



Screening and isolation of arsenic tolerant *Rhizobacteria* from arsenic contaminated areas of Bangladesh

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Abstract

An experiment was carried out to collect and isolate arsenic (As) tolerant rhizobacteria from As contaminated areas of Faridpur district. A total of 32 root samples of 17 plant species were collected from six locations of Bhanga and Nagarkanda upazila. From the plant roots, 16 rhizobacterial isolates were separated and their pure cultures were prepared. The collected rhizobacterial isolates were then characterized for morphological and biochemical traits. Among the bacterial isolates four were gram negative and 12 were gram positive. About 12 bacterial isolates had catalase producing capacity and the remaining three were negative to catalase test. Four bacterial isolates gave the positive HCN test. These bacterial isolates were grown on Pikovskays media to determine their phosphorus (P) solubilizing capacity. Four bacterial isolates had P solubilizing capacity and their Phosphorus Solubilizing Index (PSI) ranged between 3.00 and 4.66. However, isolate FB4 gave the highest P solubilization (4.66 PSI). For the evaluation of As tolerance, nutrient agar medium was prepared with different concentrations of As ranging from 30 to 600 ppm and the bacterial isolates were allowed to grow on the As contaminated medium for 36 hours. Isolate FB9 and FB4 showed maximum tolerance against As upto 600 ppm. Hence, these two isolates, both collected from Bhanga upazila and from wild brinjal species (*Solanum capsicoides*). Further research is needed to assess their effect on As dynamics at the rhizosphere, selection of suitable plant species for bacterial association and their potentials for field applications.

Key words: *Rhizobacteria*, arsenic tolerance, phosphorus solubilization, Bangladesh

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Introduction

Carcinogenic, mutagenic and teratogenic (Plant *et al.*, 2003) arsenic (As) is a major constituent in more than 245 minerals and is ubiquitous in the environment (Mandal and Suzuki, 2002). 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). Both acute and chronic poisoning to humans has raised great concerns, especially in heavily contaminated areas such as Bangladesh and West Bengal, India. The serious health problems in Bangladesh were described as “the greatest mass

poisoning in human history” by World Health Organization (Vaughan, 2006).

Arsenic (As) contamination of soil and drinking water is a serious issue in Bangladesh (Vaughan, 2006). About 80 million people in Bangladesh are exposed to As poisoning through food chain and drinking water contamination. Extensive irrigation with contaminated water and over use of contaminated agrochemicals are the principal reasons of As contamination in food.

Arsenic is stored below the ground. To achieve higher production of the modern HYVs of boro rice, our farmers use underground water in irrigation. This results in contamination of agricultural soil with As which is very significant as it is the major contributing factor for food chain contamination. Again, our rice dominated food habit enhances peoples' exposure to As. Because As accumulation in grain is enhanced by our conventional reduced condition of paddy soils (Spallholz *et al.* 2008).

There are various kind of mitigation process of arsenic from soil, physicochemical and Biological. Phytoremediation and bio-remediation are the example of biological remediation. Nowadays, bio remediation of arsenic from agricultural soil is becoming popular day by day.

Microorganisms play important roles in the biochemical cycle and arsenic-speciation (Liu *et al.* 2005). Bacteria induced arsenic transformation involves oxidation, reduction or methylation to overcome the toxic effects and survive in arsenic-rich environment. Besides interacting with plant roots, bacteria also play an important role in arsenic biogeochemistry in the environment. They affect arsenic reduction and oxidation, methylation and demethylation, and sorption and desorption in soils. As a result, bacteria have developed different detoxification strategies to withstand the growth restriction under arsenic stress (Liu *et al.* 2004). On one hand, bacterial ability to tolerate osmotic and oxidative stress contributes to their arsenic resistance. Metabolism under hyperosmotic conditions or in a low nutrient environment could cross-protect cells from other stresses such as oxidative burst, heavy metal stress, and sodium hypochlorite (Pichereau *et al.* 2000).

