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Arsenite-oxidation performance of microbes from abandoned iron ore mine

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Arsenite is considered to be more toxic than arsenate (an average of 100 times). It can be oxidized to arsenate by chemical reaction or microbiological interactions. The aim of the present study was to investigate the arsenite-oxidizing microbes that exist in iron ore mine in India. We cultured 13 morphologically distinct bacterial strains, among which 6 strains could grow in high concentrations and shows the arsenite transforming abilities. Analysis of the amplified 16S rDNA gene sequences of the isolates revealed them to belong to alpha-, gamma- proteobacteria and firmicutes particularly in genera *Paenibacillus*, *Pseudomonas*, *Ochrobactrum*, *Enterobacter* and *Bacillus*. Further, qualitative silver nitrate screening assay and quantification by HPLC-ICP-MS analysis of these strains indicated the transformation of arsenite to arsenate. Moreover, the isolates were genetically analyzed for presence of arsenic arsenite transporter gene (*arsB*) that indicates the genetic ability of bacteria to tolerate the most toxic arsenic species.

Keywords: Arsenopyrite, arsenic transformation, silver nitrate test, HPLC-ICP-MS

Introduction

Arsenic is ubiquitous in the earth's crust in the form of arsenopyrite. Arsenic bearing bedrocks and minerals are known to exist in Bangladesh, West Bengal (India), China and other countries. The introduction of arsenic in drinking water is mostly due to groundwater contamination coming from weathering of arsenic bearing rocks and minerals[1-3], acid mine drainages (AMDs) which are severe environmental conditions generated by anthropogenic activities such as mineral minings[4-5]. Arsenic toxicity is dependent on its chemical form. Inorganic arsenite once released from its complex ores and minerals is reported to be more toxic (an average of 100 times) than the less mobile arsenate[6]. In one of the interesting toxicity study based on cytogenetic study of the blood samples reported from India, revealed the development of Klinefelter syndrome and other skin related diseases in peoples consuming arsenic contaminated water[7]. Several processes including chemical oxidation have been described for arsenic removal by transformation of arsenite [As(III)] to arsenate [As(V)][8] followed by alkaline precipitation[9-12]. These chemical processes have major disadvantage, that they generate additional pollution in the form of sludge and are expensive. Microorganisms reported to play an important role in oxidation reduction of metals in earth crust and geochemical cycling of arsenic via biological oxidation[13-14]. Among the reported microbes which were isolated from differently arsenic contaminated sources (water and mining sites) were able either to oxidize or reduce the arsenic, those microbes are from the genera of *Pseudomonas*, *Bacillus*, *Psychrobacter*, *Vibrio*, *Citrobacter*, *Enterobacter*, and *Bosea*. Similarly, some of the arsenic oxidizing microbes belonging to species of *Acinetobacter*, *Lactobacillus* has demonstrated to remove the arsenic from contaminated water[15-16].

This activity of arsenic transformation and removal by microbes can be found in the ecosystem of certain mineral mines that harbor arsenic-resistant microbes. Those microbes are efficient in performing detoxification when the arsenic resistance gene such as arsenite oxidase (*aoxB*) to oxidize arsenite to arsenate[17] and arsenic transporters (*ars*) that functions as a chemiosmotic transporter[18] is present in the bacterial genome. With this background, the aim of the present study was laid to isolate bacterial species from iron mine and assay the arsenic transformation efficiency.

Material and Methods

Sample Collection and Preparation

Soil sample collection site was a six month old abandoned moist pit of active iron ore mine

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(Chhattisgarh, India). The sediment soil was collected from top soil of 10-15 cm depth and was used as inoculum for enrichment of arsenic-oxidizing microbes. Unless stated, all chemicals used in this study were of high grade (Sigma-Aldrich). Culture media used for bacterial growth was chemically defined medium (CDM). All buffers and reagents used were prepared with Milli-Q water. The stock solution of sodium arsenite was sterilized by filtration (0.22 μm pore-size) and Tris-HCl buffer by autoclaving at 120°C for 20 min. Arsenite stock solution (20 mM) was prepared from sodium arsenite ($NaAsO_2$). Silver nitrate ($AgNO_3$) stock solution (1 M) was freshly prepared each time before use.

