Growth Behaviours of Two Rhizospheric Bacterial Strains in the Presence of Different Cadmium Concentrations and Temperatures

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Abstracts

Understanding the interaction of Cd with plants, as well as the search for alternatives to minimize its effects, has caught the interest of the scientific community, due to the accelerated growth of contamination with this metal and its high toxicity. The aim of the present study was to evaluate the ability of two rhizospheric bacterial strains LC7504001.1 Burkholderia cepacia (C65RLIM) and FJ972527.1 Pseudomonas aeruginosa (C85RLIM) to three concentrations of cadmium (100, 300 and 500 mg/L) and to compare the growth behaviour with increasing temperature from 32 to 45°C. The results show different growth patterns for each strain tested against exposure to the three cadmium concentrations and the four temperatures tested. The Pseudomonas aeruginosa strain (C85RLIM) had the best adaptive behaviour to the three cadmium concentrations tested, and also showed stability when subjected to 500 mg/L CdCl2 and temperatures between 40 and 45°C. The results predict the possible behaviour of rhizospheric bacteria in the face of global warming and heavy metal remediation processes.

Keywords: rhizospheric bacteria, cadmium, temperature, growth.

Introduction

As stated by (McNear, 2013; Molina-Romero et al., 2015), rhizobacteria are bacteria that inhabit the rhizosphere, an area of soil that is attached to the root and extends a few millimetres from the surface of the root system. This zone is characterized by the unique and dynamic interaction of biogeochemical processes occurring between plant roots and soil microorganisms, which are highly influenced by root exudates, and is home to a large number of microorganisms that generally stimulate plant growth and reduce disease incidence.

When present in high concentrations of heavy metals they can form unspecific compounds creating cytotoxic and lethal effects (Soto et al, 2010). It is important to note that while some

metals have no biological influence, others such as cadmium (Cd), chromium (Cr), and mercury (Hg) are toxic (Nessner and Esposito, 2010).

As stated by Bolán et al. (2014), microorganisms play a vital role in the transformation of trace elements, including metals, as they influence their bioavailability and remediation, and can alter the toxicity, water solubility and mobility of the element.

Islam, et al., (2007), heavy metals Mercury-Hg, Cadmium-Cd, lead-Pb, Nickel-Ni among others, are a growing problem of environmental pollution worldwide, because unlike organic compounds, they cannot be biodegraded, which is why concentrations in environmental compartments are continuously increasing. Heavy metals are attributed with certain environmental pollution and toxicity effects, even as stated by Sheng et al. (2008), environmental contamination by Cd has increased as a consequence of the increase in industrial activity that has taken place at the end of the 20th century and the beginning of the 21st century, progressively affecting different ecosystems and public health.

According to Awasthi et al. (2015), in the bioremediation processes of environments contaminated with heavy metals, it seems that P. aeruginosa has a high bioremediative capacity, which prevents them, in quantities above certain limits, from being extremely harmful to the soil and the biota that support them.

Bacterial species within the genus Burkholderia represent a group of eighteen related species that are currently of interest for their extraordinary versatility as plant pathogens, saprophytes, biocontrol agents, bioremediation and human pathogens. These bacteria are naturally abundant in soil, water and on the surface of various plant species, possessing the ability to metabolize a wide range of organic compounds as a source of carbon and energy. One quality that has aroused interest in this group of bacteria is their use in bioremediation of soil and groundwater contaminated with hydrocarbons and herbicides (Orelio et al., 2014).

Projecting future scenarios of the effect of global warming, it is necessary to predict the growth behaviour of rhizospheric bacterial species in response to increases in temperature and metabolic activity to remediate heavy metals. The aim of the present study was to evaluate in vitro the growth of two rhizospheric bacterial strains at three cadmium concentrations and to predict the effect of temperature on metabolic and remediation processes.

