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RESEARCH ARTICLE OPEN ACCESS

Identification and Characterization of Arsenic Transforming *Bacillus* Species from Abandoned Mining Regions of Madhya Pradesh and Jharkhand

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Abstract

The arsenic (As) comprehensiveness in nature has aggravated the expansion of arsenic fortification and detoxification components in microorganisms. Many microorganisms discovered today with ability to oxidize arsenite (As^{3+}) into arsenate (As^{5+}) or reduce As^{5+} to As^{3+}. In this study, two bacterial strains designated 3AB3 and 5AB2 was isolated from the soil samples collected from abandoned mining region of Madhya Pradesh and Jharkhand, India and arsenic concentration has been determined in both water and soil samples. Enrichment culturing method was employed for isolating bacteria and further they are screened for their redox ability. The isolated strains exhibited maximum growth at 30°C, at pH 7.0 in arsenic stressed Luria Bertani broth, checked through UV-Vis spectrophotometer at OD-620nm. Biochemical characterization of isolated strains was performed with various confirmation tests. Phylogenetic analysis of selected bacterial strains through MEGA-X confirmed their relationship to the genus *Bacillus*. Further, they are tested for transformation ability of arsenic (MSA method) and gene identification was done in selected isolated strains (PCR method). The result of this study shows that, even after abandoning the mining activities, concentration of arsenic increases in ground water by reducing ability of bacterial strains. PCR analysis depicted the presence of genes arsR, arsB and arsC in the strain 3AB3 and gene aoxB in 5AB2 respectively.

Keywords: Arsenic, Mining, Gene, Transformation, Enrichment culture, Microplate Screening Assay (MSA), *Bacillus*

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INTRODUCTION

In recent years, improved industrial and agricultural activities have immensely enhanced a variety of chemicals in the environment. The contamination of ground water with arsenic (As) is a major concern in developing countries for the environment and to public health. Arsenic usually exist in underground region bounded with other minerals. Arsenite (As^{3+}) is more mobile and toxic then arsenate (As^{5+})[1-3]. Its appearance in ground water is toxic above 10 µg/L (as said by WHO). If arsenite contaminated groundwater is consumed on regular basis, it gets accumulated in vital organs of the body disrupting their normal functions. Both plants and animals get severely affected by As^{3+} accumulation[1,4,5]. The mobility of As^{3+} is greatly affected by mining activities. So, for public health concern, government has abandoned mining activities in many regions. But even then also its concentration in underground water is increasing per year, as reported earlier. Since, ancient times, to overcome its toxic effects, bacterial species have developed specialized mechanism; the basis of which includes oxidation, reduction and methylation[6,7].

Under anaerobic conditions, As^{5+} is reduced to As^{3+} via microbial activity and further gets methylated, forming monomethyl arsonic acid and then dimethylarsinic acid[8,9]. Solubility of arsenic is controlled by pH, biological activities, redox conditions and adsorption reaction[10,11]. The fresh water has arsenic ranges from 1-10 µg L^{-1}, rising to 100 - 5000 µg L^{-1} in sulphur and iron mining area[12,13].

At high redox abilities, arsenic becomes stable as As^{5+} oxyanions (H_3AsO_4 or $H_2AsO_4^-$) but, under low redox potential and reducing conditions As^{3+} species (H_3AsO_3) becomes predominant[14,15].

Native microbes utilizes As^{5+}, electron acceptor during their respiration process. In the absence of oxygen, microbes gain energy by reducing As^{5+}, resulting in production of toxic As^{3+} and expelling it out of cell in underground environment. Arsenate respiring organisms can affect water quality by catalyzing the mobilization of As^{3+}, coupled with affecting biogeochemical cycles of other elements. So, our study aims to identify arsenic transforming bacterial species from abandoned mining regions of Madhya Pradesh (MP) and Jharkhand (JKD), India, with following objectives:

- To check the presence of arsenic in water and soil samples.
- To isolate and identify the arsenic oxidizing/ reducing bacteria from contaminated sites.
- To characterize the arsenic transforming ability of bacterial isolates.
- To identify the genes responsible for arsenic transformation.
- Phylogenetic genetic relationship establishment using 16s rDNA gene sequencing.

