

# Adaptation Through the Production of Siderophore of Endophytic Bacteria Isolated of Rice Soils and the Capacity to Grow in the Presence of Cadmium

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## Abstract

The objective was to assess the *in vitro* capacity of siderophore production by *Bacillus cereus* strain BN5 in different concentrations of cadmium (Cd). The bacterium was isolated from the rice variety cultivated in the municipality F2000 Achi-Antioquia, Colombia. Aliquots *B. cereus* strain BN5 log phase was inoculated into minimal medium tris-MMT with different concentrations of CdCl<sub>2</sub>. The experiment was incubated with stirring at 150 rpm at 32 ° C / 90 hours; growth of bacteria in each treatment was assessed by turbidimetry technique at 600 nm. Siderophore of production was determined in the medium azurol-S (CAS). There is an inverse relationship between population density and concentration of Cd by tissue, being larger amount of bacteria in panicle compared to other tissues. *B. cereus* BN5 tolerated up to 500 mg / L of CdCl<sub>2</sub> and siderophores produced in the different treatments tested. This endophytic bacteria may be used in the future as an alternative for bioremediation and safe production of rice in soil contaminated with Cd.

**Keywords:** *Bacterium, Rice, Cadmium, Siderophores.*

## Introduction

Iron (Fe) is a cofactor of various enzymes and vital micronutrient required for many cellular processes. This micronutrient is present in the atmosphere in sufficient quantities, but in different microbial habitats insoluble complexes in the presence of oxygen and water to pH neutral and básicos<sup>1</sup>. To acquire Fe, microorganisms have developed mechanisms to solubilize and make efficient absorption. One of the mechanisms used by bacteria and fungi is the production of compounds of low molecular weight chelators Fe<sup>3+</sup>, which is synthesized under conditions of deficiency of this micronutrient called sideróforos<sup>2</sup>. Different groups of siderophores with differences in their structure, which are classified into three categories according to the functional groups used as a ligand for iron ions, finding catecholates as enterobactinas and vibriobactinas; hydroxamates as stafiloferrin and mixed type siderophores as mycobactin<sup>2</sup>.

Recent reports on several studies indicate that microbial siderophores may form stable complexes with other metals present in the environment (aluminum, cadmium, copper, gallium, lead, nickel and others) and also with uranio<sup>3</sup>. Siderophores are produced by various microorganisms groups, plant and animal pathogens, free life microorganisms and symbiotic nitrogen fixers. The siderophore production is more common in promoting rhizosphere bacteria species of plant growth, those with the ability under extreme environment conditions. The groups of bacteria are *Pseudomonas*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Serratia*, *Azospirillum*, and *steptomycetes*, *Rhizobium*<sup>1,4</sup>.

Many other bacteria associated with plants such as endophytes can synthesize siderophores, who gives a competitive advantage in colonization of plant tissues by helping to exclude other microorganisms from the same niche ecological<sup>5,6</sup>. There is evidence siderophore synthesis by bacteria when they grow in environments containing toxic heavy metals, linking these chelating agents such as those responsible for the homeostasis of said metals<sup>7</sup>. Several studies suggest that the siderophores form stable complexes with

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other metals such as Al, Cd, Cu, Ga, In, Pb and Zn<sup>3,7</sup>. According work done por8, observed that the addition of heavy metals such as Al, Cu, Ga, Mn and Ni pyoverdin induce production in *Pseudomonas aeruginosa*.

Soil contamination by heavy metals is spreading and causing serious environmental problems. Heavy metals not only destroy the ecology of soil microbes and decreases the production of agricultural crops, also it affects human health through the chain alimentaria<sup>9</sup>. Excessive use of phosphate fertilizers, dispersion sludge landfill and atmospheric deposition caused widespread contamination of cadmium (Cd) in agricultural soils<sup>10</sup>. Cadmium is a highly toxic metal harmful to living at relatively low concentrations (0.001 to 0.1 mg / L) organisms and has been classified as a carcinogen for humans<sup>11,12</sup>. Cadmium is absorbed by rice plants growing in contaminated soil through the root and there is translocated to the stem and eventually the grain from the ground. Between 22-24% of the total biomass cadmium in rice, concentrated in the grain, rice contaminated consumption can cause serious health risk humana<sup>13</sup>.

