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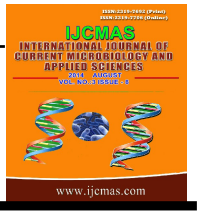


Original Research Article

In Vitro phosphate solubilization abilities of three indigenous bacteria isolated from Muscovite mine

B.Sreenivasulu[1], K.Chalapathi[1], Ch.Paramageetham[1]* and G.Prasada Babu[2]

[1]Department of Microbiology, Sri Venkateswara University, Tirupati-517502, India
[2]Department of Botany, Sri Venkateswara University, Tirupati-517502, India
**Corresponding author*

A B S T R A C T

The main objective of this study was to assess the 'P' solubilization abilities of three bacterial isolates indigenous to Muscovite mine. The results revealed that the isolate SVUNM17 was a potential rock phosphate solubilizer among the three tested bacteria. After 28DAI the isolate SVUNM17 showed two fold increase in release of 'Phosphate' from rock phosphate mineral. The rock phosphate mineral Muscovite was treated with three isolates for 14 days and 28 days. At 14th day of Incubation the isolate SVUNM16 released highest content of 'P' (74mg/l)from muscovite followed by SVUNM9(66mg/l).However at 28th day of incubation the isolate SVUNM17 was able to release highest content of 'P' from Muscovite. The isolate SVUNM17 showed two fold increase for longer incubation periods. This suggests that SVUNM17 is slow 'P' solubilizer and SVUNM16 is a fast 'P' solubilizer. Further ,the SEM analysis indicated that these isolates also secretes ExoPolysaccharides (EPS) and by producing biofilms the bacteria will bind strongly to the Muscovite.

Keywords

Rock phosphate, Muscovite mine, Phosphate solubilization, SEM, Exo-polysaccharide and Biofilms.

Introduction

In soil, phosphorus is sequestered by adsorption to the surface of soil particles and through precipitation reactions with soil cations, particularly iron, aluminum and calcium which becomes unavailable to plants (Harris *et al*., 2006). For this reason, a large amount of soluble 'P' fertilizer is commonly applied to agricultural soils in order to increase plant growth, which is likely to adversely affect both the environment and economy.

In many countries, there has been a steady increase in the use of 'P' fertilizer (Syers *et al*., 2008), which is considered as a major source of heavy metal contamination in agricultural soils (McLaughlin *et al*., 1996; Bolan *et al*., 2003). In addition, excess amounts of "P" fertilizer often leach from soil and cause eutrophication of surface and groundwater sources (He *et al*., 2003; Sharpley *et al*., 2003). Therefore, there has been increasing interest in the use of slow

release phosphate fertilizers, such as rock phosphate (Rajan *et al*., 1996; Chen *et al*., 2006). Insoluble phosphate compounds can be solubilized by organic acids and phosphatase enzymes produced by plants and microorganisms (Kucey, 1983; Duponnois *et al*., 2005). For example, phosphate solubilizing bacteria (PSB) have been shown to enhance the solubilization of insoluble 'P' compounds through the release of low molecular weight organic acids (Sahu and Jana, 2000). Phosphorous is essential for growth and productivity of plants. It plays an important role in plants in many physiological activities such as cell division, photosynthesis, and development of good root system and utilization of carbohydrates. Phosphorous deficiency results in the leaves turning brown accompanied by small leaves, weak stem and slow development. In ancient times the use of animal manures to provide phosphorous for plant growth was common agricultural practice. Organically bound phosphorous enters in soil during the decay of natural vegetation, dead animals and from animal excretions (He *et al*., 2003; Sharpley *et al*., 2003).To achieve optimum crop yields, soluble phosphate fertilizers have to be applied at a high rates which cause un manageable excess of phosphate application and environmental and economic problems. Direct application of rock 'P' materials may be more useful and environmentally more feasible than soluble 'P'(Ranawat *et al*., 2006). Rock 'P'materials are cheaper sources of 'P'. However, most of them are not readily available to the plants because the minerals are released slowly.

