Isolation Of *Bacillus Paranthracis* From The Gut Of Earthworm Fed With Arsenic Accumulated *Eichhornia Crassipes*

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ABSTRACT
The present study was intended to isolate and molecularly identify Arsenic resistant bacteria from the gut of earthworms (*Eisenia fetida*) fed with arsenic accumulated *Eichhornia crassipes*. Earthworm gut content was serially diluted and plated to isolate bacteria, followed by purification using the fourquadrant streak method. Among the isolates, one bacterial strain was high resistance to arsenic, tolerating concentrations up to 1000 ppm with no evident of inhibition zone in the disc diffusion assay. The genomic DNA of the isolate was extracted and subjected to 16S rRNA gene sequencing. The amplified sequence showed 97.85% similarity with *bacillus paranthracis* and was submitted to GenBank (Accession No. OQ568227). The high arsenic tolerance observed suggests the presence of adaptive mechanisms such as metal binding proteins or efflux systems. These findings effectively highlight the potential of *B.paranthracis* as a bioremediation agent in arsenic contaminated environments

Keywords*: Eichhornia crassipes*, Heavy metal, Earthworm gut, *Bacillus paranthracis***,** Bioremediation

INTRODUCTION

Heavy metals are group of metals and metalloids with high atomic density and toxicity, have become a global environmental concern due to their persistence, bioaccumulation, and potential to disrupt ecosystems and biological systems (Rostanski *et al.,* 2015). Among these heavymetals, arsenic (As), cadmium (Cd), and lead (Pb) are of vital concern due to their non-essential and highly toxic nature, as they cause severe threat to soil health, water quality, plant systems, and eventually, human and animal health. Arsenic, in particular, is highly dangerous for its carcinogenic and genotoxic effects, and its presence in the environment is mostly due to industrial effluents, pesticide run-off, and mining activities. These pollutants accumulate in water bodies and soils, eventually making their way into the food chain through plant uptake, especially by hyperaccumulator aquatic plants such as *Eichhornia crassipes* (water hyacinth) (Pham *et al.,* 2022).

Eichhornia crassipes, is an invasive aquatic macrophyte that has gained considerable attention in environmental biotechnology due to its remarkable capacity to absorb, accumulate, and tolerate a wide spectrum of environmental pollutants, especially heavy metals such as arsenic (As), lead (Pb), cadmium (Cd), mercury (Hg), and chromium (Cr) from contaminated water bodies (Rai, 2008)

Under selective pressure from toxic metals, many bacteria have evolved specialized mechanisms such as efflux pumps, metal sequestration proteins, enzymatic transformation, and bioaccumulation to withstand and detoxify heavy metal stress (Silver & Phung, 2005). The isolation and characterization of such metal-tolerant bacteria, from unique ecological niches like the earthworm gut, can offer hopeful perceptions of sustainable bioremediation approaches.

In this study, *Bacillus paranthracis* was isolated from the gut of earthworms fed with arsenic-contaminated *Eichhornia crassipes*. This research targets to explore the adaptive responses and potential bioremediation roles of gut associated bacteria under heavy metal stress.

MATERIALS AND METHODS

Gut bacteria were isolated from earthworms fed with heavy metal accumulated *Eichhornia crassipes* using standard serial dilution and plating techniques

Serial dilution of sample

One gram of earthworm gut content was aseptically sliced, mashed, and suspended in sterile distilled water. From this homogenate, 1 mL was transferred into a test tube containing 9 mL of sterile distilled water to obtain a 10^{-1} dilution. This serial dilution process was repeated sequentially up to a 10^{-8} dilution. From the 10^{-7} dilution, 0.1 mL was aseptically plated onto nutrient agar plates. The plates were incubated at 37 °C for 18–24 hours. After incubation, bacterial colonies developed on the plates were observed and counted. The experiments were performed in triplicates to get accurate results (Fig 1).

