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In vitro Phosphate Solubilization by *Bacillus subtilis* PSBN B4 obtained from Pineapple (*Ananas comosus*) rhizosphere

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ABSTRACT

The Phosphate solubilizing bacteria play a very significant role in the proper growth and development of plants. In the present investigation, three pineapple rhizosphere soil samples were collected from Ngariyan Hill (Andro), Huikap(Andro) and Sandrok-Khanarok (Keirao) of Imphal East district, Manipur. A total of fourteen isolates were obtained and the best isolated Phosphate solubilization named as PSBN B4 was selected. The isolated colony was observed and confirmed as *Bacillus subtilis* on the basis 16sRNA gene sequence (Genebank Accession Number KR297241). The conditions for the maximum phosphate solubilization were optimized like pH, carbon source, nitrogen source, incubation period, shaking and non shaking. It was observed that pH 7, Dextrose as carbon source, Peptone as nitrogen source and Shaking conditions at 37ºC were best suited for the phosphate solubilisation by the selected isolate.

Keywords: Phosphate solubilization, *Bacillus subtilis,* Pineapple, Rhizosphere.

INTRODUCTION:

Pine apple is one of the important fruits grown in various parts of world. It is consumed raw as well as used for production of various items like jams, ice creams, yogurt, various desserts etc. Pineapple is a very rich source of nutrients like vitamin C, vitamin B and minerals like magnesium, potassium zinc etc [1]. It has also been found to possess many medicinal properties including anti-inflammatory action and anti-oxidant properties. Pineapple leaves have been found to be effective for reducing

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high blood pressure. Different parts of pineapple plant have been reported to be helpful in an array of conditions including body aches, motion-sickness, diarrhoea, rheumatoid arthritis, sinusitis etc [2]. India is the fifth largest producer of pineapple with an annual output of about 1.2 million tonnes (http://nhb.gov.in/report_files/pineapple/PINEAPPLE.htm). The north-eastern states of India produce more than 40% of this [3]. Moreover, maximum amount of this pineapple cultivation in the north-eastern states is carried out by organic farming [4].

The growth of pine apple plant is dependent on several physicochemical factors including the appropriate levels of phosphorus in soil. The most important factors for successful cultivation of the pineapple are giving the crop with adequate fertilizers, perfect drainage, periodical turning of the soil to control weeds, and to provide mulch. The use of fertilizers aims at improving the fertility of the soil and thereby increasing the yield of the crop. The recommended dose of fertilizer for pineapple is 12 g/plant/year of N, 4 g/plant/year of P_2O_5 and 12 g/plant/ year of K_2O [5].

Phosphorous is a major element which plays critical role in the normal growth and productivity of plants [6]. Phosphorous is vital for several processes like cell division, photosynthesis, energy storage, respiration, growth of good root system and utilizing carbohydrate etc.[7, 8,9]. Efficiency of P fertilizer throughout the world is around 10 - 25 % [10], and concentration of bio available P in soil is in a minute amount correspondent to the level of 1.0 mg per kg soil [11]. Plants acquire phosphate anion which is greatly reactive and gets very easily immobilized through precipitation with cations, for example, Ca^{2+}, Mg^{2+}, Fe^{3+} , and Al^{3+}, depending on soil properties [8, 12,13]. Farmers use phosphorous fertilizers to provide phosphorus to the plants but these fertilizers are very expensive and also causes pollution of water bodies. This results in the oxygen depletion and the death of the water creatures and the development of algae in the water bodies or the other water sources. So the alternate option to remove all these kind of problems is to apply the microorganisms which can solubilize the insoluble inorganic phosphorus to the soluble form, which is available to the plants and in this way harmful effects of the immobilized phosphatic fertilizers could be reduced.

Phosphate solubilizing ability of microorganisms is one of the most significant characteristic linked to the plant phosphate nutrition. These bacteria can improve the availability of Phosphorus by converting insoluble forms of phosphate to the soluble forms and thereby making them available for uptake by plants [14, 15, 16]. Phosphate solubilizing bacteria (PSB) are a group of helpful bacteria that are capable of hydrolysing soluble inorganic phosphorus from insoluble compounds. The role of Phosphorus solubilizing bacteria is converting insoluble phosphatic compounds such as rock phosphate, bone meal and basic slag particularly the chemically fixed soil phosphorus into available form. Many PSB's have already been reported *viz*., *Pseudomonas, Bacillus, Erwinia, Rhizobium, Burkolderia, Achromobacter, Agrobacterium, Microccocus* etc. Phosphate-solubilizing bacteria (PSB) mobilize impenetrable inorganic phosphates from their mineral medium to the soil where they are made available for absorption by plant roots [17]. The chief phenomenon of mineral phosphate-solubilization by phosphate-solubilizing strains is closely related to the discharge of low molecular weight organic acids [11, 18] which, via their hydroxyl and carboxyl groups, bind the phosphate-bound cations (Al, Fe, Ca) and henceforth transforming it into soluble forms and decrease the pH in basic soils [19].

