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Screening of Phosphate Solubilizing Bacterial Strains from Rhizosphere Soil of Paddy and Ground Nut Plant Tiruvallur District, India

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A B S T R A C T

Phosphorus is an essential macronutrient for growth and development of plants involved in most important metabolic pathways. Worldwide soils are supplemented with inorganic phosphorous as chemical fertilizers to improve crop production but repeated use of these fertilizers deteriorates soil quality. There are few phosphate solubilizing bacterial strains which could convert insoluble forms of phosphorus to an accessible form which is an important trait in plant growth for increasing crop yields. These phosphate solubilizing bacteria are used as inoculants to increase the phosphorous uptake by the plants. In this present study twenty-four bacterial strains were isolated, 14 isolates were from ground nut rhizosphere soil, whereas the other 10 isolates were from root of paddy rhizosphere soil. The phosphate solubilizers were screened based on the formation of visible Halo/zone on PVK agar plates and insoluble inorganic phosphate in liquid media. The bacterial isolates were identified based on their phenotypic and 16S rRNA genes sequencing data as *Maricaulis virginensis*- APKVG-02, *Kosakonia oryzae* APKVG- 07, *Klebsiella pneumonia* APKVG- 10. Such isolated phosphate solubilizing bacterial strains could be employed as bio inoculants for the increase of crop yield.

Introduction

Phosphorus is the most important key element in the nutrition of plants, next to Nitrogen. It plays an important role in virtually all major metabolic processes in plant including photosynthesis, energy transfer, signal transduction, macro-molecular biosynthesis and respiration (Khan *et al.*, 2010) and nitrogen fixation in legumes (Saber *et al.*, 2005).

Although Phosphorus (P) is abundant in soils in both inorganic and organic forms, it is a major limiting factor for plant growth as it is in an unavailable form for root uptake. Inorganic Phosphorus occur in soil, mostly in insoluble mineral complexes, some of them appearing after frequent application of chemical fertilizers. These insoluble phosphorus, precipitated forms cannot be

absorbed by plants (Rengel *et al.,* 2005). The repeated and injudicious applications of chemical Phosphorus fertilizers, leads to the loss of soil fertility (Gyaneshwar *et al.,* 2002) by disturbing microbial diversity and consequently reducing yield of crops.

Microorganisms which are capable of solubilizing insoluble phosphate, also called phosphate solubilizing bacteria (PSB) not only provide plants with phosphorus, but also facilitate the growth of plants through (a) fixing atmospheric nitrogen (Dobbelaere *et al.,* 2002; Sahin *et al.,* 2004); (b) accelerating the accessibility of other trace elements (Mittal *et al.,* 2008); (c) producing plant hormones such as auxins (Jeon *et al.,* 2003; Egamberdiyeva *et al.,* 2005), cytokinins (Graciade Salamone *et al.,* 2001), and gibberellins (Gutierrez- Manero *et al.,* 2001); (d) releasing siderophores (Wani *et al.,* 2007), hydrogen cyanide (Kang *et al.,* 2010), enzymes and/or fungicidal compounds such as chitinase, cellulase, protease (Dey *et al.,* 2004; Lucy *et al.,* 2004; Hamdali *et al.,* 2008) which ensure antagonism against phytopathogenic microorganisms.

Soil microbes play a significant role in making the phosphorus available to plants by mineralizing the organic phosphorus in the soil.

The uptake of phosphorus by plants in tiny fraction was supplied in the form of fertilizer. This inorganic phosphorus is rapidly immobilized and unavailable to the plant (Anbuselvi *et al.,* 2015).

Several soil bacteria, particularly those belonging to phosphate solubilizing bacteria (phosphobacteria), possess the ability to solubilize insoluble inorganic phosphate and make it available to plants. The solubilization effect is generally due to the production of organic acids by these organisms. They are also known to produce amino acids, vitamins and growth promoting substances like indole acetic acid (IAA) and gibberellic acid (GA3) which help in better growth of plants.

Phosphobacteria have been found to produce some organic acids such as mono carboxylic acid (acetic, formic), mono carboxylic hydroxy acid (lactic, glucenic, glycolic), mono carboxylic, ketoglucenic, decarboxylic acid (oxalic, succinic), dicarboxylic hydroxyl acid (malic, maleic) and tricarboxylic hydroxy (citric) acids in order to solubilize inorganic phosphate compounds (Lal *et al.,* 2002).

