28. Isolation and Identification Phosphate Solubilizing *Pseudomonas Species*

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ABSTRACT:

The tremendous use of chemical fertilizers is responsible for reduction in soil fertility and environmental degradation. Mostly soils are deficient in soluble forms of phosphorous as becomes insoluble by forming salts of metals especially under alkaline conditions. Phosphorus biofertilizers in the form of microbial inoculants can help in making soil accumulated phosphates available for plant growth. Use of such plant growth promoting rhizobacteria (PGPR) and their products may boost the sustainable increase in crop yield. Among the bacterial genera recognized as phosphate solubilizing bacteria (PSB) are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aereobacter, Flavobacterium and Erwinia*.

Pseudomonas strains were isolated from alkaline rhizospheric soil on *Pseudomonas* isolation agar medium. Six strains of *Pseudomonas species* were obtained from rhizospheric soil. These strains were identified by morphological, pigmentation and biochemical characteristics. The isolated stains were screened on Pikovskaya's agar medium

The Present study isolated the Phosphate solubilising *Pseudomonas* strains from alkaline rhizosphere soil which have a potential to be exploited as biofertilizer for eco-friendly agricultural practices.

Key Word : Phosphate Solubilizing bacteria(PSB), *Pseudomonas Species* and Biofertilizer.

INTRODUCTION

The world population is increasing rapidly and is expected to reach approximately to 8 billion around the year 2020. The demand of food to feed such a growing population is also increasing. Therefore increasing the crop yield in a static confined agricultural area is a challenge before us. Use of plant growth promoting rhizobacteria (PGPR) and their products as biofertilizers may boost the sustainable increase in crop yield.

Eco-friendly biotechnological application of bio-fertilizers offer an alternatives to chemical fertilizers (Dobbelaere et al., 2003). Considering the adverse effects of chemical fertilizers and their increasing costs, use of PGPB is considered as an alternative or a supplemental way of reducing the use of eco-hazardous chemicals in agriculture (De Weger et al., 1995,Welbaum, 2004)

Phosphorus is one of the major vital macronutrients for growth and development of plants (Ehrlich, 1990). It has been valued that in some soils, up to 75% of applied phosphate fertilizer may become inaccessible to the plant because of mineral phase re-precipitation (Sundara et al., 2002). Phosphate solubilizing bacteria (PSB) are able to convert these insoluble phosphates into soluble forms and have therefore been used to enhance the solubilization of reprecipitated soil P for crop enhancement (Hilda et al., 2000ab; Shekhar et al., 2000 Babukhan et al 1995; Goldstein, 1987; Sperber 1958).

Mineral forms of phosphorus are represented in soil by apatite, hydroxyapatite, and oxyapatite (primary minerals). They are found as part of the stratum rock and their principal characteristic is their insolubility. Under appropriate conditions, they can be solubilized and become available for plants and microorganisms. Mineral phosphate can be also found with the surface of hydrated oxides of Fe, Al, and Mn, which are poorly soluble.

There are two type of phosphorous in soil, organic and inorganic phosphates. A large proportion is present in insoluble forms, and therefore, not accessible for plant nutrition. Inorganic P occurs in soil, mostly in insoluble mineral complexes, some of these appearing after the application of chemical fertilizers. These insoluble forms cannot be absorbed by plants. Organic matter, on the other hand, is an important reservoir of immobilized P that accounts for 20–80% of soil P (Richardson, 1994).

Organic Phosphate

A major component of soil P is organic matter. Organic forms of P may constitute 30–50% of the total phosphorus in most soils, it may ranges from as low as 5% to as high as 95% (Paul and Clark, 1988). Organic P in soil is largely in the form of inositol phosphate (soil phytate). It is synthesized by microorganisms and plants and is the most stable of the organic forms of phosphorus in soil, accounting for up to 50% of the total organic P (Smith, 1983).

Organic Phosphate Solubilization

Organic phosphate solubilization called mineralization of organic phosphorus, and it occurs in soil at the expense of plant and animal remains, which contain a large amount of organic phosphorus compounds. The decomposition of organic matter in soil is carried out by the action of numerous saprophytes, which produce the release of radical orthophosphate from the carbon structure of the molecule. The organophosphonates can equally suffer a process of mineralization when they are victims of biodegradation (McGrath, 1995). The microbial mineralization of organic phosphorus is strongly influenced by environmental parameters; in fact, moderate alkalinity favors the mineralization of organic phosphorus. The degradability of organic phosphorous compounds depend mainly on the physicochemical and biochemical properties of their molecules, e.g. nucleic acids, phospholipids, and sugar phosphates are easily broken down, but phytic acid, polyphosphates, and phosphonates are decomposed more slowly (McGrath, 1995;).

