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ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF AN As(V)-RESISTANT PLANT GROWTH PROMOTING RHIZOBACTERIUM ASSOCIATED WITH THE RHIZOSPHERE OF *Azolla microphylla*

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ABSTRACT

In the present study, bacterial strains were obtained from the rhizosphere of *Azolla microphylla* Kaulf. and their arsenic (As) tolerance was tested under *in-vitro* conditions. Out of the two bacteria screened for As(V) tolerance, strain R1 was found to have the highest As(V) tolerance level at 2000 mg/l and was found to retain up to 58.76% of As given in the medium solution within its cells, as revealed by inductively coupled plasma optical emission spectroscopy analysis. This efficient As(V)-resistant strain was identified using16S rRNA sequencing and fatty acid methyl ester profile analysis, and was found to be a strain of *Bacillus cereus* (GenBank accession number MH819519). Along with showing abundance of hallmark fatty acids of the *Bacillus cereus* group, some unique fatty acids were also detected in the bacterial strain R1, namely 15:1 ω5C, 17:1 iso ω10C, 17:1 ante isoA, 17:0, 18:0 iso, 18:1 ω9c, and 17:1 iso ω5C. At its maximum tolerable As(V) dose of 2000 mg/l, strain R1 showed 59.9% As(V) reduction, which was assessed through arsenate reductase enzyme activity. Not only this, under As(V) stress, strain R1 was found to produce 20.4% more cellular protein and display 30.3% antioxidant activity assayed as DPPH reduction. The bacterium is also capable of exhibiting plant growth promoting traits such as solubilisation of inorganic phosphate, production of siderophore and biosynthesis of the plant growth regulator indole acetic acid. Thus, this bacterium has the potential to remediate As(V) from environment and also assist the growth of plants growing in As-polluted ecosystems.

Keywords: Inductively coupled plasma optical emission spectroscopy, Fatty acid methyl ester profile, *Bacillus cereus* group, Arsenate reductase, Siderophore, Indole acetic acid, Antioxidant

INTRODUCTION

Heavy metal pollution in the environment is a rising global threat, primarily because of the toxic effects that these elements cause in humans and other living beings when consumed beyond a threshold concentration **(Nagajyoti *et al.*, 2010; Jaishankar *et al.*, 2014)**. One of the most notorious heavy metals is arsenic (As), the health impacts of which range from physiological to genetic alterations. Besides being a potent carcinogen, it also causes several physiological aberrations such as abdominal pain, diarrhoea, vomiting, skin discoloration, numbness in limbs, partial paralysis, etc. The occurrence of arsenic in nature is mostly geological and anthropogenic in origin. However, extensive industrialization and mining over the past few decades have resulted in a drastic increase in arsenic concentrations in ground water and soil. The W.H.O. and F.A.O. have set a permissible limit of 0.01 mg/l of As in drinking water and 0.05 mg/l of As in irrigation water **(Smith *et al.*, 1998)**. But, due to economic considerations, many developing Asian countries have a national standard of As in drinking water as 0.05 mg/l **(Chakraborti *et al.*, 2009).**

India is one of the several Asian nations which is presently facing an extreme threat of As pollution. The part of India that lies in or very close to the Ganga-Brahmaputra delta basin has been found to have an As concentration above 0.05 mg/l in the groundwater **(Chakraborti *et al.* 2004).** Thus, it becomes extremely important to device eco-friendly as well affordable strategies that can effectively reduce arsenic levels in these developing parts of the world. A requirement for As remediation of soil and groundwater is necessary. As compared to expensive *ex-situ* remediation methods, *in-situ* methods may prove to be more suitable. Some of the methods are due through sorption studies where As is immobilized through sorption to or chemical oxidation-reduction processes by iron, aluminum, manganese hydroxides and oxides, to clay minerals, cations and anions. Oxidation-reduction by sulfides is also performed. As can also be bio-transformed by chemical or microbial means as well as through phytoremediation using plants that bio-accumulate high doses of As among others. In general, bioremediation as a method is more effective over physico-chemical means and can be used for groundwater, surface water, soil and sediment remediation. The most appropriate method under bioremediation in case of reducing heavy metal pollution is microbial remediation, which makes the use of various fungi and bacteria. The main advantages served by this method are: fast reproduction time and small size making the tool an effective and portable one, compact genetic material with polycistronic DNA that produces a variety of proteins to help execute different functions despite small genome size, reduced production of waste or by products thus becoming eco-friendly and cost effective **(Shukla and Srivastava, 2017).**

In the recent years, bioremediation of heavy metals has received much attention for environmental clean-up, not only because of its economic efficacy over the conventional physical and chemical approaches, but also for its potential **(Vidali, 2001).** Many soil bacteria have already been reported to be arsenic-resistant, and they also cause a change in the oxidation states of the metal. This leads to variation in arsenic speciation and a resulting difference in solubility of the end species in areas where these resistant bacteria are present. Thus, bacteria play an important role in the biochemical cycling of arsenic in the environment **(Pàez-Espino *et al.*, 2009).**

Although extensive studies are being done on the As tolerance and removal efficacy of soil bacteria, the role of plant growth promoting rhizobacteria (PGPR) in this field has not yet been fully explored. The present study attempts to highlight the As (V) (inorganic arsenate) removal efficacy and plant growth promoting activities of a bacterial strain isolated from the rhizosphere of the aquatic macrophyte *Azolla microphylla* Kaulf.

MATERIAL AND METHODS

Collection of plant material and isolation of rhizobacteria

Freshly cultivated *Azolla microphylla* was obtained from the Vivekananda Institute of Biotechnology, Nimpith and used immediately for the study. After washing the roots of *Azolla microphylla* vigorously, 2 gm of the roots was weighed out and kept in 200 ml sterile PBS buffer (pH 7.4) on a shaker, for an hour. Serially diluted aliquots were plated with sterile Nutrient Agar media (HiMedia) (pH 7) for 24 hours, at 37˚C **(Mendes *et al.*, 2007)**. Following incubation, two morphologically distinct colonies namely, R1 and R2 were selected at random for screening their As (V) tolerance.

