

Reduction of Arsenate in a new isolate of *Bacillus megaterium*

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Abstract

In Bangladesh, the ground water of almost all the 64 districts are contaminated with arsenic and in some regions the arsenic concentration is above the World Health Organization's guideline value. Bioremediation is in demand for its removal from water especially in rural areas. In this study, four soil samples were collected from arsenic contaminated areas of Chandpur, Bangladesh. In total 58 bacterial strains resistant to arsenate were isolated. Among them I-34 has the highest arsenate reducing capability. This bacteria showed resistance to high concentration of arsenite (100 mM) and arsenate (300 mM). Its arsenate reducing enzyme is extracellular and showed highest activity at 60 °C. More interestingly, it showed auto-induction metabolism to produce arsenate reducing enzyme. Molecular characterization by 16S rDNA gene sequence analysis revealed that the bacterium is 100% identical to *Bacillus megaterium*. I-34 appears to be novel arsenic metabolizing bacteria within this genus. The bacterial isolate can be exploited for the study of possible bioremediation of arsenic containing water and have a potential impact to reduce the arsenate into arsenite form.

Key words: *Bacillus megaterium*, Arsenate, Arsenite, auto-induction, bioremediation.

INTRODUCTION

Generally arsenic is toxic to life because it disrupts ATP production through several mechanisms but some microorganisms can use arsenic compounds as electron donors, electron acceptors or can detoxify them (Ahmann *et al.*, 1994; Cervantes *et al.*, 1994). Inorganic arsenic compounds are more toxic than organic arsenic compounds (Anderson & Bruland, 1991). The most common and stable oxidation states of arsenic in the environment are the pentavalent As(V) (arsenate) and the trivalent As(III) (arsenite) forms (Cullen & Reimer, 1989). Between this two, As(III) is considered 60 times more toxic than As(V) (Ehrlich, 1996). Arsenite (AsO_2^- or AsO_3^{2-}) combined to sulfhydryl groups of proteins and dithiols such as glutaredoxin. Arsenate (AsO_4^{3-}) is structurally analogous to phosphate and can inhibit oxidative phosphorylation by producing unstable arsenilated derivatives (Anderson *et al.*, 1992; Bennett & Malamy, 1970; Da costa, 1972). Resistance to arsenic compounds of different bacteria is mediated via the ars operon. Ars operon is required for the detoxification of arsenite and arsenate. ArsA-ArsB complex responsible for production of arsenite oxidase and *ArsC* gene encodes for an arsenate reductase in bacteria (Cervantes *et al.*, 1994; Ji & Silver, 1992a; Ji & Silver, 1992b; Mobley & Rosen, 1992; Newman *et al.*, 1998).

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In Bangladesh, elevated levels of arsenic are common in ground water. According to the national drinking water quality survey report (2009), about 20 million and 45 million people of Bangladesh were found to be exposed to concentrations above the national standard of 50 µg/L and the World Health Organization's guideline value of 10 µg/L, respectively. Chronic oral exposure of inorganic arsenic leads to gastrointestinal effects, anemia, peripheral neuropathy, skin, lesions, hyper pigmentation and liver or kidney damage in humans (Flanagan *et al.*, 2012). Microbial remediation of arsenic can be used to remove arsenic compounds from environment (Say *et al.*, 2003). Identification, characterization and understanding the mechanism of metabolism of arsenic compounds of arsenic metabolizing bacteria can give insight into effective and efficient bioremediation technique to remove arsenic from environment.

The aim of the present study was to isolate bacteria from arsenic contaminated sites that could use arsenate as an electron acceptor under aerobic condition to produce arsenite and identify its various characteristics.

MATERIALS AND METHODS

Sample Collection: Four soil samples were collected from Shahrasti Upazilla, Chandpur district. Soil samples were collected from the sub-surface (from 0 to 15 cm in-depth from ground) aseptically in sterile sampling bottles and kept in 4 °C until further analysis.

Isolation of arsenate resistant bacteria: One gram of soil samples were taken in 100 mL of 0.1 M phosphate buffer solutions and mixed well by vortexing for 3 minutes. Samples were serially diluted with sterile saline water (0.85%) and plated on Yeast Extract Mannitol (YEM) agar medium {D-Mannitol (Qualikems, India), 10g; K₂HPO₄ (Scharlau, Spain), 0.5 g; MgSO₄ · 7H₂O (MERCK, India), 0.2 g; CaCl₂ (Scharlau, Spain), 0.1 g; yeast extract (TM MEDIA, India), 0.5 g; phosphate buffer solution, up to 1000 mL, pH 7.0} with sodium arsenate (Na₂HAsO₄) (LOBA Chemie, India) having final concentration of 2 mM. Then the plates were incubated at 37 °C for 48 Hrs. Pure cultures of different bacterial strains were obtained by consecutive isolation of morphologically different colonies through repeated cross streaking (Rahman *et al.*, 2018; Khanam *et al.*, 2019).

