



Original Research Article

# Isolation and characterization of arsenic resistant bacteria from agricultural soil, and their potential for arsenic bioremediation

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**Abbreviations:** AAS: Atomic Absorption Spectrophotometer, As: Arsenic, As (V): Sodium arsenate, As (III): sodium arsenite

Arsenic (As) is a toxic pollutant released into the environment either by natural phenomena or anthropogenic activities. It is a potent human toxin, which causes many diseases such as diarrhoea, bladder cancer etc. Pesticides are the major source for accumulation of As in the agricultural soils. Currently available bioremediation techniques have major disadvantages such as secondary environmental pollution and are less cost effective. Therefore, the present study was aimed to isolate arsenic resistant bacteria from terrestrial environment Tamil Nadu, South India for their potential applications in bioremediation strategies. From the isolated fifty arsenic-resistant bacteria, two bacterial isolates BC1 and BC2 were taken for further studies due to their higher resistance ability to As. The optimum pH and temperature were found to be at 6 and 37 °C respectively. The 16S rRNA gene sequence of the isolate BC1 belongs to the genera *Enterobacter asburiae* and BC2 *Enterobacter cloacae*. The results revealed that our isolates BC1 and BC2 encoded arsenite oxidizing gene *aoxA* and arsenate reducing gene *arsC* respectively. Further characterization of *aoxA* gene and *arsC* gene will be useful for the development of efficient bioremediation strategies in the detoxification of arsenic from polluted environments.

**Key words:** Arsenic; As-resistance bacteria; Agricultural soil; 16S rRNA; *Enterobacter asburiae*; *Enterobacter cloacae*; *aoxA* and *arsC* genes

## INTRODUCTION

Exposure to heavy metals such as lead, cadmium, mercury and arsenic is a major health hazard. The effect of these metals on human health have been extensively studied and periodically monitored by World Health Organization. Arsenic is a toxic pollutant released into the environment either by natural or anthropogenic activity. Thousands of arsenic contaminated sites were reported around the world, Eisler et al. (2004). Arsenic (As) was found to be abundant in rocks, soil, water, sediments and air. Arsenic is also an ingredient in many commonly used materials including wood preservatives, pigments, insecticides, herbicides, rodenticides, fungicides and animal feed additives, Mandal and Suzuki. (2002). Arsenic exists in the environment in several oxidation states such as arsenate

[As (V)], arsenite [As (III)], elemental [As (0)] and arsenide [As (-III)], Sunita et al. (2011). Although, both arsenate and arsenite are toxic, arsenite As (III) is considered to be 25–60 times more toxic than arsenate As (V), Munawar et al. (2012).

Contamination of terrestrial and aquatic ecosystems by arsenic (As) is a very sensitive environmental issue due to its adverse impact on human health. The level of arsenic accumulation in environment dramatically increased over the years that cause increasing soil contamination and thereby enter the food chain through biomagnification. It is the only known human carcinogen for which there is an adequate evidence of carcinogenic risk by both inhalation and ingestion, IARC, Monographs. (2004). Acute exposure

to high levels of inorganic arsenic by humans can be fatal while acute exposure to lower levels can result in vomiting, decreased production of red and white blood cells, abnormal heart rhythm, and damage to blood vessels. Skin lesions are a common sign of arsenic poisoning, Smedly et al. (2002). Chronic exposure especially damages the liver and leads to cirrhosis. It is also a neurotoxin, damaging peripheral and central nervous systems, Mazumder et al. (1999). At cellular level, arsenic has been shown to induce chromosomal aberrations. However, the results of genotoxicity studies have indicated that it is not mutagenic. It has been suggested that arsenic might interfere with the DNA repair system or DNA methylation state, inhibition of p53 and telomerase activities, Chou et al. (2001). oxidative stress, promotion of cell proliferation and signal transduction pathways leading to the activation of various transcription factors, Wang et al. (2001). Therefore, removal of arsenic from environment is of great significance to local agriculture and the population. The conventional techniques such as chemical precipitation, chemical oxidation and reduction, ion exchange, filtration, reverse osmosis have been employed for the removal of arsenic, Malik (2004). The disadvantage of these methods is that they are not accurate, particularly when these toxic heavy metals are present in very low concentration, Chaalal et al. (2005). The greater public awareness of arsenic poisoning in animal and human nutrition has been a growing interest in developing regulatory guidelines and remediation technologies for mitigating arsenic-contaminated ecosystems, Mahimairaja et al. (2005). The other drawbacks associated with the present techniques include secondary environmental pollution due to the chemicals used in the remediation process and the cost of the prevailing techniques.

In recent years, bioremediation of heavy metals using microorganisms has gained attention. Microorganisms play a major role in the biochemical cycle of arsenic and can convert to different oxidation states with different solubility, mobility and toxicity, Silver and Phung. (2005). Certain microorganisms in nature have evolved the needed genetic components that provide resistance mechanisms, which enable them to survive and grow in an environment containing toxic levels of arsenic. The *ars* operon located on plasmids/chromosomes of prokaryotes is well characterized and is known to involve in arsenic resistance mechanism, Xu et al. (1998). The microbial arsenic detoxification involves the reduction of arsenate As (V) to arsenite As (III) via a cytoplasmic arsenate reductase (*arsC*) and further, As (III) will be extruded by a membrane-associated *arsB* efflux pump. Other genes like *arsR*, *arsD* and *arsA* form part of *ars* operon along with *arsB* and *arsC* in most of the prokaryotes, Rosen. (2002). Hence, natural arsenic levels and arsenic from pesticides create a significant threat to human health, it is very important to remove this toxic metalloid from the soil, During et al. (2003).

In the present study, we have described detailed morphological, biochemical characterization and 16S rRNA analysis of arsenic resistant bacteria isolated from agricultural soil of Tamilnadu, India (Figure 1). The capability of the isolates to withstand high concentration of arsenic and resistance towards other heavy metals were determined. The arsenate reducing and arsenite oxidizing abilities of the bacteria using specific gene loci were also studied in depth with a view to suggesting interventions that will reduce pollution due to arsenic in the environment.

