CHAPTER 3

SCREENING OF ARSENIC RESISTANT BACTERIA AND EVALUATION OF THEIR DETOXIFICATION MECHANISMS

3.1 Introduction

Arsenic (As) is a common metalloid and well-known human carcinogen that can harm not only people's health but plants and microorganisms as well. Ongoing applications of arsenical pesticides and chemical fertilizers in Northern Thailand's agricultural highlands have increased the arsenic content of soils and stream sediments. Various farming practices, e.g. the use of animal manures, phosphate fertilizers and arsenic containing agrochemicals may increase arsenic contamination in agricultural soils (Li and Chen, 2005). The concentration of arsenic in cultivated soils of highland areas of northern Thailand greatly exceed the national environment standard (3.9 mg/kg) (Shutsrirung, 2012). In these contaminated areas, the extremely high input of agrochemicals is a common practice in farms and seemed to be a primary source of the high arsenic contamination. High arsenic levels in the topsoil, particularly in the root zone, is likely resulted an increased concentration in plant and food grains and thus pose a greater risk to human health. Inorganic arsenic has been classified as a class 1 carcinogen by the International Agency for Research on Cancer. It is responsible for bladder, kidney, liver, lung, and skin cancers and is listed as a Class A human carcinogen by the USEPA (Chen *et al*., 2002). Arsenic in the environment comes from natural and anthropogenic sources. Naturally occurring arsenic in the continental crust is present at an average concentration of 1.5 to 5 mg/kg (Ritchie, 1980; Cullen and Reimer, 1989). However, arsenic concentrations in soils with human activities vary widely among different locations. In European topsoil, arsenic concentration is estimated at the average of 7.0 mg/kg (Stafilov *et al*., 2010). The assumptions of soil arsenic concentration in areas under unrestricted use (e.g., residential) by the USEPA Regional Screening Level (RSL) is 0.39 mg/kg (USEPA, 2010). This guidance by USEPA is based on a target cancer risk of 1E-06, toxicological guidance values and standard assumptions for exposure assessment and risk assessment.

In the natural environment, the pentavalent arsenate (As(V)) and trivalent arsenite (As(III)) are the most common oxidation states of arsenic (Cullen and Reimer, 1989). Trivalent arsenic (arsenite) is generally more toxicologically potent than pentavalent arsenic (arsenate) because arsenite can form strong bonds with functional groups such as the thiolates of cysteine residues and the imidazolium nitrogen of histidine residues, of cellular proteins, and thus the bindings inactivated many cellular proteins including enzymes (National Research Council, 2001). Various microorganisms including bacteria have evolved many mechanisms to cope with arsenic exposure (Shutsrirung, 2012; Jackson, 2003) thus arsenic-resistant bacteria seemed to have a vital role in the transformation of As, movement of As in soils and the availability of arsenic to plants.

3.2 Materials and Methods

3.2.1 Soil sampling and soil arsenic concentration analysis

Rhizosphere-soils (0-20 cm) were collected from seven cultivated soils contaminated with arsenic. The areas were located on highland areas of Chiang Mai province, northern Thailand (17°30' to 19.5°30' N and 97.7°30' to 99.3°30' E) (Fig. 3.1). The soil samples were handled with caution and ground to pass a 2 mm (sieve) and collected in polythene bags and kept at 4^{o}C until analysis.

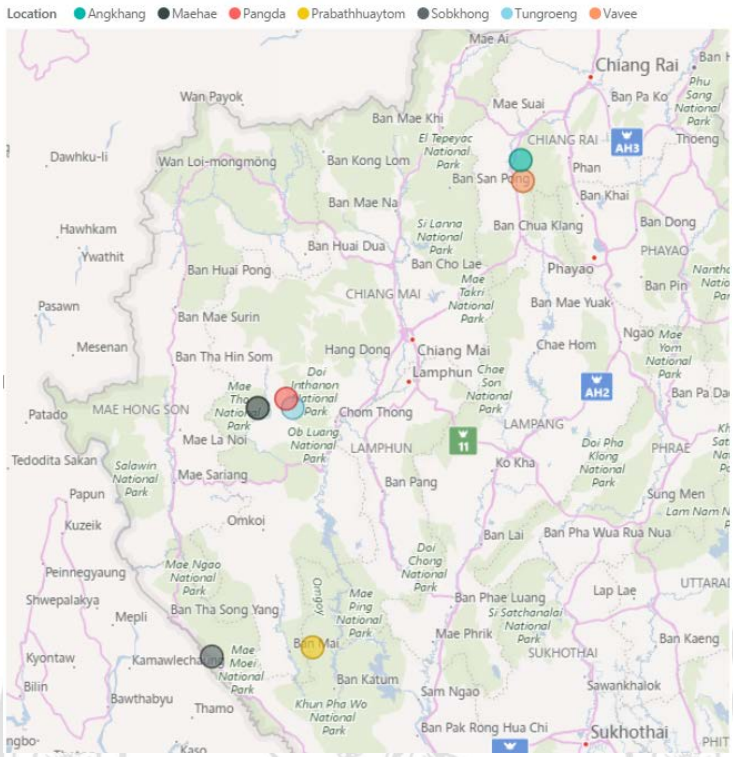


Figure 3.1 Site map of the study areas, highland areas, northern Thailand; Site1 Prabathhuaytom; Site2 Pangda; Site3 Tungroeng; Site4 Maehae; Site 5 Angkhang; Site 6 Vavee and Site 7 Sobkhong.

For determination of total arsenic content (Analyzed at the Central Laboratory (Thailand) Co. Ltd.), 0.5 g dried soil was digested in 9 ml of concentrated nitric acid (HNO_3) and usually 3 ml hydrofluoric (HF) for 15 min using microwave heating with a suitable microwave system, according to EPA Method 3052 (USEPA, 1995). After cooling, the vessel contents filtered to settle and then decanted, diluted to volume, and analyzed by Perkin Elmer Optima 4300 DV Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, USA). Final results, obtained as mean values in triplicate, were expressed as mg/kg dry weight.

The pH of the soil sample was also determined using 20 g of soil in 20 ml of distilled water and mixes every 10 min for 3 times and then incubated at room temperature for 30 min. The measurement was made after 30 min of incubation using pH meter (PHI 34 BECKMAN, USA).

3.2.2 Isolation and screening of arsenic resistant bacteria

Serial dilution (10^{-1} to 10^{-5}) and plate count technique were used to isolate bacteria from all the soil samples. An aliquot (0.1 ml) of the soil suspension (10^{-3} to 10^{-5}) of each soil sample was spread on nutrient agar (NA) (HIMEDIA: REF 002) plate (pH 6.5). Colonies which grown on the medium were purified and suspended in 25% glycerol solution (final concentration) for preservation. All isolates preserved in this solution were maintained at -20ºC until use.

For the first screening, the pure bacterial isolates were used to evaluate their resistance under high concentration of arsenic. The culture broth of each isolate was dropped on NA plate containing 50 µM aluminum (Al) (Wood and Cooper, 1988) and various concentration of sodium arsenite (($NaAsO_2$: Na-As (III)) i.e. 0, 5, 10, 20 and 40 mM. The initial pH of the medium was adjusted to 4.5 and 4.7 and 7.0.

The tolerant isolates from the first screening were selected for the second screening. In the 2nd screening, the concentration of arsenic was adjusted close to the analyzed level found in the soil. Culture solution (0.01 ml) of each isolate was dropped on NA plate containing indicator (0.05 g/L bromocersol green), 50 µM Al and various levels of Na-As (III); 0, 10, 15, 25, 50, 100, 250 and 650 mg/L (which equal to 0, 0.08, 0.12, 0.19, 0.38, 0.77, 1.9 and 5.0 mM, respectively). Four pH levels of the medium; 4.5, 4.7, 5.5 and 6.0 were used. After 7 days of incubation, the maximum tolerance concentration was determined by observing the presence or absence of visible growth detected by colony forming on agar plate and selected for further investigation. The minimal inhibitory concentration (MIC) of each bacterial isolate was also determined. MIC is defined as the lowest arsenic concentration that prevents visible growth of bacteria.

3.2.3 Evaluation of arsenite transformation and selection of high efficient isolates

The arsenic resistant bacteria were selected from the above experiment. The selected isolates were inoculated into nutrient broth (50 ml) and incubated for 3 days to obtain a standard inoculum for each of the following experiments. The growth dynamic

of the resistant isolates was determined in nutrient broth containing 0, 25, 50 and 100 mg/L of $NaAsO_2$ with 50 µM Al, the initial pH of the medium was adjusted to 4.7. The inoculums of 0.5 ml of each isolate were added into 50 mL nutrient broth and incubated at room temperature for 0, 3, 5 and 7 days. Growth dynamics of bacterial cells of each sample solution were examined by optical density (OD) at a wave length of 600 nm using an UV-visible spectrophotometer (Thermo Scientific, mod. GENESYS 20, USA) and pH change changed in the culture broth was determined by pH meter (PHI 34 BECKMAN, USA).