The insoluble forms of P such as tricalcium phosphate (Ca3PO4)2, aluminium phosphate (Al3PO4), iron phosphate (Fe3PO4), etc. may be converted to soluble Phosphorus by Phosphate solubilizing organisms inhabiting different soil ecosystems (Gupta *et al.,* 2007; Song *et al.,* 2008; Khan *et al.,* 2013; Sharma *et al.,* 2013).

Soil microorganisms in this regard have generally been found more effective in making Phosphorus available to plants from both inorganic and organic sources by solubilizing (Toro *et al.,* 2007); (Wani *et al.,* 2007) and mineralizing complex Phosphorus compounds (Ponmurugan *et al.,* 2006).

The present study aims at screening, isolation and characterization of phosphate solubilizing bacteria from rhizosphere area of groundnut and paddy plant grown soil. The isolated Phosphate solubilizing bacterial strains were optimized for its growth conditions, for higher accumulation of phosphate. Further the bacterial strains were screened and identified by biochemical characterization and molecular identification.

Materials and Methods

Collection of soil samples

Soil samples were collected from the Rhizosphere soil of groundnut and paddy cultivated field, Mappedu, Thiruvallur district.

The samples were collected in sterile zip lock covers and were immediately transferred to the laboratory and stored at 4°C.

Enrichment of the phosphate solubilizing bacterial strains (PSB)

1 g of soil samples were collected inoculated in Pikovskaya's medium without agar having the following composition (Pikovskaya, 1948) (g/l): Glucose- 10, Yeast extract - 0.5, Ammonium sulphate - 0.5, Potassium chloride - 0.2, Tri Calcium phosphate - 5, Magnesium Sulphate - 0.1, sodium chloride-0.2, ferrous sulphate-0.002, manganese sulphate-0.002, Agar – 15, pH – 4.

Isolation of phosphate solubilizing bacterial strains

The soil samples were inoculated in the PVK broth medium was serially diluted in sterile distilled water.

Dilutions from 10^{-5}, $10^{-6,}$ 10^{-7} and 10^{-8} were inoculated onto the PVK Agar medium through both spread and pour plate technique.

The plates were incubated at 37° C for 24 hours. After incubation the bacterial colonies have clear zone of phosphate solubilization were selected and streaked on new PVK agar plates to get pure colonies of the isolates.

Screening of phosphate solubilizing bacteria

Primary screening – phosphate solubilization index

0.1 ml of each of PSB cultures preserved in sterile distilled water was placed on Pikovskaya's agar (Pikovskaya, 1948) plates and incubated for seven days. Positive cultures were screened by observing transparent halo zones in Pikovskaya's medium. Solubilization Index was measured using following formula (Edi-Premono *et al.,* 1996).

$$\text{Solubilization index (SI)} = \frac{\text{Colony diameter + halo zone}}{\text{Colony diameter}}$$

Bromophenol blue dye agar method

Bromophenol media consist of pikovskaya agar and 0.5 % of bromophenol blue dye, the media were prepared and pH was adjusted to 7.0. This medium was complemented with agar 1.5 % and autoclaved at 15 psi for 15 minutes. Autoclaved medium was poured in sterile petri plates (25 ml/plate) under laminar flow hood and allowed to solidify. Bacterial colonies were inoculated on petriplates containing medium for plate assay and the plates were incubated in inverted position in incubator for up to 24 hours at 37°C. Positive cultures were screened by observing yellow halo zone on bromophenol blue medium.

Secondary screening - estimation of phosphate solubilization by broth assay

The quantitative bioassay was carried out using Erlenmeyer flasks (100 mL) containing 50 ml PVK broth. The bacteria was inoculated in the medium having pH

7.0, which was adjusted before autoclaving the medium. The flasks were incubated at 25°C in a shaker for 48 h at 100 rpm. The cultures were collected for centrifugation for 10 min at 5500 rpm. The supernatant was decanted and filtered through Whatman No. 41 filter paper (Islam *et al.,* 2007). The available P content in the supernatant was estimated by phospho-molybdate blue complex colorimetric method at 660 nm wavelength (Olsen *et al.*, 1954, Alam *et al.,* 2008).

