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Characterization and Heavy Metal Bioremediation Potential of *Halomonas* Isolates from the Bolivian Altiplano

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Abstract: The Bolivian Altiplano has an ongoing history of heavy metal pollution due to years of uncontrolled mining in this region. Heavy metals are a threat to natural environments such as lakes and soils with cultural and economic importance for the local communities. The extreme environmental conditions of the Bolivian Altiplano translate into alkaline soils with high concentration of minerals, high radiation and considerable daily temperature oscillations. Halophilic and halotolerant microorganisms isolated from such environments have interesting biotechnological applications including bioremediation of metal polluted waters and soils. Here, bacterial strains from the Bolivian Altiplano were characterized and biosorption capacity evaluated for three heavy metals (Pb^{+2}, Cd^{+2} and Zn^{+2}) in variable concentrations. Four strains were able to grow in multimetal medium with a final concentration of 100 mg. L^{-1}, with a higher tolerance to Pb^{+2}. The four isolates were selected for further characterization and were identified as different species of *Halomonas* genus. The best heavy metal biosorption rates for the four isolates were found at pH 7 and 37°C. Additionally, the fastest uptake rate for all three metals was under 120 minutes in the four chosen isolates. The biosorption process was best described by Langmuir isotherm for all isolates exposed to the three metals separately. The four *Halomonas* isolates showed a bioremediation potential for heavy metal polluted substrates, although the highest biosorption capacity values were from isolate Ss_is3 notably for Pb^{+2}. This study provides new information about the potential biotechnological capacities of *Halomonas* strains isolated from mineral soils in the Andes.

Keywords: Bioremediation, Biosorption Capacity, Halophile, Heavy Metals, Isolates

1. Introduction

Heavy metal pollution is a consequence of an increase in industrial activities, technological development, aggressive agriculture, rapid urbanization, and waste generation from domestic and industrial sources. It poses a significant threat to the environment and public health, due to its toxicity, and bioaccumulation in the food web through water and land resources persisting therefore in nature [1].

Mining is one of the main economic activities in developing countries like Bolivia. It has followed production cycles subject to external and internal factors, since colonial mining of tin, to complex mineral extraction. There is an increasing environmental concern due to solid and liquid wastes from mining operations found in soil and waterbodies, affecting biodiversity and human populations health [2]. There are reports of several heavy metals in aquatic systems of the Altiplano region (highlands) of Bolivia (i.e Lake Poopó, Lake Uru-uru, Lake Pilcomayo), most heavy metals found exceed concentration limits established by the World Health Organization (WHO) like Pb^{+2}, Cd^{+2}, Zn^{+2}, Sn^{+2}, As^{+3}, Hg^{+2}, Fe^{+3} and Cu^{+2} [2-4].

Bioremediation is a technology used to treat contaminated sites. It depends on bacteria, fungi, and plants. These organisms break down xenobiotics or mitigate heavy metals by altering them into elements with little or no toxicity, in the case of heavy metals utilizing accumulation, reducing bio-availability or toxicity through biomethylation and transformation [5]. Among the different methods described, bioaccumulation (active uptake by a biological matrix) and biosorption (passive uptake by a biological matrix) are potential for an effective removal of heavy metals using microorganisms [6].

Halophile and halotolerant microorganisms are appropriate candidates for heavy metal bioremediation processes. They have the ability to grow in a wide range of salt concentrations, have negative net charge enabling them to hold ions on their cell surface and intracellular components to retain most absorbed ions [7]. The Bolivian Altiplano, presents a series of ecosystems with unique conditions in the world like saline alkaline soils, high radiation and low temperatures, these conditions are considered adverse for a great number of organisms, including humans [8]. However, the presence of extremophilic microorganisms, like halophiles has been reported [9].

There have been few efforts to study and solve the concerning heavy metal contamination on mining areas around the country, despite awareness of the health and environmental risks that they may pose. The present study was part of a project designed to use native plants and bacteria in conjunction to recover heavy metal contaminated soils from the Bolivian Altiplano through phytoremediation. The main purpose of this work is to evaluate the biosorption capacity present in native microorganisms isolated from saline soils of the northern and central Altiplano. Hence, determining their potential as remediation agents in the removal and/or reduction of three heavy metals: Pb^{+2}, Cd^{+2} and Zn^{+2}.

2. Materials and Methods

2.1. Screening and Characterization

Bacterial isolates used for this study are part of a collection in Center of Biotechnology (CBT) at the Universidad Mayor de San Simón. These isolates were obtained from saline soils of 12 sampling sites between the northern (18°22'59.29", 67°20'1.68") and central (19°37'57.47", 67°40'48.73") Bolivian Altiplano (Figure 1). Sites were chosen based on their proximity to active and inactive mines (tail mining areas). Samples were collected through March and April (autumn/dry season). Each sample was taken using a soil sample probe and put in 50mL sterile falcon tubes. Isolation cultures were grown in Tryptic Soy Agar (TSA). Samples presented slightly alkaline pH values (8-10). Variations towards alkaline pH values is due to some chemical processes that occur in characteristic saline soils of the Bolivian Altiplano (saline, saline alkaline and alkaline soils) [10]. Therefore, optimum pH was determined to grow isolates for initial screening and characterization.

