Arsenic stress mitigation using a novel plant growth promoting bacterial strain Bacillus mycoides NR5 in spinach plant (Spinacia oleracea L.)

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Abstract

The present study aimed to identify arsenic (As)-resistant bacterial strains that can be used to mitigate arsenic stress. A bacterium Bacillus mycoides NR5 having As tolerance limit of 1100 mg L -1 was isolated from Nag River, Maharashtra, India. It was also equipped with plant growth-promoting (PGP) attributes like P solubilization, siderophores, ammonia and nitrate reduction, with added antibiotic tolerance. Further, Scanning Electron Microscopy (SEM) and Transmission Electron Micrograph (TEM) suggested biosorption as possible mechanisms of arsenic tolerance. A strong peak in FTIR spectra at 3379.0 corresponding to amine in As-treated NR5 also indicated metal interaction with cell surface protein. Amplification of the arsenic reductase gene in NR5 further suggested intracellular transformation of As speciation. Moreover, the As tolerance capability of NR5 was shown in spinach plants in which the bacterium effectively mitigated 25ppm As by the production of defence-related proline molecules. Evidence from SEM, TEM and FTIR, concluded biosorption is possibly the primary mechanism of As tolerance in NR5 along with the transformation of arsenic. B. mycoides NR5 with PGP attributes, high As tolerance and antibiotic resistance mediated enhanced As tolerance in spinach plants advocated that the strain can be a better choice for As bioremediation in contaminated agricultural soil and water.

INTRODUCTION

The rapid economic development unintentionally added a challenge for mankind to manage and clean up the environmental pollution piling up as a severe threat to environmental sustainability. One of the major environmental contaminants are the heavy metals including arsenic (As), cadmium (Cd), Lead (Pb), Nickel (Ni), Chromium (Cr), Cobalt (Co), Copper (Cu) and Zinc (Zn). Among this As in the last few decades has been found to be frequently discharged in groundwater possibly due to geogenic activity in Bay of Bengal. Studies suggests that a very minute quantity of As can cause serious health issues in human, animal and even in plants system. Arsenic is a group 1 carcinogen and thus chronic exposure may lead to neurodegenerative diseases, type 2 diabetes mellitus and even cancer (Tam et al. 2020). World Health Organization (WHO) has set 10μg L^{-1} as the permissible limit of As in potable water and up to 20 mg Kg^{-1} in agricultural soils. Recently Shaji et al. (2021) concluded that >100 countries are affected with arsenic contamination, among which Bangladesh and India from Asia are highly exposed to this metal (Chakrobarty et al. 2018; Bhowmick et al. 2018). Surprisingly, in India around 20 states along with four union territories are listed as arsenic affected area. A recent comprehensive study stated the threat of As toxicity to around 94 to 220 million population of which almost 94% is only from Asia (Podgorski and Berg, 2020). The methods of management of arsenic pollution in environment are only limited. The physico-chemical procedures that

included soil washing with nitric acid, sulfuric acid or adsorption by specific media, coagulation, precipitation, immobilization etc. are expensive and generate secondary pollutants. Among the biological methods, the phytoremediation was used either by free floating plants or rooted plants (Yan et al. 2020). Microorganisms with their inherent capabilities to tolerate heavy metals are the alternative simple, viable, cost effective and sustainable remediation tools for these contaminants. Biosorption by the bacterial cells is well known and simplest mechanisms in which heavy metals are adsorbed on the cell surface through different functional moieties, and accumulated at the cell membrane (Giri et al. 2013; Titah et al. 2018). This kind of interaction has been recognized through Fourier Transform Infra-Red Spectroscopy (FTIR) in various studies where amide, halide, nitro and hydroxyl chemical functional groups play an active role in metal sorption (Tian et al. 2012; Prasad et al. 2013; Singh et al. 2013; Altowayti et al. 2022). Molecular mechanisms pertaining to As tolerance have been considered as arsenate reduction (*ars*), arsenate respiratory reductase (*arr*), arsenite extrusion genes (*acr*), arsenite oxidation (*aio*) and methylation (Renu et al. 2020). Studies indicated that the bacteria followed above-mentioned strategies to combat arsenic toxicity. Dey et al. (2016) reported arsenite oxidation in *Bacillus* sp. and *Aneurinibacillus aneurinilyticus* isolated from As contaminated region in Burdwan district of West Bengal. Similarly, arsenic extraction efficiency of lower plants has also been increased by the inoculation of arsenic tolerant bacteria (Lampis et al. 2015). On the other hand, the importance of arsenate reductase gene (*arsC*) has been reported in majority of bacteria possessing arsenic tolerance (Sun et al. 2004; Suhadolnik et al. 2017). In addition to this, arsenic removal in few studies suggested mediation of arsenite extrusion genes (*acr*) (Gu et al. 2019) and arsenic respiratory reductase (Mirza et al. 2017). It has been established that the redox cycling of arsenic by microbes plays a crucial role in arsenic ion mobility in the environment (Stolz, 2006).

In the present study, we have reported As-tolerant bacterial strain*Bacillus mycoides* NR5 isolated from presumptive As contaminated site for its bioremediation potential and evaluated PGP ability in spinach. The study focusses on mitigation of As toxicity in spinach plants in presence of the selected bacterial strain. The study reports in-depth mechanism of As tolerance in the bacterium and its role in alleviation of As toxicity in spinach.

