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REGULAR ARTICLE

BIOLOGICAL REMOVAL OF LEAD BY *BACILLUS SP.* OBTAINED FROM METAL CONTAMINATED INDUSTRIAL AREA

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ABSTRACT

In the present study bacterial strains were isolated from soil, sediment and water samples of metal polluted environment. As a result, various 164 heterotrophic bacterial strains were isolated and studied the multiple metal tolerance profile and lead bioaccumulation potentiality. We also analyze the metal contamination of the selected study area. The average abundance order of heavy metal contents in soil, water and sediments were Zn>Cu>Pb>Cd. Zinc concentration ranged from 39.832µg/L to 310.24µg/L in water, 12.81µg/g to 407.53µg/g in soil and 81.06µg/g to 829.54µg/g in sediment; copper concentration from 25.54µg/L to 66.29µg/L in water, 8.22µg/g to 73.11µg/g in soil and 32.28µg/g to 600.61µg/g in sediment; lead concentration from 8.09µg/L to 25.23µg/L in water, 5.31µg/g to 73.11µg/g in soil and 1.02µg/g to 60.14µg/g in sediment and cadmium concentration ranged from 39.832µg/L to 310.24µg/L in water, 12.81µg/g to 407.53µg/g in soil and 81.06µg/g to 829.54µg/g in sediment. Metal resistance studies of the bacterial isolates revealed that out of 164 isolates collected about 45% of the isolates showed very high tolerance (>6000µg/ml) to lead. Tolerance to Cd and Zn were relatively low (<500 µg/ml). Resistance to Ni and Cr were in between 1000µg/ml - 1500µg/ml. A total of 18 bacterial genera were recorded from the study area; ten genera from soil and 11 from water, while only 5 bacterial genera were recorded

from sediment samples. Bioaccumulation studies revealed that with increase in time, the biomass of the selected bacterial isolates increased. Correspondingly, with increase in biomass, the heavy metal bioaccumulation was also increased. In lead removal studies, around 50% of the lead in the experimental flasks was reduced by *Bacillus sp*. In control flask, only 5% metal reduction occurs. The obtained results showed that the selected *Bacillus sp.* is good bioaccumulation medium for lead ions.

Keywords: Industrial area, metal pollution, lead, bacteria, bacillus, bioaccumulation

INTRODUCTION

Industrial activities led to extensive discharge of toxic metals into the environment. Heavy metals represent a main hazard for the human health and ecosystem (**Boopathy, 2000**). Some metals including iron, zinc, copper and manganese are micronutrients used in the redox processes, regulation of osmotic pressure, as enzymes cofactors and are also important in the maintenance of the protein structure (**Vallee and Auld, 1990**). On the other hand metals including lead, cadmium etc. do not play any known physiological role and are in fact toxic to cells. Furthermore, some heavy metals are being subject to bioaccumulation and may pose a risk to human health when transferred to the food chain (**USEPA, 1987**). The scientific literature shows that lead is one of the heavy metals that has been recognized as a potent human toxin with reports of many diseases, such as brain damages and mental disabilities associated with ingestion dating to the last century.

In the last two decades environmental interests has induced much research concentrated and focused on the effects of toxic metals on the environment because they ultimately reach and accumulate in plants and animals tissues (**Environ, 1988**). According to the water standards used by the World Health Organization, levels of heavy metals such as lead ions in wastewater must be controlled and reduced to set value (**USEPA, 1986**). Chemical methods such as precipitation, oxidation or reduction have been widely used to remove metal ions from industrial waste water. Those methods are ineffective or expensive. The activity of microorganisms is extended to environmental management, and microbes have superseded the conventional techniques of remediation. Biological methods such as biosorption and bioaccumulation using microorganisms provide promising alternative to chemical methods.

The aims of this study was to isolates and characterize bacteria from the soil, sediments and waters of Eloor-Edayar industrial zone, Kerala to study the heavy metals resistance pattern and the lead bioaccumulation potential of the selected organism. We also studied the heavy metal pollution in the selected industrial area. The proposed study would yield a data on heavy metal resistance and bioaccumulation potential of bacterial population in the heavy metal contaminated industrial area under study and possibly provide bacterial strains that could be exploited in the bioremediation of heavy metal polluted soil/ water ecosystems.

