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Arsenic-tolerant, arsenite-oxidising bacterial strains in the contaminated soils of West Bengal, India

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HIGHLIGHTS

• Arsenic resistant strains, which exhibit As(III)-oxidising ability, were isolated from arsenic-contaminated soils of India.
• Bacterial strains were identified as belonging to the genus Bacillus and Geobacillus.
• Arsenic-oxidising bacterial strains efficiently oxidise toxic As(III) to less toxic As(V).
• The prevalence of arsenite oxidase and aoxB gene raises the possibilities of alternative arsenic-mitigating options.

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ABSTRACT

As biological agents represent an affordable alternative to costly metal decontamination technologies, we isolated arsenic (As)-oxidising bacteria from the As-contaminated soils of West Bengal, India. These strains were closely related to various species of Bacillus and Geobacillus based on their 16S rRNA gene sequences. They were found to be hyper-resistant to both As(V) (167–400 mM) and As(III) (16–47 mM). Elevated rates of As(III) oxidation (278–1250 μM h⁻¹) and arsenite oxidase activity (2.1–12.5 nM min⁻¹ mg⁻¹ protein) were observed in these isolates. Screening identified four strains as superior As-oxidisers. Among them, AMO-10 completely (100%) oxidised 30 mM of As(III) within 24 h. The presence of the aoxB gene was confirmed in the screened isolates. Phylogenetic tree construction based on the aoxB sequence revealed that two strains, AGO-55 and AGH-02, clustered with Achromobacter and Variovorax, whereas the other two (AMO-10 and ADP-25) remained unclustered. The increased rate of As(III) oxidation by these native strains might be exploited for the remediation of As in contaminated environments. Notably, this study presents the first correlation regarding the presence of the aoxB gene and As(III) oxidation ability in Geobacillus stearothermophilus.

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1. Introduction

Arsenic (As)-contaminated ground water and related human suffering are reported throughout the world. However As contamination is most severe and unprecedented across a 0.173 million square kilometre-geographical region in West Bengal, India, where 36 million people are at risk for As exposure (BGS and DPHE, 2001). The indiscriminate use of As-contaminated groundwater for irrigation has rendered this carcinogenic element into the food chain (Williams et al., 2009). Edible crops are contaminated from polluted soils and irrigation water (Abedin and Meharg, 2002). This secondary source, apart from drinking water, has produced a pollution scenario that is more harmful and hazardous to human health (Guha Mazumder, 2008).

Arsenic is frequently found in nature as trivalent arsenite As(III) and pentavalent arsenate As(V). Although both As(III) and As(V) are toxic, As(III) is relatively more toxic than As(V) (Silver and Phung, 2005). The extent of toxicity varies, because As(III) has the ability to bind sulphydryl groups and dithiol groups of proteins, whereas As(V) acts as chemical analogue of phosphate and can inhibit oxidative phosphorylation (Hughes, 2002). The removal of As(III) from environmental systems is difficult due to its relatively higher solubility (Rhine et al., 2006), whereas in contrast, As(V) is poorly water-soluble and less bio-available (Mandal and Suzuki, 2002). In general, As is toxic to most living organisms. However, microorganisms have developed certain mechanisms to combat As-induced toxicity (Bhattacharjee and Rosen, 2007). For example, certain bacteria have developed the ability to oxidise As(III) to As(V), an ability that might represent a possible means of As detoxification. The oxidation of As(III) generates energy that is necessary for the growth and proliferation of autotrophic bacteria (Garcia-Dominguez et al., 2009), whereas for heterotrophic bacteria, the oxidation of As(III) represents a detoxification mechanism that is catalysed by the periplasmic enzyme...
arsenite oxidase (Muller et al., 2003). Recently, gene coding for different functional proteins that are responsible for As oxidation, *aoxA* in *Alkalimicrobium ehrlichii* MJH1-1 (Zargar et al., 2010), *aoxB* from *ß*-proteobacteria ULPAs1 (Muller et al., 2003) and *Agrobacterium tumefaciens* (Kashyap et al., 2006) have been characterised.

Currently, several As-oxidising bacteria have been identified, including *Bacillus arsenoxydans*, *Pseudomonas* sp., *Alcaligenes* sp., *Hydrogenophaga* sp., and *Thiimonas* sp. These bacteria were generally isolated from hot creeks (Salmassi et al., 2002), mines (Santini et al., 2000), sediments (Valenzuela et al., 2009), soils (Bachate et al., 2012) and ground water (Fan et al., 2008) throughout the world. Recently, a strain of *Stenotrophomonas* sp. MM-7, has been isolated from As-contaminated soils in Australia (Bachate et al., 2012).

The microbial transformation of As(III) to As(V) might represent an eco-friendly, cost-effective alternative management to conventional remediation processes (Bachate et al., 2012). Arsenic contamination in the gangetic alluvium of West Bengal (India) is characteristically different—it is geogenic in origin and widespread across waterlogged rice ecosystems, which produces a unique soil microenvironment. This investigation was aimed at isolating efficient indigenous As-oxidising bacteria from long-term contaminated soils of the severely affected Bengal delta. We evaluated the transformation efficiency of As(III) to As(V) in the presence of the arsenite oxidase enzyme, identified the *aoxA* gene and performed phylogenetic analysis. Our findings substantiate the potential application of native bacterial species in the detoxification of As in contaminated soil environments.

2. Material and methods

2.1. Field sampling, soil properties and arsenic determination

Soil samples (0–15 cm) were collected from the rice-growing areas of the Nadia district of India (N 23°02′ and E 88°34′), where As concentrations in the ground water exceed WHO-defined permissible limits (Sarkar et al., 2012). The physicochemical properties of the soil, including pH (Jackson, 1967), oxisable organic carbon (Walkley and Black, 1934), available N (Subbiah and Asija, 1956), K (Brown and Warncke, 1934) and P (Olsen and Sommers, 1982) were determined using standard protocols. Total As (Sparks et al., 2006) and NaHCO₃-extractable As (Johnson and Barnard, 1979) levels were determined using Atomic Absorption Spectrophotometer (model: Perkin Elmer Analyst 200, USA) coupled with FIAS 400. Microbial biomass carbon (Jenkinson and Ladd, 1981), total and As-tolerant microbial populations of the soil samples were also determined (Bachate et al., 2009).

2.2. Enrichment and isolation of arsenic resistant bacteria

Arsenic-contaminated soils (1 g) were suspended in Luria Bertani (LB) medium supplemented with either 1 mM As(III) or As(V) and incubated at 30 °C for 48 h (Kinegam et al., 2008). The cultures were enriched by transferring 2 ml of culture into the same medium. This process was repeated twice, and the final enriched culture was used for the isolation of bacteria. Approximately 0.1 ml of enriched culture was plated on solidified LB medium amended with As, and one hundred and eight colonies were purified.

2.3. Screening of arsenic-oxidising bacteria

Arsenic-oxidising bacterial isolates were screened using the standard silver nitrate (AgNO₃) method (Lett et al., 2001). The isolates were cultured on solidified CDM (chemically defined medium) that was supplemented with 1 mM As(III) for 48 h at 30 °C. The plates were flooded with 0.1 M AgNO₃ solutions and the colour changes of the respective colonies were recorded. AgNO₃ reacts with As(III), producing a bright yellow silver orthoarsenite (Ag₃AsO₄) precipitate, whereas the brownish silver orthoarsenate (Ag₃AsO₄) precipitate is produced at the reaction of AgNO₃ with As(V). Arsenic-oxidising ability was confirmed using a modified microplate technique (Simeonova et al., 2004). Each assay was performed in triplicate, and the isolates that produced brownish coloured precipitates were confirmed as As-oxidising strains.

2.4. Identification of the isolated strains and phylogenetic analysis

Twelve bacterial isolates exhibiting significant As-oxidising ability were selected for molecular identification by 16S rRNA gene sequence analysis. Total genomic DNA was extracted (Sambrook and Russel, 2001) and PCR amplification of the 16S rRNA gene was performed using the forward primer Y1 (401 F 5′-TGCGTCTAGGAGGAGGCCGC-3′ and the reverse primer Y2 (337) R 5′-CCCCACTGCTGCC-3′ (Chromous Biotech Private Limited, India). The PCR products were purified and maintained at 4 °C. Sequencing performed by Chromous Biotech Private Limited, India and partial 16S rRNA gene sequences were subjected to BLASTN analysis (http://www.ncbi.nlm.nih.gov/) to identify the species exhibiting the most significant homologies. The nucleotide sequences of the As-oxidising bacterial strains have been deposited in GenBank, and different morphological and biochemical analyses were conducted using standard methodologies (Holtz, 1993).

Phylogenetic trees of partial 16S rRNA sequences were generated using the neighbour-joining algorithms following the p-distance model (Saitou and Nei, 1987) in Mega IV (Tamura et al., 2007). The level of support for the phylogenies, derived from neighbour-joining analysis, was determined from 500 bootstrap replicates. Fifteen reference sequences of the reported As-oxidising bacteria were obtained from GenBank with *Escherichia coli* (FM869555), as outgroup was used for construction of the phylogenetic tree. The percentage of replicates is shown next to the branches (Felsenstein, 1985). The phylogenetic tree is drawn to scale with branch lengths shown in the same units as for inferred evolutionary distances. There were a total of 134 positions in the final dataset. The concordance of this analysis was also verified using a maximum parsimony algorithm.

2.5. Determination of resistance to As(III) and As(V)

The resistance exhibited by the selected bacterial strains to As(III) and As(V) was determined in CDM liquid medium supplemented with increasing concentrations of As(III) (from 0 to 100 mM) and As(V) (from 0 to 500 mM), followed by incubation at 30 °C for 48 h. The control for each concentrations comprised the inoculation of cells with the respective medium supplemented with different As species in culture broth by these efficient strains were determined using HPLC–ICP-MS (Majumder et al., 2013).

2.6. Arsenite transformation and species detection

Arsenite transformation by the selected bacterial isolates was determined using a modified method previously described by Bachate et al. (2012). The strains were grown in CDM without As and were then inoculated into same medium containing 30 mM of As(III), followed by incubation at 30 °C with continuous agitation. Non-inoculated medium was simultaneously incubated to verify the extent of abiotic As(III) transformation. The concentrations of As species were determined using a spectrophotometric method as previously described by Cummings et al. (1999). The four bacterial strains that exhibited increased efficiencies of As-oxidation were selected. The recoveries of the As species in culture broth by these efficient strains were determined by HPLC–ICP-MS (Majumder et al., 2013).
In a microwave teflon vessel, 10 mL of orthophosphoric acid (1 M) and 0.5 M ascorbic acid were added to 5 mL of culture broth. The mixture was digested at 60 W for 10 min and filtered through a sterile 0.22 μm Durapore filter (Millipore). The As species in the filtrates were separated using high-performance liquid chromatography (HPLC) with an anion exchange column (Hamilton PRP-X100F with a pre-column used for chromatographic separation) and were detected and quantified using a Perkin Elmer ELAN DRCe ICP-MS.

2.7. Arsenite oxidase assay

The bacterial strains exhibiting As-oxidising activity were grown in CDM in the presence of 30 mM of As(III). Cells at late log-phase were harvested by centrifugation at 10,000 rpm for 2 min. The collected cells were washed with 50 mM Tris–HCl buffer (pH 8.0) and suspended in 2 mL buffer containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and lysozyme. Cell suspensions were incubated for 2 h followed by disruption of cell membrane by sonication. Cell debris was removed by centrifugation at 10,000 rpm for 30 min (Bachate et al., 2012). Protein concentrations in the supernatants were determined by Bradford assay (Bradford, 1976) using bovine serum albumin (Sigma) as a standard. The arsenite oxidase assay was performed using a method previously described by Anderson et al. (1992).

