Isolation and Characterization of Phosphate Solubilizing Bacteria from Rhizospheric Soil of the Soybean Plants

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Abstract

Nine strain showing phosphate solubilization activities were isolated from the rhizosphere of the soybean plant. Out of which seven microorganisms were Gram-negative and two microorganisms were Gram-positive. One Gram-positive microorganism was selected for further studies and identified as *Micrococcus spp*. and three gram negative microorganisms were selected for further studies and identified as *Pseudomonas spp*., *Enterobacter spp*., *Acinetobacter*. Six carbon sources such as Glucose, Galactose, Mannitol, Maltose, Raffinose, Xylose, Lactose, Fructose, and Sucrose were utilized by various strains. These strains will be used to prepare biofertilizer. Application of *Pseudomonas spp*., *Enterobacter spp*., *Acinetobacter* and *Micrococcus spp*. culture with lignite showed plant growth promoting activity.

KEYWORDS: Phosphate solubilizing bacteria, Biofertilizer.

Introduction

Phosphorus (P) is one of the major essential macronutrients for plant and is applied to soil in the form of phosphatic fertilizers. In soil inorganic and organic forms of phosphorus is present. The inorganic forms of the element in soil are compound of calcium, iron, aluminum and fluorine. The organic forms are compounds of phytins, phospholipids and nucleic acid which come mainly by way of decaying vegetation. Therefore, soils containing high organic matter are also rich in organic forms of phosphorus (Subbarao, 1982).

Phosphobacteria not only play a significant role in supplying P to plants, but also increase plant growth and development through other plant growth promotion activities, like nitrogen fixation, siderophores, and phytohormones production (Vassilev *et al*., 2006).

In *Vidarbha* region soybean plant have great demand. Because, soybean is oil rich and soybean crop contain large amount of protein (40-44%). Therefore, it is used in many natural bodies building product. Phosphate solubilizing bacteria (PSB) play an important role for increasing crop production of soybean plant. Therefore application of phosphatic fertilizers is essential for optimum crop yield. But the utilization efficiency of phosphate fertilizers by plant is only 20-25% largely due to its chemical fixation in soil (Dave *et al.,* 2003).

The region of the soil, which is subject to the influence of plant roots, is called as rhizosphere. The average soil phosphorus concentration is about 0.05ppm and varies widely among soil. The phosphorus concentration required by most plants varies from 0.003 to 0.3ppm and depends on the crop species and level of production maximum corn grain yield may be obtained with 0.01ppm phosphorus, if the yield potential is low, but 0.05ppm phosphorus is needed under high yield potential. Organic phosphorus represents about 50% of total phosphorus in soil and typically varies between 15 and 80% in most soils. Soil organic phosphorus decrease with depth and the distribution with depth also vary among soils. The phosphorus content of soil ranges from about 1-3%. Therefore, the quantity increases with increasing organic C and N. However, the C: P, N: P ratios are more variable among soils than the C: N ratio, (Miller and Donahue, 1990).

It is generally accepted that the mechanism of mineral phosphate solubilization by phosphate solubilizing bacteria (PSB) strains is associated with the release of low molecular weight organic acids (Goldstein, 1995; kim *et al.,* 1997) like formic acid, acetic acid, lactic acid, sulphuric acid and propionic acid, which through their hydroxyl and carboxyl group chelate the cations bound to phosphate, thereby converting it into soluble forms (Kpomblekou and Tabatabai, 1994) However, P-solubilization is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the culture (Reyes *et al.,* 1999). There is experimental evidence to support the role of organic acids in minerals phosphate solubilization (Haldar et al., 1990).

However, very few attempts have been made to isolate and characterize the potential phosphate solubilizing bacteria (PSB) from the rhizosphere of soybean cultivated area in Amravati. Hence, little information is available concerning phosphate solubilizing bacteria and their ability to colonize soybean root.

The objective of this study was to isolate and characterized phosphate solubilizing bacteria (PSB) from the rhizoplane of soybean cultivated in a Parsoda, Mardi in Amravati.

Materials and Method

- ***Sample collection***

Total 40-50 soil sample were collected from soybean cultivated area in sterilized container. The soil suspension was prepared by mixing 1g of soil sample in 9ml distilled water then supernatant was discarded and soil sample was point inoculated on previously prepared and sterilized pikovaskaya's agar plates . Then the pikovaskaya's agar plates were incubated at 28±2 ^{o}C for 24-48 hours. And after completion of incubation time, Zone of phosphate solubilization was recorded. The colonies showing clear zone of solubilization were further subculture on pikovaskaya's agar plates.

