



ISOLATION AND SCREENING OF PHOSPHATE SOLUBILIZING BACTERIA FROM SUNFLOWER RHIZOSPHERE

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ABSTRACT

Phosphorus is one of the major nutrients which play an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the living plant. Chemical fertilizer is the main source of plant available phosphorus in the agriculture soils, but almost 75 to 90% of added Phosphate fertilizer is precipitated by iron, aluminum and calcium complexes present in the soils. Many soil bacteria and fungi have the ability to solubilize phosphate minerals and make it available to plants. Thus, an attempt was made to isolate and screen potential phosphate solubilizing bacteria (PSB) which can able to solubilize phosphate and be used as biofertilizer in future. Sunflower plant rhizosphere soil samples from the different sites of Surat, Bharuch district of Gujarat was collected and each sample was enriched in Pikovskaya's medium, pH 7.5 at 27°C for 5 days. About 78 isolates were obtained on same agar medium. From that 11 isolates were found to be good phosphate solubilizer. These isolates were coded as PSB1, PSB2, PSB3, PSB4, PSB5, PSB6, PSB7, PSB8, PSB9, PSB10 and PSB11. Each purified isolate was studied for zone of solubilization and quantitative phosphate solubility by Pikovskaya's agar and chlorostannous reduced molybdophosphoric acid blue method respectively. Among the isolates PSB5 gives maximum solubilization zone (18 mm) followed by PSB 9 (14 mm) and PSB6 (12 mm). However, the isolate PSB2 and PSB7 showed the least solubilization zone. The phosphate released by the strain ranged from 68 mg/l to 181 mg/l. Among the isolates PSB 5, PSB9, PSB6, PSB8, PSB11 and PSB10 were to be potential strain to solubilize the insoluble phosphate.

KEY WORDS: Phosphate solubilizing bacteria (PSB), Pikovskaya's medium, Molybdophosphoric method, Sunflower plant rhizosphere

INTRODUCTION

Phosphorus is a biocritical element in short supply in nature. Phosphorus plays an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the living plant (Gyaneshwar *et al.*, 2002). Soils are often high in insoluble minerals and organic phosphates but deficient in organophosphates (Pi) (Grover, 2003). In India, majority of the phosphorus provided in the form of chemical fertilizers which abundant use decreases the fertility of soil after long period of time. In nature, wide range of microbial biosolubilization mechanisms exist which are necessary to maintain global cycle (Whitelaw, 2000). Many microorganisms are able to solubilize unavailable forms of phosphates by excreting organic acids. These microbes present in the different forms and numbers in the soil (Kucey, 1983). A large number of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *Rhizobium* and *Serratia* have reported to enhance plant growth by with their different plant growth promoting activities including phosphate solubilization (Kumar *et al.*, 2012). The hydroxyl and carboxyl groups of microbial organic acid chelate the cations bound to phosphate, thereby converting it into soluble forms (Kpombrekou and Tabatbhai, 1994). However, Phosphate solubilization is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the culture (Reyes *et al.*, 1999). Literature reviews indicates

that an average of 10^8 CFU per gram of Phosphate Solubilizing Bacteria (PSB) was found in fertile soil of forests, organic farming and rhizosphere (Widawati *et al.*, 2004; Widawati and Suliasih, 2006; Widawati and Suliasih, 2009). Phosphate solubilizing microbes can also produce phosphatase enzyme which can benefit sustainable organic farming systems especially in coastal ecosystems, and reduce the utilization of agrochemicals in agricultural fields (Widawati, 2011). In the microorganisms bacteria can grow faster than fungus and actinomyces by utilizing simple media. Thus, an attempt was made to isolate and screen potential phosphate solubilizing bacteria from the soil for the agricultural purposes.

MATERIALS AND METHODS

Soil sampling

The soil samples were collected from different agricultural land of villages of Surat and Bharuch district located in Gujarat, India. Soil samples were collected from the rhizosphere of Sunflower plant. The samples were carefully collected in polythene bags and stored at 4°C temperature.

Isolation of Phosphate Solubilizing Bacteria (PSB) from rhizosphere soil

Soil samples were prepared by inoculating 1.0gm of each soil into 100ml of sterile distilled water. Homogenization of soil was carried out by keeping it on shaker for 30 minutes at 120 rpm at 27°C temperature. After 30 minutes, samples were removed aseptically and

centrifuged at 10,000 rpm for 15 minutes. 10ml of supernatant was inoculated into 100ml of sterile Pikovaskaya's broth and kept it at 120rpm at room temperature for 5 days for enrichment. After 5 days, serial dilution of the enriched medium was carried out and aliquots of serially diluted soil samples were subjected to spread on sterile Pikovaskaya's agar medium (Ponmurugan and Gopi, 2006). Pikovaskaya's medium contains (grams/liter); Glucose, 10.0gm; $\text{Ca}_3(\text{PO}_4)_2$, 5.0 gm; $(\text{NH}_4)_2\text{SO}_4$, 0.5 gm; NaCl, 0.2 gm; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 gm, pH 7.3 $\pm$ 2. These plates were kept at 27 $\pm$ 2°C for 24 to 48 hrs. Bacterial colonies were purified by using four flame technique on nutrient media. Each culture was maintained at 4°C.

