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Isolation and Characterization of Arsenic Resistant Bacteria from Contaminated Soil

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ABSTRACT: Arsenic contamination in soil is a serious problem in Bangladesh. The soil bacteria which are resistant to arsenic can play an important role in reduction of arsenic toxicity of soil. Hence, the aim of the study was to isolate arsenic resistant bacteria from arsenic contaminated soil. A total of three arsenic resistant bacteria *viz.* PB1, PB2 and PB3 were isolated from the suspension of arsenic contaminated soil inoculated in Basal salt media with 40 mM sodium-arsenate at 37°C under aerobic condition. The optimum growth of PB1 and PB2 were observed at 37°C while PB3 showed maximum growth at 30°C. PB2 and PB3 showed optimum growth at pH 9 while the maximum growth of PB1 was observed at pH 7. The MIC results showed that PB1, PB2 and PB3 were resistant up to 8mM and 16mM As(III) respectively. However, the all isolates showed resistance up to 256mM As(V). Analysis of 16S rRNA gene sequences revealed that isolate PB1, PB2 and PB3 were similar to *Agrobacterium* sp. PAE1 (99%), *Brevibacillus borstelensis* strain 1CK49 (99%) and *Ochrobactrum* sp. DS4 (99%) respectively.

Keywords: Arsenic; *Agrobacterium*; *Brevibacillus borstelensis*; *Ochrobactrum* and Bioremediation.

INTRODUCTION: Arsenic (As) which is one of the most toxic compounds is widely present in soils and water and causes deleterious health effects like cancer by consumption of As polluted drinking water and foods (Yang et al., 2012). Ground water arsenic contamination is a serious problem in many parts of the world especially in Bangladesh and neighboring West Bengal, India (Majumder et al., 2013). Arsenic poisoning from underground drinking water in Bangladesh was first identified in 1993 in Nawabgonj district (Smith et al., 2000). About 95 million people are vulnerable as the ground water in 47 districts out of 64 is contaminated by arsenic consequently, 80 million people are now exposed to arsenic poisoning and 10 thousand have already shown arsenicosis patients have been identified in 30 Districts (Chowdhury et al., 2001). Many people of Bangladesh are still drinking and using arsenic contaminated water for daily requirement. An estimated 200,000 to 270,000 people worldwide have died of cancer caused by drinking As-contaminated water (Meharg and Rahman, 2003). Continuous use of contaminated ground water for irrigation made the soil secondary source of As. Arsenic can enter into food chain (Ulman et al., 2004) causing wide spread distribution throughout the plant and animal kingdom. Rice grains are also containing higher concentration of arsenic (Meharg and Rahman, 2003) and represents as the most common route of arsenic poisoning through food chain (Chowdhory, 2004). Rice is the major staple food for people in West Bengal and Bangladesh, is the biggest sufferers of arsenic contamination, and so protecting crops is of major importance and serious issues at this place (Jaiswal, 2011).

Technologies for remediation of As-contaminated soils and waters have become increasingly important all over the world (Meharg, 2004, Jankong et al., 2007). Conventional methods of controlling or mitigating As-contamination were less effective, more cumbersome, time consuming and expensive than biological methods and can result in secondary pollution (Huang et al., 2004). Microbial remediation has the potential to be a less costly and environmentally friendly *in-situ* remediation technology.

Since heavy toxic pollutants are ubiquitously present in our environment, microorganisms have developed mechanisms to resist the toxic effects of these pollutants (White and Gadd, 1986). Although arsenic is generally toxic to life, it has been demonstrated that microorganisms can use arsenic compounds as electron donors, electron acceptors or possess arsenic detoxification mechanisms (Ahmann et al., 1994). Soil and water microorganisms capable of mitigating arsenic are thought to play a significant role in the reduction of arsenic in environments, and several bacteria capable of degrading high concentration of arsenic have been isolated from contaminated soil and water, *viz. Aeromonas*, *Exiguobacterium*, *Acinetobacter*, *Bacillus*, *Pseudomonas* (Anderson and Cook,

2004), *Acidithiobacillus*, *Deinococcus*, *Desulfitobacterium* (Dopson et al., 2001, Suresh et al., 2004).

Hence, isolation and characterization of suitable arsenic resistant bacteria is a prerequisite for developing a sustainable mechanism of arsenic bioremediation. Therefore, the present study was carried out for the isolation and characterization of arsenic resistant bacteria from arsenic contaminated soil so that they can be used in mitigating of soil contamination.

MATERIALS AND METHODS:

Soil collection and determination of arsenic concentration: Soil sample were collected in polyethylene bags with the help of spade from Bera Upazila under Pabna District, where soil, sediment, and ground water in the area have been contaminated with arsenic for many years. Surface soils (0-15cm depths) were collected and dried at room temperature (30± 3°C) and finely ground (<0.1 mm). The analysis of arsenic concentration of soil was conducted in the Environmental Engineering Laboratory of Bangladesh University of Engineering and Technology (BUET), Dhaka by using US EPA 206.2, SM 3113B method.
The pH of soil was also determined by soil pH and moisture tester (model pH-707 soil pH and moisture tester).

