Evaluation of arsenic removal potential of arsenic resistant bacteria with the role of physiological and genomic factors

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Arsenic contamination in drinking ground water has reached an alarming situation in parts of Bengal Delta, India. Development of methods for removal of arsenic contamination from the drinking water will be of great importance for the mankind. The main objective of this study was to isolate and characterize arsenic resistant bacteria (ARB) from the surface water of Kabar Wetland (6.066±0.23 mg/L As) located in Begusarai district of Bihar province (25°35'N and 86°10'E), India. Bacteria were isolated from water sample by enrichment culture technique and based on their 16S rRNA gene sequences, isolates tolerating 150mM arsenate/As(V) were identified as Paracoccus sp strain NC-A and Alcaligenes faecalis strain NC-B, whereas isolate resistant to 30mM arsenite/As(III) was identified as Stenotrophomonas sp. strain NC-C. Bioinformatic analysis of genome of reference strains revealed the presence of different arsenic resistance (ars) genes. Arsenic removal efficiency of isolates was evaluated and it was observed that As(V) resistant strains of Paracoccus and Alcaligenes removed up to 84.5% and 93% arsenic, respectively from the external growth medium, whereas, As(III) resistant strain of Stenotrophomonas sp. removed 79.6% arsenic from the culture broth. The optimum pH, temperature and nutrient levels (peptone water) for arsenic removal by these isolates were 7.0, 35°C and (PW/60), respectively. These observations suggest that the strains described in this study are capable of removing arsenic from the dilute solutions, and hence being natural isolates, these strains can be easily used in the purification of arsenic contaminated water and thereby check the increasing arsenic toxicity in human food chain.

Keywords: Alcaligenes faecalis, ars genes, Arsenic toxicity, Paracoccus sp., Stenotrophomonas sp., Water pollution

Arsenic (As), a toxic metalloid, is released into the environment either by natural phenomena (weathering, volcanic eruptions) or anthropogenic activities (insecticide, pesticide, burning of gasoline, coal, mining). Arsenic mainly exists in two inorganic forms, oxyanion arsenate As (V) and arsenite As (III) species. As (V) is predominantly found in oxygenated aqueous environment, whereas As (III) species predominantly under anoxic or reduced conditions, being 100 times more toxic than As (V)2,3. Arsenate, being a phosphate analogue inhibits oxidative phosphorylation. On the other hand, arsenite binds to sulphydryl groups of proteins and dithiols such as glutaredoxin groups apart from disrupting hormone activity and cell-signalling functions4-6. Arsenic contamination has become one of the most severe threats to aquatic environment and human health. In Bangladesh about 40 million people are drinking arsenic contaminated ground water2. Bengal delta, which includes Bangladesh, eastern Indian states including Bihar has been reported as most arsenic contaminated region on earth, due to the presence of toxic level of arsenic in ground water, where 36 million people are exposed to risk of arsenic contamination7,8. Thus, removal of arsenic from contaminated drinking water is of immense importance for human health. Some of the conventional techniques involved in removal of toxic metals from dilute solutions are chemical precipitation, oxidation and reduction, ion exchange, filtration, reverse osmosis, etc. However, these methods are not only expensive but also generate secondary contaminants. The need for low cost ecofriendly technologies for water treatment has stimulated studies on bioremediation of metals9-11. Microorganisms play a major role in the biogeochemical cycle of arsenic, through its conversion to species of different oxidation states with altered solubility, mobility, bioavailability and toxicity12,13. Due to natural abundance of arsenic in environment, microorganisms have developed various resistance mechanisms such as reduction, oxidation and methylation for arsenic compounds2. The mechanism of
reduction of arsenate or oxidation of arsenite is grouped into two categories comprising of either detoxification mechanism that confer arsenic resistance, or redox reactions which conserve the energy required for the growth of microorganisms. Basically, two types of ars operons are found in bacteria, one containing five (ars RDABC) genes and other having only three (arsRBC) genes. The arsR gene encodes a transcriptional regulator (ArsR) that binds the promoter and regulate ars operon, arsA gene codes for a arsenite activated ATPase subunit (ArsA) which increases the efficiency of arsenite carrier protein (arsB) which is a membrane bound arsenite transporter that pumps out arsenite from cell and arsC encodes a reductase which converts arsenate into arsenite. In addition, arsD encodes for an arsenic metalo-chaperone that transfers As (III) to Ars A and arsM encodes for methylase which are involved in methylation and volatilization of arsenite. Both arsenate respiration and detoxification process are governed by microorganisms and contributes towards the mobilization of arsenic in soil and groundwater.

