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Isolation and Characterization of Phosphate Solubilizing Bacteria from Calcareous Soil

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The present investigation was conducted with aim to isolate and characterize phosphate solubilizing bacteria from calcareous soil. Four efficient PSB isolates were obtained from fourteen soil samples collected from different locations. Based on morphological and biochemical characterization, highly phosphate solubilizing isolates were identified as *Pseudomonas fluorescence, Bacillus subtilis, Bacillus thuringiensis* and *Pseudomonas aeruginosa* respectively. The amount of Pi released from TCP by the isolates at 10 DAI ranged from 23.2 to 30.5 % and zone of solubilization recorded between 6 -13 mm. Among them PSB isolate 1 (*Pseudomonas fluorescence)* recorded highest Pi released from TCP broth *i:e* (30.5 %) and zone of solubilization recorded (13 mm) than the other isolates tested.

Keywords: Isolation; pseudomonas fluorescence; bacillus subtilis, bacillus thuringiensis; pseudomonas aeruginosa; phosphorus fixation; calcareous soil.

1. INTRODUCTION

Calcareous soils are those that have free $CaCO_3$ in their profile. In the reference base soil classification system, calcareous soil mainly occurs in the reference group of calcisols. Calcareous soils are prevalent over more than 30 per cent of the earth surface [1]. The total global

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extent of calcisols is estimated to be 800 mha, mainly concentrated in arid or Mediterranean type climatic zones. In calcareous soils where pH is high and $CaCO_3$ is dominated, plants suffer low availability of P and K would cause problems more serious than their deficiencies. Increasing availability of these nutrients is one of the important objectives in plant nutrition.

Phosphorus (P) is one of the major essential macronutrients for plants, which is applied to the soil in the form of phosphatic manure. However, a large portion of the applied phosphorus is rapidly immobilized, being unavailable to plants. In average, the content of phosphorus of soil is about 0.05% (w/w); however, only 0.1% of them are usable for plants.

In calcareous soil, the solubility of phosphorus is depressed which results in the decrease of P availability. Phosphorus is often lacking in calcareous soil. A considerable amount of P is rapidly transformed into less available forms by forming a complex with Fe and Al in acid soils or Ca in calcareous soils before plant roots have a chance to absorb it [2]. Under such a situation phosphate solubilizing microorganisms play an important role in making P available to the plants which increase the yield of crop plants. The issue of inaccessibility of phosphorous can be overcome by utilizing advantageous microorganisms that guarantees P accessibility to plants by synthesis of different types of organic acids (Poonamgautam et al., 2003) [3]. Some of the acids produced by PSB are gluconic acids, citric acid, malic acid, succinic acid, lactic acid, fumaric acid, tartaric acid etc. [4]. Therefore, the present study was aimed towards isolation and characterization of phosphate solubilizing bacteria from calcareous soil which are highly phosphate solubilizers and not only will reduce the dependency on chemical phosphatic fertilizer but also reduce the cost of cultivation in addition to maintain the soil health.

2. MATERIALS AND METHODS

2.1 Collection of Soil Samples

The soil samples were collected from different locations i:e Ahmednagar, Satara, Pune, Solapur districts. The soil samples collected with soil auger. Auger turn into the soil at desired depth up to 15 cm in zigzag pattern. The samples were brought in polythene bags. The collected Rhizospheric soil samples were air dried, thoroughly mix, sieved (2mm) and use for isolation of Phosphate solubilizing bacteria.

2.1.1 Isolation and purification of phosphate solubilizing bacteria from rhizosphere soil

2.1.1.1 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 02.g, Magnesium sulphate 0.1g, potassium chloride 0.2g, Yeast extract 0.5g, Manganese sulfate and ferrous sulfate in trace amounts, Agar 15g, distilled water 1 liter and pH adjusted to neutral. The media was sterilized by autoclaving at 121°C for 15 minutes and cooled to 50°C.

