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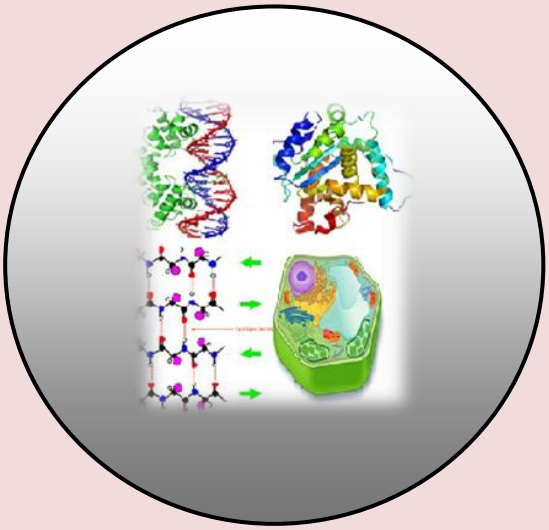

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Isolation of a Phosphate Solubilising *Comamonas* Sp from Rhizosphere Soil of *Vigna vexillata*

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ABSTRACT

Phosphate is an essential nutrient for plants and often acts as a limiting factor in growth due to its reduced availability. Phosphate accessible to plants can be enhanced if it is solubilised. Phosphate Solubilising Bacteria (PSB) are competent tools for this and act as biofertilisers to improve the nutrient quality of soil. PSB can be used as a biological agent for the remediation of phosphate contaminated sites. Rhizosphere of plants is a rich source of microbial activity. Phosphate solubilising bacteria was screened from rhizosphere soil of leguminous plant Vigna vexillata and was found to solubilise insoluble tricalcium phosphate in Pikovskaya Agar medium. Among the three isolates sv3 was the most effective. Phosphate solubilisation efficiency was calculated from the Solubilisation Index and the colour change observed in BPB medium indicated organic acid production. 16S r RNA characterization of the bacterial isolate indicated the identity of the strain as Comamonas sp. The phosphatase production from the bacteria was also estimated. The bacteria were able to solubilise rock phosphate and the effect of pH and substrate concentration on rock phosphate solubilisation was studied.

Key words: ***Phosphate Solubilising Bacteria, Comamonas sp SV3, Phosphatase, Rock phosphate solubilisation.***

INTRODUCTION

Biogeochemical cycle is the determinant of material mobilization on earth and it operates through microorganisms. Phosphorus cycle is the movement of phosphorus in the lithosphere and hydrosphere and it is one of the slowest recycling pathways. Phosphorus has crucial roles in plant metabolism involving in photosynthesis, respiration, energy storage and transfer (Sarma et al., 2013). The largest component of phosphorus absorption takes place in the form of phosphate (Beever and Burns. 1980). But the concentration of soluble phosphate in soil is very low usually making it a biocritical element. This reduces plant growth and to rectify this phosphorus is provided in the form of chemical fertilizers.

The continuous application of these deteriorates soil quality by the emission of poisonous Hydrogen flouride gas and deposition of heavy metals like Cadmium (Sarma et al. 2013). Therefore phosphate solubilisation in soil by natural measures is of great ecological and economic importance. Microbial solubilisation of phosphate is a favourable alternate which increases the availability of phosphate in the soil (Mikanova and Novakova. 2002). Phosphate solubilising microorganisms are present in soil including bacteria and fungi. Phosphate Solubilising Bacteria(PSB) of different species like *Pseudomonas, Bacillus, Rhizobium, Alcaligenes, Arthrobacter, Azospirillum* etc are reported to accelerate plant growth through different mechanisms(Karpagam and Nagalakshmi. 2014).

Rhizospheric microorganisms are associated with soil processes like exudation of substances, storage and release of nutrients and water, mobilization of compounds, phosphate solubilisation, nitrogen fixation etc. Employment of phosphate solubilising microorganisms proves to be a sound technology for improving phosphate availability through which quality of agricultural lands can be elevated (Walpola and Yoon 2012).

In the present study rhizospheric soil of leguminous plants were examined for the presence of a promising microbial phosphate solubiliser. The rock phosphate solubilisation efficiency of the microbe was studied and the organism was characterized.

