

Original Article

Zinc from Aqueous Solutions can be Absorbed by Immobilized *Bacillus subtilis*

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Abstract

Heavy metals have been introduced through industrialization in the environment. It is well known that heavy metals can be found in the environment and endanger the living organisms. Industrial effluents contain various types of microorganisms. Different strategies have been adapted by these organisms to cope with the harmful effects of the metals. These strategies may be independent or dependent on metabolism. Biosorption, the binding of metal ions with proteins found on the cell wall is one of the strategies. Bacteria, algae, fungi, yeast all have biosorption capabilities. In this study, the use of *Bacillus subtilis* immobilization in sodium alginate to remove zinc from aqueous solution was investigated. The biosorbent's A batch biosorption experiment was conducted. The zinc removal efficiently was 75%, 51.24%, 45.89%, and 41.44% at initial concentrations of zinc aqueous solutions of 25ppm, 50ppm, 75ppm, 100ppm respectively, under ideal conditions (pH 7 biosorbent dosage 2g/l) for 24hours at 37^o C temperatures. This suggests that compared to similar biosorbent, *Bacillus subtilis* was found to be effective biosorbent zinc removed.

1. Introduction:

Environmental pollutants are being released in greater quantities as a result of rapid industrial contamination and urban growth. Particular attention should be paid to metal contamination because of its toxicity and potential bioaccumulation through the food chain [1]. Heavy metals are the metals having greater densities than 5g/cm³ [2]. They fall into two categories such as essential and non-essential metals. The metals such as Copper, Zinc is essential metals that are necessary in low concentration. Metals like lead, Cadmium is referred to as non-essential metals and their biological role is unknown.

However, at any concentration these metals are hazardous [2].

Heavy metals contaminate water bodies not only through direct discharge of effluents from industrial plants, refineries and sewage treatment facilities but also from soil washes, leach in groundwater deposited from the atmosphere by the rain water [3]. One of the significant heavy metals contaminants is zinc metal which is found in the aerosphere of the zinc smelters, scares and zinc refineries as consequences of human activities [4]. Zinc is an essential metal to our body. However, in large concentrations, it may result in harm

to the body such as damaging the lining of the stomach and severe haemolysis anaemia. Plants, fishes and invertebrates are also extremely poisoned by zinc ions [5].

Many physical and chemical techniques have been used over the past several decades to remove metals in the environment. Chemical techniques include oxidation/reduction and physical methods include ion exchange, evaporation recovery, and filtration. The removal of toxic metals, radionuclides and precious metals from waste water using conventional methods has several disadvantages including incomplete metal extraction, high cost and the possibility of production of toxic by-products [6]. As an alternative to it, biosorption is an economical and environmentally beneficial technology process, in which dead microbial biomass can remove and concentrate metal ions from aqueous solution through a metabolic independent method [7]. Biosorption removes toxic metals, precious metals, radio nuclides, dyes, fluorides, phthalates and pharmaceuticals from waste water released by industries [8].

Microbial biomass such as bacteria, fungi, yeast, cyanobacteria, algae, food industry, and biotechnological process, active sludge, chitin and chitosan containing biomass can be utilized [9]. Due to the presence of numerous functional groups with varying charges and geometries (carboxyl, hydroxyl, amine, sulphate, sulfhydryl groups, etc.), the microbial cell wall is crucial in the removal of metal ions from the aqueous solutions. There are various benefits to dead microbial biomass over living cells in such a way that it is cost effective, lacks nutrient medium, maintenance of high sorption and desorption as well as a pure microbial culture rate. It also operates across a broad pH range employing basic equipment regeneration [10].

The gram positive rod shaped bacteria; *Bacillus subtilis* shows a great capacity to bind the metals ions through the complexation and ion exchange processes, due to its thick peptidoglycan cell wall [11]. Hence, this study has been undertaken to isolate a *Bacillus subtilis* strain that is resistant to zinc, immobilized dead biomass in the sodium alginate beads and methodically strain zinc removal under ideal batch conditions.

