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Original Research Paper

Characterization of Arsenic Resistant Bacteria from Shallow Tubewell and Evaluation of their Remediation Capacity

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ABSTRACT

The present study was conducted to characterize arsenic resistant bacteria and to evaluate their arsenic remediation capacity. Water samples were collected from the shallow tubewell of Deganga, West Bengal. The arsenic content of shallow tubewell water was 45.07 µg/L. 16S rDNA analysis characterized the isolated arsenic resistant bacteria from the shallow tubewell water to be under the genus *Pseudomonas* and *Acinetobacter*. Minimum inhibition concentration (MIC) of arsenic for the selected bacterial isolates *Pseudomonas* sp. and *Acinetobacter* sp. was found to be 7 and 17.5 m*M* As(III), respectively. The selected bacterial isolates were capable of removing arsenic in the range of 1.54-5.95% from the nutrient broth supplemented with 25, 50, 75 and 100 mg/L As(III). Analysis of variance (ANOVA) indicated a significant difference ($P<0.05$) in arsenic removal between the arsenic concentrations, but there was no significant difference ($P>0.05$) between the selected bacterial isolates. The selected bacterial isolates could thus be useful for developing a technology for biological removal of arsenic by standardizing certain parameters for the optimum removal of arsenic by the selected bacterial isolates.

INTRODUCTION

Arsenic is a metalloid which is mostly found in the environment as the oxidised pentavalent form, arsenate [As(V)] and reduced trivalent form, arsenite [As(III)]. Arsenic is one of the widely distributed elements in the environment (Smedley & Kinniburgh 2002). Arsenic is toxic to humans and many other living organisms; it causes many health related problems in many parts of the world (Wang et al. 2001, Mohan & Pittman 2007). Arsenic pollution is a global issue due to the prevalence of arsenic toxicity in groundwater in many parts of the world, with the risk of harmful human exposure through drinking water (Smedley & Kinniburgh 2002, Lindberg et al. 2006). Because of its toxic effects, WHO has set a provisional guideline with the permissible level of 10 µg/L for arsenic in drinking water (WHO 2011).

Various methods and techniques are available for the treatment of metal bearing pollutants, which can usually be divided into two broad divisions: abiotic and biotic methods. Abiotic methods for treating metal bearing pollutants have many drawbacks, which can be summarized as expensive, not environment friendly and usually dependent on the concentration of the pollutants (Crini 2006). Hence, the search for efficient, eco-friendly and cost-effective remedies for metal bearing pollutant treatment has been very important. In recent years, special attention has been given by many researchers on biological methods for the treatment of waste water, some of which are in the process of commercialization (Prasad & Freitas 2003).

The arsenic resistant bacteria are common and widespread as many arsenic resistant bacteria were isolated from different environmental conditions like hot springs, soil, mine, tubewell water, sea sediment and lakes (Santini et al. 2000, Jackson et al. 2005a, Kulp et al. 2006, Hetzer et al. 2007, Chen & Shao 2009, Sutton et al. 2009). Several bacteria have various kinds of mechanisms to resist the effects of arsenic toxicity. Many bacteria have the capacity to use either the oxidized form of inorganic arsenic [As(V)] or the reduced form of arsenic [As(III)] in their metabolism, and several bacteria have the capacity of resisting arsenic toxicity through the *ars* genetic system (Stolz & Oremland 1999, Santini et al. 2000, Jackson & Dugas 2003). Bacteria and many other microbes accumulate metal through several processes such as biosorption onto cell wall, trapping in extracellular capsule, oxidation-reduction reaction and transportation across the cell membrane (Malik 2004). Metal accumulating bacteria are frequently observed among metal resistant bacteria (Srinath et al. 2002, Hussein et al. 2005, Takeuchi et al. 2007). Many arsenic resistant bacteria were observed to be capable of removing arsenic (Patel et al. 2007, Takeuchi et al. 2007, Aksornchu 2008, Chen & Shao 2009). Arsenic resistant bacteria could thus be useful for arsenic removal and could be used as an alternative or to supplement existing methods of arsenic remediation (Takeuchi et al. 2007, Chen & Shao

2009). Hence, the present study focused to isolate and characterize arsenic resistant bacteria from shallow tubewell and to evaluate arsenic remediation capacity of the selected bacterial isolates.