Materials and Methods

a) Sampling

Initially, bibliographic information was collected on areas contaminated with heavy metals due to anthropogenic activities in the department of Sucre. Once the areas with soils contaminated with heavy metals had been identified, site visits were made and a diagnosis was made of the current state, crops grown, number of hectares and vulnerable areas. Next, the contaminated soil sampling stage was carried out. This consisted of a random sampling, which was divided into three plots according to the topographical characteristics of the land. In each of these plots, three samples of rhizospheric soil from established rice crops were taken at random, covering the first

20 cm of depth. Approximately 3000 g of sample were taken per site. These were distributed into two sub-samples of 1500 g each (cadmium concentration and the other for microbiological analysis). The soil samples were then stopped, carefully packed in labelled and identified plastic bags, stored and preserved for transport and processing.

b) Isolation of rhizospheric bacteria

It was carried out using the methodology proposed and modified by Ayubb et al., (2017), which consisted of: prepare A 1:100 dilution of soil was inoculated on selective medium with 1-carboxylic acid-1-aminocyclopropane (ACC), the bacteria that grew on this medium were characterized for their growth-promoting capacity. This medium contains the mineral salts of the Dworkin and Foster medium whose composition per litre is as follows (4g KH_2PO_4, 6 g $NaHPO_4$, 0.2 g $MgSO_4$, 1 mg $FeSO_4$, 10 µg H_3BO_3, 10 µg $MnSO_4$, 50 µg $CuSO_4$, 10µg MoO_3, 70 µg $ZnSO_4$), glucose 0.2%, gluconic acid 0.2%, citric acid 0.2% and bacteriological agar at 2% and ACC 3 mM (Sigma).

c) In vitro evaluation of cadmium tolerance by rhizospheric bacteria

Bacterial isolates were seeded on nutrient agar supplemented with $CdCl_2$ at concentrations of 100, 150, 200, 250, 300, 350, 400 up to 1000 mg/L; then incubated at 29°C for 7 days (Sorkhoh et al. 2010).

d) Molecular identification of cadmium-tolerant rhizospheric bacteria

The extraction of genomic DNA from cadmium-tolerant rhizospheric bacteria was performed using the protocol proposed by Oliveira et al. (2013). For the amplification of the 16S rRNA gene, three specific primer sets were used: F BLS342 (5‘ CAGCAGTAGGGAATCTTC3’) and R 1392 (5‘ ACGGGGGCGGTGTGTGTACA 3’), for the class Firmicutes; F 948β (5‘ CGCACAAGCGGTGGTGGATGA 3’) and R 1492 (5‘ TACGG(C/T) TACCTTGTTACGACTT 3’), for the class Betaproteobacteria; FD2 (5‘ AGAGTTTGATCATGGCTCAG 3’) and RP1 (5‘ ACGGTTACCTTGTTACGCTT 3’), for the gammaproteobacteria class. The conditions used in each amplification reaction were based on the protocol described by Oliveira et al. (2013), using a mastercycler nexus eppendorf thermal cycler. The amplification products obtained were purified and sequenced at Macrogen Korea. The sequences obtained were compared with those stored in Genbank. Base alignment was performed in the Clustal w program, analysis and correction in the Mega 5 program, phylogenetic inferences were obtained by the maximum similarity method, based on the kimura-2-parameter model.

e) In vitro evaluation of cadmium and temperature tolerance

The in vitro tolerance assessment of rhizospheric bacteria to different concentrations of cadmium ion was carried out in tris-MMT minimal medium (Rathnayake et al., 2013) prepared with three concentrations of Cd in the form of CdCl2. The initial concentration of Cd used in the present study was 0.01 mg/mL and from these, concentrations of 100; 300 and 500 mg/L) were prepared. Aliquots of rhizospheric bacterial suspensions (C65RLIM and C85RLIM) were used in log phase and inoculated onto MMT medium supplemented separately with each of the cadmium concentrations. MMT medium without CdCl2 was used as a control. The experiment was

performed in triplicate, which was incubated in shaking at 150 rpm at various temperatures (32, 37, 40 and 45°C) separately and for 4 days (Zhang, et al., 2011). The growth of rhizospheric bacteria was determined by turbidimetry at 600 nm every 2 hours for four days.

In the same experiment, a control without cadmium supplementation was used, but was incubated under stirring at 150 rpm at various temperatures (32, 37, 40 and 45°C) °C for 26 hours.

