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Isolation of Phosphate Solubilizing Bacteria's (PBSs) from soil and to estimate the amount of phosphate imbibed in the reservation granules by Fiske–Subbarow (F-S) method

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Abstract

Phosphate solubilizing microorganisms (PSMs) are one among the biofertilizers, because of their potential to convert insoluble phosphate to a soluble form by hydrolysis, by which the plants are able to gain phosphate readily. The process of hydrolysis of insoluble phosphate to soluble form is very clear whereas the amount of phosphate reserved in the cell is still missing. Reservation compounds are diverse by having a multiple chemical nature; they play a major role in phosphate storage, structural, biochemical by regulatory function. The most common phosphate reservoir includes polyPhosphate and Teichoic acid. In the present work, we have isolated the PSMs from soil and estimated the amount of phosphate imbibed in the phosphate storage/reservation granules by Fiske- Subburows method (F-S). We could make out as phosphate concentration increases from 0.028M (standard) growth of bacteria decline this could be due to change in pH of the media (basic), whereas when the concentration of phosphate decreases from the standard, bacteria starve and finally die this could be due to deficiency of phosphate. Similarly, when the concentration of phosphate is huge, bacterial cell consumes more phosphate from their surrounding and accumulate them in their reservation granules for cellular metabolism, growth, and development, whereas when phosphate is low in their surrounding bacterial growth cease.

Keywords: Phosphate, solubilization, Pikovskaya, biofertilizer, granules

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1. Introduction

Phosphorus is the second most important and vital macronutrient required by the plants, next to nitrogen. Phosphorus plays a vital role in plant growth, development and maturation; deficiencies can cause abnormal growth or death of plants [1]. Phosphorus accounts for an average range between 0.2 and 0.8% of the dry weight of plants, and it is a major part of nucleic acids, enzymes, coenzymes, nucleotides, and phospholipids. It is essential in every aspect of plant growth and development, including photosynthesis [2]. Though soil contains total Phosphorus in the form of organic and inorganic compounds, most of them remain insoluble and thus are unavailable to plants. [3] Therefore, about 95 to 99%, of phosphate present in the soil is in an insoluble form.

To overcome this loss, Phosphate solubilizing microorganisms (PSMs) are appointed a group of favorable microorganisms which are capable of hydrolyzing insoluble phosphorus to soluble Phosphorus that can easily be taken up by plants [4]. The availability of phosphorous is limited due to its complex structure as insoluble phosphates of iron, aluminum, and calcium in the soil. [4,5].

Phosphorus gets immobilized by cations (M^+) such as calcium, aluminum, and iron in soils to form complexes such as calcium phosphate, Aluminum phosphate, and ferrous phosphate respectively [3,6]. These forms of phosphorous are insoluble and consequently unavailable for the plants. Therefore, PMS aid in the hydrolysis of insoluble phosphorous into soluble forms.

Among the entire microbial community in soil, Phosphate solubilizing bacteria comprise about 1–50% and Phosphate solubilizing fungi comprises about 0.1 to 0.5% [6]. As mentioned PSMs are a group of beneficial microorganisms that are capable of hydrolyzing organic and inorganic insoluble phosphorus to soluble Phosphorus that can easily be taken up by plants, [4] so they are been appointed as one of the members of biofertilizers. The process of hydrolysis of organic and inorganic insoluble phosphorus to soluble Phosphorus is very clear in scientific data, though a comparison between amounts of phosphate stored or imbibed the cells is unclear.

Various mechanisms are put forward by researchers on phosphate reservation in the cells; one such mechanism is the inorganic phosphate (Pi) transport systems with different specifications and mechanisms of action. Another mechanism of microbial adaptation to the changes in phosphorus accessibility in their surrounding is the formation of storage compounds intracellular, which are utilized under excess or deficiency of phosphorus sources in the surrounding. These reservation compounds are diverse and have multiple chemical natures and not only play the role of phosphorus reserves but also perform structural, biochemical, and regulatory functions.