Screening of isolated bacteria for As (V) tolerance

The bacterial strains R1 and R2 were cultured in sterile Nutrient Broth media (HiMedia) for 16 hours and thereafter inoculated in As (V) supplemented Nutrient Broth media, with As (V) supplementation given in gradually increasing order.

These freshly prepared cultures were incubated for 24 hours at 37˚C. In order to screen for the maximum tolerable As(V) dose of the two strains, concentration of As(V) supplemented in the growth media ranged from 0 mg/l to 4000 mg/l for bacterial strain R1, and 0 mg/l to 40 mg/l for bacterial strain R2. Sodium arsenate ($Na_2HAsO_4.7H_2O$) (LobaChemie) was used as the source of As(V), while deionized water of an electrical conductivity of 0.2 µS was used to make all the preparations. The inoculum was given at a concentration of 1% (v/v) of the final media volume, having a bacterial cell count of about 4.3×10^8 cfu/ml, which was measured through colony counting on pour-plated Nutrient Agar and multiplying with the appropriate dilution factor. A negative control was also kept, having no As(V) supplementation. Growth was assessed spectrophotometrically at OD_{580} (Optical Density at 580 nm wavelength), after an incubation period of 24 hours at 37˚C, with respect to the control **(Hussein *et al.*, 2004; Courvalin *et al.*, 1985)**. The bacterial strain showing maximum As(V) tolerance was considered as the most efficient As(V)-tolerant strain and taken for further studies.

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) analysis of the amount of As retained in the cells of the most efficient As(V)-tolerant strain

The most efficient As(V)-tolerant bacterial strain (R1) was made to grow at its maximum tolerable As(V) dose for 24 hours, followed by centrifuging the culture at 7.826 ×*g* for 10 minutes. The cell pellet was collected, dried and acidified to a pH less than 2 using conc. HNO_3, 16 hours prior to ICP-OES analysis. For the analysis, Perkin Elmer model Avio 220 Max was used. Arsenic (As) was measured spectrophotometrically at the UV wavelength of 193 nm. The As content of the sample was obtained with reference to an As standard calibration curve. For all preparations, deionized water of an electrical conductivity of 0.2 µS was used.

Morphological and biochemical characterization of the bacterial strain R1

Purified culture was grown on Nutrient Agar plates and the colony morphology was recorded. Gram staining was done and cellular morphology was studied under a total magnification of 400X, using Dewinter microscope digital camera, DIG1510, 5.1 MP 1/ 2.5 ′′ CMOS sensor. Biochemical characterization was also performed, according to Bergey's Manual of Systematic Bacteriology, to test for carbohydrate utilization, nitrate reduction, H_2S production, citrate utilization and presence of enzymes such as oxidase, catalase, gelatinase and amylase following standard protocols **(Kreig, 1984; Cappuccino and Sherman, 2004)**.

16S rRNA sequence analysis of R1 bacterial strain

gDNA, isolated from the pure culture of bacteria, was checked for its quality on 1% Agarose gel. The16S rRNA gene was amplified using 27F and 1492R primers. A discrete PCR amplicon band corresponding to 1500bp was observed upon resolution on Agarose gel and was thereafter purified (Figure 3). This amplicon was then subjected to forward and reverse DNA sequencing reactions using the specific primers, by means of BDT v3.1 cycle sequencing kit, on ABI 3730xl Genetic Analyzer. Contigs were generated from the forward and reverse sequence data using the BioEdit software. Thereafter, it was used to carry out BLAST with the NCBI GenBank database. Maximum query coverage and % identity were used as parameters to estimate the species of the bacteria. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7. While constructing the phylogenetic tree, the Maximum Likelihood method, based on Kimura 2 parameter model, was used to infer the evolutionary history. To represent the evolutionary history of the taxa analyzed, bootstrap consensus tree, inferred from 1000 replicates, was taken. Branches that correspond to partitions reproduced in less than 50% bootstrap replicates have been collapsed. The percent of replicate trees, in which the associated taxa are clustered together in the bootstrap test (1000 replicates) have been shown next to the branches. Initial tree(s) for the heuristic search have been obtained automatically using the Neighbour-Join and BioNJ algorithms. The algorithms were applied to a matrix of pair-wise distances that were estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. 11 nucleotide sequences have been used for analysis. The codon positions that have been included are: $1^{st}+2^{nd}+3^{rd}$+Non-coding. All positions containing gaps and missing data have been eliminated. There are a total of 1434 positions in the final dataset. MEGA7 has been used to conduct the evolutionary analyses **(Kimura, 1980; Kumar *et al.*, 2015; Felsenstein, 1985)**.

Fatty acid methyl ester (FAME) profile analysis

In order to extract the fatty acids from the pure bacterial culture, saponification using sodium hydroxide (diluted in methanol) was carried out, followed by derivatization using hydrochloric acid (diluted in methanol) to obtain the respective Fatty Acid Methyl Esters (FAMEs). The FAMEs were thereafter extracted from the aqueous phase using a combination of organic solvents (hexane and methyl tert-butyl ether).This extract was subjected to GC analysis, using Agilent HP-ULTRA 2 column, having a length of 25m, diameter of 0.200mm, with a 0.33µ film and a temperature limit of 60˚C to 325˚C. The analysis was carried out using hydrogen gas as the mobile phase and Flame Ionization detector, adjusted at a flow rate of 1.3 ml/min. The FAME profile thus generated was identified using the MIDI software (TSBA database, version 6.0) and a similarity index of ≥ 0.7 was considered for good species match, while that between 0.4 and 0.6 was considered as species match only when good separation from the others listed was found.

