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Original Research Article

Isolation and characterization of arsenate reducing bacteria from the waste water of an electroplating industry

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Keywords

Arsenate reduction; *Achromobacter;* Electroplating industry.

A B S T R A C T

Three arsenic reducing bacterial strain designated as AR_2, AR_4 and AR_{10} were isolated from the waste of electroplating industry. The strains were identified by 16S rDNA sequencing and were found to be *Bacillus oceanosediminis* (AR_2) and *Achromobacter pulmonis* (AR_4 and AR_{10}). All the strains resisted both As(III) and As(V), common inorganic species of arsenic found in nature. Strains reduced As(V) to As(III) aerobically thus suggesting the presence of arsenic detoxification mechanism coded by *ars* operon. Strains AR_4 and AR_{10} which belonged to *Achromobacter* genus did not show As(III) oxidation, unlike previously reported *Achromobacter* species, thus suggesting that arsenic transformation capabilities may differ among different species of same genus.

Introduction

Arsenic is a well-known toxic chemical and listed as a known carcinogen by the Environmental Protection Agency (EPA) and the World Health Organization (WHO, 2001). It is the 20th abundant (0.0001%) compound in the earth's crust but is widely distributed in the nature (National research council, 1977). It is released in the environment by natural phenomena such as weathering, biological activity, and volcanic activity, as well as by anthropogenic inputs (for example electroplating industry, mining activities) from where it is redistributed on the earth's surface by rain and dry fallout (Cullen and Riemer, 1989).

Arsenic can exist in four oxidation states: Arsenate As(V), arsenite As(III), elemental As(0) and arsenide As(-III). The main species of arsenic found in the environment are the As(III) and As(V). As(V) is the less mobile form, mainly because it is strongly adsorbed to the surface of several common minerals, such as ferrihydrite and alumina, whereas As(III) is more mobile as it adsorbs less strongly and to fewer minerals (Smedley and Kinniburgh, 2002).

Microbes have been exposed to toxic heavy metals and metalloids including

arsenic since beginning of life. In spite of toxicity, microorganisms have developed different resistance mechanisms to counteract detrimental effects of arsenic (Mukhopadhyay *et al.*, 2002). The *ars* operon, either chromosomally or plasmid encoded, is the most widespread resistance mechanisms found in both Gram negative and Gram positive bacteria. The *ars* operon consists of minimum of three co-transcribed genes *arsRBC.* The *arsR* gene codes for transcriptional repressors, *arsC* for a reductase for the conversion of As(V) to As(III) and a*rsB* for a membrane-located As(III) efflux pump (Rosen, 1999). The *ars* operon imparts resistance to both As(V) and As(III). As(V) which is analogue of phosphate enters into the microbial cells via transmembrane phosphate transport proteins (Cervantes *et al.*, 1994). Microorganisms can not distinguish As(V) from phosphate unless it is converted to another form which is then pumped out of cell. Some bacteria have phosphate specific transport system which restricts entry of As(V) thus imparting resistance to metalloid (Willsky and Malamy, 1980). When As(V) enters inside the cell it is reduced to As(III) by cytoplasmic arsenate reductase. As(III) is then pumped out of cell by efflux pump coded by *arsB* gene. Some Gram negative bacteria, for example *E. coli* plasmid R773, contain *ars* operon with five genes *arsRDABC* (Hedges and Baumberg, 1973). ArsD is thought to be an additional trans-acting co-repressor present with well characterized ArsR (Li *et al.*, 2002) and *arsA* gene product is an intracellular soluble ATPase protein that binds to ArsB protein as a dimer. As (III) efflux pump can thus function alone as ArsB or as an ATPase with ArsA (Mukhopadhyay *et al.*, 2002). Waste of electroplating industry often contains different heavy metals including arsenic as contaminants and microorganisms inhabiting in the environments contaminated with such waste may develop resistance to different toxic heavy metals. The aim of the present study was to isolate and characterize arsenic resistant bacteria from metal contaminated waste water of a electroplating industry.

