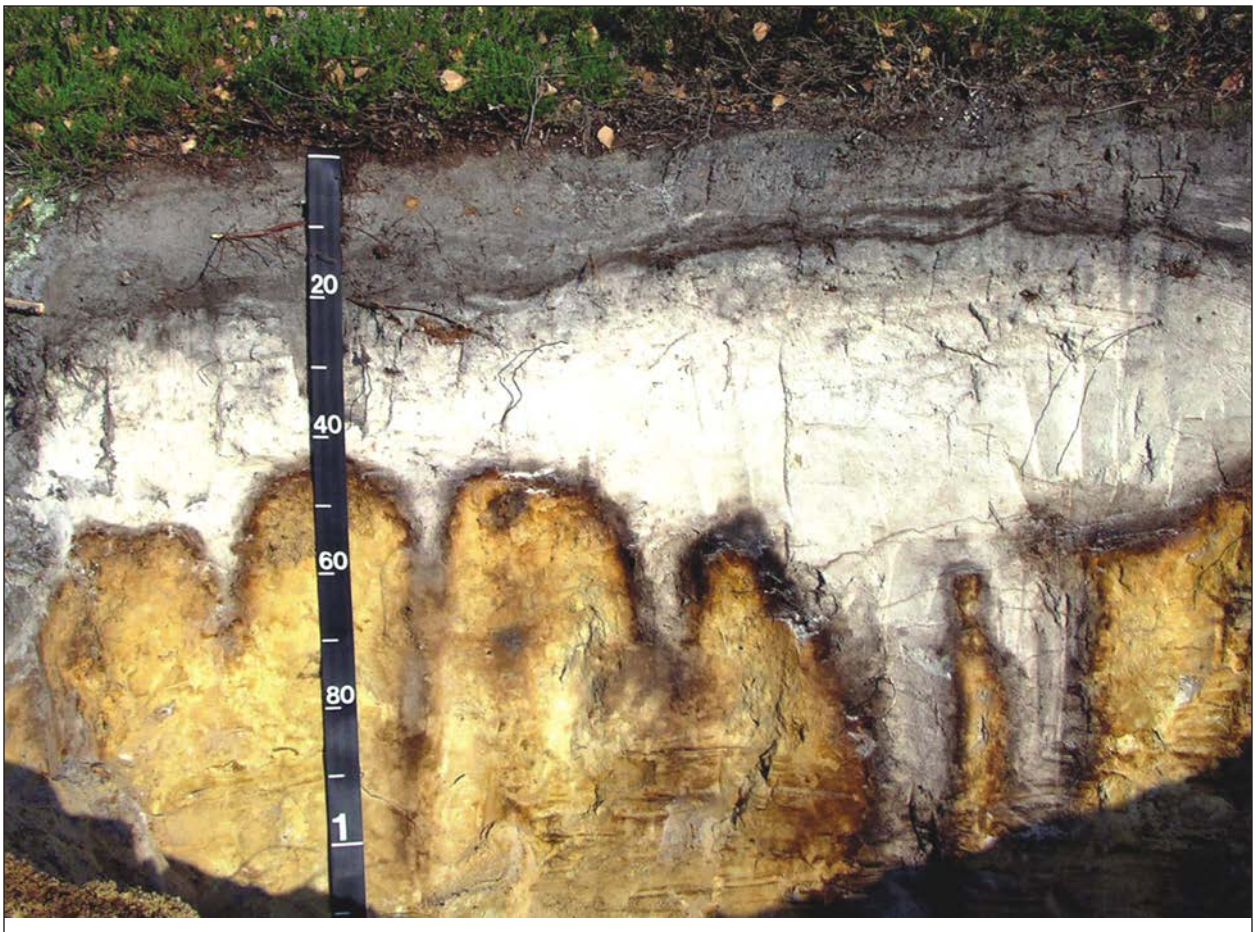

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Insoluble Phosphate Solubilization by Bacterial Strains Isolated from Rice Rhizosphere Soils from Southern India

