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Brief *In Vitro* Analysis on Improvement of Available Phosphorous In Low Soluble Eppawala Rock Phosphate

Rhan H*and Walpola BC

Department of Soil Science, University of Ruhuna, Sri Lanka

Abstract

Rock Phosphate (RP) is any naturally occurring geological material that contains one or more phosphate minerals suitable for commercial use. There is a major RP deposit in Eppawala, Sri Lanka and this RP identify as Eppawala Rock Phosphate (ERP). Large proportion of soluble inorganic phosphate added to soil is rapidly fixed as insoluble forms soon after application. Development of an economical and environmentally friendly alternative for successful soil P management is major concern in crop production and Phosphate Solubilizing Microorganism (PSM) attracted attention in this regard. This study was conducted to identify the Phosphorus solubilizing bacteria. Soil samples were collected from different Agricultural crop lands. Soil were Spread cultured in NBRIP medium after a serial dilution. The bacterial colonies that produced clear zone of activity were considered as potential phosphate solubilizes. They were sub-cultured for the further purification. The experiment has been carried out adding 28% P2O5 containing Eppawala Rock Phosphate with NBRIP medium. Bacterial growth in 660 wave length by using shimadzu UV spectrophotometer, P solubilization, and pH were measured respectively 1, 3, 5 and 7 days after inoculation. Clear zone producing bacteria in phosphate enriched medium has been selected as P solubilizing bacteria with time period all bacterial strains has shown improvement of solubilized P in the culture media. Among them PSB-7 strain has shown a vigorous growth and maximum P Solubilization 237.61 ppm *in vitro* during experiment. Despite of environment unfriendly chemical solubilization methods of naturally existing Rock Phosphate this biological solubilization generate maximum amount of plant available phosphorous.

Keywords: Insoluble rock phosphate; Phosphorus solubilizing microorganisms; Biological solubilization; Environment friendly crop improvement and biofertilizer

Introduction

Rock Phosphate (RP) contains one or more phosphate minerals suitable for commercial use. The term RP is globally accepted but not precise describing any naturally occurring geological material. Most of the world's phosphate fertilizers are produced from RP resources and almost all of these resources contain some form of mineral apatite [1,2]. There is a major RP deposit and occurrence in Eppawala in Anuradhapura district in Sri Lanka. This RP identify as Eppawala Rock Phosphate (ERP). The ERP deposit display some feature of a sedimentary phosphate secondary deposit formed over parent carbonatite rock [3,4]. The effectiveness of the direct application of RP depends on the following factors mineralogical composition of RP [5,6]. Soil physicochemical properties and the cultivated crop [7,8]. When considering natural RP deposits only a small percentage of the total phosphorous available to plants out of the total phosphorous of the RP deposit [1,9]. Although RP solubilization rarely occurs in nonacidic soils, it may occur when these soils are deficient in exchangeable Ca, because this characteristic enhances P solubilization [10]. Although a large proportion of soluble inorganic phosphate added to soil is rapidly fixed as insoluble forms soon after application and becomes unavailable to plants [11]. Next to nitrogen Phosphorous is an essential macronutrient for the plant growth and development [12]. To increase P content in soil ERP solubilization is being done by lowering of soil pH by using chemical solubilization but this is not environmentally viable [13,14].

Many soils used for agriculture are infertile with low levels of essential plant nutrients. The key components of the soil-plant system is microbial populations where they are immersed in a interactions affecting plant development [10,15]. The application of efficient microbial inoculants and slow release fertilizers, like RP are low input technological practices which might lead to the development of sustainable soil plant systems [16].

The insoluble phosphate is expected to convert plant available form of Phosphate by enhancement of multification of beneficial microbes and the P solubilizing organisms present in the soil to react with RP [4,17]. It is well known that many microorganisms isolated from the soil are able to dissolve different kinds of rock phosphates in a liquid culture [18,19]. Phosphate Solubilizing Microorganism (PSM) specially Phosphate solubilizing bacteria attracted attention in this regard development of an economical and environmentally friendly alternative for successful soil P management is major concern in crop production [20,21].

