Indian Journal of Experimental Biology
Vol. 60, October 2022, pp. 798-804
DOI: 10.56042/ijeb.v60i10.37308

Biosorption of Zinc using *Bacillus subtilis* (MTCC 2423)

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Received 09 March 2021; *revised 27 October 2021*

Heavy metals such as zinc in untreated industrial effluents cause diseases and disorders in living organisms. They cannot be degraded like organic contaminants and hence have to be removed. Though physical and chemical methods are available for their removal, most of them are not economical and eco-friendly. Hence, a suitable technique is necessary to minimize the deleterious effects of dispersion of heavy metals in ecosystems. Though zinc serves as a micronutrient, it becomes toxic in higher concentrations. Bacteria can be used in the removal of zinc and the process is economical and ecofriendly. Hence, in the present study, we tested zinc removal efficiency of *Bacillus subtilis* (MTCC 2423) for various concentrations viz. 100, 200, 300, 400 and 500 ppm of zinc in nutrient broth for a period of 10 days. Samples were tested for the zinc level every two days in each concentration and the maximum removal was noticed after six days of treatment. With the increase in zinc concentration, both biomass and zinc removal efficiency showed an increase. Autoclaved cells showed maximum zinc removal when compared with other cell types. Among the other heavy metals tested, iron enhanced the biomass of *B. subtilis* during zinc treatment and the results are discussed.

Keywords: Adsorption, Bioremediation, Heavy metal pollution, Immobilized cells, Industrial effluents, Isotherms

Rapid urbanization and industrialization without proper environmental planning led to the discharge of toxic heavy metals in the environment[1]. These toxic metals discharged in to the environment may result in geoaccumulation, bioaccumulation and bio-magnification[2]. Contamination of heavy metals in the environment is a major global concern because of their toxicity and threat to human life and environment[3]. Zinc is an important heavy metal which occurs in the atmosphere around zinc smelters, scrap zinc refineries and most often as a result of industrialization and human activities[4]. Zinc can cause damage to the stomach lining and severe hemolytic anemia. Zinc ions are highly toxic to plants, invertebrates and fish. Therefore, studies on the removal of zinc were encouraged[5].

Metals can be removed from the environment by conventional processes such as chemical precipitation, chemical oxidation or reduction, electrochemical treatment, evaporative recovery, filtration, reverse osmosis, ion exchange and membrane technologies by making use of conventional adsorbents such as silica gel, active alumina, zeolite, and metal oxides[6]. But they have certain disadvantages like unpredictable metal ion removal, high quantities of reagents and sludge generation and disposal along with high cost of installation and operation[7]. The alternative use of microbe-based biosorbents for the removal and recovery of toxic metals from industrial effluents can be economical and effective for metal removal[8]. Several microbes including bacteria, fungi, yeasts, cyanobacteria and algae have been reported to remove a variety of heavy metals from waste water[9]. Among bacteria, *Bacillus subtilis*, a Gram positive and rod shaped bacterium has been identified with a high potential for metal sequestration due to uptake of metals[10]. Hence, in the present study, we planned to characterize zinc biosorption behaviour of *Bacillus subtilis*.

Materials and methods

Zinc sulphate salt was dissolved in sterile distilled water to prepare various concentrations of zinc.

Strain procurement and maintenance

The bacterial strain, *B. subtilis* (MTCC 2423) used in the present study was obtained from Microbial Type Culture Collection (MTCC), CSIR-IMTECH, Chandigarh, India. A pinch of the obtained culture was seeded into nutrient broth. After eighteen hours, a loopful culture was streaked onto nutrient agar slants and incubated at 37°C for 24 h. Later they were stored at 4°C[11].

