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RESEARCH ARTICLE

Seasonal Variation in Arsenic Concentration and its Bioremediation Potential of Marine Bacteria Isolated from Alang-Sosiya Ship-Scrapping Yard, Gujarat, India

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ABSTRACT

This work investigates seasonal variations in arsenic concentration at Alang-Sosiya, world's largest ship-scrapping yard situated on the Gulf of Khambhat. Annually, hundreds of ships have been dismantled, which lead to discharge large amounts of detrimental and persistent pollutants at this location. In all seasons, the arsenic concentration was significantly elevated in sediments and seawaters in the intertidal zone of Alang-Sosiya ship-scrapping yard as compared to the reference station at Ghogha, 42 km away towards the northeast. The highest arsenic concentrations in seawater and sediment samples were observed during the Winter season and Summer season respectively. The marine environment affected by ship-scrapping activity and contaminated with arsenic is the potential location to get arsenic hyper-tolerant bacterial isolates. Out of 16 isolated bacterial strains, KKDK-1 and KKDK-2 sustained 600 mM and 500 mM arsenate respectively. The 16S rRNA ribotyping identified strains KKDK-1 and KKDK-2 as Halomonas species. The strain KKDK-1 showed the maximum arsenic accumulation of 21.7±3.3 mg g-1 cell dry weight at exponential phase (60 h), followed by sudden extrusion of arsenic during stationary phase (84 h) of bacterial growth. Whereas, strain KKDK-2 accumulated 6.8±1.12 mg Arsenic g-1 cell dry weight during exponential phase (72 h), which remains almost invariable during stationary phase (96-144 h) of bacterial growth. These results indicate the hypertolerance of arsenic by KKDK-1 and KKDK-2 with its higher accumulation capacity, signifying them as potential candidates for arsenic detoxification of arsenic contaminated sites.

Keywords: Ship scrapping yard, arsenic hyper-tolerant, marine bacteria, *halomonas sp*., arsenic accumulation, bioremediation

1. INTRODUCTION

Arsenic is a notoriously toxic element that causes serious threats to the environment and human health in numerous places around the world. The long-term exposure to even low concentrations of inorganic arsenic can cause various adverse health effects, such as 'arsenicosis' and cancer[1]. Due to its severity, the United States Environmental Protection Agency (U.S.EPA) classified it as a priority pollutant with carcinogenicity classification A (a human carcinogen)[2]. Natural sources, as well as anthropogenic sources (such as industrial effluents, mining discharges, and ship scrapping activities), are the major contributors of arsenic pollution in the coastal environment. Coastal areas and estuaries of many countries, including Australia, France, Korea, Japan, China, UK, and USA have been reported elevated arsenic pollution[3-11].

Ship-scrapping activities lead to discharge large amounts of detrimental and persistent pollutants, which cause serious threats to the marine ecosystem. Hossain and Islam[12] reported elevated concentrations of heavy metals in fishes from the ship-scrapping area, Chittagong, Bangladesh. One of such ship-scrapping yards in India is Alang-Sosiya ship-scrapping yard near Bhavnagar, Gujarat. The Alang-Sosiya ship-scrapping yard is one of the largest ship-scrapping yards in the world. Yearly around 350 ships have been dismantled, which generates enormous quantities of waste, including broken wood, plastics, rubber, metals, paints, leather, etc[13]. Wood was constituting the significant portion (~16.5 per cent) of total solid waste generated at ship-breaking locations[13]. Around 90 per cent industrial uses of arsenic compounds are as wood preservatives[14]. High concentrations of various heavy metals (iron, manganese, cobalt, copper, zinc, lead, cadmium, nickel and mercury)[15,16], total petroleum hydrocarbons (PACs) and total polycyclic aromatic hydrocarbons (PAHs)[17] have been reported at Alang-Sosiya ship-scrapping yards. However, to our knowledge the arsenic contamination and seasonal variation in its concentration at this location have not been studied yet.

