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Organic Acid Production as a Function of Phosphate Solubilization by *Pseudomonas aeruginosa* Strain An-G Isolated from Temperate Zone of Himachal Pradesh

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An efficient phosphate solubilizing *Pseudomonas aeruginosa* strain An-G isolated from apple rhizosphere of Himachal Pradesh, exhibited solubilization of tricalcium phosphate (TCP) in Pikovskaya's broth (47μg/ml). A decline in the pH of the medium was observed after three days of incubation at 28±2°C from 7.0 - 4.02 during the solubilization of phosphate substrate (TCP). Among the different organic acids which were estimated through HPLC-MS/MS, succinic acid was produced in higher concentration (2.016μg/ml). Other major organic acids produced were citric acid, malic acid and malonic acid in concentration 0.162 μg/ml, 0.20 μg/ml and 0.39μg/ml respectively. The production of fumaric acid, tartaric acid, quinic acid, lactic acid and schimic acid were observed in small amount during the solubilization of tricalcium phosphate.

Key words: *Pseudomonas aeruginosa,* Phosphate solubilizing bacteria, TCP and Organic acids

Phosphorus is one of the major plant nutrient second to nitrogen required in optimum amount for plant growth and development (Dave & Patel, 1999)[1]. Much of the inorganic P applied as phosphatic fertilizer is rapidly converted to unavailable forms with low solubility in the soil (Vassilev and Vassileva, 2003)[2]. However a large portion of soil as chemical fertilizers is immobilized rapidly and becomes unavailable to the plants (Goldstein, 1986)[3].

Even in phosphorus rich soils, most of this element is insoluble form and only a small proportion (0.1%) is available to the plants (Stevenson and Cole, 1999)[4]. Phosphate-solubilizing rhizobacteria (PSRB) improve soil fertility and soil health by converting insoluble forms of P to soluble forms that is accessible by plants. Consequently, PSRB application has increased tremendously in agriculture (Arcand and Schneider, 2006)[5]. The screening of PSRB from P-deficient soils appears a good strategy for selecting the promising strains for application in sustainable agriculture. *Pseudomonas fluorescens*, *P. poae* and *P. trivialis* are the few efficient PSRB reported from phosphorus deficient and Ca-rich soils from the cold deserts of the Indian trans-Himalayas (Gulati *et al.*, 2008)[6].

In the present study, multiple organic acids production were observed during solubilization of inorganic phosphate (Tricalcium phosphate).

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MATERIALS AND METHODS

Bacterial strain

Pseudomonas aeruginosa strain An-G (NCBI GenBank accession no. KJ500025) isolated from the rhizosphere of apple in temperate zone of HP. The *Pseudomonas* culture was maintained in 20% glycerol at -20ºC was revived on nutrient agar and employed for the present study.

Solubilization of inorganic phosphate substrate

The bacterial strain was grown in triplicate in 10 ml of PVK broth supplemented with 0.5 % of tri-calcium phosphate (TCP) and incubated at 28±2ºC for three days at 150 rpm in a refrigerated incubator shaker. Cultures were centrifuged at 10,000 rpm for 10 min. and passed through 0.22 µm nylon filter. Phosphorus content in culture filtrates was estimated by the method of Olsen *et al*., 1954[7]. The uninoculated autoclaved media was used as control. The values of Phosphorus-liberated are expressed as µg/ml over control. The change in the pH of the culture was recorded using pH meter (E Merck, USA).