The transformation of insoluble phosphate into soluble form is carried out by a number of microbes present in the soil. A large fraction of soil microbes can dissolve insoluble inorganic phosphates present in the soil and make them available to the plants. Microorganisms play an important role for transformation of phosphorous in water and sediments and the phosphate ions are reported to be strongly adsorbed by sediments with a high content of silt and clay (Seshadri *et al.,* 2002). Bacteria are the predominant microorganisms that can solubilize phosphate compared to the fungi and actinomycetes (Yin, 1988). There are some species of bacteria which have potential to mineralize and solubilize organic and inorganic phosphorus (Khiari and Parent, 2005). Strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers (Rodriguez and Fraga, 1999).In this context, the aim of this study was to evaluate the bio-solubilization of the muscovite a rock 'P" mineral for Phosphate release from the muscovite mine.

Materials and Methods

Isolation of Bacterial isolates

Muscovite ore samples were collected from Muscovite mining sites situated in Gudur division ,Nellore dist. Muscovite ore was collected by employing cable tool drilling from underground mines. The collected samples were pooled and stored at 4^{o}C for further processing. The isolation of native microorganisms present in the samples were done by dilution plate technique using nutrient agar medium. Three morphologically distinct colonies were selected and grown on nutrient agar slants for pure culture preparation. Nutrient agar plates were incubated at 37^{o}C for 24hr. Pure culture isolates were named as $SVUNM_{9}$, $SVUNM_{16}$, $SVUNM_{17.}$

Morphological and Biochemical Characteristics of the isolates

Identification of pure cultures were made by using morphological and biochemical characteristics (Sneath Peter,1994). The

colony characteristics were studied basing on their shape, size, elevation ,margin, surface, colour and structural characteristics and Gram` +`ve or Gram `-`ve nature. Various biochemical tests were also carried out to characterize the isolated bacterial strains. The biochemical tests include O/F test , Catalase, Indole production , methyl red, Voges proskauer reaction, Citrate utilization, Urease activity, Starch hydrolysis, Gelatin liquefaction, carbohydrate utilization, nitrate reduction and antibiotic resistance test .

InVitro Phosphate solubilization assay

Bacterial Cultures

The Bacterial Strains $SVUNM_9$, $SVUNM_{16}$,$SVUNM_{17}$ strains which were indigenous to 'muscovite' a rock phosphate mineral mine used for the In vitro solubilization assay . These three isolates were inoculated into 100 ml Nutrient sterile medium and incubated on a rotary shaker at 150 rpm/min and 28^{o}C for 24 hours.

Mineral Processing

The rock phosphate Muscovite mineral was crushed and sieved to collect grains as large as 20-40 mesh, washed with de-ionized water and dried at 50^{o}C to constant weight. The mineral sample was cut into pieces measuring 1 cm x 1 cm x 1 mm in size and burnished with 5um abrasive.

Experimental settings for phosphate Mineral degradation

A batch of experiments were made to explore different aspects.

Release of Phosphorus from phosphate Minerals

In phosphate minerals phosphorus is locked up in the phosphate lattice. During interaction with phosphate minerals the bacteria breaks the lattice and release phosphorus. Therefore, in this study phosphorus solubilization was quantified. A loop of 48 hrs old grown bacterial cultures were individually inoculated into 25 ml modified mineral salt medium (PSM) $(NH_4)_2SO_4$: 0.10 gms / ltr; $MgSO_4.7H_2O$: 0.125 grms / ltr; $MgCl_2$. $6H_20$: 2.5 grms / ltr; KCl : 0.10 grms / ltr containing 0.50% Muscovite. Mineral salt medium containing muscovite was sterilized at 121^{o}C for 20 minutes followed by inoculating 2 ml culture broth of the bacterial strains individually and incubated on a rotary shaker at 150 rpm/min and at 28^{o}C. The experimental setting has two batches incubated for 14 days and 28 days respectively. Control experiments were carried out in parallel to the experimental runs, whose experimental conditions were identical to those of the treatment experiments, but with autoclaved medium without bacterial culture. After, every 14 and 28 days of incubation, Phosphorus (PO^4) content was measured using Induction Coupled Plasma (ICP-OES Optima 4300 DV, Perkin Elmer, Waltnam, MA.USA).