2.1.2 Isolation of phosphate solubilizing bacteria (PSB) from rhizosphere soils

The isolation of phosphate solubilizing bacteria on Pikovskaya's medium was carried out by serial dilution of soil and agar plating method [5]. Ten gram rhizosphere soil sample was suspended in 90 ml of sterilized water blank. Serial dilutions were made from 10^{-1} to 10^{-6}. One ml aliquot of dilutions from 10^{-3} to 10^{-6} was transferred to sterilized petriplates separately. The sterilized Pikovskaya's medium before solidification (45°C temperature) was poured in each petriplates and mixed the contents in plates by rotating the plates gently taking care that medium should not touch the lid. After solidification, plates were kept at 28$\pm$2°C in BOD incubator for 2-3 days of incubation, colonies forming halo zones were selected and subculture on Pikovskaya's agar medium for further characterization of study.

2.1.3 Purification of PSB isolates

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

2.1.4 Screening of isolates of Phosphate solubilizing bacteria

Phosphate solubilization ability of PSB isolates was determined by measuring the zone of P solubilization on the Pikovskaya's agar medium and by estimating percent P solubilization in Pikovskaya's broth medium [6,7].

2.1.5 Zone of solubilization

For determining zone of P solubilization, Pikovskaya's agar medium was poured on the petriplates, after solidification 10µl broth culture was spot inoculated on the plates. The plates were incubated at 28±2°C for about 3-5 days and solubilization zone was observed around the colony. P solubilization efficiency was calculated by using the following formula.

PSE = Solubilization diameter /Growth diameter *100

2.1.6 Quantitative Pi released from tricalcium phosphate estimation of for bacterial isolates

The bacterial isolates positive for P solubilization on Pikovskaya's agar medium were subjected to quantification of Pi released from TCP in broth medium. The Erlenmeyer flasks containing 50 ml Pikovskaya's broth [8] were inoculated with 500 µl overnight culture of each isolate in two replicates and incubated for 10 days at 28 ± 2°C. The amount of Pi released in the broth was estimated at 10 days of incubation in comparison with the uninoculated control. The reduction in pH of the broth from the initially adjusted pH of 7.0 was also noted after 10 days of incubation so as to monitor the amount of acidity produced and study its correlation with the Pi and insoluble phosphate and the available P content of the supernatant was estimated by using phosphomolybdic blue colour method [9] as described below.

2.1.7 Cultural characterization of PSB isolates

The colony morphology was studied on the basis of their form, color, pigmentation, and optical characteristics. The pure effective isolates were further cultured on new plates for colony morphology.

2.2 Biochemical Characterization of PSB Isolates

2.2.1 Gram staining

The isolate was characterized for gram staining as per the following procedure given by Sharma et al. [10]. A loopfull culture was taken on a cleaned dry slide; smear was prepared, air dried and heat fixed. 1-2 drops of crystal violet was added and kept for 30 seconds. The slide was washed with distilled water. Then, 1-2 drops of Gram's iodine was added and kept for 60 seconds. The slide was washed with 95% ethyl alcohol. Later, safranin was added and kept for 30 seconds and then washed with distilled water. The slide was dried with blotting paper and observed under microscope. The pink colonies indicate gram negative bacteria and the purple colonies show the gram positive bacteria.

2.2.2 Biochemical test

The biochemical test of the isolates was carried out as per the procedures outlined by Cappuccino and Sherman [11] in their 10th edition of Microbiology: A Laboratory Manual. Catalase test, Oxidase test, Indole production test, Methyl red test, Voges-Proskaeur (VP) test, Urea hydrolysis, Nitrate reduction test, Gelatin hydrolysis test, Starch hydrolysis, Casein hydrolysis and H_2S production test were performed.

3. RESULTS AND DISCUSSION

3.1 Isolation of Phosphate Solubilizing Bacteria from Rhizosphere Soils

The isolation procedure was carried out for all the fourteen rhizosphere soil samples which were collected from various locations like Ahmednagar, Pune, Satara, Solapur districts. The soil pH varying 7.0-8.5 and soil was moderately calcareous in nature. The isolation of phosphate solubilizing bacteria on Pikovaskaya's medium was carried out by serial dilution of soil and agar plating method [5]. The plates were observed for the appearance of bacterial colony showing clear zone of solubilization of tricalcium phosphate purified (TCP) on Pikovaskaya's medium. On the basis of formation of halozone, four effective isolates were obtained as PSB isolate 1, PSB isolate 2, PSB isolate 3 and PSB isolate 4 maintained on the slants of Pikovaskaya's agar for further use.