## MATERIALS AND METHODS

### Chemicals and stock solutions

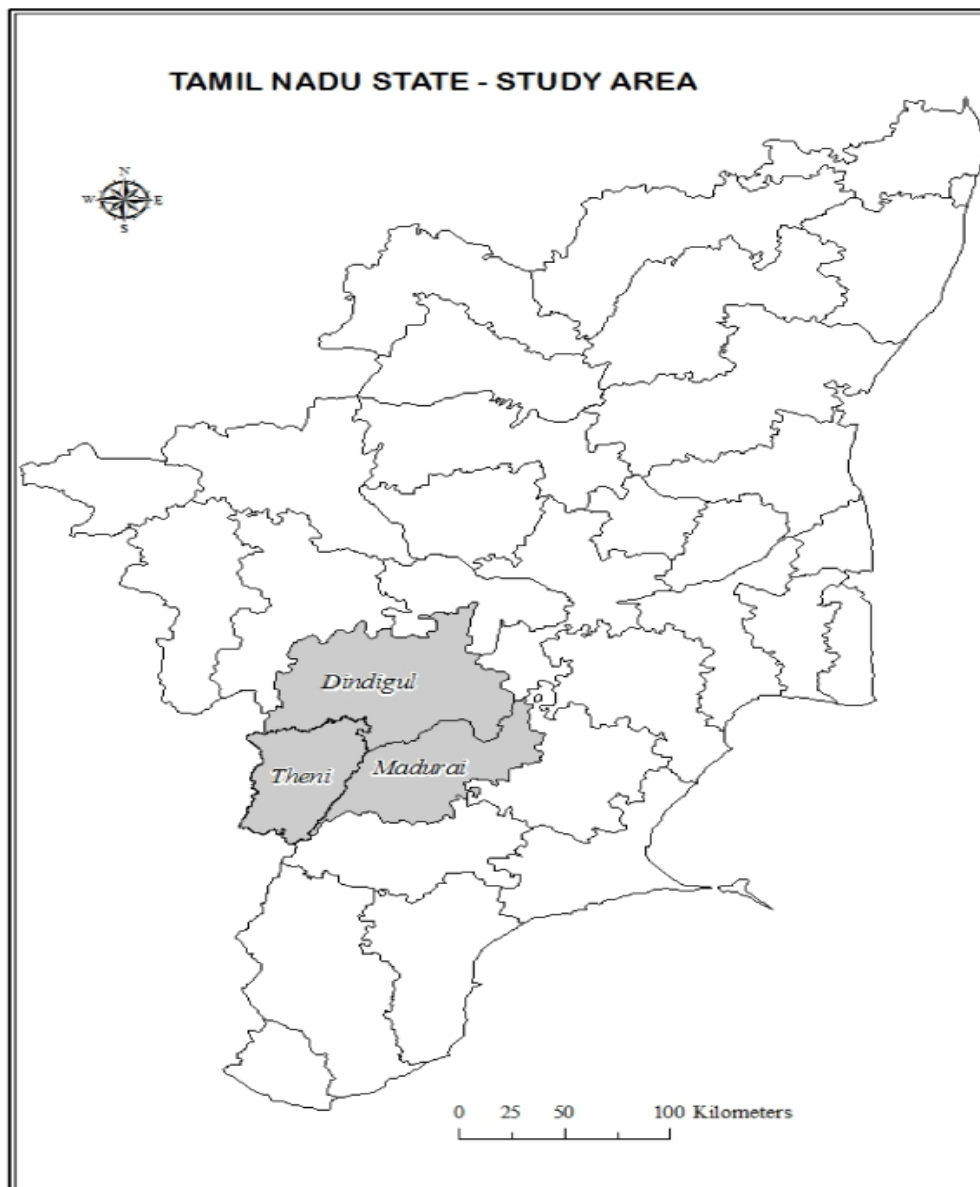
Chemicals used in this study were purchased from Himedia, India. As [III] stock solutions were prepared freshly before use from sodium arsenite ( $\text{NaAsO}_2$ ) and As [V] stock solutions from sodium arsenate ( $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ ) and were stored at 4 °C in the dark. Stock solutions of Silver nitrate ( $\text{AgNO}_3$ ) (1 M) were prepared freshly before use.

### Sample Collection

Agricultural soils were collected from three different districts in Tamilnadu, South India namely Dindigul, Madurai and Theni. The latitude and longitudes of the sites are, site A (10.3500°N and 77.9500°E), site B (9.6100°N and 77.7433°E), site C (10.0667°N and 77.7500°E). From each site five samples were collected one week post treatment with pesticide (from 0 -5 cm in depth) in sterilized and sealed polythene bags. The samples were then carefully brought to the laboratory and stored at 4 °C for further use. The pH of soil sample was determined by shaking 10 g of soil in 20 mL of distilled water for 25 min after that measurement with pH meter, Aksornchu et al. (2008).

### Atomic absorption spectrophotometric analysis of the soil sample

Arsenic content in the soil samples were analyzed by atomic absorption spectrophotometer (AAS) (Varian AA240 system). For arsenic determination, the samples were pre-reduced prior to analysis. This was accomplished by the addition of 1 mL of concentrated hydrochloric acid (HCl) and 1 mL of reducing solution (10% (w/v) potassium iodide) to the 1 mL of the sample. The reduction rate was improved by increasing the acid concentration. The solution was left to stand for 30 - 45 min at room temperature thus allowing the conversion of arsenate As (V) to arsenite As (III) in the sample in order to provide increased sensitivity. All solutions were filtered using Whatman No 1 filter paper to remove any fine suspended particulates. Standard as solution was prepared in the



**Figure 1:** Site map of the study area Dindigul, Madurai and Theni. Samples were collected from the agricultural soil

range 10-60 mg L<sup>-1</sup>. Each sample was analyzed in triplicate and the values were presented mean  $\pm$ SD.

#### Isolation and enumeration of arsenic resistant bacteria

The soil sample (1g) was serially diluted with saline and plated on Luria -Bertani (LB) agar plate and minimal medium plate containing 1 mM of sodium arsenate (Na<sub>2</sub>HAsO<sub>4</sub>·7H<sub>2</sub>O) and 1 mM of sodium arsenite (NaAsO<sub>2</sub>). The plates were incubated at 37 °C for 72 hrs. After the growth was observed, the colonies were patched on fresh LB agar plate. A number of morphologically different colonies were randomly selected. Based on this preliminary

screening, the colonies showing resistance to arsenate or arsenite were selected and used for further studies.

#### Determination of minimum inhibitory concentrations (MICs)

The levels of resistance were determined by MIC. The minimum inhibitory concentration (MICs) is the lowest concentration that completely inhibits bacterial growth. Isolated bacterial cells were streaked on LB and minimal medium supplemented with varying concentrations of sodium arsenate and sodium arsenite. The bacterial strains

grown in LB medium with As (V) and As (III) showed growth up to 400 mM and 40 mM where as in the minimal medium showed growth up to 240 mM for arsenate and 40 mM for arsenite.

### Identification and characterization of arsenic-resistant bacteria

The bacterial isolates that could tolerate arsenate and arsenite concentration were selected and identified by their morphological features and biochemical properties. Biochemical characterization was determined by Indole production, MR-VP test, Citrate utilization, Oxidase test, Catalase test, Starch hydrolysis, Gelatin hydrolysis, Triple sugar iron test, Mannitol salt agar, Urea hydrolysis test, Dextrose, Sucrose, Maltose, Rhamnose, Arabinose and Sorbitol tests according to Bergey's Manual of Systematic Bacteriology, Claus and Berkeley.(1986).