The same selected isolates in the above experiment were used for evaluation of arsenite transformation in nutrient broth containing 50 and 100 mg/L of $NaAsO_2$ with 50 µM Al, the initial pH of the medium was adjusted to 4.7. Growth dynamics of bacterial cells of each sample solution were also examined by optical density (OD) as described above. The pH changed in the culture broth was determined by pH meter (PHI 34 BECKMAN, USA). The culture broths of all the isolates were centrifuged at 5000 rpm for 15 min (ORTO ALRESA, Spain). The supernatant of each isolate was used to determine the total arsenic content (Analyzed at the Central Laboratory (Thailand) Co. Ltd.). In brief, the supernatant was detected by digested microwave system, according to EPA Method 3052 and analyzed by Perkin Elmer Optima 4300 DV Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, USA). Arsenic species were determined at the Central Laboratory (Thailand) Co. Ltd.). The same supernatant samples were used to analyze arsenic species. The separation of arsenic species was performed using the high-pressure liquid chromatography (HPLC) system consisted of an Agilent Series 1100, Japan, column CAPCELL PAK C18 MG, particle size 5 µm, 4.6 mm i.d. x 250 mm (Shiseido Ltd., Tokyo, Japan) with a mobile Phase HPLC eluent, isocratic, flow rate 0.75 ml/min. The flow rate was adjusted when necessary, to make it suitable for clear resolution of As (III) and As (V). Detection by coupled plasma mass spectrometry (ICP-MS), condition, was monitoring ion-m/z 75, single ion monitoring (Agilent 7500ce, Japan). The amount of 20 µl of the standard solution was injected in to HPLC-ICP-MS to verify the clear resolution of the four peaks (As(V), As(III), MMA and DMA).

3.3 Results

3.3.1 Soil analysis and bacterial isolation

The pH values of the soils varied from location to location. The surface soil pH value of the area was low (Table 1) and ranged from very strongly acidic to slightly acidic. The soil pH of Maehae, Angkhang and Vavee was 5.09, 4.80 and 4.67, respectively and could be classified as very strongly acidic (Havlin *et al*., 2014). In contrast, the soil pH of Pangda Tungroeng and Sobkhong was slightly acidic. All the soil samples contained higher arsenic level than the standard background level (3.9 mg/kg) (National Environment Board No.8, 1994). The soils from Tungroeng and Vavee contained around ten times higher arsenic (39.48 and 30.52 mg/kg, respectively) than the standard level (Table 1). The rest of the soils samples also contained a high level of arsenic with values ranging from 5.45 to 16.06 mg/kg. Although all the soil samples contained quite a high level of arsenic, resistant bacterial isolates (47 isolates) (Table 1) could be obtained. The largest number of resistant bacterial isolates was obtained from Angkhang soil (ten isolates; 21.27% of the total isolates). There seemed to be no relationship between arsenic concentration in soils and number of resistant bacterial isolates. Although 47 bacterial isolates were obtained, only 40 isolates were kept and used for further investigations because seven isolates were contaminated and died during preservation.

Table 3.1 Arsenic concentrations and bacterial isolates obtained from arsenic contaminated soil.

Location	Coordinates	AMSL[1] (m)	pH	Total Arsenic conc. (mg/kg)[2]	No. of isolates	% of total isolates
Prabathhuaytom (vegetables)	17°43'32 N, 98°57'14 E	528	5.63	5.61	6	12.76
Pangda (vegetables)	18°51'20 N, 98°45'38 E	620	6.45	8.83	5	10.63
Tungroeng (vegetables)	18°47'28 N, 98°48'36 E	847	6.62	39.48	7	14.89
Maehae (vegetables)	18°47' 31N, 98°32'14 E	1178	5.09	5.45	8	17.02
Angkhang (vegetables)	19.54°39' N, 99.5°2'41 E	1468	4.80	6.66	10	21.27
Vavee (vegetables)	19°45'54 N, 99.5°33'26 E	1597	4.67	30.52	5	10.63
Sobkhong (vegetables)	17°39' 49N, 98°11'57 E	1765	6.39	16.06	6	12.76
				Total	**47**	**(100%)**

[1]Height above mean sea level

[2]The standard background level for arsenic in soil is set at 3.9 mg/kg, National Environment Board No.8, (1994)

3.3.2 The first screening of arsenic resistant bacteria

In this experiment 40 isolates were evaluated for their resistance to arsenic by growing them separately in nutrient agar plate containing aluminum (50 µM) with increasing concentration of sodium arsenite ($NaAsO_2$) at 0, 5, 10, and 20 mM, initial pH of the medium was adjusted to 4.5, 4.7 and 7.0. The minimum inhibitory concentration was determined by observing the presence or absence of visible growth detected by colony forming in agar plate.

At low pH (pH 4.5 and 4.7), the similar pattern of bacterial growth and tolerant ability was observed. The results showed that at low pH all the isolates could grow on the media without $NaAsO_2$ (0 mM) except for isolate BAs1, BAs10, BAs12 and BAs16

(Table 3.2 and 3.3). In the medium containing 5 mM $NaAsO_2$ only 12 and 14 isolates were able to grow under pH 4.5 and 4.7, respectively. Only three bacterial isolates (BAs 20, BAs 29 and BAs 30) were able to grow on the medium with high level of $NaAsO_2$ with the MIC of >20 mM. Isolate BAs36 could tolerate arsenic up to 10 mM $NaAsO_2$ and no growth was observed at 20 mM $NaAsO_2$ thus its MIC was considered as 20 mM.

Table 3.2 Growth of bacterial isolate under different levels of $NaAsO_2$ at pH 4.5

Isolate BAs	areas	$NaAsO_2$ Concentration (mM)				Isolate BAs	areas	$NaAsO_2$ Concentration (mM)			
		0	5	10	20			0	5	10	20
1	Vavee	NG	NG	NG	NG	21	Tungroeng	+	NG	NG	NG
2		+	NG	NG	NG	22		++++	NG	NG	NG
3		+	NG	NG	NG	23		+	+	NG	NG
4		+++	NG	NG	NG	24	Prabathhuaytom	++	NG	NG	NG
5	Pangda	++	+	NG	NG	25		+	NG	NG	NG
6		++	+	NG	NG	26		+	NG	NG	NG
7		+++	+	NG	NG	27		++	+	NG	NG
8	Sobkhong	+	NG	NG	NG	28		++	NG	NG	NG
9		++	NG	NG	NG	29		++++	++++	+++	++
10	Maehae	NG	NG	NG	NG	30		++++	++++	+++	+++
11		++++	+++	NG	NG	31		+	NG	NG	NG
12		NG	NG	NG	NG	32	Angkhang	++	NG	NG	NG
13		++	NG	NG	NG	33		+	NG	NG	NG
14		++	+	NG	NG	34		+	NG	NG	NG
15	Tungroeng	++	+	NG	NG	35		+	NG	NG	NG
16		NG	NG	NG	NG	36		++++	+++	+++	NG
17		++	NG	NG	NG	37		++	NG	NG	NG
18		++	NG	NG	NG	38	Maehae	+	NG	NG	NG
19		+++	++	NG	NG	39		++	NG	NG	NG
20		+++	++	++	+	40		++	NG	NG	NG

Growth ++++; very good, +++; good, ++; fair; +; little, NG; No Growth

Table 3.3 Growth of bacterial isolate under different levels of $NaAsO_2$ at pH 4.7

isolate BAs	areas	$NaAsO_2$ Concentration (mM)				Isolate BAs	areas	$NaAsO_2$ Concentration (mM)			
		0	5	10	20			0	5	10	20
1	Vavee	NG	NG	NG	NG	21	Tungroeng	++	+	NG	NG
2		+	NG	NG	NG	22	Prabathhuaytom	++++	+	NG	NG
3		+	NG	NG	NG	23		++	+	NG	NG
4		+++	NG	NG	NG	24		+	NG	NG	NG
5	Pangda	++	+	NG	NG	25		++	NG	NG	NG
6		++	+	NG	NG	26		++	NG	NG	NG
7		+++	+	NG	NG	27		+	NG	NG	NG
8	Sobkhong	+++	+	NG	NG	28	Angkhang	++	NG	NG	NG
9		++	NG	NG	NG	29		++++	++++	+++	++
10	Maehae	NG	NG	NG	NG	30		++++	++++	+++	+++
11		++++	+++	NG	NG	31		++	NG	NG	NG
12		NG	NG	NG	NG	32		++	NG	NG	NG
13		++	NG	NG	NG	33		+	NG	NG	NG
14		++++	+	NG	NG	34		+	NG	NG	NG
15	Tungroeng	++++	+	NG	NG	35		+	NG	NG	NG
16		NG	NG	NG	NG	36		++++	+++	+++	NG
17		++	NG	NG	NG	37		+++	NG	NG	NG
18		++	NG	NG	NG	38	Maehae	+++	NG	NG	NG
19		++++	++	NG	NG	39		+++	NG	NG	NG
20		++++	++	++	+	40		+++	++	NG	NG

Growth ++++; very good, +++; good, ++; fair; +; little, NG; No Growth

When the pH of the agar medium was raised to 7.0, it was observed that most of the isolates show a good growth without addition of $NaAsO_2$ (Table 3.4). However, the four isolates; BAs1, BAs10, BAs12 and BAs16 could not grow on the media without $NaAsO_2$ (0 mM), no matter which pH level was used (Table 3.2, 3.3 and 3.4). There were two isolates; BAs7 and BAs27 could grow on the medium at 10 mM $NaAsO_2$ and no growth was observed at 20 mM $NaAsO_2$ thus its MIC was considered as 20 mM. The highest tolerant ability under pH 7.0 was detected in eight isolates; BAs8, BAs11, BAs 19, BAs20, BAs22, BAs29, BAs30 and BAs36. Thus, the MIC of the eight isolates was >20 mM.