Materials and Methods

Enrichment procedure and strain isolation

Waste water was collected from electroplating industry located at Pirangut in Pune district (Maharashtra). Waste water (10%) was inoculated in the 250 ml flasks containing 27 ml Tris-mineral medium at low phosphate content[11], (Mergeay *et al*., 1985), supplemented with 0.04% yeast extract (TMM) and 0.6% glucose (TMMG) and 5 mM of As(V). Flasks were incubated on shaker at 30°C for 5 days and 3 ml of this enrichment culture was inoculated in fresh medium. This procedure was repeated two times and arsenic resistant bacteria were isolated by plating enrichment cultures on $1/10^{th}$ diluted tryptic soya agar (TSA) containing 5 mM of As(V). The cultures were routinely maintained on TSA with 1 mM of As(V).

Identification of isolates

The isolates were identified by sequencing the 16S rRNA gene fragment. The DNA from the isolates AR_2, AR_4 and AR_{10} were extracted via enzymatic lysis using extraction buffer (100 mM Tris–HCl, pH 8.0, 100 mM EDTA, and 1.5 M NaCl) containing Proteinase K (100 µg/ ml). The isolated DNA was purified and amplification of 16S rRNA gene sequences was carried out by polymerase

chain reaction (PCR, Eppendorf Mastercycler gradient system) using the primers F27 (5′-CCAGAGTTTG ATCMTGGCTCAG–3′) and R1495 (5′-CTACGGCTACCTTGTTACGA-3′).

Each reaction mixture contained approximately 10 ng of DNA; 1.75 mM $MgCl_2$; 1× PCR buffer (TAKARA, India); 200 µM of each dNTPs; 0.2 µM of each forward and reverse primer; and 1 U of Taq DNA polymerase (TAKARA, India) in a final volume of 20 µl. The PCR was performed with a cycle of initial denaturation at 94°C for 5 min; 30 cycles of 94°C, 60°C, and 72°C for 1 min each; and final extension at 72°C for 10 min, and the mixture was held at 4°C. The amplified PCR products were checked by electrophoresis and purified by PEG–NaCl method. The purified PCR product was sequenced using Big Dye terminator kit (Applied Biosystems Inc., Foster city, CA).

Sequence analysis and phylogenetic tree construction

16S rDNA sequences were searched for homology by using the NCBI-Blast2 – Nucleotide Database Query program (http://www.ebi.ac.uk/blastall/index.html). Phylogenetic analysis of 16S rDNA sequences were performed using MEGA version 5 software[12] (Tamura *et al*. 2011). Phylogenetic tree was constructed using the neighbour-joining distance method based on number of differences. A total of 100 bootstrap replications were calculated.

Biochemical characteristics

Biochemical characteristics of the isolates were studied by using standard methods. The tests included Gram nature, motility, utilization of different carbon sources (arabinose, citrate, glucose, mannitol, maltose, and xylose), catalase and oxidase test, nitrate reduction, hydrolysis of starch and gelatin.

Arsenic transformation by isolates

To test the ability of the isolates to reduce As(V) or oxidize As(III), the isolates were grown overnight in TMMG without arsenic and then inoculated (2%) into flasks containing 20 ml of TMMG either with 2 mM of As(V) or 2 mM of As(III). The flasks were incubated in dark on rotary shaker with 140 rpm at 30°C. Control flasks without cells were incubated to check abiotic transformation of arsenic. At each sampling time, suspension was removed to measure cell growth by OD_{600nm} and to determine As(V) and As(III) concentration spectrophotometrically by molybdenum blue method[13] (Cummings *et al*.,1999). As(V) concentration was determined by acidifying 100µl sample in 100 µl of HCl (24 mmol) a 100 µl of the acidified sample was then added to 900 µl of the reaction mixture containing the following: ammonium molybdate (6 g/l), ascorbic acid (10·8 g/l), potassium antimonyl tartrate (0·136 g/l) and concentrated H_2SO_4 (67·3 ml/l). Each component was stored as a separate solution. Samples were heated in a water bath at 78°C for 10 min and placed on ice for 5 min. The absorbance at 865 nm was compared to acidified As(V) standards. As(III) concentration was determined by oxidizing a second sample in 100 µl of KIO_3 (5 mM) and HCl (48 mM) for 10 min and then reading the OD_{865nm}. Blanks of milli-Q water were used to calibrate the spectrophotometer. Standard curves were prepared from concentrations of (0–100 µM) for both As(V) and As(III). The difference between oxidized and

unoxidized samples represented the concentration of As(III).