Thus, the present investigation was carried out to isolate phosphate solubilizing bacteria associated with rhizosphere of pineapple plants growing in Manipur (part of North-eastern India). Relatively less information is available regarding the microflora of this region although the region boasts of a good pineapple yield.

MATERIALS AND METHODS:

Soil sample and isolation:

Three rhizhosphere soil samples of Pineapple plants were taken from the three different location of Imphal – East district, Manipur viz. Ngariyan Hill (Andro), Huikap (Andro), Sandrok- Khanarok (Keirao) and stored in air tight polybags. The samples were serially diluted on Pikovskaya's medium [20] plates and incubated at 37º C. The various isolates obtained were pentagonally streaked on the Pikovskaya's medium for purification and subsequently stored in the form of slants of the same medium.

Screening:

Primary screening was done by plate assay method by spotting the isolates on the Pikovskaya's medium plates and measuring the colony radius and zone of phosphate solubilisation. The Phosphate Solubilizing Efficiency (PSE%) was calculated as per the formula: PSE (%) = Z - C/C × 100 (where, Z = zone of phosphate solubilisation C = Colony radius) [21]. Secondary screening was done by using the method described by Narveer *et al.* [22] and quantitative estimation of phosphate solubilisation was done using John's method [23].

Optimization:

For optimization the conditions like time of incubation, pH, carbon source, nitrogen sources were varied (one factor at a time).

Characterization:

Characterization of selected isolates: The selected bacterial isolate was characterized on the basis of various morphology and biochemical characters as per Bergey's Manual Systemic Bacteriology. The 16s rDNA sequencing was carried out by Samved Biotech (Ahmedabad, Gujarat, India) with the help of 27F and 1492R primers. Consensus sequence of 1106bp rDNA gene was generated from forward and reverse sequence data using the software aligner. The 16S rDNA gene sequence was used to perform BLAST with the nrdatabase of NCBI genbank database

RESULTS AND DISCUSSION:

Isolation and screening of phosphate solubilizing bacteria

Three rhizosphere soil samples of Pineapple plant were collected from the three different places of Imphal-East district viz. Ngariyan-hill (Andro), Huikap (Andro) and Sandrok (Keirao). A total of fourteen different phosphate solubilising bacterial isolates were obtained from these three samples. The Ngariyan-hill sample resulted in six isolates (designated as PSBN B1 to PSBN B6) and the sample from Huikap yielded three isolates (PSBH B1 to PSBH B3) while from the pineapple rhizosphere soil sample collected from Sandrok, five phosphate solubilizing bacterial isolates (named as PSBSK B1 TO PSBSK B5) were obtained. Gulati *et al*. [24] isolated *Pseudomonas flourescens, P. Trivialis, P. poae* and *Pseudomonas spp.* having high phosphate solubilizing capability from the calcium abundant soils and alkaline soils in the cold-desert region of Spiti and Lahaul in the trans- Himalayan regions of India. Ren *et al*. [25] found two strains of Phosphate solubilizing bacteria and identified as *Acinetobacter calcoaceticus* YC-5a and *Enterobacter agglomerans* KMC-7. Javadi Nobandegani *et al*. [26] identified 4 best isolates on basis of phosphate solubilizing bacteria from the soil of Malaysian oil palm field (Serdang) as *Alcaligenes faecalis, Bacillus cereus, Vagococcus carniphilus* and *Serratia plymuthica.* Suarez *et al*. [27] isolated phosphate solubilizing Gram negative bacteria *Rhizobium, Labrenzia, Stappia, Aureimonas,* and *Mesorhizobium* and mostly related to *Rhizobium rhizoryzae* from rhizospheric soil of *Plantago winteri* from a natural salt meadow.

