ABSTRACT

Arsenic is a toxic metalloid which cause serious health effects to human population worldwide. Considered as a Group I carcinogen, it exists naturally in soil and ground water, however, human activities lead to high amount of arsenic pollution in these environments. Arsenic finds its entry into human food chain through different agricultural crops grown in arsenic polluted agricultural fields or fields where arsenic contaminated ground water is used for irrigation. In India, West Bengal tops the list of most contaminated states with several reports of arsenic toxicity in human. Therefore, there is an immense necessity for the implementation of efficient arsenic remediation strategies without harming the natural environment as done by the conventional methods for arsenic removal. In this consideration, arsenic remediation using microorganisms can be a cost effective and safer approach. Additionally, since several years, microorganisms have been used for controlling the agricultural crop loss caused due to the attack by plant pathogens. Rhizosphere microorganisms as biocontrol agents are being extremely used as an alternative to the use of synthetic chemicals for the better management of crop production. Moreover, nowadays, rhizobacteria are also receiving much attention in terms of their application in bioremediation of toxic compounds from soil. Therefore, through this study we aimed to focus on the rhizosphere inhabiting bacteria which can be applied as a potent multifunctional agent for arsenic bioremediation as well as in the biocontrol of plant pathogens.

The work was conducted with a primary aim of isolating some highly arsenic resistant siderophoregenic strains with strong antagonism against plant pathogens. We then focused on the arsenic removal efficiency of potent isolates considering their field trials for arsenic bioremediation. Another aspect of this study was to prepare a stable and effective bioformulation for management of plant disease. We also isolated and characterized siderophore, suggesting its role as an antifungal metabolite as well as arsenic chelator. Overall, the work was conducted with the following objectives: (i) Isolation of siderophoregenic bacterial strains from agricultural soils and their screening for antifungal activity and arsenic resistance; (ii) Identification and phylogenetic analysis of the selective isolates based on 16S rRNA gene sequence; (iii) Evaluation of antagonistic potential of selected bacterial strains against plant pathogenic fungi; (iv) Characterisation of siderophore

and metal complexation studies between siderophore and arsenic; (v) Analysing the presence of genetic determinants of arsenic resistance and antifungal property in the bacterial isolates; (vi) Batch study for arsenic removal from the soil by potent isolate; (vii) Studying the biocontrol potential of selected bacterial strain under greenhouse conditions.

For bacterial isolation, rhizosphere soils were collected from agricultural fields of different regions of Darjeeling, Jalpaiguri, Cooch Behar, Alipurduar, Malda and Purulia districts of West Bengal. A total of 821 isolates were obtained from which 603 isolates were screened as siderophore producers. 500 siderophorogenic isolates with prominent haloes were tested for their resistance towards both forms of arsenic i.e., arsenate (AsV) and arsenite (AsIII) from which 230 isolates were able to grow in presence of both AsV and AsIII. Further, *in vitro* antifungal activity of these isolates was tested by dual culture method against six plant pathogens viz. *Lasiodiplodia theobromae*, *Colletotrichum gloeosporioides*, *Curvularia eragrostidis*, *Fusarium oxysporum*, *Fusarium solani* and *Rhizoctonia solani*. Here, thirty isolates were found to show strong antagonism against all test pathogens among which, three isolates BM3, CDG7 and GST18 were the most efficient antagonists. All thirty isolates were tested for the production of antifungal metabolites including extracellular lytic enzymes and HCN followed by detection of PGPR traits such as phosphatase and IAA production. Maximum isolates showed amylase production followed by lipase and amylase whereas a smaller number of isolates produced chitinase, cellulase and pectinase. Only two isolates showed HCN production. Four isolates were detected with both phosphatase and IAA production where highest amount of IAA was produced by BM3. Biofilm formation, which is also an important mechanism of biocontrol agents, was observed in two different media i.e., Luria Bertani (LB) and M9 yeast extract (M9YE) by all thirty isolates. Comparatively, maximum biofilm production occurred in LB medium where seven isolates appeared as strong biofilm formers.

The identity of thirty potential isolates was revealed through morphological, biochemical and phylogenetic characterization. Through 16S rRNA gene sequencing and BLAST similarity search, isolates were distinguished into eight different genera where majority of the isolates (60%) belonged to genus *Bacillus* followed by *Pseudomonas, Serratia, Microbacterium, Lysinibacillus, Proteus, Ensifer* and *Micrococcus.* The sequences of all thirty isolates were

deposited in NCBI GenBank and accession numbers were assigned as follows: MN133951, MN133999, MN865204, MN133963, MT032417, MN923204, MN148541, MN120803, MN865978, MN809373, MN809382, MN122130, MN809526, MN809529, MN108490, MN148539, MN809348, MN809357, MN809577, MN912102, MN120791, MN814034, MN865987, MN915155, MN814036, MN918097, MN809367, MN865968 and MN809363. The isolates were also subjected to hemolysis test considering the safety during field application where eighteen isolates were found to be non-hemolytic.

