



## STUDIES ON PHOSPHATE SOLUBILIZATION AND PLANT GROWTH ENHANCEMENT BY RHIZOSHERIC BACTERIA

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### ABSTRACT

A total of 28 samples were processed, of which 20 isolates were identified as phosphate solubilizing bacteria. Among 20 isolates, 12 (60 %) belonged to *B. subtilis*, 3 (15%) were *B. brevis*, 3 (15%) belonged to *Pseudomonas aeruginosa* and 2 (10 %) were identified as *Enterobacter aerogenes*. All the isolates exhibited phosphate solubilization (1.009 µg/ml to 1.609 µg/ml), produced Indole acetic acid (5.46 mg/ml 14.89 mg/ml), protease activity and ammonia production. The isolates produced hydrogen cyanide in the range of 1-5 and were able to tolerate high concentration of insecticide chemorise i.e. upto 5% (v/v). *B. subtilis* PSB 16 isolates gave the highest values for all plant growth promoting attributes and exhibited growth promotion in *Hordeum vulgare* by showing enhancement in mean root and shoot length of the plant. PSB isolates showing plant growth promoting attributes has potential to be used as biofertilizer agent.

**KEY WORDS:** Phosphate solubilizing bacteria, biofertilizer, *Bacillus* sp.



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## INTRODUCTION

Phosphorus is essential for growth and productivity of plant. Phosphorus is the second most important macro-nutrient required by the plants, next to nitrogen<sup>1</sup>. It plays an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in a living plant. It helps plants to survive winter rigors and also contributes to disease resistance in some plants<sup>2</sup>. Phosphorous deficiency results in the leaves turning brown accompanied by small leaves, weak stem and slow development<sup>3</sup>. A great part of soil phosphorus, approximately 95-99% is present in the form of insoluble phosphates and hence cannot be utilized by the plant<sup>4</sup>. To increase the availability of phosphorus for plant, large amount of chemical phosphate fertilizers are used on a regular basis which is the main limitation in intensive crop production<sup>5</sup>. Insoluble phosphate is transformed into soluble form by microbes which are present in the rhizospheric microenvironment. Soil organisms play an important role in solubilization of raw phosphates which is either in the form of organic or inorganic phosphorous compounds<sup>6</sup>. Bacteria are more effective in phosphate solubilization among phosphate solubilizing microorganisms<sup>7</sup>. Phosphorous solubilizing bacteria (PSB) play an important role in plant nutrition not only through increase in phosphorus uptake by the plant, but also by exhibiting many other plant growth promoting attributes<sup>8</sup>. Therefore, their use as biofertilizer or control agents for agricultural improvement is the focus of numerous researchers. Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that can be found in the rhizosphere, in association with roots which can enhance the growth of plant and yield via various plant growth promoting substances<sup>9</sup>. The mechanisms by which PGPR can exert a positive effect on plant growth can be of two types: indirect and direct. Indirect growth promotion is the decrease or prevention of deleterious effect of pathogenic microorganisms, mostly due to the synthesis of enzymes or hydrogen cyanide. Direct growth promotion can be through the synthesis of phytohormones, N<sub>2</sub>

fixation and increasing the availability of many nutrients<sup>10</sup>. Phosphorous solubilizing bacteria with ability to exhibit PGPR can be the most feasible strategy for development of sustainable agriculture. The aim of the present study was to isolate phosphate solubilizing bacteria and to explore these isolates for various plant growth promoting attributes. The isolates were also checked for insecticidal tolerance. The isolate positive for maximum plant growth promoting attributes was used for seed inoculation.

## MATERIALS AND METHODS

### 1. Isolation of Phosphate Solubilizing Bacteria

Phosphate solubilizing bacteria were isolated using Pikovskaya's agar (HiMedia, Mumbai) from plant rhizosphere<sup>11</sup>. Approximately 1gm of soil sample was dissolved in 9 ml of sterile distilled water and mixed thoroughly. Then ten fold serial dilutions were made by dissolving 1ml of sample from the first test tube to next tube containing 9 ml of sterile distilled water. Various dilutions were spread inoculated on Pikovskaya's agar plates which were then incubated at 37°C for 7 days. The plates were observed daily and the colonies exhibiting halozone around them were considered as PSB. PSB were further purified after subculturing.