The simplest and most common reserve of phosphorous compounds of the microbial cell is low-soluble phosphates: $MgPO_4OH \cdot 4H_2O$ formed in the halophilic archaea, Halobacterium and Halorubrum sps and $NH_4MgPO_4 \cdot 6H_2O$ formed in bacteria of the genus Brevibacterium. The halophilic archaea store inorganic phosphate (Pi) from aqueous solutions during their growth and in excess, a

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considerable part of Pi accumulates in the biomass. The Phosphate content in the biomass increases nearly 10-folds higher than that of inorganic polyphosphate. An increase in the accumulation of Pi leads to abnormal cell development and results in cellular lysis [7,8].

In contrast to the archaea, Brevibacteria was also been identified to accumulation of Pi intracellular. The phosphorous compound was extracted from the *Brevibacteria antiquum* by high-pressure extrusion and was identified as $NH_4MgPO_4 \cdot 6H_2O$ [8]. Storage of phosphate as low-soluble salts was also identified in several species of Brevibacteria, during their growth most consumed Pi was from the medium at its concentration of about 11 mM [9,10].

Once the bacterial cells were inoculation into Pi rich medium, like Pikovskayas Agar (PVK), the cells grow under excess Pi give more biomass yield (hyper compensation, or "phosphate overplus"), similarly when re inoculation into Pi deficient/poor medium, the cells starved and finally die. This confirms that both intracellular and extracellular phosphate performs the function of phosphate storage for the cell population and biomass.

In a large number of microorganisms, the role of phosphate storage is performed by inorganic polyphosphates (polyP), linear polymers of orthophosphoric acid, containing more than 3 to several 100-phosphate residues [11]. PolyP, act as an energy reserve because the energy of their phosphodiester bond is similar to that of ATP molecule. The amount of PolyP is much lower under phosphate deficiency in the medium and higher with sufficient phosphate content in the medium. When we talk about PolyP it is necessary to know about polyphosphate kinase, a key enzyme of PolyP synthesis in prokaryotes.

With respect to inorganic phosphorus reserves, in some microorganisms, organic phosphorus reserves were also identified. One among them is Teichoic acids, polymeric compounds of the cell walls of Gram-positive bacteria, which consist of repeating polyol or glycosyl polyol residues linked by phosphodiester bonds. They function similar to that of inorganic phosphorus reserves, they are involved in bacterial morphogenesis and participate in cell adhesion and regulation of the ionic composition of the cell wall [12].

Phosphate storage compounds in microorganisms are more often mineral compounds. Organic phosphorous reserve compounds occur rarely. Hence, we get a clear picture that; Phosphorus deficiency suppresses the growth and development of microorganisms, while their excess has a negative impact on the regulation of phosphate metabolism and cell biochemistry. Microorganisms habituated in the varying environment have mechanisms of adaptation to phosphate deficiency and excess.

For isolation and characterization of PSBs, various growth mediums are being appointed the most effective and reliable approach used for screening and isolation of PSM was first described by Pikovskaya hence the name Pikovskayas selective media for isolation of PSBs. [13]

The media is composed of Yeast extract, Dextrose, Calcium phosphate, Ammonium sulfate, Potassium chloride, Magnesium

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sulfate, Manganese sulfate, Ferrous sulfate, and, Agar (Sundara Rao W. V. B. and Sinha, 1963) [14]. The phosphate solubilizing ability of a microorganism can be assessed in terms of the solubilization index (SI), that is the ratio of total diameter,(clearance zone and the colony diameter).[15]

$$\textbf{SI} = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

Plants gain phosphate by the various mechanism of phosphorus solubilization which includes lowering soil pH, chelation, and mineralization. [16,17] In our work we aim to modify the molarity (M) of Calcium phosphate used in the standard medium, to study the effect of change in M on microbial growth and to estimate the amount of phosphate imbibed in the storage granules by Fiske–Subbarow method.