Microorganisms remove metals from dilute solution either by adsorption (bioadsorption) on their cell surfaces or accumulation inside the cytoplasm (bioaccumulation). Arsenate [As (V)] adsorbing bacteria has been used in batch reactor together with immobilizing material for removal of arsenic from waste water.

The present study describes isolation and molecular identification of arsenic resistant bacteria from Kabar wetland located in the Begusarai district of Bihar province in eastern India. Arsenic content in this district is 1.39 ppm (18.5 µM), higher than the WHO permissible limit of 0.05 ppm (0.66 µM) for developing countries. Arsenic removal by the isolate was detected by monitoring the decrease in the arsenic content of the culture broth at a regular interval.

**Materials and Methods**

Chemical and stock solutions

All the chemicals used in this study were of analytical grade and stocks solutions were prepared in deionised water. Sterilization of solution and medium used for bacterial growth were done either by filtration or autoclaving at 120°C for 15 min. Sodium arsenate (NaHAsO₄), Sodium arsenite (NaAsO₂) and media components were purchased from Merck (Germany). All the components used for PCR amplification were purchased from Genei, Bangalore, whereas for gene sequencing, BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) was used in this study.

Description of study site and sample collection

Kabar Wetland is a residual ox-bow type lake located in the Begusarai district of Bihar province in eastern India (25°35’N and 86°10’E) (Fig. 1). Stretched across 6043 ha in 2002, it is the largest wetland of northern Bihar and is a significant migratory bird reserve. It was declared a Protected Area in 1986 and has also been assigned as an...
Important Bird Area (Site Code IN BR 04). For the collection of water samples in the month of August 2010, about 25 sampling points were randomly selected to cover the entire span of the wetland. Samples were collected in sterile polyethylene containers and brought to the laboratory under cooling conditions. Samples were processed within 24 h of collection. The pH of the water sample was measured using laboratory digital pH meter (Systronics, India).

**Isolation of arsenic resistant bacteria**

Initial cell count was taken by plating 100 µL of water sample on peptone agar (PA) plates containing no arsenic. Bacteria were isolated from water sample by enrichment culture techniques. Briefly, 1 mL of water sample was inoculated into the 99 mL of peptone water (PW) containing 5 mM sodium arsenate (NaHAsO₄) and 1 mM sodium arsenite (NaAsO₂). Plates were incubated at 35°C and flasks were placed in a classic shaker incubator (CS 36a) at 35°C for 24-36 h for appearance of colonies and turbidity. About 100 µL of inoculum from the enrichment culture was spread on PA plates amended with 10, 50 and 100 mM of sodium arsenate, As (V) and 10, 15 and 20 mM of sodium arsenite, As (III).

All the plates were incubated at 35°C for 36 h and final colony count of was noted. Morphologically distinct colonies that appeared on the PA plates with highest concentrations of As (V) and As (III) were tested for tolerance of higher concentrations at 125, 150 and 175 mM As (V) and 30, 35 and 40 mM As (III).

**Growth of isolates under varying concentration of arsenic**

Isolates which were able to grow on PA plates amended with 150 mM As (V) and 30 mM As (III) was selected for further study. For testing the arsenic resistant capability of isolates, growth of the isolates was monitored in peptone water (PW) without and with different concentration of As (V) and As (III). Briefly, PW without and with different concentration of As (V) and As (III) was inoculated with 1 % of overnight grown culture of isolates. The culture flasks were then incubated at 35°C and 180 rpm in shaker incubator. Growth of the isolates were measured by monitoring the absorbance at 600 nm (double beam spectrophotometer, Systronic), at a regular interval of 2 h until stationary phase was reached.

**Identification of hyper-tolerant isolates based on 16S rRNA gene sequence**

Isolates able to tolerate 150 mM As (V) and 30 mM As (III) in culture broth were selected for identification on the basis of 16S rRNA gene sequence. Isolates were identified using following protocol used for identification.

