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Condition Optimization, Free Energy Considerations and Isothermal Studies of Zinc Sorption by the Cyanobacterium *Anabaena variabilis* MEGCH1

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Abstract: ***Heavy metal contamination has long been recognized as a significant public health threat, especially in developing nations, and their toxicity consequences are well realized. Traditional remediation methods are either expensive or produce hazardous by-products derogatory to both physical and biological environments. Microbial biosorption has proven to be an effective method of removing heavy metals from contaminated water. The cyanobacterium Anabaena variabilis MEGCH1 was isolated from a coal mine region of Chiehruphi, Jaintia Hills, Meghalaya, and its ability to remove Zn^{2+} from aqueous solutions was investigated in this study. Zn^{2+} binding on the cyanobacterial biomass was confirmed by SEM-EDX, and FTIR analysis indicated several negatively charged functional groups on the cell surface that assisted in metal binding. Zn^{2+} biosorption was impacted by a number of experimental variables, including pH, temperature, inoculum age and size, shaking rate, and contact time. The sorption percentage was dramatically increased when the experimental circumstances were optimized. Thermodynamic analyses demonstrated that the biosorption process was energetically feasible, with a negative free energy change at various temperatures [-6.703 kJ/mol at 308 K (35°C), -6.595 kJ/mol at 303 K (30°C) and -6.486 kJ/mol at 298 K (25°C)]. According to the isotherm modeling research, the Freundlich isotherm (R^2 value- 0.9559) matched better than the Langmuir isotherm (R^2 value- 0.9427), implying that Zn^{2+} binding to the organism biomass was a multilayer sorption process. In addition, various other thermodynamic and isothermal parameters indicate favorable binding of Zn^{2+} on the biomass surface. Further, the organism also revealed a considerable tolerance toward Zn^{2+} exposure.***

Keywords: *Anabaena variabilis* MEGCH1, zinc sequestration, SEM-EDX and FTIR, Thermodynamics, Freundlich and Langmuir isotherms

1. Introduction

Heavy metals in the environment are released through mining activities, industrial discharges, volcanic activities, combustion of fossil fuels, use of pesticides/insecticides in agriculture, etc. [1, 2]. All heavy metals at high concentrations are toxic to the environment because of their non-biodegradable nature and can have implications for the well-being of humans, animals, as well as plants and microorganisms [3]. However, trace amounts of heavy metals like Zn^{2+}, Co, Cu, Mn, Ni, Mo, Fe, etc., have various roles in living organisms acting as protein structural components and as cofactors of several enzymes [4]. Some heavy metals like Mn, Fe, Cu play an important role in the electron transport chain of photosynthetic organisms [5]. Other heavy metals such as Fe, Cu and Zn^{2+} are responsible for scavenging free radicals as these metals are cofactors of enzymatic antioxidants (superoxide dismutase, catalase, ascorbate peroxidase, etc.) [6–8]. Zinc is required for the normal growth of the organism. It mainly serves as a cofactor for many important enzymes such as carbonic anhydrase, which plays a role in the carbon concentrating mechanism of photosynthesis. It is also an essential component of zinc fingers and in structural motifs found in transcription factors [9, 10]. However, Zn^{2+} when present in high concentrations in water, induces oxidative stress leading to protein, lipid and DNA damage thereby influences the growth and development of the organism [11, 12]. The presence of high Zn^{2+} concentration in water causes harsh effects on the aquatic organisms as well that pose serious threats to human beings via their accumulation in the food chain [13, 14]. Exposure to high concentrations of zinc can cause severe health problems to humans such as skin irritations, vomiting, dizziness, nausea, stomach pains, pancreas impairment, anemia, edema and sleeping difficulties [2, 15, 16]. The recommended concentration of Zn^{2+} in drinking water by the World Health Organization (WHO) is 5 mg/L [17].

Anthropogenic activities over the years to meet the human population demands on natural resources such as coal, petroleum, natural gas etc., has led to the accumulation of various heavy metals in the environment. Due to their non-biodegradable nature and obvious detrimental effects on living organisms, it is imperative to find measures for cleaning up of these metal ions from the environment. Over the last few decades, researchers have been experimenting with various chemical and physical treatments to reduce the concentrations of these metals in the environment. Among them, chemical precipitation, ion exchange, membrane filtration, coagulation and flocculation, electrochemical treatment, solvent extraction and evaporation are often used [18–22]. However, these methods have their own disadvantages as they are expensive, have low removal

potential and because of their chemical-based process they can even lead to more pollution [23, 24].

Thus, alternative methods for removal of various contaminants including heavy metals that are more friendly, low cost and non-polluting such as the biosorption process using biological matters are being extensively researched [25–29]. For example, the biosorption method using plants and microorganisms such as water hyacinth, bacteria and cyanobacteria have been used for the last decades as this process is more viable economically and shows a high ability to remove environmental pollutants without producing toxic substances [22, 24, 30–32].

Cyanobacteria are a group of prokaryotic organisms that have the ability to fix both carbon and nitrogen. They originated 3.8 billion years ago and can survive in a diverse range of environments [33, 34]. They have many industrial applications such as biofertilizers, in the production of dietary supplements, dye for food and cosmetics, immunodiagnostic probes, in fluorescence microscopy and biofuels etc. They are also being researched as potential medicines as various cyanobacteria have anticancer, antifungal, antituberculosis, antibacterial and antitumor activities [35–37].

In another application, cyanobacteria are being intensely researched for their potential in the biosorption of heavy metals. This is because cyanobacteria are ubiquitous and exhibit good biosorbent potential. Being both photosynthetic and nitrogen fixing, their biomass production and maintenance is also cost-effective [38, 39]. Various cyanobacteria that grow and thrive in heavy metal polluted sites show metal tolerance and therefore are being investigated for their metal removal potential aiming toward environmental clean-up and wastewater treatments [32, 40–43].