The need for the remediation of Arsenic contaminated sites

Increased amount of arsenic was found on affected sites above acceptable limits. The World Health Organization (WHO 2001) has accepted limits of As in soil at 10 mg/kg and in water at 10 µg/L. This limit is far lower than what is realistic in numerous old mine locales and agricultural lands. In mining regions even when the mining activities are abandoned, then also the concentration of As increases.

The more mobile and toxic As^{3+} is converted to less mobile and less toxic As^{5+}, by native bacterial species, in order to minimize the toxic As^{3+} concentration. The risk associated with mobile arsenic contaminants lies in its bioavailability through food chain in plants, microorganisms and consequently reaching to humans[16-20]. It is essential to decrease the arsenic level in soil, which can further decrease its ground water leaching and subsequent plant and human exposure. Many options were accessed for remediating contaminated sites such as landfill excavation, phytoremediation, solidification, and stabilization/immobilization, which were not proved to be significant and has their own limitations[21]. When the affected sites were visited, it was identified by talking to the local residents that, none of the person survived above 56 years of age. Even the crops grown on As contaminated soil also gets affected and when these plants are used as fodder for cattle, they gradually becomes weak and milk yielding capacity also decreases. In some animals As is also found present in milk[22-25].

To deal effectively with arsenic, it is important to have a correct and proper understanding of factors and mechanisms controlling its accessibility in underground water

and soil. This way, the problematic issues associated with arsenic can be efficiently controlled.

MATERIALS AND METHODS

Regions Selected For Study

The different regions were selected based on the availability of high arsenic concentration either in soil or water. The selected regions are listed in Table S1 (supplementary file). The regions were selected based on the fact that even after abandoning the mining regions, arsenic concentration is increasing in contaminated regions[26].

Sampling

A total of 112 samples (water + soil) were collected from contaminated abandoned mining regions of MP and JKD. Then, 250 ml and 400 gm of water and soil samples were collected aseptically in sterile capped bottles and transported to laboratory for analysis. The As content was found highest upto 3916 µg/Kg in soil and 1520 µg/l in water. On- spot qualitative analysis of arsenic was done using arsenic test kit (HiMedia Laboratories, India). Quantitative assessment of As was conducted by inductively coupled plasma - optical emission spectrometry (ICP-OES), technique through FICCI Research and Analysis Centre, Dwarka, New Delhi, India.

Physico-chemical analysis of water and soil samples

A total of 12 parameters were used to determine the physico-chemical properties of collected water samples (Table 2). These parameters were also used to determine water quality index (WQI), which is an important tool for determining quality of water for drinking purpose (Table S2 and S3, supplementary file). The graphical comparison between WQI of collected water samples from various regions is given in Fig. 1.

The water samples collected from the selected area is found acidic to alkaline in nature. The ionic balance of water is dominated by Mg^{2+}, Ca^{2+}, HCO_3^- and SO_4^{2-}. In majority of the samples, the analyzed parameters are well within the desirable limits, but higher concentration of EC, TDS, SO_4^{2-} and TH in many water samples make it unsafe for drinking purpose. The results suggested that the chemical composition of the water of study area is mainly controlled by environmental factors, weathering and with contributions from mining sources. The presence of arsenic tainted water at abandoned mining sites creates operational and stability problems, requiring effective water management strategy for drinking purpose.

Establishment of Enrichment Culture

Enrichment culturing technique was used to isolate microbial strains. Soil sample was inoculated in minimal salt medium (MSM) enhanced with 5 mM of sodium meta-arsenite ($NaAsO_2$) after three successive serial dilutions.

Table 1. Arsenic concentration in collected water and soil samples – ICPOES (inductively coupled plasma atomic emission spectroscopy)

S.no	Region	Place of Sample Collection	Source of sample	Water Sample (mg/L)	Soil Sample (mg/Kg)
1	Madhya Pradesh (MP)	Rajnandgaon	Water Supply Station	1	12.40
2		Abandoned Mining area Khairi	Borewell	2.23	375.65
3		Abandoned mining area Kaudikasa	Borewell	1.72	2.26
4	Jharkhand	Rajmahal	Drinking water Pond	0.02	6.73
5		Udhawa	Abandoned Mining area	1.72	28.38
6		Dehari	Abandoned Tubewell Mining area	1.2	31.84
7		Barhait	Abandoned Mining area Handpump	0.05	0.51