**PCR amplification**

For amplification of 16S rRNA gene total genomic DNA of the isolates was extracted with Wizard genomic DNA purification kit (Promega, Madison, WI, USA). 16S rRNA gene was amplified using universal bacterial primer 8f (5'-AGA GTT TGA TAT GAC TTT GTT ACG ACT T-3') and 1495r (5'-CTA CGG CTA CCT TGG TAC G-3')²⁰. A 50 µL reaction mixture included 50 ng of bacterial DNA as template, 200-250 µM of each primer, and 1.0 unit of Taq DNA polymerase (Genei, Bangalore). The Polymerase chain reactions were performed on Veriti 96 well Thermal cycler (Applied Biosystem USA) under the reaction condition of an initial denaturation of 5 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 51°C, and 1 min at 72°C , with a final extension of 5 min at 72°C. The 16S rRNA gene amplicons were analyzed in 0.8% agarose gel at 5 V cm⁻¹ and visualized under UV light with Alpha imager (Alpha Innotech Corporation, UK).

**Sequencing**

For sequencing, the 1.5 kb amplicon of 16S rRNA genes were purified with Wizard SV gel PCR purification kit (Promega, USA) and quantified using ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA). Direct sequencing was performed at SBT, BHU with three primers 8f (5'-AGA GTT TGA TAT GAC TTT GTT ACG ACT T-3'), 1495r (5'-CTA CGG CTA CCT TGG TAC G-3') and 561f (5'-ATTACTGGGCGTAAAG -3')²¹ using the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) in an ABI Prism™ 310 automated DNA Sequencer (Applied biosystems, Rotkreuz, Switzerland).

**Analysis of 16S rRNA Gene Sequence**

The obtained 16S rRNA gene sequences were edited using Bioedit software version 3.1 to make a complete sequence. The almost complete sequence (1,400 nt) was compared with the nucleotide sequences present in the NCBI database using the standard nucleotide Basic Local Alignment Search Tool (BLAST)²². The programme ClustalW was used to align the 16S rRNA gene of the isolates with the similar sequences retrieved from the NCBI database to construct a phylogenetic tree.
Bioinformatic analysis of genes conferring resistant to arsenic

Based on 16S rRNA gene sequence isolates were identified as Paracoccus sp., Alcaligenes sp. and Stenotrophomonas sp. With the view to understand the arsenic resistant ability of isolates described in this study, genome sequence of Paracoccus denitrificans PD1222 (Accession no: NC_008686, NC_008687), Alcaligenes faecalis subsp. faecalis NCIB 8687 (Accession no: NZ_AKMR00000000.1) and Stenotrophomonas maltophilia (Accession no: NC_010943) available at NCBI (http://www.ncbi.nlm.nih.gov/genome/browse) was selected as reference. Genome of these reference strains was screened for the presence of genes involved in arsenic resistant mechanism viz arsM (methylases or methyl-transferases), arsA arsenite activated ATPase, arsB (arsenic resistant transmembrane transport protein), arsC (arsenate reductase), arsH (arsenic resistant proteins arsR (transcriptional regulator)23.

Evaluation of arsenic removal efficiency of isolates

Arsenic removal ability of the isolates was monitored by determining the reduction in the arsenic concentration of the culture medium after incubation10. One percent of overnight culture of hypertolerant isolates was inoculated individually in 1000 mL PW amended with 150 mM sodium arsenate and 30 mM sodium arsenite, respectively. The culture flasks were incubated under shaking condition (180 rpm) at 35°C. 100 mL samples were aseptically extracted from each flask at a regular interval of 24 h and centrifuged at 8000 rpm for 10 min to pellet down the cells. Now, this 100 mL of cell free culture broth (CFB) was digested as per APHA guidelines with 5 mL concentrated nitric acid, and its arsenic content was estimated by Hydride Generation-Atomic Absorption Spectrophotometry (Elico SL173, New Delhi). For testing the accuracy of the digestion and analytical procedures, samples containing known amounts of arsenic were also analyzed. Analysis of variance (ANOVA) were performed by using software STATISTICA for the statistical evaluation of the variations in As(V) and As(III) removal efficiency due to temperature, pH and nutrient level with the differences considered significant at P <0.05.

Factors affecting arsenic removal efficiency of isolates

Effect of pH

To determine the effect of variation in the pH on arsenic removal ability of isolates, PW medium adjusted with different pH (5.5, 7.0 and 9.5) and amended with suitable amount of arsenic (150 mM arsenate; 30 mM arsenite) was used. One percent of overnight grown culture was aseptically inoculated in the medium and flasks were incubated at 35°C under shaking condition. Residual arsenic in the CFB was determined as described above.