Table 3.4 Growth of bacterial isolate under different levels of $NaAsO_2$ at pH 7.0

Isolate BAs	areas	$NaAsO_2$ Concentration (mM)				Isolate BAs	areas	$NaAsO_2$ Concentration (mM)			
		0	5	10	20			0	5	10	20
1	Vavee	NG	NG	NG	NG	21	Tungroeng	++++	+	NG	NG
2		++++	NG	NG	NG	22	Prabathhuaytom	++++	++++	+++	+++
3		++++	NG	NG	NG	23		++++	+++	NG	NG
4		++++	++	NG	NG	24		++++	NG	NG	NG
5	Pangda	++++	++	NG	NG	25		++++	NG	NG	NG
6		++++	+	NG	NG	26		++++	NG	NG	NG
7		++++	+	+	NG	27		++++	++++	++++	NG
8	Sobkhong	++++	+++	++	+	28	Angkhang	++	NG	NG	NG
9		++	NG	NG	NG	29		++++	++++	++++	++
10	Maehae	NG	NG	NG	NG	30		++++	++++	+++	+++
11		++++	++++	++	+	31		++++	NG	NG	NG
12		NG	NG	NG	NG	32		++	NG	NG	NG
13		++	NG	NG	NG	33		++	NG	NG	NG
14		++++	+	NG	NG	34		++	NG	NG	NG
15	Tungroeng	++++	+	NG	NG	35		++++	NG	NG	NG
16		NG	NG	NG	NG	36		++++	++++	++++	+++
17		++++	NG	NG	NG	37		++++	NG	NG	NG
18		++++	NG	NG	NG	38	Maehae	++++	NG	NG	NG
19		++++	++	+	+	39		++++	NG	NG	NG
20		++++	++	++	+	40		++++	+	NG	NG

Growth ++++; very good, +++; good, ++; fair; +; little, NG; No Growth

3.3.3 The second screening of arsenic resistant bacteria and the minimum inhibitory concentration

From the results of the first screening (3.3.2), eight tolerant isolates (BAs8, BAs11, BAs19, BAs20, BAs22, BAs29, BAs30 and BAs36) were selected for the second screening. An arsenic sensitive isolate, BAs7 was also selected for comparison. All the nine isolates were tested in another set of $NaAsO_2$ 0, 10, 15, 25, 50, 100, 250 and 650 mg/L. The MIC of all the isolates was determined after 7 days of incubation. Our results indicated that at pH 4.5 the highest MIC (250 mg/L) was found in isolates BAs8, BAs11, BAs19 and BAs29. At pH 4.7, the MIC of isolates BAs8, BAs19 and BAs29 was the same as at pH 4.5. However, when the pH of the medium was increased from 4.5 to 4.7, the MIC of isolates BAs11, BAs22 and BAs36 was dramatically increased and the highest MIC was found in BAs11 (650 mg/L) (Table 3.5). Most of the selected tolerant isolates showed the MIC of >650 mg/L when the pH of the medium was 5.5 and 6.0.

Table 3.5 The minimum inhibitory concentration (MIC) of $NaAsO_2$ against selected isolates.

Bacterial isolates	MIC of isolates exposed to $NaAsO_2$ at 0, 10, 15, 25, 50, 100, 250 and 650 mg/L			
	pH 4.5	pH 4.7	pH 5.5	pH 6.0
BAs 7	15	25	25	25
BAs 8	250	250	>650	>650
BAs 11	250	650	>650	>650
BAs 19	250	250	>650	>650
BAs 20	15	25	250	650
BAs 22	15	250	>650	>650
BAs 29	250	250	>650	>650
BAs 30	15	15	100	250
BAs 36	50	100	250	650

3.3.4 Responses of arsenic resistant isolates to various arsenic concentrations

Responses to different pH levels (4.5, 4.7, 5.5 and 6.0) under various Na-As (III) concentrations (0, 10, 15, 25, 50, 100, 250 and 650 mg/L) of the nine isolates were recorded after 7 days of incubation. Bromocresol green was incorporated into the medium to indicate pH change. Bromocresol Green is also a pH indicator, changing from yellow (pH 3.8) to blue color (5.4). A standardized color chart was used as an aid in determining the color changes. The medium is yellow at pH 3.5 to 3.8, yellowish at pH 4.0 to 4.2, green at pH 4.5 to 4.6, ocean blue at pH 5.0 and bright blue at pH 5.5 to 6.0. Observation of pH change was made by comparing with the controlled plate without cultures of each pH level.

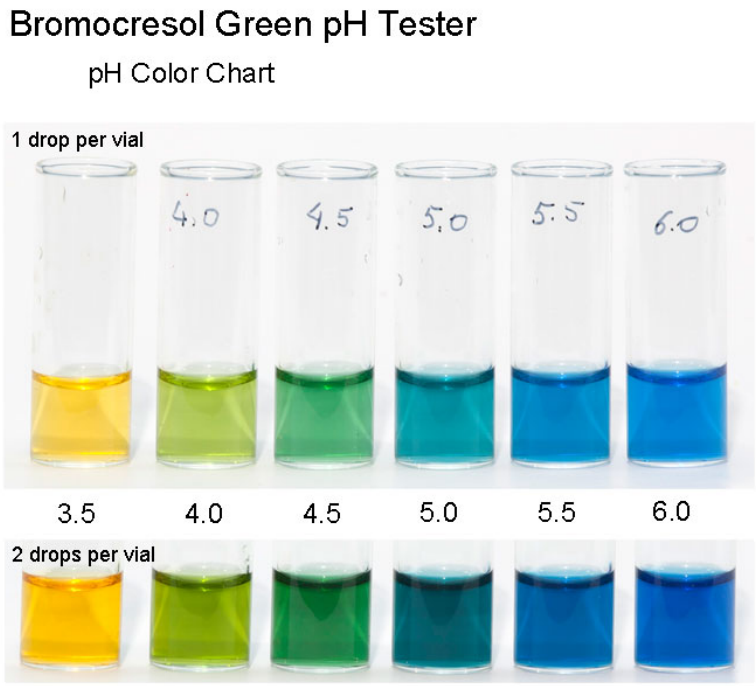


Figure 3.2 Standardized pH color chart for Bromocresol green
Source: http://mslavenda.com/bromocresol_green.htm

At pH 4.5 with 0 and 10 mg/L Na-As (III), all the tested isolates showed a good growth and performed the same response as pH of the medium was greatly increased from 4.5 (green) at the beginning to around ≥ 5.0 (ocean blue – bright blue). Four isolates; BAs8, BAs11, BAs19, and BAs29 were able to tolerate higher concentration of Na-As (III) (15, 25, 50 and 100 mg/L) and turning the pH of the medium to ≥ 5.0 (Table 3.6). No growth was obtained in isolate BAs7, BAs20 and BAs22 on the medium with Na-As (III) higher than 10 mg/L although isolate BAs7 were able to turn the pH of the medium blue. BAs36 could grow and withstand Na-As (III) concentration up to 25 mg/L. All the isolates were not able to grow on the medium containing 250 and 650 mg/L.

