Bioremediation of Zinc Using *Bacillus* sp. Isolated from Metal-Contaminated Industrial Zone

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M.P. Krishna, Rinoy Varghese, V. Arun Babu, S. Jyothy, and A.A. Mohamed Hatha

Abstract

The production of heavy metals has increased quickly since the industrial revolution. Heavy metals frequently form compounds that can be toxic, carcinogenic, or mutagenic, even in very small concentrations. The usual techniques of removing metals from wastewaters are in general expensive and have many restrictions. Alternative methods of metal removal and recovery based on biological materials have been measured. Among various agents, the use of microbes for the removal of metals from industrial and municipal wastewater has been proposed as a promising alternative to conventional heavy metal management strategies in past decades. Thus, the present study aims to isolate and characterize bacteria from soil, sediment, and waters of metal-contaminated industrial area to study the zinc resistance patterns and the zinc bioaccumulation potential of the selected microorganism. Zinc analysis of the samples revealed that concentrations varying from 39.832 μg/L to 310.24 μg/L in water, 12.81 μg/g to 407.53 μg/g in soil, and 81.06 μg/g to 829.54 μg/g in sediment are present. Bacterial zinc resistance study showed that tolerance to Zn was relatively low (<500 μg/ml). Ten bacterial genera were represented in soil and 11 from water, while only 5 bacterial genera were recorded from sediment samples. *Bacillus, Pseudomonas*, and *Enterobacter* were found in soil, sediment, and water samples. Highly zinc-resistant *Bacillus* sp. was selected for zinc removal experiment. Zinc removal studies revealed that at pH 5 about 40% reduction occurs; at pH 7, 25% occurs; and at pH 9, 50% occurs. Relatively an increased removal of Zinc was observed in the first day of the experiment by *Bacillus* sp. The metal bioaccumulative potential of the selected isolates may have possible applications in the removal and recovery of zinc from industrial effluents.

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Introduction

Heavy metal contamination by industrial process and technological development is posing major threats to the environment and public health because of its toxicity, nonbiodegradability, and bioaccumulation [1]. Zinc and its compounds are found in the earth's crust and are present in most rocks, certain minerals, and some carbonate sediments. As a result of weathering of these materials, soluble compounds of zinc are formed and may be released to water [2]. Zinc is one of the metals found in effluents discharged from industries involved in galvanization, electroplating, manufacturing of batteries, and metallurgical industries. Zinc in its metallic form has limited bioavailability and poses no ecological risk. However, zinc can react with other chemicals like acids and oxygen to form compounds, which can be potentially toxic and can cause serious damage to biological systems [3].

All through the previous two decades, wide consideration has been paid on management of environmental pollution and its control due to hazardous materials like heavy metals. Decontamination of heavy metals in the soil and water around industrial plants has been a challenge for a long time. A lot of physicochemical strategies, such as filtration, electrochemical treatment, oxidation/reduction, ion exchange, membrane technology, and reverse osmosis, have been developed for removing heavy metals from the polluted water [4]. But nearly all of them showed to be expensive, less efficient, labor-intensive operation, or lack of selectivity in the treating process [5].

Some reports have shown that indigenous microbes and plant–microbe symbionts tolerate high heavy metal concentrations in different ways and may play a significant role in the restoration of contaminated soil [6]. It is important to study the indigenous microorganisms in heavy metal-polluted sites. It may provide new insight into bacterial diversity under unfavorable conditions, new isolates, and probably new genetic information on heavy metal resistance, which could be exploited in decontamination process in the future [7].

Bioremediation, which involves the use of microbes to detoxify and degrade environmental contaminants, has received increasing attention in recent times to clean up a polluted environment [8]. Bioremediation, being in situ treatment, offers several advantages over the conventional chemical and physical treatment technologies, especially for diluted and widely spread contaminants [9]. The objective of the present study is to isolate and characterize bacteria from soil, sediment, and waters of metal-contaminated industrial area to study the zinc resistance patterns and the zinc bioremediation potential of the selected microorganism.

