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Study of an arsenic metabolizing bacteria from arsenic contaminated soil of Chandpur district, Bangladesh

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Abstract

Arsenic is a toxic metal found as inorganic oxyanion arsenate As(V) and arsenite As (III) species. The disposal of toxic heavy metals such as arsenic poses high risk to environment. The present study was undertaken to isolate arsenic-metabolizing bacteria from arsenic contaminated soil of Chandpur district, which is one of the most arsenic contaminated area in Bangladesh and later these bacteria were screened for their ability to metabolize arsenate. Out of ninety eight isolates, ten were found to be capable of metabolizing arsenic in Yeast Extract Mannitol (YEM) medium containing 2 mM arsenate at 37ºC. One of the bacterial isolates designated as I-25 was found to produce an extracellular enzyme which can reduce As(V) into As(III) and able to grow in presence of up to 500 mM arsenate. Subsequent molecular identification of this enzyme producing bacterial isolate using 16s rRNA sequence analysis was correlated with previously identified isolate as *Bacillus aryabhatti*. Further characterization of the enzyme showed that optimum pH of the extracellular enzyme by the bacterial species was 7 and optimum temperature for the enzyme activity was 60ºC. The bacterial isolates can be exploited for the study of possible bioremediation of arsenic contamination.

Key words: Arsenic metabolizing bacteria, *Bacillus aryabhattai*, Bangladesh

INTRODUCTION

Arsenic is one of the world's most notorious poison. Consumption of arsenic contaminated water and plants grown on arsenic-contaminated soils can lead to arsenic toxicosis in animals and melanosis, gangrene, cancer and ultimately death in humans. It is a natural toxic element released into the environment by natural phenomenon (geogenic) or by anthropogenic activities (Cullen & Reimer, 1989). Arsenic contamination in ground water and irrigation fields is a serious problem in many parts of the world, especially in Bangladesh. It exists both in toxic inorganic and comparatively less toxic organic species in the environments. The most common species of inorganic arsenic are trivalent arsenite [As (III)] and pentavalent arsenate [As(V)]. Mitigation of As (III) from soil and groundwater is of great challenge and due to its higher toxicity and mobility than As (V).

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Arsenite can bind to sulfhydryl groups of proteins and dithiols such as glutaredoxin. On the other hand, arsenate is a chemical analog of phosphate and can inhibit oxidative phosphorylation (Ordonez *et al.*, 2005). It may interfere with the DNA repair system or DNA methylation state, inhibition of p53 and telomerase activities (Chou *et al*., 2001; Wang *et al.*, 2001), oxidative stress, promotion of cell proliferation and signal transduction pathways leading to the activation of transcription factors (Wu *et al.*, 1999). In addition, it has been found that arsenic induces DNA damage via the production of reactive oxygen species (Matsui *et al*., 1999).

Although arsenic is generally toxic to life, microorganisms can use arsenic compounds as electron donors, electron acceptors or show arsenic detoxification mechanisms (Ahmann *et al*., 1994). The bacteria which possess the ability to convert arsenate to arsenite are of environmental significance due to the formation of uncharged (H_3AsO_3) state which has higher mobility than arsenate. Arsenic mobility in natural environment is a major concern in arsenic-rich and contaminated areas. Dissimilatory arsenate reduction bacteria (DARB) may be involved in the solubilization, fate and transport of arsenic by reducing arsenate to arsenite (Ahmann *et al.*, 1994).

Wide varieties of microorganisms are capable of growth in presence of heavy metal ions and can tolerate high concentrations (Nies, 1992; Gaballa & Helmann, 2003; Rehman *et al.*, 2007). It has been reported that the strains of *Aeromonas*, *Exiguobacterium*, *Acinetobacter*, *Bacillus* and *Pseudomonas* that can tolerate high concentrations of arsenic up to 100 mM arsenate or up to 20 mM arsenite (Anderson & Cook (2004). The heavy metals are present in the environment naturally; therefore, microorganisms have evolved mechanisms to resist their toxic effects (White & Gadd, 1986). Various bacteria such as *Acidithiobacillus*, *Bacillus*, *Deinococcus*, *Desulfitobacterium* and *Pseudomonas* have been reported to be resistant to arsenic (Cervantes *et al.*, 1994; Oremland *et al.*, 2001; De-Vicente *et al*., 1990; Dopson *et al*., 2001; Niggemyer *et al*., 2001; Suresh *et al*., 2004).