Isolation of arsenic tolerant bacteria: One gram of soil sample was dissolved in 20 ml saline solution (0.9% NaCl) and shaken gently. Then 5 ml of soil suspension was added into 250 ml Erlenmeyer flask containing 50 ml Basal salt minimal medium (BSMY) with 5 mM sodium-arsenate, which was incubated for 3 days at 37°C and subjected to shaking on an orbital shaker. Control flasks without inoculates were also prepared and incubated at 37°C with an orbital shaker. The cultures that were found turbid after a period of 0 up to 3 days were used as inocula in subsequent experiments.

Arsenic tolerance capacity of the isolates: The ability of arsenic tolerance of the isolates were determined by comparing their growth in BSMY media containing different concentration of sodium arsenate (5mM to 40mM) after incubation at 37°C for 3 days in an orbital shaker. Bacterial isolates that could tolerate the highest arsenate concentration were selected and identified by their morphological features and biochemical properties (Aksornchu et al., 2008).

Microscopic examination and identification of bacterial cells: For the identification of the arsenic resistant organisms, morphological characterization, microscopic observation, growth characteristics, biochemical tests and antibiotic sensitivity tests were performed. The microorganisms were identified according to Bergey's Manual of Systematic Bacteriology (Holt, 2005).

Identification of the isolates by 16S rRNA gene sequence: Genomic DNA of bacterial isolates was isolated according to Mohanta et al., 2012. Gene fragments specific for the highly variable region of the bacterial 16S rRNA gene was amplified by PCR using universal PCR primer as described by Loffler et al., 2000 (Sigma USA) in a thermal cycler (MJ Research Inc., Watertown, USA). The sequence of the forward primer was 16SF (8f) 5'- AGA GTT TGA TCC TGG CTC AG-3' and the sequence of the reverse primer was 16SR (805r) 5'- GAC TAC CAG GGT ATC TAA TC-3'. The PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator for the presence of about 1500 bp PCR products. Amplified 16S rRNA gene PCR products were purified using StrataPrep PCR purification kit (Stratagene USA) according to the manufacturers protocol. Sequencing reactions were carried out using ABI-Prism Big dye terminator cycle sequencing ready reaction kit and the PCR products were purified by a standard protocol. The purified cycle sequenced products were analyzed with an ABIPrism 310 genetic analyzer. The chromatogram sequencing files were edited using Chromas 2.4. The homology of the 16S rRNA gene sequences was checked with the 16S rRNA gene sequences of other organisms that had already been submitted to GenBank database using the BLASTN (http://www.ncbi.nih.gov/BLAST/) algorithm.

Phylogenetic Analysis: The phylogenetic trees were constructed by the pairwise alignments of all the strains and the related species using Neighbor-Joining algorithms (Saitou and Nei 1987) using the Jukes-cantor model in NCBI website (http://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi?). Downloads the guide tree into a text file in Newick format which is recognized by a phylogenetic software named Mega VI software (version 6.0) (Tamura et al. 2013).

Effect of temperature and pH on bacterial growth: Temperature and pH influence bacterial growth. For the effect of pH, culture medium (nutrient broth, Himedia, India) was adjusted to pH 6.0, 7.0, 8.0 and 9.0. Incubation temperature was varied at 25°C, 30°C and 37°C. Bacterial cell density of liquid cultures was determined by measuring optical density at 660 nm with photoelectric colorimeter (AE-11M, ERMA INC, TOKYO) (Mohanta et al., 2012).

Enumeration of Viable Cell Count: Enumerations of arsenic resistant heterotrophic bacteria were deter-

mined using plate technique. Varying concentrations (20mM, 40mM) of arsenate ion (sodium arsenate) were added to Basal Salt Medium (BSMY) from a 1 M stock solution ($HgCl_2$). All samples were serially diluted in autoclaved distilled water up to 10^5. Aliquots of 0.1 ml from each dilution was spread on Basal Salt Medium (BSMY) plates supplemented with 20mM, 40mM of sodium arsenate and without sodium arsenate. The plates were incubated at 37°C for 24 hours. After incubation period, the appeared colonies on both BSMY containing As (V) and without As (V) were enumerated using total viable plate count method (Prescott and Harley 2002) and expressed as colony forming units (cfu)/mg.

RESULTS AND DISCUSSION:

Arsenic concentration and pH of soil sample: Arsenic concentration of the collected soil was 8.44 mg/kg and the pH was in between 5.8-6.0.

Isolation, identification and phylogenetic analysis of the bacterium: Bacteria were isolated by plating onto an agar solidified BSMY medium supplemented with sodium arsenate. The plates were incubated at 37°C for 3 days and bacterial colonies were found to grow on the medium. Results of microscopic analysis of bacterial cells and their growth characteristics are presented in Table 1 while the biochemical and antibiotic sensitivity tests of the bacteria are presented in Table 2, 3 respectively. Isolated bacterial strains were identified by both morphological and biochemical tests and this is further confirmed by 16S rRNA gene sequence analysis. Analysis of 16S rRNA gene sequences (data not shown here) revealed that the isolates similar to *Agrobacterium* sp. PAE1(99%), *Brevibacillus borstelensis* strain 1CK49 (99%) and *Ochrobactrum* sp. DS4(99%).

