CHAPTER 3

CHARACTERIZATION OF PSMs ISOLATED USING BUFFERED MEDIA CONDITION

3.1 : INTRODUCTION

PSMs were envisaged as potential biofertilizers to provide phosphorus to plants thus eliminating the use of chemical superphosphate (Kucey *et al.,* 1989; Subba Rao, 1982; Goldstein, 1986). The inoculations of plants with PSMs gave variable results which were attributed to various factors such as the type of soil, variety of the plant etc. (Kucey *et al.,* 1989; Subba Rao, 1982). There is also no direct evidence of P release by PSMs from the soil (Kucey *et al.*, 1989).

In the preceding chapter, we have shown that the PSMs tested were unable to solubilize phosphate from alkaline soil. This inability is due to the buffering action of the soil as the soil required about 20-50 times more acid than was being secreted by these PSMs. Also the buffering of the media severely reduced the phosphate solubiizing potential of these PSMs. These results indicated that the limitation of the screening procedure that is currently employed to isolate PSMs which does not have a buffering component. The results also showed that the concentration of the acids required to release P from alkaline soil are 20 -50 times more than that secreted by the PSMs tested.

Screening of various rhizospheric and non-rhizospheric soils resulted in isolation of PS bacteria capable of showing zone of pH reduction in buffered media (Table 2.5) from the rhizosphere of Pigeon pea (*Cajanus cajan*). In this chapter we report the P

solubilization characteristics of these PS bacteria. These isolates, in contrast to those isolated under unbuffered media conditions employed in conventional screening procedure, could solubilize mineral phosphates under buffered media conditions and could also release P and Fe from alkaline soil.

3.2 : MATERIALS AND METHODS

Screening Procedure :

The local plant varieties were taken and the microorganisms from the bulk soil, rhizosphere and rhizoplane were screened for their ability to grow and reduce the pH of their surrounding on RP plates buffered with 100mM Tris pH 8.0. The plates contained 100 mM glucose, 10mM NH_4Cl or 10mM KNO_3 as indicated and 15mM NaCl. The pH reduction was monitored by change in the color of methyl red dye, which was added at 0.05% in the medium. Soil to be screened was suspended in saline at 1g/ml concentration and various dilutions of the supernatant plated on the above medium.

Identification of the Isolates :

Identification of the isolates was done as per Bergey's manual of determinative bacteriology (Holt, 1993).

Culturing Procedures :

The bacteria were cultured on media containing 100mM glucose, 25 μM $MgSO_4$, 10mM NH_4Cl and the following micronutrients (mg/L) $FeSO_4.\ 7H_2O$ (3.5), $ZnSO_4.\ 7H_2O$ (0.16), $CuSO_4.\ 5H_2O$ (0.08), H_3BO_3 (0.5), $CaCl_2.2H_2O$ (0.03) and $MnSO_4.\ 4H_2O$ (0.4). Phosphate source was 0.1mM KH_2PO_4. The media was buffered with 100mM Tris-HCl pH 8.0. These cells were used to inoculate to fresh media as described below.

Solid Medium Experiments :

The soil isolates were grown on 1.5% agar plates containing 100mM glucose, 10mM NH_4Cl or KNO_3 as indicated and either DCP or RP as the P source. The pH of the

media was adjusted to 8 by NaOH in the case of unbuffered conditions and was buffered with 100mM Tris-HCl pH 8, unless otherwise indicated. Rock phosphate was added to the plates before pouring the media. Di-calcium phosphate was precipitated *in situ* as described earlier (Goldstein & Liu, 1987). Methyl red indicator dye was added at 0.05 g% in the medium.

The effect of nitrogen source and the buffering capacity of the media on P solubilization by the isolates was recorded by measuring the diameter of the red zone or the zone of clearance in the case of rock phosphate and DCP respectively, around the colony. To determine the effect of NaCl on the RP solubilizing ability of the isolates, different concentrations of NaCl was added to the buffered media and the zone of pH reduction was monitored.

Rock Phosphate Solubilization with Different Carbon Sources :

Different carbon sources were used at 100mM concentration in liu of glucose in the above medium. The medium was buffered with either 50mM or 100mM Tris-HCl pH 8.0 and the zone of pH reduction was monitored at different time intervals.

Liquid Medium Experiments :

Effect of Buffering of the Media and the Nitrogen Source on the Growth and Phosphate Solubilization by Isolates :

The cells grown overnight on the culturing media were inoculated in liquid media containing 100mM glucose, 10mM NH_4Cl or KNO_3 as indicated. 1mg/ml of rock phosphate was provided as P source. The pH of the medium was adjusted to 8.0 by NaOH in case of unbuffered media and was buffered with 100mM Tris-HCl pH 8.0 in case of buffered media. After different time of incubation on rotary shaker at room

temperature, media was centrifuged and the supernatent was used for pH and P estimations.

Solubilization of Soil Phosphates by the Isolates :

The bacteria grown on the above media were inoculated into media containing 100mM glucose, 10mM NH_4Cl or KNO_3 as indicated. 1g/ml of alkaline soil collected locally was provided as P source. The growth of the bacteria was monitored by culturable counts at different time points. Culture was collected after different time intervals and centrifuged in a microfuge at 10,000 rpm for 5 minutes. The supernatent was used for pH, P and Fe estimations.

