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Research Article

Arsenic bioremediation and bioactive potential of endophytic bacterium *Bacillus pumilus* isolated from *Pteris vittata* L.

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ABSTRACT

Endophytic microorganisms have been found to colonize virtually every healthy plant species that exist on the earth. In an attempt to study the endophytic bacterial diversity, the common fern *Pteris vittata* L. were collected from the different regions of West Bengal including Darjeeling hills. A total of 15 phenotypically distinguishable bacterial endophytes were isolated from surface sterilized frond, rhizome and root tissues of *P. vittata* using nutrient agar (NA), tryptic soy agar (TSA) and glycerol aspargine agar (GA) media. Shannon Weaver diversity index has clearly revealed more diverse types of endophytes in rhizome (1.75) than in roots (1.72) and frond (1.09). The isolated endophytes were screened for their heavy metal tolerance and the isolate DSKP8 was found to tolerate 8 mM of As, Ni, Co and Cd. Based on morphological, physiological and biochemical characters along with 16S rDNA sequence analysis, the isolate was identified as *Bacillus pumilus* (Genbank Accession No. KJ881418). To determine the role of the endophytic bacterium DSKP8 on arsenic remediation *P. vittata* L. was grown in arsenic spiked soil inoculated with the bacterium DSKP8 and plant growth and arsenic uptake were analyzed. The uptake of arsenic was significantly higher in bacterial inoculated plants, 2.58 mg kg^{-1} in shoot and 1.06 mg kg^{-1} in root in comparison to non-inoculated arsenic treated plants. It is apparent that the integrated use of *P. vittata* with the microbe *B. pumilus* can enhance the plant growth and uptake of arsenic, and have potential application for the remediation of arsenic contaminated sites.

Keywords: Bacterial endophytes/ Heavy metal bioremediation/ Arsenic uptake/ Bioactive compounds/ *Pteris vittata* L/ Antimicrobial activity

1.INTRODUCTION

Since ancient time, ferns and fern allies have been used as ornamentals and in human health benefits as herbal medicine in many regions of world [32,31]. More recently the functional activities of these plants in human health care are being explored through advance scientific approaches [21]. *Pteris vittata* L. the evergreen perennial lithotrophic fern species having worldwide distribution has received much attention in the recent past due to its remarkable

capacity of arsenic hyperaccumulation, antimicrobial and antioxidant activities [30,25,44,22]

Pt. vittata is not only capable of producing various matabolites of pharmaceutical and industrial importance but also are able to cope up with metal toxicity specifically due to arsenic [22,34]. As the technologies for remediation of arsenic contaminated soil and water by chemical or physical means have been found to be very costly and result in secondary pollution. Phytoremediation by *Pt. vittata* has emerged as a less costly and environment friendly *in-situ* remediation technology. Microbe assisted phytoremediation has been established as a promising method for cleaning-up of soil contaminated with metals and organic pollutants. For bioaugmentation, it is essential to inoculate the contaminated sites with suitable microbes so that they can thrive in the soil and degrade the target compound(s) with considerable efficacy [1,35].

Ma et al., [22] and several others [37,35], have revealed that the rhizospheric microflora have the capability to play a very important role in plant growth and bioremediation process because the metabolic activity of microbes can enhance plant nutrition uptake, promote plant tolerance to heavy metals, and increase the synthesis of plant growth factors, etc. [26,36,37,35]. While the current research activities have focused attention on symbiotic or parasitic plant-microbe interactions, other types of associations between plants and microorganisms, such as endophytic microbial associations have often been overlooked. Endophytes are microorganisms, mainly fungi and bacteria, which live inside the plant tissues without causing any disease symptoms [28,2]. However, endophytic bacteria of *Pt. vittata* appear to be an underexplored and less studied group of microorganism and represent an open field of study for exploiting them in bioremediation of heavy metals and production of novel bioactive metabolites.

The present study was carried out to isolate the endophytic bacterial diversity of *Pt. vittata* L. and evaluate the bioremediation and bioactive potential of these endophytic bacterial strains to improve tolerance and accumulation of heavy metals and metalloids by the host plant and to enhance the synthesis of bioactive metabolites.

2. MATERIAL AND METHODS

2.1. Collection of plants

Healthy and disease free *Pteris vittata* L. were collected from natural populations growing in different parts of West Bengal, India during July-September 2013. Collected whole plant samples were placed in zip lock plastic bags, preserved at 4^{O}c during transportation and until being used for the isolation of endophytic microorganisms.

2.2. Isolation of endophytes

The collected fern samples were washed thoroughly in running tap water and cut into small pieces for surface sterilization. Surface sterilization of plant organs was accomplished by performing a stepwise washing in i) 70% ethanol for 30s, ii) sodium hypochlorite solution (3% v/v available chlorine) for 3 min, iii) 70% ethanol for 30s followed by three rinses in sterile distilled water. To confirm that the plant surfaces were effectively sterilized, 100 µl aliquots of the washings were plated onto tryptic soy agar (TSA) (Tryptone 1.5%, soytone 0.5%, glucose 0.25%, NaCl 0.5%, agar agar 2.0%, pH 7.3) and incubated at 30^{o}C.

