Secretion of Organic Acids by Phosphate Solubilizing Bacteria Isolated from Oxisols

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ABSTRACT

Secretion of Organic Acids by Phosphate Solubilizing Bacteria (I. D. Prijambada, J. Widada, S. Kabirun, and D.Widianto): Phosphorus availability is a major limiting for crop production. Bacterial solubilization of insoluble inorganic phosphate has been studied as a means of providing available phosphorus for crop production. Bacterial abilities to solubilize calcium phosphate and rock phosphate have been identified to be related with their abilities to produce gluconic acid and ketogluconic acid. However, there is no information regarding the relationship between bacterial ability to solubilize aluminum phosphate and their ability to produce organic acids. This study was conducted to investigate the relationship between bacterial ability to solubilize calcium and aluminum phosphates with their ability to produce organic acids. Bacterial ability to solubilize calcium and aluminum phosphates were determined as the concentration of soluble phosphate in the filtrate of bacterial cultivation media, while bacterial ability to produce organic acids were assessed from the accumulated organic acids in its. The results showed that bacterial abilities to solubilize calcium and aluminum phosphates well related to their abilities to produce organic acids. Organic acids related with the solubilization of calcium phosphate differ from the ones related with the solubilization of aluminum phosphate. Moreover, there is similarity in the production of organic acids related to the solubilization of aluminum phosphates and iron phosphate.

Keywords: Aluminum phosphate solubilization, calcium phosphate solubilization, organic acids production, phosphate solubilizing bacteria

INTRODUCTION

Phosphorus is an essential nutrient for biological growth and development. Plants obtain their needed phosphorus from their external environment in a soluble ionic form. This circumstance has metabolic consequences since the availability of phosphorus in soil is highly influenced by soil pH. Phosphorus is fixed as insoluble iron and aluminum phosphates in acidic soils or as calcium phosphates in alkaline soils (Tunesi *et al.*, 1999; Lopez-Pineiro and Garcia-Navarro, 2001; Zhang *et al.*, 2001). Phosphorus is most readily available at pH around 6 to 7 (Curtin and Syers, 2001; and Beauchemin *et al.*, 2003).

Although mechanism of phosphate solubilization is still not fully understood, the production of organic acids seems to be the main mechanism (Alam *et al.*, 2002; Siddique and Robinson, 2003). Organic acids were found to be responsible for tricalcium phosphate and rock phosphate solubilization (Kumari *et al.*, 2008). Addition of EDTA was found to increase solubilization of tricalcium phosphate (Sridevi *et al.*, 2007). Organic acids were also found to mediate the release of phosphorus bound to iron oxides (Johnson and Loeppert, 2006). The ability of *Pseudomonas corrugata*, *Burkholderia cepacia*, *Azospirillum brasilense*, and *A. lipoferum*, rock phosphate solubilizing bacteria, were associated with their ability to produce gluconic acid and ketogluconic acids (Trivedi and Sa, 2008; Rodriguez *et al.*, 2004; and Song *et al.*, 2008). However, there has been no report relating the ability of bacteria to solubilize aluminum phosphate with their ability to produce organic acids.

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The objective of the present study was to examine the production of organic acids and its relation to the ability to solubilize insoluble inorganic phosphates by calcium and aluminum phosphate solubilizing bacteria.

MATERIALS AND METHODS

Preparation of Bacteria and Medium

The phosphates solubilizing bacteria were isolated from Oxisols in Central Java, Indonesia (pH(H_20) 5.6 – 6.0, pH (KCl) 5.2 – 5.9, exchangable Al 0.02 – 0.23 meq, and available P 9.3 ppm) by pour plate method on a modified Pikovskaya medium containing (L^{-1}) glucose (10.0 g), $(NH_4)_2SO_4$ (0.5 g), NaCl (0.5 g), KCl (0.2 g), $MgSO_4.7H_2O$ (0.1 g), $MnSO_4$. H_2O (0.001 g), yeast extract (0.5 g), $Ca_3(PO_4)_2$ (5.0 g), and agar (15.0 g) (Rao and Sinha, 1963). To prepare Pikovskaya broth, the agar was removed. When needed, $Ca_3(PO_4)_2$ is replace with $AlPO_4$ (4.0 g L^{-1}) or $FePO_4$ (5.2 g L^{-1}).