A variety of microorganisms (iron and sulfur reducing bacteria) are known to play an important role in the biochemical cycle of arsenic, through its conversion with different solubility, mobility, bioavailability and toxicity (Silver & Phung, 2005). Arsenite (the reduced form) is more toxic and more soluble than arsenate, which is relatively insoluble, non-bioavailable compounds. Speciation of arsenic is controlled by not only oxidation and reduction processes, but also by methylation, and adsorption to other particles (Aurilio *et al.*, 1994). Bacteria, fungi, ciliates, algae, mosses, macrophytes and higher plants have several mechanisms for the conversion and removal of arsenic and other heavy metals (Holan & Volesky, 1994; Rehman *et al*., 2007).

The present study reports *B. aryabhatti* with the ability to metabolize arsenic and reduce As (V) into As (III) for the first time in Bangladesh. This *B. aryabhatti* has been previously identified and reported by our group to produce various extracellular enzymes such as α-amylases, cellulases, β-glucosidases, lipases and proteases (Rahman *et al*., 2018). However, the ability of this bacterium for arsenic metabolizing activity was never reported and there had been no report about the presence of this bacterium in arsenic

contaminated samples. The present study also investigates optimization temperature, pH and optimum arsenate concentration for maximum arsenate reduction for the extracellular enzyme produced by this bacterium.

MATERIALS AND METHODS

Sample collection: Soil sample was collected from Saharasti upazilla of Chandpur district, Bangladesh. Sample from subsurface soil (0-15 cm in depth) were collected, placed in plastic bag and kept on ice or at 4ºC until further analysis.

Chemicals and stock solutions: All buffers and solutions were prepared with distilled water and sterilized by autoclaving at 121ºC for 30 min. As(III) stock solutions (10 mM) were prepared from stock sodium arsenite ($NaAsO_2$) and As(V) solutions (10 mM) from sodium arsenate heptahydrate ($Na_2HAsO_4.7H_2O$). Stock solutions were stored at 4ºC in the dark. A starch-iodine complex (mixture of aqueous starch and Lugol's iodine solution) was prepared freshly before use.

Bacterial culture preparation and identification of isolate: For the bacterial culture preparation, 1 g of each soil sample was taken in 100 mL of 0.1 M phosphate buffer solution and mixed well by vortexing for 3 min. Each sample was serially diluted with sterile saline water and plated on Yeast Extract Mannitol (YEM) agar medium. After 16 h of incubation, stocks were prepared by dissolving the culture in equal volume with 40% glycerol. After single colony isolation, the morphological, biochemical and molecular characteristics of the isolated bacterial strains were also evaluated as previously described by Rahman *et al.* (2018).

Screening of arsenic metabolizing Bacteria: Precultures were prepared from each single colony in 5 mL YEM (without arsenate) broth and incubated at 37ºC for 24 h at 120 rpm. Then preculture was centrifuged, washed twice with sterile buffer solution and resuspended in 2 mL sterile water. Twenty µL preculture was inoculated into YEM broth medium containing 2 mM arsenate and incubated under the above condition. Then cultures were centrifuged at 10,000 × g for 5 min and supernatant was taken for further analysis. Next, 1mL of supernatant was added into test tube followed by addition of 30 µL of starch-iodine complex (Mandal *et al.*, 2007). The test tubes were incubated in dark for 10 min. Then optical density (OD) was measured by using spectrophotometer (Mecasys Optizen Pop UV/Vis Spectrophotometer, Korea) and highly active bacteria were identified from these bacterial isolates.

