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SCREENING OF PHOSPHATE SOLUBILIZING BACTERIA FROM SOILS OF DHARAN, SUNSARI, NEPAL

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ABSTRACT

Phosphorus is one of the essential macronutrients for plant growth and reproduction. Plant absorbs phosphorus from soil solution as phosphate anions which are highly reactive and may be immobilized by precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, present in the soil and thus it becomes insoluble and unavailable for plants. PSB can be used as biofertilizer since they increase phosphorus availability in the soil for growing plants. The main objective of this study was to isolate and identify PSB morphologically and biochemically from the agricultural soil sample. For this purpose, total 16 samples were collected. Isolation was performed on Pikovskaya's (PVK) media by spread plate technique. PSB were isolated from soil sample of Dharan, taken from agricultural field. The isolated organisms were identified according to colony morphology, gram staining reactions and various biochemical properties. The solubilization index (SI) and solubilization efficiency (SE) were calculated on the basis of the halozone and colony diameter. Only two PSB were isolated. Both were gram positive, spore forming rod shaped bacteria. According to colony characteristics, staining reactions and biochemical tests, the isolates were *Bacillus megaterium* and *Streptomyces* spp. Their phosphate solubilizing ability was analyzed qualitatively on PVK plate. Their solubilizing indexes (SI) were found to be 1.75 and 1.33 while solubilizing efficiency (SE) were calculated 75% and 33% respectively. Both PSB isolates were spore former, as the soils of Dharan generally contain comparatively low moisture (drought) in April to June.

Keywords: Phosphate solubilizing bacteria (PSB), Solubilization Index (SI), Solubilization Efficiency (S.E.), Pikovskaya's medium.

1. INTRODUCTION

Phosphorus is the second essential macronutrient next to nitrogen for growth and development of plants involved especially in important metabolic pathways like photosynthesis, biological oxidation, nutrient uptake and movement and cell division [1].

Phosphorus (P) profoundly affect the overall growth of plants [2] by influencing various key metabolic processes such as cell division and development, energy transport, signal transduction, macromolecular biosynthesis, photosynthesis and respiration of plants [3] and nitrogen fixation in legumes [4].

Soils contain a big amount of the insoluble Phosphorus in the form of phosphate such as calcium phosphate [Ca_3 $(PO_4)_2$], ferrous phosphate [$FeSO_4$], aluminium phosphate [$AlSO_4$], rock phosphate, apatite etc. Since such phosphates are unavailable for plants, Inorganic Phosphorus mostly in the form of superphosphate is used as chemical fertilizers to support crop production worldwide but repeated use of such fertilizers deteriorates soil quality [5]. Superphosphate is soluble phosphate and easily absorbed by plants as it is soluble in water. However, only 5-30% of applied phosphate is utilized by plants and rest are immobilized into insoluble phosphate due to presence of Fe, Al and Ca ions in soil and becomes unavailable for plants [6].

The insoluble forms of P may be converted to soluble P by P-solubilizing organisms inhabiting different soil ecosystems [7]. Many fungi and bacteria e.g., *Aspergillus, Penicilium, Bacillus* and *Pseudomonas* are known to solubilize insoluble organic and inorganic phosphates. Phosphate solubilizing microorganism produces organic acids such as formic acid, propionic acid, acetic acid, lactic acid, glycolic, fumaric and succinic acid. These acid form chelate with Fe and Al ions and enhance the solubilization of phosphate. Phosphate solubilizing bacteria include the strains from the genera such as *Pseudomonas, Azospirillum, Burkholderia, Bacillus, Enterobacter, Rhizobium, Erwina, Serratia, Alcaligens, Arthrobacter, Acenetobacter and Flavobacterium* [8].

Most of these bacteria are associated with rhizosphere of the plants and plays beneficial role in the plant growth. Therefore, they are also termed as plant growth promoting rhizobacteria [9]. PSM can be produce commercially and use on the agriculture for better yield of the crops as they are less economic, ecofriendly, than chemical fertilizer and can benefit human and the environment [10].

The present study was focused on isolation and identification of phosphate solubilizing bacteria from soils of Dharan which might have a significant role in solubilizing complex phosphate and providing phosphorus to the plants.

2. MATERIALS AND METHODS

2.1. Sample collection

Total 16 Soil samples were collected in the plastic bags. Bags from about 10 cm deep from the agricultural surface fields of Dharan (26° 48' 44.93" N and 87° 17' 0.78" E), Sunsari (Nepal) in April to June 2017. Soils from different points of a site were collected, mixed and air dried for a single sample. The sample was kept in the plastic bag and transported.