Phosphate Solubilizing Capability

Solubilization of phosphate in agar medium was assayed by inoculating one loop of cells onto Pikovskaya agar and then incubated at room temperature for 14 d. Phosphate solubilization capability of bacterial isolates was indicated by the appearance of clear zone around the bacterial colony. The size of the clear zone indicates phosphate solubilizing capability. The results were expressed as solubilization efficiency (E):

$$E = \frac{\text{Solubilization diamter (s) - Growth diameter (g)}}{\text{Growth diameter (g)}}$$

Solubilization of phosphate in liquid medium was assayed in triplicate by inoculating 100 mL Pikovskaya broth with 1 mL cells (10^9 cells mL^{-1}) and then incubated at room temperature on a rotary shaker for 14 d. The cultures were then centrifuged at 4,000 rpm for 15 min. Autoclaved uninoculated medium was served as control. The cultures were harvested by filtration with Whatman paper filter no. 42. Soluble phosphate in the supernatant was determined by molybdenum blue method of Olsen and Sommers (1982).

Organic Acid Analysis

After centrifugation at 4,000 rpm for 15 min, the supernatants were filtered through a 0.2-µm millipore filter. Low-molecular-weight aliphatic organic acids in the supernatants were analyzed by high performance liquid chromatograph (Shimadzu CTO-6A) equipped with ORH-801 Organic Acid Column and a UV-VIS SPD-6AV detector. About 20 µL of the filtered supernatant were injected into the chromatograph using a glass syringe and eluted isocratically with 0.02 *N* H_2SO_4 at a constant flow rate of 0.6 mL min^{-1} for 10 min at 25°C. Peaks of organic acids were detected at a wave length of 214 nm. The organic acids in the supernatant were identified by their retention time, corresponding with their standards.

RESULTS AND DISCUSSION

Isolates of Phosphate Solubilizing Bacteria

Thirty four phosphate solubilizing bacterial isolates were obtained from Oxisols in Central Java, Indonesia. Twelve of them, which appeared to have much higher clear zone, were selected for further examination. Table 1 shows solubilization efficiency, cell morphology, and Gram staining characteristic of the selected isolates.

Solubilization of Tricalcium and Aluminum Phosphate

To examine the ability of the twelve isolates in solubilizing insoluble inorganic, the isolates were grown in Pikovskaya broth in which tricalcium or aluminum phosphates were used as their phosphate sources. The results of the examination were shown in Table 2.

U1 and G2 isolates show similar capabilities in solubilizing tricalcium phosphate. The concentration of soluble phosphate in Pikovskaya broth supplemented with insoluble tricalcium phosphate and inoculated by U1 and G2 isolates were 5.67 and 5.57 mg mL^{-1}, respectively. However, capabilities of the two isolates to solubilize aluminum phosphate were differed significantly. The concentration of soluble phosphate in Pikovskaya broth supplemented with insoluble aluminum phosphate and inoculated by U1 and G2 isolates were 5.47 and 0.26 mg mL^{-1}, respectively. The U1 and G2 isolates were rod shape bacteria having positive and negative Gram staining characteristic, respectively. T1 isolate, which is also rod shape bacterium having negative Gram staining characteristic, has a similar capability to U1 isolate in solubilizing both tricalcium as well as aluminum

Table 1. Solubilization efficiency, cell morphology, and Gram staining characteristic of the selected isolates.