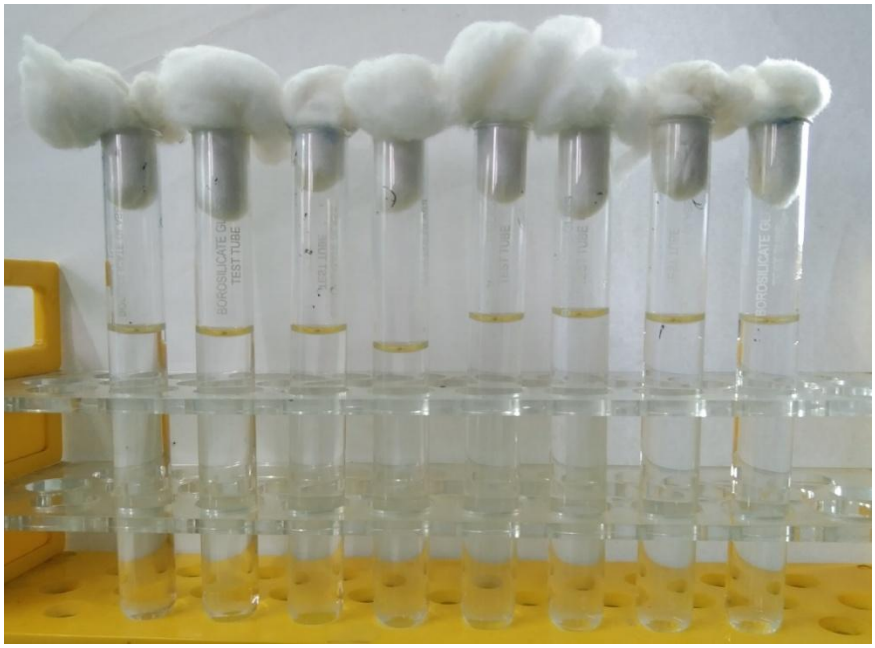


Fig.1 Serial dilution of earthworm gut sample

Preparation of Agar plates

Petri plate containing solid growth medium (agar) is divided into four quadrants. A loopful of the mixed bacterial culture is streaked across the first quadrant to create a dense area of growth. The inoculating loop is sterilized (flamed) and allowed to cool before transferring cells from the edge of the first quadrant into the second quadrant. This process is repeated for the third and fourth quadrants, sterilizing the loop between each streaking step. The bacterial concentration decreases with each successive quadrant. By the fourth quadrant, the inoculum is sufficiently diluted to allow the development of distinct isolated colonies after incubation (Plate 1).

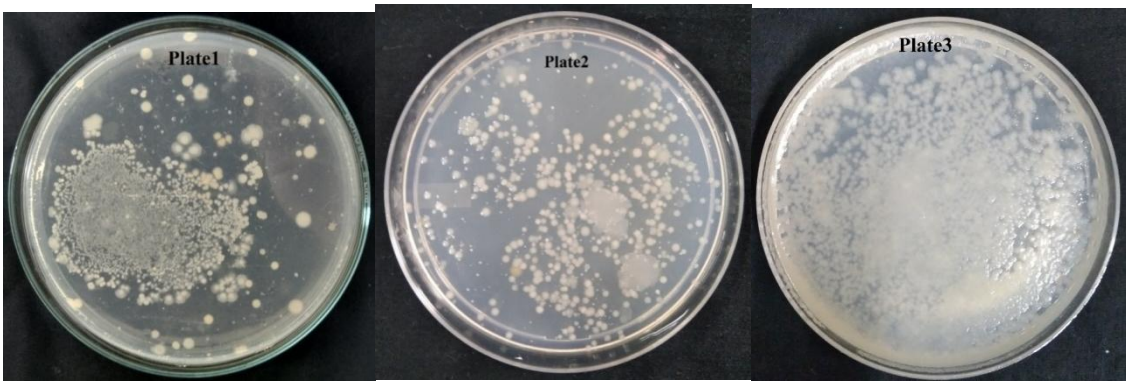
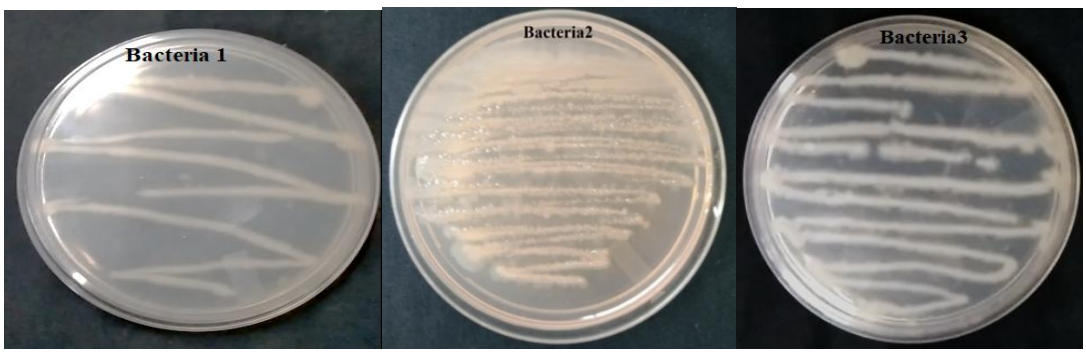


