CHAPTER 2

EFFECT OF BUFFERING ON THE PHOSPHATE SOLUBILIZING ABILITY OF MICROORGANISMS

2.1 : INTRODUCTION

It has been realized that Phosphate Solubilizing Microorganisms (PSMs) could be effective as biofertilizers in enhancing crop yields in phosphate deficient soils (Subba Rao, 1982; Goldstein, 1984; Kucey *et al.*, 1989). Many soil microorganisms display the ability to solubilize dicalcium phosphate (DCP), tricalcium phosphate and hydroxyapatite (HAP) when provided as the sole P source in laboratory media. On the other hand, only a few have the ability to solubilize ferric and aluminum phosphates (Sperber, 1957; Bolan *et al.*, 1987; Kucey *et al.*, 1989; Halder *et al.*, 1991; Halder & Chakrabartty, 1993). A majority of PSMs solubilize calcium phosphate (CaP) complexes by extrusion of protons (Roos & Luckner, 1984) or by the secretion of mono-, di- and tri-carboxylic acids (Kucey *et al.*, 1989; Cunningham & Kuiack, 1992; Nahas, 1996). These microorganisms have been considered to play a significant role in supplying P to plants especially those growing in alkaline soils rich in CaP complexes, like vertisols. It is paradoxical that although PSMs are abundant in soils and in the rhizosphere of most plants (Kucey *et al.*, 1989), phosphorus is still one of the major limiting nutrient for plant growth.

Inoculation of PSMs into soils has been shown to increase the population of PSMs in the rhizosphere but only a few studies show consistent enhancement of phosphorus uptake by plants as well as of plant growth (Subba Rao, 1982; Tandon, 1987; Kucey *et al.*, 1989). The variations in growth enhancements are attributed to the differences in

the composition and properties of soils, the nature and distribution of soil microflora, and the type of the crop (Kucey *et al.*, 1989). It is also possible that the inconsistency in growth enhancement of inoculated plants could arise due to the inability of some PSMs to release phosphate from soils. Such a view can be considered if the phosphate solubilization ability of microorganisms in soils is different than that found under laboratory conditions. This proposition is supported by several observations. Most of the PSMs have been isolated under unbuffered conditions (Kucey *et al.*, 1989) whereas soils rich in Ca-P complexes have a very strong buffering capacity (Ae *et al.*, 1991). The buffering capacity of soils could limit solubilization of soil phosphates by microorganisms as it has been shown that solubilization of CaP complexes is mainly mediated by lowering the pH of the medium (Sperber, 1957; Kucey *et al.*, 1989; Halder & Chakrabartty, 1993; Nahas, 1996). It has also been shown that phosphate solubilization varies with respect to the composition of the medium and to the nature of the mineral phosphate provided. This phenomenon is attributed to the buffering effect of these components (Cunningham & Kuiack, 1992). Solubilization of mineral phosphates by the culture filtrates of phosphate solubilizing *Rhizobium* sp. was abolished after neutralizing the pH of the medium by NaOH (Halder & Chakrabarty, 1993) and there is so far no direct evidence for PSM mediated release of P from soils (Kucey *et al.*, 1989).

Therefore, in a systematic effort to resolve the controversy regarding the effectiveness of PSMs, we examined the efficiency of various PSM isolates at releasing P from vertisols and mineral phosphates under buffered conditions. The inability of PSMs to release P from alkaline soils is due to the high buffering capacity of these soils. PSMs isolated using unbuffered screening conditions secrete organic acids at a concentration which is 20-50 fold less than that required to release P from soil.

2.2 : MATERIALS & METHODS

Bacterial strains and Media :

Citrobacter koseri was obtained from the Biofertilizer Unit, Gujarat State Fertilizer Company, Baroda. *Bacillus coagulans* was a gift from Dr. T. K. S. Gowda, Department of Agricultural Microbiology, University of Agricultural Sciences, Bangalore. Packets of the commercially supplied PSMs by Tamilnadu Agricultural University and T. Stanes & company were bought from the market and the PS bacteria isolated from the sample. These PSMs have been isolated from agricultural soils on the basis of their ability to grow on solidified media containing calcium phosphate complexes as a sole P source.

Culturing Procedure :

The bacteria were routinely 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), Zn SO_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_2PO4. The media was buffered with 100mM Tris-HCl pH 8.0. These cells were used to inoculate to fresh media as described below.

Determination of Buffering Capacity of Alkaline Vertisol Soil :

Buffering capacity of the alkaline soil used in the study was determined as described by Ae (Ae *et al.*, 1991). 10 g of soil was taken and titrated with 0.01 N acetic acid and pH value measured.

Effect of Buffering of Media on PS Abilities of PS Bacteria :

Solid-medium Experiments :

The phosphate solubilizing bacteria were grown on 1.5% agar plates containing 100mM glucose, 10mM NH_4Cl or 10mM KNO_3 as indicated and the above mentioned micronutrients and DCP or 'Senegal' rock phosphate as the P source. The pH of this media was adjusted to 8.0 with NaOH. Sterilized rock phosphate (RP) was added to the plates before pouring the media. For buffering the medium, 100mM Tris-HCl pH 8.0 was used. Methyl red indicator dye was used at 0.01% in plates. DCP was precipitated *in situ* as described earlier (Goldstein & Liu, 1987).

The effect of nitrogen source and buffering of the media on phosphate solubilization was recorded by measuring the diameter of the red zone around the colonyin the case of rock phosphate plates and the zone of clearance in DCP plates. Measurements were taken in triplicate center-point inoculated plates after different time intervals as indicated.

Liquid Medium Experiments :

The cells grown overnight were inoculated in liquid media containing 100mM glucose, 10mM NH_4Cl or 10mM KNO_3 as indicated. P was provided in the form of either 1.67mM HAP or 1mg/ml rock phosphate (RP). The pH of the media was adjusted to 8.0 by NaOH in the case of unbuffered media and was buffered with 100mM Tris-HCl pH 8.0 in the case of buffered media. Tris-HCl buffer concentration was decided based on the buffering capacity of the soil used in the study. The soil showed buffering capacity similar to 100 mM Tris-HCl pH 8.0. After 48 h of inoculation on the rotary shaker at room temperature, cells were centrifuged and the supernatent was used for pH and P estimations.

