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INVITRO SELECTION OF PHOSPHATE SOLUBILIZING BACTERIA AND THEIR ROLE IN PLANT GROWTH PROMOTION

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Abstract

Phosphate solubilizing bacteria (PSB) are known to be able to solubilize different forms of inorganic phosphates. A total of twenty four phosphate Solublizing bacterial colonies were isolated on the Pikovskaya's (PKV) agar medium, containing insoluble tri-calcium phosphate (TCP), from different pond water sediment sample in and around Tirunelveli district, Tamil Nadu, India. The colonies showing clear halo zones around the bacterial growth were considered as phosphate solubilizers. Out of 24 bacterial isolates, 6 isolates showing highest phosphate solubilisation index (SI) ranged from 1.6-2.5 were selected for the further study as qualitative as well as quantitative estimation of phosphate. Among these 6 potent isolates, *Bacillus* sp PSB6 showed the maximum phosphate solubilization index of 2.5 in Pikovskaya's agar plates. A pot culture experiment was conducted to investigate the effects of isolated PSB on early development of plants. Seeds were treated with bacterial strain, and seedlings were harvested 5weeks after inoculation. Pots treated with strains showed a positive effect on plant growth. A significant increment in plant shoot, Root height (21.49, 08.64 cm) and dry weight of Shoot and Root (2.39, 2.21 cm) were determined in plants treated with *Bacillus licheniformis (*PSB6) has remarkably increased compared to the uninoculated control. This growth promoting action was confirmed by the molecular analysis of genomic DNA by RFLP technique.

Key Word: PSB, *Bacillus Licheniformis*, Pot Study & RFLP

Introduction:

India is primarily an agriculture based country with more than 60-70 percent of its population dependent on agriculture. India's fast growing population is projected to cross 1.3 billion by 2020 (Kanekar *et al.,* 2004). Feeding and clothing this population from almost exhausted arable land and fast depleting water resources would be a great concern and challenge. A tremendous pressure is, thus, exerted on the annual food grain production and minimizing crop losses. In recent years, plant protection has become one of the essential inputs in crop production. In the context of changing cropping patterns, introduction of high yielding varieties, application of high doses of fertilizers, with enhanced irrigation facilities, pests have assumed a special significance and more and more pesticides are being applied. Phosphorus (P) is one of the major essential macronutrients for plants and is applied to soil in the form of phosphatic fertilizers. However, a large portion of soluble inorganic phosphate applied to the soil as chemical fertilizer is immobilized rapidly and becomes unavailable to plants (Hu *et al.,* 2006)

An estimate reviewed that 5.7 billions of hectares worldwide contain too little of available phosphorus for crop production (Batjes, 1997; Gijsbers, 2001). Analysis of 3.65 million samples of Indian soils showed that 42 percent of soil falls in low level of phosphate (Muginova, 2007). Certain beneficial bacteria can remobilize the insoluble phosphate into soluble form through various mechanisms. These phosphate solubilizing bacteria (PSB) play an important role for sustaining the level of plant utilizable soluble phosphate in the soil in an environmental friendly and sustainable manner. PSBs are autochthonous and their population is not sufficient to compete with other soil bacteria. Therefore, application of these PSB as bioinoculants in agricultural practice is expected to improve crop yield and productivity. (Gupta, *et al.*, 2009; Kostadinova, S., and Marhova, M. (2008).

In the present study, we described the isolation and characterization of PSB6 (*Bacillus licheniformis)* and its plant growth promotion activity were screened in the presence and absence of supplemented tricalcium phosphate in the soil.

2. Material and Methods:

2.1 Isolation and Identification of Phosphate Solubilizing Bacteria:

Pond sediment sample was suspended in 100 mM phosphate buffered saline solutions and were shaken for 6 h on a rotary shaker. Serially diluted samples were plated on nutrient agar (HiMedia, Mumbai). After 24 h incubation individual colonies were screened for their ability to solubilize inorganic phosphate by spotting the inoculum on Pikovskaya's (PVK) agar. The sizes of the phosphate solubilization by means of clear zone around the colonies were measured at 28°C after 7 days of incubation. Colonies with clear zones around them were confirmed as phosphate solubilizing bacteria (PSB) and further it maintained in nutrient agar slants. Selected

PSB isolates were identified on the basis of its physiological growth parameters and biochemical characteristics as per Bergey's Manual of Systematic Bacteriology.