### Physiological characterization

#### Determination of optimal growth conditions

The optimal growth conditions with reference to pH and temperature were determined. The strains were grown in LB medium, in the presence and absence of arsenic with varying pH (5, 6, 7, 8 & 9) incubated at 25, 30, 37 and 40 °C. The optical density of the growing cultures in LB medium was observed at OD 600 nm using an UV visible spectrophotometer, Doenmez and Aksu. (2001).

#### Silver nitrate test

Silver nitrate test was performed as described by Simeonova et al. (2004). In brief, LB agar plates were supplemented with sodium arsenite and sodium arsenate. A single line streak of the isolated organism across the centre of the LB plate was made and incubated at 37 °C for 48 hrs. After incubation, the plates were flooded with 0.1 M silver nitrate solution. A brownish precipitate indicated the presence of arsenate in the medium (arsenite oxidizing bacteria) and the presence of arsenite were detected by a bright yellow precipitate (arsenate reducing bacteria).

#### Determination of antibiotic resistance

Antibiotic sensitivity of the arsenic resistant isolates was determined by the disc diffusion method. Antibiotic-impregnated discs were placed on Muller Hinton Agar (MHA) plates spread with bacterial culture and incubated at 37 °C for 24 hrs. Inhibition zone was noted after 24 hrs incubation, resistance was recorded as positive. The diameters of the inhibition zones around the discs were measured. The antibiotic concentrations of the disc used were Ampicillin (10 µg), Tetracycline (30 µg), Chloramphenicol (30 µg), Erythromycin (10 µg),

Kanamycin (10 µg), Neomycin (10 µg), Nalidixic acid (5 µg), Rifampicin (3 µg) and Streptomycin (20 µg).

### Effect of arsenic on bacterial growth

Growth of arsenic resistant bacterial strains was determined in LB medium. From an overnight pure culture, 1% inoculum was added to 50 ml of LB medium supplemented with 1 mM and 350 mM sodium arsenate and 1 mM and 40 mM sodium arsenite. The cultures were incubated at 37 °C in an orbital shaker at 120 rpm for 72 hrs. The growth of the isolate was monitored by measuring optical density after every 6 h intervals at OD 600 nm using spectrophotometric method.

### Atomic force microscopy analysis

BC1 and BC2 cells cultured in LB medium supplemented with sodium arsenate 350mM and sodium arsenite 40 mM were incubated at 37 °C with agitation for 72 hrs. Cells were harvested by centrifugation at 4000 rpm for 15 min and washed twice with double distilled water. Samples for AFM analysis were mounted on the cover glass and air dried as described by Bolshakova et al. (2001). Samples were scanned at different areas using AFM (Shimadzu SPM 9500-2J). For high resolution, contact mode micro cantilever was used for the analysis. Digital images were stored in the computer and processed

### Resistance to other heavy metals

The arsenic bacterial isolates resistant to various heavy metals like Pb, Zn, Hg, Cd, Ni, Cu, and Cr were determined with 1M stock solutions. Overnight culture was streaked in LB medium with different concentrations of heavy metals. The culture was evaluated after 72 hrs of incubation at 37 °C.

### 16S rDNA sequence determination

Genomic DNA was extracted from BC1 and BC2 as described by Sambrook et al. (2001). Bacterial 16S rRNA gene was amplified by PCR using the universal 16S rRNA primers, forward primer (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer (5'-GGT TACC TTG TTA CGA CTT-3'). PCR was carried out with 50 µL reaction containing 1X PCR buffer with 0.6 mM MgCl<sub>2</sub>, 0.2 mM dNTP, *Taq* DNA polymerase 1U and 100 ng template DNA using a Gene Amp PCR system 2700 (Applied Biosystems) with the following cycling conditions, including initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 5 min. A negative control without the DNA template was used for amplification along with experiment. The PCR products were analyzed in 1.5% (w/v) agarose gel in 1X TAE buffer,

**Table 1.** Arsenic concentration and pH value of the soil samples

Sample Sites A,B and C	pH	Total arsenic concentration (mg As/kg soil)
Site -1	6.72	1.43± 0.047
Site -2	7.63	1.06±0.065
Site -3	7.02	0.49±0.030
Site -4	7.35	1.52±0.045
Site -5	7.97	0.88±0.072
Site -6	7.88	1.05±0.035
Site -7	7.53	0.79±0.040
Site -8	7.71	1.07±0.075
Site -9	7.85	0.95±0.070
Site -10	7.22	1.98±0.086
Site -11	7.95	1.17±0.110
Site -12	7.09	1.02±0.090
Site -13	7.51	0.69±0.035
Site -14	7.39	1.67±0.045
Site -15	6.90	1.02±0.040

stained with ethidium bromide (0.5 mg/mL) and observed under ultraviolet light before being subjected to further analysis.

### Nucleotide sequencing

The PCR product was purified by using sigma-Gen Elute™ PCR Clean-Up Kit according to the manufacturer's instructions. The purified products were sequenced by Eurofins genomics India Pvt., Ltd, India. 16S rRNA sequence of each isolate, and also the whole sequences were compared to reference sequences in the GenBank database using BLASTN. The phylogenetic tree was constructed using online phylogenetic tool phylogeny.fr with MUSCLE and PhyML. 16S rRNA gene sequences of BC1 and BC2 deposited in GenBank.

### Isolation and identification of *arsC* and *aoxA* gene

Plasmid DNA was isolated by using Himedia - HiPurA™ plasmid DNA Miniprep Purification Kit method from BC1 and BC2 strains. The primers used for the isolation of *aoxA* gene and *arsC* gene is *aoxA* F 5'-ATG GAA CAT CAA ACT AGT CG-3' and *aoxA* R 5'-ACA GAA TGT TGG ATT GAC G-3' [23] *arsC* F: 5'-GTA ATA CGC TGG AGA TGA TCC G-3' and *arsC* R: 5'-TTT TCC TGC TTC ATC AAC GAC-3' respectively, Chang et al. (2007). The plasmid DNA (100ng/μL) amplification was carried out by PCR method (Gene Amp PCR system 2700, Applied Biosystems) with the following cycling conditions. The PCR protocol for amplification of *aoxA* gene is comprised of denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, annealing at 55 °C for 1 min, and an extension at 72 °C for 2 min. A final extension was carried out for 5 min at 72 °C and that for *arsC* gene PCR was performed by initial denaturation 94 °C for 3 min followed by 35 cycles of denaturation 94 °C for 30 s, annealing 52 °C for 30 s and extension 72 °C for 30 s and a

final extension at 72 °C for 5 min. The *aoxA* and *arsC* gene amplicon was eluted and sequenced. The obtained gene sequences were confirmed based on homology analysis by using NCBI BLAST software Altschul et al. (1990). The *aoxA* and *arsC* gene sequences were submitted to GenBank.