Phosphate Solubilization From Alkaline Soil and Identification of Organic Acids Secreted by *C. koseri* and *B. coagulans* :

The bacteria grown on the above media were inoculated into media containing 100mM glucose, 10mM NH_4Cl or KNO_3 as indicated. 1 g/ml (30 g/30 ml) of alkaline soil (pH 8.0) collected locally, was provided as P source. The growth of the bacteria was monitored by determinig culturable counts at different time points. Culture was collected at different time intervals and centrifuged in a microfuge at 10,000 rpm. The pH and P were determined from the supernatant. Organic acid analysis was performed with the supernatent collected after maximum drop in pH.

Enumeration of acid Producing PSMs From Rhizospheric and Non-Rhizospheric Alkaline Soils :

The rhizospheric and nonrhizospheric soils was resuspended in saline and different dilutions were plated on above mentiond media with (RP) as P source. The plates contained methy red indicator to moniter pH drop. The media was adjusted to pH 8.0 with NaOH in case of unbuffered media and the buffered media contained 50 mM Tris-HCl pH 8.0 as the buffering component. The total number of microorganisms were estimated by culturable counts on plates having same media composition as buffered media and containing 10mM free P. The number of acid producing PSMs were calculated by counting the microorganisms showing a zone of pH reduction as monitored by change of colour of methyl red from yellow to red.

Effect of Nutrient Supplementation on Growth and P Release by Native Microorganisms from Alkaline Vertisol :

Rhizospheric and non-rhizospheric alkaline soils were collected locally and resuspended in sterile media containing 100mM glucose, 10mM amonium chloride at a concentration of 1g/ml (30 g/30 ml). The organisms in the soils were allowed to

grow aerobically at 30°C on a rotary shaker at 200 rpm. The sample were collected at different time intervals and the total number of microorganisms were determined by culturable counts on M9 media. The supernatant solution was collected by centrifuging the sample at 10000 rpm and was used for determining pH and P.

Effect of Organic Acids on P Release from Alkaline Soil :

Local alkaline vertisol soil was taken at 1g/ml (30 g/30 ml). Concentrated stocks of organic acids were added so as to result in different final concentration. The solution was filtered after 15 minutes through watman no. 1 filter paper. The filtrate was used for estimating pH, P and Fe.

Analytical Methods :

(i) **Phosphate solubilization** : Phosphate estimations were done by the ascorbate method (Ames, 1964). The range of the method is 30-300 nmoles of P.

(ii) **Fe estimations** : Fe estimations were done by α-α-Di-pyridyl method (Ramsay, 1957).

(ii) **Organic acid analysis** : Organic acids were analyzed by HPLC using Shimadzu SCR-101 column. The mobile phase was 0.1% phosphoric acid at a flow rate of 1ml/min. and the column temperature was 65°C. The acids were detected by absorbance at 210 nm by UV detector.

2.3 : RESULTS

Effect of Buffering on the P-solubilization Abilities of *C. koseri* and *B. coagulans* and Other Commercially Supplied PSMs :

C. koseri, *B. coagulans* and the PSMs used in this study have been isolated on the basis of their ability to show a zone of clearance on unbuffered HAP plates. In order to check the effect of buffering on their P solubilization potential, experiments were performed using liquid as well as solid media. In solid media the effect of N source on P solubiliztion was also studied.

As seen in Table 2.1, the zone of clearance on the buffered DCP plates is approximately half the size of that on unbuffered plates. This holds true for all the bacteria using NH_4Cl as N source. In the case of *B. coagulans*, on DCP plates with KNO_3 as the N source there was no such decrease upon buffering. However, when RP was provided as the P source and the zone of acidification was monitored, buffering seemed to drastically reduce not only the zone size but also the growth of these bacteria. In this case the observation was the same with both the N sources. In order to confirm whether the lack of of growth on buffered RP plates was due to P limitation, buffered plates containing 1mM KH_2PO_4 but otherwise similar to those used earlier, were inoculated with the two bacteria. Both grew well but did not produce any zone of acidification zone even after 72h indicating that the lack of growth on buffered RP plates was indeed due to P limitation and the buffer *per se* did not inhibit the growth of the bacteria (Fig. 2.1). *C. koseri* and *B. coagulans*, however could solubilize DCP in the presence of buffer in the medium (Fig 2.2). The bacteria supplied by Tamilnadu Agricultural University could solubilize DCP and RP under unbuffered conditions but not under buffered media conditions whereas the PS

Table 2.1

Effect of buffering on the zones of DCP solubilization and pH reduction by *C. koseri* and *B. coagulans*

P Source	Buffer	Bacteria	N Source			
			NH_4Cl		KNO_3	
			Zone Size (cm)		Zone Size (cm)	
			48 h	72 h	48 h	72 h
DCP	-	*C.k.*	2.4	3.5	ND	ND
	-	*B.c.*	2.6	3.8	1.5	2.5
	+	*C.k.*	1.0	2.0	ND	ND
	+	*B.c.*	1.8	2.0	1.8	3.0
RP	-	*C.k.*	1.8	2.6	ND	ND
	-	*B.c.*	2.5	3.2	1.3	1.8
	+	*C.k.*	UD	UD	ND	ND
	+	*B.c.*	UD	UD	UD	UD

Note : UD - Undetectable zone with poor or no growth.
ND - Experiment was not done since *C. koseri* is known not to assimilate NO_3