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Estimation of metal tolerance

The maximum concentration of zinc for bacterial growth was determined by inoculation of the selected bacterial strain onto the nutrient agar medium (peptic digest of animal tissue 0.5 g, beef extract 0.15 g, yeast extract 0.15 g, sodium chloride 0.5 g and agar 1.5 g in 100 mL of distilled water) containing wide range of zinc concentrations (50, 100, 500, 1000, 2000, 3000 and 4000 ppm). The plates were incubated at 37°C and were observed for growth after 24 h[12].

Sample preparation

The overnight culture of the bacterial strain maintained in nutrient broth (peptic digest of animal tissue 0.5 g, beef extract 0.15 g, yeast extract 0.15 g and sodium chloride 0.5 g in 100 mL of distilled water; pH 7.4) was inoculated (10^9 cells) into nutrient broth having various concentrations of zinc (200, 400, 600, 800 and 1000 ppm). The flasks were incubated at 30°C on a shaker for intermittent mixing and the samples were then subjected for the estimation of residual zinc concentration after every two days up to 10 days[12].

Estimation of residual zinc concentration

About 10 mL of the sample from the culture flask was taken in a centrifuge tube and was subjected to centrifugation at 2500 rpm for 15 min. The supernatant was taken in an Eppendorf tube and subjected to atomic absorption spectrophotometric (AAS) analysis (Model: MSA030351; Thermo Fisher Scientific Ltd., India) and the readings were recorded[13].

Biomass estimation

Pellet obtained from the previous step was transferred into a Petri dish which was dried in a hot air oven at 80°C for 3 h. The final dried biomass was weighed[14].

Preparation of different cell types

For preparing immobilized cells, the bacterial cells harvested from nutrient broth were centrifuged at 8000 rpm for 20 min. The cells were washed and suspended in 0.1% NaCl. Then 3.5% of sodium alginate was added to the cell suspension and mixed thoroughly without forming any air bubble in the slurry. The slurry containing cells was extended as drops through a tube (2 mm diameter) into 4% $CaCl_2$ solution. The drops formed into spherical beads of 2 mm size. The gel beads were kept in 4% $CaCl_2$ solution, at 5°C for about an hour for complete gelation[14]. Then the beads were washed with sterile distilled water and used for zinc biosorption study. For obtaining dead cells, the bacterial culture (24 h) in nutrient broth was autoclaved at 121°C for 30 min. The third type of cells used for the study was live cells, obtained from overnight culture of *B. subtilis* in nutrient broth.

For testing the biosorption of different cell types, 100 mL of minimal broth containing 1000 ppm of zinc in 250 mL Erlenmeyer flasks was prepared. To such flasks, different preparations were inoculated individually (10^9 cells for live and dead preparations and 10 beads in the case of immobilized cells) and samples were taken after every 30 min up to 150 min[14].

Atomic absorption spectrophotometry

About 10 mL of the sample containing minimal broth, 1000 ppm concentration of zinc and different preparations of inoculum was centrifuged at 2500 rpm for 15 min, after every 30 min up to 150 min and subjected to AAS analysis. The values so obtained from AAS analysis represented the residual concentration of zinc in solution[15].

Statistical analysis

Two way analysis of variance (ANOVA) was performed on the factors like residual zinc concentration, percent removal of zinc and biomass of *B. subtilis* during zinc treatment for the two variables namely zinc concentration and treatment period. It was also carried out for residual zinc concentration and percent removal of zinc by different cell preparations with treatment period and cell types as variables, using Microsoft MS- Excel package (Version: 12.0.6219.1000)[13].

Calculation of zinc biosorption

Zinc adsorption by the biosorbent was calculated using the following mass balance equation for the biosorbent[16]:

$$q = [V (C_i - C_f)]/S \quad \text{... (1)}$$

where, q = zinc uptake (mg metal/g cell dry weight); V = volume of metal-bearing solution contacted with the biosorbent (L); C_i = initial concentration of metal in solution (mg L^{-1}); C_f = final concentration of metal in solution (mg L^{-1}); S = dry weight of biosorbent added (g).