Further, the level of arsenic resistance was checked by determining the minimum inhibitory concentration (MIC) of AsV and AsIII against thirty isolates in two different media i.e., LB and minimal salt (MS) medium. Highest MIC value of AsV was observed against *M. luteus* BPA2 irrespective of the growth medium. This strain also showed maximum resistance towards AsIII in MS medium while in case of LB medium, highest MIC value of AsIII was observed for *B. amyloliquefaciens* BM3. Arsenic transformation has been one of the mechanisms adopted by microorganisms to cope with arsenic toxicity. In this study, we tested arsenic reducing as well as arsenic oxidizing ability of all thirty isolates where nine isolates reported arsenate reduction and eight isolates showed arsenite oxidation in silver nitrate test. *M. luteus* BPA2 showed both the abilities of arsenic transformation. Detection for the genetic determinants of arsenate reduction (*arsC* gene for arsenate reductase) and arsenite oxidation (*aoxB* gene for arsenite oxidase) was also done inorder to correlate with the arsenic resistant phenotype of four highly resistant isolates. Successful PCR amplification of *arsC* gene was observed in all four isolates *M. luteus* BPA2, *B. amyloliquefaciens* BM3, *P. putida* BPA1 and *L. macroides* SUT34. However, *aoxB* gene was amplified in three isolates except *L. macroides* SUT34. The amplicons were cloned in pGEMT Easy vector and sequenced. The obtained gene sequences were submitted to GenBank through BankIt tool and the provided accession numbers are: *arsC* (OR875842, OL405606, OR195443 and OR228422) and *aoxB* (OR875841, OL405605 and OR875843).

The growth study performed for *M. luteus* BPA2 and *B. amyloliquefaciens* BM3 (with highest MIC values) under arsenic stress in LB and MS media revealed higher impact of AsIII on the growth of both bacteria than AsV. Moreover, arsenic stress lowered the growth rate more in MS medium than LB medium. In comparison, *M. luteus* BPA2 showed better

growth under arsenic stress in both media than *B. amyloliquefaciens* BM3. Scanning electron microscopy showed slightly irregular surface morphology of *M. luteus* BPA2 cells whereas an increase in cell size was observed in *B. amyloliquefaciens* BM3 under arsenic stress. *M. luteus* BPA2 and *B. amyloliquefaciens* BM3 showed efficient arsenic removal ability in both *in vitro* and *in vivo* studies.

The quantitative study for siderophore production done through CAS shuttle assay revealed varied level of siderophore production by thirty isolates with *B. amyloliquefaciens* BM3 producing highest amount of siderophore (94.15 p.s.u). The chemical nature of siderophore was also detected where eighteen isolates produced hydroxamate type, thirteen isolates showed carboxylate type and only seven isolates were detected with catecholate type of siderophore. Siderophore from *B. amyloliquefaciens* BM3 was extracted and purified though Amberlite XAD2 and Sephadex LH20 column chromatography. Following purification, thin layer chromatography was done where a single spot was observed with Rf 0.84 under UV light (365 nm) and also by spraying with $FeCl_3$. The compound was identified as bacillibactin through spectroscopic analyses including FT-IR, NMR and LCMS. Bacillibactin was studied for its metal chelating ability through qualitative and quantitative CAS assay which revealed that this siderophore could chelate both forms of arsenic apart from iron and the binding affinity was in the order Fe> AsV>AsIII. This observation was further confirmed by fluorescence spectroscopic analysis.

SEM study of the interaction between our most potent biocontrol agent *B. amyloliquefaciens* BM3 and *F. oxysporum* shows several morphological abnormalities in the mycelia of fungal pathogen. The genes of three antifungal metabolites i.e, chitinase (*chiA*), bacilysin (*bacAB*) and surfactin (*srfA*) were successfully amplified through PCR in *B. amyloliquefaciens* and subsequently cloned and sequenced. The obtained gene sequences were submitted to GenBank and the following accession numbers were provided: *chiA* (OL335882), *bacAB* (MT740320) and *srfA* (OR228422). Soil inoculation of talc based formulation of *B. amyloliquefaciens* BM3 was considerably effective in controlling the *Fusarium* wilt disease in brinjal. The disease control efficiency of this strain was found to be more or less similar to that of the fungicide (thiophanate methyl). Moreover, this strain remained viable in talc formulation upto seven months at room temperature.

In conclusion, the current study reports the recovery of useful strains from soil sample with multipurpose potential. The major findings of this study suggest the application of rhizospheric strains for cost effective removal of arsenic from soil. Moreover, we can also consider the development of formulation with the recovered strain as biocontrol products in eco-friendly agriculture. This study also witnesses the first report of arsenic chelation by bacillibactin thereby leading a new insight towards the application of this compound in combating arsenic toxicity in soils.