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Isolation and Characterization of an Arsenic Resistant Bacterial Strain from Changki, Nagaland

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ABSTRACT

The present study focused on isolating arsenic (As) resistant bacteria from acid mine tailings of Changki, Nagaland and evaluating their bioremediation potential. The isolation was performed using enrichment culture approach and was further characterized using standard procedures. The obtained bacterial strain AS3 was found to be resistant to As^{3+} and As^{5+} ions up to 1562 µg/mL and 125000 µg/mL, respectively. The 16S rRNA gene sequence of the strain was found to be identical to Lysinibacillus sp. The growth behaviour of the strain in the presence of selected heavy-metals (HMs) showed a prolonged lag phase, especially in As^{5+}. Moreover, the strain appeared to be resistant to several antibiotics. SEM and EDX analyses, revealed the presence of HM ions on the outer surface of AS3 strain. Available functional groups on the surface of the AS3 strain cells engaged in the metal-binding process were identified using FTIR, suggesting their active participation in adsorption. AAS showed that the strain had the potential to remove As^{3+} and As^{5+} ions with removal efficiencies of 99.94% and 99.49% respectively. Based on the findings, strain exhibits intriguing biotechnological potential for HM bioremediation.

INTRODUCTION

Numerous technological developments and growth in industries have resulted in the substantial deposition of hazardous waste in the environment, which includes HMs that are reported to have several toxic effects on the environment and pose a threat to living beings (Elahi et al. 2019). The HM, metalloid arsenic (As) is known to be amongst lethal toxic element which pose a significant public health risk (Bermanec et al. 2021; Haroon et al. 2023). As usually occurs in different environmental matrices including water, soil and air in the form of trivalent arsenite (As^{3+}) or pentavalent arsenate (As^{5+}) (Goswami et al. 2015). According to previous reports, it even controls a variety of redox processes between the oxidation states of As^{3+} and As^{5+}, which can have harmful and dispersed effects on the environment (Maizel et al. 2016). This toxic metalloid As is extensively spread in the environment, originating from both natural and anthropological sources (Takeuchi et al. 2007). As usually occurs in numerous minerals and can also be present in sulphide minerals of mine waste that can be easily oxidised with redox conditions, pH, and microbial activity, resulting in very high arsenic levels in mine tailings (Matlakowska et al. 2008). Microorganisms are known to be crucial to the mineral cycle. It may even be able to perpetuate the As cycle (Goswami et al. 2015).

The presence of As in groundwater has become a major cause of apprehension as humans depend on groundwater as a drinking water source, thereby increasing the chances of exposure to As-contaminated water all over the globe (Maizel et al. 2016). The Indo-Bangladesh Gangetic Basin, also known as the Bengal Basin, reported very high As contamination from soil sediments (Goswami et al. 2015). There have been reports of serious health impacts from groundwater As-pollution for residents of developing nations like Bangladesh and India, including skin cancer, lung cancer, arsenicosis, and Bowen's disease (Ahmad et al. 2018). Millions of individuals in several nations, including Argentina, Cambodia, China, Hungary, Nepal, Mexico, Romania, and others, have been claimed to be impacted by As exposure. According to previous studies, the nations with the highest levels of As pollution in drinking water are Bangladesh and India, followed by Vietnam and Cambodia (McCarty et al. 2011).

Microorganisms that dwell in HM-rich environments frequently evolve diverse HM resistance and detoxifying systems (Chien et al. 2013). Various As-resistant bacteria have been previously isolated from As-contaminated soils (Turpeinen et al. 2004) and hydrothermal vents (Jeanthon & Prieur 1990). Bacterial species can become resistant to arsenic due to presence of phosphate-specific transport system that prevents the uptake of arsenate (Willsky & Malamy 1980); due to the efflux pathways mediated by the ars operon (Takeuchi et al. 2007). The ars operon is an integration of various genes such as *arsH*, *arsM* and several others responsible for arsenic resistance in bacterial systems. This operon is responsible for detoxification of arsenic (Gogoi et al. 2023). Moreover, bacteria have been known to produce siderophores, biofilm and EPS which facilitate in effective removal of HM ions (Gogoi et al. 2023). Several researchers were able to isolate As-resistant bacterial species from diverse environments, such as *Corynebacterium glutamicum*, which was isolated from As-contaminated soil and was able to remove 60 mM As^{3+} (Mateos et al. 2006); *Marinomonas communis* IAM 12914,

which was isolated from marine environment in Japan, was able to accumulate 2290 µg As per gram dry weight of arsenic in the presence of 5 mg As/ L of arsenate (45.8%) (Takeuchi et al. 2007); *Stenotrophomonas* sp., *Microbacterium* sp. which were isolated from Crven Dol mine of North Macedonia exhibited hyper-resistance to As^{3+} (209 mM) together with extremely high resistance to arsenate (564 mM) (Bermanec et al. 2021).

Although various physical and chemical-based remediation approaches are already available, most of them are either expensive or ineffective (Khalid et al. 2017). Moreover, the toxic chemical sludge produced by these treatment processes is not ecologically sustainable (Zakaria et al. 2024), requires high disposal or treatment costs and cannot entirely reduce the quantities of HMs to acceptable levels (Khalid et al. 2017). Thus, it becomes imperative to economically and sustainably reduce the concentration of such pollutants to a level that does not disturb the environment. The recent trend in developing newer environment benign technologies have drawn attention throughout the scientific community and technologists for the remediation of HMs (Takeuchi et al. 2007). Due to the lesser chemical involvements throughout the treatment process, low overhead expenses, greener, more affordable alternative to current methods, and even efficacious at decrease contamination levels are some major advantages of bioremediation of HMs (Khalid et al. 2017).

In this perspective, the current study attempted to isolate and identify bacteria from mine tailings area of Changki Hills, of Mokokchung district, Nagaland which was resistant to arsenic. The main objectives of this study were: (i) isolation, characterization and identification of the bacterial strain, (ii) heavy metal accumulation study, (iii) determining the strain's susceptibility to antibiotics.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Chemicals, reagents and salts including Na_3AsO_4, $NaAsO_2$, NaCl, Na_2HPO_4, NaH_2PO_4, KCl, Glutaraldehyde (2%, $C_5H_8O_2$), ethanol, Acetone were of analytical grade (AR) purchased from Qualigens fine chemicals, Thermo electron LLS India Private Limited, Mumbai. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Luria Bertani broth (LBB), Agar, and H_2O_2 were purchased HiMedia Laboratories Private Limited, Mumbai, India. Whatman Filter paper (Grade 1) was purchased from Cytiva, Global Life Science Solutions Operations UK Limited.

2.2. Study area and sample collection

The study examines subsurface soil in a stream near coal mines in Changki, Nagaland. Six sampling locations were used, with three upstream and three downstream. Subsurface soil was collected, stored at 4°C in sterile containers.