Biosorption models

Freundlich[17] and Langmuir[18] isotherm models were used for interpreting zinc biosorption equilibrium. The classical Freundlich equation is given below:

$$q = K_f C_e^{1/n} \quad \text{... (2)}$$

where, q = heavy metal adsorbed on biosorbent (mg/g dry weight); C_e = final concentration of metal (mg L^{-1})

in solution; K_f = an empirical constant that provides an indication of adsorption capacity of biosorbent; n = an empirical constant that provides an indication of intensity of adsorption.

Equation [2] can be linearized as follows:

$$\text{Log } q = \log K_{f+} (1/n) \log C_e \quad \ldots (3)$$

The adsorption constants (K_f and 1/n) were determined by plotting log q as a function of log C_e.The classical Langmuir equation is given below:

$$q = (Q_{max}\, b\, C_e)/ (1+ b\, C_e) \quad \ldots (4)$$

where, q = heavy metal adsorbed on biosorbent (mg G^{-1} dry weight); C_e = final concentration of metal (mg L^{-1}) in solution; Q_{max} = maximum possible amount of metallic ion adsorbed per unit weight of adsorbent; b = equilibrium constant related to affinity of binding sites for the metals.

Equation [4] can be linearized as follows:

$$1/q = (1/q_{max}) + (1/q_{max}b)\,(1/C_e) \quad \ldots (5)$$

The adsorption constants (Q_{max} and b) were obtained by plotting 1/q as a function of $1/C_e$.

Results

Bacillus subtilis (MTCC No.2423) was found to be tolerant up to 500 ppm of zinc which was identified by the ability of the organism to grow in nutrient agar medium containing various concentrations of zinc. The residual concentrations of zinc after treatment with *B. subtilis* are given in Table 1. The least residual concentration was found during the sixth day of treatment at 400 ppm zinc concentration. This indicates that removal of zinc was very effective at this particular stage.

Figure 1 shows the percent removal of zinc when treated with *B. subtilis*. The highest percent removal was observed at 400 and 200 ppm during the 6th and 8th days of treatment, respectively. Fig. 2 depicts the amount of biomass (g/mL) of *B. subtilis* during zinc treatment. A gradual increase in the biomass was observed with respect to the treatment period till the 8th day of treatment and then there was a decrease in biomass by 10th day of treatment. When the biomass was high, the efficiency of zinc removal was also high.

Residual concentration values of zinc after treatment with different preparations of *B. subtilis* are given in Table 2. There was a decrease in residual concentration of zinc with an increase in treatment period. Autoclaved cells were found to be very effective by showing low residual concentrations of