Table 3.6 Growth of bacterial isolate under different levels of $NaAsO_2$ at pH 4.5

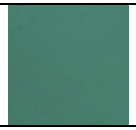
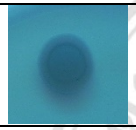
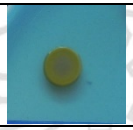
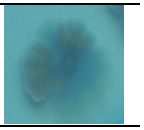
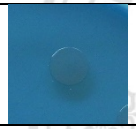
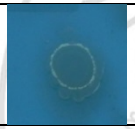
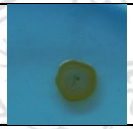
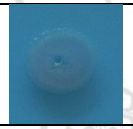
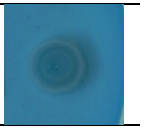
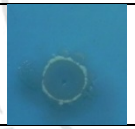
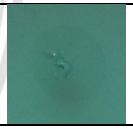
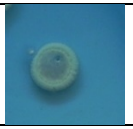
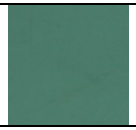
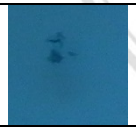
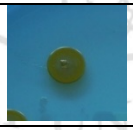
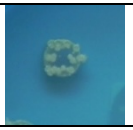
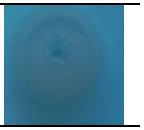
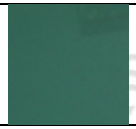
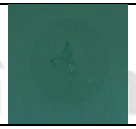
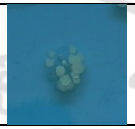
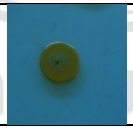
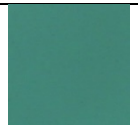
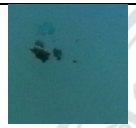
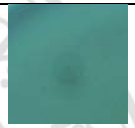
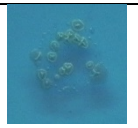
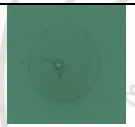
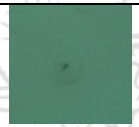
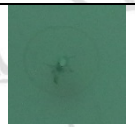
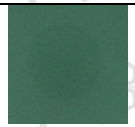
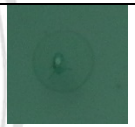
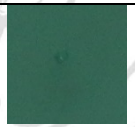
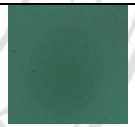
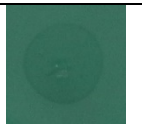
$NaAsO_2$ concentration (mg/L)	pH 4.5								
	control	**BAs 7**	**BAs 8**	**BAs 11**	**BAs 19**	**BAs 20**	**BAs 22**	**BAs 29**	**BAs 36**
0									
		++++	++++	++++	++++	++	++	++++	++++
10									
		+++	++++	++++	++++	+	+	++++	++
15									
		NG	++++	++++	++++	NG	NG	++++	++
25									
		NG	++	+++	++	NG	NG	+++	+
50									
		NG	++	+++	+	NG	NG	++	NG

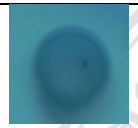
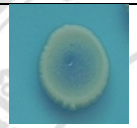
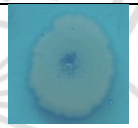
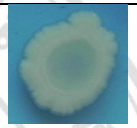
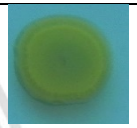
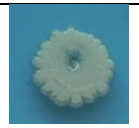
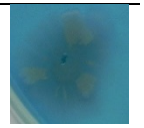
Table 3.6 (continued)

$NaAsO_2$ concentration (mg/L)	pH 4.5								
	control	**BAs 7**	**BAs 8**	**BAs 11**	**BAs 19**	**BAs 20**	**BAs 22**	**BAs 29**	**BAs 36**
100									
		NG	+	+++	+	NG	NG	+	NG
250									
		NG	NG	NG	NG	NG	NG	NG	NG
650									
		NG	NG	NG	NG	NG	NG	NG	NG

Growth ++++; very good, +++; good, ++; fair; +; little, NG; No Growth

A slightly increase in the pH of the medium from 4.5 to 4.7 resulted in higher arsenic tolerant of several isolates. Most of the tested isolates were able to grow on the medium containing up to 100 mg/L of Na-As (III) (Table 3.7). The growth appearance of the tested isolates was observed when they were able to alter the pH of the medium to be around ≥ 5.0 (ocean blue – bright blue). Although, BAs7, BAs20, BAs36 could increase the pH of the medium to be more alkali (blue), no growth of these isolates was observed at 25 and 50 mg/L of Na-As (III). Five isolates; BAs8, BAs11, BAs19, BAs22 and BAs29 were able to tolerate up to higher concentration 100 mg/L of Na-As (III) and turning the pH of the medium to ≥ 5.0 (Table 3.7). Only BAs11 were able to grow in the medium with 250 mg/L Na-As (III). All the isolates were not able to grow on the medium containing 650 mg/L.

Table 3.7 Growth of bacterial isolate under different levels of $NaAsO_2$ at pH 4.7

$NaAsO_2$ concentration (mg/L)	pH 4.7								
	control	**BAs 7**	**BAs 8**	**BAs 11**	**BAs 19**	**BAs 20**	**BAs 22**	**BAs 29**	**BAs 36**
0									
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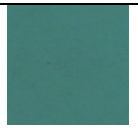
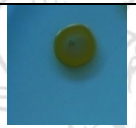
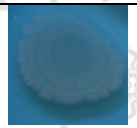
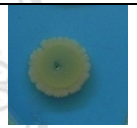
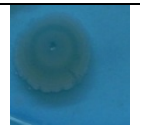
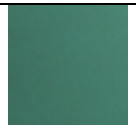
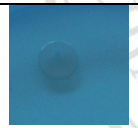
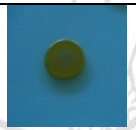
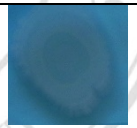
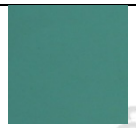
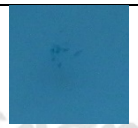
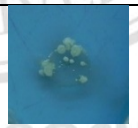
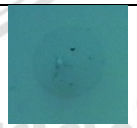
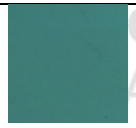
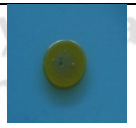
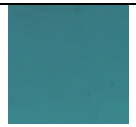
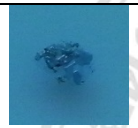
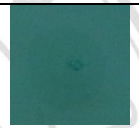
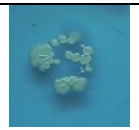
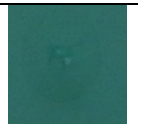
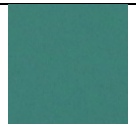
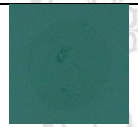
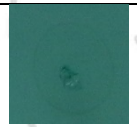
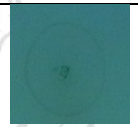
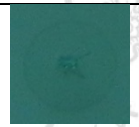
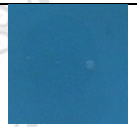
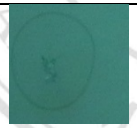
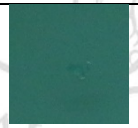
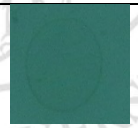
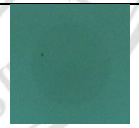
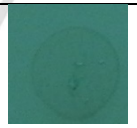
10									
		+++	+++	++++	++++	++	+++	++++	+++
15									
		+	+++	++++	+++	++	+++	++++	++
25									
		NG	++	++++	++	NG	++	+++	NG
50									
		NG	++	+++	++	NG	++	++	NG

Table 3.7 (continued)

$NaAsO_2$ concentration (mg/L)	pH 4.7								
	control	**BAs 7**	**BAs 8**	**BAs 11**	**BAs 19**	**BAs 20**	**BAs 22**	**BAs 29**	**BAs 36**
100									
		NG	+	+++	++	NG	+	++	NG
250									
		NG	NG	++	NG	NG	NG	NG	NG
650									
		NG	NG	NG	NG	NG	NG	NG	NG

Growth ++++; very good, +++; good, ++; fair; +; little, NG; No Growth

When the pH of the medium was set as 5.5, almost all of the tested isolates exhibited good to very good growth up to 100 mg/L of Na-As (III). Only BAs7 appeared to be sensitive to high arsenic concentration although the pH of the medium was raised to 5.5. No growth of BAs7 was observed on the medium with Na-As (III) higher than 15 mg/L (Table 3.8). Three isolates; BAs7, BAs20 and BAs36 were not able to grow on the medium containing 250 and 650 mg/L of Na-As (III). It was observed that isolates Bas8, Bas11, Bas19, Bas22 and Bas29 exhibited high level of resistance to Na-As (III) at the highest tested concentration of 650 mg/L.

Table 3.8 Growth of bacterial isolate under different levels of $NaAsO_2$ at pH 5.5

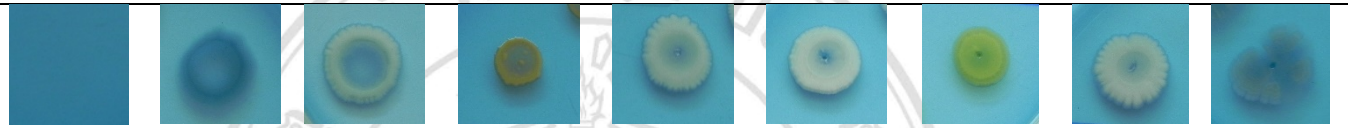
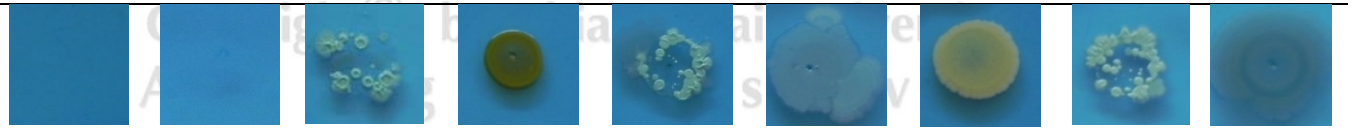
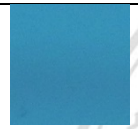
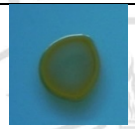
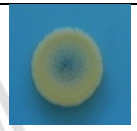
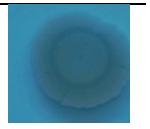
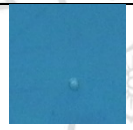
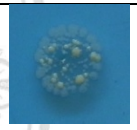
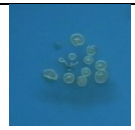
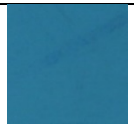
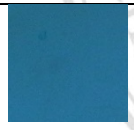
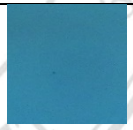
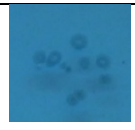
$NaAsO_2$ concentration (mg/L)	pH 5.5								
	control	**BAs 7**	**BAs 8**	**BAs 11**	**BAs 19**	**BAs 20**	**BAs 22**	**BAs 29**	**BAs 36**
0		+++	++++	++++	++++	++++	++++	++++	++++
10		++	++++	++++	++++	++++	++++	++++	++++
15		+	+++	++++	+++	++++	++++	++++	++++
25		NG	++	++++	++	++++	++++	+++	++++
50		NG	++	++++	++	++++	++++	+++	++++