2.3. Isolation of As-resistant bacteria

The study isolated culturable As –resistant bacteria from soil samples using an enrichment culture technique (Bowman et al. 2018). The soil samples were mixed and sieved, then introduced to sterilized As^{3+} and As^{5+} loaded LBB medium. The culture medium was incubated for three cycles at 37 ºC, followed by serial dilution and spread plate method. After 48 hours, As –resistant bacterial colonies were documented based on their distinct morphological characteristics. The process was repeated up to three cycles, with each cycle involving concentrations of Na_3AsO_4 (300 µg/mL) and $NaAsO_2$ (300 µg/mL) (Sanders 2012).

2.4. Screening of potential As-resistant bacteria

The study involved loading a 96-well microtiter plate with diluted As^{3+} and As^{5+} solutions, then adding fresh bacterial inoculum. Sterile LBB media with fresh bacterial inoculum and devoid of HMs served as control. The plate was incubated for 24 hours at 37 ºC, then MTT was added, and absorbance measured at 600 nm using a Multiskan SkyHigh Microplate Spectrophotometer. The growth pattern of the selected bacterial strain was observed based on OD and colour changes from bluish to pale yellowish upon the administration of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and the MIC (Minimum Inhibitory Concentration), MBC (Minimum Bactericidal Concentration), and MTC (Maximum Tolerable Concentration) were determined (Agarwal et al. 2020).

2.5. Morphological, biochemical and molecular characterization of bacterial isolate

The study examined bacterial colony characteristics using Bergey's Manual of Determinative Bacteriology, Gram staining, spore staining, and motility tests (Bergey 1994). Biochemical characterization was performed using standard procedures (Banerjee et al. 2011). The Sanger dideoxy sequencing method was used for molecular identification of the 16S rRNA gene of the selected bacterial strain. BLAST analysis was performed on the NCBI Gene Bank database, and multiple sequence alignments were made using muscle alignment. Later, the Molecular Evolutionary Genetics Analysis (MEGA) software (version 4.0) was used to create a consensus maximum likelihood tree (Tamura et al. 2021).

2.6. Growth kinetics

The study used Ka-ot and Joshi's modified approach to study the growth behavior of a selected bacterial strain (Ka-ot & Joshi 2022). Two sets of conical flasks were used, each with specific HM salts (Na_3AsO_4 and $NaAsO_2$ at their MTC values. Culture broth devoid of HMs served as control. The cultures were kept in a shaker incubator at 160 rpm and 37°C throughout the exponential phase, and the OD was measured using a UV-VIS spectrophotometer at 600 nm. The growth curves of each treatment and control were compared.

2.7. Antibiotic susceptibility test

The Kirby-Bauer disc diffusion method (Bauer et al. 1966) and the well diffusion method (Balouiri et al. 2017) were utilized to ascertain the antibiotic sensitivity of the chosen As-resistant strain against fourteen distinct antibiotics. Inhibition zones, whose diameters are indicated in the standard antibiotic disc chart, have been shown to aid in classifying organisms as either susceptible or resistant to antibiotics (Dey et al. 2016).

2.8. Cellular study of the selected strain

2.8.1. Scanning electron microscopy-energy dispersive X-ray spectroscopy (SEM-EDX)

The influence of arsenic on the surface properties of the cell and changes in cell morphology under stress were examined using SEM. With a few modifications, the sample preparation procedure was carried out as stated by Pandey & Bhatt (2015). In separate Erlenmeyer flasks, the cultures were grown in 50 ml LB broth supplemented with 100 µg/mL Na_3AsO_4 and 100 µg/mL $NaAsO_2$, and they were incubated at 37 ºC for 48 h. Subsequently, the cultures were centrifuged at 6000 rpm, and the bacterial pellets were collected and rinsed three times with PBS (pH 7). The bacterial pellets were then stored at 4 ℃ for overnight period in a fixative solution containing 2% (v/v) glutaraldehyde in Na-phosphate with a pH of 7.2. The bacterial pellets were then centrifuged three times and then rinsed finally using Na-phosphate buffer. Afterwards, the cells underwent a succession of ethanol dehydration (30–100%) and drying. In order to study the morphology and microstructure of the bacterial strain, the samples were then prepared for SEM examination using a 3kV accelerating voltage. The SEM Microscope (Carl Zeiss NTS GMBH, Germany; JSM 6390LV, Japan) was utilized for mapping and point ID on the samples for energy dispersive X-ray spectroscopy (EDX) analysis of elemental composition.

2.8.2. Fourier transform infrared (FTIR) spectroscopy of bacterial biomass

With minor adjustments, the Singh et al. (2016) approach was used for FTIR analysis. The bacterial strain was cultured along with the heavy metal salts in the same way as previously mentioned. A bacterial biomass pellet was obtained after centrifuging the fully-grown culture for 10 min at 6000 rpm. The pellets underwent two rounds of washing in sterile distilled water before being dried for 48 hours at 40 °C. A Perkin Elmer FTIR spectrophotometer (Spectrum Two, Perkin Elmer) was used to analyse the dehydrated samples further. After mixing potassium bromide with the powdered bacterial sample, a manual hydraulic press was used to press the mixture into a translucent pellet disc and was used for the analysis. The 4000-400 cm^{-1} scan range was used to produce the FTIR spectrum.

2.9. Bioremediation potential of the selected strains

In order to assess the bioremediation potential of the selected As-resistant strain, the method described by Pandey & Bhatt (2015) was followed with modifications (Fig. 1) Firstly, the strain was cultured in Erlenmeyer flasks with 100 ml of LBB medium supplemented with selected HMs at their MTC values individually. The cultures were incubated for 72 hours with an agitation of 160 rpm at 37 ºC. Post incubation period, the

cultures were centrifuged at 6000 rpm and pelleted. The supernatant was passed through membrane filter (0.22 µm) and further undergone microwave digestion method (Xing 2022) for the quantification of available HM in the test sample using an Inductively Coupled Plasma Mass Spectrometry (Agilent 7850 ICP-MS). The removal percentage was then calculated using the formula:

$$\text{Removal \%} = (C_i - C_f)/C_i \times 100$$

Where C_i is initial concentration and C_f is final concentration/concentration of sample.