Total protein content and arsenate reductase assay

Two sets of Nutrient Broth media were used, wherein one was the negative control and the other was supplemented with As(V) at the maximum tolerable dose of R1 strain. Overnight grown cultures were taken to obtain the cell pellets, followed by washing twice in reaction buffer (10mM Tris, pH 7.5, with 1mM Na_2EDTA and 1mM $MgCl_2$) (HiMedia). The pellet was finally suspended in the reaction buffer and lysed by sonication, followed by cold centrifugation and collecting the supernatant. To measure the total protein content, Thermo Fisher Scientific- Qubit 3.0 Fluorometer (Invitrogen Ref.: Q33216) was used. To the Qubit working solution, prepared by using Qubit buffer and reagent, 10 µl of the crude protein extract was added. The mixture was used to measure the protein content in the already calibrated fluorometer. Assay for the arsenate reductase enzyme was carried out using the NADPH oxidation method. To initiate the NADPH oxidation at 37˚C, 50 µl of the crude extract was mixed with 820 µl of reaction buffer, 30 µl 10 mM DTT, 50 µl 2 mM arsenate, and 50 µl 3 mM NADPH. OD_{340} was obtained spectrophotometrically (UV-Vis spectrophotometer, Optizen) to estimate the percentage reduction of arsenate with reference to NADPH oxidation (NADPH has an absorbance of 1.0 at 340 nm) **(Anderson and Cook, 2004)**. Enzyme activity was also calculated.

Antioxidant activity

Overnight grown bacterial cultures grown in Nutrient broth media were used to collect cell pellet. One set was given the As(V) treatment at maximum tolerable dose while the other was not (control). The pellet was re-suspended in distilled water. To 0.5 ml of this soup, 0.5 ml of 0.004% 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich) (in methanol) was added. Following 30 minutes of incubation, OD_{517} was obtained spectrophotometrically (UV-Vis spectrophotometer, Optizen) for the control and treatment sets. % reduction of DPPH in treatment with respect to control was calculated **(Shen *et al.*, 2010)**.

Qualitative detection of Siderophore production

Siderophore production was assessed using the method described by Milagres et al. A petri-plate was divided into two halves. One half of the petri-plates contained Nutrient Agar while the other half contained Chrome Azurol S- Agar (HiMedia), which is a blue-coloured solution having a dye and iron (III) complex. The method of siderophore detection described here uses the principle that an iron (III) and dye (Chrome Azurol S-HDTMA) complex changes its blue color when the iron (III) is taken out by the competing siderophore molecule, which has greater affinity for the metal. Bacterial culture was streaked on the Nutrient Agar half of the plate, and was incubated for a period of 7 days at 37°C. The distance of discharge of the blue colour on the Chrome Azurol S- Agar half of the plate was measured and considered as an indicator of siderophore production efficiency of the bacterial strain **(Milagres *et al.*, 1999)**.

Quantification of Indole Acetic Acid (IAA) production

Bacterial cultures grown in King's B bacterial media (Peptone: 20 gm/L, K_2HPO_4: 1.15 gm/L, $MgSO_4.7H_2O$: 1.15 gm/L, Glycerol: 1.5 % v/v), with and without the supplementation of Tryptophan (0.5 gm/L), were used to collect the supernatant. One ml of this soup was treated with one ml of the Salkowski reagent (4.5 gm $FeCl_3$ in 1 L 10.8 M H_2SO_4), followed by incubation in dark for 30 minutes. OD_{530} was measured spectrophotometrically (UV-Vis spectrophotometer, Optizen), which was plotted against a pre-standardized IAA (HiMedia) standard curve to obtain the concentration of IAA produced **(Glickmann and Dessaux, 1995)**.

Quantification of Phosphate solubilisation

Sterile Pikovskaya broth media (10 g/l glucose, 5 g/l tricalcium phosphate, 0.5 g/l ammonium sulphate, 0.2 g/l KCl, 0.25 g/l MgSO4.7H2O, 7 mg/l MnSO4.H2O, 11 mg/l FeSO4.7H2O, 0.5 g/l yeast extract, pH 7.2) was inoculated with the bacterial strain and incubated for 10 days at 28ºC, with intermittent shaking. The cultures were then filtered and centrifuged and 1 ml aliquot was collected. To this aliquot, 2.5 ml of freshly prepared Barton's reagent (ammonium molybdate and ammonium metavanadate) was added. Following an incubation of 10 minutes, OD_{430} was measured spectrophotometrically (UV-Vis spectrophotometer, Optizen) and plotted against a pre-standardized phosphate standard curve to obtain the amount of phosphate that was solubilised **(Cappuccino and Sherman, 2004)**.

Antibiotic sensitivity test

Actively growing cultures of the bacterial strains were spread on sterile Mueller-Hinton Agar (HiMedia) plates. After plating, the plates were allowed to dry. Commercially available antibiotic discs were placed atop the plates having the bacterial cultures and then gently pressed using sterile forceps. Plates were incubated at 37°C for 18 hours **(Barry *et al.*, 1969)**. Thereafter the Zone of Inhibition was measured and the response classified as Resistant, Sensitive and Intermediate based on comparing with standard reference table provided by the manufacturer, i.e. HiMedia catalog.

Statistical Analysis

All the experiments were run in triplicates and their mean with the Standard Error of the Mean (S.E.M.) is provided. The software GraphPad Prism 5 was used for graphical representation of the data and to include the S.E.M.

RESULTS

Screening of isolated bacteria for As(V) tolerance and the most efficient As(V)-tolerant bacterial strain

Out of the two bacterial strains isolated, strain R1 was found to have a much greater tolerance to As(V) than strain R2. Strain R1 could withstand a maximum As(V) dose of 2000 mg/l, while at 3000 mg/l the growth reduced significantly. Its growth was completely ceased at an As(V) concentration of 4000 mg/l. The effect of As(V) on growth (OD_{580}) of this bacterial strain has been shown in Figure 1. On the other hand, strain R2 could only tolerate a maximum dose of 20 mg/l of As(V) (data not shown here). Thus, due to its virtue of exhibiting a significantly greater As(V) tolerance, strain R1 was considered as the most efficient As(V)-tolerant bacterial strain and was considered for further analyses. When grown at 2000 mg/l of As(V), this bacterium could retain 1175 ±0.47 mg/l As in its dried cell pellet as revealed by the ICP-OES analysis data. Thus, the strain R1 can effectively retain up to 58.7% (w/v) of As in its cells under in vitro conditions.

