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ISOLATION AND SCREENING OF PHOSPHATE SOLUBILIZING BACTERIA FROM AGRICULTURAL FIELDS AROUND JAIPUR

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ABSTRACT

An essential macronutrient for plant growth and crop productivity is phosphorus (P). However, maximum portion of phosphorous in mineral soils is associated with Al and Fe oxides or hydroxide in soils of low pH and is unusable. Hence, in order to help plants fulfill their ideal nutritional needs for development and to maintain the sustainability of the soil, phosphorus is added to the soil as fertilizer. But this fertilizer overuse exacerbates soil degradation issues, lower agricultural output, pollutes the environment, degrades water quality, and destroys biodiversity. Thus as an alternative, phosphate solubilizing bacteria (PSB) possessing the ability to solubilize this immobilized phosphate and can serve as biofertilizer are focused nowadays. In current study, an effort was made to isolate and screen these microbes. Eight bacterial isolates forming solubilization zone ranging in size from 5.0mm to 15.6mm on pikovskaya agar plates and having solubilization indices of 5.16 to 3.79 were recovered from soils of Jaipur. The isolates were subjected to biochemical test and were identified as Pseudomonas, Bacillus, Pantoe and Enterobacter sp. Thus, these strains can be further exploited as biofertilizer, which can be a boost for agrarian community.

Keywords: *Biofertilzer, Halo Zone, Phorphorus Solubilizing Bacteria, Bioinocula.*

Introduction

Phosphorus (P) is a macronutrient, necessary for crop yield and plant growth. It is permanently classified as a macronutrient alongside nitrogen, yet unlike nitrogen; there is no large atmospheric source of phosphorus that may be obtained biologically (Guignard*et al.,* 2017). It is important for the growth, development and physiological activities of plants like cellular division, photosynthesis, root machine improvement, carbohydrate utilization, seed generation, cell structure, and inducement of early maturity. It also acts as the primary source of energy for microbial oxidation (Maharajan *et al.,* 2018). If the soil is lacking in phosphorus, it will not produce large yields (Meng *et al*., 2021). Hence, phosphorus deficit disrupts chlorophyll production, resulting in leaf chlorosis (Viégas*et al.,* 2018), browning of leaves, weak stems, and sluggish growth.

The amount of total phosphorus in soils varies between 50 and 3000 mg kg^{-1}, depending on a variety of factors. Organic and inorganic phosphorus exist in soil, both of which are crucial to plants due to phosphorous availability. Inorganic phosphorus makes up about 50 to 75% of the total phosphorus in soils, and it can vary from 10 to 90% in some circumstances whereas organic phosphorus makes up about 30 to 50 % of the total phosphorus in soils. Organic phosphorus fractions include inositol phosphorus, nucleic acid, and phospholipids, whereas inorganic phosphorus fractions include soluble and loosely bound phosphorus, Al- phosphorus, Fe- phosphorus, Ca- phosphorus, reductant soluble phosphorus, and occluded phosphorus (Johan *et al.,* 2021; Maharajan *et al.,* 2018).

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However, because maximal phosphorus in mineral soils is coupled with Al and Fe oxide or hydroxide in acidic soils and with Ca in calcareous soils, a considerable percentage of this phosphate is present in an unusable form. Even fertiliser that is applied externally is brought on and falls into the same category. Soluble phosphorus awareness in soil is typically quite low, with levels of 1 ppm or much less than 1 ppm being prevalent (Johan *et al.,* 2021; Neal *et al.,* 2017).

Materials and Method

- **Soil Sample Collection**

Soil samples were taken from rhizospheric soil of four distinct of agricultural fields nearby Jaipur, Rajasthan, India and were assigned the letters A, B, C, and D as code (Table 1).

