Original Article

Characterization of Dietzia natronolimnaea ASO3 Isolated from Arsenic Anriched Water Sources for its Potential to Arsenic Resistance and Removal

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Abstract

Introduction: Arsenic is a known toxic metalloid ubiquitous in nature and exposure can occur from natural and anthropogenic sources. Inorganic arsenic both arsenite and arsenate constitute the highest toxicological risk associated with arsenic in drinking water. This study presents the arsenic resistance and removal capacity of a bacterial strain indigenous to arsenic enriched water of Rautahat district, Nepal.

Methods: Identification was carried out by phenotypic and 16S rDNA sequence analysis. The optimal growth conditions regarding temperature, hydrogen ion concentration and salinity; growth kinetics in presence and absence of arsenic, determination of maximum arsenic tolerance concentration, sensitivity to antibiotics, plasmid mediated resistance as well as arsenate reduction and arsenite oxidation and finally arsenic removal potential was determined.

Results: The bacterium, Dietzia natronolimnaea ASO3 showed relatively high resistance to arsenate up to 37,460 mg/l and arsenite up to 374.6 mg/l. It showed optimal growth at 30°C in pH 8. The bacterium conferred resistance to penicillin and removed 47% of arsenite and 51 % of arsenate from the medium amended with 200mg/l arsenate and 74.92mg/l arsenite respectively.

Conclusion: The higher arsenic tolerance to both arsenate and arsenite species with potential for their removal can be explored further for arsenic mitigation and mobilization study.

Keywords: arsenate, arsenite, arsenic removal, arsenic resistant bacteria, Dietzia natronolimnaea ASO3

Introduction

Arsenic has been known as the 'silent toxin' since ancient time and contamination of drinking water resources by geogenic and anthropogenic arsenic were described in 105 countries and their territory with over 226 million people at risk[1]. Arsenic occurs naturally in sedimentary and hard rock aquifers, which are in many developing countries used as source for drinking water. The natural presence of arsenic in groundwater has become a worldwide public-health problem and a natural disaster. With Nepal arsenic standard of 50µg/l and 10µg/l by World Health Organization (WHO) for drinking water, Rautahat district (study site) is categorized as a high risk districts to be looked after for the Arsenic problem where the arsenic

concentration was substantially above the safe limit. The prevalence of arsenicosis was also high around 10% among middle aged people[2]. As a consequence of the biggest calamity in the world, which was detected more than twenty years back in West Bengal, India; Bangladesh and other parts of Southeast Asia including Nepal, there has been an exponential rise in scientific interest towards its release mechanism, mobilization and removal. Microbial activity has been of concern in arsenic mobilization from sediments. Living cells especially microbes carry out redox chemisty and are important players in the arsenic geocycle[3]. Arsenic is released from sediments through the coupling of microbial decomposition of organic matter and reductive dissolution of arsenic-bearing iron minerals. Direct microbial reduction of arsenate to the more mobile arsenite is known for bacterial, algal, and fungal species[4,5]. This study presents findings of isolation and characterization of *Dietzia natronolimnaea* ASO3 from arsenic enriched water sources of Rautahat, Nepal for its potential to arsenic resistance and removal.

Methods

Isolation of arsenic resistant microorganisms

Four water samples 100 ml each were collected randomly from groundwater as well as surface water located at Toribari Jungle (groundwater), Toribari non-Jungle (groundwater), Bagahi village (groundwater), Mardhar village (riverwater) in Rautahat District of Nepal. Water samples were handled aseptically in sterile sampling bottles and brought to Microbiology laboratory at Kathmandu University stored in ice box. Isolation of the arsenic resistant microorganisms was carried out by standard pour plate method[6].

Identification and characterization of the bacterial isolate

One of the potent strains that showed growth in 160 mg/l of arsenate was selected for further characterization. Arsenic resistant bacterium obtained was initially characterized in terms of colony morphology (color, shape, size, elevation, margin, consistency, opacity) and basic microscopic observations (Gram stain, spore stain, dimensions). The isolate was analyzed for different biochemical tests with the help of Hi media test kits. These tests were used to identify the isolates referring to the Bergey's Manual of systematic bacteriology, Determinative Bacteriology and then further confirmed by 16S rDNA sequence analysis[7].

