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Determination of phosphate solubilization and plant growth promotion of bacterial isolates from paddy rhizosphere

Vinithra Muthaiyan, Saravanan Ramalingam*

Department of Biotechnology, Bio - Medical Engineering Research Foundation, Periyar University, Harur main road, Kuppanur, Salem, Tamilnadu, India

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Abstract

A study investigating bacterial isolates having inorganic phosphate solubilization along with plant growth promoting abilities was attempted. The isolates were from different paddy rhizosphere soil samples of Salem region. A total number of 34 bacillary isolates were successfully isolated from 315 morphologically distinct colonies and evaluated for their 'P' solubilization ability. The colonies showing clear halo zones (≥ 2mm) around them, on the Pikovskaya agar medium containing Tri-calcium Phosphate (TCP) were selected as phosphate solubilizers. Among these isolates 11 strains (BMERF-PSB3, BMERF-PSB4, BMERF-PSB7, BMERF-PSB8, BMERF-PSB12, BMERF-PSB14, BMERF-PSB16, BMERF-PSB19, BMERF-PSB20, BMERF-PSB23 and BMERF-PSB25) had shown superior (≥ 5-29 mm) phosphate solubilization ability. Among these 11 superior (32%) PSB isolates, 4 (12%) (BMERF-PSB3, BMERF-PSB7, BMERF-PSB16 and BMERF-PSB19) were found to be having plant growth promoting characteristics by producing Auxin.

***Corresponding Author:** Saravanan Ramalingam ✉ saravana67@gmail.com

Introduction

The United Nations World Health Organization (WHO) defines human health as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity. In South East Asia Region (SEAR) countries, prevalence of anemia among pregnant women was the highest in India. National Family Health Survey (NFHS) indicated that Vitamin-A deficiency led to 2% of total loss of vision in childhood. From this viewpoint, agriculture should not only be a means of producing food for man's survival but also a protector of their health (Singh, 2009). The replacement of organic manure with inorganic fertilizers, especially after green revolution, is held responsible for the depletion of soil organic content. This affected the soil health and nutrition status which is usually made up with an excessive use of chemical fertilizers leading to soil and water pollution. Therefore the total "negative nutrient balance" will surely aggravate health hazards for human and livestock. Biofertilizers are supposed to be a safe and cost effective alternative to these inorganic fertilizers to minimize the ecological chaos and jeopardy.

This particular study concentrated on the microbial fertilizers which erases the Phosphate deficiency in Indian soils without pollution. After nitrogen (N), Phosphorus (P) is the most important element limiting plant growth. Although abundant in soil, P is one of the essential macronutrients required for plant growth and development, because it is in the unavailable form for the plants. Normal plant growth cannot be achieved without phosphorus. Phosphorus deficient plants are characterized by stunted growth with poorly developed roots, dark green leaves with a leathery texture, etc. (Ray tucker, 1999). In 1908, Tri-Calcium (TCP) solubilization by isolated bacteria from soil was reported and these bacteria were generally called Phosphate Solubilizing Bacteria (PSB) which have the ability to solve this problem. It can convert the insoluble phosphates into soluble form by acidification, chelation, exchange reaction and production of gluconic acid. Apart from insoluble phosphate solubilization, it has been reported that PSB's can produce plant growth promoting substances that can contribute to the plant's overall growth, development and excellent yield of the produce.

Phosphorus deficiency can be removed by supplying phosphate to the soils but increasing phosphorus uptake and decreasing P fixation is complicated but important (Hassan *et al.*, 2012). Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that enhances plant growth and yield via various plant growth promoting substances. Rhizosphere and PGPR's go hand-in-hand for erasing this difficulty of P solubilization. In India, the common method to deal with 'P' deficiency is using rock phosphate/ mono-ammonium phosphate/ di-ammonium phosphate/ superphosphate. This practice in addition to high cost and low efficiency can endanger our environment by irreparable damage to our soil health due to soil sterility, phosphate toxicity and pollution problems.