The phylogenetic positions of all isolates within different subgroups were investigated by comparing their 16S rDNA sequences to those representatives of various genera. Three different groups can be seen from the tree: *Agrobacterium* (Figure1), *Brevibacillus* (Figure 2) and *Ochrobactrum* (Figure 3). It is evident from the phylogenetic tree that PB1 is closely related to *Agrobacterium* sp. PAE1, PB2 to *Brevibacillusborstelensis* 1CK49 and PB3 to *Ochrobactrum* sp. DS4. The distance was indicated at the branches and its nodes.

Effect of temperature and pH on bacterial growth: To determine the effect of temperature and pH of growth medium on the growth rate of the bacteria was tested a series of investigation. The results of the investigations are presented in Figures. 4, 5, respectively. The optimum pH for the growth of PB2 and PB3 was 7.0 and BP1 showed optimum growth at pH 7.0 (Figure 4). The optimum temperature for the growth of isolates was found to be 37°C and growth rate was moderately low in other temperature *viz.* 30^0C and 25^0C (Figure 5).

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration: The MIC of sodium arsenite and sodium arsenate for three strains is given in Table 4. After detecting MIC value of the bacterial isolates 500 µL of bacterial suspension added to 5 mL of each fresh medium tube for determining the MBC value (Table 4).

Effect of arsenic on bacterial growth: The growth curve pattern was studied by growing the organism in the presence of sodium arsenate and comparing with the control culture in which no metal ions were added. Although the growth pattern of the isolate was significantly different from those of control that indicates arsenic effect on the growth of the isolated bacteria (Figure 6).

Bacterial Enumeration: Total viable counts were 10.12×10^4 (cfu/mg) in collected soil sample. Comparison of total heterotrophic bacteria (THB) and Arsenic Resistant Bacteria has been shown in Table 5.

Table 1: Cultural characteristics and microscopic observation of the bacterial strains.

Characteristics of the strain	PB1	PB2	PB3
Colony colour	White	White	Yellow
Cell shape	Circular	Circular	Irregular
Surface	Smooth	Smooth	Smooth
Elevation	Convex	Convex	Umbonate
Edges	Entire	Entire	Undulate
Opacity	Opaque	Opaque	Opaque
Gram staining	Gram positive	Gram positive	Gram negative
Motility	Non-motile	Non-motile	Non-motile

Table 2: Biochemical test results for the isolated bacterial strains.

Biochemical test	Isolates		
	PB1	**PB2**	**PB3**
Triple Sugar Iron (TSI)	-	-	-
Citrate utilization	-	-	-
Oxidase	+	+	-
Catalase	+	+	+
Sulfide Indole Motility (SIM)	-	-	-
Methyl red	-	-	-
Voges-Proskauer reaction	+	-	-
Urea hydrolysis	+	+	-
MacConkey Agar	+	+	-
Sugar uttilization			
Glucose	-	-	+
Fructose	-	-	-
Galactose	-	-	-
Maltose	-	+	+
Sucrose	-	+	+
Lactose	+	+	-
Arabinose	-	-	+
Xylose	-	-	+
Mannitol	-	-	-

(+ = microbial growth, - = no growth)

Table 3: Antibiotic sensitivity tests.

Antibiotics	Disc distance (mm)		
	PB1	**PB2**	**PB3**
Neomycin (30 µg)	20 (S)	21 (S)	19 (S)
Azithromycin (15 µg)	23 (S)	34 (S)	18 (S)
Tetracycline (30 µg)	30 (S)	23 (S)	15 (S)
Ceftriaxone (30 µg)	30 (S)	12 (I)	10 (R)
Erythromycin (15 µg)	21 (S)	26 (S)	07 (R)
Cefradine (25 µg)	12 (I)	14 (I)	07 (R)
Gentamicine (10 µg)	28 (S)	06 (R)	22 (S)
Kanamycin (30 µg)	13 (I)	11 (I)	08 (R)
Cephradine (30 µg)	25 (S)	22 (S)	07(R)
Ampicillin (10 µg)	09 (R)	30 (S)	12 (I)

(5-10mm) = Resistance to antibiotic (R); (15-20mm) = Sensitive to antibiotic (S); (10-15mm) = Intermediate resistance (I)

Table 4: MIC and MBC value of the Bacterial Isolates.