Identification of localization of the active enzyme: Firstly, 24 hours cultures were grown in YEM medium which contained arsenate. Then they were subjected to centrifugation at 10000 × g for 5 min and supernatant was removed. Pellet was washed twice with autoclaved phosphate buffer. Then the pellet was resuspended into 1 mL of autoclaved YEM medium (without arsenate). 100 µL of resuspended aliquots were inoculated into autoclaved 5 mL YEM medium (without arsenate) for 24 h shaking at 37ºC, 120 rpm. After 24 h of incubation, the tubes were centrifuged and supernatant (4 mL) was taken into another autoclaved test tubes. Then 1 mL arsenate (10 mM) was added into 4 mL of supernatant. After 2 h of incubation in shaking water bath (37ºC, 120

rpm), 2 mL supernatant was withdrawn and 60 µL starch-iodine complex was added into it and incubated in dark. If color change occurred, the enzyme responsible for arsenic metabolism was considered as extracellular one.

In the event of no color change, sonication was used for cell disruption. In this case, pellets were washed twice with autoclaved buffer solution by centrifugation at 10000 × g for 2 min. Then supernatant was aspirated and the pellet was dissolved into 2 mL autoclaved YEM medium. Then the cells were disrupted by using ultrasonicator at 37 ºC for 3 h. After sonication, Lugol's iodine was added and incubated in dark. Color change indicated that the enzyme responsible for arsenic metabolism was intracellular one.

Determination of optimum metabolizing condition: For determining the optimum growth of the bacterial strains, different arsenate concentrations (0 mM, 2 mM, 16 mM, 64 mM, 128 mM, 250 mM and 500 mM) in YEM medium were considered in different test tubes and autoclaved at 121°C for 30 min. Then freshly prepared bacterial culture inoculated into these concentrations and incubated for 24 h. Finally, the absorbance was measured at 600 nm.

For optimization of the temperature requirement on enzyme activity, large amount of supernatant were used. For this, 100 mL 2 mM arsenate containing YEM medium were prepared in a conical flask with 50 mL medium. Then the flask was autoclaved at 121ºC for 30 min and 500 µL of freshly prepared bacterial culture was inoculated into each flask. After 24 h incubation, 10 mL of culture was taken into 15 mL falcon tube and centrifuged at 10,000 × g for 20 min. Then supernatant was collected into test tubes (5 mL each) and stored at -20ºC. Test tubes were kept in different temperatures (30ºC, 40ºC, 50ºC, 60ºC, 70ºC, 80ºC and 90ºC) for 10 min and then cooled at room temperature. Finally starch iodine complex was added and after an incubation period, optical density was measured at 580 nm using spectrophotometer.

RESULTS AND DISCUSSION

Screening of arsenic metabolizing bacteria: For the first instance, 98 bacterial isolates was isolated from arsenic contaminated soil samples and it was found that 40 bacterial strains were able to grow in presence of arsenic. However, ten isolates showed arsenic metabolizing ability and only four strains designated as I-3, I-6, I-25, and I-112, respectively showed higher arsenic metabolizing activity of all. In all cases, soil samples were cultured into YEM medium which contained 2 mM arsenate and starch iodine complex was used as an indicator for growth. Among the different isolates, I-25 is found to be a novel arsenic metabolizing strain that was previously identified as *B. aryabhatti* according to the morphological, biochemical and molecular characteristics as reported earlier. In addition to that the ability of this strain to produce α-amylases, cellulases, β-glucosidases, lipases and proteases was reported before by our group (Rahman *et al.*, 2018). In the present investigation, the bacterial species *B. aryabhatti* showed highly active arsenic metabolizing activity as it was able to change the color of starch iodine complex within 5min of incubation at dark condition.