Isolation of Arsenite Transforming Heterotrophs

Enrichment, isolation and sub-culturing of isolates were performed using a minimal medium as described by Santini *et al* (2002)[19]. As(III) solution (2-5 mM sodium arsenite) in the medium was filter sterilized and pH was adjusted to 7.0. Duplicate enrichment cultures were established by adding the sediment soil as inocula (10% w/v) in 50 mL medium and were dispensed into 125 mL Erlenmeyer flasks. Further, it was incubated in the dark at 28°C on an orbital shaker (100 rpm) for 48 h. Thereafter, 10 mL of the suspended culture was mixed by vortex and 100 μL of mixture was inoculated into fresh medium and incubated as described above. Results were recorded after 48 hrs of incubation as either growth or no growth. This sub-culturing cycle by diluting the active cultures was repeated thrice to ensure sediment-free cultures. After getting sediment-free culture, an aliquot of the cultures was spread onto CDM plates containing 2-5 mM sodium arsenite to test the tolerance. After incubation for 3-5 days, different individual colonies based on colony morphology were transferred to fresh plates supplemented with 2-5 mM sodium arsenite. Growth and transforming ability of individual isolates were monitored routinely by (qualitative test) $AgNO_3$ method[20]. These isolates were analyzed for colony characters, Gram-staining and micro-morphological characters. Further, biochemical characterization and antibiotic susceptibility test of these isolates was carried out using biochemical test kit and antibiotic hexa discs (HiMedia Laboratories, India).

Quantitative Analysis of Arsenite Oxidation

After confirmation of qualitative test for arsenite oxidation of pure cultures, all selected bacterial isolates were inoculated separately in 10 ml media and allowed to grow for 72 h to attain optical density at 600 nm of 0.4–0.6. The liquid culture media were then centrifuged to separate the bacterial cells, the pellets were washed with Tris-HCl buffer (5 mM; pH 7.2) and centrifuged again. To the bacterial pellets 10 mL of buffer (Tris-HCl, 5 mM, pH 7.2) was added and incubated for 8 h with arsenite treatment (5 ppm). Sample preparation of bacterial culture for quantitative analysis was carried out according to Weeger *et al* (1999)[21]. Quantitative analysis has been carried out using HPLC systems coupled with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) according to Reuter *et al* (2003)[22].

Genetic Analysis of Isolated Bacteria

Bacterial genomic DNA isolated from each individual culture obtained from single colony of purified cultures as described by Liao *et al* (2011)[1]. Amplification of 16S rDNA was performed using universal 16S rDNA primer 27F/1492R (5'-AGAGTTTGATCCTGGCTCAG/5'-GGTTACCTT GTTACGACTT)[23], whereas arsB1F/arsB1R primer (5'-GGTGTGGAACATCGTCTGGAAYGCNAC/5'-CAGGCCGTACACCACCAGRTACATNCC) was used for amplification of arsenic transporters (ars) gene[24]. PCR amplification reaction was performed in 25 μl reaction volume that includes ReadyMix *Taq* PCR reaction mix (Sigma-Aldrich) in a MJ Mini thermal cycler (Bio-Rad). PCR products were purified using AxyPrep PCR clean up kit (Axygen Scientific, USA). Purified amplicons were further sequenced using 16S rDNA primers. The obtained sequences were searched for homologies at NCBI GenBank (http:// www.ncbi.nlm.nih.gov/Blast/). Phylogenetic analysis of these sequences was performed using MEGA ver. 6 software with implementation of ClustalW algorithm[25]. The nucleotide sequences of the isolates were deposited in NCBI GenBank database (KJ459928-KJ459933).

Results and Discussion

Isolation and Screening of Bacterial Strains

After successive removal of sediments and sub-culturing, morphologically different colonies observed onto the agar plates were picked and purified it again on a fresh agar plate by picking single colony. Inventory of arsenite oxidizing bacterial populations revealed that the isolates were of aerobic chemoheterotrophs. About 13 arsenic resistant bacteria were obtained. These isolates

were screened with $AgNO_3$ assay in agar plates. Among the initial 13 isolates, which later on confirmed as 6 strains, on the basis of possibility to transform arsenite to arsenate (silver nitrate test) and 16S rDNA sequences (Table 1). Among the isolates, strain SCW-5, SCW-7 and SCW-8 were found to tolerate up to 2 mM concentration whereas SCW-1, SCW-3 and SCW-4 were found to be highly tolerant and resist up to 5 mM concentration.

