Research Paper
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Evaluation of Phosphate Solubilizing Potential of HalotolerantRhizobacteria

Shah Rupal K., Priyanka J. Patel, Goral R. Trivedi, Meenu Saraf*

Department of Microbiology and Biotechnology,
School of Sciences, Gujarat University, Ahmedabad,Gujarat.
shahrupal1989@gmail.com

*Corresponding Author

Abstract

Phosphorus is an important macronutrient that is essential for plant growth and development. Inorganic phosphorus (P), which can make up to 70% of the total P content in soils, can exist in calcium-, aluminum- or iron-complexed forms that are unavailable for plant use. As a result, mineral phosphorus, P_2O_5 , is often used as a fertilizer to supplement the nutrient for crop growth. Soil microorganisms play a role in maintaining the ecological balance by active participation in nutrient cycles in nature. Phosphate Solubilizing Bacteria(PSB) as a phosphorus bio fertilizer improves soil fertility by solubilizing insoluble phosphate salts and increase crop production. This research aimed in isolation salt-tolerant or halophilic PSB will facilitate the development of saline-alkali soil-based agriculture. The fastest growth was observed when the culturing temperature was 28°C and the concentration of NaCl was 6% (w/v). It was found that the isolates can survive at a concentration of NaCl up to 20%. Halo zone formation and plate screening method was used for phosphate solubilization evaluation on Pikovskaya s agar medium, containing insoluble tri- calcium phosphate (TCP). Isolates showing highest Phosphate Solubilization Index (PSI) were selected for further study as qualitative as well as quantitative activities. The growth of the isolates was related with a significant decrease of pH of the medium which indicates the production of acids like acetic acid, formic acids etc. These isolates were further identified by 16S rRNA, their morphological and biochemical properties respectively.

Keywords:Phosphorus,PSB,NaCl,Phosphate

Introduction

Phosphorus is an important plant nutrient next only to nitrogen.It is a master key element in crop production (Pierre, 1938). It is a component of key molecules such as nucleic acids, phospholipids and ATP.It stimulates the root growth at early stages of plant growth, helps in early maturity of crops and inadequate availability of it markedly reduces plant growth, reduces plant size and

unusually deep green colouration. Plants acquire P from soil as phosphate anions. P is abundant in soils in both organic andinorganic forms, P deficiency is a serious concern for agricultureproductivity and sustainability around the globe, Despite having rich total P contents, the available P in evenmost fertile soils are too low to meet most plants' demands dueto precipitation with Ca^{2+}in alkaline soils (Rahmatullah et al.1994) and with Al^{3+} and Fe^{3+} oxides in acid soils (Plaxton andCarswell 1999; Raghothama 1999)

Since phosphorus content is quite low in saline soil so the application of phosphatic fertilizer is essential for better crop yield. However, P reacts very strongly with soil constituents,as a result only about 5–25% of applied P can be utilized bythe plants while the remaining 75–95% of applied P is beingrapidly fixed and transformed into insoluble forms unavailableto plants (Yin et al. 2015).More than 2/3 of this fertilizer is rendered unavailable due to fixation in soil complex.They also causes surface and ground water pollution and various other deleterious effects.

Several reports indicated that various bacterial species have the ability to solubilize insoluble inorganic phosphate compounds like TricalciumPhosphate(TCP),Dicalcium Phosphate(DCP),hydroxyapatite and Rock phosphate. Solubilization of insoluble P by microorganisms was reported by Pikovskaya (1948).These specific groups of soil microbes known as PSB(Phosphate Solubilizing Bacteria) which increase the availability of phosphates to plants, not only by mineralizing organic phosphorus compounds but also by rendering inorganic phosphorus compounds more available to them.(Gaur,1979). PSB vary in efficiency in solubilizing inorganic phosphate are known to renderinsoluble phosphate into a soluble form through the process ofsolubilization (inorganic P)/ mineralization (organic P) byreleasing low molecular mass organic acids, phosphatases orother complex agents (Duponnois et al. 2005). The concentration of iron ore, temperature, and C and N sources greatly influence the P-solubilizing potentials of these microbes. Among the various nutrients used by these microorganisms, ammonium salts has been found to be the best N source followed by asparagine, sodium nitrate, potassium nitrate, urea and calcium nitrate(Ahuja et al. 2007).The role of PSB is shown in Figure:1.