SEM analysis of Muscovite for structural changes

To observe the structural changes in the phosphate minerals if any as affected by phosphate dissolving bacterium another set of treatments were selected. For this part, the Muscovite samples were collected during harvesting and washed three times to remove culture medium with sterile distilled water. These mineral samples were air dried at room temperature. This dried sample was fixed by applying 15 ml of fixing solution (2.5% Glutaraldehyde in 0.0075 M phosphate buffer) on to the ore inside Greiner tube. Mine samples were washed

three times for 15 min each with 0.0375M phosphate ($Na_3(PO_4)$) buffer. The samples were then dehydrated at different alcohol concentrations (50, 70, 95, 100%) at 10 min each. The dehydrated samples were repeatedly soaked in 100% alcohol twice. This stage was followed by drying of the samples that were later sputter coated in a Polaron equipment limited SEM coating unit E5200 with gold prior to observation under the scanning electron microscope (SEM). They were then assembled for observation under the microscope at 5 KV on a JEOL 5800 LV Scanning electron microscope (Tokyo, Japan).

Results and Discussion

From the isolated colonies in the petriplates three different strains were selected on the basis of district morphological characteristics such as colony shape, size, margin, colour and elevation as given in table-1. These selected strains were sub cultured repeatedly several times to obtain the pure culture. There strains were pure cultured and their microscopic and bio chemical characteristics were studied. The Gram staining was carried out for six bacterial strains to differentiate between two principle groups of bacteria, such as bacillus or cocci *etc*. Further various biochemical tests related to the characterization of bacteria were carried out and presented in Table-2.

The isolate M9 was found to be small in size, white in colour, with irregular form, entire margin having raised elevation. This isolate was found to be Gram positive rod, showing motility. From the bio chemical test it was found that the isolate showed positive results for catalase production, methylred, Voges proskauer reaction, citrate utilization, urease activity, starch hydrolysis, zelatin liquefication, nitrate reduction and resistant to streptomycin, chloramphenicol, and tetracyclin. The isolate also showed positive results for oxidative test. The isolate produced acid from D-glucose and sucrose and gas from lactose . However, the isolate is unable produce Indole. The isolate was resistant to

The isolate M16 was found to be small, circular, brown colour with white margin, having flat elevation. This isolate was found to be Gram `-`ve *cocci* from the biochemical tests it was found that the bacteria was motile fermentative. The isolate also showed positive results for the production of catalase, Voges proskauer reaction, citrate utilization, urease activity, starch hydrolysis, gelatine liquification, nitrate reduction and able to produce acid from D-glucose, gas from lactose. However neither gas nor acid were produced from sucrose. The isolate showed resistance to streptomycin, chloramphenicol and tetracycline.

The isolate M17 was found to be pinhead in size, with circular form, entire margin, raised elevation and purple colour. This isolate was found to be Gram positive cocci with appendges. The biochemical tests were positive for fermentative, Voges proskauer, citrate utilization, urease activity, starch hydrolysis, gelatine liquification, nitrate reduction, resistance to streptomycin, choloramphenicol and tetracycline negative for catalase, indole production and methyl red.

In vitro phosphate Solubilizing assay

The Muscovite, rock phasphosphate was subjected to phosphate solubilization assay. The amount of P released from Muscovite in a broth by the isolates was studied at 14 and 28 days of Incubation (DAI). The results indicated that the amount of P released from Mica by three strains

increased with increased incubation and was maximum at 28 DAI. The P – release from mica by all the three strain at 14 DAI ranged from 44.8 – 66.50mg/l . Similarly the amount of 'P' released from Mica by three isolates after 28 DAI ranged from 74-96.10mg/l. Among the three isolates SVUNM16 released maximum amount of (74.3mg/l) from Mica followed bySVUNM9 (66.50mg/l). The isolate SVUNM 17 released only 44.8mg/l 'P' only after 14days of Incubation.

In case of 28 DAI the amount of 'P' release increased slightly for SVUNM9 ,SVUNM16 isolates as 74.0mg/l and 84.30mg/l respectively. In case of M17 longer incubation times decreased the 'P' release (96.10mg/l). This may be due to the biosorption of P by bacterial isolate. The 'P' release from potash mineral by three isolates after 14days ranged from 52-721.50 for 2/4dAI and 20-103.14mg/l after 28 days. The isolate SVUNM17 released good amount of P after 14days. However the isolate SVUNM16 released good amount of P (184.4mg/l) after -28days.

