Isolation and Characterization of Phosphate Solubilizing Microorganisms from *Saccharum officinarum* Rhizosphere

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Abstract
Deficits in phosphate can hinder plant growth and development because it is essential to all aspects of plant growth and development. The present study focused on isolation and evaluation of soil bacterial isolated from 4 different locations. The 21 isolates recovered were subjected to phosphate solubilization and siderophore productions assay. As per the results 9 bacterial isolated (V1, V2, V3, V11, V12, V13, CV1, CV2 and CV11) were found best phosphate solubilizers on the basis of qualitative phosphate assay. Out of 9 screened bacterial isolated only 3 were best suited for siderophore production (V2, V11 and V13). The pqq genes responsible for phosphate solubilization were also checked through amplification and as per the observation, 5 bacterial isolates showed positive amplification (V1, V2, V3, V11, V13 and CV11).

The identified isolates present potential phosphate solubilizing ability of bacterial isolates which can further be applied in the field of biofertilizer development and commercialization.

Keywords: Biofertilizer, phosphate solubilization, *pqq*D gene.

Introduction

The Phosphorous is one of the most essential primary elements for plant growth in addition to nitrogen and potassium [38]. It is a fundamental structural component of plant cell like DNA, RNA and ATP and serves as catalyst in several biochemical processes occurring in plants. Phosphorus nutrition is also associated with resistance development against diseases in plants[32,33]. Phosphorous is present in soil in both organic and inorganic forms; its content varies in soil from 0.02–0.5% with an average of 0.05%[36]. Phosphorous (P) is the least mobile nutrient in the soil and plants when compared with other macronutrients. It is uptaken by the plants from the soil in the form of phosphate $(PO_4)^{-3}$.

Phosphorous is essential for plant growth as it triggers the growth of young plants, it accelerates a vigorous start and hastens the maturity. Insufficient supply of P reduced the plant growth. Phosphorus is acquired by microorganisms such as mycorrhizal fungi and PSMs. Phosphorus solubilizing microorganisms (PSM) have been found in the rhizospheric soil since 1903[14]. In the last few decades, soil-plant-microbe interaction has attracted much attention. Many species of microorganisms have been discovered in soil, particularly in the rhizosphere and they are recognised to play an essential part in the growth and development of plants[15,16,18].

Plant growth promoting rhizobacteria (PGPR) are key to agriculture which help in mobilization of micro and macronutrients, provide growth hormones, activate defense response within plant system, provide external shield against diseases etc. These PGPR due to huge importance are extensively studied[3-6,18]. Phosphorus solubilization in soil is one of those important traits acquired by PGPR. Bacterial species are more efficient than that of fungi in the solubilization of phosphorus[17]. Phosphorus solubilizing bacteria (PSB) make 1–50 percent of the total microbial population in soil while phosphorus solubilizing fungi (PSF) make just 0.1–0.5 percent[7].

Phosphate solubilizing microorganisms (PSMs) isolated from *Saccharum officinarum* rhizosphere have shown potential as plant growth-promoting rhizobacteria. Studies have identified various bacterial species with phosphate solubilization abilities including *Acinetobacter*, *Staphylococcus*, *Bacillus* and *Pseudomonas*[1,13,24]. These PSMs can convert insoluble phosphates into soluble forms through organic acid and phosphatase production[29].

In addition to phosphate solubilization, many isolates exhibit other plant growth-promoting traits such as indole-3-acetic acid (IAA) production, nitrogen fixation and siderophore production[9,23]. The use of these native PSMs as biofertilizers enhance sugarcane growth and provide a sustainable alternative to chemical fertilizers. Further research on these beneficial microorganisms may lead to improved sugarcane cultivation practices and reduced environmental impact.

Material and Methods

Collection of Soil Samples: Soil samples were collected from different geographic locations of western Uttar Pradesh in India namely Meerut, Muzaffarnagar, Baghpat and Ghaziabad. Sugarcane farming is the main factor that governs the economy of the farmers of this region. Surface layer soil, not deeper than 15 cm, from at least three locations of each site, was collected, composited, homogenized by sieving and stored at 4^0C till further use[27]. The details of the sample collection sites are given in table 1.

Table 1
Geographical Details of the Soil Sampling Locations.

