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Isolation and characterization of bacteria with arsenic tolerance from garden soil and their potential role in soil biology

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Abstract
Arsenite (III) is the most toxic form of arsenic salt that is a widespread concern in many developing countries. Our study was carried out primarily to detect arsenite tolerance level of bacteria isolated from garden soil of Stamford University Bangladesh. Only 8 bacterial isolates were presumptively isolated and identified as *Bacillus* spp. (C1, C3, and D3), *Staphylococcus* spp. (C6, D2, and D6) and *Moraxella* spp. (D4, and D7) with 0.5 mg/ml concentration of arsenite tolerence. However, only five isolates including *Bacillus* spp. (C1, C3, and D3), and *Staphylococcus* spp. (D2 and D6) were able to convert highly toxic arsenite to less toxic arsenate. Amongst, isolate C1 (*Bacillus* sp.) showed the highest bioconversion *in vitro*. Those 5 isolates showed minimum inhibitory concentration (MIC) from 1 to 4 mg/ml and minimum bactericidal concentration (MBC) from 4 to 16 mg/ml for arsenite salt. Moreover, MIC and MBC of NaCl salt were ranged from 1.7% to 3.4% and 1.7% to 6.8%, respectively. None of those isolates showed any role in ammonification, nitrification and nitrogen fixation. A degree of drug resistance was observed against those 8 isolates. Above all, the resident bacterial isolates from garden soil can be examined to distinguish their potential in bioremediation.

Keywords: Arsenite (III), *Bacillus* spp., *Staphylococcus* spp., *Moraxella* spp., NaCl tolerance

Introduction
Arsenic is a toxic metalloid, which may be found in a variety of forms namely arsine (-III), elemental arsenic (0), arsenite (III), and arsenate (V) [1]. In the soil, it exists as insoluble sulphides and sulfosalts such as arsenopyrite, orpiment, realgar, lollingite, and tennantite [2]. Only arsenite and arsenate are ampler in the natural environment than the other two [3]. Moreover, several environmental factors affect arsenic solubility and bioavailability of different arsenic forms in soil, such as pH, redox potential, the presence of other ions, organic matter content, soil texture, fungal or bacterial activities [4].
Arsenate and arsenite both are very toxic in the environment, but arsenite is more toxic than arsenate. While the adopted range of arsenic in industrialized countries is 0.01 mg/l according to the world health organization (WHO) but due to economic consideration and lack of adequate measurement tools and techniques, most Asian countries keep the permissible limit as 0.05 mg/l [5]. They can persuade several sorts of cellular damage in the living system [6]. Several biological methods are vulnerable to arsenic toxicity. Arsenic can severely inhibit plant growth, compromising plant reproductive capacity through losses in fertility, yield, and fruit production, as well as can inhibit root extension and proliferation [7]. Cellular membranes turn out to be damaged in plants exposed to arsenic, causing electrolyte leakage [8]. Arsenic results in various toxic outcomes in human health. The most severe symptoms include skin itching, weight loss, loss of appetite, weakness, lethargy, partial paralysis, numbness in hand and feet, blindness, chronic respiratory disorder, gastrointestinal disorders like anorexia, nausea, pain in the abdomen, enlarged liver and spleen, cancer of the bladder, lung, skin, kidney, nasal passage, liver, and prostate [9,5].
Irrigation is frequently done in Bangladesh by arsenic-contaminated groundwater at agricultural fields, mostly in the rice paddies (*Oryza sativa*). A survey reported the presence of arsenic level is above 1·7 $\mu g\ g^{-1}$ in rice grains grown in arsenic-contaminated soil [10]. The uptake of different forms of arsenic is dependent on several factors like plant types, chemical forms of arsenic, and the presence of other ions as phosphate [11].
In the future, the microbial approach might be very prolific to reduce the accumulation of highly toxic arsenite by bioconversion mechanism. It might be imperative to implement microbial resources to develop a sustainable healthy agriculture system. Consequently, our study was performed with some interest to detect whether indigenous garden soil bacteria can resist highly toxic arsenic concentration. Therefore, we isolated and identified arsenite tolerant bacteria and detect their tolerance against increasing concentrations of arsenite along with some prospective role in the soil environment.

