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Full Length Research Article

Isolation and Biochemical Characterization of Phosphate Solubilizing Bacteria from Soils of Some Regions of Satara District

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Abstract

The Phosphate Solubilization is very essential for recycling the nutrient in the nature. Phosphate is available in organic and inorganic form and its solubilization results into the availability to the plants. It is one of the plant growth promotion activities. The bacteria involved are species of *Pseudomonas*, *Mycobacterium*, *Bacillus*, *Flavobacterium*, Micrococcus etc., seven isolate of phosphate solubilizing were obtained by using Katzelson and Bose medium. Soil Samples were streaked on Katzelson and Bose medium incubated at room temperature for 48 – 72 hrs. and clear zone was observed and such colony was used for further morphological, cultural and biochemical characterization. From morphological, cultural and biochemical characters studies isolate no. 2,3,7 was tentatively identified as *Micrococcus lylae, Micrococcus sedentarius, Bacillus megaterium* and *Bacillus cereus* respectively and remaining isolate no. 1,4,5,6 they may be different strains of *Micrococcus* and *Bacillus.* The phosphate solubilization index of isolate 1 and 3 ranged between 2.4 to 2.6. Microbial Consortia of above isolates, we formulate the biofertilizer and it can be used for sustainable agriculture.

***Key words*:** Katzelson and Bose medium, *Bacillus*, Phosphate solubilization index

Phosphorus (P) makes up about 0.2% - 0.8% of the plant dry weight, is the second major common limiting macronutrient after nitrogen that is required for plant growth and development as it is involved in the basic biological functions of the plants such as structures of DNA, RNA, Phosphoproteins, ADP, ATP etc. The Plant absorb phosphorus as phosphate anions from soil which are extremely reactive and can be immobilized through precipitation with cations such as Fe_3^+, Mg_2^+, Al_3^+, and Ca_2^+ and this proportional supply cause deficiency of phosphorus [1].

Phosphate solubilizing bacteria (PSB) such as *Bacillus*, *Pseudomonas*, *Azospirullum*, *Rhizobium*, *Azotobacter*, *Agrobacterium* etc., convert insoluble inorganic phosphate compounds into soluble forms. Phosphate solubilization takes place through the process of acidification, chelation and exchange reactions leading to the production of organic and inorganic acids which in turn lower the pH of the soil causing increase in solubility and release of phosphorus [2-3]. Occurrence of Phosphate solubilizing bacteria (PSB) in the soil are ubiquitous in different forms and an elevated Phosphate solubilizing bacteria (PSB) population are seen in agricultural soils [4]. The present study was designed and conducted with the objectives to isolate, characterize the Phosphate solubilization capabilities and used for plant growth promoting activities.

MATERIALS AND METHODS

Collection of samples

The soil samples selected as a source of phosphate solubilizing bacteria were from the rhizosphere of *Cajanus cajan* field of Patil Shivaji Ganapati from Marali near to Pal, Tal: - Karad. The soil samples were collected in polythene bag and after proper labeling, brought to the laboratory. The samples were kept in refrigerator till further use.

Isolation of phosphate solubilizing bacteria

One gram of each soil sample was mixed with 10 ml sterile distilled water and mixed vigorously and stood still for 10 min. to allow the soil to settle. Supernatant was further serially diluted and loopful of suspension was streaked on sterile Katznelson and Bose agar plates. The plates were incubated at room temperature (25 – 27 °C) for 48-72 hours. The colonies, which produced the clear zone around them, were selected. The isolates were then purified by repeated isolation of the bacteria from the obtained colonies on the same medium. The morphological characters of each colony were studied and recorded [5]. After proper labeling all the isolates were preserved at refrigeration temperature on Nutrient agar slants. Gram nature of the isolates was studied by Hucker and Cohn

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[6] modified Grams staining method and Motility of isolates was observed by hanging drop method [6].

Study of enzymatic properties

The production of various enzymes and their activities were studied by using standard methods and materials as described below:

i) *Catalase test*: One ml of 10% hydrogen peroxide was taken in clean small test tube and the growth of each isolated was picked up with sterile glass rod from the Katznelson and Bose Agar plate and was dipped in it and observed for evolution of gas bubbles for positive test.

ii) *Oxidase test*: A piece of Whatman filter paper was soaked with freshly prepared 1% aqueous solution of tetra methyl p-phenylene diamino hydrochloride. On this impregnated filter paper the growth of each isolate from Katznelson and Bose agar was rubbed with help of sterile glass rod and observed for appearance of purple colour on filter paper strip [7].

iii) *Starch hydrolysis test*: A loopful of culture of each isolate was spot inoculated out a sterile starch agar plates and the plates were inoculated at room temperature (25°C-27°C) for 48-72 hrs. After incubation, the plates were flooded with Lugol's iodine solution and observed for zone of hydrolysis of starch around the growth [8].

iv) *Gelatin liquefaction test*: A loopful of culture of each isolate was spot inoculated at room temperature (25°C - 27°C) for 48-72 hrs. After incubation, the plates were flooded with Frazier's regent to detect the liquefaction of gelatin.