MATERIALS AND METHODS

Collection of water sample and analysis of arsenic: Water samples were collected from shallow tubewell of Deganga, West Bengal, India. The arsenic content in the sample water was analysed by hydride generation method using Flow Injection Analysis System, Atomic Absorption Spectrometer, A-Analyst 800 (Perkin Elmer, USA).

Isolation and characterization of arsenic resistant bacteria: The water samples were serially diluted with normal saline water and plated on nutrient agar (Hi-Media Laboratories India) supplemented with sodium(meta)arsenite ($NaAsO_2$) (Hi-Media, Laboratories, India) to have the arsenic concentration of 2 m*M* As(III) by spread plate method and were incubated for 48 hours at 30°C. Arsenic resistant bacteria were isolated by picking up the distinct bacterial colony from the nutrient agar plate containing 2m*M* As(III). Confirmation of the arsenic resistant bacteria was done by inoculating the selected bacterial isolates into the nutrient broth (Hi-Media Laboratories, India) with 2 m*M* As(III) and incubated at 30°C in the shaking incubator at 130 rpm for 3 days. The selected bacterial isolates were subcultured continuously until pure cultures were obtained.

Genomic DNA from the selected bacterial isolates were extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany) by following the manufacturer's instructions. 16S rDNA of the bacterial isolates were amplified by using primers, 27F (AGAGTTTGATCCTGG) and 1492R (GGTTACCTGTTACGACTT). The PCR reaction was carried out in a thermocycler (Quanta Biotech U.K) of thirty five amplification cycles by following the manufacturer's instructions. Amplified 16S rDNA from each isolate was screened for differences using individual digests with restriction enzymes RsaI, HhaI and TaqI (Fermentas, Lithuania). Isolates that showed differences with regard to restriction enzyme patterns were considered to be individual isolates and isolates that were identical to other isolates based on the restriction enzyme patterns were grouped as one. The PCR products were sequenced through Bangalore Genei Pvt. Ltd., Bangalore, India. The 16S rDNA sequence data were searched against Genbank database (http://www.ncbi. nlm.nih.gov/) by using the BLAST algorithm.

Evaluation of arsenic remediation capacity of the selected bacterial isolates: Evaluation of the arsenic remediation capacity of the selected bacteria was done by comparing the arsenic remaining in the nutrient medium inoculated with bacterial isolates and the arsenic remaining in the nutrient medium without bacterial isolates after 48 hours of incubation.

A loopfull of the selected bacterial isolates were inoculated in 20 mL of nutrient broth (Hi-Media Laboratories, India) and kept in the shaking incubator at 30°C for 48 hours at 130 rpm. Nutrient broth was prepared which was supplemented with $NaAsO_2$ to have a different concentration of 25, 50, 75 and 100 mg/L of As(III). In each case filter sterilization was done by using membrane filter (Millipore, France). 0.5 mL of each nutrient broth with the bacterial growth was inoculated to 20 mL of nutrient broth containing 25, 50, 75 and 100 mg/L As(III). For each concentration of arsenic, control was maintained separately without inoculating the bacterial culture. pH was measured in each case by pH meter (Eutech, Malaysia) and the experiment was carried out in the shaking incubator at 30°C at 130 rpm for 48 hours. The experiment was carried out in triplicate. After 48 hours of incubation, each of the nutrient broth with the bacterial growth was centrifuged for 15 minutes at 15°C in the refrigerated centrifuge. The supernatant was taken for the estimation of arsenic remaining in the nutrient broth. Arsenic remaining in the supernatant was analysed by Atomic Absorption Spectrometer. The arsenic removal was calculated by subtracting the amount of arsenic remaining in the nutrient broth with the bacterial growth from the amount of arsenic remaining in the control nutrient broth. Analysis of variance (ANOVA) and cluster analysis was performed using software package SPSS 16 and PAST 2.17c for the comparative analysis of arsenic removal between the arsenic concentrations and between the selected bacterial isolates.

Minimum inhibitory concentration (MIC): MIC of arsenic for the selected bacterial isolates was conducted by incorporating $NaAsO_2$ to the nutrient broth to have a different concentrations from 5 to 25 m*M* As(III). The bacterial isolates were inoculated into 5 mL of nutrient broth containing different concentrations of arsenic and incubated in the shaking incubator for five days at 30°C at a speed of 130 rpm. The lowest concentration of arsenic at which the bacterial growth was inhibited was considered as the MIC. Turbidity in the nutrient medium was considered as growth.