Sampling sites	Longitude/ Latitude	Altitude (Meters)
Meerut	28°98°45°N 77°70'64"E	247
Muzaffarnagar	29°47'27"N 77°70'85"E	243
Baghpat	28°94'28"N 77°22'76"E	253
Ghaziabad	28°66'92"N 77°45'38"E	214

Culture Isolation: All the soil samples were subjected to serial dilution and agar plating method for isolation of organisms. Dilution factors were taken ranging from 10^{-1} up to 10^{-10} and the diluted soil samples were spread on sterile agar NBRIP plates and incubated at 37°C for 24 hours. Agar for bacterial isolation employed the standard procedure of Somasegaran and Hoben[35]. The individual colony was picked and purified on separate media using streak plate method. Glycerol stocks in 50% glycerol were made and stored at 4^0C for preservation.

Qualitative P Solubilization: Qualitative P solubilization of all bacterial isolates was done on Pikovskaya medium to screen P solubilizing positive isolates. Sterilized Pikovskaya medium was poured in to sterilize Petri plates and after solidification of the medium, a pinpoint inoculation of bacterium was made on the Petri plate under aseptic condition. The plates were incubated at 30 ± 1^0C for 7 days. Nine P solubilizing positive bacterial isolates were selected based on the presence of halo zone around the bacterial colony. Comparative solubilization index was determined by the following formula[25]:

$$Solubilization\ Index\ (SI) = \frac{\text{colony diameter} + \text{halozone diameter}}{\text{colony diameter}}$$

Morphological and biochemical Characterization: Colonies developed after streaking on their respective medium as well as on nutrient agar were examined for their morphological characteristics like size, shape, color, elevation and margin etc. For microscopic examination, loopful culture was smeared on a clear slide and stained through Gram's staining procedure as per Bergey's Manual of Determinative Bacteriology[11]. The slide was observed under oil immersion objective lens (100X magnification) of compound microscope and studied for Gram's reaction, shape and arrangement of the cells.

Quantitative Estimation of Phosphorous: Phosphorous solubilized by bacterial isolates was quantified by the method of Fiske and Subbarow[10]. Quantification of P on the basis of blue color developed was done in NBRIP-BPB broth medium, as described by Nautiyal et al[26] followed by Das et al[3]. Bacterial isolates were grown in 25 ml NBRIP medium for 72h at 28±1°C, 120 rpm. Cultures were centrifuged at 6000 rpm for 5 min. One ml of supernatant at different interval was mixed with 0.4 ml of molybdate solution (2.5% ammonium molybdate in 5 N H_2SO_4) + 0.2 ml of color reagent (10 ml of 5% sodium bisulphite, 20% sodium sulphite and 25g 1-amino-2-naphthol-4-sulphonic acid) along with 0.4 ml of 10% TCA and 4 ml TDW. After incubation at room temperature for 5 minute, a blue color was developed which was read at 640nm in spectrophotometer. The standard curve was drawn with the help of stock of phosphorous (KH_2PO_4) at the rate of $1mgml^{-1}$.

Siderophore Production: The chrome azurol sulfonate (CAS) assay was used for screening siderophores producers, since it is comprehensive, exceptionally responsive and most convenient[31,34]. CAS agar was prepared using 60.5 mg CAS, dissolved in 50 mL dH_2O and mixed with 10 mL iron (III) solution (1 mM/L $FeCl_3.6H_2O$, 10 mM/L HCl). Under stirring, this solution was slowly added to 72.9 mg HDTMA dissolved in 40 mL dH_2O. The resultant dark blue mixture was diluted 20-fold and autoclaved at 121°C for 15 min., agar (2%, w/v) being used as gelling agent. For the qualitative assay, cultures were spot inoculated onto the chrome azurol blue agar and incubated at 28^0C for 24 to 48h. The results were interpreted based on the color change due to transfer of the ferric ions from its intense blue complex to the siderophore. The sizes of yellow orange haloes around the growth indicated siderophore activity.

Identification of P solubilization potential through *pqq*D and *pqq*E Gene amplification: Genomic DNA was isolated from all the isolates using HipurA™ Bacterial and Yeast. Genomic DNA Purification Spin Kit (MB505), Himedia Laboratories Ltd., Mumbai, India was used as per the manufacturer's instructions. Agarose gel electrophoresis was done for checking the purity and homogeneity of sample. For amplification of *pqq*D gene, 0.1 µM each of forward and reverse primer were used (primer F-5′CATGGCATCGAGCATGCTCC3′ and primer R-5' CAGGGCTGGGTCGCCAACC3')[20]. The PCR program included initial denaturation at 94°C for 3 min. followed by 35 cycles of denaturation (94°C for 1 min), annealing (58°C for 1 min) and extension (72°C for 2 min) and a final extension of 72°C for 10 min. The quality of amplicons was checked electrophoretically on 1.5 % (w/v) agarose gel prepared in 1X TAE buffer (pH 8.0).