16S rDNA sequence analysis

16S rDNA sequence determination

A colony PCR method was used for amplification of 16S rDNA. A single colony of bacterial isolate was suspended into 50µl sterile distilled water. Boiled for 15 mins at 95˚ C and centrifuged at maximum speed in microcentrifuge for 10 minutes (min) to pellet cell debris. 5 µl of the supernatant was used as DNA templates for PCR. Bacterial 16S rDNA was amplified from the extracted genomic DNA by using the universal bacterial 16S rDNA primers Bact8F forward primer (5'-AGA GTT TGA TCC TGG CTCAG-3') and Bact1492R reverse primer (5'-GGT TACC TTG TTACGA CTT-3'). PCR was performed with a 25 µl reaction mixture containing 5 µl of DNA extract as the template, 100nm of primers Bact 8F and Bact1492R, 0.2 mM of dNTPs and 1 U of Taq polymerase with its supplied 10X buffer (Fermentas, Hanover, Germany). The thermal cycling was performed in MJ minicycler (MJ research, PTC 100, USA). It consists of an initial 95°C denaturation for 3 min followed by 30 cycles of 95°C for 3 sec, 55°C for 1 min, 72°C for 2 min, followed by a final extension at 72°C for 6 min. PCR products were analysed by electrophoresis in 1.5% (w/v) agarose gel in 1x TAE buffer with ethidiumbromide (0.5 µg/ml) before being subjected to further analysis. The amplification products were purified with spin column (Centricon 100 columns, Amicon) and send for DNA sequencing. The DNA sequencing was carried out in Gene way Research, USA.

Nucleotide sequencing, alignment, and phylogeny

Sequences were matched with previously published bacterial 16S rDNA sequences in the NCBI databases using BLASTN. Based on the scoring index the most similar sequences were aligned with the sequences of other representative bacterial 16S rDNA regions by using ClustalX 2 software.

Determination of Minimum Inhibitory concentration

Minimum inhibitory concentration (MIC) with As was evaluated by growing the bacterium in nutrient medium supplemented with the respective arsenic salt concentration i.e. As (III) 0 –1,498.4 mg/l; As(V) 0 – 37,460 mg/l for 96 hrs at 30˚C.

Determination of optimal growth conditions

The optimal growth conditions with reference to pH, temperature and salinity were determined as described previously in our published paper[6]. The pH range of 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5; temperature range of 25°C, 30°C, 45°C and 55°C; salinity concentrations NaCl of 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% were used for determination of optimal growth.

Growth kinetics of *Dietzia natronolimnaea* ASO3

The bacterium was grown at 30 °C in nutrient broth medium with continuous shaking at 150 rpm in the orbital shaker in the presence and absence of 74.92 mg/l arsenite and 200 mg/l arsenate. Optical density was measured after different time intervals at 600 nm using UV-visible spectrophotometer. The growth rate constant (k) for the log phase of growth was determined by plotting the log10 of the optical density against time.

Screening for as (III) oxidation and as (V) reduction Activity

The isolate was tested for the abilities to oxidize As (III) ($NaAsO_2$) or reduce As (V) ($Na_3AsO_4.7H_2O$) using a qualitative KMnO4 screening method[8]. The arsenic resistant bacterium was cultured to nutrient broth medium containing 1mM either $NaAsO_2$ or $Na_3AsO_4.7H_2O$. After forty eight hours of incubation 1 ml of the culture was pipette out in sterile eppendorf in triplicate. The cell pellet was separated by centrifugation at 5000g for 15 mins. The pellet was washed twice in distilled water and suspended in 20 µl of sterile distilled water and 80 µl of sterile Tris HCl buffer pH 7.4. 0.1 µl of 1M sodium arsenite or arsenate was added to bring the final concentration of 1 mM and incubated at 30 °C for 48 hrs. Then, 20 µl of 0.01 M KMnO4 was added to the culture. A pink colour of the mixture indicate positive arsenite oxidation reaction [formation of As (V)], and a yellow colour indicate positive arsenate reduction reaction [formation of As (III)].

Screening for the arsenic resistant gene location using plasmid curing technique

Curing experiments was carried out using acridine orange. 1% inoculum (v/v) of bacteria from 48 h culture is added to sterile nutrient broth containing 10 µg/ml acridine orange and 0.1 M Tris then, incubated at 30ºC for 24 h. The curing was screened by sub-culturing in the presence and absence of arsenic amended nutrient agar plate. The plasmid curing was confirmed by plasmid isolation before and after acridine orange treatment. The absence of plasmid and failure to grow in the presence of arsenic after curing was confirmed for plasmid mediated resistance. The test was performed in triplicate.

