JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2007. Vol. **1**(2), p. 269-280

Isolation of Tri-calcium Phosphate Solubilizing Bacterial Strains from Semi-arid Agricultural Soils of Rajasthan, India.

Anamika, Jyoti Saxena and Vinay Sharma*

Department of Bioscience and Biotechnology,
Banasthali Vidyapith, Banasthali - 304 022, India.

(Received: 01 July 2007; accepted: 26 July 2007)

The present study deals with the isolation and *in vitro* activity of tri-calcium phosphate (TCP) solubilizing bacteria. Bacterial strains were isolated by serial dilution and enrichment methods from the rhizosphere of green gram, sesame and pearl millet cultivated soil and from the non-rhizosphere. The rhizosphere of these plants inhabited with diversified and larger bacterial population than those of non-rhizosphere soil. Enrichment method yielded a high percentage (ca.79%) of TCP solubilizing bacteria than serial dilution method (ca. 44%). Total 83 bacterial isolates were obtained from different soil samples on the basis of colony morphology, Gram's reaction and zone of P- solubilization on Pikovskaya's agar plates. P- solubilization activity of these bacterial isolates was determined at 30^{0}C upto 5 days in Pikovskaya's broth. They solubilized 7.6-13.5% of the TCP supplied in the medium. The phosphate analysis of selected soils showed low to medium amount of available P (1.12 Kg ha^{-1} – 20.71 Kg ha^{-1}). On the basis of P- solubilization efficiency within the first 24 hours of inoculation we selected ten most efficient strains, which may possibly be used as biofertilizers in P poor soils in arid and semi-arid areas such as Rajasthan.

Keywords: Phosphate solubilizing bacteria, tri-calcium phosphate, Pikovskaya's broth, P- solubilization, Rhizosphere.

Phosphorus (P) is a critical element of natural and agricultural ecosystems and is essential for plant growth. Most agricultural soils contain large reserves of unavailable P, a considerable part of which accumulates as a consequence of regular application of chemical fertilizers. After the application of chemical fertilizers to the soil, its large soluble inorganic phosphate proportion is rapidly converted to insoluble forms that are unavailable to plants (Rodriguez and Fraga 1999). Since plants can absorb only inorganic phosphates, its low availability and relative immobility become limiting factors for plant growth (Bhat and Nye 1974). Further, phosphorus fixation and precipitation in soil is generally highly dependent on pH and soil type. P is fixed as water-insoluble iron and aluminium phosphates in acidic soils and as calcium phosphate in alkaline soils, therefore reduces the efficiency of P fertilizers (El-Komy 2005).

Several bacterial species generally the inhabitant of the rhizosphere region exert a beneficial effect on plant growth (Glick 1995). This group of bacteria has been termed as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). These include some phosphate solubilizing bacteria (PSB), which have already been used as commercial biofertilizers for agricultural improvement (Subba Rao 1993; Rodriguez and Fraga 1999). These PSB are known to solubilize insoluble phosphate through the production of organic acids and chelating oxo acids from sugars (Asea *et al.* 1988; Halvorson *et al.*

* To whom all correspondence should be addressed.
E-mail: vinaysharma30@yahoo.co.uk

1990). Although PSB occur naturally in soil, usually their number is not high enough to compete with other bacteria commonly established in the rhizosphere. Therefore, for agronomic utility, inoculation of plants by target PSB at a much higher concentration than that normally found in soil is necessary to take advantage of their beneficial properties for plant yield enhancement. However, a lack of consistent performance under different environmental conditions in the field has precluded their wider use.

Although exotic strains of the microbes can increase crop growth and yield, molecular technique based evidence suggest that genotypes of these beneficial bacteria may be endemic to a biogeographical region (Cho and Tiedje 2000). The endemic bacterial pool of a region may contain highly efficient genotypes and is likely to perform better than the exotic strain. Therefore, the bacterial strains effective in most part of the globe are needed to be identified and characterized.

The state of Rajasthan in India has an arid to semi-arid environment. Due to low and erratic rainfall distribution and lack of nutrients especially nitrogen and phosphorus, the production level in these regions is affected substantially. The enhancement of soil fertility in such drought prone areas thus assumes significance for sustainable agriculture. Very little information is available towards the development of PSB based biofertilizers in Rajasthan, India. Only few reports are available on occurrence of PSB in alkaline soils (Johri *et al.* 1999) and their abundance and distribution in arid region (Rao and Tarafdar 2002). Soil of Rajasthan has high pH, EC, ESP and preponderance of $NaHCO_3$ and Na_2CO_3 and therefore, suffers from the problem of alkalinity (Sharma, 1998) which subsequently leads to P-fixation as calcium phosphates. This prompted us to isolate the phosphate solubilizing bacteria from the rhizosphere and non- rhizosphere soils of three agriculturally important crop plants (viz., *Pennisetum glaucum, Sesamum indicum* and *Phaselous aureus*) cultivated in Banasthali and nearby regions in Tonk district, which is located in South-east Rajasthan, and screen them for tri-calcium phosphate solubilization activity in *in vitro* conditions.

MATERIALS AND METHODS

Study site and collection of soil samples

Three different sites termed as I, II and III, located at a distance of around 5 km from Banasthali Vidyapith campus (latitude $25^0$41' N to $26^0$34' N and longitude $75^0$70' to $76^0$19' E) were chosen as study sites. From each site three fields of the crop species viz., *Pennisetum glaucum* (cv. Raj 171), *Sesamum indicum* (cv. RT 46) and *Phaseolus aureus* (cv. RMG 492) were randomly selected and the plants were uprooted during the rainy season in August and September 2003. During this period, the soil temperature at different collection sites varied from 28°-39°C and moisture content was 3 -14 %. Five plants (four plants from the corners and one from the centre of the field) were sampled from each field. Soil region in the immediate vicinity of the roots of each plant (i.e. rhizosphere) alongwith the roots was collected in sterile polythene bags and carried to the laboratory in an ice-box. Loosely adhering soil was removed from the roots by washing with sterile distilled water. The composite mixture was then prepared by mixing the five samples thoroughly. Soil samples were also collected from a non-vegetated patch of all 3 sites in similar manner and were termed as non-rhizosphere soil. Sampling was done taking all possible aseptic measures and stored at 4°C. The samples were processed for isolation of phosphate solubilizing bacteria and testing their phosphate solubilization efficiency. Composite soil sample of each soil class was air dried and physicochemical properties such as pH and phosphorus concentration were determined using 1 mm sieved soil samples.