2. Materials and Methods

2.1 Collection of soil sample

Soil samples were collected from composting area of Sri Dharmasthala Manjunatheshwara Post graduate College, Ujire, DK, Karnataka-574240. Soil samples were collected in sterilized polythene seal bags. The bags were properly sealed to avoid contamination, labeled, and stored at room temperature for isolation of phosphate solubilizing bacteria.

2.2 Isolation of PSB from soil sample

One gram of soil sample was suspended in 9 ml sterile distilled water, vortexed, and serially diluted up to 10^{-8}. The dilutions were spread over Pikovskaya's agar medium (Pikovskaya's, 1948) pH 7.3±2. These plates were incubated at 35±2 °C for 7 days in order to isolate the PSB [13]. The bacterial colonies exhibiting the clearance zone around the colonies were selected, purified, sub-cultured and stored on the slants for further studies.

2.3 Estimation of the amount of Phosphate imbibed in the storage granules by Fiske–Subbarow method

Pikovskayas medium is composed of Yeast extract 0.500/L, Dextrose 10.000/L, Calcium phosphate 5.000/L, Ammonium sulfate 0.500/L, Potassium chloride 0.200/L, Magnesium sulfate 0.100/L, Manganese sulfate 0.0001/L, Ferrous sulfate 0.0001/L and Agar 15.000/L

For the studying of the effect of phosphate on the growth of purified PSB's and to estimate the amount of Phosphate imbibed in the storage granules by Fiske–Subbarow method, we first modified the morality of Calcium phosphate with respect to the stranded Pikovskaya's agar medium (Sundara Rao W. V. B. and Sinha, 1963) as in Table. 1

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Quantity of $Ca_3(PO_4)_2$ (g/L)	Molarities (mol/L)
0.868	0.0028
4.342	0.014
6.824	0.022
8.685	0.028*
24.18	0.08
124.08	0.4
310.20	1.0

Tab1 indicates the modification of molarities of $Ca_3(PO_4)_2$ the medium

0.028 M* is the standard Calcium phosphate put forward by Sundara Rao W. V. B. and Sinha, 1963

With respect to the above-mentioned moralities, 10 ml of Pikovskaya's broth was modified for each isolate, and inoculated with respective isolated cultures and incubated at 37^{0}C, simultaneously a uninoculated broth (control) was maintained for all the molarities, after the incubation period turbidity was observed in each broth culture.

The broth was centrifuged at 10,000 rpm for 10 minutes, the supernatant and pellet obtained was collected separately, the pellet was washed thrice with deionized water, followed by sonication (Sonics Vibra-cell vcx 750) for 2 to 3 minutes at 15KHz, then both the samples were estimated colorimetrically (Equiptronics EQ 650) by Fiske–Subbarow method.

3. Results

3.1 Isolation of PSB from soil sample

Within a week of incubation, a clear halo zone around each colony was observed (Fig 1). This is preliminary confirmation of PSMs. Four distinctive colonies were isolated and purified and labeled as A, B, C and D respectively. The Colony morphology of each isolates were studied and listed in Table 2.

