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Microbial Assisted Reduction of Lead by River Isolate

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ABSTRACT

The present study deals with isolation and identification of heavy metal lead reducing microorganism from Mula-Mutha river, Pune, India. A strain was characterized based on its morphological and biochemical screening test. It was confirmed as *Bacillus cereus* ATCC 14579 by MALDI-TOF and 16S rRNA sequencing. The selected isolate successful in reducing lead up to 500 ppm. It showed maximum reduction potential up to 89 % in supernatant and 88% in pellet. The reduction of lead by that isolate was estimated by using atomic absorption spectroscopic studies. The isolate showed extracellular as well as intracellular bioaccumulation mechanism for the reduction of lead.

Key words: *Bacillus cereus* ATCC 14579, Bioreduction, Lead, Heavy metal.

1. INTRODUCTION

Around the globe the developing countries are facing the problem of heavy metal pollution. The persistent and non-degradable nature of heavy metal cause a serious threat to human health and the accumulation of these metals at different levels of the food chains also adds threat to plants, animals, aquatic life, and humans. Heavy metal ions in water are characterized by their toxicity, mobility to living beings even at low concentrations. Heavy metals cause significant environmental problems by their presence in water and soil, further which is aggravated by different anthropogenic activities. These anthropogenic activities convert metals into various forms that are highly toxic and persist for longer time in the environment [1].

Heavy metals refer as the metals which having specific gravity greater than 5.0 (or density 5.0g/cm3). There are 23 types of heavy metals which can have ill-effects because of exposure are: Au, Ga, V, U, Zn, Sn, TI, Te, Ag, Pt, Ni, Hg, Mn, Pb, Fe, Co, Cu, Cr, Ce, Cd, Bi, As, Sb. Since the toxicity of a metals is linked with its different forms, it is worthwhile to know about different forms that are found in water bodies [2]. The heavy metals concentration in water bodies is increasing day by day. Even at low concentrations some heavy metals are highly toxic to human health and cause adverse effects on environment. These metals are silent, subtle, and stalking killers. The heavy metals like Fe, Mo and Mn have low toxicity while Zn, Ni, Cu, V, W, Cr, CO having average toxicity and some such as Sb, Cd, Hg, Pb, U, Ag are highly toxic. Toxic effects of some heavy metals on humans are given below (figure 1.)

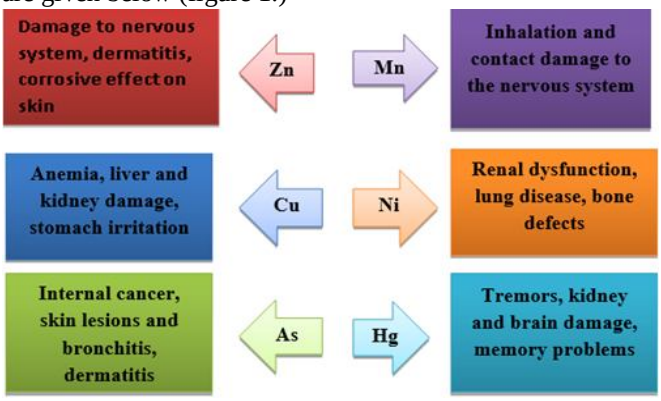

Figure 1: Effects of various heavy metals on human health.

lead is one of the non bioessential heavy metal which persist in environment for longer period and shown ecotoxicity in humans. Exposure to lead can result in wide range of biological effects which basically depends on the level and durations of exposure [3]. When exposure rate is high ultimately it results in toxic biochemical effects in humans which in turns cause problems in the synthesis of hemoglobin, effects on joints, reproductive system, kidneys, gastrointestinal tract and chronic or acute damage to nervous system. Even at minute concentrations lead being toxic, it is regarded to be one of the most toxic pollutants with primary sources from metal smelting industries, plumbing pipes, and manufacturing of insecticides [4]. Some natural processes like volcanic emissions, soil erosion and mineral mobilization also

cause environmental contamination of lead. Heavy metals pollution occurs directly by effluent that comes out from industries, wastewater treatment plants and refineries and indirectly by some contaminants that enter water supply from ground water system, soils, and from atmosphere via rainwater [5]. The small concentrations of heavy metal reduce food quality and crop production due to the excessive application of agricultural inputs like pesticides, fertilizers and have resulted in heavy contamination of soil.