Materials and Methods

Study Area

Eloor-Edayar industrial area – largest industrial belt of Kerala – is a part of Ernakulam District of Kerala and lies between 76º 17′ 32.9″ and 076º 18′ 31.8″ E longitudes and 10º 04′ 51.6″ and 10º 04′ 38″ N latitudes and is a chronic polluted area and one of the biggest exporting centers of fertilizers and chemicals. Eloor is the house of Fertilizers and Chemicals Travancore (FACT), Travancore Cochin Chemicals (TCC), Indian Rare Earths (IRE), Hindustan Insecticides Limited (HIL), and many other small and big industries situated in the lower flood plains of the river Periyar, and it is an island of 14.21 km^2 formed between two distributaries of river Periyar.

Collection of Samples

Soil, sediment, and water samples were collected from abandoned paddy fields, canals, and river of the selected industrial area. 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 15–20 cm from the surface after removing the top layer. For each of the sampling sites, subsamples 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 using 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.

Zinc Analysis

Zinc contamination in water was 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, <200 μm and <63 μm, using stainless steel sieves. An aliquot of 0.25 g of powdered sediments of <63 μm was digested with Selectipur nitric acid using a microwave digester (MARSXpress, CEM, USA) as per USEPA 3051a for heavy metals [10, 11]. The digested solution was filtered through Whatman no. 1 filter paper, and finally the volume was made up to 25 ml with ultrapure water (ELGA ultrapure water system, UK). Cadmium was then determined by voltammetric trace-metal analyzer – 797 VA Computrace, Metrohm [12, 13].

Isolation and Identification of Bacteria

Isolation and enumeration of bacteria were carried out by standard serial dilution plate technique. Serially diluted samples were plated to Nutrient Agar and incubated at 37°C for 24–48 h. 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 [14] and 8th edition [15]. 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.

Bacterial Zinc Resistance Test

Resistance of the bacterial isolates to varying concentrations of zinc was determined by agar dilution method [16]. Fresh overnight cultures of the isolates grown in peptone water were aseptically inoculated into nutrient agar plates, which were supplemented with increasing concentration of zinc metal ions (500 μg/ml–1.5 mg/ml). The plates were incubated at room temperature and observed for bacterial growth. The lowest concentration of zinc at which no growth occurred when compared with the control plates was considered as the minimal inhibitory concentration (MIC). Metal salts were added to the medium after autoclaving and cooling to 45–50°C, from filter sterilized stock solutions. The metal salt used for this study was zinc sulfate ($ZnSO_4$).

Bioaccumulation Experiment

The bacterial strain TCC51 growing in the most zinc concentration was selected for the study, and it was identified as *Bacillus* sp. Bioaccumulation method at different pH (5, 7, and 9) with living bacterial cells was used for the removal of zinc. To study heavy metal removal with live cells, nutrient broth amended with initial concentration of zinc (20 mg/L) was inoculated from overnight grown cultures of selected bacterial isolates. The inoculated flasks were incubated at room temperature for 72 h in a shaking condition. An aliquot of 5-mL sample was taken daily (24-h interval) from each flask. Control flasks without bacterial biomass were running simultaneously with the experiment flasks. Samples were centrifuged to remove suspended biomass, and concentration of heavy metals was determined in the supernatant.

Results and Discussion

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 2.1). *Bacillus, Pseudomonas*, and *Enterobacter* were found in soil, sediment, and water samples. Zinc analysis of the samples revealed that Zn concentrations varying from 39.832 µg/L to 310.24 µg/L in water, 12.81 µg/g to 407.53 µg/g in soil, and 81.06 µg/g to 829.54 µg/g in sediment were present. Zinc levels in the soil sediment and water samples of the study area are represented in Table 2.2. Zinc resistance studies of the bacterial isolates showed that out of 164 isolates collected, most of them showed low resistance (<500 µg/ml) and many isolates showed high resistance of >1,500 µg/ml.

Comparatively highly zinc-resistant *Bacillus* sp. was selected for zinc removal. Results of zinc removal study revealed that with increase in time, the biomass of the selected *Pseudomonas* sp. increased (Fig. 2.1). Correspondingly, with increase in biomass, the zinc bioaccumulation was also increased. Zinc removal studies revealed that at pH 5 about 40% reduction occurs; at pH 7, 25% occurs; and at pH 9, 50% occurs. Relatively enhanced removal of zinc was observed in the first day of the experiment by *Bacillus* sp. (Fig. 2.2, 2.3, 2.4, 2.5).