Plate 1: Bacterial colonies of earthworm gut

Isolation of pure bacterial colonies by simple streak method

A simple streaking technique was used to isolate bacterial colonies from the mixed culture. A loopful of the serially diluted sample (from 10^{-7} dilution) was streaked on nutrient agar plates using the fourquadrant streak method to separate individual colonies. The plates were incubated at 37 °C for 18–24 hours to allow bacterial growth (Plate 2).



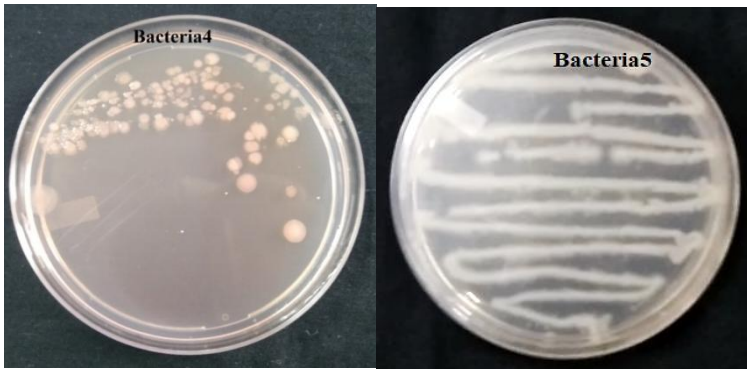


Plate 2: Pure culture of bacterial colonies

After incubation, the plates were observed for morphologically distinct colonies. Bacterial colonies that appeared uniform in shape, colour, elevation and margin were selected for further purification.
From the initial streaked plates, five distinct colonies were carefully picked using a sterile inoculating loop. Each selected colony was then streaked separately onto fresh nutrient agar plates using the same four quadrant method to ensure the development of pure cultures. These plates were again incubated under the same conditions. After incubation, each plate was examined to confirm the growth of isolated pure colonies. These pure bacterial cultures were maintained for biochemical characterization and metal resistance studies.

Heavy metal degradation assay by disc diffusion method (Kirby& Bauer, 1966) Procedure: The test bacteria was inoculated in peptone water and incubated for 3– 4 hours at 35ºC. Nutrient agar plates was prepared and poured in sterile petri plates. 0.1 ml of bacterial culture was inoculated on the surface of Nutrient agar plates and spread by using L-rod. The inoculated plates were allowed to dry for five minutes. The disc loaded with different concentrations 250, 500, 750, 1000 ppm of arsenic was placed on the surface of inoculated petri plates using sterile technique. The triplet plates were incubated at 37ºC for 18-24 hours. The plates were examined for inhibitory zone and the zone of inhibition was measured in mm. Ampicillin is used in positive control.

DNA extraction, PCR 16S rRNA amplification and sequencing
Bacterial genomic DNA was extracted using the standard phenol–chloroform extraction method (Sambrook and Russell, 2006). The 16S rRNA gene was amplified using the universal primer pair 27F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′ TACGGCTACCTTGTTACGACTT 3′). The PCR reaction mixture contained 10 pmol of each primer, 50–100 ng of DNA template, and 12.5 µl of 2× Dream Taq Green PCR Master Mix (Thermo Fisher). Amplification was carried out in a thermal cycler (Applied Biosystems, USA) using the following cycling conditions: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 60 s, annealing at 58 °C for 60 s, and extension at 72 °C for 75 s; followed by a final extension at 72 °C for 10 min (Karlsen *et al.,* 2014). PCR products were subjected to agarose gel electrophoresis, stained with ethidium bromide (EtBr), and visualised using a gel documentation system (Azure Biosystems, USA). Amplified products were sequenced in both forward and reverse directions using the same primer set on an Applied Biosystems AB 3730 capillary sequencer.
The raw sequences obtained were edited using BioEdit sequence alignment editor version 7.2.5.2 (Hall, 1999). Homology comparison was performed using multiple databases, including the European Nucleotide Archive (ENA; https://www.ebi.ac.uk/ena), the Ribosomal Database Project (RDP; https://rdp.cme.msu.edu), EzBioCloud16S (www.ezbiocloud.net), and the NCBI GenBank database using the BLAST tool. For species-level identification, only sequences showing ≥99% similarity with a classified reference species were considered. A phylogenetic tree was constructed using the neighbour-joining method in MEGA X software (Tamura, 2011). Distance matrices were calculated using Kimura's two-parameter model, and bootstrap analysis with 1000 replicates was performed to assess the robustness of the phylogenetic groupings.