Organic Acid Analysis :

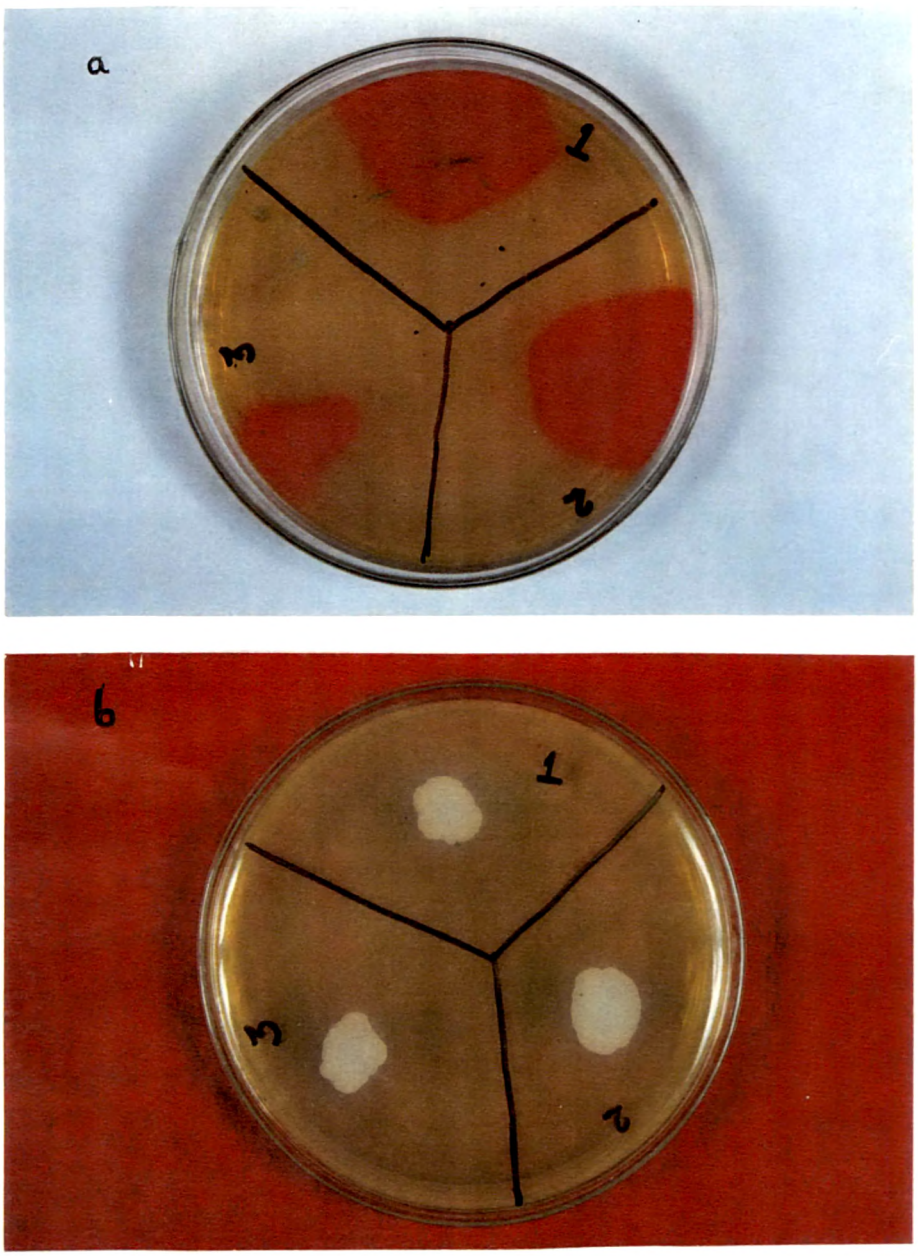
The inoculated soil sample was taken after maximum drop in pH and centrifuged at 10,000 rpm in a microfuge for 10 minutes. The supernatant was used for organic acid analysis by HPLC using Shimadzu SCR-101 column. The mobile phase was 0.1% H_3PO_4 at a flow rate of 1 ml/min. and the column temperature was 65°C. The acids were detected by absorbance at 210 nm by UV detector.

3.3 : RESULTS

Screening of Local Alkaline Soils for PSMs Under Buffered Media Conditions : Soil samples were taken from local agricultural fields, within the vicinity of plant roots and were screened for PSMs that could grow on buffered plates containing rock phosphate as P source and could also show zone of pH reduction. Three bacterial isolates were found to form red zone with both NH_4Cl and KNO_3 as nitrogen source were isolated from the rhizosphere of *Cajanus cajan* (Table 2.5). While isolates 1 & 2 showed red zone formation within 24 h on either of the nitrogen sources, isolate 3 could show red zone on KNO_3 only after 48 h (Fig 3.1-3.4). The red zone was abolished in the presence of 1mM free P in the medium indicating the acid secretion was phosphorus starvation inducible in all three isolates (Fig 3.1). These isolates also showed solubilization zone with DCP under buffered media with both ammonium chloride and potassium nitrate as N source (Table 3.1). Apart from glucose, the isolates could show growth on rock phosphate plates with various carbon sources but could show the zone of pH reduction only when lactose and galactose were used as C source and there was difference in the ability to reduce the pH with the C source provided. With lactose, the isolates showed pH reduction zone in media buffered with 100mM Tris-HCl pH 8.0 whereas with galactose the zone could be seen only in media buffered with 50mM Tris-HCl pH 8.0 (Table 3.2). The isolates could show RP solubilization in the presence of upto 500 mM NaCl but could not do so when NaCl concentration was raised to 750 mM (Fig. 3.5).

