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Isolation and Characterization of Arsenic Resistant Soil Bacteria

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ABSTRACT

Arsenic contamination in drinking water is a serious problem in Bangladesh. The soil bacteria which can degrade arsenic can play an important role in reduction of arsenic toxicity of soil. Hence, this study was planned to isolate arsenic degrading bacteria from arsenic contaminated soil. A total of two arsenic resistant bacteria *viz.* AD1 and AD2 were isolated from the arsenic contaminated soil inoculated. The optimum growths of both isolates were observed at 35 ^{0}C. AD1 showed optimum growth at pH 7 while the maximum growth of AD2 was observed at pH 8. The MIC results showed that AD1 and AD2 were resistant up to 28 mM and 14 mM As(III) respectively. However, both isolates showed resistance up to 220 mM As(V).

1 INTRODUCTION

Arsenic contamination in groundwater has been reported as a severe health threat for the population who are using contaminated water for drinking and/or for cooking their meal (Zaman, 2015; Yang *et al.,* 2012). About 95 million people are vulnerable as the groundwater 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, 2001). Groundwater arsenic contamination is a serious problem in many parts of 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). 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 (Harvey *et al.,* 2002; Meharg and Rahman, 2003). Continuous use of contaminated groundwater for irrigation made the soil secondary source of As. Arsenic can enter into food chain (Ulman *et al.,* 2004) causing widespread distribution throughout the plant and animal kingdom. Rice grains are also containing higher concentration of arsenic (Meharg and Rahman, 2003) and represent 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 in this countries (Jaiswal, 2011). Technologies for degradation of As-contaminated soils and waters have become increasingly important all over the world (Smith *et al.,* 2000; 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.

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). 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). 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. Agrobacterium*, *Aeromonas*, *Exiguobacterium*, *Acinetobacter*, *Bacillus*, *Pseudomonas* (Saha, 2016; Anderson and Cook, 2004), *Acidithiobacillus*, *Deinococcus*, *Desulfitobacterium* (Dopson *et al.*, 2001; Suresh *et al.*, 2004).

Therefore, isolation and characterization of suitable arsenic resistant bacteria is essential for developing a sustainable mechanism of arsenic bioremediation. The present study was carried out for the isolation and characterization of arsenic resistant bacteria from arsenic contaminated soil.

2 MATERIALS AND METHODS

2.1 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 groundwater in the area have been contaminated with arsenic for many years. Surface soil (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 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).

2.2 Isolation of arsenic tolerant bacteria

It was done as described by Saha and their colleagues, 2016. Briefly, 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.

2.3 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).

2.4 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).

2.5 Effect of temperature and p^H on bacterial growth

Temperature and pH influence bacterial growth. For the effect of p^H, culture medium (nutrient broth, Hi-media, India) was adjusted to p^H 6.5, 7.0, 7.5 and 8.0. Incubation temperature was varied at 25ºC, 30ºC, 35ºC and 40ºC. Bacterial cell density of liquid cultures was determined by measuring optical density at 660 nm with photoelectric colorimeter (AE-11M, ERMA INC, TOKYO) (Saha ***et al.,*** 2016; Mohanta ***et al.,*** 2012).

2.6 Enumeration of Viable Cell Count

Enumerations of arsenic resistant heterotrophic bacteria were determined 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 0.1ml from each dilution was spread on 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.

3 RESULTS

3.1 Arsenic concentration and pH of soil sample

Arsenic concentration of the collected soil was 7.23 mg/kg and the pH was in between 5.6-6.3.

3.2 Isolation of Identification of bacteria

Bacteria were isolated by plating onto an agar solidified BSMY medium supplemented with arsenic. 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 as *Agrobacterium.*

3.3 Effect of temperature and p^H 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 Fig. 1. The optimum pH for the growth of AD1 was 7.0 while AD2 showed optimum growth at pH 8.0 (Fig.1). The optimum temperature for the growth of isolates was found to be 35^0C while minimum growth rate was found at 25^0C (Fig. 2).

3.4 Antibiotic sensitivity test

Sensitivity of antibiotic to the isolated bacteria was performed as described by Saha and their colleagues (Saha *et. al.* 2009). Briefly, 1ml of fresh broth culture of bacterial isolate was spread uniformly on a nutrient agar plate with a sterile glass spreader. The plate was air-dried for few minutes and then antibiotic discs were placed on inoculated nutrient agar plates which were incubated at 37°C for 24 hours. After incubation, clear zones indicated inhibition of growth of the bacterial isolate.