RESULTS
The results of microbial activity assessment using the disc diffusion method to analyse heavy metal tolerance and the molecular identification of the isolated bacterial strain through 16S rRNA gene sequencing are presented below.
Heavy metal degradation assay by disc diffusion method (Kirby& Bauer, 1966)
Disc diffusion method of analysis showed that the positive control with ampicillin produced a large inhibition zone and the blank and empty disc showed no inhibition in all the three bacterial plates (Plate 3).
The bacterial isolate exhibited no inhibition zones at arsenic concentrations of 250 ppm, 500 ppm, and 750 ppm. At 1000 ppm concentration of arsenic also inhibition zone was not observed. This result indicates a high level of resistance to arsenic

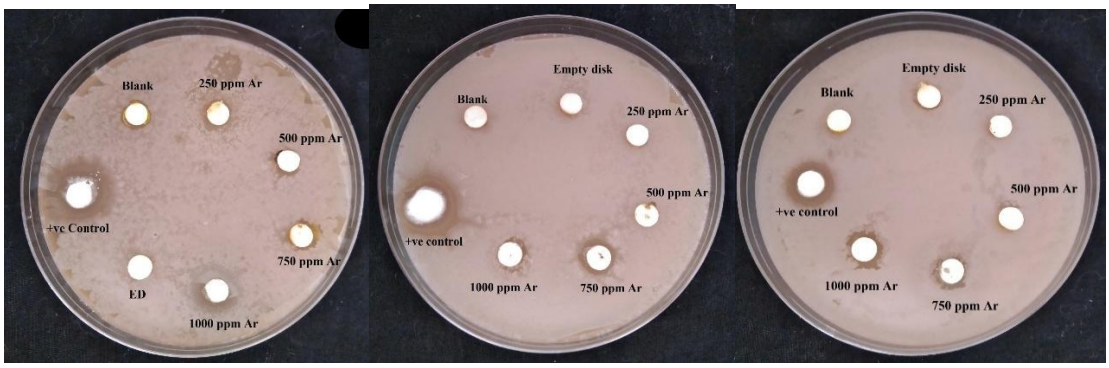


Plate 3: Heavy metal degradation assay

Molecular Identification of *Bacillus paranthracis* via 16S rRNA Gene Sequencing

Gut associated bacterial isolates were obtained from earthworms fed with arsenic-accumulated *Eichhornia crassipes* biomass. Genomic DNA was extracted from the bacterial culture, and the 16S ribosomal RNA (rRNA) gene was amplified using universal primers. The amplified 16S rDNA sequence was then subjected to quality checks, including chimera screening using BioEdit software version 7.2.5.

The BLAST analysis revealed that the isolate shared 97.85% sequence identity with reference sequences of *Bacillus paranthracis*, strongly supporting its taxonomic classification.

Based on this high sequence similarity, the isolate was confidently identified as *Bacillus paranthracis*. The validated partial 16S rRNA gene sequence, comprising 1516 base pairs, was submitted to the NCBI GenBank database and has been assigned the accession number OQ568227.

This molecular identification confirms the presence of *Bacillus paranthracis* in the gut microbiome of earthworms exposed to heavy metal-contaminated plant material, suggesting its potential role in microbial tolerance and adaptation to arsenic stress (Fig. 2).