Results and Discussion

Subsequently, 21 different bacterial isolates were isolated from soil of all the four regions.

Screening of microbial isolated on the basis of Qualitative P solubilization: Based on qualitative P

solubilization study, out of 21 isolates, 09 isolates have shown the considerable potential in their phosphate solubilizing potential, which was evidently visible on plates, where a clear halo zone was produced around the colony after 3-4 days. (Table 3; Figure 1). Under the qualitative P solubilization assay on Pikovskaya medium, the activity of the isolate was evaluated by determining the solubilization index (SI) i.e. ratio diameter of halo zone over the diameter of the colony. Solubilization index is considered as an indicator for the isolates efficiency. The higher are the values of SI, the greater are the activities of the tested isolate. But liquid culture experiments involved quantification of the amount of phosphate solubilized[15,16,18]. Similarly Rajwar et al[30] also isolated the bacterial isolates from different rhizospheres of Uttarakhand and screened them for P solubilization capacity.

Morphological Characterization of Selected Isolates: Colonies developed after streaking on nutrient agar medium were examined for their morphological characteristics like size, shape, color, elevation and margin etc. (Table 2)[21,22].

Quantitative Estimation of P Solubilize by Bacterial Isolates: 9 bacterial isolates showing positive P solubilization were subjected to quantitative P solubilization test using NBRI-BPB liquid medium that contained known amount of insoluble phosphate in the form of tri calcium phosphate (TCP). The NBRI-BPB liquid medium for bromo phenol blue dye as a pH indicator was used for the test of purity and stability[26].

Significantly, highest P solubilization potential of V-2 was documented as 415.08 µg/ml which corresponds with its largest SI on Pikovskya agar plate. V-11 solubilized 440.14 µg/ml phosphorus followed by V-13(380.43 µg/ml). V-12 (268.75 µg/ml), CV-11 (216.29 µg/ml), CV-1 (293.17 µg/ml), V-1 (210.92 µg/ml) V-3(167.83 µg/ml) and CV-2 (215.92µg/ml) (Figure 2).

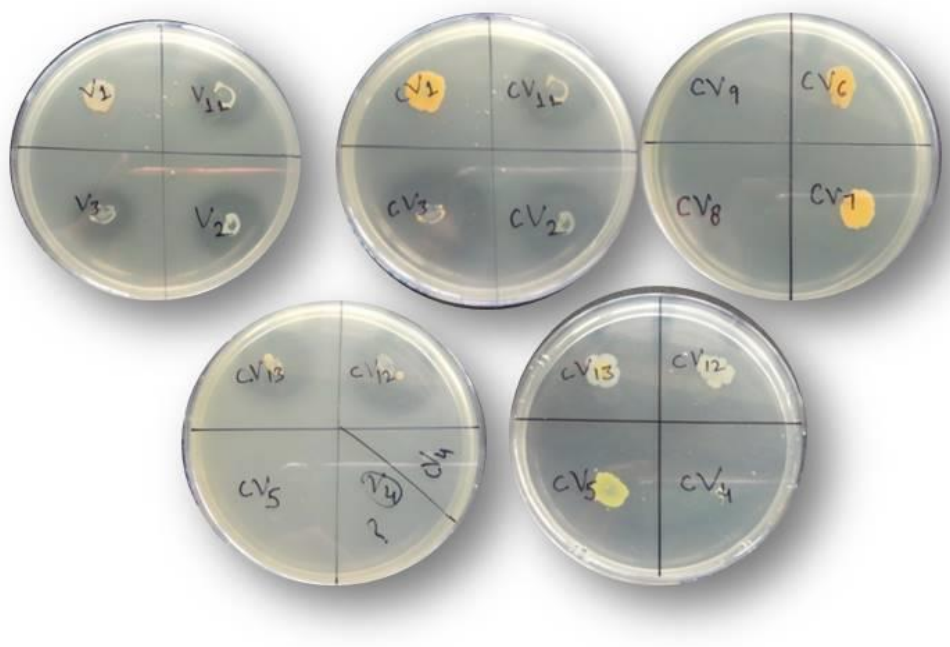

Figure 1: Isolated cultures showing clear zone on Pikovaskya Agar Plate

Table 2
***In vitro* Tricalcium Phosphate Solubilization by Bacterial Isolates in NBRI-BPB Broth medium (pH 7.0) at 30°C and Siderophore Production Screening**