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ABSTRACT

Phosphates solubilizing bacterial strains were isolated from various rice- rhizosphere soils of southern peninsular region of India. Thirty efficient PSB isolates were selected from 226 colonies based on their ability to form clear zone on Pikovskaya's agar medium. Phosphorus release and phosphatase activity were quantified using ELISA on 3rd, 5th and 7th day of incubation to determine the efficiency of the strains. The isolated phosphate solubilizing bacterial strains released high amount of phosphorus from tricalcium phosphate and ranged from 22.4 to 825.8 μg P mL^{-1} and the amount of phosphatase secreted into the medium ranged from 11.6 to 64 U. From the results we conclude that the efficiency of the strain to solubilize phosphate or release phosphorus depends on the specificity of the enzyme phosphatase than the amount of phosphatase released into the medium. The efficient strains isolated from rice rhizosphere soils were identified as *Enterobacter, Micrococuss, Pseudomonas, Bacillus, Klebsiella* and *Serretia*. Among all the strains, A4 strain (*Enterobacter aerogenes*) released high amount of phosphorus. All the isolated PSB were efficient phosphate solubilizers and can be used as bioinoculants to increase the available phosphorus in the soil for rice plant growth.

Key words: Phosphate solubilization, bacteria, tricalcium phosphate, phosphatases, biofertilizers

INTRODUCTION

Phosphorus (P) deficiency is a major constraint for crop production. Plants absorb inorganic form of P which acts as an essential element for plant growth and development making up to 0.2% of plant dry weight. The level of inorganic P is very low in the soil and the available P is in insoluble form. The beneficial microorganisms in the soil convert insoluble P into soluble form for plant growth (Rodriguez and Fraga, 1999) by acidification, chelation and exchange reactions (Gerke, 1992) in the periplasm, which act as an indicator for routine isolation and selection procedure of phosphate solubilizers (Illmer and Schinner, 1992). Bacteria are the predominant microorganisms that can solubilize phosphate compared to fungi and actinomyces (Yin, 1988). Bacteria use several direct and indirect mechanisms of action to improve the plant growth such as phosphate solubilization (Kim *et al*., 1998), aminocyclopropane-1-carboxylate (ACC) deaminase

(Penrose and Glick, 2003), nitrogen cycle (Ahn *et al*., 2007) and phytohormone production (O'Sullivan and O'Gara, 1992). Bacteria belonging to *Mesorhizobium*, *Rhizobium, Klebsiella, Acinetobacter, Enterobacter, Erwinia, Achrobacter, Micrococcus, Pseudomonas* and *Bacillus* isolated from different soils have been reported as efficient phosphate solubilizers (Villegas and Fortin, 2002). The beneficial effects of phosphate solubilizing bacteria on crops have been well documented (Richardson *et al*., 2001).

Rice field soils possess considerable accumulation of phosphorous due to regular application of chemical phosphate fertilizers and a large proportion of the applied fertilizers are converted into insoluble form and become unavailable to plants (Rodriguez and Fraga, 1999). Microorganisms solubilize the insoluble phosphates and maintain the soil health and quality (Richardson, 2001). Considering the multiple applications of phosphate solubilizing bacteria, it is important to study the efficiency of the strain isolated from rice field soils and design strategies to use these native strains as bioinoculants for organic agriculture of rice crop without causing harm to the environment and the farmers. The objective of the present investigation was to isolate and identify efficient Phosphate Solubilizing Bacteria (PSB) from various paddy rhizosphere soils of different regions of southern peninsular India and to understand the mechanism involved in the release of phosphorus from phosphates by measuring the P-release and phosphatase activity by the strains.