Therefore, the usage of biological solutions for this peril is getting greater importance around the globe. Using soil micro-organisms which have the potentiality of solubilizing insoluble phosphates and changing them into soluble forms is one of the most effective ways to increase the uptake of 'P' in alkaline soils. Free living PGPR have shown promise as bio fertilizers by putting themselves as antennas carrying the signaling molecules which communicate for the plant growth and excellent yield. The enhancement in various agronomic yields due to it, has been reported because of the production of growth stimulating phytohormones such as Indole Acetic acid (IAA), Cytokinins, Giberrellic Acid (GA), Zeatin, Ethylene and Abscisic Acid (ABA) (Jay Shankar Singh, 2013). By keeping all the lacunae in mind, the present study was designed for the isolation and identification of the bacteria with high potential of inorganic insoluble 'P' solubilization and along with growth promoting traits such as IAA phytohormones production.

Materials and methods

Collection of soil sample

The soils samples used for bacterial isolation were collected from Edappadi located at 11.58°N 77.85°E at an average elevation of 288 m (945 feet) above mean sea level and Udayapatti region placed at 11°46′46″N 78°12′12″E of Salem district.

The top 3 Cm soil was taken out and soil samples were collected at a depth of 0-30 Cm from randomly selected sites in each area. The samples were then transferred to a sterile polythene bags and transported immediately to the laboratory and were preserved at 4°C until microbiological isolation and analysis were performed.

Isolation of PSB

To obtain standard soil suspension 10g of soil samples were shaken with 90ml of sterile physiological saline solution for 10-20 minutes. Thus 10-fold serial dilutions of the samples were prepared and serially diluted, spread plated on Pikovskaya's agar medium to isolate different strains of Phosphate Solubilizing Bacteria. One ml of soil suspensions from aliquot dilutions was aseptically added to sterile Petri plates containing sterile medium and incubated at 28±2°C for 24-48h and after incubation, well separated individual colonies were marked and detected by viewing under digital colony counter (EI). The individual colonies were picked and transferred to Sperber's, cetrimide, sorbitol, Polymyxin, Mannitol phenol-red polymyxin selective media for the isolation of common phosphate solubilizers *Bacillus megaterium, Pseudomonas, Bacillus subtilis, Bacillus polymyxa, Bacillus licheniformis* respectively.

Biochemical test of isolates

The bacterial isolates were studied for their morphological and biochemical characteristics as prescribed in Bergey's Manual Systematic Bacteriology, 1982.

Screening of phosphate solubilizing activity (Qualitative estimation)

Qualitative test of inorganic phosphate solubilization for assessing the ability of the bacterial isolates to solubilize inorganic insoluble phosphate was carried out by the observation of a clear halo around the colony and measuring the halo diameter to colony diameter ratio. The bacterial strains were tested for their ability to solubilize insoluble phosphate on Pikovskaya's agar (Pikovskaya, 1948), supplemented with Tri-calcium Phosphate (TCP).

Quantitative estimation of phosphate solubilization

The amount of phosphorous present in the isolates was determined by Subba Rao. One ml of sample was taken in two test tubes and its volume was made up to 8.6ml with distilled water. 1ml of ammonium molybdate was added to the tubes and vortexed. The color intensity was read out after 10mts in samples at 660nm. Concentration of phosphorous in sample was calculated (Subba Rao *et al.*, 1982).

Screening of IAA (Qualitative estimation)

Bacterial strains were subjected to qualitative analysis for the production of IAA (Brick *et al.*, 2004). Bacteria producing IAA were identified by the formation of red halo around the colony. The spotted colonies were overlaid on Whatmann No.1 filter paper discs. After adequate incubation, the discs were treated with Salkowski's reagent. The paper discs were then viewed under UV light. The spots giving green fluorescence were taken as positive for IAA production (Tien *et al.*, 1979).