3.5 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).

3.6 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 (Fig. 3).

3.7 Bacterial Enumeration

Total viable counts were 12.15×10^4 (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	**Isolate AD1**	**Isolate AD2**
Colony colour	White	White
Cell shape	Circular	Circular
Surface	Smooth	Smooth
Elevation	Convex	Convex
Edges	Entire	Entire
Opacity	Opaque	Opaque
Gram staining	Gram positive	Gram positive
Motility	Non-motile	Non-motile

Table 2 Biochemical test results for the isolated bacterial strains

Biochemical test	**Isolate AD1**	**Isolate AD2**
Triple Sugar Iron (TSI)	-	-
Citrate utilization	-	-
Oxidase	+	+
Catalase	+	+
Sulfide Indole Motility (SIM)	-	-
Methyl red	-	-
Voges-Proskauer reaction	+	-
Urea hydrolysis	+	+
MacConkey Agar	+	+
Glucose	-	-
Fructose	-	-
Galactose	-	-
Maltose	-	+
Sucrose	-	+
Lactose	+	+
Arabinose	-	-
Xylose	-	-
Mannitol	-	-

(+ = microbial growth, - = no growth)

Table 3 Antibiotic sensitivity tests

Antibiotics	Isolate AD1		Isolate AD2	
	Inhibition zone (mm)	Sensitivity	Inhibition zone (mm)	Sensitivity
Ampicillin	18	S	18	S
Azithromycin	22	S	22	S
Bacitracin	00	R	00	R
Cephradine	12	I	14	I
Ceftriaxone	26	S	26	S
Doxycycline	17	S	17	S
Erythromycin	20	S	20	S
Neomycin	17	S	17	S
Sulphamethoxazole/ Trimethoprim	18	S	19	S
Tetracycline	20	S	20	S

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

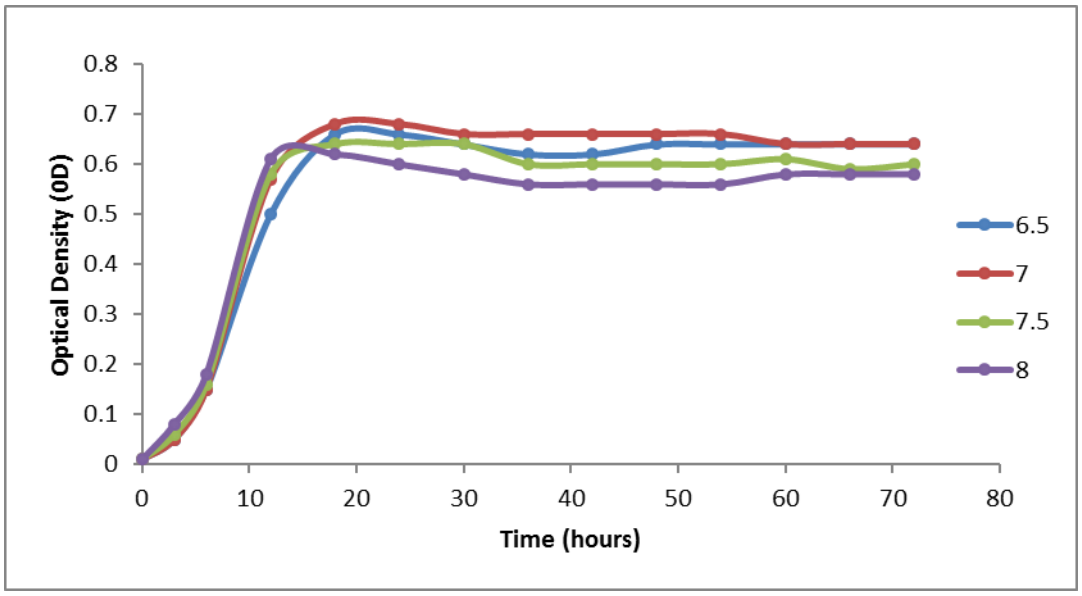
Table 4 MIC and MBC value of the Bacterial Isolates

Bacterial Isolates	MIC		MBC	
	As (III) mM	As (V) mM	As (III) mM	As (V) mM
Isolate AD1	28	220	30	220
Isolate AD2	14	220	30	220

Table 5 Total heterotrophic bacteria

Dilution factor	Total heterotrophic bacteria(CFU/mg)	Arsenic resistant bacteria (cfu/mg)		(%) of arsenic resistant bacteria
		20mM	40mM	
10^{-2}	12.15×10^{4}	2.55×10^{4}	2.01×10^{4}	18.23%

A.