2.2 Inoculum Preparation for Pot Experiment:

Bacterial culture *B. licheniformis* (PSB6) were transferred to 500 mL flasks containing nutrient broth; colonies were then grown aerobically in flasks on a rotating shaker (150 rpm) for 48 h at 30 °C. The bacterial suspension was then diluted in sterile distilled water to a final concentration of 10^8 CFU mL^{-1} and resulting suspensions were used to treat oryza seeds.

2.3 Microbial Inoculation in Pot Experiment:

Physico chemical properties of rhizosphere soil was completely done in experimental pot in with pH 8.2, EC - 0.28 dS m^{-1}, organic carbon content of soil 0.47%, low available N and P (23 and 1.67 kg ha^{-1}) and K (31.34 kg ha^{-1}) Each experiment carried out in plastic pots containg 3 kg soil – sand (acid washed) **(5:2)** steam-sterilized mixture. 2 g of charcoal based bacterial culture *(B. licheniformis)* containg (10^8 cells /g) was inoculated. Tricalcium phosphate powder (0.1 mm mesh), at a concentration of 5 g / kg was added to all treatments. Water was added to 50% water holding capacity and the whole material mixed thoroughly. The seeds were surface sterilized with 0.1% $HgCl_2$ for 3 min and successively washed with sterile distilled water.
The experimental plan was based on FOUR different pots as given below.

Pot I- Control- (Soil+ without TCP + without PSB 6 coated seed)
Pot II – (Soil + TCP)
Pot III - Soil + PSB6 coated seed
Pot IV – Soil + PSB6 coated seed + TCP

All pots were cultivated in a greenhouse for five weeks under a day/night cycle at 30/35 ºC. Throughout the experiment, the pots were weighed every day and water loss from field capacity was replaced by top watering. After five weeks of incubation, plants were harvested and determined Shoot, root length (cm) and Dry matter of shoot and root were calculated (Navari-Izzo and Quartacci, 2001). Finally, the results were compared with the control pot.

2.4 Chlorophyll Determination:

The chlorophyll content of plant leaves was estimated by the method of Arnon, (1949). 100 mg of leaf samples were ground with 80% of acetone followed by centrifugation at 3000 rpm for 5 min. Absorbance of the supernatant was detected at 645 nm.

2.4 RFLP Analysis of Microbial Community in Plant Rhizosphere Soil:

Soil DNA Isolation:

After the experimental studies microbial community from plant rhizosphere soil was isolated by soil DNA elution kit. This technique is supported to confirm the psb6 strain has involved promoting the plant growth as well as enhancing the soil fertility. Soil DNA from approximately 0.5 g of soil was extracted in triplicate using the Fast DNA Spin KitR for soil (Eppendorf, Germany) following the manufacturer's instructions. Resolution of extracts on a 0.7% agarose gel containing 0.01% ethidium bromide was used to estimate DNA quantity and quality (Mori, S *et al.,* 1999).

2.5 PCR-RFLP Analysis:

Restriction Fragment Length Polymorphism (RFLP) analysis was carried out in PCR amplified product of 16S rDNA sequence (Tringe *et al.,* 2005). The required quantity of 16S rDNA was digested with respective restriction endonuclease in the presence of appropriate 1X reaction buffer. Reaction mixture was incubated at 37 °C for 3 h and if necessary, the reaction was stopped by heat inactivation as recommended by the manufacturer. The DNA was analyzed by agarose gel electrophoresis.

3. Results and Discussion:

3.1 Isolation and Identification of PSB:

The collected pond water sediment samples were evaluated for Phosphate solubilizing Bacteria in Pikovskaya's agar medium. A total of 24 Phosphate Solubilizing bacterial colonies were isolated on the Pikovskaya's agar medium, containing insoluble tri-calcium phosphate (TCP) from pond water samples. Out of 24 microbial isolates 6 isolates showed highest Phosphate Solubilization Index (PSI) ranged from 1.6 - 2.5 **(SI)**. Phosphate solubilizing microbes are detected by the formation of clear halo zone around their colonies. The halo is produced due to solubilization of insoluble phosphates, which in turn is mediated via the production of organic acid in the surrounding medium (Gaur,1990).The maximum activity of 6 isolates were further characterized by a series of biochemical reaction and identified as *Bacillus sp* (Table 1 and 2). The morphological and biochemical characteristics of these isolates were shown in the Table 3. It proves that bacteria was well known identified as phosphate solubilizer by compare the findings of Hilda, R., and Fraga, R., 1999.