There are various techniques developed to remove heavy metals from environment but none of them are that much effective. Various conventional methods are developed for removal of heavy metals, such as reverse osmosis, filtration, chemical precipitation, membrane technology etc. but these methods are not eco-friendly and expensive too [6], the one major disadvantage of these methods is production of sludge, and these methods are ineffective if there is low concentration of heavy metals. So, there is need to develop the eco-friendly and low-cost effective methods. The biological methods are the best alternative to physio-chemical methods for removal of heavy metal from environment [7], these methods are bioaccumulation, bioventing, biosorption, phytoremediation etc.

Bioremediation is type of biological method that remove organic waste by using microorganisms such as bacteria, fungi, these microbes convert the organic waste into non-toxic or less toxic form. These methods are effective when surrounding environment promotes the growth of microbes and optimal activity. There are different factors that affect the rate of bioremediation such as the degradation ability of bacteria, then environmental factors like temperature, pH, the anaerobic and aerobic conditions, nutrients. Some researchers isolated the several bacterial strain which showed the great ability of bioremediation [8]. We can used bacteria for removal of one metal or mixture of metals. Usually, the strain has good reduction potential towards multiple heavy metals are of great interest. So, there is need to isolate such type of bacteria which reduces multiple heavy metals becomes very important.

Table 1: Microorganisms used for bioremediation of Lead

Types of microorganisms	Sources	Concentration of lead	Mechanism	References
Bacteria				
Stenotrophomonas sp.	Hattar industrial state Pakistan	200 ppm	Bioaccumulation	[9]
Bacillus thuringiensis	Jammu and Kashmir	900 ppm	Bioremediation	[10]
Enterobacter cloacae	South Korea		Biomineralization	[11]
Cellulosimicrobium sp. (KX710177)	Lucknow	200 ppm		[12]
Fungi				
Aspergillus niger RH18	Rawalpindi, Pakistan	7007.9 ppm	Biosorption	[13]
Aspergillus terreus		100 to 1000ppm	Bioremediation	[14]
Penicillium chrysogenum	Nigeria	4.19 ppm	Biosorption	[15]
Algae				
Nannochloropsis oculata	Jepara, Indonesia	1.3 ppm	Bioremediation	[16]
Scenedesmus sp.		30000 ppm	Biosorption	[17]

2. MATERIALS AND METHODS

2.1 Collection of samples

Water sample collection was done from Mula-Mutha river, Pune, India (Figure 2). Sample was collected in pre-sterilized bottle and transported to the laboratory aseptically. Then sample was stored at 4 ^{o}C until further examination.