Table 3.8 (continued)

$NaAsO_2$ concentration (mg/L)	pH 5.5								
	control	**BAs 7**	**BAs 8**	**BAs 11**	**BAs 19**	**BAs 20**	**BAs 22**	**BAs 29**	**BAs 36**
100									
		NG	+	+++	++	+++	+++	+++	++++
250									
		NG	+	++	++	NG	++	++	NG
650									
		NG	+	+	+	NG	+	+	NG

Growth ++++; very good, +++; good, ++; fair; +; little, NG; No Growth

Under pH 6.0, on the average, all the isolates showed good to very good growth up to 250 mg/L of Na-As (III) except for BAs7. It was observed that Bas7 could tolerate the tested concentration of Na-As (III) only up to 10 mg/L at pH 4.5 and up to 15 mg/L at pH 4.7, 5.5 and 6.0 (Table 3.6 to 3.9) suggesting arsenite sensitivity of this isolate. Although the pH of the medium was raised to 6.0, BAs7 could not grow in the medium with Na-As (III) higher than 15 mg/L (Table 3.9). The same results as found at pH 5.5 was obtained in that only three isolates; BAs7, BAs20 and BAs36 were not able to grow on the medium containing 650 mg/L of Na-As (III). At pH 6.0, only the five isolates, BAs8, BAs11, BAs19, BAs22 and BAs29 were able to grow on the medium at the highest tested concentration of 650 mg/L Na-As (III).

Table 3.9 Growth of bacterial isolate under different levels of $NaAsO_2$ at pH 6.0

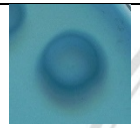
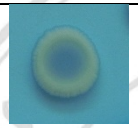
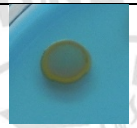
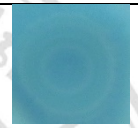
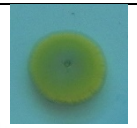
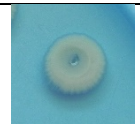
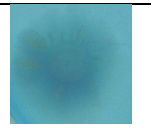
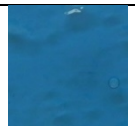
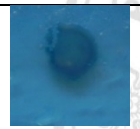
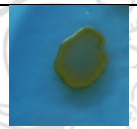
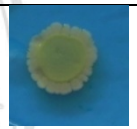
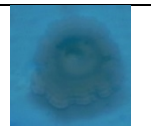
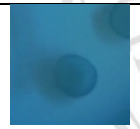
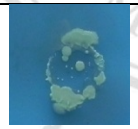
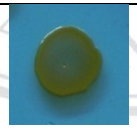
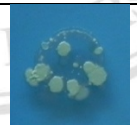
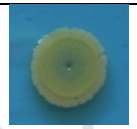
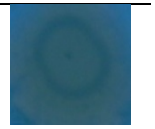
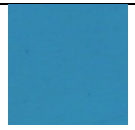
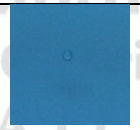
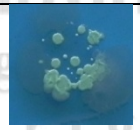
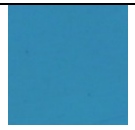
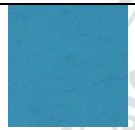
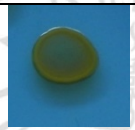
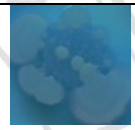
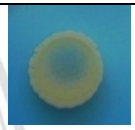
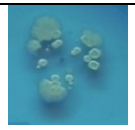
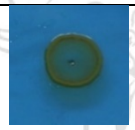
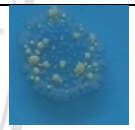
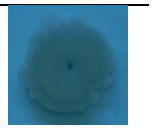
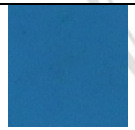
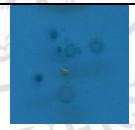
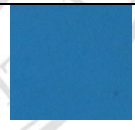
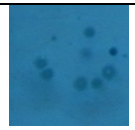
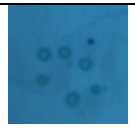
$NaAsO_2$ concentration (mg/L)	pH 6.0								
	control	**BAs 7**	**BAs 8**	**BAs 11**	**BAs 19**	**BAs 20**	**BAs 22**	**BAs 29**	**BAs 36**
0									
		++++	++++	++++	++++	++++	++++	++++	++++
10									
		++++	+++	++++	++++	++++	++++	++++	++++
15									
		++	+++	++++	+++	++++	++++	++++	++++
25									
		NG	++	++++	++	++++	++++	+++	++++
50									
		NG	++	++++	++	++++	++++	+++	++++

Table 3.9 (continued)

$NaAsO_2$ concentration (mg/L)	pH 6.0								
	control	**BAs 7**	**BAs 8**	**BAs 11**	**BAs 19**	**BAs 20**	**BAs 22**	**BAs 29**	**BAs 36**
100									
		NG	++	++++	+++	+++	+++	+++	+++
250									
		NG	++	+++	++	+++	++	++	+++
650									
		NG	+	++	+	NG	+	+	NG

Growth ++++; very good, +++; good, ++; fair; +; little, NG; No Growth

3.3.5 Population density and pH change under different levels of Na-As (III)

From the above experiment, four isolates; BAs8, BAs11, BAs19, and BAs29 exhibit much higher tolerant ability under various concentration of Na-As (III) than the rest of the isolates both at low and high pH levels. Therefore, the four isolates were selected for further experiments. BAs7 was highly sensitive to $NaAsO_2$ and was also selected to test as a sensitive isolate along with the high tolerant isolates.

In this experiment, the five selected isolates were inoculated into nutrient broth containing 0, 25, 50 and 100 mg/L of $NaAsO_2$ with 50 µM Al and the initial pH was 4.7. After 7 days of incubation, each sample solution was determined for growth (optical density) and pH change. Without Na-As (III) addition (0 mg/L), the population density (OD) of all the isolates was increased after 3 days of incubation, and the highest population was obtained with BAs8 BAs19 and BAs29 (OD 1.8, 1.6 and 1.6, respectively) (Fig. 3.2). These three isolates increased the pH of the medium from 4.71 to >8.0. The population of BAs7 and BAs11 was lowest at 3 days of incubation and they could increase the pH of the medium only up to 6.7 and 6.37, respectively. Isolate BAs11 showed the highest population at 5 and 7 days of incubation (OD 1.87 and 1.98, respectively) with higher pH increase of 7.87 and 8.08, respectively. Although BAs7 showed an increase in population density up to 5 days of incubation however its population was the lowest as compared to the rest of the isolates. BAs11 exhibited the highest population after 5 days of incubation (OD 1.87) and reach maximum growth at 7 days (OD 1.98).

In the culture broth with 25 mg/L $NaAsO_2$, the population of all the isolates was decreased except for BAs11 that showed similar growth as in the media without $NaAsO_2$. Isolates BAs8 BAs19 and BAs29 showed highest population at 3 days after incubation with the dramatically pH increase from 4.72 to 8.13. The lowest populations at all incubation period was recorded in BAs7 and this isolate could increase the pH of the medium only up to around 5.5. Isolate BAs11 performed the highest populations after 5 days of incubation and at the isolate could raise the pH of the medium from 4.72 to much higher value (8.23).

When the higher concentration of $NaAsO_2$ (50 and 100 mg/L) was added to the culture broth, no growth was observed in a sensitive isolate BAs7. The pH value of BAs7 culture broth was also remain the same at initial pH value (around 4.7) (Fig. 3.2). Isolates

BAs29 showed a better growth than BAs8 and BAs19 under high levels of $NaAsO_2$ (50 and 100 mg/L). At these high arsenite concentration, after 5 days of incubation, isolates BAs8, BAs19 and BAs29 raised the pH of the broth medium from 4.71 to around >8.2. Isolate BAs11 performed the highest populations after 5 days of incubation at both 50 and 100 mg/L of $NaAsO_2$ with the optical density of 1.87 and 1.74, respectively. At 7 days of incubation, this isolate could raise the pH of the medium from 4.72 to much higher value (>8.2).