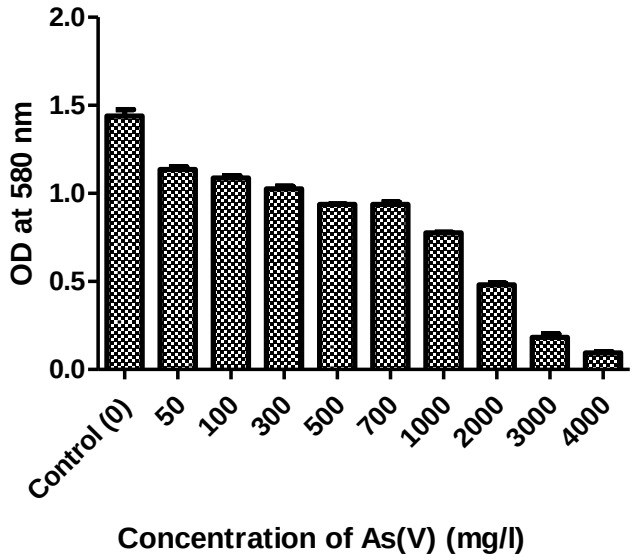


Figure 1 Effect of As(V) on the growth (OD_{580}) of R1 strain

Morphological and biochemical characterization of R1 strain

The result of biochemical tests has been shown in Table 1. Table 2 shows the response of the bacteria to the various antibiotics.
Colony morphology was whitish, slimy, circular with entire margin, flat curvature and moderate in size (Figure 2). The cells were Gram positive, rods. The bacterium could utilize all the three types of sugars for growth after 48 hours of incubation, i.e. glucose, sucrose and lactose; however, only the use of glucose and sucrose resulted in fermentation of acids, as revealed by red colouration in the methyl-red containing growth medium, with no bubble formation in the Durham's tube indicating absence of gas formation during fermentation. Motility was observed under the compound light microscope which could be attributed to flagella. Possible presence of amylase resulted in starch hydrolysis, while there was a lack of gelatinase activity. An important enzymatic factor which is involved in oxidative stress tolerance, namely catalase, was also present.

Figure 2 Colonies of R1 bacterial strain on Nutrient Agar plate

Among the antibiotics tested, strain R1 showed strong resistance against the beta-lactam antibiotics piperacillin, penicillin, oxacillin, cephalothin (1st generation cephalosporin), amoxyclav, cephotaxime and ceftazidime (3rd generation cephalosporins) and aztreonam (which is similar in action to penicillin), indicating strong β-lactamase activity. However, it was found to be sensitive toward the protein synthesis inhibitors such as amikacin, gentamycin and erythromycin. Being a Gram positive bacterium, strain R1 also showed sensitivity toward the glycopeptide antibiotic teicoplanin. The highly β-lactamase resistant carbapenem antibiotic imipenem also had sensitive response from the bacterium.

Table 1 Biochemical tests of R1 bacterial strain

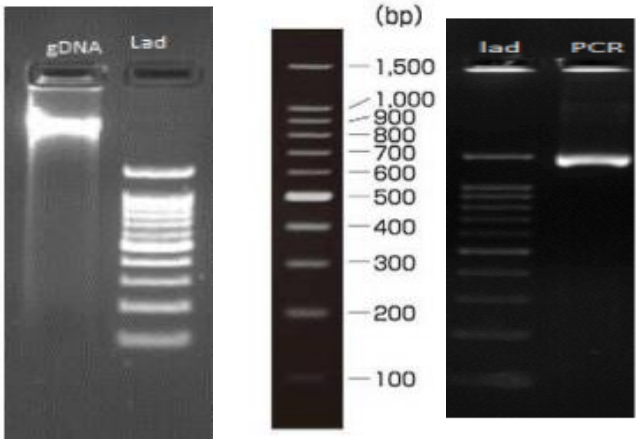
			Response
Motility test			+
Nitrate reduction test			+
H_2S production test			-
Indole test			-
Methyl red test			-
Voges-Proskauer test			+
Citrate test			+
Starch hydrolysis test			+
Gelatin liquefaction test			-
Catalase test			+
Oxidase test			-
Oxidation-Fermentation and Acid production from different carbohydrates	Glucose	Growth (Turbidity)	+
		Gas production	-
		Acid production	+
	Lactose	Growth (Turbidity)	+
		Gas production	-
		Acid production	-
	Sucrose	Growth (Turbidity)	+
		Gas production	-
		Acid production	+

Table 2 Antibiotic sensitivity test of R1 bacteria strain.

Antibiotic	Zone of Inhibition. (mm)	Response
Piperacillin (100 µg)	20±0.00	Resistant
Linezolid (30 µg)	20±0.09	Intermediate
Ciprofloxacin (5 µg)	30±0.15	Intermediate
Teicoplanin (30 µg)	23±0.24	Sensitive
Vancomycin (30 µg)	25±0.31	Intermediate
Gentamicin (10 µg)	22±0.03	Sensitive
Penicillin G (10 units)	None	Resistant
Oxacillin (1 µg)	None	Resistant
Cephalothin (30 µg)	None	Resistant
Clindamycin (30 µg)	18±0.07	Intermediate
Erythromycin (15 µg)	17±0.44	Sensitive
Amoxyclav (30 µg)	None	Resistant
Cephotaxime (30 µg)	13±0.26	Resistant
Levofloxacin (5 µg)	30±0.21	Sensitive
Aztreonam (30 µg)	16±0.01	Resistant
Imipenem (10 µg)	25±0.10	Sensitive
Amikacin (30 µg)	24±0.32	Sensitive
Ceftazidime (30 µg)	11±0.41	Resistant

Identification of R1 strain through 16S rRNA sequence analysis

In order to identify the bacterial strain, the gDNA was isolated and the 16S rRNA gene amplified using the universal primers (Figure 3).



