Isolation And Identification Of *Bacillus Anthracis* From The Gut Of Earthworm Fed With Chromium Accumulated *Eichhornia Crassipes*

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ABSTRACT
This study aimed to isolate and identify gut bacteria from *Eisenia fetida* fed with chromium contaminated *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 chromium, tolerating concentrations up to 1000 ppm with no evident of inhibition zone in the disc diffusion assay. Genomic DNA was extracted from the bacterial isolate obtained from the earthworm gut and subjected to 16S rRNA gene sequencing. The amplified sequence was analyzed and showed 99.86% similarity with *Bacillus anthracis*. The nucleotide sequence was submitted to the NCBI GenBank database under the Accession No. OQ568517. These findings suggest that *B. anthracis* may be part of the microbial community associated with chromium tolerance or transformation in the earthworm gut environment.

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

INTRODUCTION

Heavy metal pollution is a serious environmental concern due to its hazardous impacts, even at very low concentrations. Heavy metals (HMs) are non-biodegradable, tend to bioaccumulate in tissues, and undergo biomagnification across trophic levels (Gray, 2002). Natural processes such as weathering of geological bedrock and volcanic eruptions can release heavy metals into the surrounding environment (Wuana & Okieimen, 2011).
In aquatic ecosystems, macrophytes play a dual role: they serve as a food source for aquatic invertebrates and act as effective accumulators of heavy metals (Vardanyan & Ingole, 2006). Due to their extensive fibrous root systems, macrophytes possess a large surface area that enhances their bioaccumulation capacity (Bragato et al., 2009). Their ability to tolerate high concentrations of heavy metals in water and sediments makes them ideal candidates for biomonitoring and permanent biofilters (Eid et al., 2019).
Microorganisms contribute to heavy metal immobilization and function as natural sinks by employing several mechanisms such as biosorption, bioaccumulation, bioconversion, and intra- or extracellular precipitation (e.g., as oxalates of Zn, Cu, Co, Cd, and Ni) (Mosa et al., 2016). Microbes that inhabit highly contaminated environments develop resistance to heavy metals. The extracellular polymeric substances (EPS) on microbial cell walls can bind heavy metals through various processes, including proton exchange and micro-precipitation (Comte et al., 2007).
This research targets to

- To isolate bacterial DNA from the gut of earthworms (*Eisenia fetida*) fed with *E.crassipes*
- To identify the bacterial species through molecular technique
- To submit the isolated sequence to NCBI to know the bacterial species

MATERIALS AND METHODS

Gut bacteria were isolated from earthworms fed with chromium 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).

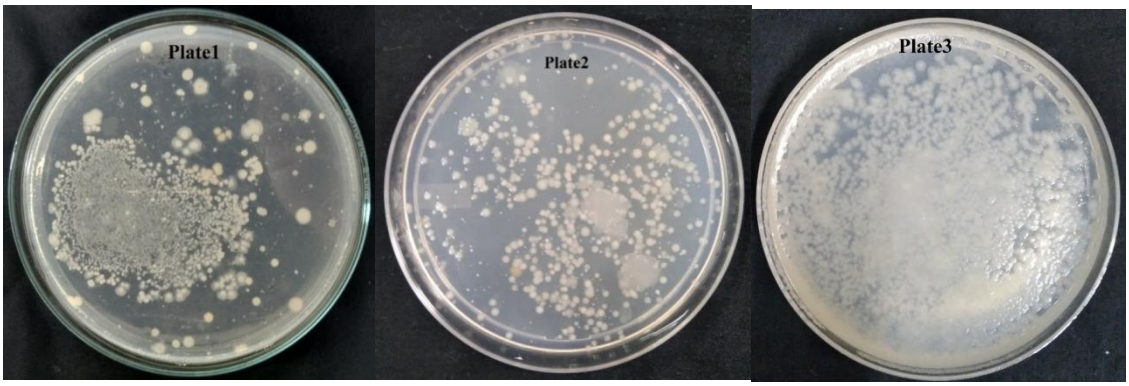
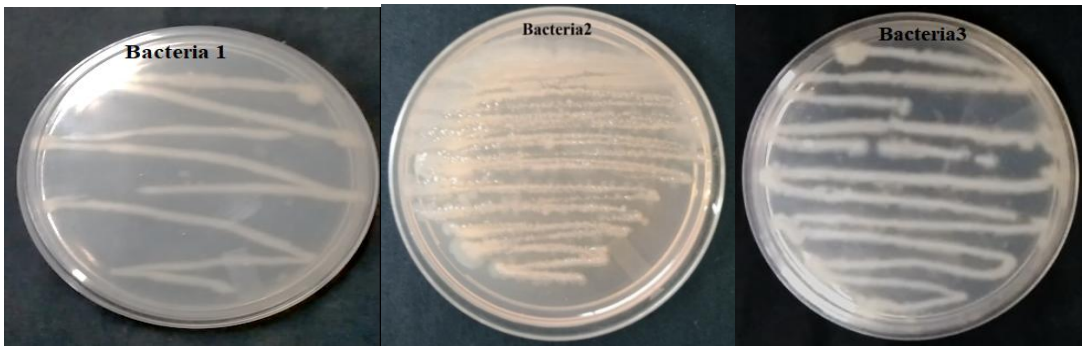


Plate 1: Bacterial colonies of earthworm gut

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).