Isolate	Colony diameter (mm)	Clear zone diameter (mm)	Solubilization efficiency	Cell morphology	Gram staining
T1	4	31	6.8	Rod	Negative
O1	6	20.5	3.5	Coccus	Negative
S6	4	18	3.0	Coccus	Negative
S7	5.5	19.5	2.6	Rod	Negative
I3	7	21	2.5	Rod	Negative
N1	4.5	18	2.5	Coccus	Negative
U3	5	18	2.4	Coccus	Negative
U13	5	17.5	2.0	Coccus	Negative
U1	7.5	19.5	1.6	Rod	Negative
J9	8	20	1.5	Rod	Negative
T2	8	20	1.5	Rod	Positive
G2	8	19.5	1.4	Rod	Negative

Table 2. Tricalcium and aluminum phosphates solubilization by the selected isolates.

Isolate	Soluble phosphate concentration in the broth (mg mL^{-1})	
	Calcium phosphate	Aluminum phosphate
T1	4.76	5.95
O1	4.92	5.63
I3	5.93	2.58
S6	6.58	3.37
S7	6.48	2.05
N1	6.38	9.32
U3	4.00	6.74
U13	7.72	7.89
U1	5.67	5.47
J9	2.31	0.84
T2	1.17	0.58
G2	5.57	0.26

phosphates. The concentration of soluble phosphate in Pikovskaya broth inoculated by T1 isolate and supplemented with insoluble tricalcium or aluminum phosphates were 4.76 and 5.95 mg mL^{1}, respectively. As the ability to solubilize insoluble phosphates is associated with the capability to produce organic acids, the organic acids secreted by the isolates into the Pikovskaya broth supplemented either with tricalcium or aluminum phosphates were then examined.

Organic Acids Accumulated in the Pikovskaya Broth

Accumulation of organic acids by the isolates in the Pikovskaya broth supplemented either with tricalcium or aluminum phosphates were observed using HPLC. Malic, tartaric, citric, succinic, lactic, formic and asetic acids, which were found to present in soil and be associated with rock phosphate solubilization (Hue *et al.*, 1986; Bolan *et al.*, 1994; Kumari *et al.*, 2008), were used as standards. Figure 1 shows HPLC chromatogram of standard organic acids. Citric, tartaric, malic, succinic, lactic, formic, and acetic acids have retention times of 5.7, 6.4, 7.7, 9.6, 9.9, 10.4, and 11.4 min, respectively (Figure 1). An organic acid which has a retention time of 4.4-4.5 min was employed as an internal standard.

HPLC chromatograms of organic acids accumulated in Pikovskaya broth supplemented with tricalcium phosphate and inoculated with G2, T1 and

