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SCREENING OF PHOSPHATE SOLUBILIZING BACTERIA FOR PLANT GROWTH PROMOTING FACTOR FROM RHIZOSPHERIC SOIL

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ABSTRACT

Phosphorus is essential for the growth and productivity of plant which can absorb phosphate only in soluble form. The phosphate solubilizing bacteria (PSB) mobilize insoluble phosphate into soluble form through enzymatic and organic acid production. Thus, PSB can be used as biofertilizers and are able to improve the soil fertility. The phosphorous is most abundant in soil both in organic as well as inorganic form and is the major limiting factor for the growth of the plant. In the present study, 6 phosphate solubilizing bacteria were isolated from various agricultural fields and designated as NR1-NR6. NR-1, NR-2 and NR-5 were found to be strong phosphate Solubilizer, with halo zone diameters of 13, 12 and 12 mm respectively on Pikovskaya agar. All the isolates were further screened for plant growth promoting factor such as siderophore, HCN, ammonia and cell wall degradation enzyme production. All the isolates were found to be positive for siderophore production and their frequency ranged from 5mm -11 mm. NR-1, NR-3, NR-4 and NR-5 were positive for HCN production. NR-1, NR-2, NR-3 and NR-5 produced ammonia. While isolates NR-1, NR-2, NR-4 and NR-6 produced cellulase and protease which are cell wall degrading enzymes. All the isolates were also characterized in term of morphological and biochemical test and after comparison with the Bergy's Manual of Systematic Bacteriology the isolates NR-1, NR-2, NR-3 and NR-5 tentatively assigned as Pseudomonas spp. while NR-4 and NR-6 assigned as Bacillus spp.

Keywords: Ammonia, Phosphorus, Phosphate Solubilizers, HCN, Siderophore.

INTRODUCTION

Phosphorus is one of the major plant nutrients limiting plant growth factor. Most of the essential plant nutrients, including phosphorus, remain in insoluble form in soil. Phosphorous is important for growth and productivity of plants. Phosphorous plays an important role in many physiological activities such as cell division, photosynthesis, and development of good root system and utilization of carbohydrate [1]. Phosphorous is optimizing the crop production and minimize the phosphate loss from soil. Phosphate solubilizing micro-organism had attracted the attention for agriculture as soil inoculants to improve the plant growth [2-3].Phosphate solubilizing bacteria play important role

in supplying phosphate to plants. The Phosphate solubilizing bacteria are known to mobilize insoluble phosphate to soluble forms through enzymatic actions. Phosphate solubilizing bacteria (PSB) are known to be able to solubilize different forms of inorganic phosphates. Phosphate is used as biofertilizer that is eco-friendly. Biofertilizer are bio-organic substance that is able to improve the soil fertility. The Phosphate solubilizing bacteria solubilize the fixed soil phosphorous and applied phosphates resulting in higher crop yields [4]. The Phosphate solubilizing bacterial strains exhibit inorganic phosphate solubilizing abilities and organic phosphorous mineralizing abilities [5]. The Phosphate solubilizing in conjunction with single super phosphate and rock phosphate reduce the phosphate dose by 25 and 50 %, respectively [6]. Phosphorous solubilizing bacteria mainly are Bacillus, Pseudomonas and Enterobacter are very effective for increasing the plant available in the soil as well as growth and yield of crops. So exploitation of phosphate solubilizing bacteria through bio fertilization has numerous potential for making use of ever increasing fixed phosphorous in soil and natural reserve of phosphate rock [7]. Microbial inoculants are in use for improving soil fertility. Soil phosphate is dynamics and characterized by physicochemical such as sorption-desorption and biological such as immobilization-mineralization processes. Organic forms of phosphorus are changed into inorganic forms by microbial activity in the soil that is called mineralization process. When inorganic forms of Phosphorous are changed into organic forms of Phosphorous that plants cannot use that is known as immobilization process [9]. The present study was emphasized with the isolation of PSB bacteria, their screening for plant growth factor production and partial identification of the isolates.