V) *Casein hydrolysis test*: A loopful of culture of each isolate was spot inoculated onto a sterile milk agar plate and plates were incubated at room temperature (25°C - 27°C) for 48 - 72hrs. After incubation, appearance of clear zone around growth indicating hydrolysis of casein was observed [9].

vi) *Nitrate reduction test*: A loopful of suspension of each isolate was incubated separately in tubes containing 5 ml sterile peptone nitrate broth. All the tubes incubated at room temperature (25°C-27°C) for 48 72 hours. After incubation production of nitrate to nitrite was detected by addition of sulphanilic acid and alpha napthyl amine reagents.

vii) *Arginine hydrolysis test*: A loopful of suspension of each isolate was inoculated separately in tubes containing 5 ml sterile Arginine broth and were incubated at room temperature (25°C-27°C) for 48-72 hrs. After incubation, red precipitate observed at bottom of tube by addition of Nessler's reagent was reported as positive test [9].

viii) *Hugh-Leifson's test*: The growth of each isolate was inoculated into the sterile Hugh-Leifson's agar butts in a tube with the help of straight and incubated at 27 °C for 24 hrs. aerobically and anaerobically. Tubes Were observed for colour change from blue to yellow for Fermentative activity.

Study of fermentation of carbohydrates

Fermentation of carbohydrates was studied using the basal medium of Norris [10] and 1% of carbohydrates as:

Pentose - Xylose
Hexose - Glucose, Galactose, mannose
Disaccharides - Lactose, fructose

Qualitative measurement of phosphate solubilization

Bacterial isolates were screened for their Tri-calcium phosphate solubilizing activity on KB plates. Isolates were spot inoculated on the center of agar plate aseptically. All the plates were incubated at 25 – 27 °C for 48 hrs. A clear zone around a growing colony indicated phosphate solubilization and was measured as phosphate solubilization index (SI). SI was calculated as the ratio of the total diameter (colony + halo zone) to the colony diameter [11].

$$PSI = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

RESULTS AND DISCUSSION

Isolation of the phosphate solubilizing bacteria

Considering the aim of this research project, 7 bacterial isolates were obtained from the soil sample on showing zones of clearance Katznelson and Bose agar medium (Fig 1). Those isolates were purified and then labeled as 1 to 7. There colony characteristics were studied on as Katznelson and Bose agar medium.

Studies on morphological, motility and staining properties

Cell morphological characters were studied by performing Gram Staining (by Hucker and Conn [6] modified Gram staining method) and motility (by hanging drop method). The colony characters as well as results of cell morphologies, motility, and staining properties were as shown in (Fig 1-5, Table 2-3). From (Table 2-3) it was observed that isolate No. 2 produced circular, off white, entire, flat, smooth, opaque, moist colony on agar medium and was Gram positive rods in chains and were non-motile. The isolate No. 1,3,4,5,7 produced circular, off white, entire, convex, smooth, opaque, moist colony on agar medium and was Gram positive Cocci in clusters and were non-motile. The isolate No. 6 produced circular, off white, irregular, convex, rough, opaque, dry colony on agar medium and was Gram positive Cocci in clusters and were non-motile.

Table 1 Colony characters of phosphate solubilizing bacterial isolates on Katznelson and bose agar (Incubation temp. 27°C, time 48-72 hours)

Isolate No	Colony characters of bacterial isolates							
	Size	Shape	Colour	Margin	Elevation	Surface	Opacity	Consistency
1	2mm	Circular	Off white	Entire	Convex	Smooth	Opaque	Moist
2	1 mm	Circular	Off white	Entire	Flat	Smooth	Opaque	Moist
3	2mm	Circular	White	Entire	Convex	Smooth	Opaque	Moist
4	1mm	Circular	Off white	Entire	Convex	Smooth	Opaque	Moist
5	1mm	Circular	Off white	Entire	Convex	Smooth	Opaque	Moist
6	1mm	Circular	Off white	Irregular	Convex	Rough	Opaque	Dry
7	1mm	Circular	Yellow	Entire	Convex	Smooth	Opaque	Moist