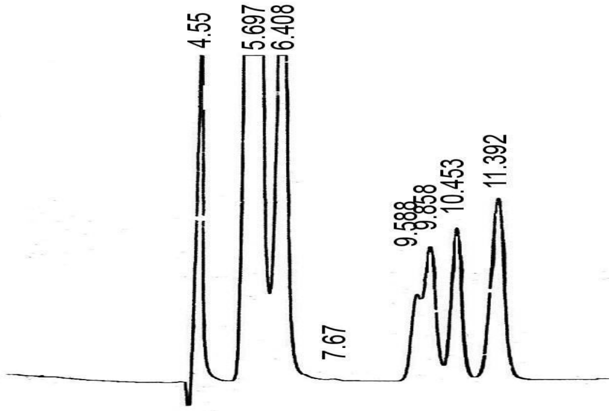


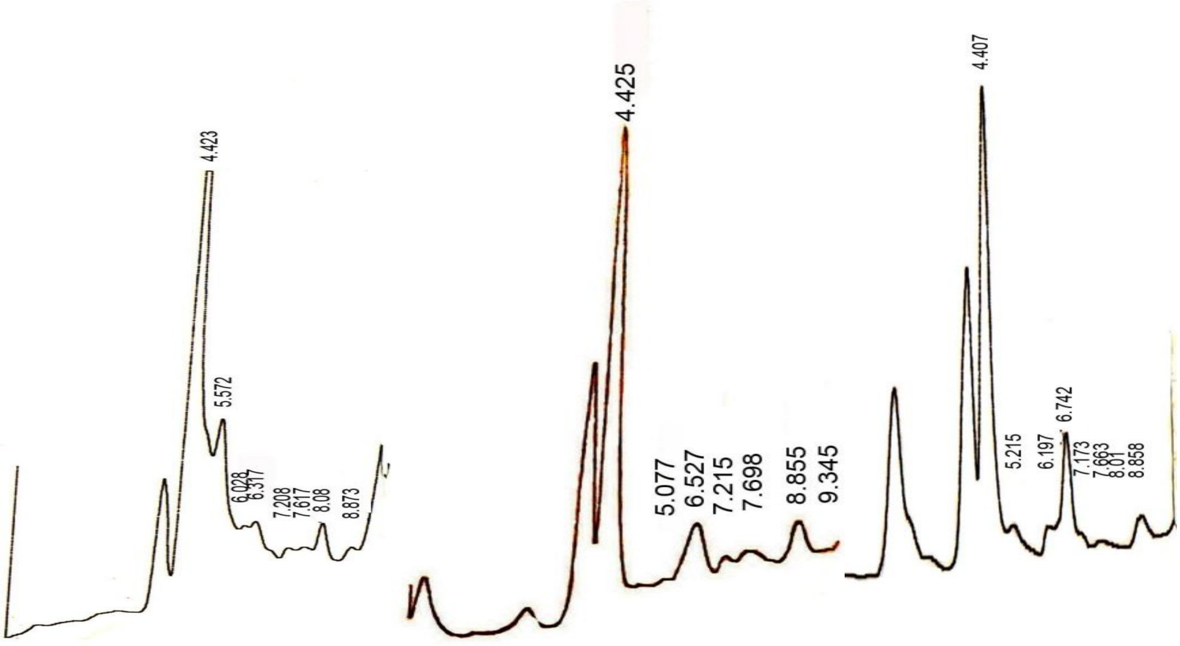
Figure 1. HPLC chromatogram of standard organic acids. Citric, tartaric, malic, succinic, lactic, formic, and acetic acids which have retention times of 5.7, 6.4, 7.7, 9.6, 9.9, 10.4, and 11.4 min, respectively, were used as standard organic acids.

U1 isolates were shown in Figure 2. The three isolates were found to accumulate organic acids having retention times of 7.2, 7.6 (approximating the retention time of malic acid), 8.0, and 8.8 min (Figure 2). Similarity in the accumulated organic acids coincides with similarity in the capability of the three isolates to solubilize tricalcium phosphate in Pikovskaya broth supplemented with tricalcium phosphate.

Bolan *et al*. (1994), Sagoe *et al*. (1997), Alam *et al*. (2002), and Kumari *et al*. (2008) have reported that malic, tartaric, oxalic, and citric acids have high capability to release soluble phosphate from insoluble tricalcium phosphate as well as rock phosphate. U1 and G2 isolates also accumulate tartaric and citric acids, respectively. Both isolates were able to release more soluble phosphate than T1 isolate. These results suggest that the ability of our isolates to solubilize tricalcium phosphate was attributed to their ability to produce organic acids, especially malic, tartaric, and citric acids.