SEM analysis: The SEM analysis of residue of Muscovite mineral left after the 28 DAI showed noticeable structural changes. The SEM image of SVUNM9 treated Muscovite showed visible forms of biofilm formation and SVUNM16 treated Muscovite showed copieus amounts of biofilm and strong attachments to the surface of mica. The isolate SVUNM17 treated Muscovite SEM image represents the high possibility of EPS , secretion that binds the mica ore strongly together. All the Images obtained were at the end of 28DAI of the Experiment. (Plate-1a,1b,1c and 1d)

Soil microorganisms play a key role in soil 'P' dynamics. Release of 'P' by PSB from insoluble and fixed/adsorbed forms is an important aspect regarding 'P availability in soils.

Table.1 Morphological characteristics of the isolates

Name of the isolate	Size	Pigmentation	Form	Margin	Elevation	Colour
SVUNM9	Small	-	Irregular	Entire	Raised	White
SVUNM16	Small	-	Circular	Brown with white margin	Flat	Brown
SVUNM17	pinhead	-	Circular	Entire	Raised	purple

Fig.1 Release of P from Muscovite using three bacterial strains after 14and 28 days

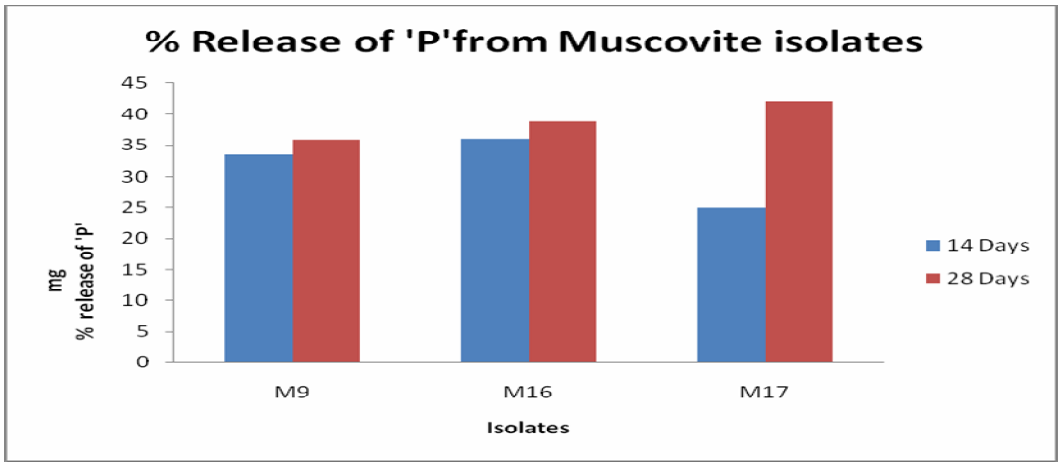


Table.2 Biochemical Characteristics of the isolates

Name of the test	SVUNM9	SVUNM16	SVUNM17
Gram staining	'+'ve, rods	'-'ve, rod	'+'ve, cocci with appendages.
Motility	Motile	Non motile	Motile
O/F test	Oxidative	Fermentative	Fermentative
Catalase	+	+	-
Indole production	-	-	-
Methyl red	+	-	-
Vogesproskauer reaction	+	+	+
Citrate utilization	+	+	+
Urease activity	+	+	+
Starch hydrolysis	+	+	+
Gelatin liquification	+	+	+
D-Glucose-acid	+	+	+
D-Glucose-Gas	-	-	-
Lactose-acid	-	-	-
Lactose-Gas	+	+	+
Sucrose-Acid	+	-	-
Sucrose-Gas	-	-	-
Nitrate reduction	+	+	+
Antibiotic resistance test			
A. Sreptomycin	+	+	+
B.Choloramphenicol	+	+	+
C. Tetracyclin	+	+	+

