Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2582-2845.8544

ISSN: 2582 – 2845

Ind. J. Pure App. Biosci. (2021) *9*(1), 99-107

Indian Journal of Pure & Applied Biosciences

Peer-Reviewed, Refereed, Open Access Journal

Research Article

Isolation, Characterization and Identification of Efficient Phosphate Solubilizing Bacteria from Different Chickpea Growing Areas of Karnataka

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Received: 13.12.2020 | Revised: 16.01.2021 | Accepted: 24.01.2021

ABSTRACT

Chickpea is an important pulse crop extensively grown in different parts of the world. India is the largest producer of chickpea whereas in Karnataka northern Karnataka has larger area under chickpea cultivation compared to southern regions of Karnataka. Phosphorus is the key element required in the nutrition of plants next to the nitrogen and these days phosphatic fertilizers are becoming costly and less available. In this context, an attempt was made to isolate and characterize the efficient phosphate solubilizing bacteria (PSB) from different areas of south Karnataka. Four different soil samples were collected from Arasikere, Chamarajanagara, Mysore and Devanahalli. PSB were isolated using Pikovskaya's media and total of twenty four isolates were obtained. Based on zone of solubilization ten isolates were selected for screening process and from the result of screening tests three best isolates were identified by their morphological and biochemical characteristics as described in Bergey's Manual of Determinative Bacteriology. From 16SrRNA sequencing, the top three most efficient PSB isolates were identified as Bacillus licheniformis(ACP-3), Arthrobacter sp. HPG166 (ACP-2) and Pseudomonas chlororaphis (CCP-2). "Bacillus licheniformis" was the best isolate which was further tested under field condition with different fertilizer levels of SSP and rock phosphate

Keywords: *PSB, Isolation, Characterization, Identification*

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important pulse crop, also known as Bengal gram, simply gram in English. Chickpea is originated in Western Asia. Although it is predominantly consumed as a pulse its flour is used in the preparation of variety of sweets and condiments. Phosphorus (P) is known as the 'energy currency' as it is associated with the several vital functions of life and P is the key element in the nutrition of plants next to the nitrogen.

Cite this article: Dakshayini, G, Anantha Rama, A., Arunkumar P., Muthuraju, R., Narayanaswamy, B., & Nagaraju, K. (2021). Isolation, Characterization and Identification of Efficient Phosphate Solubilizing Bacteria from Different Chickpea Growing Areas of Karnataka, *Ind. J. Pure App. Biosci. 9*(1), 99-107. doi: http://dx.doi.org/10.18782/2582-2845.8544

Phosphorus exists in nature in a variety of organic and inorganic forms. P availability can be very low in soil, because of its fixation as insoluble phosphates of aluminum, calcium and iron. Since P deficiency is the most important key limiting factor restricting the plant growth, chemical phosphate fertilizers are widely used to increase crop yields, where soluble forms of P fertilizer used are easily precipitated as insoluble form, which leads to excessive and repeated application of P fertilizers (Alam et al. 2002).

Phosphorus solubilizing activity is carried out by a large number of saprophytic bacteria and fungi through various microbial processes or mechanisms including organic acid production and proton extrusion (Karpagam and Nagalakshmi, 2014). Phosphate solubilizing microorganisms (PSM) play a significant role in making phosphorus available to plants by bringing about favorable changes in soil reaction in the soil microenvironment leading to solubilization of inorganic phosphate sources. Some microorganisms associated with different pant rhizosphere are able to solubilize inorganic insoluble P salts. *Pseudomonas* and *Bacillus* are two important genera of soil bacteria with promising activity of phosphate solubilization (Reyes et al.,1999; Yadav and Tarafdar, 2011).

Root development, stem, branches, flower and seed formation, crop maturity and quality crop production, and also resistance to plant diseases are the attributes associated with phosphorus nutrition. In the recent years, the cost of chemical fertilizers especially phosphatic fertilizers is increasing which is away from reach of small farmers in the developing countries like India. This has led to the finding of alternative ways for supplying the P to the crop plants in a cost effective way. One such way is development and use of phosphorus solubilizing biofertilizers.