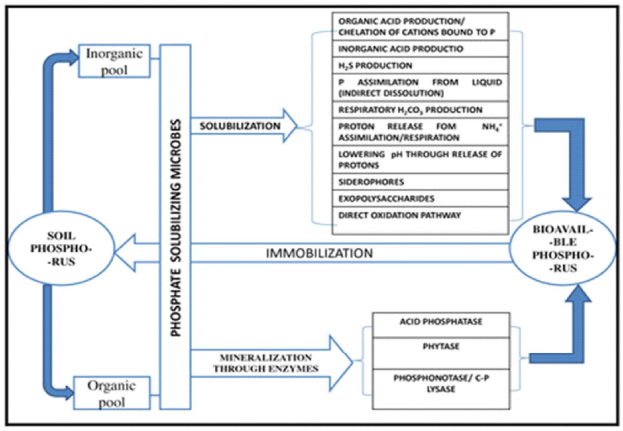

Figure: 1 Schematic representation of PSB mechanism in increasing soil phosphorus(Seema et al.2013)

Application of phosphate solubilizing microorganismsincluding bacteria, fungi and yeast to agriculture ecosystemshave been considered as a low-cost and low-energy managementstrategy to enhance the agronomic effectiveness and efficiencyof both soluble and insoluble P fertilizers, especiallyinsoluble RP (Biswas and Narayanasamy, 2006; Xiao et al.2013; Abbasi et al. 2015; Bakhshandeh et al. 2015). PSB constitute 1-50%.Moderately halotolerant bacteria are capable of growing at 4 to 20%(0.8 to 3.4 M) NaClconcentration.Thesehalotolerant bacteria are capable to solubilize phosphate in saline areas and make it available to plants in saline areas. They are the promising strategy to improve world food production without causing any environmental hazards.

Materials and Methods

Study sites and rhizospheric soil sampling

3 different sites from Kachchhdistrict ,Gujarat,India were selected for sampling of soil in 2015. The air temperature of the sites varied between 25°C and 30°C, soil temperature 20°Cand 25°C. Before sampling,grass, forest litter or any other material on the soil surface were removed.Within each site, rhizospheric soil samples of *Cumin cyminum* from 15 cm were collected by soil auger and mixed as one composite sample and stored at 4°C for isolation of PSB. A subsample of about 0.5 kg was air-dried and passed through 2-mm sieve and used for the determination of physical and chemical characteristics.

Isolation of Bacterial Isolates

Composite Rhizospheric soil sample stored at 4°C was used for bacterial isolation.Rhizospheric bacteria were isolated by adding 1g soil sample in 99 ml sterile distilled water and kept the flask on shaking for 20 min to separate microorganisms completely from the soils,allowed to stand for 1 hour .Then the supernatant was used for serial dilution plating on nutrient agar plates having NaCl concentration of 6% The plates were incubated at 28 ±2°Ctill the appearance of bacterial colonies. Individual colonies were picked and streaked on nutrient agar plates for further purification. Morphological characteristics of colonies were recorded after 24 h of growth on nutrientagar plates at28 ± 2°C . Bacterial colonies having different morphological characteristics were screened and 30 isolates were selected for evaluating theircell morphology The slides of screened strains were prepared to study Gram positive or negative characteristics by staining techniques(Murray et al. 1994).

Culture Maintenance

Bacterial cultures were maintained on nutrient agar slants having NaCl concentration as per in their respective plates and preserved at 4°C under refrigerator

Phosphorus Solubilization Assay

A) Qualitative Assay

Phosphorus solubilizing capacity of isolates on Pikovskaya'sagar plate medium having bromophenol blue dye(Gupta et al,1992) and Nacl concentration upto 6 %.The potential of isolates to solubilize insoluble phosphates wasexamined on the Pikovskaya's medium (Pikovskaya 1948).Each bacterial culture in its isolated single colonial form wasspot inoculated in the center of agar plates of

Pikovskaya'smedia, containing tricalcium phosphate [$Ca_3(PO_4)_2$] as insolublephosphate source. The plates were incubated at 28±2°C for 6 days.Isolates capable of solubilizing insolublephosphates will form halos.Using the diameter of clearing halozones, P solubilization index (PSI) was calculated by the formula mentioned below (Edi-Premono et al. 1996).

$$PSI = \frac{\text{colony diameter+halozone diameter}}{\text{colony diameter}}$$

Enrichment Method for Isolation

1g of soil sample was added to 100ml Pikovskaya's broth containing flask and three successive transfers were made weekly intervals to enrich the medium.Then from the final flasks,PSBs were isolated by streaking a loopful of the culture on solid phosphate medium.The colonies showing halo zones were purified and preserved.