Isolation and preliminary screening of phosphate solubilizing bacteria

Bacterial isolation was done by (standard) serial dilution and soil enrichment techniques. Enrichment in liquid medium was done by adding 1 g of soil sample to 100 ml of Pikovskaya's broth (containing 5% TCP) and incubated at 30°C on an orbital incubator shaker at 150 rpm (Metrex, MO-250 ^{0}C, Mercantile Engineers, New Delhi, India). Pikovskaya's broth contained the following ingredients (g/l of distilled water): dextrose, 10; $Ca_3(PO_4)_2$, 50; $(NH_4)_2SO_4$, 0.5; $MgSO_4.7H_2O$, 0.1; KCl, 0.2; Yeast extract, 0.5; $MnSO_4.7H_2O$, 0.002;

and $FeSO_4.7H_2O$, 0.002. The pH of the media was adjusted to 7.0 before autoclaving. Three successive transfers were made at weekly intervals to enrich the medium. From the final flask, culture was withdrawn, diluted to 10^5 times and spread on Pikovskaya's agar (Highmedia Laboratories Pvt. Ltd. Mumbai, India) plates containing Nystatin as an antifungal agent. Well isolated colonies were further streaked on Pikovskaya's agar plates to obtain pure strains and were stored on Pikovskaya's agar (Pikovskaya 1948) slants at 4°C for further studies. Bacterial isolates obtained from both the methods were spot inoculated on Pikovskaya's agar plates containing 0.5% TCP and incubated at 30°C for 10 days. The development of a halo/clear zone at the inoculation site on the culture plates was noticed as an index of phosphate solubilization. Since isolation from each soil sample was done by two methods i.e. serial dilution and enrichment, matching of strains on the basis of colony morphology, Gram's reaction and solubilization zone was done to avoid the duplicacy. The isolates were designated as BAM (BAM-1 to BAM-83). These isolates were further tested for solubilization activity in liquid medium.

Determination of TCP solubilization efficiency on Pikovskaya's agar plate

All the TCP solubilizers were point inoculated on Pikovskaya's agar plates and incubated for 5 days at 30°C. Size of the halo/clear zones around the colonies showing phosphate solubilization was noted. Solubilization efficiency was calculated according to Nguyen *et al.* (1992) as given below:

$$\text{Solubilization efficiency (E)} = \frac{\text{Solubilization diameter}}{\text{Growth diameter}} \text{ (S) x 100}$$

Determination of TCP solubilization in liquid medium

Quantitative determination of phosphate solubilization in broth was carried out in 100 ml of Pikovskaya's broth. 110 mg of TCP was added to all the flasks as the source of insoluble phosphate and inoculated with 0.2 ml of bacterial suspension adjusted to 0.25 optical density at 590 nm. Autoclaved, uninoculated medium served as a control. The flasks were incubated at 30°C on an orbital incubator shaker for 5 days at130 rpm. Culture samples were harvested aseptically after every 24 hours and centrifuged at 8000 rpm for 30 min. Water soluble phosphate in the supernatant was determined spectrophotometrically (Systronics UV-VIS Spectrophotometer 119, Naroda, Ahmadabad, India) upto 5 days according to the method of Olsen and Sommers (1982). and was expressed as ppm (µg/ml). Change in pH of culture broth was recorded by digital pH meter after every sampling. The amount of P-solubilized and pH of reference strain *Pseudomonas striata* (obtained from the Division of Microbiology, IARI, New Delhi, India) was also carried out under similar conditions along with the other isolates.

Determination of soil pH and phosphorus concentration

Soil pH was determined by adding 25 ml of 0.01 M $CaCl_2.2H_2O$ to 10 g air dried and (1 mm) sieved soil. It was shaken for 5 minutes, left undisturbed for 24 hours at room temperature and then centrifuged at 5,000 rpm for 5 minutes. Finally, supernatant was taken to measure the pH using digital pH meter. Total phosphorus concentration of soil samples was determined after digestion with perchloric acid[15]. Volume of the acid digested samples was made upto 100 ml. The samples were then treated with 8 g of $NaHSO_3$ (from Ranbaxy Fine Chemicals Ltd., N. Delhi, India) for removing the arsenate impurities and further processed for phosphate estimation. To determine the available P concentration the samples were first extracted with 0.5 M $NaHCO_3$ (Olsen's reagent) and then available P was determined according to Olsen et al (1954). The P concentration was calculated as µg/ml and was then converted to µg/g of soil.

Statistical analysis

The correlation coefficient (r) between soluble P and pH value was calculated using SPSS software. One-way and two-way analysis of variance (ANOVA) was calculated using MS-Excel.