Results and Discussion

Rhizospheric bacteria isolated from cadmium-contaminated rice soils were used in the present study. A total of 29 isolates were obtained from the rhizosphere of rice crops adapted to high cadmium concentrations (figure 1). Each isolate was evaluated in vitro and tested for tolerance to different cadmium concentrations ranging from 100 to 100 mg/L. The results obtained from in vitro evaluated tolerance show that the strains coded as: C65RLIM and C85RLIM and identified by molecular sequencing techniques correspond according to the phylogenetic analysis of 16S rRNA sequences with a high homology to sequences similar to LC7504001.1 Burkholderia cepacia (C65RLIM) and FJ972527.1 Pseudomonas aeruginosa (C85RLIM) (figure 2).

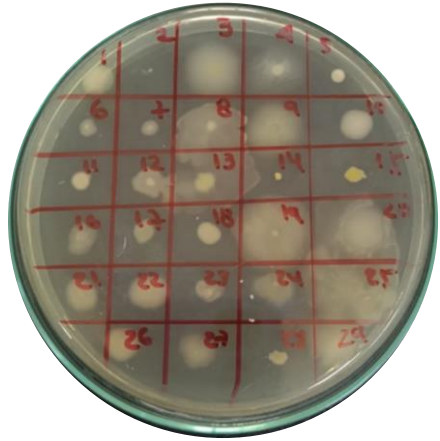

Figure 1. Cultural characteristics of rhizospheric bacterial isolates present in rice soils of the San Jorge sub-region, department of Sucre, contaminated with cadmium.

The results of the growth curve show in figure 3, the growth behaviour of the strain (C85RLIM) in medium supplemented with concentrations of 100, 300 and 500 mg/L CdCl2, compared to the control (growth of the strain without cadmium). The growth curve shows a uniform behaviour depending on the concentration to which the C85RLIM strain was subjected (100, 300 and 500 mg/L $CdCl_2$).

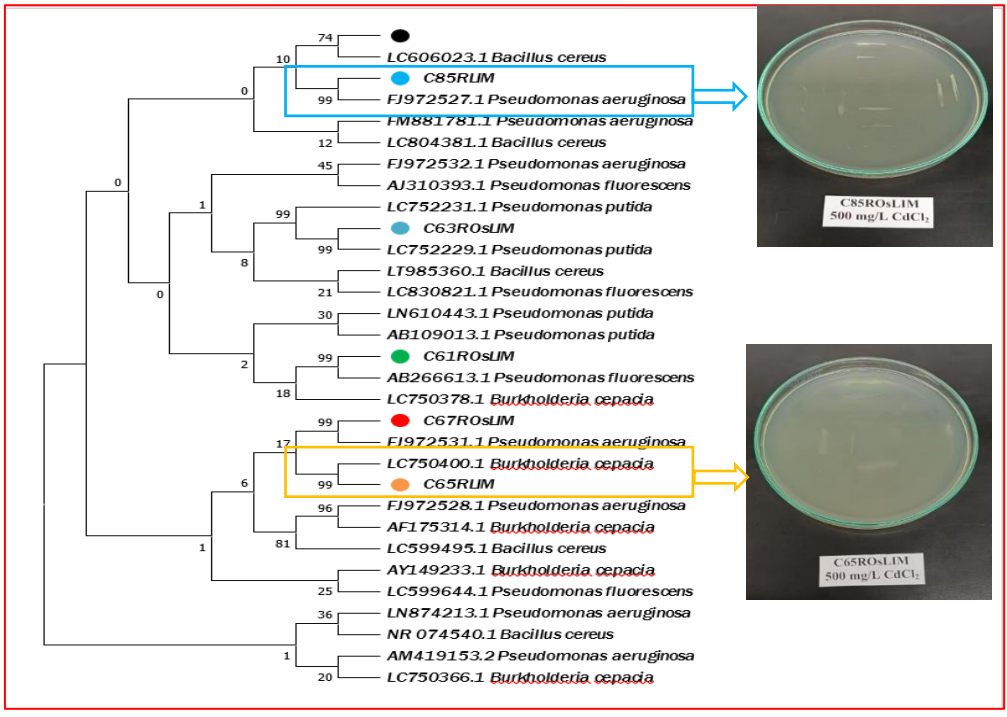

Figure 2. Phylogenetic tree based on a partial 16S rRNA gene sequence of bacteria's rhizospheric.