Similar to other heavy metals, As causes oxidative stress by inducing reactive oxygen species (ROS). Therefore, it is not surprising to see the correlation between arsenic resistance and hydrogen peroxide (H₂O₂) resistance (Liu *et al.* 2001). On the other hand, arsenic-resistant bacteria usually have a specific

genetic system, which is directly involved in arsenic transformation and sequestration. One example is the reduction–detoxification mechanism, which has been found in bacteria isolated from arsenic-contaminated soils and mine tailings (Jones *et al.* 2000; Macur *et al.* 2001). Several arsenic-resistant bacteria from aquifer sediments of Bengal Basin (Chowdhury *et al.* 2009; Gault *et al.* 2005; Islam *et al.* 2005; Rowland *et al.* 2009; Salam *et al.* 2009) have been documented. However, reports focusing on the As tolerant rhizobacteria from agricultural soils and their potentials for bioremediation is yet limiting. With the increased detection of As from different areas of Bangladesh, the local microbiota is expected to adapt to high concentrations and perhaps even gain the capability of maintaining the arsenic cycle as well. In reference to this, the present study has been aimed to isolate and characterize rhizobacteria collected from arsenic contaminated soils of Bangladesh and evaluate the arsenic tolerance of the rhizobacteria to identify the tolerant bacterial lines.

Materials and Methods

Collection of rhizobacteria: Bhanga and Nagarkanda are the two main As contaminated upazilas under Faridpur district. After consultation with the SRDI regional officer and local NGO personnel and farmers, the specific As contaminated sites were selected in each upazila. From the two Plant and soil samples were collected from arsenic contaminated areas of Faridpur district. The specific sampling locations are presented in Table 1. In order to isolate rhizospheric bacteria, plant samples with their roots were collected from different portions of the field of each As contaminated sampling site. In total 32 plant samples of 16 plant species were collected from the sampling sites presented in Table 2. Immediately after collection, each sample was kept in a labelled air tight plastic zipper bag and stored at 4°C inside an ice box.

Isolation of the Rhizobacteria: Rhizobacteria were isolated from both plant root and soil of the

contaminated sites assuming that they have the capacity to tolerate arsenic stress. The isolation was done using nutrient agar medium. The ingredients were dissolved in deionized water. The pH of the solution was adjusted to 6.5 with 1% NaOH and 1mM HCl before autoclaving. To isolate bacterial isolate from each plant root all plant roots were washed with sterilized distilled water in a test tube. A series of dilution (10^{-1} , 10^{-2} and 10^{-3}) were made to reduce the density of the bacterial population. Each diluted sample was allowed to culture separately on a 9 cm petri dish. In this isolation process a solid nutrient rich agar medium was used and each media was autoclaved at 121°C with 15 psi for 20 minutes before inoculation. After inoculation the samples were spread with the help of a sterile spreader and then incubated in an incubator at 28°C for 2 days.

After 2 days of the incubation of the bacterial isolate morphologically different size and shape bacteria were selected for the further culture with the help of a toothpick and pin pointed sterile needle. Pure cultures of the bacterial isolates were obtained by repeated sub-culture method. In this method, the bacterial isolates were grown repeatedly until a pure culture of a strain is obtained. The pure isolates were maintained on NA media and also kept in 10% glycerol and stored low temperature refrigerator.

Morphological characterization of the bacterial isolates: For the morphological characterization the colony colour, shape and edge shape of the pure cultured bacterial isolate was determined. In order to characterize all the bacterial isolate was placed on the nutrient broth agar medium with help of a loop. Before inoculation all media and petri dishes were autoclaved at 121°C with 15 psi for 20 minutes. After the inoculation all the bacterial isolates were incubated in an incubator for 2 days at 28°C. Two days after incubation all the bacterial colonies were observed with the help of a hand magnifying glass to identify their colony colour, shape and edge shape. The total number of bacterial isolates were stained with crystal violet,

gram iodine, safranin. Then the prepared staining slides were kept under the microscope and captured its photos.