B.

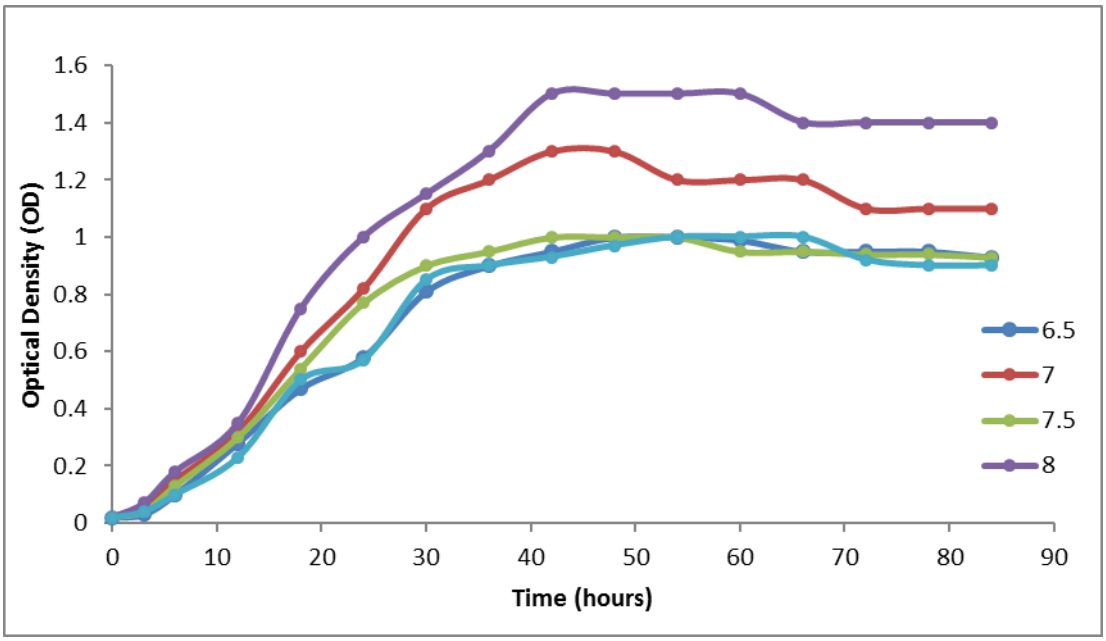
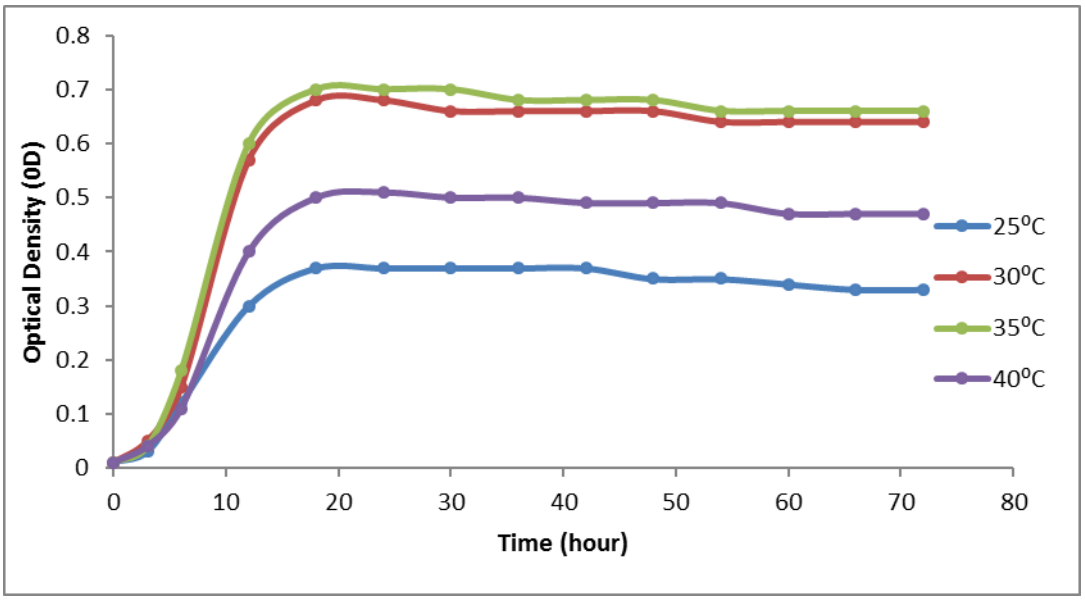


Figure 1 Effects of pH on bacterial growth (A= Isolate AD1; B= Isolate AD2).