Basic components of MSM (mM l^{-1}) were KH_2PO_4, 3.60; $Na_2HPO_4.7H_2O$, 29.4; NaCl, 3.6; $MgSO_4$, 1.66; $FeSO_4.7H_2O$, 0.0035; $CaCl_2$, 0.009; H_3BO_3, 0.0098; NH_4Cl, 18.69; CH_3COONa, 60.95; Tryptone and Yeast extract (1.0 g l^{-1}); Glucose (20.0 g l^{-1}). The inoculated medium was incubated at 30°C and 120 rpm in rotary shaker. After seven days (168hr) of incubation, 5ml of culture was transferred to autoclaved MSM and incubated (30°C and 120 rpm) in rotary shaker. This process was repeated three times. The turbidity (denseness) of culture was analyzed by UV-Vis spectrophotometer, optical density at 620 nm (OD620) prior every sub-culturing. Later, cultures were consecutively diluted and inoculated on solid (agar containing) MSM for obtaining bacterial colonies.

Table 2. Summary of measured parameters in collected water samples

Parameters	Minimum	Maximum
pH	5.2	8.4
Electrical conductivity (EC)	615	1440
Total Dissolved Solute (TDS)	438	1060
F^-	0.45	2.23
Cl^-	11.2	66.7
HCO_3^-	180	565
SO_4^{2-}	28	490
NO_3-	0.6	42.3
Ca_2^+	26.4	130
Mg^{2+}	17.6	194.7
Na^+	14.8	188.4
K^+	1.3	9.2
Total Hardness (TH)	137	697

All concentration in mg/L, except pH and EC (µS/cm)

Isolation of arsenic-resistant bacteria

After subsequent enrichment and growth of bacterial cells over solid MSM, they are then further grown on Luria agar (LA) media supplemented with 5mM of As^{3+} and As^{5+} respectively, followed by 96 hours of BOD incubation at 30°C. After 96 hours, white colored bacterial colonies were obtained. The physiological characteristics of bacterial colonies obtained are given in Table 3.

Biochemical depiction

Morphological and functional characterization of isolated strains was executed through the standard approaches of Pelczar et al.[27]. Diverse biochemical properties of isolates like enzyme activity (indole, catalase, urease and oxidase), methyl red test, Voges–Proskauer test, citrate test, etc. were tested (Table 4), by following standard procedures[27]. Both the isolates were white in color, having flat colonies and smooth surface.

Verification of Arsenic transformation – MSA Method

The ability of bacterial isolates to transform (reduce or oxidize) As^{3+} and As^{5+} respectively was determined by using microplate screening assay (MSA)[28]. Isolates were cultured on Luria agar (LA) plate containing, tryptone 1 g/l, beef extract 1 g/l, yeast extract 1 g/l and agar powder 15 g/l) with either As^{5+} or As^{3+} and

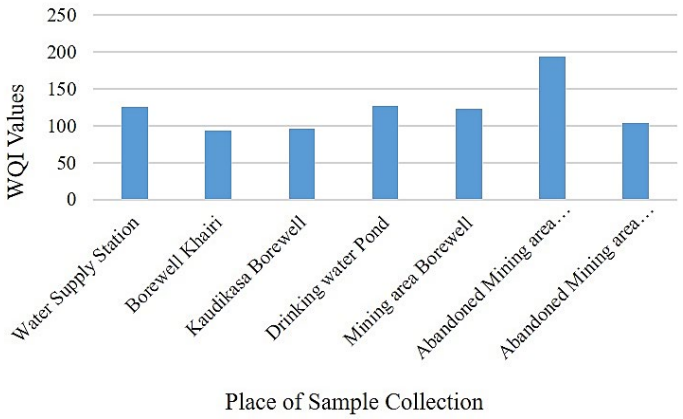


Fig. 1. Graphical representation of water quality index of collected water samples.