3.2 Phosphate Solubilizing Ability of the PSB Isolates

3.2.1 Zone of solubilization

All the four efficient PSB isolates on the basis of halozone exhibited by bacterial colony were tested for their ability to solubilize inorganic phosphate both qualitatively and quantitatively and their results are presented in (Table 1). Quick analysis of P-solubilization was carried out on Pikovaskaya's agar medium. All the four

isolates were able to form zone of P-solubilization on the medium. The diameter of the zone of P-solubilization ranged from 6-13 mm in different isolates.

3.2.2 Quantitative estimation of Pi released from TCP for bacterial isolates

The amount of Pi released from tri-calcium phosphate by the PSB isolates along within Pikovaskaya's broth was estimated at 10 days after inoculation. The amount of Pi released from TCP by the isolates at 10 DAI ranged from 23.2 to 30.5 per cent (Table 1). The PSB isolate 1 recorded highest P-solubilization (30.5 %) than the other isolates tested.

3.2.3 Decrease in pH of medium during phosphate solubilization

The decrease in pH of TCP broth from initially adjusted pH of 7.0 was also noted at 10 days after inoculation. The maximum reduction in pH of the medium *i:e* pH 3.48 was recorded by PSB isolate 1 followed by PSB isolate 4, PSB isolate 2, and PSB isolate 3 (3.8, 4.09 and 5.11 respectively) as shown in (Table 1). The decrease in pH of the medium with the amount of Pi released had positive correlation.

3.2.4 Morphological Characterization of Efficient Phosphate Solubilizing Bacterial Isolates

PSB1, PSB2, PSB3 and PSB4 were isolated from the rhizospheric soil samples taken from the different districts like Ahmednagar, Pune, Satara and Solapur respectively. Among those PSB isolate 1 were Gram -ve long rods arranged in chains and were having the creamy white colony morphology. PSB isolate 2 and PSB isolate 3 were found Gram +ve rod shaped having fuzzy white colony morphology and white with uneven border respectively. PSB isolate 4 found Gram –ve rod shaped arranging in single chain having bluish green colony morphology. The colony form of the PSB1, PSB2 and PSB4 was circular whereas the PSB3 was observed as irregular. PSB1 produces fluorescent green pigment, while PSB4 produces bluish green pigment in culture medium; with all PSB isolates are motile (Table 2).

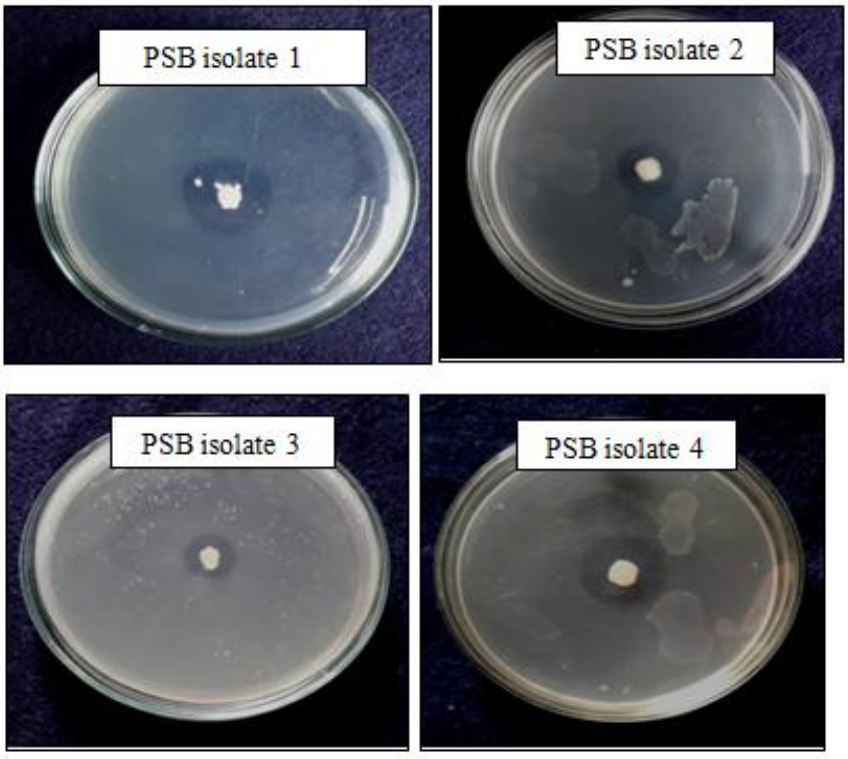

Fig 1. Formation of zone of solubilization on Pikovaskaya's agar plate by diff. effective PSB isolates