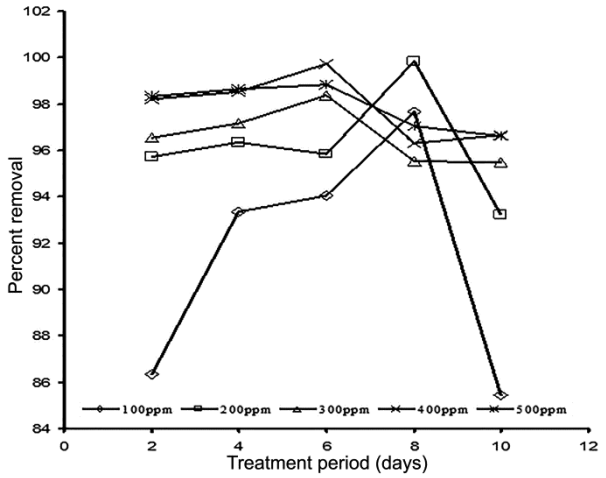

Fig. 1 — Percent removal of zinc after treatment with *Bacillus subtilis*

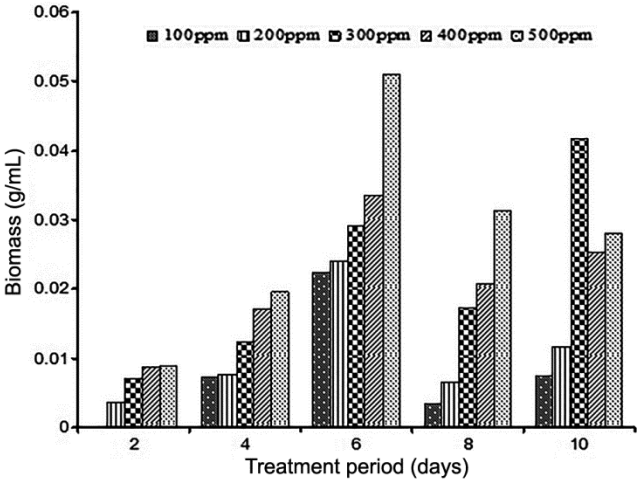

Fig. 2 — Biomass (g/mL) of *Bacillus subtilis* during zinc treatment

Table 1 — Residual concentration of zinc (ppm) after treatment with *Bacillus subtilis*

Treatment period (days)	Zinc concentration (ppm)				
	100	200	300	400	500
2	13.6763	8.5748	10.4189	7.1866	8.3782
4	6.6468	7.3059	8.4902	5.8803	6.7836
6	5.9443	8.2735	4.8783	1.0616	5.7290
8	2.3390	0.2711	13.4582	14.7134	14.6876
10	14.5611	13.5319	13.5338	13.3527	16.9346

Table 2 — Residual concentration of zinc (ppm) after treatment with *B. subtilis* of different preparations

Treatment period (minutes)	Cell types		
	Live	Autoclaved	Immobilized
30	16.4693	19.8199	19.7264
60	21.0509	16.6128	18.2199
90	19.9249	19.8430	23.8509
120	22.1836	15.9997	18.9463
150	17.6592	12.9189	19.6852

zinc. Fig. 3 illustrates the percent removal of zinc during the treatment with different preparations of *B. subtilis*. The efficiency of zinc removal by autoclaved cells was found to be more with the increase in treatment period when compared to live and immobilized cells. The influence of heavy metals on the biomass (g/mL) of *B. subtilis* during zinc treatment is shown in Fig. 4. The highest amount of biomass was observed for iron and the lowest for cadmium. This shows that iron has the ability to influence the adsorption of zinc.

Two way analysis of variance for the factor, residual concentration of zinc with the variables, treatment period and cell types indicated that variations in residual concentration of zinc due to treatment period and cell types were not statistically significant at 5% level. Variation in residual concentration of zinc due to zinc concentration was statistically significant but not significant due to treatment period at 5% level. Variation in biomass (g/mL) of *B. subtilis* due to zinc concentration was statistically significant at 5% level but not significant due to treatment period. Variations in percent removal of zinc due to cell types and treatment period were not statistically significant at 5% level. Variation in percent removal of zinc due to zinc concentration was not statistically significant at 5% level but significant due to treatment period (Table 3). The Freundlich and Langmuir adsorption isotherm details for zinc biosorption by *B. subtilis* after every two days of treatment period are given in Table 4. In Freundlich isotherm models, R^2 was the maximum after two days of treatment and it showed a decline with the increase in treatment period. Kf was the highest after fourth and eight day of treatment while '1/n' was the maximum after two days of treatment. In case of Langmuir models, R^2 was the highest after eight days and Qmax was the highest after two days of treatment while 'b' was the highest after four days of treatment.

