaCl_2.2H_2O$, $MgSO_4$, $MnSO_4.7H_2O$, and $FeSO_4.7H2O$. Copper was added from 1000mg/l stock solution of $CuSO_4.5H_2O$. Flasks were kept in incubator shaker at37°C, 200rpm. pH was adjusted to 7.0 before inoculation. pH and optical density at 600nm were measured daily to analyze bacterial growth. Further, 1ml. bacterial culture was transferred to metal concentration of 25mg/l, and subsequently to 50mg/l, 100mg/l upto 800mg/l to get potential microorganism. 100µl of samples of each concentration were plated on minimal media containing metal solutions of respective concentration. Single colony was isolated at higher concentration and identified as potential microorganism for bioaccumulation copper.

Identification of isolated microorganism by 16SrDNA analysis

16SrDNA analysis was done by using predetermined universal primers of 16SrDNA. DNA isolated from pure culture was used as template. PCR was performed with a 50µl reaction mixture containing primer 16S, DNA template buffer, $MgCl_2$, 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).

DNA 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

The present research study has been carried out at the waste disposal site located at Mira Road, Bhayander, Mumbai. The small scale industries carried out the various processes and operations which generates wastes containing metals. The potential microorganism has been identified from the microbial consortium present in the waste disposal area for remediation of the metals. The physico-chemical parameters studied include: -pH, Temperature, Moisture content, Bulk density, Phosphate, Sulfate, Total Organic Carbon, Biological Oxygen Demand, Chemical Oxygen Demand, Total Nitrogen, Alkalinity, Total Organic Matter. The concentrations of each parameter obtained are presented in table 1.

Table 1: Physical and chemical parameters

Sr. No.	Parameters	Sample				Avg.
		I	II	III	IV	
1.	pH	5.1	6.2	5	5.3	5.4
2.	Temperature	30	28	30	32	30
3.	Odor	Pungent	Odorless	Earthy	Pungent	Pungent
4.	Color	Black	Brownish	Black	Black	Black
5.	Bulk density	1.21	1.12	1.12	1.14	1.15
6.	Moisture content	2.14	1 7.3	12.12	15.25	11.71
7.	Alkalinity	172.6	240	250.6	138	200.3
8.	Dissolved oxygen	7.8	6.5	7.6	7.6	7.4
9.	Biological oxygen demand	4.7	2.5	2.5	5.5	3.8
10.	Chemical oxygen demand	54.6	52	129.3	22.6	64.6
11.	Phosphate	24.3	26.5	19.8	20.0	22.65
12.	Sulfate	374.5	59.5	1200	603	559.3
13.	Total organic carbon	.006	.007	.003	.012	.007
14.	Nitrogen	0.44	0.33	0.004	0.02	0.20
15.	Total organic matter	0.012	0.134	0.28	0.22	5.6

Table 2: Microbial characterization of soil

Sr. No.	Parameter	Specialized Agar	Results	
			(Sample I)	Sample II
1.	Total Viable count/g	Mac Conkey Broth	42000	69000
2.	Total coliform count	Plate Count Agar	980	1360
3	Total yeast and mould count/g	Violet Red Bile Agar	1030	1130
4	Pseudomonas	Eosin Methylene Blue Agar	Present	Absent
5	E.coli count	Sabourauds Chloramphenicol Agar	absent	Absent
6	Claustridia count/25g	Baird Parker Agar	Absent	Absent
7	Vibrio cholorae count/25g	Clostridium Botulinum Isolation Agar	Absent	Absent
8	Salmonella/25g	TCBS Agar	Absent	Absent
9	S.aureus/25g	Cetrimide Agar	Absent	Absent
10	Shigella/25g	Slanetz and Bartley Medium	Present	Present
11	Streptococcus/25g	Bismuth Sulphite Agar	Present	present