Screening of Potential Phosphate Solubilizing Bacteria

Each of the isolates was screened for their ability to solubilize calcium phosphate present in the Pikovaskaya's medium (Pikovaskaya, 1948; Gupta *et al.*, 1994). A loopful of pure culture was placed on the center of the same agar plates and incubated for 27 $\pm$ 2°C for 5 days. The solubilization zone was determined by subtracting the diameter of bacteria colony from the diameter of total zone.

Quantitative estimation of soluble phosphate in Pikovaskaya's broth

The isolates showing zone of solubilization on Pikovaskaya's agar were further examined for their ability to release phosphate in broth media. Thus, 1.0ml (O.D₆₀₀, 1.0) of each isolates culture (18hrs old) was inoculated separately into 100ml of sterile Pikovaskaya's broth in the 250ml Erlenmeyer flasks. Each flask was incubated at 28 $\pm$ 2°C at 120 rpm for 5 days. Simultaneously, the uninoculated control was also kept under similar

conditions. All the experiments were carried out in triplicate. To estimate the amount of phosphate released by the isolates, 10ml of each sample was withdrawn at regular intervals of 24 hrs and was examined for soluble phosphorus and pH. The cultures were harvested by centrifugation at 10,000 rpm for 15 minutes. The soluble phosphorus in the supernatant was measured by chlorostannous reduced molybdophosphoric acid blue method (Jackson, 1973). For that, to a 100 μ l aliquot of supernatant, add 10ml of chloromolybdic reagent and dilute it upto 40ml with distilled water. Add 5 drops of chlorostannous acid reagent and mix it properly by making final volume 50ml with distilled water. The absorbance of the resultant color was read at 600 nm against blank in UV/visible spectrophotometer. pH of the supernatant was measured by pH electrode.

RESULTS

Isolation and Screening of phosphate solubilizing bacteria

Wide number of bacterial isolates was found from the different agricultural lands of Surat and Bharuch district in Gujarat, India. Among them about 78 isolates was found from the different rhizosphere of Sunflower plants. Each of the isolates was purified successfully on nutrient agar media and maintained at 4°C. On the basis of zone of solubilization in Pikovaskaya's agar medium, total 11 bacterial strains were screened for further studies. These data are represented in the table 2. Other isolates which shows hazy zone or without zone were not selected for further studies. The distribution of locations of rhizosphere soil, number of samples and isolates has been shown in table no. 1.

TABLE 1: Description of bacterial isolates isolated from different rhizosphere of sunflower plant.

Sample Numbers	Locations of rhizosphere soil	Number of Isolates
Sample 1	Garden soil of University, Surat	10
Sample 2	Kribhco, Surat	12
Sample 3, 4, and 5	Different Garden soil of Surat	14
Sample 6	Kathore village, Kamrej, Surat	07
Sample 8,9	Farms nearby Bardoli, Surat	12
Sample 10	Farm, Bharuch	10
Sample 11	Garden , Bharuch	13

TABLE 2: Bacterial isolates showing zone of solubilization and quantitative P-solubilization in Pikovaskaya's medium.

Sr. No.	Isolates Code	Zone of Solubilization (mm)	Phosphate solubilization (mg/l)
1.	PSB1	6.0	74.0
2.	PSB2	4.0	68.0
3.	PSB3	8.0	119.8
4.	PSB4	6.0	78.0
5.	PSB5	18.0	181.0
6.	PSB6	12.0	156.3
7.	PSB7	3.0	91.90
8.	PSB8	8.0	137.3
9.	PSB9	14.0	167.4
10.	PSB10	7.0	120.3
11.	PSB11	8.0	132.7

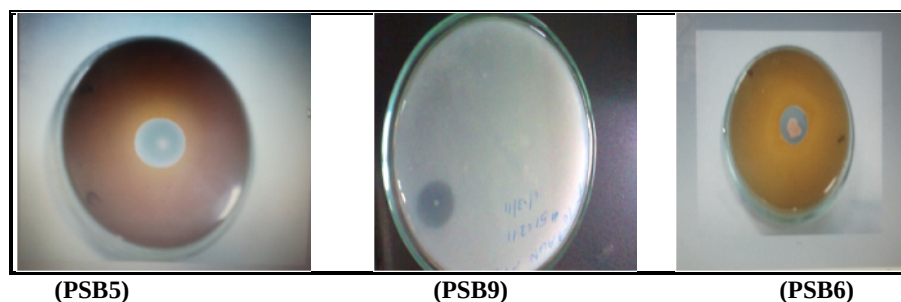


FIGURE 1: Bacterial isolates PSB5, PSB9 and PSB6 shows maximum zone of solubilization on Pikovaskaya's Agar medium