The times in hours used for each test are listed in table 1 with their equivalence in number used for the construction of the respective growth curves.

Table 1. Time in hours used in growth curve assays to evaluate adaptation to three cadmium concentrations (100, 300 and 500 mg/L) and temperatures (32, 37, 40 and 45°C) for each strain (C65RLIM and C85RLAM).

Hours	0.0	2.0	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0	22.0	24.0	26.0
N°	1	2	3	4	5	6	7	8	9	10	11	12	13	14

With respect to the growth behaviour of the strain, it is observed that at a concentration of 500 mg/L the strain showed an adaptation phase until two hours later, after which it entered the logarithmic phase, which was maintained from 4 hours until 18 hours after the experiment began, and between 20 and 22 hours it entered the stationary phase, and after 24 hours it began the transition to the death phase. With respect to the concentrations 100 and 300 mg/L it was similar and lower than the behaviour shown by the control. If we compare the behaviour of the C85RLIM strain in the three concentrations evaluated, we observe a different compartment when it was subjected to concentrations of 100 and 300 mg/L and only at 18 hours (10 in the graph) the strain in the 500 mg/L concentration had the same growth behaviour as the other two concentrations evaluated (100 and 300 mg/L).

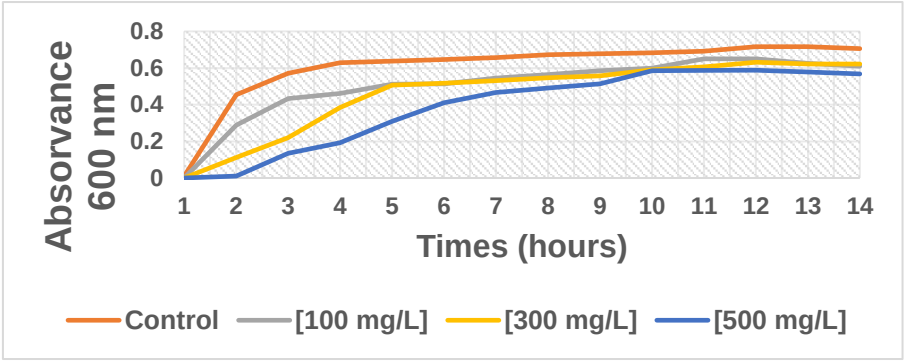

Figure 3. Growth assay of rhizospheric strain (C85RLIM) in three cadmium concentrations.

Despite being subjected and exposed to high concentrations of cadmium, strain C85RLIM showed a similar growth pattern to the control.

Figure 4 shows the growth behaviour of C65RLIM at three cadmium concentrations (100, 300 and 500 mg/L). The observed growth pattern of C65RLIM at the same concentrations to which C85RLIM was subjected, showed a different and more marked compartment between each of the concentrations analyzed. At concentrations of 100 mg/L, the C65RLIM strain had an adaptation phase of up to 2 hours, while at concentrations of 300 mg/L it was up to 4 hours, in comparison with the growth behaviour at 500 mg/L, where it is observed that the adaptation phase was up to 6 hours after the start of the experiment. The same figure shows that at a concentration of 100 mg/L, C65RLIM had a pattern very close to the control (without cadmium). It can also be seen that at a concentration of 500 mg/L the C65RLIM strain grew until 16 hours and then entered the stationary phase (18 to 20 hours) and after this time it entered the decline or death phase.