Zinc is commonly found in the earth's crust, and natural release to the environment can be significant. In addition, zinc is one of the most widely used metals in the world. The major industrial sources of zinc include electroplating, smelting and ore processing, and drainage from both active and inactive mining operations [17]. Furthermore, zinc is an important component of

Table 2.1 Bacterial genera found in the soil, sediment, and water samples of the selected industrial area

	Sample		
Sl no.	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	*Pseudomonas*		*Klebsiella*
10	*Enterobacter*		*Aeromonas*
11			*Thiobacillus*

Table 2.2 Zinc concentration in the soil, water, and sediment samples of selected industrial area

	Zinc concentration		
Sample name	Soil (µg/g)	Water (µg/L)	Sediment (µg/g)
TCC	193.29	86.211	244.48
BPM	378.88	279.836	309.28
KDM	12.81	252.022	829.54
BR	174.76	41.555	280.64
WMH	237.16	39.832	165.49
RFH	117.31	79.381	81.06
WB	407.31	310.246	378.14

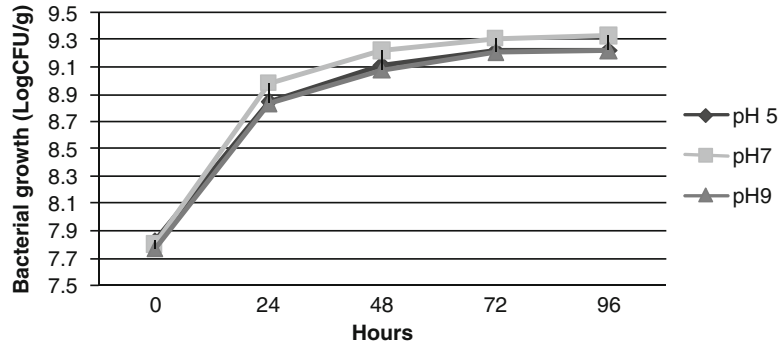


Fig. 2.1 Growth kinetics of *Bacillus* sp. at different pH with 20 mg/L initial concentration of zinc