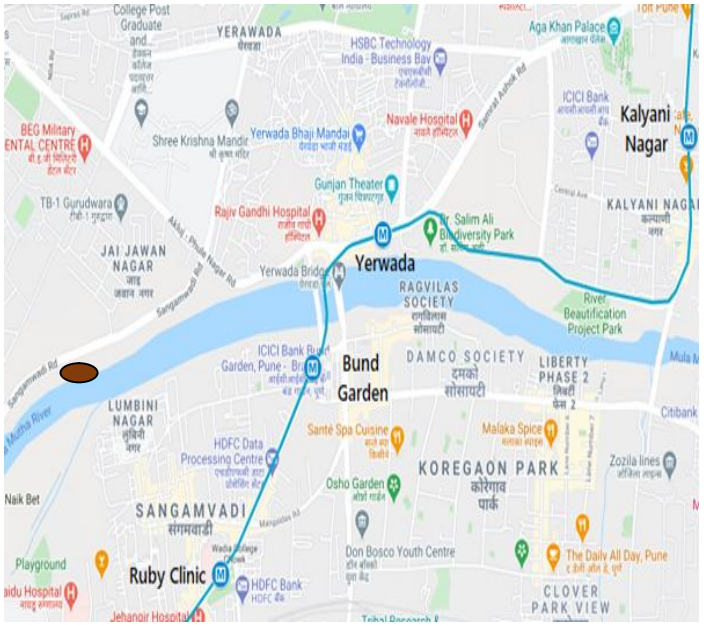

Figure: 2 Study area map indicates sample collection site

Figure 3: Mula-Mutha river

2.2Screening and isolation of bacteria

The lead reducing microorganism was isolated on LB (Luria Bertine) agar plates incorporated with 100 ppm of lead acetate (use as sources of lead). Firstly, the LB agar was sterilized at 121 ^{o}C for 15 min., after cooling the heavy metal lead (lead acetate) was added to agar then poured into petri plates. The water sample was serially diluted for that 9ml saline was taken in 6 test tubes and then 1ml sample was added to first tube to make dilution 10^{-1}repeat the same process up to reached dilution 10^{-6}, then 0.1ml of dilution was spread on LB agar plates and incubated at 37 ^{o}C for 24 hrs. after incubation the colonies that are differing in morphology were selected for further studies and subculture on same media. After primary screening of lead reducing microbes for isolation of purified colonies streak plate technique was used. Control plates also prepared with LB media without including lead to make comparison.The colonies which were differing morphologically were selected for further studies and sub cultured on the same media.

2.3 Minimum inhibitory concentration (MIC)

To determine the MIC values of the selected isolate, the isolate was cultured on LB agar plates with varying concentrations of Lead that is 100 ppm, 200 ppm, 500 ppm. For AAS analysis the isolate showed the highest MIC values was selected.

2.4 Morphological and Biochemical characterization

The selected isolate was studied for its morphological and biochemical characterization, The morphological characteristics included shape and the colour of the microbial colonies. The gram staining was also done and observed under the microscope. Then the isolate was biochemically tested for the activities of oxidase, catalase, nitrate reductase, urease, and indole production along with citrate utilization test. The isolate was provisionally identified up to genus level by following the Bergey's manual of systemic bacteriology [18].

2.5 Molecular characterization

Molecular identification has been carried out using MALDI-TOF MS technique and MALDI Biotyper database [19]. And 16S rRNA sequencing and phylogenetic analysis. The 16s rRNA gene was amplified by PCR using universal primers: 27F (5'AGAGTTTGATCCTGGCTCAG-3') and 149R (5'- GGTTACCTTGTTACGACTT-3'). The PCR product was then purified with Pure Link TM Quick PCR Purification Kit (Invitrogen) and run on 0.8% agarose gel [20]. The 16s rRNA gene sequence obtained were compared with known sequence from the NCMR EzBioCloud database. Then the sequence was aligned with ClustalW, and phylogenetic tree was constructed by the neighbor-joining method.

2.6 Lead bioreduction assay using atomic absorption spectroscopic (AAS) analysis

The percentages of Lead bioaccumulation by selected isolate were calculated through atomic absorption spectroscopic (AAS) analysis. The isolate with maximum heavy metal tolerability was selected for further investigations. For this, the bacterial isolates were grown into the Lead containing medium and incubated for 72 hrs., while pH and temperature was maintained 7.0 and 37◦C, respectively. After this, the supernatant was separated from the bacterial cell pellets by centrifuged them at 8000 rpm for 10 min. and digested with a double volume of HNO_3. After digestion the extract was filtered by using Whatman filter paper and collected into volumetric flask after collection then it was diluted with deionized water [21].

% Of Lead bio reduction =

$$\frac{Initial\ conc. - Final\ conc.}{Initial\ conc.} \times 100$$

3. RESULTS

3.1 Isolation of lead reducing bacteria

The isolates were selected based on their ability to grow in medium that contains lead. The selected isolate was further screened out through a preliminary screening process that was based on their MIC values, that shown in Figure.4