C.k. *Citrobacter koseri*

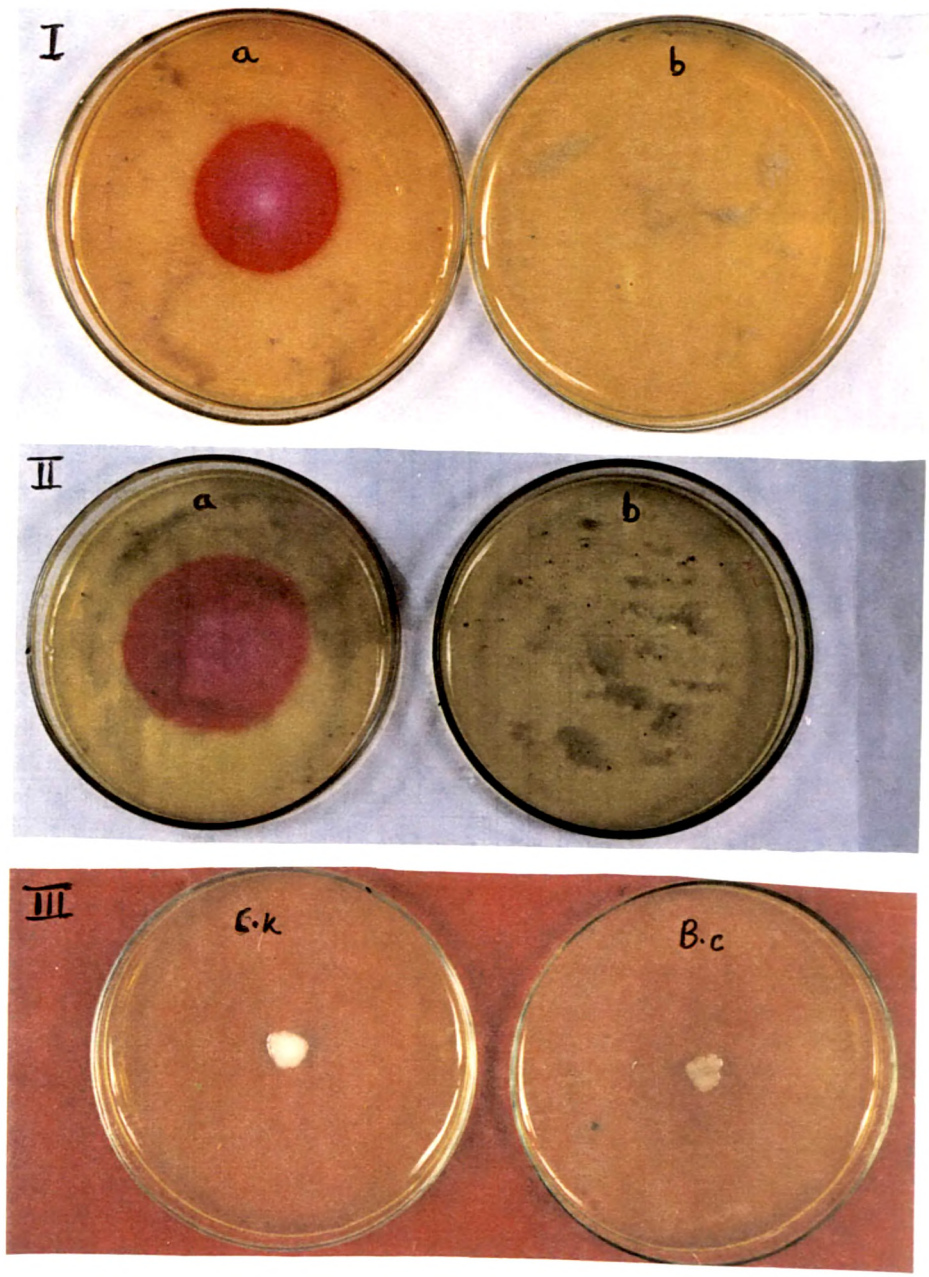
B.c. *Bacillus coagulans*

DCP Dicalcium phosphate

RP Rock phosphate

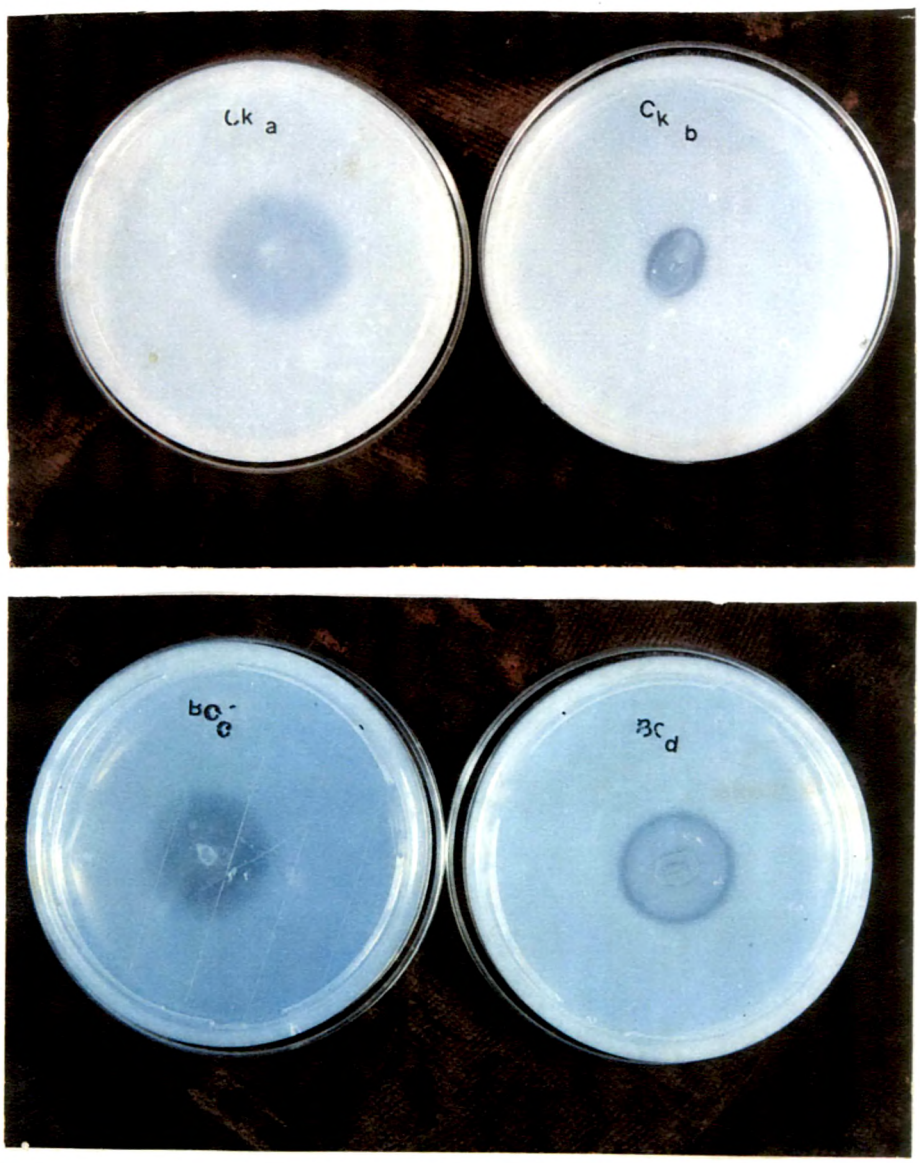
The buffer used was 100 mM Tris-HCl pH 8.0 (+ indicates presence and - indicates absence of buffer). The zone size presented is an average of three independent measurements.