2. Materials and Methods:

2.1 Sample collection and preparation

Soil sample was collected from Nakshatra garden, Baramati, Maharashtra, India. For the enrichment and isolation of bacteria, dilution of soil sample up to 10⁻⁵

was done. The diluted soil sample was inoculated into 100ml sterile nutrient broth containing 50ppm of zinc under aseptic conditions. The mixture was then incubated at 37 °C for 24 hours. A loopful of enriched culture was streaked onto sterile nutrient agar petri plates. Plates were then incubated at 37 °C for 24 hours and bacterial colony growth was monitored (figure.1) [12].

2.2 Screening, identification of resistant isolate

A single isolated colony was selected, and suspension was made with 10 ml of sterile saline (0.85% NaCl). Using an inoculating loop, the suspension was streaked on a Petri plate containing sterile nutritional agar with zinc (50 ppm). The plate was then incubated at 37 °C for 24 hours and the colonies were monitored for growth [13]. The identification of zinc resistant isolates was done by morphology characterization including size, shape, colour, margin, elevation, consistency, opacity. Microscopic examination includes motility, gram staining, endospore staining method and biochemical characteristics including oxidase, catalase, according to Bergey's Manual of Determinative Bacteriology [14].

2.3 Zinc intake's relative impact on bacterial growth

Bacillus subtilis was cultivated in sterile nutrient broth supplemented with different concentrations of zinc (50–200 ppm) in a rotary shaker set at 150 rpm and 37 °C for 6 to 8 hours. After incubation, the impact of heavy metal concentration on bacterial growth was evaluated by measuring the optical density at a wavelength of 600 nm using a UV Spectrophotometer [15].

2.4 Determination of Minimum Inhibitory Concentration (MIC) of zinc

The minimum inhibitory concentration (MIC) of zinc was calculated using the plate diffusion method. A 0.1 ml inoculum of isolated zinc-resistant isolate was spread over the entire surface of the sterile nutrient agar plate. A well measuring 6 mm in diameter was aseptically punched using a sterile cork borer. A 0.1 ml of zinc metal solution with different concentrations (50 ppm to 200 ppm) was added to the well under aseptic conditions. The plates were refrigerated for ten minutes to allow the zinc metal solution to diffuse. After that, the plates were incubated for 24 hours at 37 °C. The zone of growth inhibition (measured in millimetres) was noted after incubation. [16].

2.5 Zinc stock solution preparation and zinc aqueous solutions with varying concentrations

To prepare a stock solution of zinc with a

concentration of 1000 parts per million, 1 gram of zinc dust was dissolved in 30 millilitres of 5M HCl and making the final volume was 1 litre in a volumetric flask filled with deionized water. For various concentrations, a suitable dilution of a 1000 ppm zinc stock solution with deionized water was used to make 100 ml working solutions of zinc at concentrations of 25 ppm, 50 ppm, 75 ppm, and 100 ppm [17].

2.6 Preparation of bacterial biomass for biosorption and sodium alginate immobilized biosorbent

a) Bacterial Biomass preparation for biosorption Optical Density Measurement and Cell Quantification

The single colony of zinc-resistant bacteria that had grown on a nutrient agar plate was aseptically inoculated with 100 ml of sterile nutrient broth in a 150 ml conical flask, and it was then incubated for 48 hours at 37 °C in a rotary shaker set at 150 rpm.

Following incubation, biomass was extracted from the medium by centrifuging it for ten minutes at 9000 rpm. To ensure that no media remained on the cell surface, the supernatant was disposed and the cells were suspended in purified water for washing before being centrifuged once more. The biomass was heated to 60 °C for 24 hours in hot air oven. [18].

b) Preparation of sodium alginate immobilized biosorbent

A 2% (w/v) concentration of sodium alginate was dissolved in hot distilled water. Heat-inactivated bacterial biomass cell suspension was combined with sodium alginate solution in a 1:1 ratio using a magnetic stirrer until the mixture was completely homogenized. After cooling at 4 °C, the suspension was then extruded dropwise to a gelling solution of 0.1mol/L CaCl_2 using the syringe method. After allowing the beads to polymerize for half an hour, they were filtered out of the solution using a funnel and cleaned twice with distilled water. For biosorption, the biosorbent beads were kept in distilled water at 4 °C [19].