For isolation of bacterial endophytes, the surface sterilized frond, stem and root segments were cut into 4 x 4 mm pieces sections and placed aseptically on the surface of the previously prepared nutrient agar (NA), glycerol asparagines agar (GA), and tryptic soy agar (TSA) plates. The nutrient agar (NA) contained (g/l) peptic digest of animal tissue 10.0, beef extract 5.0, sodium chloride 5.0, agar agar 20.0, pH 7.0). Glycerol asparagine agar (GA) contained (g/l) asparagine 0.5, glycerol 1.0, K_2HPO_4 0.5, agar agar 20, pH 7.2; while tryptic

soy agar (TSA) was composed of (g/l) tryptone 1.5%, soytone 0.5%, glucose 0.25%, NaCl 0.5% agar agar 2.0%, pH 7.3. The plates were incubated at 30ºC for 2–4 days and observed for growth of bacterial colonies surrounding the leaf, stem and root sections. Pure cultures of bacterial endophytes were developed by dilution-streaking on the same media and morphologically distinguishable isolates were indexed and maintained on slopes of the same media by repeated sub-culturing at monthly interval and stored at 4°C until used.

2.3. Diversity analysis

Colonization rate was calculated as the total number of plant segments infected by bacteria divided by the total number of segments incubated. Isolation rate was determined as the number of bacterial isolates obtained from plant samples divided by the total number of samples incubated. The Shanon Weaver diversity index H' was calculated as: $H' = -\Sigma\ Pi$ X ln Pi, where, Pi is the proportion of individuals that species "*i*" contributes to the total.

2.4. Screening of endophytes for heavy metal tolerance

To check the heavy metal tolerane, the isolated bacterial strains were allowed to grow on nutrient agar (NA) plates containing different concentrations (1-9 mM) of arsenic ($Na_2HAsO_4.7H_2O$), cadmium ($CdCl_2$), nickel ($NiCl_2.6H_2O$), cobalt ($CoCl_2.6H_2O$), zinc ($ZnSO_4.7H_2O$) and chromium ($K_2Cr_2O_7$). The plates were incubated at 32ºC for 24 h and visually observed for the extent of growth. After screening, DSKP8 strain was able to grow upto 8 mM concentraton of As, Cd, Ni, and Co. This bacterial isolate could tolerate the highest concentration was selected and identified by its morphological, physio-biochemical and functional properties.

2.5. Characterization and identification of selected bacterial isolate

The selected bacterial isolate was subjected to morphological, physiological and biochemical tests following standard microbiological method. A genotypic study was used to confirm the identity of the isolate. For the amplification of 16S rRNA gene, pure culture of the bacterial isolate was grown overnight in liquid nutrient broth. For isolation of genomic DNA the method as described by Hiney et al., [14] was used. A PCR product of 1200 base pairs16S rDNA was sequenced with a pair of the universal primers (Forward primer 5'-AGAGTTTGA-TCCTGGCTCAG-3' and reverse primer 5'-CTTGTGCGGGCCCCC-GTCAATTC-3'), and data were analysed as described earlier by Hiney et al., [14]. Sequence data of the isolate have been deposited in the GenBank, with accession number KJ881418. Sequence analysis of the isolate was compared with16S rDNA sequences using BLAST search in the NCBI, GenBank database (http://www.ncbi.nlm.nih.gov).

The 16S rDNA sequencing of PCR product was carried out in 454 sequencing from Life Sciences at CSIR-NBRI. Related sequences were searched using BLAST programme from the GenBank database (http://www.ncbi.nlm.nih.gov/blast/). The multiple sequence alignment was made using Multalin software version 5.4.1. Molecular phylogenetic tree was created by using phylogeny programme of Information Genomique et. Structurale software (http://www.phylogeny.fr) to evaluate its extent of difference in homology with other organisms.

2.6. Functional characterization of isolate

Assessment of plant growth promoting properties such as phosphate solubilization, indole-3-acetic acid (IAA) production and siderophore production by the isolated endophytic bacterial strain was done following the methods as described by Srivastava et al., [37].

2.7. Antibiotic sensitivity profile

Antibiotic sensitivity profile of the isolate DSKP8 was determined following the Kirby Bauer disc-diffusion method using antibiotic impregnated discs (6 mm dia. Himedia India) [4]. Based on the diameter of inhibition zone

recorded to nearest mm, the organism were categorized as resistant, intermediate and sensitive following DIFCO Manual 10th edition [10]. Antibiotics used include bacitracin, erythromycin, vancomycin, penicillin, chloramphenicol, methicillin, nystatin, chlorotetracycline, gentamycin and kanamycin.

2.8. Evaluation of antimicrobial activity

Antimicrobial activity of selected bacterial endophyte DSKP8 was assessed against *Bacillus subtilis, Bacillus cereus, Escherichia. coli, Pseudomonas cepacia* and *Staphylococcus aureus* following cross-streak method on nutrient agar (NA) plates by the method as described by Pal et al., (2012) [27]. The isolate which inhibited growth of any of the test isolate(s) was considered having antibacterial activity and the length of inhibition zone was measured to nearest mm.

2.9. Evaluation of effect of bacterial endophyte on *Pt. vittata*

2.9.1. Bacterial suspension preparation

Bacterial endophyte (DSKP8) was grown in 250 ml Erlenmeyer flasks containing 50 ml of sterilized nutrient broth on a shaker (120) at 30^{o}C. Bacterial cells were harvested from the exponential phase of growth by centrifugation (at 8000g, 25 min, 4^{o}C), and the cell pellets were washed twice and suspended in sterile distilled water to an absorbance of 0.5 at 600 nm (equivalent to approximately 7.6×10^{6} cfu ml^{-1}).

