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Isolation and characterization of phosphorus solubilizing bacteria from manganese mining area of Balaghat and Chhindwara

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Plants require optimum amount of available phosphorus to support their growth and development. Phosphorus is known to have significant role in root subdivision, vitality and disease resistance of plants. Different types of bacteria involved in phosphorus solubilization can be used as biofertilizer in reclamation of mining area. The present study deals with isolation and identification of phosphorus solubilizing bacteria from the manganese mining area of Balaghat and Chhindwara districts of Madhya Pradesh, India. rDNA (16s) based molecular identification was performed assisted by MEGA phylogenetic analysis. *Pseudomonas putida*, *Bacillus licheniformis*, *Pseudomonas taiwanensis* and *Pseudomonas aeruginosa* were explored as potential phosphorus solubilizers from the selected sites.

Keywords: Mining area, phosphorus solubilizing bacteria, 16s rDNA.

PHOSPHORUS is one of the essential elements in soil but present in very low concentration in available form. Iron, calcium and aluminium rapidly immobilize inorganic available phosphorus and convert it into unavailable format of tricalcium phosphate, iron phosphate and aluminium phosphate. About 20% of soil phosphorus occurs in organic form and the general concentration of available phosphorus exists about 2 μM and rarely exceeds 10 μM (ref. 1). But plant tissues require a minimum of 5–20 mM of available phosphorus for rich growth and development[2,3]. To increase the available phosphorus of soil certain chemical fertilizers with inorganic phosphorus need to be added, but this affects the soil quality[4]. Numerous microbes, specially bacteria, are potential solubilizers of phosphorus and used as biofertilizers in agricultural lands. The role of microorganisms in solubilization of soil attached or soil precipitated phosphorus is already a focused area in recent studies. Most of the phosphate solubilizing bacteria (PSB) belong to genera: *Pseudomonas*, *Enterobacter*, *Rhizobium*, *Bacillus*, *Burkhoderia*, *Azotobacter*, *Azospirillum*, *Mesorhizobium* and *Erwinia*[5–9]. The general mechanism of phosphorus solubilization

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includes generation of organic acids, ion-exchange, chelation and enzymatic degradation of organic compounds[10–12]. Other than phosphorus solubilization, PSB such as *Pseudomonas flurescens*, *P. aeruginosa* and *Chromobacter imviolaceum* also secretes antibiotics and provides protection to plants from soil pathogens.

Study of PSB in soil of mining areas can assist in remediation, reclamation, biocontrol and biomineralization. These PSB also help in improving rock phosphorus availability through acidification, substitutions and chelation reactions. Different phenotypic and molecular based studies help in exploring heterogeneous microbial communities, their behaviour, existence and mechanism involved in phosphorus solubilization[13–15]. 16s rDNA sequence analysis perceives the better significance of biological functions to maintain biosphere of soil for improving fertility[16–19]. The present study deals with phosphate solubilizing bacteria from the manganese mining areas of Balaghat and Chhindwara districts of Madhya Pradesh. For the same 16s rDNA sequencing approach, i.e. molecular level identification followed by phylogenetic analysis with MEGA 6.06 was used.

Four different soil samples were collected from the manganese mining areas of Bharweli manganese mine Balaghat and Chhindwara Mine Pvt. Ltd of Madhya Pradesh during March 2013. All soil samples were collected with soil augur randomly after removal of upper stony layer and stored in sterile bags at 4°C till use. The soil of selected manganese mining areas was sieved through 2 mm sieve and designated as SB1 and SB2 from Balaghat and SC1 and SC2 from Chhindwara[20].

One gram of soil sample was suspended in 10 ml of phosphate buffer saline (pH 7.2) and serial dilutions were made up to 10^{-8}. Respective serial dilutions were spread on sterile Pikovskaya's agar plates (PVK composition in gm/l: yeast extract, 0.50; dextrose, 10.00; calcium phosphate, 5.00; ammonium sulphate, 0.50; potassium chloride, 0.20; magnesium sulphate, 0.10; manganese sulphate, 0.0001; ferrous sulphate, 0.0001; agar, 15; pH, 6.8) and incubated for seven days at 30°C. The morphological distinct colonies with clear zone were selected and further sub-cultured on PVK agar for pure culture isolation. A total of 10 PSB were obtained and the best four phosphate solubilizing colonies of bacterial isolates were selected based on size of the zone and ability to retain phosphate solubilization in sub-culturing. These were identified with 16s rDNA molecular marker.

Phosphorus solubilizing bacterial colonies with different morphological and gram-staining characters were analysed by growing cultures on sterile nutrient agar plates. Different biochemical tests such as catalase, oxidase, IMViC, motility, urease, nitrate reductase, starch hydrolysis, citrate utilization, triple sugar iron and carbohydrate metabolism test were performed.