Fig. 2.1 : P solubilization from rock phosphate by *C. koseri* and *B. coagulans*.



I) *C. koseri*; (II) *B. coagulans* ; a : Unbuffered; b : Buffered; (III) *C. koseri* (C.k.) and *B. Coagulans* (B.c.) on buffered media with 1 mM K_2HPO_4.

Fig. 2.2 : P solubilization from di-calcium phosphate by *C. koseri* and *B. coagulans*.



C.k.: *Citrobacter koseri*; *B.c.* : *Bacillus coagulans*
(a) Unbuffered NH_4Cl; (b) Buffered NH_4Cl; (c) Unbuffered KNO_3;
(d) Buffered KNO_3.

bacteria supplied by T. Stanes and Company did not show pH reduction zone even in unbuffered media conditions (Fig 2.3 and 2.4).

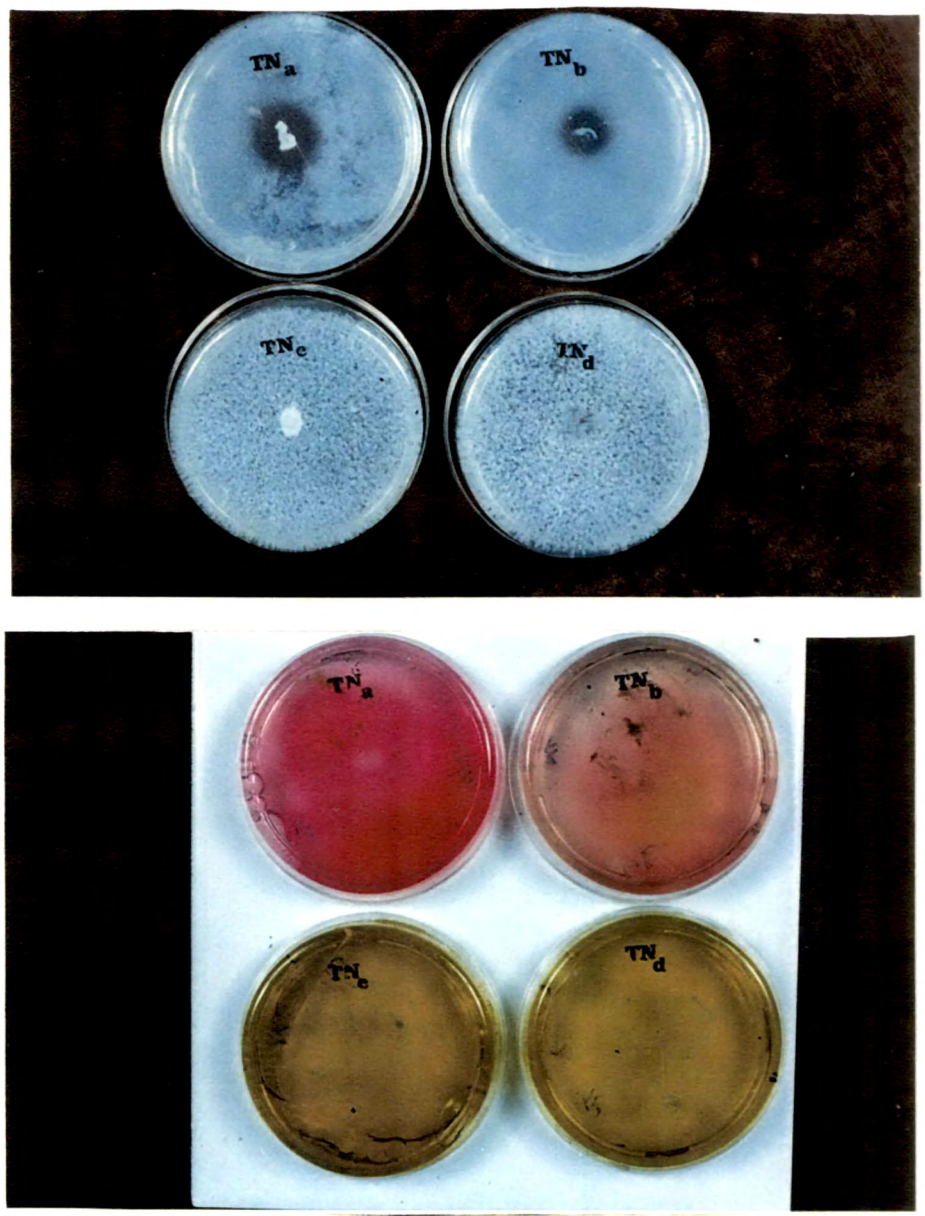
To further study the effect of buffering on mineral phosphate solubilization, the P solubilizing potential of *C. koseri* and *B. coagulans* from two mineral phosphates *viz* HAP and RP was studied in liquid media. Table 2.2 shows the results of these experiments. Under unbuffered conditions, both the bacteria grew well using HAP as P source. The pH of the extracellular medium dropped to values below 3 after 48h and a high amount of soluble P was released by both the bacteria. RP containing medium, on the other hand, did not support as much growth as HAP medium, especially in the absence of buffer. Nevertheless, there was a considerable reduction in medium pH and P was liberated. When 100 mM Tris-HCl pH 8.0 was added to the media, there was no significant change in pH brought about by both the bacteria using either of the P source. Also the P solubilization was negligible.