2.8. Detection of the aoxB gene and phylogenetic affirmation

Bacterial genomic DNA was extracted (Sambrook and Russel, 2001), and PCR amplification of arsenite oxidase (aoxB) gene was performed using the forward primer 69F 5′-TCYATYGTNCCGNTY GGNTAYMA-3′ and the reverse primer 1374R 5′-TANCYCTYTGRTG NCCNCC-3′ (Rhine et al., 2007). The reaction mixture was prepared in 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 1 M of each primer, 25 ng of DNA template, and 2 units of Taq DNA polymerase. The final volume of each reaction was supplemented to 25 μL with sterile deionised water. The PCR products were purified and maintained at 4 °C. All of the amplified PCR products were gel-eluted using a Wizard SV Gel and PCR clean-up system (Promega, Madison, WI).

Sequencing of the aoxB gene of the four bacterial strains that exhibited maximum arsenite oxidase activity (ADP-25, AMO-10, AGH-02 and AGO-55) was performed by Chromous Biotech Private Limited (India) using primer 69F. The partial aoxB gene sequences of the isolated strains were compared with those that were available in the public databases using the BLASTN algorithm to identify sequences with a high degree (≥98%) of similarity. The in-silico translated sequences were aligned using the ClustalW function in MegAlign (DNastar) and the phylogenetic trees of the arsenite oxidase-derived protein sequences were generated using the neighbour-joining algorithms (Saitou and Nei, 1987) in Mega IV (Tamura et al., 2007). Nineteen reference aoxB sequences from GenBank were used for the construction of the phylogenetic tree. There were a total of 269 positions within the final dataset. The concordance of this analysis was also verified using a maximum parsimony algorithm.

3. Results

3.1. Characterisation of arsenic contaminated soils

In this study, soil samples were collected from four different locations within the Chakdah block of West Bengal (Fig. 1), where As-contaminated groundwater has been used for irrigation for years. The physicochemical properties and As status of the experimental soils are presented in Table 1. Total and extractable As concentrations of the soils varied from 7.4 to 13.4 mg kg⁻¹ and from 0.7 to 2.3 mg kg⁻¹, respectively. The studied soils were neutral in reaction (pH 6.9 to 7.7), moderate-to-high in organic C (5.1–17.1 g kg⁻¹), and low in available N (119–188 kg ha⁻¹). The level of available P (21–28 kg ha⁻¹) was moderate-to-high, whereas that of the available K (123–146 kg ha⁻¹) was low-to-moderate. The microbial biomass carbon (211–439 μg g⁻¹) varied significantly among the experimental sites. Total microbial count (6.31–6.39 log CFU) and As resistant microbial count (3.28–4.35 log CFU) of the experimental soils did not exhibit significant differences. Of the different physicochemical properties, only soil pH exhibited significant correlation with total microbial (r = 0.837) and As resistant microbial (r = 0.811) populations.

3.2. Isolation and purification of arsenic resistant bacteria

One hundred and eight indigenous bacteria were isolated from the contaminated soils by enrichment in LB medium the spiked with increasing concentrations of As. Among these isolates, 55 exhibited
different colony morphologies and grew in the presence of both As(III) and As(V). These isolates were selected and purified for further studies.

3.3. Screening of arsenic oxidising bacteria

Twelve bacterial isolates were screened as As-oxidising bacteria using a standard silver nitrate (AgNO₃) method, which produced a brown precipitate as a reaction product between As(V) and AgNO₃ after 3 days of incubation. Arsenite transforming ability of the isolates was confirmed by microplate screening assay. The AGH-02, ADP-25, AGO-S5 and AMO-10 strains emerged as the most efficient strains to oxidise As(III) to As(V) in the experimental media (data not shown).

3.4. Identification and phylogenetic analysis of arsenic oxidising bacterial isolates

The As-oxidising bacterial isolates were identified as firmicutes based on their 16S rRNA gene sequences (Fig. 2). The isolates exhibited significant sequence homologies with different members of the genus Bacillus (Bacillus flexus, Bacillus megaterium, Bacillus pumilus) and Geobacillus (Table 2). A comparative sequence analysis of the 16S rRNA gene fragment, concordant with sequences obtained from previously reported As-oxidising bacteria, revealed that all of the isolates form a monophyletic cluster along with the genus Bacillus. The phylogenetic tree exhibited an optimal branch length sum of 1.13010145. The

Table 1
Physiochemical and microbiological properties of experimental soils.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Soil-1</th>
<th>Soil-2</th>
<th>Soil-3</th>
<th>Soil-4</th>
<th>Soil-5</th>
<th>Soil-6</th>
<th>Soil-7</th>
<th>Soil-8</th>
</tr>
</thead>
<tbody>
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<td>Soil pH</td>
<td>7.5 a</td>
<td>7.2 a</td>
<td>7.5 a</td>
<td>7.6 a</td>
<td>7.0 a</td>
<td>7.7 a</td>
<td>7.2 a</td>
<td></td>
</tr>
<tr>
<td>Organic C (g kg⁻¹)</td>
<td>7.3 cd</td>
<td>5.1 d</td>
<td>11.4 b</td>
<td>9.9 bc</td>
<td>6.1 d</td>
<td>17.1 a</td>
<td>11.4 b</td>
<td>6.8 cd</td>
</tr>
<tr>
<td>Available N (kg ha⁻¹)</td>
<td>119 e</td>
<td>136 d</td>
<td>174 ab</td>
<td>172 ab</td>
<td>146 cd</td>
<td>188 a</td>
<td>160 bc</td>
<td>146 cd</td>
</tr>
<tr>
<td>Available P (kg ha⁻¹)</td>
<td>28 a</td>
<td>23 ab</td>
<td>27 ab</td>
<td>21 b</td>
<td>25 ab</td>
<td>26 ab</td>
<td>25 ab</td>
<td>24 ab</td>
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<td>Available K (kg ha⁻¹)</td>
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<td>134 b</td>
<td>123 c</td>
<td>127 c</td>
<td>132 b</td>
<td>146 a</td>
<td>137 b</td>
<td>135 b</td>
</tr>
<tr>
<td>Total As (mg kg⁻¹)</td>
<td>13.4 a</td>
<td>11.6 a</td>
<td>8.4 ab</td>
<td>7.4 ab</td>
<td>8.9 ab</td>
<td>12.0 a</td>
<td>13.2 a</td>
<td>11.4 a</td>
</tr>
<tr>
<td>Available As (mg kg⁻¹)</td>
<td>2.3 a</td>
<td>1.4 b</td>
<td>6.7 c</td>
<td>1 bc</td>
<td>1.4 b</td>
<td>1.9 ab</td>
<td>1.4 b</td>
<td>1.3 b</td>
</tr>
<tr>
<td>MBC (μg g⁻¹ of soil)</td>
<td>439 a</td>
<td>355 b</td>
<td>359 b</td>
<td>352 b</td>
<td>391 b</td>
<td>241 c</td>
<td>243 c</td>
<td>211 c</td>
</tr>
<tr>
<td>Total bacterial populations (log CFU)</td>
<td>6.37 a</td>
<td>6.34 a</td>
<td>6.34 a</td>
<td>6.39 a</td>
<td>6.31 a</td>
<td>6.33 a</td>
<td>6.36 a</td>
<td>6.33 a</td>
</tr>
<tr>
<td>As resistant bacterial populations (log CFU)</td>
<td>4.35 a</td>
<td>4.31 a</td>
<td>4.31 a</td>
<td>4.34 a</td>
<td>3.28 a</td>
<td>3.30 a</td>
<td>4.34 a</td>
<td>4.30 a</td>
</tr>
</tbody>
</table>

Figures denoted by letters are statistically different at 5% probability level by DMRT (N = 3).

Fig. 2. Phylogenetic tree based on partial 16S rRNA gene sequences, including 16S rRNA gene sequences of screened arsenic oxidising bacterial isolates from arsenic contaminated soil and other arsenic oxidising bacterial isolates from the database. The database accession numbers are indicated after the name of bacteria.
3.5. Resistance to As(III) and As(V)

The bacterial isolates exhibited considerable resistance to As toxicity with varying levels of tolerance (Table 4). The twelve As-oxidising bacterial isolates examined were found to be hyper-tolerant of As(V) [i.e., resistant to As concentrations in excess of 100 mM (Jackson et al., 2005)]. The MIC of inorganic As for the selected strains varied within a range of 167–40 mM and 16–47 mM for As(V) and As(III), respectively. The MIC of As(III) and As(V) for the As-oxidising bacterial strains was observed to be significantly correlated \( r = 0.764 \) with each other. Relatively reduced tolerance to As(III) was exhibited by the selected isolates when compared to their tolerance for As(V). This might be attributed to the increased toxicity of As(III). The highest tolerance to As(V) (400 mM and As(III) (47 mM) was exhibited by AMO-10. Isolate AGH-02 (which exhibited 98% sequence similarity with Geobacillus stearothermophilus) also emerged as an As hyper-tolerant bacteria, exhibiting resistance of up to 380 mM and 40 mM for As(V) and As(III), respectively.

3.6. Arsenite transformation and species detection

The rate of As(III) oxidation exhibited by the selected bacterial isolates varied between 278 and 1250 \( \mu \text{M h}^{-1} \), which accounted for 22–100% of the oxidation of the total As(III) present in media (Table 4). The AMO-10 strain was able to completely oxidise 30 mM of As(III) within 24 h. The AGH-02, AMO-10, AGO-55, and ADP-25 strains could oxidise 83%, 100%, 74%, and 80% of As(III), respectively, within 24 h and were subjected to As speciation analysis.

Asper speciation studies of the culture broth recorded the sum of As species recovered as 66.6–98.8% of the total As present in the media, which justified the methodology for these speciation studies.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>GenBank accession number</th>
<th>Most closely related organism based on 16S rRNA gene sequence analysis</th>
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<td>AGH-02</td>
<td>HQ834293</td>
<td>Bacillus stearothermophilus</td>
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<td>HQ834294</td>
<td>Bacillus sp.</td>
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<td>HQ834295</td>
<td>Bacillus flexus</td>
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<td>AGH-31</td>
<td>HQ834297</td>
<td>Bacillus sp.</td>
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<td>AMO-7A</td>
<td>HQ834303</td>
<td>Bacillus flexus</td>
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<td>AMO-10</td>
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<td>HQ834292</td>
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<table>
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<tr>
<th>Bacterial isolates</th>
<th>Sugars utilization</th>
<th>Starch hydrolysis</th>
<th>Nitrate reduction</th>
<th>Catalase activity</th>
<th>Gelatinase activity</th>
<th>Indole production</th>
<th>Methyl red reaction</th>
<th>VP reaction</th>
<th>Citrate utilization</th>
<th>Oxidase activity</th>
<th>Growth on TSI agar slant</th>
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<tr>
<td></td>
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Table 2
Identification of arsenic oxidising bacterial isolates.

Table 3
Biochemical characteristics of arsenic oxidising bacterial isolates.
using a HPLC-coupled with ICP-MS (Table 5). The transformation of As(III) to As(V) in the culture media varied from 0 (non-inoculated control) to 52.2% (media inoculated with AMO-10) by the selected bacterial strains (Table 5). The efficiency of AMO-10 for oxidising As(III) in culture media as determined using the spectrophotometric method (Cummings et al., 1999) was further substantiated by speciation studies that involved chromatographic separation and mass spectrometric detection. Thus, the relative efficiencies of the strains for oxidising As(III), were as follows: AMO-10 > AGH-02 > AGO-S5 > ADP-25. The percent recoveries of the transformed species [As(V)] were significantly reduced as determined by HPLC–ICP-MS analysis, probably due to recoveries that were below instrument detection levels. The AMO-10 and AGH-02 strains appeared as the optimal strains in terms of their ability to oxidise As(III) to less vulnerable As(V).