### 2. Characterization of PSB

Gram staining was performed on each purified culture for confirming their Gram nature and cell shape<sup>12</sup>. Various biochemical tests were performed for the characterization of bacterial strains isolated from rhizosphere soil as given in Cappuccino and Sherman<sup>13</sup>.

### 3. Quantitative estimation of Phosphorous

Quantitative estimation of Phosphorous solubilization was done by method as suggested by Chen *et al.*<sup>14</sup>. The bacterial isolates were inoculated to Pikovskaya's broth containing insoluble tricalcium phosphate and incubated at 35°C for 5 days. After incubation the broth was centrifuged at 10,000 rpm for 10 mins. After

centrifugation, the amount of soluble phosphate was determined by Mo-blue method<sup>14</sup>.

### **3. To screen the PSB for plant growth promoting activities**

All the PSB isolates were studied for different PGPR activities such as indole acetic acid production, ammonia production, protease enzyme production and hydrogen cyanide production

#### **(i) Indole acetic acid production**

The production of Indole acetic acid (IAA) by PSB and the effect of L-tryptophan on IAA production were assayed by following the method as given by Glickman and Dessaux<sup>15</sup>. Briefly, the bacterial isolates were inoculated into the nutrient broth containing varying concentration of L-tryptophan viz., 0, 50, 100, 200, 300 mg/l. After 48 h of incubation at 37°C, the broth was centrifuged and 1ml of the supernatant was mixed with 4 ml of Salkowski's reagent. The reaction mixture was incubated at room temperature for 20 min and then the absorbance was measured immediately at 535 nm using UV-VIS Spectrophotometer (Systronics, India). The amount of IAA produced was calculated using IAA as standard.

#### **(ii) Ammonia production**

The isolates were studied for the production of ammonia using peptone water. The cultures were inoculated into 10 ml peptone water and each tube was incubated for 48 hrs at 37°C. After incubation, 0.5 ml of the Nessler's reagent was added to each tube. Development of brown to yellow color indicated positive test for ammonia production<sup>13</sup>.

#### **(iii) Protease enzyme production**

All bacterial isolates were screened for protease production using skim milk agar<sup>16</sup>. Briefly, the bacterial cells were spot inoculated on the skim milk agar plate and the plates were incubated for 4 days at 37°C. Proteolytic activities were identified by clear zone formation around the colony.

#### **(iv) Hydrogen cyanide production**

Hydrogen cyanide (HCN) production was assayed by the method as suggested by Lorck<sup>17</sup>. For the production of HCN, bacteria were streaked onto nutrient agar slants supplemented with glycine (4.4g/l). After this, a piece of filter paper impregnated with 0.5% picric acid and 2% of sodium carbonate was placed in the test tube along with cotton plug. Then the tubes were incubated at 35°C for 96 hrs. Discoloration of the filter paper from orange to brown after incubation was considered as microbial production of cyanide. The results were assessed using 1 to 5 scales as followed by Donate *et al.*,<sup>18</sup>.

#### **4. Assay for insecticidal tolerance**

All the isolates were tested for their tolerance/resistance to commonly used insecticide viz. Chemorise. Chemorise (Cheminova, Denmark) is an insecticide which contains 25% buprofezin w/v. Briefly the bacterial isolates were inoculated into a nutrient broth containing varying concentration of Chemorise viz., 1%, 2%, 3%, 4%, 5%. The tubes were incubated at 35°C for 24 hrs. After incubation, absorbance was measured at 600 nm<sup>19</sup>.