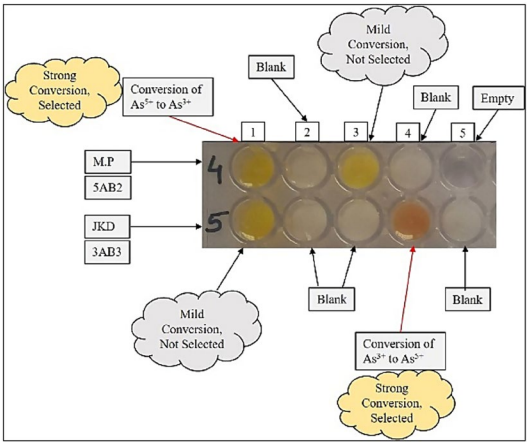
incubated at 30°C for 96 hr. After incubation standard protocol was followed and small amount of silver nitrate (0.1M) was added to microplate. If the media turned brown, it confirms the presence of silver arsenate and if it turns yellow, the presence of silver arsenite was confirmed. Among the isolated strains 3AB3 and 5AB2 was confirmed found to reduce and oxidize arsenic respectively[29].

16S rDNA sequencing for identification of bacterial isolate

PCR amplification of 16S rDNA (>1200 bp) and sequencing services were outsourced from National Centre for Microbial Research (NCMR), Pune. The obtained 16S rDNA sequence is submitted to Genbank database with accession number MH729060 of 3AB3 and MH729214 of 5AB2. Multiple Sequence Alignment and comparison with the 16S rDNA sequences available in GenBank database was performed using CLUSTAL W and BLAST programs, respectively. The phylogenetic tree was constructed using pairwise distance through MEGA X[30].

Gene Identification

Gene identification was performed by PCR method which was carried out in Allele Life Sciences, Noida, India and the respective results were obtained.



Well 1 (row - 4): inoculated with bacterial isolate 1 (designated as 5AB2), Well 2 (row- 4): blank without bacterial cells, Well 2 & 3 (row - 5): blank without bacterial cells, Well 4 (row - 4): blank without bacterial cells, Well 4 (row - 5): inoculated with bacterial isolate 2 (designated as 3AB3).

Fig. 2. Microplate Screening Assay.

Table 3. Physiological characteristics of bacterial colonies obtained.

Physiological characteristic	Isolate 1 3AB3	Isolate 2 5AB2
Shape	Circular	Circular
Elevation	Plane	Plane
Surface	Smooth	Smooth
Opacity	Opaque	Opaque

RESULTS

The presence of arsenic in water and soil samples was determined using inductively coupled plasma atomic emission spectroscopy (ICPOES) technique (Ficci Frac Laboratory, New Delhi) which was ranged between 1000 μgL^{-1} to 1520 μgL^{-1} in water and 2000 μgKg^{-1} to 3916 μgKg^{-1} in soil samples. All the samples collected was found alkaline to basic property with an average of pH 6.8. The bacterial cells were isolated through enrichment culturing technique using MSM media. The bacterial colonies (Fig. S1, supplementary file) were obtained on LA plates, standardized to pH 7.2.

Physico – chemical Properties of water and soil

Drinking water of good quality is the basic requirement for human physiology. Even

Table 4. Biochemical Properties of the Bacterial Isolates

Parameters	Isolate 1 3AB3	Isolate 2 5AB2
Catalase test	+	+
Indole test	-	-
Citrate test	-	-
Oxidase test	+	-
Urease test	-	-
Methyl red test	-	-
Voges-Proskauer Test	+	+
Hydrogen sulphide production	-	-
Glucose fermentation	+	+
Arabinose test	+	+
Sucrose fermentation	+	+
Fructose	+	+
Inositol	+	-
Maltose	+	-
Mannose	+	+
Gram Staining	+	+

the government has also started the provision of mobile water supply to rural as well as urban population to meet its necessity and to prevent health problems. Groundwater is generally considered fresh and safe source of drinking water, but rapid population growth and increased industrialization have resulted in greater contamination of quality water.

A total of 112 samples (water and soil) were collected from abandoned mining regions of MP and JKD in sterile bottles. The physiological properties of both water and soil is determined upon pH, temperature, TDS etc. (Table 2). The most important factor i.e. As concentration in the abandoned mining regions has also been determined commercially by ICPOES technique. The concentration of As in both water and soil samples is given in Table 1.

Isolation of Arsenic resistant bacterial isolates

Enrichment cultures were established in 500 ml Erlenmeyer flask containing 100 ml of autoclaved MSM, supplemented with 5 mM of As^{3+} and As^{5+} salt, separately. The flask were then kept at 120 rpm on a rotatory incubator shaker at 30°C. The enrichments were sub-cultured weekly into a freshly prepared MSM containing same concentration of arsenate and arsenite. After the second sub-culture, the enriched bacterial cultures were serially diluted and plated onto L.A medium (1.5%) containing 5 mM of As^{3+} and As^{5+}. Single colonies were picked and streaked onto same medium to obtain pure colonies Fig. S1 (supplementary file).