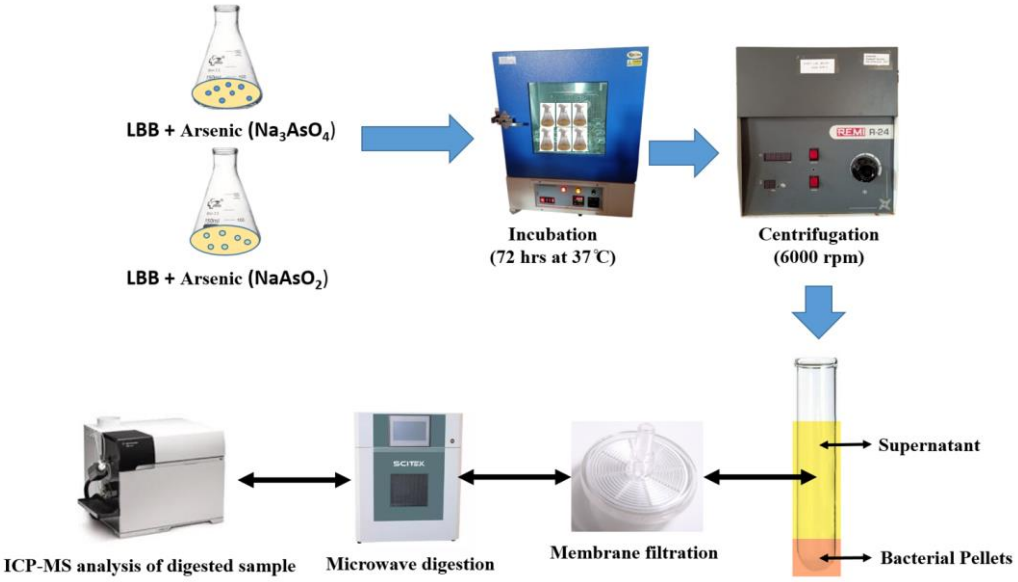


Fig. 1: Bioremediation study of AS3 in presence of Na_3AsO_4 and $NaAsO_2$

2.10. Statistical analysis

All the experiments were conducted in triplicate and analysed statistically using SPSS software. The obtained values were expressed as Mean values ± Standard Deviation (SD). The experimental data were checked for one-way analysis (ANOVA) at $P \leq 0.005$ confidence level.

3. RESULTS

3.1. Study area and sample sites

The collected soil samples were wet and had yellowish precipitation of AMD. The six sampling sites (26º26'4.10''N; 94º25'18.23''E, 26º26'3.28''N; 94º25'16.09''E, 26º25'59.38''N; 94º25'18.04''E, 26º24'57.87''N; 94º22'52.17''E, 26º24'55.95''N; 94º22'51.80''E, and 26º24'53.03''N; 94º22'47.61''E) were mapped with the help of Google Earth and the same is shown in Fig. 2A and Fig. 2B.

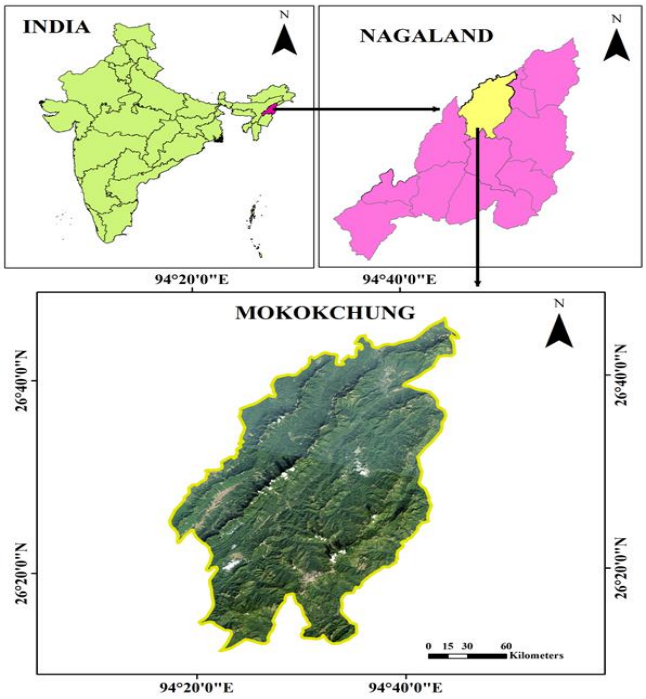


Fig. 2A: Map of Mokokchung

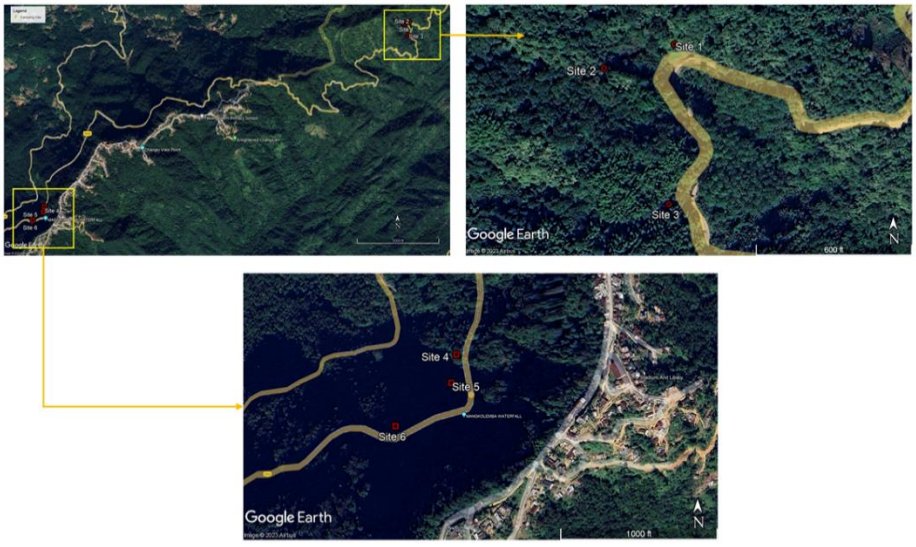


Fig. 2B: Sampling sites of the study area

3.2. Isolation of arsenic resistant bacteria

After completing the third cycle, followed by serial dilution (Fig. 3) and spread plate technique, ten bacterial isolates appeared from Site 1, 2 and 3 that could grow in an arsenic amended medium. These ten isolates were further screened for their tolerance for selected HMs; based on the obtained MTC values of As, three bacterial isolates, namely AS3, AS4 and AS11, were screened. For the present study, bacterial isolate AS3 was selected for further investigation.

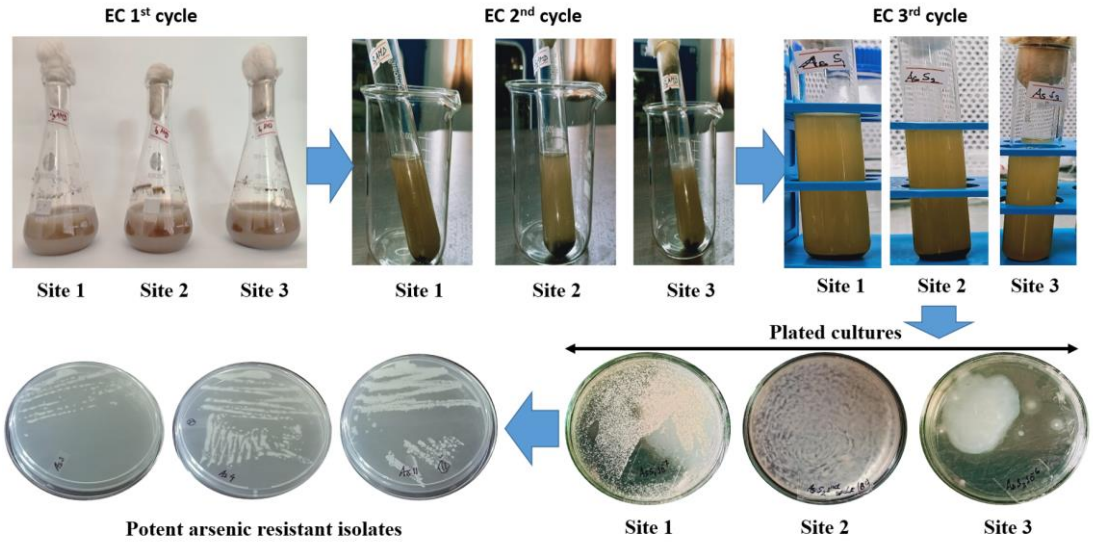


Fig. 3: Enrichment culture method for isolation of arsenic resistant isolates

3.3. MIC, MBC and MTC of selected bacterial strain

According to the results, the bacterial isolate AS3 was shown to be least tolerant of As^{3+} HM ions up to a concentration of 781.25 µg/mL and extremely tolerant of As^{5+} HM ions up to a concentration of 62500 µg/mL. Table 1 shows the MIC, MBC, and MTC values of the chosen isolates against selected HMs.