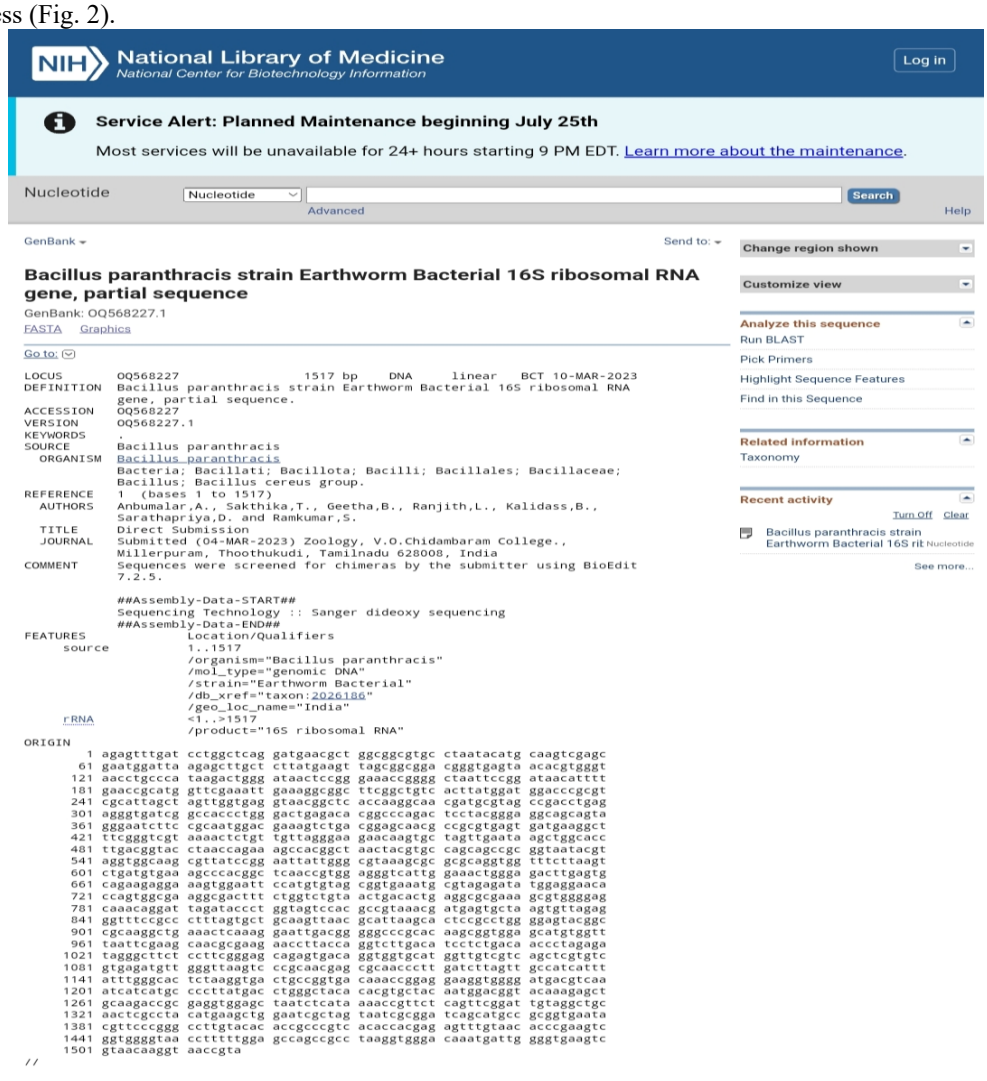


Fig 2. *Bacillus paranthracis* strain AR 16SrRNA gene partial sequence

DISCUSSION

This study reports the isolation and molecular identification of an arsenic resistant bacterium, *Bacillus paranthracis*, from the gut of earthworms fed with arsenic-contaminated *Eichhornia crassipes*.

Microorganisms play a fundamental role in the biogeochemical cycling of arsenic, directly influencing its mobility, speciation, and toxicity in the environment through complex redox and methylation-based pathways (Hussain *et al.,* 2021). Arsenic exists in multiple oxidation states, and microbial activity governs its transformation among these states. For instance, arsenic-oxidizing and -reducing bacteria can convert highly toxic inorganic arsenic forms (As(III) and As(V)) into less toxic or more mobile organic derivatives such as monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), thereby impacting its fate in soil and aquatic ecosystems (Shi *et al.,* 2021; Raturi *et al.,* 2023).