Phosphorus can be released from organic compounds in soil by three groups of enzymes:Nonspecific phosphatases, which perform dephosphorylation of phosphoesteror phosphoanhydride bonds in organic matter Phytases, which specifically cause P release from phytic acid Phosphonatases and C–P Lyases, enzymes that perform C–P cleavage in organophosphonates The main activity apparently corresponds to the work of acid phosphatases and phytases because of the predominant presence of their substrates in soil.

Inorganic Phosphate Mineralization

Several reports have suggested the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein, 1986). In two thirds of all arable soils, the pH is above 7.0, so that most mineral P is in the form of poorly soluble calcium phosphates (CaPs). Microorganisms must assimilate P via membrane transport, so dissolution of CaPs to Pi (H2PO4) is considered essential to the global P cycle. Evaluation of samples from soils throughout the world has shown that, in general, the direct oxidation pathway provides the biochemical basis for highly efficacious phosphate solubilization in Gramnegative bacteria via diffusion of the strong organic acids produced in the periplasm into the adjacent environment. Therefore, the quinoprotein glucose dehydrogenase (PQQGDH) may play a key role in the nutritional ecophysiology of soil bacteria. MPS bacteria may be used for industrial bioprocessing of rock phosphate ore (a substituted fluroapatite) or even for direct inoculation of soils as a 'biofertilizer' analogous to nitrogenfixing bacteria. Both the agronomic and ecological aspects of the direct oxidation mediated MPS trait. (Gold stein et al., 2003)

Among the bacterial genera with this capacity are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aereobacter, Flavobacterium and Erwinia* (Babukhan et al 1995; Goldstein, 1987; Sperber 1958).

Mechanism of Phosphate Solubilization

Various theories have been proposed for the mechanism of phosphate solubilization as follows.

- Acid production theory
- Proton and enzyme theory

MATERIALS AND METHODS:

Isolation and biochemical characterization of *PSB*:

Pseudomonas strains were isolated from rhizospheric soil on *Pseudomonas* isolation agar medium. The pigmentation and biochemical reactions were determined as described in Bergey's Manual of Determinative Bacteriology. All the 6 isolates of *Pseudomonas were* biochemically characterized for H2S production, citrate utilization, amylase, oxidase, urease, catalase and lipase production, indole production and pigment production activities.

Isolation of Phosphate solubilizing bacteria - *Pseudomonas* sp. :-

Phosphate solubilizing trait of all isolates was screened on Pikovskaya's medium containing per liter of Yeast extract (0.50 g) Dextrose (10 g), Calcium phosphate (5 g) , Ammonium sulphate(0.5 g), potassium chloride (0.2 g) magnesium sulphate (0.1 g), magnese sulphate(0.0001 g)ferrous sulphate(0.0001 g) Agar- agar (15 g). (Pikovskaya 1948).The spot inoculation of appropriate soil dilutions on Pikovskaya's medium was followed by incubation at 30 ± 1 ºC for 2-3 days. The colonies forming halo zone of clearance around them were counted as P-solubilizers. All the bacterial colonies exhibiting hallow zones were selected, isolated and maintained on nutrient agar slants for further studies.

The solubilization index of the microorganisms were calculated using following Formula:

$$\text{Solubilizing efficiency index (S.E)} = \frac{Z}{C}$$

Z = Solubilization zone (mm)
C = Colony diameter (mm)

The qualitative estimation of phosphate solubilization potential of selected isolate was measured *in vitro* by determining available soluble phosphate in the Pikovskaya's broth supplemented with 0.5% Tri Calcium phosphate. The broth medium was inoculated with phosphate solubilizing bacterial strain RSMP2 and incubated at 28 °C for 5 days on rotary shaker at 180 rpm followed by centrifugation at 8,000 rpm for 10 min. Phosphomolybdate method was used for determination of available soluble phosphate in supernatant (F.S. Watanabe, et al 1965). The pH of the broth medium was also measured with a digital pH meter after every 24 hour.

RUSULT AND DISCUSSION:

Isolation and biochemical characterization of *Pseudomonas isolates*:

Screening of the isolates was done on Pikovskaya's agar medium by spot inoculation, reveled the zone of clearance by all the strains. (Photo: 1and Table:) The strain RSMP2 exhibiting maximum zone (8mm) was subjected for further study of estimation of phosphate solubilizing efficiency.