2.9.2. Effect of DSKP8 on growth and As uptake by *Pt. vittata*

Greenhouse experiments were conducted to evaluate the effect of bacterial isolate DSKP8 on growth and arsenic uptake of *Pt. vittata*. Sterilized, (autoclaving at 121^{o}C at 15 lbs pressure for 1 h for three consecutive days) garden soil (750 g) was filled in each surface sterilized pots (4 inches) supplemented with 25 mg kg^{-1} As by adding adequate amounts of an aqueous solution of sodium arsenate ($Na_2HAsO_4.7H_2O$). The pots filled with experimental soil were left in the greenhouse for 2 weeks for proper acclimatization of As. The experimental design was a randomized complete block design with three replicates for each treatment. Twelve pots with three replicates for endophytic bacterial strain with arsenic were labelled as PtBAs and three for negative control PtAs [with arsenic and without bacterial inoculant] and three for one positive control (Pt) [without arsenic and without bacterial inoculant] and other three positive control as PtB [without arsenic and with bacterial inoculant]. After two weeks of acclimatization, two frond stage sapling of *Pt. vittata* was transferred in each pot. After one week of plant growth, bacterial suspension (50 ml pot^{-1}) was sprayed on the soil surface. Control plants received 50 ml of sterile distilled water. Inoculated and non-inoculated plants in pots, were kept under greenhouse conditions [18°C (night) and 24°C (day), 60% relative humidity, 11 hours photoperiod]. The soil was moistened with sterile water and moisture was maintained at 60% level by covering the pots with plastic hood. After 90 days of sowing, harvesting was done and growth parameters like frond length, root length, fresh and dry weight were recorded. *Pt. vittata* fronds and fresh roots were kept at -80°C until further analysis.

2.9.3. Estimation of arsenic

After harvesting, the plants were sampled for quantifying As accumulation in *Pt. vittata*. Plants were partitioned in frond and roots and washed thrice with distilled water in order to remove surface adsorbed metalloid ions. Excess water was removed by the blotting paper. Plant roots were kept in EDTA (20 mM) solution for 15 min to remove adhered metalloid on the root surface [46]. All the samples were oven dried at 75^{o}C till constant weight were achieved. Samples were powdered in a grinder and sieved with 2 mm sieve. Powdered material (0.1 g) of each sample was digested in BURGHOF-speedwave-MSW-3^{+} microwave digestion unit with 5 ml of HNO_3 (70%) and the digested sample was filtered through Whatman filter paper no. 44. Volume of

each digested sample was maintained to 50 ml with the addition of Milli Q water. The As content of in the digested samples was determined by Inductively coupled plasma mass spectrometry [ICP-MS] (Agilent-7500cx).

Translocation factor (TF) was calculated by using the following formulae:

TF (root to shoot) = mean accumulation of metal by shoot part/mean accumulation of metal by root part.

Bioconcentration factor (BCF) was calculated by using the following formulae:

BCF= Concentration of metal in plant/concentration of metal in experimental soil.

2.9.4. Quality control assessment

The standard reference material of As (consisting of 998±4 mg l^{-1} As-NIST and BAM-CRM traceable; EMerck, Germany) was used for each analytical batch. Analytical data quality was ensured with repeated analysis of quality control samples (n=3) and the results were within (±2.82 mg l^{-1}) limit of the certified values. Standard AA03N-3 (Accustandard, USA) was used as a matrix reference material which was spiked with known concentration (0–50 µg l^{-1} As) of standard reference material and the recovery of total As was within 85.3% (±2.8; n=5) to 89.5%.

2.9.5. Statistical analysis

Groups were compared by using one way analysis of variance (ANOVA) followed by Duncan multiple range test (DMRT).

3 RESULTS

3.1. Isolation and characterization of culturable endophytic bacteria from *P. vittata*

Endophytic bacteria were isolated after 48 h of incubation of surface sterilized segments of *Pt. vittata* on nutrient agar, glycerol asparagine agar and tryptic soy agar. Avoiding the repetitive strains, a total of 15 phenotypically distinguishable endophytic bacteria were isolated in pure form from 146 segments (81 frond, 21 rhizome and 44 root) of *P. vittata*. Out of these 15 isolates, 3 were derived from frond, while 6 isolates each were derived from root and rhizome segments (Table 1).

Colonization frequency was higher (73%) in frond while isolation rate was higher in rhizome (0.29%) and root (0.14%) portion. Shannon-weaver Diversity index has defined that rhizome (1.75) and root (1.72) carried more diverse type of endophytic bacteria than frond (1.09). Out of 15 bacterial endophytes 11 were Gram-positive and only 4 were Gram-negative. Majority of these endophytes were motile and rod shaped, while only three of them were cocci.

3.2. Screening of heavy metal tolerance of endophytic bacteria

In order to select the multi-metal tolerant strains, the endophytic isolates of *P. vittata*, were grown in different concentrations (1-9 mM) of six metal(loid)s such as As, Cd, Co, Ni, Cr, and Zn. Screening results indicate that out of these 15 bacterial endophytes, only one endophytic isolate designated as DSKP8 was able to grow up to 8 mM As, Cd, Ni and Co (Fig. 1) and was selected for further studies.

3.3. Characterization and identification of isolate DSKP8

The selected bacterial isolate DSKP8 was characterized on the basis of its morphological, physio-biochemical and functional features (Table 2). Colonies of DSKP8 were whitish in color, cells rod-shaped, Gram-positive, measuring 2.5 x 0.62 µm and produced endospore. The isolate produced catalase, amylase, gelatinase, pectinase and have the capability to ferment sugars like sucrose, lactose and raffinose. It was also able to grow at a temperature 4-40^{o}C and pH range of 7-10 (Table 2). Identification was carried out through PCR amplification of 16S rDNA. Approximately 1200 bp of gene fragment was purified and sequenced. The BLAST analysis indicated that the strain DSKP8 was closely related to the genus *Bacillus*. The 16S rDNA sequences determined in this study have been deposited to NCBI GenBank with the accession number of KJ881418.