Screening for arsenate reduction activity: Screening was done by following the method described previously (Mandal *et al.*, 2007; Rahman *et al.*, 2018 and Khanam *et al.*, 2019). 1% isolated arsenate resistant bacterial cultures were inoculated in arsenate containing YEM medium having sodium arsenate final concentration of 2 mM and grown 24 Hrs at 37 °C, 120 rpm in shaking water bath (VS-1205SW1, Vision Scientific Co. Ltd., Korea). After centrifugation at 10,000 rpm by table top centrifuge (Model: Eppendorf 5418, Gernmeny) for 10 min, supernatants were collected and kept on ice. 30µL starch- Lugol's iodine complex (10% Lugol's iodine solution in 1% starch) were added to each arsenate resistant bacterial 1.5 mL supernatant and mixed well by vortexing, kept in dark for 10 min at 37 °C. Then optical density (OD) at 570 nm was measured of these supernatants immediately after 10 min. 1.5 mL of 2 mM sodium arsenite (NaAsO₂) and 2 mM sodium

arsenate (Na_2HAsO_4) solution was used as positive and negative controls. 30 μL of starch-Lugol's iodine complex was added to the positive and negative controls. Mixed well by vortexing and kept in dark at 37 °C for 10 min. Then OD_{570} were measured immediately after 10 min.

Identification of bacterial isolate: After screening, the bacteria that has the highest arsenate reducing activity was selected and its morphological, biochemical and molecular characteristics were evaluated. Gram stain and cell morphology were investigated under microscope (Optika B-350, Italy) (1000X magnification). Biochemical properties of the selected bacteria were evaluated according to Bergey's Manual of Systemic Bacteriology (Bergey *et al.*, 1984). Various biochemical tests were performed: Methyl Red (MR) test, Voges-Proskaur (VP) test, catalase test, glucose, fructose, sucrose, maltose, mannitol fermentation test, indole, citrate, gelatin and nitrate reduction test (Cappucino & Sherman, 2001).

Molecular identification was done by 16S rDNA sequence analysis. Genomic DNA was extracted by automated DNA extractor (Maxwell 16, Promega, USA) and qualified by Nanodrop Spectrophotometer (ND2000, Thermo Scientific, USA). The 16S rDNA from the extracted DNA was amplified by PCR using primers such as F (5'-AGAGTTTGATCCTGGCTCAG-3') and R (5'-ACGGCTACCTTGTTACGACTT-3'). The reaction mixture composed of 12.5 μL Hot Start Master Mix (Promega, USA), 1 μL extracted genomic DNA, 1 μL R primer (concentration 10 pMol), 1 μL F primer (concentration 10 pMol), 9.5 μL nuclease free water. PCR was done by Gene Atlas (G2, Astec, Japan). PCR profile was as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation (95 °C, 30 sec), annealing (48 °C, 30 sec), extension at 72 °C for 1 min) and final extension at 72 °C for 5 min. To confirm amplification gel electrophoresis was done using agarose, ethidium bromide, 100bp DNA ladder, TAE buffer (Promega, USA). Gel documentation was done by Alpha Imager (Mini, Protein Sample, USA). Sequencing was performed by ABI 3700 Genetic Analyzer (1st Base Laboratory SdnBhd, Malaysia). The 16S rDNA gene sequence was BLAST searched against GenBank database (<http://www.ncbi.nlm.nih.gov/>). At the same time, phylogenetic analysis was executed using Mega6- tree explorer after multiple alignment by BioEdit software (Hall, 1999; Rahman *et al.*, 2018).

Determination of As(III) and As(V) tolerance: The lowest concentration at which the bacterial growth completely inhibited is termed as maximum tolerance concentration (MTC). To determine As(V) MTC, YEM medium supplemented with different sodium arsenate (Na_2HAsO_4) concentrations (0 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM) were inoculated with cells from fresh overnight culture grown in YEM medium and incubated for 24 Hrs in shaking water bath at 37 °C, 120 rpm. After 24 Hrs, OD was measured at 600 nm.

To determine As(III) MTC, the cells were first grown 24 Hrs in YEM medium having arsenate concentration 2 mM. Then centrifuged at 10,000 rpm for 10 min and pellet was washed with autoclaved phosphate buffer solution through centrifugation at 10000 rpm

for 10 min. Pellet was resuspended in 5 mL phosphate buffer solution and inoculated in YEM medium supplemented with different sodium arsenite (Na_2AsO_2) concentrations (0 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM) and incubated for 24 Hrs in shaking water bath at 37 °C, 120 rpm. Then OD were measured at 600 nm wave length. In each case OD_{600} of the inoculated cells were measured just after inoculation.