Phytoremediation is considered one effective, cheap and friendly technology environment with much attention worldwide, because it brings great benefits as opposed to the traditional technology of accumulating heavy metals from soil. Such advantages are its low cost and negligible impact for human beings and ecosystems<sup>14,15</sup>. Phytoremediation success depends on the ability of the plant to tolerate high concentrations of metals and produce many biomasa<sup>16</sup>.

The endophytic bacteria found along cough different internal plant tissues play a major role, which is to contribute to the adaptation of plants to contaminated sites, and thus potentiate their fitorremediadora capacity and tolerance the present contaminants in soil resources such as the heavy metals<sup>17</sup>. Similarly, these bacteria also have effects on plant development promoting plant growth and increasing the biomass through the production of phytohormone such as indoleacetic acid, in turn improve the nutritional status thereof by nitrogen fixation, solubilization of phosphates and production of siderophores for uptake of essential nutrients in its desarrollo<sup>18</sup>.

*Bacillus cereus* It has been reported as endophytic bacteria of Cyperus and Paspalum plant isolated *in vitro* ability to tolerate up to 400 ppm (0.4 mg / L) mercury as HgCl<sub>2</sub> and besides rice plants ability to tolerate up to 400 ppm of Pb as Pb(NO<sub>3</sub>)<sub>2</sub> and siderophore produced<sup>19,20</sup>. In order to contribute to the search for new ecological alternatives to carry out processes phytoremediation, and ensure that rice crops can grow and adapt to contaminated environments cadmium, so it is proposed to isolate endophytic bacteria from different tissues of plants rice, evaluating *in vitro* the ability of different concentrations of tolerance cadmium and simultaneously the production of siderophores.

## Materials and Methods

- Study area. Commercial variety of rice Fedearroz 2000 (F2000) was collected from rice farms belonging to the municipality of Nechí located in the subregion of the low Cauca department of Antioquia, Colombia.
- Sampling. was performed randomly shaped zig-zag collecting whole plants (root, stem, leaf, and panicle) of the Fedearroz 2000 (F2000) rice variety and soil sample to a depth of 20 cm. For the collection and selection of plants were considered those I showed good phytosanitary state without the presence of symptoms of phytotoxicity. Plant samples were stored in refrigerators Icopor for conservation and were identified with the respective range, date of collection, and municipality. Soil samples and a part of the collected tissues were sent to the University of Cordoba for the determination of cadmium and the other samples were transported to the laboratory Microbiological Research, University of Sucre, for processing within 24 hours after you collected.

- Isolation of endophytic bacteria and molecular identification by sequencing. Isolation of endophytic bacteria F2000 plant, was carried out following the methodology proposed by<sup>21</sup>. The amount of bacteria expressed as population density of tissue bacteria (CFU / g tissue) was estimated by direct counting on plates. During counting were observed and selected colonies are distinguished in shape, surface appearance, color and size. The selected isolates were purified and maintained on agar R<sub>2</sub>A<sup>21</sup>.

From endophytic bacteria isolated from the roots proceeded to perform molecular identification and sequencing: For the extraction of DNA samples pure bacteria were taken and were activated in agar Luria Bertani (LB), (Bacto tryptone 10 g, yeast extract 5 g, NaCl 10 g, agar 15 g, milli-Qate 1000 mL, pH 7.0) and incubated at 28 °C for 24 hours, after this time were taken again and pure colonies were transferred to tubes containing 10 ml LB broth and incubated again for 24 hours at 28 °C with constant stirring at 150 rpm in a controller IKA 260.1 Basic. The DNA was extracted using proposed protocol by<sup>22</sup>. Amplification of 16S rDNA fragments was carried out using specific oligonucleotide eubacterias groups<sup>22</sup>.

The amplification products were sent to sequencing the company Macrogen (Seoul, South Korea) on an automated capillary sequencer 3730XL. Entities of the nucleotide sequences obtained were compared with those stored in databanks of the National Center for Biotechnology Information (NCBI). The alignment of the bases was performed by CLUSTAL W, phylogenetic inferences were obtained by maximum similarity method based on Kimura-2-parameter test bootstrapping (1000 replicates) with 7 MEGA program model.

