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Isolation And Characterization of Phosphate Solubilizing Microorganisms From Maize Rhizosperic Soils

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ABSTRACT
Among the crops corn (Zea mays) is an important in temperate climatic region because of the increasing demand for food and livestock feed. Nitrogen and phosphorus are essential nutrients for plant growth and development in corn. The beneficial microbes like phosphate solubilizing bacteria (PSB) are known to play an important role in supply of phosphorous (P) to plants in a sustainable manner in P deficient soils. The mineral nutrition of plants mainly depends on soil P content that can be assimilated as a soluble phosphate. P is involved in all major metabolic processes in plants such as biosynthesis of macromolecules, energy transfer, photosynthesis, signal transduction and respiration.In the present study twenty four (24) phosphate solubilizing bacteria (i.e., sixteen Bacillus and eight Pseudomonas) isolated from Maize research station and college farm, Rajendranagar, PJTSAU, Telangana and characterised by morphological, cultural and biochemical tests.
Keywords : *Corn crop, Solubilising Bacteria*

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INTRODUCTION

The use of phosphate solubilizing bacteria as inoculants simultaneously increases phosphorus uptake by the plant and crop yield. Strains from the *genera Pseudomonas, Bacillus, Rhizobium, Azospirillum, Burkholderia, Enterobacter, Erwinia, Serratia, Alcaligenes, Arthrobacter, Acinetobacter* and *Flavobacterium* are among the most powerful phosphate solubilizers (Rodríguez *et al.* 1999).In the soil, phosphorus is one of the major plant nutrients that is least available. Phosphorus is essential for morphological, physiological and biochemical development of plants. It plays an important role in plant growth promotion. It is an essential nutrient for plants which is required synthesis of nucleosides, nucleotides, phospholipids etc. Plant will not properly grow without sufficient quantity of phosphorus (Busman *et al.* 2006). An adequate supply of phosphorus in the early stages of plant growth promotes physiological functions including early root formation and is important for laying down the primordia for reproductive parts of plants. Without phosphorus, crops do not reach full yield, and animals do not prosper. Low Phosphorus levels in soils reduce crop yields by well over 50%. Lack of access to phosphorus (and other fertilisers) is one of the significant problems of agriculture soils in some areas (Zaidi *et al.* 2006).

MATERIALS AND METHODS

Soil sampling and Isolation

Samples were collected from Maize Research Station, Hyderabad and College Farm, College of Agriculture, Rajendranagar, Hyderabad. Pikovaskya's medium was used for the isolation of Phosphate solubilizing bacterial isolates.

Morphological Characterization of Phosphate solubilizing bacterial isolates

All the Phosphate solubilizing bacterial isolates were checked for their purity and then studied for the colony morphology and pigmentation. The cell shape and gram reaction was also recorded as per the standard procedures given by Barthalomew and Mittewar (1950).

Colony Morphology

Morphological characteristics of the colony of each isolate was examined on Nutrient agar and specialized medium and incubated for according to isolate. Cultural characterization of isolates were observed by different characteristics of colonies such as shape, size, elevation, surface, margin, color, odour, pigmentation, etc. were recorded.

Cultural characterization

All the bacterial isolates were studied for their colony morphology, pigmentation and spore production as per Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994)

Biochemical and Physiological Characterization of Phosphate solubilizing bacterial isolates

Starch Hydrolysis

Sterile starch agar plates were spotted with 10 µl overnight broth cultures of the isolates and incubated at 28 ± 2°C for 24 - 48 h. After incubation, the plates were flooded with iodine solution. The formation of a transparent zone around the colony was taken as positive reaction for the test.

Indole Production

Sterilized SIM agar slants were inoculated with the overnight cultures of the isolates and incubated for 48 h at 28 ± 2°C. Following incubation, 10 drops of Kovac's Indole reagent was added to each tube. The isolates showing production of red colour was recorded as positive for Indole production.

Catalase Test

This test was performed to study the presence of catalase enzyme in bacterial colonies. Fresh cultures of pure isolates were taken on glass slide and one drop of H_2O_2 (30%) was added. Appearance of gas bubble indicated the presence of catalase enzyme.