## RESULTS

### Analysis of Arsenic concentration and pH of soil samples

The pH and arsenic concentrations of collected soil samples were found to be in the range of 7.02- 7.22 and 0.49 -1.98 (mg As /kg of soil), respectively (Table 1).

### Isolation and biochemical characterization of arsenic resistant bacterial strains

Fifty arsenic-resistant strains were isolated from three different sites (Site A -20, B -18 and C -12 strains). The preliminary identification of strains indicates that 16 isolates were Gram- negative rod shaped bacteria and 34 isolates were gram positive cocci. From these, 2 bacterial isolates (BC1 and BC2) were selected based on their higher arsenic resistance. The strains BC1 and BC2 were found to be Gram-positive cocci, motile and formed yellow and white colonies respectively. Table 2 shows the detailed analysis of morphological and biochemical characterization of two potential arsenic resistance strains BC1 and BC2.

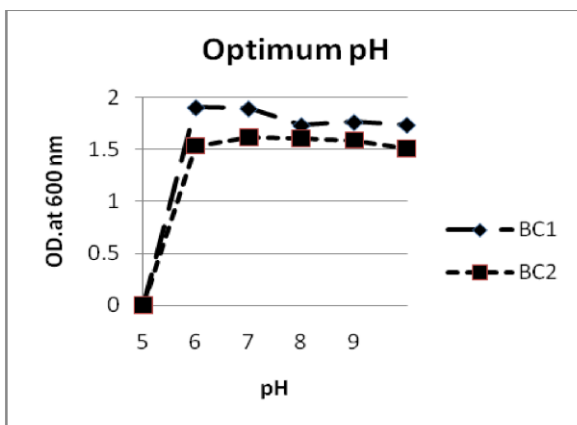
### Minimum inhibitory concentrations

Microbial resistance to arsenate [As (V)] and arsenite [As (III)] were determined by visible growth after 24 h in LB medium and the minimal medium plates supplemented with varying concentrations of sodium arsenate and

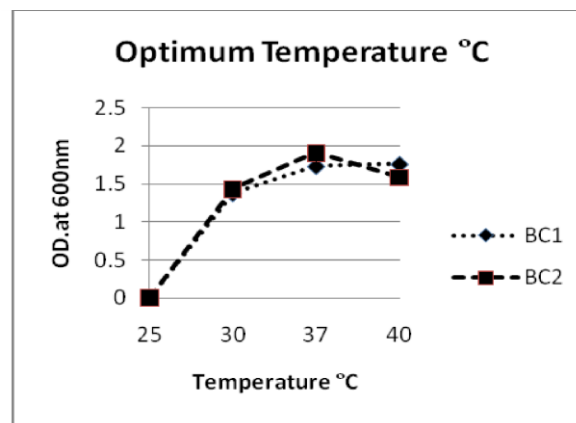
**Table 2.** Biochemical characterization of arsenic resistant bacteria

Characteristics	BC1	BC2
Colony color	Yellow	White
Gram staining	Gram +	Gram +
Cell Morphology	Cocci	Cocci
Motility	Motile	Motile
Oxidase	+ve	- ve
Catalase	+ ve	+ ve
Indole	- ve	- ve
MR-VP	+ ve	- ve
Citrate utilization test	+ ve	+ ve
Carbohydrate fermentation Sucrose, Glucose, Lactose (A/G)	+ /+ve	+ /+ve
Starch hydrolysis test	+ ve	- ve
Mannitol salt Agar	-ve	-ve
Gelatin hydrolysis	-ve	-ve
Urea hydrolysis	+ ve	-ve
Dextrose	+	-
Sucrose	-	+
Maltose	-	+
Rhamnose	-	-
Arabinose	+	+
Sorbitol	+	+

+: Indicates positive result - : Indicates negative result



**Figure 2a:** Growth responses of the selected isolates BC1 and temperature BC2 at different pH



**Figure 2b:** Growth responses of the selected isolates BC1 and BC2 at different

sodium arsenite. Among the tested strains, two isolates BC1 and BC2 could tolerate higher level of arsenate and arsenite concentration. Both arsenic resistant isolates exhibited MIC at a range 400 mM and 40 mM for sodium arsenate and sodium arsenite in LB medium, whereas, in minimal medium it was found to be 240 mM and 40 mM respectively. The higher values of MIC for arsenic [As (V) and As (III)] indicated that these bacterial isolates could be used for efficient bioremediation purposes.

### Physiological characterization

pH plays a major role in growth and metal accumulation

properties of the bacterial strains. Temperature is another environmental factor that affects bacterial growth. In the present study, BC1 and BC2 isolates showed growth at pH 5-9 and temperature at 25, 30, 37 and 40°C. The optimum pH and temperature for growth was observed to be 6 and 37°C respectively (Figure 2a and b).

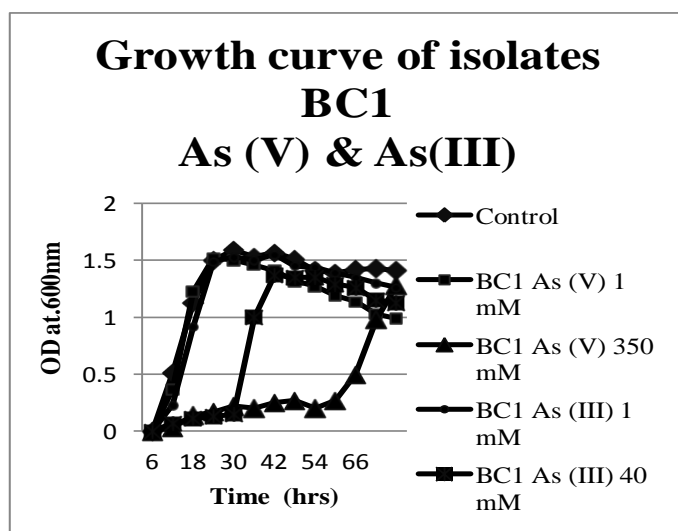
### Silver nitrate test

A qualitative silver nitrate ( $\text{AgNO}_3$ ) screening technique was used to detect the oxidation of arsenite to arsenate or the reduction of arsenate to arsenite. The results revealed that, the strains BC1 is arsenite oxidizing bacteria and BC2