Screening for arsenate reductase activity: Four isolates (I-3, I-6, I-25, and I-112) were able to convert arsenate into arsenite in which, it broke up the I_5^- of starch Lugol's iodine complex into iodine and iodide and resulted in disappearance of blue color. It indicated the presence of arsenate reductase enzyme in the supernatant. Among the 4 arsenate reducing bacterial isolates, I-25 (i.e. *B. aryabhattai*) was found to be the most potent (Fig. 1 and Fig. 2). Here, positive and negative controls were sodium arsenite and sodium arsenate respectively.

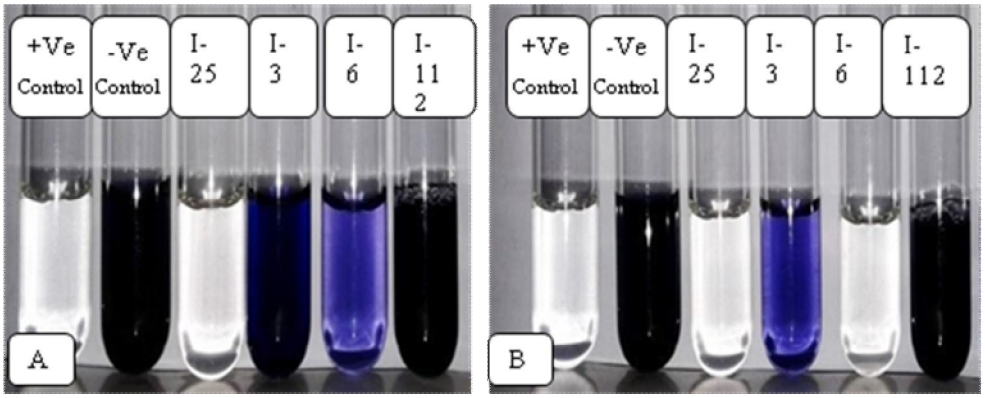

Fig. 1. Induction mechanism for arsenic metabolizing bacteria (A. Screening before conversion of arsenate into arsenite. B. Screening result after 10 min)

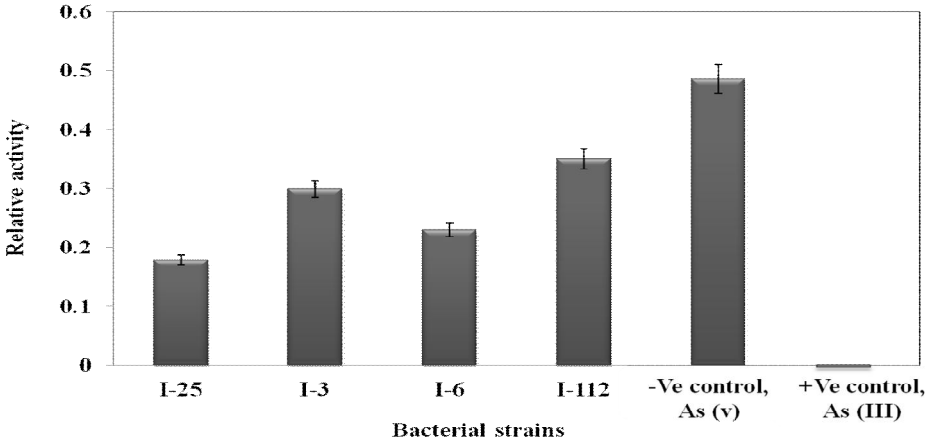

Fig. 2. Screening of the most potent arsenate reducing bacteria (n≥3)

Localization of active enzyme: The enzyme produced by the bacterial isolate I-25 (i.e. *B. aryabhattai*) was extracellular in nature and it was interpreted that it contained arsenate reducing enzyme. It was because of the fact that the supernatant of the isolate was able to reduce starch-iodine complex and produced a colorless compound from a colored product (Fig. 3) where as pellets did not show any change in color after the addition of the starch-iodine complex (Fig. 4).