Oxidase Test

The overnight cultures of the test isolates were spotted on plates poured with sterile Trypicase Soy Agar (TSA) and the plates were incubated for 24 h at 28 ± 2°C. After incubation, 2-3 drops of N, N, N', N'-tetramethyl - p - phenylene diamine dihydrochloride (Wurster's reagent) were added on to the surface of growth of each test organism. The isolates showing change of colour to maroon were noted as oxidase positive.

Gelatin liquefaction

The overnight cultures of the test isolates were inoculated into sterilized nutrient gelatin deep tubes and incubated for 24 h at 28 ± 2°C. Then the tubes were kept in the refrigerator for 30 min at 4°C. The isolates showing liquefied gelatin were taken as positive and those which resulted in solidification of gelatin on refrigeration were recorded as negative for the test.

Methyl Red Test

Sterilized glucose - phosphate broth tubes were inoculated with the test culture and incubated at 28 ± 2°C for 48 h. After incubation five drops of methyl red indicator was added to each tube and gently shaken. Red colour production was taken as positive and yellow colour production was taken as negative for the test.

Voges - Prausker's Test

To the presterilized glucose-phosphate broth tubes, test cultures were inoculated and incubated at 37°C for 48 h. After incubation ten drops of Baritt's reagent A was added and gently shaken followed by addition of 10 drops of Baritt's reagent B. Development of pink colour in the broth was taken as positive for the test.

Citrate Utilization

Isolates were streaked on Simmon's citrate agar slants and incubated at 28 ± 2°C for 24 h. Change in colour from green to blue indicates the positive reaction for citrate utilization.

Carbohydrate Utilization

All pure bacterial isolates were screened for the carbohydrate fermentation abilities using 4 different carbohydrates (Lactose, sucrose, dextrose and mannitol) in peptone broth medium. Bacterial isolates were inoculated in broth containing specific carbohydrate. The change in colour of peptone broth was observed for utilization of particular carbohydrate present in broth.

Qualitative method of estimation of phosphorus

Sterilized Pikovskaya's agar was poured as a thin layer on to the sterilized petriplates and incubated for 24 h. After incubation the Pikovskaya's plates were spot inoculated with isolates and incubated at 28 ± 1°C for 4-5 days. Formation of a clear zone around the colonies were considered as positive result for phosphate solubilization.

PSE (Phosphate Solubilization Efficiency) = Z / C x 100

Z - Clearance zone including bacterial growth

C - Colony diameter

Quantitative method of estimation of phosphorus by Broth assay

One ml of filtrate/ suspension added in a 50 ml volumetric flask to which 10 ml of chloromolybadic acid was added and with the help of distilled water volume was made up to 45 ml. 2-3 drops of chlorostannous acid was immediately added and the volume was made up to 50 ml with distilled water. A blue colour was developed and the absorbance at 600 nm was recorded using spectrophotometer.The standard graph was constructed by using KH_2PO_4 (0, 3, 6, 9, 12 and 15 ppm)as substrate (Gaur, 1990).

RESULTS AND DISCUSSION

Isolation of Phosphate Solubilizing Bacteria (PSB) from maize rhizospheric soils:

Soil samples were collected from the Maize rhizosphere soils of Maize research station and College Farm, College of Agriculture, PJTSAU, Rajendranagar, Hyderabad. Different phosphate solubilizing bacteria and fungal isolates were isolated from the rhizospheric soils using serial dilution and plating method on Pikovskaya agar plates (supplemented by inorganic phosphate source). *Aspergillus* spp (Asp1) was collected from Department of Agricultural Microbiology and Bioenergy. All the isolates were named and designated as shown in Table 1. All the isolates were studied for their cultural and morphological characterization.