Solubilization of Soil Phosphates by *C. koseri* and *B. coagualns* :

In order to check the efficacy of *C. koseri* and *B. coagulans* at releasing P from alkaline soil, an experiment was carried out using soil (1g ml^{-1}) as the sole P source as well as buffering component in the minimal medium and the growth of the bacteria, change in pH and release of P was monitored for 48 h at different time intervals. Results (Table 2.3) showed that both the bacteria grew well (approx. 8-9 doublings) in 48h. However, both the bacteria failed either to cause any significant decrease in pH or to release P in the soil solution.

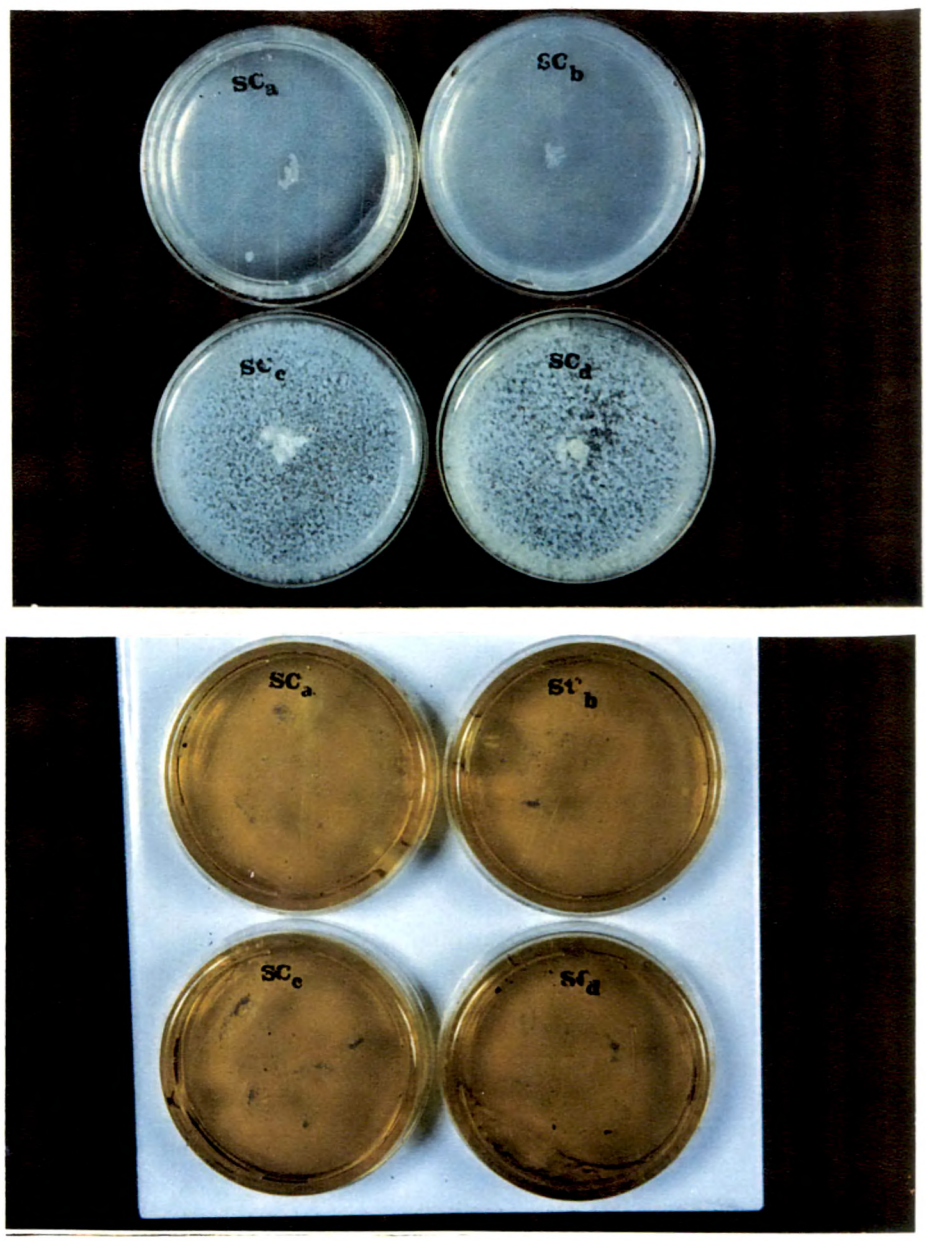
Since it was possible that the P solubilized from soil phosphates would have reprecipitated, 10^8-10^9 cfu of *C. koseri* and *B. coagulans* were added to the media containing alkaline soil and the pH drop and solubilized P was determined at 1 h

Fig.2.3 : Mineral phosphate solubilization by PS bacteria supplied by TNAU, Madurai.



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

Fig. 2.4 : Mineral phosphate solubilization by PS bacteria supplied by T. stanes & Co., Coimbatore.



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

Table 2.2

Effect of buffering on the growth and P-solubilization from mineral phosphates by *C. koseri* and *B. coagulans*

P Source	Buffer	Bacteria	Growth ($O.D._{600\ nm}$)	pH	Soluble P
HAP	-	*C.k.*	0.43 ± 0.01	2.71 ± 0.08	3.96 ± 0.20
	-	*B.c.*	0.14 ± 0.02	3.01 ± 0.13	3.60 ± 0.02
	+	*C.k.*	0.38 ± 0.03	8.0 ± 0.20	0.10 ± 0.02
	+	*B.c.*	0.26 ± 0.03	8.0 ± 0.30	0.02 ± 0.01
RP	-	*C.k.*	0.08 ± 0.01	3.0 ± 0.10	3.47 ± 0.04
	-	*B.c.*	0.06 ± 0.01	2.8 ± 0.20	3.40 ± 0.24
	+	*C.k.*	0.13 ± 0.02	7.6 ± 0.03	UD
	+	*B.c.*	0.25 ± 0.05	7.5 ± 0.04	UD

Note : UD - Less than 30 μM

C.k. *Citrobacter koseri*

B.c. *Bacillus coagulans*

HAP Hydroxyapatite

RP Rock phosphate

The buffer used was 100 mM Tris-HCl pH 8.0 (+ indicates presence and - indicates absence of buffer). All the parameters were monitored after 48 hours of incubation. Results are expressed as mean ± S.D.