Determination of enzyme location: To determine enzyme location, the cells were grown in arsenate containing YEM medium for 24 hours at 37 °C, 120 rpm. Then the culture was centrifuged at 10,000 rpm for 10 minutes, removed the supernatant and washed the pellet two times with autoclaved phosphate buffer solution. The pellet was resuspended into 1 mL autoclaved YEM medium (without arsenate) and incubated for 24 hours at 37 °C, 120 rpm. After 24 hours the culture was centrifuged at 10,000 rpm for 10 minutes, supernatant was collected, cell pellet was washed with autoclaved phosphate buffer solution and resuspended in 2 mL autoclaved YEM medium. To determine whether the enzyme was extracellular or not, 1.2 mL supernatant was mixed with 300 μL 10 mM sodium arsenate, incubate at 37 °C for 2 hours, and then added 30 μL of starch-Lugol's iodine complex. Mixed well by vortexing and kept in dark at 37 °C for several minutes to observe color change and measure OD at 570 nm.

To determine whether the enzyme was intracellular or not, the resuspended cells were disrupted by bench top ultrasonicator (Power sonic 603, Hwashin Technology, Seoul, Korea) for 9 Hrs at 30,000 Hz, 37 °C. To confirm cell disruption cells were spread plated before and after ultrasonic treatment. After ultrasonic treatment, it was centrifuged at 10,000 rpm for 10 minutes and supernatant was collected. Then 1.2 mL supernatant was mixed with 300 μL 10 mM sodium arsenate, incubate at 37 °C for 2 Hrs, and then added 30 μL of starch-Lugol's iodine complex. Mixed well by vortexing and kept in dark at 37 °C for several minutes to observe color change and measure OD at 570 nm. Positive and negative controls were 1.5 mL 2 mM sodium arsenite and 2 mM sodium arsenate solution respectively. 30 μL of starch-Lugol's iodine complex was added to the positive and negative controls. Mixed well by vortexing and kept in dark at 37 °C for several minutes to observe color change and measure OD at 570 nm (Khanam *et al.*, 2019).

Determination of induced metabolism: Bacterial cells were grown in normal YEM medium (for non-induction) and with sodium arsenate containing YEM medium having final arsenate concentration 2 mM (for induction) for 24 Hrs at 37 °C, 120 rpm. After centrifugation at 10,000 rpm for 10 min, supernatants (both non-induced and induced) were removed and cell pellets were washed two times with autoclaved phosphate buffer solution. Then the pellets were resuspended in autoclaved YEM medium and inoculated into 5 mL YEM medium containing tubes. The tubes were incubated at 37 °C, 120 rpm for 24 Hrs. After 24 Hrs, induced and non-induced cultures were centrifuged at 10,000 rpm for 10 minutes and supernatants were collected. Then 1.2 mL of induced and non-induced supernatants were kept in two tubes, 300 μL , 10 mM sodium arsenate were added to each tube and incubated for 2 hours at 37 °C. 30 μL starch-Lugol's iodine complex was added to each tube, mixed well by vortexing and kept in dark at 37 °C for several minutes

to observe color change and measure OD at 570 nm wave length. Positive and negative controls were 1.5 mL 2 mM sodium arsenite and 2 mM sodium arsenate solution respectively. 30 μ L of starch-Lugol's iodine complex was added to the positive and negative controls. Mixed well by vortexing and kept in dark at 37 °C for several min to observe color change and measure OD at 570 nm.

Determination of optimal temperature for enzyme activity: The cells were grown in arsenate containing YEM medium having arsenate concentration 2 mM for 24 Hrs at 37 °C, 120 rpm. Then the culture was centrifuged at 10,000 rpm for 10 min and supernatant was collected. 1.2 mL supernatants were mixed with 300 μ L 10 mM sodium arsenate and incubate at different temperatures (20 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C) for 2 Hrs. After that these were kept on ice for stopping the enzyme activity. Then 30 μ L starch-Lugol's iodine complex was added, mixed well by vortexing and kept in dark for 10 min at 37 °C. Then immediately OD₅₇₀ were measured. Also 30 μ L starch-Lugol's iodine complex was added to 1.5 mL supernatant and measured OD₅₇₀ following the same procedure. Positive and negative controls were 1.5 mL 2 mM sodium arsenite and 2 mM sodium arsenate (Na₂HAsO₄) solution respectively. 30 μ L of starch-Lugol's iodine complex was added to the positive and negative controls. Mixed well by vortexing and kept in dark at 37 °C for 10 min. Then OD₅₇₀ were measured immediately after 10 min.