2.2. Screening and Identification of phosphate solubilizing bacteria

Isolation of phosphate solubilizing bacteria was done by spread plate technique by performing the serial dilution of soil samples on Pikovskaya's medium

Table 3. Biochemical tests for *Streptomyces* spp.

Isolate code	Organisms isolated	Colony + halozone diameter	Colony diameter	Halozone diameter	Solubilising Index (S.I.)	Solubilising Efficiency (S.E.)
P1	*Bacillus megaterium*	3.5	2	1.5	1.75	75%
P2	S*treptomyces* spp.	1.6	1.2	0.4	1.33	33%

plates as selective media. The plates were allowed to stand for about 10 minutes. The plates were incubated at 30° C for 7 days and observed for transparent clear zones around the colonies of bacteria grown on the surface of media [11].

The pure culture of phosphate solubilizing bacteria from the positive culture was obtained by subculturing on the sterile Pikovskaya's medium plates and was then incubated at 30° C for 7 days [12]. After obtaining of pure colonies of microorganisms, they were identified by using standard techniques as described in the Bergey's Manual [13] which involves morphological appearance of colonies, staining reactions and biochemical properties.

2.3. Qualitative analysis of phosphate solubilization activity

The bacterial isolates with halo zone were selected for the analysis of phosphate solubilization activity. Qualitative analysis of phosphate solubilization activity of the isolate was conducted by plate screening method. The isolated bacteria were stab inoculated on the Pikovskaya's medium and incubated for 5 days. Solubilization Index (S.I.) and Solubilization Efficiency (S.E.) were calculated [14].

S.I. = (colony diameter + halozone diameter) / (colony diameter)

S.E. = (halozone diameter)/ (colony diameter)*100

3. RESULTS AND DISCUSSION

Only 2 phosphate solubilising bacteria (P1 and P2) were screened from 16 soils samples collected from Dharan. The colony of the bacteria showed varying levels of phosphate solubilising activity as clear zones around their colonies on Pikovskaya's medium (figure 1)

Table 1. Biochemical tests for *Bacillus megaterium*

Biochemical tests	Result
Catalase test	+
Oxidase test	+
Indole test	-
Methyl red test	+
Vogesproskaur test	-
Citrate utilization test	-
Starch hydrolysis	+
Gelatin hydrolysis	+
Nitrate reduction	-
H_2S production	+
Motility	-

The P1 isolate showed creamy white, circular and translucent colony while the isolate P2 showed white colored, circular and opaque colony on PVK medium plate.In gram staining, P1 isolate showed gram positive rod (figure 2) while P2 showed gram positive mycelium (figure 3).

Table 2. Biochemical tests for *Streptomyces* spp.

Biochemical tests	Result
Catalase	+
Oxidase	-
Indole	-
Methyl Red test	-
Vogesproskaur test	-
Glucose utilization	+
Fructose utilization	+
Sucrose utilization	+
Lactose utilization	-
Citrate utilization test	+
Starch hydrolysis	+
Gelatin liquefaction	+
Urea hydrolysis	+
Esculin hydrolysis	+
Nitrate reduction	-
H_2S production	-
Motility	-

Figure 2. Micrograph of *Bacillus megaterium* under oil immersion

According to colony characteristics, staining reactions and biochemical tests, both isolates were identified as *Bacillus megaterium* (Table 1) and

Figure 3. Micrograph of *Streptomyces spp.* under oil immersion

Streptomyces spp (Table 2) respectively.

Phosphate solubilising ability of both bacteria was analyzed qualitatively on PVK medium plate. Their

Figure 1. Colonies of Phosphate solubilizing bacteria on Pikovskaya's medium

solubilising Indexes (S.I.) were found to be 1.75 and 1.33 while Solubilising efficiencies (S.E.) were calculated 75% and 33% respectively (Table 3). Both PSB isolates were gram positive and spore former, as the soils of Dharan generally contain comparatively low moisture (drought) in April to June.

Since Dharan is a dry city of Eastern Nepal during the month of April to June, only two phosphate solubilising bacteria were screened on Pikovskaya's medium from 16 soils samples and identified on the basis of biochemical tests as *Bacillus megaterium* and *Streptomyces* spp. In this study, *Bacillus megaterium* followed by *Streptomyces* spp were isolated having high capacity to solubilize insoluble phosphates.