Quantitative estimation of IAA

The tested strains were grown in LC broth medium in the presence of tryptophan and incubated at 37°C. The IAA production was measured after 10 days of incubation. After incubation, about 2ml of Salkowski's reagent was added to 1ml of supernatant fluid along with 100µl of Orthophosphoric Acid. The absorbance of the developed pink color was read at 530nm after 25min in a UV-Vis spectrophotometer (Elico, CL-27). IAA concentration was determined by using a standard curve of pure IAA (Sharma, 2012).

Results

P Solubilization test

Among these 34 isolates, 21 isolates were related to the soil samples of Edappadi region which were coded

from BMERF-PSB1 to BMERF-PSB21 and 13 isolates were from the soil of Udayappatti region and they were coded from BMERF-PSB 22 to BMERF-PSB 34. Among these 34 strains, 11 strains were selected for further studies because of having the highest clear halo diameter to the colony diameter (≥ 5mm) and supposed as superior and the most efficient strains. Among the isolates, 8 strains were from Edappadi soil samples and 3 strains were from Udayapatti soil samples. The qualitative and quantitative test results are illustrated in Table 1 and Table 2. The bacteria isolated from Edappadi region were superior to Udayapatti soil.

Table 1. Zone of P Solubilization.

S.No	Bacterial Isolates	Diameter of Zone (mm)		
		3rd day	5th day	7th day
1	BMERF-PSB3	1.2	4	4
2	BMERF-PSB4	0.93	2.8	3
3	BMERF-PSB7	1	3.5	3.5
4	BMERF-PSB8	0.85	3	3
5	BMERF-PSB12	ND	2.2	2.7
6	BMERF-PSB16	ND	4.5	4.5
7	BMERF-PSB19	0.68	4.1	4.1
8	BMERF-PSB20	0.66	3.3	3.3
9	BMERF-PSB23	ND	1.9	2
10	BMERF-PSB25	ND	2.3	2.5
11	BMERF-PSB31	ND	1.9	1.9

Table 2. Phosphate solubilization efficiency of the isolates.

S.No	Strain name	Solubilization zone diameter mm [z]	Bacterial colony diameter mm [C]	Phosphate solubilization efficiency (PSE) = (Z-C)/C X 100
1	BMERF-PSB3	4	1.2	233.33
2	BMERF-PSB4	3	1	200
3	BMERF-PSB7	3.5	1	250
4	BMERF-PSB8	3	1	200
5	BMERF-PSB12	3.2	1	220
6	BMERF-PSB16	4.5	1.6	181.25
7	BMERF-PSB19	4.1	1.4	192.85
8	BMERF-PSB20	3.3	1	230
9	BMERF-PSB23	2	1	100
10	BMERF-PSB25	2.5	1	150
11	BMERF-PSB31	1.9	1	90

Examination of optimum IAA production

Highest IAA producing ability was related to the strains BMERF-SB16 and BMERF-SB19 with IAA production of 45.11 and 41.25µg/ml which statistically had remarkable significance than the other strains. Following PSB isolates BMERF-PSB3, BMERF-PSB7, BMERF-PSB16 and BMERF-SB19. Strain BMERF-SB4, BMERF-SB8, BMERF-SB12, BMERF-SB20, BMERF-SB25, BMERF-SB31 produced 33.14, 34.25, 30.63, 35.18, 31.05, 33.08 µg/ml IAA respectively and were located in the second place after highest IAA producing strains. Strain BMERF-PSB23 significantly had lower ability to produce IAA (29.82 µg/ml) in the race (Table 3).

Table 3. *In-Vitro* P Solubilization capability of the bacterial isolates.

S.No	Bacterial Isolates	Available P (µg/ml)
1	BMERF-PSB3	39.53
2	BMERF-PSB4	26.38
3	BMERF-PSB7	32.25
4	BMERF-PSB8	27.68
5	BMERF-PSB12	21.68
6	BMERF-PSB16	45.01
7	BMERF-PSB19	43.29
8	BMERF-PSB20	29.01
9	BMERF-PSB23	20.02
10	BMERF-PSB25	24.09
11	BMERF-PSB31	27.33

Table 4. Quantification of IAA production by the isolates.