2.7 Biosorption experiment

A batch biosorption experiment was conducted in 300 ml plant tissue culture bottles with 100 ml of a single working solution of zinc metal ions at concentrations of 25 ppm, 50 ppm, 75 ppm, and 100 ppm at 37 °C on a rotary shaker (150 rpm) for 24 hours at pH 7.0 and a biosorbent dosage of 2 g/L. The biosorbent was separated by filtration method and by using an atomic absorption spectrometer, the residual metal ion concentration in the solution is measured [20].

2.8 Evaluation of the efficiency of metal uptake

The following equation was used to calculate the heavy metal uptake using a straightforward method: $q = (C_i - C_f) V/W$ using a sterile cork borer. A 0.1 ml of zinc metal solution with different concentrations (50 q = the metal uptake in mg/g.

C_i , C_f = the initial and final metal ion concentrations, expressed in mg/L

V = the volume of the solutions, L;

W = the dosage of the biosorbent, g.

The following formula was used to determine the removal of metal ions:

$R = [(C_i - C_f) / C_i] \times 100$ ppm to 200 ppm) was added to the well under aseptic conditions. The plates were refrigerated for ten minutes to allow the zinc metal solution to diffuse. After that, the plates were incubated for 24 hours at 37 °C. The zone of growth inhibition (measured in millimetres) was noted after incubation. [16].

3. Results:

3.1 Isolation of bacteria from soil sample

The bacteria from the enriched soil sample were successfully isolated on a nutrient agar plate (figure 1).



Figure 1: Isolated colonies of bacteria was observed on nutrient agar plate

3.2 Screening of zinc resistance isolate

The selected isolate showed zinc resistance as the growth of bacteria was observed on a nutrient agar plate with zinc (50 ppm) after incubating at 37 °C for 24 hours (figure 2).



Figure 2: Screening of zinc resistant isolate at 50ppm

3.3 Morphological Characterization

The zinc resistant isolate shows distinct colony morphology traits on the nutrient agar plate as summarized in Table 1. The isolate formed small colonies with an approximate size of 1.1 mm in diameter.

Table 1: colony morphology of zinc resistant isolate

shape	color	margin	elevation	consistensy	opacity
Circular	white	regular	flat	dry	opaque

3.4 Microscopic identification

Microscopic examination was done using Gram staining method shows gram positive, rod shaped, endospore forming bacteria (figure 2). The presence of endospore was further verified by endospore staining (figure 3).

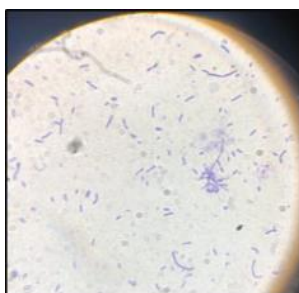


Figure 3: Gram staining of zinc resistant isolate shows gram positive, rod shaped bacteria

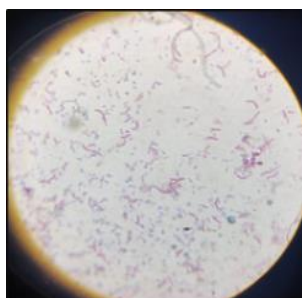


Figure 4: Endospore staining of zinc resistant isolate

3.5 Biochemical Identification

Biochemical characterization revealed that the zinc resistant isolate was positive for oxidase, catalase, starch hydrolysis, Voges-proskauer, citrate utilization test and growth in 6.5% NaCl. On the basis of morphological, microscopic and biochemical characteristics, the zinc-resistant isolate was identified as *Bacillus subtilis* according to Bergey 's Manual of Bacteriology.