India is the largest producer of Chickpea in the world, contributing around 70 per cent of the world's production occupying an area of 9.01million hectares with the production of 7.58 million tones and productivity of 841 kg/ha. In Karnataka northern Karnataka has larger area under chick pea compared to southern Karnataka, Dharwad, Gadag, Gulbarga, Bidar, Yadgiri, Bellary, Raichur and Davanagere are the major Chickpea growing districts in Karnataka. In this point of view the present experiment was conducted for the isolation and characterization of efficient PSB from different chickpea growing areas of Southern parts of Karnataka

MATERIALS AND METHODS

The evaluation of efficient phosphorus solubilizing bacteria on growth and yield of Chickpea was done at GKVK, Bengaluru. The laboratory experiments were conducted in the Department of Agricultural Microbiology, UAS, GKVK, Bengaluru.

Sample collection

Soil samples were collected from different Chickpea growing areas like Arasikere, Chamarajanagara, Mysore and Devanahalli. Samples were collected at the depth of 0-15cm and brought to lab in polythene bags. The collected soil samples were subjected for the isolation of phosphate solubilizing bacteria.

Media preparation

The Pikovskaya's medium was used for the isolation of phosphorus solubilizing bacteria. Medium composition for one liter : Glucose 10g, Tricalcium phosphate(TCP) 5g, Ammonium sulphate 0.5g, Sodium chloride 0.2g, Magnesium sulphate 0.1g, Potassium chloride 0.2g, Yeast extract 0.5g, Manganese sulphate and Ferrous sulphate in trace amounts, Agar 15g, distilled water one liter and pH adjusted to neutral. The media was sterilized by autoclaving at 121 $^{\circ}$C for 15 minutes and cooled to 50 $^{\circ}$C.

Isolation of phosphate solubilizing bacteria

PSB were isolated from each sample by serial dilution and spread plate method. One gram of soil sample was suspended in 9ml sterilized distilled water blank and shaken thoroughly which gives 10^{-1} dilution. From this solution again 1ml is transferred into 9ml sterile water blank to obtain 10^{-2} dilution. Similarly $10^{-3,}$ 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions were made for

each sample. 0.1 ml of the aliquot was spread on the Petri dish containing Pikovskaya's agar medium (Sonam et al., 2011) and the plates incubated at 28±2 °C. After 2-3 days of incubation, colonies forming halo zones were selected and subcultured on Pikovskaya's agar medium for further characterization study.

Purification of PSB isolates.

All the PSB isolates were purified on Pikovskaya's medium. The colonies that form clear halo zones were selected and purified by streak plate method and preserved on agar slants for further screening and characterization.

Biochemical characterization of phosphorus solubilizing bacteria

All the selected isolates were examined for the colony morphology, cell shape, Gram reaction and subjected to different biochemical tests as detailed below.

Biochemical tests

Gram staining

All the bacterial cultures were examined for colony morphology, cell shape and size, Gram reaction and ability to form spores as per the standard procedures given by Barthalomew (1950).

Gelatin liquefaction

The gelatin liquefaction ability of the bacterial isolates was determined by following the procedure given by Blazevic and Ederer (1975). To the pre sterilized gelatin agar plates, the test cultures were inoculated and incubated at 30 °C for three days. After incubation, the culture plates were flooded with 12% $HgCl^2$ and allowed for 20 minutes. Clear zone around the growth of organism was taken as positive for gelatin liquefaction.

Catalase test (Blezevic and Ederer, 1975)

Nutrient agar slants were inoculated with test culture and were incubated at 30 °C for 24 hr. After incubation the tubes were flooded with one ml of three per cent hydrogen peroxide and observed for production of gas bubbles. The occurrence of gas bubble was taken as positive for catalase activity.

Starch hydrolysis

The ability of the isolates to hydrolysis was examined by the procedure of Eckfod (1927). Petri plates containing two per cent starch agar were inoculated with test cultures and incubated at 30 °C for three days. After incubation the plates were flooded with Lugol's iodine solution and allowed for 15-20 minutes. The clear zone around the colony was considered as positive.

Urease test (James and Natalie, 1992).

The urease activities of the bacterial isolates were determined by inoculating the cultures to five ml of pre-sterilized urea broth containing phenol red as pH indicator. The tubes were incubated for 24 to 48 hours at 30 °C. The formation of dark pink color was taken as positive for urease activity.

Citrate utilization test (James and Natalie, 1992).

Bacterial isolates were streaked on citrate agar slants containing bromo-thymol blue as indicator and incubated overnight at room temperature. After incubation slants were observed for the formation of blue color in the medium and it was taken as positive for the citrate utilization test.

Oxidase test

The isolates to be tested were spotted on the trypticase soya agar plates and incubated for 24 hours at 28 ± 2 °C. After incubation, two to three drops of tetra methyl phenylene diamine dihydrochloride was added to the growth surface of the test organism. The color changed to maroon was taken as oxidase positive.