It's generally accepted that there are three major mechanism of the mineral phosphate solubilization. The first one is release of complexing or mineral dissolving compounds like organic acid anions, siderophores, protons, hydroxyl ions, CO_2. Second one is liberation of extracellular enzymes by biochemical P mineralization. The third one is release of P during substrate degradation by biological P mineralization [1,10,18,22]. Some identified PSB namely *Achromobacter sp*, *Aerobacter* sp., *Agrobacterium tumefaciens*, *Bacillus* sp., *Enterobacter* sp., *Pseudomonas* sp., *Serrtia* sp., *Brevibacterium* sp., *Burkholderia* sp.

In spite of the number of studies already done, further research is needed to differentiate clearly the ability of P solubilizing bacteria to solubilization of ERP.

***Corresponding author:** Rhan H, Department of Soil Science, University of Ruhuna, Sri Lanka, Tel: 041-2222681/2222682; Fax: 041-2227001; E-mail: nuwanrha@gmail.com

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Materials and Methods

Rhizospheric soil sampling

The soil samples were collected from rhizosphere, near to the root zone of the plants. About 45 soil samples were collected for the purpose of bacteria isolation. They were collected from different Agricultural crop lands of Sri Lanka including terrestrial, aqua and swamp regions including Matara, Galle and Hambantota districts of Sri Lanka by using opportunity sampling method.

Phosphate solubilizing bacteria (PSB) isolation

The collected soil samples were spread cultured in National Botanical Research Institute phosphate (NBRIP) medium after a serial dilution. A 10g of soil sample of each sample was dissolved in 90 ml of distilled sterilized water. Then 1ml of above soil solution was diluted in an aliquot of 9 ml distilled water. This procedure was performed until 10^{-5} concentration of soil solution was obtained. The bacterial colonies which producing clear zones around the colony were known to be great phosphate solubilizes and they were sub cultured in the petri dishes which containing NBRIP for the purpose of further purification. Ten phosphate solubilizing bacteria were used which produced clear zone around the colony [18].

Quantitative enumeration of bacteria

The experiment has been carried out using 28% P_2O_5 containing Eppawala Rock Phosphate as Eppawala phosphate deposit to represent different weathering stages and varying mineralogy [8].

After that isolated bacterial cultures were inoculated into NBRIP broth cultures (pH=7.0) which containing 500 ml flasks of 250 ml ERP instead of $Ca_3(PO_4)_2$. The composition of culture was 10 g glucose, 8.03 g ERP, 5 g $MgCl_2$, 0.25 g $MgSO_4.7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ in 1 L distilled water [23]. Except ERP all the chemicals used in this research was Analytical regents products of Daejung Chemicals & Metals Co., Ltd. The samples containing flasks were kept in a rotary shaker at 150 rpm speed at 30°C. There bacterial growth, P solubilisation and pH were measured respectively 1, 3, 5 and 7 days after inoculation.

Bacterial growth was measured colorimetrically by using a spectrophotometer at 660 nm wavelength. The bacterial broth cultures were mixed with 1 N HCl at 1:1 ratio and subjected to spectrophotometric measurements [24,25].

P solubilization was determined by using following procedure. About 10 ml of bacterial solution was centrifuged at 10 000 rpm. After that the solution was carefully taken into a colour development procedure. P absorptions were determined at 660 nm wavelength by using shimadzu uv spectrophotometer.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) using SAS [26]. The Duncans's Multiple Range Test (DMRT) was used to test the significance between treatment mean at P<0.05, P<0.01 and P<0.001.

Results and Discussion

Identification of phosphorus solubilizing bacteria

Basically clear zone producing bacteria in a phosphate enriched medium has been selected as P solubilizing bacteria. They were transferred in to ERP enriched broth medium and those bacteria were monitored for pH, growth and P solubilization.

Comparatively high solubilized P could be identified in an ERP solution. Normally around 5 ppm in an ERP solution. But after 7 days of introduction of PSB to the media the solubilized P is increasing to hundreds (Tables 1 and 2).