Table 2: Biochemical characterization

Test	Result
Oxidase test	Positive
Catalase test	Positive
Starch hydrolysis test	Positive
Voges-Proskauer's test	Positive
Citrate utilization test	Positive

3.6 Relative effect of zinc consumption on bacterial growth

The relative impact of zinc consumption on bacterial growth; as zinc concentration rises, *Bacillus subtilis* growth declines (figure5).

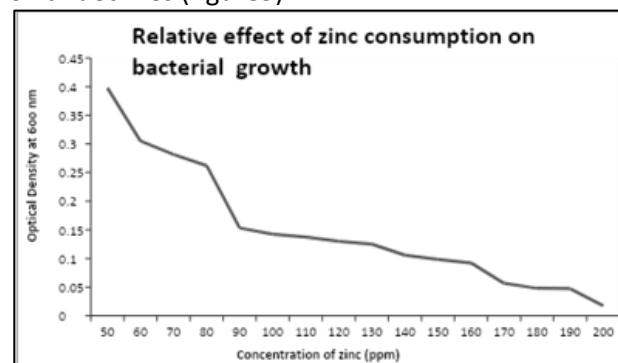


Figure 5: Graph showing Zinc was absorbed from water by dead biomass of bacteria

3.7 Determination of Minimum Inhibitory Concentration of zinc

The Minimum Inhibitory Concentration (MIC) of zinc against isolated zinc-resistant *Bacillus subtilis* was found to be 130 ppm by plate diffusion method (figure 6). and subsequent proliferation.

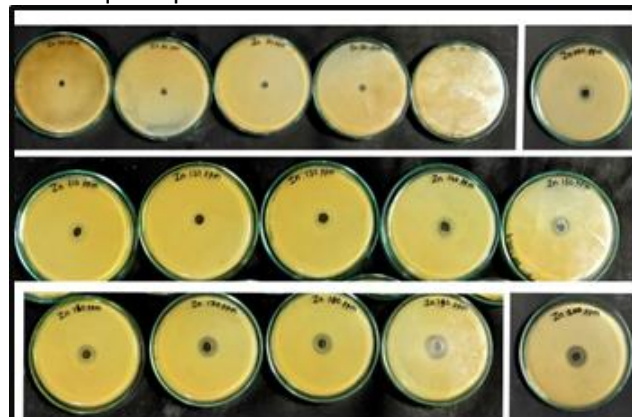


Figure 6: Minimum Inhibitory Concentration (MIC) of zinc.

3.8 Biosorption experiment

The dead biomass of *Bacillus subtilis* using sodium alginate was successfully prepared for zinc removal and used for biosorbent (figure 7). Batch biosorption experiment was conducted using zinc solutions at various concentrations (figure 8).

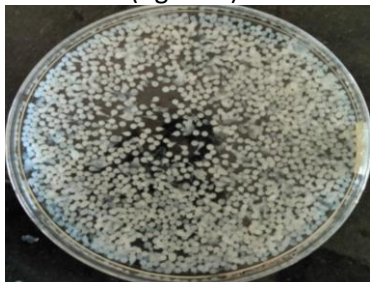


Figure 7: Sodium alginate immobilized with *Bacillus subtilis* biosorbent

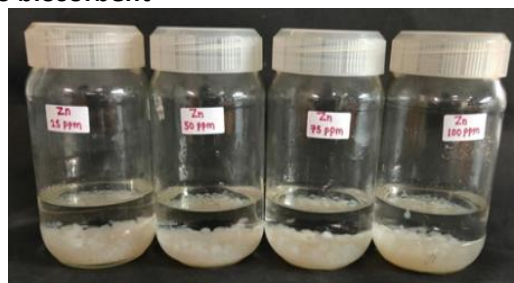


Figure 8: Zinc removal from aqueous solutions at concentrations 25 ppm, 50 ppm, 75 ppm, and 100 ppm.