The 16s rDNA sequencing was done to identify isolated PSB. Genomic DNA was extracted with modified Marmur's method[21]. Further extraction and purification was done with Qiagen DNeasy Plant Mini Kit followed by spectrophotometric estimation of DNA. 16sF AGAGTTTGATCCTGGCTCAG and 16sR GGTTACCTTGTTACGACTT primers were used for 16s rDNA amplification. A total of 50 µl of mixture was prepared having 1× standard *Taq* reaction buffer, 200 µM dNTPs, 1 µM of each primer, 125 units of *Taq* DNA polymerase and 1 ng to 1 µg of template DNA. The PCR conditions involved initial denaturation at 95°C for 30 sec, followed by 30 cycles of 95°C for 30 sec, 45°C for 60 sec, 68°C for 2 min and final extension at 68°C for 5 min. The amplified 16s rDNA product was purified with Centricon®-100 and the purity explored by agarose gel electrophoresis. 16s rDNA sequencing was done with big dye terminator reagent v3.1 cycle sequencing kit and ABI 3730 XL DNA analyzer (applied biosystem).

The 16s rDNA sequences obtained were compared with saved gene sequences of NCBI with Basic Local Alignment Search Tool (BLAST) analysis of GenBank®. Multiple sequence alignment was executed by CLUSTAL W with homologous sequences obtained by BLAST analysis. Phylogenetic tree was constructed with Neighbour-Joining method with bootstrap value of 1000 under Molecular Evolutionary Genetics Analysis version 6.06.

Table 1. Physicochemical characteristics of soil samples

Sampling Site	pH	Total alkalinity (mg/l)	Chloride content (mg/l)	Soil phosphorus (mg/l)	Soil organic carbon (%)
SB1	6.16	30.33	4.996	0.745	0.241
SB2	6.03	30.33	7.497	0.83	0.320
SC1	7.63	120.36	12.995	0.99	1.55
SC2	7.83	149.5	14.996	1.01	2.44
SF1	6.83	50.5	0.616	18.00	2.63
SF2	6.93	70.47	0.611	21.20	2.71

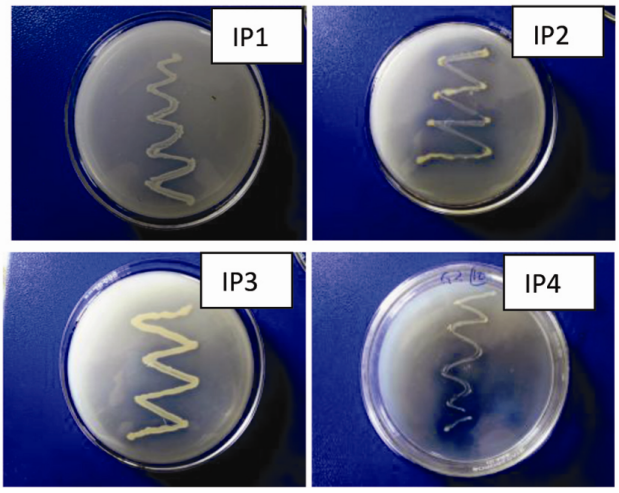

Figure 1. Pure culture of PSB on Pikovskay's agar.

Table 2. Colony morphological characteristics

Sampling sites	Bacteria code	Colour	Form	Elevation	Margin	Texture
SB1	IP1	Creamy	Circular	Convex	Entire	Dry
SB2	IP2	Greenish yellow	Circular	Raised	Entire	Moist
SC1	IP3	Creamy	Circular	Raised	Entire	Moist
SC2	IP4	Creamy	Irregular	Flat	Undulate	Moist

Table 3. Results of various biochemical tests of bacterial isolates

Biochemical test	IP1	IP2	IP3	IP4
Gram staining	–ve	+ve	+ve	+ve
Urease	+ve	–ve	–ve	–ve
Catalase	+ve	–ve	–ve	–ve
Oxidase	–ve	+ve	+ve	+ve
Starch hydrolysis	–ve	–ve	+ve	+ve
Indole	–ve	+ve	–ve	+ve
MR	+ve	+ve	+ve	–ve
VP	+ve	–ve	+ve	+ve
Motility	–ve	+ve	+ve	+ve
Citrate utilization	+ve	–ve	+ve	+ve
Nitrate reductase	+ve	–ve	–ve	–ve
Glucose	+ve	+ve	–ve	+ve
Fructose	–ve	+ve	–ve	+ve
Lactose	–ve	–ve	–ve	–ve
Sucrose	–ve	–ve	–ve	–ve
H_2S production	–ve	–ve	+ve	–ve