Materials and Methods
Sample collection and processing
Soil samples were collected in a sterile beaker using a sterile spatula at a depth of 2-3 cm from a garden soil of Stamford University Bangladesh, Shiddeswari campus, Dhaka, Bangladesh. One gram of soil sample was dispensed into 100 ml of sterile distilled water and homogenized. One ml of homogenized soil sample was transferred into 9 ml sterile distilled water and serial dilution was carried out up to 10^{-6} dilution.

Growth media for arsenic tolerant bacteria isolation
Nutrient agar (NA) media was prepared with the desired arsenite concentrations for isolation and characterization of

arsenic tolerant bacteria. At first, a stock solution of sodium arsenite salt was prepared using distilled water. Afterward, nutrient agar plates were prepared with 0.1, 0.3, and 0.5 mg/ml of final arsenite concentrations using the stock solution. Growth media were sterilized by autoclaving at 121 °C for 15 min. After autoclaving, the plates were prepared in triplicate for each arsenite concentration and each dilution series.

Isolation and identification of arsenite tolerant bacteria

In brief, 0.1 ml of each dilution was spread onto NA medium containing the previously mentioned arsenite concentrations. Following incubation at 37 °C for 24 h, bacterial isolates that could tolerate the highest arsenite concentrations were selected and identified presumptively by their colony characteristics, morphological features, and biochemical properties.

Morphological characterization

Colony morphology was observed in terms of color, forms, margin, elevation, and size [12]. Gram staining was performed to observe the physiological properties of those isolates [13].

Biochemical characterization of isolates

A loop-full of isolates was streaked onto freshly prepared nutrient agar plates and incubated for 24 h at 37 °C. A pool of biochemical tests was performed by those young cultures. The presence or absence of changes in the media was documented as positive or negative, respectively, and the results were understood using Bergey's Manual of Determinative Bacteriology [14].

Bioconversion of arsenite salt to arsenate by the isolates

The capability of those bacterial isolates to oxidize arsenite was tested by using a silver nitrate solution [5]. Isolates were cultured in the agar plates (peptic digest of animal tissue 5 g/l, beef extract 1.5 g/l, yeast extract 1.5 g/l, and agar powder 15 g/l) for 72 h at 37 °C containing 0.5 mg/ml arsenite salt. Followed by incubation, a small amount of silver nitrate was supplemented to the media. The presence of silver arsenate and silver arsenite in the media is confirmed by the alteration in the color of the media to brown and yellow, respectively.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of arsenite

The broth dilution technique was performed in which identical volumes of broth containing increasing concentrations of arsenite salt were prepared in vials and then, inoculated with a known volume of bacterial culture. In brief, a dilution series of arsenite salt concentrations were prepared in Mueller Hinton broth (MHB) followed by the addition of an equal volume of our culture of interest. Therefore, the final concentrations of arsenite salt ranged from 0.25 mg/ml to 32 mg/ml. The vials were incubated at 37 °C for 24 h and turbidity was measured in comparison to the control vial where there was no culture added. The MIC was measured as the lowest concentration where the visible growth was absent.

The MBC was considered as the lowest concentration of arsenite salt where no bacterial growth was detected. This was performed by sub-culturing those bacterial cultures from each vial into arsenite-free nutrient agar media and incubated at 37 °C for 24 h. Following incubation, the MBC was detected. All those experiments were performed in triplicates.

MIC and MBC of NaCl

MIC and MBC of NaCl salt were performed using a similar protocol as mentioned in the previous section for arsenite salt. The final concentration of NaCl attained from 0.85% to 13.6%.

Production of Ammonia

Isolates with the ability to release simple ammonia from complex proteins are considered as a potential for plant growth. To detect ammonia excretion, freshly grown arsenite tolerant bacterial cultures were inoculated into 5 ml sterilized peptone water containing test tubes and incubated at 37 °C for 1 week. Following incubation, 1 ml of Nessler's reagent was added to the incubated peptone water on the 3rd, 5th, and 7th days. The tubes were observed for any color change from no change to brown precipitate that depicts the presence of no ammonia to a large amount of ammonia, respectively [15]. To verify the experiment, previously identified *Bacillus* sp. and *E. coli* isolates from our laboratory were used as positive and negative controls, respectively.