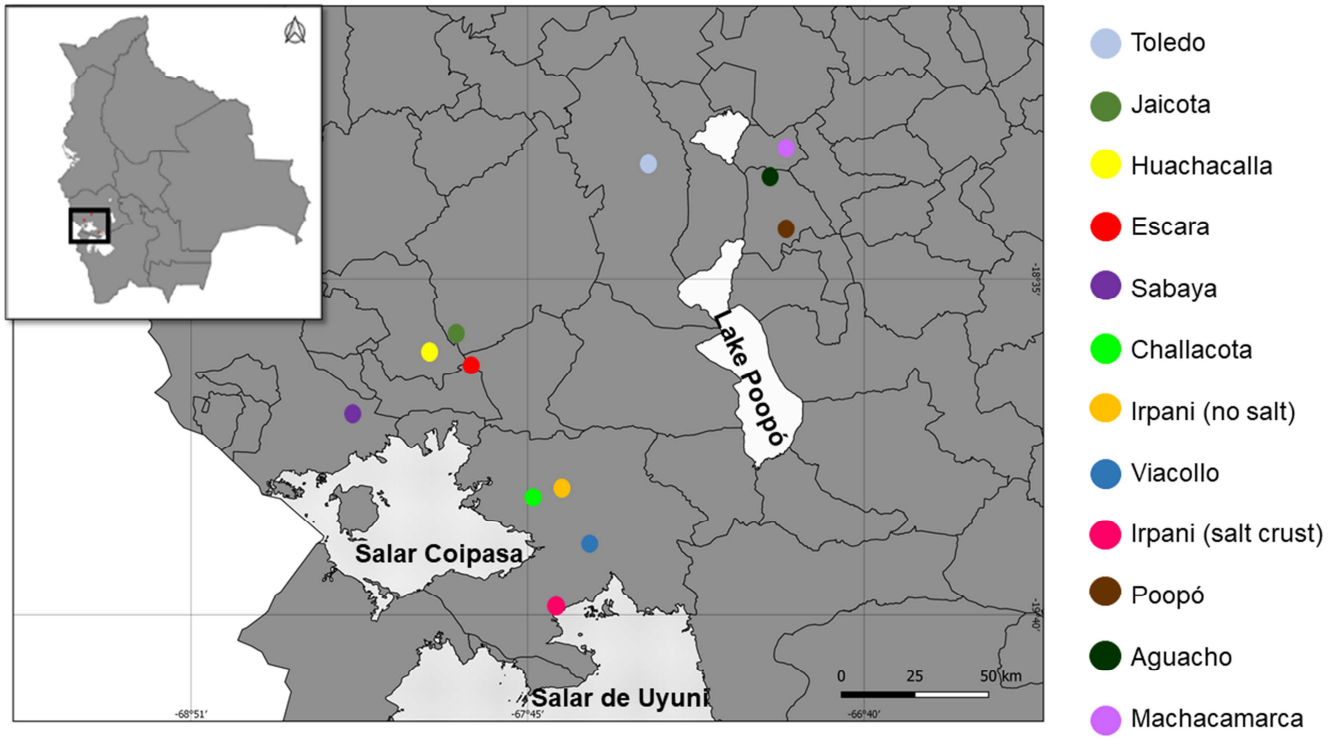

Figure 1. *Sampling sites map of the collection in CBT used for this study. Colored dots represent communities or small towns where soil samples were taken from and named by.*

To determine salt tolerance, all isolates were grown in Tryptic Soy Broth (TSB) until an optical density of λ=0.6

was achieved. From the previous cultures 20 µL of each isolate was spot inoculated on to TSA at seven different NaCl concentrations (0, 5, 7.5, 10, 12.5, 15, 20), all concentrations were evaluated by triplicate. Plates were incubated at 25° ± 2°C. Growth was evaluated by presence or absence of colonies after 72 hours.

2.2. Metal Tolerance Assays

To determine tolerance to heavy metals; Pb^{+2}, Cd^{+2} and Zn^{+2} were evaluated separately. Stock solutions of each heavy metal were prepared, and concentrations were adjusted according to each experiment. Pb $(NO_3)_2$, Cd $(NO_3)_2 \cdot 4H2O$ and $ZnSO_4 \cdot 7H_2O$ salts were individually added to deionized water to obtain a final concentration of 10000 mg. L^{-1}. To determine tolerance each heavy metal was added individually to TSA medium to achieve final concentrations of 25, 50, 100, 150, 200, 250 and 500 mg. L^{-1}. A 0.85% of NaCl was added to avoid cellular lysis from this point forward. 20µl of overnight cultures with optical density of λ=0.6, were spot inoculated to plates in triplicates and incubated at 25° ± 2°C for 72 hours.

Tolerant isolates were selected based on a presence or absence of growth. The following two parameters were determined: Minimum inhibition concentration (MIC) which is the lowest concentration that completely inhibits visible growth of an organism [11], and maximum tolerable concentration (MTC) was designated as the highest metal concentration which allows growth [12].

Bacteria that demonstrated tolerance were used for multimetal tolerance and subsequent biosorption assays. Isolates with heavy metal tolerance were separately grown in TSA medium supplemented with Pb^{+2}, Cd^{+2} and Zn^{+2}. Each trial was designed to increase concentration in mg. L^{-1} of the three heavy metals gradually and evaluate its effect on microbial growth. Seven trials were carried out, starting with an initial equal concentration of 25 mg. L^{-1} of each metal, escalating gradually to 50 and 100 mg. L^{-1} (Table 1). Overnight cultures were spot inoculated. Plates were grown at room temperature and the multi metal tolerance was later assessed based on presence or absence of growth.