S.N.	Strain I.D.	P solubilization index*	P solubilization (µg/ml)	Siderophore production
1	**V-2**	**7.5**	**415.08**	+++
2	**V-13**	**6.2**	**380.43**	+
3	V-3	5.3	167.83	-
4	V-12	4.5	268.75	-
5	CV-1	3.5	293.17	-
6	CV-2	3.2	215.32	-
7	V-1	2.8	210.92	-
8	CV-11	2	216.29	-
9	**V-11**	**7.1**	**395.29**	++

Qualitative assay of Siderophore Production: Out of the nine bacterial isolates, only three V-02, V-11and V-13 were found to produce siderophore (Table 3). Siderophore producing bacteria promote plant growth by depriving indigenous microflora of Fe**.** Siderophore producing bacteria are also indirectly responsible for P solubilisation**.** Applied P fertilizers readily form complexes with soil cations such as Fe, Ca and Al. Siderophore producing PGPR enhance the availability of P as they have a high affinity for Fe and release P from iron bound complexes. Siderophores producing PGPR scavenge Fe from minerals and organic compounds under conditions of iron starvation and thus indirectly release P in soil[31,34].

Amplification of Partial *pqq*D and *pqq*E Gene from Bacterial Isolates: Pyroloquinoline quinine (PQQ), a cofactor required for gluconic acid synthesis, is involved in P solubilization and antifungal action[12]. Out of nine bacterial cultures, five have shown positive amplification for partial *pqq*D and *pqq*E gene as ideal markers for identification of P solublizers[2,20].

Table 3
Cultural Characteristics of Bacterial Isolates

S.N.	Isolate I.D.	Gram Reaction	Cell Morphology	Arrangement	Colony characteristics				
					Shape	Size	Elevation	Surface	Chromogenesis
1	V-3	Positive	Small rods	Solitary	Circular	Small	Convex	Smooth	Cream
2	V-1	Positive	Small rods	Solitary	Irregular	Large	Raised	Smooth	Yellow
3	V-11	Negative	Small rods	Solitary	Circular	Large	Convex	Smooth	Cream
4	V-2	Negative	Cocci	Solitary	Irregular	Small	Umbonate	Smooth	Cream
5	CV-11	Positive	Filamentous	Solitary	Circular	Small	Convex	Smooth	Brown
6	CV-2	Negative	Filamentous	Solitary	Circular	Small	Convex	Smooth	Cream
7	CV-1	Negative	Small rods	Solitary	Circular	Small	Convex	Smooth	Yellow
8	V-12	Negative	Small rods	Solitary	Circular	Large	Convex	Smooth	Yellow
9	V-13	Negative	Small rods	Solitary	Circular	Small	Flat	Smooth	Cream

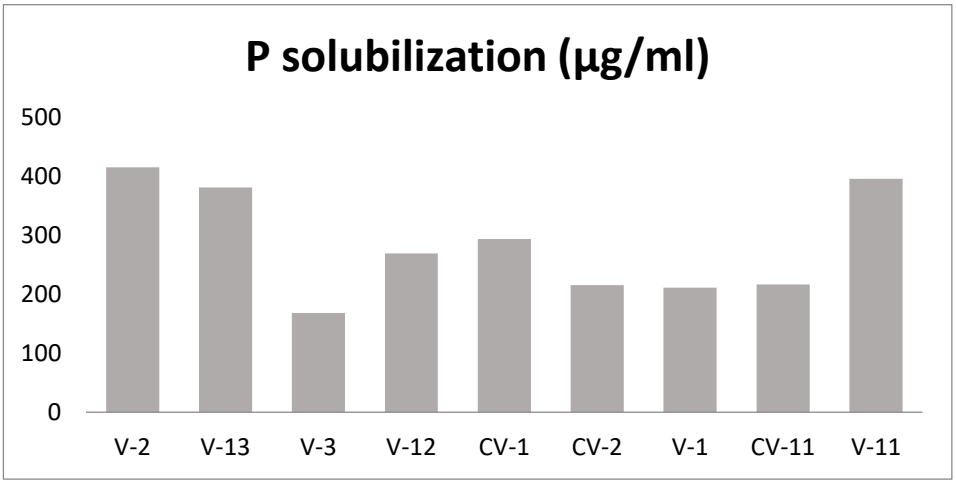

Figure 2: Effect of bacterial strains on P solubilization in NBRIP broth at 30˚C for eight days. Each value is the mean of three replicates