Table 1: MIC, MTC and MBC of selected isolate AS3 against selected HMs

Type of HMs tested	MIC (µg/mL)	MBC (µg/mL)	MTC (µg/mL)
Na_3AsO_4	125000	125000	62500
$NaAsO_2$	1562.50	1562.50	781.25

3.4. Morphology and biochemical characterization of selected bacterial isolate

The morphological characteristics of the chosen bacterial isolate AS3 and its colony, which include size, shape, and surface texture, are listed in Table 2a. The strain was found to be motile, spore forming, rod-shaped,

and Gram-positive. Moreover, it was found to be positive for- Catalase and Oxidase and negative for H_2S production, Indole, Citrate utilization, Methyl Red, Voges–Proskauer, Nitrate reduction, Glucose fermentation and Lactose fermentation.

The bacterial colony had an irregular shape, undulated margin, raised, shiny creamy colour texture and appeared to be optically opaque. Through 16s ribosomal RNA gene sequencing, the chosen bacterial strain was further identified as *Lysinibacillus* sp. strain AS3 (Fig. 4). The obtained sequence was later submitted to the NCBI database with the accession number OQ202230.

Table 2a: Morphological characterization of the arsenic resistant isolates

Sl No	Name of isolates	Shape	Margin	Elevation	Texture	Colour	Opacity
1	As 3	Irregular	Undulated	Raised	Shiny	Creamy	Opaque

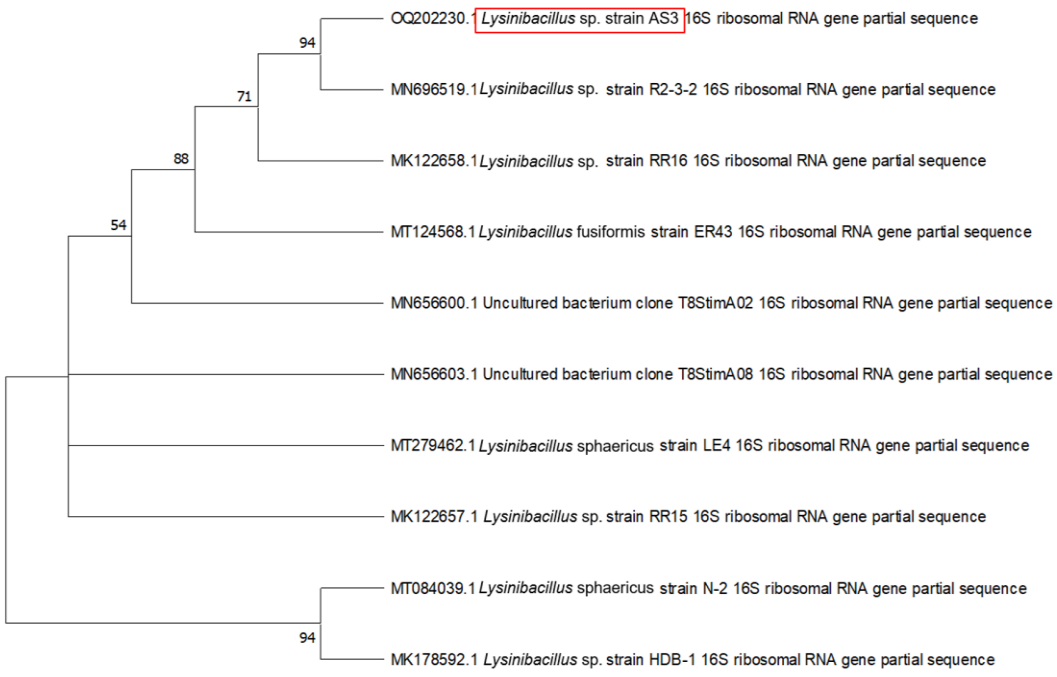
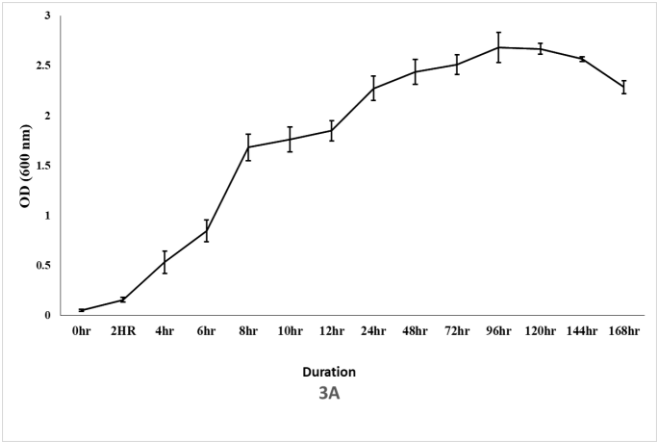
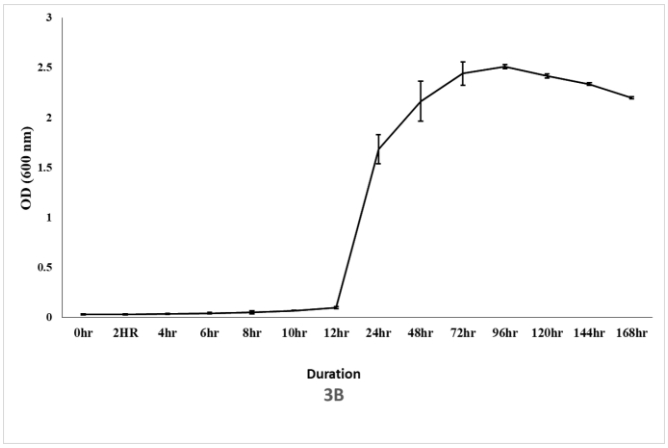


Fig. 4: 16s rRNA based phylogenetic analysis of the bacterial strain AS3

3.5. Growth characteristics of the selected bacterial strain

The growth curves of AS3 strain indicated that, in presence of As^{3+} and As^{5+} HM ions the lag phase was prolonged, or late exponential phase was observed (Fig. 5). Moreover, the lag phase in As^{5+} HM ions was more prolonged (12 hours) as compared to As^{3+} HM ions (6 hours). Additionally, the stationary phase in both As^{5+} and As^{3+} was achieved after 72 hours with gradual decline of growth after 120 hours.





