JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2010. Vol. 4(2), p. 717-723

Screening and Isolation of Phosphate Solubilizing *Pseudomonas stutzeri* (EGB_3) from Gut of Earthworm (*Eisenia foetida)*: Solubilization as Influenced by Organic Acids

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(Received: 14 April 2010; accepted: 30 May 2010)

Crops absorb phosphorous in the form of soluble orthophosphate ion. The solubility of phosphate is inhibited by the presence of iron and aluminium in acidic soils and calcium in neutral and alkaline soils. This leads to fixation of phosphorous, making it not available to crop plants. The phosphate solubilizing bacteria (phosphobacteria) secretes organic acids like Gluconic acid, Oxalic acid, Acetic acid etc., which act on insoluble phosphates and convert the same into soluble form, thus providing phosphorous to plant. An experiment was conducted for the detection of organic acids using HPLC technique. Results clearly indicates that Gluconic acid and Oxalic acids were produced by *Pseudomonas stutzeri* in PKVS media.

Key words: Phosphate solubilization, *Pseudomonas stutzeri,* organic acids, Gluconic acid, Oxalic acid.

Many microbial species have the ability to solubilize sparingly soluble phosphorous (P) *in vitro*[1]. Inoculation with effective P solubilizers increases P uptake. Solubilization of inorganic P is one of the direct influences of plant growth promotion[2,3]. The use of P solubilizing microorganisms (PSM) to increase plant nutrient availability and subsequently benefiting the plant growth has been reported for different crops and regions. There have been a number of reports on plant growth promotion by bacteria that have the ability to solubilize inorganic and/or organic P from soil [4,5].

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Phosphate solubilization of microorganisms can be evaluated by two techniques, one is Precipitated phosphate agar plate assay which is used widely in the screening of P solubilizing microorganisms[6-9] and the other is liquid media/culture broth assay, which is a contrast method to the earlier, a direct measurement of phosphate solubilization in liquid media is considered as more accurate[10-12]. The rate of P solubilization is typically estimated by subtracting the final culture solution P from the un-inoculated control of P substrate [2].

It is generally accepted that the major mechanism of mineral phosphate solubilization is by the action of organic acids synthesized by microorganisms[13-19]. Production of organic acids results in acidification of the microbial cell and its surroundings. Consequently, Pi may be released from a mineral phosphate by proton substitution for Ca21 [20]. The production of organic acids by phosphate solubilizing bacteria has been well documented among them, gluconic acid seems to

be the most frequent agent of mineral phosphate solubilization. It is reported as the principal organic acid produced by phosphate solubilizing bacteria such as *Pseudomonas* sp.[21], *Erwinia herbicola* [22] and *Pseudomonas cepacia*[23]. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also been identified among phosphate solubilizers[21,16]. Goldstein AH [20] has proposed that the direct periplasmic oxidation of glucose to gluconic acid, and often 2-ketogluconic acid, forms the metabolic basis of the mineral phosphate solubilization phenotype in some Gram negative bacteria.

Acid is produced as the principle organic acid by phosphate solubilizing bacteria such as *Pseudomonas* sp.[21] and 2- ketogluconic acid is produced by *R. leguminosarum*[14]. Organic acids that solubilize phosphates are mainly: citric, lactic, gluconic, 2-ketogluconic, oxalic, tartaric, acetic, etc [24].

The present investigations were taken up to study (1) the presence and isolation of P-solubilizing organisms from earthworm gut. (2) testing of P-solubilizing ability of the identified isolate and their mechanism of solubilisation under *in vitro* conditions with regard to organic acids.

MATERIAL AND METHODS

Collection of earthworms

Earthworm species (*Eisenia foetida*) used in the study were collected from the top ploughed horizon (0-20cm) of vermicomposting farm yards of S.V Agriculture college, Tirupati.

Preparations of earthworm gut homogenates

Homogenization buffer

The homogenization buffer contained the following per liter: Mineral solution: 50ml, $Na_2HPO_4.2H_2O$ (1.678g), $Na_2HPO_4.H_2O$ (0.948g), mineral solution consists of KH_2PO_4 (10g); NaCl (8g); $MgCl_2.7H_2O$ (1g); $CaCl_2.2H_2O$(0.2g) per liter.