Table 1: Screening of Phosphate Solublizing bacteria from pond water sediment sample

S.No	Isolates	Zone of inhibition (cm)	S.No	Isolates	Zone of inhibition (mm)
1	PSB1	0.4	13	PSB13	0.5
2	PSB2	0.3	14	PSB14	0.5

3	PSB3	0.7	15	PSB15	0.4
4	PSB4	0.2	16	PSB16	0.3
5	PSB5	0.4	17	PSB17	0.5
6	PSB6	1.6	18	PSB18	0.3
7	PSB7	0.6	19	PSB19	0.3
8	PSB8	0.5	20	PSB20	0.9
9	PSB9	1.3	21	PSB21	0.5
10	PSB10	0.5	22	PSB22	0.3
11	PSB11	0.6	23	PSB23	0.5
12	PSB12	0.4	24	PSB24	0.4

Table 2: Phosphate Solubilizing Activities of Six most P solubilizing bacterial strains

S.No	Isolates of PSB	Colony diameter (cm)	Zone measurement (cm)	Solubilization Index(SI)
1	PSB3(*Bacillus sp.*)	0.5	0.7	1.9
2	PSB6(*Bacillus sp.*)	1.4	1.6	2.5
3	PSB7(*Bacillus sp.*)	0.6	0.6	1.6
4	PSB9(*Bacillus sp.*)	1.2	1.3	2.3
5	PSB13(*Bacillus sp.*)	0.5	0.6	1.7
6	PSB20(*Bacillus sp.*)	0.7	0.9	2.0

***SI**= (Colony diameter + Halo zone)/colony diameter

Table 3: Morphological and Biochemical characteristics of Phosphate Solubilization bacterial strain PSB6

Characteristics	Selected Bacterial Isolates PSB6
Colony property	On nutrient agar, colonies are circular, smooth round, waxy, pale, no pigment.
Gram's stain	Positive, rod
Motility	Motile
Catalase	+
MR – VP test	+
Citrate utilization	–
Gelatin Liquefaction	+
Casienase	+
Nitrate reduction	+
H_2S production	–
Adonitol fermentation	–
Lactose	–
Xylose	–
Maltose	+
Fructose	–
Dextrose	+
Galactose	–
Sucrose	–
Melezitose	+
Arabinose	–

3.2 Phosphate Solubilization Under *In Vitro* Conditions:

The effect of PSB strains inoculated soil pH, available P content, and total PSB population are shown in Table 4. A more significant decrease ($P \leq 0.05$) in soil pH was recorded from PSB-inoculated soils than uninoculated soils. Furthermore, available P content in the rhizosphere soil inoculated by PSB were found to be significantly ($P \leq 0.05$) higher than for uninoculated soil. This was further improved by adding TCP. The highest available P content (177.35 ± 3.01 mg kg-1 soil) recorded from of PSB strains with TCP was higher than for uninoculated soil. A remarkable increase in the PSB population was observed in PSB-inoculated rhizosphere soil when compared with uninoculated soil. The highest PSB population (5.41CFU g-1 soil) recorded from PSB strains with TCP were approximately four times higher than for uninoculated soil. Phosphate solubilization potential has been attributed to the strains' ability to reduce pH of the surroundings, either by releasing organic acids or protons (Hariprasad and Niranjana, 2009). Organic acids, such as gluconic acid, oxalic acid, and citric acid, secreted by PSB can directly solubilize mineral phosphate as a result of anion exchange or indirectly chelate both Fe and Al ions associated with phosphate. This leads to increased P availability, which ultimately increases plant P uptake. Results of the present study as to maximum plant growth and P uptake recorded with inoculation of PSB strains with TCP are in line with the findings of Qureshi *et al.* (2011), who also observed

similar results when co-inoculating phosphate solubilizing and noduleforming bacteria *Rhizobium phaseoli* and *B. megaterium* in mung bean plants. A similar increase in the PSB population and available P content was observed by Yu *et al.* (2011).

Table 4: Effect of PSB6 on soil pH, available P content, and population of phosphate solubilizing bacteria (PSB) in rhizosphere soil of experimental plants

S.No	Treatment	Soil pH	Soil available P mg kg-1	nr of PSB CFU g-1 soil
1	Pot I Control	7.52 ± 0.32	106.91 ± 1.35	1.17×10^2
2	Pot II -(Soil + TCP)	7.53 ± 0.21	104.39 ± 1.22e	1.26×10^2
3	Pot III- (Soil + PSB6 coated seed)	7.81 ± 0.35	169.81 ± 2.34	4.61×10^4
4	Pot IV – (Soil + PSB6 coated seed + TCP)	7.41 × 105c	177.35 ± 3.01	5.41×10^5