Feeding on arsenic accumulated *Eichhornia crassipes* to earthworm creates a selective environment within the gut, potentially favoring the survival and enrichment of metal tolerant microbial taxa. The presence of *B. paranthracis* in such a niche suggests its adaptive potential in arsenic-rich environments, where it may play a role in biotransformation or detoxification of heavy metals.

Former studies have identified numerous *Bacillus* species with robust heavy metal resistance and detoxification abilities, including *B. thuringiensis*, *B. aryabhattai*, *B. toyonensis*, *B. tequilensis*, and *B. amyloliquefaciens*, among others (Tarekegn & Abioye, 2020). These species have demonstrated efficiency in the bioremediation of cadmium, lead, and chromium, supporting the utility of *Bacillus* spp. as bioresources for microbial remediation strategies. Similarly, *B. subtilis*, *B. megaterium*, and fungi such as *Aspergillus niger* and *Penicillium* spp. have also been shown to remove multiple heavy metals from contaminated environments (Njoku *et al.,* 2020).

The ability of microorganisms to perform arsenic oxidation, reduction, methylation, and even adsorption and precipitation processes (Mondal *et al.,* 2021; Ulhassan *et al.,* 2022) positions them as central agents in sustainable and ecofriendly bioremediation efforts.

Conclusion

This study highlights the potential of gut microbial communities in earthworms, particularly *Bacillus paranthracis*, as valuable resources in the bioremediation of arsenic. The sequence submission to GenBank not only validates the molecular identity of the isolate but also enables future studies involving experimental validation of arsenic detoxification mechanisms.

REFERENCES

- Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol*. 1966;36:493–496.
- Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95–98.
- Hussain MM, Bibi I, Niazi NK, Shahid M, Iqbal J, Shakoor MB, Ahmad A, Shah NS, Bhattacharya P, Mao K, et al. Arsenic biogeochemical cycling in paddy soil-rice system: Interaction with various factors, amendments and mineral nutrients. *Sci Total Environ*. 2021;773:145040.
- Mondal S, Pramanik K, Ghosh SK, Pal P, Mondal T, Soren T, Maiti TK. Unraveling the role of plant growth-promoting rhizobacteria in the alleviation of arsenic phytotoxicity: A review. *Microbiol Res*. 2021;250:126809.
- Njoku KL, Asunmo MO, Ude EO, Adesuyi AA, Oyelami AO. The molecular study of microbial and functional diversity of resistant microbes in heavy metal contaminated soil. *Environ Technol Innov*. 2020;17:100606.
- Pham VHT, Kim J, Chang S, Chung W. Bacterial biosorbents, an efficient heavy metals green clean-up strategy: Prospects, challenges, and opportunities. *Microorganisms*. 2022;10(3):610.
- Rai PK. Heavy metal pollution in aquatic ecosystems and its phytoremediation using wetland plants: An ecosustainable approach. *Int J Phytoremediation*. 2008;10(2):133–160.
- Raturi G, Chaudhary A, Rana V, Mandlik R, Sharma Y, Barvkar V, Salvi P, Tripathi DK, Kaur J, Deshmukh R, et al. Microbial remediation and plant-microbe interaction under arsenic pollution. *Sci Total Environ*. 2023;864:160972.
- Rostanski A, Cabala J, Slota M. Environmental exposure to heavy metals. In: Wierzbicka M, editor. *Ecotoxicology. Plants, Soils, Metals*. Warsaw, Poland: Warsaw University Press; 2015. p. 522–541. ISBN: 978-83-235-1854-9.
- Shi W, Song W, Zheng J, Luo Y, Qile G, Lu S, Lu X, Zhou B, Lu C, He J. Factors and pathways regulating the release and transformation of arsenic mediated by reduction processes of dissimilated iron and sulfate. *Sci Total Environ*. 2021;768:144697.
- Silver S, Phung LT. Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. *Appl Environ Microbiol*. 2005;71(2):599–608.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731–2739. https://doi.org/10.1093/molbev/msr121
- Tarekegn M, Zewdu SF, Ishetu AI. Microbes used as a tool for bioremediation of heavy metal from the environment. *Cogent Food Agric*. 2020;6:1783174.

- Ulhassan Z, Bhat JA, Zhou W, Senan AM, Alam P, Ahmad P. Attenuation mechanisms of arsenic induced toxicity and its accumulation in plants by engineered nanoparticles: A review. *Environ Pollut*. 2022;302:119038.