MATERIALS AND METHODS

Study area

Eloor-Edayar industrial area - largest industrial region of Kerala; parts of Ernakulam Districts, India located in 76º 17' 32.9" - 076º 18' 31.8" E longitudes and 10º 04' 51.6" - 10º 04' 38" N latitude and are a chronic contaminated area and one of the major exporting centers of fertilizers and chemicals.

Collection of Samples

Samples were collected from abandoned paddy fields, canals and river of the selected industrial region. Totally seven sampling sites were identified and from each sampling site soil, sediment and water samples were collected. Soil samples were collected at a depth of 15 to 20 cm from the surface after removing the top layer. For each of the sampling sites, sub-samples of soil were collected from different locations, pooled together and homogenized so as to obtain representative sample. Samples were collected using a spade that is thoroughly cleaned and disinfected between sampling so as to prevent cross-contamination. Sediment samples were collected by Grab sampler and transferred to sterilized plastic bags. Water samples were collected using sterilized plastic bottles. Soil, sediment and water samples were transferred to an ice box and transported to the laboratory.

Heavy metal analysis

Heavy metals such as Zn, Cd, Pb and Cu in water were determined with Anodic Stripping Voltammetry (797 VA Computrace, Metrohm) after acid digestion of sample as per the method described in **APHA, (1998)**. For sediments and soil, samples were air dried first and grounded to fine powder using pestle and mortar. Then the samples were separated into two different granulometric fractions, < 200µm and < 63µm, using stainless steel sieves. An aliquot of 0.25g of powdered sediments of < 63µm were digested with Selectipur Nitric acid using a microwave digester (MARS X PRESS, CEM, USA) as per USEPA 3051a for heavy metals (**Kingston and Jassie, 1988; Kingston *et al.,* 1997**). The digested solution was filtered through Whatman No: 1 filter paper and finally the volume were made upto 25 ml with ultrapure water (Elga ultrapure water system, UK). Heavy metals were then determined by Voltammetric Trace Metal Analyser- 797 VA Computrace, Metrohm (**Ireland- Ripert *et al.,* 1982; Lo and Lee., 1994**).

Isolation and identification of bacteria

Isolation and enumeration of bacteria were carried by standard serial dilution plate technique. Serially diluted samples were sow in Nutrient Agar and incubated at 37°C for 24-48 hours. Bacterial colonies from Nutrient agar were isolated, purified and maintained as a pure culture for further study. Bacterial isolates which are maintained as pure culture on Nutrient Agar were characterized and identified up to genus level by morphological tests as per Bergey's Manual of Determinative Bacteriology: 9th edition (**Holt, *et al.,* 1994**) and 8th edition (**Buchanan and Gibbons, 1974**). Morphological tests carried out for the identification of the isolates are Gram's staining, cell shape and arrangement, pigment production, O/F glucose tests, Endospore staining, Motility, Catalase, Oxidase etc.

Heavy metal resistance test

Resistance of the bacterial isolates to varying concentrations of heavy metals such as lead, zinc, chromium, nickel and cadmium were determined by agar dilution method (**Luli *et al.,* 1983**). Fresh overnight cultures of the isolates grown in peptone water were aseptically inoculated into nutrient agar plates, which were supplemented with increasing concentration of the aforesaid metals individually (5 µg/ml to 6 mg/ml). The plates were incubated at room

temperature and observed for bacterial growth. The lowest concentration of heavy metals at which no growth occurred when compared with the control plates was considered as the Minimal Inhibitory Concentration (MIC). All metal salts were added to the medium after autoclaving and cooling to 45-50^{o}C, from filter sterilized stock solutions. The metal salts used for the study includes Lead nitrate $(PbNO_3)_2$, Potassium dichromate ($K_2Cr_2O_7$), Nickel Sulphate ($NiSO_4.6H_2O$), Zinc sulphate ($ZnSO_4$) and Cadmium nitrate ($Cd(NO_3)_2.4H_2O$).