The state of Meghalaya situated in the North-Eastern India is rich in coal, limestone and uranium that is being mined unscientifically leading to the release of various heavy metals into the nearby water bodies. There are very few scientific studies relating to heavy metal removal from these areas. However, in the field of bioremediation there are few studies that explored the potential of different bacteria and cyanobacteria isolated from these sites for metal removal and sequestration [32, 41, 43–49]. The evaluation of the potential of these indigenous microbial species shaped the idea that they could be used for bioremediation of metal-polluted areas. As pointed out earlier, this may be because microbes present in the mining sites have already adapted to the high concentrations of heavy metals and therefore, metals sequestration by these microbial strains may be more effective compared to other strains from non-polluted areas. Among various microbial strains cyanobacteria shows promising application potential due to their cost-effectiveness and high surface binding potential towards metals.

Keeping this in mind, the present work was carried out to access the biosorption potential of a cyanobacterium native to a site contaminated with heavy metals. The study included assessment of surface binding potential, optimization of experimental conditions, thermodynamics and characteristics of metal binding using Langmuir and Freundlich adsorption isotherms modeling.

2. Materials/ Methods

2.1. Growth conditions

The cyanobacterium *Anabaena variabilis* MEGCH1 (GenBank accession No: **MF589225**) was isolated from coal mine areas of Chiehruphi, Jaintia Hills, Meghalaya. The organism was grown in BG-11_0 medium at pH 7.5 in the culture room at a temperature of 25 ± 2 °C and the photon fluence rate was kept at 50 µmol m^2/s [50].

2.2. Test cultures

$ZnSO_4.7H_2O$ was used as the source of Zn^{2+} metal. A 10 µM Zn^{2+} solution was prepared by diluting a stock solution (1 mM) with BG-11_0 media. For treatment, the cyanobacteria were exposed to the metal ion for 24 h before elucidating different biosorption parameters, whereas the unexposed cell culture was taken as control.

2.3. Elemental analysis using Scanning Electron Microscopy-Energy dispersive X-ray (SEM-EDX) spectroscopy

Samples for elemental analysis of metal ions taken up by the organism were studied by Energy dispersive X-ray (EDX) spectroscopy (INCA Penta FETX3) combined with scanning electron microscopy (JSM, 6360, JEOL, Tokyo, Japan) according to the standard protocol described in Nongrum and Syiem, and Goswami et al. [51, 52]. The samples were fixed with 4 % glutaraldehyde for 4 h and then were washed three times with 0.1 M sodium cacodylate buffer at an interval of 15 min each at 4 °C. Further, the samples were dehydrated using acetone (10 – 90 %) at 4 °C. The dehydrated samples were mounted on brass stubs and gold-coated before viewing under SEM and the presence of Zn^{2+} ion was analysed using Energy Dispersive X-ray spectroscopy.

2.4. FTIR spectroscopic analysis

Fourier Transform Infrared (FTIR) Spectroscopy was used for comparative determination of functional groups available for Zn^{2+} binding [53]. 1 mL of both control and treated cell cultures were added onto the petri-plates and was dried in an oven under slow heat. After the medium was evaporated, the dehydrated cultures were mixed with dried spectroscopic-grade potassium bromide in the ratio 1:10 (w/w) for pellet formation. The pellets were then analyzed using Fourier Transform Infrared Spectroscopy (FTIR) (Perkin Elmer Spectrum 400 FT-IR/ FT-FIR Spectrometer; MODEL: SP400) operating in the range of 4000 cm^{-1} to 500 cm^{-1}.

2.5. Analysis of water samples

The water sample collected was pre-digested with concentrated nitric acid and the presence of heavy metals in

the water sample was analyzed by atomic absorption spectrophotometer (AAS 240, Varian, USA), SAIF, NEHU.

2.6. Metal biosorption studies

The metal uptake by the cyanobacterium *Anabaena variabilis* MEGCH1 and the cellular distribution of the quantity of the metal taken up was determined according to the method of Mohammed, and Nongrum and Syiem [51, 54]. For metal uptake, the cyanobacteria were inoculated in metal supplemented media and incubated in different conditions- pH, temperature, inoculum age, inoculum size, shaking rate and time as well as in varying metal concentration. After 24 h of incubation in different conditions, for metal uptake and distribution study, Zn^{2+} metal was added into the cyanobacterial culture in a final volume of 30 mL and after 24 h, the solution was centrifuged at 2500 rpm for 5 min and the supernatant was used for estimating the total metal ions removed using AAS. To the pellet, 30 mL of the double-distilled water was added, mixed and centrifuged at 2500 rpm for 5 min. The supernatant was used for determining the amount of metal ions precipitated on the cell surface by AAS. The pellet was then resuspended in 30 mL of 0.01 N HCl and again centrifuged at the same speed. The supernatant from this step analysed using AAS showed the amount of metal ions adsorbed on the cell surface. The last step was done by adding 30 mL of double distilled water to the pellet and sonicate. The mixture was centrifuged for 5 min at 2500 rpm to determine the amount of metal ions accumulated inside the cell.

The percent removal of metal was calculated using the Eq.

$$\%\, removal = \frac{C_I - C_F}{C_I}\ X\ 100$$

C_I: Initial concentration of metal present in the medium
C_F: Metal concentration remained in the supernatant.

2.7. Optimization of experimental conditions

2.7.1. pH and temperature

Different pH of the medium was used (6, 7, 7.5, and 8) to study the effect of pH on the removal of Zn^{2+} metal. For temperature, its effect on metal removal was studied by setting the experiments at three different temperatures- 298 K (25 °C), 303 K (30 °C), and 308 K (35 °C). The incubation period was kept for 24 h and the total amount of metal ions removed was determined using AAS.

2.7.2. Initial inoculum size and age

The experimental cultures with initial chlorophyll *a* concentration 2, 3, 4, 5 and 10 µg/mL was prepared along with 10 µM of Zn^{2+} in a total volume of 30 mL. After the 24 h incubation period, the amount of metal removed and the inoculum size with the highest metal removal was estimated. Similarly, the initial inoculum age: 2, 4, 6, 8……32-day-old was taken and grown in 30 mL of Zn^{2+} supplemented medium. After 24 h incubation, the amount of metal removed by each of the inoculum size employed was calculated.

2.7.3. Shaking rate

To determine the most suitable shaking speed, the Zn^{2+} supplemented cultures were subjected to different shaking rate on an orbital shaker. After 24 h of experimentation, the effect of shaking was determined by calculating the total amount of metal ions removed by the cultures.