By Using an atomic absorption spectrophotometer and atomic absorption spectroscopy (AAS), the residual zinc metal ion concentration in the solution is successfully determined at 25ppm, 50ppm, 75ppm, 100ppm. For initial zinc concentrations of 25, 50, 75, and 100 mg/L, the residual zinc concentrations following treatment were 06.25, 24.38, 40.58, and 58.56 mg/L, respectively.

Table 3: The residual zinc metal ion concentration following biosorption.

Initial concentration of zinc (ppm)	Residual/final concentration of zinc (ppm)
25	06.25
50	24.38
75	40.58
100	58.56

3.9 Metal uptake of zinc (q)

The following formula was used to determine the heavy metal uptake:

$$q = (C_i - C_f) V/W$$

The biosorption capacity was directly impacted by the initial zinc concentrations (q). The specific metal uptake was 0.9375, 1.281, 1.721, and 2.072 mg/g at initial concentrations of 25, 50, 75, and 100 mg/L, respectively. Due mostly to the increased availability and collision frequency of zinc ions in the solution, this upward trend shows that higher zinc levels increase the total mass of metal bound per unit of biosorbent.

Table 4: Metal uptake of zinc (q)

C _i Initial concentrati on of Zn (mg/L)	C _f Final concentrati on of Zn (mg/L)	V Volume of workin g solutio n (L)	W Bios orbe nt dosa ge (g).	q - metal uptake, (mg/g)
25	06.25	0.1	2	0.9375
50	24.38	0.1	2	1.281
75	58.56	0.1	2	1.721
100	58.56	0.1	2	2.072

3.9 Efficiency of zinc metal removal (%)

The following formula was used to determine the removal of metal ions:

$$R = [(C_i - C_f) / C_i] \times 100$$

At the lowest concentration of 25 mg/L, the maximum removal efficiency was 75%. The removal of zinc gradually decreased to 51.24%, 45.89%, and 41.44% as the starting concentrations increased to 50, 75, and 100 mg/L. This decrease implies that the fixed active binding sites on the immobilized bacterial biomass were quickly crowded and saturated by higher zinc concentrations, leaving a greater percentage of ions unabsorbed in the liquid phase.

Table 5: Efficiency of zinc metal removal (%)

Ci Initial concentration of Zn (mg/L)	Cf Final concentration of Zn (mg/L)	R Removal efficiency (%)
25	6.25	75
50	24.38	51.24
75	40.58	45.89
100	58.56	41.44

4. Discussion

The current study showed that *Bacillus subtilis* biomass immobilized in sodium alginate is a useful biosorbent for removing zinc ions from aqueous solutions and showed growth at 50 ppm of zinc concentration. The isolate was identified as Gram-positive, rod-shaped, endospore-forming bacteria. *Bacillus subtilis* are well known for their tolerance and interaction with heavy-metal ions and represent as a microbial resource for heavy-metal bioremediation because of their diverse metal-resistance and biosorption mechanisms [22, 23].

The present experiment revealed a concentration-dependent pattern. For initial zinc concentration of 25 ppm, residual zinc concentration was 6.25 ppm with 75% removal efficiency. When the initial concentration increased to 50ppm, 75ppm and 100 ppm, the percentage removal decreased to 51.24%, 45.89% and 41.44% respectively. The decrease in percentage removal at higher metal concentrations may be attributed to the progressive saturation of the binding sites of the biosorbent. At lower Zn concentrations, there are relatively more binding sites available compared to the amount of metal ions [24, 25].

The percentage removal decreased with increasing Zn concentration, the calculated Zn uptake increased from 0.9375 mg/g at 25 ppm to 2.072 mg/g at 100 ppm. This is common in biosorption systems. Higher initial concentrations increase the gradient between the aqueous phase and biosorbent, enhancing Zn (II) ion transfer to binding sites. Therefore, more zinc is retained per gram of biosorbent, even if the percentage removed decreases. Aryal also notes the importance of initial metal concentration in biosorption capacity and equilibrium behaviour [25].