Effect of temperature

In order to determine the effect of temperature on the arsenic removal ability of isolates, arsenic (150 mM arsenate; 30 mM arsenite) amended PW medium were inoculated with 1% overnight grown culture of isolates and flasks were incubated under shaking conditions at three different temperatures of 20, 35 and 50°C. Residual arsenic in the CFB was determined as described above.

Effect of nutrients

For evaluation of effect of macronutrients, PW was diluted 1:20, 1:40, 1:60, 1:80 and 1:100 times to prepare dilute PW medium (PW/20, PW/40, PW/60, PW/80 and PW/100). The diluted PW medium containing suitable amount of arsenic (150 mM arsenate; 30 mM arsenite) was inoculated with 1% overnight grown culture and residual arsenic was determined in the CFB as described above.

Results

Isolation and characterization of arsenic resistant bacteria

The pH, temperature and arsenic content of the water sample collected from Kabar wetland were found to be 6.8±0.2, 32°C and 6.066 ± 0.23 mg/L, respectively. When water sample was spread on PA plates without arsenic initial cell count was found to be 19 × 10^7 CFU/mL and when inoculums from the enrichment culture was spread on arsenic amended PA plates numbers of colonies which appeared on PA plates amended with 10, 50 and 100 mM As (V) were 11, 7 and 5, respectively, and on PA plates amended with 10, 15 and 20 mM As (III) were 4, 3 and 2, respectively. The five colonies from 100 mM AS (V) and 2 colonies from 20 mM AS (III) plates were streaked on PA plates with higher concentration of 150 mM and 175 mM of As (V) and 30 mM and 40 mM of As (III), only 2 isolates (designated as PUEBK17 and PUEBK32) were able to grow on 150 mM As (V) while one isolate designated as PUEBK2 tolerated up to 30 mM As (III). The morphology of all the three isolates selected for further study was studied in details. All of these isolates were microscopically studied and morphology such as shape and gram reaction was observed by
gram staining. Two of the isolates were gram-negative rod shaped except PUEBK17 which was gram negative coccoid. Colony color, size, margins and motility of each isolate are described in (Table 1).

Identification and phylogenetic analysis of arsenic resistant bacteria

The arsenic resistant isolates were identified using 16S rRNA gene sequencing. The identification based on comparison of the 16S rRNA gene sequence of the isolates with that of other bacterial sequences existing in the GenBank database showed that As (V) resistant isolates PUEBK17 and PUEBK32 showed maximum identity with Paracoccus sp. HXG-22 (99%) and Alcaligenes faecalis strain CD234 (99%) respectively. As (III) resistant isolate PUEBKPT2 was identified as Stenotrophomonas maltophilia strain TCCC11216 (96%). The 16S rRNA gene sequences of the arsenic resistant isolates described in this study have been deposited in GenBank under accession numbers KC608760 (PUEBK17), KC608761 (PUEBK32) and KC608762 (PUEBKPT2). Phylogenetic analysis of the isolates revealed that AS (V) resistant isolates PUEBK17 clustered with α-proteobacteria and PUEBK32 clustered with β-proteobacteria. As (III) resistant isolate PUEBKPT2 grouped together with γ-proteobacteria, indicating the wide distribution of arsenic resistant bacteria in the environment (Fig. 2).

Table 1—16S rRNA gene sequence based identification and colony characteristics of hyper tolerant isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Identity [%]</th>
<th>Colony characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUEBK17</td>
<td>Paracoccus sp. HXG-D22 [99%]</td>
<td>Orange, 2.5 mm, coccoid, non-motile</td>
</tr>
<tr>
<td>PUEBK32</td>
<td>Alcaligenes sp. JF3 [98%]</td>
<td>Light yellow, 1 mm, regular, motile</td>
</tr>
<tr>
<td>PUEBKPT2</td>
<td>Stenotrophomonas sp. NC-C [99%].</td>
<td>Translucent, 4 mm, irregular, motile</td>
</tr>
</tbody>
</table>