Growth and As(V) reduction by isolates

The isolates were grown in TMMG without arsenic and then inoculated (2%) into flasks containing TMMG with 2 mM of As(V). The flasks without inoculum served as abiotic control. The flasks were incubated at 30°C on a rotary shaker at 140 rpm. The samples were removed at particular time interval for growth and arsenic measurement as described above.

Determination of Minimum inhibitory concentration

The minimum inhibitory concentrations for As(V) and As(III) were determined for isolates AR_2, AR_4 and AR_{10} by growing them in TMMG liquid medium amended with increasing concentrations of As(V) (from 0 to 300 mM) or As(III) (from 0 to 50 mM). Two flasks for each concentration were inoculated with appropriate cell suspension grown in TMMG without arsenic to obtain initial optical density of approximately 0.005. The growth was evaluated by measuring the OD_{600nm} after 72 h incubation at 30°C.

Results and Discussion

Isolation and identification of arsenic resistant bacteria

In all, a total number of 11 bacterial cultures resistant to arsenic were isolated from the enrichment culture and were designated as AR_1 to AR_{11}. Based on the results of the screening, only three isolates, viz.; AR_2, AR4 and AR_{10} were characterized and identified by partially sequencing the 16S rDNA. The isolate AR_2 was identified as *Bacillus* species and isolates AR_4 and AR_{10} were identified as *Achromobacter* species. The phylogenetic analysis of the 16S rDNA sequence of the isolates and most closely related species was carried out to identify their correct phylogenetic position (fig. 1 and fig. 2). Isolate AR_2 which belonged to *Bacillus* genus was most closely related to *Bacillus firmus.* Isolates AR_4 and AR_{10} were most closely related to *Achromobacter pulmonis.* These isolates formed different clade from *A. pulmonis* and *A. xylosoxidans*, therefore may be representing novel species within *Achomobacter* genus. However further studies are necessary to confirm its correct phylogenetic position.

Biochemical characteristics

The isolates were studied for their biochemical characteristics. The isolate AR_2 was Gram positive, motile, reduced nitrate and showed positive catalase test. It used glucose, arabinose and xylose as sole carbon source but not maltose and mannitol. The strain also hydrolysed gelatin but not starch. The isolates AR_4 and AR_{10} were Gram negative and were positive for nitrate reduction, catalase and oxidase. They used acetate and citrate as sole carbon source but not glucose, arabinose, mannitol and maltose. Isolate AR4 hydrolysed gelatine but not starch, while isolate AR10 did not show hydrolysis of starch and hydrolysis of gelatine. The resistance of all the isolates towards As(V) and As(III) was also tested. The strain AR2 resisted up to 50 mM of As(V) and 5 mM of As(III), whereas strains AR4 and AR10 resisted >300 mM of As(V) and up to 15 mM of As(III). These levels of arsenic resistance are comparable to the previously reported *Achromobacter* species (Cai *et al*., 2009; Bachate *et al*., 2012).

Table.1 Gram nature, motility, Biochemical Characteristics and arsenic resistance pattern of the isolates

Characteristics	Isolate		
	AR2	**AR4**	**AR10**
Closest match (%)	*Bacillus oceanosediminis* (98%)	*Achromobacter pulnonis* (97%)	*Achromobacter pulnonis* (97%)
Gram nature	positive	negative	negative
Motility	motile	motile	motile
Oxidase	-	+	+
Catalase	+	+	+
Nitrate reduction	+	+	+
Sole carbon sources			
Acetate	-	+	+
Arabinose	+	-	-
Glucose	+	-	-
Xylose	+	-	-
Mannitol	-	-	-
Maltose	-	-	-
Citrate	+	+	+
Hydrolysis of:			
Gelatin	+	+	-
Starch	-	-	-
Resistance to arsenic (mM)			
As(V)	~50	>300	>300
As(III)	5	15	15