RESULTS AND DISCUSSION

Isolation of arsenate resistant bacteria: Fifty eight (58) bacteria were isolated that were grown in arsenate containing YEM medium having final concentration 2 mM. These bacteria were resistant to arsenate and the following screening procedure was done to identify the bacteria that were capable of reducing arsenate to arsenite.

Screening for arsenate reduction activity: Among the 58 isolated arsenate resistant bacteria only four of them showed the ability of converting arsenate to arsenite. These bacteria converted arsenate to arsenite and the arsenite acted as a reducing agent. It broke up the polyiodide ion (I₅⁻) of starch-Lugol's iodine complex into iodine and iodide resulting in blue color disappearances. The bacteria, that produces more arsenite from arsenate can be identified by observing faster disappearance of blue color of starch-Lugol's iodine complex. Among the four arsenate reducing bacteria, I-34 is the most potent. OD₅₇₀ indicated that its culture's supernatant disappeared the blue color of starch-Lugol's iodine complex faster than that of other three strains (Figure 1). From the results, two interpretations can be made: Firstly, I-34 produces a potent arsenate reducing enzyme that can convert arsenate into arsenite very fast. Secondly, the bacteria produces high amount of arsenate reducing enzyme that can convert more arsenate into arsenite in a short period of time. Further identification and characterization was done only for this strain (I-34).

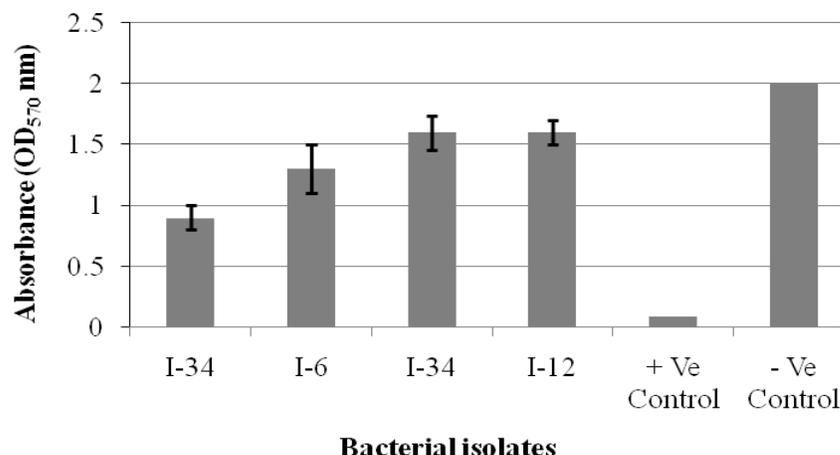


Fig. 1. Comparison of four potent arsenate reducing bacterial isolates. After 10 minutes of adding starch-Lugol's iodine complex I-34 supernatant disappeared blue color more than others, its OD₅₇₀ is less than that of other isolates. Results shown are the mean of three experiments

Identification of bacterial isolate: Based on the morphological and biochemical characteristics (Table 1), I-34 was found to be closely related to the members of genus *Bacillus* according to the Bergey's Manual of Systematic Bacteriology (1984). Molecular characterization by 16S rDNA analysis indicates that its nearest phylogenetic relative is *B. megaterium* (Figure 2).

Table 1. Morphological and biochemical characteristics of I-34

Tests	Results
Gram stain	+Ve
Shape	Rod
Methyl Red (MR) Test	+Ve
Voges-Proskaur (VP) Test	+Ve
Catalase Test	+Ve
Glucose fermentation test	+Ve
Fructose fermentation test	+Ve
Sucrose fermentation test	-Ve
Maltose fermentation test	+Ve
Mannitol fermentation test	+Ve
Urease Test	-Ve
Oxidase Test	-Ve
Indole Test	-Ve
Citrate Test	-Ve
Gelatin Test	+Ve
Nitrate Reduction Test	-Ve