Cultural and Morphological Characterization:

More number of Phosphate solubilizing microorganisms were present in the rhizosphere and they are metabolically more active than others. Morphological and Cultural characteristics of all the isolates were studied viz., shape, size, margins and color etc., The morphological features on nutrient agar plate was studied and they showed small to medium size, dull white or off white, flat, smooth, irregular colonies and there was no pigment production (Table 1). These isolates were found to be gram positive, short stumpy, rod shaped cells when observed under microscope. On the basis of biochemical reactions it was found that *Bacillus* spp. The sixteen isolates were named as PSB 1 to PSB 16. (Table 1). The other eight isolates were examined morphologically and concluded as *Pseudomonas* spp. The culture has showed the features like small to medium, smooth, glistening yellowish green color with light green pigmentation around the colonies on King's B agar medium (Table 1). These isolates were gram negative, small, single isolated rods without sporulation when observed under microscope. These isolates were named as PSB 17 to PSB 24. (Table 1) Fungal culture (Asp1) was characterized by the morphological and cultural characteristics and confirmed as *Aspergillus* spp*.* Based on microscopic examination and cultural characteristics, Preeti *et al.* (2011) identified four isolates as *Pseudomonas* spp and others as *Bacillus* spp.

Biochemical characterization:

All the isolates were tested for biochemical characterization viz., IMViC tests, oxidase, catalase, starch hydrolysis, gelatin liquefaction, carbohydrate utilization. Results (Table 2) revealed that all the sixteen isolates were *Bacillus* and positive for oxidase test, catalase, starch hydrolysis, negative for gelatin liquefaction. For citrate utilization except PSB 10 all the isolates were positive in Voges Prausker's test PSB 7 only showed negative result. For methyl red test all the isolates showed positive reaction except PSB 1. For Indole test all the isolates showed positive reaction except PSB 5, PSB 13 and PSB 15. For carbohydrate utilization i.e., for glucose test all the isolates were showed positive results expect PSB 8, PSB 11 and PSB 12. For Galactose test all the isolates were showed positive results expect PSB 3. For lactose test all the isolates showed positive results expect PSB 7 and PSB 11. All the eight isolates of *Pseudomonas* showed positive results for oxidase tests, Gelatin liquefaction, where as they were negative for indole production test. For catalase test all isolates are positive except PSB 18, for methyl red PSB 17, PSB 18 and PSB 19 were negative and for Voges Prausker's test only PSB 23 and PSB 24 were positive. For citrate utilization all the isolates were positive except PSB 18. For starch hydrolysis all showed negative expect PSB 23 and PSB 24. For carbohydrate utilization i.e., for glucose test all the isolates showed negative results expect PSB 20. For Galactose test all the isolates showed positive results expect PSB 21, PSB 22 and PSB 23. For Lactose test all the isolates showed positive results expect PSB 18 and PSB 19. *Aspergillus* spp(Asp1) isolate was showed positive results for starch hydrolysis, Voges Prausker's, methyl red, catalase, citrate utilization, oxidase, starch hydrolysis, gelatin liquefaction, carbohydrate utilization and negative for Indole production.

Anitha and Kumudini (2014) isolated *Pseudomonas* from fifteen rhizospheric samples from different regions of India. They characterized morphologically and biochemically and concluded as genus *Pseudomonas*.One hundred and eighteen *Bacillus* spp*.* were isolated from the rhizosphere of soybean plant of Cirebon, Indonesia and further examined for plant growth promoting activities (Wahyudi *et al.,* 2011).

Screening of isolates for their Plant Growth Promoting (PGP) characters

Phosphate Solubilization

Qualitative method

All the sixteen Bacillus isolates were able to form clear zone of phosphate solubilization on Pikovaskaya's agar plate ranged from 15.50 - 6.10 mm. Among them PSB 6 of *Bacillus* spp detected the highest solubilization zone (15.50 mm) followed by PSB 5 (14.80 mm) and the lowest solubilization zone was observed with PSB 3 (6.10 mm).

All the eight *Pseudomonas* isolates were able to form clear zone of phosphate solubilization on Pikovaskaya's agar plate ranged from 12.00 - 6.40 mm. Among them PSB 24 of *Pseudomonas* spp detected as highest solubilization zone 12.00 mm followed by PSB 20 (11.00 mm) and the lowest solubilization was showed by PSB 22 (6.40 mm).