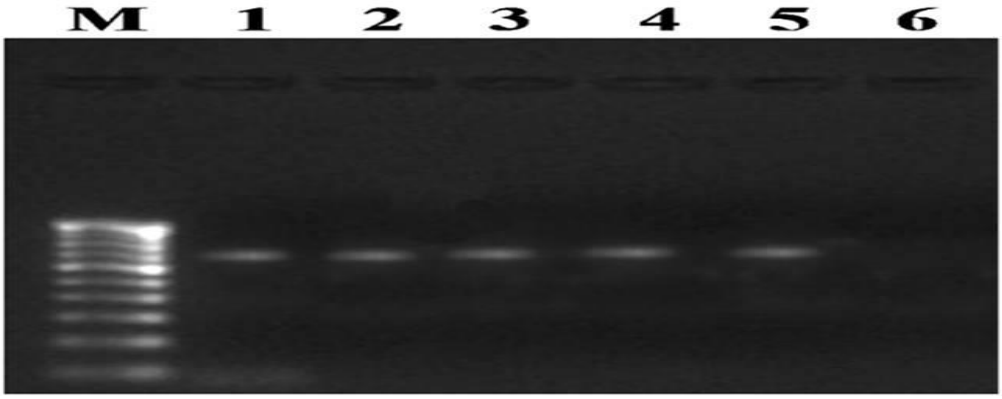

Figure 3: PCR amplicons of partial *pqq*D and *pqq*E fragment amplified using primer set[20]. Lanes: M. 100 bp DNA ladder; 1. V-1; 2. V-2; 3. V-3; 4. V-13; 5.CV-11; 6. -ve Control, respectively

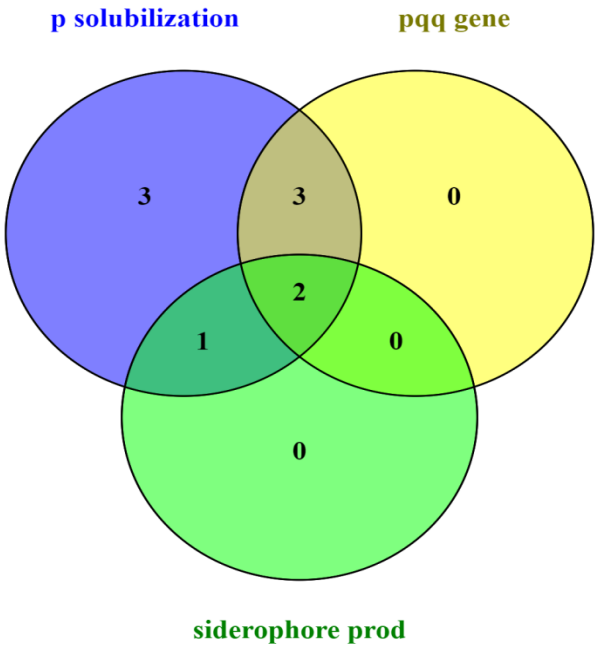

Figure 4: Vein Diagram to show overall results (Venny 2.0)

An amplicon of the expected size i.e. approximately 670 bp was found in genomic DNA of bacterial cultures of V-2, V-11, V-13, CV-11, V-1 (Figure 3). Rajwar et al[30] also validated the P solubilization potential among the isolated bacterial strains with the help of Pqq genes.

Relationship between different parameters studied: The vein diagram compiles all the PGP traits assayed in the study. As per the results, a total of 9 bacterial isolates were positive P solubilizers, out of which only 3 showed siderophore production and 5 gave positive Pqq amplification. In addition to the above mentioned results, 2 bacterial isolates (V-2 and V-11) were the best isolates as they gave maximum possible results (Figure 4).

Conclusion

To meet the ever-increasing demand of food due to population pressure, Green revolution came into existence. It however, brought remarkable gain in food production but with unnoticed concerns for sustainability due to disproportionate use of chemical fertilizers. Moreover, future reliability on chemical fertilizers will persist to cause loss in soil fertility, pollution and a lot of saddle on the fiscal system. To conquer this matter, the Government of India is promoting a practice of using biofertilizers alone or in combination with fertilizers. Due to slow growth rate and delicate handling, relatively insufficient attention has been paid to biofertilizers.

Therefore, efforts were made to isolate and characterize phosphate solubilizing bacteria and field implications of selected potential strains. Hence, on the basis of above observed growth promoting parameters, it is concluded that V02, V03, V11, V12, V13, CV12 and CV13 may have good plant growth promoting potential and can efficiently be used for agriculture. In the present scenario, where acceptance of genetically modified food crops is a big question mark, farming practices using biofertilizers may be a boon to humanity. PGPR mediated organic farming would pave the way to a prosperous, healthy and sustainable Nation towards sustainable agriculture systems for holistic growth.

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