Note: +Ve = positive result -Ve = negative result

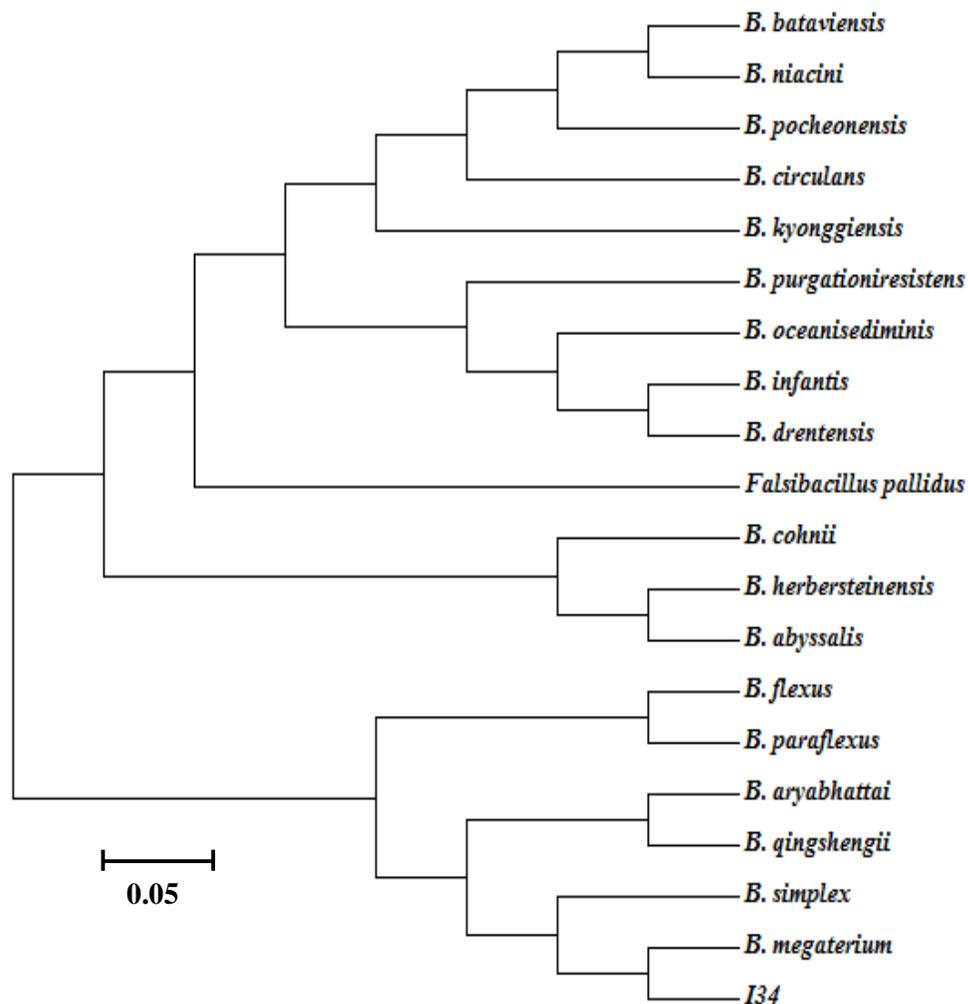


Fig. 2. Phylogenetic relationship of arsenate reducing bacteria I-34 with different species. The scale representing 0.05 substitutions per sequence site.

Determination of As(V) and As(III) tolerance: I-34 bacterial strain was grown in different arsenate and arsenite concentrations for 24 Hrs. From the results (Figure 3, 4), it can be concluded that I-34 can tolerate up to 300 mM arsenate and 100 mM arsenite. Above these concentrations the bacteria could not grow, so OD₆₀₀ of cells at 0 H and after 24 Hrs remain the same

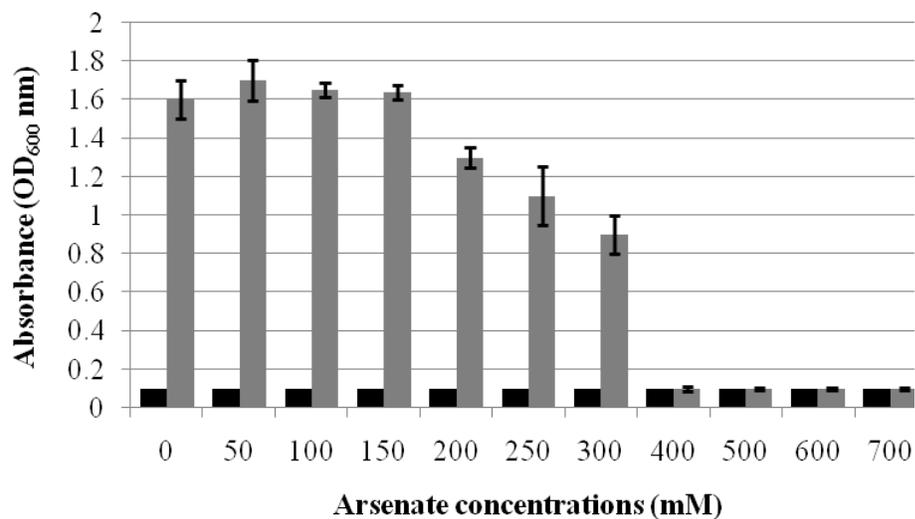


Fig. 3. Arsenate tolerance of I-34 at different arsenate concentrations. Absorbance at 600nm of initial cells at 0 hour (■) and cells after 24 hours (■). Results shown are the average of three independent experiments