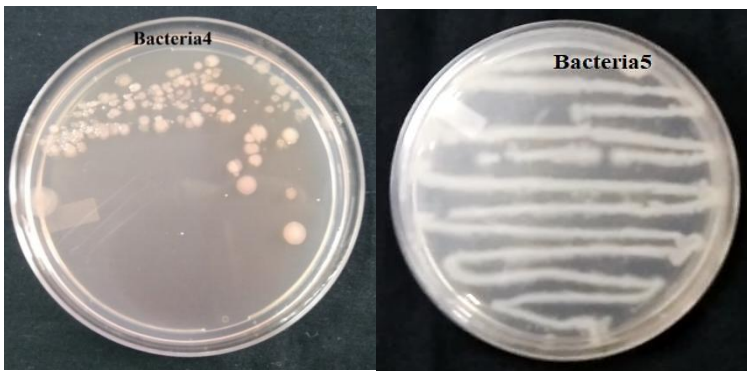


Plate 2: Pure culture of bacterial colonies

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 chromium also inhibition zone was not observed. This result indicates a high level of resistance to chromium.

Molecular Identification of *bacillus anthracis* 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 **99.86% sequence identity** with reference sequences of *Bacillus anthracis*, strongly supporting its taxonomic classification.

Based on this high sequence similarity, the isolate was confidently identified as *Bacillus anthracis*. 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 **OQ568517**

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

Fig 2. *Bacillus anthracis* strain CR 16SrRNA gene partial sequence

DISCUSSION

This study reports the isolation and molecular identification of chromium resistant bacterium, *Bacillus anthracis*, from the gut of earthworms fed with chromium accumulated *Eichhornia crassipes*.

Microorganisms and plants have developed various strategies to counteract the toxic effects of Cr (VI) (Sharma P.et al., 2021). Among the various methods, microbes enzymatic reduction of Cr (VI) into Cr (III) is the best understood mechanism for such bioremediation (Singh Set al.,2008). Chromium resistant bacteria are responsible for or contribute to the biological reduction of Cr (VI) to less mobile Cr (III), and precipitation of these bacteria could be a useful method for cleaning up polluted Cr (VI) areas.

In this study, the partial 16S rDNA sequence obtained (1516 bp) demonstrated 99.86% similarity to the reference sequence of *bacillus anthracis* in the GenBank database, confirming its taxonomic identity. *Bacillus anthracis* is Gram-positive rod shaped bacteria that is an obligate, endospore forming pathogen.

Feeding on chromium 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. anthracis* in such a niche suggests its adaptive potential in chromium-rich environments, where it may play a role in biotransformation or detoxification of heavy metals.

Chen et al. 2012 investigated the Cr(VI) uptake mechanism in *B. cereus* that reduced Cr(VI) into Cr(III). The reduced Cr(III) was coordinated with carboxyl and amido functional groups of the bacterial cell and the Cr(III) precipitates were accumulated on bacterial surfaces. Das et al, 2014 studied the mechanism of Cr(VI) reduction in *B. amyloliquefaciens* strain CSB 9 isolated from chromite mine soil of Sukinda, India. The reduced product Cr (III) was removed via surface immobilization and accumulated inside the bacterial cells.

Studies by Silva et al., 2009 have demonstrated that microbial metal biosorption is highly influenced by external pH, which affects metal ion availability and binding efficiency. For instance, *Pseudomonas aeruginosa* AT18 exhibited complete chromium removal at pH 7–7.2 highlighting the critical role of pH in chromium uptake. Elsilk et al 2014 reported that, *B. anthracis* PS2010 showed varying uptake of metals like Zn^{2+} and Pb^{2+}, with optimal uptake at 35°C and 25°C, respectively.

Conclusion

The augmentation of *Bacillus anthracis* in the gut of earthworms exposed to a chromium rich environment indicates strong selective pressure favoring chromium tolerant bacteria. This highlights the adaptive potential of *B. anthracis* in heavy metal-contaminated ecosystems. The submission of its identified nucleotide sequence to GenBank (Accession No. OQ568517) not only adds to the repository of environmental isolates but also provides a valuable resource for future comparative and functional genomics studies related to metal resistance and bioremediation.

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