Hydrogen sulphide production (Cowan and Steel, 1970)

Tubes containing SIM agar were sterilized. The sterilized tubes were stabbed with the test cultures. The tubes were incubated for 48 hours at 28 ± 2 °C. After incubation, the development of black color along the line of the stab was noted and considered as positive for the test.

Molecular characterization of efficient PSB isolates

After screening, the most promising three pure cultures of PSB isolates were sent to Macrogen institute (South Korea) for 16SrRNA sequencing.

RESULTS AND DISCUSSION

Isolation of PSB from the soils of Chickpea growing areas

A total of ten isolates were selected from four different soil samples collected from different Chickpea growing areas of southern parts of Karnataka. The details of population of PSB are given in Table 1. The population of PSB ranged from 11×10^6 to 23×10^6 cfu per gram of soil. All the four soil samples showed the presence of PSB (with solubilizing zones). The highest PSB population was found in Arasikere sample where as the lowest in Devanahalli. Even though PSB population was found in Devanahalli sample high zone of solubilization was not observed. Therefore, based on the zone of solubilization and results of screening tests three most efficient phosphate solubilizing bacteria were selected for the further biochemical characterization and identification process.

Characterization of efficient PSB based on biochemical tests

The three most efficient PSB isolates were identified up to generic level based on morphological and biochemical characteristics as described in the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The results obtained are furnished in Table 2 and 3 respectively. Out of three isolates, ACP-3 was the best efficient P solubilizer, it was identified as *Bacillus*, and other two organisms were ACP-2 and CCP-2 which were identified as *Arthrobacter* and *Pseudomonas* respectively. The occurrence of *Bacillus* as the predominant genus in the many soil has been reported (Vikram, 2001; Elliot and Lynch, 1985) and its predominance could be due to the versatility of the genus to use different substrates as nutrient source. The occurrence of *Psuedomonas* sp., *Bacillus* sp., *Enterobacter aerogenes* and *Vibrio proteolyticus* in the rhizosphere earlier as mineral phosphate solubilizers (Guar et al., 1973; Nair and Subba Rao, 1975; Illmer and Schinner, 1992; Thakkar et al., 1993; Vazquez et al., 2000) and the results of the present investigation are in line with these reports. The PSB were found to belong to the genera *Arthrobacter* (ACP-2), *Bacillus* (ACP-3) and *Pseudomonas* (CCP-2).

Molecular characterization of efficient PSB isolates

From 16SrRNA sequencing, the top three PSB isolates were identified as *Bacillus licheniformis*(ACP-3), *Arthrobacter* sp. HPG166 (ACP-2) and *Pseudomonas chlororaphis* (CCP-2). "*Bacillus licheniformis"* was the best isolate which was furthur tested under field condition with different fertilizer levels of SSP and rock phosphate.

16SrRNA SEQUENCING AND DENDOGRAM OF EFFICIENT PSB ISOLATES

16SrRNA sequencing result of isolate ACP-2 (*Arthrobacter* sp. HPG166)