Table 1. *MTC and MIC values from 4 isolates showing growth in the presence to Pb^{+2}, Cd^{+2} and Zn^{+2}.*

Isolate	MIC (mg. L^{-1})			MTC (mg. L^{-1})		
	Pb^{+2}	Cd^{+2}	Zn^{+2}	Pb^{+2}	Cd^{+2}	Zn^{+2}
Ss_e1	250	150	200	200	100	150
Ss_s2	250	200	150	200	150	100
Ss_is3	250	150	200	200	150	150
Ss_i4	250	200	200	200	100	150

2.3. Secuencing of 16S rDNA of Tolerant Isolates

Bacterial DNA of multitolerant strains were extracted and sequenced using Eurofins Genomics services. Identification was done through 16S rDNA sequencing using universal primers 27F and 1492R. The obtained sequences were inserted into GenBank, BLAST search program was used to assert prevailing similarity of the genetic makeup of submitted sequences with the database available in National Centre of Biotechnology Information (NCBI).

2.4. Biosorption Studies

Since the ability of microorganisms to remove heavy metal ions, is influenced significantly by environmental conditions [13] temperature, pH and contact time were evaluated in batch cultures. A pH range was established between 5 and 8. Three temperature 25, 30 and 37°C [14] and contact time from 30-480 minutes. All conditions were evaluated at a 50 mg. L^{-1} initial concentration of each heavy metal. Once each biosorption experiment concluded 20µL were taken from each flask and inoculated onto solid medium, hence, determining the viability of bacterial cells.

Biosorption rate was determined through the bacterial capacity of metal biosorption in seven time periods (5, 10, 15, 30, 60, 120, 240 min). To determine adsorption equilibrium and maximum capacity, each isolate was subjected to an increasing concentration in mg. L^{-1} of each heavy metal (60, 70, 80, 90, 100). Both kinetics and equilibrium assays were carried out using optimum parameter results obtained from previous batch experiments.

Overnight pre-cultures of each isolate were grown in TSB medium until exponential phase. Cultures were subsequently transferred to 50 ml tubes and centrifuged at 6000 rpm for 15 minutes, supernatant was discarded, pellet was resuspended in 50 ml of saline solution of 0.85% (w/v) with each heavy metal solutions added separately in Erlenmeyer flasks, to procure the desired concentration for the targeted experiment and left incubating following experiment established conditions. Flasks were centrifuged, and pellets harvested for heavy metal analysis. Acid digestion with HNO_3 was carried out to break the pellet, dissolve organic matter and obtain the heavy metal uptake or biosorption percentage (Ad%). All experiments were carried out with negative controls and done by triplicate [14]. Quantifying heavy metal uptake was calculated as follows according to Volesky & May-Phillips (1995) [15].

$$Ad\% = \frac{C_O - C_{eq}}{C_O} \times 100 \quad (1)$$

Where C_o is the initial concentration of the metal ion (mg. L^{-1}) and C_{eq} is the residual concentration of the metal in the solution in equilibrium (mg. L^{-1}).

2.5. Data Analyses

To determine heavy metal uptake, supernatant and acid digested pellets were analyzed using atomic adsorption spectrometer Perkins Elmer Analyst 200 at Universidad Católica Boliviana. Samples were appropriately diluted with deionized water to ensure that the heavy metal concentration in each sample was within the detection range.

To determine biosorption rate (kinetics) of the three heavy metals in the four bacterial isolates, pseudo first order and pseudo second order models were applied [16]. The pseudo-

first order kinetic model is described by the following equation:

$$\log(q_e - q) = \log q_e - \frac{k_l t}{2.303} \quad (2)$$

Where qe and q are the biosorption capacity at equilibrium and at time t (mg. g^{-1}) and k_l (min^{-1}) is the rate constant of pseudo first order adsorption.

Ho's pseudo-second order kinetic model is described by the following equation [17]:

$$\frac{t}{q} = \frac{1}{k_2 q_e^2} + \frac{1}{q_e} \quad (3)$$

Where k_2 (g. mg^{-1}. min^{-1}) is the rate parameter of pseudo-second order equation, qe and q are the adsorption capacity at equilibrium and at time t (mg. g^{-1}).

Adsorption isotherms are used to establish ratio between equilibrium concentration of solute in the solution and equilibrium concentration of solute on the sorbent at constant temperature [18]. Langmuir and Freundlich isotherm models were applied [19].

$$\frac{C_e}{q_e} = \frac{1}{K_L \cdot q_{max}} + \frac{1}{q_{max}} C_e \quad (4)$$

$$\frac{1}{q_e} = \frac{1}{q_{max}} + \left(\frac{1}{K_L \cdot q_{max}}\right) \frac{1}{C_e} \quad (5)$$

Where C_e is the concentration of the metal ions in the solution (mg. L^{-1}), q_{max} (mg. g^{-1}) is the maximum amount of metal ion per unit weight of bacteria to form a complete monolayer on the surface and K_L (L. mg^{-1}) is the equilibrium adsorption constant which is related to the affinity of the binding sites.

When experimental data is adequately described by the Langmuir model, it is essential to predict whether the biosorption system is a favorable or unfavorable, it can be expressed in terms of a dimensionless constant called separation factor (R_L) [20] it is calculated as follows:

$$R_L = \frac{1}{1 + K_L C_O} \quad (6)$$

Where C_o is the initial metal concentration, K_L is the Langmuir equilibrium constant. The value of R_L indicates the shape of the isotherm if: R_L>1 is not favorable, R_L=1 is linear, 0< R_L<1 it is favorable or R_L=0 is irreversible.