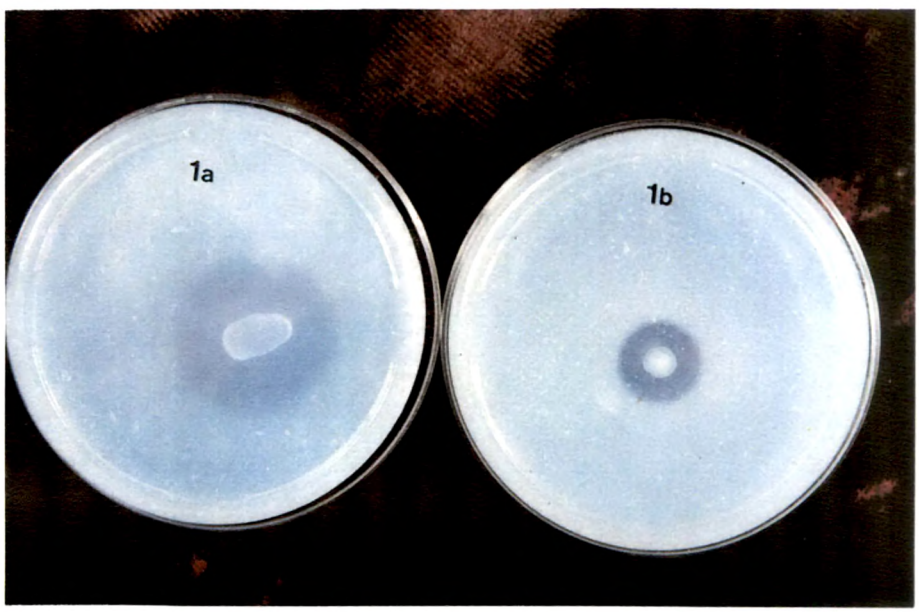
Identification of the isolates : The three isolates showed different antibiotic sensitivity implying that all of them were different bacterial strains (Table 3.3). All the three isolates were gram negative. Biochemical and morphological tests were

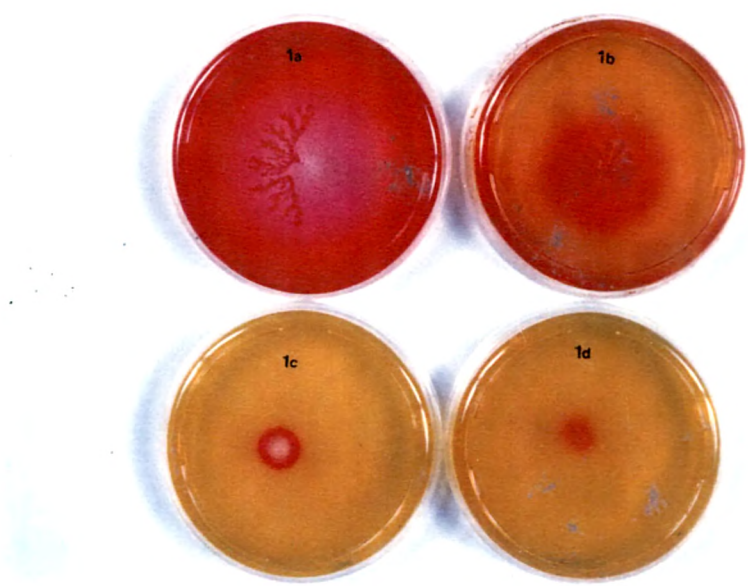
Fig. 3.1 : Acidification of buffered media by PS isolates.



a RP
b 1 mm K_2HPO_4

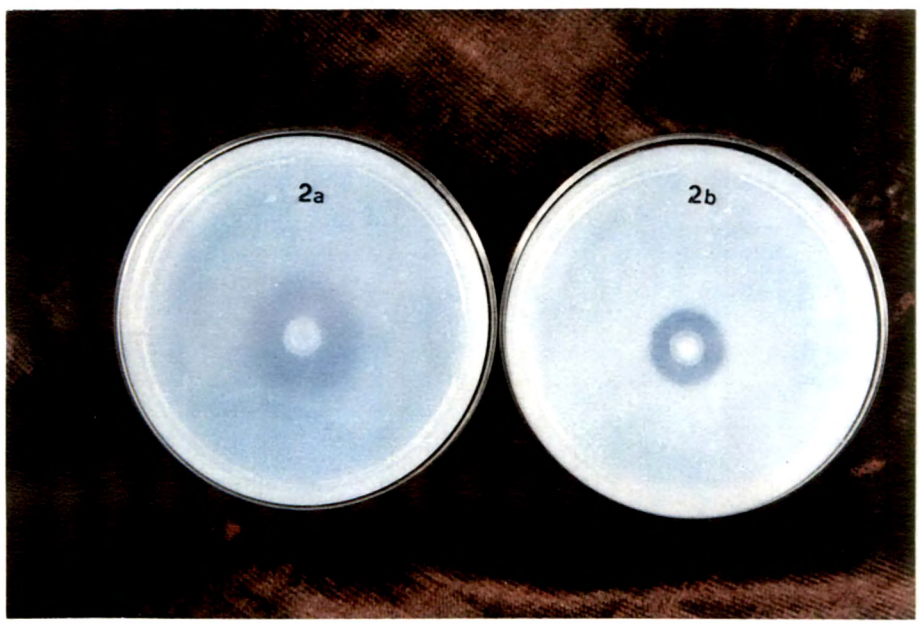
Fig.3.2 : Growth and P solubilization from RP and DCP by isolate number 1 under various media conditions.