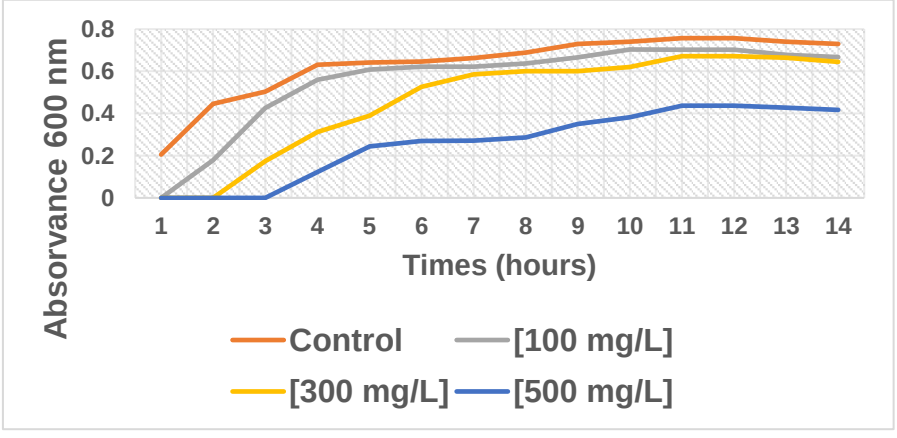

Figure 4. Growth assay of rhizospheric strain (C65RLIM) in three cadmium concentrations.

When comparing the growth model between strain C85RLIM and C65RLIM, it is observed that strain C85RLIM had a more uniform growth compartment in the three concentrations evaluated (100, 300 and 500 mg/L) compared to figure 2, which shows the growth behaviour of strain C65RLIM at the same concentrations.

Figure 5 shows the growth performance of the control strain C65RLIM (without cadmium) at different temperatures (32, 37, 40 and 45°C), compared to the growth performance of the same strain C65RLIM in culture supplemented with 500 mg/L CdCl2 (figure 6) and at the same growth temperature as the control.

The growth behaviours of the control strain at different temperatures was uniform, showing better growth between 32 and 40°C with respect to the growth at 45°C where less growth was observed, entering the lysis or death phase after 20 hours.

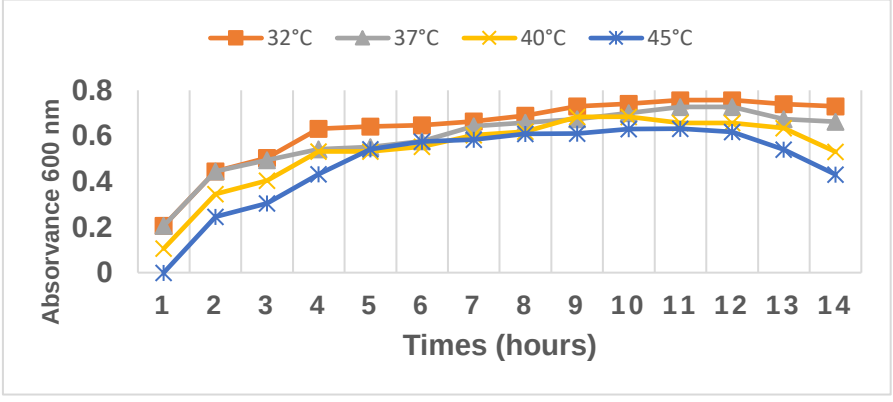

Figure 5. Growth performance of the control strain (C65RLIM) without cadmium at different temperatures.

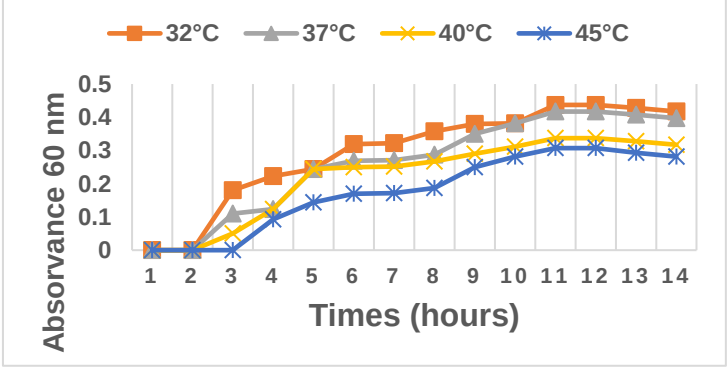

Figure 6. Growth performance of strain (C65RLIM) supplemented with 500 mg/L $CdCl_2$ and at different temperatures.