Bacrerial Isolates	MIC		MBC	
	As (III) mM	**As (V) mM**	**As (III) mM**	**As (V) mM**
PB1	32	256	32	256
PB2	16	256	32	256
PB3	16	256	64	512

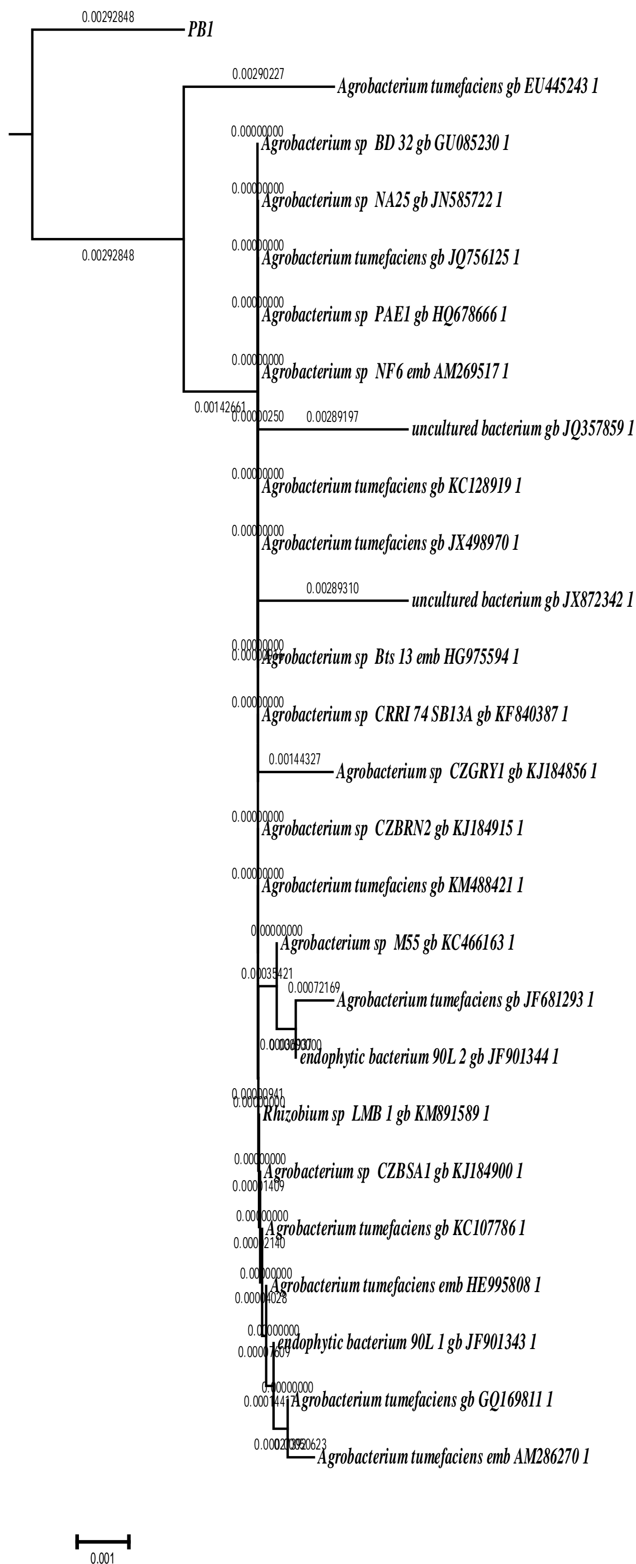

Figure 1: Phylogenetic tree showing the genetic relationship among the cultivated bacteria PB1 and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.001 = 0.1% difference among nucleotide sequences.