Removal of arsenic has become a necessity and global area of interest. The bacterial cells capable of removing arsenic from their surroundings could be used as an alternative or to supplement existing physicochemical methods of arsenic removal, and the ideal candidate for bioremediation. Increasing focus of researcher on marine bacteria for bioremediation purpose, because marine

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environments are one of the most adverse system owing to their varying nature of salinity, temperature, pH, Coastal inputs, sea surface temperature, precipitation regimes, currents, and wind patterns[18]. Therefore, the bacteria present in such a harsh environment are more capable of the adaptations. And consequently, they may be better the candidates for bioremediation. The high ability of some marine bacteria to tolerate arsenic and to accumulate arsenic was first reported by Tokeuchi[19], *et al.* Due to high pollution of contaminants at Alang-Sosiya ship-scrapping yard, it may be the prime location to get potential marine bacteria capable of arsenic bioremediation. The present study aims:

(i) To determine seasonal variations in arsenic concentration
(ii) To isolate potential marine bacteria that are better candidates for arsenic bioremediation.

2. MATERIALS AND METHODS

2.1 Site Description

Alang-Sosiya ship-scrapping yard is one of the largest ship-scrapping yards in the world. Geographically it is situated 21°5' 21°29' towards the north and 72°5' 72°15' towards the east on the western coast of the Gulf of Khambhat (Figure 1). The coastal region is a 14 km long coastal strip in which there are 112 ship scrapping yards in Alang and nearly 80 yards in Sosiya. The yard has a gentle slope of around 10 degrees with a firm and hard rocky bottom, which is suitable for bringing ships right up to the scrapping yard afloat with minimum investment and risk factors[17]. Nearly half of the world's ocean-going ships are being dismantled and recycled in India, of which around 95 per cent are scrapped in Alang-Sosiya yards[20]. Annually, hundreds of ships mainly including cargo vessels, oil tankers, passenger liners, and warships have been dismantled, which adds up to millions of Light Displacement Tonnage.

2.2 Sample Collection

The samples of coastal surface sediments were collected by digging at the depth of 30 to 45 cm in acid washed sterilized plastic bags while coastal surface seawater samples were collected in acid-washed sterilised bottles. Samples were collected under an aseptic condition during the summer (June 2012), Monsoon (August 2012) and Winter (January 2013) season. We selected total six stations (AS1-AS6) covering entire Alang-Sosiya coast (Fig. 2), and one station from reference site Ghogha (R1) ~ 42 km northeast of the Alang-Sosiya coast for sample collections. The reference site, Ghogha was preferred as a reference location due to the absence of considerable anthropogenic activities affecting arsenic contamination as well as it is quite far from ship scrapping industries. The Sediment and seawater samples were collected at low tides and high tides respectively from the intertidal zone. Each collected sample was used for arsenic analysis as well as for the isolation of arsenic hyper-tolerant marine bacteria.

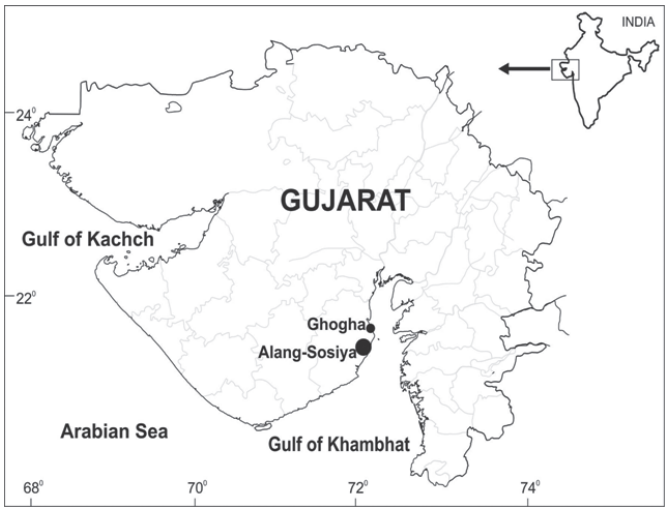

Figure 1. Location map of Alang-Sosiya (study area) and Ghogha (reference station).

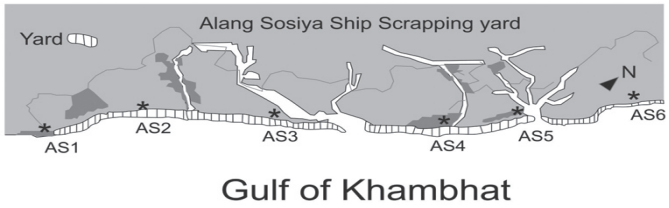

Figure 2. Location of sampling stations at Alang-Sosiya ship scrapping yard.