RESULTS AND DISCUSSION

Isolation and screening of PSB

The results of isolation of PSB from soil samples are given in Table 1. The number of bacterial isolates (210) obtained by dilution method was greater than by enrichment method

(90). Interestingly, however, the number of TCP solubilizers isolated by both these methods was nearly same. The enrichment method yielded a very high percentage of TCP solubilizers as compared to the dilution method. This may perhaps indicate that enrichment of soil samples favoured the growth of PSB, and is a better technique for their isolation. Over the years, the enrichment method has been established as a routine technique for the isolation of various microorganisms**.** The liquid enrichment media tend to select the microorganisms of highest growth rate among all the members of the introduced population that grow under the given conditions (Stanier *et al.* 1987). Many group of workers have adopted enrichment method for the isolation of rock phosphate dissolving microorganisms (Bardiya and Gaur 1974; Gaind and Gaur 1991; Pal 1998). By using a combination of these methods, 83 bacterial isolates (ten gram positive and seventy three gram negative) were obtained on the basis of colony morphology and zone of P-solubilization (Fig. 1). Of these, 61 isolates were common but a few of them were unique as they were restricted to either of the isolation methods. This finding suggests that more than one method of isolation should be followed to enhance the probability of getting more number of efficient isolates.

In this study, the highest bacterial population was found in the rhizosphere of *Phaseolus aureus* followed by *Pennisetum glaucum* and *Sesamum indicum* (Table 2). This is understandable since *P. aureus* is a leguminous species known for its association with symbiotic nitrogen fixing bacteria and other rhizobacteria. Rhizosphere soil inhabited larger bacterial population in comparison to the non- rhizosphere soil. For example the rhizosphere bacterial population was nearly 2.15, 1.93 and 2.52 folds higher than the non- rhizosphere soil in *P. glaucum, S. indicum* and *P. aureus* respectively. This is understandable as the rhizosphere area is considered as a region of high microbial activity. The microorganisms are influenced in many ways by growing plants and the microbial processes are more rapid in rhizosphere than in the non-rhizosphere soil (Gaur 1990). In the only other study from Rajasthan, Rao and Tarafdar(2002) performed a comprehensive study on microbial ecology of desert soil and reported very low bacterial population in most of the regions (0.2 to 12.1 x 10^5 g^{-1} dry soil). However, in general, the average bacterial population of non- rhizosphere soil in this study is more. It may however be noted that in Rao and Tarafdar's study, most of the samples were chosen from Western Rajasthan which is a typical desert with arid climate. In our study, the samples were taken from the region in and around Banasthali University, which is located in Southeast Rajasthan. In fact we are not aware of any other work on the microbial flora of the soil samples of this region. Moreover, the soil samples were obtained during rainy season, and thus had high moisture content and this probably favoured the bacterial growth and multiplication in the plant rhizosphere.

Statistical interpretation of Table 2

The one-way ANOVA (Analysis Of Variance) showed that total bacterial population in *P. glaucum* rhizosphere was significantly higher (significant at 5%) than the non- rhizosphere soil ($F = 33.91$, $p = 0.04$). Similarly, the bacterial population in the rhizosphere of *P. aureus* was also significantly higher (significant at 1%) than the non-rhizosphere ($F = 46.926$, $p = 0.002$). Whereas the ANOVA showed that statistically there was no significant difference ($F = 5.181$, $p = 0.085$) in the bacterial population of *S. indicum* rhizosphere and the non-rhizosphere soil. Also, the two-way

Table 1. Phosphate solubilizing bacteria isolated by serial dilution and enrichment methods.

Isolation method	No. of bacterial isolates	No. of TCP solubilizers	% of TCP solubilizers	Unique isolates*
Serial dilution	210	73	44	12
Enrichment	90	71	79	10

***** The isolates restricted to either of the two methods i.e. serial dilution and enrichment.

ANOVA showed that there was significant difference (significant at 1%) among the rhizobacterial population of different plants and the non- rhizosphere by featuring the values F = 9.296, p = 0.011, whereas no significant difference could be observed in the bacterial population at different sites, as featuring values F = 1.41 and p = 0.314.

The diversity of bacterial population as depicted in Table 3, shows highest diversity in *S. indicum* (85 isolates) followed by *P. glaucum* (62 isolates) and *P. aureus* (41 isolates). On the other hand, *P. glaucum* has the highest percentage of TCP solubilizers (ca. 45%) followed by *P. aureus* (ca. 41%), *S. indicum* being the poorest (26%). Further although *P. aureus* has maximum bacterial population, it is not as diversified as in *S. indicum* and may perhaps contain nitrogen fixing bacteria in abundance whose growth may be stimulated by PSB as the latter are known to produce growth regulating substances (Brown 1972; Barea *et al.* 1978). However, *P. aureus* also has a relatively higher percentage of TCP solubilizers (Table 3). In an interesting study, coinoculation of immobilized PSB and N2 fixing bacteria led to improved N and P in wheat plants (El-Komy 2005). The non- rhizosphere soils are poor in bacterial diversity as well as the number of TCP solubilizers in comparison to the rhizosphere soil. It has been observed that a high proportion of P solubilizing microorganisms is concentrated in the rhizosphere of plants (Gaur 1990). Since phosphate activities are found to be much higher in rhizosphere soil than in bulk soil,

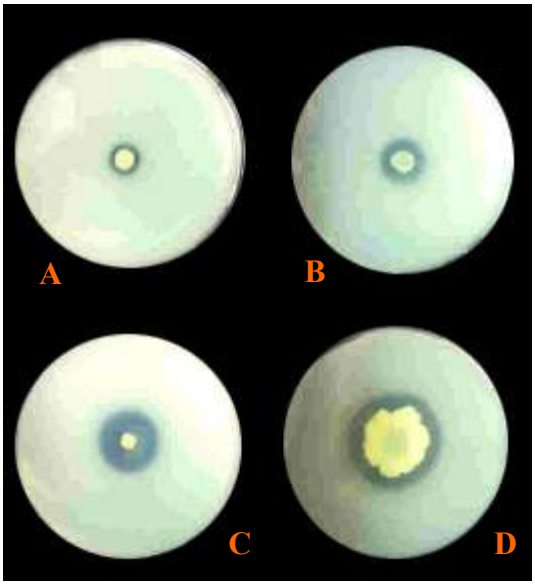

Fig. 1. P-solubilization in Pikovskaya's agar plate by bacterial isolates (A) BAM–1 (B) BAM–6 (C)BAM–4 and (D) BAM–12 after 5 days of growth at 30^0C.