Morphological, Biochemical and Molecular identification of PSB strains

The bacterial strain isolated was studied for morphological and biochemical characteristics. Initially, Gram staining and motility test were performed after which biochemical characterization was done (with Himedia, India) to identify the phenotypic characters of the bacterial strains. Catalase, Oxidase, Urease, IMViC and Triple sugar iron agar tests were performed to determine the biochemical characteristics of the isolate.

After 24 h incubation at 37 °C, the colour change observed was determined as positive/negative result. Genus level identification of the unknown bacterial strain was accomplished by using Bergey's Manual of Systematic Bacteriology (Brenner 2005). Molecular characterization of chromosomal DNA was done from the pure strain by the standard phenol/chloroform extraction method. The 1.2 kilo base partial sequence of the 16S rRNA gene was amplified from the chromosomal DNA using polymerase chain reaction (PCR) with universal Eubacteria-specific primers 16F27 (5'- CCA GAG TTT GAT CMT GGC TCA G - 3') and 16R1525XP (5'- TTCTGCAGT CTA GAA GGA GGT GWT CCA GCC - 3'). The PCR conditions used were an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min and sequenced on an ABI310 automated DNA sequencer using the BigDye Terminator kit (Applied Biosystems 3730 x DNA Analyzer). The amplified 16S rRNA gene PCR products from these isolates were directly sequenced after purification by precipitation with polyethylene glycol and NaCl. The primers used to obtain the complete sequence of 16S rRNA gene of the isolates were the same as for PCR amplification (16F27N and 16R1525XP).

The isolated bacterial strains were identified by 16s rRNA sequencing which was done in Amnion Bio-sequencing, Bangalore and construction of phylogenetic tree. The evolutionary relationship between the bacterial strains was determined by constructing a phylogenetic tree. The sequences obtained were converted to FASTA format and BLASTn was done for the nucleotide sequences of the isolated strains. The homologous 16s rRNA sequences were downloaded in FASTA format and the phylogenetic tree was constructed using the Maximum likelihood method in MEGA 7.0 software.

Results and Discussion

Enrichment of the phosphate solubilizing bacterial strains

Enrichment of the bacterial strains was performed in PVK medium containing tricalcium phosphate as the phosphorus source. A total of 24 isolates were isolated, out of which 14 isolates were obtained from groundnut cultivated soil and 10 were from paddy cultivated soil. The isolated bacterial strains were studied for the phosphate solubilizing activity in the PVK Medium.

Isolation of phosphate solubilizing bacterial strains

The isolated 24 bacterial strains were able to survive and grow on PVK medium from 10^{-5} to 10^{-9} dilution by spread plate method. From the isolated strains, effective phosphate solubilizing bacterial strains were used for further screening. The bacterial strains, which were able to produce effective phosphate solubilization with zone of inhibition was further analysed on their screening properties (Fig. 1).

Screening of phosphate solubilizing bacterial strains

Primary screening – phosphate solubilization index

Primary screening was conducted with phosphate solubilizing bacterial strains (24 isolates) showed that the tricalcium phosphate present in the PVK agar medium in insoluble form was converted into the soluble form by the isolates (phosphate solubilizers). It was noted that the halo zone was produced by the bacterial strains in the range of 1- 7.5 cm while effective solubilization index where obtained with three bacterial strains. It was estimated as APKVG 02 - 7.5 cm, APKVG 07 - 4.2 cm and APKVG 10 - 5.5 cm respectively. Hence, the three effective bacterial strains were used for further plant growth studies (Fig. 2).

Bromophenol blue dye agar method

To prove the phosphate solubilizing ability of the efficient phosphate solubilizing bacterial strains they were studied for organic acid production in bromophenol blue agar method. The isolated efficient PSB strains produced organic acid which lead to change in pH of the medium. It was indicated by change in the colour of the medium from blue to yellow around the colony of the inoculated PSB strains (Fig. 3).

Secondary screening – estimation of phosphate solubilization by broth assay

The isolated phosphate solubilized bacterial strains showed phosphate solubilization of the PSB strains APKVG02 (79 mg/ml) followed by APKVG07 (48 mg/ml) and APKVG10 (65 mg/ml) (Fig. 4).

