# Efficiency of Rhizospheric Bacteria in the Production of Ammonium in the Presence of Arsenic

Alexander Pérez Cordero[1], Donicer E. Montes Vergara[1], Yelitza Aguas Mendoza[2]

[1]Universidad de Sucre, Facultad de Ciencias Agropecuarias, Colombia
[2]Universidad de Sucre, Facultad de Ingeniería, Colombia
Email: alexander.perez@unisucre.edu.co

## Abstract

The aim of the present study was to isolate rhizospheric bacteria and to evaluate in vitro their arsenic tolerance and ammonium production. Bacteria were isolated from arsenic-contaminated environments, tolerance to the highest arsenic concentration was evaluated in vitro and ammonium production capacity was determined. A total of 6 rhizospheric bacterial strains were able to tolerate 500 mg/L AsCl2 and simultaneously were found to produce ammonium ion concentrations of 0.87 to 1.3 mg/L. The ability of rhizospheric bacteria to tolerate high concentrations of arsenic makes them direct candidates for contributing to remediation of arsenic-polluting environments and the ability to produce ammonium is an indirect strategy to promote plant growth and promote plant tolerance to toxic metals.

**Keywords:** Metalloids, rhizosphere, bacteria, nitrogen fixation, remediation.

## 1. Introduction

Arsenic is the 20th most abundant metalloid in the earth's crust; it occurs naturally at a concentration of 1.5 to 3 mg/m$^2$. More than 200 arsenic minerals have been identified (Mohsin et al. 2023). The main source of arsenic found at the earth's surface is activity from igneous rocks (Cervantes et al. 1994). Arsenic is classified as a class 1 carcinogen by the International Agency for Research on Cancer. The World Health Organization states that the acceptable level of arsenic in drinking water is up to 10 μg/l (Mohsin et al. 2023). Due to its abundance and toxicity, it is essential to control and remove arsenic from water, soil and food.

Conventional methods to remediate arsenic-contaminated media have high cost, low efficiency and secondary contamination limitations. Phytoremediation and microbiology are emerging alternatives. Several strains of bacteria that have the ability to resist arsenic have been found to contribute to the remediation of arsenic contamination (Laha et al. 2022). Arsenic is a non-degradable element, but it can be removed and detoxified by altering its solubility and state

through the action of microorganisms (Rahman and Singh 2020). The presence of arsenic in the environment has led to the evolution of arsenic defense mechanisms in microbes, with the ars resistance operon being the most common mechanism (Cervantes et al. 1994).

Rhizobacteria inhabit the rhizosphere, an area of soil attached to the root and extending a few millimeters 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 (McNear, 2013) and is home to a large number of microorganisms that generally stimulate plant growth, reduce disease incidence and contribute to abiotic stress due to the presence of contaminants (Molina-Romero et al., 2015).

The aim of the present study was to isolate rhizospheric bacteria from environments contaminated with arsenic metalloid and to evaluate in vitro their ability to tolerate the maximum concentration of arsenic and the capacity to fix nitrogen expressed in the efficiency of ammonium production.

## 2. Materials and Methods

Isolation of rhizospheric bacteria from arsenic-contaminated soil. The counting of the rhizospheric bacteria population was carried out as follows: A 10 g sample of soil was added to 90 mL of a phosphate solution (g/L) with the composition (0.2 $KH_2PO_4$, and 0.8 $K_2HPO_4$, at pH 7.0). The solution was stirred at 100 (rpm)/30 min as suggested by (Mauricio et al., 2010). Serial dilutions of 10-1 -10-10 were then prepared and the bacteria were isolated on the surface on plates with modified M9 mineral medium (g/L) (6.0 $Na_2HPO_4$, 3.0 $KH_2PO_4$, 1.0 NH4Cl, 0.5 NaCl, 0.5 yeast extract and 10 glucose; in addition, (mL/L) were included: 2.0 $MgSO_4 \cdot 7H_2O$ 1 M, 0.1 $CaCl_4$ 0.1 M, and 0. 1 thiamine at a concentration of 1 mg/mL (sterilized by 0.2 µm filtration), adjusted to pH 7 and following the protocol proposed by (Sambrook and Green 2012).