(a) **(b)**

Figure 3 (a) Single high molecular weight band of gDNA of R1 bacterial strain was observed on 1% agarose gel. (b) A prominent 1500 bp band of 16S rDNA gene was observed on amplification using 27F and 1492R primers

Thereafter, the amplicons were subjected to sequencing. The consensus sequence generated for the 16S rRNA gene of the R1 bacterial strain was subjected to nucleotide BLAST using the NCBI GenBank database. The consensus 16S rRNA gene sequence of R1 strain was also used to construct phylogenetic tree based on the Kimura 2 parameter. The top 10 bacterial sequences retrieved from GenBank database that showed maximum similarity/identity with the 16S rRNA sequence of R1 were considered for sequence alignment during the tree construction. Details of these bacterial isolates are provided in Table 3.

Table 3 Details of the isolates considered in phylogenetic analysis.

Accession	Species	Strain	% Identity
JF705198.1	*Bacillus cereus*	HN	99%
HQ200405.1	*Bacillus anthracis*	B	99%
AM292032.1	*Bacillus thuringiensis*	CMBLBT-5	99%
AY138279.1	*Bacillus cereus*	2000031513	99%
KU902033.1	*Bacillus cereus*	SB2	99%
KU870751.1	*Bacillus cereus*	BFM4	99%
KJ812439.1	*Bacillus cereus*	MCCC1A02143	99%
JQ799109.1	*Bacillus cereus*	KJW12	99%
JF772063.1	*Bacillus anthracis*	LRC82	99%
JF907013.1	*Bacillus cereus*	MSU	99%

The phylogenetic tree, highlighting the reference species has been shown in Figure 4. The closest similarity of R1 strain was found to be with *Bacillus cereus* strain HN (accession: JF705198.1).

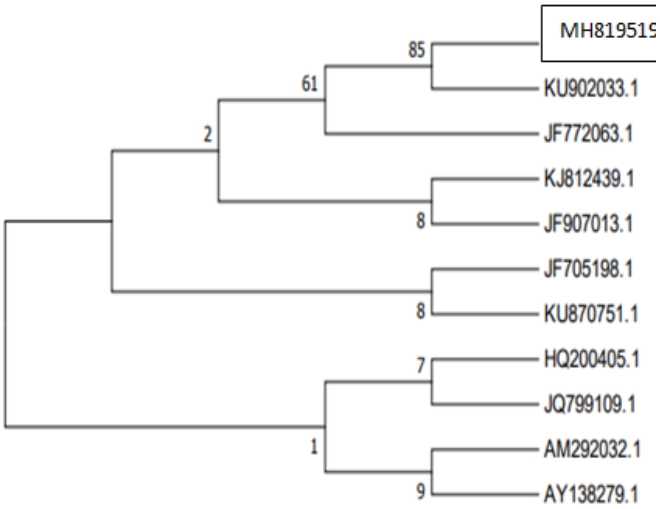


Figure 4 Phylogenetic tree showing the relatedness of R1 strain (MH819519) with the reference species

The consensus sequence of R1 strain was submitted at the NCBI GenBank database under the accession number **MH819519**.

FAME profile analysis

From Tables 3, it can be seen that the 16S rRNA gene sequence of R1 strain shows the same value of % identity with all the sequences retrieved in BLAST analysis. This makes the species identity confirmation ambiguous, and therefore the FAME profile of R1 bacterial strain was also analysed. The results of the analysis are provided in Figure 5 and Table 4.

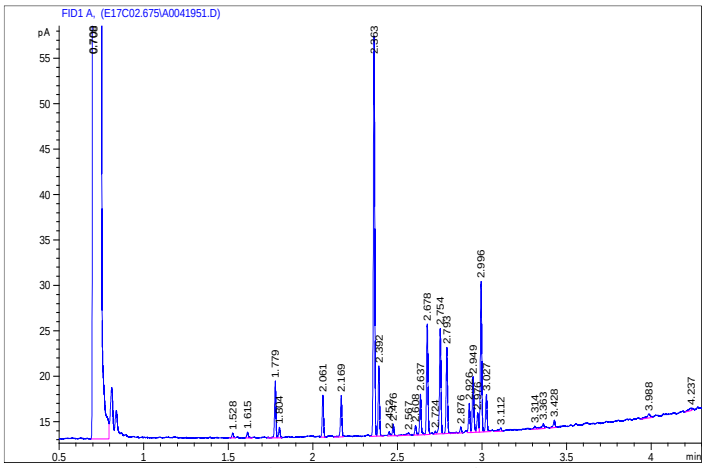


Figure 5 Gas chromatogram of the FAMEs generated for R1 bacterial strain

The FAME profile shows that 10 out of the 24 FAs detected were major, while the rest were minor components, with their content being 2% or lesser (Table 4). On comparison with the TSBA library, the maximum similarity was generated with *B. cereus* GC subgroup A. Few of the FAs have been found to be unique, namely 15:1 ω5C, 17:1 iso ω10C, 17:1 ante isoA, 17:0, 18:0 iso, 18:1 ω9c, and 17:1 iso ω5C.

Table 4 Percentage of fatty acid composition of R1 bacterial strain

Peak name	Percent
12:0 iso	0.43
12:0	0.56
13:0 iso	4.25
13:0 anteiso	0.83
14:0 iso	3.05
14:0	3.18
15:0 iso	29.46
15:0 anteiso	5.07
15:1 ω5C	0.33
16:1 ω7C alc	0.76
16:0 iso	8.41
16:1 ω11C	0.22
16:0	6.52
15:0 2-OH	0.62
17:1 iso ω10C	2.23
17:1 anteisoA	1.53
17:0 iso	11.18
17:0 anteiso	3.07
17:0	0.46
18:0 iso	0.32
18:1 ω9C	0.65
18:0	0.69
17:1 iso ω5C	4.63

Total protein content and arsenate reductase assay

For the strain R1, there was 20.4% increase in the total protein content under As(V) stress at 2000 mg/l, with respect to the control. There was 23.4% increase in the arsenate reductase enzyme activity under As(V) treatment, with respect to the control. The results have been shown in Figures 6 and 7 respectively. There was 59.9% oxidation of NADPH under *in-vitro* conditions, indicating significant reduction of arsenate to arsenite under the As(V) stress, with respect to the control, during the redox reaction. Both the protein content study and arsenate reductase assay indicate that the genes for reducing and detoxifying arsenic may be constitutively expressed and that, the associated functions are further enhanced in presence of As(V) stress.