Table 2. Bacterial population in rhizosphere of three crops growing in Banasthali region.

S. No.	Plant species	Total bacterial population g^{-1} of dry soil (x 10^5) Site I	Site II	Site III	Average
1.	*Pennisetum glaucum*	56	47	49	51
2.	*Sesamum indicum*	31	43	61	45
3.	*Phaseolus aureus*	53	65	59	59
4.	Non- rhizosphere soil	16	28	27	23

Table 3. Culturable bacterial diversity* in the rhizosphere of 3 crops species.

S. No.	Plant species	No of bacterial isolates	No. of TCP solubilizers	% of TCP solubilizers
1.	*Pennisetum glaucum*	62	28	45
2.	*Sesamum indicum*	85	22	26
3.	*Phaseolus aureus*	41	17	41
4.	Non- rhizosphere soil	22	6	27

*diversity was measured using serial dilution method.

inorganic phosphate solubilizers are more concentrated in rhizosphere of plants than in bulk soil (Vesquez *et al.* 2000).

Solubilization efficiency of PSB on Pikovskaya's agar plates

The results of qualitative screening of PSB on Pikovskaya's agar plate are summarized in Table 4. Different levels of solubilization, i.e. from inconspicuous to clear zone were observed in all the 83 bacterial strains during the study. Fairly high activity of the isolates in quantitative assays (Table 5) was directly related with high solubilization efficiency (123-283%) in PA plates. A positive relation between qualitative and quantitative assays of isolates is commonly observed. For example, Mehta and Nautiyal (2001) reported similar findings while developing an efficient method for qualitative screening of PSB. In the present study only few isolates showed anomalous behaviour where the results of qualitative assay did not match with quantitative assay. Bacterial isolate BAM-4 showed less solubilization efficiency (123%) in plate as compared to BAM-6 and BAM-8 (132 % and 141% respectively), but solubilized highest amount of TCP (148.52 ppm) in broth. On the other hand, BAM-47 showed high solubilization efficiency (210%) in plate but did not show comparable amount of solubilization in broth (only 85.84 ppm). This result reveals that such bacterial strains may be considered as efficient solubilizers as also supported by the study of El-Komy (2005) in *Pseudomonas fluorescence, Bacillus megaterium* and *Azospirillum lipoferum* (with solubilization efficiency 350%, 186% and 157% respectively). Although P solubilzing capability remained stable in most isolates, some isolates (BAM-3, BAM-30, BAM-32 and BAM-81) showed a sharp decline in solubilization after 4-5 cycles of inoculation and cultivation. Kucey (1983) also observed a similar pattern. Such behavior may be attributed to the genetic instability of these isolates. Thus, the persistence of P solubilizing capacity after 5 or more subcultures should be the first criteria in selecting efficient bacterial strains as microbial inoculants (Igual *et al.* 2001). On the other hand an increase in the ability of some strains to solubilize TCP was also observed, which might mean that they gradually adapted themselves to P-deficient conditions. Such observations have been made by other workers too. Illmer and Schinner (1992) isolated microorganisms from forest soils and screened them for inorganic phosphate solubilization and observed a similar increase in TCP solubilization capacity. Isolates BAM-35,

Table 4. Solubilization efficiency of 83 bacterial isolates on Pickovskaya's agar plate.

S. No.	Level of solubilization	Solubilization efficiency(%range)	No. of bacterial isolates	% of bacterial isolates
1.	Hala zone	100-367	56	67
2.	Clear zone	123-283	22	26
3.	No. solubilization zone	nil	5	6

Table 5. TCP solubilization by 83 bacterial isolates in Pikovskaya's broth.

Category	Amount of TCP solubilized(in ppm)	No of bacterial	% of bacterial isolates
I	90-150	17	20
II	80-90	6	7
III	70-80	26	31
IV	60-70	11	13
V	<60	23	28
Uninoculated control	25	-	-
Pseudomonas Stirata	91	-	-

BAM-39, BAM-64, BAM-65 and BAM-80 did not show any solubilization in the plate assay, but solubilized good amount of TCP in broth (48 ppm, 80 ppm, 73 ppm, 46 ppm and 75 ppm respectively). This result is in agreement with the findings of Levyal and Barthelin (1989) and Louw (1959). Thus, existing plate assay fails where halo is inconspicuous or absent. This may be because of varying diffusion rates of different organic acids secreted by an organism (Johnston 1952). The data confirm our observation that the criterion for selecting a good PSB strain based on the formation of a halo/clear zone on agar is not a confirmatory technique (Nautiyal 1999). Therefore, all 83 strains including those not showing activity in plates were proceeded for quantitative assay in liquid medium in order to select the efficient strains.

Determination of TCP solubilization in liquid medium

All the isolates showed varying degree of solubilzation ranging from 21 ppm to 149 ppm (Table 5). Highest number of bacterial isolates was found in the category III (70-80 ppm) followed by category V (< 60 ppm). However, 17 isolates were found in the category I (90-150 ppm) that solubilized higher amount of P than the reference strain of *Pseudomonas striata*. The results of 23 bacterial strains belonging to categories I and II showing P solubilization in the range of 80-150 ppm are depicted in the table 6. Of these 23 strains investigated, 12 strains showed maximum P solubilization after 1 day of inoculation, while the others could attain peak activity in several days. This shows that maximum P solubilization by many efficient bacterial strains may occur in and around 1 day of growth.

A fall in pH during the growth of phosphate solubilizers in liquid medium containing insoluble P has often been reported (Gerretson 1948; Bajpai and Rao 1971; Gaind and Gaur 1989;

Table 6. TCP solubilization and corresponding change in pH during the solubilization process by twenty three bacterial isolates from category I and II on the basis of their solubilization efficiency in Pikovskaya's broth.