Sensitivity to antibiotics

Antibiotic sensitivity of the arsenic resistant bacteria was determined by the standard disc-agar diffusion method. The antibiotic used were ampicillin (10 µg), penicillin (6.25 µg), tetracycline (30 µg), chloramphenicol (30 µg), cotrimoxazole (10 µg), and gentamycin (10 µg).

Arsenic removal by Dietzia natronolimnaea ASO3

Dietzia natronolimnaea sp ASO3 was cultured in 250 ml conical flasks containing 50 ml of nutrient broth medium amended with arsenic salt at the concentration of 74.92 mg/l arsenite or 200mg/l arsenate in water bath shaker (150 rpm) at 30° C for 72 h. At selected intervals of time, biomass was harvested by centrifugation at 5,000 rev/min for 10 minutes. The supernatants were collected and stored at –20° C for arsenic analysis. The arsenic was analyzed by an Atomic Absorption Spectrophotometer (Thermoelectron, UK).

Results

Isolation and Identification of arsenic resistant strain

A bacterial strain showing growth up to 160 mg/l with distinct pinkish orange colony was selected as one of the potent strains for the investigation. The preliminary characterization was based on morphological and biochemical properties. The isolate was aerobic, Gram-positive, non-motile cocci without endospore measuring approximately 0.9–1.2 µm. Colonies on nutrient agar are pinkish orange pigmented, circular, raised, smooth, convex and 1–2 mm in diameter. The putative type strains of *Dietzia* species was distinguished using a combination of phenotypic characteristics (Table 1) and 16S rDNA sequence analysis indicated that the isolate belongs to the genus *Dietzia* with nearest species *Dietzia natronolimnaea* with maximum identity of 99%.

Table 1: Phenotypic properties of the putative type strains *Dietzia natronolimnaea* ASO3

Tests	Result	Tests	Result	Tests	Result
Gram's stain	+	Arginine utilization	+	Maltose	+
Spore staining	-	Lysine utilization	+	Mannitol	+
Catalase	+	Ornithine utilization	+	Melezitose	-
Oxidase	-	ONPG	+	Melibiose	-
Oxidative/Fermentative	O/F	Oxidase	-	Mannose	-
Indole	-	Phenylalanine Deamination	-	Methyl-D glucoside	-
Methyl red	-	Esculin hydrolysis	-	Methyl-D mannoside	-
Voges Proskauer's	+	Arabinose	-	Raffinose	+
Citrate utilization	+	Adonitol	-	Ribose	-
Triple Sugar Iron	R/Y	Dextrose	+	Rhamnose	+
Nitrate reduction	-	Dulcitol	-	Saccharose	
Urease	-	Fructose	+	Sodium gluconate	-
H2S production	-	Glucosamine	-	Sucrose	+
Trybutyrin Test	+	Glucose	+	Salicin	-
Motility Test	-	Glycerol	-	Sorbitol	-
Starch hydrolysis	-	Galactose	+	sorbose	-
Gelatin liquefaction	-	Inositol	-	Trehalose	+
Casein hydrolysis	-	Inulin	-	Xylitol	-
Malonate utilization	+	Lactose	-	Xylose	-
Alkaline phosphatase	+	Dextrose	+		

Note: +, positive; -, negative; O/F, oxidative/fermentative; R/Y, red/yellow

Determination of Maximum tolerance concentration (MTC)

The tested bacterium *Dietzia natronolimnaea* ASO3 showed resistant to both inorganic arsenics; arsenate as well as arsenite. The bacterium tolerated arsenate at concentrations up to 37,460 mg/l [37,460 mg/l As, approaching the solubility limit of As (V) in nutrient broth medium]. Similarly, *Dietzia natronolimnaea* ASO3 exhibited tolerance upto 374.6 mg/l of arsenite.

Determination of optimal growth conditions

The strain showed growth in the range of 25–45 °C with optimal growth at 30 °C (Fig 1), pH 6 – 9 with optimal growth at pH 8 (Fig 2) and tolerated up to 9% of sodium chloride (NaCl).