2.3 Analysis of Arsenic Content in Collected Samples

For the analysis of arsenic contents, oven-dried (90 - 100°C) sediment samples (1 g) were digested with a mixture of nitric acid (Qualigens, India) and Perchloric acid (Qualigens, India) (10:1 by volume) in microwave digestion system (MarsXpress, CEM). Digested material was diluted with MilliQ water to a constant volume and analyzed by inductively coupled plasma-mass spectrometry (ICP-MS, ELAN 9000, Perkin Elmer).

Initially, the seawater samples were filtered through 0.45 µm membrane filter (Sartorius A G, Goettingen, Germany), acidified to pH 2.0 with concentrated Nitric acid and were stored at a freezing temperature. The samples were preconcentrated using APDC and MIBK solutions[21,22] and estimated by inductively coupled plasma Optical Emission Spectroscopy (ICP-OES, Optima 3300RL, Perkin Elmer). For the calibration and accurate analysis, NASS-5 and CASS-5 (Seawater reference materials) were used in this study.

2.4 Isolation of Arsenic Tolerant Marine Bacteria

For the isolation of arsenic tolerant marine bacteria, 1 g of sediment (1 ml in case of seawater) samples were diluted in 10 ml (9 ml in case of seawater) sterile MilliQ water. All samples were kept on shaking at 130 rpm for 10 min. The aliquots were serially diluted and plated on marine agar (Zobell marine agar 2216, HiMedia, India) medium embedded with 0.14 mM arsenic concentration.

The well-defined isolated colonies were selected on the basis of colonial morphological characteristics. Arsenate (as $Na_2HAs•7H_2O$) and arsenite (as $NaAsO_2$) were procured from Loba-Chemie, India and a stock solutions of 1 M and 0.1 M respectively, were prepared by dissolving it in milliQ water, sterilized through membrane filter (0.22 µm, Sartorius A G, Goettingen, Germany) and then used to add to the medium to give final concentration 0.28 – 200 mM for arsenate and 0 – 2 mM for arsenite. Morphologically distinct bacterial colonies capable of tolerating above levels of arsenic were further screened for Maximum Tolerance Concentration (MTC).

2.5 Identification of Arsenic Hyper-tolerant Marine Bacteria

Selected arsenic hyper-tolerant marine bacterial isolates were identified by sequencing 16S rRNA gene fragment. The DNA from arsenic hyper-tolerant bacterial isolates KKDK-1 and KKDK-2 were extracted by Bacterial DNA Purification Kit (GeNeiPure™, India). The amplification of 16S rRNA gene sequences was carried out by polymerase chain reaction (PCR) using universal primers 27f (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492r (5´-GGTTACCTTGTTACGACTT-3´). Purified PCR products were sequenced using internal overlapping primers[30]. Sequences were initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov/) using Blast (blastn) tool, and corresponding sequences were downloaded. Phylogenetic analyzes were performed using MEGA 5.10 software, and the phylogenetic tree was constructed using the neighbor-joining distance method[32].

Phylogenetic analysis of strain KKDK-1 and KKDK-2 consistently place the organisms in the genus Halomonas. The partial 16S rRNA gene sequences of Halomonas sp. strain KKDK-1 and strain KKDK-2 were submitted to GeneBank under the accession number KF682368 and KF741279 respectively.

2.6 Growth Kinetics and Determination of Arsenic Accumulation Capacity of Arsenic Hyper-tolerant Marine Bacterial Isolates

Halomonas sp. strain KKDK-1 and Halomonas sp. strain KKDK-2 showing maximum tolerance for arsenate, were grown at 30 °C in 200 ml of arsenic embedded and arsenic free marine broth media (Zobell marine broth 2216, HiMedia, India) with continuous shaking at 130 rpm on the orbital shaker (MAXQ 6000, Thermo Scientific, USA). The culture broth of 10 ml was collected at different time intervals till it reached stationary phase, and bacterial growth was scrutinized by measuring the O.D. (Optical Density) of cultures at 600 nm using the spectrophotometer. The growth rate constant (k) for the log phase of growth was determined by plotting the log10 of optical density against time.