S. No. Bacterial strains	Days after inoculation 0 P-solubilized	pH	1 P-solubilized	pH	2 P-solubilized	pH	3 P-solubilized	pH	4 P-solubilized	pH	5 P-solubilized	pH
	(ppm)		(ppm)		(ppm)		(ppm)		(ppm)		(ppm)	
Uninoculated Control	19	6.8	20	6.7	22	6.7	25	6.7	24	6.7	24	6.7
1. BAM-1	21	6.7	144	3.0	102	2.5	125	2.5	112	3.7	116	3.5
2. BAM-4	21	6.7	148	2.5	99	2.0	127	2.5	108	3.0	110	3.0
3. BAM-6	22	6.8	146	3.3	110	2.2	132	2.2	119	3.5	119	2.8
4. BAM-8	20	6.7	107	4.0	92	5.7	41	5.7	76	6.0	94	6.0
5. BAM-9	19	6.8	82	3.0	79	3.3	96	3.0	85	3.0	88	3.5
6. BAM-11	20	6.8	71	4.7	58	4.5	90	4.0	80	4.0	87	4.0
7. BAM-12	21	6.7	149	2.7	128	2.7	68	2.7	90	2.5	138	3.0
8. BAM-13	20	6.8	149	2.7	129	2.7	70	2.7	94	2.5	144	3.3
9. BAM-14	20	6.8	148	2.7	139	2.7	67	2.7	94	2.5	142	3.6
10. BAM-15	21	6.7	88	5.0	87	4.3	44	4.0	82	3.7	122	3.6
11. BAM-18	20	6.8	108	4.0	88	5.7	33	5.7	24	5.7	83	5.7
12. BAM-19	21	6.8	88	3.7	66	5.8	61	5.8	70	5.3	72	5.7
13. BAM-20	18	6.7	71	3.3	77	3.3	79	3.3	97	3.0	86	3.3
14. BAM-23	17	6.8	69	3.2	74	3.2	77	3.5	94	3.0	80	3.3
15. BAM-33	20	6.7	87	3.0	95	2.8	94	2.8	92	2.8	76	3.2
16. BAM-37	20	6.7	83	3.5	95	2.3	82	2.3	101	2.3	81	3.0
17. BAM-40	19	6.7	104	3.2	82	5.3	60	5.3	56	5.5	52	5.7
18. BAM-42	19	6.8	64	3.3	84	3.0	68	3.0	62	3.5	68	3.6
19. BAM-47	18	6.6	74	3.3	80	3.4	86	3.4	66	3.4	81	3.7
20. BAM-50	18	6.6	80	3.2	80	4.7	88	5.5	75	4.3	83	4.3
21. BAM-55	20	6.8	112	2.3	107	2.1	108	2.2	81	2.3	62	3.0
22. BAM-77	18	6.6	72	3.7	78	3.2	74	3.2	75	3.7	126	3.6
23. BAM-83	24	6.6	84	4.3	79	4.7	76	4.7	77	4.3	71	5.9
Pseudomonas striata	21	6.6	91	2.4	83	2.4	80	2.4	81	2.9	85	2.9

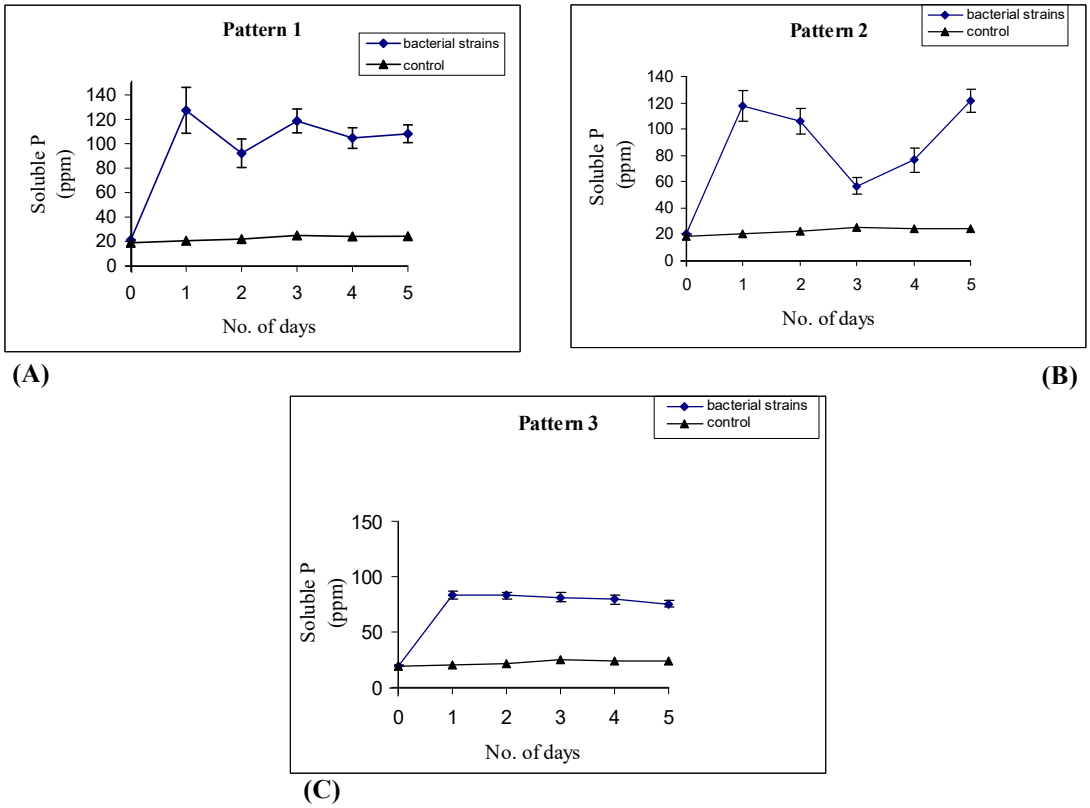

Fig. 2 Three representative patterns {(A), (B) and (C)} of TCP solubilization by 23 bacterial strains. Pattern 1 shows the average values of P solubilized by bacterial strains BAM-1, 4, 6 and 11 on different days after inoculation. Pattern 2 shows the average values of P solubilized by bacterial strains BAM-8, 12, 13, 14, 15, 18 and 77 on different days after inoculation Similarly, pattern 3 shows the average values of P solubilized by bacterial strains BAM-9, 19, 20, 23, 33, 37, 40, 42, 47, 50, 55 and 83 on different days after inoculation. Control shows the P- solubilization in absence of bacterial inoculation. Bars represent the standard error (SE).