16s r-RNA sequencing and phylogenetic analysis

The identification of the bacterial strains was done by using BLAST tool available in the NCBI database. The sequences of the individual bacterial obtained from the 16s rRNA sequencing were loaded in the BLAST software and the organisms were identified as APKVG02 – *Maricaulis virginensis*, APKVG07 – *Kosakonia oryzae*, APKVG10 – *Klebsiella pneumonia.*

The evolutionary relationship between the bacterial strains was determined by constructing a phylogenetic tree. The sequences obtained were first converted into a FASTA format and the phylogenetic tree was then converted by using the software MEGA 6.0 (Fig. 5).

Phosphorus (P) is the second important key plant nutrient after nitrogen. An adequate supply of Phosphorus is therefore required for proper functioning and various metabolisms of plants. Majority of Phosphorus in soils is fixed, and hence, Phosphorus for plant is scarcely available despite the abundance of both inorganic and organic Phosphorus forms in soils. A group of soil microorganisms capable of transforming insoluble Phosphorus into

soluble and plant accessible forms across different genera, collectively called phosphate-solubilizing bacteria (PSB), have been found as the best ecofriendly option for providing inexpensive Phosphorus to plants (Saghir khan *et al.*, 2014).

Fig.1 Enriched bacterial isolates grown on PVK agar

Fig.2 Halo zone formation showing P solubilization by APKVG02, APKVG07 and APKVG10

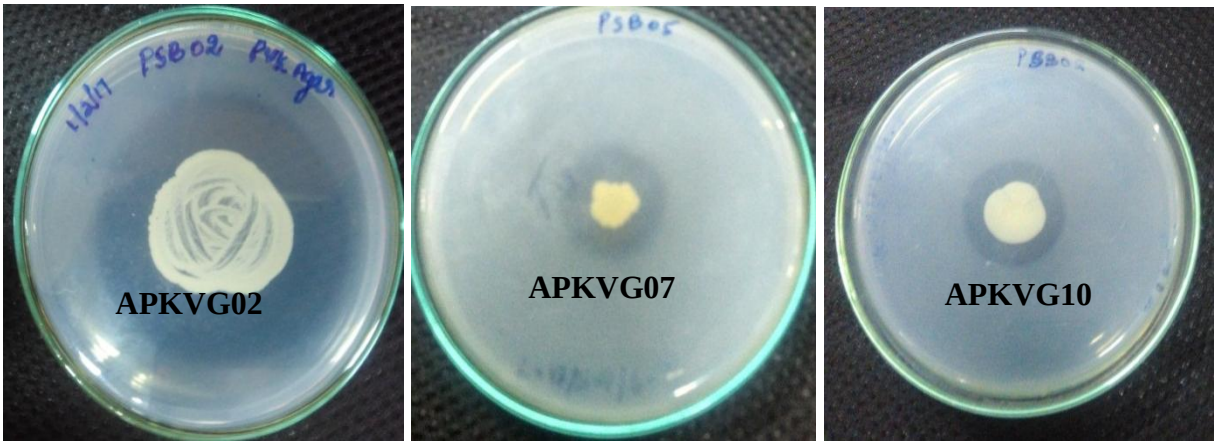

Fig.3 Organic acid production by PSB strains showing a yellow zone in bromophenol medium