With respect to the behaviour of the C65RLIM strain with cadmium at different temperatures, a different behaviour is observed for 32, 37 and 40°C, with respect to the control strain (figure 5). The figure shows that the strain grows up to 45°C in the medium supplemented with 500 mg/L of cadmium, observing an adaptation phase up to 6 hours, with a slow logarithmic phase at the beginning up to 12 hours, after which it shows an increase and behaviour similar to that presented at 40°C.

Figure 7 shows the growth performance of the control strain C85RLIM (without cadmium) at different temperatures (32, 37, 40 and 45°C), compared with the growth performance of the same strain C85RLIM in culture supplemented with 500 mg/L CdCl2 (figure 8) and at the same growth temperature as the control. The growth performance of the control strain at different temperatures was uniform, showing better growth at 32 and 37°C, very similar at the times evaluated, and we also observed a growth performance between 40 and 45°C, with a slight decrease in growth at 45°C.

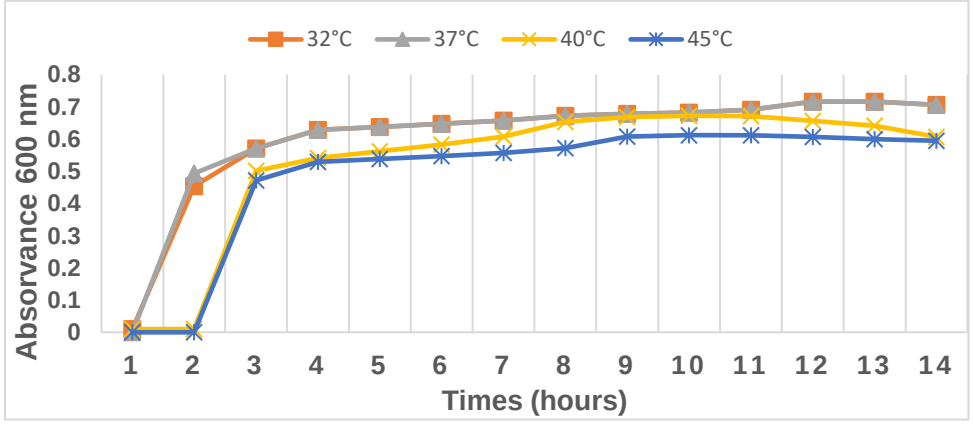

Figure 7. Growth performance of the control strain (C65RLIM) without cadmium at different temperatures.

Figure 8 shows the growth behaviour of strain C85RLIM in culture medium supplemented with 500 mg/L CdCl2. In this figure, a uniform and dispersed growth pattern is observed for each temperature, showing an adaptation phase of 6 hours at the temperature of 45°C, with respect to the other temperatures. With respect to the growth of strain C85RLIM compared to that shown by strain CR65RLIM (figure 6), there was greater differential growth for C85RLIM subjected to the same condition.

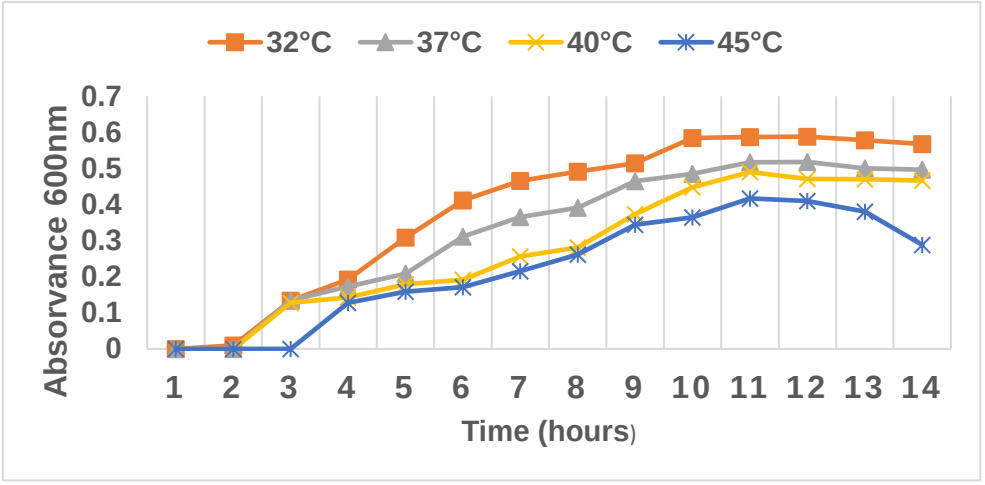

Figure 8. Growth performance of strain (C85RLIM) supplemented with 500 mg/L CdCl2 and at different temperatures.