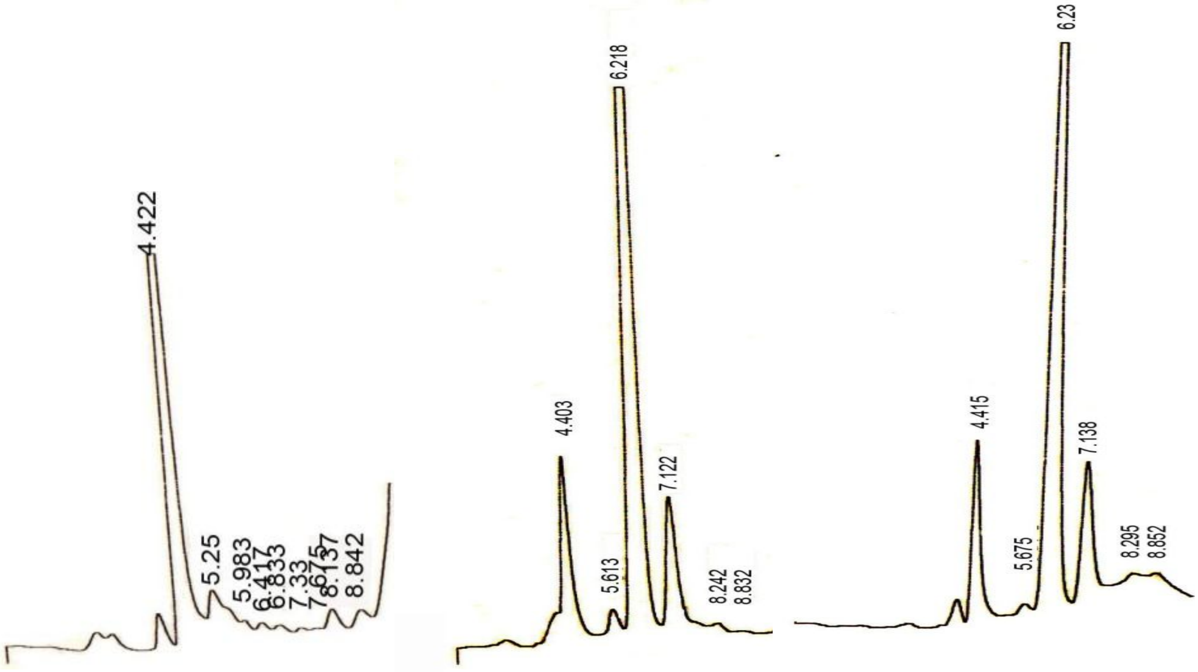
HPLC chromatograms of organic acids accumulated in Pikovskaya broth supplemented with aluminum phosphate and inoculated with G2, T1 and U1 isolates were shown in Figure 3.

T1 and U1 isolates, which has high ability to solubilize aluminum phosphate, accumulated organic acids having retention times of 5.6 (approximating the retention time of citric acid), 6.2 (approximating the retention time of tartaric acid), 7.1, 8.3, and 8.8 min (Figure 3). Tartaric acid and an organic acid having retention time of 7.1 min were the dominant ones. G2 isolate, which has a very low capability in solubilizing aluminum phosphate, did not accumulate