MATERIALS AND METHODS

Rhizosphere soil collection: Soil samples were collected during the year 2008 from wetland rice field soils of Tamil Nadu, India. The Rhizosphere soil samples were collected by random sampling procedure in three replicates, taken from the same plot at different sites, carefully removed along with the plant and surrounding soil. The soil samples were analyzed for its physiochemical properties

Isolation of strains: Ten gram of soil from each sample was aseptically weighed and transferred to 250 mL Erlenmeyer flask containing 90 mL of sterile double distilled water. The flasks were shaken in orbital shaker (Scigenics, India) at approximately 200 r min^{-1} for 30 min. The samples were allowed to settle for 10 mins. Aliquots of 1 mL of the supernatant from the sample were transferred to 9 mL of 0.85% NaCl dispensed in test tubes and serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Aliquots of 0.1 mL of the sample from each of these dilutions were spread on to a Petri dish containing Pikovskaya's medium containing Tricalcium phosphate (0.5 g Yeast extract, 10 g Dextrose, 5 g $Ca_3(po_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.20 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.0001 g $MnSO_4.H_2O$, 0.0001 g $FeSO_4.7H_20$, 18 g Agar in 1000 mL distilled water) (Pikovskaya, 1948). The pH of the media were adjusted initially to 7.0 with 1 mol L^{-1} NaOH or HCl and sterilized by an autoclave for 20 min at 115°C. The plates were incubated aerobically at 28°C±2 for 7 days in an incubator (Scigenics, India).

Colonies showing clear zone of P-solubilization were counted (De Freitas *et al*., 1997) and expressed as colony forming unit (cfu) per gram of soil. Different types of single, well separated colonies, from each sample site, which grew on plates showing clear zones were picked and restreaked onto fresh Pikovskaya's solid medium. This procedure was repeated until pure culture with high P solubilization or mineralization was obtained. The strains which showed clear zones were inoculated into nutrient broth and incubated at 28°C±2 for 24 h at 200 r min^{-1} until it reached 2 OD. Gram staining was done to confirm if the isolated strains were gram negative or gram positive bacteria using gram staining kit (K001-1KT, Hi media). The cultures obtained were

centrifuged and the pellets were lyophilized in lyophilizer (Christ, Germany) and stored at -20 (Sanyo, Germany).

***In vitro* Assay of phosphorus (P) release, phosphatase activity and strain identification:** The lyophilized strains were inoculated into nutrient broth to revive the culture and incubated at 28°C±2 for 24 h until the Optical Density (OD) reached 2 OD (A600) (Biophotometer, Eppendorf, Germany).To estimate the amount of phosphorus release and phosphatase activity, 1 mL of the cell suspension of the revived culture was inoculated into 30 mL of sterilized Pikovskayas broth amended with 300 mg L^{-1} of tricalcium phosphate (Ca_3PO_4) dispensed in two different 250 mL Erlenmeyer flask for each strain. The flasks were incubated in the dark at 28°C±1 for 7 days. The amount of phosphorus released into the culture media and phosphatase activity of each strain was estimated on 3rd, 5th and 7th day.

To estimate the amount of phosphorus released from tricalcium phosphate by Phosphate solubilizing bacterial strains, 1 mL of the culture was taken on each day and centrifuged at 10,000 rpm for 10 min (Microfuge, eppendorf, Germany) and the supernant was used to estimate the P release. Potassium di hydrogen phosphate was used as standard. The P released in the supernatant was measured by Molybdenum blue method using ELISA at 450 nm (Murphy and Riley, 1962; Watanabe and Olsen, 1965).

To estimate phosphatase activity, aliquots of 150 µL of the supernatant were assayed for phosphatase activity (De Freitas *et al*., 1997). Aliquots of each sample were added to 0.48 mL universal buffer 0.1 M, pH 6.5 and 0.12 mL of 0.05 M p-nitro phenyl phosphate (pNPP) solution, followed by 1 h incubation at 37 C. p-nitro phenol (pNP) was used as the standard. The yellow color was measured at 405 nm (Tabatabai and Bremmer, 1969) and expressed in terms of units (U). One unit of phosphatase activity is the amount of enzyme required to release 1 µg pNPP mL^{-1} of culture filtrate under assay conditions (1U = 1 µg pNPP mL^{-1}). The pH of the media was determined on 7th day.

The genus and species of the efficient Phosphate Solubilizing Bacterial (PSB) strains, which released high amount of P from tricalcium phosphate in each soil sample were identified using Hi Assorted Biochemical Identification Test Kit (KB002, Himedia).

Statistical analysis: All data were subjected to statistical Analysis of Variance (ANOVA) using NCSS 2007 model. Newman's Kuels multiple comparison test was used to compare all the strains. Differences obtained at $p<0.05$ level were considered significant.