The *Aspergillus* spp (Asp1) showed 10.1 mm Phosphate solubilization zone. (Table 3) *Bacillus* and *Pseudomonas* spp differ in the ability to produce phosphatase enzyme and production of organic acids and hence showed different solubilization efficiency. Tensingh *et al.* (2015) identified the selected strains were *Bacillus* and *Pseudomonas*. The isolated strains were characterized under *in vitro* conditions. They showed solubilization zone ranges from 2 - 5 mm at 28 - 30^{0}C. The highest solubilization was observed with *Pseudomonas putida* (5 mm) followed by *P. flourescens* (4 mm) and the lowest solubilization was observed in *Bacillus megaterium* (2 mm). Similarly Uma *et al*. (2012) reported phosphate solubilization by *Bacillus* spp from groundnut rhizosphere (*Arachis hypogaea* L).

Quantitative estimation of available phosphorus in Pikovaskaya's broth

All the sixteen Bacillus isolates were able to solubilize the available phosphorus in Pikovaskaya's broth with known amount of Tri - calcium phosphate as a substrate. Among them PSB 6 recorded the more available phosphorus content of 0.89 mg L-1 (pH: 7.10). Second best was showed by different isolates PSB 8 and PSB 10 i.e., 0.82 mg L-1 (pH: 7.00 and 6.00). The lowest was shown by PSB 16 with 0.57 mg L-1 (pH: 7.89).

All the eight *Pseudomonas* isolates were able to solubilize the available phosphorus in Pikovaskaya's broth with known amount of Tri - calcium phosphate as a substrate. Among them PSB 24 was recorded the highest available phosphorus content of 0.82 mg L-1 (pH: 7.00). Second best was observed by the isolate PSB 18 and PSB 23 i.e., 0.81 mg L^{-1} (pH: 6.80 and 7.00). The lowest was recorded by PSB 21 with 0.54 mg L-1 (pH: 6.00) phosphorus solublization.

The *Aspergillus* spp (Asp1) showed available phosphorus concentration i.e., 0.78 mg L-1 (pH: 6.70) respectively. (Table 2) similar results were observed by Karpagam and Nagalakshmi. (2014) i.e., thirty seven Phosphate Solubilizing Microbial isolates were isolated on the Pikovskaya's agar medium. Out of 37 microbial isolates eight isolates were showed highest Phosphate Solubilization Index (PSI) ranged from 1.13 - 3.00 mg L-1 and they were selected for the qualitative as well as quantitative study. Among these eight potent isolates, 3 strains (PSM 1, PSM 2 and PSM 6) showed maximum PSI on agar plates along with high soluble phosphate production of 0.37 mg L^{-1}, 0.30 mg L^{-1} and 0.28 mg L^{-1} in Pikovaskaya's broth.

CONCLUSION

A key advantage of beneficial microorganisms is to assimilate phosphorus for their own requirement which in turn available to its soluble form in soil. *Pseudomonas, Bacillus* and *Aspergillus* spp reported to be active in the solubilization process. Initially PGP strains viz., *Pseudomonas* spp*, Bacillus* spp and *Aspergillus* spp were isolated and grown in respective media. All the isolates were characterized morphologically and biochemically. The efficient isolates were subjected to further characterization.The isolates were selected based on their good performance of PGPR characters. For phosphate solubilization the individual isolates were able to form a solubilization zone ranged from 15.50 - 6.10 mm. The highest efficiency (258.33 %) was recorded in PSB 6 and lowest efficiency was observed in PSB 3 (117.30 %).