#### **5. Plant growth promotion assay**

Effect of PSB on plant growth promotion was studied by seed inoculation by following the method as suggested by Ahmad and Khan<sup>20</sup>. Seeds of *Hordeum vulgare* were washed with distilled water and were surface sterilized by soaking in hydrogen peroxide for 10 mins. The sterilized seeds were bioprimered with PSB by soaking the seeds in liquid culture medium for 2 days. The non coated seeds were soaked in sterile water only and served as control. A total of 6 non-inoculated and 6 inoculated seeds were sown in disposable glass used as pot, which contained soil. The pots were watered with tap water when required and maintained in open natural conditions. The pots were observed daily for progress in plant growth.

## **RESULTS AND DISCUSSION**

Phosphorus, in Indian agriculture, occupies a unique position both in conventional as well as in alternative agriculture<sup>5</sup>. The requirement of

phosphorous for plants is usually met using chemical fertilizers. Owing to the negative impact of chemical fertilizers and their increasing costs, the scenario is shifting towards the use of biological fertilizers for a more sustainable agriculture. Phosphate solubilizing bacteria (PSB) enhances phosphorus availability in soils through dissolving insoluble inorganic phosphates into soluble organic phosphorus and makes them available to plant for their better growth and development<sup>21, 22</sup>. A considerably higher concentration of PSB is commonly found in the rhizosphere soil in comparison with non rhizosphere soil<sup>1</sup>. Population of PSB depends on different soil properties such as physical,

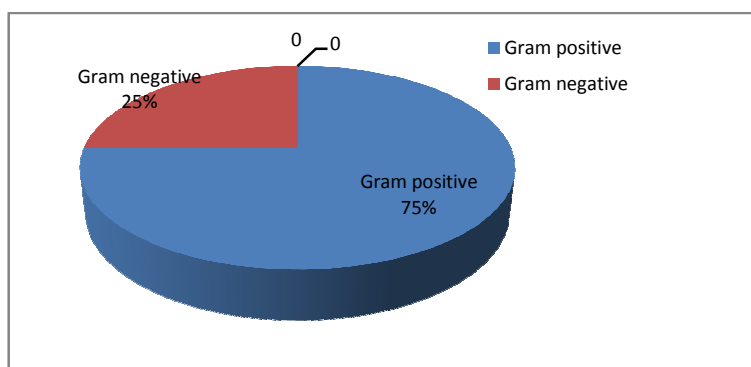
chemical properties and cultural activities<sup>3</sup>. The present study was carried out to isolate and characterize PSB and to screen them for their plant growth promoting activities. Apart from this the isolates were also checked for insecticidal tolerance. The isolate positive for maximum plant growth promoting attributes was used for seed inoculation. In the present study, a total of 28 soil samples of plant rhizosphere from different areas of Punjab were processed for isolation of PSB using Pikovskaya's agar. Twenty isolates showed halozones around them (Fig. 1) and were designated as PSB 1 to PSB 20. After purification each isolate was stored on nutrient agar slants for further studies.



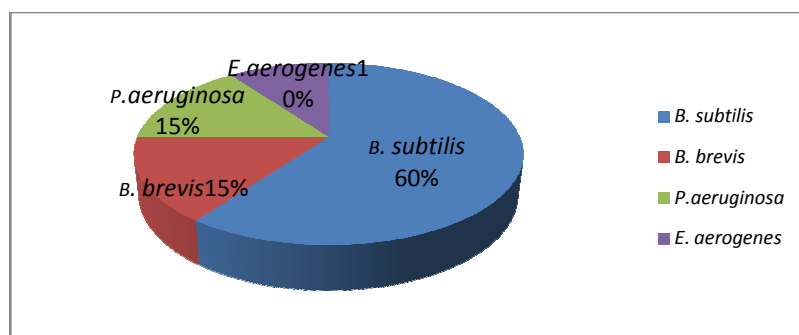
**Figure 1**  
**Bacterial colonies exhibiting halozone on Pikovskaya agar**

After purification isolates were characterized morphologically and biochemically. After morphological characterization, it was observed that 75% of the isolates were Gram positive while 25% were Gram negative (Fig. 2). After

biochemical characterization, it was observed that 12 (60 %) of the bacterial isolates belonged to *B. subtilis*, 3 (15%) were *B.brevis*, 3 (15%) were *Pseudomonas aeruginosa* and 2 (10 %) were *Enterobacter aerogenes* (Fig. 3).