Detection and quantification of organic acids during phosphate solubilization

The culture filtrates of the three replicates obtained on solubilization of various phosphate substrates as described above were pooled for the analysis of different organic acids by using an agilent HPLC 1260 coupled with an ABsciex QTRAP 5500 as detector. A 2 ml of three day old cell free supernatant of *Pseudomonas aeruginosa* strain An-G obtained after solubilization of tri-calcium phosphate (0.5%) of sample was homogenized using a vortex mixer and then centrifuged at 10,000 × g at 4ºC for 10 min. The supernatant was filtered through 0.2 µm syringe filter and injected into HPLC-MS/MS system. The samples in autosampler were kept at 4ºC.The organic acids were separated by using a Hi Plex H (7.7 × 300 mm × 8 µm), (Agilent Technologies Pvt. Ltd., Chandigarh, India) column and a guard column (Hi–Plex H 3 × 5mm×8 µm) (Agilent Technologies) maintained at 60°C at a flow rate of 0.6 mL/min. The samples were run isocratically using 0.1 % formic acid in water for 20 min. The MS/MS analysis was performed with a hybrid triple quadrupole/ion trap mass spectrometer, QTRAP 5500 (ABSciex India Pvt. Ltd., Gurgaon, India). The mass spectra were acquired using TurboIonSpray ionization in negative ion mode and scheduled MRM using analyst software.

The compound–dependent MS parameters were determined by infusion of each compound. The curtain gas was adjusted to 30 psi. The ion spray voltage, ion source gas 1, and ion source gas 2 were – 4.5 kV, 50 psi, and 50 psi, respectively. The temperature of the source was ûxed to 550ºC. The authentic standards of organic acids were used for preparation of calibration curve. The organic acids in the samples were determined by comparing the retention times and peak areas of chromatograms with the standards for malic acid, malonic acid, citric acid, tartaric acid, succinic acid, formic acid, lactic acid, quinic acid and schimic acid. The quantification of organic acids was conducted using MultiQuant software.

RESULTS AND DISCUSSION

Solubilization of inorganic phosphate substrate

Phosphorus solubilzing microorgansims produce a variety of organic acids from simple carbohydrates (Bajpai and Sundara Rao, 1971)[8] by virtue of which they solubilize insoluble inorganic phosphate (Banik and Day, 1983; Vanquez *et al*., 2000)[9&10]. The release of soluble phosphate from tri-calcium phosphate usually involves the production of organic acids and a decrease in pH of the medium (Chen *et al*., 2006; Mohammadi, 2012)[11&12]. The result on phosphate solubilization in PVK broth suplemented with TCP by *Pseudomonas aeruginosa* strain An-G was 47µg/

Table 1. Organic acid production during solubilization of phosphate substrate (TCP) by *Pseudomonas aeruginosa* strain An-G after three days of incubation at 28±ºC

Phosphate source	Organic acids (µg/ml)								
	Succinic	Fumaric	Tartaric	Citric	Malonic	Malic	Quinic	Schimic	Lactic
TCP	2.016	0.013	0.065	0.161	0.390	0.200	0.153	0.008	0.091

Values are means of three replicates

ml in three day old cell free supernatant obtained after centrifugation. A decline in the pH of the medium after three days was observed from 7.0 to 4.02 during the solubilization of phosphate substrate (TCP).

Production of organic acids

Total thrity PSBs produced several kinds of organic acids indicating the ability of P solubilzers to produce organic acids like lactic, glycolic, succinic, acetic, oxalic, citirc and malonic acids by PSB have been reported earlier (Illmer and Schinner, 1995)[13]. Goldstein *et al.*, 1993[14] which showed that organic acids like glycolic, gluconic, succinic, oxalic, citirc and malonic acids also have been identified in phosphate solubizers namely *Bacillus firmus*, *Pseudomonas cepacia and Pseudomonas* sp.

HPLC-MS/MS system analysis of culture filtrate showed the presence of multiple organic acids during the solubilization of tricalcium phosphate. Among the different authentic organic acids used for the comparison, production of succinic acid was higher followed by malonic acid, malic acid and citric acid given in Table 1 and Fig 1. Fumaric acid, tartaric acid, quinic acid, lactic acid and schimic acid were produced in small amount during the solubilization of tricalcium phosphate.