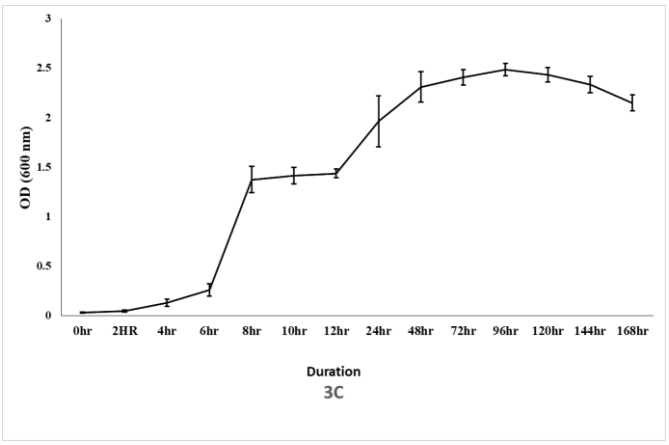


Fig. 5: Growth curve of AS3 strain in (A) Control, (B) Treated with As^{5+}

3.6. Antibiogram study

Based on the antibiotic susceptibility testing, AS3 was found to be resistant to 8 major antibiotics, while it was found to be susceptible to 6 major antibiotics. The diameter of the appeared zone of inhibition around the antibiotic loaded well/discs were measured and the same is presented in Table 3 and Fig. 6.

Table. 3: Antibiotic susceptibility of AS3 strain

Sl No.	Antibiotics	Class of antibiotics	Spectrum of activity	Zone of inhibition	Effect on isolates
1	Tetracycline (30 mcg)	Tetracycline	Broad	0	Resistant
2	Ampicillin (10 mcg)	β-lactam	Broad	0	Resistant
3	Penicillin (1 unit)	β-lactam	Narrow	0	Resistant
4	Cefaloridine (30 mcg)	β-lactam	Broad	0	Resistant
5	Kanamycin (5 mcg)	Aminoglycoside	Broad	0	Resistant
6	Cloramphenicol (30 mcg)	Chloramphenicol	Broad	20±0	Sensitive
7	Streptomycin (10 mcg)	Aminoglycoside	Broad	0	Resistant
8	Cephalexin (30 mcg)	Cephalosporin	Broad	0	Resistant
9	Ciprofloxacin (5 mcg)	Quinolone	Broad	25.67±0.57	Sensitive
10	Azithromycin (15 mcg)	Macrolide	Broad	0	Resistant
11	Norfloxacin (10 mcg)	Quinolone	Broad	14.67±0.58	Sensitive
12	Clarithromycin (15 mcg)	Macrolides	Broad	30.67±0.57	Sensitive
13	Erythromycin (15 mcg)	Macrolides	Broad	19±0	Sensitive
14	Amoxicillin (10 mcg)	Penicillin-type	Broad	34.33±0.58	Sensitive

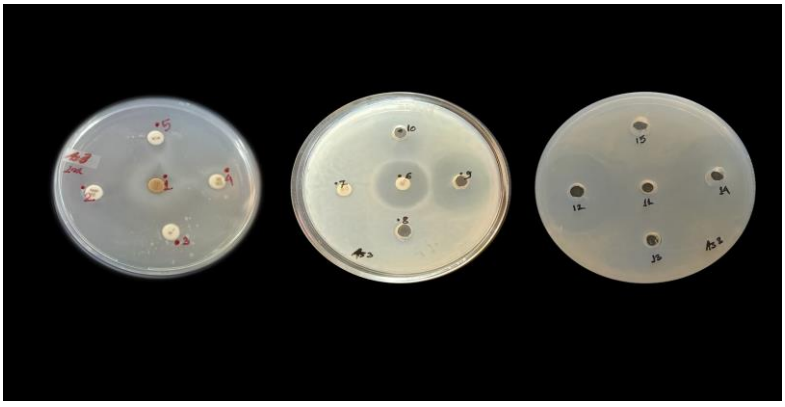


Fig. 6: Inhibition zones produced in antibiotic susceptibility test

3.7. Intracellular and extracellular study of the selected strain

3.7.1. SEM-EDX analysis of selected strain under different treatments

The SEM-EDX results confirmed the presence of As^{5+} ions over the surface of AS3 bacterial cell, which were previously grown in Na_3AsO_4 supplemented medium (Fig. 8). However, the strains did not show adsorption of As^{3+} metal ions on their surfaces (Fig. 8). Furthermore, distinct changes in morphological features were observed in both As^{5+} and As^{3+} treated samples (Fig. 8) as compared to control (Fig. 7) such as decrease in population size and increase in cell volume. In As^{3+} treated samples, the bacterial surface was rough as compared to the control.