Transformation of arsenic (MSA method)

Tris-based buffer was used to standardize the micro-plate assay, irrespective of growth medium, to prevent interference problems with other ions, present in growth (MSM) media, which could result in color formation with silver nitrate ($AgNO_3$) under the same conditions similar to arsenic ions.

After adding $AgNO_3$ in required wells of microtitre plate, it was incubated for 96 hr. The 3AB3 culture plate media having arsenite, gradually turned brown (Fig. 2) confirming the existence of silver arsenate formed by conversion of As^{3+} to As^{5+} in the media. At the point when silver nitrate was mixed with arsenate containing culture, it turned yellow confirming formation of silver arsenite by conversion of As^{5+} to As^{3+}.

Some other isolates were also obtained which didn't show any type of transformation. Among two isolated bacterial strains, the strain present in Well number 1 (row- 4) has given positive result and designated as 3AB3 and in Well number 4 (row - 5) designated as 5AB2 (Fig. 2). Hence, it was confirmed that bacteria named 5AB2 possess the skill to reduce As^{5+} to As^{3+} and bacteria named 3AB3 oxidized As^{3+} to As^{5+}. A clear representation of mechanisms intended by

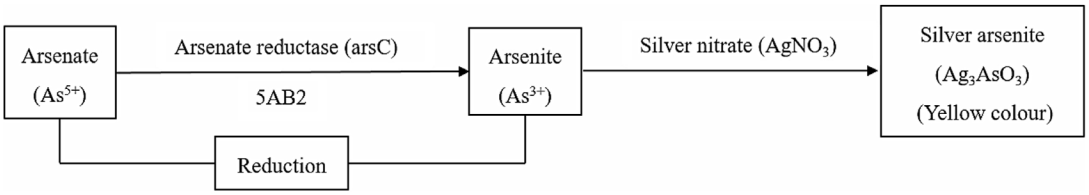


Fig. 3(a). Mechanism of arsenate to arsenite transformation by 5AB2 bacterial isolate

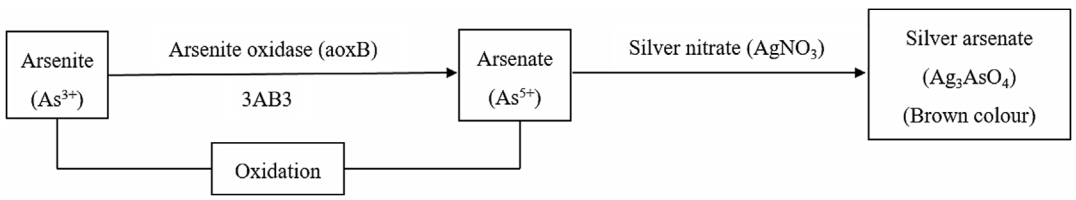


Fig. 3(b). Mechanism of arsenite to arsenate transformation by 3AB3 bacterial isolate

Fig. 3. Mechanism of arsenic species transformation by 5AB2 (3a) and 3AB3 (3b) bacterial isolates. This transformation mechanism was studied using microplate screening assay

bacterial isolates for arsenic transformation are given in fig. 3.

Phylogenetic analysis

The phylogenetic history of 3AB3 and 5AB2 named bacterial isolates was retrieved (Fig. 4 and 5) using Neighbor-Joining method[31]. The phylogenetic tree was constructed using 500 duplicates representing evolutionary past[32]. The evolutionary distances was intended using Maximum Composite Likelihood method using MEGA X software[33,34]. The phylogenetic analysis revealed that the two isolated strains 3AB3 and 5AB2 are closely related to *Bacillus* sp.

Gene Identification

PCR method was used with specified primers, designed by Primer blast tool of NCBI and the experiment was performed commercially. For conduction of PCR, the primer sequences and respective bacterial samples were brought to Allele life sciences, Noida and following (Fig. 6) results were obtained.