- ***Microscopic study of Bacteria** :*

Size, shape, arrangement and gram's nature of the isolates were studied for gram's staining. Smear was prepared from the isolated culture on clean glass slide, heat fixed and stained. The stained smear was observed under microscope. (Oil immersion lance-100x)

- ***Identification of Bacterial isolated through Biochemical test :***

Biochemical test were performed as suggested by **(**Garrity *et al.,* 2001**)** which included following tests Grams, IMViC reaction, catalase test, starch hydrolysis test oxidation fermentation test, Phenyl alanine deamination test, Nitrate reduction, Gelatin hydrolysis test, Urea hydrolysis test, Dehydrogenase test, Casein hydrolysis test, Citrate utilization test, Indol production test, Triple sugar iron (TSI) test, Carbohydrate fermentation test (Glucose, Fructose, Sucrose, Arabinose, Mannitol , Lactose, Treholase, Galactose, Raffinose), Motility test, Endospore staining and capsule staining.

- ***Phosphate solubilization by plate Assay:-***

Solubilization of tricalcium phosphate was detected in Pikovskaya's Agar medium (Sundara-Rao and sinha., 1963) each isolate was point inoculated in at the center of Pikovskaya's Agar plate and inoculated for 24 – 48 hr the development of clear around the colony indicated phosphate solubilizing activity.

Bioassay- based determination of the plant growth promotion ability of the isolate was conducted using Soybean seedling in sterile soil under glasshouse condition. The soybean seed were sterilized in 70% alcohol for 2 min and 2% Sodium hypochlorite for 2 min and followed ten times washing in sterile tap water.

For this experiment, pure cultures were grown in nutrient broth at 28°c and diluted to a final concentration of 10^8 colony – forming units (cfu) ml in sterile saline water (0.85%). The surface sterile seeds were inoculated by immersion in the PGPR suspension (ca -18^8cfuml^{-1}) for 45 min on rotary shaker (140 rev min^{-1}), air dried and sown immediately into 2 pots, out of this 1 pot contain single super phosphate. Control seed were treated with sterile distilled water. Seed were sown in plastic pots (15 cm diameter) containing 1 kg of sterile soil and place in room, which having temp is (25 to 45°C) room temp, pH - 6.8 – 7.2 and then supply daily sterile tap water as suitable.

Observe the growth of soybean plant and record the height, root, leaf area, shoot and production of soybean.

Results

On the basis of cultural character, Morphological character and biochemical character phosphate solubilising bacteria was identifying. Following character was compare with 'BERGEY'S MANUAL' and five phosphate solubilising bacteria were identified. That is PSB1&PSB3 – *Pseudomonas spp.*, PSB4- *Acinetobacter*, PSB7 - *Enterobacter spp.* and PSB9 - *Micrococcus spp* as shown in Table 1.

Out of this nine isolate *Micrococcus spp.* having efficiency of Phosphate solubilization was more as compare to other eight isolated phosphate solubilizing bacteria, that is (280). But *Enterobacter spp.* having efficiency of Phosphate solubilization was less as compare to other eight isolated phosphate solubilizing bacteria, that is (170). Efficiency of Phosphate solubilization was determined by plate assay using Pikovaskaya's Agar Medium.

Table 1 Morphological Cultural and Biochemical Characteristics

Morphological Cultural and Biochemical Characteristics																																	
Isolates	**Shape**	**Size**	**Margin**	**Elevation**	**Color**	**Surface**	**Texture**	**Appearance**	**Pigmentation**	**Optical property**	**Gram ch.**	**Shape**	**Size**	**Capsulation**	**Spore**	**Motility**	**Indol**	**MR**	**VP**	**Citrate**	**Oxidase**	**Catalase**	**Nitrate**	**Dextrose**	**Galactose**	**Treholase**	**Mannitol**	**Sucrose**	**Lactose**	**Fructose**	**Arabinose**	**Raffinose**	**Testively isolated spp.**
PSB 1	F	L	Fi	Fl	P W	F	Ru	Sh	N	Op	-	Rod	1	No	A	+	-	-	-	-	+	-	+	+	-	-	+	-	-	+	-	-	***Pseudomonas spp.***
PSB 2	F	L	Fi	Cr	W	F	Ru	Sh	N	Op	-	Rod	1	No	A	+	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	**No Identify**
PSB 3	F	L	Fi	Fl	P W	F	Ru	Sh	N	Op	-	Rod	1	No	A	+	-	-	-	-	+	-	+	+	-	-	+	-	-	+	-	-	***Pseudomonas spp.***
PSB 4	Cu	M	Un	Ra	W	Wr	Ru	Du	N	Op	-	Cocci	1	No	A	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	***Acinetobacter,***
PSB 5	Ir	S	Er	Co	Y W	Ru	Ru	Du	Y	Op	+	Rod	1	No	A	+	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	-	**No Identify**
PSB 6	R	L	Er	Co	P W	Ru	Ru	Du	N	Op	-	Rod	1	No	P	+	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	**No Identify**
PSB 7	Ro	L	En	Co	W	Sh	Sm	Sh	N	Tr	-	Rod	1	No	A	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	***Enterobacter spp.***
PSB 8	Ro	M	Un	Fl	W	Sm	Sm	Du	N	Op	-	Rod	1	No	P	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	**No Identify**
PSB 9	Ro	S	En	Co	PY	Sm	Sm	Sh	Y	Op	+	Co.Cl	1	No	A	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	***Micrococcus spp.***