- Determination of cadmium concentration in F2000. Samples of different tissues of F2000 were washed with distilled water to remove mineral particles adsorbed on its surface. Then each tissue separately deposited in paper bags and dried in oven at 60 °C for 24 h. To determine the total cadmium in the samples 0.5 g of dry material were taken and thereto is added an acid mixture HNO<sub>3</sub> / H<sub>2</sub>O<sub>2</sub> (5 + 2 mL). Moreover, the predried soil 0.5 g were taken they were added 10 mL of 65% HNO<sub>3</sub>. Samples were processed in a Milestone ETHOS microwave oven TOUCH series 127,697 and sent to specialized for determining the cadmium concentration by laboratory tissues.
- Tolerance of *Bacillus cereus* strain BN5 in different cadmium concentrations. The *in vitro* assay of *B. cereus* strain BN5 tolerance was carried out in minimal medium tris-MMT proposed by<sup>23</sup> with eight treatments (concentrations) different cadmium in the form of CdCl<sub>2</sub>. The initial concentration of cadmium used was 0.01 mg / ml and from these different treatments were prepared: T1: 100 (0.1); T2: 150 (0.15); T3: 200 (0.2); T4: 250 (0.25); T5: 300 (0.3); T6: 350 (0.35); T 400 (0.4); T7: 450 (0.45) and T8: 500 (0.5 mg / mL). Aliquots of *B. cereus* in log phase was inoculated into the MMT medium. As a control medium without MMT CdCl<sub>2</sub> was used. The experiment was performed in triplicate, which was incubated under stirring at 150 rpm at 32 °C for 120 hours<sup>24</sup>. The growth of each bacterium was determined by turbidimetry at 600 nm every hour for four days.

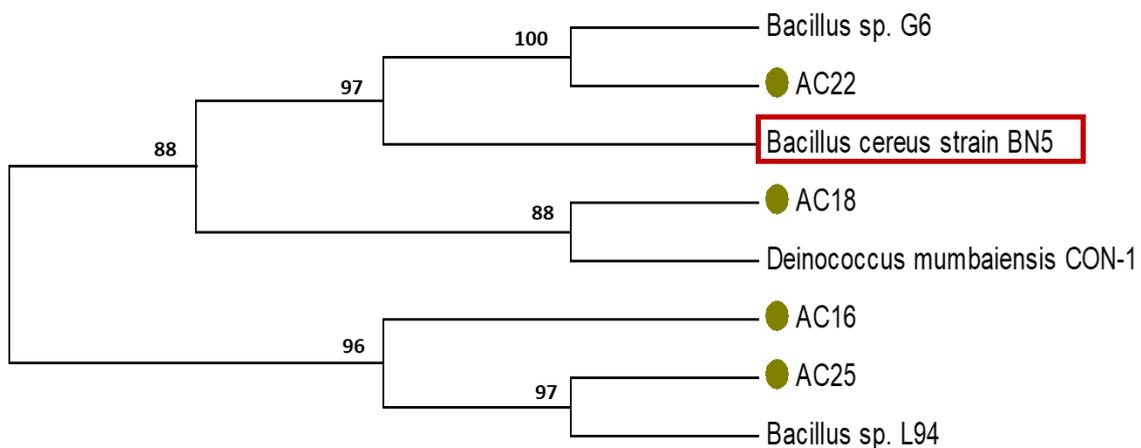
Siderophore of production. At the end of the experiment, each treatment sample was taken to determine the qualitative production of siderophores. Qualitative analysis was carried out in half azurol-S (CAS) by<sup>25</sup> used. For this purpose, 60.5 mg of CAS was dissolved in 50 ml of distilled water, the above was combined with 10 ml of a solution of iron (III) (1 mM FeCl<sub>3</sub>·6H<sub>2</sub>O and 10 mM HCl). Under stirring, this solution was mixed with 72.9 mg of HDTMA dissolved in 40 ml of water. The resultant blue liquid was sterilized at 121 °C for 15 minutes. In another container a mixture of 750 ml of water, 15 g agar, 30.24 g of pipes, and 12 g of a 50% solution (w / w) NaOH to reach pH 6.8 was also sterilized. The medium will be added 4 g of glucose as carbon source. The bacteria was incubated for 7 days at 30 °C.

Analysis of data. The information obtained in the experiment were organized through figures for further interpretation of the results. Analysis of variance and multiple range Tukey test was used to establish significant differences between the variables analyzed. Assays were performed in triplicate and results expressed in half. Data were analyzed in the InfoStat software.