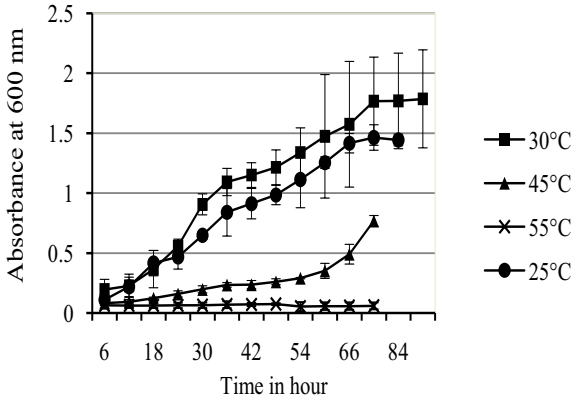

Figure 1: Growth of arsenic resistant Dietzia natronolimnaea ASO3 in nutrient broth at corresponding temperatures.

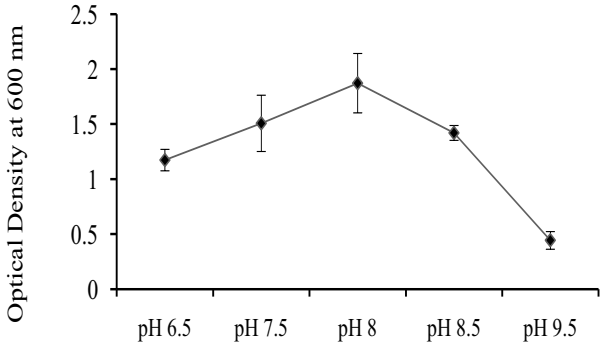

Figure 2: Growth of arsenic resistant Dietzia natronolimnaea ASO3 in nutrient broth after 48 hours of incubation at 30°C and corresponding pH.

Determination of growth kinetics

Growth comparisons of the cells grown in arsenic free media and arsenic containing media revealed an approximately twofold decrease in growth following arsenic treatment as compared to the cells grown in arsenic free media. The growth rate calculated in the absence of arsenic was 0.25 h^{-1} with a doubling time of 2.7 h, in the presence of arsenate it was 0.096 h^{-1} with a doubling time of 5.01 h, and in the presence of arsenite it was 0.15 h^{-1} with a doubling time of 4.76 h, resulting in 38% and 60% reductions in the cellular growth of the bacterial isolate by arsenate and arsenite, respectively (Fig. 3).

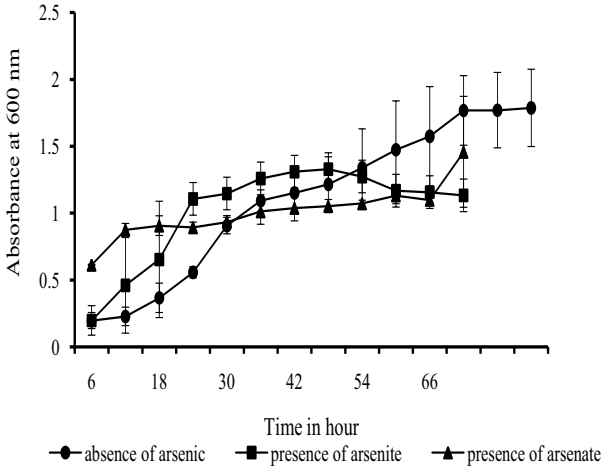

Figure 3: Growth of Dietzia natronolimnaea ASO3 in the absence of arsenic, in the presence of 200 mg/l arsenate and 75 mg/l arsenite respectively.

The pH of the extracellular medium was found to increase gradually from 7.5 to 8.3 from the lag to the stationary phase in absence of arsenic while it increased gradually from 6.6 to 8.6 with arsenate and 7.5 to 8.9 in presence of arsenite (Fig. 4A, 4B and 4C). The change in optical density versus extracellular pH over 72 h is shown in the figures.