A sequence alignment and phylogenetic tree was constructed based on 16S rDNA sequences. Fig. 2 showed the phylogenetic relationship of the bacterial strain DSKP8 with the bacterial strain *Bacillus pumilus*. As can be seen from the phylogenetic tree, the 16S rDNA sequence of DSKP8 showed closest relation to *Bacillus pumilus* and forming a cluster in which the homology between these species was highest (98%). Taking in to account these results we consider the bacterial isolate DSKP8 as *Bacillus pumilus* DSKP8.

3.4. Plant growth promoting properties of isolate DSKP8

To explore the plant growth promoting (PGP) activities, the isolate DSKP8 was tested for its ability to produce indole acetic acid (IAA), solubilization of phosphate and production of siderophore**.** Table 2 clearly shows that the endophytic bacteria DSKP8 possessed all three PGP traits. Maximum IAA production (71.66 ± 5.77 µg mg^{-1}) was observed after 120 h of incubation. The solubilization of phosphate (95 µg ml^{-1}) by DSKP8, was achieved after 120 h of incubation. Further incubation had no effect on the extent of solubilization. The isolate DSKP8 also showed the production of iron chelating compound siderophores.

3.5. Antibiotic sensitivity and antimicrobial activity of isolate DSKP8

Disc-diffusion assay was performed for determination of antibiotic sensitivity pattern of the endophytic bacterial isolate (DSKP8) against nine different antibiotics (Bacitracin; Erythromycin; Vancomycin; Penicillin; Chloramphenicol; Methicillin; Chlorotetracycline; Gentamcin; Kanamycin). It was observed that bacterial endophyte DSKP8 was resistant to bacitracin and methicillin, while it was mostly sensitive to erythromycin followed by penicillin, chlorotetracycline, and gentamicin. On the contrary, this isolate showed sensitive to intermediate response towards chloramphenicol and kanamycin (Table 2).

Isolated endophyte DSKP8 was assessed for antimicrobial activity by cross-streak method on nutrient agar plates against five bacterial test organisms, *B. subtilis, B. cereus, E. coli, P. cepacia*, and *S. aureus*. The isolate DSKP8 showed inhibitory activity against *S. aureus, B. subtilis, B. cereus* followed by *E. coli* (Table 2).

3.6. Influence of isolate DSKP8 on growth and arsenic uptake by *P. vittata*

Inoculation of soil by the bacterial endophyte DSKP8 significantly (P<0.05) enhanced the fern growth as reflected by root length, frond length, fresh weight and biomass formation of *P. vittata* (Table 3). In *P. vittata* significant reductions in morphological data were clearly observed in only arsenic spiked soil (25 mg kg^{-1}) (Table 3). In the presence of arsenic, the frond length, root length, fresh weight and biomass were significantly reduced while in the presence of inoculated bacteria (DSKP8) in arsenic spiked soil (PtBAs) and without arsenic treated soil (PtB), all the morphological data of plant were significantly increased. These morphological results clearly explained the plant growth promoting ability of the inoculated endophytic bacteria (DSKP8).

In case of metal uptake, arsenic addition significantly increased As concentrations in both fronds and roots, and the effect was more pronounced in fronds than in roots (Fig. 3). When the bacteria inoculated arsenic treated plants (PtBAs) were compared with those of non-inoculated arsenic treated plants (PtAs), it was evident that percent increment of arsenic content was 210% higher in fronds and 178% higher in root portion of the bacterial inoculated plants. These were significantly higher from non-inoculated arsenic treated plants (PtAs).

The metal accumulation efficiency in plants can be evaluated using the bioconcentration factor (BCF), which is defined as the ratio of metal concentrations in the plant and that of the soil. In this study, the BCF of the bacterial treatments was more than 1 (BCF>1) (Fig. 4). Inoculated *P. vittata* plants have significantly high BCF ratio in

comparison to non-inoculated arsenic treated plants. Translocation efficiency in plants from root to shoot can be evaluated using translocation factor (TF), which is defined as the ratio of metal concentrations in the shoot and that of the root. In this experiment the TF was greater than 1 in both treatments (Fig. 5).

4. DISCUSSION

This study appears to represent the report regarding the isolation and characterization of bacterial endophytes from *P. vittata*. Shannon-weaver diversity index clearly explained that the endophytic bacterial community was higher in root and rhizome in comparison to frond of the plant (Table1). Distribution of endophytic bacterial population may vary according to different habitat. In this study the plant samples were collected from the Darjeeling areas of the Eastern Himalayas which are very rich in fern vegetation [5].

This study is unique in the sense as very few reports are available on the isolation of heavy metal resistant bacterial endophytes from *P. vittata*. Since *P. vittata* is a well known As hyperaccumulator plant [22], it is expected to harbour metal-resistant endophytes in its internal environment. The heavy metal resistance assay of the isolated endophytes resulted in the selection of bacterial isolate DSKP8 capable of tolerating 8 mM of As, Cd, Co and Ni (Fig. 1). Such resistance of heavy metals in DSKP8 might be due to its adaptation in heavy metal containing environment as well as presence of detoxification mechanisms inherent in bacterial genome.