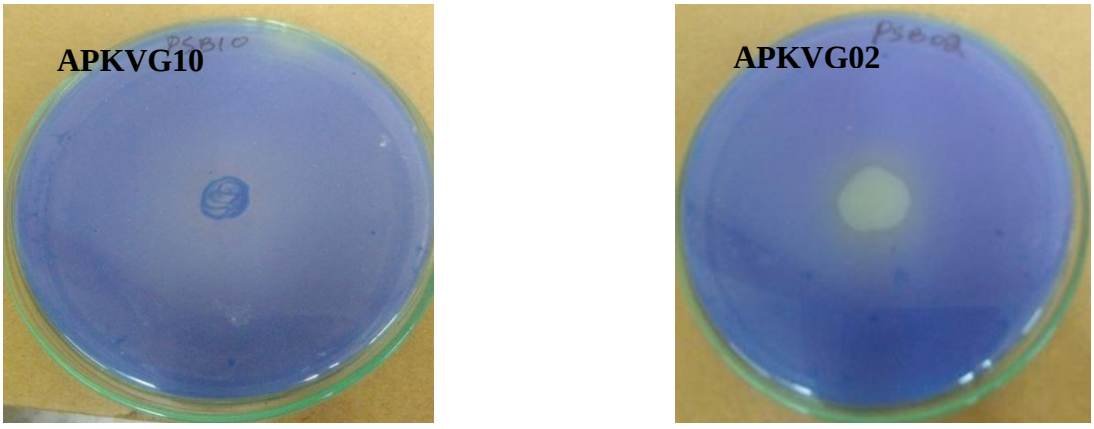

Fig.4 Broth assay on Phosphate solubilization

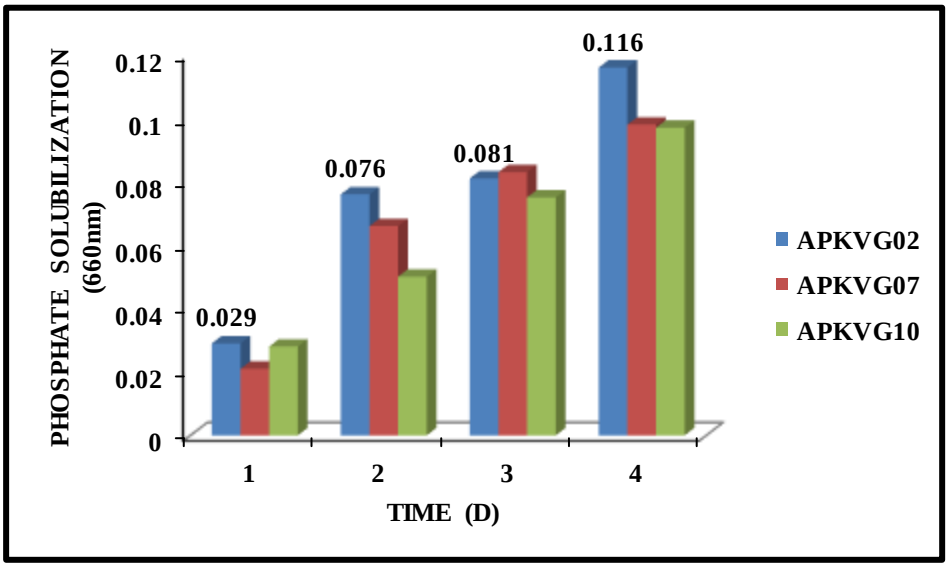

Fig.5 Phylogenetic tree of the PSB strains

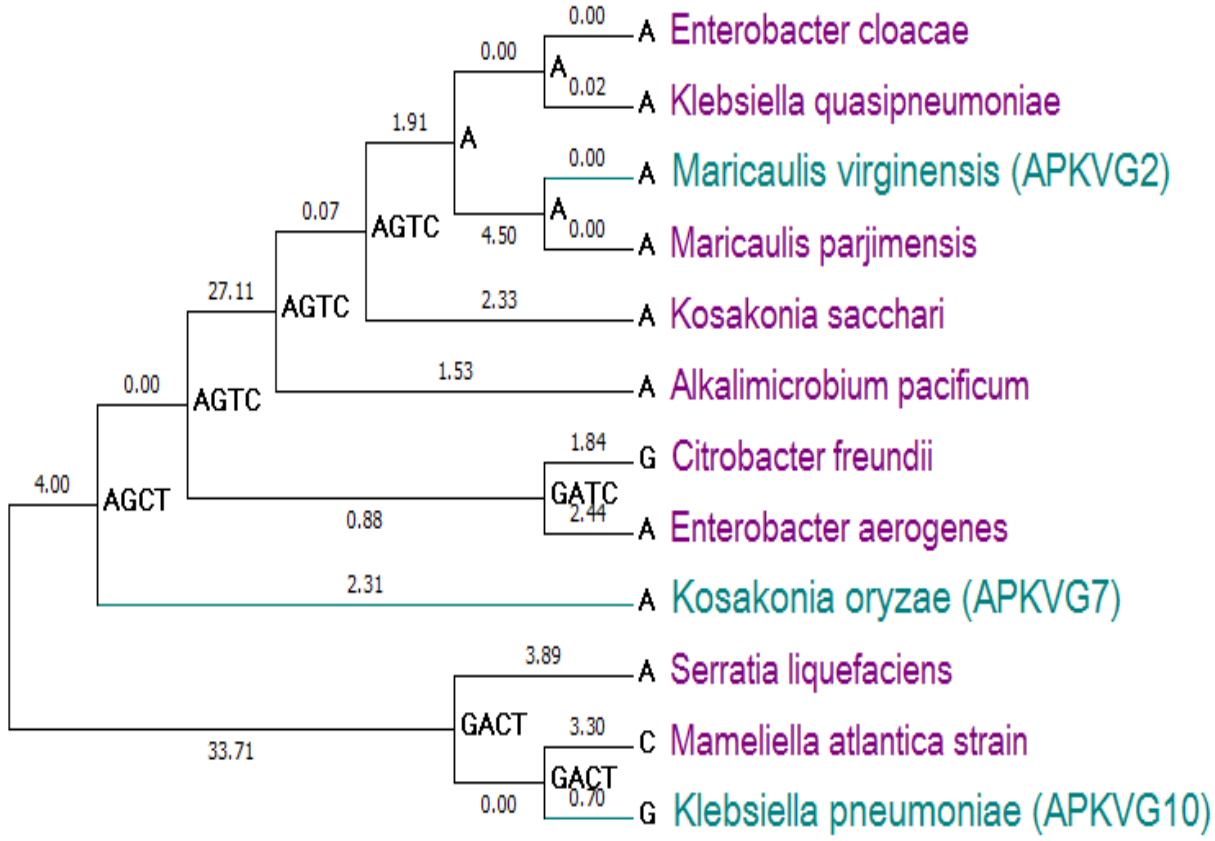

Table.1 Analysis of solubilization index and P solubilization by the bacterial

S.NO	ISOLATES	SOLUBILIZATION INDEX (cm)	P SOLUBILIZATION (mg/ml)
1	APKVG01	3.3	63
2	APKVG02	7.2	79
3	APKVG03	3.5	39
4	APKVG04	2.5	48
5	APKVG05	3.9	22
6	APKVG06	1	53
7	APKVG07	4.2	48
8	APKVG08	2.4	33
9	APKVG09	2.2	59
10	APKVG10	5.5	65
11	APKVG11	2.5	40
12	APKVG12	1	27
13	APKVG13	2.4	20
14	APKVG14	2.7	28
15	APKVG15	2.4	40
16	APKVG16	2.9	16
17	APKVG17	2.9	29
18	APKVG18	2.9	31
19	APKVG19	-	12
20	APKVG20	-	19
21	APKVG21	3.5	43
22	APKVG22	1.6	21
23	APKVG23	-	11
24	APKVG24		

Karpagam and Nagalakshmi (2014) reported 37 Phosphate Solubilizing bacterial (PSB) strains isolated from rhizosphere soil out of which 8 bacterial strains showed highest solubilization index ranging from 1.13 to 3.9 cm and high soluble phosphate production of 0.37 mg/ ml in liquid media. In the present study a total of 24 PSB strains were isolated from rhizosphere of paddy and ground nut cultivated soil out of which three isolates showed highest solubilization index ranging from 7.5 to 4.2 cm and high soluble phosphate utilization of 63 to 120 mg/ml in liquid media.