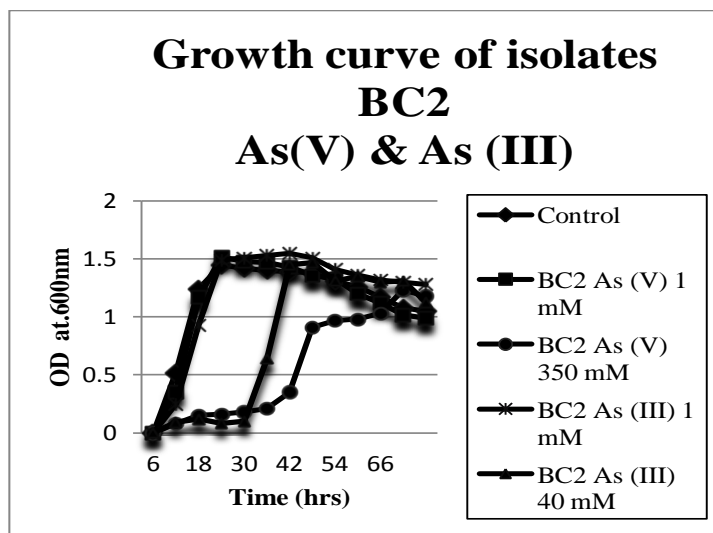
**Table 3.** Antibiotic resistance of *Enterobacter asburiae* BC1 and *Enterobacter cloacae* BC2

Antibiotics	Inhibitory zone BC2	Inhibitory zone BC1
Ampicillin	(I)	(R)
Chloramphenicol	(S)	(S)
Nalidixic acid	(I)	(I)
Neomycin	(I)	(I)
Kanamycin	(I)	(I)
Rifampicin	(R)	(I)
Streptomycin	(R)	(I)
Tetracycline	(I)	(R)

R= resistant; S = sensitive; I= intermediate



3a



3b

**Figure 3a and 3b:** Growth pattern of selected isolates BC1 and BC2 in arsenite and arsenate

is arsenate reducing bacteria. There by it was inferred that the isolates BC1 and BC2 are capable of producing arsenite oxidase and arsenate reductase enzymes respectively.

#### Antibiotic resistance for arsenic resistance bacteria

Antibiotic susceptibility test revealed that both isolates BC1 and BC2 were sensitive to chloramphenicol. BC1 was found to be resistant to rifampicin and streptomycin and intermediate resistance to kanamycin, neomycin and nalidixic acid. BC2 was resistant to ampicillin and tetracycline. However, it shows intermediate resistance to kanamycin, neomycin and nalidixic acid (Table 3).

#### Effects of arsenic on bacterial growth

To determine the effects of As[V] and As[III] ions on bacterial growth, the two arsenic resistance bacterial

isolates BC1 and BC2 were grown in arsenic free media supplemented with arsenate (1mM and 350 mM) and arsenite (1 mM and 40 mM) respectively. The growth of the isolates was monitored periodically by measurement of optical density. The growth curves of BC1 and BC2 were found similar to the control (without arsenic) and with 1 mM of As (V) and As (III) containing media. In contrast to this, the growth rates of BC1 and BC2 showed significant difference in 350 mM arsenate and 40 mM arsenite containing media (Figure 3a and 3b).

#### Multiple metal resistances

The arsenic resistant bacterial strains BC1 and BC2 found resistant to other heavy metals such as Zn Cr, Ni, Pb, Cu, Hg, and Cd. The MIC values of BC1 were Zn (17.5 mM), Cr (16.5 mM), Pb (16 mM), Ni (15.5 mM), Cu (11.5 mM) Hg (6.5 mM) and Cd (2 mM) and for BC2 were Zn (20.5 mM), Ni (18.5

Table 4. MIC of bacterial isolates in other heavy metals (mM)

Metals	Selected isolates	MIC
Zn	BC1&BC2	17.5& 20.5mM
Cr	BC1&BC2	16.5& 17.8mM
Pb	BC1&BC2	16& 18mM
Ni	BC1&BC2	15.5& 18.5mM
Cu	BC1&BC2	11.5& 13.5mM
Hg	BC1&BC2	6.5& 7.5mM
Cd	BC1&BC2	2/1.8mM

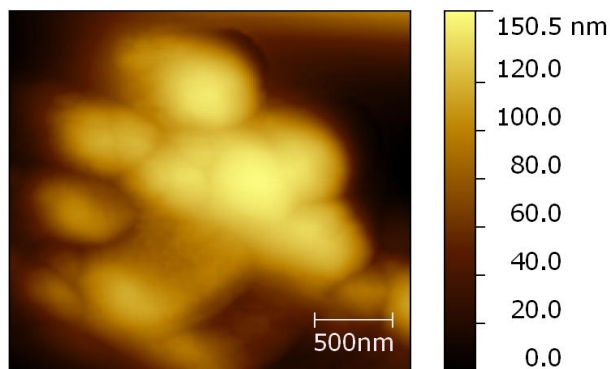


Figure 4a : Control BC1 at magnification 500µm

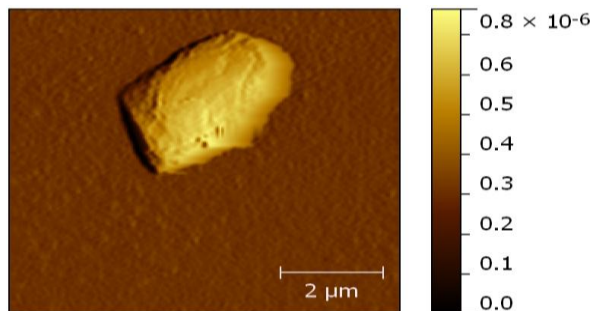


Figure 4d: Control BC2 at magnification 2µm

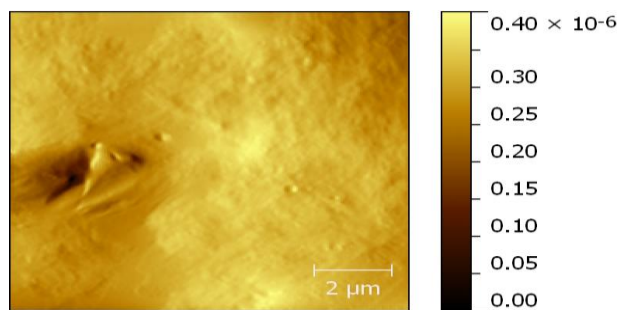


Figure 4e: BC2 at 350 mM of arsenate (magnification at 2µm)

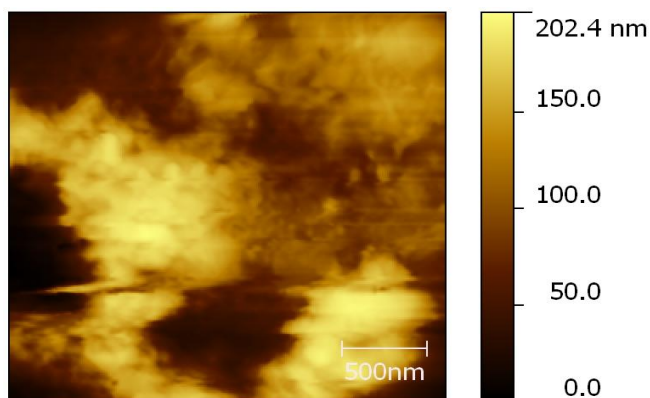


Figure 4b: BC1 at 350 mM of arsenate (magnification at 500µm)