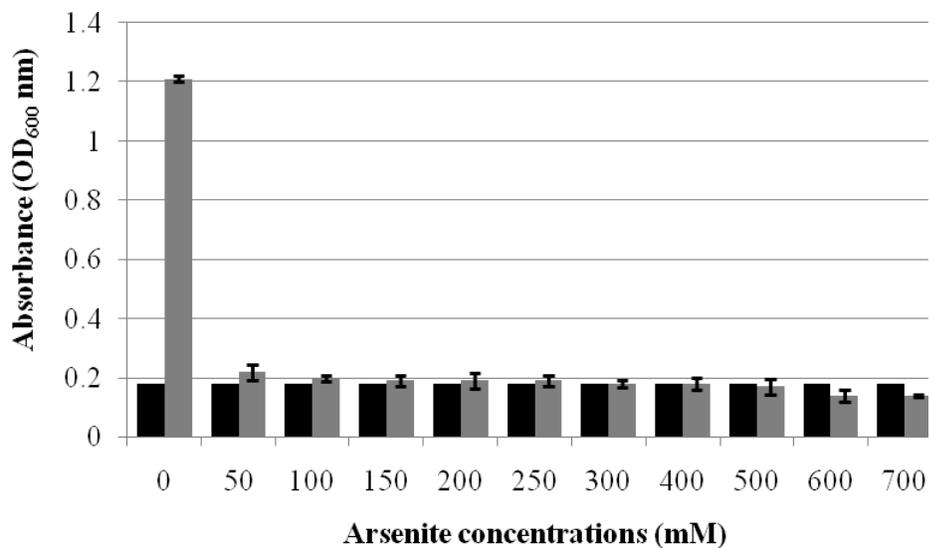


Fig. 4. Arsenite tolerance of I-34 at different arsenite concentrations. Absorbance at 600 nm of initial cells at 0 hour (■) and cells after 24 hours (■). Results shown are the average of three independent experiments

Determination of enzyme location: The conversion of arsenate to arsenite is believed to be done by arsenate reducing enzyme. The location of the active arsenate reducing enzyme of I-34 was determined. Experiments revealed that active arsenate reducing enzyme of I-34 is extracellular because extracellular supernatant disappeared the color of starch-Lugol's iodine complex as time passed by but the complex's color was not disappeared by the intracellular fluid collected after cell disruption (Figure 5). From the result, it can be interpreted that the extracellular supernatant of I-34 contained arsenate reducing enzyme, thus it converted arsenate into arsenite and arsenite broke up the polyiodide ion (I_5^-) of starch-Lugol's iodine complex into iodine and iodide resulting in blue color disappearance. After cell disruption, the intracellular fluid did not contain any arsenate reducing enzyme therefore, no color disappearance of starch-Lugol's iodine complex's occurred. OD_{570} of positive and negative controls were constant at different time after 10 minutes.

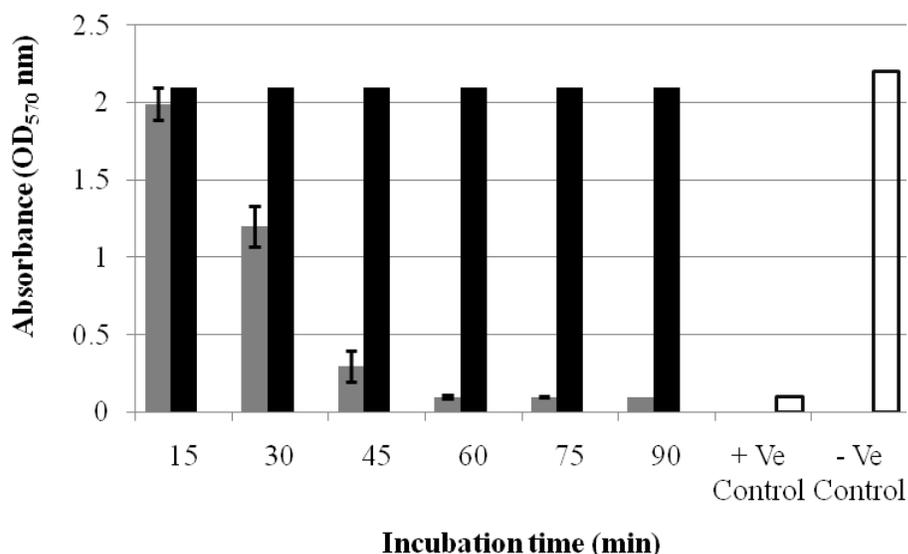


Fig. 5. Reduction of arsenate by culture supernatant (■) and cell pellet (■) of the isolate I-34. Absorbance was taken at 570 nm was investigated extracellular enzyme activity and for intracellular enzyme activity and results shown are the average of three independent experiments