2.8. Influence of initial metal concentration on biosorption

The initial concentration of chlorophyll *a* concentration of 3 µg/mL was taken and incubated with different Zn^{2+} concentrations ranging from 1 – 12 µM. At the end of 24 h incubation period, the total amount of metal removed was determined to study the influence of metal concentration on biosorption.

2.9. Effect of contact time on metal biosorption

Different time intervals of 0, 5, 15, 30, 60 and 120 min was used for studying the influence of contact time on biosorption. The cultures were incubated in Zn^{2+} supplemented medium and the total amount of metal ions removed at above specified time intervals was estimated.

2.10. Determination of thermodynamic parameters

The thermodynamic parameters- ΔG (change in free energy), ΔH (change in heat content or enthalpy) and ΔS (change in entropy or randomness) for the removal of Zn^{2+} at different temperatures, 298 K (25 °C), 303 K (30 °C), and 308 K (35 °C) were calculated according to the formula below (Reddy et al., 2012)

$$\Delta G = -RTlnK \ or\ \Delta G = -2.303RTlogK \qquad \text{(Eqn. 1)}$$

Where, ΔG is the change in the free energy content (kJ/mol); R is the molar gas constant (8.314J/K/mol) and T is the absolute temperature (in K). The equilibrium constant K can be defined as the mass action ratio between the concentration of metal adsorbed (µg/mL) on the cyanobacterial biomass and the concentration of remaining metal (µg/mL) in the medium (C_F) at equilibrium.

$$K = \frac{C_B}{C_F}$$

Thermodynamically the free energy change of a reaction is related to the change in enthalpy and the change of entropy. This is given in the following equation

$$\Delta G = \Delta H - T\Delta S \qquad \text{(Eqn. 2)}$$

Where, ΔG is the free energy change (kJ/mol); ΔH is the change in enthalpy (kJ/mol) and ΔS is the change in entropy (kJ/mol)

Combining Eqn. 1 and Eqn. 2 gives

$$lnK = \frac{\Delta S}{R} + \frac{\Delta H}{R}\ X\ \frac{1}{T} \qquad \text{(Eqn. 3)}$$

The Eqn. 3 is also known as Van't Hoff equation. From the slope ($\frac{\Delta H}{R}$) and the intercept ($\frac{\Delta S}{R}$) of a Van't Hoff plot between lnK and $\frac{1}{T}$, the change in enthalpy and the change in entropy can be calculated. Hence, a plot between lnK vs $\frac{1}{T}$ for Zn^{2+}

sorption was plotted in order to obtain $\frac{\Delta H}{R}$ from the slope and $\frac{\Delta S}{R}$ from the intercept and from these two values, the change in enthalpy (ΔH) and the change in entropy (ΔS) can be calculated.

2.11. Isotherm modeling for evaluation of sorption capacity

The popular isotherm modeling for metal removal, i.e, Langmuir and Freundlich adsorption isotherms were used by various researchers for biosorption studies [55–61]. Isotherm modeling for analyzing metal sorption by cyanobacteria, a fixed concentration of chlorophyll *a* (3 µg/mL) was taken and the Zn^{2+} concentration taken for the experiment ranged from 10 – 160 µg/mL.

The amount of metal taken up by the cyanobacterial biomass was expressed as q. q (mg of metal ion taken up per g of the biomass) was calculated using the following equation

$$q = \frac{C_I - C_F.V}{w}$$

Where, V is the volume of medium in mL and w is the weight of biomass in g.

Langmuir equation is based on the assumptions that:

1) Finite numbers of energetically uniform sites are present on the adsorbent
2) There are no interactions among the adsorbed species and
3) A monolayer of adsorbate are formed on the adsorbent surface beyond which no further adsorption takes place.

Langmuir's isotherm is mathematically expressed as

$$q_F = \frac{Q_{max}.K_L.C_F}{1 + K_L.C_F}$$

Which can be linearized to the following equation

$$\frac{C_F}{q_F} = \frac{1}{Q_{max}.K_L} + \frac{1}{Q_{max}}C_F$$

Where,

q_F: metal adsorbed per g of biosorbent at equilibrium;

C_F: equilibrium concentration;

Q_{max}: maximum adsorption capacity related to the amount of metal ion per unit weight of the biosorbent required to form a complete monolayer on the surface;

K_L: equilibrium adsorption constant related to the binding affinity.

From the Langmuir isotherm equation, a parameter R_L which is defined as a dimensionless equilibrium parameter also known as a separation factor can be obtained that further determines the nature of the interaction between the biomass and the metal ions.

For calculation of the separation factor (R_L), the following equation was used:

$$R_L = \frac{1}{1 + K_L.C_I}$$

If the calculated value of R_L > 1 indicates unfavourable interaction; R_L = 1 shows linear interaction; R_L < 1 indicates favourable interaction and R_L = 0 means the interaction is irreversible [59, 62, 63].

Freundlich's adsorption isotherm defines the adsorption characteristic of a heterogeneous surface. It assumes that the enthalpy of adsorption is independent of the amount adsorbed. It is mathematically expressed as

$$q_F = K_F.C_F{}^{1/n}$$

K_F and n are Freundlich constants characteristic of the system indicating sorption capacity and intensity, respectively and can be determined from the linearized logarithmic form of the equation given below:

$$\log q_F = \log K_F + \frac{1}{n}\log C_F$$

From a plot of $\log q_F$ vs $\log C_F$, the slope gives the value of $\frac{1}{n}$ and the intercept the value of $\log K_F$. In Freundlich isotherm, the value of $\frac{1}{n}$ specifies the favourability and capacity of adsorbent/adsorbate system. A value of n > 1 indicates a favorable sorption process [63]. Thus, to evaluate the maximum sorption capacity of the organisms for the Zn^{2+}, various parameters were calculated from these isotherms.

2.12. Statistical analysis

All data represented are calculated by taking the mean from three independent experiments (mean ± SD) values. All the plots and thermodynamic parameters were calculated using statistical functions of Microsoft Excel, 2010 (version Office XP, Microsoft Corporation, USA).