Bioaccumulation of Lead

The bacterium (strain TCC52) growing in the most lead concentration was selected for the study and it was identified as *Bacillus*. Bioaccumulation method at different pH (5, 7 and 9) with living bacterial cells was used for the removal of lead. To study heavy metal removal with live cells, nutrient broth amended with initial concentration of lead (20mg/L) was inoculated from overnight grown cultures of selected bacterial isolates. The inoculated flasks were incubated at room temperature for 72 hrs in a shaking condition. An aliquot of 5mL sample was taken daily (24hrs interval) from each flask. Samples were centrifuged to remove suspended biomass and concentration of heavy metals was determined in the supernatant.

RESULTS

A total of 18 bacterial genera were recorded from the selected industrial area. Ten bacterial genera were represented in soil and 11 from water, while only 5 bacterial genera were recorded from sediment samples (Table 1). *Bacillus, Pseudomonas* and *Enterobacter* were found in soil, sediment and water samples.

Table 1 Bacterial genera found in the soil, sediment and water samples of the Eloor- Edayar Industrial belt

Sl No.	Sample		
	Soil	**Sediment**	**Water**
1	*Bacillus*	*Kurthia*	*Staphylococcus*
2	*Caryophanon*	*Pseudomonas*	*Acinetobacter*
3	*Listeria*	*Bacillus*	*Azotobacter*
4	*Kurthia*	*Enterobacter*	*Bacillus*
5	*Agromyces*	*Escherichia*	*Pseudomonas*
6	*Arthrobacter*		*Xanthobacter*
7	*Cellulomonas*		*Enterobacter*
8	*Deinococcus*		*Escherichia*
9	*Psedomonas*		*Klebsiella*
10	*Enterobacter*		*Aeromonas*
11			*Thiobacillus*

Heavy metal contamination

The average abundance order of heavy metal contents in soil, water and sediments were Zn>Cu>Pb>Cd. Zinc concentration ranged from 39.832µg/L to 310.24µg/L in water, 12.81µg/g to 407.53µg/g in soil and 81.06µg/g to 829.54µg/g in sediment; Copper concentration from 25.54µg/L to 66.29µg/L in water, 8.22µg/g to 73.11µg/g in soil and 32.28µg/g to 600.61µg/g in sediment; Pb concentration from 8.09µg/L to 25.23µg/L in water, 5.31µg/g to 73.11µg/g in soil and 1.02µg/g to 60.14µg/g in sediment and cadmium concentration ranged from 39.832µg/L to 310.24µg/L in water, 12.81µg/g to 407.53µg/g in soil and 81.06µg/g to 829.54µg/g in sediment. Metal contamination in the soil sediment and water samples of the study area is represented in Table 2, 3 and 4

Table 2 Heavy metal concentration in the water samples of selected industrial area

Sample Name	Metal concentration (µg/L)			
	Zn	**Cd**	**Pb**	**Cu**
TCC	86.211	3.465	nd	26.535
BPM	279.836	5.774	13.754	28.003
KDM	252.022	2.316	25.213	52.912
BR	41.555	nd	nd	nd
WMH	39.832	nd	nd	nd
RFH	79.381	5.086	nd	66.291
WB	310.246	8.962	8.091	25.548

Legend: Wetland near Binanipuram (WB), Wetland near Merchaam and HIL (WMH), Kuzhikandam canal (KDM), Canal near Binanipuram (BPM), Binananipuram river (BR), Pathalam bund river (TCC), River near FACT and HIL (RFH)

Table 3 Heavy metal concentration in the soils of selected industrial area

Sample Name	Metal concentration(mg/kg)			
	Zn	**Cd**	**Pb**	**Cu**
RFH	117.3138	nd	5.3122	70.0649
WB	407.537	5.957	61.7072	26.2226
TCC	193.294	1.22795	19.3604	48.8075
BR	174.7672	2.4732	60.36925	73.11965
KDM	12.81555	0.5549	nd	8.2268
BPM	378.8874	25.44565	27.6793	66.6585
WMH	237.1605	1.3467	28.79235	57.76925