EXPERIMENTAL PROCEDURES

Survey and collection of samples from heavy metal contaminated sites

After a thorough survey of heavy metal contaminated soil, the bulk soil/rhizosphere soil of plants growing on metal-contaminated sites along with water samples from polluted water-bodies were collected from different locations of Jharkhand, Wardha; Nagpur, Maharashtra, India and Bhanpura, Bhopal (MP). All the samples were collected in sterilized polybags and tubes, and transported and stored in laboratory at 4. Samples were analysed for their pH level and the heavy metal content was estimated using ICP-OES (Perkin Elmer, USA).

Test chemicals and media

The stock solution of arsenic and other heavy metals was prepared by dissolving heavy metal salts (1,000 mg L^{-1}) in Milli-Q water and filter sterilized through 22-μm filter (Axiva). For arsenate (V) Sodium arsenate dibasic heptahydrate ($Na_2HAsO_4{\cdot}7H_2O$), and for arsenite (III) Sodium (meta)arsenite ($NaAsO_2$) salts were used. Beside these, salts like Cadmium chloride ($CdCl_2$), cobaltous nitrate hexahydrate ($Co(NO_3)2.6H_2O$), copper chloride ($CuCl_2$), potassium dichromate ($K_2Cr_2O_7$), lead nitrate ($Pb(NO_3)_2$), nickel sulphate hexahydrate ($NiSO_4.6H_2O$), and zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) were used to prepared stock solution of respective heavy metal. Working test metal solution (20 mg L^{-1}) was prepared by diluting the prepared concentrated stock solution. All the glasswares were acid washed with 10% nitric acid before use to avoid binding of metals. All the chemicals and culture media were of analytical grade and obtained from Himedia, Merck, Sigma, and BioRad.

Isolation and morphological characterization of Bacteria

Bacteria from contaminated samples were obtained by serial dilution method followed by growth on nutrient agar (NA) medium at 37degC +-1 for 24-48 h. Further pure bacterial colonies were subjected to morpho-

logical characterization and colonies with different colour, size, shape, margin, and texture were recorded through stereomicroscopy. (Renu et al. 2020).

Minimum Inhibitory Concentration (MIC) and Multi-metal screening

A total of 16 bacteria showed arsenic tolerance in preliminary examination were further evaluated for their minimum inhibitory concentration (MIC) level by spot inoculation of 100 µl of 10^8 cells/ml bacterial suspension on nutrient agar plates amended with sodium arsenate up to 1500 ppm and sodium arsenite solution up to 500 ppm separately, through pour plate method following incubation at 35°C (Dey et al. 2016). Comparative MIC value of 16 bacterial isolates for other heavy metals like Cadmium (Cd), Cobalt (Co), Copper (Cu), Chromium (Cr), Lead (Pb), Nickle (Ni), and Zinc (Zn) using respective salts was also determined. Based on high arsenic tolerance among 16 bacterial isolates NR5 was selected for further studies.

Adsorption isotherm

NR5 was raised in 100 ml nutrient broth medium (pH 7.0) with 100mg of arsenic (V), and flask without bacteria served as control. All the flasks were incubated at 30 for 48 h and 250rpm. Subsequently, bacterial culture was harvested at different time interval of 6h and bacterial pellet and liquid phase stored for arsenic content estimation using ICP-OES. The equilibrium arsenic concentration was plotted against arsenic content adsorbed on bacterial cell (mg/g), and calculated by using control. In order to understand adsorption behaviours and mechanism of arsenic removal by NR5, adsorption isotherm with consideration of Langmuir (Langmuir 1918) and Freundlich (Freundlich 1906) was studied. Langmuir described the monolayer adsorption pattern and hypothesized that numerous binding sites on the surface of biosorbent are available equally for metal sorption, calculated by using equation 2.

$$\frac{C_{\mathrm{eq}}}{q_{\mathrm{eq}}} = \frac{1}{q_{\mathrm{max}} b} + \frac{C_{\mathrm{eq}}}{q_{\mathrm{max}}}$$

where, C_{eq} is the arsenic content remained in liquid phase, q_{eq} is the amount of arsenic per unit mass of NR5, and the 1max is the maximum capacity of NR5. Here, b represents Langmuir constant (mgL^{-1}).

On the other hand, Freundlich isotherm represents multilayer sorption, summarize in equation 3 shows an empirical relation suggest non-liner relationship of arsenic concentration and amount.

$$q_{\mathrm{eq}} = K_f C_{\mathrm{eq}} \frac{1}{n}$$

where, q_{eq} is the amount of arsenic per unit mass of NR5. Kf is the Freundlich constant coefficient (mg/g), while n is the arsenic adsorption intensity (mg/g). In order to determine the rate of metal sorption, adsorption kinetic was investigated using following kinetic models Lagergren pseudo-first, Lagergren pseudo second-order, and Elovich. Table 1 demonstrating, arsenic adsorption capacity (qt) at equilibrium, k1, k2 as are rate constant for first- and second-order reactions.