Phenotypic Characterization and Taxonomical Identification

All selected arsenite transforming bacterial strains were analyzed for colony characters, Gram-staining, micro-morphological characters (Table 2). Further, biochemical and antibiotic susceptibility tests were recorded (Table 3). The investigated bacterial isolates observed to form round and smooth colonies onto the surface of agar plates, except SCW-1 found with irregular form. Most of the colonies were white to light yellow in color and mostly rod shape. Isolates

Table 1 — Identified bacterial strains and response to silver nitrate test and arsenic resistance gene

Bacterial isolates	GeneBank Accession No.	Taxonomic placement	Silver nitrate test	Detection of arsenic resistance gene (*ars*B)
SCW-1	KJ459928	*Paenibacillus* sp.	+	+
SCW-3	KJ459929	*Ochrobactrum* sp.	+	+
SCW-4	KJ459930	*Enterobacter* sp.	+	+
SCW-5	KJ459931	*Bacillus* sp.	+	-
SCW-7	KJ459932	*Bacillus* sp.	+	-
SCW-8	KJ459933	*Pseudomonas* sp.	+	-

Table 2 — Phenotypic characterizations of studied bacterial isolates

Bacterial isolates	Gram stain response	Cell shape	Spore formation	Colony shape	Colony color	Arsenite tolerance (mM)
SCW-1	+	Rod	Endospore	Irregular	Light yellow	5
SCW-3	-	Short rod	Non-sporulating	Round	White to non-pigmented	5
SCW-4	-	Rod	Non-sporulating	Round	White to non-pigmented	5
SCW-5	+	Rod	Spore forming	Round	White	2
SCW-7	+	Rod	Spore forming	Round	White	2
SCW-8	-	Rod	Non-sporulating	Irregular	White	2

Table 3 — Biochemical characterizations and antibiotic susceptibility of studied bacterial isolates

Test	Bacterial isolates					
	SCW-1	SCW-3	SCW-4	SCW-5	SCW-7	SCW-8
Citrate utilization	+	+	+	+	-	+
Lysine utilization	-	-	-	+	-	-
Ornithine utilization	-	-	+	+	-	-
Urease	-	+	-	+	-	-
Phenylalanine deamination	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	-
H2S production	-	-	-	-	-	-
Glucose	-	-	+	-	+	-
Adonitol	-	+	-	+	+	-
Lactose	-	-	+	-	+	-
Arabinose	-	+	+	+	+	-
Sorbitol	-	-	+	-	-	-
Gentamycin	+	+	+	+	+	+
Vancomycin	-	-	-	-	-	-
Chloramphenicol	+	-	+	-	-	+
Methicillin	-	-	-	-	-	-
Rifampicin	-	-	+	-	+	+
Ciprofloxacin	+	+	+	+	+	+

(+) positive; (-) negative

belonging to Firmicutes were Gram positive, spore forming including SCW-1, whereas, other isolates belonging to *Proteobacteria* were Gram negative, non-spore forming.

In support of colony morphology, microscopic and biochemical study, molecular diversity for taxonomical identification was also investigated by employing 16S rDNA sequences. The molecular taxonomic analysis of the isolated arsenite oxidizing strains indicated that these belonged to a separate line of descent in the alpha- and gamma- *Proteobacteria* and Firmicutes (Fig. 1). The analysis of unambiguous nucleotide positions showed that strain SCW-4 and SCW-8 belonged to gamma-*Proteobacteria* branch. These two strains supported the sub-branch of *Pseudomonas* and *Enterobacter* with the sequence similarity to *Pseudomonas stutzeri* (100%) and *Enterobacter kobei* (97%), respectively. Similarly, these strains share the biochemical test with *Pseudomonas alcaligenes* and *Enterobacter cloacae*. Only one isolate was found to belong to alpha-*Proteobacteria* branch (SCW-3) with the sequence similarity to *Ochrobactrum lupini* (99%) and also supported sub-branch of the *Agrobacterium-Rhizobium* branch with 95-96% sequence similarity. Similarly, strains namely, SCW-1, SCW-5 and SCW-7 belonged to Firmicutes and supported sub-branch of the *Paenibacillus-Bacillus*. The sequence similarity of these three strains is in the range of 81-98%, and their nearest known phylogenetic relatives are *Paenibacillus alvei*, *Bacillus thioparans* and *B. firmus* with sequence similarity of 92%, 81% and 98%, respectively.