Legend: Wetland near Binanipuram (WB), Wetland near Merchaam and HIL (WMH), Kuzhikandam Thodu (KDM), Canal near Binanipuram(BPM), Binananipuram river (BR), Pathalam bund river (TCC), River near FACT and HIL (RFH)

Table 4 Heavy metal concentration in the Sediments of selected industrial area

Sample Name	Metal concentration(mg/kg)			
	Zn	**Cd**	**Pb**	**Cu**
WMH	165.498	0.6067	21.184	88.013
BPM	309.2965	21.4885	1.0271	45.87835
TCC	244.4824	0.45655	60.1493	76.29165
WB	378.1402	12.7355	36.21895	32.2815
RFH	81.0671	22.9079	8.7451	59.0251
KDM	829.5499	2.7852	46.1046	600.6127

Legend: Wetland near Binanipuram (WB), Wetland near Merchaam and HIL (WMH), Kuzhikandam Thodu (KDM), Canal near Binanipuram (BPM), Pathalam bund river (TCC), River near FACT and HIL (RFH)

Metal resistance of the isolates

Metal resistance studies of the bacterial isolates revealed that out of 164 isolates collected about 45% of the isolates showed very high tolerance (>6000µg/ml) to lead. Tolerance to Cd and Zn were relatively low (<500 µg/ml). Resistance to Ni and Cr were in between 1000µg/ml - 1500µg/ml.

Bioaccumulation of lead

Bioaccumulation studies revealed that with increase in time, the biomass of the selected bacterial isolates increased (Figure 1). Correspondingly, with increase in biomass, the lead bioaccumulation was also increased. In the case of lead removal, around 50% of the lead in the experimental flasks was reduced by *Bacillus sp*. In control flask, only 5% lead reduction occurs (Figure 2-4). Somewhat lead removal showed higher reduction in pH 5 (Figure 5), about 10% increased reduction occurs in pH 5. Relatively an increased removal of lead was observed in the first day of the experiment.

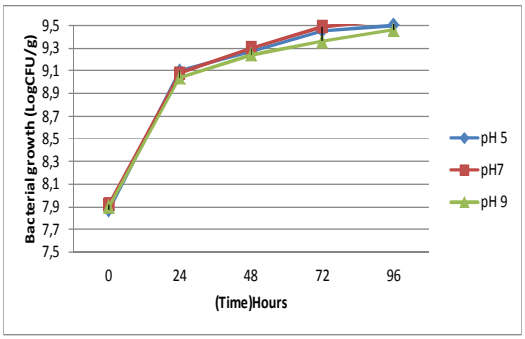

Figure 1 Growth Kinetics of *Bacillus sp.* at different pH with 20mg/L initial concentration of lead