Denitrification potential

Denitrification is a step where nitrates are reduced to nitrites, ammonia, nitrous oxide, and finally to elemental nitrogen in the form of nitrogen gas. In brief, freshly grown bacterial isolates were inoculated into nitrate broth containing Durham tubes. The broth cultures were incubated at 37 °C for 1 week and the test cultures were examined every day for the presence or absence of air bubbles in the Durham tube [15].

Nitrogen fixation capability

To detect whether the isolates can fix atmospheric nitrogen, freshly grown bacterial cultures were grown in nitrogen-free mannitol agar plates. The media were incubated at 37 °C and checked each day for any growth till the 8th day of incubation [15]. Here, previously isolated *Azotobacter* spp. and *E. coli* from our laboratory were kept as positive and negative controls, respectively.

Antibiotic susceptibility test

This test was performed following the well-standardized Kirby-Bauer disk diffusion test using Mueller Hinton agar (MHA) [16]. However, the zone of inhibition (in mm) was measured and recorded as sensitive and resistant as per CLSI (Clinical and Laboratory Standards Institute) guideline. Antibiogram was performed to determine the susceptibility pattern of the isolated bacteria against commercially available standard antibiotics such as ceftazidime, cotrimoxazole, kanamycin, amoxicillin, cefotaxime, cefuroxime, azithromycin, cephradine, levofloxacin, nalidixic acid, colistin, gentamicin, and piperacillin/tazobactam.

Results

Isolation and characterization

A total of 8 isolates was found on nutrient agar media containing 0.5 mg/ml of arsenite concentration. Those isolates were named as C1, C3, C6, D2, D3, D4, D6, and

D7. The isolates were presumptively found to belong to the genus *Bacillus* spp. (C1, C3, and D3), *Staphylococcus* spp. (C6, D2, and D6) and *Moraxella* spp. (D4, and D7) following characterization by morphological (Table 1), cultural (Table 2), and biochemical properties (Table 3).

Table 1: Microscopic features of the arsenite tolerant isolates.

Sample	Shape	Arrangement	Gram reaction
C_1	Long rod	Chain	G +
C_3	Rod	Single	G +
C_6	Cocci	Cluster	G +
D_2	Cocci	Cluster	G +
D_3	Long rod	Single	G +
D_4	Short rod	Diploid	G -
D_6	Cocci	Cluster	G +
D_7	Short rod	Diploid	G -

Table 2: Colony characteristics of the arsenite tolerant isolates on nutrient agar media.

Sample	Color	Forms	Margins	Elevation	Size
C_1	Off white	Irregular	Undulate	Flat	Medium
C_3	Off white	Circular	Entire	Convex	Small
C_6	Off white	Circular	Entire	Convex	Small
D_2	Off white	Circular	Entire	Convex	Small
D_3	Watery	Circular	Entire	Convex	Medium
D_4	Creamy white, Opaque	Circular	Entire	Convex	Small
D_6	white	Circular	Entire	Convex	Medium
D_7	White, Opaque	Circular	Entire	Convex	Small

Table 3: Biochemical characteristics of the arsenite tolerant isolates.