Table 3: Biochemical characterization of *Citrobacter freundii*

Parameters	Result	Parameters	Result
Lactose	d	Indole	-
Sucrose	d	Methyl red	+
Manitol	+	Voges-proskaver	-
Dulcitol	d	Simmonus citrate	+
Salicin	d	Hydrogen sulfide	-
Adonitol	-	Urease	dw
Inositol	-	KCN	+
Sorbitol	+	Gelatin	-
Arabinose	+	Lycin decarboxylase	-
Raffinose	d	Arginine dehydrolase	d
Rhamnose	+	Ornithine decarboxylase	d
		Phenylalanine deaminase	+

Table 4: Metals present in contaminated 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

Figure 1: 16SrDNA sequence of *Citrobacter freundii* - Sequence BI359

>BI359

GGGTCGCGGGCTAAAACATGCAAGTCGAACGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGTGGCGGACGGGTGA
GTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGAC
CAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCA
CCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGG
GTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCACAGCAATTGACGTTACCCGCAGAAGAAGC
ACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTGGA
GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGC
GGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACGGATTAAAACGCGGGGGGTCCACAAA

Figure 2: Phylogenetic Tree showing evolutionary relationships of *Citrobacter freundii*

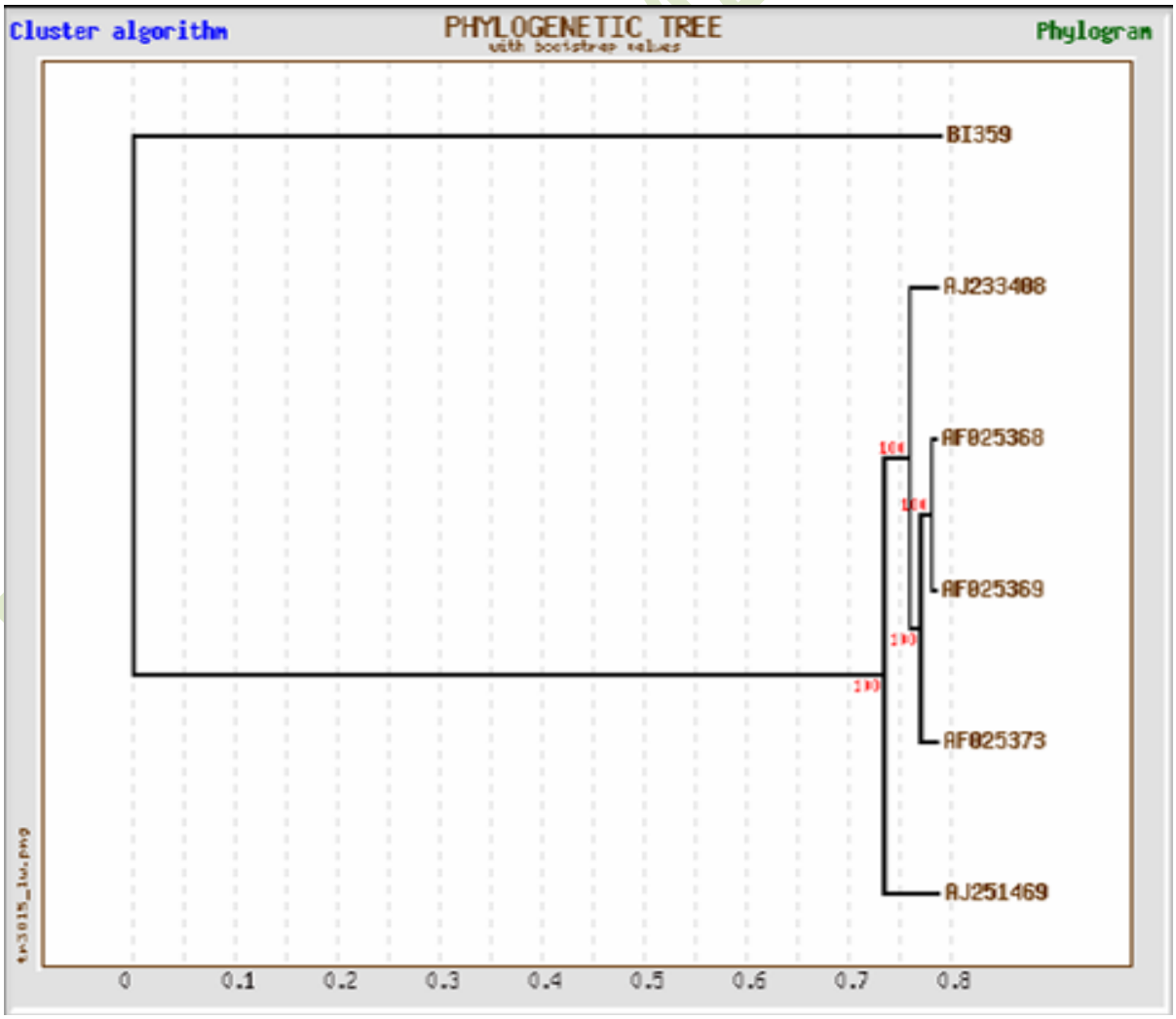

Table 5: Hit list and classification of the nearest neighbors

	Gene Bank Entry	Domain	Phylum	Class	Order	Family	Genus	Species
Sample BI 359	AJ233408	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Citrobacter*	*freundii*
	AF025368	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Citrobacter*	*braakii*
	AF025369	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Citrobacter*	*murliniae*
	AF025373	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Citrobacter*	*cloacae*
	AJ251469	*Bacteria*	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Enterobacter*	*werkmanii*

Microbial characterization studied is presented in Table 2. Microbial characterization of soil/sediment sample shows that *S. aureus*, *Clostridia, Pseudomonas, streptococci, salmonella* and *shigella* are the prominent microorganisms present in environmental contaminated area of the small scale industries. The concentrations of Sulphate (559.3mg/l), Phosphate (22.65 mg/l), Nitrogen (0.20mg/l) show that the microbial consortium has the sufficient amount of supply of nutrients that made it possible for them to survive and grow in the metal contaminated environment.