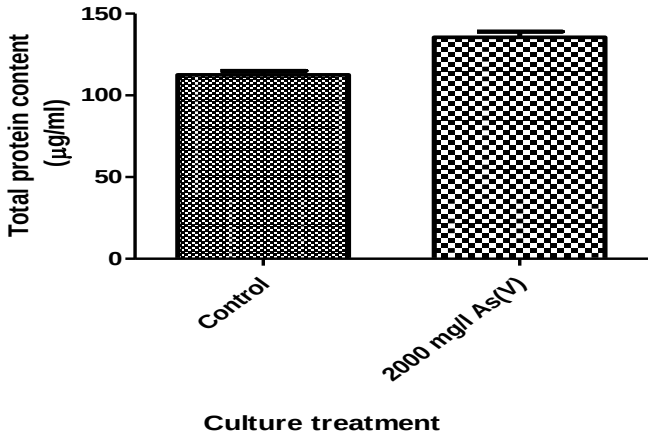


Figure 6 Variation in total cellular protein of R1 strain with As(V) stress

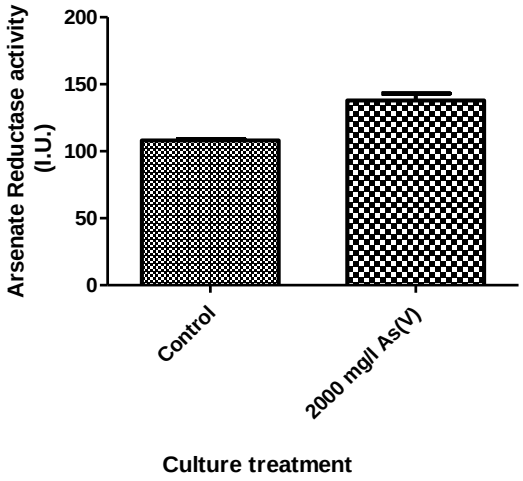


Figure 7 Variation in arsenate reductase enzyme activity with As(V) stress

Antioxidant activity

The culture of R1 strain grown in the absence of As(V) stress could reduce 27.3% DPPH, while that grown in presence of As(V) stress at 2000 mg/l could show 30.3% reduction of DPPH. Thus, there was an increase in radical scavenging activity seen under the heavy metal stress.

Siderophore production

The ability and efficacy of siderophore production by bacterial strain R1 was assessed by observing the disappearance of blue color in the CAS-HDTMA-iron III complex-based half of the plate. It was observed that the discharge of the blue color was higher in that region of dye front where the growth of microorganism in the corresponding Nutrient Agar front was denser (Figure 8).

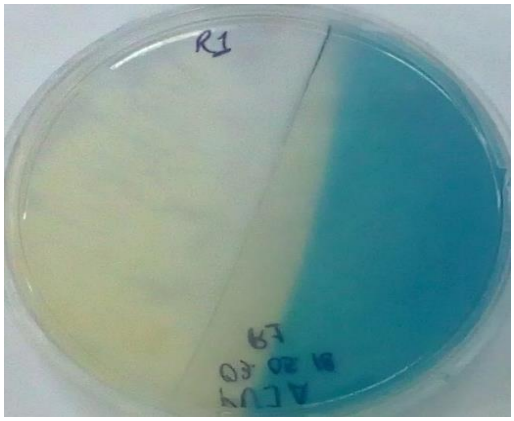


Figure 8 Siderophore production by R1 strain and discharge of blue colour on the CAS agar half of the plate

This clearly demonstrates the chelation of iron (III) by the siderophores produced by R1 strain in the Nutrient Agar half of the plate, which gradually diffused to the Chrome Azurol S half of the plate. The result has also been tabulated in Table 5, giving more precise information on the siderophore production efficacy of bacterial strain R1.

Table 5 Assessment of siderophore production ability by R1 bacterial strain.

Strain	Advance of colour change (mm/day)	Efficacy of chelation*
R1	4 ± 0.05	+

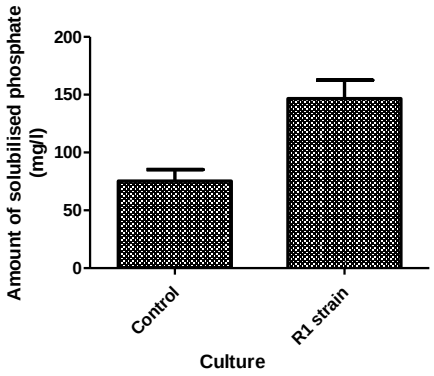
*__Index:__ <1mm/day→±; 1-4mm/day→+; 4.1-8mm/day→++; >8mm/day→+++

Indole Acetic Acid (IAA) production

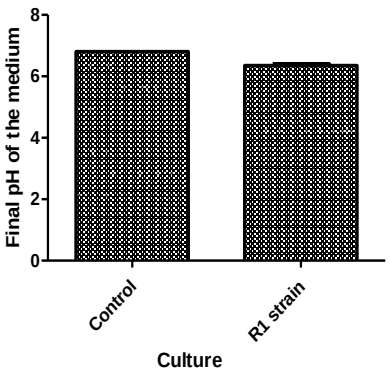
R1 bacterial strain could produce IAA in the medium with and without precursor tryptophan supplementation as seen by the development of rosy pink coloration upon addition of Salkowski reagent. There was 4.33±0.57 µg/ml IAA produced by the bacterium when 0.5 gm/L tryptophan was added in the culture medium. In the absence of tryptophan, there was 3.83±0.61 µg/ml production of the phytohormone.