Identification of arsenic tolerant NR5

Genomic DNA of NR5 was extracted using Nucleopore bacteria DNA extraction kit (Genetix Biotech, India), and isolated DNA was visualized in 0.8% agarose gel to ensure the proper extraction and purification. Yield and purity of extracted DNA was also checked by Nabi-UV/Visible Nano Spectrophotometer (MicroDigital, Korea) by measuring absorption ratio of A260/A230, and A260/A280. Subsequently, 16S rRNA gene was amplified in extracted genomic DNA through universal primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1542R (5' AAGGAGGTGATCCAGCCGCA 3'). A 25 µL reaction mixture was prepared using 1.0 µL of 100pM concertation of forward and reverse primers by Integrated DNA Technologies (IDT, Coralville, IA), dNTPs (200nM), and 2 µl of 100 ng template. In MyCycler (BioRad thermal cycler). PCR reaction was setup with the conditions - initial denaturation 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 30 seconds, 52°C for 30 seconds and 72°C for 1 minute and final extension at 72 °C for 5 minutes. The amplified

product was visualized on 1.5% agarose gel, and the bands obtained were compared to standard 1Kb DNA ladder (genetic biotech, India). The amplified band was further purified through Nucleopore PCR clean up kit (NP-36105, Genetix Biotech, India) as per protocol provided by the manufacturer. Sequencing PCR was performed with universal primers as mentioned above and Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) in a 3130×l Genetic Analyzer (Applied Biosystems, USA). The amplicon sequence obtained from ABI sequencer were analyzed for quality check and contig was prepared manually using Finch TV software (Geospiza, Inc). Sequence similarity was carried out by EZBioCloud database and also analyzed by NCBI-BLASTn algorithm (Yoon et al. 2017). Maximum percent identity score of first 20 sequence were selected and used for multiple sequence alignment using EZEditor2 (Jeon et al. 2014). Subsequently phylogenetic tree was constructed by Neighbor-Joining method with 1000 bootstrap value in MEGA X (Kumar et al. 2018). 16S rRNA gene sequence of the bacterium was submitted to NCBI GenBank, and well-characterized strain was submitted to ICAR-National Agriculturally Important Microorganisms Culture Collection (NAIMCC), Mau, India.

In vitro **evaluation of antibiotic resistance and PGP traits in NR5**

NR5 **was evaluated for its resistance/susceptibility towards** 20 antibiotics *viz* . Amikacin (AK) 30µg, Ampicillin (AMP) 10µg, Amoxycillin (AMX) 10µg, Cefadroxil (CFR) 30µg, Cefoperazone (CPZ) 75µg, Ceftazidime (CAZ) 30µg, Ceftriaxone (CTR) 30µg, Chloramphenicol (C) 30µg, Ciprofloxacin (CIP) 5µg, Cloxacillin (COX) 1µg, Co-Trimoxazole (COT) 25µg, Erythromycin (E) 15µg, Gentamicin (GEN) 10µg, Nalidixic Acid (NA) 10µg, Netillin (NET) 10µg, Nitrofurantoin (NIT) 300µg, Norfloxacin (NX) 10µg, Penicillin (P) 10Unit, Tobramycin (TOB) 10µg, and Vancomycin (VA) 30µg. Bacterial culture of *NR5* **was plated onto nutrient agar plate and antibiotic discs (Icosa 006, HiMedi** a Pvt. Ltd.) were placed over it. Plate was incubated at 30°C for 24 h to observe the bacterial tolerance against different antibiotics by measuring zone diameter.

Bacillus mycoides NR5 **was** screened *in vitro* for various plant growth promoting (PGP) traits like - Siderophore production by CAS agar method and Universal Chemical Assay [CAS] by Schwyn and Neilands (1987); Nitrate *reduction* Cappuccino & Sherman (1992); ammonia production by Ahmad et al. (2008); phosphate solubilization (Gaur,1990) and IAA production by Gutierrez et al. (2009) and Hydrocyanic acid (HCN) production as per method given by Miller et al. (1970).

Determination of biosorption capacities to assess the mobility and the bioaccumulation of As using different methods

Determination of growth pattern of NR5 and biosorption potentials under in vitro conditions

Based on MIC results out of 16 **potentials As tolerant bacteria** NR5 was **selected for a batch culture study over the period of 7 days and were grown in** presence of 90 mgL^{-1} As(V) in nutrient broth (NB) medium. **NB without bacteria served as control 1 and** NB without As served as control 2. All the flasks were incubated on a rotary shaker and O.D. was measured every 2 hours, and growth was recorded against a reference.

Scanning Electron Micrograph-Energy Dispersive Spectroscopy (SEM-EDS) and Transmission Electron Microscope (TEM)

Morphology of bacterial cells before and after arsenic treatment was examined using SEM (JSM 6490, JOEL, USA). The NR5 were raised in normal and 25 mg L^{-1} As amended nutrient broth. Cells were harvested by centrifugation at 3000 rpm for 10 minutes and collected in sample size of 2-4mm. Fixation of bacterial cells was carried out using 2.5% glutaraldehyde solution for 2-6 hours at 4°C, and subsequently washed with 0.1M phosphate buffer. Post fixation was done by treating samples with 1% osmium tetroxide for 2 hours at 4°C, and again, samples were washed with 0.1M phosphate buffer to remove unreacted fixative. Specimens were dehydrated using increasing concentrations of ethanol to remove water. Specimens were dried and mounted on aluminium stubs with carbon tape. Sputter coater used for coating and to make samples conductive. Finally, samples were visualized under SEM. Content of As in control and arsenic treatment NR5 was estimated using

energy dispersive spectroscopy (EDS). To perform TEM analysis of control and arsenic loaded bacterium, cells initially fixed in 2.5% glutaraldehyde and later with osmium tetroxide in sodium cacodylate buffer. Samples were dehydrated, thin sections were prepared using microtome, and visualized under (TEM Jeol 1011, Japan, 80KVA.)

Fourier transform infrared spectroscopy (FTIR)

Bacillus mycoides NR5 was grown on 25 mg L^{-1}amended medium and without As was taken as control. Cells were harvested by centrifugation and 2.0 mg dried biomass was mixed and ground with 98.0 mg of potassium bromide (KBr) in an agate mortar. Translucent discs were prepared by pressing the KBr grounded pellets with the aid of 14 tons of pressure beneath the bench press. The KBr pellets were immediately analysed with a spectrophotometer in the range of 400-4000 cm^{-1} with a resolution of 5 cm^{-1}.