Determination of arsenate induced metabolism: If a molecule induces *i.e.* initiates or enhances the expression of an enzyme, the phenomenon can be termed as enzyme induction. If the molecule induces enzymes that are responsible for its own metabolism, then it can be termed as auto-induction and it inhibits the enzyme, it can be termed as auto-inhibition. These processes are particular forms of gene expression regulation (Zhu, 2010). In our study, when I-34 was at first grown in arsenate and then in YEM medium without arsenate its supernatant showed enzymatic activity (Figure 6) because I-34 was induced by the arsenate in the medium to produce arsenate reducing enzyme. This

enzyme converted arsenate into arsenite when the supernatant was incubated with arsenate for 2 Hrs. These arsenite then disappeared the starch-Lugol's iodine complex's blue color. Auto-induction occurred in this case because arsenate enhanced the production of arsenate reducing enzyme that is responsible for arsenate metabolism. However, when bacterial cells were not grown in arsenate containing medium at first, no blue color disappearance of starch-Lugol's iodine complex's was observed by the supernatant of the culture (Figure 6). From the results, it can interpret that the production of arsenate reducing enzyme by I-34 is a result of auto-induction.

Arsenate induction necessity was also observed during bacterial growth in various concentrations of arsenite. At first when I-34 was inoculated in various arsenite concentrations, no growth of the bacterium was observed in none of the arsenite concentrations because arsenite is very much toxic. However, when the bacteria at first grown in arsenate containing medium then collected the cells and inoculated again in various arsenite concentrations, its growth observed in medium containing up to 100 mM arsenite concentration (Figure 7). When the bacteria grown in arsenate containing medium it grow well and produced arsenite using the arsenate in the medium and get resistant to this produced arsenite. Therefore, when these cells were inoculated in arsenite medium, the cells were grown well up to 100 mM arsenite concentration. OD_{570} of positive and negative controls were constant at different time after 10 minutes.

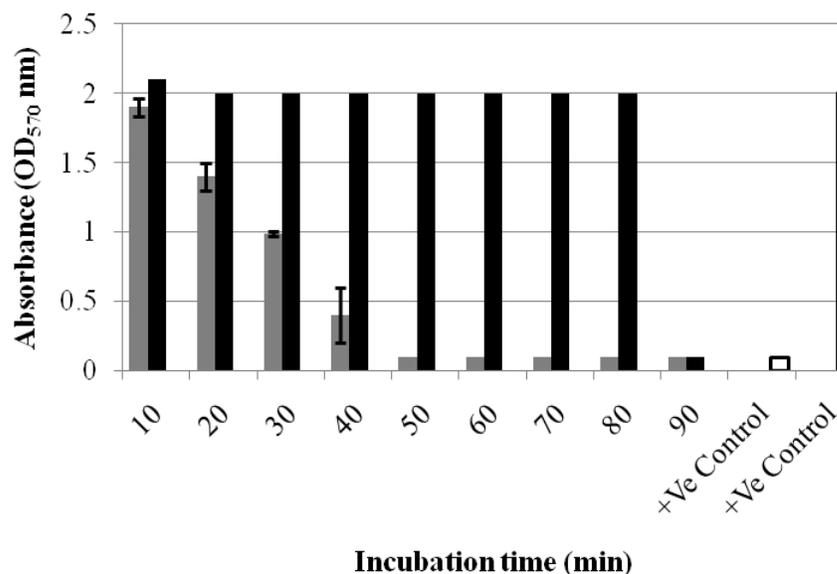


Fig. 6. Arsenate reduction at different incubation period by the isolate I-34. Results shown are the average of three experiments. Absorbance at 570 nm for induction (■) and non-induction (■)