- **Screening of Phosphate Solubilizing Bacteria**

Soil samples were processed as quickly and efficiently as possible by plating up on nutrient agar and Pikovskaya agar medium (Hi-media), which contains the phosphorus source insoluble tricalcium phosphate ($Ca_3(PO_4)_2$). After incubation, bacterial colonies showing zone of solubilization on media were picked and streaked again on fresh medium to check their phosphorus solubilizing ability. The phosphorus solubilizing bacteria were streaked and maintained on nutrient agar slant (Yang *et al.,* 2014).

- **Isolation of Phosphate Solubilizing Bacteria**

Each region's soil samples were used to make a stock solution, which consisted of 10 g of soil in 100 ml of sterile distilled water. The stock soil suspension was then subjected to a serial dilution. 1ml of suspension of 10^{-3} to 10^{-7} dilutions was taken from the serially diluted suspension for culturing on Nutrient agar and Pikovskaya agar medium by pour plate technique. The bacterial colonies on the Nutrient agar plates were observed after 5 days of incubation, whereas the Pikovskaya agar medium plates were examined after 7 days for further analysis. The best bacterial colonies were then screened using phosphate solubilization zone and solubilization efficiency. The colonies were selected from Pikovskaya agar medium and then were transferred on to nutrient agar slant for maintaining pure culture and for further studies (Cappucino and Sherman, 2009).

- **Identification of Bacterial Isolates**

The bacterial isolates was identified up to genus level by morphological and biochemical characteristics as prescribed by the standard protocols. The primary identification was done according to the Bergey's Manual of determinative Bacteriology (Claus and Berkeley, 1986).

- **Quantitative Analysis of Phosphate Solubilization by Phosphate Solubilizing Bacteria**

The potential of bacterial isolates to dissolve inorganic phosphorus from Pikovskaya's broth containing tri calcium phosphate was investigated (TCP). The colorimetric chlorostannousreduedmolybdo phosphoric acid blue technique was used to determine the soluble phosphorus. (Dave and Patel, 1999; Eileen Inghame*t al.,*1979).

- **Assay Procedure for Phosphorus Solubilizing Ability**

Stock culture suspension was prepared by inoculating a small loop of pure culture from nutrient agar slant in to a tube containing 10 ml of sterile buffer solution. 1 ml of bacterial isolate stock culture suspension was inoculated into 100 ml of Pikovskaya's broth (16.3 g/1000 ml) distilled water. The medium was autoclaved for 15 minutes at 15 lbs pressure (121°C) to thoroughly disinfect it. This was done in a 250 ml conical flask containing 50 mg/100 ml tri calcium phosphate (Ca3(PO4)2), which was maintained for 7 days on a rotating shaker at 30°C and 150 rpm under aerobic conditions. As a control, uninoculated media was employed. For 10 days, samples were taken at two-day intervals and examined to determine phosphate solubilization in the culture supernatant. The cell free supernatant containing soluble phosphorus was determined using a colorimetric technique after centrifugation of the withdrawn broth culture at 15000 rpm for 30 minutes. Before calculating soluble phosphorus, the pH of the cell free supernatant was determined.

Results

- **Isolation and Screening of Phosphate Solubilizing Bacteria**

The nutrient agar plates inoculated with the suspension from 10^{-3} and 10^{-4} dilution from all four regions after day 5, showed maximum growth but were too numerous to count. The easily identifiable colonies were observed in 10^{-5} dilution plates which were 258, 211,198 and 201 bacterial colony forming units in soil of region A, B, C and D respectively while in 10^{-6} dilution plates the count was 31, 29, 26 and 28 in soil of region A, B, C and D respectively .Also, in 10^{-7} dilution plate, very less number of colonies

was found which were 5, 5, 2, and 4 in soil of region A, B, C and D respectively. From 10^{-5} dilution plated on PVK, 16 best colonies forming clear phosphate solubilizing zone were isolated by sub culturing (Table 2). Among these 16 colonies, four colonies each from region (A to D) were selected and were designated as isolate 1 to 16 respectively. From them further eight strains (Figure 1) were screened for further analysis. The phosphate solubilizing zone formed by eight bacterial isolates on Pikovskaya agar plates were observable and varied from 5.0mm to 15.6mm (Table 3).These eight isolates were assigned codes as B1, B2, B3, B5, B6, B8, B11 and B16 for further studies. The solubilization index for these eight isolates was found to be ranged from 5.16 to 3.79. Isolate B5 showed the maxima while isolate B8 showed the least value (Table 3).

