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In vitro Solubilization of Inorganic Phosphate by Phosphate Solubilizing Microorganisms (PSM) from Maize Rhizosphere

SADIA ALAM, SAMINA KHALIL†, NAJMA AYUB AND MALIHA RASHID
Department of Biological Sciences, Quaid-I-Azam University, Islamabad–Pakistan
†*Applied Microbiology Section, NARC Islamabad–Pakistan*
Corresponding address: khalilhumayun@hotmail.com

ABSTRACT

Ten most efficient phosphate solubilizing bacteria and three fungi (PSM) isolated from maize rhizosphere were studied for biochemical characteristics. PSM were grown *in vitro* for seven days on Pikovskaya's medium and following analyses were carried out i.e. solubilization index, pH change, phosphorus (P) solubilized, P immobilized and organic acids produced under *in vitro* conditions. P solubilization index of these isolates ranged from 1.63-3.29. Drop in pH of the medium ranged from 7 to 3.2 with the continuous growth of these isolates for seven days. Study showed that more P was immobilized (0.2-0.46%) than solubilized (0.088-0.22%). Bacteria were found to be more active than fungi in conversion of insoluble P to soluble P. Citric and oxalic acids were two common organic acids produced by PSM. Correlation studies suggested that besides organic acids there might be other factors responsible for P solubilization.

Key Words: Phosphate solubilization; PSM; Maize

INTRODUCTION

Phosphorus is the second most important plant nutrient after nitrogen (Donahue *et al.*, 1990). It exists in nature in variety of organic and inorganic forms. P availability is low in soils because of its fixation as insoluble phosphates of iron, aluminium and calcium. Since deficiency of P is the most important chemical factor restricting plant growth, chemical phosphatic fertilizers are widely used to achieve optimum yields. Soluble forms of P fertilizer used are easily precipitated as insoluble forms, this leads to excessive and repeated application of P fertilizer to cropland. P supply through biological means is a viable alternative, phosphate solubilizing bacteria (PSB), phosphate solubilizing fungi (PSF) and actinomycetes that have been reported to be active in conversion of insoluble phosphate to soluble primary and secondry orthophosphate ions by many investigators (Chabot *et al.,* 1993; Pal, 1998). Although mechanism of phosphate solubilization is still not fully understood, several mechanisms have been implicated in the process. The production of organic acids seem to be the main mechanism (Illmer *et al.,* 1995). In liquid media studies many workers have shown that phosphate solubilization is due to organic acid production (Gupta *et al.,* 1994; Illmer & Schinner, 1992, 1995; Illmer *et al*., 1995).

Antarikanonda *et al*. (1991) found fungi more active in solubilizing phosphate than bacteria. Omar (1998) tested 36 fungal species on agar plates. Most of them were non solubilizers. *Aspergillus niger* and *Penicillium citrinum* caused a marked drop in pH of liquid culture media and solubilized considerable amounts of phosphate.

Kumar and Narula (1999) evaluated different mutant phosphate solubilizing strains of *Azotobacter chroococcum* for their ability to solubilize insoluble phosphate and indole acetic acid (IAA) production on the basis of clear zone produced on Pikovskaya's and Jensen's media. Nautiyal *et al.* (1999) stated that organic acid production is an important mechanism in 'P' solubilization but not the sole mechanism. Chabot *et al.* (1993) found that PSM constitute 26-46% of microbial population of four Quebec soils studied. They isolated 10 bacteria and three fungi capable of phosphate solubilization on the basis of large clear zone on solid media containing different insoluble phosphates. Khalil (1995) reported that phosphate availability from rock phosphate was increased in soil from 0.67 ppm in control to 17.78 ppm with PSM inoculation in 20 days.

In an effort to develop a PSM inoculum for improving P uptake by maize crop, author isolated numerous PSM from the rhizosphere of maize plants. This study reports biochemical characteristics of the most efficient 10 PSB and three PSF among these isolates.