Evaluation of arsenic tolerant capacity of rhizospheric bacteria. Bacterial isolates were seeded on nutrient agar supplemented with $AsCl_2$ 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).

In vitro evaluation of arsenic 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 $AsCl_2$. The initial concentration of As 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 were used in log phase and inoculated onto MMT medium supplemented separately with each of the cadmium concentrations. MMT medium without $CdCl_2$ was used as a control. The experiment was performed in triplicate, which was incubated in shaking at 150 rpm at various temperatures (32°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.

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‘ CAGCAGTAGGGAATCTTC 3’) 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.

## 3. Results and Discussion

Figure 1 shows the in vitro growth behaviours of rhizospheric bacterial strains growing at 500 mg/L AsCl2. As shown in the figure, strains C85RosLIm and C84RosLIM showed similar growth behaviours to the control strain (no arsenic present). Strain C63RosLIM was the only one that showed growth below the behaviours presented by the other five strains evaluated at the same concentration of arsenic 500 mg/L.

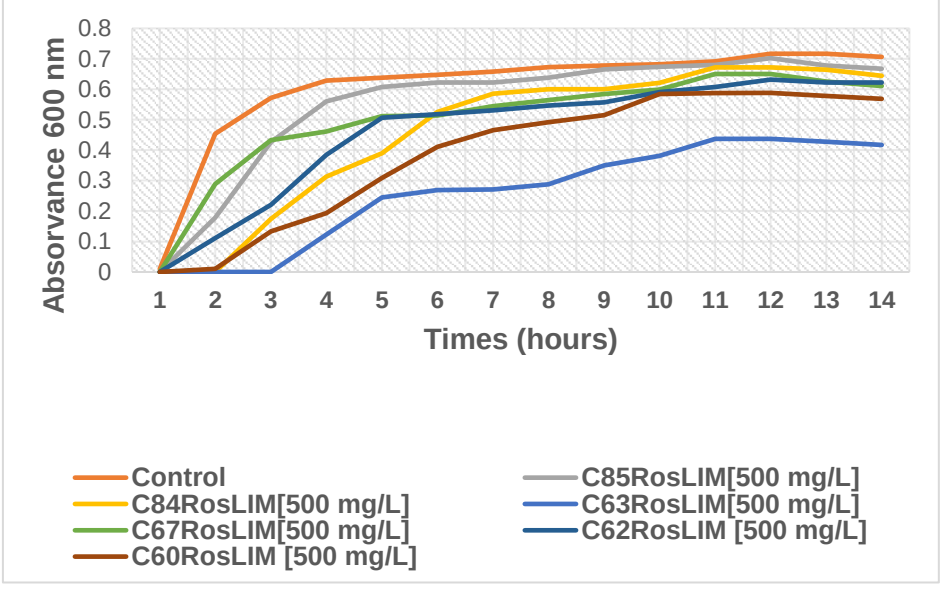


Figure 1. In vitro growth behaviours of rhizospheric bacterial strains at 500 mg/L AsCl2. C: Strain; R: rhizosphere; Os: Oryza sativa; LIM: Genomic bank - Microbiological Research Laboratory.

Different forms of arsenic enter bacterial cells in different ways via specific transporters in bacterial cells. However, due to its structural analogy with other essential molecules, such as phosphate, toxic arsenic enters the cell via existing transporters (Yin et al. 2022). Several arsenic-resistant bacteria use cell extrusion or sequestration into intracellular compartments to resist and detoxify arsenic (Mohsin et al. 2023).

Bacterial metabolism of arsenic has been well documented in various research, some study results indicate that biosorption and bioaccumulation are two of the most important bioremediation techniques in recent years and play a vital role in bioremediation strategies (Kabiraj et al. 2022; Preetha et al. 2023; Yin et al. 2022). Microbes have evolved several metabolic mechanisms to combat toxic arsenic, Microbes have evolved several metabolic mechanisms to combat toxic arsenic. These mechanisms include reduction, oxidation, methylation and demethylation (Irshad et al. 2021). In addition, arsenic bioremediation has been used for biosorption, bioaccumulation, bioleaching and biomineralization (Kabiraj et al. 2022).