In vitro **arsenic transformation assay**

Ability of *Bacillus mycoides* NR5, of transforming As (V) to As (III) was evaluated by the method of Simeonova et al. (2004). Bacterium grown on nutrient agar plates containing 100 mgL^{-1} and 20 mgL^{-1} As(V) and As (III) respectively. After growth agar plates were flooded with 100mM silver nitrate ($AgNO_3$) and placed in dark in incubator.

Molecular mechanism of arsenic transformation by NR5

Arsenic reductase (*arsC*) gene responsible for arsenic reduction from As (V) to As (III) was amplified in *B. mycoides* NR5 DNA. A primer set was designed from region of arsenic reductase gene using primer3 plus, and named *arsC* 1-F (5'GGAATTGAAGCACACGGAGT3') and*arsC* 1-R (5' CATCAAATCCCCAGTGAACA 3'). PCR condition for were initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 45 sec, 57°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 6 minutes.

Evaluation of application of *B. mycoides* NR5 on alleviation As toxicity in Spinach *(Spinacia oleracea L.)*

The effect of arsenic (V) on spinach plant was studied using pot experiment. Sandy loam textured soil was collected from non-contaminated fields, air dried, sieved through a 2mm mesh, homogenized and a total of 3.0 kg of air-dried sterilized soil was placed in each pot. As 25mg Kg^{-1} supplied in the form of **Sodium arsenate** dibasic **heptahydrate** ($Na_2HAsO_4.7H_2O$) was mixed with soil and kept for a week to stabilize the mixture. Plants without arsenic served as control. **Pot experiment was performed in** Completely Randomized Design (CRD) in three replicates**.** Seeds of spinach (*Spinacia oleracea* L.) *var* . F1-MULAYAM were disinfected with 1% sodium hypochlorite solution, washed with deionized water three times to completely remove disinfectant and then placed in Petri dishes inside the folds of wet filter papers at 25±1^{o}C for germinated. Four days later germinated seeds were withdrawal from wet paper towel and coated with 1% carboxy methyl cellulose (CMC) and air dried. Five coated seeds were placed in each pot and a control, and marked properly. This experiment was carried out in controlled conditions. The position of pots was changed on a regular basis to ensure similar light and temperature exposure. The plants were irrigated with deionized water (100 mL) twice per day and one set (from each concentration) of pot harvested after maturation at 45, 60, and 75 days after inoculation. The plants were washed with tap water and then deionized water and were cut down into shoots and roots. Fresh weights as well as length and shoot diameters and other physical appearance were recorded. Roots and shoots were oven dried at 70 ^{o}C for 48 h, powdered and sieved through 2 mm mesh size.

Measurement of growth and physiological parameters

Growth (number of leaves, germination percentage, roots and shoots length, root and shoot fresh weight and dry weight) and physiological (chlorophyll content and proline) content were assayed.

$$Germination\ \% = \frac{\text{Number of seed germinated}}{\text{Total number of seed used}} x100$$

Estimation of Biomolecules

The chlorophyll content in the fresh leaves was estimated by using the method of Arnon (1949). One gram of fresh leaf was weighed, homogenized and extracted in chilled 80% acetone (v/v), and the optical density of supernatant was read at 645nm and 663nm wavelengths in a spectrophotometer. Proline concentration was estimated by the method of Bates et al. (1973). Fresh leaves (250 mg) were homogenized in 10 ml of 3% aqueous sulphosalicylic acid and homogenate was centrifuged at 8000 × g for 15 min. A total of 2 ml aliquot of the supernatant was added with equal volume of glacial acetic acid and acid ninhydrin (prepared by dissolving 1.25 g ninhydrin in 30 ml acetic acid and 20 ml of 6N H_3PO_4) and incubated at 100°C for 1 h. The complete reaction was terminated by keeping mixture in ice bath and chromophore was extracted with 4 ml of toluene. This mixture shook vigorously for 15-20s and the chromatophore-containing toluene was drawing out from the aqueous phase and absorbance was determined spectrophotometrically at 520 nm with toluene for a blank. Proline content was calculated by following formula:

$$\underline{[(mg\ proline/ml\ \times ml\ toluene)/115.5\ \ mg/mmole]}$$

english[(g sample)/5]=μmole proline/g of fresh weight material

Statistical Analysis

All experiments were conducted in three replicates and data recorded was statistically analysed using Statistics 24.0 (SPSS Inc, Chicago, IL, USA). Mean of treatments was compared by analysis of variance (ANOVA) followed by Duncan's multiple range post hoc with significance level p

RESULTS

Heavy metal content in samples collected from polluted sites

The pH of samples collected from contaminated soil and water ranges 5.8 (BB) to 11.5 (MA), and EC 0.8 to 3.0 mS. Heavy metal content of polluted soil and water samples for various heavy metals like Cd, Co, Cu, Fe, Mn, Ni, Pb, and Zn was also measured by ICP-OES (data not given).