1. MATERIALS AND METHODS

1.1. Collection of soil samples

A total 10 soil samples were collected aseptically from the different agricultural field such as wheat, sugarcane and mustard field of Tangori village, Punjab (India) is located geographical coordinate of 30.5952^{o} N and 76.7064^{o} E. The Soil sample were collected in a polythene bag and brought to the laboratory and stored at 4°C until further processing.

1.2. Isolation of bacteria from soil

The soil samples were first dried to remove the moisture and mashed to fine powder. The isolation of bacterial strain was carried by serial dilution method. 1 gram of sieved soil was dissolved in 9 ml of sterile normal saline to make the stock of 1:10. Then the stock was serially diluted upto 10-8. 0.1 ml of last three dilutions i.e. 10-6, 10-7 and 10-8 were spread over the nutrient agar plate. All the plate were then incubated at 35± 2°C for 24 h.

1.3. Screening for phosphate solubilizing bacteria

Morphologically different colonies were screened for phosphate solubilising activity. The morphologically distinct colonies were spot inoculated on Pikovskaya agar plate and incubated at 35± 2oC for 3 days. Bacterial colonies forming halo zones were considered to be phosphate solubilises [10].

1.4. Culture preservation and Maintenance

All the bacterial isolates positive for phosphate solubilising ability were purified by sub culturing in Pikovskaya agar. The purified isolates were then preserved in nutrient agar slant and stored at 4oC. These isolates were maintained in nutrient agar slant by reviewing the culture in 30 days interval.

1.5. Screening for plant growth promoting factor

1.5.1. Siderophore production

Phosphate Solubilises Bacterial isolates was assayed for siderophore production on the chrome azurole S agar (CAS) [11]. Chrome azurole S agar plates were prepared and spot inoculated with test organism and incubated at 30° C for 5 days. Development of yellow - orange halo around the colony was considered as positive for siderophore production. Their zone diameter was measured.

1.5.2. HCN production

Nutrient agar was supplemented with the Glycine (4%). A piece of filter paper was dissolved in the picric acid and put on the lid. Isolate was streaked on the plate. The dish was sealed with paraffin and held at 28^0C for 96 hrs. Appearance of yellow color indicated positive test for HCN Production [12].

1.5.3. NH_3 production

Isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated into 10 ml peptone water in each tube and incubated for 48 hrs. at 30^0C. Nessler's reagent (0.5 ml) was added to each tube. Appearance of brown to yellow colour indicated positive test for ammonia production [13].

1.5.4. Cell wall degrading enzyme

Protease activity (casein degradation) was determined from clear zone in skimmed milk agar. The skimmed milk agar was prepared and autoclaved the test organism was spot inoculated and the plate was incubated at 37°C for 24-48 h. The CMC agar plates were prepared and spot inoculated with the isolate and incubated at 30°C for 5 days. Appearance of halo zone around the colony was considered as positive for cell wall degrading enzyme [14].

1.6. Partial identification of the isolates

1.6.1. Morphological characterization

Morphological characterization of isolate viz., Colony morphology such as colour, shape, margin, elevation, pigmentation and surface were studied. The cell morphology such as size, shape, arrangement was also studied by gram reaction [15].

1.6.2. Cultural Characterization

Cultural characterization of isolate was studied by growing on the culture media such as Nutrient agar and Pikovskaya agar [16].

1.6.3. Biochemical Characterization

The biochemical characterization of the phosphate solubilizers were studied by different biochemical tests such as IMViC, Oxidase, Catalase, Nitrate reduction, Sugar fermentation Urease , triple sugar iron Gelatine liquefaction[15].

2. RESULTS AND DISCUSSION

A total 10 morphologically distinct colonies were selected for the phosphate solubilizing activity.

2.1. Screening of the isolates for phosphate solubilising activity

Phosphate solubilising bacteria were screened by using the plate assay method on Pikovskaya agar. Bacterial colonies forming clear hallo zone around the growth were depicted phosphate Solubilizer. Out of 10 morphologically distinct colonies 6 phosphate solubilizing strain were recovered selectively from Pikovskaya agar. These isolates were assigned as NR-1, NR-2, NR-3, NR-4, NR-5 and NR-6 (Table 1). Among the isolates, NR-1, NR-2 and NR-5 found to be having strong phosphate solubilizing activity with halo zone size 13, 12 and 12 mm respectively (Fig 1.). The phosphate solubilizers produce organic acids that solubilize the insoluble phosphate in soluble form which is indicated by clear halo zone around the bacterial colony. Similar observation was reported with 5 bacterial isolates forming clear halo zone on Pikovskaya agar [17].