>150518-27_A07_ACP-2_785F.ab1711

AGGGCGCCGTTGGGATAGGTGTGGGGGAATTCCACGTTTTCCGCGCCGTAGCTAACGCAT
TAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAG
GCTTGACATGGACTGGATCGCATCAGAGATGGTGTTTCCCTTCGGGGCTGGTTCACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
AACCCTCGTTCCATGTTGCCAGCGCGTAATGGCGGGGACTCATGGGAGACTGCCGGGGT
CAACTCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCA
CGCATGCTACAATGGCCGGTACAAAGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAA
AAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTTGGAGTCGCTA
GTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG
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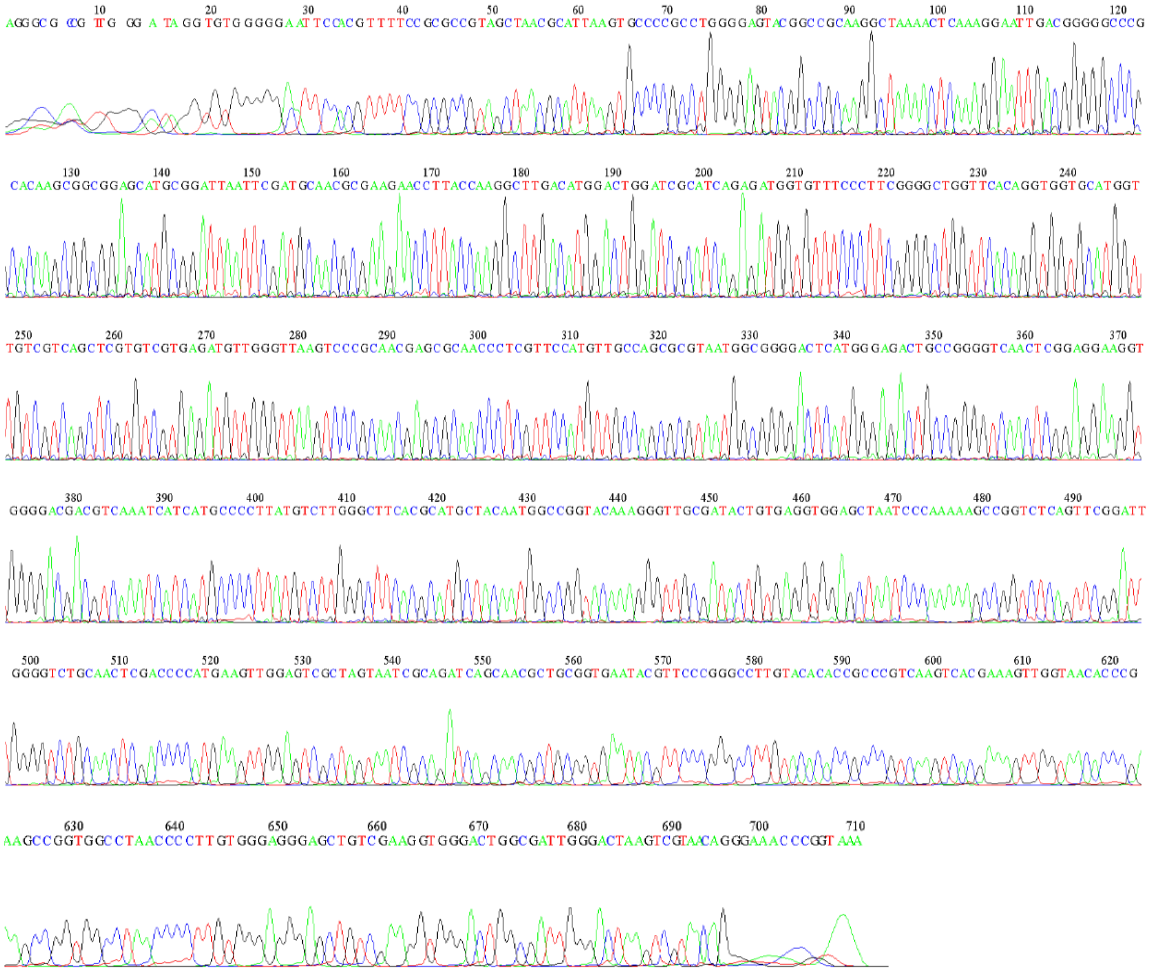