Isolation and morphological characterization of bacteria

A total of 77 bacterial isolates from different contaminated soil and water samples were isolated by serial dilution method, morphologically characterized and tested for tolerance against arsenic (Table S1). All the bacteria obtained from contaminated samples tested for growth condition and the optimum temperature for growth was 37°C, while optimum time to grow ranged from 12 to 48 h. In addition to this colony color was recorded from white to yellow to brown, and colony shape was generally round with pointed at end, sometimes irregular and colony thread-like size ranged from 0.01 to 1 cm; surface smooth or rough and elevation was either flat or convex or raised. **A total to 16 different morphotypes obtained from contaminated samples showed tolerance against arsenic.**

Minimum Inhibitory Concentration (MIC)

MIC of the 16 most potential bacteria ranged from 500 -1100 mgL^1 for arsenate (V) and 100-600 mgL^1 for arsenite (III). Out of 16 arsenic tolerating bacteria, NR5 exhibited maximum tolerance i.e. 1100 mgL^{-1} against As (V) followed by NR1, MB1, MA7 and MA8 (1000 mgL^{-1}) (Table 2). As (III) tolerance of NR5, NR1 and MB 1 was recorded as 400mgL^{-1} followed by MA7 (200 mgL^{-1}).

Growth pattern and multi-metal screening of NR5

Growth pattern of the most potential bacterium NR5 was observed under varying concentration of As (V). It was noticed that increasing concentration of arsenic extended the log phase of growth (Figure 1b). NR5 was found to tolerate multiple heavy metals at different concentrations, As (1100), Cd (100), Co (150), Cu (200), Cr (500), Pb (1000), Hg (100), Ni (150) and Zn (400) mgL^{-1}. We reported that heavy metal tolerant bacterium NR5 exhibited tolerance against all 9 tested heavy metals, and explored for the possible mechanism of metal tolerance of the organism. Further, high concentration tolerance of arsenic in NR5 makes it a candidate bacterium to further explore its role in bioremediation of As in spinach crop plant.

Adsorption Isotherm

In order to determine the sorption capacity of NR5 biosorbent it is required to generate an equilibrium data set which can further be subjected to Langmuir and Freundlich adsorption isotherm equation. Biosorption potential of NR5 was evaluated at concentration of 100 mgL^{-1} of As (V), and found that concentration of arsenic (V) on NR5 cell surface increased with time progression, and reached at a saturation point. This possibly because of high availability of arsenic in medium and thus result in enhanced arsenic biosorption capacity. Further when all surface is entirely covered with As (V) adsorption potential reached to limit, and can be defined as maximum biosorption capacity. The results suggested that both Langmuir and Freundlich isotherm fitted, although Langmuir was comparatively more demonstrative than Freundlich (Figure 2a) with a higher correlation coefficient value (R^2). Freundlich exhibits some degree of linearity but Langmuir model was perfectly fit (Figure 2b). Thus, results advocated of monolayer adsorption possibly due to topology of bacterial (NR5) cell and binding sites. In addition to this when adsorption behavior was evaluated using Elovich, Pseudo-first order reaction couldn't explain adsorption precisely due to its low correlation coefficient value, although on the other hand second-order kinetic model pronounced adsorption fittingly with correlation coefficient $R^2 > 0.992$. Thus, pseudo second order kinetic model was best fitted in arsenic removal

Identification of arsenic tolerant NR5

Based on arsenic and multiple metal resistance evaluation, NR5 was found the best candidate for further screening and experimentation of arsenic removal study *in vitro* and *in vivo* . The colony characteristics of NR5 was rhizoid, large, flat, non-pigmented colonies with filiform margins (Figure 1a). Gram staining described the bacteria as Gram positive strain. Genomic DNA isolated from NR5 was visualized on 1% agarose gel electrophoresis. Spectroscopic estimation results DNA yield as 250ng/µl. A260/A280 and A260/A230 ratio were 1.78 and 1.29 respectively, and results confirmed of no RNA and protein contamination in extracted DNA. The gDNA was also free from phenolic contamination. Sequence data obtained from ABI Sanger sequencer was subjected to quality check. Peaks in the data were sharp with no noise, and there was no chimeric sequence found in the sequences. EzTaxon represents that submitted sequences were belonging to *Bacillus mycoides* and thus NR5 labeled as *Bacillus mycoides* NR5 (Figure 3). The sequence was deposited in GenBank with accession number KX268123. The culture was submitted to National Depository ICAR-NAIMCC, Mau, India, with the accession number NAIMCC-B-02103.

In vitro **evaluation of Antibiotic resistance and plant growth promoting traits in NR5**

B. mycoides NR5 was tested against 20 antibiotics and was found resistant to 16 antibiotics. NR5 was found to be highly resistant recorded against Amikacin, Ciprofloxacin, Co-Trimoxazole, Erythromycin, Gentamicin, Nalidixic Acid, Netillin, Nitrofurantoin, Norfloxacin, and Tobramycin, moderately resistance to Cefadroxil, Ceftriaxone, Cloxacillin, and Vancomycin, and low resistance to Cefoperazone and Ceftazidime. There was no growth inhibition in Ampicillin, Amoxycillin Chloramphenicol and Penicillin (Table S2).

B. mycoides NR5 was evaluated for having plant growth promoting attributes such as phosphate solubilization, IAA production, siderophore, ammonia production and nitrate reduction. The qualitative test for P-solubilization was found positive, and the subsequent quantitative estimation of *B. mycoides* NR5 was calculated as 115.51 ± 0.47 μgL^{-1}. The NR5 was also found to possess siderophore production ability and the index for production of siderophore was calculated to be 28.61% unit (Table S3). Similarly, ammonia production, which is also an important PGP trait, was also high and the bacterium was a good ammonia producer. NR5 was also found positive for nitrate reduction assay but the efficiency was not high.