### Arsenite oxidase assay

The specific activity of arsenite oxidase enzyme in crude cellular lysates of the isolates varied from 2.1 to 12.5 nM min⁻¹ mg⁻¹ protein (Fig. 3). The AMO-10 strain exhibited the highest enzyme activity (12.5 nM min⁻¹ mg⁻¹ protein), followed by AGH-02 and then the others. The bacterial strains exhibiting higher As(III) oxidation capacity also exhibited more favourable arsenite oxidase activity.

### Determination of arsenite oxidase aoxB gene and phylogeny

The aoxB gene, which is responsible for the expression of the arsenite oxidase, was detected in four selected bacterial isolates, which exhibited maximal As(III) oxidation in culture media. A fragment of about 1200 bp was amplified from the genomic DNA of selected bacterial strains (Fig. 4). The sequence of the large subunit of arsenite oxidase aoxB gene was translated into its respective amino acid sequence, and a phylogenetic tree was constructed (Fig. 5) along with previously deposited arsenite oxidase sequences. The nucleotide sequences were submitted to GenBank, and the accession numbers for these sequences were provided in Fig. 5. Phylogenetic analyses of aoxB in AGO-S5 (JX188051), through neighbour-joining and maximum parsimony algorithms, demonstrated that the arsenite oxidase of this group clustered with Achromobacter spanius aoxB (AB638437) and the AGO-02 (JX188050) with Variovorax sp.4-2 (EU304263). The aoxB sequences present in the AMO-10 (JX188052) and ADP-25 (JX188049) strains were largely unclustered but derived from a common ancestor (Salmeron et al., 2011).

### Discussion

The unique characteristic of the As contamination within the experimental sites examined (the deltaic region of river Ganges) is its geogenic origin. Agricultural soils (sink) are polluted via irrigation with contaminated underground water (Saha and Ali, 2006). The level of contamination and the abundance of As species in such contaminated soils are principally governed by soil reactions, redox situations and organic matters (Mandal and Suzuki, 2002), which also influence the soil microbial populations (Zouboulis et al., 2004). The area investigated in this study is principally a rice-based ecosystem, which renders the soil matrices as a waterlogged and anoxic condition for most of the growing season. This promotes conditions that favour the predominance of reduced As species, which are more soluble, mobile and toxic.

Historically, the diversity of As resistant bacteria has been found to be increased in long-term contaminated soils, where resident microbial communities adapt to metal or metalloid stress (Oliveira et al., 2009). In this investigation, the study areas were predominated by heterotrophs (probably due to increased soil organic fractions (Bachate et al., 2009)), which were exposed to As contamination for prolonged periods of time. Hence, these bacteria are expected to develop intrinsic mechanisms of resistance/tolerance to acute metal (As) contamination.

An example of such a metabolic mechanism is the biological oxidation of toxic As(III) to less mobile and less toxic As(V) by different bacterial species. *B. arsenicoxidans* is likely the first reported As-oxidising bacterial strain that was identified in cattle-dipping fluids in South Africa (Green, 1918). Most of the other As-oxidising bacteria were identified and isolated from contaminated mines (Santini et al., 2000), hot creeks (Salmassi et al., 2002), ground water (Fan et al., 2008), sediments...
of the genus Stenotrophomonas (Bahar et al., 2012) have been reported. Members of the genus Achromobacter (Cai et al., 2009), Bacillus (Salmassi et al., 2002), Agrobacterium (Salmassi et al., 2002), Bacillus thuringensis (Pepi et al., 2007) and other members (Ike et al., 2008) represent the most frequently reported As-oxidising bacteria, similar to those observed in this investigation. Cuebas et al. (2011) isolated a strain of Geobacillus kaustophilus from geothermal soil, which exhibited significant As tolerance. In this investigation, similar findings were obtained when a strain of G. stearothermophilus, uniquely isolated from As-contaminated rice soil, exhibited significant As resistance.

Microbial resistance to toxic As is quite common in natural environments (Valenzuela et al., 2009). Bacterial strains that exhibit resistance to As(V) are generally increased in population numbers than those that exhibit resistance to As(III), which is likely due to the increased toxicity of the latter in soil matrices. Microbes that are able to resist As(V) levels in excess of 100 mM are considered hyper-tolerant (Jackson et al., 2005). All of the isolates examined in this study were found to be resistant to both As(III) and As(V) up to concentrations of 47 and 400 mM, respectively. The levels of resistance to As(III) and As(V) were significantly increased as compared to reported As-oxidising bacteria from ground water (200 mM As(V) and 5 mM As(III); Liao et al., 2011), mines (10 mM As(III); Botes et al., 2007), soil (183 mM As(V) and 6 mM of As(III); Srivastava et al., 2012), and estuaries (400 mM As(V) and 10 mM As(III); Jackson et al., 2005).

Corynebacterium glutamicum has previously been established as a model microorganism for maximal resistance to As(III) and As(V) (400 mM As(V) and 60 mM As(III); Mateos et al., 2006). The strains isolated in this study were also found to be hyper-tolerant and almost as efficient as other previously reported strains. The increased resistance to As(III) exhibited by the native bacterial strains, might be due to the anaerobic soil environment of submerged paddy soil that is enriched in As(III) (Zha et al., 2010).

The AMO-10 strain, which was isolated in this investigation, was identified as Bacillus megaterium (based on 16S rRNA gene sequence homology) and oxidised 1250 μM of As(III) h⁻¹ (100%) in the presence of relatively increased As(III) concentrations (30 mM) in the culture media. The As oxidation rates of the isolated strains were relatively increased compared to the rates of previously identified strains, such as UPB-24 and UPB-31 (416 μM of As(III) h⁻¹ at a concentration of 5 mM; Bachate et al., 2012) and Agrobacterium albertinamagi A015 (oxidising 585 μM of As(III) in 24 h; Salmassi et al., 2002). The complete oxidation of As(III) within 40 h at relatively reduced As(III) concentrations (1 mM) by the Alcaligenes sp. RS-19, has been previously reported by Yoon et al. (2009). The increased efficiencies of As(III) oxidation by the bacterial strains in this investigation have been also quantitatively substantiated through As speciation studies.

The specific activities of enzyme arsenite oxidase in the bacterial strains assessed in this investigation were consistent with the activities of Arthrobacter sp. (10 nm min⁻¹ mg⁻¹ protein; Prasad et al., 2009) and two different strains of β-proteobacteria (12 nm min⁻¹ mg⁻¹ protein; Bachate et al., 2012). However, the constitutive expression of arsenite oxidase has been reported (Osborne et al., 2010), and it is generally inducible in nature (Anderson et al., 1992). Pronounced enzymatic activity in the presence of enhanced As(III) level in the media established the inducible character of this enzyme. Lebrun et al. (2003) previously reported that this heterodimeric enzyme is located on the outer surface of the inner membrane, which is transported to the cytoplasmic membrane. In the course of assessing the specific activity of arsenite oxidase in different strains of Bacillus sp, we report for the first time such activity in a new member G. stearothermophilus.

(Valenzuela et al., 2009) and soil (Bahar et al., 2012). Diverse groups of bacteria including Alcaligenes (Osborne and Ehrlich, 1976), Bordetella (Bachate et al., 2012), Agrobacterium (Salmassi et al., 2002), Achromobacter (Cai et al., 2009), Thermus (Gihring et al., 2001), and Stenotrophomonas (Bahar et al., 2012) have been reported. Members of the genus Bacillus, such as Bacillus arsenicus (Shivaji et al., 2005), Bacillus fusiformis and Bacillus thuringensis (Pepi et al., 2007) and other members (Ike et al., 2008) represent the most frequently reported As-oxidising bacteria, similar to those observed in this investigation. Cuebas et al. (2011) isolated a strain of Geobacillus kaustophilus from geothermal soil, which exhibited significant As tolerance. In this investigation, similar findings were obtained when a strain of G. stearothermophilus, uniquely isolated from As-contaminated rice soil, exhibited significant As resistance.

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Efficacy of indigenous soil microbes in arsenic mitigation from contaminated alluvial soil of India

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Efficacy of indigenous soil microbes in arsenic mitigation from contaminated alluvial soil of India

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Abstract Selected arsenic-volatilizing indigenous soil bacteria were isolated and their ability to form volatile arsenicals from toxic inorganic arsenic was assessed. Approximately 37 % of AsIII (under aerobic conditions) and 30 % AsV (under anaerobic conditions) were volatilized by new bacterial isolates in 3 days. In contrast to genetically modified organism, indigenous soil bacteria was capable of removing 16 % of arsenic from contaminated soil during 60 days incubation period while applied with a low-cost organic nutrient supplement (farm yard manure).

Keywords Soil bacteria · Arsenic · Volatilization · Bioremediation

Introduction Arsenic (As) is an environmental threat of geogenic and anthropogenic origin. It is prevalent in many regions of the world (Tripathi et al. 2007; Mandal and Suzuki 2002), especially in the lower Ganges river Plain of Bengal, India (Rahman et al. 2005) and Bangladesh (Smith et al. 2000). Use of As-contaminated groundwater for irrigation made the soil a major sink, affecting farmland ecosystems (Williams et al. 2009).

The solubility, mobility, and bioavailability (and hence toxicity) of arsenic in soil systems depends on the chemical form, primarily the oxidation state. Estimation of total As fails to determine the species-dependent toxicity problem of the metalloid As (Heikens et al. 2007). Trivalent arsenic inactivates enzymes and affects the respiratory system, while pentavalent arsenic is rapidly excreted from the body. Generally, inorganic arsenic species are believed to be more toxic than organic forms to living organisms, including humans and other animals (Goessler and Kuehnett 2002; Meharg and Hartley-Whitaker 2002). The toxicity of different arsenic species were often reported to vary in an order of arsenite > arsenate > mono-methylarsonate (MMA) > dimethylarsinate (DMA) (Penrose 1974; Stugeron et al. 1989). For many years it was believed that the acute toxicity of inorganic arsenic was greater than organic arsenic and hence, the methylation of inorganic arsenic was a detoxification reaction. This dogma was held because DMA\textsuperscript{V}, the primary excreted metabolite of inorganic arsenic, is less acutely toxic than inorganic arsenic, until it was reported recently that derivatives of MMA\textsuperscript{III} (LD\textsubscript{50} for mice is 2 mg As kg\textsuperscript{-1} oral feed) are much more toxic than arsenite (LD\textsubscript{50} for mice is 26 mg As kg\textsuperscript{-1} of oral feed) (Hughes 2002).

Problems related to arsenic contamination have drawn attention worldwide and several physical and chemical remediation processes have been developed (Hering et al. 1996). However, these are expensive and have limited use as poverty and contamination coexist in most As-contaminated areas in the world (Zhu et al. 2009). Considering the limitations of conventional remediation techniques, biological methods using microbes, could be explored as alternative mitigation options (Singh et al. 2008; Chandraprabha et al. 2011).

Bioremediation of As by microorganisms has been widely hailed because of their potential advantage in providing a cost-effective, eco-friendly technology for heavy-metal removal (Valls and De Lorenzo 2002). Conversion of metalloid(s) to their volatile derivatives by organisms is a well-known phenomenon in nature (Challenger et al. 1945). During arsenic volatilization, some species of fungi and bacteria methylate inorganic As species to relatively less toxic volatile methylarsenicals (Rodriguez 1999; Cernansky et al. 2009).
Arsenic biovolatilization starts with a reduction of AsV to AsIII and through a series of methylation reaction forms less toxic volatile organo-arsenicals (Turpeinen et al. 1999; Bentley and Chasteen 2002).