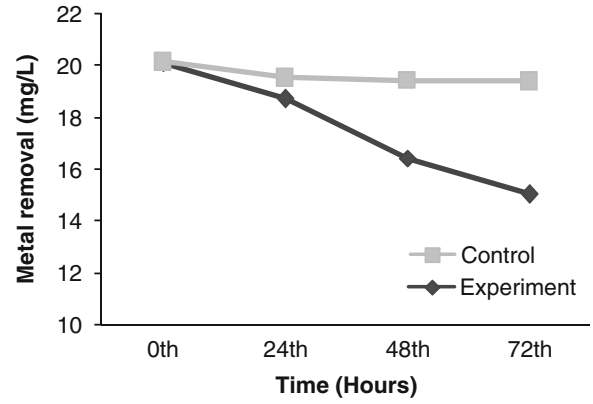


Fig. 2.2 Bioaccumulation of zinc using *Bacillus* sp. at pH 7

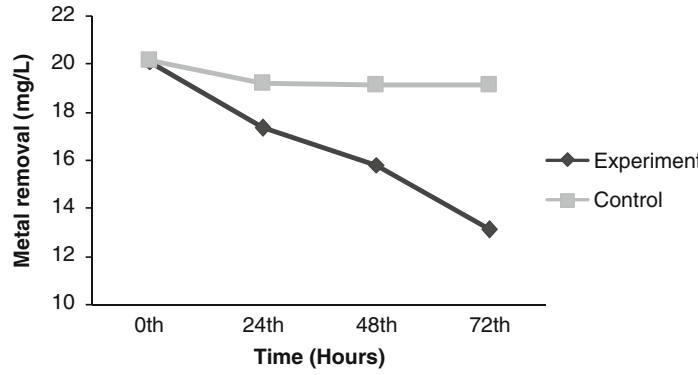


Fig. 2.3 Bioaccumulation of zinc using *Bacillus* sp. at pH 5

brass, bronze, die casting metal, other alloys, rubber, and paints. The environmental releases of zinc from sources of human origin far exceed the releases from natural sources [18]. The permissible concentrations of Zn in water and sediment/soil as per international standards are 5.0 mg/L and 300–600 mg/kg, respectively. In the present study, the values of Zn in water samples are below the permissible limits. In the case of soil and sediment samples, zinc showed enrichment levels exceeding the normally expected distribution. High levels of zinc are observed in several pockets, very nearer to industries which specify that the source of these elements could be the industrial effluents.

Zinc resistance studies of the bacterial isolates showed that out of 164 isolates collected, most of

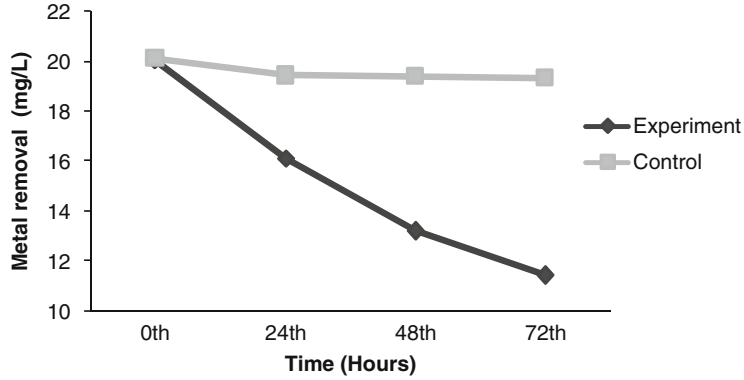
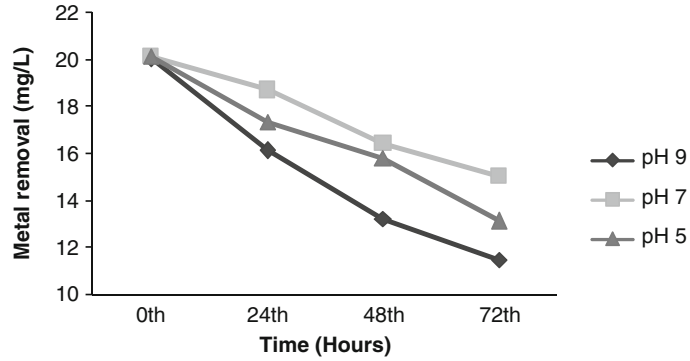


Fig. 2.4 Bioaccumulation of zinc using *Bacillus* sp. at pH 9

Fig. 2.5 Effect of pH on zinc removal using *Bacillus* sp.



them showed low resistance (<500 μg/ml) and many isolates showed high resistance of >1,500 μg/ml. The high levels of resistance and the widespread tolerance that was found among the isolates are probably attributed to zinc contents in the soil [19]. The level of tolerance among the 164 cultures varied may be due to the difference in the concentration of zinc 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 [20].

Results of heavy metal removal studies showed that with increase in time, the biomass of the bacterial strains increased. Likewise, with increase in biomass, zinc 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 [21]. The selected metal-resistant strains showed that their growth was only slightly affected with different pH. Therefore, it is clear that growth of the newly isolated strains 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 [22]. 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 [23].

Many researchers reported the efficiency and mechanisms of bacteria to remove different metal ions, and many of them are comparable with the present study. Richard et al. [24] 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 [25, 26]. This could be generally explained by the fact that the negatively charged groups (carboxyl, hydroxyl, and phosphoryl) 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 [27].

Since the pH values in metal-containing water and wastewater can vary, 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 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 inconsistency in literature regarding the influence of pH on biosorption seems to indicate the way pH would alter the adsorption of metal ions to biomass, and it varies with the type of adsorbents (biomass) and also the type of adsorbates (metal ions). The obtained results showed that the selected *Bacillus* sp. is a good bioaccumulation medium for zinc ions and had high adsorption yields for the treatment of wastewater containing zinc. Consequently, bacterial bioaccumulation technologies are still being developed and much more work is required.

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