Photo: 1 screening of the isolates

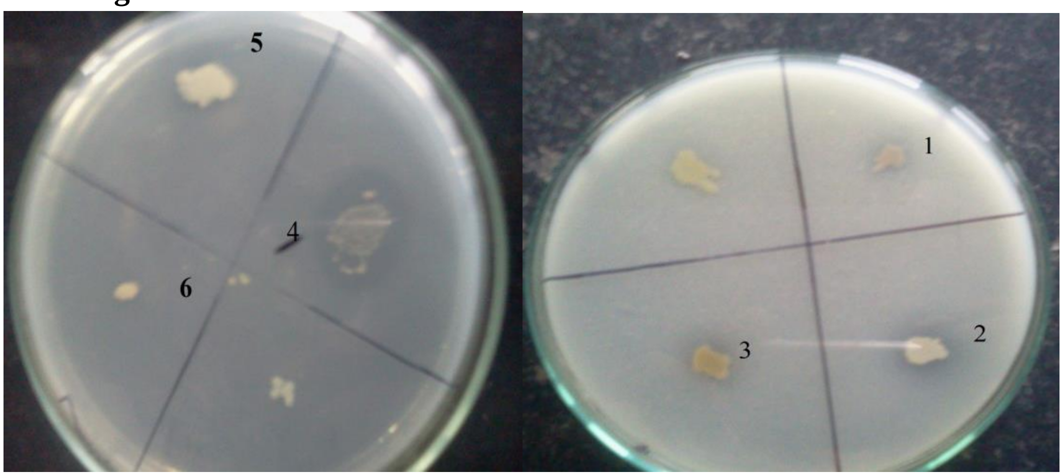

Table 1: Solubilization index

Strain	Colony diameter (c)in mm	Solubilization zone (z) mm	Solubilization index
RSMP1	3	6	2
RSMP 2	4	8	2
RSMP 3	4	7	1.75
RSMP 4	3	6	2
RSMP 5	3	6	2
RSMP 6	2	4	2

The cultural and biochemical characterization of six isolated strains from rhizospheric soil were found Gram-negative, citrate-positive, oxidase-positive, indole-positive, produced fluorescence, were able to hydrolyze starch and use glucose, mannitol, fructose and starch as carbon source identifying them as species of genus *Pseudomonas* as described in Burgey's manual of systematic bacteriology (Table:2). Estimation of the phosphate solubilization ability of RSMP2 was done using solubilization index method by measuring zone of clearing around the microbial colonies.

Significant decrease in pH of culture in liquid medium, from an initial pH of 7.0 after 96 h of incubation period by all strains was recorded. The drop in pH value was correlated with elevated levels of phosphate solubilization. For PSB strain RSM2 the pH was declined to 4.6 from initial pH 7.0 indicating maximum phosphate solubilization. Park et al. also reported that in case of *Pseudomonas fluorescens* RAF15, the maximum drop of pH was recorded upto pH 4.0. The acidification of culture supernatants clearly indicated the production of organic acid seemed to be generally the main mechanism for phosphate solubilization.

Table 2: Morphological and Biochemical Characterization

SN	**Character**	RSMP**1**	RSMP **2**	RSMP **3**	RSMP **4**	RSMP **5**	RSMP **6**
1.	**Size (in mm)**	2	3	2	2	1	3
2.	**shape**	Rod	Rod	Rod	Rod	Rod	Rod
3.	**color**	Yellowish	Greenish		Greenish	colourless	colourle
4.	**margin**	Entire	Entire	Entire	Entire	Entire	Entire
5.	**surface**	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
6.	**elevation**	flat	flat	flat	flat	flat	flat
7.	**opacity**	opaque	opaque	opaque	opaque	opaque	opaque
8.	**consistency**	sticky	sticky	sticky	sticky	smooth	smooth
9.	**Gram's nature**	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
10.	**motility**	motile	motile	motile	motile	motile	motile
11.	**Glucose**	A	A	A	A	A	A
12.	**lactose**	-	-	-	-	-	+
13.	**Mannitol**	A	A	A	A	A	A
14.	**Maltose**	-	A	A	-	A	A
15.	**Catalase**	+	+	+	+	+	+
16.	**Oxidase**	+	+	+	+	+	+
17.	**Indole**	+	+	+	+	+	+
18.	**MR**	+	+	+	+	-	+
19.	**VP**	-	-	-	--	-	-
20.	**Citrate utilization**	+	+	+	+	+	+
21.	**H_2S**	+	+	+	+	+	+
22.	**Nitrate reduction**	-	-	-	-	-	-
23.	**Identified As**	*Pseudomonas* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.
24.	**pH fall after 96 hr**	*5.7*	4.6	*6.2*	*6.7*	*5.9*	*6.8*

+: Test Positive - :Test Positive, A: acid Production

CONCLUSION:

The considerable phosphate solubilizing potential of Pseudomonas strain RSMP2 among the isolated six strain indicate that the strain can be exploited as a PSB for development of a biofertilizer after further investigations.

The use of this native strain as bio-fertilizer may remain more comfortably active with the local soil micro flora. It will help in reducing the use of chemical fertilizers and also effective in reducing the cost of cultivation and maintaining the natural fertility of soil. Use of these PSB as bio-inoculants will increase the available P in soil, reduces environmental pollution and promotes sustainable agriculture.

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