Biosorption is a process that does not require energy or biological metabolism. It mainly concerns physicochemical interactions between metalloids and cellular compounds. In general, arsenic can bind to cell surface functional groups, such as $^{-}COOH$, $^{-}NH_2$, $^{-}SH$, $^{-}OH$ and $^{-}PO_4^{3-}$, through ion exchange, chelation and physical adsorption (Sher and Rehman 2019). Bioaccumulation reduces the toxicity of free and bioavailable arsenic by associating with proteins or peptides via glycerol and phosphate transporters (Yin et al. 2022).

For years, bacteria have been isolated from arsenic-contaminated environments that are able to assimilate arsenic through their metabolic pathways, achieving a detoxifying effect (Kruger et al., 2013), and recent studies have focused on increasing the ability of microorganisms to resist arsenic through the use of genetic engineering (Lorenzo et al., 2016).

Figure 2 shows the results of ammonium production (mg/mL) of rhizospheric bacteria that showed qualitative nitrogen fixation activity. The results show that the most efficient strain in ammonium production in vitro corresponded to C85ROsLIM and C84ROsLIM. These strains were able to grow in vitro up to 500 mg/L $AsCl_2$. The efficiency of nitrogen fixation was determined by the production of ammonium ion, using the Berthelot method, which consists of the change in coloring to an intense indolfenol blue due to the reaction produced between the ammonium ion and phenolic compounds in the presence of an oxidizing agent. Additionally, a nitrogen-free Burk's medium was used for the test, which allows observing the capacity of rhizospheric bacteria to convert atmospheric nitrogen and expel it to the medium. All strains tested showed ammonium ion production at different concentrations, however 6 of the strains showed ammonium ion production at concentrations higher than 0.8 mg/L.

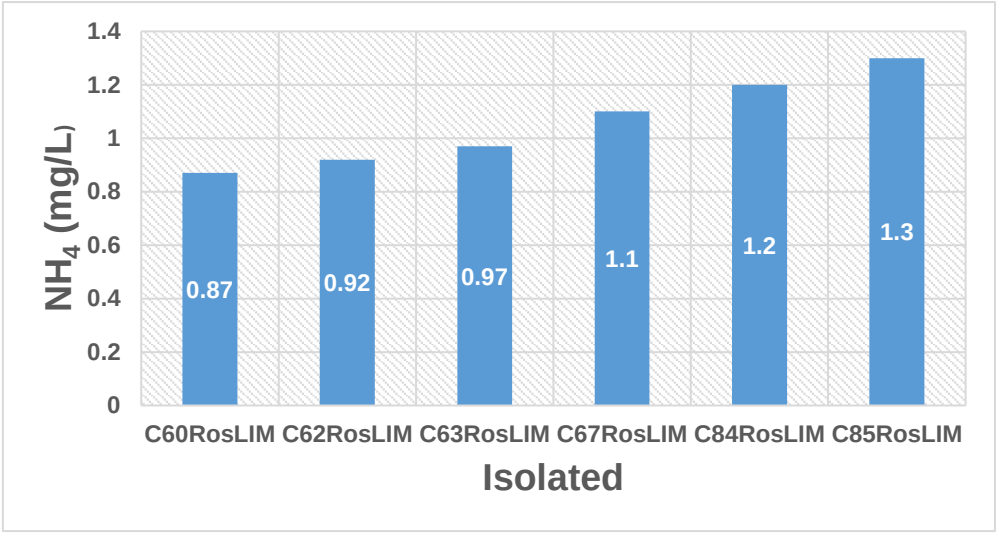


Figure 2. In vitro ammonium production (NH4 mg/L) of rhizospheric bacterial strains at 500 mg/L AsCl2. C: Strain; R: rhizosphere; Os: Oryza sativa; LIM: Genomic bank - Microbiological Research Laboratory.