MATERIALS AND METHODS

Sample collection and screening

Fertile soil from rhizosphere of the leguminous plant *Vigna vexillata* was used for bacterial isolation. Soil samples were collected in clean containers from three different regions of Pathanamthitta District namely Poovanpara, Vakayar and surroundings of SAS SNDP Yogam College, Konni (Table 1). For the screening of phosphate solubilising bacteria, Pikovskaya's agar with the following compostion was used: Dextrose - 10g/L, Potasiumdihydrogenphosphate-1g/L, Di potassium hydrogen phosphate-3g/L, Magnesium sulphate - 2g/L, Ammonium sulphate -1g/L, Tricalciumphosohate-5g/L, Agar -2g/L (Metha and Noutiyal. 2001). The bacterial strains obtained from the primary screening were subjected to repeated quadrant streak culture on PVK agar plates. The same were cultured in bromophenol blue agar plates with the following composition : Dextrose - 10g/L, Potassium dihydrogen phosphate-0.5 g/L , Ammonium sulphate-0.025 g/L, Pottassium chloride-0.0020 g/L, Magnesium sulphate - 0.0010 g/L, Bromophenol blue -0.05 g/L, Tricalcium phosphate - 0.5 g/L, Agar - 2 g/L(Onyia and Uzoma. 2013). Phosphate solubilisation efficiency was calculated from the colony diameter by finding out the Solubilisation Index.(Mardad etal. 2013). Bacterial isolates were tested for identity using the16s rRNA sequencing method.

Calculation of phosphate solubilisation efficiency

Solublisation efficiency was compared by calculating SI, Solubilisation Index as proposed by Mardad et al (2013). Solubilisation Index was measured by using following formula:

SI= (Colony diameter +halo zone diameter)/ Colony diameter

Production of phosphatase by the isolated PSB

22 hour old bacterial culture harvested at 7000 rpm for 10 minutes was suspended in physiological saline so that the OD is 1 at 600nm. This was used as inoculum for phosphatase production. 2 % inoculum was introduced to PVK broth and incubated under standard conditions of temperature and pressure at 180 rpm. After 24 hours bacterial culture was pelletised (7000 rpm, 10 minutes) and the supernatant was used for quantification of phosphatase. Phosphatase production was estimated by a spectrophotometric method (Moss, 1982) which quantifies enzyme production at 540 nm.

Solubilisation of rock phosphate

Solubilisation of rock phosphate was tested by adding 50 mg of rock phosphate in 100 ml PVK medium deficient in calcium phosphate (modified PVK). Phosphatase production was tested after 24 hours.

Effect of substrate concentration on Rock phosphate solubilisation

To study the effect of substrate concentration on rock phosphate solubilisation, 250 ml PVK broth containing rock phosphate 100mg, 200mg, 400mg, 600mg, 800mg and 1000 mg were used. 2% inoclum was added to each and incubated under standard conditions. Phosphatase activity was measured after 24 hours.

Effect of pH on Rock phosphate solubilisation

To measure the effect of pH on Rock phosphate utilization PVK broth with pH ranging from 2 to 12 were inoculated with 2% cell suspension and phosphatase activity was measured after 24 hours of incubation under standard conditions**.**

RESULT AND DISCUSSION

Today, as a result of increasing environmental awareness, there is a tendency to develop green technologies in the fields of industry, agriculture and basic research. Practical approaches towards these demands intelligent initial screening and cost effective designing of strategies with standardization trials. An example of this approach is the use of biofertilisers. Phosphate solubilising microorganisms are excellent biofertilizers to increase the availability of phosphate which acts as a limiting nutrient. The phosphate solubilising bacteria are seen in the rhizosphere abundantly and they can upgrade the availability of insoluble phosphate to the soil to enhance soil fertility. Many PSB can produce organic acids and they exhibit the ability to chelate P from Al^{3+}, Ca^{2+}, Fe^{3+} etc (Ordonez, et al. 2016).

Three different soil samples collected were from the rhizosphere regions of the roots of wild pea plants (*Vigna vexillata*). The microbial community in soil is more concentrated to certain niches like rhizosphere. Rhizosphere harbours many useful microorganisms including phosphate solubilisers and acts as a hotspot of beneficial microbial activity.

Screening was done in Pikovskaya agar medium which is a specially designed medium with known components and is recommended for the isolation of PSB. The phosphate source is insoluble TCP (Tricalcium phosphate). The bacterial isolates were also cultured in PVK plates containing bromophenol blue. Such selective media are significant in the isolation of microorganisms with undetected enzymatic activities. The bacterial isolates with potential for phosphate solubization were determined by observing the clear zone produced in the plates. Two bacterial isolates Sv1 and Sv3were able to produce clear zones and remarkable colour change in bromophenol blue medium (plate 1 and 2). Color change was observed in the medium from 18 hour onwards. The insoluble calcium phosphate incorporated in the medium is the key component and it screens the bacteria that solubilise phosphate. The presence of BPB is especially helpful in screening out phosphate utilization with production of organic acids. The color change from purple to yellow is observed in the medium around the colony due to a drop in pH by the organic acids (Krishnaveni, 2010).