Table 1. Zone of P solubilization on Pikovaskaya's agar and per cent Pi released from TCP broth by the PSB isolates

Sr. No.	PSB isolate	Zone of P solubilization on TCP (mm)	% Pi released from TCP after 10 days	Decrease in pH of medium (from initial pH 7.0) after 10 days
1	PSB isolate 1	13	30.5	3.48
2	PSB isolate 2	8	26.8	4.09
3	PSB isolate 3	6	23.2	5.11
4.	PSB isolate 4	11	28.9	3.8

Table 2. Colonial Morphology of four efficient Phosphate solubilizing bacteria

Strain	PSB 1	PSB 2	PSB3	PSB4
Gram nature	Gram negative	Gram positive	Gram positive	Gram negative
Shape	Straight Rod shaped	Rod shaped	Rod shaped	Rod shaped
Cell arrangement	Single	Pairs	Single	Single
Colour of the colony	Yellowish	Fuzzy white	White	Bluish green
Form	Circular	Circular	Irregular	Circular
Pigmentation	Pigmented	-	-	Pigmented
Motility	Motile	Motile	Motile	Motile

Table 3. Biochemical tests of the efficient PSB strains.

Sr. No.	Biochemical test	PSB 1	PSB 2	PSB 3	PSB4
1	Catalase test	+	+	+	+
2	Oxidase test	+	–	–	+
3	Starch hydrolysis test	–	+	+	–
4	Urea hydrolysis test	+	–	–	+
5	H2s production test	–	–	–	–
6	Indole production test	–	+	+	–
7	MR test	–	–	+	–
8	Voges- Proskaeur test	–	+	+	–
9	Citrate utilization test	+	+	+	+
10	Casein hydrolysis test	+	+	+	+
11	Nitrate reduction test	+	+	–	+
12	Endospore formation	–	+	+	–
13	Growth on carbon source				
	Glucose	+	+	+	–
	Lactose	–	–	+	–
	Sucrose	+	+	+	–

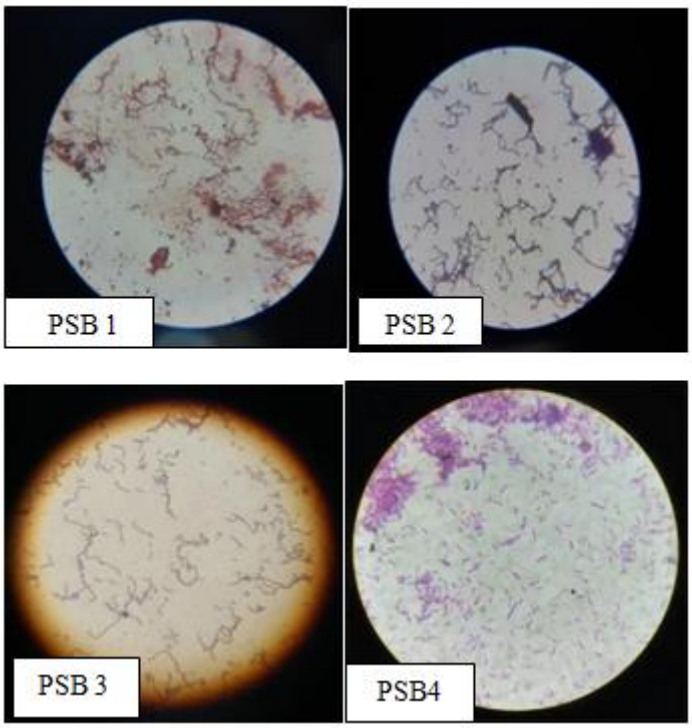

Fig. 2. Cell morphology of four effective PSB isolates from diff. soil samples (Gram staining)