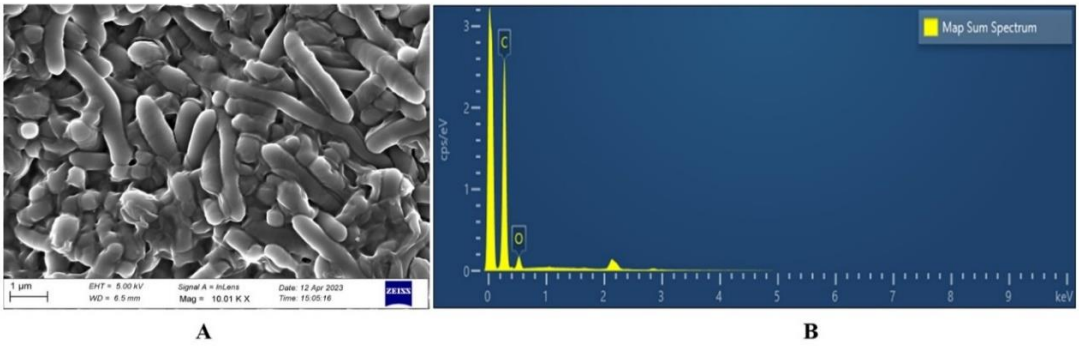


Fig. 7: (A) SEM micrograph of AS3 control and (B) EDX graph of AS3 Control

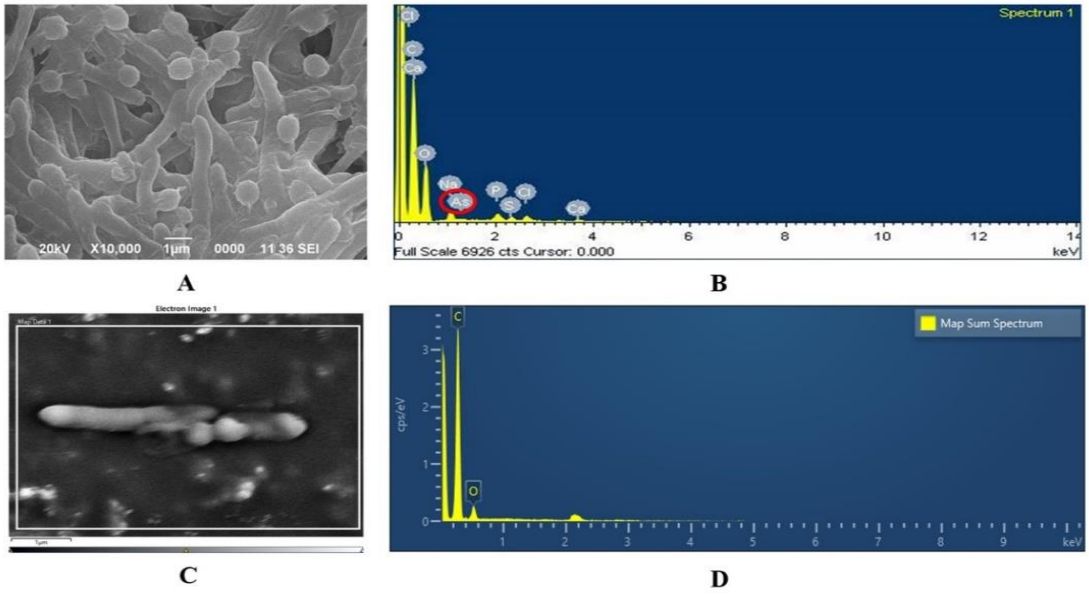


Fig. 8: (A) SEM micrograph of AS3 treated with As^{5+}, (B) EDX graph of AS3 treated with As^{5+}, (C) SEM micrograph of AS3 treated with As^{3+}, and (D) EDX graph of AS3 treated with As^{3+}

3.7.2. IR spectroscopic analysis of the selected bacterial strain

The IR spectra of the dry bacterial biomass of strain AS3 in the presence of various HMs at its MTC in comparison to a control without HM are presented in Fig. 9. The resulting spectra varied noticeably depending on the HMs present compared to the control. In this study, it was observed that, the peak values shifted from

3462 cm^{-1} (control) to higher wavenumbers in both the As treatments in the range of 3,200–3,600 cm^{-1}, corresponding to O-H stretching vibrations Krishnan (1961; Sarwan et al. 2024). The range between 3000-2800 cm^{-1}, corresponding to methyl, methylene and –CH stretching vibrations, the peaks were observed around 2969 cm^{-1} in control along with the As treatments (Xu et al. 2021; Shi et al. 2020). In the range of 1,550–1,700 cm^{-1}, 1647 cm^{-1} peak was recorded in the control, corresponding to N-H bending vibrations. However, the peak was not found in As treatments (Xu et al. 2021). A significant peak was observed at 1546 cm^{-1} in control was attributed to N-H in-plane bending or the CN stretching vibration. The peak value was found to shift to a lower wavenumber in As treatments (Gupta et al. 2022). Another significant peak at 1400 cm^{-1} was observed which was attributed to COO− stretching vibrations (Saraeva et al. 2023). The peak was absent in As amended treatments. Moreover, the peaks around 1236-1240 cm^{-1}, which were attributed to P=O asymmetric vibrations, was observed in control but was not observed in arsenic treatments (Parikh & Chorover 2006; Quilès et al. (2010). In the range 1000–1200 cm^{-1}, the peak value in control treatment was observed at 1092 cm^{-1} corresponding to C–O–C and C–O stretching vibrations (Makarem et al. 2019; Atykyan et al. 2020). This peak was shifted to higher wavenumber in As^{5+} (1096) and As^{3+} (1097) HM treatments. Another significant peak at 872 cm^{-1} was found in both As^{5+} and As^{3+} treatments (Fan & Zhang 2019).

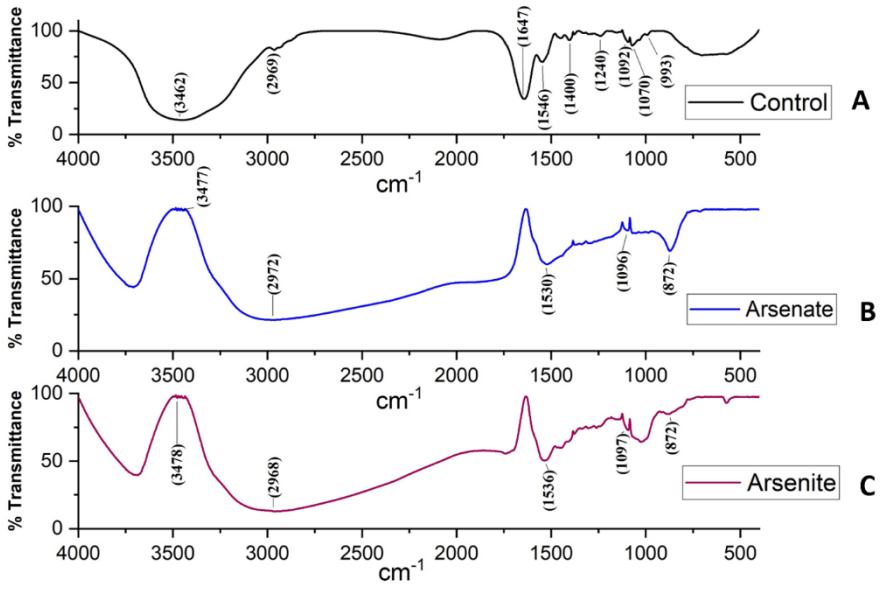


Fig. 9: FTIR study of AS3 strain in the presence of (A) control, (B) As^{5+}, and (C) As^{3+}

3.8. Bioremediation potential of the selected strain

The accumulating capacity of the selected HM ions by the AS3 strain was determined through ICP-MS and found out that the strain exhibited significantly high accumulating capacity for both As^{3+} and As^{5+} ions, with 99.94% ± 0.005 and 99.49% ± 0.59 respectively.

4. DISCUSSION

4.1. Study area and sample sites

Due to its proximity to human activity, a coal mining area, and a passing highway, the Changki study location that we have chosen is vulnerable to naturally occurring acid mine drainage (NOAMD). Prior records of Changki indicate the presence of HMs like zinc and copper beyond permissible limit (Semy & Singh 2021). Due to its proximity to human activity, a coal mining area, and a passing highway, the Changki research zone that we have chosen is vulnerable to naturally occurring acid mine drainage (NOAMD). Changki has been linked to HMs like Zn and Cd levels that are higher than allowed (Semy & Singh 2021). Reports reveal the presence of harmful HMs, such as arsenic, in NOAMD (Morgante et al. 2015). Furthermore, bacteria have persistently evolved in response to heavy metal exposure, thereby acquiring resistance to various HMs through mechanisms including metal resistance systems (Diba et al. 2021), extracellular metal sequestration (Voica et al. 2016), biosorption (Sevak et al. 2021), reduction of heavy metal ions (Ukkund et al. 2021), and morphological alterations (Mathivanan et al. 2021). Further, heavy metal resistant rhizobacteria that may demonstrate resistance against Cu, Cr, Zn, Cd, Ni, Sb, and As were reported from these area in a study conducted by Tatung & Deb (2024).