3. Results

3.1 Zn^{2+} binding to *Anabaena variabilis* MEGCH1

3.1.1 Zn^{2+} binding assessment by SEM-EDX

Zn^{2+} binding on control and treated cells was analyzed using SEM coupled to EDX. The percent abundance of Zn^{2+} in the control cells was found to be 3.34 %, whereas the same was 5.61 % in Zn^{2+} treated cells [Fig 1a (i & ii)].

3.1.2 Functional group analysis

Various functional groups available for Zn^{2+} binding was determined by FTIR spectroscopy study. Vibrational frequency shifts recorded from 3413 cm^{-1} to 3401 cm^{-1}, 3300 cm^{-1} to 3307 cm^{-1} and 2858 cm^{-1} to 2925 cm^{-1} showed the presence of anionic groups like amine, hydroxyl and carbonyl groups, respectively. Frequency shifts from 1802 cm^{-1} to 1810 cm^{-1} and 520 cm^{-1} to 529 cm^{-1} indicated the involvement of ester and phosphate groups, respectively (Fig 1b).

3.1.3 Zn^{2+} sequestration and its cellular distribution

The total amount of Zn^{2+} ions removal by the cyanobacterium from the test solutions containing 10 µM Zn^{2+} was estimated after 24 h of Zn^{2+} inoculation. Within this period, the organism could sequester 99 % of the supplied 10 µM Zn^{2+} from the solution. Of the 99 % of the total amount of Zn^{2+} removed, 81 % of the ions was adsorbed on the biomass surface, 4 % was precipitated on the biomass and 14 % of the ions was internalized [Fig 1c (i & ii)].

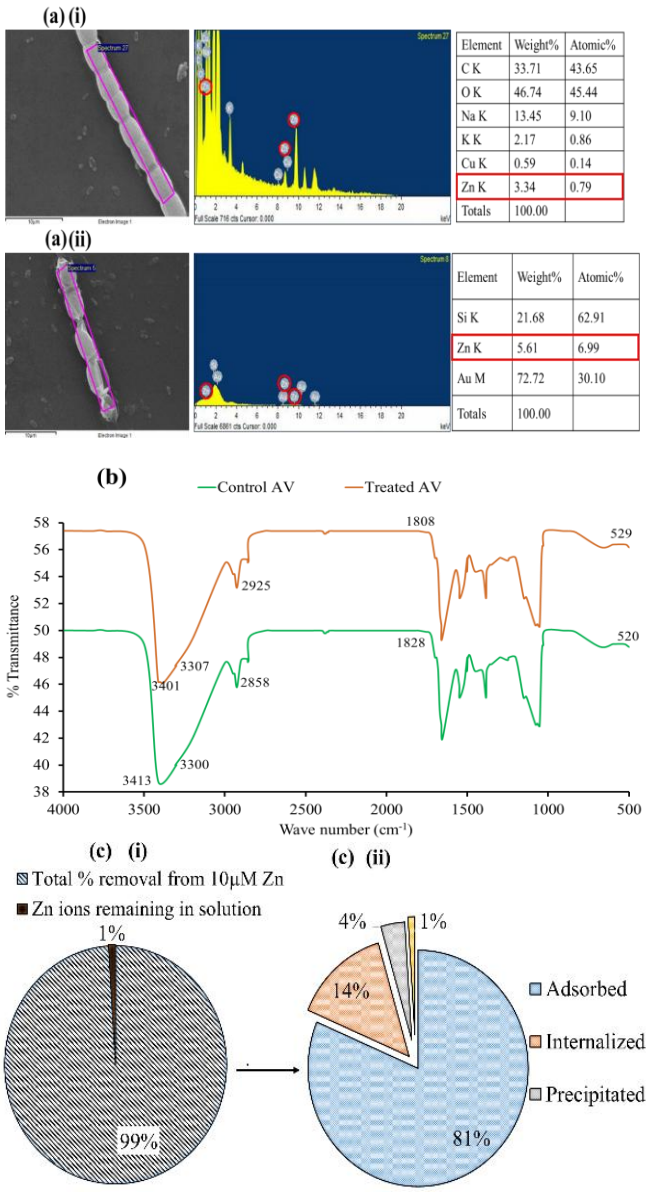

Fig. 1 (a) SEM-EDX spectra presenting the percent abundance of elements in **(i)** control and **(ii)** in Zn^{2+} treated samples of *Anabaena variabilis* MEGCH1. For both control and Zn^{2+} treated cells, the data presenting the percent abundance is given in micrographs along with the spectra and tabular form. The peaks for Zn^{2+} in the spectra and the metal's percent abundance in the table have been highlighted in red; **(b)** FTIR spectral analysis of *Anabaena variabilis* MEGCH1 showing the shifts in vibrational frequencies and changes in transmittance intensities in signature peaks for different functional groups; **(c) (i)** The total amount of Zn^{2+} ions being removed by the cyanobacterium *Anabaena variabilis* MEGCH1 from 10 µM Zn^{2+} supplemented medium after the incubation period of 24 h **(ii)** Distribution pattern of the total ions removed: **Adsorbed**- ions that physically stick to the surface; **Internalized**- ions that were taken up by the organism; **Precipitated**- ions that were deposited on the already bound metal ions; and **Unaccounted**- ions that were lost out during the experimental process.

3.2 Optimization of conditions for Zn^{2+} removal

3.2.1Temperature and pH

The removal of Zn^{2+} ions by *Anabaena variabilis* MEGCH1 was studied at different temperatures of 298 K (25 °C), 303 K (30 °C), and 308 K (35 °C) and different pH of 6, 7, 7.5, 8 and 9. Amongst the three temperatures studied, the cyanobacterial biomass showed best Zn^{2+} removal at a temperature of 35 °C (Table 1). At pH 7, the percent Zn^{2+} removal was 75 %, 76 % and 88 % at 25 °C, 30 °C and 35 °C. The percent Zn^{2+} removal at 25 °C, 30 °C and 35 °C at a pH of 7.5 was 79 %, 90 % and 99 % and at pH 8, the percent removal was 79 %, 95 % and 99 % at 25 °C, 30 °C and 35 °C respectively. The percent Zn^{2+} removal at pH 9 were 74 %, 79 % and 82 % at 25 °C, 30 °C and 35 °C. At pH 6, the Zn^{2+} removal percentage was comparatively low at the three different temperatures studied with 50 %, 56 % and 66 % percent removal. The organism showed a maximum percentage (99 %) of Zn^{2+} removal at a temperature of 35 °C under pH of 7.5 and 8. Therefore, the pH of 7.5 and temperature of 35 °C were used for further biosorption studies.