16srRNA sequencing result of isolate ACP-3 (*Bacillus licheniformis)*

>150518-27_M07_NB_isolateACP-3_785F.ab1703

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GCACAAGCGGTGGAGCATGTGGTTTAATTCAAAGCAACGCGAAAAACCTTACCAGGTCT
TGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTG
GTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACAC
GTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAA
ATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
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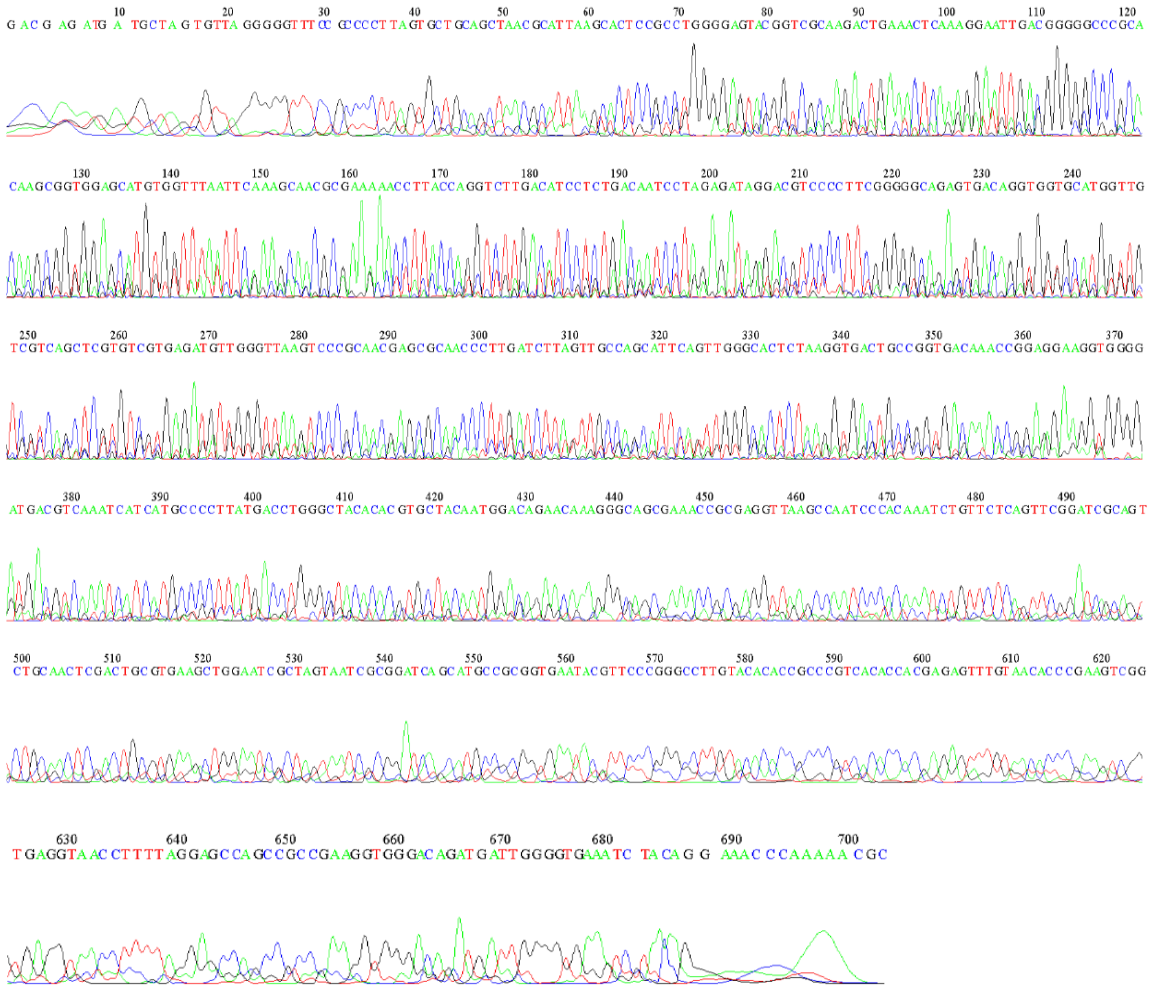
GAAGGTGGGACAGATGATTGGGGTGAAATCTACAGGAAACCCAAAAACGC



16SrRNA sequencing result of isolate CCP-2 *(Pseudomonas chlororaphis)*

>150518-27_A07_CCP-2_785F.ab1711

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AGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAA
CTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCA
GCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTACTTACCTAA
TACGTGAGTATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGA
GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCC
CCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTG
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CCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAG
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AGAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACATTGAGACAG
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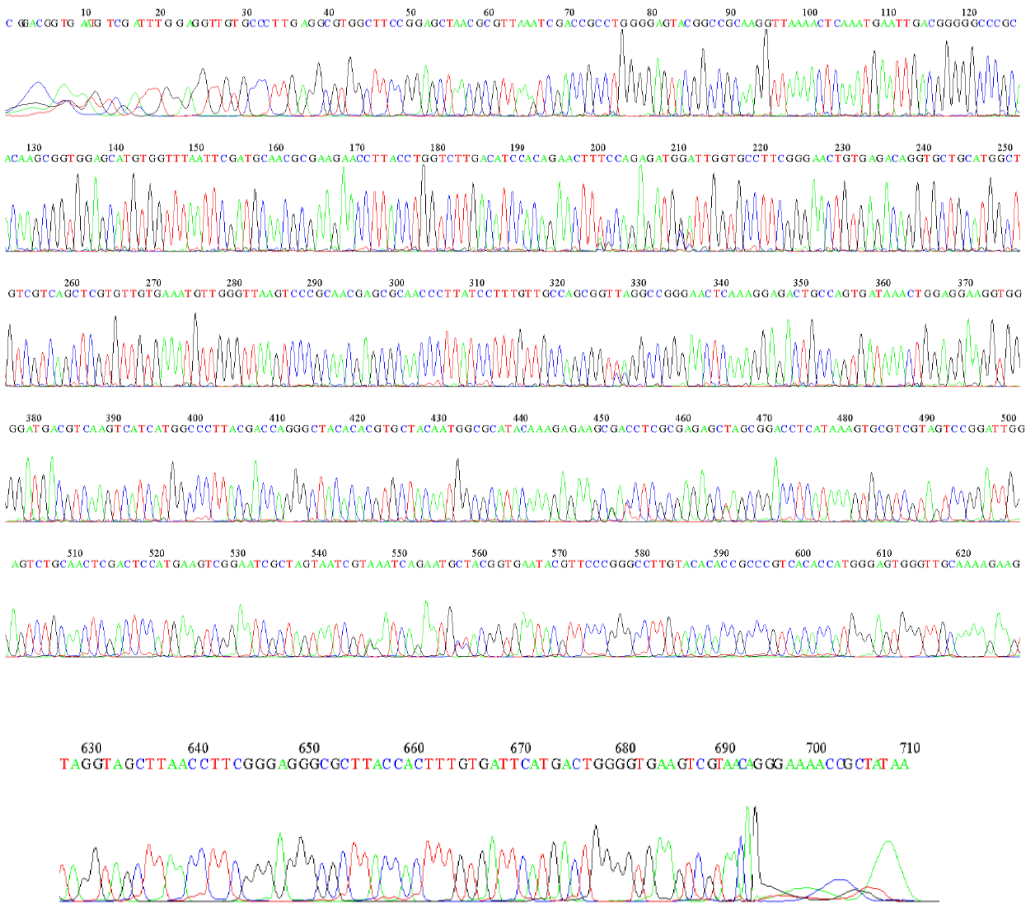