Biosorption capacities to assess the mobility and bioaccumulation of heavy metals

Determination of biosorption potential under in vitro conditions

To observe morphological changes due to arsenic stress, micrograph of normal cell (Figure 4a) and arsenic loaded (Figure 4c) was compared using SEM. *B. mycoides.* NR5 expressed distinct morphology and appeared as tangled mass because of its cellular morphology. The cells with no arsenic appeared normal while in arsenic stress condition, visible changes on cell surface morphology were seen as swollen and large in size which possibly indicated the accumulation of heavy metal. Along with SEM micrograph estimation of arsenic through EDS also showed the presence of arsenic metal over microbial cell surface (Figure 4d) whereas, no arsenic was recorded in control bacterial cell surface (Figure 4b). On the other hand, Transmission Electron Micrograph of*Bacillus mycoides* NR-5 having large irregular cells also shows surface deposition as well as intracellular accumulation of arsenic (Figure 5 a & b). Result of TEM analysis also supports observation of SEM that such morphological changes might have happened due to interaction of cells to arsenic.

Fourier transform infrared spectroscopy (FTIR)

In order to recognize the surface functional molecules involved in biosorption of As (V), FTIR spectra of normal and As treated bacteria was studied. Infra-Red spectra of *Bacillus mycoides* NR-5 (control) reveals three short peaks at 3588.8, 3277.9 and 2923.7 cm^{-1} (wave number) representing presence of alcohol and alkyl groups. Arsenic biosorption on the cell surface results in a a single broad and strong peak at 3379.0 Cm^{-1}corresponding to amine and indicated interaction of surface protein-metal complex. In zone 5, two peaks at 1654.0 Cm^{-1} and 1541 Cm^{-1} attributed to the amide (I and II) bands were shifted to 1652.9 and 1541.0 Cm^{-1} strengthening the possibility of metal interaction with surface proteins (Figure 6 a & b).

In vitro **arsenic transformation assay**

In present study *B. mycoides* NR5 when tested for arsenic ion transformation found that bacterium possess arsenate reduction potential and left yellow precipitate of silver ortho-arsenite (Ag3AsO3) in the medium. Further, to confirm the results obtained from agar plate were repeated through micro plate method and interestingly found the similar results. The quantitative reaction of silver nitrate with arsenic ions resulted in the formation of yellow coloured precipitate that indicates the arsenate reduction potential of NR5.

Molecular mechanism of arsenic transformation by NR5

Amplification of arsenic reductase gene (arsC) in *Bacillus mycoides* NR5 indicating partial contribution in arsenic tolerance mechanisms of bacterium. Primer (arsC1) designed using arsenic reductase gene sequences amplified 202bp amplicon. The amplified product was sequenced and analyzed which shared homology to arsenic reductase. The nucleotide sequence was submitted to Genbank with Accession No OP086227.

Evaluation of *B. mycoides* NR5 on alleviation of As toxicity in Spinach *(Spinacia oleracea L.)*

Exposing leaf vegetables to arsenic resulted in the growth retardation. A delayed development in arsenic stressed plants compared to the control was observed, and found that As 25mg kg^{-1} soil significantly reduced the shoot (32.39%) as compared to control plants respectively (Table 3). Although root length was slightly increased in arsenic treatment plant. On the other hand, shoot fresh weight was reduced in metal stressed plant, but bacteria augmented metal stressed plants could lower down the arsenic toxicity and thus achieved the improved growth than stressed plant with no bacteria augmentation.

Similarly, dry weight of root and shoot also confirm the plant growth retardation in arsenic stressed plants. Apparently, the plants grown with bacteria were having bulgy roots which confer the exhibition of plant growth promoting traits in bacteria augmented plants under heavy metal stress. Physiology of spinach plant was also altered under heavy metal stress. Chlorophyll content estimation revealed the changes in amount of pigment content in expanding leaves. Chlorophyll-a concentration was significantly lower than that of chlorophyll-b in both tested plants. The pigment contents in plants showed an almost linear decrease in

response to arsenic concentration increases in the soil. The total chlorophyll content of control plants at 45, 60, and 75 DAS was 82.22±3.71, 93.55±1.32, and 99.11±1.07 μgml^{-1}respectively, which reduced on arsenic treatment i.e., 55.24±0.92, 48.59±2.17, and 77.65±5.42μgml^{-1} respectively (Table 4), however when plant added with arsenic and NR5 exhibited 39.1% improved growth as compared to plants with As but no NR5, and thus confirm NR5 potential in reducing metal uptake and toxicity in plants (Figure 7).