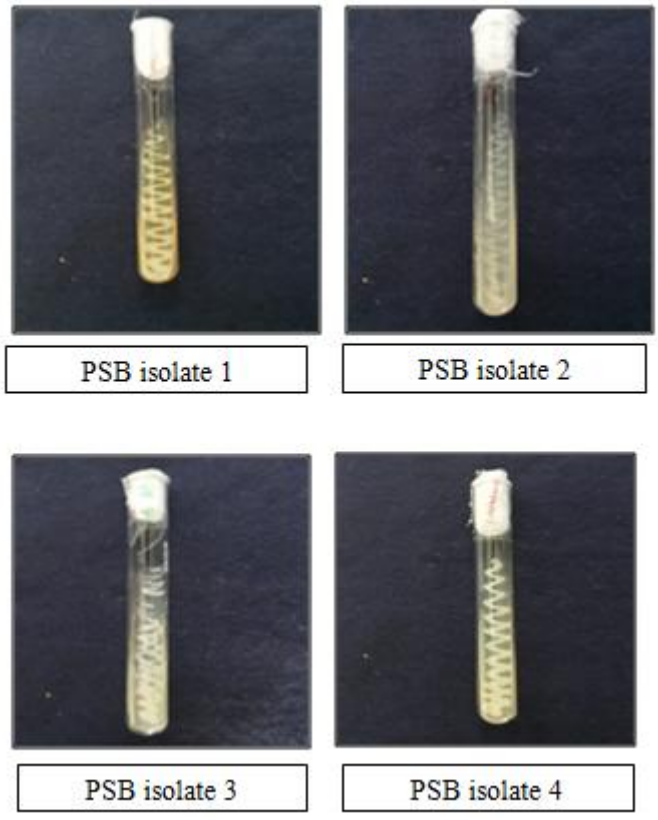

Fig 3. Isolation and maintenance of pure culture of effective PSB isolates (Morphological characterization