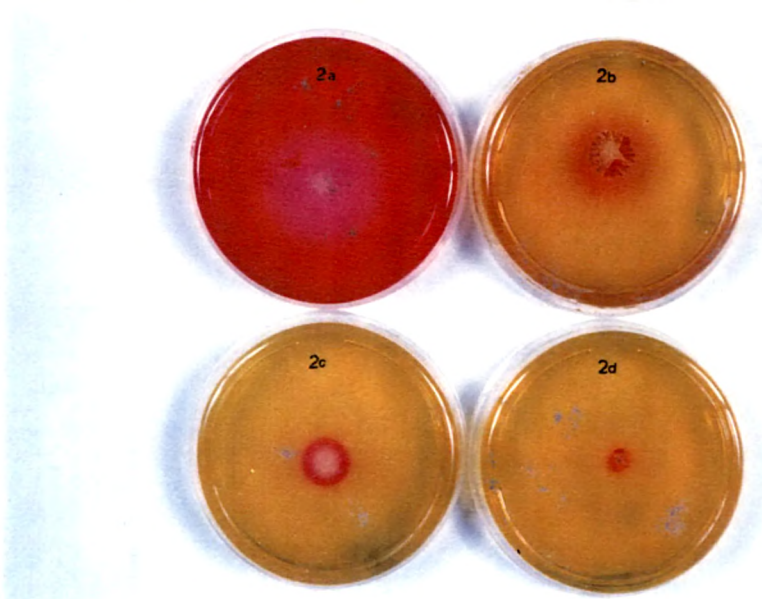




(a) Unbuffered NH_4Cl; (b) Buffered NH_4Cl; (c) Unbuffered KNO_3;
(d) Buffered KNO_3.

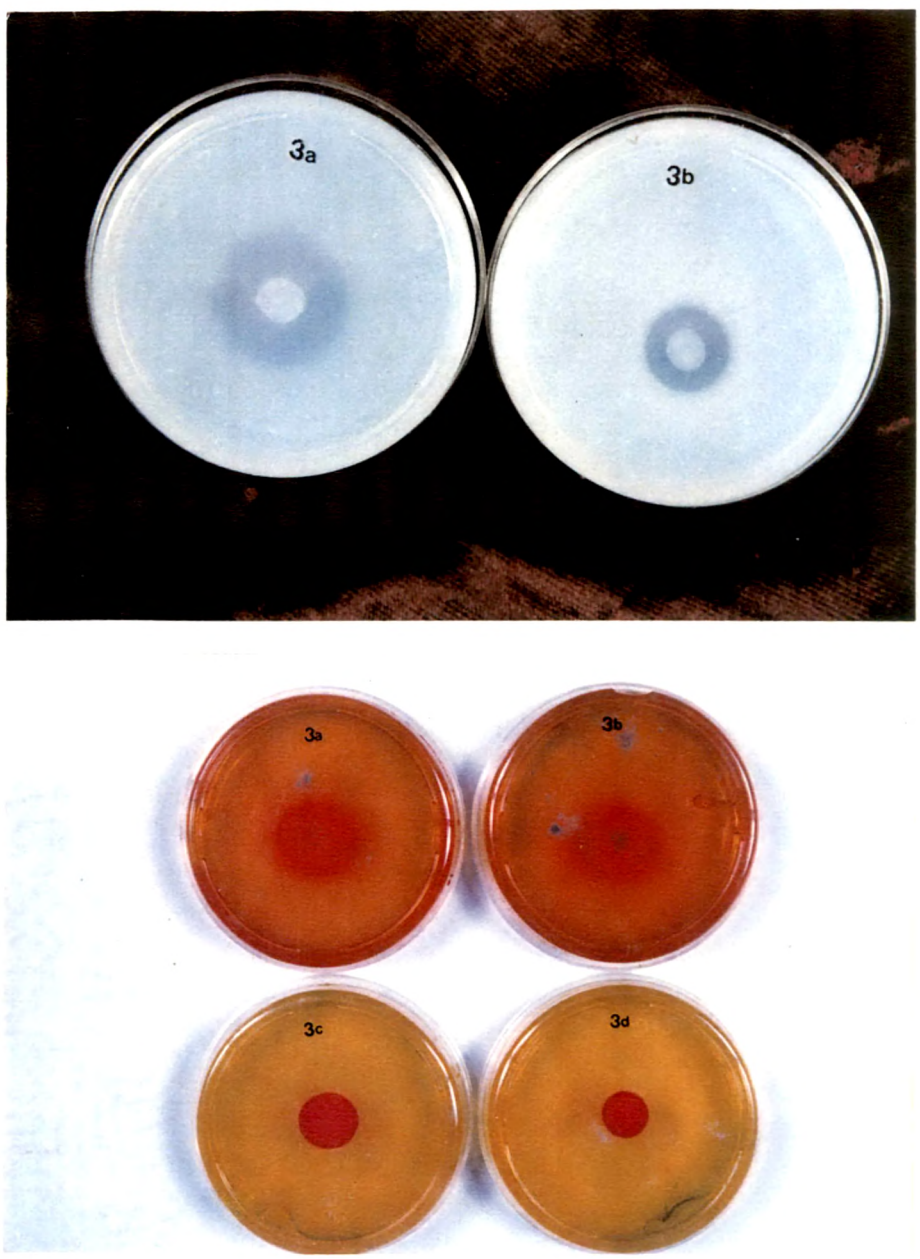
Fig.3.3 : Growth and P solubilization from RP and DCP by isolate number 2 under various media conditions.