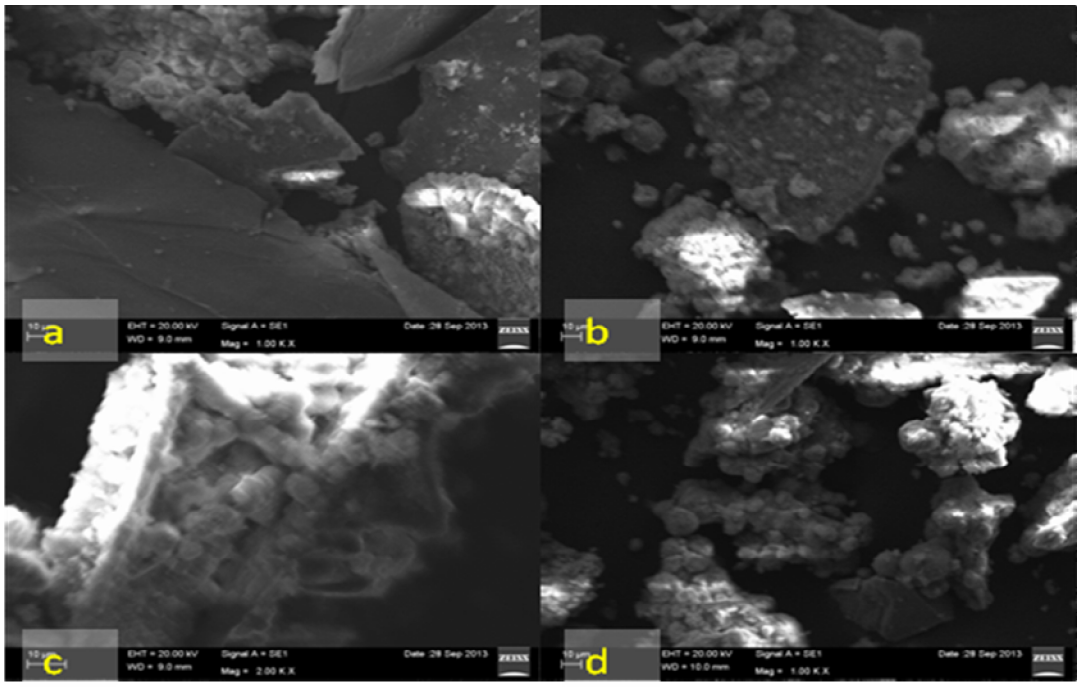
Table.3 Content of P from Mica using three bacterial strains after 14 and 28 days

Name of the Culture	Content of 'P' in Control	Content of 'P' after 14 days	Content of 'P' after 28 days
M9	131.80	198.30	205.80
M16	131.80	206.00	216.10
M17	131.80	176.60	227.9

Table.4 Release of P from Mica using three bacterial strains after 14and 28 days

Name of the Culture	Content of 'P' in Control	'P' release after 14 days	'P' release after 28 days
M9	131.80	+66.50	+74.0
M16	131.80	+74.2	+84.30
M17	131.80	+44.8	+96.10

Plate.I Scanning Electron Microscope (SEM) images showing the action of Microbes on Mica sheets



Ia: SEM image represents the mica control.
Ib: SEM image represents the isolate SVUNM9 with visible forms biofilm formation.
Ic: SEM image represents the isolate SVUNM16 with copious amounts of biofilm and strong attachments to the surface of mica.
Id: SEM image represents the isolate with visible SVUNMM17 with high possibility of EPS secretions that binds the mica ore strongly together

There are strong evidence that soil bacteria are capable of transforming soil 'P' to the form available to plants. Rock phosphate minerals are too insoluble. However some bacterial species have mineralization and solubilization potential for organic and inorganic phosphorous respectively. Application of insoluble rock phosphate alone in the soil caused limited increase in the 'P' uptake, this increase in 'P' content could be due to the role of indigenous microorganisms in the soil. In the present study microorganisms indigenous to muscovite mine were isolated and evaluated for their rock phosphate solubilization. This solubilization may be due to the production of strong acids (Schilling et al.,1998). In our study also three indigenous microorganisms were able to increase 'P'release from rock phosphate by about 0.5-2.0 fold in comparison to the control. Similarly, Amer *et al*., (2010) reported the *B.subtilis* and *P.florescence* also increases 'P' release. PSB have been used to improve rock 'P" value because they convert insoluble rock 'P' into soluble forms. This conversion is through acidification, Chelation and exchange reaction and produce strong organic acids in the periplasm. Acid phosphatase also play a major role in the mineralization of rock phosphate minerals. In our study three indigenous bacterial isolates were able to solubilize 'P' from

muscovite significantly. Similarly, Styriakova *et al*.,(2003) reported that the activity of potassium dissolving bacteria played a pronounced role in the release of 'K' from Feldspar, a rock phosphate mineral. Similarly in our study also these three bacterial isolates are good candidates for Phosphate solubilization. In conclusion due to their phosphate solubilizing capacity , they can also be used as biofertilizers.

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