Freundlich-linear equation [19] (Ho, 2014) is given as follows:

$$\log q_e = \log K_F + \frac{1}{n} \log C_e \quad (7)$$

Where K_F (mg. g^{-1}(L. mg^{-1})$^{1/n}$) is the Freundlich constant related to bonding energy. K_F is the adsorption or distribution coefficient and represents the quantity of metal biosorbed onto biosorbent for unit equilibrium concentration. 1/n is the heterogeneity factor and n is a measure of deviation from linearity of adsorption.

Statistical analysis to determine the optimal conditions for adsorption from the batch experiments was carried out using IBM SPSS Statistics V.19. Data gathered from biosorption uptake based on isolate and factor variations from the batch experiments was studied using a one-way analysis of variance (ANOVA) at 95% of confidence interval to determine the degree of significance between treatments. Mean values were then compared using Tukey's Test.

3. Results

3.1. Screening and Sample Characterization

Of the collection, 43 bacterial isolates were able to grow, isolated and classified according to their salt tolerance: 21 moderate halophiles, 5 obligated halophiles and 17 halotolerant bacteria. According to a classification from Kushner (1993) mentioned in a more recent study [21].

Most bacterial isolates presented tolerance to Pb^{+2}. However, only four isolates presented tolerance to Cd^{+2} and Zn^{+2}. Such were the cases of: isolate Ss_e1 (Saline soil from Escara), isolate Ss_s2 (Saline soil from Sabaya), isolate Ss_is3 (Saline soil from Irpani salt crust) and isolate Ss_i4 (Saline soil from Irpani no salt crust) (Table 2). These four isolates grow optimally at 5% NaCl concentration, therefore, are moderate halophiles. They all are rod shaped (bacilli), with an optimum pH around 8.5, except for Ss_is3, which grew best at pH 9.5.

Table 2. *Multitolerance trials of Pb^{+2}, Cd^{+2} and Zn^{+2} with concentration variation of each metal.*

Metal (mg. L^{-1})	Trial A	Trial B	Trial C	Trial D	Trial E	Trial F
Pb^{+2}	25	25	50	25	50	100
Cd^{+2}	25	25	25	50	50	100
Zn^{+2}	25	50	25	25	50	100
Total concentration	75	150	100	100	150	300

The four tolerant isolates to the three heavy metals (Pb^{+2}, Zn^{+2} and Cd^{+2}) individually, presented a good tolerance to most of the multimetal trials. Isolate Ss_e1 was able to grow in most of the trials (A – D). MTC for the four isolates was found in trial C with 50 mg. L^{-1} of Pb^{+2}, and 25 mg. L^{-1} of Cd^{+2} and Zn^{+2} each (Figure 2). Trial F was determined as MIC, given that no isolate was able to grow at this combined concentration of the heavy metals.

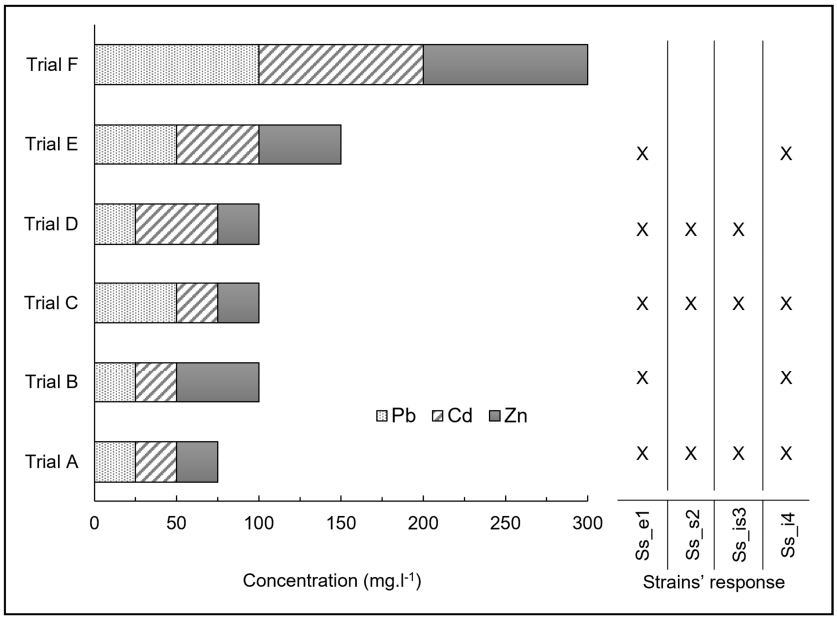

Figure 2. *Multimetal tolerance trials with varying concentrations of Pb^{+2}, Cd^{+2} and Zn^{+2}, determining absence/presence of growth in bacterial isolates Ss_e1, Ss_s2, Ss_is3 and Ss_i4.*