Growth pattern revealed the arsenic resistance ability of isolates

For evaluation of arsenic resistant ability, isolates identified as Paracoccus sp. and Alcaligenes sp. were grown in PW amended with 0, 25, 50, 75, 100, 125,
150 and 175 mM of As (V) and isolate identified as *Stenotrophomonas* sp. was grown in PW amended with 0, 15, 20, 25, 30 and 35 mM of As (III). It was observed that in normal PW (without arsenic, control) all the three isolates acquired similar growth pattern. However, in PW amended with different concentration of arsenic, concentration dependent toxic effect of As (V) and As (III) was observed. A gradual decline in growth of all three bacteria with gradual increase in the concentration of As (V) and As (III) showed the inhibitory effect of arsenic on the growth of the isolates. In the presence of arsenic growth pattern of all the three isolates exhibited extended lag phase when compared to control. Growth of isolates was reduced to half at 150 mM As (V) and 30 mM As (III) when compared to control and no growth was observed at 175 mM As (V) and 35 mM As (III) suggesting that 150 mM As (V) and 30 mM As (III) is the maximum tolerance level (minimum inhibitory concentration) for the isolates identified as *Paracoccus* sp., *Alcanigenes* sp. and *Stenotrophomonas* sp., respectively (Fig. 3).

Genome analysis revealed the presence of gene involved in arsenic resistance

Bioinformatic analysis of genomes of the organisms which are closely related to our isolates revealed the presence of several arsenic resistant genes encoding proteins/enzymes conferring arsenic resistance to isolates. Genome analysis of reference strain *Paracoccus denitrificans* PD1222 (Accession no: NC_008686, NC_008687) revealed the presence of genes encoding arsenical resistant proteins (YP_914024), transcription regulatory protein (YP_913978), arsenate reductase (YP_916059) and methylases (YP_915329). Reference strain *Alcanigenes faecalis* subsp. *faecalis* NCIB 8687 (Accession no: NZ_AKMR0000000.1) harbours genes encoding arsenate reductase (EJC65569), arsenical pump proteins (EJC65626), transcription regulatory protein (EJC65628), arsenite oxidase subunit AioA (EJC61961), arsenite oxidase subunit AioB (EJC61955), arsenite activated ATPase (EJC61994). In reference strain *Stenotrophomonas maltophilia* (Accession no: NC_010943) possessed genes encoding arsenite transmembrane efflux pump transporter protein (YP_001970100), arsenate reductase (YP_001970101), transcription regulatory protein (YP_0019700102), arsenic resistant protein (YP_00972208) and arsenic resistant transmembrane transport protein (YP_00972211).

**Evaluation of arsenic removal efficiency of isolates**

For characterizing the arsenic removal ability, isolates were grown at 35°C in PW (pH 7.0) amended with 150 mM As (V) and 30 mM As (III) respectively. When the residual amount of arsenic was detected in the cell free culture broth, it was observed that As (V) resistant strains of *Paracoccus* sp. and *Alcanigenes* sp. were capable of removing 84.5 and 93% of arsenic, respectively from the external growth medium within 35 days. Whereas, As (III) resistant strain of *Stenotrophomonas* sp. strain NC-C removed 79.6% arsenic from the culture broth (Fig. 4).
Effect of physiochemical parameters on arsenic removal efficiency

Since, *Alcaligenes faecalis* strain NC-B and *Stenotrophomonas* sp. strain NC-C, were most efficient in removal of As (V) and As (III), respectively from the growth medium, these two strains were selected to evaluate the effect of different parameters on arsenic removal. At 35\textsuperscript{th} day, As (V) removal efficiency of *Alcaligenes* sp. was highest (93%) at 35°C when compared to 19.4% of As (V) removal at 20°C and at 50°C negligible amount of arsenic removal was observed. Similarly, *Alcaligenes* sp. removed 93% of As (V) at pH value of 7.0 and its efficiency to remove As (V) from culture broth was adversely affected when pH was shifted from pH 7.0. Also, *Alcaligenes* sp. was able to remove As (V) maximally (97.25%) at PW/60 as compared to PW/40 (94.76%), PW/20 (93.84%) and PW/100 (26.58%) (Fig. 5). Effects of temperature and nutrient levels were statistically significant, but change in pH and incubation period did not have significant effect on As (V) removal efficiency of *Alcaligenes* sp. (Table 2).

*Stenotrophomonas* was tested to standardize the best conditions for As (III) remediation. It was observed that the growth of the isolate and its subsequent As (III) removal efficiency at pH 9.5 was significantly reduced to 25% when compared to 85.33% at pH 7.0. *Stenotrophomonas* sp. was able to remove maximum 88% of As (III) at PW/60 and 79.6% at a temperature of 35°C (Fig. 6). The effect of pH, nutrient levels and incubation period had a significant effect on the As (III) remediation efficiency of *Stenotrophomonas* sp., although, temperature was perceived not to have statistical significance (Table 2).