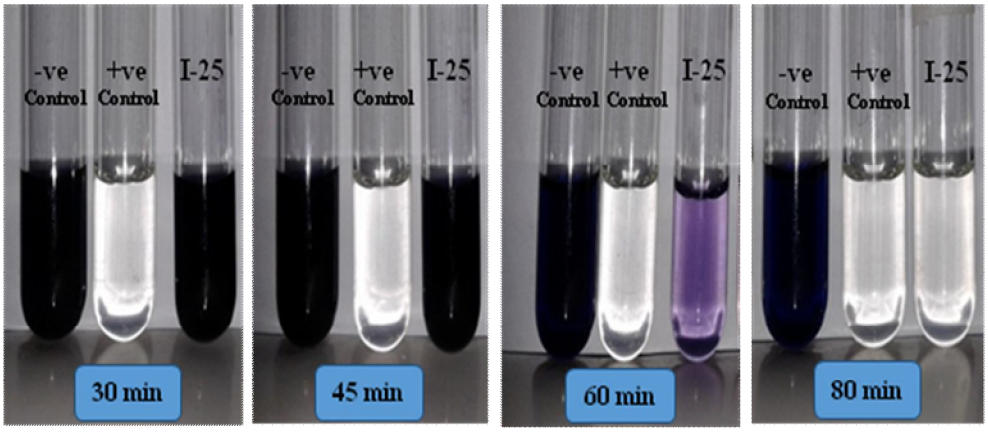

Fig. 3. Effect of supernatant activity on starch iodine complex reagent

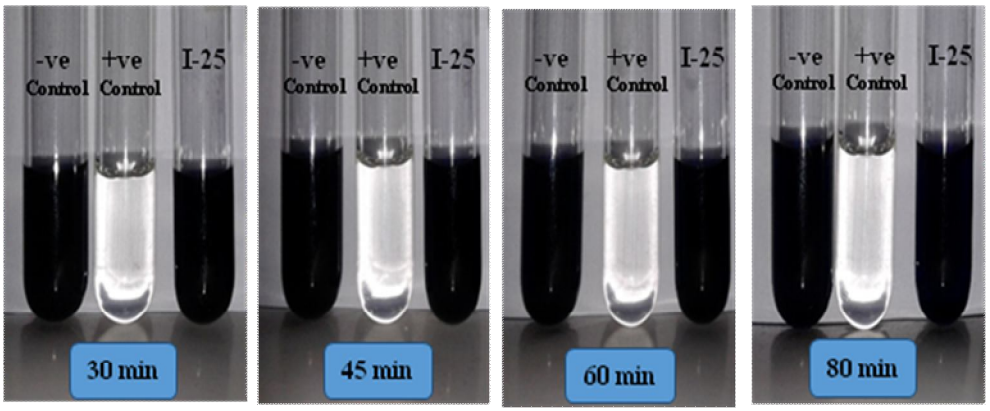

Fig. 4. Effect of cell (pellet) activity on starch iodine complex reagent

Determination of optimum metabolizing conditions: For arsenic metabolizing strains of *B. aryabhattai*, optimum arsenate concentration was between 64-128 mM. Higher OD gives the higher arsenate concentration on bacterial growth (Fig. 5). Optimum temperature on arsenate reducing enzyme was 60 ºC. In this case lower OD indicated higher effect of temperature on bacterial enzyme activity (Fig. 6) and could tolerate about 500 mM arsenate on bacterial growth.