MATERIALS AND METHODS

Solubilization index (SI). 0.1 mL of each PSM culture preserved in sterile distilled water was placed on Pikovskaya's agar (PA) (Pikovskaya, 1948) plates [containing insoluble tricalcium phosphate 2.5 g, glucose 13 g, $(NH_4)SO_4$ 0.5 g, NaCl 0.2 g, $MgSO_4.7H_2O$ 0.1 g, KCl 0.2 g, Yeast Extract 0.5 g, $MnSO_4$ trace, $FeSO_4.7H_2O$ trace, Agar 15 g, pH adjusted to 7.2 and dissolved in 1000 mL distilled water] and incubated for seven days. Solubilization Index was measured using following formula (Edi-Premono *et al.,* 1996).

$$SI = \frac{\text{colony diameter + halozone diameter}}{\text{colony diameter}}$$

pH change. 1 mL of three day old culture in sterile distilled water (containing about 1x 10^3CFU) was added to sterile 100 mL Pikovskaya's broth (PB) medium in 250 mL conical flask and kept on shaker for seven days. Sterile uninoculated medium served as control. Initial pH and change in pH was noted each day for one week by digital pH meter.

'P' Analysis in broth

i. Phosphorus solubilized. PSM cultures were grown in PB for seven days with continuous shaking (reciprocating shaker at 150 cpm) at 35^0C. A 10 mL sample of each culture was taken in centrifugation tube and centrifuged for 15 min at 1500 rpm. P in solution was extracted with Ammonium bicarborate diethylene triamine penta acetic acid (Soltanpour & Workman, 1979). Supernatant was decanted and 5 mL of supernatant was added to 20 mL of AB-DTPA extracting solution. The mixture was then shaken on a reciprocating shaker for 15 min at 180 cpm in open flasks and extract was stored in plastic bottles.

Test for available P. Broth P was determined by Ascorbic acid method (Watanabe & Olsen, 1965). One mL of broth sample extract was taken in 50 mL conical flask and 9 mL of distilled water + 2.5 mL of freshly prepared colour reagent [12 g ammonium molybdate $\{(NH_4)6Mo_7.4H_2O\}$+250 mL distilled water and 0.2908 mg antimony potassium tartrate $\{K(SbO)C_4H_4O_6.1/2\ H_2O\}$ in 1000 mL of 5N H_2SO_4(148 mL conc.$H_2SO_4L^{-1}$)]. Both the solutions were mixed and the volume was raised upto 2 L. 140 mL of this mixture was added to 0.74 g Ascorbic acid and stirred gently. The optical density of the blue colour developed after 15 min was measured at 880 nm by spectrophotometer and the concentration of available P (ppm) was calculated.

ii. Phosphorus immobilized. Pellets of centrifuged cultures after decanting supernatant were taken in a 50 mL calibrated pyrex Kjeldahl flask. Ten mL of percholoric acid and nitric acid mixture (1: 2) was added to the pellet. Samples were digested on a micro Kjeldahl digestion rack using gentle heat until clear solution was obtained. The volume of samples was reduced up to 2-3 mL. When effervescence stopped, volume of digest was made up to 100 mL and stored in plastic bottles.

Estimation of P. Immobilized P was estimated by "Vanadomolydbdo phosphoric acid colour method" (Jackson, 1958). To 5 mL plant digest, 5 mL colour reagent (50 gL^{-1} Ammonium molybdate + 2.5 gL^{-1} Ammonium vanadate + 20 mL conc. HNO_3 + 250 mLL^{-1} HNO_3) was added. Same procedure was repeated with P standards of 5, 10, 15, 20, 25 and 30 ppm. After 30 min, optical density of yellow colour was noted with spectrophotometer at 430 nm and concentration of percent immobilized P was calculated.

Organic acids determination. Organic acids produced by PSM strains in broth medium were analyzed by high performance liquid chromatography (HPLC). Supernatant of samples, centrifuged at 1500 rpm for 15 min was taken. Samples were passed through 0.45 μm syringe filters and injected with 20 μL injection loop into the column. These were determined by Biorad's ion-exchange column of aminex 97-H (25x4.6 mm), mobile phase 0.001 N H_2SO_4 at the flow rate of 0.6 mL min^{-1}. The column was set at 45^0C temperature. The samples were detected at Refractive Index (RI) detector. The RI impulse was read at turbochrom navigator programme in gL^{-1} after running standards of organic acids (Lopez & Gomez, 1996).

Statistical analysis. Simple correlation was run to determine correlation coefficients. The result means were depicted diagrammatically using computer programme MSTATC and Microsoft Excel version 5.0 graphic facility.