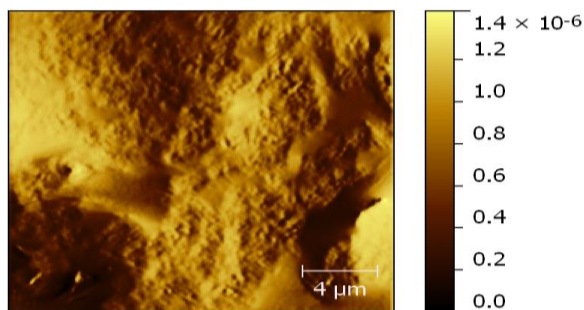


Figure 4f: BC2 at 40 mM of arsenite (magnification at 4µm)

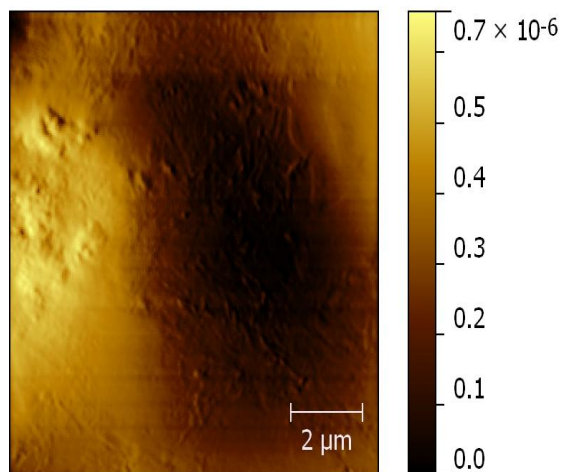


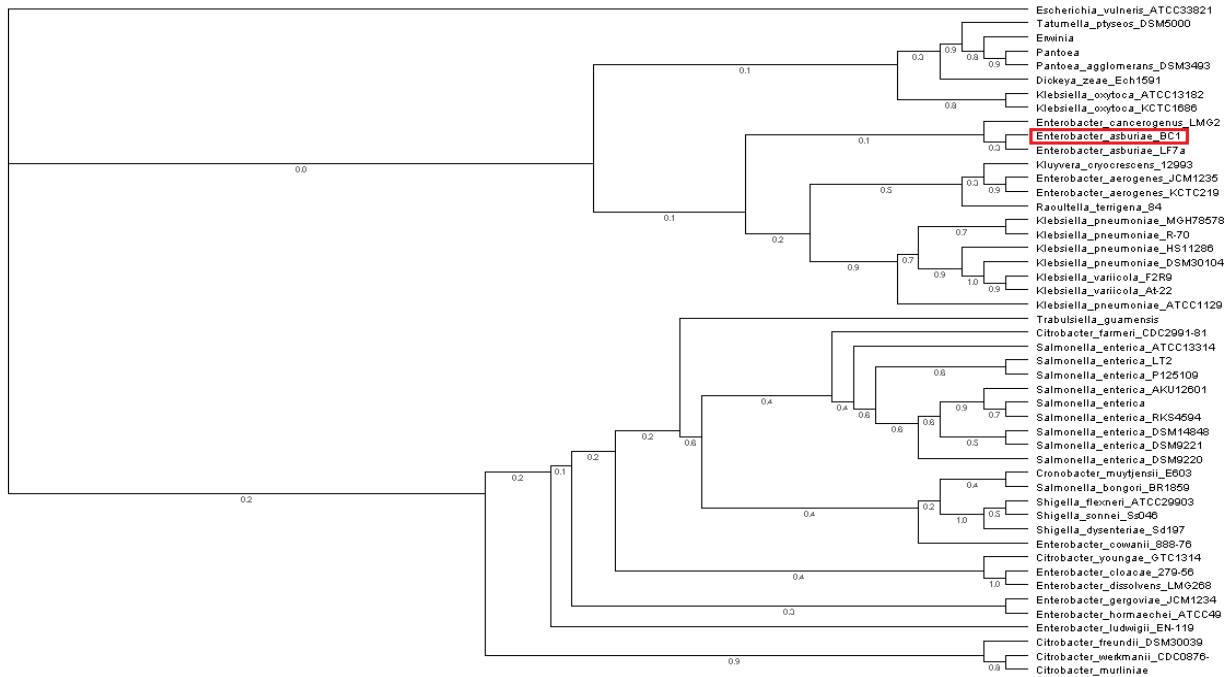
Figure 4c: BC1 at 40 mM of arsenite (magnification at 2µm)

mM), Pb (18 mM), Cr (17.8 mM) Cu (13.5 mM), Hg (7.5 mM) and Cd (1.8 mM) respectively. The resistance of the isolates BC1 and BC2 to other heavy metals is shown in Table 4. Among the heavy metals tested Cd was highly toxic for BC1 and BC2. Other heavy metals were toxic to both BC1 and BC2 with the order of resistance Zn > Cr > Pb > Ni > Cu > Hg > Cd and Zn > Ni > Pb > Cr > Cu > Hg > Cd respectively.

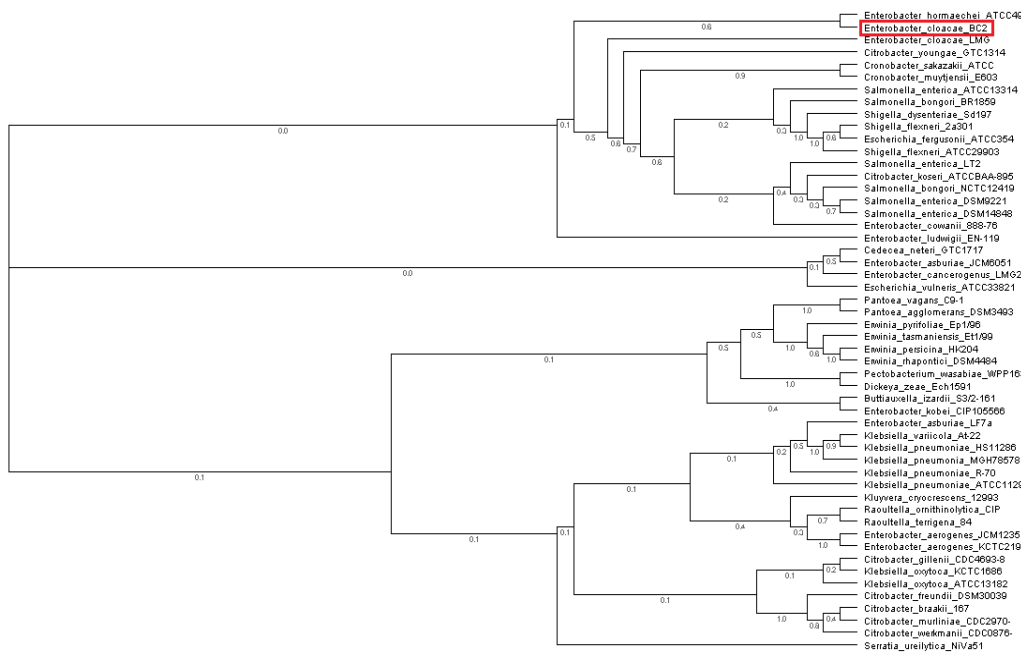
**Microscopic observations of bacterial cells (AFM)**

*Enterobacter asburiae* (BC1) and *Enterobacter cloacae* (BC2) cells were imaged with AFM operated in contact mode in liquid conditions. Cells analyzed under AFM showed significant difference in their cell wall morphology and cell dimensions from solution with and without arsenic (Figure 4 a, b, c, d, e and f).