Tests	Media	Observation							
		C1	C3	C6	D2	D3	D4	D6	D7
Starch hydrolysis	Starch agar plate	+++	+	-	-	++	+++	-	+
Methyl red test	GPB broth	+	+	+	+	+	-	+	-
Voges-proskauer test	GPB broth	+	+	+	+	+	+	+	+
Citrate utilization	Simmons citrate agar slant	+	-	+	+	-	-	+	-
Indole production	1% peptone	-	-	-	-	-	-	-	-
H_2S production	2% peptone	+	+	-	-	-	-	-	-
Motility test	MIU media	+	+	-	-	+	-	-	+
Gelatin hydrolysis	Gelatin media	-	-	-	-	-	-	-	-
Sugar hydrolysis	Triple sugar iron agar slant	K/A	A/A	A/A	A/A	K/A	A/A	K/A	K/A
Oxidase	Nutrient agar	+	+	-	-	+	+	-	+
Catalase	Nutrient agar	+	+	+	+	+	+	+	+
Carbohydrate fermentation	Glucose (acid/gas)	+/+	+/+	+/-	+/-	+/+	-/-	+/-	-/-
Carbohydrate fermentation	Fructose (acid/gas)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Carbohydrate fermentation	Maltose (acid/gas)	-/-	-/-	+/-	+/-	-/-	-/-	+/-	-/-
Carbohydrate fermentation	Lactose (acid/gas)	+/-	+/-	-/-	-/-	+/-	-/-	-/-	-/-
Carbohydrate fermentation	Sucrose (acid/gas)	-/-	-/-	+/-	+/-	-/-	-/-	+/-	-/-
Carbohydrate fermentation	Mannitol (acid/gas)	-/-	-/-	+/-	+/-	-/-	-/-	+/-	-/-

Note: GPB, MIU, K and A stand for glucose phosphate broth, motility indole urease, alkaline and acidic.

Bioconversion of arsenic salt

After the addition of silver nitrate, only 5 out of 8 isolates were found to turn the media color into brown (Fig. 1) which indicated the presence of silver arsenate in the media. It was observed that 3 bacterial isolates (C6, D4, and D7) were unable to oxidize arsenite to arsenate but others (C1, C3, D2, D3, and D6) differentially oxidized arsenite to arsenate. Amongst, isolate C1 showed the greatest conversion potential.

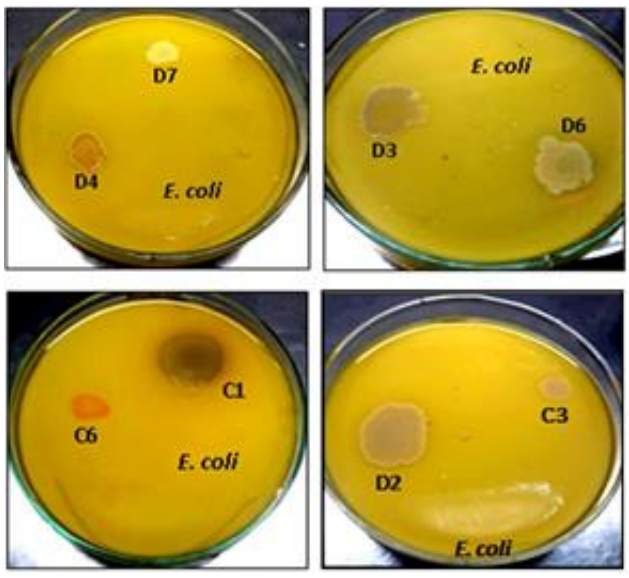

Fig 1: Bioconversion of arsenite to arsenate by arsenite tolerant bacterial isolates.

MIC and MBC of sodium arsenite and NaCl salt

The MIC of sodium arsenite against 5 isolates that oxidized arsenite to arsenate was presented in Figure 2. After 24 h of incubation under the aerobic condition at 37 °C, no turbidity was observed at 2, 4, 2, 1, and 4 mg/ml concentrations when comparing with the control by the isolates C1, C3, D2, D3, and D6, respectively. Following incubation of isolates on arsenite-free nutrient agar media, MBCs were observed at 4, 8, 16, 8, and 8 mg/ml concentrations by the isolates C1, C3, D2, D3, and D6, respectively. Moreover, the MICs and MBCs of NaCl salt by arsenite oxidizing isolates were ranged from 1.7% to 3.4% and 1.7% to 6.8%, respectively (Figure 3).

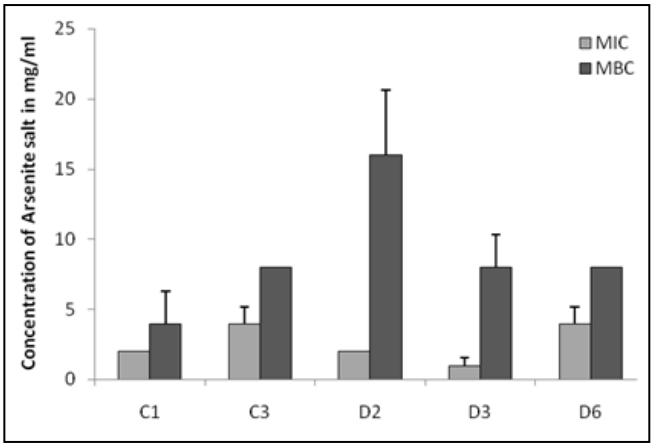

Fig 2: MIC and MBC of arsenite salt by 5 isolates which was able to oxidize arsenite salt.