- **Identification of Isolated Phosphorus Solubilizing Bacteria**

All the eight phosphate solubilizing bacteria were subjected to various morphological and biochemical tests for preliminary identification up to genus level. The isolated bacterial cultures were identified as *Pseudomonas sp.* (B5, B8 and B16)*; Bacillus sp*. (B1, B2 and B6); *Pantaoe sp*. (B3) and *Enterobacter sp.*(B11) by cultural, morphological and biochemical characteristics (Table 3 and 4).

- **Quantitative Assay for Phosphate Solubilization by Wild Isolates**

The phosphorus solubilizing activity of all eight isolates varied on day 3, 6 and 9.The range of soluble phosphate varied on day 3 from 48.5 µg/ml(B5) to 114 µg/ml (B2) , while on day 6 it varied 55µg/ml (B5) to 117 µg/ml (B2), and 69.1 µg/ml (B8) -167 µg/ml(B6)on day 9 using tricalcium phosphate as a source of insoluble phosphate. The phosphate solubilization was accompanied by decrease in pH of the medium of isolates from 3rd day to 9th day; maximum pH value was 6.5 on day 3rd while minimum was 3.1 on day 9th(Figure 2).

The isolates were grouped according to the soluble phosphate on day 9 as high phosphate solubilizers (>150 µg/ml; Isolate B6), medium phosphate solubilizers (150-100 µg/ml; Isolate B2, B16, B3 and B1) and low phosphate solubilizers (<100 µg/ml; Isolate B5, B8 and B11).

Discussion

Due to the requirement for increased agricultural productivity, application of higher amount of chemical fertilizer to rhizpspheric soil is practiced. As a result of fixation, a substantial chunk of these insoluble phosphates have accumulated in the soil.Out of 53 isolates, best sixteen were streaked on to Pikovskaya's agar medium for isolation of pure colony. The phosphate solubilizing zone formed by bacterial isolates on Pikovskaya agar plates, varied from 5.0mm to 15.6mm and solubilization index varied from 3.79 to 4.98. These findings were in accordance with the results of Suleman *et al.,* 2018 who reported the solubilization of inorganic phosphate by fifteen isolates with solubilization index ranging from 2.4 to 5.8 in plate assay. Similarly, Pande *et al.,* 2017; also isolated eight phosphate solubilizing bacterial colonies from Indian agriculture field (Nainital region, Uttarakhand) and observed the halo zone diameter in a range of 5.33- 5.96 and solubilization index in a range of 4.88 ± 0.69 to 4.48 ± 0.30.Likewise, In addition, Sadiq *et al.* 2013 also discovered that the solubilization index of various bacterial isolates from the rhizosphere of various plants in the Lahore District, Pakistan, including *Bacillus spp*., ranged from 2.56 to 4.50.It is well known that phosphate-solubilizing bacteria help plants access phosphorus (Ponumurgan and gopi, 2006).