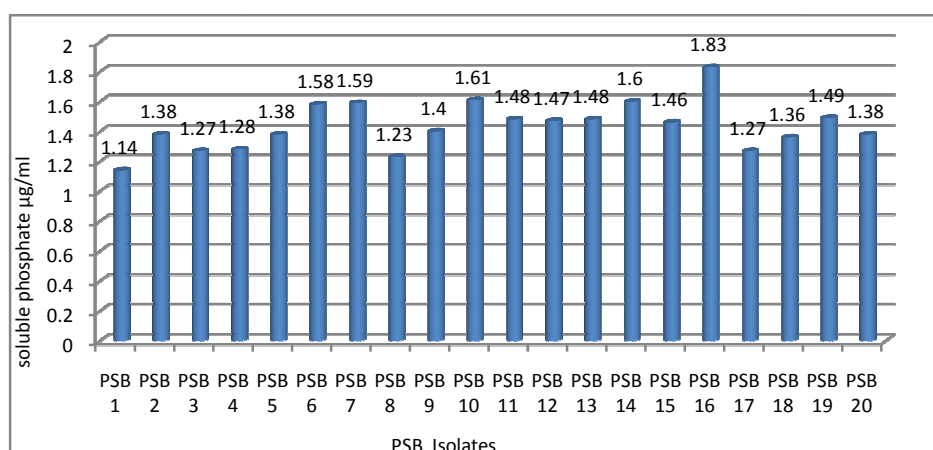
**Figure 2**  
**Gram nature of PSB isolates**



**Figure 3**  
**Bacterial isolates in rhizosphere soil**

The bacterial isolates which have shown bacterial solubilization in solid media were also studied for quantitative solubilization of phosphate. After 5 days of incubation the isolates showed phosphate solubilization ranging from 1.009 µg/ml to 1.609 µg/ml as shown in Fig. 4. *B. subtilis* showed phosphate

solubilization in the range of 1.009 to 1.609 µg/ml, *B. brevis* 1.090 to 1.309 µg/ml, *P. aeruginosa* 1.119 to 1.419 µg/ml and *E. aerogenes* 1.118 to 1.400 µg/ml Maximum phosphate solubilization was shown by *B. subtilis* PSB 16 i.e. 1.609 µg/ml.



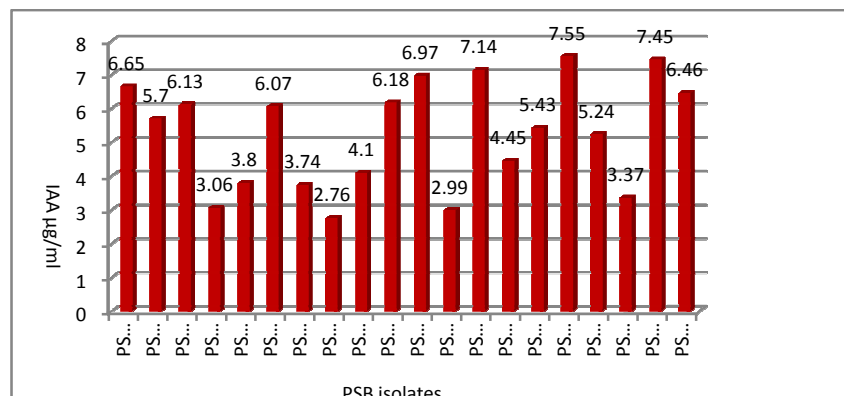
**Figure 4**  
**Phosphate solubilization by PSB**