Solublisation efficiency was compared by calculating Solubilisation Index (SI) as proposed by Mardad et al (2013). Solubilisation Index was measured by using following formula:

SI= (Colony diameter +halo zone diameter)/ Colony diameter.

The calculation indicated that the culture Sv3 had highest efficiency in solubilisation. Both PVK medium and modified PVK (with bromophenol blue) showed increased capacity of SV3 to carry out phosphate solubilisation (Table. 2). Phosphate solubilisation operates through different mechanisms of which a major one is the production of organic acids. The production of organic acids gradually decreases the pH of the medium within 24 h. Kapri and Tewari (2010) reports a drop in pH of the medium in the case of Trichoderma sp. during phosphate solubilisation in the initial hours. In the present work, studies are progressing to identify the profile of organic acids produced by the micro organism. On 16S rRNA sequencing, the culture Sv3 was identified as *Comamonas sp* and was designated as *Comamonas sp.* Sv3.

Phosphatase estimation was done by quantifying the amount of phenol liberated by the activity of Phosphatase from disodium phenyl phosphate. The enzyme activity is expressed as units and one unit of enzyme is defined as the amount of enzyme which liberated 1 mg of phenol in 15 minutes. In Pikovskaya medium, *Comamonas sp Sv3* produced 0.74 mg of phenol and the Enzyme activity is expressed as 0.74 Units. The efficiency of the bacteria for solubilising rock phosphate was also examined. It was found that the bacteria could show active growth and could utilize the rock phosphate. It was estimated that about 0.53 Units of enzyme was produced in 50mg rock phosphate medium. In the present study the there was a pH drop in the medium due to organic acid production initially at 24 h. At 72 h the phosphatase production was maximum in the alkaline pH ranges. Phosphatase activity was increased gradually as the pH changes from acidic to alkaline.

The maximum solubilisation occurred at pH 9(Figure. 1). Phosphatases show optimum activity at alkaline and acid pH forming two broad groups of alkaline phosphatases and acid phosphatases. Maximum activities of alkaline phosphatases were reported from Trichoderma spp. At 96h of incubation in PVK broth with 10gL^{-1} tricalcium phosphate (Kapri and Thewari, 2014).
The substrate concentration is related to the efficiency of substrate utilization. After 24 hours of incubation time it was noted that as the concentration increases the utilization efficiency was decreased (Figure 2). This may be due to the inhibition at increased concentrations. The prolonged incubation may result in gradual utilization of phosphate.

Table 1. List of soil samples used for the isolation of Phosphate Solubilising Bacteria.

SAMPLE	PLACE
Sample Sv1	Vakayar
Sample Sv2	Poovan para
SampleSv3	Konni

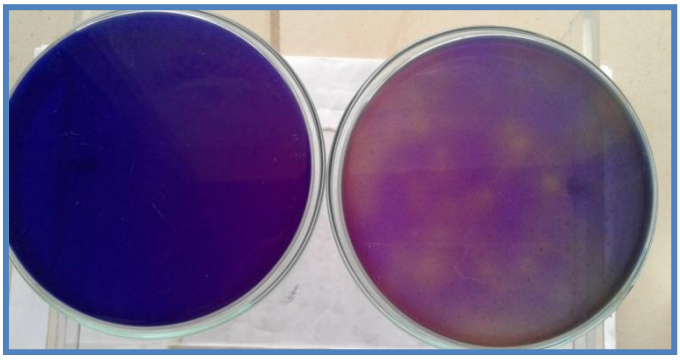

Plate 1. BPB medium – control and inoculated plates. Yellow color observed in the inoculated plate.

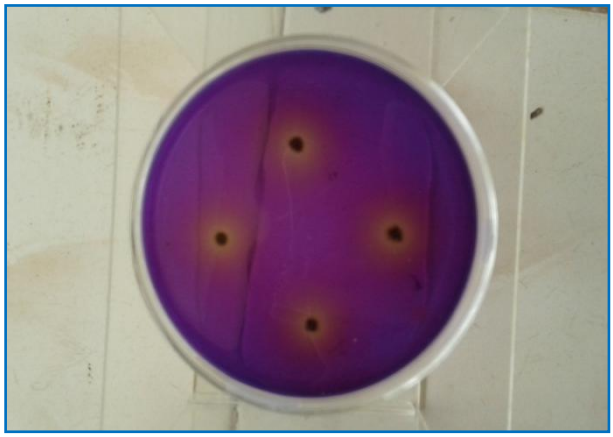

Plate 2. Colour change and clear zone in the BPB medium.