RESULTS AND DISCUSSION

Analysis of the water sample: The pH of the water samples from the shallow tubewell was found to be 7.4. The arsenic content of water samples was found to be 45.07 µg/L. The present study clearly indicated that the shallow tubewell water was contaminated with arsenic as the ar-

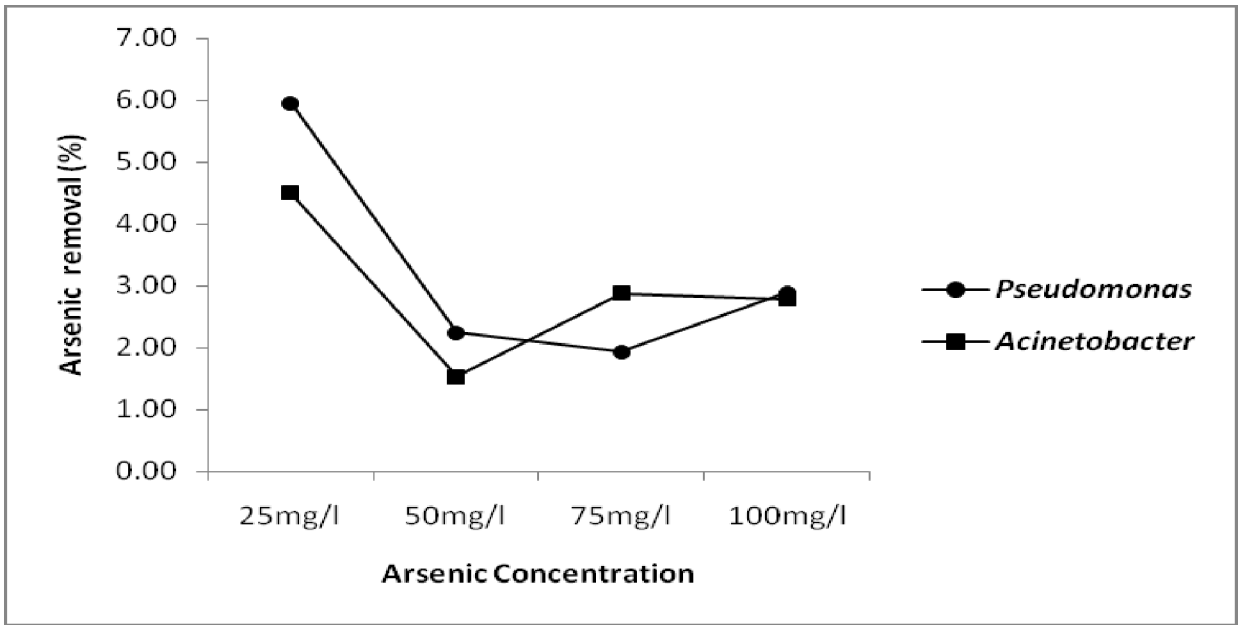

Fig. 1: Arsenic removal by the selected bacterial isolates at different arsenic concentrations.

senic content was above the permissible limit (10 µg/L) of arsenic in drinking water given by WHO. Arsenic pollution in tubewell is prevalent in West Bengal, a 20 years study since 1998 revealed the prevalence of tubewell waters arsenic pollution (> 10 µg/L) in 19 districts of West Bengal (Chakraborti et al. 2009).

Isolation and characterization of arsenic resistant bacteria: Three distinct bacterial colonies were picked up from the shallow tubewell water and named as ARS-1, ARS-2 and ARS-3. The three bacterial isolates were inoculated in to the nutrient broth with the arsenic concentration of 2 m*M* As(III) to confirm that the bacterial isolates were resistant to arsenic. All the three bacterial isolates were able to survive in the nutrient broth with the arsenic concentration of 2 m*M* As(III).

The water samples from shallow tubewell contained significant numbers of culturable arsenic resistant bacteria, which clearly indicated that arsenic resistant bacteria are common and widespread. Many researchers also observed arsenic resistant bacteria from different environments (Santini et al. 2000, Jackson et al. 2005a, Jackson et al. 2005b, Kulp et al. 2006, Hetzer et al. 2007, Chen & Shao 2009, Sutton et al. 2009).