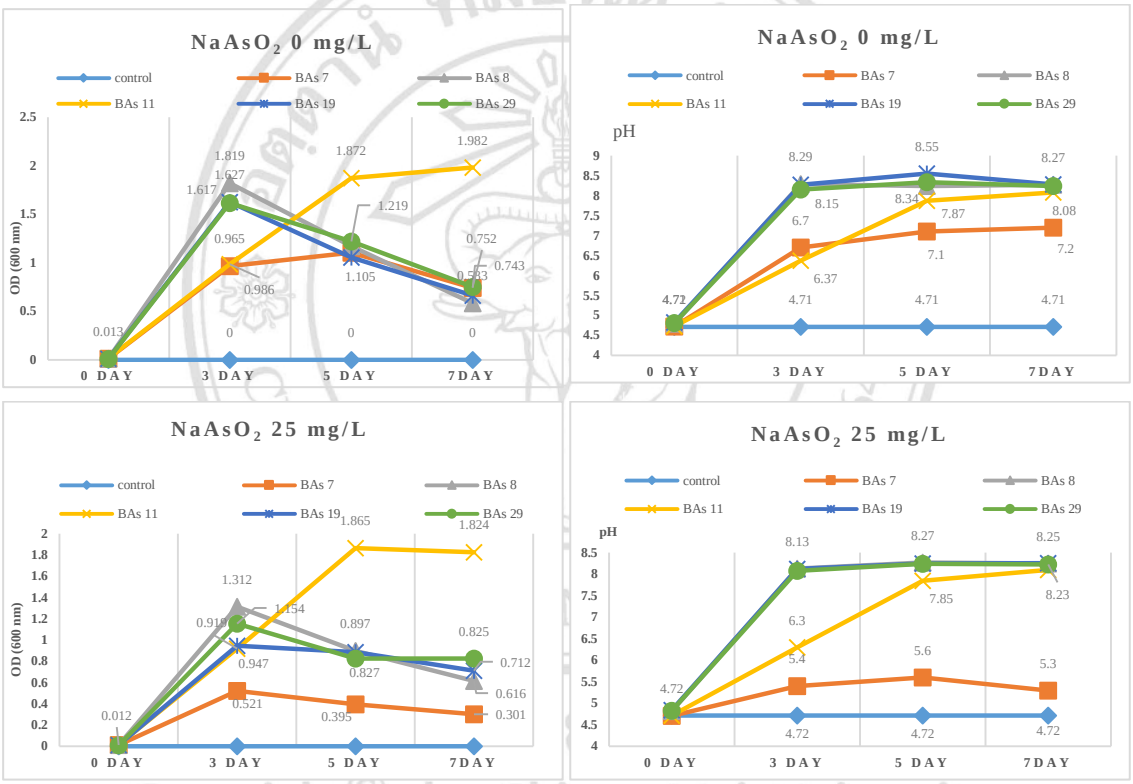


Fig 3.3 Effect of different arsenite concentration on growth of high arsenic-resistant isolates and pH change of the culture broth.

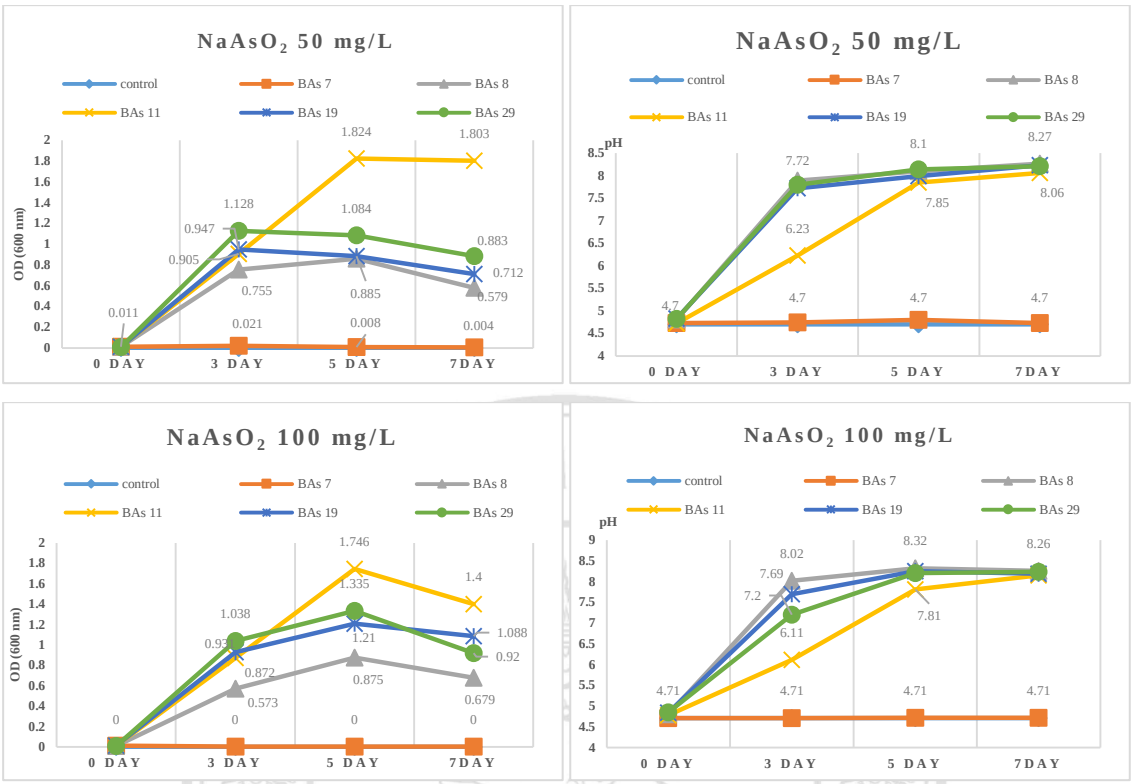


Fig 3.2 (continued)

3.3.6 Arsenite transformation by high arsenic-tolerant isolates

The five selected isolates; BAs7, BAs 8, BAs 11, BAs 19 and BAs 29 were used in this experiment. All the isolates were grown in acidic broth media (pH 4.7) containing 50 and 100 mg/L of sodium arsenite ($NaAsO_2$). Optical density (OD 600 nm) and changes in the pH of the culture broth medium of each isolate were recorded. The same culture broth of each isolate was also used to analyze different species of arsenic.

After 7 days of incubation, no growth was observed in the culture broth of sensitive isolate BAs7 at both concentrations of Na-As(III) and the pH of the culture broth remain unchanged. In contrast to BAs7, the high tolerant isolates; BAs8, BAs11, BAs19 and BAs29 were able to grow under high concentration of arsenite (50 and 100 mg/L) (Fig. 3.3). The same results were obtained at both arsenite concentrations, the highest population density was observed in BAs11 followed by BAs29, BAs19 and BAs8. The population density of isolates BAs8, BAs19 and BAs29 was slightly higher at 100 mg/L

than at 50 mg/L of arsenite. These three isolates increased the pH of the culture broth to much higher value; 8.37, 8.27 and 8.43 (50 mg/L of arsenite), and 8.13, 8.34 and 8.23 (100 mg/L of arsenite), respectively. BAs11 showed lower population density at 100 mg/L than at 50 mg/L of arsenite and the pH the culture broth was 6.79 and 7.85, respectively.

In addition to pH and optical density measurement, the same supernatant of the culture solution was also analyzed for different species of arsenic. The standard arsenic species; arsenite (As(III)); arsenate (As(V)); monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) was applied. The peak area was used for quantification of each arsenic specie. Figure 3.4 showed the chromatogram of arsenic species performed by each tested isolate. No organic form of arsenic (MMA and DMA) was observed in the culture broth of the tested isolates. The peak height of the control (without bacterial isolate) and sensitive isolate BAs7 showed the same pattern indicating that BAs7 could not transform As(III) to (As(V)) (Fig. 3.4). The resistant isolates; BAs 8, BAs 11, BAs 19 and BAs 29 exhibited a much higher peak of (As(V)) as compared to the control thus arsenite transformation was occurred in the culture broth.