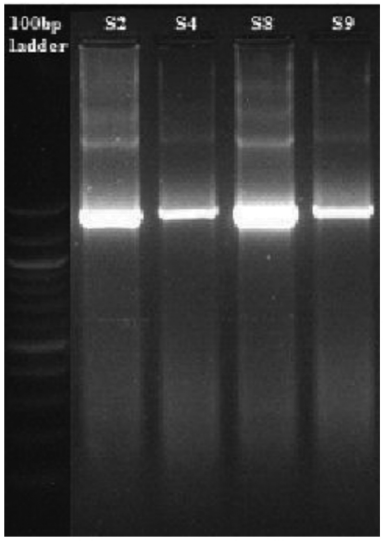

Figure 2. 16s rDNA agarose gel photograph (S2 = IP1, S4 = IP4, S8 = IP2, S9 = IP3).

Most of the mining areas are deficit in available phosphorus which results in retarded plant growth and development during reclamation processes. The present study is mainly focused on recovery of phosphate solubilizing bacteria from the selected mining areas. The physicochemical characteristics of soil represent neutral to alkaline nature of soil and are very low in fertile components (Table 1). From the selected sites, based on plate assay, four best phosphate solubilizing bacteria were recovered on Pikovskay's agar medium from which two belong to Balaghat (IP1, IP2) and two to Chhindwara (IP3, IP4) manganese mining area (Figure 1). Phenotypic based colony morphological and biochemical characteristics analysed are presented in Tables 2 and 3.

These four PSBs (IP1, IP2, IP3 and IP4) were subjected to 16s rDNA molecular marker-based identification. Amplified 16s rDNA product was checked for purity by agarose gel electrophoresis and found pure and free of any diffuse band on 1.2% agarose gel (Figure 2).

As per chromatograms raw sequences were generated and both forward and reverse raw sequences were further analysed. All gaps and missing data were eliminated from the data sets and the trimmed sequences were generated with Bio Edit analyzer. Further contig assembly was done with EG assembler online to generate 16s rDNA sequence. The 16s rDNA sequence obtained was of length 1381 bp (IP1), 1441 bp (IP2), 1358 bp (IP3) and 1402 bp (IP4). The complete 16s rDNA sequences were submitted to the International Nucleotide Sequence Database, National Center for Biotechnology Information (NCBI), GenBank. The registered GenBank accession numbers of bacterial strains are SUB1244156 Seq1 KU308257, SUB1244156 Seq2 KU308258, SUB1244156 Seq3 KU308259 and SUB1244156 Seq4 KU308260.

Phosphate solubilizing bacteria IP1 was 100% identical with *Pseudomonas putida* having accession id KF765789.1 with maximum score of 2425. *P. putida*, found to have identity with IP1, was involved in bioconversion of styrene to poly (hydroxyalkonoate), i.e. PHA and was submitted on 14 April 2015 as a new organism. Anand Mohan Chakrabarty invented this organism for biodegradation of toluene. Parani *et al.*[22] demonstrated prospects of using phosphate solubilizing *P. putida* as biofertilizer which makes it important in reclamation of mining area. Another phosphate solubilizing bacteria IP2 was found to be 100% identical with *Bacillus licheniformis* having accession id KT588646.1 with maximum score of 2479. *B. licheniformis* is a common Gram-positive mesophillic soil bacterium. The compared resultant *B. licheniformis* have been submitted as Bacterial Polymeric Bioflocculent and are being explored for the degradation of feathers for agricultural processes. Tahir *et al.*[23] also identified *B. licheniformis* as phosphate solubilizer by 16s rDNA sequence analysis.