Table 1: Population of phosphorus solubilising bacteria in the soil from different Chickpea growing areas

Sl. No.	Locations	Sample code	PSB population (cfu×10^6/g soil)	Number of isolates obtained	Isolates selected for screening
1	Arasikere	ACP	23×10^6	7	ACP-1
					ACP-2
					ACP-3
					ACP-4
2	Chamarajanagara	CCP	21×10^6	6	CCP-2
					CCP-3
					CCP-3
3	Mysore	MCP	16×10^6	6	MCP-1
					MCP-2
					MCP-3
4	Devanahalli	DCP	11×10^6	5	-
5	Total	-	-	24	10

Note: ACP - Arasikere Chickpea PSB

CCP- Chamarajanagara Chickpea PSB

MCP- Mysore Chickpea PSB

Table 2: Morphological and biochemical characterization of the efficient phosphate solubilizing bacteria

Sl. No.	Isolates	Morphological characters			
		Colony characters	Gram reaction	Shape and size	Endospore staining
1	ACP-2	Creamy white, smooth, widely spreading	+ve	Rod, small	-
2	ACP-3	Orange, widely spreading,	+ve	Rod, large	+
3	CCP-2	White, slimy, widely spreading,	-ve	Ellipsoidal, large	-

Table 3: Biochemical characterization of the efficient phosphate solubilizing bacteria

Sl. No.	Isolates	Biochemical tests							Genus
		1	2	3	4	5	6	7	
1	ACP-2	+	+	-	-	-	+	-	*Arthrobacter*
2	ACP-3	+	+	+	+	+	+	-	*Bacillus*
3	CCP-2	-	-	-	+	+	+	-	*Pseudomonas*

1-Gelatin liquefaction, 2- Catalase test, 3- Starch hydrolysis, 4- Urease test, 5- Citrate utilization test, 6- Oxidase test, 7- Hydrogen sulphide test

Table 4: Molecular characterization of the efficient phosphate solubilizing bacteria

Sl. No.	Efficient PSB isolates	Organism identified
1	ACP-2	*Arthrobacter* sp. HPG166
2	ACP-3	*Bacillus licheniformis*
3	CCP-2	*Pseudomonas chlororaphis*

CONCLUSION

The four soil samples collected from different Chickpea growing areas were found to harbor PSB in good numbers. A total of 24 PSB isolates were isolated from these samples. Molecular characterization was done by using 16SrRNA sequencing for the promising three isolates for further identification up to species level. The most efficient isolate ACP-3 was identified as *Bacillus licheniformis*. Other two organisms ACP-2 and CCP-2 were identified as *Arthrobacter* sp. HPG166 and *Pseudomonas chlororaphis* respectively. Further green house and trial has to be conducted for knowing their working efficiency.

Acknowledgement

I express my sincere gratitude to Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bengaluru for providing all the facility and it makes me immensely proud and honored to be a student of this prestigious university.

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