Phosphate solubilisation

To estimate phosphate solubilisation efficiency, two culture treatments of the inorganic phosphate-containing growth medium were taken: one having no bacterial inoculation (control) and the other having bacterial strain R1 inoculum. The reaction mixture, consisting of culture aliquot (after incubation) and Barton's reagent, was used to measure OD_{430} values, which was then used to obtain the amount of soluble phosphate from the standard curve. It was found that bacterial strain R1 was able to transform insoluble inorganic phosphate into soluble phosphate, which was indicated by a heightened OD_{430} value of R1-treated reaction mixture and a consequently high amount of solubilised phosphate. This was in sharp contrast to the significantly lesser amount of soluble phosphate in the untreated control reaction mixture. The strain R1-treated culture displayed an appreciable 14.7% solubilisation of phosphate. There was also a simultaneous drop in the final pH of the R1-treated culture after the incubation period, from its initial pH of 7.2, indicating that the production of organic acids through utilization of sugar molecules assists in the process of phosphate solubilisation (Figure 9). No significant pH drop was seen in the untreated control culture. The production of organic acids through the utilization of carbohydrates by bacterial strain R1 was already understood through the biochemical study of the bacterium.



(a)



(b)

Figure 9 (a) Solubilisation of phosphate by R1 strain, (b) along with a simultaneous drop in the final pH of the culture medium

DISCUSSION

The primary objective of this study was to identify the most efficient As(V)-resistant bacteria associated with the rhizosphere of *Azolla microphylla*, a floating macrophyte that naturally inhabits rice paddy fields. This would mark the initial step towards identifying potentially As-resistant PGPR that could be further used in bioremediation in cultivable wetlands polluted with As and harbouring this aquatic fern, *Azolla* sp., with simultaneous boost-up in the plant growth. The strain R1 was confirmed to be a strain of *Bacillus cereus* based on 16S rRNA gene sequence and FAME profile analyses. Acccording to the recent developments and research, the *Bacillus cereus* group (also called the *Bacillus cereus sensu lato*) comprises of 19 valid or published species. They are: *B. cereus sensu stricto*, *B. albus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. clarus*, *B. cytotoxicus*, *B. luti*, *B. mobilis*, *B. nitratireducens*, *B. pacificus*, *B. paramycoides*, *B. paranthracis*, *B. proteolyticus*, *B. pseudomycoides*, *B. toyonensis*, *B. tropicus*, *B. wiedmanni*, *B. weihenstephanensis*. The *B. cereus* group is defined as a group of facultative anaerobic and spore-forming bacterial species that are found in diverse environments **(Liu *et al*., 2017; Acevedo *et al*., 2019)**. The primary structures of the 16S rRNA of these species within the closely-related group have been reported to show >99 % similarity- an observation that is consistently confirmed by DNA-DNA hybridization studies **(Ash *et al*., 1991; Liu *et al*., 2017)**. For this reason, the FAME profile analysis was also carried out during the identification of R1 strain. The abundance of 15:0 iso, hallmark of *B. cereus* group, is consistent with previous findings on FAME profile of *B. cereus* **(Sharmili and Ramasamy, 2016; Dikbas, 2010; Kaneda, 1967)**. The major FAs 15:0 iso, 16:0 iso and 17:0 iso have earlier been reported as frequent major FAs of *B. cereus* **(Kaneda, 1967)**. An interesting observation was that, when the 16S rRNA sequence of the R1 strain was used to perform BLAST analysis using Ez-BioCloud database, it showed maximum similarity with the type strain *Bacillus paranthracis* Mn5, reported by Liu *et al.* in 2017, which was isolated from sediments of Pacific Ocean. But the FAME profiles of these two strains, i.e. R1 and Mn5 show marked variations. A plausible explanation for this observation could either be that the initial environment of the bacterial strains (from which they were isolated) influenced their physiology, or perhaps there was a spontaneous mutation causing a switch from one species to another closely-related species.

The finding that R1 bacterial strain could reduce As(V) in the medium is in tandem with the previous report on As(V) resistant *Bacillus cereus* associated with rhizosphere of another As-hyperaccumulating fern *Pteris vittata* **(Jia *et al.*, 2019)**. This particular strain could tolerate up to 1,500 mg/l of As(V), with the reduction efficiency going as high as 90%. The As(V) resistance was due to the genes showing high similarity with arsC and acr3 genes of *Bacillus* sp. Some other reports have indicated that members of *Bacillus* genus do have 100 % reduction efficiency **(Cai *et al.*, 2016; Guo *et al.*, 2015; Yamamura *et al.*, 2003)**. *Bacillus* sp. reported from garbage leachates have been found to have tolerance up to 150 mg/l and bioeremediation efficacy up to 80% under the most optimized conditions **(Taran *et al.*, 2019)**. Although Jackson *et al.* **(2003)** has indicated an As(V) resistance of >7,500 mg/l (roughly 100 mM) as very high, the As(V)-resistance of R1 strain at 2000 mg/l was regarded as fairly high during this current piece of research work. This is because, its extent of As(V) tolerance and uptake was found to be the highest between the two bacterial strains that were studied during the investigation. Also, the concentrations of As(V) used for stress imposition under *in-vitro*/laboratory conditions were much higher than the concentrations found in the As-polluted aquifers and adjoining soils of Bengal Basin- an infamous stretch of As polluted zone in India. A modified suggestion given by Sarkar *et al.* **(2013)** indicates that, bacteria that can withstand an external As(V) stress of up to 0.5 mg/l (roughly 6.67 µM) can very well qualify as resistant bacteria, because this is the average As concentration prevailing in the As-polluted groundwater or aquifers. Thus, based on such an assumption, it is clear that strain R1 shows high As(V) resistance.