Sample collection

Worms were washed with sterile tap water and allowed on moist filter paper for starvation for 24hours. After starvation they were washed thrice with double distilled water, weighed and sedated with ethanol (40%). The gut behind the gizzard was dissected using aseptic techniques and the gut contents were pooled weighed (1g wet weight) and homogenized for 5min, using vertex mixer by adding 10ml homogenization buffer. Solid matter was separated by centrifugation at 10,000rpm for 15mins and the supernatant was filtered with filter paper of pore size 0.2µm which was then used to isolate earthworm gut bacteria.

Identification of isolates

Based on morphological and biochemical characteristics the isolate EGB_3 was identified as a strain of *Pseudomonas* in our laboratory. Subsequently the isolate was submitted to Bioserve (Hyd) for 16S rRNA sequencing and compared with the sequences of pseudomonas sp. available in the data bank. The sequence analysis for 16SrRNA and Phyllogenetic comparisons was performed by Bioserve (Hyd).

Sequencing of the 16S rRNA

Purified DNA product was adjusted to 100mg/µl concentration in MQ water (pH 8) and sequencing was carried out using primers. Sequencing was carried out in a 313 OXL capillary DNA sequencer (Applied Biosystem) utilizing thermocycling reaction Big Dye termination version 3:1. The 16S rRNA gene was sequenced in both directions by primer walking using primers directed to the conserved regions within the gene.

Phylogenetic analysis

The DNA sequences of the 16S rRNA from the isolate of interest were edited manually and blast searched individually to fish out sequences of homology. The sequences were aligned using the programs CLUSTAL W[25]. The aligned sequences were applied to genetic distance by using Neighbor-Joining method for phylogenetic inference. Phylogenetic tree was visualized using tree program

Screening for the Phosphate Solubilization by EGB3 Isolate

The EGB_3 isolate were evaluated for their ability to solubilize phosphate was studied by using Pikovskay's medium PKVS[16].

Solubilization Index

A pin point inoculation of the EGB_3 on solid formulations was made under aseptic conditions. The plates were incubated at 28 ± 1^0C for 7 days and observed regularly for solubilization zone. Solubilization index (SI) was calculated according to the ratio of the total diameter (colony + halo zone) and colony diameter [26].

Mechanism involved for phosphate solubilization

Screening for the production of organic acids by EGB3 Isolate

Screening for organic acids was done by inoculating the EGB_3 isolate, in PKVS+BPB (Bromo Phenol Blue) medium (modified method of Sangeeta Mehta and Chandra Shekar Nautiyal[12]. Ingredients of the media except agar were taken in 50mL quantity in each 150mL Erlenmeyer flasks and autoclaved at 121^0C, 15 lbs P for 15 min. Each flask containing 50mL of PKVS+BPB media was inoculated with 0.1 mL of *Pseudomonas stutzeri* of 24 hrs old broth cultures set in triplicates. pH was adjusted to 7 ± 1 before sterilization. Autoclaved, un-inoculated broth served as negative control in each case. The inoculated Erlenmeyer flasks with controls were incubated at 28±1^{0}C on rotary shaker at 180 rpm for 20 days. The cultures were harvested by centrifugation at 10,000 rpm for 10 minutes. The culture supernatant obtained was used for qualitative assay. Absorption spectra of bromophenol blue (0.025 mg/mL) was observed at 600 nm using Shimadzu Spectrophotometer.

Detection of Organic acids by HPLC

EGB_3 was grown in PKVS medium (13g/L glucose) [27] on a rotator shaker at 180 rpm and on 5th day cultures were centrifuged at 1000 rpm for 10 min. Supernatant of culture was purified by filtration through sterile micro filter with 0.45 µm pore size and 20µl purified solution was injected to column of HPLC Gemini C18 (silica derivative) system with reverse phase liquid chromatography. Standard solutions of Gluconic acid [28,21], Acetic acid and Oxalic acid [21,16] were also injected into the HPLC column and retention times were recorded with reference to test sample. This experiment was performed with the help of Regional Agricultural Research Station, Tirupati. The operating conditions consisted of

Injection volume	-	20µl
Oven Temperature	-	30^0 C
UV Wavelength	-	226 nm
Flow rate	-	0.5 mL/ min
Run time	-	60 min
Mobile Phase	-	0.2 M
Phosphate buffer		
pH	-	5.7

Retention times between 5-15 peaks were eluted.