In the present study it was found that Gram positive bacteria showed higher level of phosphate solubilization as compared to Gram negative bacteria. This is in contrast to a study by Ranjan *et al.*<sup>1</sup> who reported that Gram-negative bacteria mobilize insoluble phosphate very efficiently, by producing gluconic acid during the extracellular oxidation of glucose catalyzed by quinoprotein glucose dehydrogenase. Dutta *et al.*<sup>23</sup> reported high phosphate solubilization by Gram negative bacteria isolated from soil samples from sunflower and rice. Nath *et al.*<sup>24</sup> who isolated

*Penicillium* sp., incubation up to eight days showed remarkable phosphate solubilization. Selvi *et al.*<sup>25</sup> observed maximum amount of phosphate solubilization by *Bacillus* sp. on 3<sup>rd</sup> day which decreased after 9 days. Karpagam and Nagalakshmi,<sup>8</sup> reported that Gram negative bacteria showed highest phosphate solubilization. The isolates which showed phosphate solubilization were also studied for plant growth promoting attributes viz. IAA production, ammonia production, protease production and HCN production. All the isolates produced IAA in media supplemented with

different concentrations of tryptophan. It was observed that with the increasing concentration of tryptophan, increased IAA production was shown by all the isolates. The isolates produced very little amount of IAA with no

tryptophan, ranging from 0.047 mg/ml to 0.98 mg/ml. The maximum production of IAA was shown by *B. subtilis* PSB 16 i.e. 14.89 mg/ml at 300 mg/ml tryptophan (Fig. 5).



**Figure 5**  
**IAA production by PSB (300 mg/ml of tryptophan)**

Bacteria producing IAA are considered as an effective tool for screening of growth promoting microorganisms as their beneficial effect on plant growth has been reported in many studies<sup>26, 27</sup>. They promote lateral and adventitious root formation which can facilitate high root surface area for nutrient absorption from soil<sup>28</sup>. Ammonia production is another important trait of PGPR that may directly influence the plant growth. All the 20 isolates of PSB were positive

for ammonia production (Table 1). The colour of the media turning yellow was the indication of ammonia production. Rhizobacteria through nitrogen fixation are able to convert gaseous nitrogen to ammonia making it an available nutrient to the host plant which can support and enhance plant growth. The host plant provides the bacteria with amino acids so they do not need to assimilate ammonia<sup>29</sup>.

**Table 1**  
**Plant growth promoting activities of PSB**

PSB	Ammonia production	HCN production*	Protease production
<i>B.subtilis</i> PSB 1	Positive	2	Positive
<i>B.subtilis</i> PSB 2	Positive	3	Positive
<i>E. aerogenes</i> PSB 3	Positive	3	Positive
<i>P. aeruginosa</i> PSB4	Positive	1	Positive
<i>B.subtilis</i> PSB 5	Positive	2	Positive
<i>B.subtilis</i> PSB 6	Positive	2	Positive
<i>E. aerogenes</i> PSB 7	Positive	4	Positive
<i>B.brevis</i> PSB 8	Positive	4	Positive
<i>B.brevis</i> PSB 9	Positive	4	Positive
<i>P. aeruginosa</i> PSB 10	Positive	3	Positive
<i>B.brevis</i> PSB 11	Positive	3	Positive
<i>B.subtilis</i> PSB 12	Positive	2	Positive
<i>B.subtilis</i> PSB 13	Positive	2	Positive
<i>B.subtilis</i> PSB 14	Positive	1	Positive
<i>B.subtilis</i> PSB 15	Positive	2	Positive
<i>B.subtilis</i> PSB 16	Positive	5	Positive
<i>P. aeruginosa</i> PSB 17	Positive	3	Positive
<i>B.subtilis</i> PSB 18	Positive	3	Positive
<i>B.subtilis</i> PSB 19	Positive	2	Positive
<i>B.subtilis</i> PSB20	Positive	2	Positive

HCN Scale	1	2	3	4	5
Indicator colour	Yellow	Cream	Orange	Light brown	Brick like colour
HCN production	_____	Minimum	Relatively little	Relatively high	Maximum

Production of protease activity was analyzed as this is an important mechanism of inhibition of harmful organisms. All the 20 isolates of PSB were found positive for the production of protease enzyme (Table 1). The cell wall

degrading capability of the isolates is very important as it helps to degrade those organisms which are not beneficial to the plant. Halozone produced around colony indicated positive result (Fig. 6).