Biochemical characterization of the bacterial isolates: On glass slide a loop full of bacteria from a well grown colony was mixed with a drop of 3% aqueous KOH. Mixing was continued for less than 10 seconds. A toothpick was used for picking bacteria from a colony as well as for mixing it. The toothpick was raised a few centimeters from the glass slide. Strands of viscid material confirmed the bacterium was gram-negative (Ahmed, 2015). Catalase is the enzyme that breaks hydrogen peroxide (H_2O_2) into H_2O and O_2 . Hydrogen peroxide is often used as a topical disinfectant in wounds, and the bubbling that is seen is due to the evolution of O_2 gas. H_2O_2 is a potent oxidizing agent that can wreak havoc in a cell; because of this, any cell that uses O_2 or can live in the presence of O_2 must have a way to get rid of the peroxide. One of those ways is to make catalase. A small amount of bacterial isolate was placed from culture onto a clean microscope slide. A few drops of H_2O_2 were added onto the smear. A positive result is the rapid evolution of O_2 as evidenced by bubbling. A negative result is no bubbles or only a few scattered bubbles. To determine the production of HCN, bacteria were streaked onto NB agar plates supplemented with glycine ($4.4g L^{-1}$). The petri dishes were inverted and piece of filter paper was impregnated with 0.5% picric acid and 2% sodium carbonate was placed on the upper lid. Petri dish was sealed with parafilm and incubated at 28°C for 7 days. Discoloration of filter paper colour from yellow to orange brown was considered to be the indication of HCN production.

Screening of PSB: For screening of the phosphate solubilizing bacteria, the bacterial isolates were grown in Pikovskaya's medium. The ingredients were dissolved in deionized water adjusted to pH 7.0 with 1% NaOH and 1mM HCl before adding agar. Mineral phosphate solubilization activities of isolated bacterial isolates were tested by plate assay. A colony was taken

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from each isolate using sterile toothpicks and inoculating loop and plated three ways onto Pikovskaya's agar medium containing inorganic phosphate, making a groove on the medium and incubated at 25°C for 72 hours. Solubilization of mineral phosphate was characterized by a clear halo around bacterial colony with phosphate solubilization capacity. The halo zone of solubilized P and colony

diameters were measured after 14 days of incubation of plates at 25°C.

Phosphate solubilizing capacity was calculated in terms of PSI ($PSI=A/B$, where A is the total diameter of the halo zone, and B is the colony diameter). The isolates showing $PSI > 2$ have been considered as phosphate solubilizing bacteria. The ratio of total diameter (colony and halo zone) and the colony diameter was measured (Edi, 1996).

Table 1. Sampling locations in Faridpur district for collecting rhizobacteria

Upazila	Union	Village	Latitude	Longitude	No of plant sample
Bhanga, Faridpur	Chandra	Singherdak	23.37991	90.06121	6
		Jibonpur	23.38971	90.07141	7
		Hijolkhali	23.39801	90.08125	5
Nagarkanda, Faridpur	Kaichail	Pach-kaichail	33.59026	89.82580	8
		Alimpur	33.60015	89.84231	6
		Norkona	33.61721	89.85137	5

Table 2. Plant species collected from arsenic contaminated sites of Faridpur district.

Local name	Scientific name
Wheat	<i>Triticum aestivum</i>
Rice	<i>Oryza sativa</i>
Lentil	<i>Lens culinaris</i>
Coriander	<i>Scoparia dulcis</i>
Wild eggplant (<i>tith begun</i>)	<i>Solanum capsicoides</i>
Ground nut	<i>Arachis hypogea</i>
Onion	<i>Allium cepa</i>
Bhat	<i>Clerodendrum viscosum</i>
Shama ghas	<i>Echinochloa colona</i>
Bermuda Grass (<i>Dubra</i>)	<i>Cynodon dactylon</i>
Halud nakful	<i>Acmella uliginosa</i>
Water caltrop (<i>Painal</i>)	<i>Trapa natans</i>
Nunia	<i>Portulaca oleracea</i>
Helencha	<i>Enhydra fluctuans</i>
Badla grass	<i>Achnatherum hymenoides</i>
Pitla grass	<i>Aegilops sp.</i>

Identification of maximum tolerance of the bacterial isolates to As:

The maximum tolerance of the As by the bacterial isolate was measured by growing them on a nutrient agar broth medium which contain As. In order to measuring As tolerance, a series of As solution was prepared using $NaAsO_2$. The concentration of the series of the As ranges from 30-1000 ppm. First a nutrient agar medium was prepared which contained different concentration of As solution in a conical flask. Then the pH of those medium was fixed at 6.5 using 1% NaOH and 1mM HCl. Neck of the conical flask was covered with cotton and brown paper to avoid contamination. Then all the media and petridishes were autoclaved at 121°C with 15 psi for 20 minutes. After autoclaving the about 15mL of the media was poured into a petri dish and left it for few minutes for cooling and solidification of the media. Then pure bacterial isolate was streaked on the media using a sterile toothpick and incubated in an incubator at 28°C for 24 hours. After 24 hours all bacterial isolates were observed properly to identify their growth condition. Only the surviving strains were selected for

the next trail with higher As concentration and the remaining strains were discarded. This procedure was continued until the maximum tolerant limit of As was identified. All the operations after autoclaving and before incubation were carried out inside a biohazard safety cabinet. Deionized water was used for the media preparation.

Results and Discussion

Selection of bacterial strains: Among 25 bacteria, 16 potential bacteria were selected on the basis of their growth performance and observations and were used for the present study. The selected bacteria along with their collection locations are presented in Table 3. The pure culture of the selected bacterial strains were prepared by repeated streak culture method are shown in Figure 1 and 2.

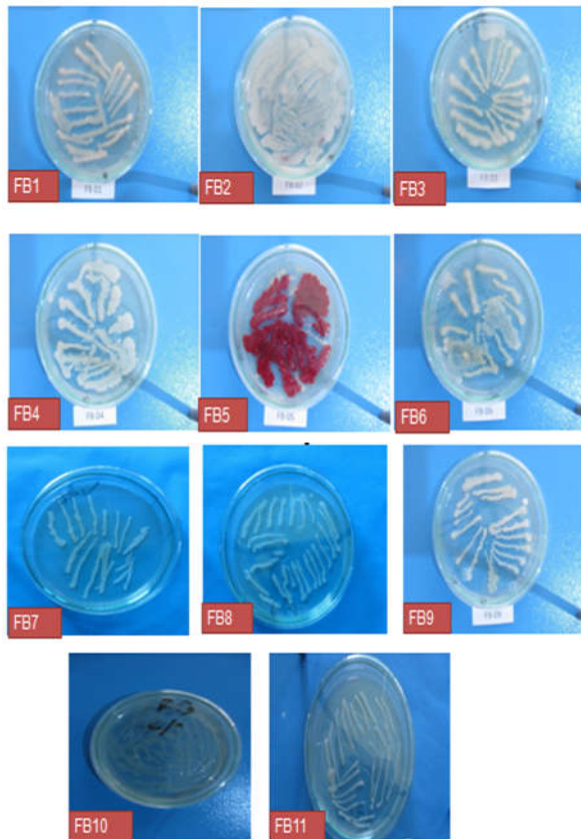


Figure 1. Isolated pure cultures of rhizobacteria collected from Bhanga upazila



Figure 2. Isolated pure cultures of *Rhizobacteria* collected from Nagarkanda upazila

Table 3. Selected bacteria along with their collection location.

Micro organism	Collection location	Bacterial strain
<i>Rhizobacteria</i>	Bhanga	FB1, FB2, FB3, FB4, FB5, FB6, FB7, FB8, FB9, FB10, FB11
	Nagarkanda	FN1, FN2, FN3, FN4, FN5

Morphological characteristics: The morphological characteristics of rhizobacteria isolates were observed in single colony study which was widely varied. All the bacterial isolates produced different size, shape, colour and elevation production capacity. Some of the rhizobacterial isolates were colourless while others were creamy or white in colour. The morphological characteristics of bacterial isolates are given in the Table 4. It is interesting to note that isolate FB5 is red pigment producing bacteria. It has been reported that colour producing bacteria are generally termed as chromobacteria and these bacterial pigments are very useful in heavy metal and antibiotic resistance, protection from phagocytosis, radiation, help in survival and also have many industrial applications (Soliev, 2011).

Table 4. Morphological characterization of the bacterial isolates.