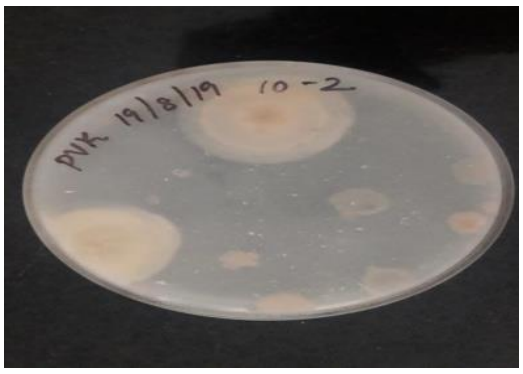

Fig 1 Clear halo zone around each colony are preliminary confirmed as PBMs

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Colony morphology	Isolate A	Isolate B	Isolate C	Isolate D
Shape	Circular	Circular	Circular	Circular
Color	Creamish white	Creamish white	Creamish white	Creamish white
Margin	Entire	Entire	Entire	Entire
Elevation	Raised	Raised	Raised	Raised
Opacity	Opaque	Opaque	Opaque	Opaque
Texture	Slightly Rough	Slightly Rough	Slightly Rough	Slightly Rough
Constancy	Wet	Wet	Wet	Wet
Gram reaction	Gram + ve	Gram + ve	Gram + ve	Gram + ve

Tab 2 Colony morphology of isolates

3.2 Estimation of the amount of Phosphate imbibed in the storage granules by Fiske–Subbarow method

Colorimetric analysis at 700nm was carried out for both pellet and supernatant to determine the concentration of phosphate imbibed and present in the broth. A standard graph was plotted wherein X-axis is the concentration of the standard (mg/ml) and Y-axis is OD at 700nm. From the standard graph (Fig 2) the concentration of both pellet and supernatant was found for all four isolates as in the figures below (Fig 3-9).

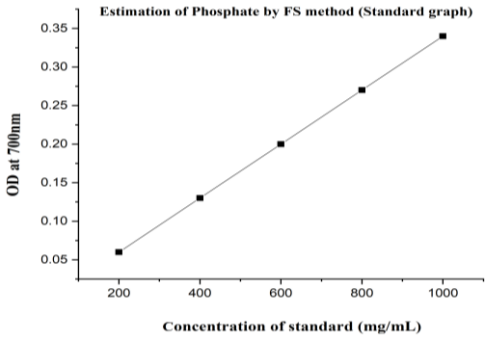

Fig 2 Standard graph of Ca3 (PO4)2

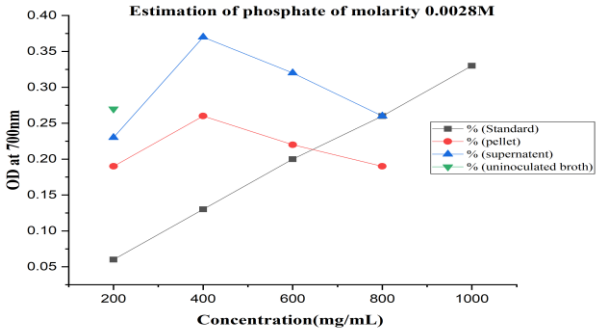

Fig 3 Estimation of phosphate of molarity 0.0028M

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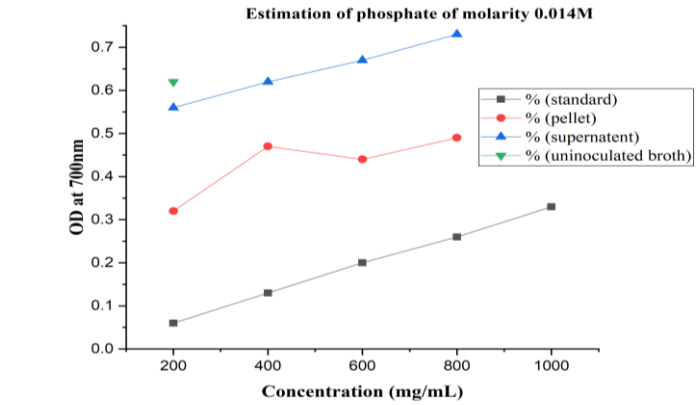