Abbreviation = F-Filamentous, Cu –Curled, Rh- Rhizoid, Ro-Round, L-Large, M-Moderate, S-Small, Tr-Translucent, Un-Undulated, Er-Erose, En-Entire, Fl-Flat, Cr-Crateriform, Ra-Raised, Co-Convex, Wr-Wrinkled, Ru-Rough, Sm-Smooth, Sh-Shiny, D-Dull, O-Opaque, P W-Pale White, Y W-Yellowish White, P Y-Pale yellow, W-White, MR-Methyl red, VP-Vogus proskauer, Ci-Citrate, I- Indo, NR- Nitrate reductase, Absent, P-Present, D-D-Glucose, Ga-Galactose, Tr-Treholase, Ma-Mannitol, Su-Sucrose, La-Lactose, Fr-Fructose, Ar-Arabinose, Ra-Raffinose, (+)-positive test, (-)-Negative test, Ox-Oxidase, Ca-Catalase. Co-cl- Cocci in cluster,

2. **To isolate phosphate solubilizing bacteria from rhizosphere soil of the soybean plants**
   - 9 colonies showing zone of clearance were observed on Pikovaskaya`s agar plates.
   - The ability to solubilize precipitated phosphate was positively exhibited by *Pseudomonas spp. and Enterobacter spp., Acinetobacter, Micrococcus spp.* and four other *phosphate* solubilizers.
   - All nine phosphate solubilizing bacteria was selected and subcultured on Pikovaskaya`s agar plates for further studies
3. **Determination of Efficiency of Phosphate solubilization, solubilize by. *Pseudomonas spp. and Enterobacter spp. , Acinetobacter, Micrococcus spp.* and four other phosphate solubilizer**

   % of Efficiency of PSB was calculated by using following formula

$$Efficiency\ of\ phosphate\ solubilization = \frac{Solubilization\ diameter}{Diameter\ of\ colony} \times 100$$

Table 2 represents the percentage efficiency of different PSB using *Pseudomonas spp. And Enterobacter spp., Acinetobacter, Micrococcus spp.* and four other phosphate solubilizer

Table 2 Efficiency of Phosphate solubilization

SN	PSB Strain	Colony Diameter	Solubilization Diameter	% Efficiency 48 Hr
1.	*Pseudomonas spp.*	0.5	1.2	240
2.	PSB 2	1.2	1.6	133
3.	*Pseudomonas* ***spp.***	0.6	1.4	233
4.	*Acinetobacter*	0.8	1.1	137
5.	PSB 5	1.2	1.8	150
6.	PSB 6	0.5	1.1	220
7.	*Enterobacter spp.*	1.1	1.4	127
8.	PSB 8	0.9	1.4	155
9.	*Micrococcus spp.*	0.5	1.4	280