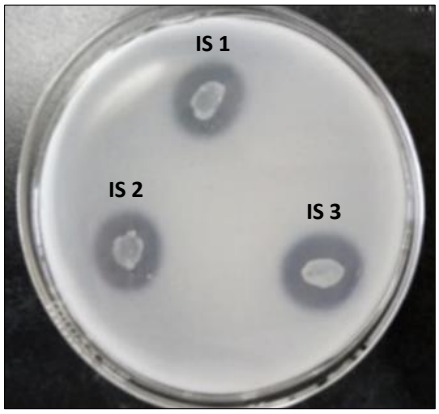

Fig 1 Zone of clearance by phosphate solubilizing some bacterial isolates on Katznelson and bose agar after 48-72 hrs of incubation at 25-27 °C

Fig 2 Colonial morphology of Isolate No.3 on after 48-72 Katznelson and bose agar hrs. of incubation at 25-27 °C

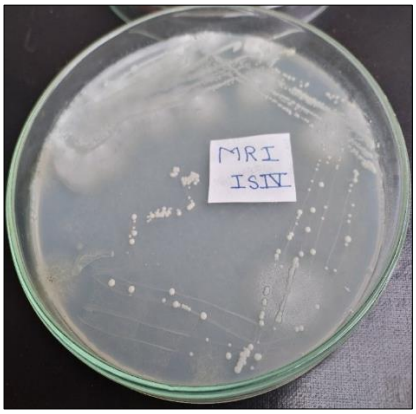

Fig 3 Colonial morphology of Isolate No.4 on after Katznelson and bose agar 48-72 hrs. of incubation at 25-27 °C

Fig 6 Colonial morphology of Isolate No.5 on after 48-72 Katznelson and bose agar hrs. of incubation at 25-27 °C

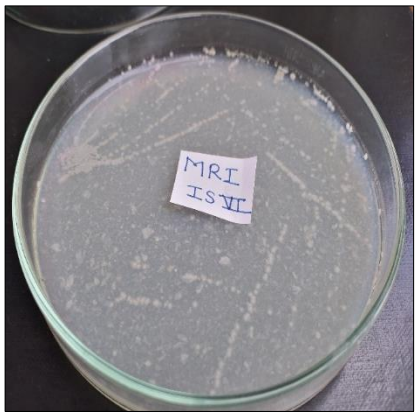

Fig 7 Colonial morphology of Isolate No.6 on Katznelson and bose agar on after 48-72 hrs. of incubation at 25-27 °C

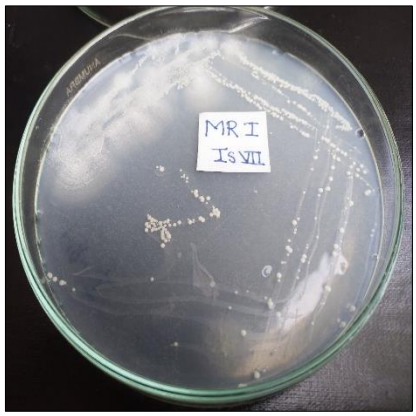

Fig 8 Colonial morphology of Isolate No.2 Katznelson and bose agar on after 48-72 hrs. of incubation at 25-27 °C

Table 2 Gram nature and motility of phosphate solubilizing bacterial isolates

Isolate No.	Gram nature	Motility
1	Gram positive rods in clusters	Non-motile
2	Gram positive cocci in chain	Non-motile
3	Gram positive cocci in clusters	Non-motile
4	Gram positive cocci in clusters	Non-motile
5	Gram positive cocci in clusters	Non-motile
6	Gram positive cocci in clusters	Non-motile
7	Gram positive thin rods	Non-motile

Studies on the enzymatic and biochemical properties

Further the enzymatic and bio chemical studies were also done and their result were noted in (Table 4-6). According to (Table 4-5) all the isolates showed catalase positive and isolate No. 1, 2, 7 showed oxidase positive and remaining isolate showed negative oxidase test. The starch hydrolysis was showed positive by isolate No. 4, 6, 7 and gelatin liquefaction was shown positive by isolate No. 1, 2, 3, 4, 6, 7 result respectively. The isolate No. 4 have ability to convert nitrate to nitrite and isolate No. 1, 3, shows arginine di hydrolase test positive where isolate showed negative tests respectively While isolate No. 1,7 have ability to hydrolyze casein.