## Results and Discussion

Samples of commercial rice variety Fedearroz 2000 (F2000), were collected in rice farms located in the municipality of Nechí-Antioquia, Colombia. The soils of this municipality showed average values of cadmium 5.5 mg / kg, considering these according to international standards as toxic category. With respect to the values present cadmium tissues averages found by this heavy metal by well tissue: root (2,3); stem (1,92); leaves (1,27) and panicle (0,97 mg / kg tissue). From these samples this is proceeded to the isolation and determination of population density of endophytic bacteria by tissues. From the isolated endophytic bacteria of root samples where higher values of cadmium (2,3 mg / kg) were presented,

The sequencing results showed that the isolated AC22 from the root of the commercial variety F2000 kept 97% similarity with sequences stored in GenBank with the bacteria *Bacillus cereus* strain BN5 (Figure 1).



**Figure 1.** Phylogenetic tree of the *Bacillus cereus* strain BN5 isolates the variety fice F2000 and their relationships with species of bacteria of the phylum Firmicutes.

Upon compliance with the criteria of ANOVA, we proceeded to the analysis of variance, which indicates significant statistical differences ( $p$ -value  $< 0,05$ ) between the amount of endophytic bacteria by tissue and the cadmium concentration. The results of the Tukey test show statistically significant difference ( $p$ -value  $> 0,05$ ) between population density and cadmium, being greater presence in panicle ( $1,12 \times 10^8$  CFU / g tissue) when there values in the same tissue under Cd (0,97 mg / kg) and lower in roots ( $5,8 \times 10^5$  CFU / g tissue) when found higher values of Cd (2,3 mg / kg). The results indicate an inverse relationship to the concentration of Cd in plant tissues with respect to the density of endophytic bacteria, showing that in those tissues as the roots where higher value Cd (2,3 mg / kg) there fewer bacteria, whereas in tissues with lower concentration of Cd (0,97 mg / kg) there is a high presence of these bacteria (Figure 2).

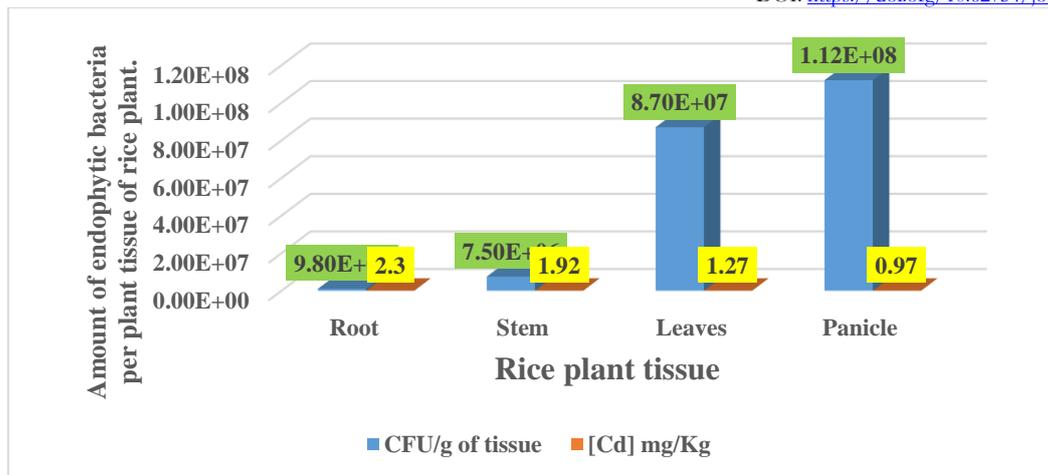


Figure 2. Population density and concentration of cadmium for plant tissue

The average values of cadmium in tissues of the rice variety F2000 found in this following study (2,3 mg / kg) and panicle (0,97 mg / kg), classified according to the reference values internationally to plant tissue as excessive for agricultural crops. Rice plants can absorb  $Cd^{2+}$  ions, from the ground, studies indicate that there are different processes for making from the root from the root to the stem through the xylem, the redirection by xylem vessel and remobilization from leaf to the granos<sup>26</sup>. Therefore, prospects for reducing the content of cadmium in rice and other cereals is the concern that has worldwide to prevent contamination in humans and improve the health of these<sup>27</sup>. Phytoremediation assisted by microorganisms has increased interest in the last 10 years due to costs- effective and friendly to the ambiente<sup>28,29</sup>. Microbial remediation of heavy metals involves bioaccumulation, bioabsorption, bio-mineralization and bio-transformación<sup>30</sup>.