Rose 1957). Several authors have attributed solubilization of inorganic P solely to the production and release of organic acids (Sperber 1957; Halder *et al.* 1992; Goldstein 1994; Rashid *et al.* 2004). However, this may not be the sole mechanism for P solubilization (Asea *et al.* 1988) as confirmed by only a weak or even lack of linear correlation between pH and the amount of P solubilized. In our study TCP solubilization was followed by a sharp decline in pH from initial value of 6.7 to 2.0 within 2 days. The statistical analysis showed a high negative correlation between pH value and the amount of soluble P present in the broth inoculated with PSB ($r = -0.831$ to -0.994). These results were highly significant ($P = 0.000$ to 0.041). Among 83 bacterial isolates, 20 isolates showed significant results at 5% ($P < 0.05$), whereas 43 isolates showed significant at 1% level ($P < 0.01$) and only 20 isolates showed non-significant results indicating that there may be other mechanisms of P solubilization besides acidification of the medium.

The selected 23 strains showed broadly three representative patterns of P-solubilization (Fig. 2). In pattern 1 (shown by isolates 1, 4, 6 and 11), the P-solubilization steeply increased during the first 24 hours and thereafter declined on the second day followed by a rise and then steady levels. In the second pattern (shown by isolates 8, 12, 13, 14, 15, 18, and 77), the steep rise during the 1st day was followed by a decline upto 3rd day and then gradual rise. In the third pattern (shown by isolates 9, 19, 20, 23, 33, 37, 40, 42, 47, 50, 55 and 83) too, there was rise in the first 24 hours but was followed by a gradual

decline on the subsequent days. Thus, in all cases, the P-solubilization increased during the first 24 hours, however subsequently it either declined or remained steady and then increased again. The data are in agreement with the previous studies of Illmer and Schinner (1992) and Seshadri *et al.* (2000). Thus possibly in the present study too, the initial rise of P in solution was caused by acid production, and then cells might have to alter their metabolism due to lack of C, release of organic metabolites such as citrate, lactate, NH_4^+ etc. into the medium and an organo-P compound might be formed thus reducing the amount of soluble P in solution. Due to permanent alteration of composition of the medium, cells might be forced to use this compound again as energy or nutrient source, which may result in a second release of P. This may explain rise in P-solubilization during later days. The whole process would hardly influence the pH and could be repeated several times with always different organo-P compounds until the culture dies from a lack of nutrients. These 23 bacterial strains solubilized 8-15% of TCP supplied in the medium in comparison to the *Pseudomonas fluorescens, Bacillus megaterium* and *Azospirillum lipoferum* strains reported to solubilize only 2.532%, 2.13% and 1.6% of TCP respectively (El – Komy 2005). Similarly, P-solubilized by *Pseudomonas corrugata* 1 and *Pseudomonas corrugata* 7 (Pandey and Palni 1998) was also less (2.38% and 3.24% respectively) than our bacterial strains. A comparable amount of P-solubilzation (i.e. 10.2%) has been shown by the bacterial strain NBR13 in NBRIP growth medium (Johri *et al.* 1999).

Determination of soil pH and phosphorus concentration

Soil physicochemical analysis showed that most of the soil samples had neutral or near neutral pH in the range of 6.3 to 7.5. The results of total and available P are presented in Table 7. Tata and Wadhwani (1992) assigned three values to differentiate among low, medium and high available P in soil, i.e. <10 Kg ha^{-1} as low, 10-25 Kg ha^{-1} as medium and >25 Kg ha^{-1} as high available P. Based on this classification, we compared our values and found that the rhizosphere soil of site I has low available P (4.60 Kg ha^{-1} - 9.16 Kg ha^{-1}), whereas non-rhizosphere soil contained very low amount of available P (1.12 Kg ha^{-1}). Rhizosphere as well as non- rhizosphere soils of site II had medium available P (13.71 Kg ha^{-1} - 20.71 Kg ha^{-1}). The rhizosphere soil of site III contained low to medium amount of available P (7.55 Kg ha^{-1} - 12.40 Kg ha^{-1}), whereas non-rhizosphere soil had low available P (8.69 Kg ha^{-1}). In the rhizosphere soil, microbial population (w.r.t. PSB) is more as compared to the non-rhizosphere and it may account for the high P availability in this region as compared to the non-rhizosphere soil. Similar observations were made by Helal and Sauerbeck (1984), and Riley and Barber Riley and Barber (1971). They found that microorganisms are actively engaged in P transformations in plant rhizosphere region. After

Table 7. Total and available phosphorus concentration of rhizosphere and non-rhizosphere soil samples of agricultural fields.