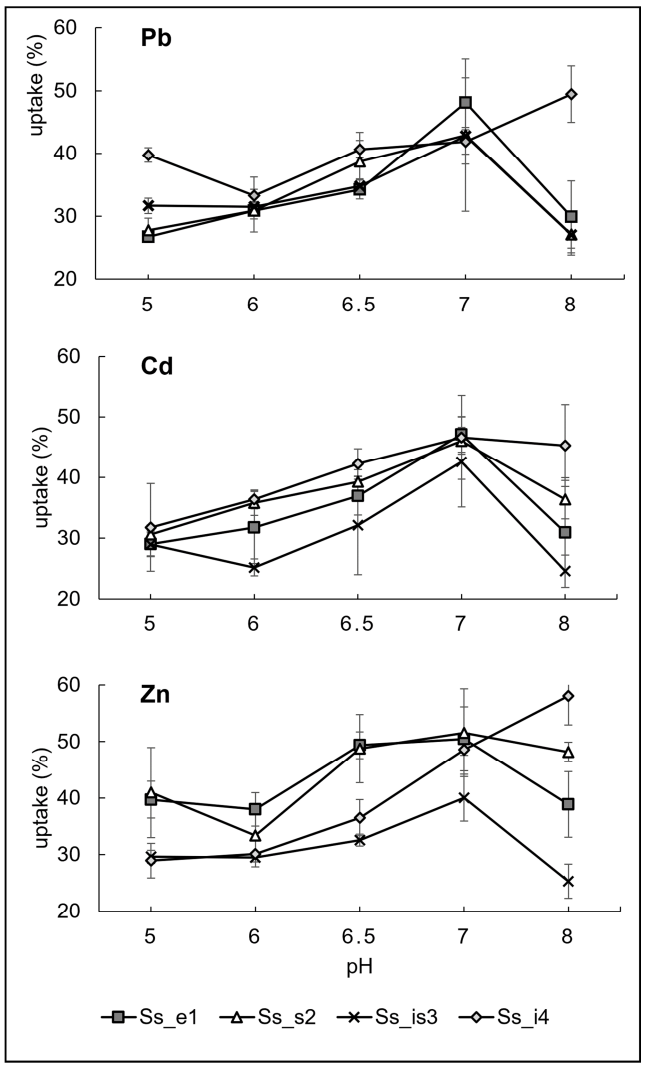

Figure 3. *Effect of pH on Pb^{+2}, Cd^{+2} and Zn^{+2} uptake by bacterial isolates Ss_e1, Ss_s2, Ss_is3 and Ss_i4.*

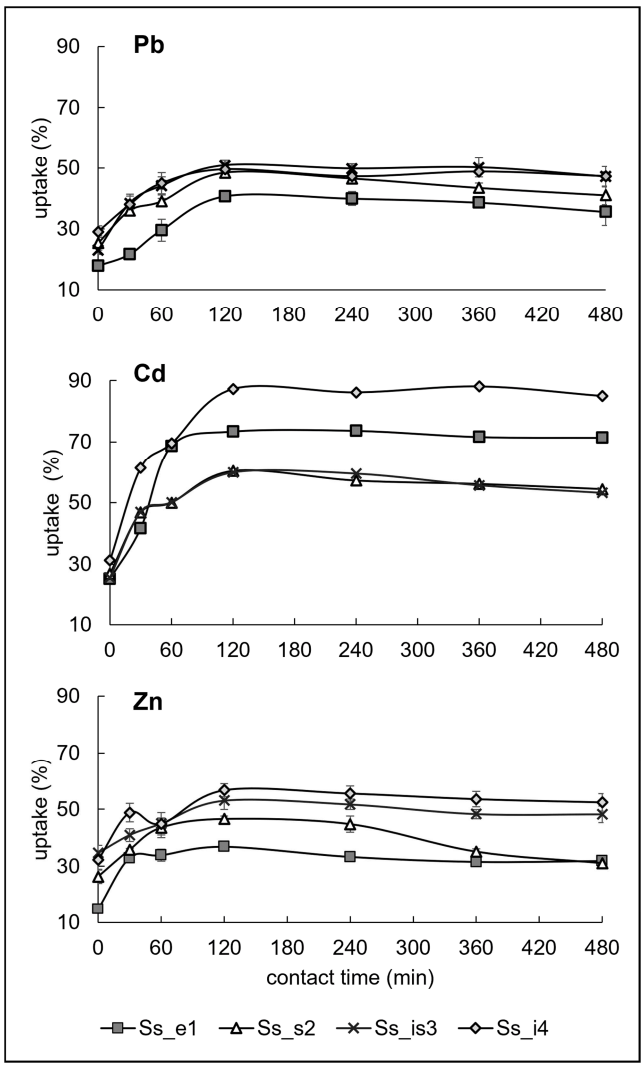

Figure 4. *Effect of contact time on Pb^{+2}, Cd^{+2} and Zn^{+2} uptake by bacterial isolates Ss_e1, Ss_s2, Ss_is3 and Ss_i4.*

3.2. Molecular Characterization

PCR amplifications of the 16S rDNA sequences of the four selected isolates produced approximately 1000 base pairs in size. Comparisons with BLAST analysis revealed that all isolates belong to the genus *Halomonas*. Isolate Ss_e1 and Ss_s2 have a 97.59% and 98.97% sequence identity respectively with *Halomonas* accession CPO11052_s. Isolate Ss_is3 has 99.68% with *Halomonas pacífica* and Ss_i4 has 98.94% with *Halomonas zhanjiangensis*.

3.3. Optimizing Heavy Metal Uptake Through Batch Experiments

3.3.1. pH Effect

In all cases, metal uptake increased with a rising pH, reaching maximum biosorption uptake values up to 58% of Zn^{+2} in Ss_i4. When reaching pH higher than 7 biosorption tended to drop. Isolates Ss_e1, Ss_s2 and Ss_is3 reached a maximum metal uptake of Pb^{+2}, Cd^{+2} and Zn^{+2} at pH 7. In contrast Ss_i4 presented a maximum heavy metal uptake for Pb^{+2} and Zn^{+2} at pH 8 (49% and 58% respectively) (Figure 3). Due to physicochemical properties of the heavy metals, pH was maintained at 7 for all isolates.