4.2. Isolation of arsenic resistant bacterial strain

Finding bacterial species that are resistant to hazardous heavy metal ions, like As, was the key objective of the current study. During the process of isolation and screening, the enrichment culture method facilitates the growth and multiplication of certain microorganisms that possess the desired traits, hence increasing the population of these target species (Gupta 2023). In addition, the Enrichment Culture method was performed not only to generate a population of bacteria resistant to HMs, but also to eliminate the auxotroph (Roncero 1984).

Through the combination of ribotyping techniques and routine biochemical testing, the selected potential bacterial strain was found to be *Lysinibacillus* sp. The strain was found to be 94 % identical to *Lysinibacillus* sp. strain R2-3-2 (Accession ID: MN696519) that had previously been reported. As per our knowledge the present study reports on the first instance of *Lysinibacillus* species from North-East India and Nagaland that exhibit great tolerance against HMs including As, Cd, and Pb ions. Several bacteria from the *Lysinibacillus* genus have been documented to exhibit resistance to HMs, including As, such as *L.* strain B1-CDA (Rahman et al. 2014), *L.* sp. DMAB5 (Mandal et al. 2022), and *L. boronitolerans* P2IIIb (Aguilar et al. 2020). Moreover, *Lysinibacillus* species exhibit resistance to Cd, including *L. fusiformis* L13 (Ma et al. 2023) and *L. varians* strain

KUBM17 (Pal & Sengupta 2019). In addition, this species was also reported to exhibit resistance to Pb^{2+} heavy metal ions, including *L. varians* strain KUBM17 (Pal & Sengupta 2019).

4.3. MIC, MBC and MTC of selected bacterial strain

The capacity to the bacterium to proliferate at elevated concentrations was utilized to ascertain its tolerance threshold to a particular heavy metal in the medium(Explain). Piotrowska-Seget et al. (2005) mentioned that as the quantity of HMs in the natural habitats grows, the prevalence of tolerant bacteria in metal-contaminated environments escalates. The limit of tolerance to the highest concentration of a particular heavy metal in the media was ascertained by measuring the bacterium's growth on the resulting higher concentration. The frequency of resistant bacteria in metal-polluted environment increases as the quantity of HMs in these habituates grows (Piotrowska-Seget et al. 2005).

Due to its repeatability (Hasselmann 2003), speed, affordability, and suitability for several heavy metal assays (Agarwal et al. 2020; Wiegand et al. 2008), the MIC for AS3 was determined using Luria Bertani Broth. The MIC values observed in this study, as seen in Tables 4, exceeded those reported by previous researchers. Typically, elevated levels of HMs correlate with diminished bacterial proliferation, mostly due to compromised membrane functions and the binding of metal ions to surfaces. Nevertheless, heavy metal-resistant strains typically endure in this environment owing to various intrinsic mechanisms, including the synthesis of exopolysaccharides (EPS), the presence of efflux and transporter proteins, metal adsorption on cellular envelopes, methylation, reduction to less toxic forms, the existence of multiple heavy metal resistance genes and operons, and several additional pathways (Haferburg & Kothe 2010; Gogoi et al. 2023).

Furthermore, compared to arsenite ions, arsenate exhibits greater minimum inhibitory concentrations (MIC) because to its lower toxicity. As Table 4 discusses, the findings are quite consistent with those of other research. According to Abbas et al. (2014), this is explained by the greater solubility of metal arsenites relative to metal arsenates. This implies that the isolated strains would be highly suitable for bioremediation of an environment contaminated with arsenic.

Table 4: MICs of bacteria against As^{5+} and As^{3+} HM ions

Sl No.	Bacterial strains	MIC against arsenate ions	MIC against arsenite ions	Type of media	Reference
1	KG1D	2500 μg mL^{-1}	500 μg mL^{-1}	Minimal salts	Roy et al. (2024)
2	PF14	1800 μg mL^{-1}	600 μg mL^{-1}		

3	MNZ1	-	300 μg mL^{-1}	Medium (MSM) broth Acetate	
4	MNZ4	_	300 μg mL^{-1}	minimal medium broth	Abbas et al. (2014)
5	MNZ6	_	370 μg mL^{-1}		
6	*Sporosarcina luteola* M10	70000 μg mL^{-1}	1300 μg mL^{-1}	Minimal Medium broth	Salam et al. (2020)
7	*M. paraoxydans*	36428.58 μg mL^{-1}	4800.78 μg mL^{-1}	Luria Bertani Agar Medium	Mandal et al. (2024)

4.4. Growth characteristics of the selected bacterial strain

A definite sign of metallic stress applied to the strain is shown in the growth curves of the AS3 strain, which showed that the lag phase was prolonged. According to Cristani et al. (2012), there may be a shift in the way that bacteria develop when they are in the presence of metals due to a physico-chemical interaction that occurs between the bacteria and the metals. Unambiguously, the protracted lag phase and late exponential phase of the growth curve show that the strain is under metallic stress or deliberately starts to oxidize As^{3+} as a defensive mechanism (Bachate et al. 2012; Bertrand 2019). Additionally, studies reveal that As damages the bacterial cell wall, causing the bacteria to respond towards arsenic stress with an extended lag phase during which cells strive to adjust to their new surroundings (Abbas et al. 2014). The growth pattern for As^{3+} ions was comparable to the control, suggesting that the bacteria were not under much stress. It required over twelve hours to adjust to the stress in the presence of As^{5+} HM ions, though.

4.5. Antibiogram study

Among the tested 14 different antibiotics, bacterial strain AS3 acquired resistance against 8 that include tetracycline (30 mcg) belongs to the class of tetracycline; ampicillin (10 mcg), penicillin (0.6 mcg), and cefaloridine (30 mcg) belongs to the class of β-lactam; kanamycin (5 mcg) and streptomycin (10 mcg) belongs to the class of aminoglycoside; cephalexin (30 mcg) belongs to the class of cephalosporin and azithromycin (15 mcg) belongs to the class of macrolide. Except for penicillin all other tested antibiotics are broad spectrum. Although

the strain was also found to be sensitive against 6 broad spectrum antibiotics that include chloramphenicol (30 mcg) belongs to the class of chloramphenicol; ciprofloxacin (5 mcg) belongs to the class of quinolone; clarithromycin (15 mcg) and erythromycin (15 mcg) belongs to the class of macrolides and amoxicillin (10 mcg) belongs to the class of penicillin-type.

The present study employed environmental samples taken from the immediate vicinity of an open coal mine located in the rugged landscape of Changki. This location is inhabited by a substantial number of human populations near the top of the hill. Hence, it is apparent that the pharmaceutical antimicrobial medications can be spread from human-populated places to lower sections of hills by the washing of sewage water via rain or natural waterways (Pan et al. 2023). According to Spain & Alm (2003), exposure to metal-contaminated environments appears to be the source of microorganisms that are both tolerant of metals and resistant to antibiotics, leading to coincidental selection for resistance characteristics in both. A recent study conducted Chen et al. (2019) demonstrated that bacteria in ecosystems polluted with HMs can develop resistance to both HMs and antibiotics concurrently. This phenomenon has also been observed in habitats affected with other types of pollutants (Cen et al. 2020). These studies clearly rationalize the necessity of avoiding the build-up of toxic metals in the soil and water bodies.