3.3 Growth and P Uptake in Oriza Plants:

Increased shoot length, root length, and shoot and root dry weight of oryza plants were recorded from the seedlings raised with the PSB6 inoculated seeds (Table 5). The best growth performances (21.49 cm, 08.64cm and 2.39 and 2.21 g plant-1 for shoot length, root length and shoot dry weight, and root dry weight, respectively) were recorded from the plants inoculated with *B. licheniformis* (PSB6) and amended with TCP. These results are confirmed bacterial coated seeds were proved better growth to compare all the four experimental pots. TCP resulted in better growth performances, no significant ($P \leq 0.05$) differences in shoot length, root length, and shoot root dry weight were observed among soil treatments with and without TCP (Table 5). A similar increase in growth and P uptake of rice plants due to inoculation of PSB strains was observed by Singh and Kapoor (1999), Vikram and Hamzehzarghani (2008), Ghanem and Abbas (2009).

Table 5: Effect of *B. licheniformis* (PSB6) on oryza plants growth

S.No	Treatment	Shoot length cm plant^{-1}	Root Length cm plant^{-1}	Shoot dry Matter gm plant^{-1}	Root dry Matter gm plant^{-1}
1	Pot I Control	19.86 ± 0.98	08.32 ± 0.97	2.01 ± 0.14	1.41 ± 0.24
2	Pot II -(Soil + TCP)	19.35 ± 1.12	06.01 ± 1.25	1.89 ± 0.27	1.47 ± 0.24
3	Pot III- (Soil + PSB6 coated seed)	20.16 ± 1.05	07.11 ± 0.97	2.13 ± 0.21	1.81 ± 0.21
4	Pot IV – (Soil + PSB6 coated seed + TCP)	21.49 ± 1.25	08.64 ± 1.24	2.39 ± 0.25	2.21 ± 0.11

3.4 Chlorophyll Content:

The plants treated with the PSB 6 along with TCP showed profound increase in level of leaf chlorophyll content in the rice were tabulated in Table 6. Regarding the result, the chlorophyll content of PSB treated experimental plants were 0.437; and 0.475 respectively and the corresponding control 0.412 mg gfw^{-1}**.** Similarly, Chaitra (2006) also observed increase the chlorophyll content in leaf area per plant with the application of PSB and TCP in *China aster*.

Table 6: Chlorophyll Content of the experimental plant

S.No	Treatment	**Total Chlorophyll** (mg gfw-1) In Oryza
1	Pot I Control	0.412
2	Pot II -(Soil + TCP)	0.316
3	Pot III- (Soil + PSB6 coated seed)	0.437
4	Pot IV – (Soil + PSB6 coated seed + TCP)	0.475

3.5 Rflp Analysis of Microbial Community in Plant Rhizosphere Soil:

Total genomic DNA and rhizosphere bacterial community was successfully extracted from soil (Plate 1.). RFLP technique showing different digestion pattern with restriction endonuclease (Hind III) were chosen for separate the DNA by small fragments. PCR amplification of the genomic DNA templates of soil DNA from rhizosphere soil revealed same banding pattern to compare with the DNA fragments of PSB6 on Agarose gel electrophoresis (Plate 2).

Plate 1. PCR amplification of 16S rRNA gene from soil DNA of Experimental plant soil

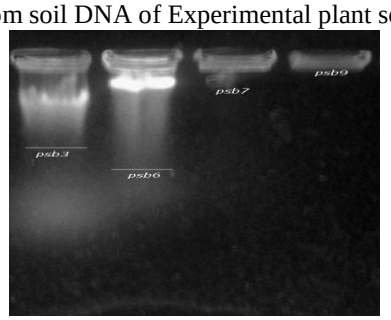
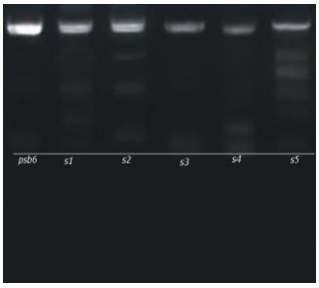


Plate 2. RFLP analysis of PCR amplified 16S rDNA product of soil DNA compare with PSB6



Lane 1: PSB6 (*Bacillus licheniformis*); Lane 2 (S1), 3(S2), 4(S3), 5(S4), and 6 (S5) were isolated cloned DNA from rhizosphere soil.

Conclusion:

The present study indicates that bacterial inoculation in Oryza seeds leads to a higher yield potential. In particular, P-solubilizing *Bacillus licheniformis (*PSB6) showed great potential for use as bioinoculants. The application of this bacterial strain had beneficial effects on growth, yield and P nutrition on oryza plants. Regarding environmental pollution due to excessive use of chemical fertilizers and high costs of P fertilizer production, the bacterial strain tested may well be used as bioinoculant to enhance sustainable agricultural production. Our study also demonstrates the importance of evaluating potential growth-promoting bacteria under a variety of experimental conditions.