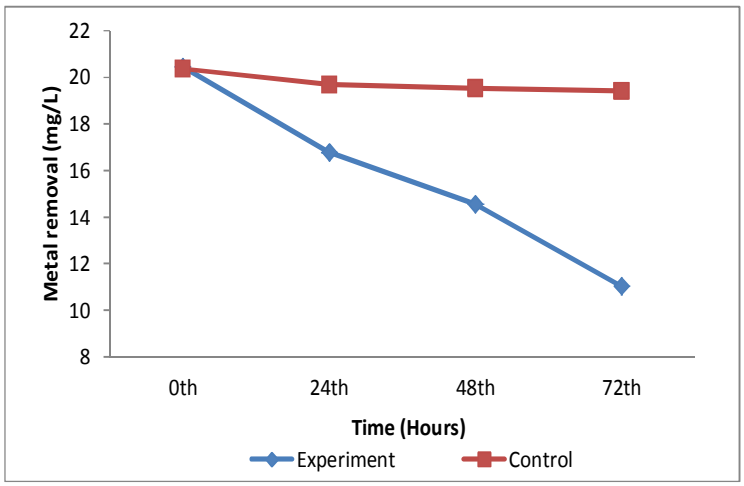

Figure 2 Bioaccumulation of lead using *Bacillus sp.* at pH 7

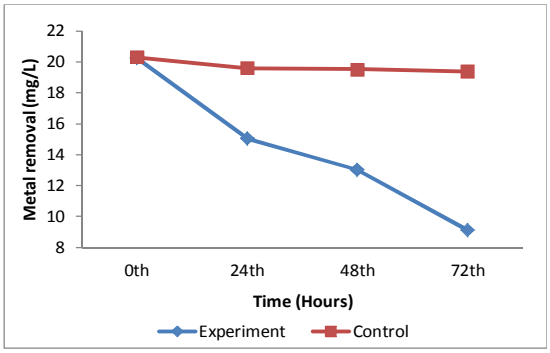

Figure 3 Bioaccumulation of lead using *Bacillus sp.* at pH 5

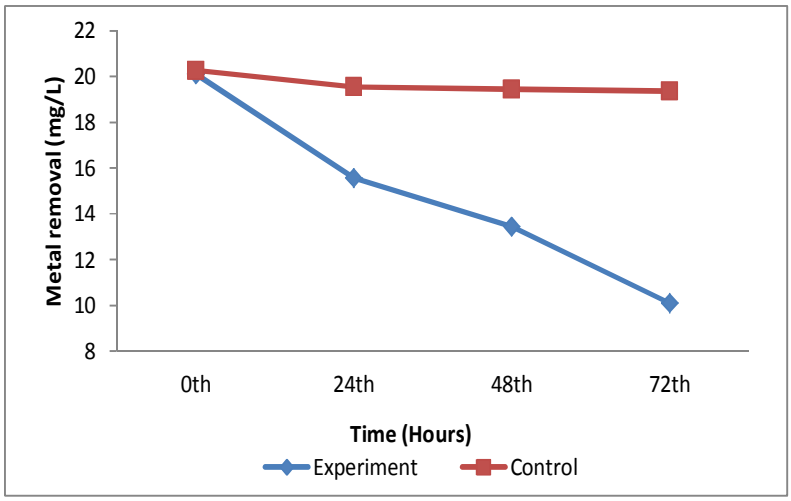

Figure 4 Bioaccumulation of lead using *Bacillus sp.* at pH 9

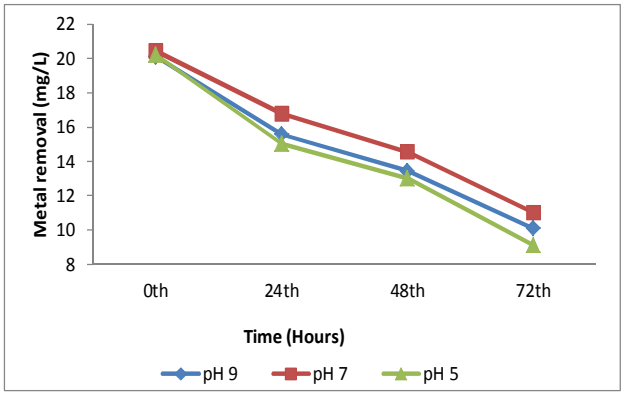

Figure 5 Effect of pH on lead removal using *Bacillus sp.*

DISCUSSION

Heavy metal contamination

The maximum allowable levels of heavy metals in drinking water (**USEPA 1979**) are (in µg/L): Cd 10, Pb 50, Cu 1,000 and Zn 5,000; the guidelines for drinking-water quality (WHO 1984) are (in µg/L): Cd 5, Pb 50, Cu 1,000 and Zn 5,000. In the present work, heavy metal content of the water samples were within permissible limit. Compared to wetland and canal water samples, river samples showed less heavy metal values. Low values of heavy

metals in water sample may be due to the speedy runoff of water in the study area due to tidal force. In the case of soil and sediment samples, copper, lead and zinc showed enrichment levels exceeding the normally expected distribution in soil giving rise to concern over suitability of soils in the study area (**Srinivasa *et al.*, 2005; Yefang *et al.*, 2006; Govil *et al.*, 2008**). High levels of these elements are observed in several pockets, very nearer to industries which specify that the source of these elements could be the industrial effluents. Copper values were found to be high in the study area. Copper accumulation in the soils and sediments of the study area was due to the industries like steel manufacture, blast furnace, and application of agrochemicals in the agro-based industry.