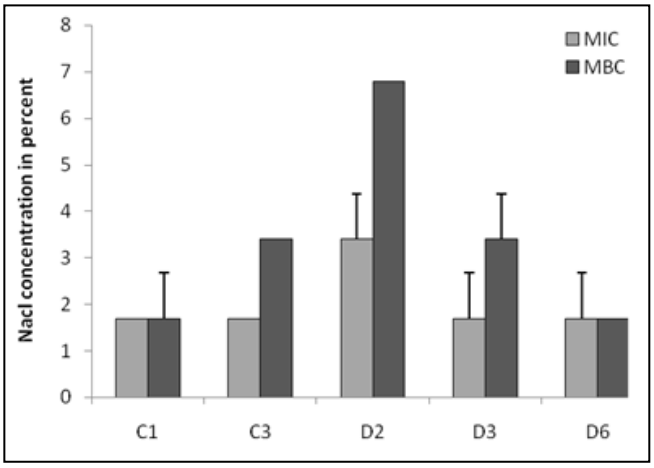

Fig 3: MIC and MBC of NaCl by the isolates which were able to oxidize arsenite salt.

Role of arsenite tolerant isolates in ammonification, denitrification, and N_2 fixation

We checked ammonification, denitrification, and N_2 fixation potentials of arsenic tolerant isolates but they did not play any significant role in those processes (Table 4).

Table 4: Ammonification, denitrification and N_2 fixation potential of arsenic tolerant isolates.

N2 cycle	C1	C3	C6	D2	D3	D4	D6	D7
Ammonification								
Denitrification			All Negative					
N2 fixation								

Antibiotic sensitivity/resistance

Antibiogram of the commercially available antibiotics by the arsenite tolerant bacterial isolates was presented in Table 5. All the isolates showed sensitivity towards cotrimoxazole, colistin, and gentamicin where all those isolates were found resistant against ceftazidime only. Moreover, differential sensitivity and resistance were observed towards the rest of the antibiotics by the isolates.

Table 5: Antibiogram of arsenite tolerant bacterial isolates

Antibiotics (Symbol & disk content)	C1	C3	C6	D2	D3	D4	D6	D7
CAZ (30 mcg)	ND	R	R	R	R	R	R	R
SXT (25 mcg)	ND	S	S	S	S	S	S	S
K (30 mcg)	ND	S	S	R	S	S	R	S
AMX (36 mcg)	ND	S	R	R	S	S	S	R
CTX (30 mcg)	S	S	R	S	R	S	S	R
CXM (30 mcg)	R	S	R	R	R	R	R	R
AZM (15 mcg)	R	S	R	R	R	R	R	R
CE (30 mcg)	S	S	R	S	R	R	S	R
LE (5 mcg)	ND	S	S	S	R	S	S	S
NA (30 mcg)	ND	S	S	S	R	R	S	S
CL (10 mcg)	ND	S	S	S	S	S	S	S
GEN (10 mcg)	ND	S	S	S	S	S	S	S
PIT (100/10 mcg)	ND	R	R	R	S	S	R	R

Note: CAZ, SXT, K, AMX, CTX, CXM, AZM, CE, LE, NA, CL GEN, PIT and ND stand for ceftazidime, cotrimoxazole, kanamycin, amoxicillin, cefotaxime, cefuroxime, azithromycin, cephradine, levofloxacin, nalidixic acid, colistin, gentamicin, piperacillin/tazobactam and not done, respectively.