(a) Unbuffered NH_4Cl; (b) Buffered NH_4Cl; (c) Unbuffered KNO_3; (d) Buffered KNO_3.

Fig.3.4 : Growth and P solubilization from RP and DCP by isolate number 3 under various media conditions.



(a) Unbuffered NH_4Cl; (b) Buffered NH_4Cl; (c) Unbuffered KNO_3; (d) Buffered KNO_3.

Table 3.1

Effect of buffering on the zone of DCP solubilization and pH reduction by soil isolates

P Source	Buffer	Bacteria	N Source			
			NH_4Cl Zone Size (cm)		KNO_3 Zone Size (cm)	
			48 h	72 h	48 h	72 h
DCP	-	Isolate 1	3.5	4.2	2.5	3.6
	-	Isolate 2	4.0	4.5	3.5	4.0
	-	Isolate 3	3.0	4.0	2.5	3.5
	+	Isolate 1	1.5	2.0	1.4	1.8
	+	Isolate 2	1.8	2.5	1.5	2.0
	+	Isolate 3	0.9	1.2	0.5	1.0
RP	-	Isolate 1	4.0	6.0	2.6	3.0
	-	Isolate 2	3.2	5.5	2.2	3.0
	-	Isolate 3	3.0	5.6	2.0	2.5
	+	Isolate 1	1.5	1.8	1.3	1.6
	+	Isolate 2	1.3	1.5	1.0	1.5
	+	Isolate 3	1.0	1.4	0.7	1.0

DCP Dicalcium phosphate
RP Rock phosphate

+ Indicates presence and - indicates absence of buffer.

The buffer used was 100 mM Tris-HCl pH 8.0;

Carbon Source : 100 mM Glucose

The zone size presented is an average of three independent measurements.

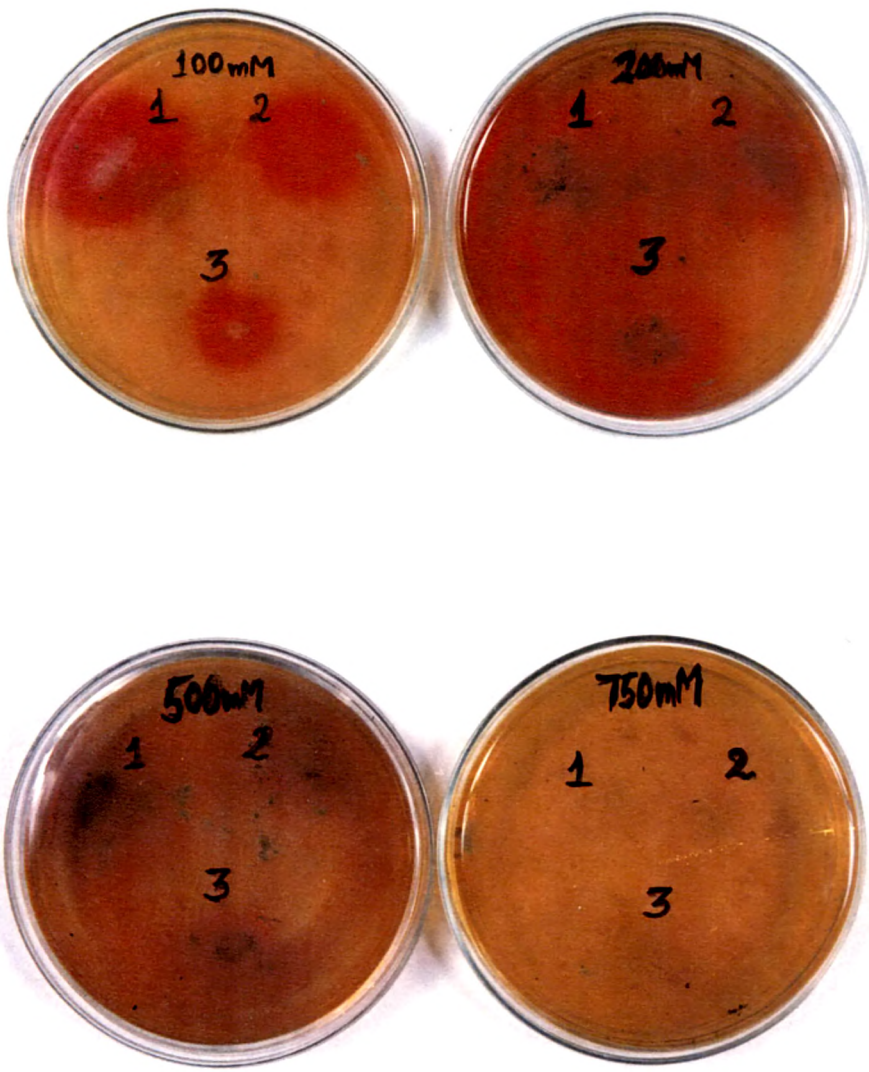
Table 3.2

Rock phosphate solubilization by soil isolates on different carbon sources with NH_4Cl as N source