S. No.	Plant species	Total phosphorus concentration (µg/g)			Available phosphorus concentration (µg/g)		
		Site I	Site II	Site III	Site I	Site II	Site III
1.	*Pennisetum glaucum*	44.71± 4.28	37.96±4.72	74.93±6.77	2.04±0.41	6.70±0.82	4.93± 1.78
2.	*Sesamum indicum*	39.33± 5.28	24.15± 3.55	50.23± 6.56	4.09± 0.33	9.24± 1.23	5.45± 0.56
3.	*Phaseolus aureus*	51.54± 1.15	31.46± 3.86	66.24± 6.78	3.41± 0.21	6.12± 1.03	3.37± 0.85
4.	Non- rhizosphere soil	52.58± 5.33	57.13± 3.45	41.40± 5.53	0.50± 0.02	10.37± 0.85	3.88± 0.56

[Values are mean ± SE of 3 replications]

analysis it can be said that the area under investigation contains low to medium amount of available P and low amount of total P. The principal reason behind this is the presence of very low amount of organic matter in arid and semi-arid soils and such soils are known to be low in microbial activities, except in the rhizosphere of growing plants (Yadav and Dadarwal 1997). The % availability of P was highest in the rhizosphere soil of *S. indicum* followed by *P. aureus* and *P. glaucum* (Table 7). When all 3 sites were compared for the % availability of P, then it was found that site II had the highest value because chemical fertilizers in the form of NPK and DAP were applied at this site.

CONCLUSIONS

Finally the present study concludes that however, enrichment is better technique for isolation of PSB than serial dilution method but both methods should be followed for enhancing the probability of obtaining more number of efficient isolates. Most of these PSB strains solubilized TCP via acidification of the medium as shown by the decline in pH value, but it appears that some isolates carried solubilization through other mechanisms too. Some of the strains showed very high degree of solubilization in broth and have the capability of being exploited as biofertilizers. However, it was only a preliminary attempt and a thorough investigation is needed. The strains BAM-1, 4, 6, 8, 12, 13, 14, 18, 55, 77 have been selected for further characterization and field studies.

ACKNOWLEDGMENTS

We are thankful to Dr. B. D. Kaushik, Head, Division of Microbiology, Indian Agricultural Research Institute, New Delhi, India, and Professor Aditya Shastri, Director, Banasthali Vidyapith, for necessary help. A financial assistance in form of Senior Research Fellowship to Ms. Anamika by Council of Scientific and Industrial Research, New Delhi, India is greatly acknowledged.

REFERENCES

1. Rodriguez, H. and Fraga, R. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv.,* 1999; **17:** 319-339.
2. Bhat, K.K.S. and Nye, P.H. Diffusion of phosphate to plant roots: III Depletion of phosphate around onion roots without root hairs. *Plant Soil*, 1974; **1:** 383-394.
3. El- Komy, H.M.A. Coimmobilization of *Azospirillum lipoferum* and *Bacillus megaterium* for plant nutrition *Food Technol. Biotechnol.,* 2005; **43:**19-27.
4. Glick, B.R. The enhancement of plant growth by free-living bacteria. *Can. J. Microbiol.,* 1995; **41:** 109-117.
5. Kloepper, J.W. and Schroth, M.N. Plant growth promoting rhizobacteria on radishes. In: Proceedings of the 4th International Conference on Plant Pathogenic Bacteria. Gibert- clarey, Tours Publishing Station de Phatologie Vegetale et Phytobacteriaologie INRA, Angers, France, 1978; **2:** 879-882.
6. Subba Rao, N.S. Biofertilizers in agriculture and forestry, Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, India, 1993.
7. Asea, P.E.A., Kucey, R.M.N. and Stewart, J.W.B. Inorganic phosphate solubilization by two *Penicillium* species in solution culture and soil. *Soil Biol. Biochem.,* 1988; **20:** 459-464.
8. Halvorson, H.O., Keynan, A. and Kornberg, H.L. () Utilisation of calcium phosphates for microbial growth at alkaline pH. *Soil Biol. Biochem.,* 1990; **22:** 887-890.
9. Cho, J.C. and Tiedje, J.M. Biogeography and degree of endemicity of fluorescent *Pseudomonas* strains in soil. *Appl. Environ. Microbiol.,* 2000; **66:** 5446-5448.
10. Johri, J.K., Surange, S. and Nautiyal, C.S. Occurrence of salt, pH and temperature tolerant phosphate solubilizing bacteria in alkaline soils. *Curr Microbiol.,* 1999; **39:** 89-93.
11. Rao, A.V. and Tarafdar, J.C. Microbial mobilization of phosphorus for higher crop production in arid soils. In: Biotechnology of biofertilizers, (Kannaiyan ed). Narosa Publishing House, New Delhi, India, 2002; pp. 323-338.
12. Sharma, R.C. Nature, extent and classification. In: Agricultural salinity managenment in India (Tyagi, N.K. and Minhas, P.S. eds). CSSRI, Karnal, India, 1998; pp. 21.