The mechanisms so far explored in different bacterial genome are classified in five main groups, efflux, oxidation, reduction, metal sequestration, and enzymatic transformation [36]. It has also been reported that bacterial isolates have *ars* operon system for arsenic detoxification [6,7] in their genome. The multi-metal tolerance of the bacterial isolate DSKP8, therefore indicates its potential for bioremediation of heavy metal contaminated soil. According to the 16S rDNA sequence analysis and as can be seen from the phylogenetic tree of DSKP8 showed closest relation to *Bacillus pumilus* and forming a cluster in which the homology between these species was highest (98%). Taking in to account these results we consider the bacterial isolate DSKP8 as *Bacillus pumilus* DSKP8. Zhu et al., [48] have also reported the presence of *Bacillus* sp. as endophytes from *P. vittata* while the same has previously been reported as endophytes from other plants. Barros et al., [3] already reported some endophytic bacterial isolates from the fern *Dicksonia sellowiana* Hook. According to their observation, *Bacillus* sp. were the most frequently isolated endophytic bacteria, which has also been reported as endophytes from variety of angiosperms [24].

The bacterium DSKP8 was further checked for its functional properties and was found to be characterized by the ability to solubilize phosphate, produce indole-3-acetic acid (IAA) and siderophore (Table 2). Possession of these plant growth promoting traits (Table 2) suggests the utility of isolate DSKP8 as a bioinoculant for plant growth promotion. Several authors have reported the isolation of plant growth promoting bacterial strains from different sources [37,29] and their role in plant growth promotion and bioremediation. Solubilization of phosphate and production of IAA also enable the plants to cope with the environmental stress and metal toxicity [37]. Similarly, siderophores also play a major role in metal detoxification and impart protection in plants against environmental stress [37]. Likewise, there are several reports on the role of endophytes as plant growth promoters [15,47]. Recently, Etesami et al., [12] have isolated plant growth promoting endophytic bacteria from rice. Khan et al., [18] and Dawwan et al.[8] have also isolated endophytic bacteria from potato plant and investigated their role on the growth of

potato plants. It has been well documented that solubilization of phosphate, production of IAA and siderophores by the endophytic bacteria contribute immensely in promoting the growth of plants. The confirmation of growth regulating factors of isolated endophytic strain DSKP8 is the contrivance for exploring the probable application of the isolate in *Pt. vittata* enhancement.

The bacterium DSKP8 being isolated from healthy tissues of *P. vittata* plants is a non-pathogenic one and showed resistance to different antibiotics which might be due to co-existance of heavy metals and antibiotic resistance genes in the bacterial genome. Further the endophytic bacterium DSKP8 showed a broad spectrum of antibacterial activity inhibiting both Gram-positive and Gram-negative bacteria so DSKP8 could be considered as a good source of antimicrobial compound. Melo et al., [24] have also clearly demonstrated that *Bacillus pumilus* colonizing the shoot and root tissues exerted antifungal activity against *Pythium aphanidermatum*. According to Strobel and Daisy [38] endophytes are novel sources of bioactive compounds.

This work showed some interesting effects of endophytic bacteria on the growth of *P. vittata* as well as on As translocation in As hyperaccumulator, *P. vittata*. In bacterial inoculated plants growth was not affected by arsenic toxicity because the toxicity effect of arsenic appeared to be invalidated by the inoculated bacterial endophyte DSKP8. Similarly, Ma et al., [23], have also observed the effect of isolated endophytic bacteria on the growth and Ni uptake in host and non-host plants and they found that isolated endophyte A3R3 significantly increased the biomass and Ni content of plants grown in Ni contaminated soil.

In the present findings, the translocation factor (TF) ranged between 2.0 to 2.5 (Fig. 5), which is similar to the upper level (0.83-1.81) reported for *P. vittata* surveyed in China [43] and within the range reported for *P. vittata* supplied with 133 or 276 μm Na_2HAsO_4 for 1 or 5 days [33]. In contrast, TF values of non-inoculated *P. vittata* plants were about 50 when plants were grown in quartz sand for 45 days and fed with 15 mg kg^{-1} As. Inoculation with *Glomus mosseae* or *Glomus intraradices* increased the TF values greatly up to 730 and 292, respectively [39]. Wang et al., [41] observed 45% As translocation from root to the above ground tissues when As resistant and plant growth promoting rhizobacterium (D14) was inoculated in *Populus deltoids* Bartr.

The bacterial inoculation enhanced As accumulation which is very similar to the As accumulation in *P. vittata* as observed by Yang et al., [45]. The TF values can be influenced by a wide range of plant and soil factors including soil properties [43], As supply level [43,30], plant growth period [33], and the associated microbes [39,13].

Studies have shown arsenite [As(III)] to be the predominant species present in the fronds of *P. vittata* and arsenate [As(V)] to be the main species in the roots [16,17,33]. Arsenate is absorbed by roots of *P. vittata* via the phosphate uptake pathway [42]. With the inoculation of the bacterium DSKP8 enhancement of As accumulation and translocation were significantly high in As-amended soil. It is reported that the IAA released by bacteria could directly promote the growth of roots by stimulating elongation of the plant cells or increasing cell division, which may enhance the root As absorption. Moreover, the siderophore production by strain DSKP8 may mobilize the As(V) in the soil during the process of iron uptake [11] which rendered As more soluble and bioavailable to plants. As a result, the bacterial inoculation enhanced As accumulation in *P. vittata*. Recently, Dell'Amico et al., [9] isolated a strain of *Pseudomonas tolaasii* (ACC23) able to increase the biomass and the total amount of Cd accumulation was increased in canola plants under Cd stress. Kumar et al., [20] isolated two

PGPR bacterial strains which were capable of stimulating plant biomass and enhanced phytoextraction of metals (Ni and Cr) from fly ash by metal accumulating *Brassica juncea* L. (Czern.).