- the genes of 5AB2 (transforms As^{5+} to As^{3+}) was identified to be arsB, arsC, arsR (Fig. 6a, 6b, 6c)
- the genes of 3AB3 (transforms As^{3+} to As^{5+}) was identified to be aoxB (Fig. 6d)

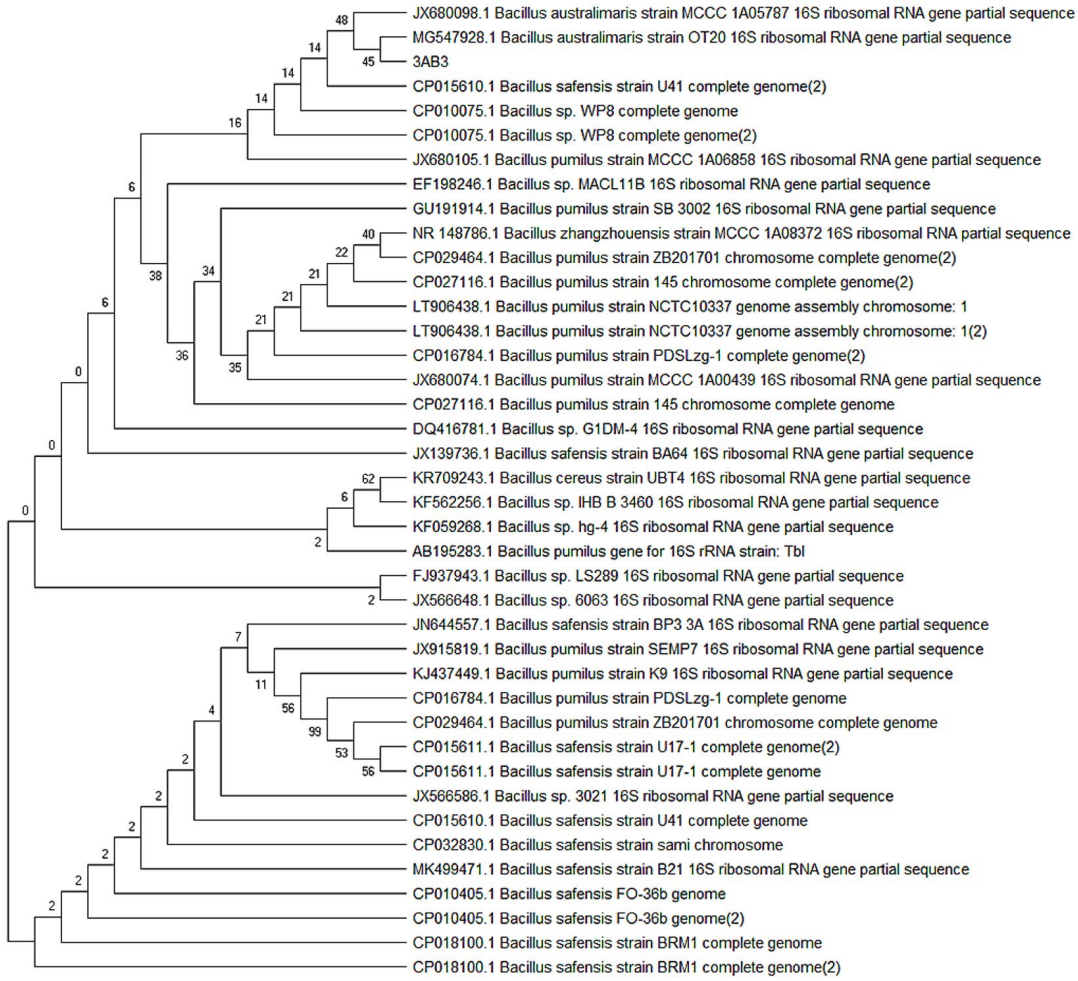


Fig. 4. Phylogenetic tree of strain 3AB3 together with its related neighbors was constructed based on 16S rDNA sequence using neighbor-joining and maximum likelihood method. GenBank accession numbers are given in parentheses.