Cells were harvested by centrifugation at 5000×g for 10 min, and the pH of the supernatant was measured. The harvested cells were washed three times with 0.85 per cent NaCl, dried and then used for the measurements of arsenic accumulation. Bacterial pellets were oven dried at 90 °C - 100 °C to constant weight and digested with concentrated nitric acid using the microwave digestion system. Samples were brought to the constant volume with milliQ water and analyzed for arsenic concentrations using ICP-MS.

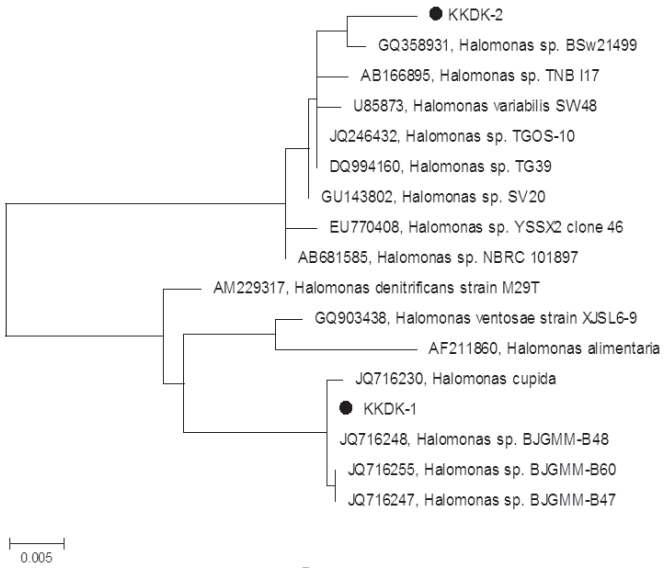

Figure 3. Phylogram of arsenic hypertolerant marine bacteria derived from 16S rRNA gene sequence data of strains KKDK-1 and KKDK-2 and closely related species.

3. RESULTS AND DISCUSSION

3.1 Seasonal Variation in Arsenic Content

The arsenic concentration in unpolluted seawaters usually ranges between 1 – 2 µg/l. However, the significant level of arsenic is found in coastal seawater influenced by anthropogenic inputs such as industrial effluents, mining discharges, and Ship-scrapping industries. In the present study, we have observed that arsenic concentrations in all samples collected in three seasons were high at Alang-Sosiya (AS1 – AS6) compared to reference station Ghogha. Table 1 and 2 depicts the seasonal variations in the average arsenic concentrations in sediments, and seawaters respectively of all stations studied here. A trend of the higher arsenic contents was observed at station AS2 – AS5 than stations AS1, AS6 in Alang-Sosiya and reference station (R1). Amongst the three seasons, the elevated arsenic content in seawaters was observed in winter season followed by monsoon and summer. The high degree of metal dispersions into waters at low temperature, low tides during the winter[17], and microbial mobilization of adsorbed arsenic from sediments may be the explanations for the elevated level of arsenic in seawaters. In the winter season, with the reduction in redox potential (Eh), increase the solubility of arsenic[23], which may increase the arsenic contents in seawater. The level of arsenic in seawaters enriched in the monsoon season may be due to the inflow of fresh contaminants from ship scrapping yards and the domestic wastes along with rain waters. The relatively low concentration of arsenic in seawaters observed in the summer season was probably due to the maximum