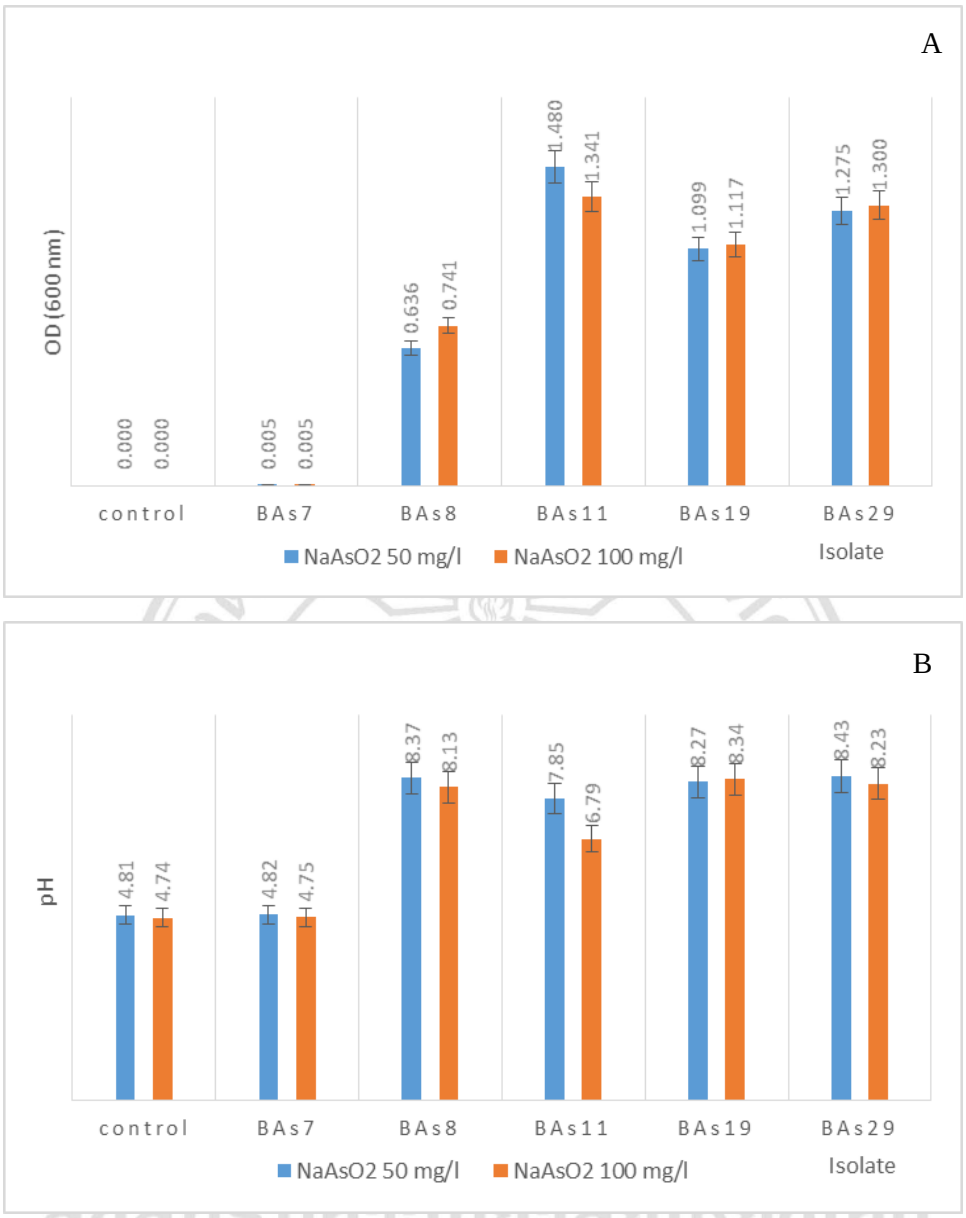


Figure 3.4 Effects of $NaAsO_2$ concentrations (50 and 100 mg/L) on (A) population density (OD) of selected isolates; BAs7, BAs11, BAs8, BAs19 and BAs29; and (B) pH change in the culture broth

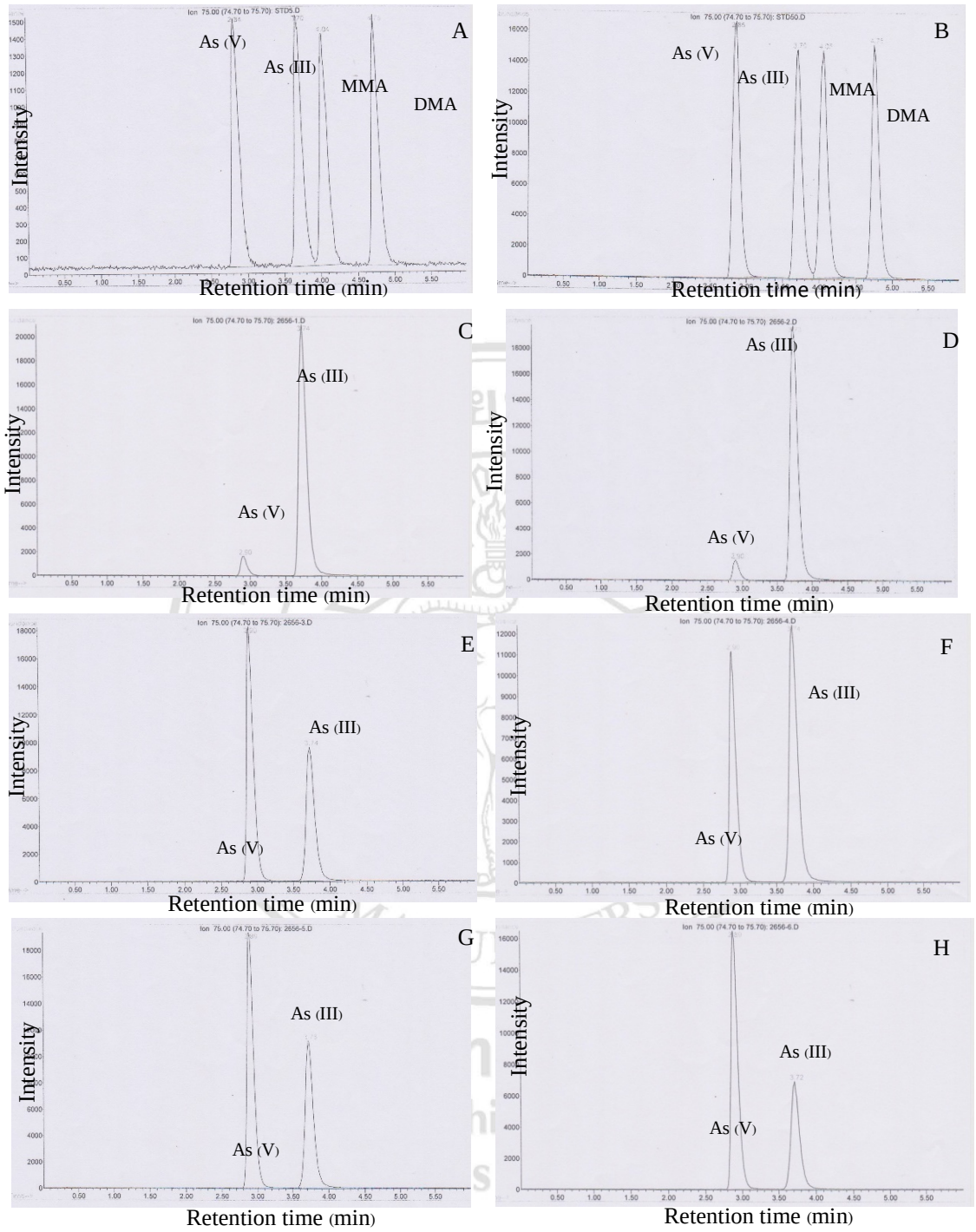


Fig 3.5 Chromatograms obtained from the HPLC-ICPMS analyses of arsenic species; arsenite (As(III)); arsenate (As(V)); monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA); (A) standard 5 ppm, (B) standard 50 ppm, (C) control, (D) BAs7, (E) BAs8, (F) BAs11, (G) BAs19 and (H) BAs29

As compared to the control without bacterial inoculum, at both concentration of Na-As(II) (50 and 100 mg/L), BAs7 appeared to be ineffective in transforming arsenite to arsenate as the values of these two forms of inorganic arsenic were almost the same as

those of the control. At the concentration of 50 mg/L of Na-As(III), isolate BAs29 showed most effective in transforming arsenite to arsenate. This isolate could oxidize highly toxic form of inorganic arsenic (As(III)) into less toxic form (As(V)), up to 64.51%; followed by isolate BAs8, BAs19, and BAs11 with transforming values of 58.98, 57.57 and 40.51%, respectively.

At the concentration of 100 mg/L of Na-As(III), it was found that all the tolerant isolates exhibited lower ability in oxidizing arsenite as compared with 50 mg/L of Na-As(III). However, similar trend of efficiency was observed, as the isolate BAs29 also performed highest ability of 47.07% while BAs11 showed lowest ability of 11.29%, in transforming arsenite. The transforming ability of BAs8 and BAs19 was also decreased to 41.74 and 35.67%, respectively.