Primary screening of the isolates was done by spotting on to Pikovskaya's medium plates. Phosphate solubilizing efficiency of the different isolates was measured. PSBN B4 and PSBN B5 showed maximum phosphate solubilizing efficiency of 100%. From sample 2 PSBH B3 showed PSE of 50% and from the sample 3 PSBSK B4 showed PSE of 50%. Over all PSBN B4 and PSBN B5 isolates showed maximum phosphate solubising efficiency however, no zone of phosphorous solubilzation was observed in case of isolate PSBH B2. Secondary screening was carried out by assessing the phosphate solublization under liquid culture condition using Pikovskaya's broth. Here, PSBN B4 isolate was found to be the best phosphate solubilizer, which solubilised 1.29µg of phosphorous/ ml and PSBN-B3 found to be the most inefficient phosphate solubilizer showing less phosphate solubilisation, 0.14µg P/ml. On comparing the results of Primary and the secondary, PSBN B4 was observed to show 100% phosphate solubilisation efficiency during primary screening and the same isolate also solubilised the maximum amount of phosphate (1.29µg P/ml) during the secondary screening. However no direct correlation was observed in PSBN B1, PSBH B2, PSBH B3 and PSBSK B5. Some other workers have also made similar observations [21, 28,29].

Optimization of conditions

On the basis of secondary screening PSBN-B4 was selected for further studies. Optimization of the selected isolate was done with respect to pH, carbon source, nitrogen sources etc. The isolate was grown at various pH like pH 4, 5, 6, 7, and 8 and it was found that isolate PSBN B4 solubilized the maximum amount of phosphorus (1.22 ± 0.015 µg P/ml) at pH 7 (fig 1).

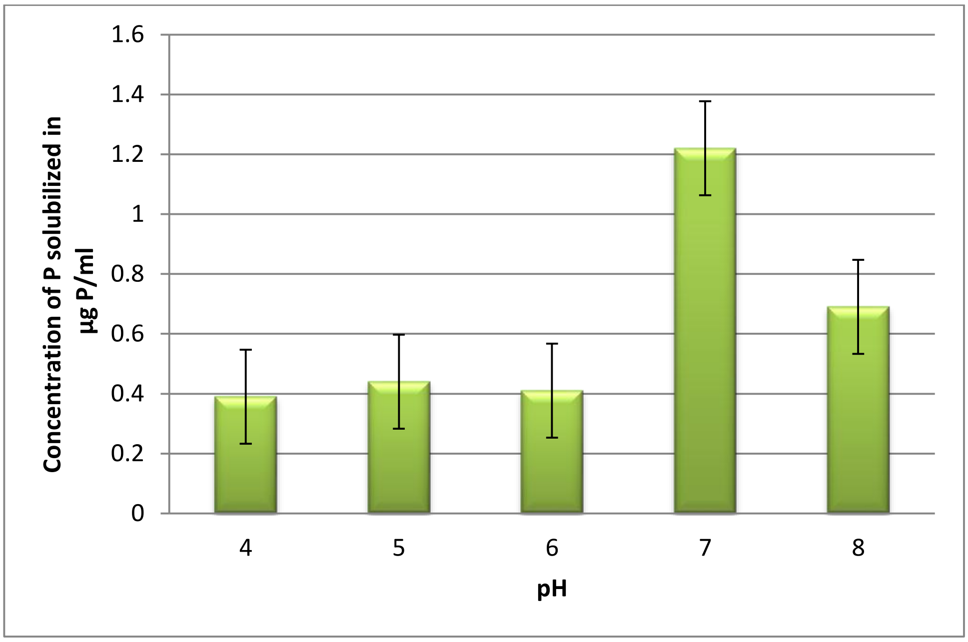

Fig 1: Effect of pH on the phosphate solubilization efficiency of isolate PSBN B4.

The least value of 0.39 ± 0.002 µg P/ml was observed in case of pH 4. This observation is in agreement with Maheswar and Sathiyavani [30](2012) who reported that for *Bacillus subtilis*, isolated from groundnut rhizosphere soil, pH 7 was most suitable for phosphate solubilisation. Similarly, Son *et al*. [31] identified *Pantoea agglomerans* to be the efficient phosphate solubilizer which solubilises 900mg/l Phosphate at pH 7.5.

The isolate was then optimized for the different carbon sources like Starch, Sucrose, Lactose and Dextrose. It was found that Dextrose was the best carbon source for the growth of the isolate and it solubilized 1.11±0.017 µg P/ml. While on the other hand, carbon source like sucrose, starch, lactose resulted into lesser phosphate solubilisation (Fig 2). This may be attributed to the fact that dextrose is the simplest sugar and hence might be easily taken up by the microbe ultimately leading to better phosphate solubilisation. Similarly, dextrose or glucose has also been reported to be favourable for bacterial phosphate solubilisation by many researchers [32, 33,34]. In case of nitrogen sources, peptone proved to be the best nitrogen source followed by ammonium sulphate while ammonium nitrate lead to the least activity (Fig 3). Pallavi and Gupta [34] observed best nitrogen sources in ammonium sulphate. However, Seshadhari *et al.* [35] observed ammonium nitrate was very effective for phosphate solubilisation by *Aspergillus*.