Arsenite Oxidation Efficiency

Quantitative analysis by HPLC-ICP-MS revealed considerable ability of isolates to transform the arsenite to arsenate (Table 4). Among the six isolates, SCW-7 and SCW-8 was found to be most efficient to transform arsenite (no arsenite detected after 8 hrs), whereas, SCW-4 was found to be least efficient (3.218 ppm arsenite detected after 8 hrs).

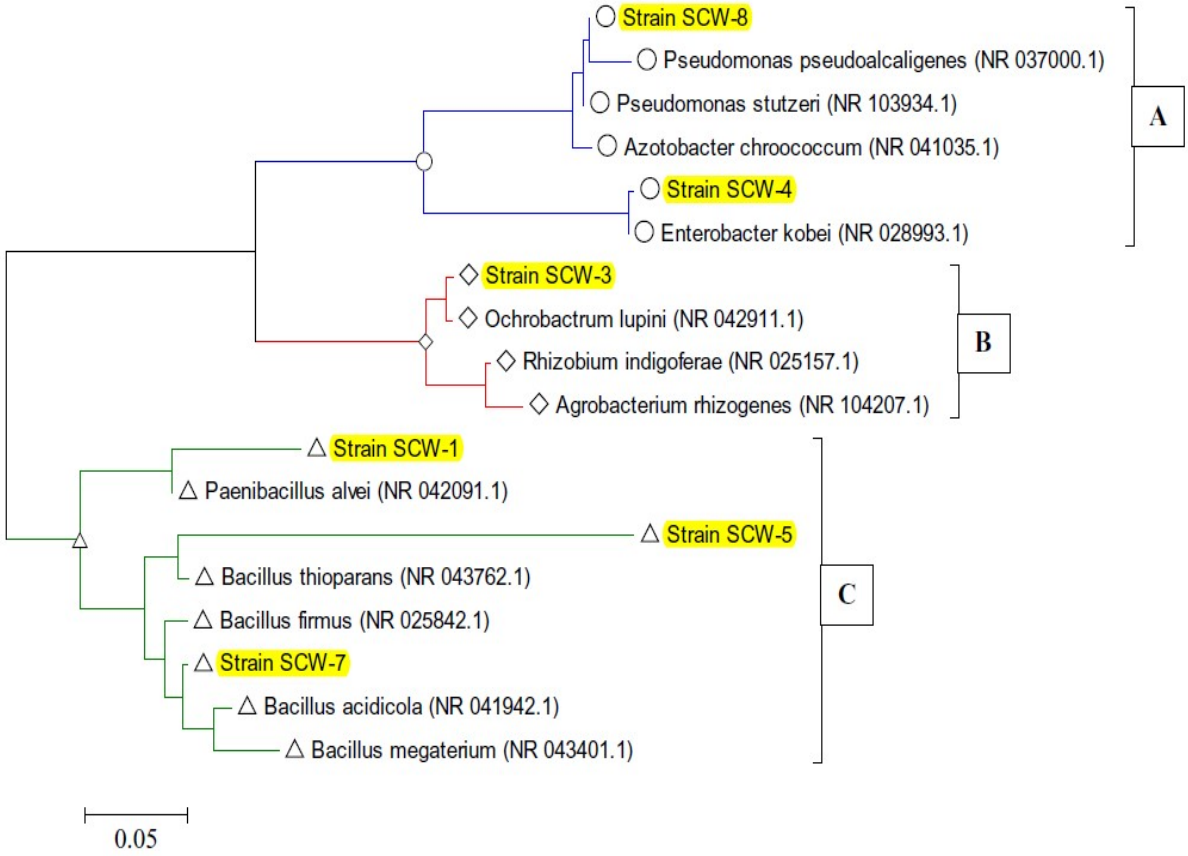


Fig. 1 — Phylogenetic tree of 6 arsenite oxidizing bacterial isolates based on 16S rRNA gene sequences revealed similarity to closely related bacterial species. Bacterial isolates belongs to A- Proteobacter and B- Firmicutes. The scale bar represents the number of substitutions per site.