Formation of gaseous As has been reported (Abedin et al. 2002; Mahimairaja et al. 2005). As summarized by WHO (WHO 2001a, b), volatilization can substantially contribute to As removal from top soils, up to 12–35 % year⁻¹. Several species of soil dwelling microorganisms have shown As-volatilizing potential (Frankenberger 2002; Turpeinen et al. 2002, Islam et al. 2007; Mohapatra et al. 2008). This includes species of Penicillium and Aspergillus, which can volatilize both organic and inorganic As compounds, while different species of Pseudomonas were capable of volatilizing inorganic As. The rate of biovolatilization of AsV was observed to be 23 % while for AsIII it was 26 % by Staphylococcus sp. in 3 days from culture media (Srivastava et al. 2012). Most of the previous reports related to As volatilization involved either aerobic (Pseudomonas, Bacillus, and Alcaligenes) or anaerobic (Clostridium, Desulfovibrio, and Methanobacterium) bacteria (Bentley and Chasteen 2002).

Earlier attempts made to assess the capabilities of microorganism in decontaminating soil matrices have often dealt with genetically engineered organisms (Liu et al. 2011) or in simulated in situ situations of anthropogenic contamination. The As contamination in West Bengal is characteristically different, geogenic in origin, and widespread across waterlogged rice ecosystems which poses an unique soil micro-environment of staggered anaerobic and aerobic spells. No specific data for anaerobic soils such as flooded paddy fields have been reported, making it yet not possible to assess the importance of As behavior in paddy fields (Abedin et al. 2002; Mahimairaja et al. 2005). Thus, the present investigation has been aimed to characterize the arsenic decontaminating microorganisms, naturally isolated from the soils of the endemic area. The mechanism which coincidentally appears to regulate As may have evolved in an arsenic-rich environment and may help stake-holders of the contaminated area, principally marginal in socio-economic stratum, to provide for a low-cost, user-friendly technology for arsenic mitigation. To reach such an outcome the present study has been designed with the specific objectives to identify efficient As-volatilizing soil microbes isolated from the polluted soil and to evaluate their volatilizing potential when enriched with farm yard manure and in contaminated aerobic and anaerobic soil systems.

Materials and methods

Isolation and screening of arsenic-volatilizing microbes

Arsenic-contaminated surface soils (0–15 cm) were collected from different sites of Chakdah block of Nadia district, India. Total and extractable As concentrations were estimated (Sparks et al. 2006). For isolation and purification of As-resistant bacterial strains, diluted soil samples prepared in sterile saline solution, were plated on solidified Luria Bertani (LB) medium (an enriched medium for growth of the isolates under stressed conditions) with either 500 mg L⁻¹ AsIII or 1,000 mg L⁻¹ AsV and incubated at 30 °C for 48 h. Arsenic volatilization capacities of 65 purified bacterial isolates were analyzed by a modified trapping method (Gao and Burau 1997). Single colonies of As-resistant bacteria were grown in closed vials containing 50 mL LB medium with 25 mg L⁻¹ AsV incubated at 30 °C for 3 days and capped with mercuric nitrate impregnated filter paper (Edvantoro et al. 2004). Trapping material (filter paper) was prepared by soaking Whatman 541 cellulose filter paper with Hg₂(ONO₂)₂·2H₂O in 10 % acetic acid water for 3 h and then air-dried (Pearce et al. 1998). Volatilized As was trapped in filter paper and analyzed for total As in an atomic absorption spectrophotometer (PerkinElmer, USA) coupled with FIAS 400 (Sarkar et al. 2012).

Tolerance to inorganic arsenic

Tolerance to AsV and AsIII were determined for each isolate by growing them separately in 20 mL LB liquid medium spiked with increasing concentrations of AsV (0 to 500 mM) and AsIII (0 to 100 mM) and incubated for 48 h at 30 °C. Controls for each concentration were inoculated with cell suspension grown in LB medium without As to obtain a cell density of approximately 10⁶ CFU (colony forming unit) mL⁻¹. Bacterial growth was monitored by measuring the optical density at 600 nm with a UV–vis spectrophotometer (Varian Cary-50 UV–vis). Bacterial isolates that could tolerate the highest As concentrations were selected and identified by their morphological features and biochemical properties (Holtz 1993).

Identification of the bacterial isolates based on 16S rDNA sequence analysis

Bacterial isolates showing considerable AsIII-volatilizing ability and higher As tolerance capacity were selected for molecular identification by 16S rDNA sequence analysis. Total genomic DNA was extracted (Sambrook 2001), and PCR amplification of 16S rDNA gene with forward primer 27F 5′-AGA GTT TGA TCM TGG CTC AG-3′ and the reverse primer 1492R 5′-GGY TAC CTT GTT ACM AG-3′ (Chromious Biotech Private Limited, India) were carried out. The reaction mixture composed of 1X PCR buffer, 0.2 mM dNTPs, 10 pmole of each primer, 60 ng of DNA template, 2 units of Taq DNA polymerase, and sterile deionized water to a final volume of 25 µL. Before amplification cycle, DNA was initially denatured at 94 °C for 5 min followed by 1 min
denaturation at 94 °C. After amplification, a final extension step (10 min at 72 °C) was performed. The cycling parameters consisted of 35 cycles initiated through denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, primer annealing at 52 °C for 1 min, and extension at 72 °C for 5 min. The PCR products were purified and held at 4 °C until verification through agarose gel electrophoresis (1 %).

The amplified and gel-eluted PCR fragments of the rDNA were sequenced in ABI 3100 Genetic Analyzer with primers 27F. Sequencing reaction was performed by using the Big Dye terminator cycle sequencing Kit V3.1 (Applied Biosystems, Foster City, USA) following the manufacturer’s protocol. The partial 16S rDNA sequences of the isolated strains were compared with those available in the GenBank database by BLASTN algorithm to identify sequences with a high degree (≥98 %) of similarity.

Biovolatilization of arsenic by efficient bacteria in culture medium

Volatilization of inorganic As species mediated through selected bacterial strains were estimated in aerobic and anaerobic situations. Volatilized As species were trapped in mercurous-nitrate-impregnated filter paper (Gao and Burau 1997). To investigate As volatilization, LB medium was spiked with AsV and AsIII (5, 10, 15, 20, 25, and 30 mgL⁻¹) and incubated with different bacterial strains in closed vials with mercurous-nitrate-soaked filter papers as As-trapping device. Sodium arsenate (Na₂HAsO₄, 7H₂O) and sodium arsenite (NaAsO₂) were used. Freshly prepared 100 μL cells of each culture media (10⁶CFU mL⁻¹) were added in 25 mL LB medium in filter paper capped closed vessel, incubated at room temperature 25±3 °C for 3 days (Liu et al. 2011). Anaerobic condition was maintained through N₂ sparging for 2 min to displace oxygen and the bottles were prepared under N₂ atmosphere. Each set of experiments was established in triplicate and total As content of the trapped material was measured (Sarkar et al. 2012).

Arsenic species identification during volatilization

A microwave digestion system (Multiwave 3000, Anton Par) with a rotor of 48 Teflon digestion vessels was used for sample digestion and extraction. For total As analysis 5 mL of culture media and trapped filter paper disk were taken in a clean Teflon digestion vessel and 5 mL aqua regia was added to it. The vessel was then digested in a microwave followed by a 5-min hold and analyzed by inductively coupled plasma–mass spectrometry (ICP-MS; PerkinElmer ELAN DRCe 6000) for total As. For speciation analysis 5 mL of culture media (culture broth of the previous experiment spiked with 10 mgL⁻¹ AsV was taken) and the trapped filter paper added with 10 mL of orthophosphoric acid (1 M) with ascorbic acid and placed in a microwave teflon vessel and the mixture was maintained at 60 W for 10 min and subjected for separation through an anion exchange column (Hamilton PRP-X100 F with a precolumn used for chromatographic separation). For the isocratic method, a PerkinElmer Series 200 Micro Pump was used instead of the quaternary pump and the detections and quantifications have been done through a PerkinElmer ELAN DRCe ICP-MS.

Volatilization of arsenic from arsenic-contaminated soil

Arsenic-contaminated soil used for this experimental study was collected from Ghentugachi (N 23°02′04.4″ and E 088° 34′55.5″) village of Nadia district, India. Total As concentration of the soil is 27 mg kg⁻¹ and extractable As concentration is 7 mg kg⁻¹ pH 7.51. To determine As volatilization by efficient bacterial strains, microcosm experiments were performed with 500 g of air-dried test soil in teflon-coated closed bottles through incubation with 5 mL culture of different bacterial strains. Formaldehyde (0.04 %) was used to kill the microbes in control soil. It inhibits the microbial activity but does not change the pH and dissolved nutrient concentration of the soil. Farm yard manure was used as organic source and added at 0, 2.5, and 5 g kg⁻¹ and thoroughly mixed to 500 g of soil. Volatilized As evolved from the soils was trapped in mercurous-nitrate-impregnated filter papers placed in the caps of closed bottles, incubated at room temperature (25±3 °C) for 30 and 60 days. Each experiment was performed in triplicate and appropriate moisture conditions of the soils were maintained according to the method of Edvantero et al. (2004). Arsenic trapped filter papers were removed at 10-day intervals and total soil As concentration was determined through the standard method (Sparks et al. 2006) at the end of the incubation period.

Results

Arsenic tolerance and molecular identification of bacterial strains

Selected bacterial strains, isolated from contaminated soil showed considerable resistance to As toxicity with varying levels of tolerance capacity (Table 1). Twelve bacterial isolates, having As-volatilizing activity, were subjected to As resistance tests and most of them were found tolerant to As concentration higher than 100 mM. The minimal inhibitory concentration (MIC) of inorganic As for selected strains were observed within a range of 106.6–400 mM for AsV and 10–53.3 mM for AsIII while highest tolerance to AsV
(400 mM) and AsIII (53.3 mM) was exhibited by the isolate AMT-08 and AMT-04, respectively. Molecular characterization of the strains has been carried out based on sequencing of 16S rDNA and compared with existing GenBank databases (Table 1).

Biovolatilization of arsenic by efficient bacteria in culture medium

Incubation studies with 12 selected As-volatilizing bacterial strains were conducted in cultures spiked with 25 mgL⁻¹ AsV for 72 h under aerobic and anaerobic condition to assess their As-volatilizing efficiency (Fig. 1). The bacterial isolates AMT-08 and AGH-09 showed higher As-volatilizing capacity under aerobic conditions, ADP-18 volatilized maximum As under anaerobic conditions while AMT-04 performed well in both conditions.

The As volatilization capacity of the selected bacterial strains has been initiated across a range of inorganic AsIII and AsV spiked in the system (Table 2). AMT-08 showed highest efficiency of removing both AsIII and AsV from culture media spiked with 5 mgL⁻¹ of AsIII/AsV in aerobic situation while such efficiencies tend to decrease with increasing concentration of As in media (Table 2). Similar efficiencies have been shown by ADP-18 under anaerobic situation which volatilized about 35 % AsIII and 30 % AsV. Interestingly, AMT-04 simultaneously supported considerable arsenic volatilization from aerobic as well as anaerobic systems. It is a characteristic feature of facultative aerobe, showing better performance under anaerobic environment but lesser activity under aerobic condition.

Arsenic species identification during volatilization under aerobic system

Arsenic speciation has been measured by high-performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (Hymer and Caruso 2004; Sanz et al. 2007). Arsenic speciation studies in culture broth (cells + LB medium; Table 3) and the filter trap (Table 4) showed recoveries up to 87 % of total As. Highest percent recovery in trapping filter paper was observed with strain ADP-18. All the As species recovered were AsIII and AsV with DMA and MMA at non-detectable levels. Significant proportions of AsIII have been recovered from the culture broth when the media has been solely spiked by AsV.