Fig 4 Estimation of phosphate of molarity 0.014M

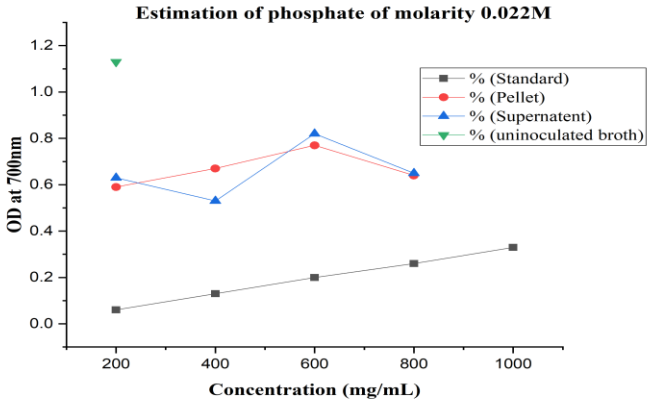

Fig 5 Estimation of phosphate of molarity 0.022M

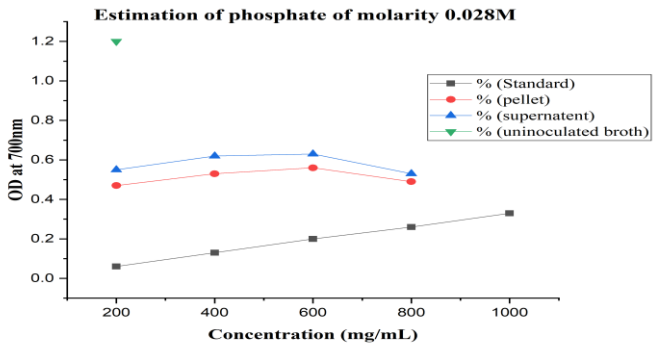

Fig 6 Estimation of phosphate of molarity 0.028M

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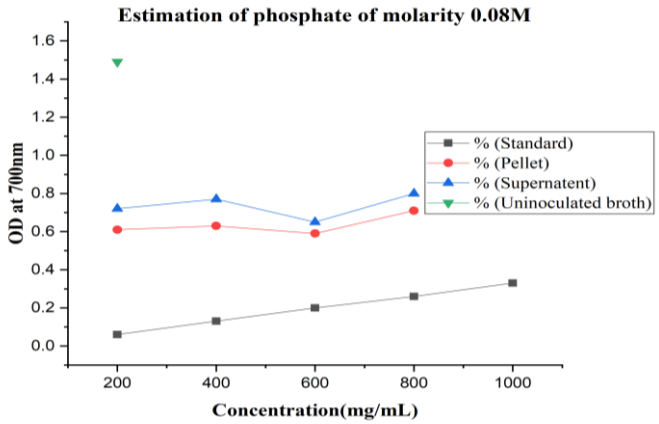