Morphological properties	Bacterial strains
Shape	
<i>ROUND</i>	FB1, FB2, FB4, FB5, FB7, FB6, FN1, FN2, FN3, FN4
<i>ROD</i>	FB3, FB10
<i>IRREGULAR</i>	FB8, FB9, FB11, FN5
Color	
<i>CREAMY WHITE</i>	FB3, FB8, FB10
<i>RED</i>	FB5
<i>YELLOW</i>	FB1, FB2, FB4, FB6, FN1, FN3
<i>YELLOWISH</i>	FB7, FB9, FB11
Elevation	
<i>FLAT</i>	FB1, FB2, FB4, FB5, FB7, FN1, FN2
<i>RAISED</i>	FB8, FB9, FB10, FB11, FN5
<i>GROWTH INTO MEDIUM</i>	FB3, FB6, FN3, FN4

Biochemical Characteristics: The gram negativity of isolates was confirmed by potassium hydroxide solubility test. The result revealed that an elastic thread or viscous thread was observed when loop raised from the bacterial solution by toothpick a few centimeters from glass slides in case of all gram negative bacterial isolates. Among the 16 rhizobacteria, three were gram negative and the rest were gram positive (Table 5).

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen (such as vegetables, fruit or animals). It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS) (Ahmed, 2015). Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second. Nine bacterial isolates were able to produce this enzyme (Table 5).

Table 5. Biochemical characterization of the bacterial isolates.

Biochemical Characterization	Bacterial isolates
Gram test	
Gram (-)	FB2, FB4, FB8
Gram (+)	FB1, FB3, FB5, FB6, FB7, FB9, FB10, FB11, FN1, FN2, FN3, FN4
Catalase test	
Catalase (+)	FB1, FB2, FB3, FB4, FB5, FB7, FB9, FB10, FB11
Catalase(-)	FB6, FB8, FN1, FN2, FN3, FN4

Phosphorous solubilizing capacity of 16 bacterial isolates were evaluated. About four bacterial isolates were capable of phosphorus solubilizing and the rest 12 bacterial isolates were not capable of phosphorus solubilization. The results are presented in Table 6. The PSI of the isolates ranged between 3.0 to 4.66 and the highest P solubilization being observed by the isolate FB2.

Table 6. Phosphorus Solubilization Index (PSI) of the bacterial isolates.

Bacterial isolates	PSI
FB2	4.66
FB4	3.00
FB6	3.25
FB10	3.00

Four of the total isolates was produced HCN. The results of qualitative test of HCN production showed that low number of isolated bacteria were capable of producing HCN. Four isolates (viz. FB1, FB7, FB9 and FB10) could produce HCN (Table 7) while the rest could not. All HCN producing isolates were collected from Bhanga upazila. HCN production by rhizobacteria is one of the key features of antifungal bacteria (Nyma 2015). Plant growth promotion by suppressing

phytopathogenic microorganisms is one of the important indirect mechanism by which they exert their beneficial effect and production of HCN is believed to play role in plant disease suppression. The four cyanogenic rhizobacteria identified can potentially be used for field application to control phytopathogens.

Table 7. List of bacterial isolates showing the results of HCN production.

HCN production	Bacterial isolates
HCN (-)	FB2, FB3, FB4, FB5, FB6, FB8, FB10, FB11 FN1, FN2, FN3, FN4
HCN (+)	FB1, FB7, FB9

Screening of As tolerant bacteria: All bacterial isolates were grown on a nutrient agar media containing sodium arsenite as an As source in the media. Different concentration of the As were prepared ranging from 60 to 1000 ppm in order to determine the maximum tolerance limit to As by the bacterial isolate. The maximum tolerance to the As was about 600 ppm was observed by the bacterial isolate FB4 and FB9 and the second highest tolerance observed by FB1, FB6 about 500ppm. The resistance limit of As by the bacterial isolate is given in Table 8.

It is interesting to note that the bacteria collected from wild brinjal (local name *tit begun*, under genus *Solanum*) and local cultivar of lentil (under the genus *Lens*) harboured four arsenic tolerant bacteria viz. FB4, FB9, FB1 and FB3. Besides the crop plants, the wild brinjal species were seen to grow abundantly in the study location. However, the plant's tolerance to As varies species to species. A number of plant species growing on arsenic contaminated soils had been identified which include *Andropogon scoparius*, *Agrostis castellana*, *A. delicatula*, *A. capillaris*, *Deschampsia cespitosa*, and *Plantago lanceolata* (Meharg and Hartley-Whitaker, 2002). Plants growing on arsenate contaminated soils will assimilate high

levels of arsenate unless they have altered phosphate transport mechanisms (Sharples *et al.*, 2000). In spite of that, arsenate resistance has been identified in those plants, resistance is generally achieved via suppression of the high affinity phosphate uptake system. Thus, arsenate sensitivity is intimately linked to phosphate nutrition, with increased phosphate status leading to reduced arsenate uptake (Meharg *et al.*, 1994).