C-Source	50 mM Tris-Cl (pH 8.0)		100 mM Tris-Cl (pH 8.0)		
	48 h	72 h	48 h	72 h	96 h
100 mM Lactose					
Isolate 1	UD	UD	UD	UD	UD
Isolate 2	0.5	0.7	UD	UD	0.5
Isolate 3	1.3	1.5	UD	UD	0.5
100 mM Galactose					
Isolate 1	UD	0.5	UD	UD	UD
Isolate 2	UD	UD	UD	UD	UD
Isolate 3	1.0	1.5	UD	UD	UD
100 mM Sucrose					
Isolate 1	UD	UD	UD	UD	UD
Isolate 2	UD	UD	UD	UD	UD
Isolate 3	UD	UD	UD	UD	UD
100 mM Glycerol					
Isolate 1	UD	UD	UD	UD	UD
Isolate 2	UD	UD	UD	UD	UD
Isolate 3	UD	UD	UD	UD	UD
100 mM Mannitol					
Isolate 1	UD	UD	UD	UD	UD
Isolate 2	UD	UD	UD	UD	UD
Isolate 3	UD	UD	UD	UD	UD

UD - Undetectable (Zone size is given as diameter in cms)

Fig.3.5 : Effect of NaCl on RP solubilization by soil isolates.



1, 2 & 3 are the three soil isolates.

Table 3.3

Antibiotic sensitivity of soil isolates

Antibiotic (µg/ml)	Isolate 1	Isolate 2	Isolate 3
Ampicillin (100)	+	+	+
Carbenicillin (100)	-	-	-
Chloramphenicol (30)	+	-	+
Co-Trimazine (25)	-	-	-
Amikacin (10)	-	-	-
Ciprofloxacin (10)	-	-	-
Norfloxacin (10)	-	-	-
Nitrofurantoin (300)	-	-	-
Streptomycin (10)	+	-	-
Tetracycline (30)	-	-	-
Kanamycin (30)	-	-	-
Cephotaxime (30)	-	-	-
Gentamycin (40)	-	-	-

\+ Indicates resistance and
\- Sensitivity to the antibiotic

conducted and based on these all three bacterial strains were classified as belonging to *Rhizobium* spp. (Table 3.4).

Characterization of Phosphate Solubilizing Properties of the Isolated PSMs :

Rock Phosphate Solubilization in Liquid Media :

All the three isolates could reduce the media pH and solubilize rock phosphate in both unbuffered and buffered media conditions with both ammonium or nitrate as the nitrogen source (Table 3.5 & 3.6) indicating that the phosphate solubilization property was independent of the nitrogen source in the media and was due to the organic acid(s) secreted by these bacteria.

Phosphate Solubilization from Alkaline Soil :

All the three isolates were able to reduce the soil pH and were able to release P from soil to different levels with both ammonium and nitrate as nitrogen source. The isolates took different time to reduce the pH. The media pH again returned to 8.0 and the solubilized P was also reprecipitated (Table 3.7 & 3.8). The isolates could also release Fe from the soil.

Analysis of the Acid Secretion by Soil Isolates :

These isolates were found to secrete mainly gluconic acid into the medium. Isolate 1, 2 and 3 could secrete 60, 45 and 55 mM gluconic acid, respectively. All these isolates also secreted succinic and acetic acids at low concentrations (Table 3.9).

Table 3.4

Identification of soil isolates

Growth on various C sources

C Source	Isolate 1	Isolate 2	Isolate 3
Glucose	+	+	+
Gluconate	+	+	+
Sucrose	+	+	+
Glycerol	+	+	+
Malate	+	+	+
Malate (-N)	+	+	+
Starch	-	-	-
Cellulose	+	+	+
Galactose	+	+	+
Maltose	+	+	+
Mannitol	+	+	+
Xylose	+	+	+
Oxalate	-	-	-
Tartarate	-	-	-
Citrate	+	+	+
Urea	+	+	+
Anaerobic growth	-	-	-
Catalase	+	+	+
Oxidase	+	+	+

\+ Growth positive
\- Growth negative

Table 3.5

Effect of buffering on the growth and RP solubilization by soil isolates using NH_4Cl as nitrogen source

Bacteria	Buffer	Growth ($OD_{600\ nm}$)	pH	Soluble P (mM)
Isolate 1	-	0.03 ± 0.01	3.30 ± 0.20	1.22 ± 0.11
	+	0.11 ± 0.01	3.66 ± 0.20	1.26 ± 0.10
Isolate 2	-	0.03 ± 0.02	3.20 ± 0.30	1.30 ± 0.10
	+	0.15 ± 0.04	3.46 ± 0.10	1.30 ± 0.05
Isolate 3	-	0.02 ± 0.01	3.90 ± 0.15	1.00 ± 0.11
	+	0.14 ± 0.04	3.10 ± 0.15	1.36 ± 0.05

\+ presence of buffer
\- absence of buffer

The buffer was used was 100 mM Tris-HCl (pH 8.0)

Carbon source was 100 mM Glucose.