According to the Tables 1 and 2 it can be identified highest P solubilization in ERP medium at day 1 and 3 has been recorded by the PSB-12 (P>0.001). But in the day 5 and 7 it has changed the highest P solubilizer. In those days highest P solubilization has been recorded by the PSB-1 (P>0.001). At the day 1 lowest P solubilization has been recorded by the PSB-3. But in day 3 lowest P solubilization has been recorded by PSB-17 (P>0.001). In the both day 5 and 7 PSB-16 (P>0.001) has recorded the lowest P solubilization.

Considering about the bacterial growth it has been recorded that PSB-8 (P>0.001) has the highest growth rate. Though the Growth of the bacteria in a higher amount its P solubilization is in the 3rd of the raw. At the 3rd day the highest bacterial growth has recorded by PSB-1. Though it's the highest bacterial growth it's P solubilization not the highest at the same date. In the day 5 as well in the day 7 PSB-7 (P<0.001) has recorded the highest bacterial growth.

In the day 1 lowest bacterial growth has shown by the PSB-15 (P<0.001). In the day 3 PSB-15 collaborates with PSB-3 to show the lowest bacterial growth. In the day 5 PSB-3 has shown the lowest growth in ERP medium. But in day 7 PSB-4, has shown the lowest bacterial growth.

Normally P_2O_5 content in Phosphate Rocks varies from 4.5-33% mainly due to mineralogical genetic process [27]. Sri Lankan ERP

	P	Growth	pH
Day 1			
PSB-1	14.59b	0.431b	3.98d
PSB-3	3.51f	0.151ef	4.19a
PSB-4	9.90d	0.236c	3.82d
PSB-6	7.34e	0.167de	4.06bc
PSB-7	14.09bc	0.252c	3.82d
PSB-8	12.70c	0.567a	3.83d
PSB-12	16.75a	0.252c	3.91d
PSB-16	7.61e	0.200d	4.18ab
PSB-15	10.45d	0.128f	3.96cd
PSB-17	8.01e	0.160ef	3.88d
Significance	***	***	***
CV%	9.26	7.78	1.74
Day 3			
PSB-1	28.19bc	0.552a	3.29c
PSB-3	30.81ab	0.161e	3.73ab
PSB-4	34.45a	0.380c	3.70ab
PSB-6	26.44bc	0.374c	3.57bc
PSB-8	25.76bc	0.381c	3.35c
PSB-7	23.37c	0.418b	3.43bc
PSB-12	34.68a	0.372c	3.96bc
PSB-15	28.15bc	0.189e	3.61bc
PSB-16	24.08c	0.270d	3.96a
PSB-17	13.89d	0.486bc	3.59bc
Significance	***	***	**
CV%	10.02	5.76	4.71

CV: Coefficient Variance, Significance at P<0.05=*, Significance at P<0.01=**, Significance at P<0.001=***, NS: Not Significant (Mean values along each column shown by same letter is not significantly difference at P>0.05 level)

Table 1: Amount of phosphorus solubilized by isolates, bacterial growth and pH variation in 1st and 3rd day after inoculation.

contain of 29% of phosphous although TSP containing 50% of P_2O_5 and Single super phosphate containing 18% of phosphorus and according to ERP contains 12% total P and 1.59% citric acid soluble P according to the vando molybdate method [21,28]. But in this experiment all the strains in day 1, 3, 5, 7 was significantly different (P<0.001) and there solubilized P was ranged from 7.61 ppm-237.61 ppm

With the time period almost all bacterial strains has shown improvement of solubilized P in the culture media (Figures 1 and 2).

When consider the bacterial growth with the pH variation according to Figure 1 it shows polynomial relationship with the R2=0.295. So there is no relationship between pH variation and bacterial growth in the ERP medium. In the day 7 lowest pH in the ERP enriched growth medium has showed by PSB-12 and PSB-4.