Discussion

Heavy metals released by a number of industrial processes are the major pollutants in marine, ground,

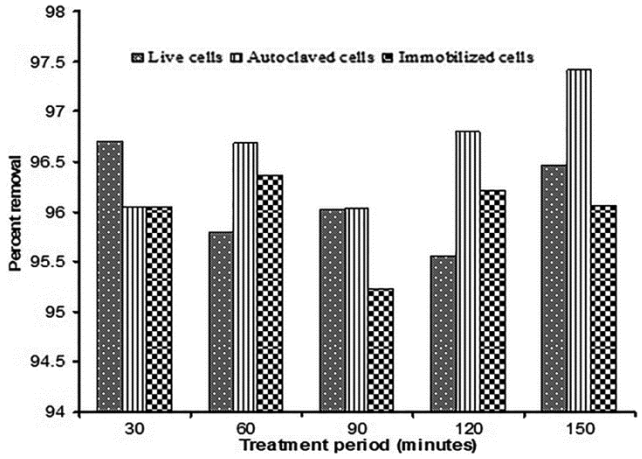

Fig. 3 — Percent removal of zinc after treatment with *Bacillus subtilis* of different preparations

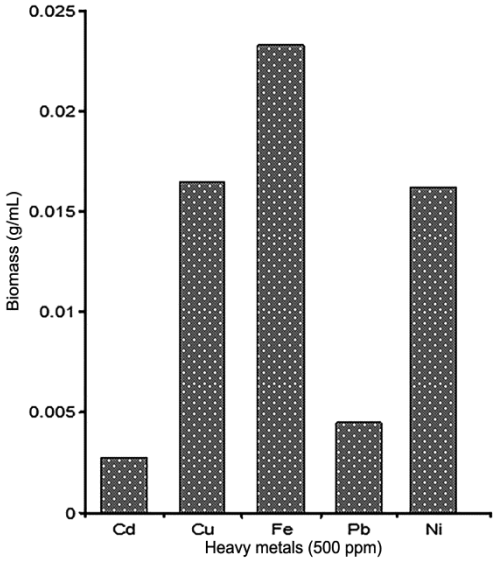

Fig. 4 — Influence of heavy metals on the biomass (g/mL) of *Bacillus subtilis* during zinc treatment

Table 3 — Results of two way analysis of variance (ANOVA) for the various factors during the bioremediation of zinc by *B. subtilis*

Factor	Source of variation	Calculated F value	F table value at 5% level	Level of significance
Residual concentration of zinc	Zinc concentration	4.14	3.00	Significant (P <0.05)
	Treatment period	0.41	3.00	Not significant (P >0.05)
Residual concentration of zinc	Cell types	1.33	3.83	Not significant (P >0.05)
	Treatment period	2.28	4.45	Not significant (P >0.05)
Biomass(g/mL) of *B.subtilis*	Zinc concentration	3.69	3.00	Significant (P <0.05)
	Treatment period	1.67	3.00	Not significant (P >0.05)
Percent removal of zinc	Zinc concentration	2.43	3.00	Not significant (P >0.05)
	Treatment period	6.16	3.00	Significant (P <0.05)
Percent removal of zinc	Cell types	1.33	3.83	Not significant (P >0.05)
	Treatment period	2.28	4.45	Not significant (P >0.05)

Table 4 — Isotherm constants for zinc biosorption by *B. subtilis*

Treatment Period(days)		Isotherm Constants	
2	Freundlich	R^2	0.6603
		Kf	3.162
		1/n	1.4281
	Langmuir	R^2	0.4721
		Qmax (mg/g)	125
		b (L/mg)	0.0074
4	Freundlich	R^2	0.0539
		Kf	17.78
		1/n	0.2125
	Langmuir	R^2	0.0422
		Qmax (mg/g)	23.80
		b (L/mg)	0.1977
6	Freundlich	R^2	0.2834
		Kf	6.607
		1/n	–0.1763
	Langmuir	R^2	0.0563
		Qmax (mg/g)	100
		b (L/mg)	–0.0401
8	Freundlich	R^2	0.0672
		Kf	17.78
		1/n	–0.0699
	Langmuir	R^2	0.4811
		Qmax (mg/g)	33.33
		b (L/mg)	–0.0923
10	Freundlich	R^2	0.1494
		Kf	10.47
		1/n	0.1405
	Langmuir	R^2	0.1286
		Qmax (mg/g)	19.23
		b (L/mg)	0.1115