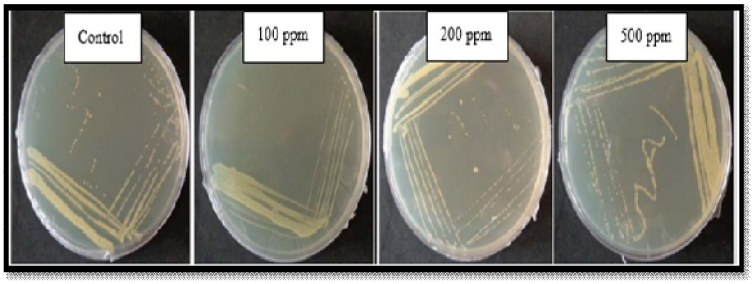

Figure :4 Isolation of bacterial strain on LB agar plates supplemented with different concentration of lead. (A) Luria agar plate (B) Luria agar +Lead (100 ppm) (C) Luria agar +Lead (200 ppm) (D) Luria agar +Lead 500 ppm

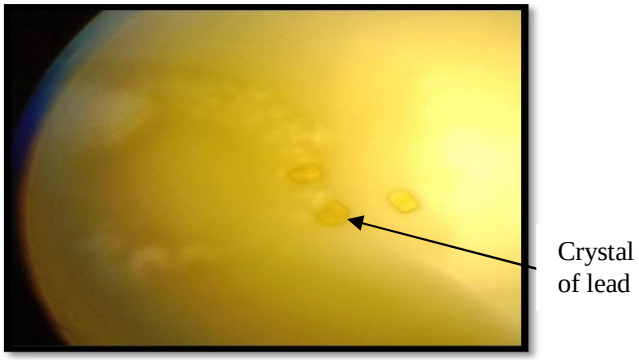

Figure :5 Stereo microscopic image of *Bacillus cereus* on Luria agar containing 500 ppm lead.

3.2 Minimum inhibitory concentration (MIC)

The selection of isolate based on their maximum MIC value (ppm), which is 100, 200 and 500 respectively. Some further studies were done on basis of MIC value of bacteria, the MIC value of Pseudomonas spp. was observed for the Lead is 1600 ppm [22]. Additionally, the MIC value of a multi metal resistant strain of Pseudomonas aeruginosa was also analyzed. Results showed that strain was able to resist up to 800 ppm of Lead amount present into the medium [23]. The tolerance capacity of microorganisms indicated by using their MIC value for heavy metal. To escape from the different toxic effect of heavy metals such as efflux pump, siderophores secretion, and cations transporter etc. microbes use different strategies etc. In addition to this, microorganism also has some metal-binding protein.

3.3 Morphological and Biochemical characterization

The strain was gram-positive, rod in shape, and gives a white colony on LB agar plate with a smooth surface. It showed positive biochemicals test results for catalase, urease, nitrate reductase. And it gives negative tests results for indole production, methyl red, and Voges-Proskauer's. Based on the morphological and biochemical characterization, the bacterial isolate as described in Table. 2 may be identified that belongs to *Bacillus* sp.

Table: 2 Morphological, biochemical characteristics of bacterial isolate

Sr. No.	Characteristics	Results
	Morphological characterization	
1.	Shape	Rod
2.	Colony morphology	Creamy white colony with smooth surface
3.	Gram staining	+
	Biochemical characterization	
4.	Catalase	+
5.	Urease	+
6.	Nitrate reductase	+
7.	Indole production	-
8.	Methyl red	-
9.	Voges Proskauer's	-

3.4 Characterization of bacterial isolate

To identify the isolate, molecular characterization was done by using MALDI-TOF MS. In this the highest intensity peak appeared at m/z 4818 Da. The species-specific peak is 3888Da and 5382 Da. Biotyper log score ≤ 2.0 indicates no reliable match. The strains which score values more than 2.0 score means reliable species-level identification, strains with score value ranging from 1.7 to 1.99 indicate genus-level identification, But the selected strains score values was 1.475 which is less than 1.7 so it could not be identified by MALDI biotyper database. The comparison done with the Bruker taxonomy database using Biotyper 3.1 software.