3.3.2. Contact Time

Cd^{+2} and Zn^{+2} uptake reached equilibrium at 2 hours (120 min) in all isolates. It was observed that all three metal concentrations decreased rapidly during the initial 5 minutes. Later reaching a constant concentration value past 120 minutes. Biosorption was quick, and saturation was obtained within 2 hours. Particularly Pb^{+2} registered a rapid biosorption in the first minutes of contact reaching 61% biosorption uptake, followed by a decrease and a second increase in uptake in isolate Ss_i4 (Figure 4). Nevertheless, all isolates reached equilibrium after 2 hours.

3.3.3. Temperature

Temperature presented a lesser considerable effect in heavy metal uptake. In general, Cd^{+2} uptakes presented more significant differences in all isolates at the evaluated temperatures. A significant difference was found at 37°C, in which all four isolates obtained the highest metal uptake in particular Ss_e1 and Ss_is4, both with 68% uptake. For Zn^{+2} and Pb^{+2}, uptake values showed few significant differences between the three evaluated temperatures.

3.4. Kinetic Experiments

The applicability of the biosorption rate models was examined through a comparison between theoretical and experimental data. Pseudo-first order obtained correlation coefficients (R^2) ranging from 0,75 for Cd^{+2} biosorption in isolate Ss_i4 and 0.99 for Zn^{+2} biosorption in the same isolate. Moreover, the theoretical q_e evaluated by the first order equations was considerably higher or lower than experimental values (Table 3). When applying pseudo-second order model (Table 4), experimental data presented high similarity with theoretical data like in the case of isolate Ss_e1, theoretical and experimental q_e for Cd^{+2} was 10,4. Furthermore, correlation coefficient values were R^2= 0.99. In comparison to the pseudo-first model, pseudo-second order described more precisely biosorption rate of the three heavy metals in all isolates.

Table 3. *Comparison between the theoretical and experimental biosorption capacity values (q_e) and correlation coefficients (R^2) associated with pseudo-first order kinetic model.*

Pseudo-first order kinetics												
Isolate	**Cd^{+2}**				**Zn^{+2}**				**Pb^{+2}**			
	R^2	**K**	**$q_{e\ theo}$ (mg. g^{-1})**	**$q_{e\ exp}$ (mg g^{-1})**	**R^2**	**K**	**$q_{e\ theo}$ (mg. g^{-1})**	**$q_{e\ exp}$ (mg. g^{-1})**	**R^2**	**K**	**$q_{e\ theo}$ (mg. g^{-1})**	**$q_{e\ exp}$ (mg. g^{-1})**
Ss_e1	0,94	0,01	2,97	10,4	0,9	0,02	5,11	12,69	0,82	0,02	2,7	9,94
Ss_s2	0,87	0,02	18,47	7,12	0,92	0,02	3,7	9,34	0,88	0,02	2,19	7,72
Ss_is3	0,98	0,02	2,93	7,56	0,94	0,01	3,6	9,45	0,89	0,02	1,89	8.28
Ss_i4	0,75	0,01	2,93	5,97	0,99	0,02	5,77	10,91	0,81	0,02	1,6	6,64

Table 4. *Comparison between theoretical and experimental data: biosorption capacity values (q_e) and correlation coefficients (R^2) associated with pseudo-second order kinetic model.*

Pseudo-second order kinetics												
Isolate	**Cd^{+2}**				**Zn^{+2}**				**Pb^{+2}**			
	R^2	**K**	**$q_{e\ theo}$ (mg. g^{-1})**	**$q_{e\ exp}$ (mg. g^{-1})**	**R^2**	**K**	**$q_{e\ theo}$ (mg. g^{-1})**	**$q_{e\ exp}$ (mg. g^{-1})**	**R^2**	**K**	**$q_{e\ theo}$ (mg. g^{-1})**	**$q_{e\ exp}$ (mg. g^{-1})**
Ss_e1	0,99	0,03	10,43	10,4	0,99	0,01	12,94	12,68	0,99	0,02	10,25	9,95
Ss_s2	0,99	0,04	7,14	7,12	0,99	0,02	9,13	9,34	0,99	0,1	7,35	7,36
Ss_is3	0,99	0,03	7,67	7,55	0,99	0,02	9,41	9,45	0,99	0,05	8,2	8,3
Ss_i4	0,99	0,06	5,84	5,97	0,99	0,01	11,14	10,9	0,99	0,05	6,59	6,64

3.5. Biosorption Equilibrium Modelling

Biosorption constants of Langmuir and Freundlich isotherm models were evaluated. Correlation coefficient (R^2) values obtained when applying Langmuir isotherm for all heavy metals and isolates were around 0,99 with the exception of Ss_s2 and Ss_is3 isolates for Zn^{+2} (0,98 and 0,97 respectively) (Table 5). In contrast, results obtained when applying the Freundlich equations and creating

isotherms, showed lower correlation coefficient values (R^2<0.95). Therefore, indicating a non-applicability of the model to describe the type of biosorption that took place between the four isolates and the heavy metals. These results clearly indicate that the experimental data fitted with Langmuir isotherm model better than Freundlich isotherm model.