Table 3 Enzymatic characters shown by phosphate solubilizing bacterial isolates

Tests	Phosphate solubilizing bacterial isolate						
	1	2	3	4	5	6	7
Catalase production	+	+	+	+	+	+	+
Oxidase production	+	+	-	-	-	-	+
Gelatin hydrolysis	+	+	+	+	-	+	+
Starch hydrolysis	-	-	-	+	-	+	+
Arginine hydrolysis	+	-	+	-	-	-	-
Casein hydrolysis	+	+	-	+	-	-	+
Nitrate reduction test	-	-	-	+	-	-	-

Table 4 Observation of Hugh and Leifson's test for the isolates

Phosphate solubilizing bacterial isolates	Hugh and Leifson's test	
	Aerobic	Anaerobic
1	+	+
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+

Acid production = + No acid production = -

Table 5 Fermentation of different carbohydrates by phosphate solubilizing bacteria

Isolate No.	Fermentation of carbohydrates					
	Glucose	Lactose	Galactose	Mannose	Xylose	Fructose
1	+	+	+	+	+	-
2	-	+	+	+	+	+
3	+	-	-	+	+	+
4	+	-	+	+	+	+
5	+	+	+	+	+	-
6	+	+	+	-	-	+
7	-	-	-	-	-	-

Acid production = + No acid production = -

Studies on utilization of various carbohydrates

The organism showed different ability to utilize different carbohydrates. Isolate No.1, 3,4,5,6 utilized glucose. Lactose was utilized by isolate 5, 6. The galactose sugar was utilized by isolate no. 1,2,4,5,6 while mannose was utilized by 1, 2,3,4,5 and remaining isolate showed negative results the Xylose was utilized by isolate no. 1,2,3,4,5 and fructose was utilized by isolate no. 2,3,4,6 while remaining isolate showed negative results respectively.

Tentative identification of the isolates

After performing these tests, by referring to the Bergey's Manual of Systematic Bacteriology – Vol-2 the organism was tentatively identified up to species level, isolate No.2 shows resemblance with the standard characters of *Micrococcus lylae* so it may be *Micrococcus lylae*, similarly isolate No.3 may be *Micrococcus sedentarius*, isolate No.7 may be *Bacillus megaterium*, while the remaining isolates may be suspected to be the different strains of *Micrococcus* and *Bacillus*.

Phosphate solubilization index of the isolates

A clear zone around a growing colony indicated phosphate solubilization and was measured as phosphate solubilization index (SI). SI was calculated as the ratio of the total diameter (colony + halo zone) to the colony diameter.

$$PSI = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

Isolate No.	Colony diameter (mm)	Halo zone (mm)	Solubilization index
1	5	8	2.6
2	6	9	2.5
3	5	9	2.8
4	7	10	2.42
5	7	10	2.42
6	7	10	2.42
7	6	11	1.83

PSB is a phosphate-solubilizing microorganism which can be routinely screened by a plate assay method using Pikovskaya medium. The bacteria will grow on this medium and form a clear zone around the colony [12-13]. These bacteria can convert tricalcium phosphate in the medium from insoluble to soluble forms [14]. Previous reports described some *Burkholderia* strains as being efficient phosphate solubilizers [15-16]. Phosphate solubilization potential has been attributed to the strains ability to reduce pH of the surroundings, either by releasing organic acids or protons [17-18]. Organic acids, such as gluconic acid, formic acid, oxalic acid, and citric acid, secreted by PSB can directly solubilize mineral phosphate as a result of anion change or indirectly chelate both Fe and Al ions associated with phosphate. This leads to increased P availability, which ultimately increases plant P uptake.

In this present work, the soil samples from nearby location were used for isolation of phosphate solubilizing bacteria and named as isolate No. 1 - 7. Out of 7 bacterial isolates, isolate No.1, 7 were Gram positive rods and isolates No. 2, 3, 4, 5, 6 were Gram positive cocci. From morphological, cultural and biochemical characters studies isolate No. 2,3,7 were tentatively identified as *Micrococcus lylae, Micrococcus sedentarius, Bacillus megaterium* and *Bacillus cereus* respectively and remaining isolate No.1,4,5,6 they may be different strains of *Micrococcus* and *Bacillus*. The bacterial isolates were qualitatively compared for phosphate solubilization here isolates No.1, 2 and 3 showed maximum phosphate solubilization within 24 and 48 hrs. respectively. While isolates No.4, 5, 6 and 7 showed moderate phosphate solubilization within 24-48 hrs. Microbial Consortia of above

isolates, we formulate the biofertilizer and it can be used for sustainable agriculture.

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