precipitation and adsorption onto the sediments. As shown in Table 1, the highest arsenic concentration in sediments was observed during the summer season followed by monsoon and winter. Suggesting, elevation in temperature during the summer, as well as high Fe (137,990 ±78,573 ppm) and Mn (4643.1±1769.5 ppm) contents in the Alang-Sosiya ship-scrapping area[16], may favor the adsorption of arsenic to the sediments[17]. The solubility of arsenic highly depends on its redox state, i.e., trivalent arsenite is four to ten time more soluble than pentavalent arsenate. In summer, trivalent arsenic is oxidized to pentavalent form leading to arsenic migration from the water to the sediment[23]. Pentavalent arsenic forms $FeAsO_4$, $Ca_3(AsO_4)_2$, $Mg(AsO_4)_2$ and other insoluble salts that precipitate and resulting in an increase in the arsenic content in the sediment[23-24]. The Hummer estuary, United Kingdom receives a large amount of Fe and Mn from industrial discharges. The presence of large amount mineral clay, iron, and manganese (oxy-) hydroxide coatings on clay scavenge dissolved arsenic from the water column and transport into the sediments[6]. This is the explanation of high seasonal variations in arsenic concentration in sediments samples compared to the seawater samples, probably due to the huge quantity of Fe, Mn, and other salts restrict the solubility of arsenic. Arsenic concentrations in uncontaminated nearshore marine and estuarine sediments were between 5 mg/kg to 15 mg/kg dry weight[25]. In the present study, we have found 2 to 3 times elevated arsenic concentration in Alang-Sosiya Ship-scrapping locations. Sediments from the coastal area receiving drainage from industries, metal mining and Ship-scrapping activities (in this study) significant contributors of arsenic pollution, which threaten the aquatic biota and has been of significant environmental concern.

Table 1. Seasonal variation in sediment arsenic concentration

Sampling Station	Seasonal arsenic concentration (mg/kg)		
	Summer (Jun. ´12)	Monsoon (Aug.´12)	Winter (Jan.´13)
AS1	28.8 ± 2.27	13.2 ± 0.67	5.47 ± 0.54
AS2	37.22 ± 0.92	15.56 ± 1.46	7.19 ± 0.16
AS3	40.01 ± 1.23	18.25 ± 0.62	7.22 ± 1.57
AS4	41.99 ± 3.74	17.64 ± 1.03	7.99 ± 1.27
AS5	40.36 ± 1.09	18.56 ± 1.53	8.77 ± 0.98
AS6	31.09 ± 2.35	15.1 ± 0.92	5.82 ± 1.58
R1	6.92 ± 0.82	3.98 ± 1.42	2.91 ± 1.04

Table 2. Seasonal variation in seawater arsenic concentration

Sampling Station	Seasonal arsenic concentration (µg/l)		
	Summer (Jun. ´12)	Monsoon (Aug. ´12)	Winter (Jan.´13)
AS1	12.94 ± 0.16	14.65 ± 0.17	15.78 ± 1.21
AS2	19.45 ± 0.05	20.01 ± 0.52	22.14 ± 0.12
AS3	15.5 ± 1.25	17.2 ± 0.12	19.44 ± 0.4
AS4	19.23 ± 0.69	22.68 ± 0.81	25.54 ± 0.2
AS5	20.34 ± 1.00	24.34 ± 0.44	28.5 ± 0.22
AS6	9.1 ± 2.72	12.34 ± 0.12	17.91 ± 1.51
R1	4.69 ± 0.19	4.98 ± 0.18	6.91 ± 0.92

3.2 Isolation and Identification of Arsenic Hypertolerant Bacteria

The present study was undertaken to isolate bacterial cells from the Alang-Sosiya ship scrapping yard that were capable of tolerating high arsenic concentration. During three seasons, 16 native bacterial strains were isolated from sediments and seawaters samples (Table 3). Out of these strains, five bacterial strains were found to tolerate high concentrations of arsenic in the media. Two of the organisms were found to grow up to 600 mM and 500 mM of arsenate and 6 mM and 2 mM of arsenite respectively in the media, which were further selected for identification. Morphological characterization showed KKDK-1 and KKDK-2 as Gram-negative, rod-shaped bacteria. 16S ribotyping identifies the bacteria as Halomonas sp. strain KKDK-1 and Halomonas sp. strain KKDK-2. Figure 2 depicts the phylogenetic tree derived from 16S rRNA gene sequence data of both the arsenic hyper-tolerant strains and other related species. The 16S rRNA gene sequences of 1451-bp (KKDK-1) and 1401-bp (KKDK-2) were aligned closely with the sequence of *Halomonas sp*. Strain BJGMM – B48 (Accession number JQ716248, sequence similarity 99 per cent) and *Halomonas sp*. Strain BSw21499 (Accession number GQ358931, sequence similarity 99 per cent).