Discussion

In our study, we screened and isolated eight isolates with 0.5 mg/ml arsenite tolerance that were presumptively belonged to three genera namely *Bacillus*, *Staphylococcus*, and *Moraxella*. One study in 2004 described 17 morphologically different arsenic tolerant heterotrophic bacteria isolated from arsenic-contaminated soil in New Zealand belonging to the genera *Exigeobacterium*, *Aeromonas*, *Bacillus*, *Pseudomonas*, *Escherichia*, and *Acinetobacter* [17]. Some other studies reported several arsenic resistant bacteria species belonging to the genera *Acidithiobacillus*, *Bacillus*, *Deinococcus*, *Desulfitobacterium*, *Pseudomonas*, *Listeria*, *Moraxella*, *Planococcus*, *Microbacterium*, *Alcaligenes*, *Staphylococcus*, *Pseudomonas*, *Corynebacterium*, *Xanthomonas*, *Acinetobacter*, *Flavimonas*, *Micrococcus*, *Aeromonas*, *Enterobacter*, and *Agrobacterium* [18,19,20]. In general, heavy metal resistant bacteria are isolated from heavy metal contaminated soil. However, we were interested to reveal the arsenic tolerance capacity of indigenous garden soil bacteria as according to our previous study, very toxic synthetic dye degrading bacterial strain was found from this garden soil [21].

Among 8 isolates with 0.5 mg/ml of arsenite tolerance, we have detected 5 isolates that were able to oxidize arsenite (III) to less toxic arsenate (V). Isolate C1 which was considered as *Bacillus* sp. showed the greatest bioconversion potential based on changes in media color. A previous study also showed the ability to convert arsenite to arsenate by *Bacillus* sp. [22]. However, arsenic tolerant bacteria have arsenic reductase enzyme by which they can oxidize arsenic but some also have arsenic oxidase by which they can reduce arsenic and some may have both [5].

The MIC of sodium arsenite against 5 isolates that oxidized arsenite to arsenate ranged from 1-4 mg/ml and MBC from

4-16 mg/ml. However, one study conducted in Thailand where arsenic resistant purple nonsulfur bacteria (PNSB), *Rhodopseudomonas palustris* strain C1 under aerobic dark conditions showed MIC and MBC of arsenite 2.5 and 3 mg/ml, respectively [23]. In another study, nine bacterial isolates were collected from sites receiving effluent from the textile (printing and dying) industries situated at Sanganer area, Jaipur (Rajasthan, India), and those isolates showed MIC of sodium arsenite ranging from 3 to 9 mg/ml [24]. Besides, we evaluated MIC and MBC of NaCl salt by those isolates to predict their probable use in soil contaminated with high salt concentration. We found our isolates with MIC value 1.7 to 3.4% and MBC value 1.7 to 6.8% where 8 to 10% tolerance was detected by *Bacillus* sp. and *Aneurinibacillus aneurinilyticus* collected from arsenic affected groundwater of Purbasthali block of Burdwan, West Bengal, India [22]. In association with the salt tolerance, we were also interested to evaluate their potentials in the nitrogen cycle but none of the isolates showed any significance in ammonification, denitrification, or nitrogen fixation.

According to our antibiogram data, none of those isolates showed complete resistance or sensitivity to all the antibiotics. However, all the isolates that were tested showed resistance to ceftazidime. Moreover, 7 out of 8 arsenite tolerant isolates showed resistance towards cefuroxime and azithromycin. In this regard, the major concern is the resistance of the isolates that were collected from a garden soil indicates the dissemination of antibiotic-resistant bacteria in the natural environment which with time might acquire resistance or tolerance to highly toxic metals.

Conclusion

Gradually, the increasing concentrations of arsenic in the soil are posing havoc to the environment due to its highly toxic nature. However, microbial approaches are well suited to address this issue. In this regard, our study was purposed to reduce highly toxic arsenite to less toxic arsenate by using indigenous garden *Bacillus* spp. (C1, C3, D3) and *Staphylococcus* spp. (D2, D6). Among these isolates, *Staphylococcus* sp. (D2 isolate) may reduce arsenic toxicity in an extreme environmental condition where NaCl concentration in soil is very high. The detoxification potential with the consideration of antibiotic multi-resistance, the isolates may contribute to bioremediation of arsenite in contaminated soil and waste treatment following a proper biotechnology approach.

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