Sharp zone of solubilization was found among eleven isolates, ranging from 3.0 to 18.0 mm. Figure 1 shows maximum zone of solubilization with PSB5 (18mm), PSB9 (14 mm) and PSB 6 (12 mm) within 48hrs. Other isolates showed the least solubilization zone. It was also observed that increasing in the incubation time, increases the in the zone size of each isolates.

Solubilization of phosphate in Pikovaskaya's broth

The amount of phosphate released in the Pikovaskaya's broth by each of the eleven isolates was quantitatively measured using chlorostannous reduced molybdophosphoric acid blue method describe by Jackson in 1973. In quantitative estimation, range of tricalcium phosphate solubilization was found between 68.0 mg/l to 181.0 mg/l which was shown in table 2. Amount of the soluble phosphate was found maximum in PSB5 (181.0mg/l), PSB9 (167.4mg/l), PSB6 (156.3), PSB8 (137.3mg/l), PSB11 (132.7 mg/l) and PSB10 (120.3mg/l) within 48 hrs. However, other isolates have been also able to solubilize phosphate but are less significant or requires more incubation time for good solubilization. Measurement of the pH was also carried out every day. It was gradually decreased with the increased incubation time. After 72 hrs, pH was decreased between 5.0-4.0.

DISCUSSION

Agricultural production in the recent past has concentrated on the use of improved high yielding varieties, which need high fertilizer application. This has resulted in enhanced application of chemical fertilizers in an effort to maximize returns. One impact of this is the accumulation of large amount of insoluble phosphates in the soil due to the chemical fixation. Ever since Gerretson (1948) demonstrated the ability of microorganism to convert fixed phosphate in the soil in to available form, extensive research has gone into isolation of efficient organisms, understanding the biochemical basis and more recently molecular basis of this phenomenon to identify the microbes (Gerreston, 1948). A large number of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *Rhizobium* and *Serratia* have been reported to solubilize phosphate (Kumar *et al.*, 2012; Reyes and Valduz, 2006). In our study, an attempt was made to isolate and screen potential phosphate solubilizing microbes from the different soil rhizosphere because abundant microbes are available and distributed in different numbers and forms. The ability of

solubilize the inorganic phosphate also depend upon the properties of soils which gives knowledge of diversified group of microbes (Widawati, 2011). In our study, total 78 isolates was found in the rhizosphere of sunflower plant. Such plants rhizosphere is known to be rich in PSB such as Casurina, Sugarcane and Sunflower etc. Thus, soil nearby root of sunflower was selected. On the basis of ability to grow and mineralize insoluble calcium triphosphate on Pikovaskaya's agar medium, only 11 strains were selected for further studies. The role of phosphate solubilizing bacteria in solubilizing a fixed form of soil phosphates and make it available for plant is well known (Vikram *et al.*, 2007). Generally, the ability of solubilization depends upon the production of organic acids and the presence of phosphatase enzyme in microbes (Park *et al.*, 2010). The ability of each PSB to quantitatively dissolve phosphate and pH of the liquid media was also measured spectrophotometrically upto 5 days. Decrease pH and increase soluble phosphate indicates that the isolates having ability to produce organic acids in the different concentration range (Jeon *et al.*, 2003; Perez *et al.*, 2007; Whitelaw *et al.*, 1999; Ramachandran *et al.*, 2007). Thus, among 11 isolates it might be possible that PSB5, PSB9 and PSB6 producing more organic acids or having an effective phosphatase enzyme by which it solubilize maximum amount of phosphate. After 48 to 72 hrs because of high acidic pH, inhibition of bacteria was observed which finally affect the solubilization efficiency of the isolates. Thus, in the present study PSB5, PSB9 and PSB6 found to be more efficient bacterial strains to solubilize the phosphate.

CONCLUSION

Our study demonstrated that many of the bacteria had P-solubilizing properties and the ability was not exclusive to specific genera, suggesting the importance of preliminary screening in vitro for a wide range of bacteria to characterize their potent P-solubilizing or mineralizing trait. At the same time in vitro potential needs to be further tests such as effect of different parameters like pH, carbon and nitrogen sources, NaCl concentration, amount and type of organic acid production by microbes, formulation of biofertilizer, in vivo studies in natural field, identification and characterization of microbes etc. This is probably the pioneer study to reveal that the rhizosphere of such plants have a good source of diverse genera of highly potential Phosphate solubilizers.

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