3.3 Growth Kinetics and Arsenic Accumulation Capacity of Selected Isolates

The growth comparisons of the cells grown in arsenic-free media and arsenic-containing media revealed 59.97 per cent reduction in the growth of strain KKDK-1 and 57.69 per cent in growth of strain KKDK-2 following the treatment of 600 mM and 500 mM arsenate containing media respectively (Figure 4(a) and 5(a)). The growth of organisms in arsenate containing media resulted in the change of extracellular pH from 7.0 to 7.32 and 7.0 to 8.5 in strain KKDK-1 and KKDK-2, respectively.

Table 3. Maximum arsenic tolerance shown by bacterial isolates

Isolate	Arsenate (mM)	Arsenite (mM)	Isolate	Arsenate (mM)	Arsenite (mM)
KKDK-1	600	6	DKKK-9	10	02
KKDK-2	500	2	DKKK-10	10	-
KKDK-3	300	6	DKKK-11	10	0.5
KKDK-4	100	-	DKKK-12	05	0.5
KKDK-5	100	4	DKKK-13	0.28	-
KKDK-6	100	-	DKKK-14	0.28	-
DKKK-7	50	-	DKKK-15	0.28	-
DKKK-8	10	-	DKKK-16	0.14	-

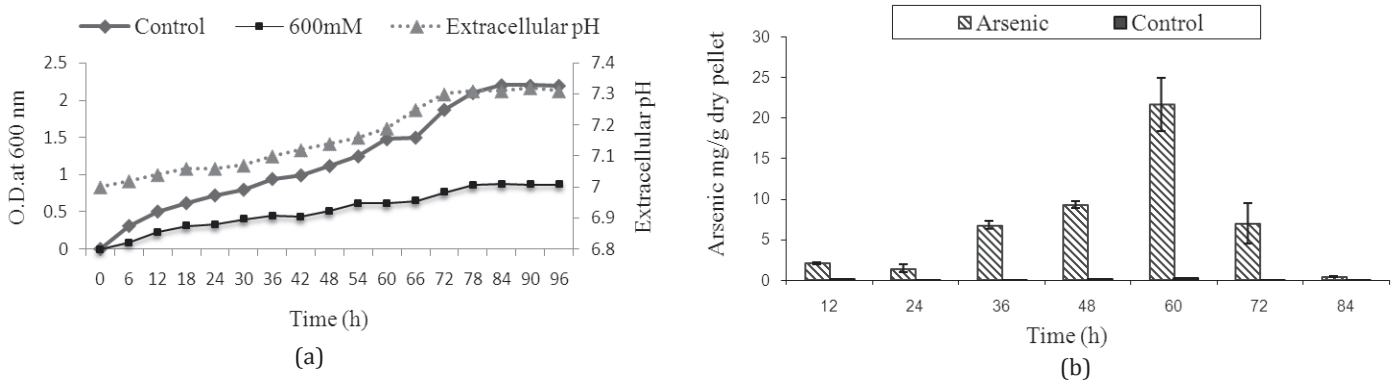

Figure 4. (a) Growth kinetics of Halomonas sp. strain KKDK-1 in 600 mM arsenate for 96 h. Change in OD600 versus extracellular pH over 96 h. (b) Time-dependent arsenic bioaccumulation in Halomonas sp. KKDK-1(Values are mean ± S.E.). Change in time versus arsenic mg/g dry pellet.