Heavy metal stress in the environment positively selects and maintains microorganisms that possess the genes encoding resistance-conferring functions. It has been reported that many microbes, besides having the potential to uptake inorganic As as an alternative substrate to useful nutrients, also possess the genetic ability that renders them resistant to both As(V) and As(III) and allows its use as natural primary substrates **(Silver and Phung, 1996; Rosen, 1999)**. It is well known that As(V)-resistance in bacteria is conferred by the arsC and arsB genes of the ars operon. The arsC gene encodes for an arsenate-reductase to reduce As(V) to As(III), while the arsB encodes for an arsenite efflux pump to throw the more toxic As(III) out of the cells. To avoid accumulation of As(III), the ArsC activity is almost always associated with ArsB activity **(Cervantes *et al.*, 1994; Gatti, Mitra and Rosen, 2000)**. Sometimes, a different class of arsenite efflux pump is encoded, by the acr3 gene **(Cavalca *et al.*, 2010)**. Nevertheless, the presence of ars genes or ars gene activities in bacteria isolated from As-polluted habitats has been stressed upon as a potential molecular bio-marker to not only assess the extent of As resistance within the bacterial community but also to indicate the levels of As pollution in that natural habitat **(Torres *et al.*, 2011; Liao *et al.*, 2011; Drewniak *et al.*, 2008; Jackson *et al.*, 2003)**. Based on this idea, it can be justified that the bacterial strain R1 reported in the present research work is well capable of showing As resistance due to the constitutive presence of arsC gene activity, i.e. As(V) reductase. Moreover, this enzyme activity is further enhanced on imposing As(V) stress at its maximum tolerable dose, suggesting its sensitive response to the external levels of As(V).

Apart from the fact that the bacterial strain R1 possesses an intrinsic ability to detoxify As(V), viz. As(V) reductase enzyme activity, it is also noteworthy that R1 shows anti-oxidant potential. The DPPH reduction calculated at λ= 517 nm confirm the anti-oxidant potential of the bacterial strain. This potential is further enhanced under As(V) stress imposition, indicating a role of the inherent ROS detoxification strategy in helping the bacterium survive better under stressed conditions. As far as our understanding and literature survey is concerned, it is for the first time that the anti-oxidant potential of *Bacillus cereus* group under As(V) stress has been reported.

Bacillus sp. associated with rhizospheric soil fractions having arsC gene activity have been reported earlier. In addition to this important attribute of As cycling, the genus has been reported to have PGP activities such as IAA and siderophore production and phosphate solubilization **(Cavalca *et al.*, 2010; Babu, Kim and Oh, 2013)**. Siderophores are low molecular weight (<1000 Da) iron-uptake systems found in microorganisms. Because iron (III) is highly insoluble at biological pH and because iron is bound to its carrier protein transferrin in many biological systems, microorganisms have evolved these highly efficient iron-chelating tools. Microbial siderophores help in the uptake of iron ions from insoluble minerals. During this process, As ions often get mobilized into the aqueous phase from the minerals, thus making it available to the bacterial cells. Thus, production of siderophores often times exposes the inorganic As to microbial arsenic-cycling in the environment and can impact the dynamics of As-cycling at naturally polluted sites **(Banerjee *et al.*, 2011).** IAA is the most abundant and effective auxin, a class of plant hormones that plays direct role in promoting plant growth and development, thus assisting the host plant in surviving under stressful condition **(Ma *et al.*, 2011)**. Thus, the PGP bacteria that can produce this molecule play a crucial role in plant's growth and their survival under varied conditions. Several PGPB that could produce IAA have been reported as potential bio-fertilizers **(Khalid *et al.*, 2004; Arkhipova *et al.*, 2005)**. Not only this, IAA has been reported to be an important signalling molecule for plant-microbe interaction **(Matsuda *et al*., 2017; Duca *et al.*, 2014)**. The role of *Bacillus* as a bio-fertilizer is well known, and as such the IAA biosynthetic machinery in this group of bacteria is also studied. It is indicated that members of *Bacillus* genus mostly use the tryptamine pathway of IAA production **(Goswami *et al.*, 2016)**. However, from our research it is indicated that R1 strain may also have a tryptophan-independent pathway for IAA biosynthesis, because of its ability to synthesize IAA in the absence of tryptophan. Moreover, the ability to synthesize IAA in an *in-vitro* set-up (that is, one lacking a potential plant host) and in absence of the precursor molecule tryptophan suggests a constitutive expression of the IAA biosynthetic pathway in R1 bacterial strain. It is noteworthy that under tryptophan supplemented conditions, R1 strain produces greater amount of IAA, indicating that tryptophan perhaps plays an inducible role on the IAA biosynthetic pathway.

The strain R1 classifies as an As(V)-resistant plant growth promoting bacilli (PGPB) because of the fact that it can display PGP traits alongside high As(V) resistance. It may be for the first time that a strain closely resembling *B. paranthracis* (i.e. R1, showing similarity with type strain Mn5) in its 16S rRNA gene chronometer has shown both As(V) resistance and PGP traits. However, it comes as no surprise because *Bacillus* sp. is a genus of popular PGP species. Many reports exist on As(V)-resistant PGPB *Bacillus* sp. that have influenced their respective host plants significantly under stressed conditions **(Das *et al.*, 2016; Ghosh *et al.*, 2018; Mallick *et al.*, 2018; Qamar *et al.*, 2017; Rahman *et al.*, 2019)**. Thus, the current investigation, which reports a unique As(V)-resistant PGPB from *Azolla microphylla* rhizosphere, can also contribute towards exploring the role of As(V)-resistant PGPB in As bioremediation from polluted ecosystems and influencing the growth of plants under As-stressed conditions. The bacterium can also be applied with the host plant *Azolla* sp. and form the basis of studying the rhizospheric interactions between PGPB and the plant rhizosphere.

CONCLUSION

The isolate R1 is a strain of *Bacillus cereus*, based on its unique FAME profile as well as 16S rRNA gene sequence. The colony morphology and biochemical characters also corroborates with that of *Bacillus* genus. The isolated strain shows extreme tolerance towards As(V). The arsC gene activity confirms the presence of functional ars operon involved in imparting arsenic resistance. This bacterial strain also possesses vital plant growth promoting activities such as siderophore and IAA production, which makes it ideal for boosting growth of the plants. The arsenic tolerance on the other hand regulates the threat of arsenic pollution. Presence of phosphate solubilising ability confirms its role as a Plant Growth Promoting Bacillus (PGPB), a group of bacilli that are routinely used as biofertilizers. Thus, this study successfully explores the potential traits of an arsenic-resistant PGPR that can be used in bioremediation, in conjunction with plant growth promotion.

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