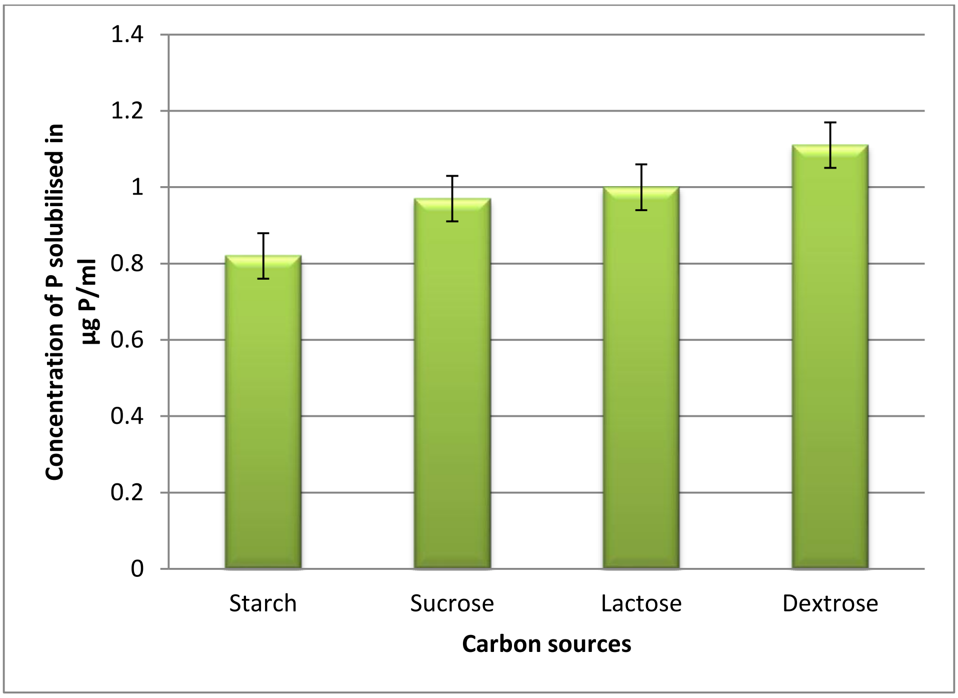

Fig 2: Effect of Carbon source on Phosphate solubilization efficiency of isolate PSBN B4.

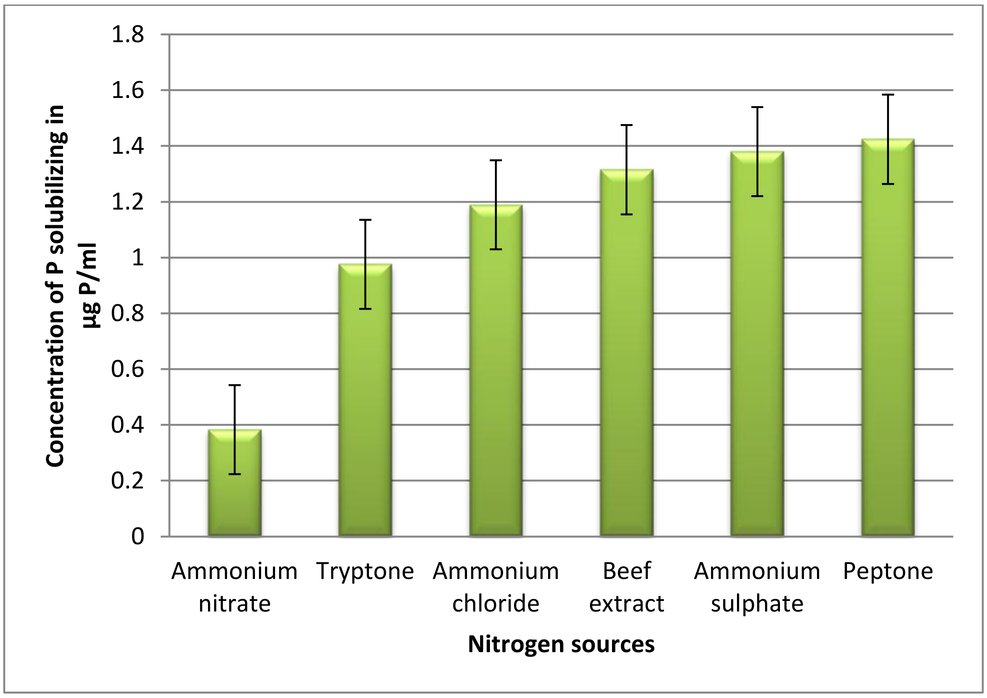

Fig 3: Effect of Nitrogen sources on the Phosphate solubilization efficiency of isolate PSBN B4.