It is interesting to note that the As trapped in the filter paper did not show any recoveries of AsIII or DMA, MMA, arsenobetaine (As-B) which envisaged that whatever As has been volatilized during incubation was in the form of AsV, although, at the same time, AsIII and AsV coexist in the

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**Table 1** Identification and arsenic tolerance of arsenic-volatilizing soil microbes

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>MIC of AsV (mM)</th>
<th>MIC of AsIII (mM)</th>
<th>GenBank accession no.</th>
<th>Most closely related organism based on 16S rRNA gene sequence</th>
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<tr>
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<td>21.3</td>
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<td>AMT-08</td>
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<td><em>Rhodobacter sphaeroides</em></td>
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<td>AB697485</td>
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<td>213.3</td>
<td>53.3</td>
<td>AB694009</td>
<td><em>Alcaligenes faecalis</em></td>
</tr>
</tbody>
</table>

**MIC** minimal inhibitory concentration, **AsV** arsenate, **AsIII** arsenite
Table 2  Arsenite (AsIII) and arsenate (AsV) volatilization (mg L$^{-1}$) through bacterial cultures exposed to varying concentrations in 3 days of incubation

<table>
<thead>
<tr>
<th></th>
<th>Aerobic</th>
<th></th>
<th></th>
<th></th>
<th>Anaerobic</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninoculated control</td>
<td>AGH-09</td>
<td>AMT-04</td>
<td>AMT-08</td>
<td>Percent volatalization</td>
<td>Uninoculated control</td>
<td>ADP-18</td>
<td>AMT-04</td>
</tr>
<tr>
<td>AsIII (mg L$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>BDL</td>
<td>1.72±0.10</td>
<td>1.42±0.09</td>
<td>1.86±0.12</td>
<td>25.00 a</td>
<td>BDL</td>
<td>1.76±0.08</td>
<td>1.5±0.06</td>
</tr>
<tr>
<td>10</td>
<td>0.2±0.02</td>
<td>2.82±0.13</td>
<td>1.88±0.10</td>
<td>3.01±0.12</td>
<td>19.78 ab</td>
<td>0.25±0.03</td>
<td>2.98±0.03</td>
<td>2.65±0.09</td>
</tr>
<tr>
<td>15</td>
<td>0.32±0.02</td>
<td>3.2±0.10</td>
<td>2.01±0.11</td>
<td>3.56±0.26</td>
<td>12.78 bc</td>
<td>0.42±0.01</td>
<td>4.3±0.26</td>
<td>3.5±0.15</td>
</tr>
<tr>
<td>20</td>
<td>0.41±0.02</td>
<td>3.74±0.10</td>
<td>2.4±0.11</td>
<td>4.37±0.16</td>
<td>13.65 bc</td>
<td>0.51±0.02</td>
<td>5.33±0.08</td>
<td>3.61±0.15</td>
</tr>
<tr>
<td>25</td>
<td>0.52±0.02</td>
<td>3.98±0.09</td>
<td>3.73±0.17</td>
<td>4.92±0.11</td>
<td>14.15 bc</td>
<td>0.64±0.04</td>
<td>3.6±0.18</td>
<td>3.73±0.10</td>
</tr>
<tr>
<td>30</td>
<td>0.65±0.03</td>
<td>3.1±0.18</td>
<td>2.82±0.10</td>
<td>3.6±0.06</td>
<td>8.52 e</td>
<td>0.8±0.03</td>
<td>2.7±0.12</td>
<td>3.03±0.19</td>
</tr>
<tr>
<td>AsV (mgL$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>BDL</td>
<td>0.98±0.04</td>
<td>0.90±0.02</td>
<td>1.27±0.02</td>
<td>15.75 a</td>
<td>BDL</td>
<td>1.5±0.22</td>
<td>1.3±0.12</td>
</tr>
<tr>
<td>10</td>
<td>0.31±0.02</td>
<td>1.35±0.11</td>
<td>0.94±0.04</td>
<td>1.85±0.09</td>
<td>11.13 a</td>
<td>0.28±0.05</td>
<td>1.8±0.03</td>
<td>1.57±0.04</td>
</tr>
<tr>
<td>15</td>
<td>0.43±0.03</td>
<td>1.85±0.11</td>
<td>1.11±0.07</td>
<td>2.65±0.15</td>
<td>10.06 a</td>
<td>0.36±0.03</td>
<td>1.98±0.05</td>
<td>1.85±0.04</td>
</tr>
<tr>
<td>20</td>
<td>0.56±0.03</td>
<td>2.73±0.18</td>
<td>1.88±0.06</td>
<td>3.4±0.05</td>
<td>10.71 a</td>
<td>0.47±0.01</td>
<td>2.6±0.03</td>
<td>1.91±0.02</td>
</tr>
<tr>
<td>25</td>
<td>0.77±0.07</td>
<td>3.5±0.31</td>
<td>2.44±0.06</td>
<td>3.8±0.12</td>
<td>10.51 a</td>
<td>0.61±0.02</td>
<td>3.85±0.04</td>
<td>2.97±0.17</td>
</tr>
<tr>
<td>30</td>
<td>0.81±0.05</td>
<td>2.5±0.28</td>
<td>1.21±0.05</td>
<td>2.74±0.03</td>
<td>4.37 b</td>
<td>0.88±0.03</td>
<td>3.12±0.11</td>
<td>2.73±0.11</td>
</tr>
</tbody>
</table>

Figures denoted by letters are statistically different at 5 % probability level. M±SD (N=3)

NB arsenic volatilizations were determined in tri-acid digested filter trap, BDL below detectable limits.
culture broth at the end of incubation inoculated with almost all bacterial strains. The unaccounted part of total As which could not be retrieved by summing up the individual species, may be due to other As species (DMA, MMA, As-B etc.) in nondetectable proportions both in filter-trapped accumulation and in the cell + LB medium.

Arsenic removal from contaminated soil by microbial volatilization

To take an account of in situ As volatilization efficiencies of selected bacterial strains, contaminated soils were incubated for 30 and 60 days with efficient As-volatilizing bacterial inoculants with or without organic supplementation through farm yard manure. Significant variations in As volatilization were observed with varying inoculants and organic supplementation as compared to respective controls (Table 5). Arsenic removal from the soil system through volatilization by bacterial strains were observed to increase with advancement of incubation period irrespective of the bacterial strains involved which has been further stimulated with increasing application of organic amendment.

AMT-08 has been found most successful in managing removal of the metalloid from soil throughout the entire incubation period (Fig. 2) and recorded a maximum 4.22 mg kg\(^{-1}\) at 60th day of incubation when the soil has been amended with 5 g kg\(^{-1}\) FYM of soil. It is interesting to note that significant arsenic recoveries in filter traps were also obtained from un-inoculated controls when amended with FYM at varying levels.

Discussion

Arsenic tolerance

Microbes developed various intrinsic As tolerance mechanism to sustain in the adverse environmental conditions. Metal resistant of bacteria often have genes located on plasmids. Genetic system named \(\text{ars}\) operon is the main functional unit for As resistance (Mukhopadhyay and Rosen 2002). \(\text{ars}^R\) is the repressor of the operon, \(\text{ars}^B\) can pump out As\(\text{III}\) present within the cell, \(\text{ars}^C\) is the As\(\text{V}\) reductase (Joshi et al. 2009). The bacterial strains isolated and studied through the present investigation have shown much higher resistance to As\(\text{V}\) than those isolated from soil, gold mines, and geothermal effluents in the related researches throughout world (Saltikov and Olson 2002; Simeonova et al. 2004). The bacterial strains under the present investigation were isolated from anaerobic soil environment (submerged paddy soil) which is predominated by As\(\text{III}\) over As\(\text{V}\) and hence showed much higher tolerance to As\(\text{III}\) as compared to findings from related research established as a model microorganism for bioremediation of arsenic and one of the most arsenic-resistant microorganisms (400 mM for As\(\text{V}\) and 60 mM for As\(\text{III}\)) described to date (Mateos et al. 2006). The selected tolerant genera, \textit{Rhodobacter sphaeroides} and \textit{Alcaligenes faecalis} have not been previously isolated from arsenic-contaminated soil environments and no report exists about their role in arsenic bioremediation. Bacteria capable of arsenic volatilization isolated herein are genetically diverse which is in conformity with previous findings (Bentley and Chasteen 2002).

Table 3 Arsenic species recoveries from cells and LB medium (media spiked with 10 mg L\(^{-1}\) As\(\text{V}\))

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>As(\text{III}) (mg L(^{-1}))</th>
<th>As(\text{V}) (mg L(^{-1}))</th>
<th>Species sum (mg L(^{-1}))</th>
<th>Total As (mg L(^{-1}))</th>
<th>Percent transformation (As(\text{V}) to As(\text{III}))</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated control</td>
<td>ND</td>
<td>6.12 a</td>
<td>6.12 ab</td>
<td>8.65 a</td>
<td>–</td>
<td>70.8 b</td>
</tr>
<tr>
<td>ADP-18</td>
<td>4.65 a</td>
<td>1.94 c</td>
<td>6.59 a</td>
<td>9.21 a</td>
<td>46.5 b</td>
<td>71.6 b</td>
</tr>
<tr>
<td>AGH-09</td>
<td>3.34 b</td>
<td>4.13 b</td>
<td>7.47 a</td>
<td>9.40 a</td>
<td>33.4 c</td>
<td>79.5 a</td>
</tr>
<tr>
<td>AMT-04</td>
<td>1.42 c</td>
<td>2.89 bc</td>
<td>4.31 b</td>
<td>8.60 a</td>
<td>14.2 d</td>
<td>50.1 c</td>
</tr>
<tr>
<td>AMT-08</td>
<td>5.40 a</td>
<td>ND</td>
<td>5.40 ab</td>
<td>8.20 a</td>
<td>54.2 a</td>
<td>65.9 b</td>
</tr>
</tbody>
</table>

Table 4 Arsenic species recoveries in filter paper trap

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>As(\text{III}) (mg L(^{-1}))</th>
<th>As(\text{V}) (mg L(^{-1}))</th>
<th>Species sum (mg L(^{-1}))</th>
<th>Total As (mg L(^{-1}))</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated control</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>ADP-18</td>
<td>ND</td>
<td>1.58 ab</td>
<td>1.58 a</td>
<td>1.80 ab</td>
<td>87.7 a</td>
</tr>
<tr>
<td>AGH-09</td>
<td>ND</td>
<td>2.06 a</td>
<td>2.06 a</td>
<td>2.81 a</td>
<td>73.3 c</td>
</tr>
<tr>
<td>AMT-04</td>
<td>ND</td>
<td>1.67 ab</td>
<td>1.67 a</td>
<td>2.54 a</td>
<td>65.7 d</td>
</tr>
<tr>
<td>AMT-08</td>
<td>ND</td>
<td>2.51 a</td>
<td>2.51 a</td>
<td>3.22 a</td>
<td>77.9 b</td>
</tr>
</tbody>
</table>
Bacteria mediated arsenic volatilization from culture media

Biovolatilization of As from culture media inoculated with 12 selected bacterial strains showed (Fig. 1) varying capabilities of As volatilization exhibited by different strains under aerobic and anaerobic conditions. Some strains (AMT-04, ADP-03) remained versatile in both the systems (aerobic and anaerobic). Similar observations have also been recorded by Mohapatra et al. (2008) who found that As volatilization decreased with increase in concentration of AsV and reached equilibrium after certain period of time.