Arsenic transformation by isolates

All the isolates were screened for their abilities to reduce and oxidize As(V) and As(III) respectively. The results of screening of the isolates for their arsenic transformation abilities are presented in Table 1. Out of 11 isolates only three isolates viz.; AR_2, AR_4 and AR_{10} were found to reduce the As (V), while none of the isolates were found to oxidize the As(III). *Bacillus* species are reported to reduce As(V) either by detoxification mechanism or by dissimilatory reduction mechanism[16] (Ahmann *et al.*, 1994). Similarly As(III) oxidizing *Bacillus* species have also been reported (Cavalca *et al*., 2010; Bachate *et al*., 2013). The unique feature of the isolate AR_2 which is identified as *Bacillus firmus* is that it is able to reduce As(V) but unable to oxidize As(III) which is in contrast to previous observations (Bachate *et al*., 2013).

Many *Ahcromobacter* species have been reported to oxidize As(III) (Santini *et al*., 2002; Fan *et al*., 2008; Cai *et al*., 2009; Bachate *et al*., 2012;). However, the strain AR_4 and AR_{10} although *Achromobacter* species, did not oxidized As(III) but showed As(V) reduction ability. This implies that different strains of the same species may have different mechanisms to resist the arsenic which could be due to differences in their genetic make-up.

Figure.1 Neighbor-joining phylogenetic tree of 16S rDNA sequence of isolate AR_2 (shown in bold face) and most closely related species. Scale bar indicates the number of base substitutions per site. Numbers above nodes represent bootstrap confidence values obtained with 100 resamplings. The GenBank accession numbers for the corresponding sequences are given after the strain name