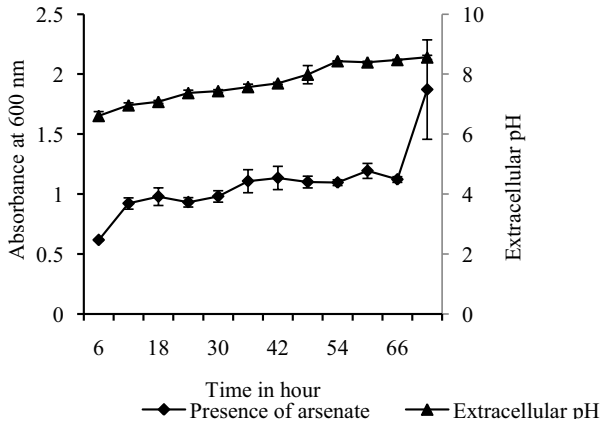

Figure 4A: Growth of Dietzia natronolimnaea ASO3 in absence of arsenic

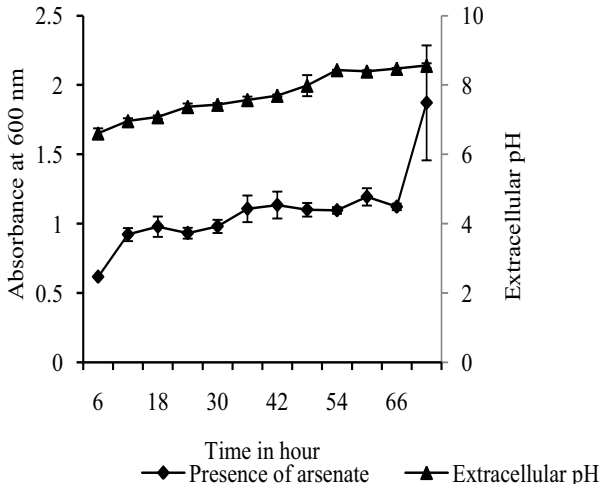

Figure 4B: Growth of Dietzia natronolimnaea ASO3 presence of 200 mg/l arsenate

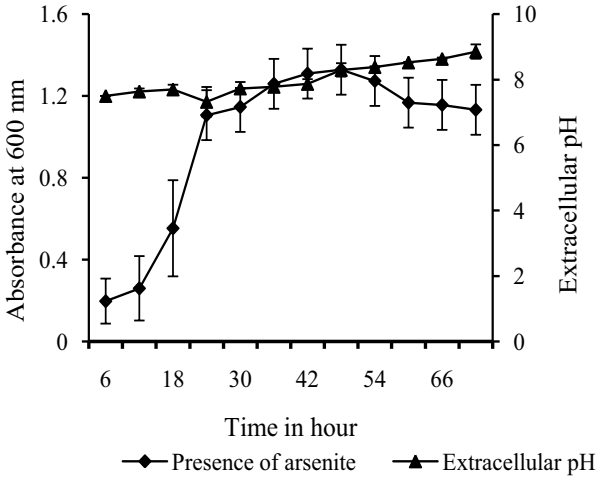

Figure 4C: Growth of Dietzia natronolimnaea ASO3 presence of 75 mg/l arsenite

Screening for Arsenite Oxidation and Arsenate Reduction Activity

In KMnO4 screening method, the putative *Dietzia natronolimnaea* ASO3 showed positive result for arsenate reduction indicating that the bacteria can influence the arsenic speciation and mobilization.

Screening for the Arsenic Resistant Gene Location using Plasmid Curing Technique

Acridine orange successfully cured the plasmid in the bacteria Dietzia natronolimnaea ASO3. Inability of the bacteria to grow in the presence of arsenic after plasmid curing is indicative of plasmid mediated arsenic resistance in Dietzia natronolimnaea ASO3.

Sensitivity to Antibiotics

The *Dietzia natronolimnaea* ASO3 was found to be sensitive to all the antibiotics tested except penicillin (Table 2).

Table 2: Antibiotic resistance profile

Antibiotic	Concentration (µg)	Growth inhibition zone (mm)
Ampicillin	10.00	45.5
Penicillin	6.25	3
Chloramphenicol	30.00	37
Cotrimoxazole	23.75	42.5
Tetracycline	30.00	32.5
Gentamycin	10.00	31.5
Novobiocin	30.00	30

Arsenic removal by Dietzia natronolimnaea ASO3

The bacterium *Dietzia natronolimnaea* ASO3 successfully removed the arsenic in both the forms as arsenate as well as arsenite. Within forty eight hours of incubation the bacteria removed approximately 51% of arsenate (1.22 mg As) and 47% of arsenite (0.44 mg As) from the medium (Fig 5A and B).