The bacterial volatilization of As also depends on substrate and AsIII volatilization rates have been found to be relatively higher than AsV both from aerobic and anaerobic systems (Table 2, Fig. 3). Some microbes can take up AsV via phosphate transporter and then reduce AsV internally to AsIII which is then extruded from the cell (Joshi et al. 2009). This may be the probable reason of getting AsIII though we started the experiment with sole AsV. In case of methylation AsIII is a reaction intermediate of AsV reduction to MMA, DMA, TMA, and supposed to be more readily reduced than AsV. The rate of biovolatilization was 23 % (AsV) and 26 % (AsIII) in case of Staphylococcus sp (Srivastava et al. 2012) also supported the present findings. Fungal strains, Aspergillus niger, Aspergillus clavatus, and Neosartorya fischeri on the contrary, did not show any mentionable difference in volatilization output from AsIII or AsV system (Cernansky et al. 2009). This investigation identified the bacterial strain having As volatilization potential in both aerobic and anaerobic conditions from different inorganic As species.

Bacteria mediated arsenic volatilization from soil

With a view to develop an efficient biological tool for decontaminating As in soil matrices, volatilization was checked in naturally contaminated soil. Selected bacterial
strains used in the present investigation showed varying capabilities of removing As from soil systems through volatilization which has been further stimulated when supplemented by external application of organic. These observations were supported by previous research reports that supplemented C source, which provided additional energy for microbes, was able to stimulate As volatilization (Edvantor et al. 2004; Lee et al. 2005). Unlike other incubation study experiments, where the authors had sterilized the soils prior to microcosm inoculation (Turpeinen et al. 2002), soils were not sterilized in this study. We had tried to assess volatilization of As from soils by microcosms consisting of As-volatilizing soil bacteria under natural condition in presence of native microbial residents of soil. This would imitate the true conditions when the microcosms would be applied for As mitigation in contaminated area, since it would depict the competitive existence and volatilizing activity of the applied bacterial strains in presence of other native soil bacteria.

Wide range of microorganisms, belonging to Methanobacterium, Bacillus, Pseudomonas, Streptococcus, Staphylococcus, Aspergillus, Penicillium, and Scopulariopsis strains, have been identified as potential producers of volatile As (Cullen and Reimer 1989). In the present investigation an indigenous soil bacterial strain AMT-08 have the ability to remove 10 % (in 30 days) and 16 % (in 60 days) As when supplemented with farm yard manure. Similarly, Woolson (1977) reported 18 % loss of As in 160 days under aerobic conditions, from artificially As spiked soils, whereas the reported strain AMT-08 is capable of removing twice As per day from soil. The strain AMT-08 has an increased As-volatilizing ability with exogenous nutrients (farm yard manure) and successful exploitation of these hyper-tolerant isolates may deliver an eco-friendly tool for As mitigation within manageable expenditure as compared to genetically engineered alternatives.

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Woolson EA (1977) Fate of arsenicals in different environmental substrates. Environ Health Perspect 19:73–81

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Retting of jute grown in arsenic contaminated area and consequent arsenic pollution in surface water bodies

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ABSTRACT

Arsenic (As) toxicity of ground water in Bengal delta is a major environmental catastrophe. Cultivation of jute, a non edible crop after summer rice usually reduces arsenic load of the soil. However, during retting of jute As is present in the crop and thus increase its amount in surface water bodies. To test this hypothesis, a study was carried out in ten farmers' field located in As affected areas of West Bengal, India. As content of soil and various the jute plant were recorded on 35 and 70 days after sowing (DAS) as well as on harvest date (110 DAS). During the study period, due to the influence of rainfall, As content of surface (0–150 mm) soil fluctuates in a narrow range. As content of jute root was in the range of 1.13 to 9.36 mg kg \(^{-1}\). As content of both root and leaf attained highest concentration on 35 DAS and continuously decreased with the increase in crop age. However, in case of shoot, the As content initially decreased by 16 to 50% during 35 to 70 DAS and on 110 DAS the value slightly increased over 70 DAS. Retting of jute in pond water increased the water As content by 0.2 to 2.0 mg L \(^{-1}\). The increment was 1.1 to 4 times higher than the WHO safe limit (0.05 mg L \(^{-1}\) water) as recommended by the World Health Organisation.

1. Introduction

Arsenic (As) is a toxic metalloid which exists in different inorganic and organic forms in nature. The trivalent As–III, and the pentavalent As–V, dominates in freshwater resources (Feng et al., 2001). In Bengal delta (comprising of West Bengal, India and Bangladesh), source of As pollution is geogenic (Roychowdhury et al., 2002; Saha and Ali, 2006). The aquifer water of the Lower Gangetic Plain (LGP) of West Bengal used for irrigation purpose has the total As content in the range of 0.1 to 0.35 mg L \(^{-1}\) (Sarkar et al., 2012). Use As 1300–1500 mm As contaminated water for to irrigate rice increases the As load of the soil and transform it from sink to a secondary source (Brammer and Ravenscroft, 2009).

In West Bengal after summer rice farmers grow arable crops like maize, sesame and jute in those soils where the As load is high. Due to this, a notable amount of As is present in the edible parts of maize, sesame (Sinha et al., 2011) as well as in fibre and sticks of jute (Das et al., 2004).

Jute is one of the major crops grown in Bengal Delta. Both jute fibre and sticks have commercial importance. Except the early stage no parts of jute are edible to human. Thus cultivation of jute after summer rice reduces the As load of soil and indirectly has favourable impact on As intake by human. Considering its impact on soil system, jute acts as a phytostabilizer (Zhu and Rosen, 2009) in As contaminated areas of Bengal delta; after harvest, jute stems tied into bundles and dipped into the pond water for several weeks, which is termed as retting. Retting is essential to transform the bast fibre into marketable jute fibre. Retting process produces significant amount of dissolve organic carbon (DOC) in pond water (Farooq et al., 2012). The DOC favours in release of As from jute stem to water. Thus jute increases its concentration in pond...
ecosystem in spite of reduction of As load in soil, causing harm to fish population. Elevation of organics in pond-sediment plays an important role to release As from dissolution of Fe-oxide by increasing the microbial activity (Pedersen et al., 2006). During retting microbes capable of oxidise the As, may reduce the negative impact of As toxicity in pond water. Besides, same bacterial strain having pectinolytic activity helps in producing higher quality and quantity of jute fibre (Tamburini et al., 2003). In sediment, organic material percentage is elevated which play an important role to release As from dissolution of Fe oxide by increasing the microbial activity (Pedersen et al., 2006).

Considering these the present study was carried out to assess the As dynamics in jute crop field with the concomitant study of the changes in pond water As content before and after jute retting.

2. Materials and methods

2.1. Study site

Present study was carried out in an As contaminated area where total As content in ground water and surface (0–150 mm) soil were respectively 0.106–0.573 mg L\(^{-1}\) and 7.6 and 16.25 mg kg\(^{-1}\). Ten farmers field were identified in Ghetugachi village (23 02/N, longitude 88 35/E; altitude of 8.8 m amsl) of Nadia district. Soils were silty loam type (Aeric Hapludox). Climatologically the region falls under subtropical humid climate. Jute crop is grown in the rainy season, so rainfall and pan evaporation (Fig. 1) during the cropping period play crucial role on arsenic dynamics in soil-plant continuum.

2.2. Experimental programme

Fields were selected where farmers grow jute during rainy season after irrigated summer rice. Farmers’ field were selected by considering a close proximity in status of the basic soil properties (Table 1). Each farmers plot was equally divided into three parts and each part was considered as a replication. In both 2009 and 2010 cropping years, jute was shown on 9th May and was harvested on 8th September. Farmers irrigate jute crop twice as life saving irrigation to overcome the ill effect of draught spell during the early and middle parts of the crop growing period. Arsenic status of the irrigation water ranged between 0.051 and 0.162 mg L\(^{-1}\). Thus 3.06 to 9.72 mg arsenic was added to one m\(^2\) soil area (Table 2). Both surface soil (0–150 mm) and plant samples were collected from the central zone of each plot and mixed for a representative sample. Soil and plant samples were collected on 35, 70 and 110 (harvesting date) days after sowing. After completion of retting, jute stick and jute fibre samples were collected to analyse the total arsenic content. Pond water samples were collected before and after the retting operation. From every pond three samples were collected on each occasion.

2.3. Jute retting

Retting is an important process to get jute fibre from the raw jute crop. After harvesting jute stems tied into bundles and dipped into pond water. Within the water, the process of separation and extraction of fibres from non-fibrous tissues and woody part of the stem through dissolution and decomposition of pectins, gums and other mucilaginous compounds is

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**Table 1** Important soil physicochemical properties of the jute fields.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sand</td>
<td>39.4–42.2%</td>
<td>33 ± 6.9</td>
<td>Dewis and Freitas, 1984</td>
</tr>
<tr>
<td>Silt</td>
<td>37.2–39.6%</td>
<td>38 ± 0.74</td>
<td></td>
</tr>
<tr>
<td>Clay</td>
<td>15.6–17.8%</td>
<td>16 ± 0.66</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.1–7.3</td>
<td>7.15 ± 0.58</td>
<td>Jackson, 1967</td>
</tr>
<tr>
<td>Electrical conductivity (ds m(^{-1}))</td>
<td>1.06–1.21</td>
<td>1.14 ± 0.04</td>
<td>Jackson, 1967</td>
</tr>
<tr>
<td>Organic carbon (g kg(^{-1}))</td>
<td>8.86–9.95</td>
<td>9.24 ± 0.33</td>
<td>Walkley and Black, 1934</td>
</tr>
<tr>
<td>Olsen extractable arsenic (mg kg(^{-1}))</td>
<td>1.89–2.05</td>
<td>1.96 ± 0.05</td>
<td>Johnson and Barnard, 1979</td>
</tr>
<tr>
<td>Total arsenic (mg kg(^{-1}))</td>
<td>7.49–12.53</td>
<td>10.26 ± 1.9</td>
<td>Sparks et al., 2006</td>
</tr>
<tr>
<td>Available nitrogen (kg ha(^{-1}))</td>
<td>138.7–146.6</td>
<td>142.51 ± 2.6</td>
<td>Subbiah and Ajiya, 1956</td>
</tr>
<tr>
<td>Available phosphorus (kg ha(^{-1}))</td>
<td>56.9–61.3</td>
<td>59.61 ± 1.5</td>
<td>Olsen and Sommers, 1982</td>
</tr>
<tr>
<td>Available potassium (kg ha(^{-1}))</td>
<td>146.2–152.9</td>
<td>150.1 ± 2.09</td>
<td>Brown and Warncke, 1934</td>
</tr>
</tbody>
</table>
called retting (Ahmed et al., 2002). Quality of the fibre depends on chemical composition of pond water along with its microbiological status and the duration of retting (Chi et al., 1966). In the process of retting, pectic materials are broken down and the fibres are liberated. Therefore presence of pectinolytic bacterial strains in the pond water play crucial role in jute retting. In the present study jute retting was carried out for 21 days.

2.4. Ponds used for jute retting

Farmers involved in this study used ten particular ponds of Ghetugachi village for retting of the jute crop cultivated by them. The physicochemical properties of those ponds water are presented in Table 3. Total arsenic status of the water before retting was also done (Table 4).

2.5. Analysis of total arsenic content

To estimate the total As of soil, plant and water, samples were digested with tri-acid mixture (HNO3, H2SO4, HClO4 = 10:1:4 by volume) after an overnight pre-digestion. Digestion was done in sand bath at a temperature of 120 °C for 4 h until a clear solution was obtained. The solution was then filtered with Whatman number 42 filter paper and volume was made up to desired amount. After adequate dilution, if needed, the solution was reduced with 5% mixture of KI and ascorbic acid (1:1 by volume) prior to analysis and the solution was reduced with 5% mixture of KI and ascorbic acid (1:1 by volume) prior to analysis and finally measured for total As by a Perkin Elmer ANANALYST 200 atomic absorption spectrophotometer (Perkin Elmer, USA) coupled with a same make hydride generator (FIAS 400). Data are presented in table, and figures are pooled data of both the experimental years. As year wise variation in data was not significant, pooled values are presented.