Fig 7 Estimation of phosphate of molarity 0.08M

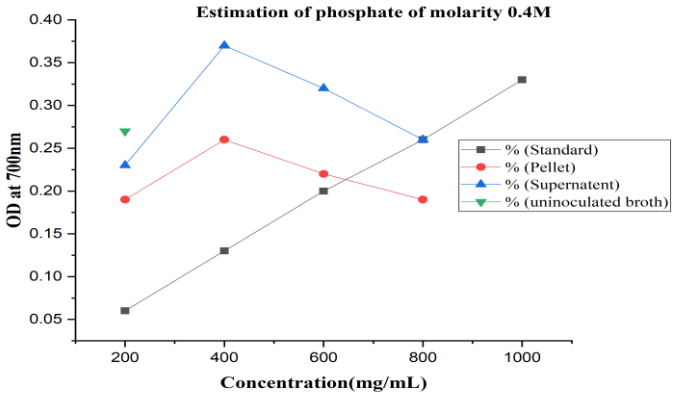

Fig 8 Estimation of phosphate of molarity 0.4M

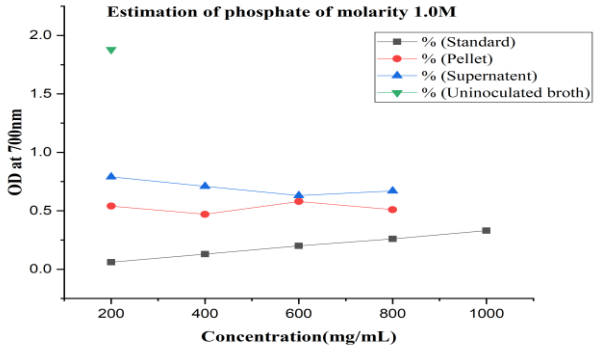

Fig 9 Estimation of phosphate of molarity 1.0M

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Discussion

Phosphate is not readily available for the plant for their normal functioning and metabolism, this is due to their insoluble nature in soil. There are many scientific methods which explain about the mechanism and methods of breakdown of organic or inorganic phosphate by phosphate solubilizing microorganism (PSM) into PO_4- ions which can be readily up taken by plants and it enhances the yield and growth of plant.

For screening and isolation of PSM, Pikovskaya's medium was used as selective media. Colony with clearance zone was selected and preliminarily confirmed as PSBs .Four different isolates were isolated and labelled as A, B, C and D respectively and the concentration of phosphate of both pellet and supernatant is determined By Fiske–Subbarow method it basically involves the measurement of phosphorus molybdate complex formed by interaction of phosphate and ammonium molybdate in the presence of sulphuric acid.

Isolated strains are allowed to grow in Pikovskaya's broth, in which Calcium phosphate molarities are taken in ascending and descending order form the standard put forwarded Sundara-Rao and Sinha (1963) as mention in table 1. We have observed a varying pattern of growth for all four isolates within varying concentration.

In the present work we have highlighted how much amount of phosphorus is imbibed in the cell, with varying morality of phosphate substrate used in the PVK media. Once the isolated culture were inoculated into varying molarities of phosphate in the media (tab1) the assorted growth pattern were observed, in low molarity the growth was seen after a week, this could be due to low phosphate concentration in media and the cell die due to starvation. Where as in high morality the growth was quick but cell died eventually this could be due to unfavourable condition for growth i.e. media turning basic.

Growth in 0.0028M and 0.014M were slow and turbidity was observed after 12 days of incubation. In 0.022M growth was better than 0.0028M and 0.014M molarities and turbidity was observed after 9 days of incubation. In 0.028M growth was better than 0.022M molarities and turbidity observed within 7days of incubation. This was the morality of standard medium. In 0.08M growth was found to be better than 0.028M and turbidity were observed after 5 days. In 0.4M growth was weaker than 0.08M and in 1.0M growth rate was weaker than 0.4M. In both the tubes turbidity was observed after 14 days of incubation.

Further the amount of phosphate imbibed in the storage granules was estimated by subjecting the cells for sonication to lyses the cells and concentration was estimated by Fiske–Subbarow method colorimetrically at 700nm. From the plot of concentration (mg/ml) vs OD at 700nm with respect to standard, concentration of both pellet and supernatant was found. It was observed that in molarity of 0.08M, 0.028M, 0.022M and 0.014M the solulization was high which resulted in high phosphate intake by cell, whereas in 0.4M and 1.0M it was less this may be due to high concentration of phosphate substrate and change in pH of media.

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Conclusion

Phosphorus with an atomic number fifteen is an essential macro element to all living organisms. As it takes the part in nucleic acid, enzymes, and cofactors, nucleotide, and phospholipid. [1,2] Though soil contains about 90% of phosphate, availability for plants is very poor and low. This is due to the combination of phosphorus with other cations. [3,6] So, to overcome this loss of phosphate, in nature PMS plays a vital role in converting insoluble to the soluble form of phosphorus [4]. Organic acid produced by such microorganisms hydrolyses organic phosphate to a soluble form, which is readily available for plants. In recent times, a lot of research is concerned with phosphate storage and solubilization. However, the amount of phosphate imbibed in the storage granules is still a mystery. In this work, we have estimated and briefed out the amount of phosphate imbibed in such storage bodies.

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Authors Contributions: Each and every author had contributed to the manuscript.

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