Bacterial metal binding has been linked to carboxyl, hydroxyl, amino, and phosphate-containing groups via mechanisms like ion exchange, electrostatic attraction, and complex formation [24, 26]. Since the biomass used in this study was heat-inactivated before immobilization, the observed Zn removal is better explained by biosorption involving physicochemical interactions near the biomass surface rather than metabolically dependent intracellular accumulation [25].

The experimental conditions in this study were pH 7.0, 37°C, 2 g/L biosorbent dosage, and 24-hour contact time. These factors significantly influence Zn (II) biosorption. In particular, pH impacts the protonation state of functional groups on the bacterial surface, affecting interactions with metal ions. For instance, Joo et al. found that Zn (II) biosorption by *B. cereus* peaked around pH 6, suggesting that optimal conditions may vary by strain and system [27].

Using dead bacterial biomass offers advantages. It doesn't rely on cellular metabolism, eliminating the need for active growth during metal removal. Bacterial cell-wall components retain metal-binding capabilities after cell death, enabling the biomass to function as a passive biosorbent. In this study, the biomass was heat-treated at 60°C before being incorporated into the sodium alginate matrix.

Previous research supports the ability of *Bacillus* biomass to interact with Zn (II). Joo et al. studied Zn (II) biosorption by *Bacillus cereus* AUMC B52, reporting a maximum adsorption capacity of 66.6 mg/g. FTIR analysis revealed amino, carboxyl, hydroxyl, and carbonyl groups important for Zn (II) binding. A review on heavy-metal bioremediation by the genus *Bacillus* also highlighted these functional groups as contributors to Zn (II) uptake. These findings explain the zinc-binding behaviour observed with *B. subtilis* in this study, although confirming the functional groups would require spectroscopic analysis [24, 28].

The lower adsorption capacity observed in this study compared to some reported systems doesn't necessarily indicate poor performance. Biosorption capacity is highly influenced by experimental conditions such as pH, initial metal concentration, biomass concentration, contact time, temperature, biomass pre-treatment, and competing ions. For instance, the reported 66.6 mg/g capacity for *B. cereus* was achieved under different conditions, including variations in pH, contact time, biomass system, and adsorption model. An important

aspect of this study is the immobilization of bacterial biomass in sodium alginate. The biomass was incorporated into 2% sodium alginate and cross-linked with calcium chloride to form beads. Immobilization provides a structure that simplifies the recovery of microbial biomass from treated water, compared to freely suspended cells. This is crucial for wastewater-treatment applications, where separating and reusing dispersed biomass can be challenging. Previous studies have highlighted the immobilization and other modifications as strategies to enhance the practical use of microbial biosorbents [27].

Overall, the results show that, under the tested batch conditions, *Bacillus subtilis* biomass immobilized with sodium alginate has a detectable capacity to remove Zn (II). At a starting zinc concentration of 25 ppm, the maximum removal efficiency was 75%, while the highest calculated uptake, 2.072 mg/g, happened at 100 ppm. The increase in Zinc uptake per gram of biosorbent and the decrease in percentage removal with increasing Zn concentration indicate that the biosorption process was influenced by both the concentration gradient and the availability of surface binding sites. These results encourage more research on immobilized *B. subtilis* for the treatment of zinc-contaminated water, especially by optimizing pH, biosorbent dosage and contact time, adsorption kinetics, equilibrium modeling, regeneration, and testing with real industrial wastewater.

5. Conclusion:

The current study examined the potential of *Bacillus subtilis* dead biomass immobilized in sodium alginate for the removal of zinc ions from aqueous solutions. This study showed that *Bacillus subtilis* cells immobilized in sodium alginate can be useful biosorbents for the removal of zinc. The initial concentration of metal ions had a significant impact on the metal's biosorption. Zinc-polluted wastewater could be treated using *Bacillus subtilis* as a sorbent and immobilized sodium alginate.

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Abbreviations:

°C: Degree Celsius

ml: Milliliter

%: Percentage

μL: Microliter

rpm: Revolutions per minute

M: Molar/Molarity

L: Liter

nm: Nanometer

mm: Millimeter

mM: Millimole

g: Gram

Ppm: Parts per million