Table 2.3

Growth and P-releasing abilities of *C. koseri* and *B. coagulans* on alkaline soil

Time After Inoculation (Hours)	*C. koseri*			*B. coagulans*		
	Viable Counts	pH	Soluble P (mM)	Viable Counts	pH	Soluble P (mM)
0	$5x10^3$	8.3	UD	$2x10^3$	8.3	UD
12	$4.8x10^5$	7.9	UD	$3x10^5$	7.5	UD
24	$1.2x10^6$	7.5	UD	$6x10^6$	7.0	UD
36	$1.8x10^6$	7.1	UD	$2x10^7$	6.5	UD
48	$2.5x10^6$	7.5	UD	$3x10^7$	7.5	UD

UD : Less than 30 μM

intervals for 6 h. Both the Ps bacteria failed to either drop the pH or solubilize P (Fig. 2.5).

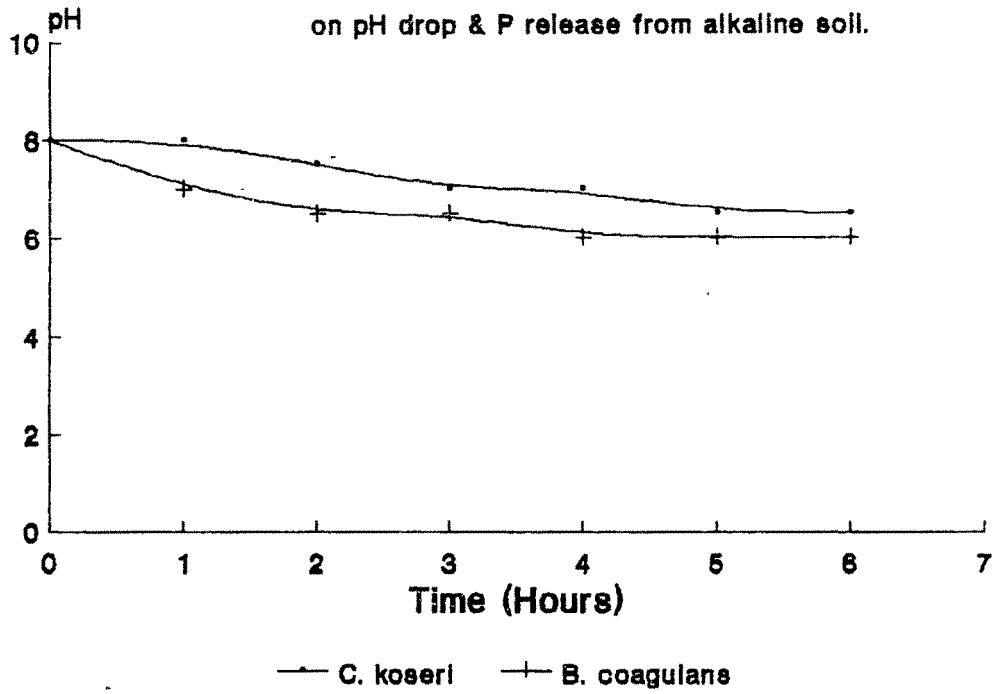
P solubilization by Native Microorganisms from Alkaline Vertisol Supplemented With C and N Sources :

It has been suggested that the PSMs present in the rhizosphere of many plants are not effective as their PS ability could be limited by the availablity of carbon and nitrogen sources in the soil conditions (Kucey *et al.*, 1989). In order to verify this, 100 mM glucose and 10mM ammonium chloride was provided and the ability of the native micororganisms to grow and release P from alkaline soil was studied. Out of the 10 soils studied, the microorganisms could grow to 10^6 / ml of the media but failed to either drop the soil pH or release P from soil (Table 2.4).

Enumeration of Acid Producing PSMs Under Unbuffered and Buffered Media Conditions from Alkaline Vertisol Soils :

The observation that native microflora of several soils failed to show solubilization of P from alkaline vertisol could be attributed to the strong buffering action of the soil. If this is indeed the case, then the earlier estimates of PSMs among native microflora of soils do not reflect the effective PSM population. In an effort to enumerate and screen for 'effective' PSMs we have developed a method based on the acidification of a buffered indicator medium containing RP as sole P source. Accordingly we expected an effective PSM to not only to grow well on such a plate but also produce a red zone indicating a drop in pH to less than 4.5. In the various soils tested, the acid producing PSMs as estimated using the unbuffered medium were in the range of 10-30% of the total number of microorganisms (Table 2.5). There was no difference in the number of PSMs between rhizospheric and nonrhizospheric soils. However, when media buffered with 50mM Tris-HCl pH 8.0 was used except for one sample no acid

Fig. 2.5 : Effect of inoculation of large No. of cells of *C. koseri* and *B. coagulans* on pH drop & P release from alkaline soil.



P was below detection limit at all time points.

Table 2.4

Growth of indigenous microorganisms in vertisol supplemented by 100 mM glucose and 10 mM ammonium chloride

Time (h)	Total No. of Microorganisms	pH	P (mM)
Sample No. 1			
0	1×10^5	8.0	UD
24	3×10^5	7.5	UD
48	8×10^5	7.0	UD
72	2×10^5	7.0	UD
Sample No. 2			
0	3×10^5	7.5	UD
24	4×10^5	7.0	UD
48	6×10^5	6.5	UD
72	3×10^6	6.5	UD
Sample No. 3			
0	2×10^5	7.0	UD
24	3×10^5	7.0	UD
48	7×10^5	6.5	UD
72	3×10^6	6.5	UD

UD : Less than 30 μM.