RESULTS

Soil characterization and strain isolation: The pH of the soil sample collected from the paddy field soil ranged from 6.00 to 8.00. The results of physiochemical property of the soil sample are tabulated in Table 1.

The bacterial population density of the rhizosphere soil varied between the soils. The colony forming unit (cfu) of PSB strains which formed clear zones on PK medium, indicating P-solubilization, relatively showed less variation between the sites, ranging from 9.46 to 13.41×10^5 cfu g^{-1}. Two hundred and twenty six bacterial strains were isolated out of which thirty strains showed clear zones on repeated plating on Pikovkayas solid medium. The bacterial strains were coded as represented in Table 2. The final pH of the culture filtrate was ranging from 5.9 to 5.0 indicating acidic nature.

Table 1: Sampling site location and physiochemical properties of the paddy rhizosphere soil samples

Sampling site	Sample code	Soil pH	Soil EC	CA (%)	N (%)	P (%)
1	A	6.68	0.01	13.56	2.14	12.38
2	B	6.67	0.01	13.44	2.15	12.74
3	C	7.15	0.02	12.37	1.97	13.32
4	D	8.40	0.05	12.96	2.47	13.44
5	E	7.25	0.02	13.17	2.17	13.38
6	F	6.33	0.01	12.16	2.08	11.37
7	G	8.34	0.05	11.31	2.15	11.88
8	H	7.21	0.02	11.32	2.11	12.23

The sampling site locations are numbered as below: 1. Kancheepuram. 2. Tiruvarur. 3. Tanjavur. 4. Tindivanum (a). 5. Adudurai. 6. Mayavarum. 7. Tindivanum (b). 8. Thiruvallur. CA- Carbon, N- Nitrogen, P- Phosphorus, EC- Electrical conductivity.

Table 2: PSB strains isolated from different soil samples

	Strain codes				
SS code	1	2	3	4	5
A	A1	A2	A3	A4	A5
B	B1	B2	B3	B4	B5
C	C1	C2	C3	-	-
D	D1	D2	D3	D4	-
E	E1	E2	E3	E4	-
F	F1	F2	-	-	-
G	G1	G2	G3	G4	G5
H	H1	H2	-	-	-

SS: Soil sample as mentioned in Table 1. A, B, C, D, E, F, G, H- different soil samples collected from different sampling locations from Southern India. 1, 2, 3, 4, 5-efficient PSB strains isolated from that particular soil sample. -: No strains

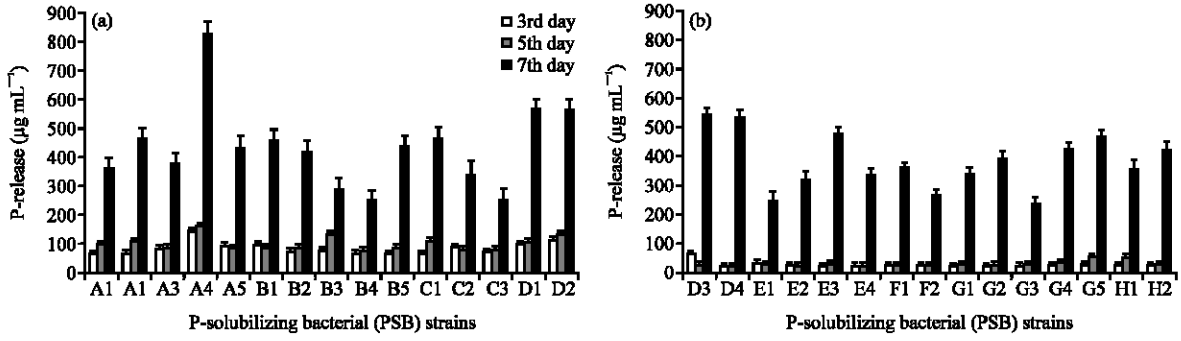

Fig. 1: Phosphorus release by PSB strains on 3rd, 5th and 7th day. (a) for sample A1-D2 and (b) for sample D3-H2