DISCUSSION

The present work focusses on the identification and transformation potential of arsenic sp. (As^{3+} and As^{5+}). To accomplish the work, water and soil samples were collected from arsenic contaminated abandoned mining regions of MP and JKD. The collected samples (water + soil) were analyzed for the presence and absence of arsenic through ICPOES (FICCI Research and Analysis Centre, New Delhi). After analysis the samples found positive for containing arsenic were processed and bacterial strains were isolated through enrichment culturing method using minimal salt medium (MSM). Isolated

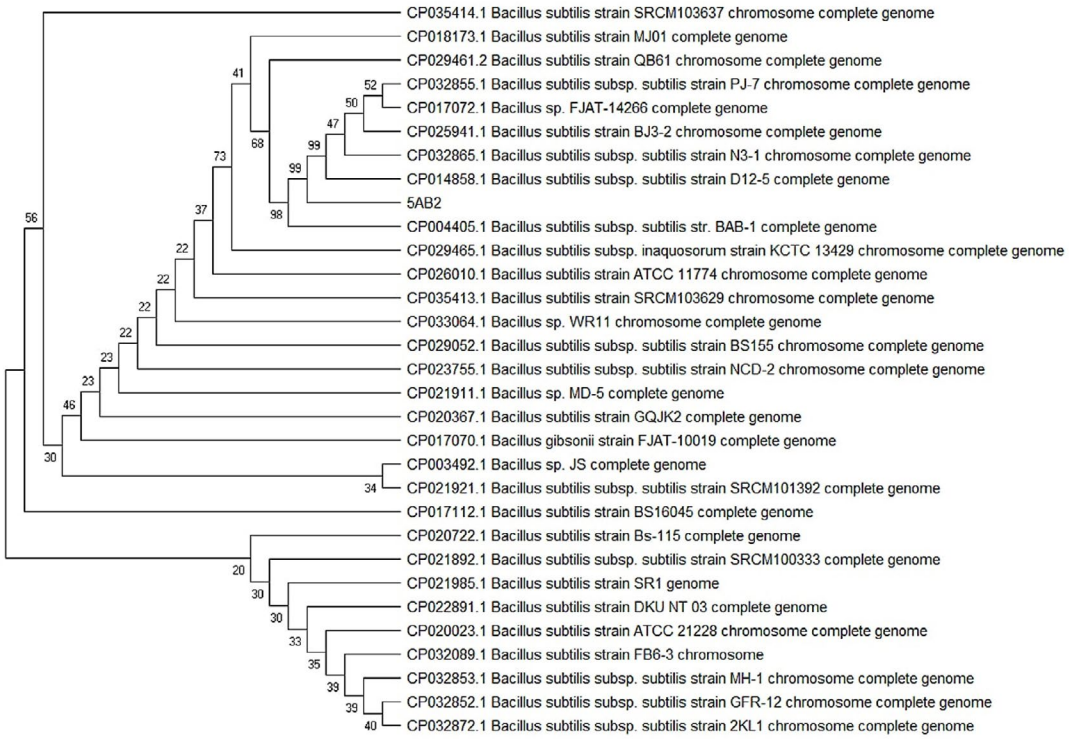


Fig. 5. Phylogenetic tree of strain 5AB2 together with its related neighbors was constructed based on 16S rDNA sequence using neighbor-joining and maximum likelihood method. Genbank accession numbers are given in parentheses.

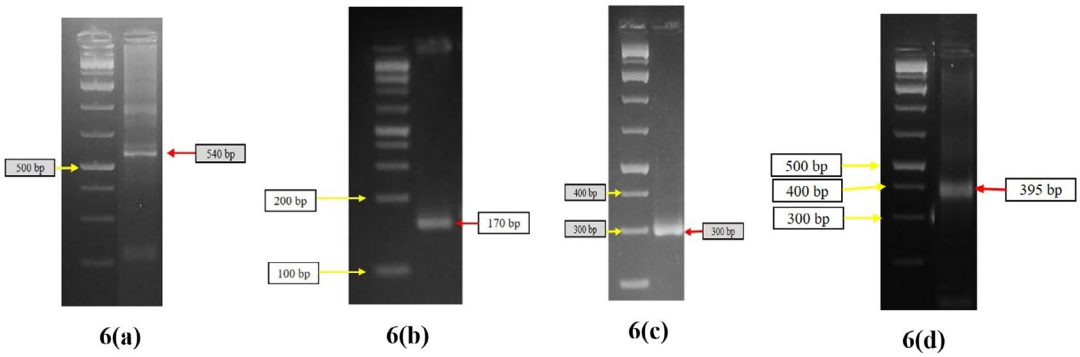


Fig. 6. Gel doc images of ars operon components. arsB (6a), arsC (6b) and arsR (6c) forms main component of ars operon. Fig 6(d) is gel doc image of aox operon component aoxB. The aox operon has two components aoxA and aoxB, aoxB is responsible for transformation of arsenite to arsenate3.