Saikrithika and Veena Gayathri (2016) reported that the growth pattern of SK1 strain showed maximum growth of 0.040 on the 2nd day of incubation and by the end of 4th day it reached the decline phase. In current work, the three bacterial strains APKVG 02, APKVG 07 and APKVG 10 showed maximum growth of 0.684, 0.758 and 0.590 respectively on the 3rd day of incubation, by the end of 5th day it reached decline phase which showed that the PSB strains was able to survive in the medium for 7 days.

Islam *et al.,* (2006) identified the phosphate solubilizing bacteria according to the Bergey's manual of determinative Bacteriology as *Acinetobacter sp., Enterobacter sp., Microbacterium sp.* and *Pseudomonas sp.* In the present study, the bacterial strains APKVG 02, APKVG 07

and APKVG 10 were biochemically characterized and molecularly identified by 16s rRNA sequencing as *Maricaulis virginensis, Kosakonia oryzae* and *Klebsiella pneumonia*.

Hence, this present study was conducted, aiming at the isolation of phosphate solubilizing bacterial strains. By testing their phosphate solubilizing properties, their growth conditions were optimized further they were used as immobilized forms as biofertilizers, which has the capacity to increase the plant growth and production. Such PSB strains will be of great importance in the form of Bioinoculants, in the field of agriculture.

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