Table 1: Percent removal of Zn^{2+} ions by *Anabaena variabilis* MEGCH1 at different temperatures and pH

pH	Temperature		
	298 K (25 °C)	303 K (30 °C)	308 K (35 °C)
	% Removal of Zn^{2+}		
6	50 ± 0.25	56 ± 0.14	66 ± 0.09
7	75 ± 0.18	76 ± 0.22	88 ± 0.5
7.5	79 ± 0.20	90 ± 0.15	99 ± 0.11
8	79 ± 0.12	95 ± 0.09	99 ± 0.20
9	74 ± 0.16	79 ± 0.21	82 ± 0.19

3.2.2 Inoculum age, inoculum size and shaking rate for optimum Zn^{2+} removal

Parameters influencing the potential of metal removal by *Anabaena variabilis* MEGCH1 such as inoculum age and size as well as the shaking rate were determined after 24 h of inoculation with Zn^{2+}. The percent metal removal by the organism under the different phases of growth (i.e., 2, 4, 6,… 32 days old) revealed that cell inoculum that has been grown for 10 days has a higher removal percentage (99 %). Inoculum size of different chlorophyll *a* concentration (2, 3, 4, 5, 10 µg/ml) extracted from a 10-day old culture was taken and it was found that the highest percentage of Zn^{2+} uptake was achieved by cultures with initial chlorophyll *a* of 3 µg/mL. Therefore, ten days old cultures at an initial chlorophyll *a* concentration of 3 µg/mL was used for further biosorption studies [Fig 2a (i & ii)].

For the shaking rate optimization, ten days old culture with an inoculum size of 3 µg/mL was taken and incubated in medium supplemented with 10 µM Zn^{2+} at different shaking speeds (0 – 250 rpm) for 24 h. The study revealed that a better removal percentage of Zn^{2+} was seen when the cultures were kept on shaking compared to cultures in stationary conditions (54 %). Shaking at the slower speeds of 50 rpm and 100 rpm the removal percentage of Zn^{2+} was 89 % and 90 % respectively. The maximum Zn^{2+} removal was seen at the shaking rate of 150 rpm (99 %). Higher shaking rates decrease in total Zn^{2+} removal percentage by 82 % at 200 rpm and 80 % at 250 rpm respectively [Fig 2a (iii)].

3.2.3 Impact of initial Zn^{2+} concentration and contact time

Different initial concentration of Zn^{2+} (0.5 – 9 µM) was taken and the total Zn^{2+} removal by the organism was

assessed after 24 h. There is an increase in total Zn^{2+} removal with increase in Zn^{2+} concentrations and it reached the full capacity by 9 µM. Percent Zn^{2+} removal showed that there is an increase in percentage at lower concentrations which thereby decline at higher concentrations. The study also revealed that the rapid Zn^{2+} removal which removes 70 % of the Zn^{2+} ions by the cyanobacterial biomass was seen in presence of 10 µM of Zn^{2+} at 15 min of exposure. Beyond this time, a flat curve was seen and a slight increase was seen around 120 min of exposure [Fig 2b (i & ii)].

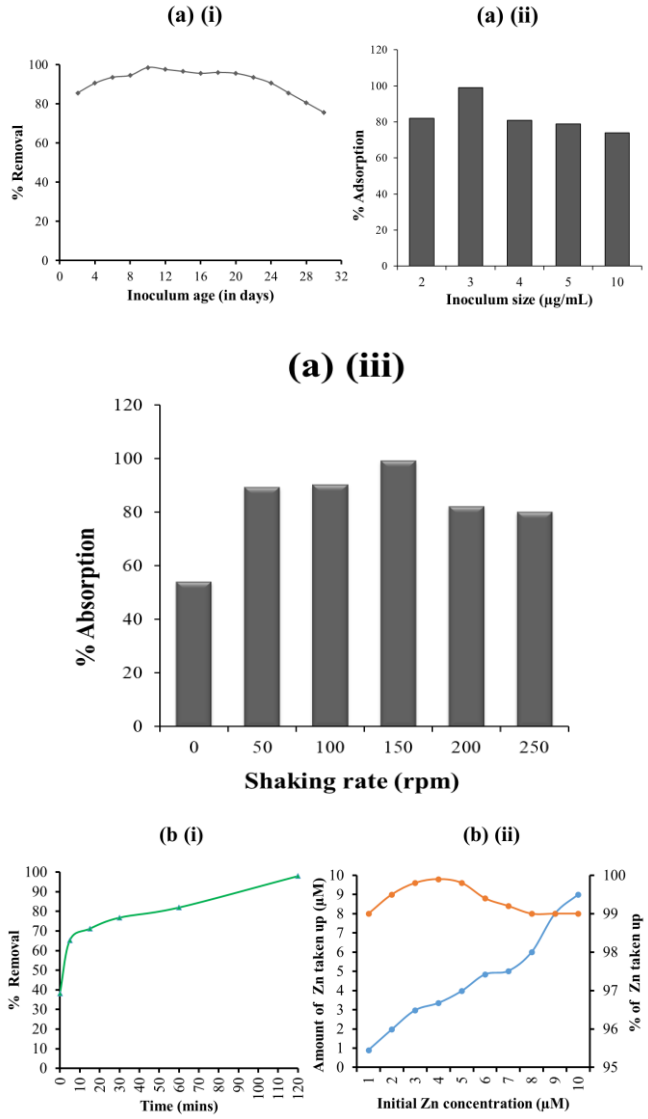

Figure 2: Zn^{2+} removal for 10 µM Zn^{2+} by the cyanobacterium *Anabaena variabilis* MEGCH1 depends on **(a) (i)** initial inoculum age, **(ii)** inoculum size and **(iii)** shaking rate; **(b) (i)** Time dependent percent Zn^{2+} removal, and **(ii)** Initial metal concentration dependent Zn^{2+} removal.