In the Figure 3, it is noted the tolerance *Bacillus cereus* strain BN5 curve in different concentrations  $CdCl_2$ . Until 4 hours of experiment, the bacteria showed tolerance percentage (growth) below 40% in all treatments was evaluated. From the 5 hours, the tolerance behavior of the bacteria increased to 14 hours, finding tolerance percentage 76,92% at 100 mg / mL of  $CdCl_2$  and 69,87% for 150 mg / mL of  $CdCl_2$ . After this time, *B. cereus* Strain BN5 enter stage decrease reaching tolerance percentages 19,41% to 25,85 and 18 hours to 100 and 150 100 mg / mL of  $CdCl_2$ , respectively. The presence of 200 mg / mL of  $CdCl_2$ , bacteria tolerated 59,55% until 12 hours. The lower percentages of tolerance for the bacteria were observed at 500 mg / mL of  $CdCl_2$ , with 5,71% tolerance until 10 hours and decreased to 1,42% at 18 hours, compared these results to that shown for control (Figure 3).

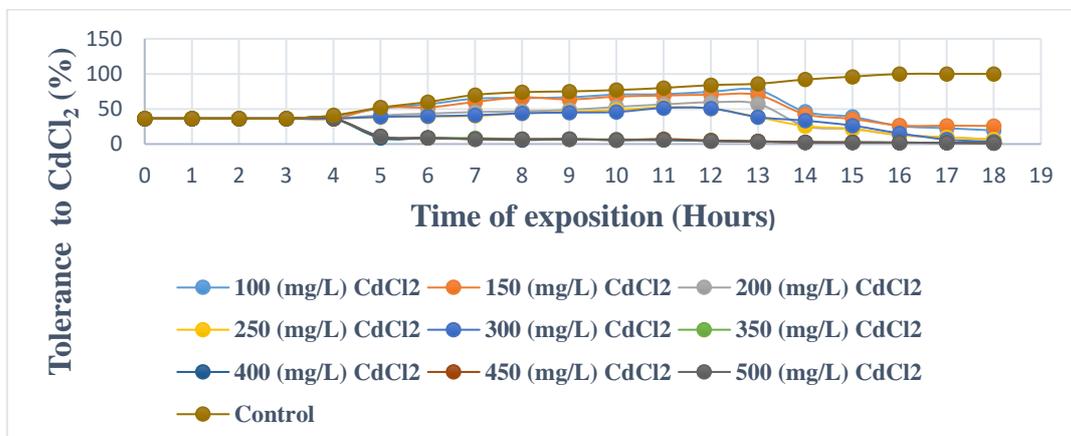
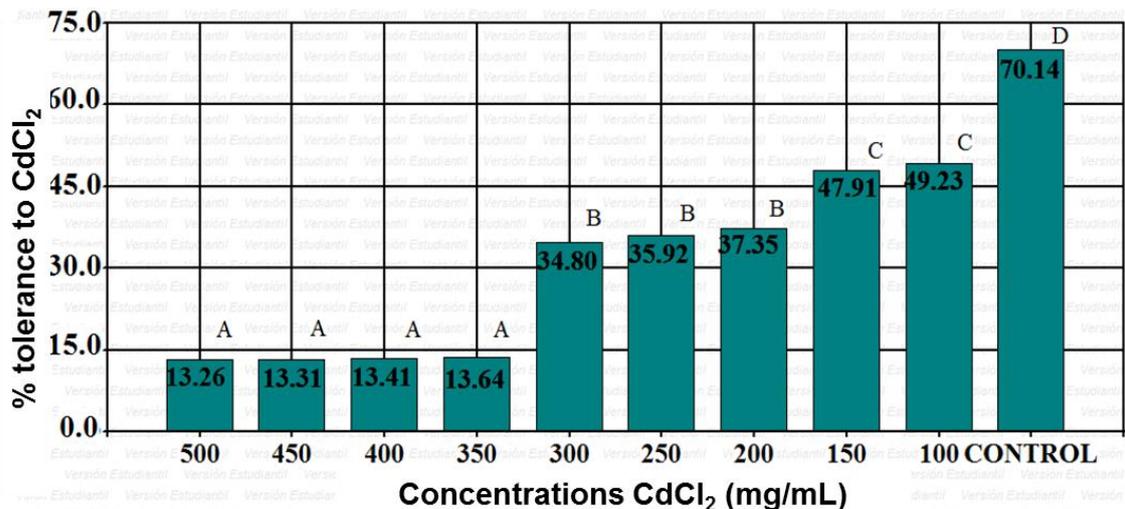