P-release by PSB strains: The PSB strains forming clear zones in pikovskayas medium were able to release P from tricalcium phosphate. P release was observed from 3rd day onwards and gradually increased until 7th day (Fig 1a, b). Statistical analyses were performed between all the strains from 3rd to 7th day of incubation. PSB strains A, B, C and D solubilized higher amount of P compared to E to H. Bacterial strain A4 released high amount of P on 3rd day (144 µg P mL^{-1}) and G2 (22.4 µg P mL^{-1}) released low amount of P. Statistical analyses on 5th day revealed comparatively higher amount of P release by A4 strain (164.4 µg P mL^{-1}) compared to other strains

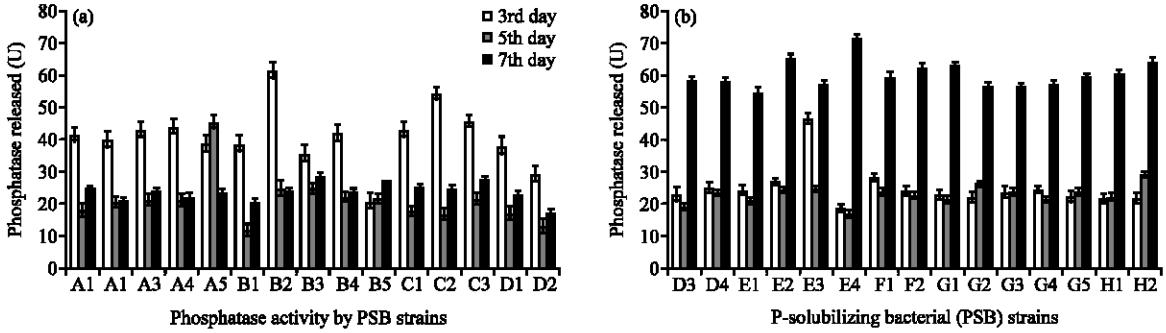

Fig. 2: Phosphatase release by PSB strains on 3rd, 5th and 7th day. (a) for sample A1-D2 and (b) for sample D3-H2

B3 (126.33 μg P mL^{-1}), G5 (53.4 μg P mL^{-1}) and H1 (57.13 μg P mL^{-1}). On 7th day, high P release was observed in all the strains isolated from A to D soil samples and maximum P release was seen in A4 strain (825.8 μg P mL^{-1}) compared to all the other PSB strains (500 to 250 μg P mL^{-1}). From the statistical analysis it is clear that P released by A4 strain was significantly higher compared to other PSB strains. All the PSB strains isolated were efficient in solubilizing tricalcium phosphate. Significant difference was observed in between the days in each strain and between the strains. The efficiency of the PSB strain to release P can be correlated with the amount of P released into the media.

Phosphatase activity by PSB strains: The acid phosphatase released into the media on 3rd, 5th and 7th day is shown in Fig 2a and b. The enzyme activity between the days were compared and found to be significantly different ($p<0.05$). It was seen that the production of phosphatase decreased from 3rd to 7th day. The amount of phosphatase released into the media was high on 3rd day. The phosphatase released by B2 (61.5 U) was high compared to other strains on 3rd day. All the strains released acid phosphatase into the liquid medium ranging between 18 to 61.5 units. The PSB strains C2 (54 U), E3 (46.4 U), C3 (45.55 U), C1 (42.75 U) also significantly released higher amount of phosphatase compared to other strains. On 5th day, A5 (45.27 U) strain released high amount of phosphatase compared to other strains which showed significant reduction of phosphatase, indicating the slow growth of the strain. A5 strain was at growth phase on 5th day, when other strains entered death phase, except D3 to H2 which produced high amount of phosphatase on 7th day indicating slow growth of the strains.