13. Pikovskaya, R.I. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Mikrobiologiya,* 1948; **17:** 362-370.
14. Nguyen, C., Yan, W., Le Tacon, F. and Lapeyrie, F. Genetic variability of phosphate solubilizing activity by monocaryotic and dicaryotic mycelia of the ectomycorrhizal fungus *Laccaria bicolor. Plant Soil.*, 1992; **143:** 193-199.
15. Olsen, S.R. and Sommers, L.E. Phosphorus. In: Methods of soil analysis, part 2 (Page, A.L., Miller, R.H. and Keeny, D.R. eds). Soil Sci. Soc. Amer. Inc., Madison, Wisconsin, USA, 1982; pp. 403-430.
16. Olsen, S.R., Cole, C.V., Watanabe, F.S. and Dean, L.A. Estimation of available P in soil by extraction with sodium bicarbonate. U. S. Dept. of Agric. Circ., 1954; pp.939.
17. Stanier, R.Y., Ingraham, J.L., Wheelis, M.L. and Painter, P.R. General Microbiology. V'th edition, Prentice Hall, Englewood cliffs, New Jersey, USA, 1987; 33 p.
18. Bardiya, S. and Gaur, A.C. Isolation and screening of microorganisms dissolving low grade rock phosphate. *Folia Microbiol.,* 1974; **19:** 386-389.
19. Gaind, S. and Gaur, A.C. Thermotolerant phosphate solubilizing microorganisms and their interaction in Mungbean. *Plant Soil,* 1991; **133:** 141-149.
20. Pal, S.S. Interactions of an acid tolerant strain of phosphate solubilizing bacteria with a few acid tolerant crops. *Plant Soil,* 1998; **198:** 169-177.
21. Gaur, A.C. Phosphate solubilizing microorganisms as biofertilizers. Omega Scientific Publishers, New Delhi, India, 1990; pp. 40-44.
22. Brown, M.E. Plant growth substances produced by microorganisms of soil and rhizosphere. *J. App. Bacteriol.,* 1972; **35:** 443.
23. Barea, J.M., Ocampo, J.A., Azcon, R., Olivares, J. and Montoya, E. Effects of ecological factors on the establishment of *Azotobacter* in the rhizosphere. *Ecol. Bull. Stockholm,* 1978; **26:** 325-330.
24. Vesquez, P., Holguin, G., Puente, M.E., Cortes, A.L., and Bashan, Y. Phosphate solubilizing microorganisms associated with the rhizosphere of mangroves in a semi-arid coastal lagoon. *Biol. Fertil. Soils.,* 2000; **30:** 460-468.
25. Mehta, S. and Nautiyal, C.S. An efficient method for qualitative screening of phosphate solubilizing bacteria. *Curr. Microbiol.,* 2001; **43:** 51-56.
26. Kucey, R.M.N. Phosphate solubilizing bacteria and fungi in various cultivated and virgin Alberta soils. *Can. J. soil Sci.,* 1983; **63:** 671-678.
27. Igual, J.M., Valverde, A., Cervantes, E. and Velazquez, E. Phosphate solubilizing bacteria as inoculants for agriculture: use of updated molecular techniques in their study. *Agronomie*, 2001; **21:** 561-568.
28. Illmer, P. and Schinner, S. Solubilization of inorganic phosphates by microorganisms isolated from forest soils. *Soil Biol. Biochem.,* 1992; **24:** 389-395.
29. Levyal, C. and Barthelin, J. Interactions between *Laccaria laccata, Agrobacterium radiobacter* and beech roots: influence on P, K, Mg and Fe mobilization from mineral and plant growth *Plant Soil,* 1989; **17:** 103-110.
30. Louw, H.A. and Webley, D.M. A study of soil bacteria dissolving certain phosphate fertilizers and related compounds. *J. Appl. Bacteriol.,* 1959; **22:** 227-233.
31. Johnston, H.W. The solubilization of phosphate: the action of various organic compounds on dicalcium and tricalcium phosphate. *NZJ Sci. Technol.,* 1952; **33:** 436-444.
32. Nautiyal, C.S. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett.,* 1999; **170:** 265-270.
33. Gerretson, F.C. The influence of microorganisms on the phosphate intake by the plant. *Plant Soil,* 1948; **1:** 51-81.
34. Bajpai, P.D. and Sundara Rao, W.V.B. Phosphate solubilizing bacteria II Extracellular production of organic acids by selected bacteria solubilizing insoluble phosphates. *Soil Sci. Plant Nutr.,* 1971; **17:** 44-45.
35. Gaind, S. and Gaur, A.C. Effect of pH on phosphate solubilization by microbes. *Curr. Sci.,* 1989; **58:** 1208-1211.
36. Rose, R.E. Techniques of determining the effect of microorganisms on insoluble inorganic phosphates. *NZ J Sci. Technol.,* 1957; **38:** 773-780.
37. Sperber, J.I. Solubilization of mineral phosphates by soil bacteria. *Nature,* 1957; **180:** 994-995.
38. Halder, A.K., Banerjee, A., Misra, A.K. and Chakraborty, P.K. Role of NH^{4+} or NO^{3-} on release of soluble phosphate from hydroxyapatite by *Rhizobium* and *Bradyrhizobium*. *J. Basic Microbiol.,* 1992; **32:** 325-330.

39. Goldstein, A.H. Involvement of quinoprotein glucose dehydrogenase in the solubilization of exogenous phosphate by Gram- negative bacteria. In: Phosphate in microorganisms: Cellular and Molecular Biology (Torriani-Gorini, A., Yagil, E. and Silver, S. eds). ASM Press, Washington DC, 1994; pp. 197-203.
40. Rashid, M., Khalil, S., Ayub, N., Alam, S. and Latif, F. Oragnic acid production and phosphate solubilization by phosphate solubilizing microorganisms (PSM) under invitro conditions. *Pak. J. Biol. Sci.,* 2004; **7:** 187-196.
41. Seshadri, S., Muthkumarasamy, R., Lakshminarasimhan, C. and Ignacimuthu, S. Solubilization of inorganic phosphates by *Azospirillum halopraeferans*. *Curr. Sci.,* 2000; **79:** 565-567.
42. Pandey, A., and Palni, L.M.S. Isolation of *Pseudomonas corrugata* from Sikkim Himalaya. World J. Microbiol. Biotechnol., 1998; **14:** 411-413.
43. Tata, S.N. and Wadhwani, A.M. Handbook of Agriculture, IVth edition, ICAR publication, Krishi Anusandhan Bhawan, New Delhi, India, 1992; pp. 71.
44. Helal, H.M. and Sauerback, D.R. Influence of plant roots on C and P metabolism in soil. Plant soil, 1984; **76:** 175-182.
45. Riley, D. and Barber, S.A. Effect of ammonium and nitrate fertilization on phosphorus uptake as related to root induced pH changes at the root- soil interface. *Soil Sci. Soc. Am. J.* 1971; **35:** 301-306.
46 . Yadav, K.S. and Dadarwal, K.R. Phosphate solubilization and mobilization through soil microorganisms. In: Biotechnological approaches in soil microorganisms for sustainable crop production (Dadarwal, K.R. ed). Scientific publishers, Jodhpur, India, 1997; pp 293-308.