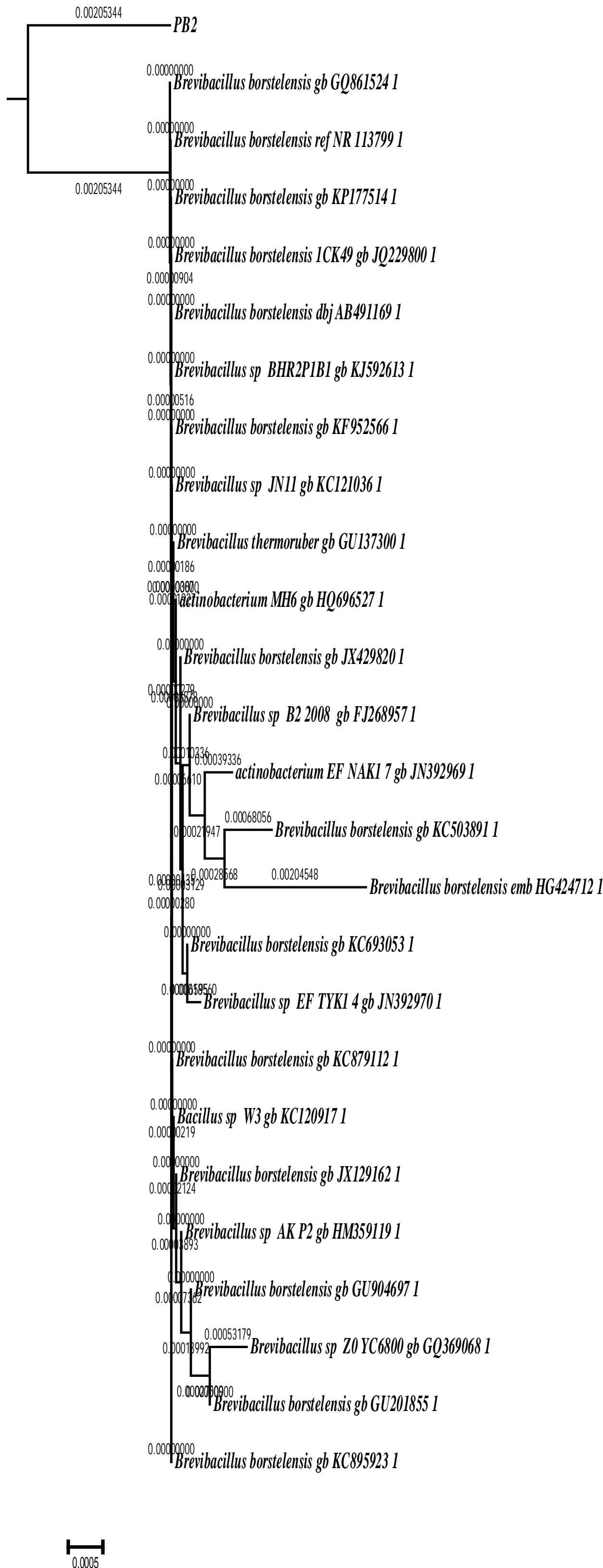

Figure 2: Phylogenetic tree showing the genetic relationship among the cultivated bacteria PB2 and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.0005 = 0.05% difference among nucleotide sequences.

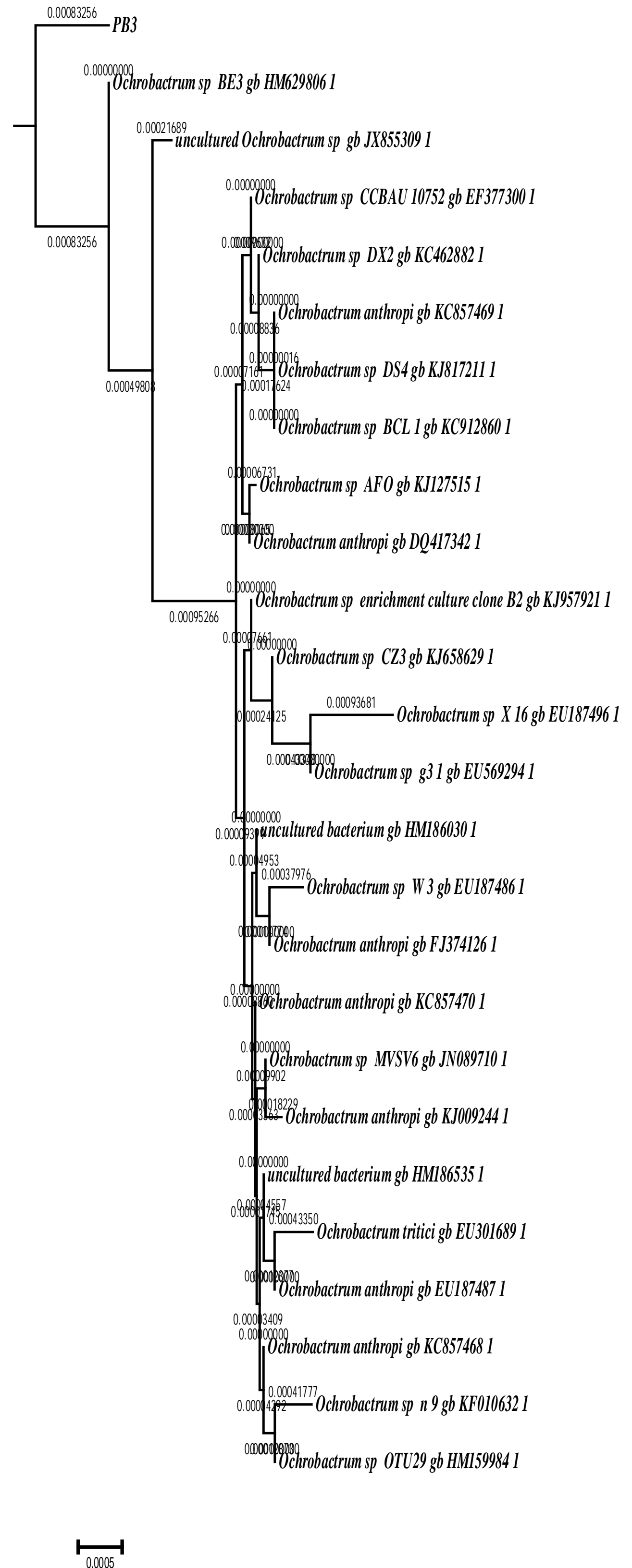

Figure 3: Phylogenetic tree showing the genetic relationship among the cultivated bacteria PB3 and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.0005 = 0.05% difference among nucleotide sequences.

Table 5: Total heterotrophic bacteria.