2.6. Arsenic resistance capacity of the isolated bacteria

Total bacterial population (log cfu) of pre and post retted pond water were recorded by serial dilution pour plate technique in LB agar plate. Arsenic resistance bacterial strains were isolated in As amended basal salt minimal media (BSMY) and resistance capacity of the isolates were estimated by growing the isolates in BSMY media spiked with AsV (1 to 15 mg L\(^{-1}\)) or AsIII (0.1 to 5 mg L\(^{-1}\)). All the culture media were incubated at 37 °C for 48 h. Bacterial growth was monitored by measuring the optical density at 600 nm with UV–vis spectrophotometer (VARIAN CARY-50 UV–VIS). Bacterial isolates that could tolerate the highest arsenic concentration were selected and identified by their morphological features and biochemical properties (data not shown) according to Holtz (1993).

2.7. Arsenic oxidizing and pectinolytic activities of the isolated strains

Arsenic oxidizing ability of the bacterial isolates were carried out by the silver nitrate method (Lett et al., 2001) and confirmed by microplate screening assay (Simeonova et al., 2004). Pectinolytic activities of the bacterial strains were determined by growing the strains in yeast-extract–pectate medium (yeast-extract 1%, pectin 1%, NaCl 0.5%, pH 7.4). Pectinolytic potency index were calculated (P) by measuring colony diameter and inhibition zone after completing the incubation period (Arguelles-Acuna et al., 1995). The isolates which showed considerable arsenic oxidizing ability along with pectinolytic capacity was chosen for molecular identification by 16S rDNA sequence analysis. Total genomic DNA was extracted as described by Sambrook and Russel (2001), and PCR amplification of 16S rRNA gene with forward primer Y1(40) F 5′-TGG CTC AGA AGG AAG GCG GCG GC-3′ and the reverse primer Y2(337)R 5′-CCC ACT GCT GCC TCC CTG AGT AGT-3′ (Chromous Biotech Private Limited, India) were carried out. Sequencing was carried out in Chromous Biotech Private Limited; India and 16S rDNA sequences were subjected to BLAST-N analysis (http://www.ncbi.nlm.nih.gov/) to identify the most similar species.

2.8. Statistical analysis

The study was laid out in a randomized block design (RBD) where each farmer’s field was considered as a treatment replicated three times. Differences among farmers field on arsenic status of soil, root, stem, leaf, jute stick and fibre were tested with Fisher’s least significant difference (P ≤ 0.05) to test using analysis of variance as mentioned in Panse and Sukhatme (1967). Similarly arsenic status of pond water at pre and post retting period were also analysed. The statistical measurements of coefficient of determination (R\(^2\)) of the equations were calculated to assess the degree of association between two variables.

3. Results and discussion

3.1. Soil arsenic status

Initial soil As content was in the range of 7.22 to 12.51 mg kg\(^{-1}\) during 2009 while in 2010 the same increased by 0.26–3.04% (Table 5). Application of 1200–1400 mm As contaminated water as irrigation to summer rice crop prior to jute is the reason for this nominal increment of initial As content (Sarkar et al., 2012) of the soil in 2010. The temporal change in soil As status from sowing to harvest did not follow the similar trends in both the experimental years. Variation in weekly total rainfall during the cropping period between the two experimental years is the
As-V into more soluble and labile form i.e. As-III. Percolation of rainwater during that period leached more amount of As-III into lower sections recorded nominal variation between two experimental years (Fig. 1). Thus, different pattern of wetting and drying made a variation in surface soil As status in between two experimental years. On 35 days after sowing (DAS), on average, As content in 2010 was 12% higher over 2009. In 2009, an amount of 109 mm rainfall experienced during after sowing (DAS), on average, As content in 2010 was 12% higher over 2009. In 2009, an amount of 109 mm rainfall experienced during 22–28 DAS. Rainwater reduced the soil environment and transformed As-V into more soluble and labile form i.e. As-III. Percolation of rainwater during that period leached more amount of As-III in to lower layers. Under lower redox levels, higher amount of As became soluble and As content in leached water increased (Khan et al., 2009). At the end part (90 DAS onward) of the cropping season, higher amount of rainfall occurred in 2010 than 2009. Due to this, on average, soil As content in 2010 was 3.1% lower over 2009. Amount of rainfall also influenced the temporal changes in As content in each experimental year. During 35 to 70 DAS, soil As status in general, increased by 9.1 to 48 and 1.9 to 6.7% respectively in 2009 and 2010 experimental years (Table 5). Application of supplemental irrigation on 40th and 60th DAS is the reason for the increment.

3.2. Arsenic status in plant parts

Unlike soil, As content in different parts of jute crop on three observation dates recorded nominal variation between two experimental years leading to pooled presentation of data for root, stem, leaf, stick and fibre of jute crop (Table 6). Jute is an arable crop and As-V is the dominant species of total inorganic arsenic in jute soil (Sinha et al., 2011). In contrast farmers grow rice under submerged condition where As-III is the dominant species. It is well-known that As-V is less mobile and less soluble over As-III (Abedin and Meharg, 2002). This has been well reflected on As content in different parts of jute plant, where the concentration was notably low in comparison to that of rice. Present study showed that in case of jute total As content in various plant parts was in the order of Root>leaf>Shoot. Sinha et al., 2011 also reported similar trend for sesame crop which is also an arable crop like jute. From the present study it might also be stated that rainfall distribution had nominal impact on As content of jute. Irrespective of plant parts, As content decreased from 35 to 110 DAS, through 70 DAS. On 35 DAS As content of jute root was in the range of 2.75 to 9.26 mg kg$^{-1}$ (Table 6). Within a span of next 35 days i.e. from 35 to 70 DAS the same decreased by 15 to 59%. During the later part (70–110 DAS) of the vegetative phase, the decreasing trend was nominal (3 to 5%). Present study showed that like rice and many other arable crops (Sarkar et al., 2012), As content in jute root showed a very strong relationship ($r = 0.81$) with soil As content (Fig. 2). The relationship was linear and from the equation it could be stated that one unit of As (mg kg$^{-1}$) in root was regulated by 1.28 mg kg$^{-1}$ As in soil.

On the first observation date (25 DAS), As content in jute leaf among different cases was in the range of 0.96–3.72 mg kg$^{-1}$. With the advancement of time from 35 to 70 DAS, As content of jute leaves decreased by 14 to 28%. Though the pattern was similar to that of root but the magnitude of decrease is markedly low in case of leaf. At harvest, because of leaf fall it was not possible to measure the leaf As content. As content of jute leaf showed a moderately strong relationship ($r = 0.74$) with the As content of soil (Fig. 3).

**Table 5**

<table>
<thead>
<tr>
<th>Farmer's name</th>
<th>Days after sowing, 2009</th>
<th>Days after sowing, 2010</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>Miyunjir Rahaman</td>
<td>7.22</td>
<td>7.13</td>
</tr>
<tr>
<td>Ajhara Tarafder</td>
<td>9.32</td>
<td>8.78</td>
</tr>
<tr>
<td>Ram Biswas</td>
<td>10.05</td>
<td>9.23</td>
</tr>
<tr>
<td>Robin Mallick</td>
<td>11.68</td>
<td>8.56</td>
</tr>
<tr>
<td>Laxman Ghosh</td>
<td>9.79</td>
<td>4.93</td>
</tr>
<tr>
<td>Alimuddin Mondal</td>
<td>11.27</td>
<td>10.94</td>
</tr>
<tr>
<td>Prabhash Mondal</td>
<td>7.49</td>
<td>7.01</td>
</tr>
<tr>
<td>Sanatan Sarder</td>
<td>11.01</td>
<td>10.13</td>
</tr>
<tr>
<td>Milan Sarkar</td>
<td>7.5</td>
<td>7.02</td>
</tr>
<tr>
<td>SEm (±)</td>
<td>0.080</td>
<td>0.113</td>
</tr>
<tr>
<td>LSD (p=0.05)*</td>
<td>0.238</td>
<td>0.336</td>
</tr>
</tbody>
</table>

* LSD: least significant difference.

**Table 6**

<table>
<thead>
<tr>
<th>Farmer's name</th>
<th>35 DAS Root</th>
<th>70 DAS Root</th>
<th>110 DAS (harvest) Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miyunjir Rahaman</td>
<td>4.27</td>
<td>3.87</td>
<td>3.13</td>
</tr>
<tr>
<td>Ajhara Tarafder</td>
<td>5.14</td>
<td>5.13</td>
<td>5.12</td>
</tr>
<tr>
<td>Ram Biswas</td>
<td>6.87</td>
<td>6.50</td>
<td>6.23</td>
</tr>
<tr>
<td>Robin Mallick</td>
<td>7.17</td>
<td>5.23</td>
<td>5.23</td>
</tr>
<tr>
<td>Laxman Ghosh</td>
<td>6.21</td>
<td>4.82</td>
<td>4.62</td>
</tr>
<tr>
<td>Robin Ghosh</td>
<td>9.26</td>
<td>7.58</td>
<td>7.26</td>
</tr>
<tr>
<td>Alimuddin Mondal</td>
<td>6.25</td>
<td>5.26</td>
<td>5.01</td>
</tr>
<tr>
<td>Prabhash Mondal</td>
<td>2.75</td>
<td>1.44</td>
<td>1.36</td>
</tr>
<tr>
<td>Sanatan Sarder</td>
<td>4.52</td>
<td>3.21</td>
<td>3.11</td>
</tr>
<tr>
<td>Milan Sarkar</td>
<td>2.87</td>
<td>1.17</td>
<td>1.13</td>
</tr>
<tr>
<td>SEm (±)</td>
<td>0.132</td>
<td>0.250</td>
<td>0.128</td>
</tr>
<tr>
<td>LSD (p=0.05)*</td>
<td>0.392</td>
<td>0.098</td>
<td>0.380</td>
</tr>
</tbody>
</table>

* LSD: least significant difference.
Irrespective of observation dates, minimum As content among the plant parts was recorded in jute shoot. On the first observation date, As content of jute shoot was in the range of 0.36 to 1.43 mg kg\(^{-1}\) (Table 6). The same was in the range of 0.26 to 1.12 on 70 DAS. The As content of shoot reduced to 22 to 47% during this period. A strong relationship \((r = 0.77)\) was recorded between As content of soil and shoot (Fig. 4). Unlike root and leaf, there was an increase in As content of jute shoot on 105 DAS over 70 DAS. Considering As content of jute stick, jute fibre as well as increase in As content of pond water after retting, the approximate amount of As in jute stick on 110 DAS was in the range of 0.61 to 2.39 mg kg\(^{-1}\). This was 24 to 69% higher over the As content of shoot on 70 DAS. After retting concentration of As in jute fibre was 66 to 111% higher than jute stick. As content of shoot showed strongest relationship \((r = 0.99)\) with the As content of leaf, majority of the observations were close to the trend line (Fig. 5).