A large number of studies also confirm the existence of cumulative effects of microbes such as solubilization of nutrients, production of phytohormones, alleviate stress ethylene production, metal biosorption, etc. [29,20,19]. These studies suggest that the inoculation of microbes possessing the ability to withstand heavy metal/metalloid stress and the potential to promote plant growth through various plant growth promoting traits in metal-contaminated soils can be a potential biotechnological tool for successful phytoremediation process.

CONCLUSION

This study indicates that the endophytic bacterium *Bacillus pumilus* DSKP8 isolated from *P. vittata* can enhance growth as well as uptake of arsenic by plants and may, therefore, have great potential for clean-up of arsenic contaminated soil by using hyperaccumulators such as *P. vittata*.

Present investigation has made a modest effort in isolating endophytic bacteria sustaining in *P. vittata* plants and assessed their efficiency of arsenic removal from contaminated soil. Antimicrobial activity of this strain revealed that the isolate could be a good antibiotic producer. Moreover, the isolate has the capability to produce different hydrolytic enzymes and thus can be used as a potential source for various biotechnological processes.

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Tables and Figures:

Table 1- Diversity of endophytic bacteria isolated from surface sterilized frond, stem and root tissues of *Pteris vittata L.*

Parameters	Plant tissue			Total
	Frond	Rhizome	Root	
Number of samples tested	81	21	44	146
Number of samples yielding isolates	59	15	16	90
Number of isolates	3	6	6	15
Colonization frequency [a] %	73	71	36	10
Isolation rate [b]	0.04	0.29	0.14	0.10
Shannon-Weaver Diversity index [c]	1.09	1.75	1.72	4.56

[a]Colonization frequency was calculated as the total number of plant samples infected by bacteria divided by the total number of samples incubated.
[b]Isolation rate was calculated as the number of bacterial isolates obtained from plant samples divided by the total number of samples incubated.
[c]Shannon Weaver diversity index H' was calculated as: $H' = -\sum Pi \ln Pi$, where, Pi is the proportion of individuals that species "i" contributes to the total.

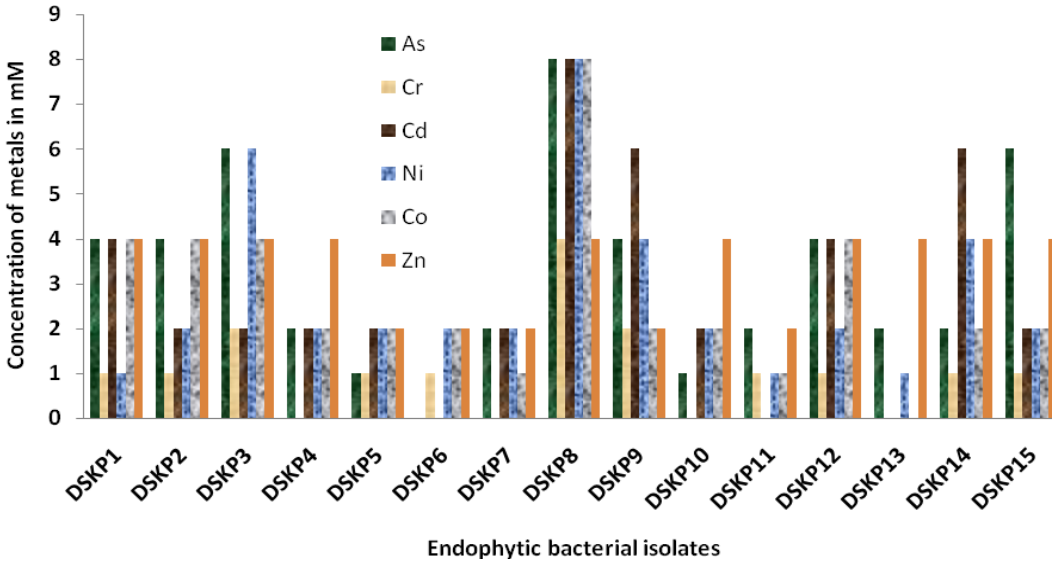


Fig. 1- Screening for heavy metal tolerance of isolated endophytic bacterial strains of *P. vittata*

Properties	Response	Properties	Response
Morphological characterization		**Biochemical characterization**	
Gram staining	+ve	Production of nitrate reductase	▪
Configuration	Elongated	Production of amylase	✓
Surface	Moist	Production of pectinase	➢
Pigment	Whitish	Production of gelatinase	✓
Cell shape	Rod	Production of catalase	✓
Size (µm)	2.5x0.62	Production of oxidase	✓
Motility	Motile	Fermentation of sucrose	✓
Anaerobic	✓	Fermentation of xylose	✓
Endospores formation	✓	Fermentation of maltose	✓
Physiological characterization		Fermentation of fructrose	✓
Growth at 4-40^{o}C	✓	Fermentation of dextrose	✓
Growth at pH 7.2 - 10	✓	Fermentation of glycerol	✓
Growth on NaCl 2-6%	✓	Mannitol	✓
Functional characterization		Cellobiose	✓
IAA [µg mg^{-1} l]	71.66^{a}±5.77^{b}	Esculin	✓
Phosphate solubilization [µg ml^{-1}]	94.66^{a}±7.57^{b}	D-Arabinose	✓
Siderophore production	✓	Citrate	✓
NCBI GEN bank accession number	KJ881418	Lactose	✓
16s rDNA analysis	*Bacillus pumilus*	Raffinose	✓
Response to antibiotics**	**Diameter of inhibition zone (mm)**		
Resistant	$B^{10}(0), M^{5}(0), Ns^{100}(0)$		
Intermediate	$Va^{30}(17), C^{30}(18), K^{30}(14)$		
sensitive	$E^{15}(25), P^{10}(20), CT^{30}(28), G^{10}(23)$		
Antimicrobial activity	Diameter of inhibition zone (mm)		
Bacillus subtilis	*15*		

Staphylococcus aureus	*30*
Escherichia coli	*7*
Bacillus cereus	*13*
Pseudomonas cepacia	*(-)*

Table 2- Morphological, physio-biochemical and functional properties of the endophytic bacterial isolate DSKP8.