Dilution factor	Total hetero-trophic bacteria(CFU/mg)	Arsenic resistant bacteria (cfu/mg)		(%) of arsenic resistant bacteria
		20mM	40mM	
10^{-2}	10.12×10^{4}	2.86×10^{4}	2.18×10^{4}	21.54%

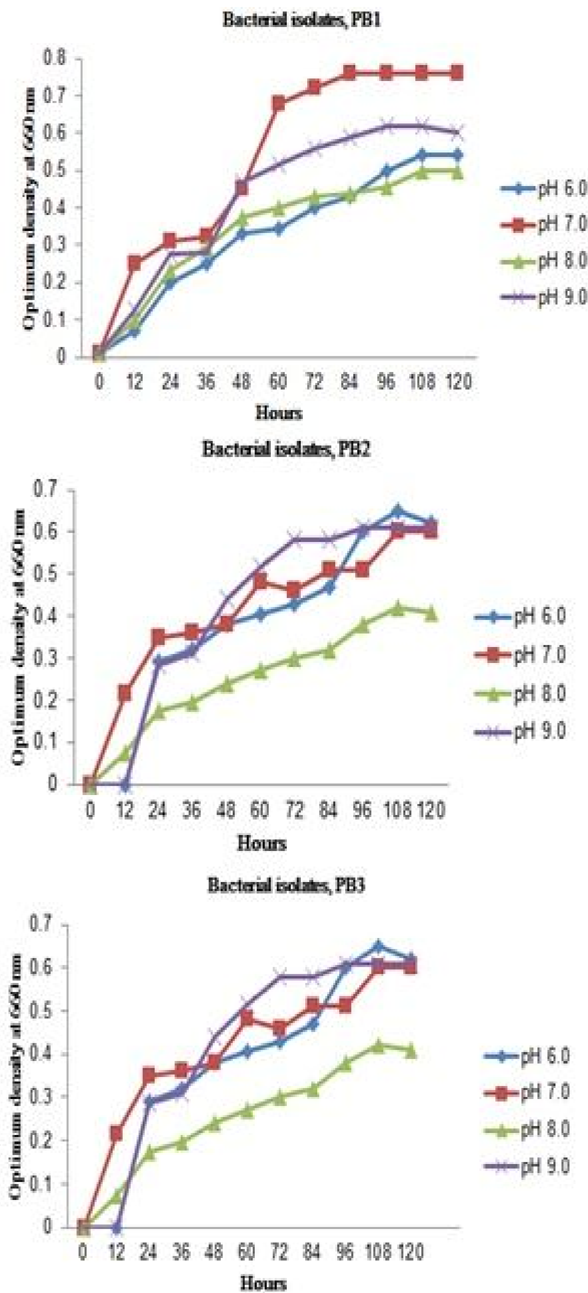

Figure 4: Effects of pH on bacterial growth. For the effect of pH, culture medium (nutrient broth, Hi-media, India) was adjusted to pH 6.0, 7.0, 8.0 and 9.0. Then, the media were inoculated and incubated for 120 hours at 37°C. During incubation, bacterial cell density of liquid cultures was determined by measuring optical density at 660 nm with photoelectric colorimeter (AE-11M, ERMA INC, TOKYO) at every 12 hours interval.