**Discussion**

Microbial removal of arsenic contamination from water has been proved very efficient and eco-friendly approach and has drawn our attention towards isolation of native bacteria from highly arsenic contaminated region. Chakraborti and colleagues\textsuperscript{18} have reported arsenic contamination of this studied region, a part of Bengal delta, above

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**Table 2**—Statistical evaluation of the variations in AsV and AsIII removal efficiency of *Alcaligenes faecalis* and *Stenotrophomonas* sp. respectively, due to temperature, pH, nutrient levels and incubation period using analysis of variance (ANOVA)

<table>
<thead>
<tr>
<th>Factors</th>
<th>As (V)</th>
<th>As (III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>0.0046*</td>
<td>0.0510</td>
</tr>
<tr>
<td>pH</td>
<td>0.0872</td>
<td>0.0089*</td>
</tr>
<tr>
<td>Nutrient Levels</td>
<td>0.0000*</td>
<td>0.0000*</td>
</tr>
<tr>
<td>Time</td>
<td>0.2306</td>
<td>0.0226*</td>
</tr>
</tbody>
</table>

[Values marked with asterisk indicate significant differences at *P* < 0.05]
permissible limits at 1654 µg/L. This report influenced us to choose one of the site for the study that is Kabar wetland situated in the Begusarai district (25°35’N and 86°10’E) of Bihar province in eastern India. Arsenic concentration of surface water of Kabar wetland is estimated to be 82 mM. The catchment area of the wetland is agricultural field. Thus, the origin of arsenic may be fertilizers and insecticides.

In the present study, total viable count on peptone agar (PA) plates without arsenic was not high, it seems that total fractions of native bacteria which are viable is not substantial, therefore an enrichment culture technique was used for isolation of arsenic resistant bacteria\(^2\). With increasing concentration of arsenic the percentage of isolates growing decreased gradually\(^1\). The bacterial species isolated and identified in this study have shown higher tolerance to arsenic. Isolates, identified as *Paracoccus* sp. and *Alcaligenes* sp., were capable of growing in presence of 150 mM of arsenate. Earlier, in studies of ARB investigators had reported strains of *Paracoccus* sp. resistant to 50 mM, and *Alcaligenes* sp. resistant to 350 mM of arsenate\(^2, 24\). The third isolate described in this study was identified to be *Stenotrophomonas* sp., exhibiting significant resistance to 30 mM of As (III). Similar to our findings, different other workers have reported different strains of *Stenotrophomonas* sp. resistant to arsenite\(^16, 25\). Phylogenetic analysis of isolates revealed its wide distribution within eubacteria. Bioinformatic analysis of genome of organisms closely related to our isolates revealed the presence of several arsenic resistant genes which confer arsenic resistant ability to our isolates. However, number and types of arsenic resistant genes present in our isolate may vary when compared to closely related organisms selected for this analysis.