A.



B.

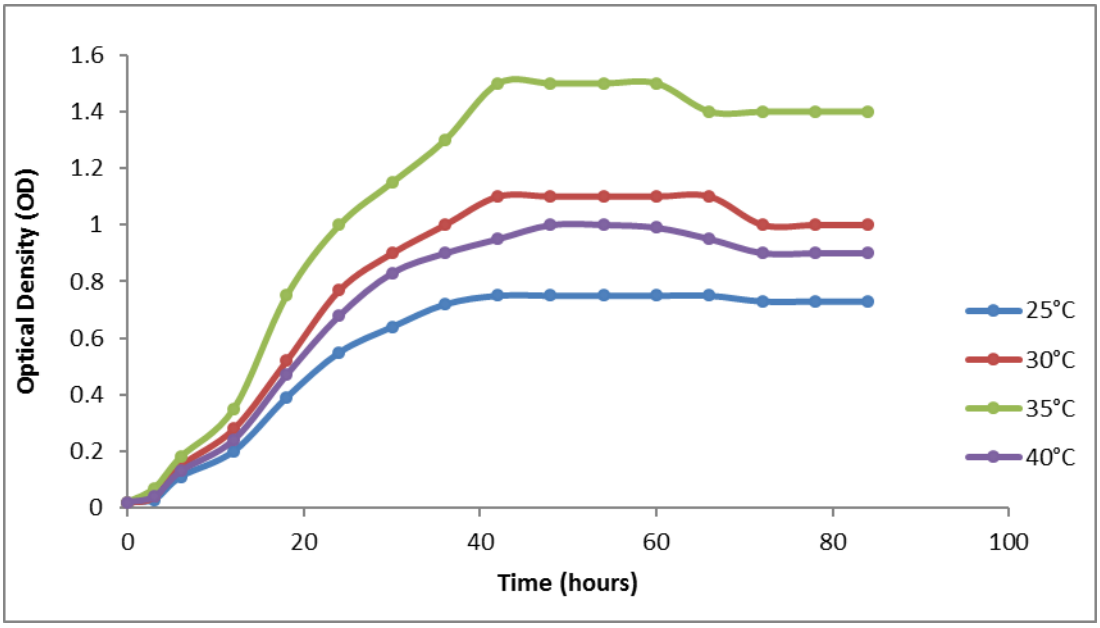
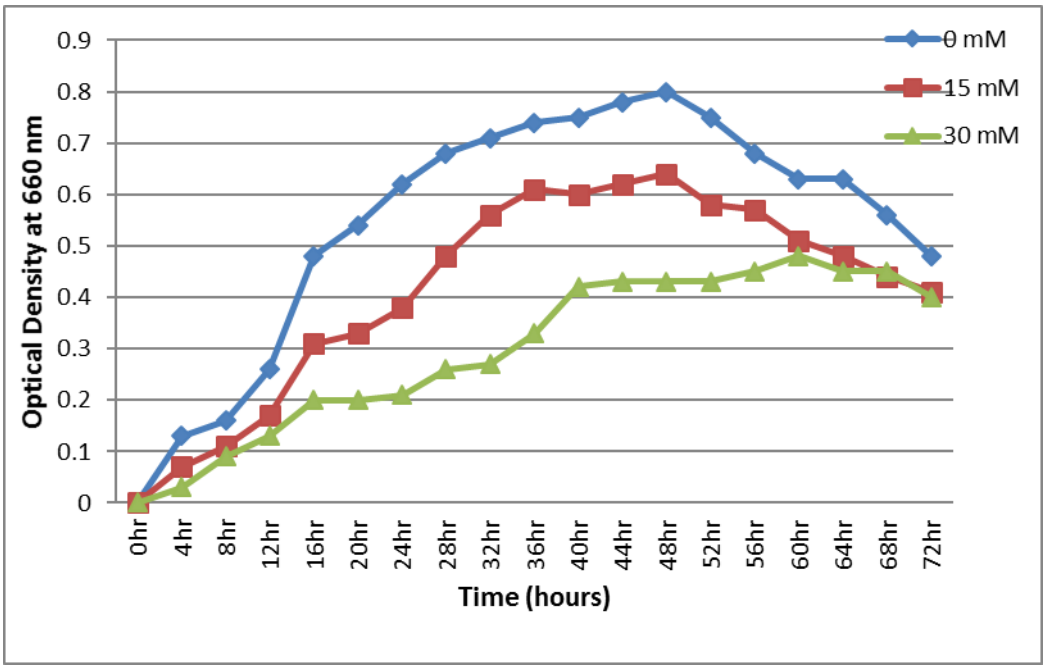


Figure 2 Effects of temperature on bacterial growth (A= Isolate AD1; B= Isolate AD2).