Optimization of the incubation period was done in which the effect of shaking and non-shaking conditions was also assessed. The shaking conditions were observed to be good for the solubilization of the phosphorus by the bacteria. This may be because shaking resulted in better aeration and distribution of nutrients. Maximum phosphate solubilization was observed in case of 72 hrs of incubation in case of both shaking and non-shaking conditions (Fig 4). Similar observations have also been made by many workers [36, 37,38].

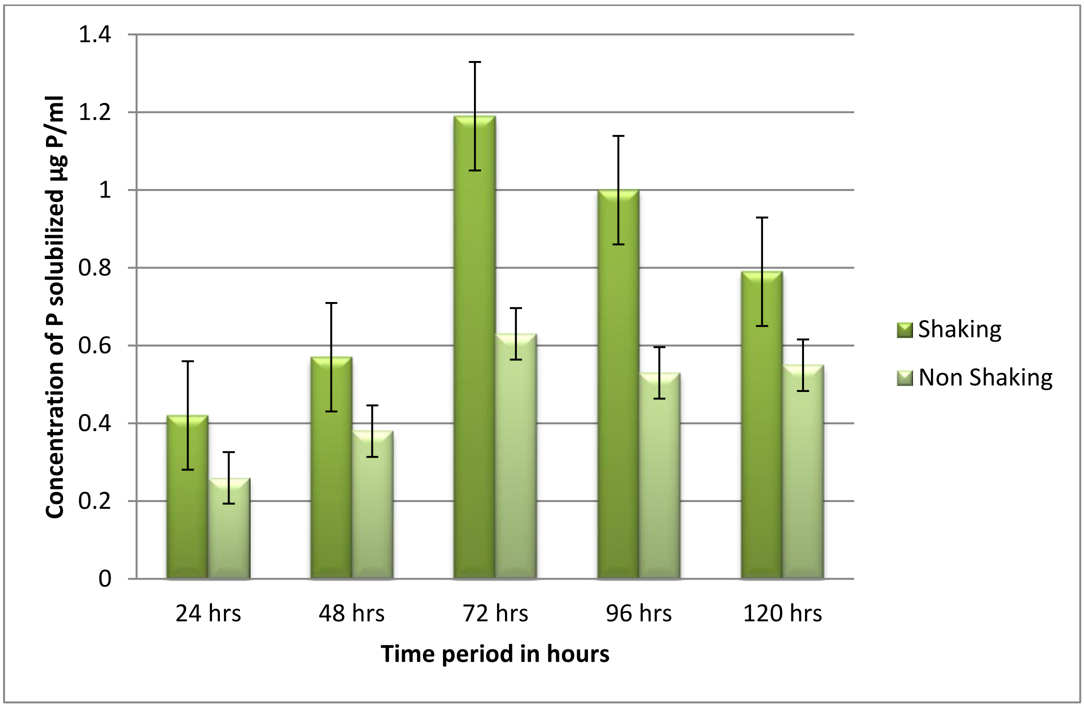

Fig 4: Effect of incubation time with respect to Shaking verses Non Shaking on the Phosphate solubilization efficiency of isolate PSBN B4.

Characterization of selected isolate

Biochemical tests were done for the partial characterization of the isolate. From the biochemical tests it was observed that the isolate is Gram's Positive in nature and is having Rod like shape. It was a sporulating bacteria possesing motility. The isolate was also found to be Catalase positive. The isolate was also characterized on the basis of 16s rDNA sequence (Genebank Accession Number- KR297241) and it appears to be *Bacillus subtilis*.

CONCLUSIONS

The frequent use of chemical fertilizers has lead to the depletion of nutrients from the soil. Thus, we need alternative strategies to improve soil health with causing damage to the environment as well as in the soil. So Bio-fertilizers are the products consisting of selected and the beneficial living microorganisms like Phosphate Solubilizing

Bacteria which are having the ability to solubilize the insoluble inorganic phosphorus to the soluble inorganic phosphorus and which are very important now a days, in farmers for future higher production. Phosphate solubilizing microbes are known to enhance the plant available form of phosphorus by converting the insoluble phosphate to the soluble form. In the present investigation, out of the fourteen isolates *Bacillus subtilis* PSBN B4 was found to be the most efficient phosphate solubilizing strain obtaineded from the pineapple rhizosphere. This isolate displayed appreciable amont of phosphate solubilizing activity under in vitro conditions. So, although the isolate seems to a potential candidate for biofertilizer production, the phosphate solubilisation activity of this isolate needs to be assessed under in vivo conditions.

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