Table1:Cultural and Morphological characteristics of Phosphate solubilizing bacterial isolates

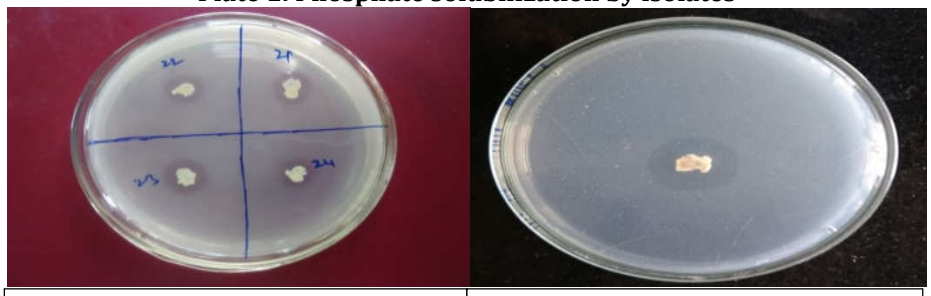
Isolate Code	Cultural and Morphological Characteristics	Identification of isolate
PSB1	Irregular, creamy whitish flat, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB2	Dull whitish, irregular, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB3	Creamy white, regular, medium, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB4	Light cream color, flat Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB5	Big, white, irregular, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB6	Small, white, irregular, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB7	Dull white, flat, regular, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB8	Off white, irregular, flat, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB9	Creamy whitish, irregular, flat, Gram +ve, purple, rod shaped	*Bacillus* spp.

PSB10	whitish, small, irregular, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB11	Dull white, medium, flat, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB12	Whitish, medium, flat, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB13	Light cream color, small, regular, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB14	Whitish, large, flat, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB15	Cream, small, regular, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB16	Dull white, irregular, flat, Gram +ve, purple, rod shaped	*Bacillus* spp.
PSB17	Yellowish green, round, fluorescent, Gram-ve, rod shaped, pink	*Pseudomonas* spp.
PSB18	Yellowish green, irregular, glistening, pigmented, Gram-ve, rod shaped, pink	*Pseudomonass*pp.
PSB19	Yellow colored, medium, spreaded, Gram-ve, rod shaped, pink	*Pseudomonas* spp.
PSB20	Yellowish, button shape small, round, Gram-ve, rod shaped, pink	*Pseudomonass*pp.
PSB21	Yellowish green, regular round, Gram-ve, rod shaped, pink	*Pseudomonas* spp.
PSB22	Yellowish green, small, irregular, Gram-ve, rod shaped, pink	*Pseudomonass*pp.
PSB23	Yellow colored, regular, round, Gram-ve, rod shaped, pink	*Pseudomonass*pp.
PSB24	Yellowish, glistering, medium, irregular, Gram-ve, rod shaped, pink	*Pseudomonass*pp.
Asp1	Black colonies with a white leading edge, Globose conidial head, smooth and colorless conidiophore, conidia forms chains	*Aspergillus* spp.

Table 3: Phosphate solubilisation efficiency of different bacterial isolates

Isolate Code	Zone diameter		Solubilization efficiency (%)	Soluble P concentration (mg L^{-1})	pH
	Solubilization zone (mm)	Culture diameter (mm)			
PSB1	9.10	5.00	182.00	0.63	5.20
PSB2	8.60	6.30	136.50	0.60	7.60
PSB3	**6.10**	**5.20**	**117.30**	0.62	6.40
PSB4	8.00	4.10	195.10	0.71	6.12
PSB5	14.80	6.00	246.60	0.79	6.68
PSB6	**15.50**	**6.00**	**258.33**	**0.89**	7.10
PSB7	12.30	8.00	153.75	0.76	5.63
PSB8	9.10	4.00	227.50	0.82	7.00
PSB9	6.60	4.00	165.00	0.68	7.36
PSB10	14.50	6.40	226.60	0.82	6.00
PSB11	10.40	6.40	162.50	0.75	5.14
PSB12	13.50	9.00	150.00	0.78	6.32
PSB13	9.30	7.00	132.80	0.66	6.00
PSB14	12.00	10.00	120.00	0.77	7.00
PSB15	10.80	5.50	196.30	0.60	8.32
PSB16	11.40	5.70	200.00	**0.57**	7.89
PSB17	8.30	6.40	129.60	0.74	6.45
PSB18	9.70	6.00	161.60	0.81	6.80
PSB19	8.70	6.90	126.00	0.65	5.00
PSB20	11.00	9.20	119.50	0.65	7.98
PSB21	8.40	6.70	125.30	**0.54**	6.00
PSB22	**6.40**	**5.60**	**114.28**	0.77	6.34
PSB23	9.40	6.00	156.60	0.81	7.00
PSB24	**12.00**	**6.00**	**200.00**	**0.82**	7.00
Asp1	10.10	5.80	174.10	0.78	6.70
CD	**0.313**			**0.035**	
SE(d)	**0.155**			**0.018**	
SE(m)	**0.110**			**0.012**	
CV	**1.855**			**2.969**	