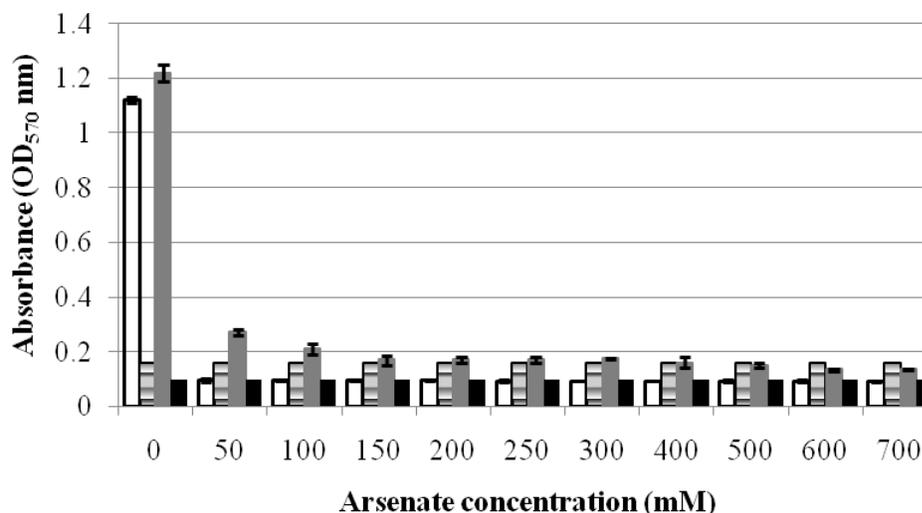


Fig. 7. Effect of arsenate concentration on growth of I-34 against arsenite. Results shown are the average of three experiments. Absorbance at 570nm of induced initial cell (▨), induced cell after 24 hours (▩), non-induced initial cells (□) and non-induced cells after 24 hours (■)

Determination of optimal temperature for enzyme activity: To determine the optimal temperature for enzyme activity, culture supernatant was studied in a range of temperature from 20 °C to 70 °C. From this experiment, it was revealed that arsenate reducing enzyme of I-34 showed good activity at 30 °C to 60 °C (Figure 8). The enzyme lost its activity at 20 °C and below temperatures and 70 °C and upper temperatures. The optimum temperature for the enzyme's best activity is 60 °C. Culture supernatant also contained arsenite because cells were grown in arsenate containing medium. Supernatant's OD₅₇₀ after adding starch-Lugol's iodine complex was also measured to show color change responsible for arsenite that the supernatant contained. OD₅₇₀ of positive and negative controls were constant at different time after 10 minutes.

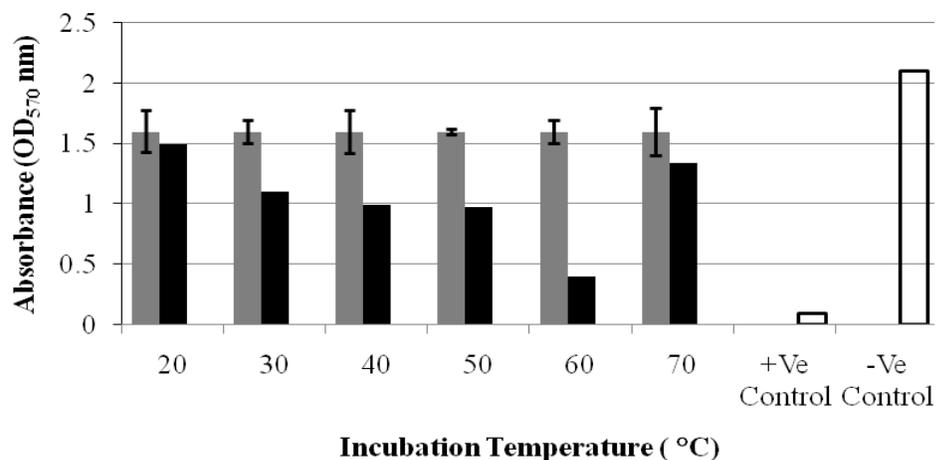


Fig. 8. Effect of incubation temperature for arsenate reducing enzyme activity of I-34. Absorbance at 570nm of supernatant at 0 hour (■) and supernatant after 2 hours incubation (■). Results shown are the average of three experiments

The ground water of almost all districts of Bangladesh is contaminated with arsenic. In some region the arsenic concentration is above the specifications of World Health Organization's guideline value (10 µg/L). Therefore, it is very important to remove these arsenic compounds from soil and ground water. Bioremediation can be a good solution of this problem. By isolating and characterizing the arsenic metabolizing bacteria and investigating their arsenic metabolism mechanism, a good insight into bioremediation process can be gained. In this study, the isolated strain I-34 is a potent arsenate reducing bacteria which is identified as *Bacillus megaterium*. Under aerobic condition, it uses arsenate for their growth and produces arsenite. This bacteria can resist high concentration of arsenite. The arsenate reductase enzyme is found extracellular and shows better activity on relatively elevated temperature. From our experiment it shows that arsenate itself induces its reduction by producing its arsenate reducing enzyme. The bacterial isolate can be exploited further for the study of possible bioremediation of arsenic containing water and have a potential impact to reduce the arsenate into arsenite form.

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