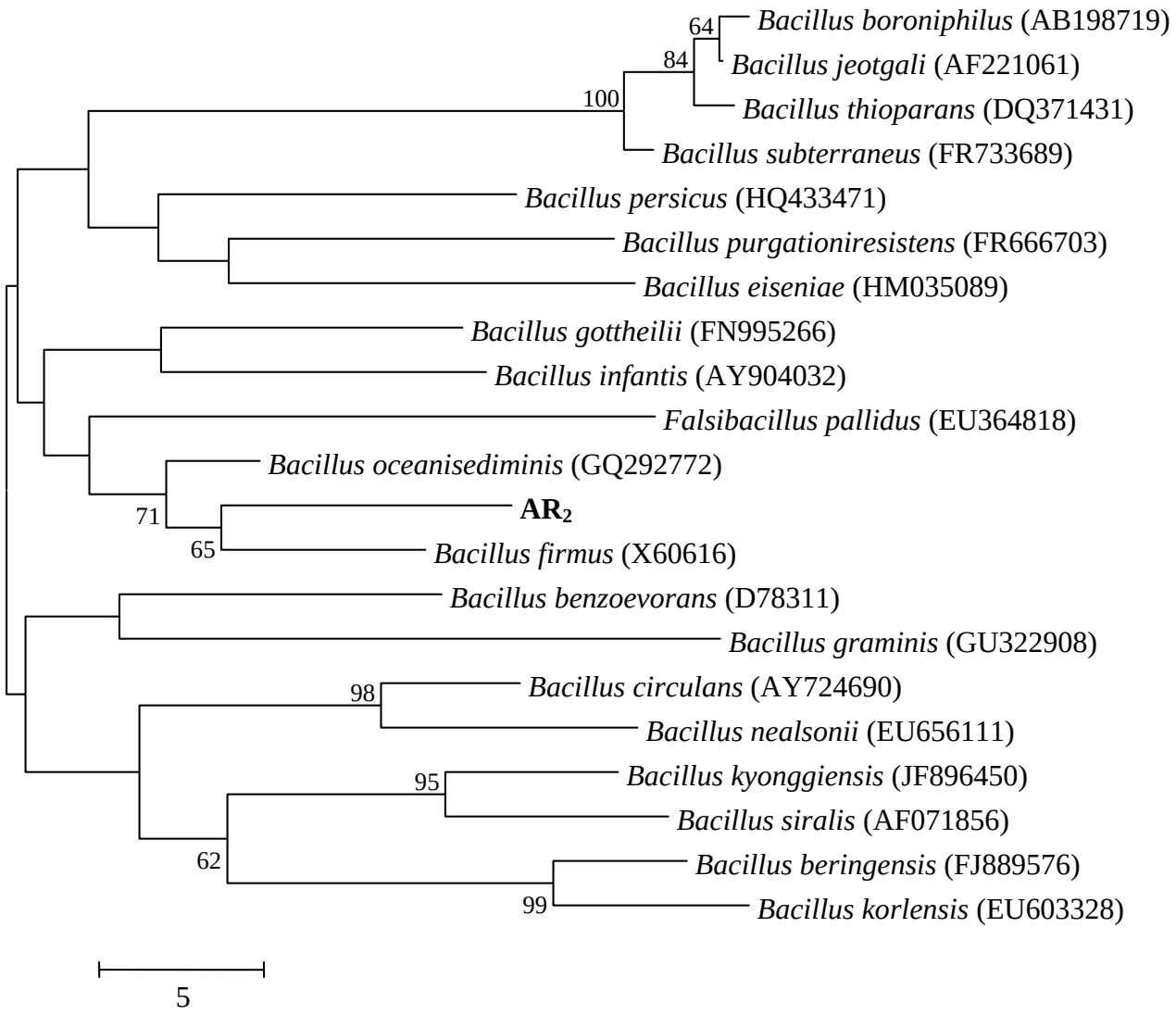