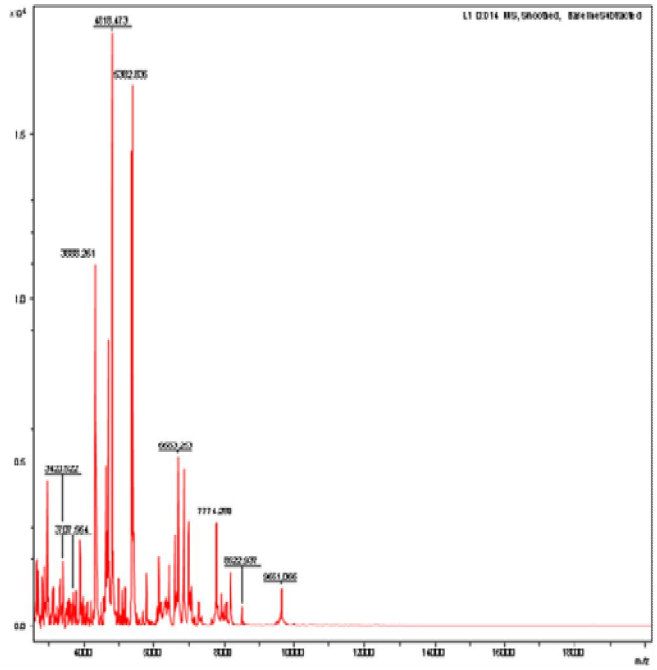

Figure: 6 MALDI-TOF MS spectra of Microbial strains indicating the protein profile (2-20KDa)

Furthermore, we done 16S rRNA gene sequencing and phylogenetic analysis to identify isolate. The 16s rRNA gene sequence was obtained with Accession No. AE016877. It showed 100% similarity with *Bacillus cereus* ATCC 14579. A taxonomically united database of 16S rRNA and whole genome assemblies are given below.

>A_JAN_22_251

GAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGC

GGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAAT

ACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCC6

GCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGA

TCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG

ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGA

AGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGC

CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGT

TTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCA

GAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGG

CGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA

GTCCACGCCGTAAACGATGAGTGCTAAG

The constructed phylogenetic tree of strain with closets neighbor strain is described in Figure. 7

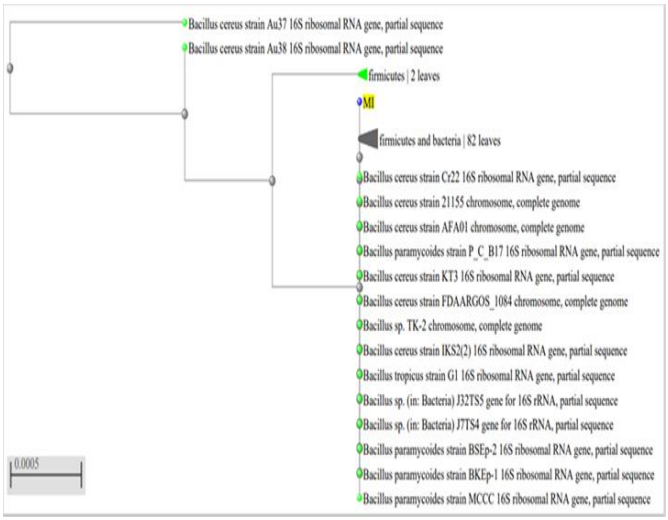

Figure :7 Neighbor-joining evolutionary tree showing relationship of 16s rRNA gene (Accession No. AE016877) with other sequences used in Multiple Sequence Alignment analysis.

3.5 Lead bioreduction assay

The AAS was used to measure the amount of residual Lead present in the supernatant and pellet after the reduction of lead by bacterial culture. The results showed that the isolate, has the maximum reduction potential of Lead that is 89% in supernatant and 88% in pellet in lead concentration of 500 ppm while in other concentration that is in 100 ppm it shows 75% in supernatant and 80% in pellet and in 200 ppm 82.5% in supernatant, 77.5% in pellet as represented in Figure.8.