Table 5. *Biosorption constants and parameters from equilibrium experiments with best fit to Langmuir model.*

Isolate	Metal	Isotherm	R^2	q_{max} (mg. g^{-1})	K_L (L.mg^{-1})
	Cd^{+2}	Langmuir I	0,99	18,83	0,15
Ss_e1	Zn^{+2}	Langmuir I	0,99	28,25	0,08
	Pb^{+2}	Langmuir I	0,99	22,7	0,03
	Cd^{+2}	Langmuir I	0,99	19,01	0,23
Ss_s2	Zn^{+2}	Langmuir I	0,98	22,12	0,28
	Pb^{+2}	Langmuir I	0,99	33,11	0,12
	Cd^{+2}	Langmuir II	0,99	39,2	0,01
Ss_is3	Zn^{+2}	Langmuir II	0,97	39,7	0,02
	Pb^{+2}	Langmuir II	0,98	65.8	0.01
	Cd^{+2}	Langmuir I	0,99	22,52	0,08
Ss_i4	Zn^{+2}	Langmuir I	0,99	20,2	0,13
	Pb^{+2}	Langmuir I	0,98	27,25	0,05

Separation factors for the Langmuir model of the four isolates for the three heavy metals were calculated as Langmuir isotherm model was established to present a better fit. These results indicated that the biosorption process is favorable.

4. Discussion

To the extent of our knowledge there are no studies regarding halophilic and halotolerant bacteria found in the Bolivian Altiplano soils, nor their interaction with heavy metals that are also present in the region.

Selected isolates with the highest heavy metal tolerance presented a high sequence identity to the *Halomonas* genus, which is found in a great variety of saline environments. From sea water to salt flats like the athalassohaline environment of Salar de Uyuni in Bolivia [9, 22-24]. This genus is comprised of halophilic and halotolerant Gram-negative aerobic bacteria [25]. It has been studied for many years due to its industrial uses. *Halomonas pacifica* and *Halomonas zhanjiangensis* have been isolated from marine environments. Both can tolerate up to 20% of NaCl and grow in a range of approximately 4 – 40°C and a pH range of 6 – 10 [26]. This coincides with the data obtained in the present study given that both exhibit a slightly basic optimum pH (8.5 and 9.5 respectively) and have a high tolerance to NaCl (up to 20%). The four isolates grow optimally at 5% (w/v) concentration of NaCl which is standard of moderately halophilic bacteria.

Halophilic and halotolerant microorganisms isolated from similar sites [27-29] often show a tolerance to metals, which in turn is associated to their tolerance to high salt concentrations. *Halomonas* genus has been largely studied for bioremediation and production of desirable compounds. It is known to have strains that can tolerate high Pb^{+2} and Cd^{+2} concentrations of above 1000 and 500 mg. L^{-1} respectively [30, 31].

The four isolates had MIC values that fit in a range of tolerance to Pb^{+2} found in other studies [32-35]. Although concentrations found in the present study are considerably lower than the majority of studies, it is worth mentioning that tolerance assays were carried out at 0.85% salt concentration. Optimum NaCl concentration was not applied in biosorption experiments with heavy metals. Other studies concerning heavy metal tolerance and accumulation at different NaCl concentrations show mixed results like in [30] Amoozegar et al., (2012). Which found that decreasing NaCl concentration increased Pb^{+2} tolerance and the opposite in the case of Cd^{+2}. In the end, this might have affected the ability of bacteria to tolerate higher metal concentrations. Sowmya et al., (2014) demonstrated the effect of salt concentrations on the MIC of their isolates, with higher salt concentrations leading to higher MIC in the same strain [35]. Originally Ventosa et al., (1998) indicated that certain halophilic microorganisms have a salt-sensitive tolerance to certain heavy metals [36]. In general, sensitivity increased when NaCl concentration was lowered, in turn tolerance increased with higher salt concentration determining that moderate halophiles are good candidates for heavy metal removal due to their natural need for high concentrations of cations and anions. Therefore, the effect of different NaCl concentrations on heavy metal tolerance and removal should be considered in future studies as a significant factor.

The way in which pH shifts biosorption of metal ions depends on the type of biosorbent and on the type of sorbate [37, 38]. The four isolates presented a high uptake of all heavy metals at pH 7 and decreased at more alkaline values. Zouboulis et al., (2004) found that for *Bacillus laterosporus,* optimum pH for Cd^{+2} biosorption was 7, given that Cd^{+2} precipitates to hydroxide in the cell wall at higher pH values [39]. Mwandira et al., (2020) evaluated that for biosorption of Pb^{+2} and Zn^{+2} *Oceanobacillus profundus* a pH value of 6 yielded better biosorption percentages and lower removal percentages at low pH were due to protonation of functional groups [40]. Volesky & Holan (1995) determined that cellular surface is positively charged at low pH and attraction between metal ions and cell wall decreases [41], in contrast when pH increases, the groups responsible for metal

retention are negatively charged, hence favoring retention. Although Ss_is4 presented higher uptake at pH 8, as described by Pardo et al., (2003), at higher pH values a series of hydroxylated complexes can form, which might compete for the active sites of the cells, therefore, biosorption would decrease [42]. Therefore, pH in this study was maintained at 7 for all biosorption experiments. In terms of temperature, Prakash et al., (2013) and Green-Ruiz et al., (2008) found better heavy metal uptake at temperatures in a range of 30 - 37°C [43, 44]. Suggesting that heavy metal biosorption seems to be more affected by larger temperature ranges. In contrast Muzammil et al., (2021) found that between 25, 37 and 42°C, maximum removal capacity was reached at 37°C [45]. Therefore, for this study temperatures were maintained at 37°C to obtain good growth and heavy metal uptakes.