Efficient strains: The efficiency of the PSB strain was analyzed by comparing P-release and phosphatase activity. The amount of P release was significantly different from phosphatase activity. P released from tricalcium phosphate dependents upon the amount of phosphatase produced by the strains. Surprisingly, on 7th day the amount of P released by A4 (825.8 U) was high but the phosphatase released (22.2 U) into the media was less compared to other strains. Similar trend was seen in other strains. However the efficiency of the released phosphatase varied from strain to strain resulting in variation of P released into the medium. The amount of P released into the medium measured at a particular point varied between strains indicating PSB strains were at different stages of the growth cycle. This current investigation leads to the conclusion that the efficiency of the strain to release P from soil phosphate depends on the specificity of phosphatase

enzyme rather than the amount of enzyme released into the media. This evidently shows that all the isolated PSB strains from paddy rice field soils were efficient phosphate solubilizers and can be used as bioinoculants in paddy rice field soil to increase the available P in soil, minimize P-fertilizer application, reduce environmental pollution and promote the plant growth. The efficient PSB from each soil sample was identified by biochemical test and found to be A4 (*Enterobacter aerogenes*), B1 (*Micrococcus* sp.), C1 (*Pseudomonas aeruginosa*), D1 (*Bacillus* sp), E3 (*Bacillus* sp.), F1 (*Bacillus* sp.), G5 (*Bacillus* sp.) and H5 (*Kluyvera ascorbata*).

DISCUSSION

All the PSB strains isolated from southern Indian rice rhizosphere soils showed efficient solubilization of insoluble phosphate compared to the earlier reports (Villegas and Fortin, 2002). Microbial solubilization of phosphate in soil is correlated with the ability of microbes in producing selected organic acids and extra cellular polysaccharides (Kim *et al*., 1998; Halvorson *et al*., 1990). It is generally accepted that the mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids (Goldstein, 1995; Kim *et al*., 1997), which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Kpomblekou and Tabatabai, 1994). The organic acids produced by the organism changes soil pH to acidic. Keeping this into account, acid phosphatase activity of all the strains was analyzed.

In the present study the concentration of P released into the media varied from strain to strain which could be a consequence of P precipitation of organic metabolites as reported earlier (Babenko *et al*., 1984; Khan and Bhatnagar, 1977) and/or the formation of organo-P compound formed with secreted organic acids which subsequently used as an energy or nutrient source (Illmer and Schinner, 1992). An alternative explanation could be the difference in the rate of P release and uptake. When the rate of uptake is higher there is a decrease in the concentration of P in the media and vice versa. P release is a complex phenomenon and depends on many factors such as nutritional, physiological and growth conditions of the culture (Reyes *et al*., 1999).

The results presented in this study are concurrent with the earlier reports on the mechanism of P release and phosphatase activity by PSB strains. In the present study, some of the strains produced high amount of phosphatase but released less amount of P from insoluble phosphate and vice versa. This explains clearly that though the amount of phosphatase released into the medium was high or low, the amount of P released is determined by the specificity of phosphohydrolases on P substrate. This finding indicates that there is a correlation between P release and phosphatase activity. Phosphate assimilation from organic compounds by microorganisms takes place through the enzyme phosphatase. P is released as a by product by the action of phosphatases, providing the cell with essential nutrients (Goldstein, 1994) for plant growth and development. Mineralization of insoluble phosphates is carried out by several phosphatases (phosphohydrolases). These dephosphorylating reactions involve the hydrolysis of phosphoester or phosphoanhydride bonds. The acid phosphohydrolases shows optimal catalytic activity at acidic to neutral pH than alkaline phosphatases. Moreover these phosphatases are classified into specific or nonspecific according to the substrate specificity. Though some strain produced high amount of phosphatase they seem to be nonspecific (Rossolini *et al*., 1998) as seen in E4 strain on 7th day.

From this study, we conclude that we have isolated efficient phosphate solubilizing bacteria (*Enterobacter aerogenes, Micrococcus* sp., *Pseudomonas aeruginosa, Bacillus* sp. and *Kluyvera ascorbata*) which released high amount of P. It was observed that the amount of P release depends

on the binding specificity of the enzyme rather than the amount of enzyme released into the medium. From the observation, we conclude that the efficiency of isolated PSB strain can be determined by measuring the phosphatase activity on the P substrate. It requires further investigation on the bacterial strains to exploit these strains as biofertilizers for rice field soils to enhance the growth of rice crops.

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