2.2. Screening of rhizospheric bacteria for plant growth promoting activity

Rhizospheric bacteria are known to influence the plant growth. All the recovered isolates positive for phosphate solubilizing activity were further screened for plant growth promoting factor like Siderophore production, HCN production, Production of ammonia and Cell wall degrading enzymes (Table 2).

2.2.1. Siderophore production

All the 6 isolates viz. NR-1, NR-2, NR-3, NR-4, NR-5 and NR-6 were found to be positive for siderophore production test (Table 2) and (Fig 3.). The frequency of siderophore production among the recovered isolates was ranged from 5 to 11mm. The size of orange halo zone for NR-1, NR-2 and NR-5 was 9 mm, 11mm and 8 mm respectively. The range was 5mm, 7mm and 6mm for NR-3, NR-4 and NR-6 respectively (Fig 2.). Similarly, some co-workers reported that Siderophore producing bacteria are good candidates for plant growth promotion, especially in neutral to alkaline soil. They screened 220 P- solubilizing bacteria and found have good siderophore prospects to improve plant growth, especially in the soil with large amount of precipitation. Iron is a limiting bioactive metal in soil and essential for the growth of soil microorganisms[18]. The iron concentration in the soil is low (10-7M) enough to limit the growth of soil microorganism (10-8 to 10- 6 M). Rhizobacteria developed some strategies to acquire iron and the major strategy is the production and utilization of siderophores. The rhizobacteria that can produce siderophores could compete for iron with soil borne pathogens [19].

2.2.2. HCN production

Table 1. Different isolates and their phosphate solubilizing activity

Sr. No.	Isolates ID	Phosphate Solubilizing Activity
1	NR-1	+++
2	NR-2	+++
3	NR-3	+
4	NR-4	++
5	NR-5	+++
6	NR-6	++

Table 2. Results of plant growth promoting factor

Plant growth promoting factor	NR-1	NR-2	NR-3	NR-4	NR-5	NR-6
Siderophore Production	+	+	+	+	+	+
HCN production	+	-	+	+	+	-
NH_3 production	+	+	+	-	+	-
Cell wall degrading enzyme (cellulase and protease)	+	+	-	+	-	+

Table 3a. Cultural characterization of bacterial isolates

Cultural characteristics on Nutrient agar				
Isolate	**Elevation**	**Surface**	**Pigment**	**Shape**
NR-1	Convex	Smooth	Green	Irregular
NR-2	Flat	Smooth	Green	Spherical
NR-3	Raised	Smooth	No pigment	Irregular
NR-4	Convex	Dotted, spherical	No pigment	Spherical
NR-5	Flat	Smooth	Green	Spherical
NR-6	Convex	Smooth	No pigment	Irregular

Table 3b. Cultural characterization of bacterial isolates

Cultural characteristics on Pikovskaya agar				
Isolate	**Elevation**	**Surface**	**Pigment**	**Shape**
NR-1	Convex	Smooth	Creamy white	Irregular
NR-2	Flat	Smooth	Creamy white	Spherical
NR-3	Raised	Smooth	white	Irregular
NR-4	Convex	Dotted, spherical	white	Spherical
NR-5	Flat	Smooth	Creamy white	Spherical
NR-6	Convex	Smooth	white	Irregular

Table 3c. Microscopic Examination of isolated strains

Isolate	**Gram reaction**	**Shape**	**Arrangements**
NR-1	-	Small, Rod	Scattered
NR-2	-	Small, Rod	Scattered
NR-3	-	Small, Rod	Scattered
NR-4	+	Large, Rod	Diplobacilli, scattered
NR-5	-	Small, Rod	Scattered
NR-6	+	Large, Rod	Diplobacilli, scattered

All the isolates were screened for the production of hydrogen cyanide (Fig 4.). However, production of HCN was observed only in case of NR-1, NR-3, NR-4 and NR-5. The NR-2 and NR-6 strains were negative for HCN production (Table 2). Similarly, some co-workers isolated 98 bacteria from the rhizosphere of wheat and potato on TSA. Four could produce hydrogen cyanide and grew well on the Pseudomonas sp. selective King's B+ medium. Cyanide-producing microorganisms were abundant in the rhizosphere of both potato and wheat and in both short and long potato rotation soil. The main reason of HCN production by the species was its antagonistic property [20].