As Schedlbauer (2015) states, the presence and evolution of metal-resistant bacterial species occurs as a consequence of their adaptation to environments with high concentrations of these elements, both anthropogenic and natural.

According to Marrero-Coto et al. (2010), heavy metals cannot be degraded when the oxidation state is changed, as occurs with toxic organic compounds. There are only three mechanisms by which a system can confer resistance to heavy metals: (a) decrease of the accumulation of a given ion by its active transport out of the cell; (b) segregation of cations by molecules containing thiol groups; (c) reduction of some heavy metal ions to a less toxic oxidation state.8-11 However, (b) and (c) cannot function as unique detoxification mechanisms in a cell. Therefore, the 'metabolism' of heavy metals is primarily carried out by a transport 'metabolism' or ultimately by a combination of mechanisms.

Likewise, the authors (Marrero-Coto et al., 2010) go on to say, many bacterial cells possess different types of heavy metal cation capture systems. One is fast and non-specific, constitutively expressed and utilized by a variety of substrates. Fast systems are generally dependent on the chemosmotic gradient across the bacterial cytoplasmic membrane. The second type of capture system has high substrate specificity, is slower and generally uses ATP hydrolysis as an energy source in addition to the chemosmotic gradient.

As stated by (Marrero et al., 2010; Martínez et al., 2010), when a bacterial cell is confronted with large concentrations of a heavy metal, it transports them to the cytoplasm in the form of cations via transporters of the cellular non-specific system; in addition, other mechanisms are activated such as the use of membrane transporters that expel these ions into the environment, enzymatic modifications to change the redox state and the incorporation of metal ions into the cells; however, it is possible that mutations are generated due to the pressure exerted by the contaminated medium.

Similarly, as expressed by Lovley (200), some examples of metal sequestration in the cytosol are very interesting, such as the case of uranium accumulation by Pseudomonas aeruginosa, which was detected only in the cytoplasm, as in the yeast S. cerevisiae.

The results obtained show that as the concentration of cadmium was increased, not only did the number of bacterial cells decrease, but also growth was retarded, requiring more time for the bacteria to adapt and recover their ability to grow in a Cd-contaminated medium, as corroborated by the work done by Kamika and Momba (2013). However, the interaction between metal and bacteria may be a key mechanism contributing to the bioavailability and toxicity of the metal (Kamalakannan and Krishnamoorthy, 2006).

Temperature affects growth rate (and thus generation time). Each bacterium (assuming all other environmental conditions are held constant) shows a characteristic growth rate curve as a function of temperature. To date, there are no references of other studies evaluating the effect of temperature on the metabolic activity of cadmium-remediating bacteria. However, it is important to know the behaviour of rhizospheric bacteria in situ in a future scenario of how global warming may affect the growth and bioremediation activity of rhizospheric bacteria.

Conclusion

In our study, we isolated a highly effective Cd-tolerant P. aeruginosa and Burkholderia cepacia bacterium from rice soils in the region of San Jorge, department of Sucre, contaminated with cadmium., which was able to tolerate up to 500 mg/L $CdCl_2$ and support growth up to 45 °C. The results obtained indicate the presence of P. aeruginosa and Burkholderia cepacia bacterium screened has good environmental adaptability, making it a good candidate for environmental remediation and adaptation to climate change.

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Author contribution. Alexander Perez Cordero: experiment execution, data analysis. Donicer Montes V and Yelitza Aguas M, conceptualization, writing - revision and editing. All authors have read and approved the manuscript.

Conflict of interest. All the authors of the manuscript declare that they have no conflictof interest.

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