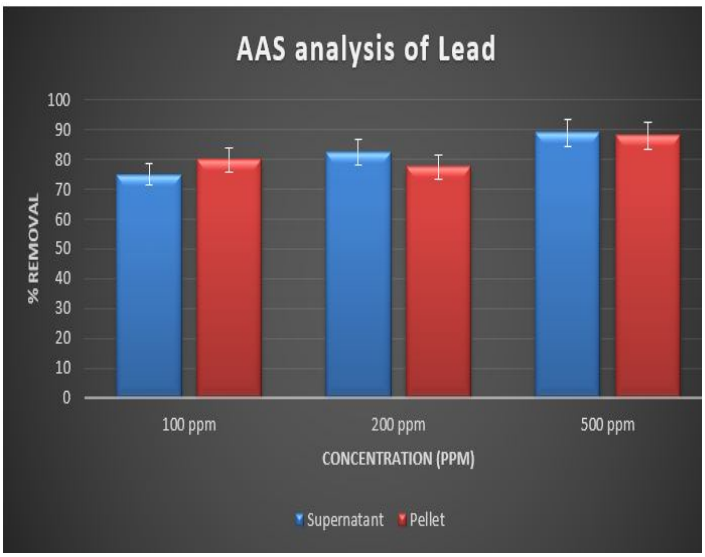

Figure :8 Graphical representations of Bioreduction of Lead

4. CONCLUSION

Heavy metals are non-essential thing even at very low concentration they are toxic to humans and environment. Higher concentration of both the essential metals and non-essential metals are toxic. They damage the DNA and block the normal function of cells which ultimately leading to the cell death [24]. Nowadays to protect natural water resources from heavy metals pollution studies on river water quality is very important.

In present study, we have isolated the bacterial strain, which have led reducing ability from Mula-Mutha river, Pune, India. Thermax Ltd. And Impact India Foundation done the water quality assessment of Mula-Mutha rivers in 1997. The results obtained from 1976 to 1994 clearly indicate that there was gradual deterioration of river water quality. After the observation of river water quality, it was concluded that the rate of DO in river water get lowering that indicates the increasing load of biodegradable matter and increasing value of BOD. This has resulted in eutrophication, which has brought changes in biological community such as rapid growth of aquatic weeds and algal biomass [25]. Lead considered to be more toxic elements as far as environmental agencies are concerned, they asses the studies of lead in the Mula-Mutha river the observed values of lead in their studies ranges of 00 to 2.4 mg/l which is higher than WHO set limit which 0.001 mg/l after their observation they concluded that there more neurotoxic effects of lead at lower level [26]. Another, studies were done for quantitative analysis of lead in Mula-Mutha river after their observation the estimation mean of lead to be 0.028, 0.039 and 0.108 which in excess amount as compared with permissible limit declared by WHO i.e., 0.05 mg/l [27].

The removal of lead from water resources has been reported by several literatures. The *Bacillus cereus* NWUAB01 was isolated from a mining soil and the strain tolerate 1000 mg/l of Pb. Another strain of *Bacillus cereus* NSPA8 isolated from solar salterns showed significant level of lead biosorption with maximum 87-90% [28], demonstrated the biosorption potentialities of *Bacillus cereus* up to 500 mg/l of pb. The isolated strain *Bacillus cereus* ECD were able to reduce chromate up to 76% at concentration of 100 µg ml^{-1}, this strain showed high resistance to chromate and other metals under aerobic conditions. This strain also used for the treatment of metal based industrial effluents [29]. The strain *Bacillus cereus* RC^{-1} under heavy metal stress the bioaccumulation by growing cells under varying range of pH. It has highest resistance to Cd^{2+}. Removal of Cd^{2+} Pb^{2+} coincide with uptake of Na^{+} and Mg^{2+} respectively. Growing cells kept metal homeostasis might through the Cd-efflux system induced by Na^{+} [30].

The results from present study clearly demonstrate that *Bacillus cereus* ATCC 14579 has strong potentialities to reduced lead and this strain can be employed as a bio-agent for lead detoxification from the contaminated water. The isolated strain *Bacillus cereus* ATCC 14579 from Mula-Mutha river showed significant capabilities to reduced lead. The maximum reduction potential of *Bacillus cereus* ATCC 14579, 89 % in supernatant and 88% in pellet. The isolate showed extracellular as well as intracellular bioreduction mechanism for the reduction of lead.

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