Table 1. Morphological and biochemical tests for identification of EGB_3

Test	EGB_3
Colony morphology	
Configuration	Wrinkled, cream, round , concentric
Margins	Smooth
Surface	Butyraceous
Pigmentation	-
Turbidity	+
Opacity	Translucent
Gram's reaction	Negative
Cell shape	Rods
Size(µm)	1 -3 µm in length, width 0.5 µm in width
Arrangement	Singles
Spores	-
Motility	+
Physiological tests	
Growth at temperature (°C)	
4 °C	+
37 °C	+
41 °C	+/w
45 °C	-
Growth in NaCl (%)	
2	+
5	+
7	w
10	w
Growth at pH	
4	-
5	-
6	W
7	+
8	W
Growth under anaerobic condition	+
Biochemical tests	
Indole test	-
Methyl red test	+
Voges proskauer test	-
Citrate utilization test	+
H_2S production	+
Nitrate reduction	-
Gelatin hydrolysis	w
Urea hydrolysis	+
Starch hydrolysis	+
Lectinase	–
Lipase (Tween 80 hydrolysis)	+
Catalase test	+
Oxidase test	+
Denitrification	+
Levan formation from sucrose	-
Arginine dihydrolase	-
Nutritional characteristics	
Starch	+
Maltose	+
Glucose	+
Glycerol	+
suucinate	+
β-Alanine	-
L-histidine	-
L-Arginine	-
L-lucine	-
D-alanine	+

RESULTS

Identification of *Pseudomonas stutzeri* EGB_3

Morphological studies revealed that the isolate EGB_3 was non-pigmented, wrinkled, concentric colonies. The growing cells were Gram negative, aerobic, motile, rod shaped (1.4 to 1.6 µm) turbid, nonspore former. No bacterial growth was observed at pH 5.7 and weak growth was seen at pH 6 in both liquid and solid media used. The isolates grew well in nutrient broth at pH range of 7.5 to 8.5 and showed salt tolerance at NaCl concentration up to10% (w/v). Bacterial growth was observed in the temperature range from 28°C to50°C. With an optimum growth around 37°C. The isolate was positive for the utilization of glucose, arabinose, hydrolysis of gelatin, urea and casein, enzymes test of catalase, oxidase and methyl red, H_2S and citrate. Negative results were observed for indole, nitrate reduction, VP, utilization of L-arginine and Xylose. (Table 1). Partial sequencing of 16SrRNA was performed by Bioserve, India Hyd. BLAST analysis of the sequence data revealed, 100% identity with pseudomonas stutzeri when the sequences were compared with *pseudomonas stutzeri*. EGB_3 was confirmed as pseudomonas stutzeri based on morphological, physiological and biochemical characteristics and also by 16S r RNA sequence analysis.

Phosphate solubilization

The phosphate solubilization capacity of the *P.stutzeri* was detected by clear zones around the colonie on PKVS medium and the diameter of zone was measured.

Mechanism involved for phosphate solubilization

Production of organic acids

Screening for the presence of organic acids was done with EGB3 isolates inoculated in PKVS +BPB (Bromo Phenol Blue) medium [12] on rotator shaker at 180 rpm for 10 days and harvested by centrifugation at 10,000 rpm for 10 minutes. The culture supernatant obtained was used for qualitative assay. Absorption spectrum of 0.021 mg/mL bromophenol blue was observed at 600 nm. The decolourization of -0.16 O.D was observed at 600 nm in culture supernatant of EGB_3.

Detection of organic acids by HPLC

Detection of organic acids from EGB_3 was done by inoculating this isolate in PKVS medium with 13g/L glucose [27] and incubated on a rotator shaker at 180 rpm. On 5th day cultures were centrifuged at 10,000 rpm for 15min. Supernatant of culture was filtered through sterile micro filter with 0.45 µm pore size and 20µl purified solution was injected to column of HPLC-Gemini C18 (silica derivative) system with reverse phase liquid chromatography. This experiment was performed with facilities from of RARS (Regional Agricultural Research Station), Tirupati.

This experiment was performed under the operating conditions of 20µl injection volume, oven temperature-30^0 C, UV wavelength-226 nm, flow rate-0.5 mL/ min, run time-60 min, mobile phase-0.2 M Phosphate buffer and pH -5.7, A typical chromatogram for acid mixture of Acetic acid, Oxalic acid and Gluconic acid appeared (Fig. 1) compared with chromatograms of the standards were obtained under the optimum conditions.