RESULTS AND DISCUSSION

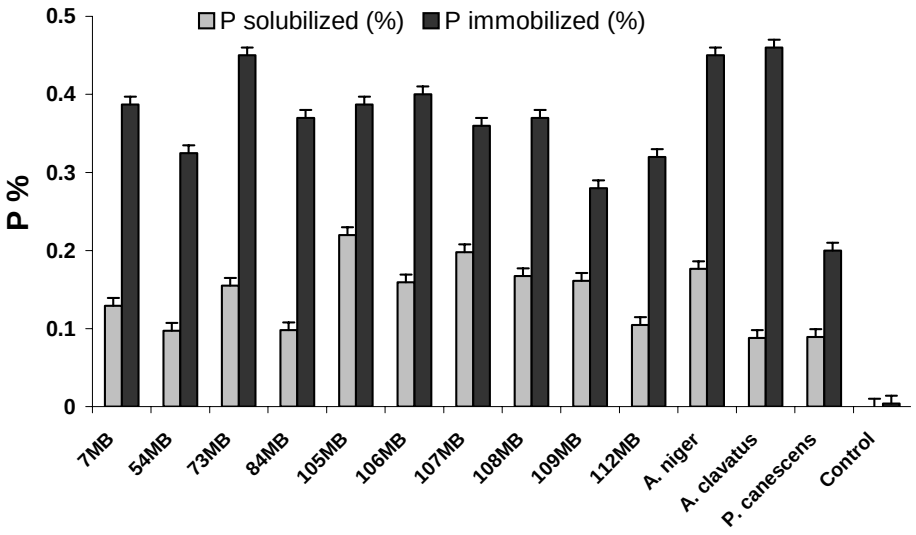
Solubilization index (SI). Solublization index based on colony diameter and holozone for each PSM isolate is presented in Table I. Results showed that among PSB, 107MB was most efficient phosphate solubilizer on PA plates with SI = 3.29. Where as among fungi *P. canescens* showed highest solubilizing index. Studies on agar plates revealed that phosphate solubilizing microorganisms formed clear zones by solubilizing suspended tricalcium phosphate. Measurements ranged from 2.9-7 cm for bacteria and 4.07-8.84 cm for fungi (data not shown). Generally, halozone increased with increase in colony diameter. Fluctuations in solubilization index were observed during the seven days observation period. In most of the cases it gradually increased while in few cases (73MB, 105MB, 106MB, 107MB and 109MB) it first decreased and then increased (7MB, 54MB and *Aspergillus clavatus*). Fungi produced larger halozones than bacteria. These results are in accordance to Chabot *et al.* (1993) and Nahas (1996). Similar results were found by Kucey (1983, 1987), Edi-Premono *et al.* (1996) and Kumar and Narula (1999). But solubilization index (SI) of most efficient bacteria (73MB, 84MB, 106MB, 107MB, 108MB, 109MB and 112MB) was greater than that of fungi. Most of PSM strains lost their ability to form halozone on PA medium on repeated subculturing. This result is in accordance to Kucey (1989), and Illmer and Schinner (1992).

pH. Most phosphate solubilizing microorganisms studied lowered the pH of PB medium as compared to uninoculated sterile PB control incubated for seven days under conditions as inoculated (Table II). Fungi and bacteria both were found to be equally active in lowering the pH. Drop in pH by PSM ranged from 4 to 3.2 at the end of incubation period. Few PSM isolates (73MB, 109MB and *A. clavatus*) although showed larger halozones but, did not lower the pH accordingly. Fluctuations in pH drop were also noted. pH studies showed a drop of pH from 7.01 (control) to 3.2 (107MB, *A. niger*). Similar results were observed by Bar – Yosef *et al.* (1999), and Khalil and Sultan (2000).

Solubilized and immobilized P. P solublization and

immobilization data indicates that bacterial isolates solubilized more P than fungi, which in turn immobilized more P (Fig. 1). Results also indicate that more P was immobilized than solubilized. These results are in contrast to the findings of previous studies (Kucey, 1983; Nahas, 1996). This contradication may be due to the fact that fungi immobilize more P because they form larger colonies and their biomass is greater than bacteria (Illmer & Schinner, 1995). Based on these results it may be concluded that 105MB and *A. niger* were the most efficient strains among PSB and PSF, respectively, where as *P. canescens* was least efficient. Solubilized P by all studied PSM strains was higher than uninoculated control. Values of solubilized P ranged from 0.088 - 0.22% and the values for P immobilized ranged from 0.20 - 0.46%. Illmer and Schinner (1995) noted that P contents of PSM biomass varied from 0.4 - 0.8%. Johri *et al*. (1999) found 0.036 - 0.44% P solubilized by PSM.This difference might be due to different strains used by them.