Hence, the most dominant phosphate solubilizing bacteria of *Bacillus* sp., found to be gram positive aerobic and spore former which can overcome several unfavourable conditions. Spore forming bacteria's are well known to resist adverse conditions such as high temperature and dryness [15].

The most dominant phosphate solubilizing bacteria found were aerobic and among which some were spore forming bacteria. Identification of this group showed that *Bacillus* sp. was the most predominant PSB especially *B. cereus, B. subtilis* and *B. megaterium*. Other PSB involved were *Klebsiella* sp., *K. aerogenes*, *K. pneumoniae, Proteus sp., P. mirabilis, P vulgaris, P. inconstans, Enterobacter sp., E. aerogens,* and *Pseudomonas* sp. [16]. Total 10 PSB were isolated from soil of Tamil Nadu and among these 6 strains were *B. megaterium*, 2 strains were *P. putida* and 2 strains were *P. flourescences* [17]. Out of total 44 isolates, 35 (79.5%) were able to solubilise calcium Phosphate [$Ca_3 (PO_4)_2$] and 14 bacteria were screened for further analysis on the basis of diameter of the solubilising halos on PVK medium ranged between 0.6 cm and 1 cm. According to the gram stain, all were gram negative bacteria [18].

According to several studies, the bacteria isolated from the rhizosphere of different plants have ability to dissolve insoluble Phosphorus [19]. Phosphorus is one of the very important element required for the plant growth and development because it is a component of nucleic acid, phospholipid and ATP. It involves in the regulation of various metabolic pathways in plants [20]. Organic acids are produced by various bacteria causing acidification which has a significant role in mineral P solubilization [21]. However, Hamdali et al. (2008), have reported alkalinisation during the solubilization of natural P by Actinomycetes isolated from Moroccan phosphate mines and absence of organic acids in the growth medium [22]. This proves that there is other mechanism in the process of inorganic P solubilization as the production of siderophores [23].

Chemical fertilizers disturb the microbial diversity on the soil and decrease the soil fertility [24]. The main source of chemical P fertilizer is the Phosphatic rock, which has been depleting day by day and high cost manufacture [7]. About, 75% of total phosphate fertilizer applied on the soil becomes unavailable due to immobilization [25]. Therefore, PSM can be used as the substitute of the chemical phosphate fertilizer.

For a more sustainable agriculture, application of phosphate solubilising bacteria increases soil fertility due to their ability to convert insoluble P to soluble P by releasing organic acids, chelation and ion exchange [26]. The positive effect of P solubiliser has been reported on food and fodder crops [27].

Most agricultural soil contains large amount of P, but it is often present in unavailable forms. So chemical fertilizers are used in the agricultural fields to overcome the scarcity of available phosphorus for plant growth. This fertilizer contains soluble P and provides P to increase the plant productivity. But not all applied fertillizer can be used by the plants because in the soil more than 80% of the P is unavailable for plants due to precipitation, adsorption or conversion to the organic form [28].

Many soil microorganisms are known to solubilize insoluble organic and inorganic phosphates by producing phosphatase enzymes and organic acids [29]. Among 35 isolated bacteria, only two bacteria showed highest solubilization efficiency namely *Bacillus megaterium* (S.E. 80) and *Pseudomonas synxantha* (S.E. 70) [30].

In addition to P-solubilization, PSB also produced the other secondary metabolites like IAA and siderophore. Several evidences related to plant growth promotion by PSB through the production of IAA [31] and siderophore [23] makes the PSB more suitable as biofertilizers which are ecofriendly, low cost and cause less polution to the environment than the chemical fertilizers. Many literatures show only 15-20% of applied fertilizer can be utilized by plants and rest of the others are immobilised on the soil and becomes unavailable. This mean thousand of tons of fertilizers were deposited on the soil year by year. Therefore, PSB as biofertilizer can be used in soil to provide required amount of the phosphorus for plants.

4. CONCLUSION

Phosphate solubilizing bacteria were successfully screened at the laboratory of microbiology of Central Campus of Technology from soil of Dharan. The isolated bacteria were identified as *Bacillus megaterium* and *Streptomyces* spp. Hence Soil of Dharan contains phosphate solubilizers. The solubilization index and solubilization efficiency was obtained on the basis of phosphate solubilization.

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6. CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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