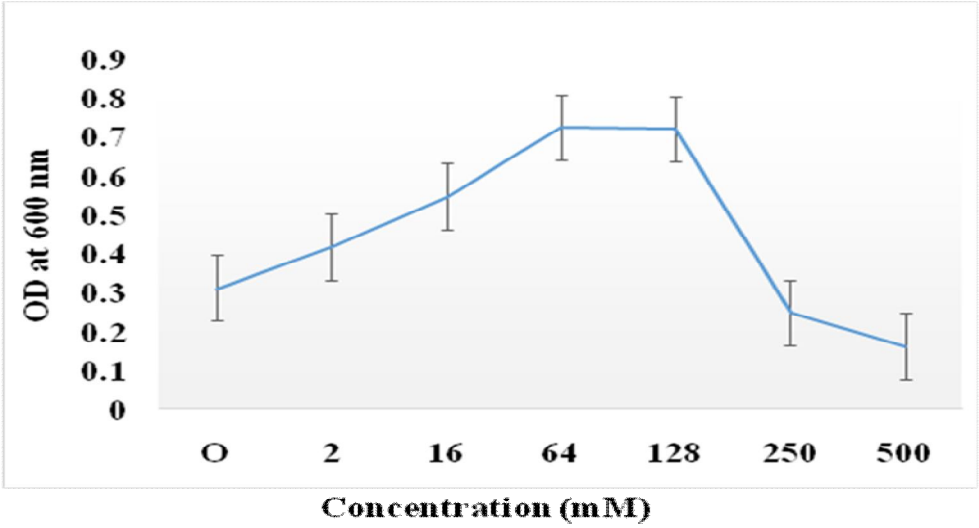

Fig. 5. Effect of optimum arsenate concentration on bacterial growth

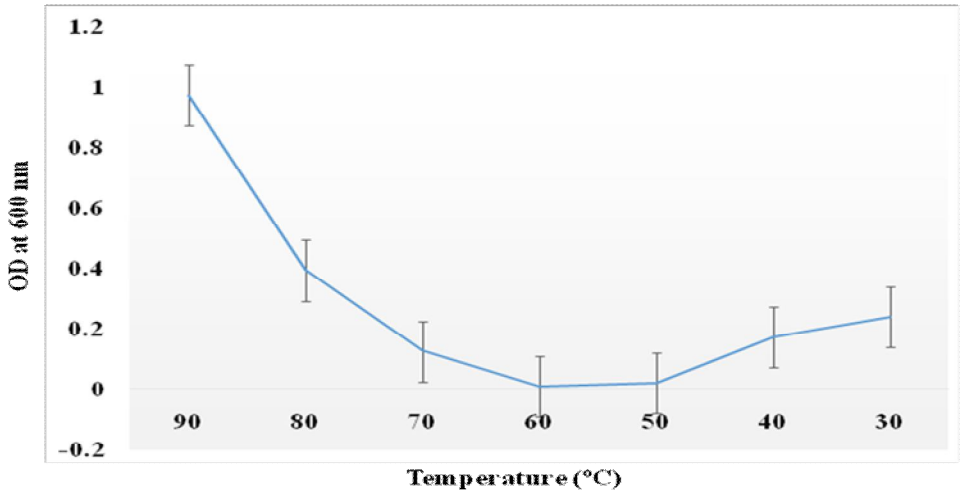

Fig. 6. Effect of temperature on enzyme activity of bacterial isolates growing in YEM medium

The screening method of this study is simple, rapid, cost effective and reliable compare with other methods such as HG-AAS, ICP-MS or ICP-AES (Mandal *et al.*, 2007). This technique is easy to perform, and the reagents and equipment are readily available in most of the laboratories. In this study, the arsenic-metabolizing bacterial isolate was identified as *B. aryabhattai* based on phylogenetic analysis of 16S rRNA sequence. During the present investigation *B. aryabhattai* could tolerate As (V) up to 500 mM on their growth at pH 7.0. During the present investigation, the enzyme of *B. aryabhattai* showed the

ability to reduce As (V) into As (III). It seems counter-intuitive to convert a less toxic compound to a more toxic form, but arsenate activity is closely coupled with efflux from the cells so that intracellular arsenite never accumulates (Shakoori *et al.,* 2010).

This study provides insight into identification, levels of arsenic resistance and reduction of arsenate by novel bacterial isolates which could play an important role in arsenic cycling in the arsenic-contaminated soils in Bangladesh. Isolated bacteria from arsenic contaminated soil were capable of metabolizing arsenic from the culture media. Highly As (V) resistant isolates could be a excellent candidate for the study of arsenic bioremediation. Further research is necessary to explore the possibility to use these bacteria in the other culture conditions to remove arsenic from the contaminated soil.

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