Determination of arsenic resistance by growing on solid medium alone may not give a true picture of arsenic resistant bacteria as it is possible that bacteria grown on solid medium may not be fully exposed to arsenic because of colonial growth. Some bacterial isolates were observed to show very low arsenic resistance in liquid medium, despite growing well on arsenate supplemented R2A plates (Jackson et al. 2005b). In the present study, the arsenic resistant bacteria obtained from the solid medium were confirmed by growing the isolates in the As(III) amended nutrient broth for 3 days. All the bacterial isolates were able to grow in liquid medium, which clearly confirmed that the bacterial isolates were resistant to arsenic.

Restriction enzymes digestion of the 16S rDNA of the three bacterial isolates with the three restriction enzymes *Rsa*I, *Hha*I and *Taq*I suggested that some of the isolates were likely the same taxa. The restriction pattern of the 16S rDNA of the three bacterial isolates using the three restriction enzymes *Rsa*I, *Hha*I and *Taq*I suggested that the isolates ARS-1 and ARS-3 were the same taxa and the isolate ARS-2 was assigned as a distinct bacterial isolate. The bacterial isolates ARS-1 and ARS-2 were selected for sequencing and the 16S rDNA sequence data of the isolates ARS-1 and ARS-2 were searched against Genbank database by using the BLAST algorithm.

The BLAST analysis of the bacterial sequences data against database of 16S rDNA assigned the isolates ARS-1 and ARS-2 from shallow tubewell water to be under the genera *Pseudomonas* and *Acinetobacter*. Some researchers

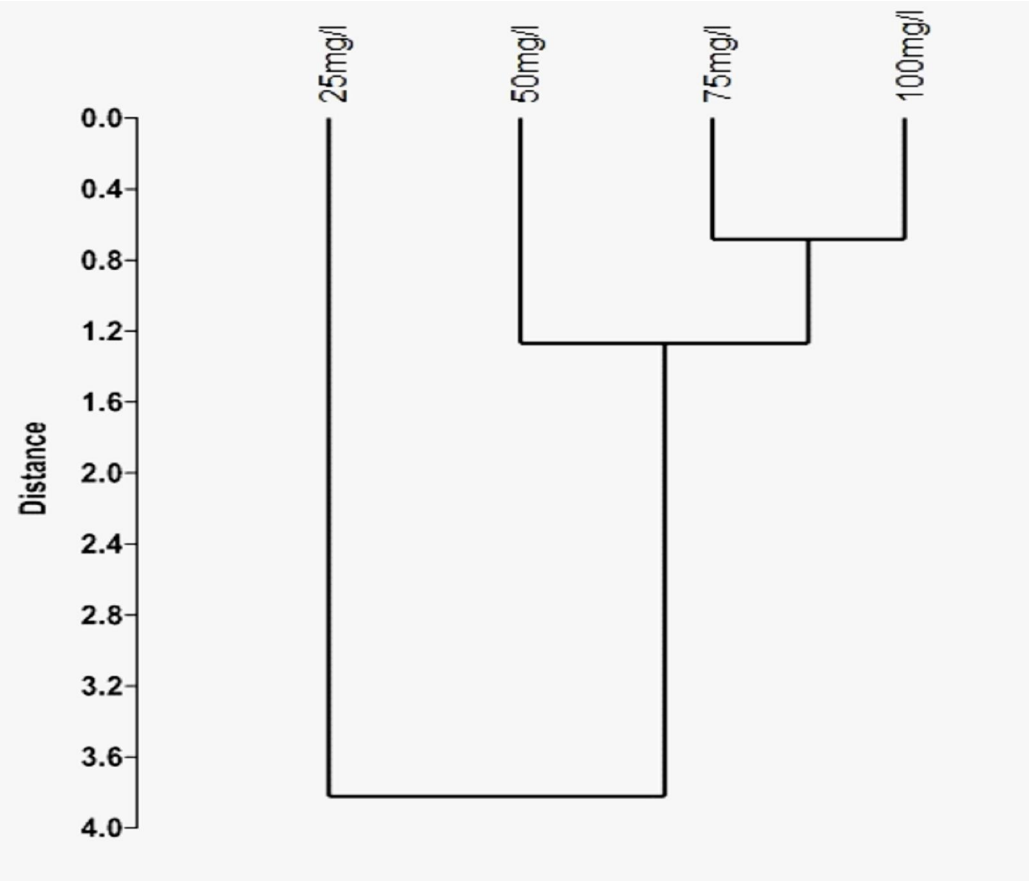

Fig. 2: Cluster analysis of arsenic removal at different arsenic concentrations.

have isolated and characterized the genera *Pseudomonas* and *Acinetobacter* as the arsenic resistant bacteria. *Pseudomonas putida* was isolated and characterized as arsenic resistant bacteria from the deep sea sediments of the Southwest Indian Ocean (Chen & Shao 2009) and *Acinetobacter* sp. was also isolated and characterized as arsenic resistant bacteria from shallow tubewell in Bangladesh (Sutton et al. 2009).