3.3 Thermodynamics of Zn^{2+} removal

Thermodynamic parameters such as ΔG (change in free energy), ΔH (change in heat content or enthalpy) and ΔS (change in entropy or randomness) for the Zn^{2+} removal by the *Anabaena variabilis* MEGCH1 were calculated at different temperatures, 298 K (25 °C), 303 K (30 °C), and 308 K (35 °C). These parameters were calculated with the help of the Van't Hoff plot. From the plot between lnK vs $\frac{1}{T}$, ΔH can be calculated from the slope ($\frac{\Delta H}{R}$) and ΔS from the intercept ($\frac{\Delta S}{R}$). The change in enthalpy ΔH was found out to be - 0.0000216 kJ/mol and the change in entropy ΔS was 0.021764 kJ/mol/K (Fig 3). The calculated Gibbs free energy ΔG for the three different temperatures studied was -6.703 kJ/mol at 308 K (35°C), -6.595 kJ/mol at 303 K (30°C) and -6.486 kJ/mol at 298 K (25°C) (Table 2).

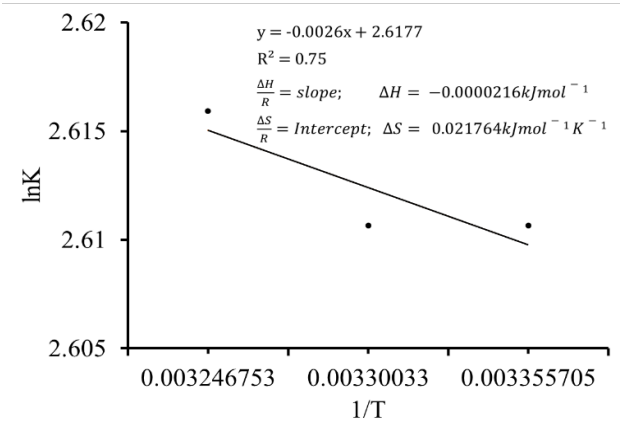

Figure 3: Van't Hoff plot at 298 K (25 °C), 303 K (30 °C) and 308 K (35 °C) for Zn^{2+} removal by *Anabaena variabilis* MEGCH1.

Table 2: Calculated thermodynamic parameters from Van't Hoff plot for the removal of Zn^{2+} by *Anabaena variabilis* MEGCH1.

Temperature	ΔH and ΔS calculated from Van't Hoff plot	Gibbs Free Energy ($\Delta G = \Delta H - T\Delta S$)
298 K	ΔH = -0.0000216 kJ/mol ΔS = 0.021764 kJ/mol/K	-6.486 kJ/mol
303 K		-6.595 kJ/mol
308 K		-6.703 kJ/mol

3.4 Langmuir and Freundlich isotherm modeling of Zn^{2+} sorption

Isotherm modeling- Langmuir and Freundlich isotherms, the two commonly used isotherm models, were used for the study of removal capacity of Zn^{2+} ion by *Anabaena variabilis* MEGCH1. The graph plotted using Freundlich isotherm revealed a better fitting (R^2 value- 0.9559) compared to that of Langmuir isotherm (R^2 value- 0.9427) (Fig 4). The various Langmuir and Freundlich isotherm parameters such as Q_{max}, K_L, K_F, R_L and n were calculated and the calculated values were given in Table 3.

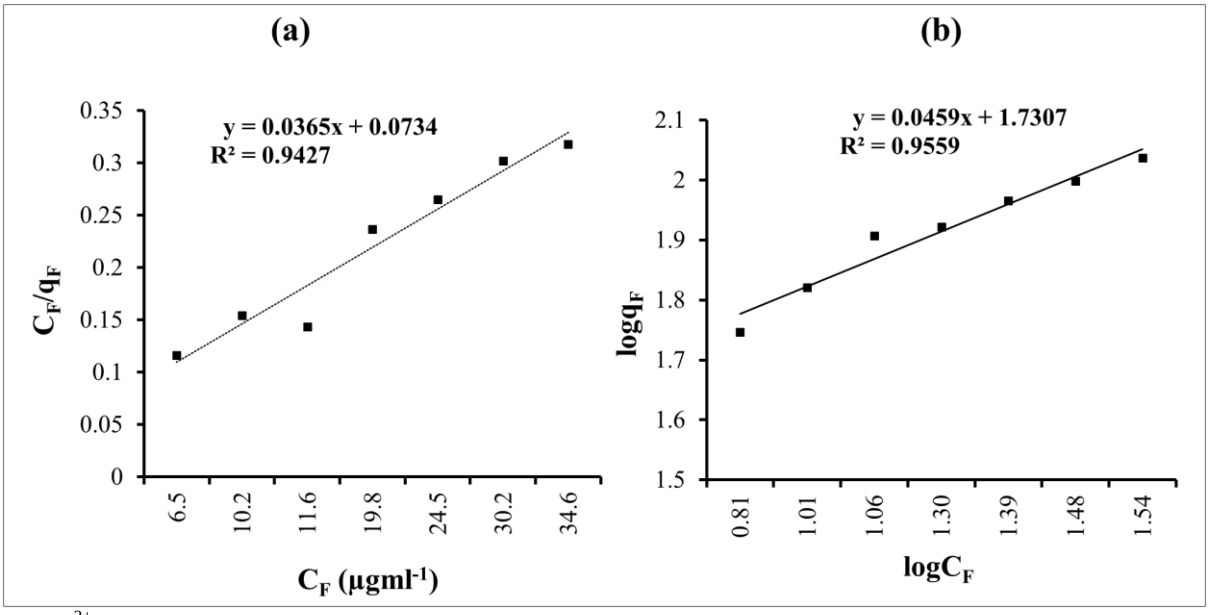

Figure 4: Zn^{2+} biosorption studies on biomass of *Anabaena variabilis* MEGCH1 at 308 K (35 °C) using (a) Langmuir and (b) Freundlich isotherm models

Table 3: Calculated values of various Langmuir and Freundlich parameters for Zn^{2+} sorption by *Anabaena variabilis* MEGCH1.