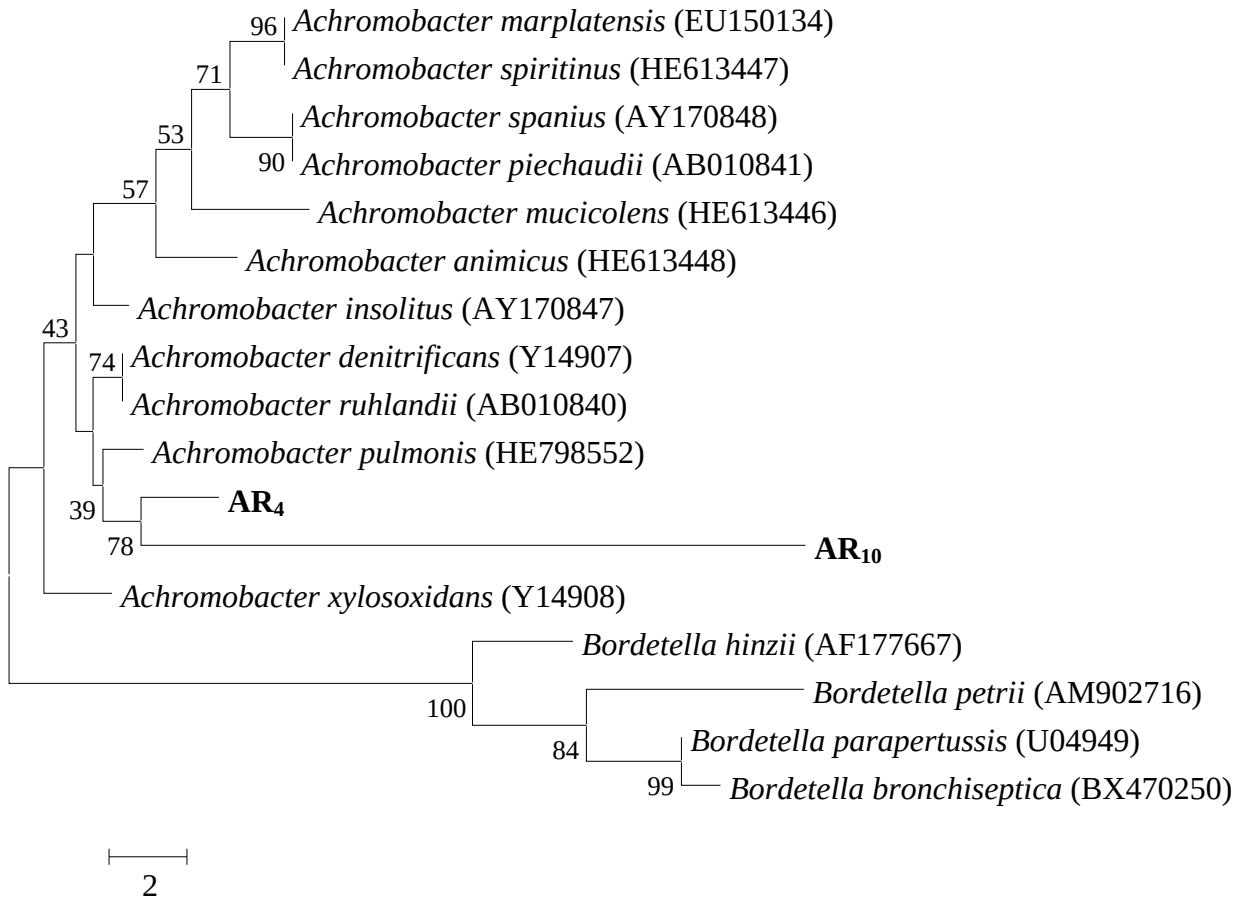