✓ = Positive
▪ = Negative
➢ = weakly positive

[a] Values represent average of three replicates, [b] Values represent standard deviation. **B^{10}-Bacitracin; E^{15}- Erythromycin; Va^{30}-Vancomycin; P^{10}-Penicillin; C^{30}-Chloramphenicol; M^{5}-Methicillin; NS^{100}-Nystatin; Ct^{30}-Chlorotetracycline; G^{10}-Gentamicin; K^{30}-Kanamycin ; (-) = no inhibition

Antibiotic susceptibility was tested on nutrient agar plates using antibiotic impregnated discs (6 mm) from HIMEDIA, India.

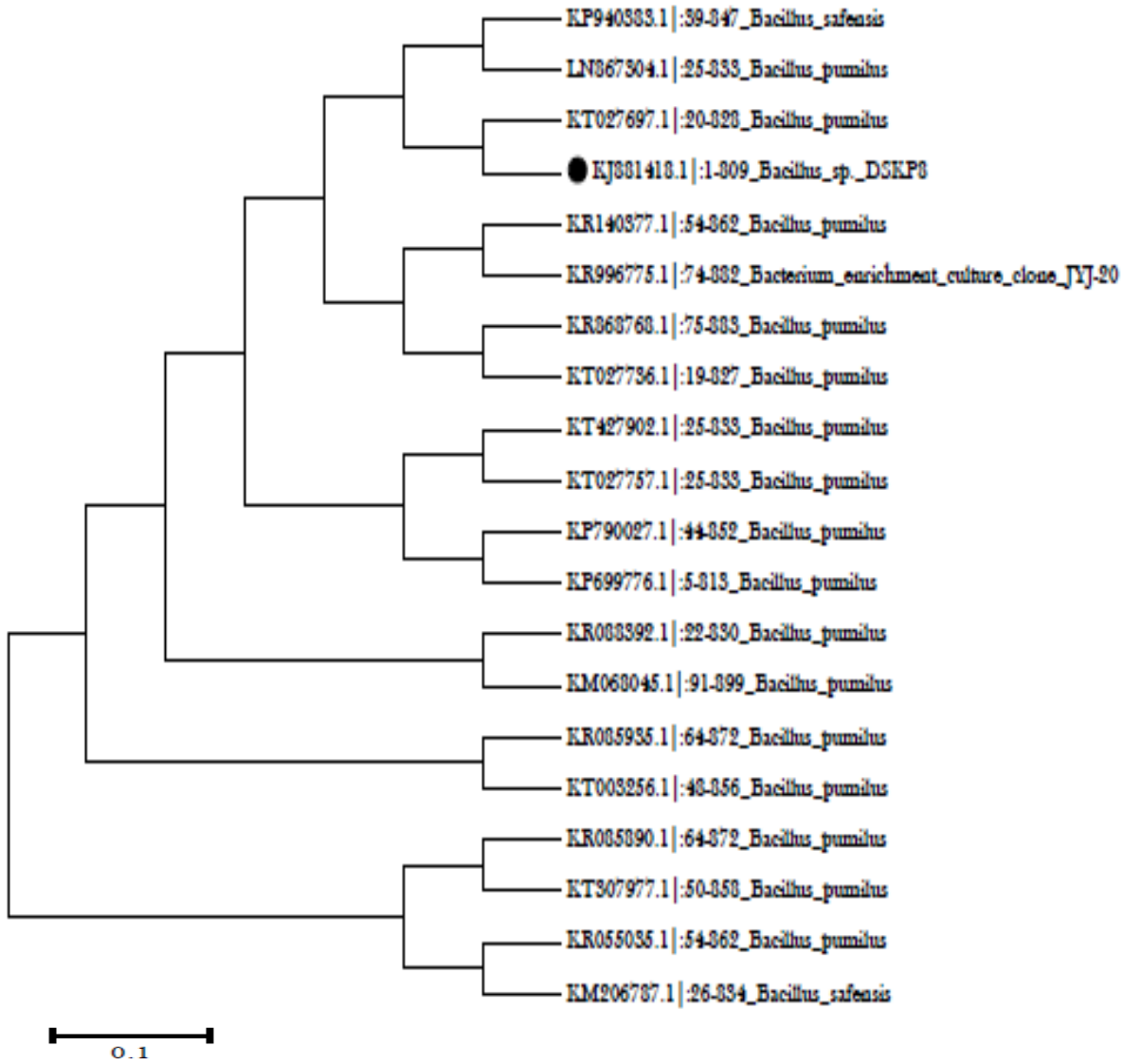
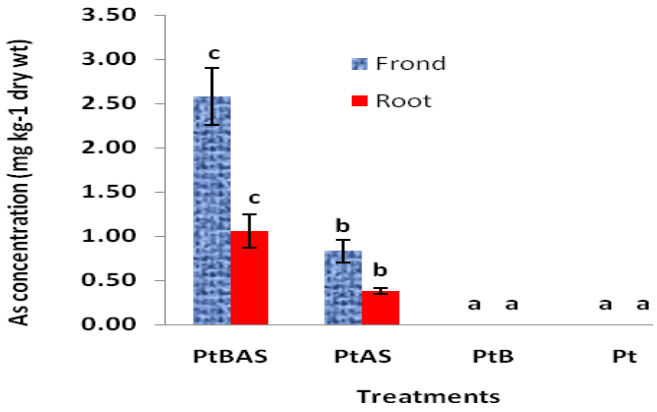


Fig. 2- Phylogenetic tree showing the closest relationship of isolate *DSKP8* (*KJ881418)* with *Bacillus pumilus* based on 16S rDNA sequences. Phylogenetic analyses were conducted by phylogeny program of MEGA-6.