Langmuir isotherm					
Q_{max} (mg/g)	K_L (L/mg)	R^2	Range of R_L values		
			40 µg/mL	70 µg/mL	100 µg/mL
27.4	0.497	0.9427	0.0479	0.0279	0.0197

Freundlich isotherm			
K_F (mg/g)	1/n (L/g)	n (g/L)	R^2
53.79	0.0459	21.8	0.956

4. Discussion

Microbes are known to inhabit all types of environments, and they are known to possess astounding metabolic pathways, which allows them to use diverse compounds from their surrounding for their growth and development [64]. In polluted areas, microorganisms maintain homeostasis by evolving different mechanisms that enable them to resist numerous polluted compounds including heavy metals [65, 66]. Microorganisms, for instance cyanobacteria have developed strategies such as bioaccumulation, biosorption, biomineralization, and biotransformation for maintaining their growth and development in heavy metal contaminated areas. Because of their ability to grow in polluted environments, microbes have been used either *in-situ* or *ex-situ* for precipitation, absorption, oxidation as well as reduction of various heavy metals present in the environment [67]. They can also be used as biosorbents for the removal of heavy metals because of the fact that microorganisms have their own defense mechanisms, which allows them to overcome the toxic effects caused by the disruption of microbial cells from the action of heavy metals [64].

The interest in the Zn^{2+} removal potential of the cyanobacterium *Anabaena variabilis* MEGCH1 was because this organism was found thriving in a highly heavy metal polluted area (coal mine). And therefore, it was evident that the organism possesses high tolerance toward heavy metal exposure. SEM-EDX scanning of the organism showed that in the presence of 10 µM $Zn^{2+,}$ there is an increase in the percent abundance of Zn^{2+} in treated cells compared to the control cells [Fig 1a (i & ii)]. This indicates that with increased Zn^{2+} concentrations in the medium, there is increased binding of the metal to the organism. FTIR spectroscopy study revealed the involvement of the shifts in vibrational frequencies of the individual peaks from 3413 cm^{-1} to 3401 cm^{-1}, 3300 cm^{-1} to 3307 cm^{-1} and 2858 cm^{-1} to 2925 cm^{-1} indicating binding involvement of –NH, –OH and carbonyl groups, respectively. Frequency shifts from 1802 cm^{-1} to 1810 cm^{-1} and 520 cm^{-1} to 529 cm^{-1} indicated the involvement of ester and phosphate groups, respectively (Fig 1b). AAS study showed that the organism could sequester 99 % of the supplied 10 µM Zn^{2+} from the solution. And 81 % out of the total ions removed were adsorbed on the surface indicating that the Zn^{2+} removal by the organism was primarily a surface phenomenon [Fig 1c (i & ii)]. A comparative study demonstrated that *Anabaena variabilis* MEGCH1 showed a very similar Zn^{2+} removal capacity (99 %) when compared to another robust cyanobacterium *Nostoc muscorum* (98.5 %) both isolated from heavy metal contaminated sites [45].

The optimum Zn^{2+} removal by the cyanobacterium was seen at 35 °C at an initial chlorophyll *a* concentration of 3 µg/mL and at a pH of 7.5. Various researchers have earlier reported that with an increase in temperature, there is an increase in metal removal by the organism. This increase in metal removal can be seen only up to a certain elevation in temperature. This is because with rise in temperature molecular agitation increases which leads to an increase escape of metal ions from the surface of the biomass to the solution resulting in reduction in metal adsorption [45, 52, 68–71]. This corroborates our finding, where the optimum temperature for Zn^{2+} removal was found at a temperature of 35 °C. The sorption study was not done beyond 35 °C as at temperature higher than this the survival rate of the cyanobacterium was greatly compromise. Similarly, for

optimum pH the best removal was found at 7.5 and 8 (Table 1). Lower adsorption of Zn^{2+} at pH lower than 7.5 and higher than 8 can be reasoned as follows: low metal removal at lower pH is may be due to the fact that at low pH the number of H^+ which has a similar charge to the metals increases in the medium that competes with the metal ions for the negative binding sites on the cell surface of the organism. On the other hand, higher pH can also adversely influence the metal binding as when OH^- ions increase in solution they may form hydroxides with the metals present in the medium that gets precipitated and become unavailable for surface binding [52, 72–74]. Similarly, other parameters such as inoculum age, inoculum size, and the shaking rate influence metal sorption. Best metal removal capacity was shown by a ten-day-old culture. This can be justified by the fact that at this period the cyanobacterial culture was in the mid-log phase and therefore was highly robust. Similarly, an initial inoculum size of 3 µg/mL chlorophyll *a* produced best Zn^{2+} sorption result as at this concentration the cyanobacterial cells were optimally spaced in the solution and all cells had maximum access to the metal of interest around them [Fig 2a (i & ii)]. The time of exposure for maximum Zn^{2+} removal was 15 min. Beyond this, the curve depicting metal removal remains stationary up to 120 min. However, a slight increase in metal uptake was recorded at 120 min [Fig 2b (i)]. This suggested that by 15 min the cyanobacterial biomass was completely saturated with Zn^{2+} binding. The small increase seen again in metal removal at 120 min may be due to the internalization of some of the Zn^{2+} ions resulting in some free anionic sites that allowed more ions to bind to the cell surface. As metal concentration increased in the solution a concomitant increase in biosorption of the metal ions was also seen up to a concentration of 9 µM within 24 h incubation period [Fig 2b (ii)]. This indicated that 9 µM Zn^{2+} was sufficient to completely saturate all available metal-binding functional groups present on the cell surface of the biomass employed.