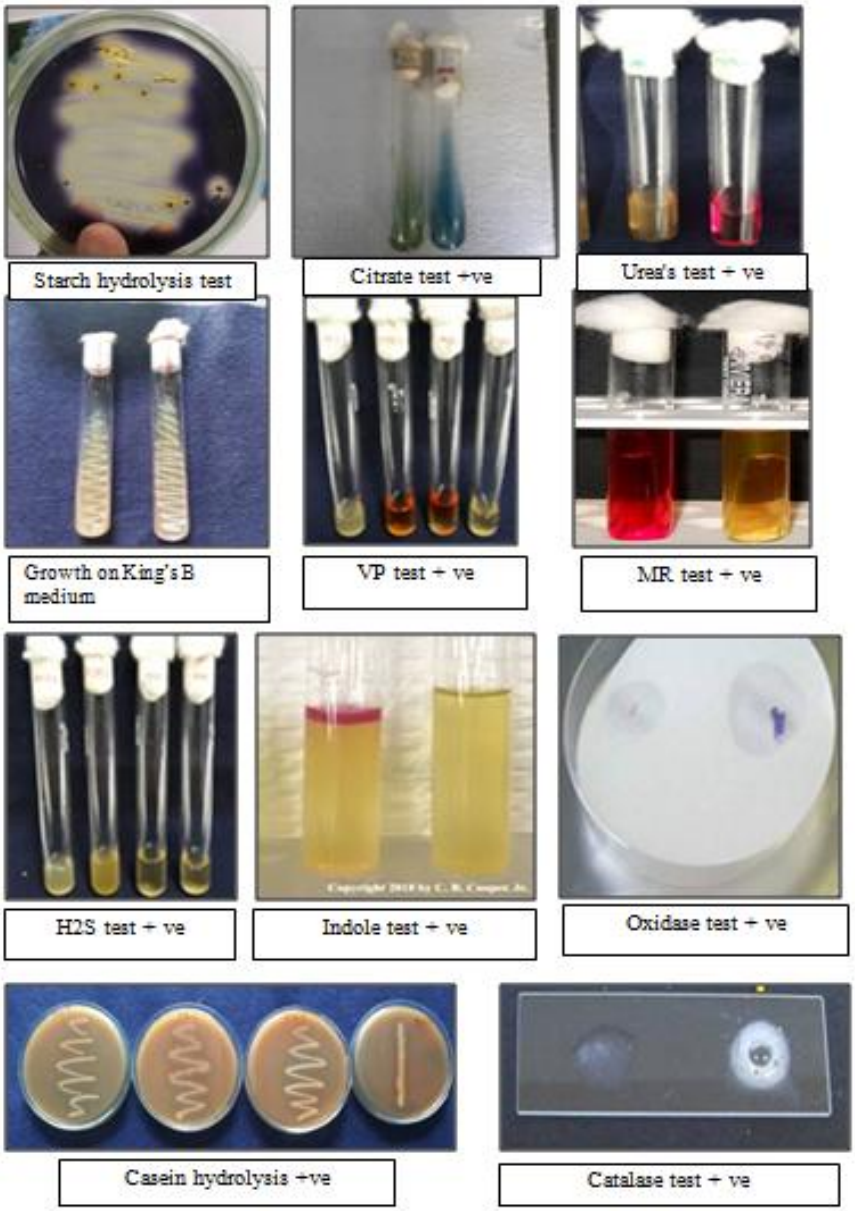

Fig. 4. Biochemical characterization of effective PSB isolates

3.2.5 Biochemical Characterization of Efficient Phosphate Solubilizing Bacterial Isolates

The effective four phosphate solubilizing bacterial isolates were tested for biochemical characterization. Among those PSB isolate 1 and PSB isolate 4 were tested for different biochemical characters *viz*., gram staining, motility test, Catalase test, Oxidase test, starch hydrolysis, H_2S production and Vogues-Proskaeur test, urea hydrolysis test, casein hydrolysis test, citrate utilization test. (Table 3) The cells of phosphate solubilizing bacterial isolate were rod shape, motile and gram negative in reaction. The phosphate solubilizing bacterial isolates were positive for, Catalase test, Oxidase test, urea hydrolysis test, casein hydrolysis test and citrate utilization test. But were negative for starch hydrolysis and Vogues- Proskaeur test, MR test and H_2S production which was supported by the reports of Lacey and Goettel [12], Yoo et al. [13], Jani [14] and Patel (2006). Based on biochemical and morphological characterization [15], the phosphate solubilizing bacterial isolates PSB1 and PSB4 were identified as *Pseudomonas fluorescence* and *Pseudomonas aeruginosa* respectively.

Table 4. Identification of effective PSB isolates from different locations

Sr. No.	Soil samples	Locations	Effective PSB isolates	Identified bacteria
1	2	Sonai	PSB isolate 1	*Pseudomonas fluorescence*
2	4	MPKV Rahuri, Campus	PSB isolate 2	*Bacillus subtilis*
3	12	Kdegaon	PSB isolate 3	*Bacillus thuringiensis*
4	14	Akluj	PSB isolate 4	*Pseudomonas aeruginosa*

Table 5. Isolation of PSB from collected soil samples

Sample number	PSB (10^6)	Zone of solubilization (mm)
1	54×10^6	5.6
2	39×10^6	13
3	48×10^6	5.6
4	42×10^6	8.0
5	56×10^6	4.9
6	38×10^6	2.3
7	40×10^6	4
8	26×10^6	4.3
9	36×10^6	2.9
10	39×10^6	2.0
11	52×10^6	1.9
12	48×10^6	6
13	43×10^6	5.0
14	29×10^6	11

PSB isolate 2 and PSB isolate 3 tested for similar biochemical test which was performed for PSB isolate1 *viz*., gram staining, motility test, Catalase test, Oxidase test, starch hydrolysis, H_2S production and Voges- Proskaeur test, urea hydrolysis test, casein hydrolysis test, and citrate utilization test (Table 3). The cells of phosphate solubilizing bacterial isolate were rod shape, motile and gram positive in reaction. The phosphate solubilizing bacterial isolates was positive for, Catalase test, Indole production test, starch hydrolysis test, casein hydrolysis test and citrate utilization test. Which was supported by the reports of Lacey and Goettel [12], Yoo et al. [13], Jani [14] and Patel (2006) But were negative for urea hydrolysis, Oxidase test, and H_2S production, PSB isolate 3 was positive for MR test while PSB isolate 2 was negative for MR test. Based on biochemical and morphological characterization [15], the phosphate solubilizing bacterial isolates PSB 2 and PSB 3 were identified as *Bacillus subtilis* and *Bacillus thuringiensis* respectively. (Table 4).