2.2.3. Assay for the production of NH_3

Among all the strains NR-1, NR-2, NR-3 and NR-5 were found positive for (Fig 6.) whereas the strain NR-4 and NR-6 were negative for ammonia production (Table 2). Another important trait of plant growth promoting regulator that may indirectly influence the plant growth, is the production of ammonia. Mostly all the isolates were able to produce ammonia. However, ammonia production was observed less frequently in Azotobacter isolates [21].

2.2.4. Cell wall degrading enzyme production

Production of fungal cell wall degrading enzymes was analysed because this is an important mechanism of fungal inhibition. Isolate NR-1, NR-2, NR-4 and NR-6 were found to produce cellulase, a fungal cell wall degrading enzyme [1]. Protease was also detected in NR-1, NR-2, NR-4 and NR-6 and produce halo zones on skim milk agar that showed protease activity (Table 2) and (Fig 5). Similar, results have been reported by some researcher while screened bacteria from

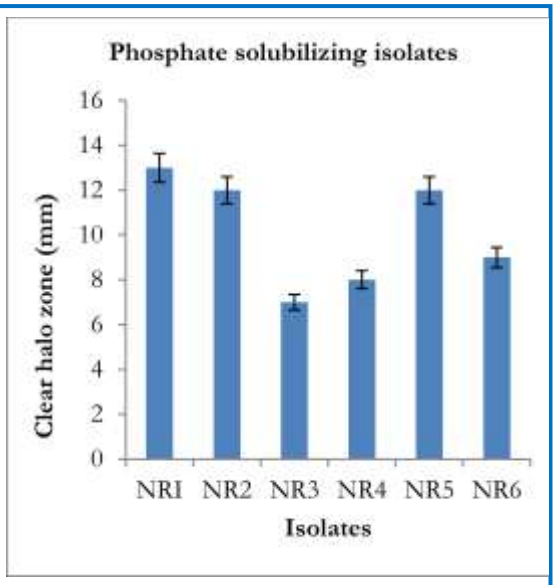

Fig 1. Phosphate solubilizing activity of different isolates

rhizospheric soil were positive for cellulose and protease activity [22].

2.3. Cultural and Morphological characterization

In the present study, six different PSB strains were isolated from the rhizospheric soil. These isolates were further characterized in term of cultural and morphological characteristics and were then identified on the basis of Bergey's manual of systemic Bacteriology [23]. Cultural characteristics were observed on the Nutrient agar and Pikovskaya. Similar, result has been reported by the Lopez-Cortes et al. while characterizing the isolates while characterizing phosphate solublizers [24].

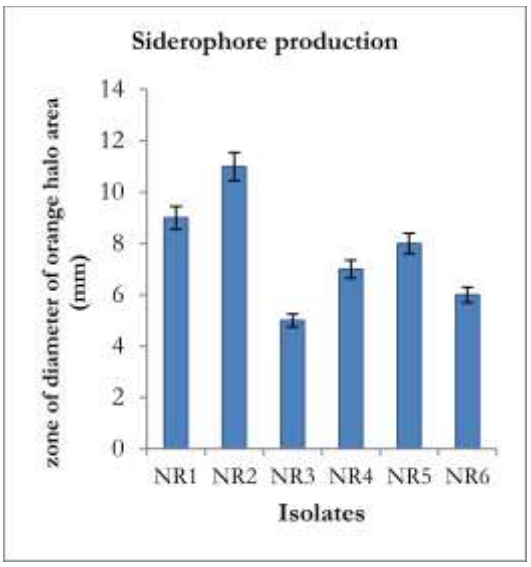

Fig 2. Phosphate solubilizing activity of different isolates

To study the cell shape, gram reaction and arrangements gram staining performed with all the isolates. Under oil emersion objectives the isolates NR-1, NR-2, NR-3 and NR-5 were observed to be gram negative, rod while isolates NR-4 and NR-6 observed to be gram positive, rod.