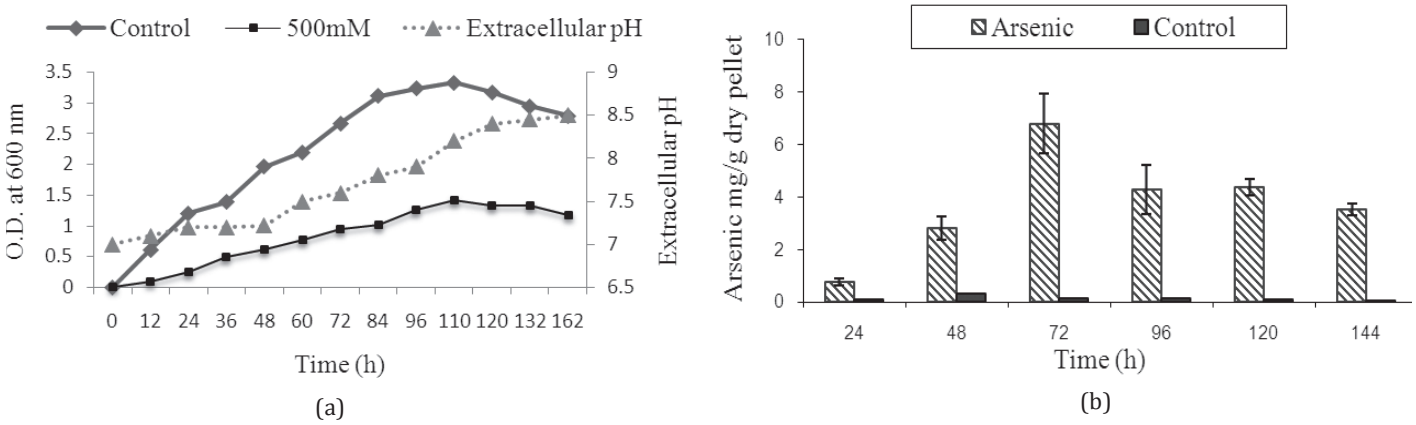

Figure 5. (a) Growth kinetics of Halomonas sp. strain KKDK-2 in 500 mM arsenate for 162 h. Change in OD600 versus extracellular pH over 162 h. (b) Time-dependent arsenic bioaccumulation in Halomonas sp. KKDK-2(Values are mean ± S.E.). Change in time versus arsenic mg/g dry pellet.

Strain KKDK-1 grown with arsenate showed maximum uptake of 21.68±3.3 mg Arsenic g-1 (dry weight) during exponential phase (60 h growth), followed by sudden extrusion of arsenic after reaches to stationary phase (84 h growth). Whereas, strain KKDK-2 showed maximum uptake of 6.8±1.12 mg Arsenic g-1 (dry weight) during exponential phase (72 h growth), which remains almost invariable during stationary phase (96 - 144 h growth). The extrusion of intracellular arsenic is governed via classical ars operon, which initially converts intracellular arsenate to arsenite mediated by ArsC (cytoplasmic arsenate reductase) protein followed by extrusion of arsenite by ArsAB/ArsB (arsenite efflux pump) protein[31]. However, the sudden extrusion of arsenic in the form of arsenite was not fully supported in strain KKDK-1 because there was no significant increase in pH of extracellular medium observed. There might be some other forms of arsenic (such as methylated arsenicals) were majorly extruded out of the cells than arsenite. The gradual efflux of arsenic by strain KKDK-2 in the form of arsenite was supported by an increase in extracellular pH (7.0 - 8.5) when grown in 500 mM arsenate embedded medium. The results clearly indicate that strain KKDK-1 has arsenic accumulation capacity 2-20 times higher than the values reported in bacteria till to date[1,27-29,31]. The highest amount of arsenic accumulation reported in non-genetically modified bacteria to date is 9.8±0.5 mg Arsenic g-1 (dry weight) in *Bacillus sp.* strain DJ-1 isolated from industrial effluent [27]. Arsenic accumulation capacity of genetically engineered *E. coli* [28, 29] cells was many folds lower than that observed in strain KKDK-1. Considering the arsenic accumulation efficiency of *Halomonas sp*. strain KKDK-1, this bacterium might be a potential candidate for bioremediation of arsenic contaminated sites.

4. CONCLUSIONS

From the present study, we come to following conclusions:

1. The concentrations of arsenic are significantly elevated in sediments and seawaters in the intertidal zone of Alang-Sosiya ship scrapping yard in all seasons compared to reference station at Ghogha. The highest arsenic concentrations in seawater and sediment samples are observed during winter season and summer season respectively.
2. The marine environment is the potential location to get arsenic hyper-tolerant bacterial isolates. Furthermore, the coastal region affected by ship-scrapping activity (in this study), mining descharge and industrial

effluents may favor the chances to get the excellent arsenic tolerant bacteria.

3. Halomonas sp. strains KKDK-1 and KKDK-2 are the potent arsenic hyper-tolerant marine bacteria competent to accumulate the significantly large amount of arsenic and may offer advantages for bioremediation of arsenic. However, future studies will require the determination of arsenate reduction and detection of genes associated with arsenic tolerance for better understandings of tolerance mechanism in these bacteria.

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Conflict of Interest : None

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