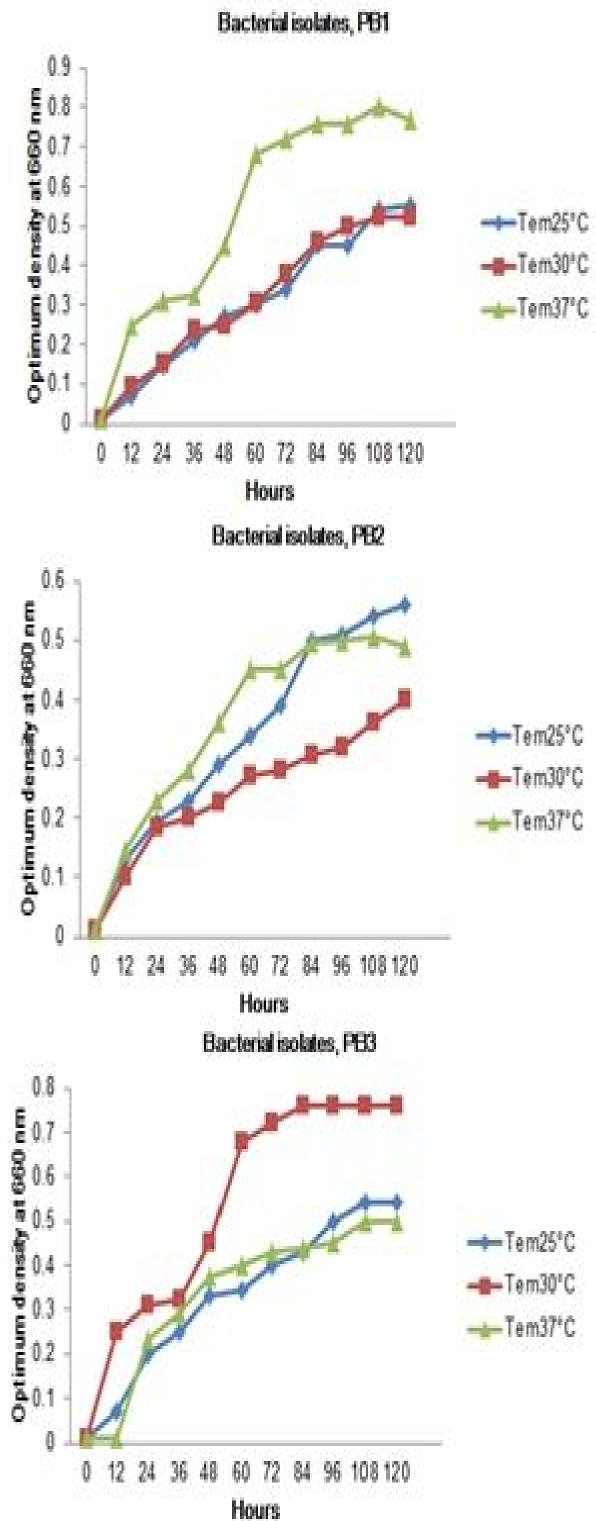

Figure 5: Effects of temperature on bacterial growth. For the effect of temperature, culture medium (nutrient broth, Hi-media, India) was adjusted to 25°C, 30°C and 37°C. Then, the media were inoculated and incubated for 120 hours at pH 7. During incubation, bacterial cell density of liquid cultures was determined by measuring optical density at 660 nm with photoelectric colorimeter (AE-11M, ERMA INC, TOKYO) at every 12 hours interval.

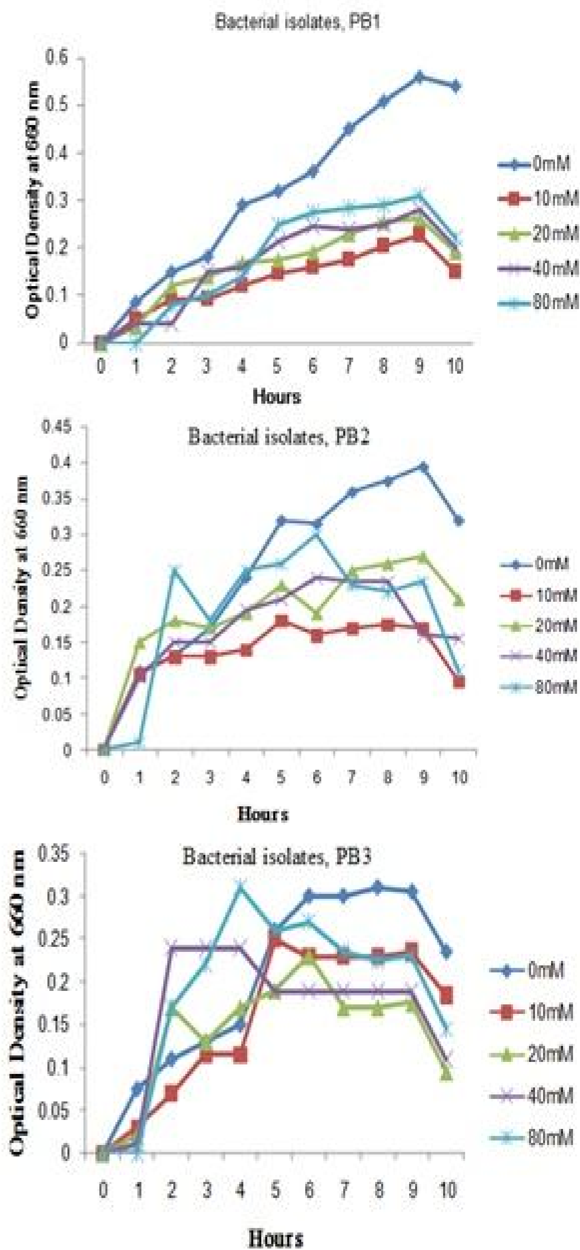

Figure 6: Effects of arsenic on bacterial growth. For the effect of arsenic on bacterial growth, culture medium (nutrient broth, Hi-media, India) was adjusted to 0 mM, 10 mM, 20 mM, 40 mM and 80 mM. Then, the media were inoculated and incubated for 10 hours at pH 7 and 37^{0}C. During incubation, bacterial cell density of liquid cultures was determined by measuring optical density at 660 nm with photoelectric colorimeter (AE-11M, ERMA INC, TOKYO) at every 1 hour interval.

Arsenic pollution in Bera Upazila under Pabna District, Bangladesh is hardly affected from a few decades. The concentration of arsenic in native polluted soil has been detected and was 8.44 mg/kg but the concentrations of Aresenic of this region have not been reported yet. According to U.S. Environmental Protection Agency (1985) the safe concentration of As for drinking water is 0.01mg/l, while the Bangladesh standard is 0.05 mg/l. The ground water in Bangladesh is heavily contaminated with arsenic (DPHE-BGS, 2000). About 40% of situated tube-wells exceeded the country standard limit of 0.05ppm where as the permissible level of arsenic in drinking water is only 0.01 ppm (WHO, 1993).