Table 5. Details of target gene, primer used and amplicon size

Gene target	Function	Primer F & R	%GC	Melting Temp. (°C)	Amplicon size (bp)	Marker (bp)
aoxB	Arsenite oxidase	F – TACGATCGGCTGCAATTCCC				
		R - GCACCATTGGGAACGATGTG	60	60	395	100
arsR	Ars operon regulator	F – TTAATGGAAAAACAATTGAAGGCTG				
		R – GCCCTCCACACATTCAGAGTT	50	57	300	100
arsB	Membrane associated efflux pump	F – TTGTGTTAGCGATGGTGCGT				
		R – ATCCTGCATTGCCAAGTCCG	60	60	540	100
arsC	Arsenate reductase	F – CCGGAAGACTGGGAAGTGT				
		R – GCTGCATCCCCACAAAGTGT	57	58	170	100

bacterial strains were biochemically characterized (Table 4) and were named as 3AB3 and 5AB2 respectively. The individual strains performing transformation, As^{3+} to As^{5+} and As^{5+} to As^{3+} was identified using Tris-HCl through MSA method. The percent transformation has also been determined in fig. S2 (Supplementary file), which depicts the time taken to transform individual arsenic forms by isolated bacterial strains. The 16s rDNA sequencing of selected transformants were performed at National Centre for Microbial Resource, Pune, India, and the result was obtained accordingly. The obtained 16s rDNA sequence of respective bacterial isolates are submitted to Genbank with accession numbers MH729060 (3AB3) and MH729214 (5AB2) respectively. The phylogenetic relationship was also established using CLUSTAL W through MEGA-X software (Fig. 4 and Fig. 5). The isolated bacterial strains shows the closest relationship with the *Bacillus* sp. The genetic identification through PCR method revealed that isolate 3AB3 contains the gene aoxB, which is responsible for conversion of As^{3+} to As^{5+}. This oxidizing operon is not well studied and needs further study, as it contains many other intermediate sequences for which the function is not known. Another isolated bacterial strain 5AB2, which is reported here for converting As^{5+} to As^{3+}, harbors the main components of ars operon viz, arsR, arsB and arsC. The details of all these genes are provided in Table 5.

The mobility and toxicity of As^{3+} is far more than As^{5+} and it is also considered to be lethal for plant and human on consumption[35-37]. The bacterial strain 3AB3 was identified for transforming toxic As^{3+} to As^{5+} and 5AB2 was identified to be converting As^{5+} to As^{3+}, which shows that isolated bacterial cells possess the mechanism to encounter arsenic toxicity and to overcome it. This transformation mechanism of bacterial cells needs further study and can also be utilized for biological remediation of toxic arsenic species.

CONCLUSION

Thus, this complete study, concludes that two microbial isolates 3AB3 and 5AB2 were identified nearest to *Bacillus australimaris (NH7I-1)* and *Bacillus subtilis sub sp. Stercoris (D7XPN1)* have 97.09% and 99.92% similarity. The isolated, biochemically characterized and identified bacterial isolates were recognized for their transformation ability. This result gives us the idea that even after quieting the mining work from the specified sites, the concentration of As^{3+} increases by bacterial transformation mechanism. The MSA test resulted oxidizing ability of isolate 3AB3 transforming harmful As^{3+} to As^{5+}. The As^{5+} has already been recognized having less toxicity than arsenite on plants and human health, if consumed. So, this test also gives us the idea of effective elimination of toxic As^{3+}, improving soil and water quality. The phylogenetic relationship was established, which depicts that 3AB3 and 5AB2 are closely related to *Bacillus sp*. containing oxidizing and reducing property. The potential bacterial mechanism of encountering As toxicity by altering its oxidation state and removing its noxious effects and acquiring resistant characteristic is an exceptional property of bacterial cells and can be utilized in the innovative strategy for bioremediation of this heavy metal.

SUPPLEMENTARY INFORMATION

Supplementary information accompanies this article at https://doi.org/10.22207/JPAM.15.1.12

Additional file: Additional Table S1- S3. Additional Figs. S1 and S2.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or the Supplementary Files.

ETHICS STATEMENT

Not applicable.

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