Table 3- Changes in morphological characters of *Pt. vittata* plant as influenced by arsenic amendment (25 mg kg^{-1}) and inoculation of bacterium endophyte DSKP8.

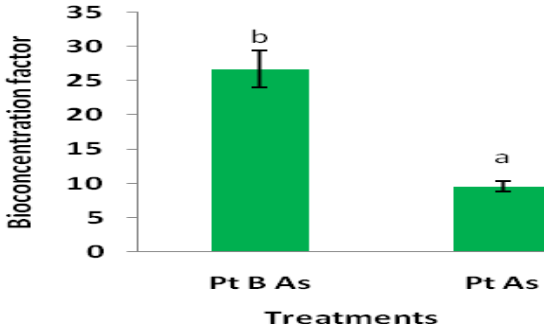
Treatments	Frond length (cm.)	Root length (cm.)	Fresh weight (g)	Biomass (g)
Pt (Control)	18.67 ± 3.21^{a}	9.33 ± 1.53^{a}	1.78 ± 0.05^{a}	0.045 ± 0.003^{a}
PtB	29.74 ± 0.59^{b}	15.27 ± 0.26^{b}	4.39 ± 0.36^{b}	0.071 ± 0.01^{b}
PtAs	13.00 ± 4.36^{a}	7.73 ± 1.55^{a}	0.98 ± 0.02^{a}	0.045 ± 0.003^{a}
PtBAs	24.74 ± 1.43^{b}	13.60 ± 0.97^{b}	3.67 ± 0.73^{b}	0.064 ± 0.01^{b}

Pt (control) [*Pteris* without bacterial inoculant and without arsenic], PtB [*Pteris* with bacterial inoculant and without arsenic] PtAs [*Pteris* with only arsenic treatment], PtBAs [*Pteris* with bacterial inoculant and arsenic treatment], [Pt=*Pteris vittata*, As= Arsenic, B= isolated bacterial endophyte DSKP8], a, b showing significant changes in the values. Data averaged with ± standard deviation (SD). Means followed by same letter were not significantly different at $p < 0.05$ according to DMRT.



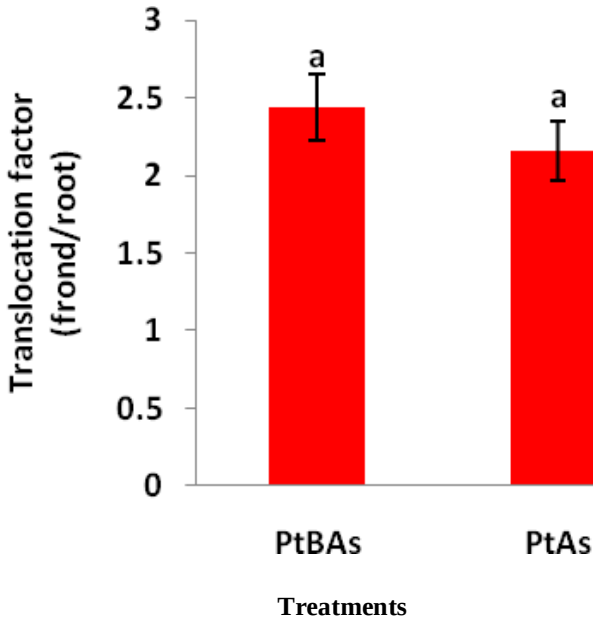
PtAs (control) [*Pteris* with only arsenic treatment], PtBAs [*Pteris* with bacterial inoculant and arsenic treatment] [Pt=*Pteris vittata*, As= Arsenic, B= isolated bacterial endophyte DSKP8], a, b,c showing significant changes in the values

Fig. 3- Arsenic concentration (mg kg^{-1} dry wt.) in *P. vittata* in all the treatments. Bars indicate means ± standard deviation [SD]. Means followed by same letter were not significantly different at $p < 0.05$ according to Duncan multiple range test (DMRT).



PtAs (control) [*Pteris* with only arsenic treatment], PtBAs [*Pteris* with bacterial inoculation and arsenic treatment], [Pt=*Pteris vittata*, As= Arsenic, B= isolated bacterial endophyte DSKP8], a, b showing significant changes in the values

Fig. 4- Bioconcentration factor (Per plant) in *P. vittata* in arsenic spiked soil. Bars indicate means ± standard deviation [SD]. Means followed by same letter were not significantly different at $p < 0.05$ according to Duncan multiple range test (DMRT)



PtAs (control) [*Pteris* with only arsenic treatment], PtBAs [*Pteris* with bacterial inoculation and arsenic treatment] a, b showing significant changes in the values.

Fig. 5- As Translocation Factor (root to frond) in *P. vittata* in arsenic spiked soil. Bars indicate means ± standard deviation [SD]. Means followed by same letter were not significantly different at $p< 0.05$ according to Duncan multiple range test (DMRT).