While studying the arsenic resistant ability of isolates, we found a gradual decline in the growth of isolates with increase in the arsenic concentration. Growth of the isolates was less in presence of arsenite when compared to arsenate indicating that arsenite was more toxic than arsenate, which was similar to other reports\(^25\). Growth of the isolates at 150 mM As (V) and 30 mM As (III) indicates the arsenic resistant ability of isolate. Both arsenate [As (V)] and arsenite [As (III)] resistant isolates were able to remove arsenic from the growth medium optimally at 35°C and pH 7.0. Since pH and temperature of the water sample was 6.8±0.2 and 32°C, respectively. Therefore, removal of maximum arsenic at the physiological temperature and pH indicates the active biotransformation of arsenic by the isolates. The pH and redox conditions are highly significant variables controlling the arsenic speciation\(^26\) and varying temperature and pH from optimum is likely to affect bacterial cell growth itself. Thus, this factor emerges as highly significant and the necessity to standardize them is of immense importance. Arsenic removal efficiency of the all the three isolates was found to be maximum at pH 7.0 and 35°C, shift from optimum pH and temperature inhibits the growth of isolates, ultimately reducing the biotransformation/removal efficiency of the isolates. Similar to our observation, growth inhibition of arsenate remediating *Neisseria* at acidic pH and inhibition of arsenite [As (III)] remediation by *Pseudomonas* at alkaline pH had been
reported previously. Mechanisms involved in removal of arsenic from culture broth may be accumulation inside the cytoplasm or adsorption of arsenic on microbial cell surfaces. In this study one of the possible mechanisms involved in arsenic removal may be adsorption of arsenate on the cell surfaces of the arsenate resistant isolates. In case of *Stenotrophomonas* sp. (arsenate resistant), arsenate is possibly oxidized to arsenate (mediated by arsenite oxidase) which is then adsorbed to cell surface and gets removed from the culture broth. For biotransformation/detoxification of arsenic microorganisms has also developed several other strategies such as arsenite oxidation, cytoplasmic reduction of arsenate respiratory arsenate reduction, arsenite methylation and sequestration by a range of cysteine-rich peptides such as γ-glutamylcysteine (γ-EC) and glutathione. Growth study of the isolates in presence of different concentration of arsenic suggests the arsenic biotransformation/bioremediation ability of isolates. When growth pattern of isolates was studied, an extended lag phase was observed under varying concentration of arsenic. During the lengthened lag phase, cells are preparing to cope up with the adverse effect of arsenic by synthesizing the required machinery (enzymes/transporter) conferring arsenic resistant to isolates. Further, isolate’s culture reached the early stationary phase at the end of 28th hours and removed maximum arsenic within seven days of growth. These observations suggest that at the end of lag phase, maximum proteins (enzymes/transporter) involved in biotransformation/bioremediation of arsenic have been synthesized and which remain active and stable for the period of first seven days only. Thus, after seven days of growth no more arsenic can be removed from the culture broth and only residual arsenic can be detected in the supernatant.

When isolates were grown in lower strength of PW (PW/60), both As (V) and As (III) removal efficiency was highest, with maximum inhibition at PW/100. Because of its structural analogy of inorganic phosphate, arsenate [As (V)] enters the cell via phosphate uptake system and gets accumulated in cytoplasm, inside the cytoplasm arsenate [As (V)] is either involved in respiratory reduction pathway or reduced to arsenite, which is further methylated to volatile trimethyl arsine gas. Therefore, high concentration of phosphate is been known to inhibit arsenic uptake by tolerant bacterial cells. With 60% dilution of PW (PW/60) the competing phosphate content of PW gets reduced, therefore more arsenate [As (V)] ions can enter the cell compared to PW, PW/20 and PW/40 ultimately decreasing arsenate [As (V)] concentration in the culture broth. However, with PW/80 and PW/100, the phosphate concentration gets reduced to a level which might be detrimental for cell growth. However, arsenite could only enter the cell via aquaglyceroporins (glycerol transporter) and gets accumulated and processed further into the cytoplasm. Hence arsenate generated by oxidation of arsenite enters the cytoplasm of *Stenotrophomonas* sp. in greater amount with 60% dilution of PW (PW/60) as compared to PW, PW/20 and PW/40, at PW/80 and PW/100 cells might not be able to grow due to low phosphate concentration. However, genes encoding arsenite oxidase was not detected in the reference strain *Stenotrophomonas maltophilia* (Accession no: NC_010943), but recently a new strain of arsenite oxidizing *Stenotrophomonas* sp. MM-7 had been reported which can oxidize arsenite to arsenate. In their study, they had shown the presence of arsenite oxidase genes using molecular methods. Including *Stenotrophomonas*, arsenite [As (III)] oxidation has been identified in bacteria such as *Alcaligenes* sp. and *Agrobacterium* sp.

This study reports high tolerance level of arsenate [As (V)] and arsenite [As (III)] by three common bacterial isolates, which can efficiently biotransform/remove arsenic from the culture broth. Isolates described in this study are naturally occurring and could be more environmental friendly and safe for depuration/removal of arsenic, from contaminated water bodies.

**Conclusion**

This study demonstrate identification, arsenic resistant ability and potential of arsenic removal by three bacterial isolates which might play an important role in cycling of arsenic in the Kabar wetland located in Bihar province of eastern India. Isolates described in this study are phylogenetically diverse and are well adapted to the level of arsenic found in the wetland. All of these observations suggest that strains described in this study are capable of removing arsenic from the dilute solutions, so being natural isolates these strains can be easily used in
development of an effective in situ bioremediation technology for the purification of arsenic contaminated water which would prevent the increased arsenic toxicity in human food chain.

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