Fig. 1. Per cent P solubilized and immobilized (+S.E) by selected phosphate solubilizing microorganisms isolated from maize rhizosphere



Organic acids. Organic acids produced by PSM isolated

Table I. Solubilization index of selected strains of phosphate solubilizing bacteria and fungi

Strains	Solubilization Index through Seven Days						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7MB*	1.21	1.27	1.20	1.35	1.44	1.52	1.63
54MB	1.45	1.40	1.30	1.36	1.47	1.63	1.91
73MB	1.55	1.80	1.98	2.17	2.28	2.33	2.50
84MB	3.30	3.13	3.45	3.67	3.27	2.77	2.48
105MB	1.45	1.46	1.47	1.44	1.65	1.77	1.77
106MB	2.34	2.34	2.73	2.70	2.71	2.73	2.39
107MB	2.24	2.25	2.33	2.62	2.81	2.96	3.29
108MB	2.44	2.50	2.31	2.11	2.21	2.20	2.21
109MB	1.25	1.50	1.84	1.93	2.22	2.22	2.24
112MB	6.30	3.75	2.80	2.59	2.22	2.40	2.54
Aspergillus niger	1.21	1.15	1.86	1.80	1.76		
A. clavatus	3.02	2.24	1.62	1.62	1.99		
Penicillium canescens	1.92	2.21	2.08	1.53	2.03		

*MB= Maize Bacteria; Solubilization index for fungi was determined for five days only because of fungal over growth.

Table II. Drop in pH by selected strains of phosphate solubilizing bacteria and fungi

Strains	pH Drop through Seven Days						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control	7.04	7.04	7.04	7.04	7.04	7.04	7.04
7MB	7.04	4.10	4.02	3.84	3.89	3.76	4.04
54MB	7.04	4.82	3.95	3.68	3.52	3.45	3.43
73MB	7.04	5.19	4.74	4.55	4.62	4.60	4.30
84MB	7.04	3.89	3.58	3.38	3.30	3.29	3.33
105MB	7.04	3.55	3.44	3.25	3.25	3.23	3.33
106MB	7.04	3.83	3.73	3.61	3.49	3.39	3.43
107MB	7.04	4.37	4.58	4.29	3.60	3.33	3.20
108MB	7.04	4.50	4.44	4.39	4.29	4.25	4.33
109MB	7.04	5.06	3.65	3.54	3.40	3.45	3.40
112MB	7.04	4.38	3.76	3.72	3.71	3.70	3.25
Aspergillus niger	7.04	6.83	5.58	4.91	4.59	3.91	3.20
A. clavatus	7.04	6.93	6.40	5.50	4.34	4.32	4.03
Penicillium canescens	7.04	7.03	5.08	4.70	3.40	3.33	3.30

from Maize *Zea mays* L. rhizosphere have been presented in Table III. Results indicated that highest acid producing strains were 112MB (3.09 gL^{-1}), 107MB (2.96 gL^{-1}), 84MB (2.54 gL^{-1}) while lowest was 73MB (0.36 gL^{-1}). Most common acids produced were citric and oxalic acid while acetic acid was produced by one strain (107MB). 112MB produced highest concentration of citric acid (2.385 gL^{-1}). Among fungi *A. clavatus* produced more acids (1.88 gL^{-1}). Lowest values were recorded for *P. canescens* (1.07 gL^{-1}). Studies related to the production of organic acids have shown that citric and oxalic acids were two major organic acids produced by PSM. An unidentified peak of unknown acid was found with citric acid in the case of strains 84MB, 107MB, 108MB, 109MB, 112MB, *A. niger, A. clavatus* and *P. canescens*. Production of oxalic acid as major organic acid from different species of *Aspergillus*, an unknown species of *Penicillium* and *Sclerotium rolfsii* was reported by Banik and Dey (1983), Gupta *et al.* (1994), and Illmer and Schinner (1995). Kim *et al.* (1998) reported oxalic acid production by *Enterobacter agglomerans* (PSB). Gluconic acid was third common acid produced by PSM except 73MB and 109MB.This finding is similar to Illmer and Schinner (1995) indicating small quantities of gluconic acid produced from *A. niger*. However, it contradicts the reports by Illmer and Schinner (1992) and Whitelaw *et al.* (1999) that gluconic acid was not produced by PSM strains used in their experiments. Besides citric, oxalic and gluconic following organic acids were produced in traces: Fumaric acid (84MB, 107MB), Succinic acid (108MB, 109MB) and Acetic acid (107MB). Venkateswarlu *et al.* (1994) detected lactic acid from *A. niger* while non of the 13 isolates produced lactic acid in the present study. Contradiction or similarities may be explained on the basis that type and quantity of acid produced depends on the PSM strain, media composition, growth conditions and several other factors. Cunningham and Kuiack (1992) reported that citric acid was major organic acid produced by *P. bilji* when nitrogen source was nitrate but it was not detected when ammonium was used. PSM used glucose as carbon source to produce organic acids. These results are similar to Nautial *et al.* (1999). They stated that more organic acids are produced by microorganisms when glucose was used as carbon source.