industrial and even treated waste waters[19]. Due to toxic nature, heavy metals like zinc pose a threat to human life and environment. Metals have been linked to birth defects, cancer, skin lesions, disabilities, and liver and kidney damage[20]. Bioremediation is the most promising and cost effective technology widely used now-a-days to clean up both soil and waste water containing organic and inorganic contaminants[21]. Bacteria are ubiquitous in nature with highly resistant cell walls that are anionic. These anionic cell walls can fix metals and provide sites for nucleation and growth of minerals[22]. Microorganisms have been used in a number of biological treatment processes for metal remediation[23]. *B. subtilis* was found to be efficient in removal of heavy metals[24]. In the present study, *B. subtilis* strain was more effective in zinc removal after six days of treatment and tolerated up to 500 ppm of zinc. In contrast to Gram negative bacteria, *B. subtilis* (Gram positive bacteria) have elevated level of heavy metal binding because of the presence of teichoic acids and other acids in cell wall[25].

Biosorption is recognized as an alternative process for eliminating toxic heavy metals from polluted soil and water[26]. Living cells (metabolically active) and dead cells (metabolically inactive) are employed in removal of heavy metals. Metal cations can be adsorbed by living and nonliving biomass in diverse manner. Dead cells can immobilize metals by biosorption, but free cells may immobilize soluble metal species both by biosorption and by other mechanisms that are part of and/or are suitable to microbial metabolism[27]. In the present work, dead cells showed 99% of zinc removal after sixth and eighth day of treatment and found to be more effective than that of live and immobilized cells. Dead cells have several advantages over live cells which include minimum processing time for large scale of wastewater, ease of access and no requirement of nutrients[28,29].

Several isotherm models have been exploited to fit the equilibrium data to reveal the nature of biosorption process. Among them, Langmuir and Freundlich isotherm models have been most commonly applied for biosorption of heavy metals employing bacteria. They are uncomplicated and explicate experimental equilibrium data splendidly[30]. Theoretical basis of the Langmuir model relies on that there are a limited number of binding sites on adsorbent surface with the same affinity for adsorption of a single molecular layer and there is no interaction among adsorbed molecules. Freundlich model assumes that adsorption energy of a metal-binding to a site on an adsorbent depends on whether the adjacent sites are previously occupied or not. The adsorption data was a fit for both Langmuir and Freundlich models for sorption of metal ions, but no one clarified how these two conflicting models fitted experimental data concurrently[31]. In the present study, Freundlich and Langmuir isotherm coefficients for zinc biosorption by *B. subtilis* were R^2= 0.6603, K_f= 3.162, 1/n= 1.4281, R^2= 0.4721, Q_{max}= 125 and b= 0.0074. Presence of iron had greater influence over biosorption ability of *B. subtilis* towards 500 ppm of zinc than the other heavy metals such as cadmium, copper, lead and nickel which was supported by the work of Chong & Volesky[32] who showed that the presence of one metal has its influence over the other metals. Slightly acidic or neutral medium was better for both bacterial growth and metal removal which shows similarity with the present work in which zinc adsorption by *B. subtilis* took place in the nutrient

broth at neutral pH[33]. Binding of the metal ions to bacterial cells is due to the presence of carboxyl, ether, alcoholic and amino groups[34]. This statement confirms the result obtained in the present work on the biosorption of zinc by *B. subtilis*.

Conclusion

Bacillus subtilis (MTCC 2423) was able to tolerate 500 ppm of zinc and zinc removal was better after six days of treatment. When cell types are compared, autoclaved cells performed better than that of live and immobilized cells with reference to zinc removal. Biomass was maximum in 500 ppm zinc after six days of treatment. With reference to the influence of other metals, iron enhanced the biomass of *B. subtilis*. It can be used in the biosorption process for removal of zinc from the industrial effluents for cleaning up of the polluted sites as it has better adsorption capacity.

Conflict of interest

Authors declare no competing interests.

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