Table 2.5

Effect of buffering on the number of PSMs in rhizospheric and non-rhizospheric soils

Sample No.	No. of Microorganisms in Unbuffered Media	No. of PSMs in Unbuffered Media	No. of Microorganisms in Buffered Media	No. of PSMs in Buffered Media
	Non-Rhizospheric Soil			
1.	3×10^5	5×10^4	1×10^3	Nil
2.	2×10^5	3×10^4	Nil	Nil
3.	5×10^5	5×10^4	Nil	Nil
	Rhizospheric Soil			
4.	4×10^5	8×10^3	Nil	Nil
5.	4×10^6	1×10^6	5×10^3	Nil
6.	8×10^5	1×10^5	4×10^3	Nil
7.	1×10^5	1×10^4	2×10^3	Nil
8.	3×10^5	1×10^4	Nil	Nil
9.	2×10^5	1×10^4	Nil	Nil
10	4×10^5	2×10^4	8×10^3	2×10^2

UD : Less than 30 μM

producing PSMs were found in other soil samples. Some microorganisms could grow under buffered conditions using rock phosphate as P source. Three independent bacterial strains were isolated from sample number 10 which was taken from rhizosphere of pigeon pea (*Cajanus cajan*). Out of the various soils screened, the PSMs in unbuffered medium were in the range of 10-30% of the total number of microorganisms. There was no differences in the number of PSMs in the rhizospheric and nonrhizospheric soils.

Organic Acids Secreted by *C. koseri* and *B. coagulans* :

It is known that majority of PSMs solubilize P by secretion of organic acids. Therefore the organic acids secreted by the two strains were identified and estimated (Table 2.6.). Culture filterates of both the bacteria showed the presence of low levels of four organic acids, their total concentration amounting to less than 10mM in either case.

Determination of Soil Buffering Properties and P Solubilization from Soil by Organic Acids :

The alkaline soil used in the study showed high buffering capacity as around 160-180 ml of 0.01N acetic acid was requried to reduce the soil pH to less than 5.0 (Fig 2.6). There were both qualitative and quantitative differences in the P releasing abilities of various organic acids to solubilize P from alkaline vertisol soil (Table 2.7) suggesting that different mechanisms operate for different acids. For instance, out of the seven organic acids used in this study, a few could release high amount of P and also showed detectable release of Fe in the soil solution (tartaric, lactic, citric and gluconic). Others could mediate P solubilization but could not liberate Fe in soil solution (oxalic). Yet others could bring about a release of P only at very high concentration (acetic, lactic and succinic). The amount of acid required were 20-50 times more than that secreted by *C. koseri* and *B. coagulans*.

Table 2.6

Major organic acids secreted by *C. koseri* and *B. coagulans*

Bacteria	Organic Acid	Concentration (mM)
C. koseri	Citric	1.2
	Oxalic	0.1
	Succinic	0.8
B. coagulans	Acetic	4.70
	Citric	1.36
	Lactic	1.40
	Succinic	1.35