References:

1. Arnon, D I. (1949). Copper enzymes in isolated chloroplasts, polyphenoxidase in beta vulgaris. Plant physiology, 24: 1-15.
2. Batjes N. H. (1997.): A world data set of derived soil properties by FAO – UNESCO soil unit for global modelling. Soil Use Manage, 13, 9–16.
3. Chaitra, R., and Patil, V. S. (2006). Effect of integrated nutrient management on growth, yield and quality of China aster (Callistephus chinensis (L.) Nees). University of Agriculture Sciences, Dharward in partial fulfillment of the requirement for the Degree of Master of science in Horticulture, 75 p.
4. Gaur, A.C. (1990) Phosphate solubilizing microorganisms as biofertilizers. Omega scientific Publishers. New Delhi.
5. Ghanem, K. H. M., and Abbas, E. E. (2009). Improvement of mung bean growth and productivity in salinity-affected soil after seed inoculation with phosphate dissolving bacteria. African Crop Science Conference Proceedings. 9: 385-389.
6. Gijsbers R., Ceulemans H., Stalmans W., and Bollen M. (2001). Structural and catalytic similarities between nucleotide pyrophosphatases/phosphodiesterases and alkaline phosphatases. J. Biol. Chem., 276: 1361–1368.
7. Gupta, D. P., Sunita and Saharan, J. P. (2009). Physiochemical Analysis of Ground Water of Selected Area of Kaithal City (Haryana) India. Researcher. 1(2): 1-5.
8. Hariprasad, P., and Niranjana, S. R. (2009). Isolation and characterization of phosphate solubilizing rhizobacteria to improve plant health of tomato. Plant Soil, 316: 13-24.
9. Hilda, R., and Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol. Adv., 17: 319-359.
10. Hu, X., Chen, J., and Guo, J. (2006). Two phosphate and potassium solubilizing bacteria isolated from Tianmu Mountain, Zhejiang, China. World J. Microb. Biot., 22, 983-990.
11. Kanekar, P. P., Bhadbhade, B., Deshpande, M. N., and Sarnaik, S. S. (2004). "Biodegradation of organophosphorous pesticides." Indian National Science Academy. 70: 57-70.
12. Kostadinova, S., and Marhova, M. (2008). Anniversary Ecological Conference (proceedings), 1: 285-292.
13. Mori, S., Okamoto, M., Nishibori, M., Ichimura, M., Sakayama, J., and Endo, H. (1999). Purification and characterization of alkaline phosphatase from Bacillus stearothermaphilus. Biotechnol. Applied Biochem., 29: 235-239.
14. Muginova, S. V., Zhavoronkova, A. M., Polyakov, A. E. and Shekhovtsova, T. N. (2007). Application of alkaline phosphatases from different sources in pharmaceutical and clinical analysis for the determination of their cofactors; zinc and magnesium ions. Analytical Sciences. 23: 357.
15. Navari-Lazzo, F., and Quartacci, M. F. (2001). Phytoremedition of metals: tolerance mechanisms against oxidative stress. Miner. Biotechn, 13: 73-83.
16. Qureshi R, Maqsood M, Arshad M, Chaudhry AK. (2011)Ethnomedicinal uses of plants by the people of Kadhi areas of Khushab, Punjab, Pakistan. Pakistan Journal of Botany. ; 43(1):121–133.
17. Singh, S., and Kapoor, K. K. (1999). Inoculation with phosphate solubilizing microorganisms and a vesicular Arbuscular mycorrhizal fungus improves dry matter yield and nutrient uptake by wheat grown in a sandy soil. Bio. Fert. Soils, 28: 139-144.
18. Tringe, S., Von Mering,C., Kobayashi,A., Salamov,A., and Chen, K.(2005) Comparative Metagenomics of Microbial Communities. Science. 308: 554–557.
19. Vikram, A., and Hamzehzarghani, H. (2008). Effect of phosphate solubilizing bacteria on nodulation and growth parameters of greengram (Vigna radiate L. Wilczek). Res. J Microbial, 3: 62-72.
20. Yu, X., Liu, X., Zhu, T.H., Liu, G. H., and Mao, C. (2011). Isolation and characterization of phosphate-solubilizing bacteria from walnut and their effect on growth and phosphorus mobilization. Biology and Fertility of Soils, 47:437-446.