In the present study, the phosphate solubilization efficacy of isolated wild bacterial strains was quantitatively measured using colorimetric chlorostannous reduced molybdo phosphoric acid blue method. The insoluble phosphate used was tricalcium phosphate. The phosphorus solubilizing activity of all eight isolates varied on day 3, 6 and 9 with soluble phosphate being greatest on day 9. All the isolates solubilized phosphors in the range of 69.1 µg/ml (B8) -167 µg/ml (B6). It was also observed that the phosphate solubilization was accompanied by decrease in pH of the medium of isolates from 3rd day to 9th day; maximum pH value was 6.5 on days 3rd while minimum was 3.1 on day 9$^{th.}$ Reduced pH shows that the isolates can generate organic acids in a variety of concentration ranges (Vikram et.al., 2007; Whitelaw, 2000; Whitelaw et.al., 1999). Hence it can concluded that the ability to solubilize is typically reliant on the formation of organic acids (Ramachandran *et.al*., 2007).The secretion of these organic acids by phosphate solubilizing bacteria's have been reported by various researchers (Chen *et al.,* 2016; Serna-Posso *et al.,* 2017). These organic acids chelate cations and compete with phosphate for sites of adsorption thus releasing the bound phosphate and enhancing the amount of available phosphorus (Mardad *et al.,* 2013).These findings suggest that the inoculation of P-solubilizing microorganisms can be a promising technique causing increase in phosphorus availability in soil enhancing the agricultural output. Also, it can be ecologically safe and healthy approach for sustainable agricultural practices.

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Table 1: Sample Collection Sites

S. No	Regions	Area	Co-ordinates
1	A	Chaksu	26.6028565N,75.9237746E
2	B	Muhana,Sanganer	26.7912222N,75.7107304 E
3	C	Chomu	27.1673038N,75.679628E
4	D	Bassi	26.8444193N,76.0280037E

Table 2: Rhizosphere Microbial Density as found on Nutrient Agar and PVK

	Nutrient Agar					PVK				
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Region A	TNTC	TNTC	258	31	5	TNTC	TNTC	18	4	-
Region B	TNTC	TNTC	211	29	5	TNTC	TNTC	17	2	-
Region C	TNTC	TNTC	198	26	2	TNTC	TNTC	10	2	1
Region D	TNTC	TNTC	201	28	4	TNTC	TNTC	8	-	-

*Mean value of duplicates; TNTC- Too numerous to count

Table 3: Zone of Clearance Formed by Isolates from different Region on Pikovskaya Agar Medium

S. No	Isolate no.	Place of Soil Sample Collection and Serial Dilution	Colony Diameter	Zone of Clearance (in mm)	Solubilization Index
1	B1	Jaipur Region A	1.9 ± 0.45	15.6 ± 1.1	4.98
2	B2		1.7 ± 0.26	11.3 ± 1.1	4.12
3	B3		1.7 ± 0.26	7.0 ± 1.0	4.65
4	B5	Jaipur Region B	2.1 ± 0.34	13.3 ± 1.5	5.16
5	B6	Jaipur Region C	2.2 ± 0.23	8.6 ± 1.5	4.22
6	B8		1.73 ± 0.37	8.3 ± 1.5	3.79
7	B11		1.53 ± 0.11	5.0 ± 1.0	4.26
8	B16	Jaipur Region D	1.9 ± 0.28	11.1 ± 1.5	4.52

*Mean of triplicates ± standard error

Table 4: Physiological Characteristics of Isolates

Isolate Number	Colony Size (In mm)	Cell Shape	Colony Form	Elevation	Colony Colour	Optical Characteristic
B1	2	Rod	Circular	Convex	Creamish	Opaque
B2	2	Rod	Circular	Convex	Creamish	Opaque
B3	3	Rod	Circular	Convex	Yellowish	Translucent
B5	2	Rod	Circular	Flat	Creamish	Opaque
B6	3	Rod	Circular	Convex	Creamish	Opaque
B8	3	Rod	Circular	Flat	Creamish	Opaque
B11	4	Rod	Circular	Convex	Yellowish	Shiny
B16	3	Rod	Circular	Flat	Creamish	Opaque

Table 5: Biochemical Tests for Preliminary Identification of Isolates

Strain/Test	IsolateB1	Isolate B2	Isolate B3	Isolate B5	Isolate B6	Isolate B8	Isolate B11	Isolate B16
Gram Reaction	+	+	-	-	+	-	-	-
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	-	+	+	+	-	+
Lactose fermentation	LF	LF	LF	NLF	LF	NLF	LF	NLF
Indole	-	-	-	-	-	-	-	-
Methy red (MR)	+	+	+	-	+	-	-	-
VogesProskauer (VP)	-	-	+	-	-	-	+	-
Citrate utilization	-	-	-	+	-	+	+	+