Fig. 2.6 : Buffering capacity of alkaline vertisol soil

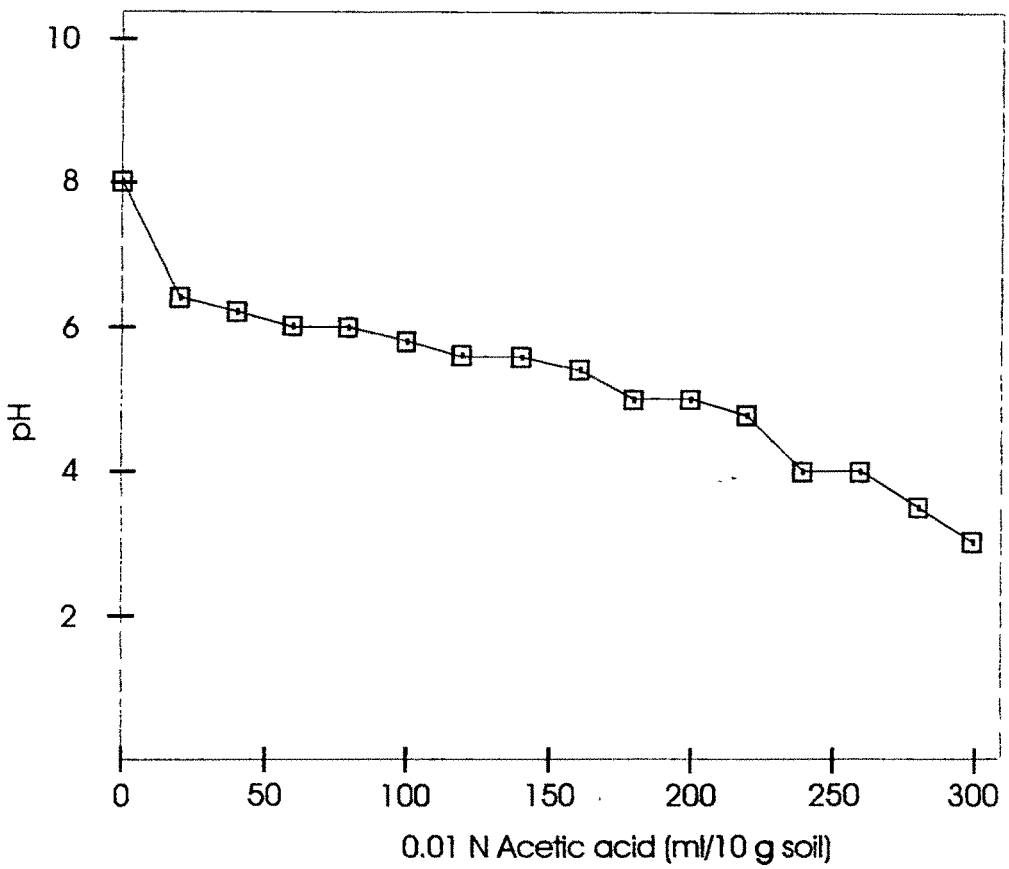


Table 2.7

Effect of organic acids on the release of P from alkaline soil

Acid	pH	P (mM)	Fe (mM)
Water	8.94 ± 0.50	UD	UD
100 mM Acetic	6.00 ± 0.40	0.18 ± 0.03	UD
100 mM Lactic	6.50 ± 0.30	0.63 ± 0.05	0.027 ± 0.01
100 mM Succinic	5.80 ± 0.20	0.53 ± 0.06	UD
10 mM Tartaric	4.83 ± 0.82	0.33 ± 0.02	0.053 ± 0.02
20 mM Tartaric	3.90 ± 1.10	0.73 ± 0.26	0.089 ± 0.02
10 mM Oxalic	6.02 ± 0.54	0.62 ± 0.25	UD
20 mM Oxalic	5.56 ± 0.40	1.29 ± 0.30	UD
10 mM Citric	4.34 ± 0.50	0.70 ± 0.20	0.083 ± 0.01
20 mM Citric	3.98 ± 0.18	1.17 ± 0.30	0.143 ± 0.05
20 mM Gluconic	6.00 ± 0.20	0.50 ± 0.10	0.050 ± 0.02
50 mM Gluconic	4.5 ± 0.50	0.65 ± 0.05	0.070 ± 0.04

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

UD : below detection limit.

2.4 : DISCUSSION

The results presented above show that the buffering capacities of alkaline soils determine the effectiveness of PSMs in releasing P from soils. These studies also show a clear difference in the phosphate solubilizing abilities of PSMs under buffered and unbuffered media conditions. While *C. koseri, B. coagulans* and other PS bacteria were effective at releasing P from mineral phosphates under unbuffered conditions, addition of buffer drastically reduced their RP and HAP solubilizing potential. It is also evident that buffering has a more pronounced effect on the growth of the bacteria using RP as P source. Presumably, in this case the drop in pH in the absence of buffer is rapid and prevents further growth. HAP, on the other hand, due to it's natural buffering capacity may allow for some growth before the fall in pH can no more sustain cell growth. Interestingly, solubilization of DCP, the most commonly used insoluble P source for identification of PSMs, was not severely affected by buffering. The two bacteria also failed to release P from alkaline soil but were proficient as far as growth using soil as sole P source was concerned.

As reported in other studies (Cunningham & Kuiack, 1992; Halder *et al.*, 1992), the PSMs showed larger zone of pH reduction in the presence of ammonium chloride as nitrogen source as compared to that of nitrate as N source under unbuffered media conditions. However, the zones were similar in the presence of buffer in the media indicating that the acidification produced as a result of ammonium utilization was not a contributing factor under buffered media condition. These results indicate the limitation of the screening procedure used for the isolation of PSMs. PSMs have been isolated using acid soluble Ca-P complexes in media devoid of any buffering component (Louw & Webley, 1958). This would result in isolation of any organism that would be able to secrete even small concentration of organic acid(s). However, it

is known that alkaline soils rich in Ca-P complexes have high buffering capacity (Ae *et al.*, 1991). It has also been suggested that the amount of organic acid required to solubilize P from soil is much higher than that required to solubilize Ca-P complexes *in vitro*. The results presented here have confirmed that the buffering capacity of alkaline soil could severely limit the P solubilization by PSMs.

High buffering capacity of alkaline vertisols could account for the differences in abundance and effectiveness of PSMs. *C. koseri* and *B. coagulans* were found to secrete various organic acid in 1-5 mM concentration whereas as the concentration of these acids required to reduce the pH of the soil was 20-50 times more. For *e.g.* these bacteria secreted citric acid at concentration of 1mM whereas 20mM citric acid was required to release 1mM P from soil. There was both qualitative and quantitative differences in the ability of various organic acids to release P form soil. Oxalic and citric were the most effective. It is also known that the organic acids differ in the efficiency to release P from phosphate rocks (Kpomblekou & Tabatabai, 1994). Release of P from alkaline vertisols is less with acetic and succinic acid whereas citric and oxalic acids are very effective at 10 mM concentration. It is interesting that *Pencilium billai* secretes 10 mM each of citric and oxalic acids and has been shown to be effective in releasing P in the field conditions (Asea *et al.*, 1987).

Enumeration of total number of PSMs under conventional unbuffered and buffered conditions showed significant differences. 10-30% of total number of microorganisms were able to show zone of pH reduction on unbuffered media which is consistent with earlier studies (Kucey *et al.*, 1989). However, except in one soil sample no PSMs could be detected when the media was buffered with 50 mM Tris-HCl pH 8.0 whereas 100 mM Tris-HCl pH 8.0 corresponds to buffering capacity of alkaline vertisol soil used in this study (data not shown).The two PSMs tested could grow on alkaline soil

but were unable to release P. Similarly some organisms could grow on rock phosphate plates buffered with 50 mM Tris-HCl pH 8.0 but were not able to acidify the medium. These results suggest that the ability to grow on buffered medium or on soil is not sufficient to release P in high levels (> 100 μM).

The native microorganisms were not able to reduce the pH of the soil even when C and N sources were externally supplied suggesting that PSMs which could be effective in alkaline soils could be much less in number than previously estimated (Kucey *et al.*, 1989) and, if so, provides an explanation for the apparent paradox that the plant growth is limited by availability of P despite the abundance of PSMs in the rhizosphere of plants. The results also show the limitation of the screening procedure used for isolating PSMs. In view of this fact, it is important to isolate organisms with the ability to secrete high concentration of organic acid(s). This could be achieved by screening for organisms capable of growth and RP solubilization in the presence of a strong buffer in the medium. The extent of pH reduction by the organisms could be monitored by incorporating a dye such as methyl red in the isolation medium and monitoring the change in color from yellow to red.

In conclusion, the results presented in this chapter demonstrate the limitation of the PSMs isolated by conventional screening procedure in releasing P from alkaline soil. This inability was due to the buffering capacity of the soil as the PS ability of the PSMs was severely reduced in the presence of buffer in the media. The alkaline soil required 20-50 times more organic acid than that secreted by the bacteria tested.