Thermodynamic parameters: ΔG, ΔH and ΔS for the Zn^{2+} removal by *Anabaena variabilis* MEGCH1 were calculated with the help of the Van't Hoff plot. The negative value of the change in enthalpy ΔH (- 0.0000216 kJ/mol) revealed that the process of removal is exothermic in nature, whereas the positive value of the change in entropy ΔS (0.021764 kJ/mol/K) showed the increase in randomness of the sorbate-sorbent system upon Zn^{2+} sorption on the organism (Fig 3). Further, the negative values of ΔG [-6.703 kJ/mol (35°C), -6.595 kJ/mol (30°C) and -6.486 kJ/mol (25°C)] indicated that the nature of Zn^{2+} biosorption process by the cyanobacterium is spontaneous and favorable in nature with the lowered free energy of the system due to the release of energy to the surrounding. The highest free energy change at 35 °C indicated the role of temperature on the binding process of sorbent and sorbate. Isotherm modeling studies revealed that the Freundlich isotherm (R^2 value- 0.9559) fits better compared to Langmuir isotherm (R^2 value- 0.9427), which suggests that the binding of the Zn^{2+} ions to the organism biomass is a multilayer sorption process. The calculated values of Q_{max} (27.4 mg/g) and K_L (53.79 mg/g) obtained from the linearized isotherms stated about the binding capacity and the binding site affinity of the metal ion to the organism. The separation factor RL values (0.0479 in 40 µg/mL to 0.0279 in 70 µg/mL to 0.0197 for 100 µg/mL), ranging between 0 and 1 signified the favorable contact between the cyanobacterium *Anabaena variabilis* MEGCH1 biomass and the Zn^{2+} ions. The n (21.8 g/L) value calculated from the Freundlich isotherm specified the highly favorable sorption for Zn^{2+} by the *Anabaena variabilis* MEGCH1 biomass.

Thus, isotherm modeling evaluation of Zn^{2+} sequestration capacity of *Anabaena variabilis* MEGCH1 in this study showed the potential scope of this organism in the application as Zn^{2+} biosorbent from the polluted environment such as wastewater. Other than that, the cyanobacterium also demonstrated a substantial tolerance towards Zn^{2+}. It registered high potential for Zn^{2+} sorption, and displayed amenability towards changes in various conditions such as pH and temperature, contact time, metal load etc. All these characteristics together contribute to the possibility of its applications in bioreactor systems for various heavy metal removal particularly Zn^{2+} in environmental remediation measures. The entire work carried out in this study could be summarized in the following figure.

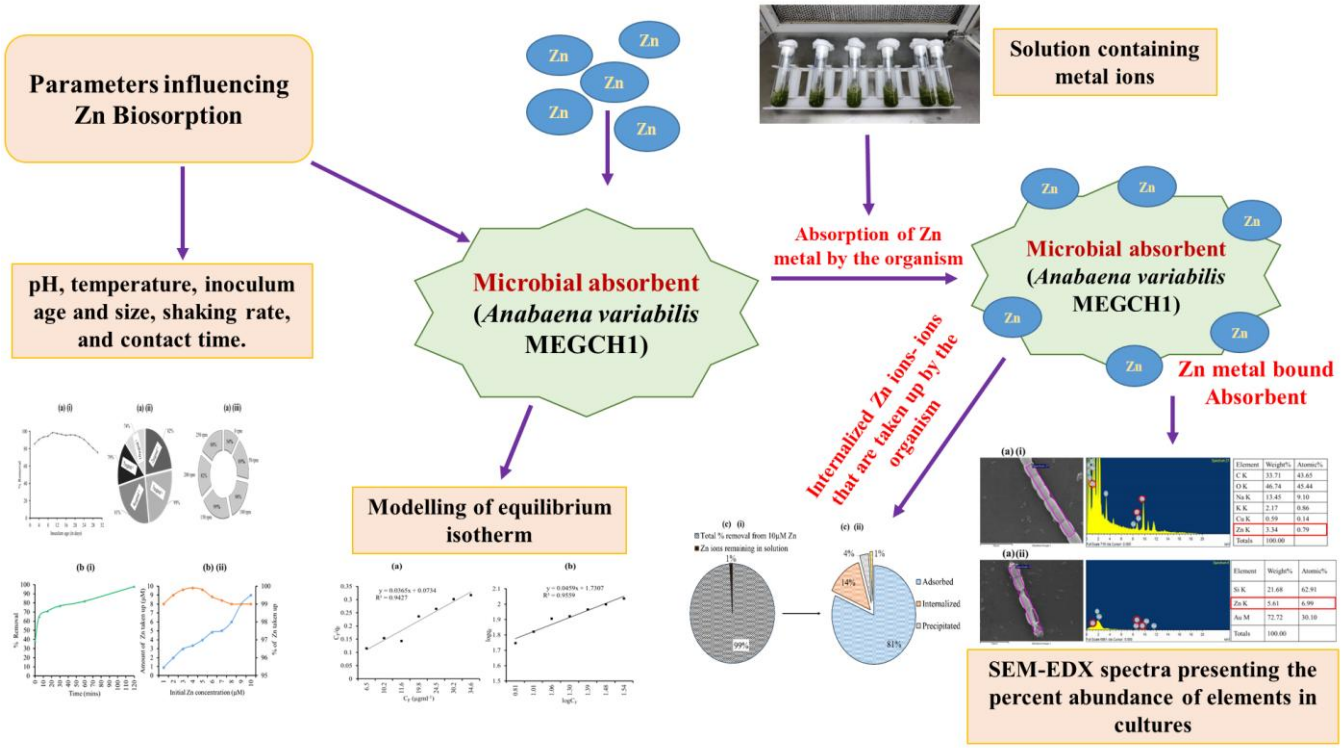

Figure 5: Effect of Zn^{2+} sequestration by the cyanobacterium *Anabaena variabilis* MEGCH1: a comprehensive summary

5. Conclusion

In this study, the cyanobacterium *Anabaena variabilis* MEGCH1, isolated from a metal-polluted environment showed high tolerance towards heavy metal Zn^{2+}. The organism could sequester 99 % of the supplied Zn^{2+} (10 µM). From the isotherm studies, the binding of Zn^{2+} to the biomass of the organism was found to be a multilayer sorption process. Because of its high tolerance towards Zn^{2+} and its amenability to various conditions, this organism showed a high potential for its application as Zn^{2+} biosorbent from wastewaters.

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Authors' Contribution
MBS: conceptualization, supervision, validation, writing, and editing. OLD: investigation, interpretation of data, figures, and validation. BLK: interpretation of data, references, writing-original draft. MS: references, writing-abstract, figures, review, and editing. All authors read and approved the final manuscript.

Competing Interests
The authors declare no competing interest

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