3.3. Arsenic status of pond water

As per Table 4, total As content in majority of the pond water used for jute retting remained within the permissible limit (0.01 to 0.08 mg L\(^{-1}\)). In contrast, As status in pond water for case nos. 5 and 6 was 5 and 3 fold higher than the permissible limit. Irrespective of cases, As status of pond water increased after the completion of jute retting (Fig. 6). Maximum increment in pond water As content occurred under case 6, where the average As content was 0.145 mg L\(^{-1}\) prior to retting. Highest As concentration of soil and different plant parts during the entire cropping season were recorded under case 6, which caused maximum, release of As from the plant system at the time of retting. During retting process, lot of dissolved organic carbon is released from the plant to pond/river water (Farooq et al., 2012). The higher concentration of organic carbon in water bodies is the reason for the release of more soluble and labile form (As-III) of inorganic As in the pond water. Present study showed that in majority of cases a notable amount of arsenic was added to the surface water bodies through jute retting. This is a very alarming situation, because the retted water used for growing fishes as well as to irrigate vegetables crop. In the year 2010, 200 mm more rainfall during the 3 weeks tenure (standard weeks 37–39) of jute retting reduced the amount of pond water As content after retting by 25 to 100% over year 2009. Very strong relationship \((r = 0.91)\) did exist in between As content of jute fibre to pond water As content after retting (Fig. 7).

3.4. Microbiological status

In 2009, the total bacterial population (log cfu) of pre-retting pond water varied from 7.18 to 7.46. The same declined by 4.2 to 8.8% in 2010 (Table 7). It was observed that in the first year due to jute retting, value of log cfu increased by 0.13 to 2.47%. In contrast in the second year the same decreased by 0.86 to 6.18%. Total bacterial population of post retting water is always higher than the pre retting samples. During
the retting of jute fibres carbohydrate compounds like sugar, glycosides and various nitrogenous compounds are released into the water. This biodegradable product creates a favourable environment for increasing bacterial population (Das et al., 2011) in post retted water. Due to 200 mm rainfall during retting period in the year 2010, this biogenic product concentration in the pond water was diluted and the total bacterial population of the water environment decreased due to unavailability of nutrients.

Twenty pure bacterial isolates were screened and As tolerance capacity of those isolates were estimated (Table 8). It was observed that out of all only six bacterial isolates could grow and withstand the As toxicity up to 8 mg mL$^{-1}$ of AsV and 1.5 mg mL$^{-1}$ of AsIII. Only one isolate (AGH-03) could grow up to 14 mg mL$^{-1}$ of AsV enriched media and two isolates (AGH-02 and AGH-03) could grow up to 2.75 mg mL$^{-1}$ of AsIII enriched media. Higher arsenic tolerant isolates (AGH-01, AGH-02, AGH-03, AGH-04 AGH-05, AGH-06) were further tested for different biochemical, morphological and some special characteristics. In general, microbial ability to grow at higher metalloid concentration is found coupled with a variety of specific mechanism of resistance and environmental factors. Smith et al. (1998) observed that many bacterial communities adapted to arsenic contaminated environment by developing resistance and tolerance mechanism. Mechanisms of resistance by microorganism include microbial surface sorption, enzymatic transformation, precipitation by oxidation/reduction reaction, and biosynthesis of metal binding proteins or extracellular polymers, whereas environmental factors may include the surrounding pH and redox potential, metal speciation, soil particulates, and soluble organic matters (Srinath et al., 2002; Zouboulis et al., 2004; Srivastava et al., 2012). Microorganisms isolated in the present study, might have the similar arsenic resistance mechanisms with different magnitudes.

3.4.1. Arsenic oxidizing and pectinolytic activities of the isolated strains

Arsenic transforming ability of the isolates were confirmed by microplate screening assay, the media showed coloured precipitate formed through reaction between AsV and AgNO$_3$. This AgNO$_3$ analysis is a confirmatory evaluation of transformation of AsIII to AsV media through bacterial strain. Two bacterial strains showed the arsenic oxidizing activity (Table 8). Ahmad (2008) isolated aerobic and anaerobic bacteria from samples of retted jute stems. They were classified in 3 genera, *Bacillus*, *Micrococcus*, and *Pseudomonas* and 13 species. All bacterial isolates isolated from jute retting water do not have pectinolytic activity, but some important species among them shows this ability. In the present study, the strain AGH-03 was capable of degrading pectin and thus accelerated the retting process. This particular strain might have some economic importance as it could convert As in its less toxic state with a crucial role in producing good quality jute fibre. To select the ecologically important bacterial strains which have pectinolytic activity, pectinolytic potency index were determined and two strains (AGH-03 and AGH-06) showed the ability to degrade pectin (Table 8). Efficient arsenite oxidizing bacterial strain AGH-03 having significant pectinolytic activities showed 99% sequence similarity with *Bacterium* C-Tj19 (HQ834294). The pectins are divided into three groups: protopectins, pectins, and pectic acids. The enzyme protopectinase hydrolyses protopectin to pectin which is broken down by the enzymepectinase to galacturonic acid and residues.
Microorganisms are capable of producing all of these enzymes used for break down the pectic substances (Chowdhury et al., 2002).

4. Conclusion

In the arsenic (As) contaminated areas of Bengal delta farmers grow jute after irrigated summer rice. Jute grows mainly as rainfed crop with one to two life-saving irrigations. Thus, soil As content in jute field either remains stable or increases marginally with time. Temporal distribution of rainfall plays a crucial role on As content of surface (0–150 mm) soil. The As concentration in various parts of jute plant were in the order of Root > Leaf > Stem. In contrast to many other crops observation recorded in this study is that, the As load in root and leaf of jute continuously decreased with the advancement of crop age. However, in case of stem, the same declined up to 70 days after sowing (DAS) and thereafter moderately increased at harvest (110 DAS). After retting of jute, farmers separate the fibre from the stick. The As content in jute fibre was two fold higher over sticks. During the process of retting, dissolved organic carbon and As from jute stem released to the pond water. This increased the pond water As content above the safe level (0.05 mg L\(^{-1}\)) prescribed by the World Health Organisation for India and Bangladesh. In the present study, we have obtained As content in post-retted pond water three to five times higher over the safe limit, even though the water As content in many of those pond was within the safe limit. During retting, bacterial strains capable of transform more toxic form of As (As-III) to less toxic form (AS-V), have also been identified. Two among these strains also possess pectinolytic activities and can improve the quality of jute fibre. Thus, cultivation of jute after summer rice may be a good option to stabilize soil As content. However, its retting pollutes surface water bodies, which will act as a new source of As toxicity in aquatic eco system.

**Table 7**

<table>
<thead>
<tr>
<th>Farmer’s name</th>
<th>Pre retting (log cfu)</th>
<th>Post retting (log cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2009</td>
<td>2010</td>
</tr>
<tr>
<td>Mujibur Rahaman</td>
<td>7.18</td>
<td>6.41</td>
</tr>
<tr>
<td>Ajabar Tarafder</td>
<td>7.20</td>
<td>6.38</td>
</tr>
<tr>
<td>Ram Biswas</td>
<td>7.30</td>
<td>7.02</td>
</tr>
<tr>
<td>Robin Mallick</td>
<td>7.43</td>
<td>6.65</td>
</tr>
<tr>
<td>Laxman Ghosh</td>
<td>7.31</td>
<td>7.01</td>
</tr>
<tr>
<td>Robin Ghosh</td>
<td>7.40</td>
<td>6.93</td>
</tr>
<tr>
<td>Alamuddin Mondal</td>
<td>7.46</td>
<td>6.93</td>
</tr>
<tr>
<td>Probalsh Mondal</td>
<td>7.45</td>
<td>6.97</td>
</tr>
<tr>
<td>Sanatan Sarder</td>
<td>7.32</td>
<td>6.50</td>
</tr>
<tr>
<td>Milan Sarkar</td>
<td>7.30</td>
<td>6.48</td>
</tr>
</tbody>
</table>

**Fig. 6.** Increase in pond water As content due to retting of jute.

**Fig. 7.** Relationship between increase in pond water As with jute fibre As content.
## Microbiological characteristics of bacterial strains isolated from the retting water during the study period.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Morphology</th>
<th>Gram character</th>
<th>Spore formation</th>
<th>Arsenic resistance capacity (mg mL(^{-1}))</th>
<th>Arsenic Oxidizing activity</th>
<th>Pectinolytic potency index</th>
<th>Pectinolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGH-01</td>
<td>Coccos</td>
<td>–Ve</td>
<td>–Ve</td>
<td>2.5 10</td>
<td>– Ve 1.88</td>
<td></td>
<td>– Ve</td>
</tr>
<tr>
<td>AGH-02</td>
<td>Short rod</td>
<td>+Ve</td>
<td>+Ve</td>
<td>2.75 12</td>
<td>+Ve ND</td>
<td>–Ve</td>
<td></td>
</tr>
<tr>
<td>AGH-03</td>
<td>Rod in chain</td>
<td>+Ve</td>
<td>+Ve</td>
<td>2.75 14</td>
<td>+Ve 3.23</td>
<td></td>
<td>+Ve</td>
</tr>
<tr>
<td>AGH-04</td>
<td>Rod in chain</td>
<td>+Ve</td>
<td>+Ve</td>
<td>1.5 8.0</td>
<td>– Ve ND</td>
<td>– Ve</td>
<td>– Ve</td>
</tr>
<tr>
<td>AGH-05</td>
<td>Coccos</td>
<td>–Ve</td>
<td>–Ve</td>
<td>2.0 12</td>
<td>– Ve ND</td>
<td>– Ve</td>
<td>– Ve</td>
</tr>
<tr>
<td>AGH-06</td>
<td>Rod in chain</td>
<td>+Ve</td>
<td>+Ve</td>
<td>1.75 10</td>
<td>–Ve 2.26</td>
<td>+Ve</td>
<td>+Ve</td>
</tr>
</tbody>
</table>

### Acknowledgements

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[NEW DELHI] A new study has identified bacterial strains capable of oxidising toxic arsenic into a less toxic form, offering a feasible and affordable solution to the problem of arsenic in soil and water.

Results of the study, to be published in the October 2013 issue of *Science of the Total Environment*, show that selected bacterial isolates of *Geobacillus stearothermophilus* could completely oxidise 30 millimolars of toxic inorganic arsenite (AsIII) into less toxic arsenate (AsV) within 24 hours.

While both forms of arsenic occur in nature, the removal of AsIII from environmental systems is difficult due to its relatively higher solubility, whereas AsV is poorly water-soluble and less bio-available.

The researchers isolated 12 strains of bacteria from arsenic contaminated soils in West Bengal and identified four of them as good arsenic oxidisers. While the strain AMO-10 performed the best, another strain AGH-02 was found effective in the bioremediation of soil.
"We have found these strains to be more effective than any bioengineered strains so far known," Aparjita Majumdar, a microbiologist working on arsenic mitigation at the Bidhan Chandra Krishi Viswavidyalaya, Nadiya district, West Bengal, and lead author of the paper told SciDev.Net.

The bioremediation method is far cheaper than the metal decontamination technologies currently used worldwide.

Majumdar said while *G. stearothermophilus* has no known pathogenic properties, more tests are required before it can be declared safe for use in bioremediation.

Arsenic toxicity in water is a major problem in the state of West Bengal and adjacent Bangladesh. The element disrupts the activity of bodily enzymes, affects skin, liver and other organs and is also known to cause cancer.

Under a World Bank-funded project, Majumdar’s team has been searching for affordable, biological ways to prevent arsenic from entering the food chain. Two types of bacteria can achieve this — one which converts arsenic into volatile methylated arsenic and another that oxidises it.

Oxidising bacteria deployed in rice paddies can reduce arsenic uptake into rice grains — a major source of toxicity in humans.

While the existence of arsenite-oxidising bacteria has been known for many years, the new paper adds to the body of knowledge in the area, says David Polya, professor of environmental geochemistry, school of earth, atmospheric and environmental sciences, University of Manchester.

Polya cautions, however, that bioremediation methods using *G. stearothermophilus* need to be studied further.

[Link to article in Science of the Total Environment](#)