B)Quantitative Assay

Phosphorus solubilizing capacity of PSB wasdetermined in 100 mL Pikovskaya's broth medium (liquidmedium) containing TCP insoluble [$Ca_3(PO_4)_2$]. Theinsoluble P source was added to 100 mL broth before sterilization.Then 1 ml suspension of each bacterial culture was added to the broth under sterilized conditions.One control i.e. one without PSB or plant growth promotingrhizobacteria (PGPR) was also maintained. The PSB were allowed to grow for 21 days at 28± 2°C on rotatory shaker.Growth medium (5ml) was withdrawn aseptically at 7 days interval from each flasks and centrifuged at 10,000rpm for 20 mins.The supernatant was analyzed for P_2O_5 content by Chlorostannous reduced molybdophosphoric acid blue method using Systronic 166 spectrophotometer (Gaur,1990).The pH of the supernatant was also checked by pH meter(and correlated with the results obtained on plates and in liquid medium.Standard graph was prepared using KH_2PO_4(2-10ppm/ml) as standard reagent.

Results and Discussion

Result of isolation of PSB is shown in **Figure:2**.The Quantitative method used foe estimation of P_2O_5 by spectrophotometer is shown in **Figure:3**.**Table :1** shows the change in pH on 7th and 14th day of incubation of liquid medium inoculated with isolated PSB along with the PSI.

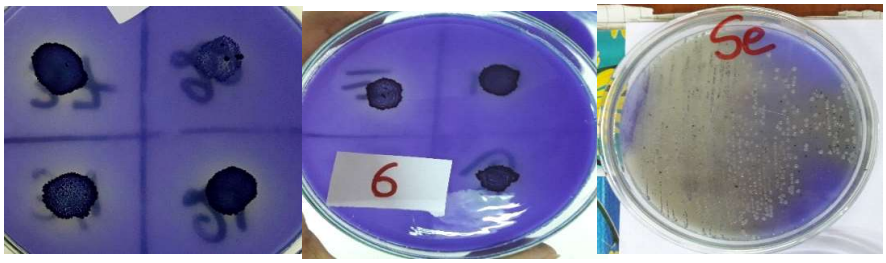

Figure :2(a) and **(b)**yellow zone of phosphate solubilization on Pikovskaya's agar plates having 6% NaCl**(c)** Enrichment technique plate

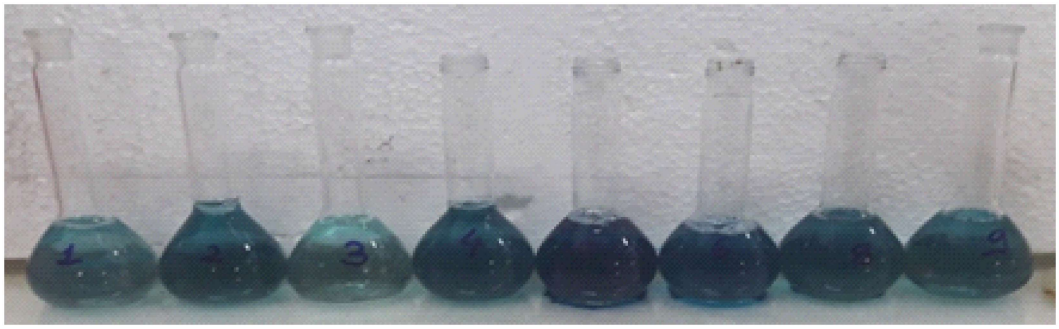

Figure :3 Quantitative estimation of phosphorus solubilized by PSB

Table:1 Change in pH of the medium and PSI of isolates

Isolate No.	pH 7th day	pH 14th day	PSI
3	5.5	3.7	6
8	5.2	3.0	5
18	5.4	3.3	8
31	5.0	3.3	8

From the above mentioned results bacteria no.3,8,18,31 growing at 6% NaCl concentration have the potential to solubilize phosphate (tricalcium phosphate).There is decrease in the pH of the medium from 7 to 3.0 which indicates the acid formation that helps in phosphate solubilization.The PSI of isolate 18 and 31 is 8.These all indicates that all the four isolates are capable of growing under saline conditions as well as can solubilize phosphate efficiently and can make available to plants for their better growth and production.The phosphate solubilization in liquid medium was found to ranging from 9.66 µg/ml to 20 µg/ml.

Future Significance

- They can be used as a means of fixing the nutrient availability in the soil .
- Maintains the phosphorus cycle.
- They can easy shows symbiotic association with plants roots.

- PSB function is in long duration ,causing improvement in soil fertility.
- They can maintain the natural habitat of soil.
- PSB can be used as biofertilizers in saline areas for crop production.
- They are easy to apply without any additive agents.
- They are cost effective biofertilizer and having low manufacturing cost**.**

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