Table 3. Solubilisation Index.

Medium	Bacteria	Colony diameter	Halo zone diameter	Solubilisation Index
Pikovskaya medium with BPB	SV1	0.6	1.9	4.16
	SV3	0.5	2	5.0
Pikovskaya medium	SV1	0.6	1.7	3.83
	SV3	0.9	3	4.33

There is always a need to screen out potent micro organisms to develop ecofriendly technologies for applying in sustainable systems. The soils of indigenous regions are rich in valuable microbes which could be formulated as biofertilisers for particular crops. The use of biofertilisers like nitrogen fixers and PSB are green strategies enhancing agricultural productivity. In the present study, rhizosphere soil of *Vigna vexillata* was screened for the isolation of phosohate solubilising bacteria. Comamonas sp Sv3 isolated from the sample displayed organic acid production and considerable levels of phosphatase activity. Rock phosphate solubilisation was observed with maximum efficiency at alkaline pH levels. Further studies are progressing to formulate the bacterium as a soil inoculant.

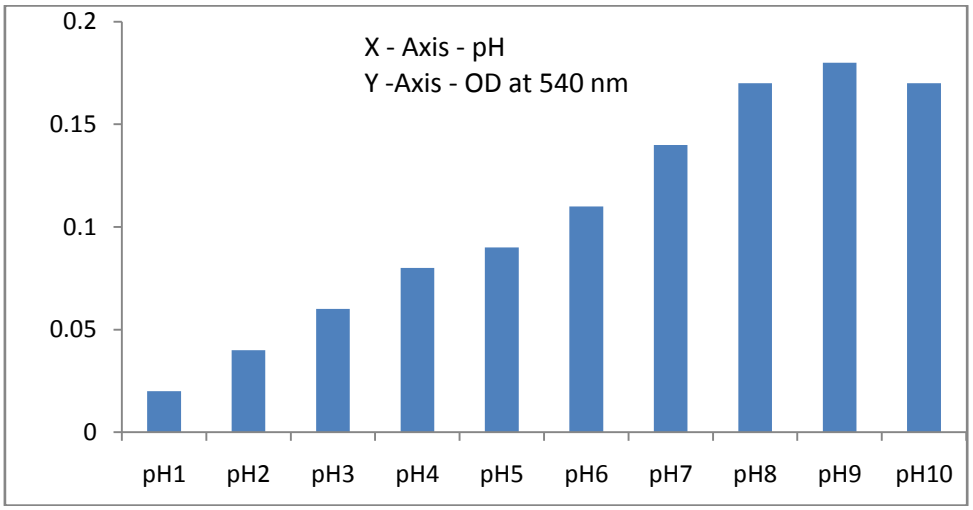

Figure 1. Effect of pH on Rock phosphate utilization.
Fig: 4. 3 Effect of pH on Rock Phosphate utilization at 72 h.

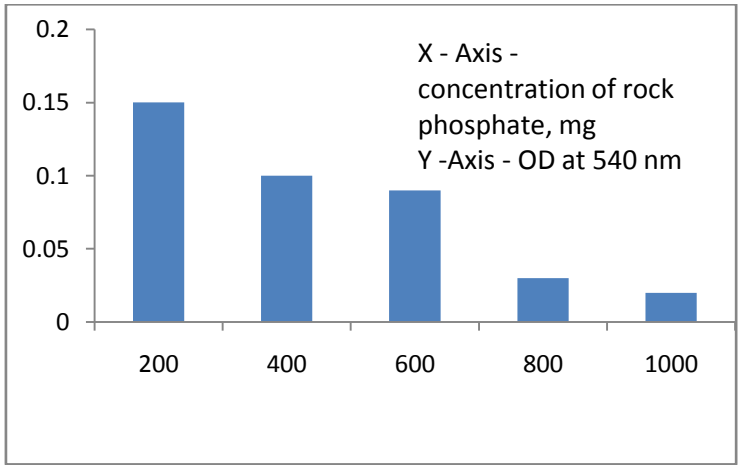

Figure 2. Effect of substrate concentration on Rock Phosphate utilization.

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