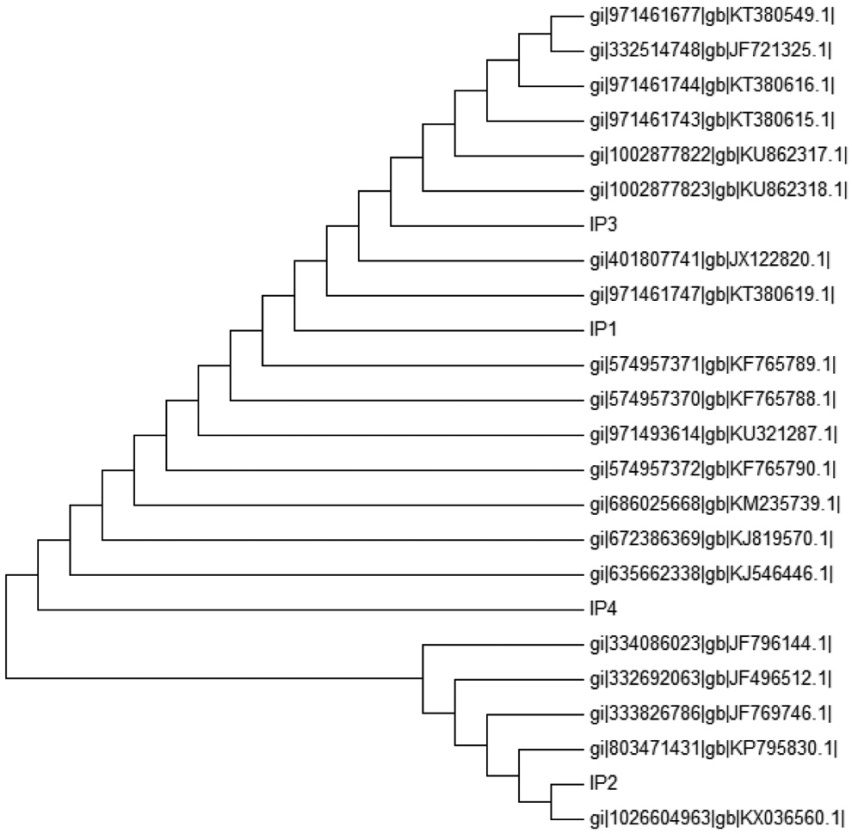

Figure 3. Phylogenetic tree using Neighbour-Joining method in MEGA.

Table 4. Identified mineral solubilizing bacteria

Bacteria code	Name of the identified bacteria
IP1	*Pseudomonas putida*
IP2	*Bacillus licheniformis*
IP3	*Pseudomonas taiwanensis*
IP4	*Pseudomonas aeruginosa*

Phosphate solubilizing bacteria IP3 had 100% similarity with 16s rDNA sequence of *P. taiwanensis* with accession id KT070311.1 and a maximum score of 1096. The KT070311.1 strain of *P. taiwanensis* had the ability to remove multiple metals including chromium (VI), copper (II) and zinc (II), which add a good sign to use IP3 as applicable bacteria. Volmer *et al.*[24] utilized *Pseudomonas taiwanensis* as biocatalysts by considering its capacity of organic solvent tolerant. So this might be a sign of utilizing this strain as biofertilizer at contaminated sites but multiplication and survival of these bacteria need to be confirmed. IP4 has shown 100% identity with *P. aeruginosa* with accession id KC633284.1 having maximum score of 2423. Jaeger *et al.*[25] have demonstrated *P. aeruginosa* application in synthetic organic chemistry because of its lipase enzyme secreting ability. Prasad[26] also found *P. aeruginosa* as phosphate solubilizer and used as biofertilizer with *Bacillus megaterium*. All isolated bacteria are listed in Table 4.

A phylogenetic tree represents nodes and branches in which node represents taxonomic units (sequences) and branch connects two adjacent nodes. Each node explains that for speciation event of the evolution beyond this point, sequences are changing and specific for each branch. The length of the branch between the nodes represents the number of changes that occur before next speciation. Terminal nodes, also termed as Operational Taxonomic Units (OTUs), are the sequences under consideration and internal nodes are inferred ancestral units and also termed as hypothetical taxonomic units (HTUs). Figure 3 shows a phenogram generated by MEGA 6.06 using Neighbour-Joining method. This represents ancestral relationship between different closely related species.

After considering BLAST and phylogenetic analysis, different mineral solubilizing strains are identified as shown in Table 3. Seventy five per cent of PSB belongs to Proteobacteria phylum and the remaining 25% to Firmicutes. Different sulphur oxidizing and phosphate solubilizing bacteria have been widely used as biofertilizer and also have successfully shown assessment in reclamation of mine area. Priyanka *et al.*[27] have recovered eight novel sulphur oxidizing bacteria and used them as biofertilizers with nitrogen fixing, antifungal activity and phosphate

solubilization. So these isolates can be tested further for both remediation, fertilizers, etc. and efficiently used in mining area dumping site for making it unpolluted and fertile. Eliya *et al.*[28] have identified phosphate and potassium solubilizers in the area around the limestone mining in Cirebon Quarry with the same type of investigation.

This study revealed the presence of phosphate solubilizing bacteria: *P. putida*, *B. licheniformis*, *P. taiwanensis* and *P. aeruginosa* in the selected manganese mining area. The analysis of BLAST similarities or differences was studied against some strains of related ones and many useful mechanisms such as heavy metal tolerance or removal of multiple metals were exploited with phosphate solubilizing activity. Although there is need for deep confirmation, these strains have the potential to be applied for many human health and wealth support programmes and reclamation of mine areas.

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