Identification of the isolates was done by the biochemical test based on the criteria laid down in the Bergey's manual of systematic bacteriology [23]. For the Indole test NR-5 was positive and NR-1, NR- 2, NR- 3, NR-4 and NR-6 were negative. For the MR test NR-5 and NR-6 were positive and NR-1, NR- 2, NR- 3 and NR-4 were negative. For Catalase test NR-1 and NR-4 were negative and NR-2, NR-3, NR-5 and NR-6 was positive. For Citrate utilisation test NR-1, NR-4 and NR- 6 were negative and NR-2, NR-3 and NR- 5 were positive. For Urease test NR-2, NR-4, NR-5 and NR-6 were negative and NR-1 and NR-3 were positive. For gelatin liquefaction NR-1 and NR-3 were positive and NR-2, NR-4, NR-5, NR- 6 were negative. For oxidase test all isolates were positive. For sugar fermentation NR-1, NR-2 and NR-5 were positive and NR-3, NR-4 and NR- 6 were negative. For Triple sugar iron test all isolates were A/A or A/A (Alkaline/Acid or Acid/Alkaline). For Nitrate reduction test all isolates were positive. All the isolates were identified on the basis of biochemical test [24].

2.4. Partial identification of the phosphate solubilizing bacteria

Fig 3. Siderophore production

Fig 4. HCN production

Fig 5. Protease production

Fig 6. NH_3 production

On the basis of cultural, morphological and biochemical characterization and the above characters was compared with Bergy's manual of systematic Bacteriology the isolates NR-1, NR-2, NR-3 and NR-5 were found to be small rod, gram negative, oxidase positive, citrate positive, nitrate positive, and catalase positive and tentatively assign in the genera of Pseudomonas sp. While the isolates NR-4 and NR-6 were found to be large rod, gram positive, oxidase positive and gelatin liquefaction positive and tentatively assign in the genera of Bacillus sp. [25].

3. CONCLUSION

Phosphorous is very abundant in various soil and major nutrient limiting factor for plant growth. The overall phosphorous used as phosphate fertilizer because formation of insoluble complex. Total 15 rhizospheric soil samples were collected. A total 6 phosphate solubilizere were screened and selected for further investigation. In the present study 6 phosphate solubilizing bacteria were screened from various agricultural fields and designated NR1-NR6. NR-1, NR-2 and NR-5 were found to be strong phosphate Solubilizer with halo zone diameter 13, 12 and 12 mm respectively on Pikovskaya agar. All the isolate were further screened for the other plant growth promoting factor such as siderophore, HCN, ammonia and cell wall degradation enzyme production. Isolate were found to be positive for siderophore production and their production frequency ranged from 5-11mm. NR-1, NR-3, NR-4 and NR-5 were positive for HCN production. NR-1, NR-2, NR-3 and NR-5 produced ammonia. While isolate NR-1, NR-2, NR-4 and NR-6 produced cellulase and protease which are cell wall degrading enzyme. All the isolates were also characterized in term of morphological and biochemical test and after comparison with Bergy's manual of systematic Bacteriology the isolates NR-1, NR-2, NR-3 and

NR-5 tentatively assigned as Pseudomonas spp. while NR-4 and NR-6 assigned as Bacillus spp. respectively. From the above study we can conclude that Bacteria isolate from rhizospheric soil are able to produce growth regulating substances and some of them capable of dissolving phosphate. The frequent application of phosphorous is very necessary for crop production. Use of phosphate solubilization bacteria as bio-inoculants and increase the available phosphorous in soil and help to minimize the phosphate fertilizer application in soil. Phosphate solubilizing bacteria also reduce the environmental pollution as well as promote the sustainable agriculture. So exploitation of phosphate solubilizing bacteria through bio fertilization has enormous potential for making use of ever increasing fixed phosphorous in the soil, and natural reserves of phosphate rocks.

4. ACKNOWLEDGEMENT

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5. CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

6. SOURCE/S OF FUNDING

No source of funding

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