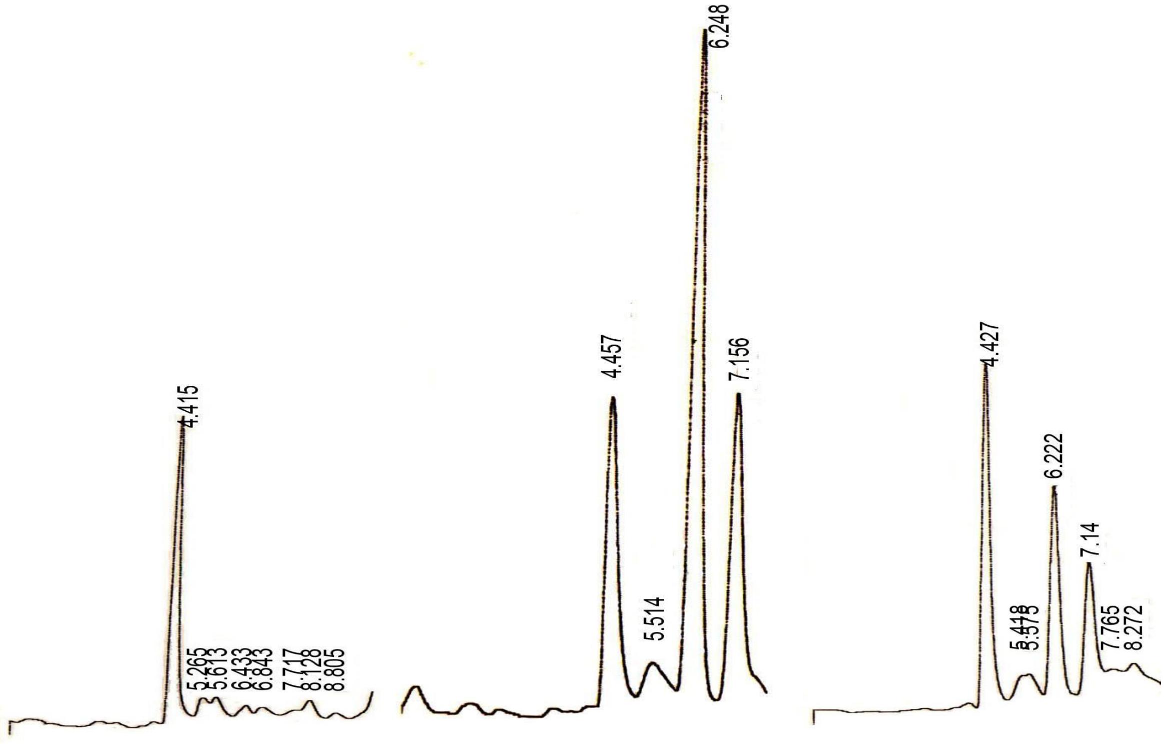
Isolate G2 Isolate T1 Isolate U1

Figure 2. HPLC chromatograms of organic acid accumulated in Pikovskaya broth supplemented with tricalcium phosphate and inoculated with G2, T1 and U1 isolates.



Isolate G2 Isolate T1 Isolate U1

Figure 3. HPLC chromatograms of organic acid accumulated in Pikovskaya broth supplemented with aluminum phosphate and inoculated with G2, T1 and U1 isolates.



Isolate G2 Isolate T1 Isolate U1

Figure 4. HPLC chromatograms of organic acid accumulated in Pikovskaya broth supplemented with iron phosphate and inoculated with G2, T1 and U1 isolates.

those acids. Fox *et al.* (1990), Johnson *et al.* (2006), and Fankem *et al.* (2008) have reported that malic, tartaric, malonic, oxalic, and citric acids were highly capable in releasing aluminum bound phosphate. These results suggest that the ability of T1 and U1 isolates to produce citric, tartaric, and organic acids having retention time of 7.1, 8.3, and 8.8 min may determine their abilities to solubilize aluminum phosphate.

Aluminum bound phosphates were often found along with iron bound phosphate in acidic soil (Sanchez, 1976). Moreover, similarity of iron and aluminum characteristics (Lopez-Hernandez *et al.*, 1979; Cline *et al.*, 1983) attract us to examine the accumulation of organic acids in Pikovskaya medium supplemented with iron phosphate and inoculated with the three isolates. Figure 4 shows HPLC chromatograms of organic acids accumulated in Pikovskaya broth supplemented with iron phosphate and inoculated with G2, T1 and U1 isolates.

G2, T1 and U1 isolates inoculated into Pikovskaya media supplemented with iron phosphate accumulated similar organic acids to the ones supplemented with aluminum phosphate. T1 and U1 isolates accumulated organic acids having retention times of 5.5 (approximating the retention time of citric acid), 6.2 (approximating the retention time of tartaric acid), and 7.1 min (Figure 4). U1 isolate also accumulated a small amount of organic acids having retention time of 7.8 min (approximating the retention time of malic acid), and 8.3 min. G2 isolate also did not accumulate those acids.

Accumulation of organic acids in Pikovskaya medium supplemented with iron phosphate coincided with the accumulation of soluble phosphate in the medium. Pikovskaya medium supplemented with iron phosphate and inoculated with G2, T1, and U1 isolates accumulated 0.39, 1.46; and 3.31 mg P mL^{-1}, respectively. These results are in agreement with Cline *et al.* (1983), Johnson *et al.* (2006), and Fankem *et al.* (2008) who have also reported that malic, tartaric, and citric acids were highly capable in releasing iron bound phosphate.

CONCLUSION

From the described results above, it can be concluded that the abilities of G2, T1, and U1 isolates to solubilize insoluble inorganic phosphate were highly related to their abilities to produce organic acids. Moreover, the results also show that the organic acids involved in the solubilization of tricalcium phosphate were differed from that involved in the solubilization of aluminum and iron phosphates.

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