The physical characterization like pH was found ranging from 5 to 6.2 with an average value 5.4. The temperature was found to have mesophilic condition for the microorganisms to survive and grow. The moisture condition (Average value: 11.71) was found suitable for the microbial population (Fulekar 2005(b)). The biological conditions such as Dissolved oxygen (7.4mg/l), Biological oxygen demand (3.8mg/l), and Chemical Oxygen Demand (64.6mg/l), were also found suitable for the microbial activities in the contaminants site that makes the microbial consortium to bioaccumulate the metals and remediate the contaminants. Metals concentration studied in waste disposal site has been presented in table 4.

In order to identify the potential microorganism from the microbial consortium, the microbial consortium was exposed to increasing concentration of copper from 5mg/l,25mg/l, 50mg/l, 75mg/l up to 800mg/l. Microbial culture was grown in Minimal salt medium in 250ml Erlenmeyer flask Flasks were kept on shaker incubator at 37ºC on 200rpm. pH and optimum density was checked to analyze bacterial growth. 100µl of samples of each concentration were plated on minimal media containing metal solutions of respective concentration. Single colony was isolated at higher concentration and identified as potential microorganism i.e. *Citrobacter freundii*. Potential microorganism was characterized by biochemical tests first (Table 3). Bacterial colony resistance at higher concentration i.e. at 800 ppm was further identified and confirmed by 16SrDNA technique and drawing Phylogenetic tree (Fig. 1 & 2) using PHYLIP software. Phylogenetic analysis indicated that isolated microorganism is a strain of *Citrobacter freundii*. Hit list and classification obtained after phylogenetic analysis has been given in table 4.

Citrobacter freundii has been listed and documented as a potential microorganism for remediation of copper. This potential organism can be used for bioremediation of heavy metals to cleanup the environment (Macaskie 2006).

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