4.6. SEM-EDX analysis of selected strain under different treatments

An attribute common to heavy metal-resistant bacterial strains is the ability to adsorb metal ions on their surfaces, which is demonstrated by the SEM-EDX investigation of the multi-metal resistant bacterial strains. These ions included arsenate, lead, and cadmium. In a study conducted in 2015, Pandey and Bhatt obtained similar outcomes with arsenate adsorption on bacterial surfaces. Without arsenite adsorption, the bacteria may have evolved an adaptation mechanism, such as the extrusion of arsenite metal ions or the presence of efflux channels that allow the clearance of arsenite metal ions (Gogoi et al. 2023). The alterations in surface shape, as shown by several researchers (Rani et al. 2009; Zolgharnein et al. 2010; Banerjee et al. 2011; Shakya et al. 2012; Pandey & Bhatt 2015), clearly show an adaptive characteristic to collect more heavy metal ions or as a reaction in a hazardous metallic environment.

4.7. IR spectroscopic analysis of the selected bacterial strain

Peak shifting to higher wavenumbers about 3200–3600 cm^{-1} was noted in the IR spectra and this might be the result of the conjugation effect (Dai et al. 2023). Such changes were seen in samples treated with arsenic,

which suggest that the AS3 strain metabolized arsenic (Watanabe & Hirano 2013). The stretching vibrations of $–CH_3$, $–CH_2$, and –CH in membrane amphiphiles, including amphiphilic lipids in bacterial membranes, may be represented by the peaks seen in the range of 3000–2800 $cm^{–1}$ (Shi et al. 2020; Sohlenkamp & Geiger 2016). The amides N-H bending vibrations around 1647 cm^{-1} are indicative of protein structure (Xu et al. 2021; Usoltsev et al. 2019; Kassem et al. 2023). Protein in general is also responsible for the peak at 1546 cm^{-1} (Gupta et al. 2022). Peptidoglycans in the bacterial capsule are shown by the peak at 1400 cm^{-1} (Saraeva et al. 2023). The absence of peak around this range in As treatments indicates degradation of peptidoglycan. According to Parikh & Chorover (2006) and Quilès et al. (2010), the peaks at 1236–1240 cm^{-1} are often associated with phosphodiester, phospholipids, LPS, nucleic acids, and ribose, which are involved in the production of bacterial membranes, nucleoid, and ribosomes, which was not found in As treatments.

The peaks at around 1096 cm^{-1} in the range of 1000–1200 cm^{-1} may be related to the vibrations of glycosidic bonds in cellulose, which is a key indicator of the cellulose-I polymorph (Makarem et al. 2019; Atykyan et al. 2020). The Ribose skelet (ARN), that forms ribosomes, may be connected to the peaks at 993 cm^{-1} (Quilès et al. 2010). According to Fan and Zhang (2019), the peak at 872 cm^{-1} may be the result of As-O bending vibrations, which suggest that arsenic was present in both treatments. It could additionally be caused by the aromatic rings of particular nucleotides and amino acids vibrations (Kassem et al. 2023). The mechanism by which metals bind to ligands on the surface of bacteria is known as metal chelation, and it is most likely the reason for the changes seen as compared to the control. Functional groups are implicated in metal binding, as demonstrated by interactions with metal ions (Singh et al. 2016; Bueno et al. 2008). When metal ions interact with negatively charged groups on the cell wall through mechanisms including electrostatic interactions, van der Waals forces, and covalent bonding, the fluctuations in peak regions imply biosorption activity. It is commonly known that carboxyl, hydroxyl, and amino groups are functional groups that interact with metal ions (Singh et al. 2016).

4.8. Bioremediation potential of the selected bacterial strains

Several species of *Lysinibacillus* have been reported to exhibit resistance to HMs. For example, *L.* strain B1-CDA, isolated from cultivated land in Chuadanga district, Bangladesh, demonstrated 50% remediation of As^{5+} HM ions (Rahman et al. 2014); *L.* sp. DMAB5, isolated from Asanpara village (Bhagobangola I block) of Murshidabad district, demonstrated 32.33 %, 31.29 %, and 31.20 % bioremediation in the presence of 2 µg/mL, 10 µg/mL, and 50 µg/mL of As^{3+} HM ions (Mandal et al. 2022); and *L. boronitolerans* P2IIIb, isolated from soil of a gold mining area in Paracatu, Brazil, demonstrated 69.38% and 85.72% bioremediation of As^{3+} and As^{5+}

HM ions (Aguilar et al. 2020). Results of the present investigation hence further suggest that the selected strain is an effective bioremediating agent against As^{3+} and As^{5+} HM ions.

4.9. Limitations and future perspectives

In our study, we have found the bacterial strain capable of tolerating high levels of As HMs. Moreover, it was resistant to a number of antibiotics with high bioremediation potential for removing As HMs. Thus, this strain shows promising results to be utilized in field conditions. However, due to this limitation of this study on field conditions, this could be a future goal of this study for its possible application as a bioremediating agent as well as beneficial bacteria for the plant systems.

5. CONCLUSIONS

The ongoing unsustainable levels of human exploitation of natural resources led to the seek for alternate methods for treating wastewater tainted with HMs. The goal of the present study was to screen bacterial isolates from natural stream lines of Changki range of Nagaland, India using an *in vitro* setting that was enriched with As HMs. The potential isolate based on the obtained MTC and MIC values against the selected HMs viz. As^{3+} and As^{5+} was selected and identified as *Lysinibacillus* sp. strain AS3. The strain was shown to be resistant to As^{3+} and As^{5+} up to 1562.50 μg/mL and 125000 μg/mL. The bacteria demonstrated resistance to commercial antibiotics, particularly to Streptomycin, Cephalexin, and Azithromycin along with five other antibiotics. Cell surface adsorption of As^{3+} and As^{5+} was verified by SEM-EDX and FT-IR analysis of the biomass. Moreover, the strain demonstrated the ability to remove 99.94 % and 99.49 % of As^{3+} and As^{5+} under *in vitro* setting, indicating its potential for bioremediation. With such notable traits, the potential As resistant native strain exhibits potential for wide scale usages covering both basic research and real-world applications especially in the bioremediation technology. In this study, the *L.* sp. strain AS3 has enormous potential as a bioremediating agent in waste water treatment plants loaded with HMs. Moreover, this strain could be processed for functional gene analysis, which could help to identify the mechanisms and genes responsible for heavy metal resistance. Another important aspect of future research could be in the checking its ability in development of a novel broad-spectrum drug which have been hot research topic in current time. Moreover, it could be a potent strain for Plant growth-promoting rhizobacteria (PGPR) activity and conducting further experiments with plants.

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