3.2.6 Preparation of PSB inoculum

Inoculum of *Pseudomonas fluorescence, Bacillus subtilis*, *Bacillus thuringiensis* and *Pseudomonas aeruginosa* was prepared in selective Pikovaskaya's medium. The media was inoculated in 500 ml conical flask containing 150 ml medium and incubated at 28 ± 2°C under shaking at 100-150 rpm for three days to give an optical density of 0.5 recorded at 535 nm. Lignite powder used as carrier was sterilized at 121°C and 1.04 kg/cm^2 pressure for one hour and inoculated with broth cultures of *Pseudomonas fluorescence, Bacillus subtilis*, *Bacillus thuringiensis* and *Pseudomonas aeruginosa* (100 ml per 500 g of lignite powder). Lignite powder based inoculum was incubated at 28 ± 2°C for three days by adding 10% sugar solution to increase the population of respective microbe. Inoculum of *Pseudomonas fluorescence, Bacillus subtilis*, *Bacillus thuringiensis* and *Pseudomonas aeruginosa* having cfu of 2 x 10^7 per gram of lignite powder.

4. CONCLUSION

Based on biochemical and morphological characterization, four efficient phosphate solubilizing bacteria were identified as *Pseudomonas fluorescence, Bacillus subtillis, Bacillus thuringiensis* and *Pseudomonas aeruginosa*. Among them PSB isolate 1 (*Pseudomonas fluorescence*) recorded highest Pi released from TCP broth *i:e* (30.5 %) and zone of solubilization recorded (13 mm) than the other isolates tested. Our study revealed that genus *Pseudomonas fluorescence* play an important role in phosphate solubilization in calcareous soil. Considering the phosphate solubilizing efficiency it may be used as bioinoculants for sustainable agriculture.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Marschner H, Obreza TA. Mineral Nutrition of Higher Plants. 2nd Edition. Academic Press, London manganese and copper. Soil Sci. Soc. Am. J. 1995;42:421-428.
2. Alam MM, Ladha JK. Optimizing phosphorus fertilization in an intensive vegetable-rice cropping system. BioFertile Soils. 2004;40:277-283.
3. Deubel A, Merbach W. Influence of microorganisms on phosphorus bioavailability in soils. In: Microorganisms in soils: Roles in genesis and functions. Springer Berlin Heidelberg. 2005;177-191.
4. Khan MS, Zaidi A. Synergistic effects of the inoculation with plant growth promoting rhizobacteria and arbuscular mycorrhizal fungus on the performance of wheat. Turkish J. Agril. Forest, 2007;31:355-362.
5. Aneja KR. Experiments in Microbiology, Plant Pathology and Biotechnology. New Age International Publishers, New Delhi, India. 2003;1-607.
6. Goldstein AH. Bacterial solubilization of mineral phosphates: historical perspectives and future prospects. American J. of Alt. Agril. 1986;1:57–65
7. Scheffer F, Schachtschabel P. Lehrbuch der Bodenkunde. Stuttgart, Germany: Ferdinand Enke Verlag; 1992.
8. Pikovaskaya's RT. Mobilization of phosphate in soil connection with the vital activities of microbial species. Microbiologia. 1948;17:362-370.
9. Jackson ML. Soil Chemical Analysis, Prentice Hall of India Private Limited, 1st edition, New Delhi, India; 1973.
10. Sharma S, Kumar V, Tripathi RB. Isolation of Phosphate Solubilizing Microorganism (PSMs) from Soil. J. Microbial. Biotech. Res. 2011;1:90-95.
11. Cappuccino JG, Sherman N. Microbiology A Laboratory Manual. 2nd Ed. The Benjamin/Cummins Publishing Co., USA. 1987;458.
12. Lacey LA, Goettel MS. Current developments inmicrobial control of insect pests and prospects for the early 21stCentury." Entomophage. 1995;40:3-27.
13. Yoo KH, Kim SY, Kang MH, Cho MH, Lee HH. Characterization of Bacillus thuringiensis isolated from soil in wonju Area. The J of Microbial. 1996;34(4):370-373.
14. Jani JJ. Studies on bacterial insect pathogens against cotton Bollworm. Unpublished Ph. D. thesis submitted to Bhavnagar University;2005.
15. Claus D, Berkeley RCW. Genus Bacillus cohn 1872. In: Bergy's Manual of Systematic Bacteriology, (Sneath, P.H.A., ed.), Williams and Wilkins Co., Baltimore. 1986;2:1105-1140.

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