All the parameters were monitored after 48 h of incubation in case of buffered and after 6 h in case of unbuffered media.

Results are expressed as mean ± S.D. of three independent experiments.

Table 3.6

Effect of buffering on the growth and RP solubilization by soil isolates using KNO_3 as nitrogen source

Bacteria	Buffer	Growth ($OD_{600\ nm}$)	pH	Soluble P (mM)
Isolate 1	-	0.02 ± 0.01	3.60 ± 0.20	1.00 ± 0.10
	+	0.14 ± 0.02	4.00 ± 0.20	0.82 ± 0.15
Isolate 2	-	0.04 ± 0.02	3.50 ± 0.50	1.10 ± 0.20
	+	0.18 ± 0.05	3.80 ± 0.30	1.00 ± 0.10
Isolate 3	-	0.03 ± 0.01	4.00 ± 0.20	0.75 ± 0.20
	+	0.12 ± 0.05	4.50 ± 0.20	0.55 ± 0.25

\+ presence of buffer
\- absence of buffer

The buffer was used was 100 mM Tris-HCl (pH 8.0).

Carbon source was 100 mM Glucose.

All the parameters were monitored after 48 h of incubation in case of buffered and after 6 h in case of unbuffered media.

Results are expressed as mean ± S.D. of three independent experiments.

Table 3.7

Growth and P release from alkaline vertisol soil by soil isolates with NH_4Cl as N source

	Time After Inoculation (Hours)				
	0	**12**	**24**	**36**	**48**
Isolate 1					
Viable Count (cfu/ml)	5×10^3	4.8×10^5	1.2×10^7	1.8×10^7	2.5×10^7
pH	8.3	4.54	4.98	7.16	7.69
Soluble P (mM)	UD	0.35 (± 0.05)	0.03 (± 0.01)	UD	UD
Isolate 2					
Viable Count (cfu/ml)	1.5×10^3	7.5×10^5	2.1×10^7	1.6×10^7	2×10^7
pH	8.3	3.18	4.22	4.76	6.86
Soluble P (mM)	UD	0.50 (± 0.05)	0.05 (± 0.01)	0.05 (± 0.01)	UD
Isolate 3					
Viable Count (cfu/ml)	1.2×10^3	2.1×10^5	2.0×10^7	2.2×10^7	2.0×10^7
pH	8.3	5.09	4.15	4.60	6.00
Soluble P (mM)	UD	0.35 (± 0.08)	0.05 (± 0.01)	0.05 (± 0.02)	UD

UD - below detection limit.
P values are given as mean ± S.D. of three independent experiments.
Replace viable counts by culturable counts.

Table 3.8
Growth and P release from alkaline vertisol soil by soil isolates with KNO_3 as nitrogen source

	Time After Inoculation (Hours)				
	0	**12**	**24**	**36**	**48**
Isolate 1					
Viable Count (cfu/ml)	4×10^3	3.8×10^5	1×10^7	1.5×10^7	2.0×10^7
pH	8.3	4.10	4.70	5.40	6.20
Soluble P (mM)	UD	0.40 (± 0.10)	0.25 (± 0.05)	UD	UD
Soluble Fe (mM)	UD	0.04 ± 0.02	0.05 ± 0.01	0.03 ± 0.01	UD
Isolate 2					
Viable Count (cfu/ml)	1×10^3	2×10^5	6×10^6	1.0×10^7	2×10^7
pH	8.3	4.00	4.80	5.30	6.50
Soluble P (mM)	UD	0.50 (± 0.10)	0.10 (± 0.01)	UD	UD
Soluble Fe (mM)	UD	0.05 ± 0.02	0.05 ± 0.03	0.04 ± 0.02	UD
Isolate 3					
Viable Count (cfu/ml)	1×10^3	1.6×10^5	1.8×10^7	2.0×10^7	2.0×10^7
pH	8.3	4.00	4.10	4.50	5.50
Soluble P (mM)	UD	0.50 (± 0.10)	0.20 (± 0.04)	0.10 (± 0.05)	UD
Soluble Fe (mM)	UD	0.05 ± 0.03	0.04 ± 0.01	0.03 ± 0.01	UD

UD : below detection limit.
P and Fe values are given as mean ± S.D. of three independent experiments.

Table 3.9

Major organic acids secreted by soil isolates

Organism	Organic Acid	Concentration (mM)
Isolate 1	Acetic	2.5
	Gluconic	60.0
	Succinic	0.35
Isolate 2	Acetic	2.7
	Gluconic	45.0
	Succinic	0.14
Isolate 3	Acetic	12.7
	Gluconic	55.0
	Succinic	0.42

3.4 : DISCUSSION

The screening procedure was modified by addition of buffer (100mM Tris-HCl pH 8.0) in the media and the pH drop was monitored by the presence of methyl red. Screening the soil for PSMs using this modified media resulted in isolation of three independent PS bacteria. The isolates obtained classified as belonging to *Rhizobium sp.* were found to secrete gluconic acid at 50-70 mM concentration in the presence of 100mM glucose in the media. It is known that the gram negative aerobic organisms like *Pseudomonas* use glucose through the Entner-Deudorff pathway and can convert glucose to gluconate in the periplasm (Lessie & Phibbs, 1984). *Rhizobium* also utilizes glucose primarily by ED pathway (Stowers, 1985).