**Figure 5a:** Phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between strain BC1 related bacteria showing Phylogenetic tree based on 16S rRNA gene sequences

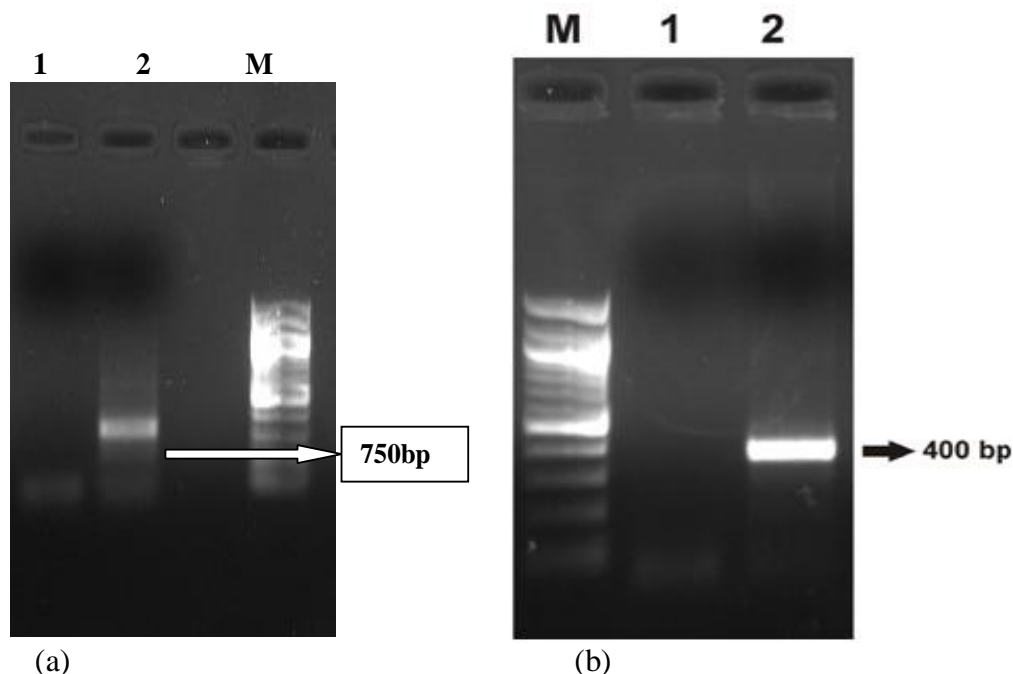


**Figure 5b:** Phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between strain BC2 related bacteria, showing the relationship between strain BC2 related bacteria

**16S rDNA sequences and Phylogenetic analysis**

The 16S rDNA sequences of BC1 and BC2 isolates were subjected to nucleotide BLAST. BC1 showed 99% similarity

to *Enterobacter asburiae* and BC2 showed 97% homology to *Enterobacter cloacae* (Figure 5a and 5b). The 16S rDNA nucleotide sequence of BC1 and BC2 were submitted in the NCBI databases under the GenBank accession numbers



**Figure 6 a, b:** PCR amplification of *arsC* and *aoxA* gene from *Enterobacter cloacae* and *Enterobacter asburiae*. Lane 1. 100 bp DNA ladder, Lane 2. PCR negative control, Lane 3 *arsC* gene from isolates BC1. Lane 1. PCR negative control, Lane 2. *aoxA* gene from isolates BC1, Lane 3 31kb DNA ladder

KF662730 and KF662731.

#### **Arsenic resistant genes (*arsC*) and (*aoxA*) sequences from isolated strains**

With prior knowledge of plasmid-borne nature of *ars* operon arsenic resistance strains, PCR amplification of arsenite oxidase (*aoxA*) and arsenate reductase (*arsC*) gene was performed for BC1 and BC2 isolate using specific primers; the expected 750 bp and 400 bp length of PCR products for *aoxA* and *arsC* gene was obtained from both wild type BC1 and BC2 strains respectively (Figure 6a & b). The BLAST analysis of the sequences from BC1 and BC2 revealed 92% and 100% homology with *Enterobacter asburiae* arsenite oxidase (*aoxA*) and *Enterobacter cloacae* arsenate reductase (*arsC*). The partial *arsC* gene (BC2) sequence was deposited in the NCBI Genbank with accession number KJ371118 and *aoxA* gene (BC1) sequence was submitted in the NCBI.

#### **DISCUSSION**

The molecular approaches are frequently being used to investigate the specific microbial communities associated with metal-contaminated environments with increased sensitivity; Miguez et al. (1997) PCR and gene probes are used to characterize the environment prevalence of

microbial community associated with the polluted environment and development of genetic model system for efficient bioremediation strategies.

The isolation of arsenic resistant bacteria is the preliminary step for identification of potential candidates for bioremediation. In the present study, two arsenic resistant bacteria *Enterobacter asburiae* (BC1) and *Enterobacter cloacae* (BC2) were isolated from arsenic contaminated agricultural soil. The soil profile analysis of samples (site A, B & C) revealed relatively acidic pH and moderately low concentration of arsenic. Arsenic concentration in the soil sample was found to be 0.49 -1.98 (mg /kg of soil) which was comparatively lower than the arsenic concentration of 16.8 mg kg<sup>-1</sup> and 2.7-202 mg kg<sup>-1</sup> as reported by Sinha et al. (2011). and De Gregori et al. (2003) in agricultural soil. The resistance limit to the highest concentration of arsenate and arsenite was evaluated based on the ability of BC1 and BC2 cells to grow on arsenic containing LB media. Both isolates BC1 and BC2 exhibited natural resistance up to 40 mM and 400 mM for sodium arsenite and sodium arsenate in solid media. Notably BC1 and BC2 strains showed the highest resistance to arsenic reported thus far. Bacterial strains used in industrial bioremediation such as *Corynebacterium glutamicum* showed resistance to arsenite upto 12 mM and 400 mM for arsenate, Mateos et al. (2006). Furthermore, previous studies have revealed that the application of bacteria resistant to arsenate (10.13 mM) in