Genetic characterization for *arsB* Gene

Isolated bacterial strains were tested with arsenic resistance gene namely, *ars*B. Primers for this gene specifically amplified about 750 bp fragment after 35 PCR cycles for three isolates (Fig. 2). The detection of amplicons thus indicated the genetic ability of these strains that shares the genetic content to arsenic-metabolic group which comprises diverse taxa. No amplification was obtained from SCW-5, SCW-7 and SCW-8.

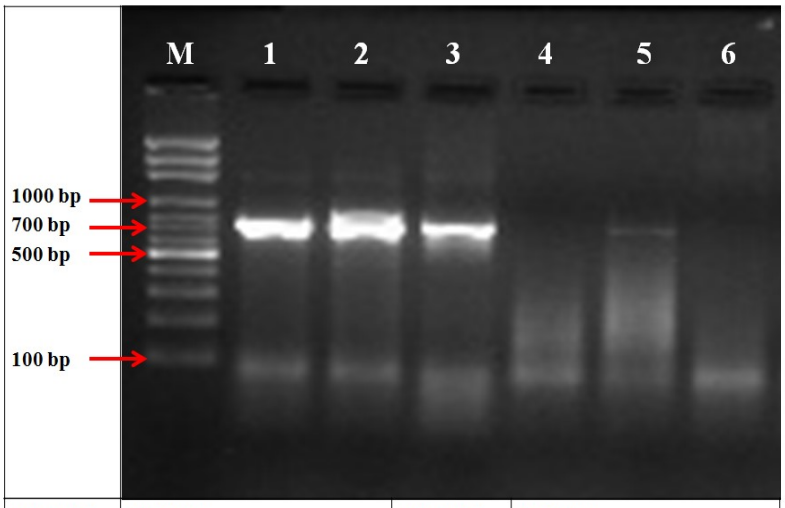
To understand the biological processes occurring in any given environment, it needs knowledge of the biodiversity of the residing microbial population and the role of each microorganism in the population. However, it is difficult to understand the role of each microbe in more diverse community[26]. In this sense, iron ore mine was selected as source of arsenic in the form of arsenopyrite and well suited environment that harbor a low microbial diversity, with few dominant taxa.

Isolation of bacteria belonging to this metabolic group have been widely reported from gold mine[19, 27-29], acid mine drainage[30-33] and arsenic contaminated soil/ water[1-2,34-36]. X-ray photoelectron spectroscopy (XPS) profiles of the oxidized arsenopyrite surfaces by both microbial and abiotic oxidation, provides insights into the changes in chemical states of the elements in arsenopyrite surface layers[37] was one of the basis to select iron ore mine as source arsenite-oxidizing bacteria in present study.

While studying phenotypic characters, advantage of silver nitrate which reacts with arsenic valence ion

Table 4 — Quantitative analysis of arsenite oxidation by bacterial isolates

Bacterial strains	Change in concentration of arsenic species after 8 hr incubation			
	Conc. of As^{+3} (ppm)	Conc. of As^{+5} (ppm)	Arsenic in cell pellet	Total arsenic
SCW-1	1.64	1.615	0.431	3.687
SCW-3	1.965	2.305	0.287	4.558
SCW-4	3.218	0.570	0.259	4.047
SCW-5	2.580	2.140	0.250	4.970
SCW-7	0.000	3.130	0.769	3.899
SCW-8	0.000	3.322	0.291	3.613
Blank (without cells)	4.680	0.002	--	4.683



Lane No.	Isolates	Lane No.	Isolates
M	100 bp DNA ladder	4	SCW-5
1	SCW-1	5	SCW-7
2	SCW-3	6	SCW-8
3	SCW-4		

Fig. 2 — PCR based detection of arsenic marker genes (*ars*B) in bacterial isolates.

was taken to screen out the bacterial isolates that demonstrated the transformation ability of arsenite to arsenate[20]. Demonstration of arsenite transformation ability further confirmed the suitability of the $AgNO_3$ test for identifying arsenite oxidizing bacteria.