Figure.2 Neighbor-joining phylogenetic tree of 16S rDNA sequence of culture 4, culture 10 (shown in bold face) and most closely related species. Scale bar indicates the number of base substitutions per site. Numbers above nodes represent bootstrap confidence values obtained with 100 resamplings. The GenBank accession numbers for the corresponding sequences are given after the strain name



Growth and As(V) reduction by isolates AR_4 and isolates AR_{10}

The isolates AR4 and AR10 showed faster growth and As(V) reduction compared to isolates AR_2, therefore isolates AR_4 and AR_{10} were further studied. Growth and As (V) reduction ability was tested in TMMG containing 2 mM As(V).

Both isolates showed fast growth and growth was not affected in the presence of As(V) (compared to control, data not shown). Both the cultures reduced only 50% of the As(V) added to culture medium (2 mM) within 40 h and there was no further reduction with the further incubation (fig. 3 and fig. 4). It is observed that there is strong correlation between the exponential phase of the growth of the isolates and reduction of As(V). Reduction is at halt when isolates enter the stationary growth phase. This implies that reduction of As(V) by the isolates is totally dependent upon the metabolic activities of the isolates in their exponential growth phase.

Figure.3 Growth curve and As (V) reduction by isolate AR_4

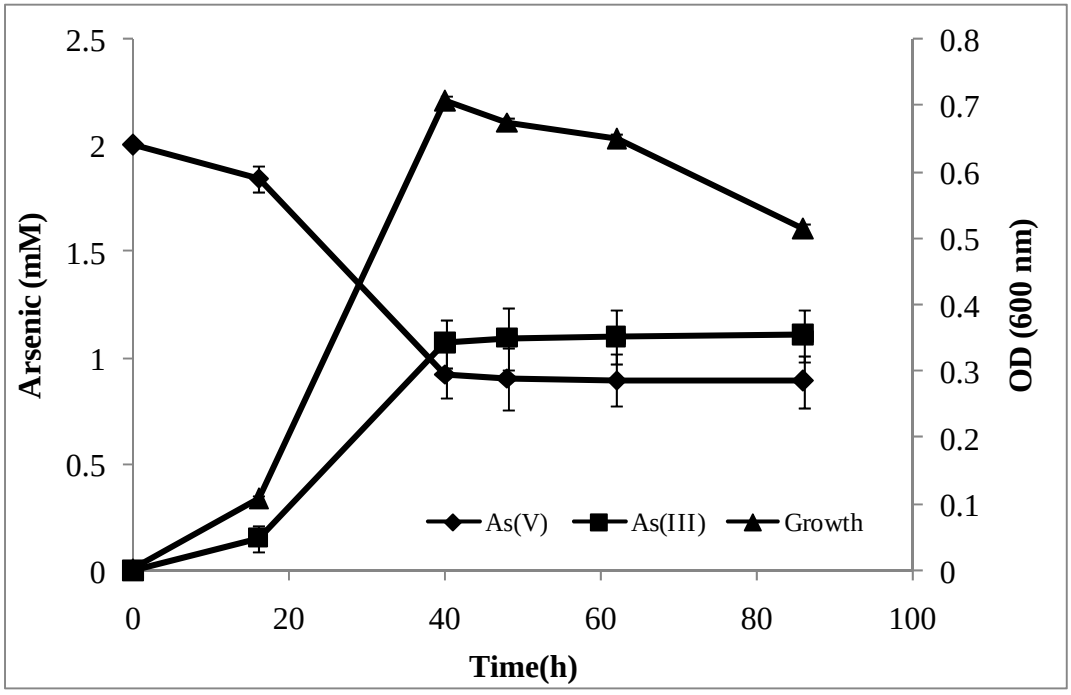
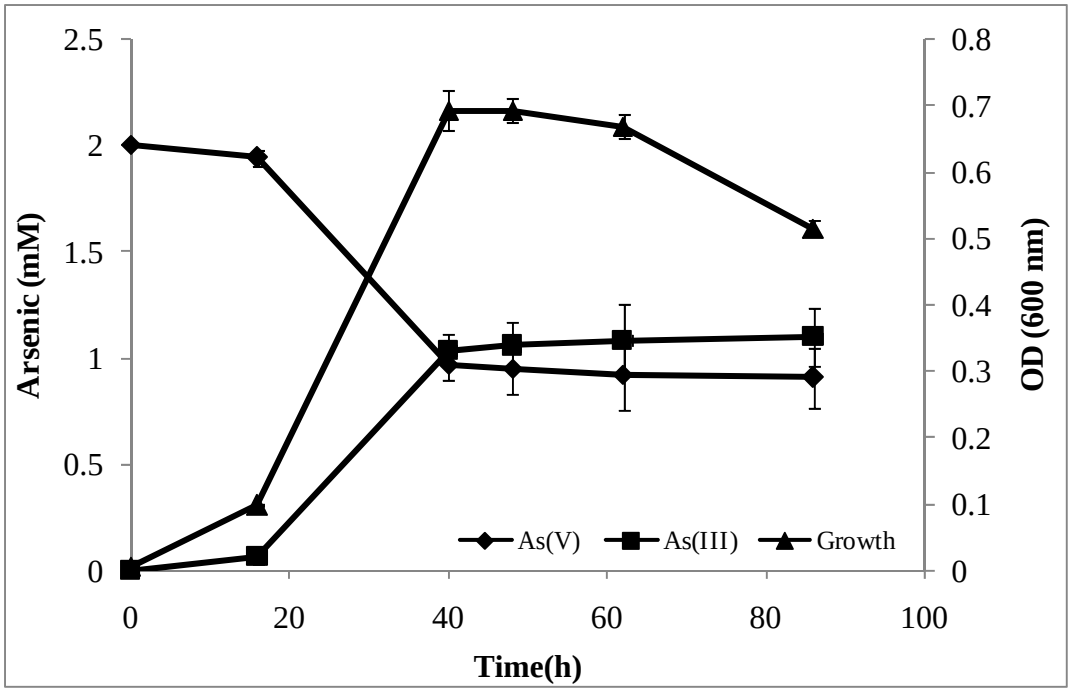


Figure.4 Growth curve and As (V) reduction by isolate AR_{10}



Three different strains of arsenic resistant bacteria were isolated from the electroplating industrial waste. As electroplating industrial waste contains different heavy metals, is the conducive for development of bacterial strains resistant to heavy metals like arsenic. Two isolates (AR4 and AR10) could be a novel species within *Achromobacter* genus. Further studies may be carried out to confirm the novelty of the isolates and its arsenic resistance mechanisms.

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