**Figure 6**  
**Protease production on Skim milk agar**

Cyanide production is one of the possible ways by which rhizobacteria may suppress plant growth in soil. Microbial production of HCN production has been reported as an important antifungal trait to control root infecting fungi<sup>30</sup>. The *in vitro* HCN production of different isolates was tested by the picric acid assay. Discoloration of filter paper indicated the level of HCN production. The HCN production was recorded at a score of 1 to 5 and results are given in Table 1. The isolates of *B. subtilis* and *B. brevis* produced HCN in the range of 1-5, however *P. aeruginosa* and *E. aerogenes* produced HCN ranging 1-3 and 3-4 respectively. *In vitro* studies revealed that the 20 PSB isolates showed IAA production, ammonia production, protease production and HCN production. These isolates were further checked for insecticide tolerance. Insecticides are organic compounds manufactured and used for insect control in agricultural practices. These chemicals may also affect the growth of beneficial microorganisms in soil and hence affect the plant growth. The rhizobacteria which can tolerate high concentration of pesticide may

be useful as inoculants. PSB isolates showed a variable tolerance to the insecticide. All the isolates were able to tolerate high concentration of insecticide i.e. upto 5% (v/v). *B. subtilis* PBS 16 showed the highest growth in the presence of insecticide. To study the effect of PSB on plant growth, PSB isolate that exhibited maximum PGPR activities was used. *B. subtilis* PSB 16 showed maximum phosphate solubility, high HCN scale, positive for ammonia and protease production was used for seed inoculation. It was observed that plants uprooted within 6 days after inoculation in all the pots (Fig. 7). The plant inoculated with rhizobacteria grew with a mean shoot length of 9.2 cm and root length of 3.5 cm as compared to controls with mean shoot length of 5.3 cm and mean root length of 1.2 cm after 10 days (Table 2). The bacteria may have enhanced plant growth through phosphate solubilization, IAA production, ammonia production and inhibiting the growth of other harmful microorganisms by producing enzymes and HCN. The effect of PGPR on plant growth has been reported in many studies<sup>31, 32, 33</sup>.



**Table 2**  
**Effect of PSB on shoot and root length of *Hordeum vulgare***

	Mean Shoot length	Mean Root length
Control	5.3 cm	1.2 cm
PSB	9.2 cm	3.5 cm

**Figure 7**  
**Plant growth with (A) and without (B) rhizobacterial inoculation**



PSB isolates are being explored in many studies with their possible role in phosphorus cycling with varying results. Kumar *et al.*<sup>29</sup> identified *Bacillus sp.*, *Pseudomonas sp.*, *Enterobacter sp.* and *Acinetobacter sp.* as efficient Phosphorous solubilizers. Selvi *et al.*<sup>25</sup> isolated PSB as *Bacillus sp.* which solubilized maximum Phosphorous. Singh and Prakash<sup>34</sup> reported *Pseudomonas sp.* and *Serratia sp.* as potent phosphate solubilizers. Susilowati and Syekhifani<sup>21</sup> identified *Bacillus sp.* and *Pseudomonas sp.* had highest Phosphorous solubilization ability. The more plant growth promoting activities shown by PSB isolates, the higher the chance of enhanced plant growth and yield. This helps for selecting the strains,

which can be used as bioinoculants and helps to increase the efficiency of agricultural strategies particularly under soil stress<sup>35</sup>. Utilization of these microorganisms as biofertilizer is environment friendly approach which can enhance the plant growth and yield and reduce chemical input.

## ACKNOWLEDGEMENT

The study was carried out by the internal grants of the Institute. We are indebted to the Management of Shaheed Udham Singh Group of Institutes for providing the infrastructure and grants required for conducting the study.

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