LF: Lactose fermenting, NLF: Non lactose fermenting; += Positive;-=Negative

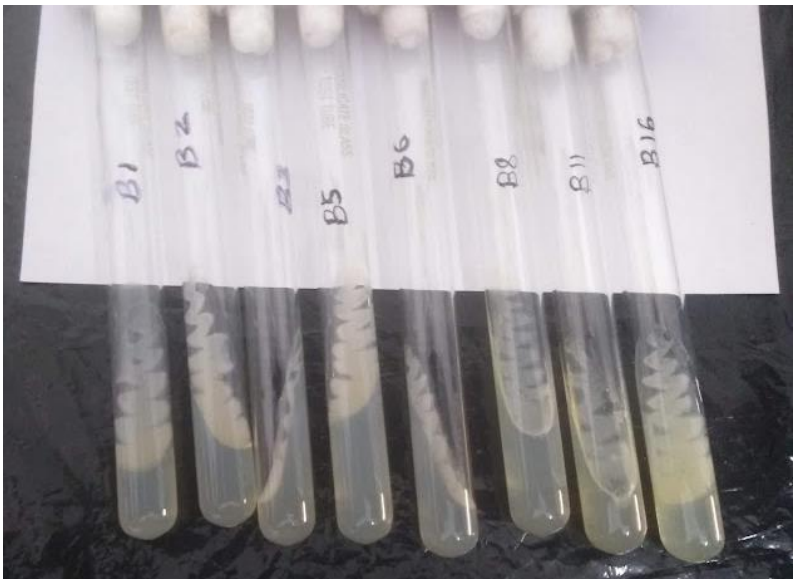

Figure 1: Pure Cultures of Eight Isolates as Maintained on Nutrient Agar Slants

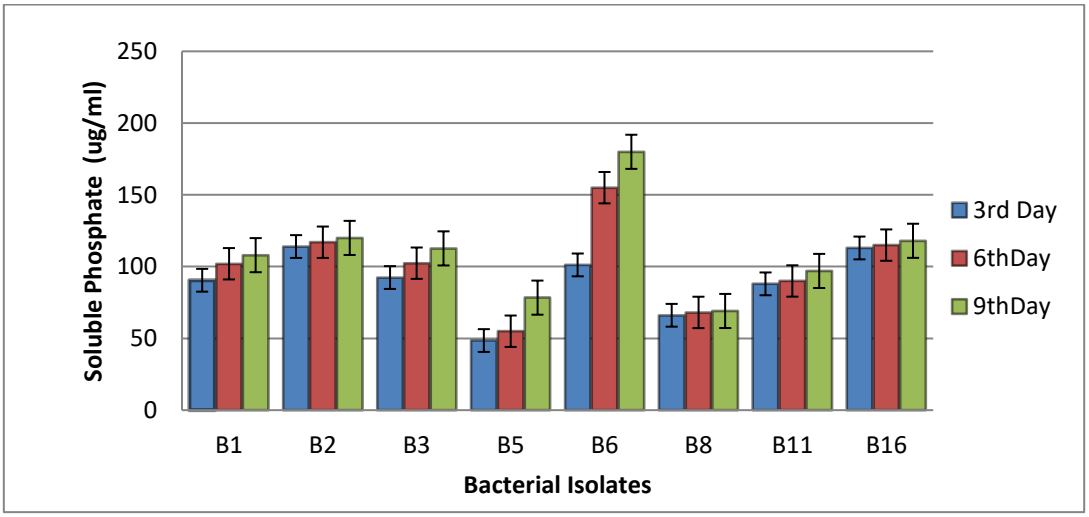

Figure 2: Phosphate Solubilization by Eight Isolates on Day 3, 6 and 9.

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