A.



B.

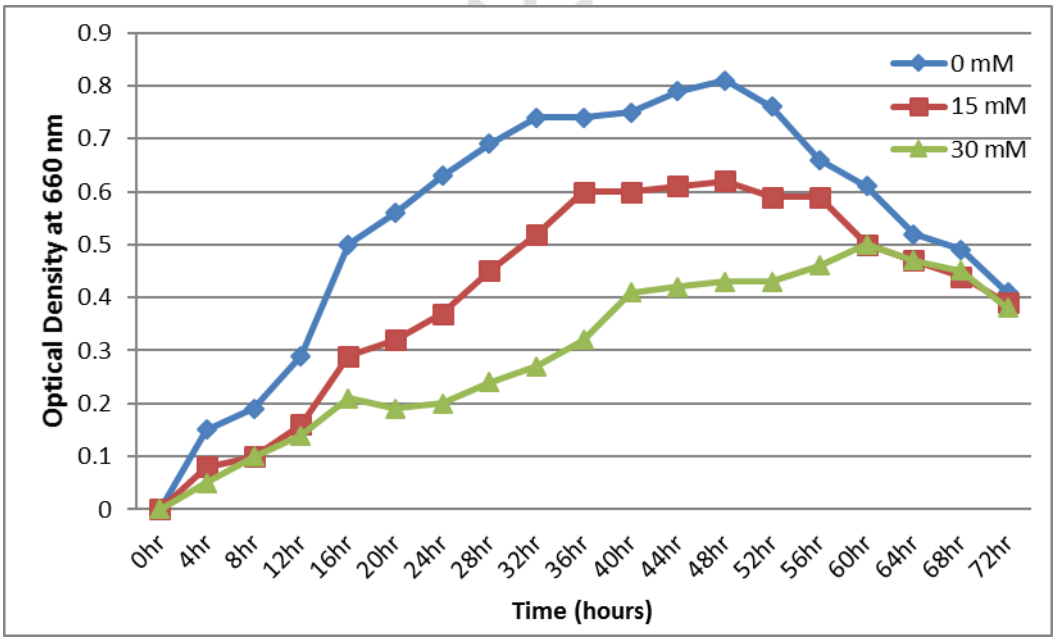


Figure 3 Effects of arsenic on bacterial growth (A= Isolate AD1; B= Isolate AD2).

4. DISCUSSION

About 40% of situated tube-wells exceeded the country standard limit of 0.05ppm whereas the permissible level of arsenic in drinking water is only 0.01 ppm (WHO, 1993). 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 groundwater in Bangladesh is heavily contaminated with arsenic (DPHE-BGS, 2000). 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 concentration of As of this region have not been reported yet.

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 arsenic. Arsenic resistant bacteria have also been isolated from As contaminated soil by several researchers (Yang *et al.*, 2012; Majumder, 2012; Selvi *et al.*, 2014). During the present investigation, it was observed that all isolates could grow and withstand the sodium-arsenate toxicity up to 30mM under aerobic condition. The MIC results of isolates AD2 showed 14mM while AD1 showed 28mM resistance to As (III). But, the all isolates showed 220mM resistance to As(V). 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. 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).

The growth of the isolated bacteria and toxic pollutants resistance were dependent on P^H and temperature. The optimum P^H for the growth of the isolates AD1 was 7.0 and AD2 showed optimum growth at extreme P^H 8.0. Optimum P^H for growth of arsenic resistant bacteria was reported at ranging from 4-9 (Shakoori *et al.*, 2010; Suchanda *et al.*, 2011). It was recovered that the optimum temperature for the best growth of isolates was found to be 35°C and growth rate was moderately low in other temperature *viz*. 30°C and 25°C. So, 35°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; 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 both isolates were sensitive to all the tested antibiotics except Bacitracin and Cephradin. 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; 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.

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