Figure 3. Tolerance Curve of *Bacillus Cereus* Strain BN5 in Different Concentrations of  $CdCl_2$

With the results of tolerance to CdCl<sub>2</sub>, a completely random design was made. Previously established the criterion of normality by means of the Shapiro-Wilks test. For the significant statistical differences, Duncan's multiple range test was performed ( $p$ -value  $\leq 0.05$ ). All tests were performed in triplicate and data was processed in software InfoStat free version. The ANOVA indicates significant statistical differences ( $p$ -value  $> 0.05$ ) between the concentrations of CdCl<sub>2</sub> (Figure 4) and the exposure times (Figure 2). Showing the highest averages of tolerance to 100 and 150 mg /L CdCl<sub>2</sub> concentration, these two concentrations did not show statistical differences. For the case of exposure times, the bacteria showed a stage of adaptation to the pollutant, not showing any growth from 0 to 3. After this stage, bacteria showed growth until day 12 with a tolerance to the pollutant of 46,3% with respect to control, after this time the growth of the bacteria in the medium decreased.

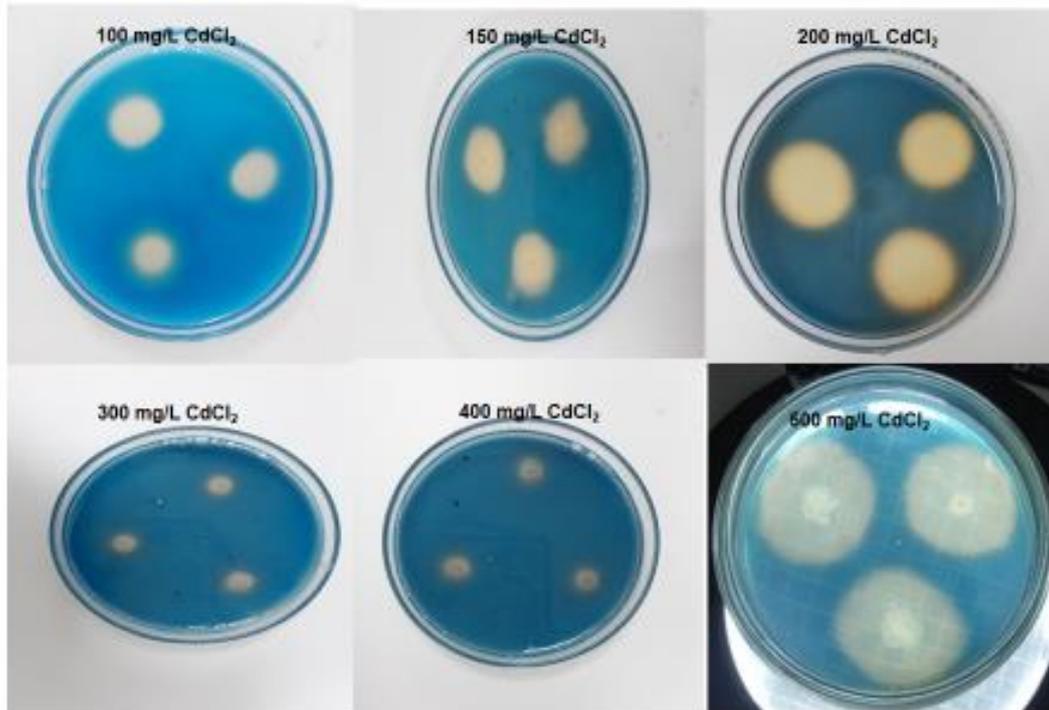


**Figure 4.** Tolerance Percentage of *Bacillus Cereus* Strain BN5 in Different Concentrations of CdCl<sub>2</sub>.

The results in this study show that with increasing concentration of cadmium in the environment decreases the tolerance percentage *Bacillus cereus* strain BN5, presenting a slight increase until 4 hours of the experiment, showing a longer adaptation the bacteria in contact with the metal and develop physiological mechanisms to survive different cadmium concentrations evaluated. According to our opinion adaptation and growth behavior of both the bacteria and accompanying this hypothesis by the findings of other studies. Cd<sup>2+</sup> cause toxicity in the cell of the bacterium in different ways: interacting with nucleic acids, binding to proteins essential for respiration producing reactive oxygen producing oxidative damage, and by displacement of Zn<sup>2+</sup> and Cd<sup>2+</sup> by protein. Cd<sup>2+</sup> ion enters the cell of the bacterium through the transport system used by divalent cations esenciales<sup>31</sup>. To avoid toxicity Cd<sup>2+</sup> quickly and efficiently be removed from the cell of the bacterium or into a biologically inactive form. In general, there are two possible basic mechanisms of resistance Cd<sup>2+</sup>, intracellular or extracellular complexation of ions of toxic metals and reduced accumulation based on the active flow of cations. The second is the main mechanism developed in prokaryotes. However, enzyme. However, the enzymatic transformations of metal ions (oxidation, reduction, methylation and demethylation) are also defense mechanisms in bacterias<sup>32,33</sup>. Bacteria resistance achieved by Cd biosorption, bioaccumulation, precipitation, complexation and metal outflow.

The siderophore production capacity according to the concentration of Cd of *Bacillus cereus* BN5 was evaluated amid azulol-S (CAS) and production in concentrations of 100, 150, 200, 300, 400 and 500 mg / L of CdCl<sub>2</sub> the siderophores was observed (Figure 5). The bacterial siderophores production can possibly contribute to the plants to reduce toxicity caused by the presence of heavy metals and also supply the need of iron as an essential element, promoting the development and growth of plants in contaminator environments<sup>34</sup>. In addition to iron chelation, siderophores serve as mechanisms for bioremediation, which has seen the role of siderophores in reducing the toxicity of cadmium and lead. These studies suggest a