bioremediation processes, Takeuchi et al. (2007). Awais et al. (2011) have identified potential strains of *Klebsiella pneumoniae* (*K. pneumoniae*) and *Klebsiella variicola* (*K. variicola*) with minimum inhibitory concentration of 26.6 and 24 mM against As (III). The MIC of arsenic in solid media was higher than those in liquid media due to the conditions of diffusion, complexation and availability of arsenic was different from those observed in solid media. Both isolates BC1 and BC2 showed optimum growth at pH 6 and temperature of 37 °C. Previous studies have shown isolates that could tolerate a wide range of pH from 4–9 and maximum growth at 37 °C, Suchanda et al. (2011). BC1 isolate was able to oxidize arsenite while BC2 was capable of reducing arsenate. Rehman et al. (2010) reported that *P. lubricans* showed high resistance against arsenite up to 40 mM and could oxidize As (III). Arsenite, frequently reported in water, is more mobile, highly soluble and more toxic than arsenate.

The most appropriate way of toxic arsenite removal is to oxidize it into arsenate which is less soluble and much more easily removed. BC1 was found to be resistant to rifampicin and streptomycin while BC2 was resistant to ampicillin and tetracycline. Farah R et al. (2010) reported that *C. freundii* and *B. anthracis* were sensitive to erythromycin, kanamycin, nalidixic acid, amoxicillin, chloramphenicol, neomycin, oxytetracycline, streptomycin and tetracycline, while *K. oxytoca* was sensitive to amoxicillin, chloramphenicol, neomycin, oxytetracycline, streptomycin. The strains BC1 and BC2 showed growth rate in 40 mM of As (III) and 350 mM of As (V) in liquid medium. Earlier studies showed inhibition of bacterial strains in the presence of As (V) 133.47 mM in LB broth (ORAs3) *Aeromonas salmonicida*, (ORAs6) *Aeromonas molluscorum*, (ORAs7) *Aeromonas salmonicida* and (ORAs9) *Bacillus cereus*. Growth of strains (ORAs4) *Aeromonas salmonicida* and (ORAs10) *Bacillus cereus* was inhibited by 13.34 mM of As (III), while strain (ORAs6) *Aeromonas molluscorum*, proved to be the most resistant to As(III), still growing in the presence of 16.68 mM of the trivalent form of the metalloid, Pepi et al. (2007). BC1 and BC2 isolates showed resistance to other heavy metals Zn (17.5 & 20.5 mM), Cr (16.5 & 17.8 mM), Pb (16&18 mM), Ni (15.5& 18.5 mM), Cu (11.5 & 13.5 mM), Hg (6.5 & 7.5) and Cd (2 & 1.8 mM). Both isolates BC1 and BC2 showed the order of resistance Zn > Cr > Pb > Ni > Cu > Hg > Cd and Zn > Ni > Pb > Cr > Cu > Hg > Cd respectively.

Previous reports have revealed that *Enterobacter cloacae* showed resistance to Cd (1.78 mM) Cr (15.38 mM) and Pb (6.76 mM), Friis and Myers (1986). *K. pneumoniae* showed resistance to heavy metals such as Cr<sup>6+</sup>(6.6 mM), Cd<sup>2+</sup>(1.3 mM), Cu<sup>2+</sup>(6.6 mM), Ni<sup>2+</sup>(5.3 mM) and Hg<sup>2+</sup>(1.3 mM), Awais et al. (2011). and *K. variicola* isolated from industrial effluents were found to be tolerant to Cr<sup>6+</sup> (6.6 mM), Cd<sup>2+</sup>(2.6 mM), Cu<sup>2+</sup>(9.3 mM), Ni<sup>2+</sup>(5.3 mM) and Hg<sup>2+</sup>(4.0 mM). The treatment with arsenic resulted in disintegration of cell morphology which is associated with decrease in

growth and formation of localized depression in the cell surface of BC1 and BC2 was shown in the AFM micrograph. This may be due to the presence of polarizable groups on bacterial surfaces that are capable of interacting with cations of arsenic and are responsible for the reversible arsenic binding capacity of the microorganisms. Friis et al. (1986) previously reported that reduction in growth is mainly because of the interaction between the cell surface and of metal cations along with phosphate, carboxyl, and hydroxyl and amino-groups. The isolated bacteria also showed noteworthy resistance to other heavy metals too. These bacterial isolates can be helpful to explore the diversity of arsenic resistance system genes in a variety of arsenic resistant bacterial groups. The arsenic resistance system (*ars*) appears to be widely distributed among prokaryotes and it involves an arsenate reductase (*arsC*), arsenite efflux pump (*arsB* or *acr3*) and a transcriptional repressor (*arsR*) encoded by a set of genes that display large variations in their number and genomic organization, During et al. (2003). The studies of Macur et al., and Cai et al., found that the *Pseudomonas aeruginosa* chromosomal *ars* operon responsible for detoxification contained three potential ORFs encoding proteins with a significant sequence similarity to those encoded by the *arsR*, *arsB*, and *arsC* genes of *E. coli* chromosomal *ars* operons. Chang et al. (2007) isolated a strain that showed capability of completely oxidizing arsenite to arsenate. From the past two decades, PCR approach gained significance in identification and characterization of the metal tolerant genotypes in bacterial isolates inhabiting polluted environments (Kaur et al. 2009).

In the present study, we have isolated and characterized *aoxA* and *arsC* gene from the two arsenic resistance bacterial isolates of BC1 and BC2. The ability of the isolates BC1 and BC2 to oxidize the toxic As (III) to its less-toxic As (V) could be used for its potential application in bioremediation processes, since the most suitable way to remove arsenic removal from environment is oxidizing arsenite into arsenate, which is less soluble and could be removed easily from the environment. The *ars* operon is well established in other genera such as *Klebsiella*, *Escherichia*, *Pseudomonas* and *Streptococcus*. Our result also provides evidence for the presence of *ars* operon in *Enterobacter* species isolated from agricultural soils. Colin et al. (2005) had reported that the arsenic resistance among bacterial population is widespread, both environmentally and phylogenetically. Based on the above results, the isolates BC1 and BC2 could be efficiently used in the removal of arsenic from polluted sites and can be good candidates for bioremediation processes. However, the valid contribution to the exceptional arsenic tolerance of the strains *Enterobacter asburiae* (BC1) and *Enterobacter cloacae* (BC2) still remains to be unrevealed. The development of effective genetic model systems for the strains BC1 and BC2 would notably facilitate addressing these questions.

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**Conflict of interest**

The authors declare that there are no conflicts of interest associated with this work.

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