DISCUSSION

Increased industrialization raised the level of arsenic and other heavy metals in the groundwater (Mazumder et al. 2020; Shaji et al. 2021), agricultural land (Shrivastava et al. 2017) that ultimately affected plant and human life. Various studies demonstrated wide spread occurrence of arsenic in different regions. Shukla et al. (2019) revealed high concentration of arsenic of up to 300ppb in the ground water of West Bengal, India. Many districts location of Bihar, India were also found contaminated with arsenic, out of which half were highly effective (50-100ppb) and even five districts were severely affected with >100ppb arsenic (Kumar et al. 2021). Present study also reported high concentration of arsenic in agricultural soil, and water samples collected from different regions in India, and results are in accordance with previous reports. Microbes are well known for stress tolerance in environment. In order to access indigenous bacteria having arsenic tolerance we isolated a **total of 16 potential arsenic tolerant bacterial isolates from samples collected from various contaminated sites. Importance of microbes in combating stress has been discussed in different studies. High pollution level in an environment corresponds to high microbial diversity has been reported earlier (Lang and Cai et al. 2009). Previous studies demonstrated that arsenic and other heavy metal contaminated soil possess a diverse range of microbial population that has been reported as heavy metal tolerant microbes (** Turpeinen et al. 2004; Sanyal et al. 2016; Suhadolnik et al. 2017; Cavalca et al. 2019; Zhai et al. 2020)**. In addition to this,*Bacillus mycoides* NR5 exhibited resistance to multiple heavy metals. Bacteria can possess multiple metal resistance. In our study*B. mycoides NR5* also exhibited resistance to again 9 heavy metal tested. Following studies also** indicated multiple metal resistance in various potential microorganisms. Keramati et al. (2011) reported multiple metal resistance in 6 bacteria against Cd, Zn, Ni, Pb, Cu and Ag. Vallalar et al (2019) evaluated 20 bacteria against five metals Cu, Cr, Co, Ni, and Zn and found resistance among them in the range of 25 to 400 mgL^{-1}. Multiple mechanism of metal resistance in bacteria was reported by Nanda et al. (2019). The metal transport across cell membrane, accumulation of metal in cell membrane, metal entrapment and redox reaction are the basic mechanisms of multiple heavy metal tolerance in bacteria. In addition to this metallothioneins are also a key player in arsenic resistance.

Arsenic removal and adsorption behaviour by *B.mycoides*NR5 was investigated using adsorption isotherm by Langmuir (Langmuir 1918) and Freundlich (Freundlich 1906) isotherm model, that describes the monolayer/multilayer adsorption of molecule on cell surface. It was found that both models supported the adsorption behaviours of arsenic by*B. mycoides* NR5, but Langmuir was most fit in present study. Our data is also in accordance with previous studies where in monolayer sorption was recorded in bacteria mediated heavy metal and other hazardous molecules (Andjelkovic et al. 2017; Altowayti et a. 2022; Glatstein et al 2018; Sarim et al. 2019). *Bacillus mycoides is a member of Bacillus cereus group and is easily distinguishable from other closely related microbes (* Di Franco et al. 2005*). Bacillus* is well known species to possess tolerance against various kind of stresses, and multiple species of *Bacillus* have been reported as arsenic tolerant species. Shivaji et al (2020) isolated an arsenic tolerant novel species. Few other studies are as follow;*Bacillus subtilis* (Pepi et al. 2011); *B. safensis* (Raja and Omine, 2012); *B. flexus* (Marwa et al. 2019); *B. megaterium* (Islam et al. 2019) *B. cereus* (Ayangbenro and Babalola 2020), *B. firmus* (Bagade et al. 2020). Heavy metal resistance and antibiotic resistance are two aspect and their gene generally located on plasmid, extra cellular DNA material. Co-occurrence of Heavy metal (HM) and antibiotics resistance (AR) have been reported in various studies (Chen et al. 2015; Chen et al. 2019; Glibota et al. 2020). In a study by Li et al. (2017) explained that genetic distance linkage between HM and AR genes is closer and potential of co-transfer in genomes is also high, and thus play a key role in shaping resistance against both.

In order to strengthen the candidature of potential bacterium for arsenic tolerance It should also support in plant growth and development if applied in field condition. Hence, NR5 was also tested for any plant growth promoting, and found that along with multiple heavy metal and antibiotic tolerance bacteria also exhibited plant growth promoting attributes such as phosphate solubilization, IAA production, siderophore, ammonia production and nitrate reduction. The presence of heavy metal tolerance along with the plant growth promoting traits in microorganisms has been documented in some previous reports (Yu et al. 2014; Tirry et al. 2018; El-Meihy et al. 2019; Jain et al. 2020; Renu et al. 2020). As the plant growth promoting bacteria are residing in the close vicinity of plant roots and the soils contaminated with arsenic or any other heavy metal, they are supposed to develop partial resistance against heavy metals, and thus undoubtedly play an important role in plant metal toxicity remediation.

Heavy metal interaction with cell surface make many conformational changes that can have effect on cell surface topology. To Investigate changes on cell surface of NR5 scanning electron microscopy (SEM) and transmission electron microscopy (TEM) was used and observed that metal interaction causes swollen surface with deposition of metal. Alteration in cell surface topology due to exposure of arsenic has also been observed in following studies (Pandey and Bhatt, 2015; Felestrino et al. 2018; Mujawar et al. 2019). Surface chemistry probably play an active role in the interaction of metal to cell surface. Sinha and, Mukherjee (2009) and El-Helow (2001) showed that, *Bacillus* sp. containing cell wall components such as polysaccharides, teichoic and teichuronic acids or phospholipid layers and other functional groups might be responsible for the heavy metal interaction through extracellular substance secretion. The cell surface morphological changes in *Cryptococcus* sp. and *Ochrobactrum intermedium* BB12 after heavy metals exposure appeared as shrunken and distorted cell wall in the presence of Cd and depressions in the presence of Pb and Zn (Singh et al. 2013, Renu et al. 2022). The results of the present study also confirmed morphological and topological changes of bacteria exposed to heavy metals. In addition to this FTIR spectra also indicated as cell surface amine group involvement in arsenic interaction and advocated biosorption as principal mechanism of arsenic removal opted by NR5.