	P	Growth	pH
Day 5			
PSB-1	188.06a	0.730b	3.14e
PSB-3	121.62cd	0.180h	3.49bc
PSB-4	100.22ef	0.415f	3.29d
PSB-6	166.66b	0.571d	3.48bc
PSB-7	134.68c	0.913a	3.42c
PSB-8	179.95ab	0.502e	3.28d
PSB-12	91.66f	0.418f	3.18e
PSB-15	87.16f	0.215h	3.29d
PSB-16	70.27g	0.323g	3.63a
PSB-17	111.48de	0.635c	3.53b
Significance	***	***	***
CV%	7.39	5.05	1.49
Day 7			
PSB-1	173.42c	1.28a	3.19f
PSB-3	106.53e	0.613c	3.57c
PSB-4	105.85e	0.242e	3.41e
PSB-6	187.16bc	0.949b	3.63bc
PSB-7	237.61a	1.18a	3.58d
PSB-8	173.43c	0.927b	3.46de
PSB-12	124d	0.420d	3.42e
PSB-15	92.11e	0.336de	3.50d
PSB-16	66.89f	0.329de	3.86a
PSB-17	101.35e	0.926b	3.67b
Significance	***	***	***
CV%	6.32	8.41	1.09

CV: Coefficient Variance, Significance at P<0.05=*, Significance at P<0.01=**, Significance at P<0.001=***, NS: Not Significant (Mean values along each column shown by same letter is not significantly difference at P>0.05 level)

Table 2: Amount of phosphorus solubilized by isolates, bacterial growth and pH variation in 5th and 7th day after inoculation.

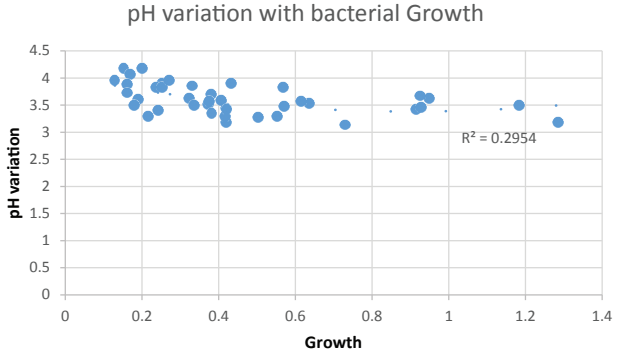

Figure 1: Relationship between bacterial growth and pH variation in ERP medium.

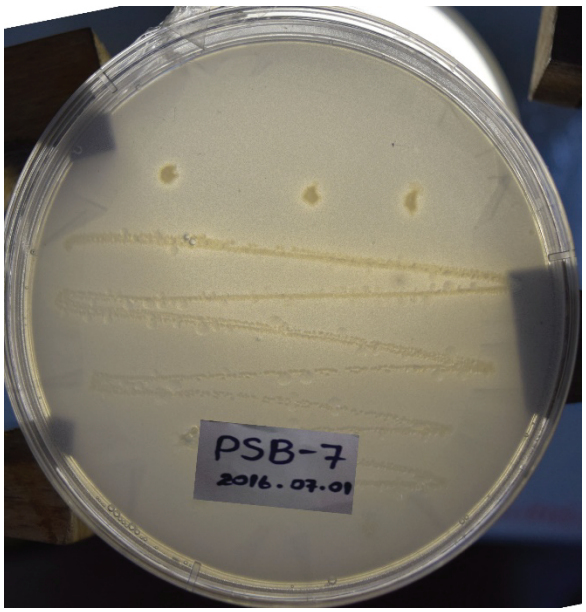

Figure 2: Halo zone produced by PSB in the Tri Calcium Phosphate containing NBRIP medium.

Within the whole experiment period there was significance change of pH (P>0.001) could be identified. The bacterial growth changes initial pH 7.0 to a normally pH 5.5-2.2 of the growth broth medium which includes ERP.

Conclusion

Despite of Environment unfriendly chemical Solubilization methods of naturally existing RP biological solubilization generate drastically higher amount of plant available phosphorous. PSB-7 strain has shown a vigorous growth and maximum P Solubilization 237.61 ppm *in vitro* during this experiment. The sedimentary phosphate deposit can be utilized under environment friendly Biological Phosphorous Solubilization by PSB. This biological method also treating the problem of rapid fixation of soluble inorganic phosphate added to soil soon after application.

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