Kinetic models can be helpful to better understand the mechanisms of metal biosorption and to evaluate performance of biosorbents for metal removal. Pseudo first order model does not fit well for the whole range of contact time and is generally applicable in the first 20-30 minutes of the biosorption process, once that window is passed, the experimental and theoretical data do not fit correctly [46, 47]. On the other hand, the pseudo-second order model has been used to describe chemisorption involving exchange of electrons between the biosorbent and sorbate as covalent forces. Furthermore, it is capable of predicting behavior over the entire contact time [48, 49]. In the present study when determining contact time biosorption rate was also established. A maximum heavy metal uptake was achieved at 120 minutes. As mentioned above, a pseudo-first order model does not fit longer periods past the initial 30 minutes. Considering correlation between theoretical and experimental data it was determined that the pseudo-second order model described best the biosorption rate of the three heavy metals in the four bacterial isolates. In most cases biosorption of heavy metals reached equilibrium past the initial 30 minutes, therefore fitting best to pseudo-second order model [20, 50-58].

Kratochvil & Volesky (1998) indicated that Langmuir isotherms are widely used due to their two constants which are easily interpretable [59]. When looking for a suitable biosorbent, high values of q_{max} and low K_L values are important. K_L values in this study were low indicating a stronger affinity of the isolates binding sites to the metal ions. In Ss_e1 and Ss_i4 which presented low q_{max} values (18.83 and 20.2 mg. g^{-1}) and K_L values of 0.15 and 0.13 L. mg^{-1} respectively. This indicates the opposite of a poor biosorbent, rather the maximum capacity values of each metal to the isolates is related to the differences between the exact composition of their cellular surfaces and in consequence to the number of available binding sites [60]. Experimental data show that the Langmuir isotherm model is suitable to describe the Cd^{+2}, Zn^{+2} and Pb^{+2} biosorption in the four bacterial isolates, following a monolayer type of biosorption [61]. Given the applicability of Langmuir isotherm model, R_L values obtained reveal that the interaction between heavy metals ions and the bacterial surfaces is strong [62].

Fathollahi et al., (2021) and Davis et al., (2003) indicated that biosorption of each heavy metal may be explained by molecular size and atom mass [63, 64]. If bigger it is easier to bind with active sites in the cell wall. Pb^{+2} has a larger cation size and therefore tolerance and biosorption capacity is higher. Loaëc et al., (1997) explained that Pb^{+2} and Cd^{+2} have a larger ionic radius, that allows them to be fixed in the polysaccharide network of the cell wall, while Zn^{+2} does not fit well due to its smaller ionic radius [65]. When comparing to maximum biosorption capacities in this study, what is mentioned by the authors is true for Pb^{+2}, which has the highest q_{max} in all isolates except Ss_e1. In the case of Cd^{+2}, it is only true for isolate Ss_i4, that presented a higher q_{max} for Cd^{+2} than for Zn^{+2}. Several factors including individual intra or extracellular biosorption properties, presence of functional groups or chelating properties, may play a role in this study's bacterial isolates biosorption capacities.

Halomonas groups are known to produce exopolysaccharides (EPS) with potential for removing metals from polluted environments as they efficiently bind to metals like Pb^{+2}, demonstrated in many studies like: Llamas et al., (2012), Mata et al., (2006) and Mukherjee, Mitra, & Roy (2019) found that EPS production in a *Halomonas* was induced in the presence of NaCl starting at 1%. Also, a higher production of EPS was linked to a higher heavy metal biosorption [66-68]. Given all isolates belong to *Halomonas* genus it is appropriate to assume that they possess the capacity to produce EPS. However, this production capacity might have been affected by the low NaCl concentration (0.85%). Lastly, maximum biosorption capacities can be observed in Ss_is3 with 65.8 mg. g^{-1} for Pb^{+2} and 39.7 mg. g^{-1} for Zn^{+2}, which is considered low, in regard to other studied bacteria (e.g., [69]). When comparing with other works published using *Halomonas* bacteria [31, 70].

5. Conclusions

In the present study, four moderate halophilic *Halomonas* strains isolated from the Bolivian Altiplano were able to grow in the presence of Pb^{+2}, Cd^{+2} and Zn^{+2}. The four bacterial isolates present a high tolerance to Pb with an MTC of 200 mg. L^{-1} (all), Cd 150 mg. L^{-1} (Ss_s2 and Ss_is3) and Zn 150 mg. L^{-1} (Ss_e1, Ss_is3, Ss_i4). Biosorption kinetics indicate that all isolates follow a pseudo-second order model for biosorption rate of the three heavy metals. Langmuir isotherm best describes the biosorption of Pb^{+2}, Cd^{+2} and Zn^{+2} in the four selected bacterial isolates with the highest q_{max} obtained by Ss_is3 at 65.8 mg. g^{-1} for Pb^{+2}. The four *Halomonas* isolates in this study have the capacity to tolerate heavy metals and keep their viability after being exposed to high metal concentrations. The ability of *Halomonas* to biosorb heavy metals could potentially be used as tools for bioremediation of soils contaminated with Pb^{+2}, Cd^{+2} and Zn^{+2} such as those present in the Bolivian Altiplano. Although the experiments were carried out under low salt concentration, all four isolates had good biosorption capacities when compared to other studies. However, we

recommend testing the effect of higher salt concentrations and EPS production on the biosorption capacity of these isolates. Finally, we recommend testing biosorption capacity of all four isolates combined as consortiums in microcosms set up with different compositions of soil, testing salt minerals and heavy metal concentrations to evaluate possible interactions and effects on the overall heavy metal uptake.

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