Plate 1: Phosphate solubilization by isolates



Phosphate solubilization by PSB bacteria	Phosphate solubilization Asp1 fungi

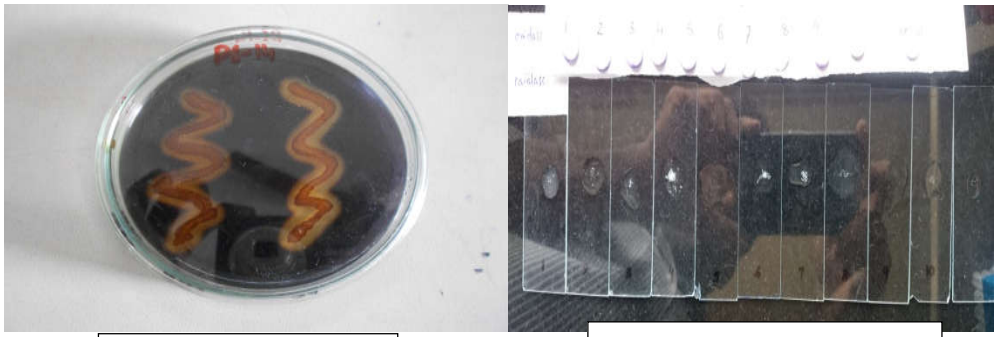
Plate: 2 Different biochemical tests for characterization of PSB isolates

Methyl red test

Indole test

Citrate utilization

Gelatine liquefaction



Starch hydrolysis

Oxidase and catalase

Table 2: Biochemical characteristics of phosphate solubilizing bacterial isolates

Isolate Code	Indole test	MR test	VP test	Citrate utilization	Catalase	Oxidase	Starch hydrolysis	Gelatin liquefaction	Carbohydrate utilization		
									Glucose	Galactose	Lactose
PSB1	+	-	+	+	+	+	+	-	+	+	+
PSB2	+	+	+	+	+	+	+	-	+	+	+
PSB3	+	+	+	+	+	+	+	-	+	-	+
PSB4	+	+	+	+	+	+	+	-	+	+	+
PSB5	-	+	+	+	+	+	+	-	+	+	+
PSB6	+	+	+	+	+	+	+	-	+	+	+
PSB7	+	+	-	+	+	+	+	-	+	+	-
PSB8	+	+	+	+	+	+	+	-	-	+	+
PSB9	+	+	+	+	+	+	+	-	+	+	+
PSB10	+	+	+	-	+	+	+	-	+	+	+
PSB11	+	+	+	+	+	+	+	-	-	+	-
PSB12	+	+	+	+	+	+	+	-	-	+	+
PSB13	-	+	+	+	+	+	+	-	+	+	+
PSB14	+	+	+	+	+	+	+	-	+	+	+
PSB15	-	+	+	+	+	+	+	-	+	+	+
PSB16	+	+	+	+	+	+	+	-	+	+	+
PSB17	-	-	+	+	+	+	+	+	+	+	+
PSB18	-	-	+	-	+	+	+	+	+	+	-
PSB19	-	-	+	+	+	+	+	+	+	+	-
PSB20	-	+	+	+	+	+	+	+	-	+	+
PSB21	-	+	+	+	+	+	+	+	+	-	+
PSB22	-	+	+	+	+	+	+	+	+	-	+
PSB23	-	+	-	+	+	+	-	+	+	-	+
PSB24	-	+	-	+	+	+	-	+	+	+	+
Asp1	-	+	+	+	+	+	+	+	+	+	+

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