Gulati *et al.*, 2010[15] showed that the strain *Acinetobacter rhizosphaerae* BIHB 723 produced different organic acids during the solubilization of

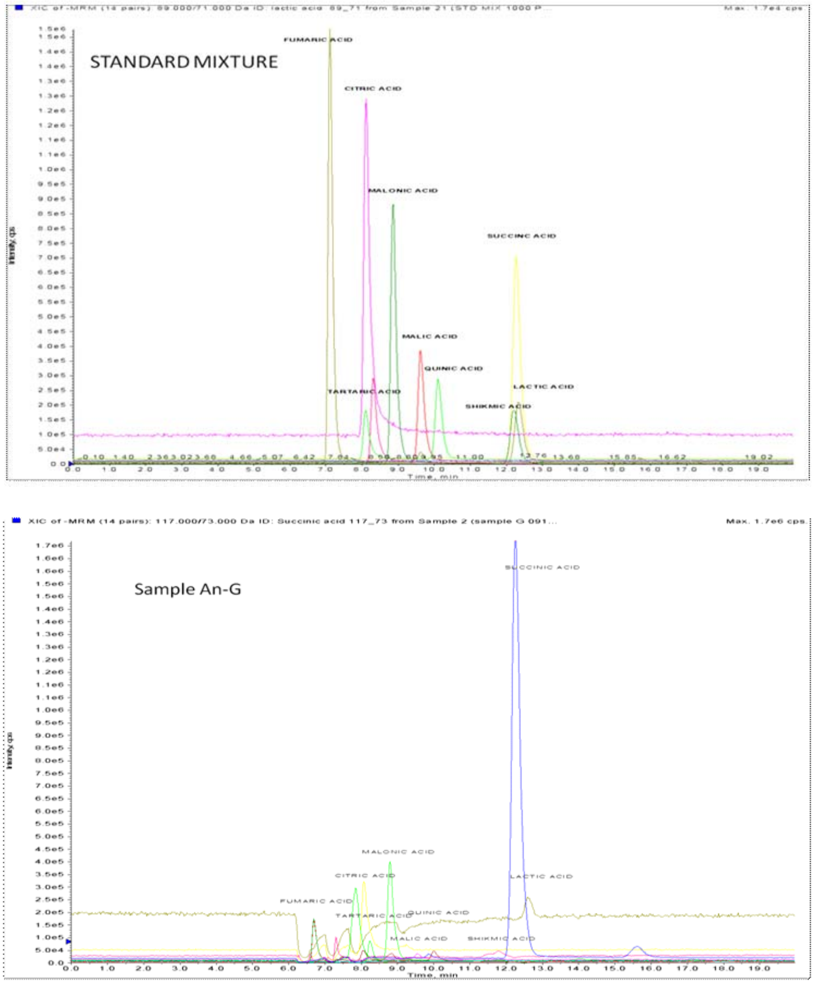

Fig. 1. HPLC chromatograms of authentic organic acids (a) and culture supernatant of *Pseudomonas aeruginosa* strain An-G after three days of incubation at 28±^{0}C in Pikovskaya's broth supplemented with tricalcium phosphate

phosphate substrates explicating the influence of substrate on the production of organic acids i.e oxalic, gluconic, 2-Keto gluconic, lactic, formic and malic acid. The higher solubilization of TCP being due to its amorphous nature and is more facile to solubilization. A decline in the pH of the medium during solubilization of phosphate substrates suggested the secretion of organic acids by *Acinetobacter rhizosphaerae* BIHB 723 as reported for other bacteria (Chen *et al.*, 2006; Illmer and Schinner, 1995)[11 & 13].

It is concluded from the results that *Pseudomonas aeruginosa* strain An-G produced multiple organic acids during P-solubilization (TCP). The phosphate solubilization is an important mechanism of plant growth promotion. The results showed the potential application of *Pseudomonas aeruginosa* strain as a bioinoculant in phosphorus-deficient soils to overcome the phosphorus deficiency.

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