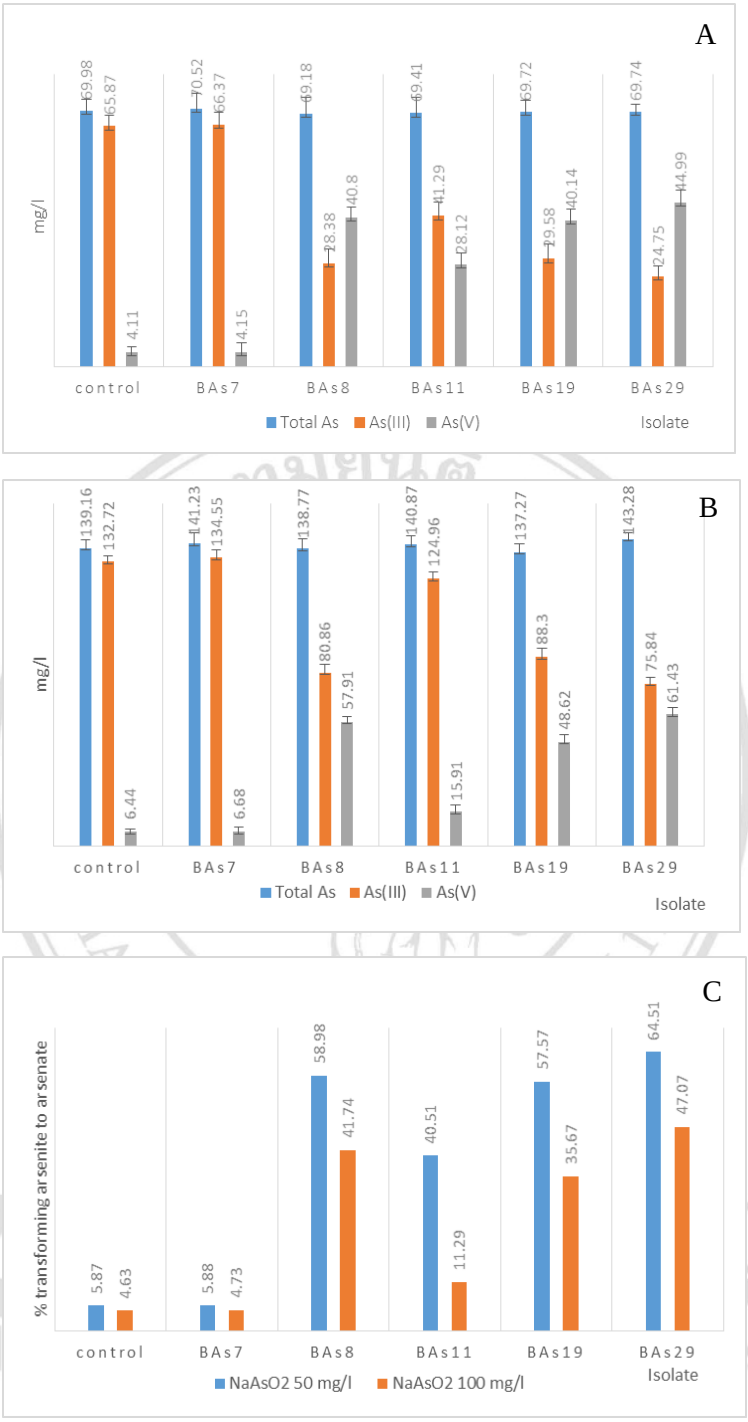


Fig 3.6 Effectiveness of arsenic-resistant bacteria in arsenite transformation; (A) and (B); Total (As), As (III) and As (V) in the culture broth of selected isolates; BAs7, BAs11, BAs8, BAs19 and BAs29; in the medium containing $NaAsO_2$ 50 and 100 mg/L, respectively; and (C) % transforming As (III) to As (V) at 50 and 100 mg/Lof $NaAsO_2$.

3.4 Discussion

High concentrations of arsenic (5.45 to 39.48 mg/kg) were found in cultivated highland soil in northern Thailand and are likely to have an adverse impact on upstream, downstream, groundwater and thus food chains. Office of National Environment Board of Thailand set an arsenic maximum concentration limits (MCL) in agricultural soil at 3.90 mg/kg (Weerasiri *et al*., 2012). Chitpirom *et al*. (2009) reported that arsenic concentration of agricultural soil samples from central Thailand were 4.11 to 4.35 mg/kg. Majumder *et al*. (2013) found that arsenic concentrations in soils of West Bengal, India varied from 7.4 to 13.4 mg/kg. The levels of soil arsenic vary widely in different countries (0.1 to 40 mg/kg). Anthropogenic sources exceed natural sources by 3 to 1 in the environment (Mandal and Suzuki, 2002). In the present study, soluble arsenic in the contaminated highland soils may be readily available for uptake by plant root leading to elevated levels of arsenic in soils. This can result in increased concentration of arsenic in plants. Eating crops grown on contaminated soils may increase the health risks. A few recent studies report 85 to 95% inorganic arsenic in rice and vegetables, which suggest more studies for standardization (Mandal and Suzuki, 2002).

In this study, observation of bacterial growth on agar medium under low pH (4.5 and 4.7) revealed that only a few isolates could tolerate high concentrations of arsenite with the highest MIC of 40 mM. The number of tolerant isolates at 40 mM MIC was doubled when the pH was raised to 7.0 indicating an important role of pH in arsenic tolerant ability. Mandal and Suzuki (2002) concluded that some isolates were hyper-resistant to arsenite at 16 to 47 mM which was similar to our results. Aksornchu *et al*. (2008) obtained high arsenic tolerant bacteria which were able to grow in medium containing 40 mM sodium arsenite from soils contaminated with 40 to 1,000 mg/kg. Thus, the few isolates obtained from this study could be considered as hyper arsenic tolerant bacteria. The high tolerant isolates (BAs8, BAs11, BAs19 and BAs29) obtained in this study were able to increase the pH of the agar medium supplemented with bromocresol green from 4.5 (green) to around ≥ 5.0 (ocean blue – bright blue). The growth appearance of the tested isolates was observed only when they were able to alter the pH of the agar medium to be around ≥ 5.0 (ocean blue – bright blue) suggesting an alkali substance(s) might be released by the isolates as a survival mechanism. When exposed to high arsenic concentration in the broth medium with high arsenic concentration under strongly acidic

condition (pH 4.7), these high tolerant isolates were able to alter the medium pH up to around >8.0 (except for BAs11, 6.8 – 7.8). It appeared that the higher pH increased in the media, the higher population density of the tolerant isolates was obtained. In contrast to the tolerant isolates, BAs7 (arsenic sensitive isolate) was not able to increase the pH of the broth medium and no growth was observed for this isolate. The results of the present study suggested that arsenic might performed quite low solubility under neutral or slightly acidic pH. The solubility of arsenic seemed to be increased considerably in strongly acidic conditions thus lower the MIC and the growth of the tested isolates. Arsenic concentration and species are influenced by pH and redox potential of soil (Mandal and Suzuki, 2002). The mobility of arsenite and arsenate is a function of their adsorption, which in turn is controlled primarily by pH (Luo *et al*., 2008; Elkhatib *et al*., 1984; McBride, 1994).

Besides pH changes and population density in the broth medium, monitoring of the redox speciation of arsenic was also performed in this study. The oxidation of As(III) to As(V) was observed in all the high tolerant isolate with the highest transforming values of 64.5% obtained in isolate BAs 29. It appeared that, under high concentration of arsenite (50 and 100 mg/L), the pH increased up to near neutral/alkali (≈7 to > 8.0) might be needed to effectively oxidize highly toxic form of arsenic (As(III)) into less toxic form (As(V)). The oxidation of arsenite is one of detoxification reactions in bacterial cell that confer arsenic resistance via the arsenic resistance system (ars) genes, which encode proteins that provide resistance through the oxidation of arsenite (Stolz *et al*. 2002; Silver *et al*., 1996). The oxidation of arsenite to arsenate by arsenic resistant bacteria and other microorganisms can highly impact the mobility and speciation of arsenic in the cultivated soils. Abiotic As(III) oxidation via reaction with O_2 under atmospheric conditions is extremely slow in the absence of a biological or a chemical catalyst therefore As(III) oxidation by microorganisms may be an important process for bioremediation (Garcia-Dominguez et al., 2008; Osborne *et al*., 2010). The results obtained in this study suggested that arsenic resistant ability of the bacterial isolates were highly related to pH conditions of specific environment which appeared to control arsenite oxidation process. High arsenic resistant bacteria thus can influence arsenic speciation in the environment and could be applied to enhance the bioremediation of arsenic-contaminated soil.

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3.5 Conclusion

Agricultural soils of highland areas of northern Thailand collected in this study had higher concentrations of arsenic (5.45 to 39.48 mg/kg) than the arsenic maximum concentration limits (MCL) (3.90 mg/kg). Only nine out of 40 isolates (22.5%) obtained from As-contaminated soils of northern Thailand, could tolerate a high level of As at pH 4.5 and 4.7 with the MIC of 40 mM. Four isolates, i.e. BAs8, BAs11, BAs19 and BAs29 performed promising tolerant ability under various pH values. The oxidation of As(III) to As(V) was observed in all the high tolerant isolate. BAs29 exhibited highest resistant ability with the maximum percentage 64.5% of arsenite transformation. High arsenic resistant isolates increased the pH of the broth medium from 4.7 to around 7 to > 8.0 while arsenic sensitive isolate (BAs7) was not able to increase the pH of the medium. No growth and no arsenite transformation was observed for BAs7. Therefore, the pH increased up to near neutral/alkaline might be needed to effectively oxidize highly toxic form of arsenic (As(III)) into less toxic form (As(V)). The results highlighted the two survival mechanisms (pH changes and arsenite oxidation) of the arsenic resistant isolates and the close relationship between pH conditions of surrounding medium and As(III) oxidation process.

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