Table 8. Arsenic tolerance of bacterial isolates.

As tolerance level (ppm)	Survived bacterial isolates
60	FB1, FB2, FB3, FB4, FB5, FB6, FB6, FB7, FB8, FB9, FB10, FB11, FN1, FN2
120	FB1, FB3, FB4, FB6, FB9
150	FB1, FB3, FB4, FB6, FB9
180	FB1, FB3, FB4, FB6, FB9
240	FB1, FB4, FB6, FB9
500	FB1*, FB4, FB6*, FB9
600	FB4, FB9

The highest As tolerant isolates FB4 (Gram negative and catalase positive) and FB9 (Gram positive, HCN producing and catalase positive) both can be characterized as yellowish color with round colony shape and can tolerate media arsenic as high as 600 ppm As. While exposed to more bioavailable arsenic, rhizosphere microorganisms tolerate higher arsenic concentration. Microbial detoxification based on arsenate reduction has been well studied in *E. coli*, and has also been documented for *Staphylococcus*, *Bacillus*, *Acidithiobacillus*, *Pseudomonas*, *Shewanella* and a large group of bacteria (Mateos *et al.*, 2006). Anderson and Cook (2004) isolated a number of arsenic resistant chemo heterotrophic bacteria from two arsenic contaminated soils in New Zealand, which tolerated up to 7,500 ppm arsenate. Rathinasabapathi *et al.* (2006) isolated bacteria from the phyllosphere of *Pteris vittata*, which exhibited resistance to arsenate, arsenite, and antimony in the culture medium. Cánovas *et al.* (2003) isolated a filamentous fungus (*Aspergillus*

sp. P37) from arsenic contaminated river in Spain, which is able to grow at 15,000 ppm arsenic. Among those documented arsenic resistant bacteria, *Corynebacterium glutamicum* is the most prominent, which tolerated up to 30,000 ppm of arsenate (Mateos *et al.*, 2006). However, tolerance level in the present exhibited much lower those reports. Most of those studies have focused on the specific arsenic resistant mechanisms, i.e., functional genes in detoxification mechanisms through transformation of arsenic species and sequestration in either vacuoles or outer membrane; However, little information is available at global metabolic levels such as the osmotic stress when arsenic is stored/accumulated in outer membrane, the oxidative stress generated during arsenic exposure, and the impacts of those stresses on cellular growth.

Both gram positive (*Bacillus selenitireducens*) and gram negative (*Chrysiogenes arsenatis*) bacteria that are resistant to arsenic was identified by Krafft and Macy, (1998). They again reported that both these bacteria encoded by typical *arr* operon protein and both contain an iron sulfur cluster, placing it in the dimethyl sulfoxide (DMSO) reductase family of mononuclear molybdenum enzyme which might induce the As tolerance.

However, in the present endeavour, the details of As tolerance mechanism was not studied. A further study needs to be carried out to identify the isolates at genus level through 16S rRNA gene sequencing.

Conclusion

All the bacterial isolates were grown on a nutrient agar media containing As, nutrient agar medium was prepared with different concentration of As ranges from 30-1000 ppm and isolates FB4 and FB9 showed maximum tolerance against As up to 600 ppm. Both isolates were collected from Bhanga upazila and from the roots of wild brinjal (*Solanum capsicoides*). Isolate FB4 was characterized by yellow colour, flat elevation, gram positive and catalase positive while FB9 can be characterized as yellowish colour with round colony shape, raised elevation, gram negative and having HCN

and catalase producing capacity. Hence, these two isolates needs to be tested further for their effect on As dynamics at the rhizosphere, selection of suitable plant species for bacterial association, bacterial effect on As uptake by plants and potentials for field applications.

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