Beside cell surface adsorption bacteria can also transform metal in to a different state so that either its impact gets reduced or it can eliminate from cell environment using porins. Presently study through silver nitrate assay where left yellow precipitate of silver ortho-arsenite (Ag3AsO3) in the medium indicate arsenate reduction might be a secondary mechanism for arsenic removal by NR5. Ability of microbes to reduce arsenic was evaluated by arsenic transformation assay using silver nitrate ($AgNO_3$) methods. Test is based on the reaction between arsenic ion and silver nitrate qualitatively (Krumova et al. 2008). Silver nitrate mediated arsenite oxidation potential (Rehman et al. 2010; Dey et al. 2016; Selvankumar et al. 2017) and arsenate reduction (Das and Barooah, 2018) have been determined in multiple studies. Bacterial cell after transforming As(V) to As (III) efflux the arsenite As(III) out of cell system through arsenite transporter proteins (Sarkar et al. 2013). Afterward the PGP trait evaluation, tentative confirmation of mechanisms of arsenic removal by NR5, bacterium was subjected to test in *in planta.* Results showed that bacterium amended plant were able to reduce arsenic toxicity and increased plant growth and development in comparison to plants without bacterium. Multiple studies have indicated that due to accumulation of heavy metal in plant parts leading to several cell function disruption there is decrease in shoot and root length (Sandil et al. 2019; Jamil et al. 2024). Metal resistant bacteria can reduce heavy metal uptake in plant through various defence mechanisms such as precipitation, volatilization, methylation, oxidation, reduction, complex formation, alkalization or biosorption (Etesami, 2018). Bacteria have developed mechanisms of extrusion of As to out of cell. A special resistance mechanism of As in plant transform arsenate to arsenate phosphoglycerate and transport this complex out of cell using efflux transporter (Zhang et al. 2022). In addition to this glutathion, thioredoxin, kinase and s-transferase are also responsible for oxidative stress tolerance (Gautam et al. 2020). Although there was a little increase in root length in As stress plants till 60 DAS, but it declined in 75 DAS. It could be due to As accumulation in roots over the period. Studies have shown that there was no significant changes in root length in case of cadmium stress (Lu et al. 2023). Overall bacteria amended plant showed improved growth close to control plants. Estimation of defence related biomolecule proline is an active indicator of stress level in plants; thus, all the plants were tested for their proline level at

different time intervals. Proline, an imino acid, is well known for accumulation under abiotic stress. Study demonstrated that proline accumulation helpful to adjust osmotic pressure at cellular level followed by protection of intracellular organelles from disintegration. In addition to this, proline also serves as nitrogen storage which can be a nutrient source to resume the cell growth after releasing stress (Chandrashekhar and Sandhyarani 1996). Increase in the osmoprotectant proline content was directly proportional to the heavy metal concentration. In comparison to control plant at 45 DAS the proline content in As treated plants was increased 2.390 µmole/g fresh weight. On the other hand, As and NR5 inoculated plants exhibited reduced intracellular proline concentration 1.726 µmole/g. Stress induced rapid enhancement in proline accumulation in plants is reported in various studies (Ghosh et al. 2017; Awasthi et al. 2018; Renu et al. 2020), and the possible mechanism of defence might be chelating ability, scavenging of free radical and increased activity of electron transport chain.

CONCLUSION

Bacillus mycoides NR5 isolated from polluted Nag River demonstrated presence of high arsenic tolerance. The primary mechanism of As resistance in NR5 was observed as biosorption. Whereas, the silver nitrate assay indicated arsenate reduction potential of NR5 and subsequently the amplification of arsenate reductase gene *arsC* 1 in NR5 genome also suggested possible role in arsenic tolerance in NR5. Moreover, *B. mycoides* NR5 also possess attributes pertaining plant growth promotion by producing Siderophore, IAA, nitrate, ammonia production and phosphate solubilization, hence, is an appropriate choice for application in crop plants for reduction in uptake of As in plants and also promote growth for sustainable environment.

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Conflict of Interest

The authors declare no conflict of interest

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Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose

Author Contributions

Renu Shukla conceptualized and supervised the experiment, Khan Mohd Sarim, Manish S. Bhoyar performed the experiment, Khan Mohd Sarim analyzed the data. Khan Mohd Sarim and Renu Shukla, and Dhanjay P. Singh wrote the manuscript. Baljeet Kaur performed Transmission Electron Microscopy. All authors read and approved the final manuscript.

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Figure 1: Colony morphology (a) and growth pattern (b) of NR5 under different arsenic concentration

Figure 2: Langmuir (a) and Freundlich (b) isotherm for arsenic biosorption by NR5

Figure 3: Neighbour-joining tree based on 16S rRNA sequence of arsenic tolerant bacteria NR5 strain by MEGA 11. The scale bar corresponds to 0.02 substitutions per position. The numbers at nodes indicated the levels of bootstrap support (%) based on 1,000 resample data sets; only values above 50% are given.

Figure 4: Scanning Electron Microscopic image of *B. mycoides* NR5 without As (V) (control) (a); with 25 mg L^{-1} arsenic (c); EDS analysis of the isolate NR5, showing no arsenic in control (b); and significant arsenic distribution in the treated bacterial strain (d).

Figure 5: Transmission electron micrograph of (A) *Bacillus mycoides* NR5 grown in (a) nutrient broth (control) (magnification, ×20,000) and (b) in media containing 25 mg L^{-1} As (V) concentration (magnification, ×20,000).

Figure 6: Infrared spectra of (A) *Bacillus mycoides* NR5 grown in nutrient broth (control) and (B) media amended with 25 mg L^{-1} As (V) concentration.

Figure 7: Growth of spinach in the soil treated with As (V) and amended with the *Bacillus mycoides* NR5 at 60 DAI (a) Control: plants grown in the soil without any treatment; (b) plants grown in soil containing 25 mg kg^{-1} As (V) (c) plants grown in the soil containing 25 mg kg^{-1} As (V) inoculated with*Bacillus mycoides* NR5

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