Minimum inhibitory concentration (MIC): The bacterial isolates in the present study were capable of tolerating high level of arsenic with the minimum inhibitory concentration of 7.5 and 17.5 m*M* As(III) by the bacterial isolates *Pseudomonas* sp. and *Acinetobacter* sp., respectively. The arsenic tolerance limits of certain bacteria observed by some of the researchers were lower than the present study. Jackson et al. (2005a) observed that most of the arsenic resistant bacteria isolated from the soil and leaf litter were not able to grow above 5 m*M* As(III), only two isolates were able to grow above 5m*M* As(III). Jackson et al. (2005b) also observed that most the bacterial strains isolated from the estuary were not able to grow above 5 m*M* As(III), only one isolate was able to grow in 10 m*M* As(III). Escalante et al. (2009) observed that MIC of some of the isolated bacteria was up to 40 m*M* As(III) from the arsenic contaminated river, which was higher than the present study. Some researchers observed higher MIC of arsenic than the present observation, which might be due to the solid medium used for the growth of bacteria while carrying out MIC test.

Arsenic removal by the selected bacteria: The mean percentage of arsenic removal ranged from 1.54-5.95% from the initial concentration of 25, 50, 75 and 100 mg/L As(III) in nutrient broth by the two selected bacterial isolates as shown in Fig. 1. The pH of nutrient broth with 25, 50, 75 and 100 mg/L As(III) was found to be 7.2, 7.2, 7.4 and 7.5, respectively. Analysis of variance (ANOVA) indicated a significant difference ($P<0.05$) in arsenic removal between the arsenic concentrations, but there was no significant differ-

ence (P>0.05) between the selected isolates in removal of arsenic. The significant difference of arsenic removal based on the initial arsenic concentrations might be due to the variation of the metabolic behaviour of the selected bacterial isolates in different concentrations of arsenic. No significant difference was observed between the bacterial isolates for the removal of arsenic, which might be due to the same behaviour with the toxicity of arsenic and the same mechanism of arsenic removal by the selected bacterial isolates. The Tukey Post Hoc tests and the cluster analysis (Fig. 2) using Ward's method indicated that the arsenic removal by the bacterial isolates at the concentration 25 mg/L was significantly different from the other concentrations used in the arsenic bioremediation. The removal of arsenic was comparatively higher at the arsenic concentration of 25 mg/L As(III) as compared to the other arsenic concentration, which might be due to the lesser toxic effect of arsenic and the better growth of the selected bacterial isolates in the lower arsenic concentration.

In accordance with the present studies, Takeuchi et al. (2007) observed that the percentage of arsenate removal by *Marinomonas communis* at the end of the experiment (after all the cultures reached a stationary phase) was in the range of 3.5-15.5% when the concentrations of added arsenic were 0.07, 5, 50 and 250 mg/L As(V). As compared to some other studies, the present study showed less amount of arsenic removal. Patel et al. (2007) observed that approximately 22% of the 5 m*M* As(V) supplied to aerobic cultures of *Pseudomonas* sp. strain As-1 was removed. Aksornchu (2008) also observed that the isolated bacteria from the arsenic contaminated soil were capable to remediate arsenic in the range of 36.87-96.93% from the initial concentration of 40 m*M* As(V), without any arsenic transforming activity. The lesser amount of arsenic removal in the present study than some of the other studies might be due to the initial arsenic concentration, species of arsenic, duration of the experiment, pH, temperature and many other factors.

The bacterial isolates could thus be useful for the development of biological arsenic removal as they could remove certain amount of arsenic from the culture medium. Further studies are needed to optimise the efficiency of arsenic removal by the selected bacterial isolates by finding out important parameters affecting the arsenic removal.

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