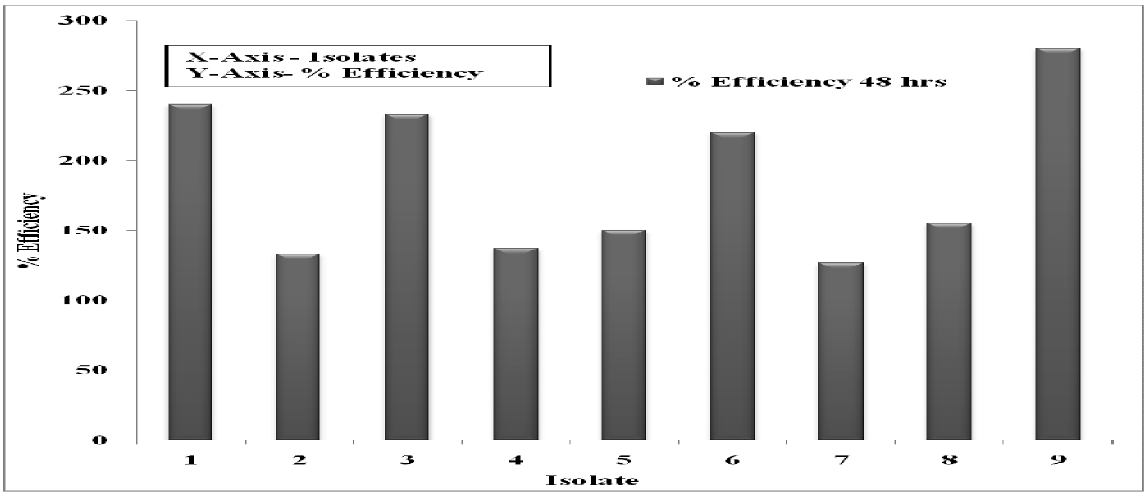

Figure 2: Result of efficiency of phosphate solubilizer

4. Effect of phosphate solubilizing Of *Pseudomonas spp. and Enterobacter spp., Acinetobacter, Micrococcus spp.* and four other *phosphate* solubilizer. on the growth of Soybean plant

7. The promote *Pseudomonas spp., Enterobacter spp., Acinetobacter, Micrococcus spp.* and four other *phosphate* s the plant growth on high extent as compared with control

Pot experiment of *Soybean* plant

Table 3 shows the growth promotion in *Soybean* test plant as compared with control after 90 days.

Table 3: Comparison of growth in cm of test plant with control

SN	**Plant of *Soybean***	**Shoot length (cm)**	**Root length (cm)**	**Leaf width**	**No. of leaf**	**No. of branches**
1.	**A1-Control (seeds)**	**63**	**9**	**3.6**	**32**	**10**
2.	**A2 (seeds+Phosphate solubilizer)**	**68**	**9.3**	**3.8**	**38**	**12**
3.	**A3 (seeds +Phosphate solubilizer + Phosphate fertilizer)**	**72**	**9.9**	**4.1**	**45**	**15**

Discussion

Phosphorus - one of the major plant nutrients limiting plant growth. Phosphorus is present in soil only in micromoles or lesser quantities. These low levels of P are due to high reactivity of soluble P with Calcium (Ca), iron (Fe) or aluminum (Al) that lead to P precipitation. Soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphate to plants. Among microbial populations in soils, phosphate solubilizing bacteria (PSB) constitute solubilization potential of between 1 to 50%. In last few decades a large array of bacteria including species of *Pseudomonas, Bacillus* and *Enterobacter* (Fernandez et al., 2007). In recent study, *Pseudomonas spp., Enterobacter spp., Acinetobacter, Micrococcus spp.* and four other phosphate solubilizers have been reported to enhance the growth of soybean plant.

The exogenous introduction of phosphobacteria in agriculture soils would help to decrease the use of chemical fertilizer while increasing yields. This would mean that a low-cost ecotechnology engineered through specific bacteria responsible for solubilization of rock phosphate could be of considerable economic importance in the developing countries. The good results obtained in vitro cannot always be dependably reproduced under field conditions. It is expected that inoculation with rhizobacteria containing PGP characteristics consequently promotes root and shoot growth. Further, evaluation of the isolates exhibiting multiple plant growth-promoting (PGP) traits on soybean plant system is needed to uncover their efficacy as effective PGPR. The present study is important in view of identification of native bacterial strains with strong potential for development as bioinoculants.

Production of enzyme like phosphates is other mechanism of phosphate solubilization (Rodriguez and Fraga 1999). PSB9 isolates, showed high activity of AP at 1 lM of P; however, the production of this enzyme was under detection limit in excess of phosphate (1.4 cm) compared to limiting condition, which could explained that the synthesis of alkaline phosphatase by these bacteria was inducible in low Pi, while it was repressed in high concentration. These results are in concordance with solubilization activity Pikovaskaya's Agar, where PSB9 isolates were strong P solubilizer. Interestingly, PSB4 strain produced a smaller drop in pH value compared to others isolates. This might suggest that this strain is capable to solubilize phosphate by other ways than the production of organic acid. Therefore, we found a positive correlation between phosphate-solubilizing capacity and phosphatase enzyme activity.

The bioavailability of P depends on the solubility and structure of chemical forms, on the soil-root environment, as well as on the susceptibility to microbial attack. Nowadays, great interest exists in the use of microorganisms as inoculants especially in areas with low P availability (Deubel and Merbach 2005). In the present Work is the preliminary step towards the use of phosphobacteria as inoculants for agro-pastoral systems in rhizosperic soils from soybean plant. The use of this technology can help minimize the P-fertilizer application, reduce environmental pollution, and promote sustainable agriculture in soybean.

Conclusion

It is concluded from the present study that all isolated phosphate solubalizing bacteria are very useful for increasing crop productivity of soybean (Glycine Max). These isolate not only solubilize phosphorous but also increases nitrogen uptake. They are symbiotically associated with soybean plants rishzosphere. There use as a PGPR is an important contribution to phosphatic bio-fertilization of agricultural crops.

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