In this study, the sample of arsenic contaminated soil were collected and used as sources of inocula for the isolation of organisms capable of mitigating arsenic. Pure cultures of arsenic tolerance bacteria were isolated by plating on agar solidified BSMY medium with sodium arsenate. Then, arsenic resistant bacteria were identified by both morphological and biochemical tests and this is further confirmed by 16S rRNA gene sequence analysis. Analysis of 16S rRNA gene sequences revealed that the isolates were similar to *Agrobacterium* sp. PAE1 (99%), *Brevibacillus borstelensis* strain 1CK49 (99%) and *Ochrobactrum* sp. DS4(99%). Arsenic resistant bacteria have also been isolated from As contaminated soil by several researchers (Yang et al., 2012, Majumder, 2012 and Selvi et al., 2014). During the present investigation it was observed that all isolates could grow and withstand the sodium-arsenate toxicity up to 40mM under aerobic condition. The MIC results of isolates PB1 showed 8mM, PB2 and PB3 showed 16mM resistance to As (III) whereas the all isolates showed 256mM resistance to As(V). Rehman et al. (2010) reported that *P. lubricans* showed high resistance against arsenite up to 40 mM and could oxidize As (III). Awais et al. (2011) have identified potential strains of *Klebsiella pneumonia* and *K. variicola* with minimum inhibitory concentration of 26.6 and 24mM against As (III). Majumder (2012) reported that twenty six As resistant bacterial strains were isolated from As contaminated soil of West Bengal, India. Among them, 10 isolates exhibited higher As resistance capacity and could grow in concentration of 12000 mgl^{-1} of arsenate (AsV) and 2000 mgl^{-1} of arsenite (AsIII) in BSMY medium. A similar study achieved by Shakoori et al. (2010) showed that *Citrobacter freundii* and *Bacillus anthracis*, could tolerate As (V) up to 290 mg/l while *Klebsiella oxytoca* was able to resist As up to 240 mg/l. The results of the study revealed that all bacterial isolates did not have the same degree of tolerance to As toxicity. This might be due to developing of As tolerance and resistant ability of the inherent individual soil microorganisms (Smith et al., 1998). Sanyal et al. (2002) reported that six arsenic resistance bacteria were isolated from As contaminated soil. The isolates were found to be *Proteus* sp., *Bacillus* sp., *Escherichia coli*, *Flavobacterium* sp., *Corynebacterium* sp., and *Pseudomonas* sp. possess varying degrees of As accumulating abilities. Selvi et al. (2014) noticed that

two As resistant bacteria *Enterobacter asburiae* (BC1) and *Enterobacter cloacae* (BC2) were isolated from arsenic contaminated agricultural soil. Both isolates BC1 and BC2 exhibited natural resistance up to 40mM and 400mM for sodium arsenite and sodium arsenate in LB solid media. In a subsequent study Anderson et al. (2004) showed that *Bacillus, Pseudomonas and E. coli* could also reduce arsenate isolated form contaminated sites of New Zealand.

The growth of the isolated bacteria and toxic pollutants resistance were dependent on PH and temperature. The optimum PH for the growth of the isolates PB2 and PB3 was 9.0 and PB1 showed optimum growth at extreme PH 7.0. Optimum P^H for growth of arsenic resistant bacteria was reported at ranging from 4-9 (Shakoori et al., 2010 and Suchanda et al., 2011). It was recovered that the optimum temperature for the best growth of isolates was found to be 37°C and growth rate was moderately low in other temperature *viz.* 30°C and 25°C. So, 37°C temperature is the most suitable temperature for the mitigation of arsenic. It has been reported that the ability of the bacterial strains *K. pneumonia*, *K. variicola*, *Citrobacter freundii* and *Bacillus anthracis* to utilize arsenic individually and in combination as a sole source of carbon and nitrogen was studied in LB medium incubated at 37°C (Selvi et al., 2014 and Shakoori et al., 2010).

The antibiotic resistance pattern is generally used for strain / identification in ecological studies. It is evident from the present investigation that PB3 were resistant to ceftriaxone, erythromycine, cefradine, kanamycin and cephradine while PB1 and PB2 were found to be sensitive to those antibiotics. PB1 showed resistance against ampicillin and PB2 showed gentamycine while PB3 was found to sensitive them. All three isolates were sensitive to neomycin, azithromycine and tetracycline. Mechanisms of resistance by microorganisms include microbial surface sorption, enzymatic transformation, and perception by oxidation/reduction reaction and biosynthesis of metal binding proteins (Srinath et al., 2002 and Zoubilis et al., 2004). Bacterial isolates capable of reducing arsenic is probable due to the presence of the novel catabolic enzyme, coded by plasmid gene. Different genes and enzymes involved in arsenic reduction were described by Silver and Phung (2005). The role of plasmid in the degradation of organic compounds has provided a lucrative ground for examining the potential and mechanisms of bacterial evolution in nature and practical consequences in terms of pollution control. Further investigation should be performed to identify the specific genes that are responsible for the degradation of arsenic.

Alternative bioremediation strategies using engineered strains also offer great promise. There are many benefits to be derived from the successful application of recombinant DNA techniques to evolve microbes that disseminate polluting xenobiotic. Also, the current revolution in genetic engineering is sure to have an impact on biodegradation technology, and in time the catabolic potential of microbes will be realized.

CONCLUSION: Soil contamination by arsenic is a major threat in Bangladesh now-a-days. The need to develop low cost and friendly technologies for remediation of arsenic contaminated soils and water has stimulated interest in arsenic resistant organisms. In the present study *Agrobacterium* sp., *Brevibacillus borstelensis* and *Ochrobactrum* sp. could tolerate sodium arsenate up to 40mM. The bacterial strains showed also high level of arsenic reduction potential and could therefore represent good cadidates for bioremediation process of arsenic contaminated environments.

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