Zinc belongs to a group of trace metals, which are vital for the growth of humans, animals and plants and are potentially hazardous for the biosphere when present in high level and the main sources of pollution are industries. (**Romic and Romic, 2003**). The zinc concentration in the present study area ranges from 12.81mg/kg to 407.53mgk/g in soil and 81.06mg/kg to 829.54mg/kg in sediment which is high. The normal threshold value prescribed in soil is 200mg/kg (**BIS, 1991**). In India 47% of soils are depleted in zinc, however the high concentration of Zn are found in the nearby industrial areas, which clearly represents the source to be anthropogenic and not natural (**Aswathanarayana, 1995**).

Bacterial metal resistance

The high levels of resistance and the widespread tolerance that was found among the isolates is probably attributed to the high metal contents in the soil (**Abou-Shanab *et al.*, 2007**). All the isolates were tolerant to multiple metal ions. However, the patterns of tolerance among the 164 cultures varied may be due to the difference in the concentration of the different heavy metals in the environment. The site from which the samples was taken has been polluted with high levels of heavy metals for many years, perhaps giving a diverse range of bacteria the chance to adapt to the environment, either by convergent evolution of resistance mechanisms or by transferring the resistance genes via a plasmid. Resistance to heavy metals, including cadmium, zinc, copper, chromate, cobalt, arsenic and nickel, is most often carried by bacteria on plasmids or transposons and it has been theorized that this allows for lateral transfer in the environment (**Silver, 1992**).

Lead removal

The results of lead removal studies showed that with increase in time, the biomass of the bacterial strains increased. Likewise, with increase in biomass, the lead bioaccumulation also increased. The increase in surface area that can be due to increase in biomass improves the adsorptive nature or increases the number of active binding sites on cell surface (**Bai *et al.*, 2002**). The selected metal resistant strains showed that their growth was only slightly affected with different pH. Therefore, it is clear that growth of our isolates is not inhibited with different pH and this fact makes them strong candidates for future application in metal bioremediation.

The active mode of metal accumulation by living cells is usually designated as bioaccumulation. This process is dependent on the metabolic activity of the cell referred to its intrinsic biochemical and structural properties, physiological and/or genetic adaptation, environmental modification of metal specification, availability and toxicity (**Cha and Cooksey, 1991**). The capacity of living cells to remove metal ions from aqueous solutions is also significantly influenced by environmental growth conditions, as temperature, pH and biomass concentrations (**Chen and Ting, 1995**).

Many researchers are reported the efficiency and mechanisms of bacteria to remove different metal ions, many of them are comparable with the present study. **Richard *et al.*** (2002) reported that Cu^{+2} and Pb^{+2} appear to bind to materials on the cell surface. Lead is precipitated in an insoluble form that is localized to the cell membrane or cell surface (**Aiking *et al.*, 1985; Levinson *et al.*, 1996; Roane 1999**). This could be generally explained by the fact that the negatively charged groups (carboxyl, hydroxyl and phosophryl) of bacterial cell wall adsorb metal cations through various mechanisms such as electrostatic interaction, van der Waals forces, covalent bonding or combination of such processes (**Chojnacka *et al.*, 2005**).

As pH values in metal-containing water and wastewater can vary, since it is necessary to use solutions of different pH values to examine the effect of heavy metal removal. In the present work, initial pH was adjusted in the range 5, 7 and 9, before the addition of the biosorbent. The greatest capacity of bioaccumulation was obtained at pH 5. The medium pH affects the solubility of metals and the ionization state of the functional groups like carboxylate, phosphate and amino groups of the cell wall. The carboxylate and phosphate groups carry negative charges that allow the cell wall components to be potent scavengers of cations. The inconsistency in literature regarding the influence of pH on biosorption seems to

indicate that the way pH would alter the adsorption of metal ions to biomass varies with the type of adsorbents (biomass) and also the type of adsorbates (metal ions).

CONCLUSION

The obtained results showed that the selected *Bacillus sp.* is good bioaccumulation medium for lead ions and had high adsorption yields for the treatment of wastewater containing lead ions. Consequently, bacteria bioaccumulation technologies are still being developed and much more work is required.

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