EGB_3 produced peaks for acetic acid, oxalic acid and gluconic acid along with some unidentified peaks. The acids were well separated within retention times respectively (Figure1). Acetic acid, Oxalic acid and gluconic acid was eluted based on their molecular weights. [21], oxalic acid and acetic acid[21,16].

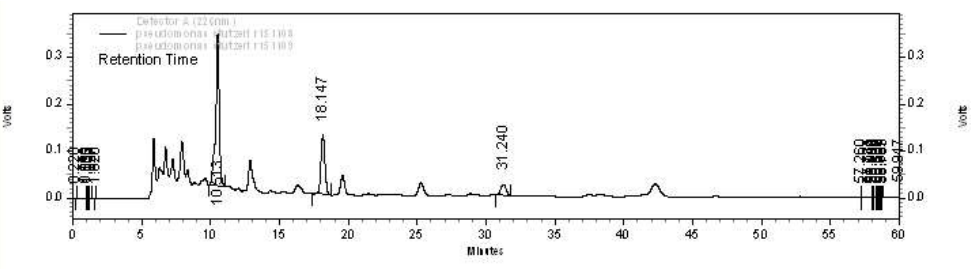

Fig. 1: Chromatogram of *P. stutzeri*

DISCUSSION

Organic acids that solubilize phosphates are mainly of citric, lactic, gluconic, 2-ketogluconic, oxalic, tartaric, acetic, etc [24]. These organic acids are sources of biotical generated H^+ ion able to dissolve the mineral phosphate and to make it available for the plant [29]. Several authors attribute the solubilization of inorganic insoluble phosphate by microorganisms by the production of organic acids from sugars [18,30]. *Pseudomonas cepacia* was isolated by B.Bar- Yosel [31] to solubilize rock phosphate who had determined the production rates of gluconic acid and 2-ketogluconic acid by the bacteria in the presence of clay minerals which prevail in soils, and the resulting rate and extent of orthophosphate release into the suspension solutions. They concluded that this bacterium efficiently produces GA and KGA which can replace the inorganic acids currently used in super phosphate production. Maliha Rashid [27] had isolated ten Phosphate solubilizing bacteria and three fungal strains which were further analyzed for their acid production ability using HPLC technique. HPLC results confirmed that few PSM isolates produced gluconic, fumaric, succinic, acetic and some other unknown acids in smaller concentrations while oxalic and citric acids in larger concentrations in broth cultures. Glucose acted as carbon source in acid production. Gaur [4] also reported that by increasing glucose concentration from one to three percent in the medium rock phosphate solubilization by phosphate solubilizing fungal strains increased acid production. James E. Cunningham and Cathy Kuiack [32] dealt with *Penicillium bilaii* and reported to solubilize mineral phosphate hence an enhanced plant uptake of phosphate. They observed that the major acidic metabolites produced by *P.bilaii* in a sucrose nitrate liquid medium were found to be oxalic acid and citric acid. Safura Babu-Khan[28] reported that mineral phosphate (MP) phenotype was associated with organic acid production and the ability of some Gram-negative bacteria to dissolve poorly soluble calcium phosphates (Mps1 phenotype) is the result of periplasmic oxidation of glucose to gluconic acid via the quinoprotein glucose dehydrogenase (GDH), a component of the direct oxidation pathway.

However, at present, there is evidence supporting the role of this mechanism in plant growth enhancement. For example, several soil microorganisms, including bacteria, improve the supply of P to plants as a consequence of their capability for inorganic or organic P solubilization [33,34,35]. There is also experimental evidence that supports the role of organic acids in mineral phosphate solubilization. Halder [13] showed that the organic acids isolated from a culture of *Rhizobium leguminosarum* solubilized an amount of P nearly equivalent to the Rodríguez.H, and Fraga.R[2] amount that was solubilized by the whole culture. Besides this, treatment of the culture filtrates from several *Rhizobium* strains with pepsin or removal of proteins by acetone precipitation did not affect phosphate release capacity, showing that this was not an enzymatic process. However, neutralization with NaOH destroyed the solubilization activity[36].

In our present study we tried to investigate the mechanism by which bacteria solubilizes phosphates under *in vitro* conditions.

CONCLUSIONS

Ability of isolate singly or in consortia to compete / co-exist with the saprophytes of rhizosphere has to be determined for successful use as bioinoculant.

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