Correlation Coefficients. Correlation coefficient (Table IV) values showed significant ($p< 0.05$) correlation between P solubilized, P immobilized and solubilization index. This result is similar to Illmer and Schinner (1995). But P solubilized was non-significantly correlated with colony diameter (r = 0.106) and colony+halozone diameter(r = 0.18). Significant ($p< .05$) and negative correlation was found between pH and P solubilized, P immobilized, solubilization index and halozone produced. Similar correlation is reported by Kumar and Narula (1999) and Whitelaw (2000). Least correlation was found with P solubilized and colony + halozone diameter (r = 0.106). Non-significant positive correlation was found between P solubilized and halozone produced. This result is similar to Kucey's (1983) study but opposite to the findings of Edi-Premono *et al.* (1996) and Kumar and Narula (1999). This is because most PSM show fluctuatios in their behaviours. Production of halozone on solid medium is important but it is not necessary that PSM with larger halozones would solubilize more phophorus. Johri *et al.* (1999) confirmed that criterion for isolation of efficient PSM based on formation of a visible zone on agar plates was not a reliable

Table III. Organic acids produced by phosphate solubilizing microorganisms Isolated from maize rhizosphere

*PSM	Total Acids	Oxalic Acid	Citric Acid	Gluconic Acid	Succinic Acid	Fumaric Acid	Acetic Acid
7MB	1.204	0.409	0.7	0.095	-	-	-
54MB	0.499	0.175	0.29	0.034	-	-	-
73MB	0.359	0.21	0.149	-	-	-	-
84MB	2.54	0.67	1.7	0.13	-	0.0386	-
105MB	1.436	1.09	0.24	0.106	-	-	-
106MB	1.746	0.67	1.01	0.06	0.0064	-	-
107MB	2.963	0.7	2.06	0.15	-	0.049	0.0035
108MB	1.74	0.554	1.14	0.026	0.0244	-	-
109MB	1.21	0.498	0.7	-	0.009	-	-
112MB	3.09	0.64	2.385	0.0724	-	-	-
Aspergillus niger	1.33	0.756	0.53	0.047	-	-	-
A. clavatus	1.88	0.745	1.087	0.049	-	-	-
Penecillium canescens	1.068	0.55	0.43	0.088	-	-	-
Control	-	-	-	-	-	-	-

* PSM:Phosphate Solubilizing Microorganisms; **MB:Maize Bacteria

Table IV. Correlation coefficients of different biochemical characters of PSM isolated from maize

	P Immobilized (%)	Solubilization Index	Colony Diameter (cm)	Halozone (cm)	pH	Organic acid (g/L)
P Solubilized (%)	0.67**	0.553*	0.106	0.18	-0.57*	0.44
P Immobilized (%)		0.575*	0.67**	0.472	-0.69**	0.37
Solubilization Index			0.058**	0.485	-0.79**	0.825**
Colony Diameter (cm)				0.515*	-0.302	0.11
Halozone (cm)					-0.625**	0.468
pH						-0.49

* significant ($p<0.05$); ** highly significant ($p<0.01$)

technique. It has also been studied that some PSM lost their ability to produce halozone on sub culturing (Kucey, 1983). Highest positive correlation was obtained with solubilization index and organic acids produced (r = 0.825). This is due to the fact that perhaps organic acids are the main factor responsible for P solubilization. A non-significant positive correlation was observed between P solubilized and organic acids produced by PSM suggesting that organic acids may play important role but are not the only possible mechanism for P solubilization (Illmer & Schinner, 1992). pH and organic acids were also found to be non significantly correlated with each other. Other reasons may be involved for drop in pH e.g microbial respiration (Illmer & Schinner, 1992).

CONCLUSION

It is concluded from the present study that PSM showed variation in their biochemical charateristics. Organic acid production was perhaps not the only possible reason for phosphosrus solubilization. Present study also showed that 105MB and 107MB are the most efficient strains on the basis of their P solubilizing activity. Further research should be continued with such efficient PSM isolates. These may be used for inoculum production and their inoculation effect on the plant growth be studied *in vivo*.

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