Fig 5A

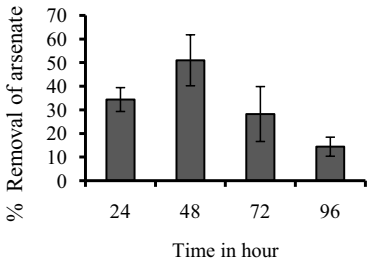

Fig 5B

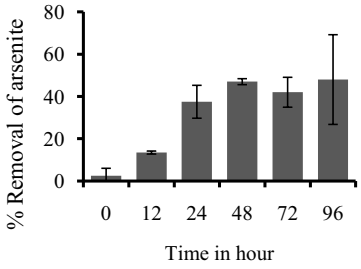

Figure 5A and B: Arsenic removal by Dietzia natronolimnaea ASO3 at corresponding time respectively

Discussion

Dietzia has only been established fairly recently. The Gram morphology and colony appearance of the species of the genus Dietzia are remarkably similar to Rhodococcus equi, the most frequently encountered actinomycetes by medical microbiologists in their daily routine practice.

Dietziae are aerobic, Gram-positive, nonsporing, catalase-positive actinomycetes that form cocci that germinate into short rods or rod-shaped cells, which exhibit snapping division and produce V-shaped forms. Circular, raised or convex, glistening, orange to coral red colonies with entire edges are formed on agar media. The genus encompasses six validly described species, namely, Dietzia maris, and the type species, Dietzia cinnamea, Dietzia kunjamensis, Dietzia natronolimnaea, Dietzia papillomatosis and Dietzia psychralcaliphila.[9] Dietzia natronolimnaea was proposed to accommodate two strains that had been isolated from an East African Soda Lake located in the Kenyan Tanzanian Rift Valley[10]. Additional alkaliphilic Dietzia strains have been isolated from a lake in Hungary; from alkaline ground water.[11,12]

Dietzia spp. may be easily missed in routine diagnostic laboratories as they have morphological properties similar to other actinomycetes, notably rhodococci. It is likely, therefore, that some Dietzia strains will be misidentified as Rhodococcus spp. Furthermore, dietziae grow relatively slowly, may require incubation at temperatures <37º C, and are hence likely to be missed if culture plates are not incubated for at least 48–72 h at an appropriate temperature.[9] The members of Dietzia spp. have been isolated from clinical specimens however out of the six validly described Dietzia spp., namely D. cinnamea, D. maris and D. papillomatosis, have been isolated from specimens taken from patients with acute infections, whereas the remaining three species have only been isolated from the environment.[9]

Microbial resistance to arsenic species is widespread in nature but resistance to concentration of As (V) higher than 7,492mg/l is considered as very high[13]. The tolerance reaching 22,476–37,460mg/l of As (V) is considered as hyper- tolerance. The bacterial strain described here show much higher resistance to As (V) than those isolated from gold mines[14,15,16] and other arsenic-tolerant microorganisms.[16,17,18] Such high resistance to As (V) has been described in the biotechnologically important bacterium Corynebacterium glutamicum, which is one of the most resistant microorganisms described. [19] High resistance to arsenate (>7,492mg/l) was also found in bacteria isolated from the Lake Pontchartrain estuary in Louisiana, USA.[13] Arsenic hypertolerant cultivable bacterial strains from ancient gold mine in Poland that exhibited hypertolerance to arsenic up to 37,460mg/l of arsenate and 1,124mg/l of arsenite.[21]

Many studies have been carried out with regard to arsenic detoxification, oxidation-reduction and methylation [4,8,13,14,15,16,20,22] but investigation with regards to *Dietzia* species has not been recorded yet.

In addition, no comprehensive investigation has been carried out on the susceptibility of representatives of *Dietzia* species to antibiotics. *Dietzia maris* has been reported to be susceptible to aztreonam, ciprofloxacin, mezlocillin, oxacillin, penicillin G, perfloxacin and ticarcillin, and resistant to sulphamethoxazole by disc diffusion testing.[23]

Conclusion

The presence of arsenic in water is frequently reported and arsenite is more mobile, highly soluble and more toxic than arsenate. The ability of the bacterium to grow over wide range of arsenic concentration as arsenate as well as arsenite, removal of the metalloid and arsenate reduction potential may offer advantage for bioremediation as well as in describing the phenomenon of arsenic mobilization.

Acknowledgments

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Conflict of interest: None declared

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