S.No	Bacterial Isolates	IAA (in µg/ml)
1	BMERF-PSB3	40.22
2	BMERF-PSB4	33.14
3	BMERF-PSB7	36.18
4	BMERF-PSB8	34.25
5	BMERF-PSB12	30.63
6	BMERF-PSB16	45.11
7	BMERF-PSB19	41.25
8	BMERF-PSB20	35.18
9	BMERF-PSB23	29.82
10	BMERF-PSB25	31.05
11	BMERF-PSB31	33.08

Morphological and Biochemical characterization

Prominent colonies appeared on Sperber's agar whereas no appreciable growth or distinct colonies were found to grow on the other selective media. The Sperber's agar selective isolates were Gram positive, rod shaped, endospore forming, non-motile, gas producing, positive for glucose, lactose, maltose, mannitol tests, catalase positive, indole negative, methyl red negative, Voges Proskauer negative and was able to withstand a high temperature (45°C and 65°C) during incubation. These characteristics render all the isolate as belonging to the genus *Bacillus* and species *megaterium*.

Discussion

Many researches on PGPR have emphasized the role of PSB's for 'P' nutrition in rice production along with available common flora of nitrogen fixing bacteria in paddy fields. Results of the present study depicted the availability of efficient 'p' solubilizer and Auxin producers in some paddy fields around Salem region. Previous studies also comply the role of soil microbes for 'p' solubilization, auxin production and plant growth promotion (Amalraj *et al*., 2012). The majority of these microorganisms live in the soil surrounding the roots, but it can also be found in the rhizoplane. Many of the isolated rhizobacteria present PGP capacity, proving that the promoting plant growth effect is the result of synergic relations established between different rhizospheric microorganisms (Ema achitei, 2010). Significant results were shown in Edappadi soil samples than Udayapatti soil samples which may be an evidence of decreasing soil fertility due to an elevated use of chemical fertilizers in Udayapatti region (Tables 1-3).

A total of thirty bacterial isolates were screened for phosphate solubilization on modified PVK agar, of which twelve isolates showed the development of sharp phosphate solubilization zones. Other isolates showed the development of hazy zones (Ajay Kumar *et al.,* 2012). However in the present work, eleven isolates showed sharp solubilization zones (maximum of 45mm) out of thirty six bacterial isolates, which did not differ much with the previous study. This difference was assumed to be caused by the changes in the soil factors and probably due to the use of ambient compounds by the isolates. In the present study, TCP was used as a substrate on PVK agar. Previous studies have reported that *Bacillus megaterium* was also capable of solubilizing zinc oxide, zinc carbonate, potassium bentonite and rock phosphate in addition to TCP (Amalraj *et al*., 2012).

Out of 34 isolates only 11 isolates were able to produce IAA in the present study. The concentration of produced IAA ranged from 23- 41 micrograms per milliliter. In a microbial suspension used in the previous study, concentration of produced IAA varied between 27–42 micrograms of IAA per milliliter between all IAA positive isolates (Inga *et al*., 2011). Srideve and mallaiah (2007) reported that the phenomenon of more production of IAA in the isolates was probably due to the better use of the ambient compounds by those isolates.

These findings had proven that the bacteria isolated from Edappadi soil were superior to the Udayapatti soil and these isolates in spite of having the ability of inorganic P solubilization, they could increase plant growth by producing IAA and guarantee the plant survival in P deficiency soils. According to the results there is no correlation between the ability of strains to solubilize the P and to produce IAA. So it was suggested that the effects of superior plant growth promoting strains like the isolates BMERF-PSB16 and

BMERF-PSB19 would be applied to increase the efficiency of phosphoric chemical fertilizers in future greenhouse, shade house and field studies.

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