possible por<sup>35</sup> application of microbial siderophores in bioremediation, for reducing metal toxicity and oxidative stress that they induce.



**Figure 4.** Qualitative Siderophores Production by *Bacillus Cereus* Strain BN5, In Different Concentrations of CdCl<sub>2</sub>.

Studies conducted by<sup>36</sup> concluded that the quantity and quality in the production of siderophores by the bacterial species *Pseudomonas fulva* plant growth promoter was due to increased exposure of Cd<sup>2+</sup> (0; 0,5; 10; 2,0 mM). The study won't know the changes in the production of siderophores. The results demonstrate that in the presence of 2.0 mM Cd<sup>2+</sup>, and synthesis of siderophores of the hidroximatos group, catecholates and phenolates occurs with respect to lower levels of Cd<sup>2+</sup> (0,5 and 1,0). In our view, possibly mechanism siderophore production around the environment where bacteria grow sequester heavy metal allows them and reduce their accumulation within the cell. Consequently, the bacteria reduce the transport of heavy metals in high concentrations to avoid poisoning cytoplasm of the cell.

*B. cereus* It is an endophytic bacteria found rice plants with the capacity to promote growth vegetal<sup>37</sup>. Also by<sup>19</sup> study conducted regarding isolation endophytic bacteria associated with the genera *Paspalum* and *Cyperus* contaminated with mercury in southern Bolivar, Colombia soil, reported the presence of endophytic bacteria *Bacillus cereus* GU056811 with in vitro ability to tolerate up to 400 ppm (0,4 mg / L) mercury as HgCl<sub>2</sub>. Also study conducted by<sup>20</sup> concluded that endophytic bacteria *Bacillus cereus* 1DH1LIM has the ability to tolerate up to 400 ppm of Pb as Pb(NO<sub>3</sub>)<sub>2</sub> and of producing siderophore.

## Conclusion

*Bacillus cereus* BN5 strain, Gram-positive bacteria tolerant cadmium endophyte was isolated from root of the rice variety that grows in soils with high levels of cadmium. *In vitro* assays indicate that can tolerate up to 500 mg / L of CdCl<sub>2</sub>, and siderophores produced at different concentrations of the metal. This bacterium lives in the root tissue with cadmium concentration of 2.3 mg / kg. The results obtained in this study emphasize explore the possibility of achieving remediation cadmium tolerant and safe production of rice in soil contaminated cadmium with endophytic bacteria.

## Acknowledgments

Microbiological research laboratory at the University of Sucre. **Competing interests:** The information is original, reviewed and prepared by people involved in it. We declare that there is no conflict of interest that jeopardize the validity of the results. **Funding:** This work was funded with resources allocated to research group Bioprospecting Agricultural University of Sucre.

**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 conflict of interest.

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