Gluconic acid secreting phosphate solubilizing *E. herbicola* and *P. cepacia* E 37 have been isolated earlier (Goldstein, 1987). However *P. cepacia* E 37 can produce only 3-5 mM gluconic acid when grown with 50mM glucose (Liu *et al.*, 1992) and probably would not be effective in alkaline soils as 50mM gluconic acid is required to liberate P from soil (Table 2.7). P solubilizing *Rhizobium* strains were shown to produce 2-ketgluconate which was implicated in PS ability of these isolates (Halder *et al.*, 1990, 1992; Halder & Chakrabarty, 1993). However, only 5-7 mM ketogluconic acid was required to solubilize HAP under same conditions indicating that these PS strains could be secreting similar amounts of acid (Halder & Chakrabarty, 1993). PQQ dependent glucose dehydrogenase (GDH) are present in a variety of bacteria like *Zymomonas*, *Acinetobacter calcoaetium, Klebsiella aerogenes* and *E. coli.* All wild type *Pseudomonas* are known to produce acid when grown on glucose. However, the results of this study indicate that only those organisms would be effective in alkaline soils that can secrete acids at a much higher concentration. The ability these bacteria to secrete gluconic acid without externally added PQQ indicates that they are capable

of synthesizing PQQ. These organism could be of great help in alkaline soils as they can convert the glucose present in the root exudate to gluconic acid. These bacteria could also be used in a more ecofriendly way to manufacture soluble phosphatic fertilizers (Goldstein, 1993).

These isolates, in contrast to those isolated under unbuffered media conditions, were able to reduce the soil pH and release P from alkaline soil. The P release from soil was independent of the nitrogen source used indicating that the major cause for decrease in pH was due to the acid secreting properties of these bacteria. The organic acids secreted were analyzed directly from the soil media indicating that these acids could be produced under soil conditions provided the carbon and nitrogen sources are available to the bacteria. The carbon could come from the plant root exudate, however, the effectiveness of gluconic acid secreting PSM would be limited by the availability of glucose from the root exudate which could be converted to gluconic acid. Another limitation of these PSM would be their use in acidic soils in which no acid soluble P is present and chelators of Fe or Al are required to solubilize P. However, these PSMs can be used in acidic soils along with phosphate sources that contain acid soluble P like CaP or rock phosphate. The effectiveness of the isolates obtained during this work for supplementing phosphates for plant growth remains to be substantiated.

Interestingly the isolates could also release Fe from alkaline soils and thus could also help in alleviating Fe-deficiency related chlorosis which results in substantial losses of certain crops in alkaline soils (Tandon, 1991). The isolates also showed high salt tolerance and could solubilize RP under buffered conditions upto 500 mM NaCl concentration. Thus, the isolates have enormous potential to be used as P biofertilizer

even in saline soils. Soil salinity in considered to be a major hindrance for plant growth (Serrano & Gaxiola, 1994).

It would be of interest to find out the biochemical and genetic basis of the high level production of gluconic acid by these bacteria. The results have shown that the acid production by all the isolates is induced under phosphate starvation condition as reported earlier for *E. herbicola* (Goldstein, 1986). The gene(s) responsible for this phenotype could be part of *pho* regulon of these bacteria. The *pho* system has been characterized in detail in *E. coli* in which P starvation results in a substantial increase in the amount of periplasmic alkaline phosphatase which could effect the ability of the bacteria to scavange the organic phosphates that are present in it's surroundings (Wanner & McSherry, 1982; Torriani & Ludtke, 1985; Wanner, 1987). Similar system also operate in most of the other bacteria (Torriani-Gorini *et al.*, 1994) including *Pseudomonas aeruginosa* (Anba *et al.*, 1990) and *Rhizobium meliloti* (Bardin *et al.*, 1996) during P starvation. It has been suggested that bacteria may have also evolved a similar gentic system to access the large inorganic pool of P present in the soil in the form of insoluble mineral phosphates and this abiiity of microorganisms has been termed as Mineral Phosphate Solubilizing (*mps*) trait (Goldstein & Liu, 1987). *mps* genes have been cloned from *E. herbicola* and *P. cepacia*. The gene from *E. herbicola* was shown to be a putative PQQ synthase, involved in the biosynthesis of PQQ, the cofactor required for GDH (Goldstein, 1992). The gene from *Pseudomonas* showed similarity to membrane transport protein *His Q* and was postulated to play some role in making PQQ accessible to GDH in the periplasm (Babukhan *et al.*, 1995) or was probably involved in regulating *E. coli* gluconic acid secretion as *E. coli* containing the clone could show gluconic acid production in the absence of PQQ. The GDH from these gluconic acid secreting bacteria remains to be cloned.

In summary the work reported in this chapter show that incorporation of buffer in the screening media resulted in the isolation of bacterial strains which released P from alkaline soil and were able to secrete high amounts of gluconic acid. This modified procedure may be useful in screening of better and "effective" PSMs from the rhizosphere of specific plants and locations.