Quantitative analysis as functional test has been carried out using HPLC-ICP-MS which is reported to be suitable for measuring arsenic species[38]. Strain SCW-4 showed 16S rDNA sequence similarity with *E. kobei* (NR 028993), was found to tolerate As(III) up to the concentration of 5 mM on growth medium, similar observation was also noted by Liao *et al* (2011)[1]. Interestingly, contrasting result was observed for this strain and found to be least efficient in arsenite transformation (3.218 ppm, untransformed arsenite out of 5 ppm) as detected in HPLC-ICP-MS analysis. This indicates that the strain SCW-4 may have avoidance mechanism to tolerate arsenic toxicity. In contrast, strain SCW-7 and SCW-8 belongs to *Pseudomonas* and *Bacillus* species was able to grow in medium containing up to 2 mM arsenite but found to be most efficient in arsenite transformation. *Pseudomonas* and *Bacillus* species were reported to be broadly represented class of arsenic-resistant microbes isolated from gold/ silver mine[39-40].

Most of the identified bacterial community in present study found to be similar with that of the bacterial community identified in arsenic-rich groundwater of shallow monitoring wells of the Choushui River alluvial fan, Taiwan[1]. All of the isolates in the present study were exhibited resistance to As(III), and some were highly resistant (up to 5 mM). Strain SCW-4 showed sequence similarity with *E. kobei* (NR 028993), as reported by Liao *et al* (2011)[1] and belongs to *Enterobacter* sp. This supports that the isolated microbes in the present study are the representatives of field site containing arsenopyrite. Acid mine drainage of Carnoules (France) is reported to contain As(III) 161.4 mg/L from where *Paenibacillus* strain Q8 have been isolated[33]. The isolated strain (SCW-1) in present study showed sequence similarity to *P. alvei* (NR 042091) was found to grow even at 5 mM concentration of As(III) indicates the high tolerance capacity to As(III). The only bacterial strain (SCW-3) which falls under class alpha-*Proteobacteria* showed sequence similarity to *Ochrobactrum* sp. *Ochrobactrum* reported to harbor the arsenic resistance (*ars*) operon (*ars*R-*ars*C1-ACR3-*ars*C2-*ars*H-*mfs*) and two non-operon-associated *ars* genes, *ars*C3 and *ars*B[41]. Occurrence of *Ochrobactrum* along with members of other genera were found to be highly resistant to As(III) and As(V)[2].

The detection of *ars*B amplicons in SCW-1, SCW-3 and SCW-4 further supports the arsenite tolerance test and indicates the genetic ability of these strains that shares the genetic content to arsenic-metabolic group which comprises diverse taxa. Three isolates in the present study were observed to be positive for *ars*B. The absence of *ars*B as observed in SCW-5, SCW-7 and SCW-8 isolates may probably indicative of variations in the gene sequence that decreased the homology of the used primer set. Some of the reasons to explain the PCR failure by used *ars*B primer for remaining isolates could be, 1) alternative mechanisms used by these isolates to deal with arsenite toxicity[24], 2) the possibility of inclusion of an aquaglyceroporin gene (*aqp*S) in place of *ars*B that confers resistance to environmental arsenate[42] and 3) unsuccessful amplification may be due to the presence of homologue with highly divergent DNA sequences to *ars*B gene[43].

The results of present study revealed that, all the six tested isolates from iron ore mine by enrichment culture method efficiently grow and transform arsenite to arsenate as indicated by silver nitrate assay and functional quantitative test except strain SCW-4. Diversity of culturable arsenite transforming bacteria from iron ore mine supports the published reports that metal-based mines serve as useful source to isolate arsenic transforming bacteria. Absence of *ars*B gene as observed in some isolates probably indicates variation in the gene in present study. These indigenous bacterial isolates may be suitable candidates for removal of most toxic arsenic species (arsenite) from water and waste water contaminated by arsenic.

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