Nitrogen (N) is one of the vital nutrients for plant growth and productivity. This element is present in amino acids in proteins, amides, chlorophyll, hormones, nucleotides, vitamins, alkaloids and nucleic acids (Ahemad and Kibret, 2014). A common feature of the microorganisms involved in FBN is the presence of nitrogenase enzymes, which reduce atmospheric nitrogen to the assimilable $NH4^+$ ion.

The results of 16S rRNA sequence analysis of rhizospheric bacteria show high homology with sequences of bacteria stored in the Gen Bank of the bacteria domain (figure 3). C85RLIM, C85RLIM and C60ROsLIM are similar to the type phylum Pseudomonas aeruginosa; C62ROsLIM to Bacillus cereus; C60ROsLIM to Pseudomonas fluorescens; C67ROsLIM to Pseudomonas putida. All strains isolated and identified were evaluated in vitro and showed the ability to tolerate up to 500 mg/L AsCl2 and to produce ammonium ion. Several studies indicate that these strains have the ability to grow in the presence of heavy metals and promote plant growth.

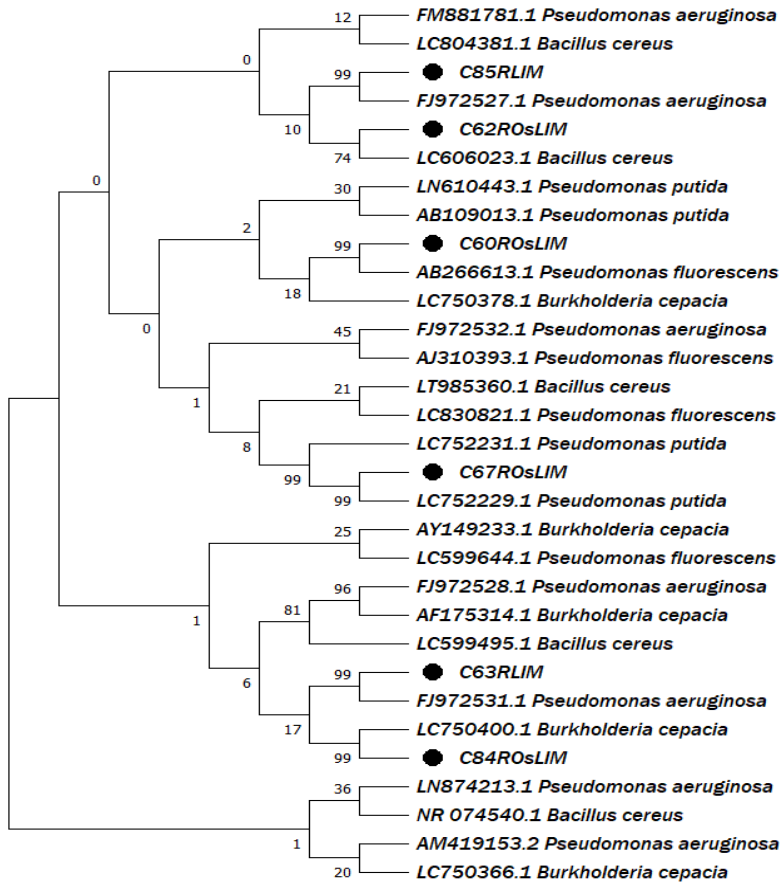


Figure 3. Phylogenetic tree based on a partial 16S rRNA gene sequence of bacteria's rhizospheric. C: Strain; R: rhizosphere; Os: Oryza sativa; LIM: Genomic bank - Microbiological Research Laboratory.

## 4. Conclusion

Bacteria from natural habitats can be a potent solution to the problem of arsenic contamination, offering a viable alternative to conventional physical, chemical and instrumental methods. In the present study, 6 strains of rhizospheric bacteria were isolated with the ability to tolerate 500 mg/L $AsCl_2$ and ammonium ion production under growth conditions in the presence of the metal.

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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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