



Original Research Article

Isolation Identification and Characterization of Phosphate Solubilising Bacteria from Soil and the Production of Biofertilizer

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ABSTRACT

Keywords

Phosphate solubilising bacteria, Biofertilizer, Rhizoid soil, *Pseudomonas*, *Bacillus*

The main aim of this research paper is the isolation of Phosphate solubilising bacteria from rhizosphere soil and hence the production of biofertilizer from it. Most soils are deficient in soluble forms of phosphorous and it is also a fact that the Phosphorous is a very important macro nutrient for the growth of plants. Apart from nitrogen phosphorous plays a very important role in the plant metabolism such as cell division, growth and development, breakdown of sugar, and nuclear transport within the plants. Phosphate solubilising bacteria's can hence be utilized for the production of biofertilizers which actually enhances the nutrient quality of soil.

Introduction

Biofertilizers may be defined as the preparations containing the living cells of different strains of microorganisms that help in enhancing the nutrient uptake by the plants and hence enriches the nutrient quality of the soil by their interaction in the rhizosphere when these biofertilizers applied either on the top soil or through seed inoculations (Isolation of azotobacter and cost effective production of biofertilizer by Gomare *et al.*, 2013). Biofertilizers may also be defined as microbial inoculants which are artificially multiplied cultures of certain soil microorganisms that can improve soil fertility and crop productivity.

Biofertilizers are low cost renewable sources of plant nutrients which supplement chemical fertilizers. Biofertilizers generate plant nutrients like nitrogen and phosphorous through their activities in the soil or rhizosphere and makes them available to the plants on the soil. Biofertilizers are gaining an importance in use because of the proper maintenance of soil health, minimize environmental pollutions and cut down the use of chemicals. The term biofertilizer is still unclear. Therefore biofertilizers may be defined as substances which contain living microorganisms which colonizes the rhizosphere or the interior of the plants and promotes growth by increasing the supply or

availability of primary nutrients to the target crops when they are applied to soils seeds or plant surfaces. Biofertilizers have an ability to mobilize nutritionally important elements from non usable to usable form. These microorganisms require organic matter for their growth and activity in soil and provide valuable nutrients to the plant. Biofertilizers are ready to use live formulates of beneficial microorganisms which on application to seed, root or soil mobilize the availability of nutrients by their biological activity in particular and help in building up the micro flora and in turn the soil health in general (Rajendra *et al.*, 1998).

Phosphorous is such an important macronutrient which is very often present in the soil in unavailable form. Many soil bacteria particularly those belonging to the genera *Bacillus* and *Pseudomonas* possess the ability to bring insoluble phosphates in the soluble forms by secreting organic acids. These acids lower the pH and bring about the dissolution of bound forms of phosphorous. These bacteria are commonly known as *phosphobacteria*. They can be applied either through seed or soil application. Phosphorus, both native in soil and applied to inorganic fertilizers becomes mostly unavailable to crops because of its low level of mobility and solubility and its tendency to become fixed in soil. The phosphate solubilizing bacteria are life forms that can soil. The phosphate solubilizing bacteria are life forms that can help in improving phosphate uptake of plantain on different ways (Rajasekaran *et al.*, 2012). Phosphorous is essential for growth and productivity of plants. It plays an important role in plants in many physiological activities such as cell division, photosynthesis, and development of good root system and utilization of carbohydrate. Phosphorous deficiency results in the leaves turning brown accompanied by small leaves, weak stem and slow development. In ancient

times the use of animal manures to provide phosphorous for plant growth was common agricultural practice. Organically bound phosphorous enters in soil during the decay of natural vegetation, dead animals and from animal excretions. At that time role of micro flora on soil fertility was hardly understood (Kannaiyan *et al.*, 2004).

Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate. Among the bacterial genera with this capacity are *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Aspergillus*, *Flavobacterium* and *Erwinia*. There are considerable populations of phosphate solubilizing bacteria in soil and in plant rhizospheres. These include both aerobic and anaerobic strains, with a prevalence of aerobic strains in submerged soils. A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere in comparison with non rhizosphere soil. The soil bacteria belonging to the genera *Pseudomonas* and *Bacillus* and fungi are more common (Role of Bio-Fertilizer in Organic Agriculture: A Review by Mishra *et al.*, 2012).

Thus in this research paper the investigation has been carried in the isolation of phosphate solubilising bacteria from rhizosphere soil obtained from hilly regions and then grown in selective media and finally the production of biofertilizer by PSB bacteria in it.

Materials and Methods

Collection of soil sample

Soil of about 1 kg was collected from hilly region from Terai district and was put inside

a plastic bag and brought to the laboratory for isolation of bacteria from it.

Serial dilution of soil samples

About 1 gm of composite soil was taken from the plastic bag and then the soil was diluted to 10 ml of water in a test-tube which served as stock solution. Remaining 9 test tubes were filled with 9 ml of water. Transferring of 1 ml of water from the stock solution to 9 ml of sterilized distilled water with the help of pipettes yielded 10^{-1} dilutions and the series continued up to 10^{-9} dilutions. Sterility is the hallmark of any bacteriological isolation so the entire process was carried in the laminar airflow.

Bacterial colony identification and external morphology study

Using the spread plate technique the bacterial colony identification and external morphology was studied for which nutrient agar media was prepared. Therefore 100 ml of Nutrient agar Media was prepared for four Petri plates. The NA media was autoclaved and then poured in four Petri dishes which were also sterilized by autoclave. Then the serial dilutions of 10^{-2} , 10^{-4} , 10^{-6} , 10^{-8} were chosen and from that 0.1 ml of culture was transferred from each serially diluted test tubes and spread on the Petri plates by means of the spreader. Then the Petri dishes were kept in the incubator for 37° Celsius for 24 hrs for the incubation and growth of bacteria.

After 24 hrs of incubation the Petri dishes were taken out of the incubators and the following bacterial external morphology were studied.

Pure culture isolation of bacteria

Well developed and separated colonies

which were identified on the nutrient agar plates were marked and then these separated colonies were chosen and by the help of inoculating needle the colonies were transferred and streaked separately on six test tubes having nutrient agar slants for the growth of the single colonies of bacterial cultures from the mixed culture of bacteria that were grown in the Petri plates. The test tubes were marked after the strains of chosen colonies from Petri plates and were left in the incubator at 37° Celsius overnight for growth and incubation.

After incubation of the pure cultures overnight different single species of bacterial culture slants developed in the test tubes which were further picked and purified.

Gram staining of the bacterial species strains from the pure culture slants

The pure cultures of different colonies that were obtained in test tubes were put for gram staining for more specific identification of the colonies. The gram staining was done in laminar airflow hood. For this purpose the six slides were taken from slide rack. The slides were washed with ethanol. Then each colony was marked on the slides. Then with the help of inoculating needle the loopful strains were picked from each test tube and made a smear on the slides and heat fixed. The slides were then taken in the staining room for staining the smears. Then smears were stained in following steps a) First applied crystal violet on each six slides. Kept for 30 secs. b) Distilled water wash. c) Iodine on the slides as mordant (1 min) then 95% alcohol wash and then washed with distilled water. d) Safranin was applied on the slides and then washed with distilled water and f) The slides air dried. All the gram staining technique was done following the Christian Gram

technique (Collee *et al.*, 1996).

Screening of bacterial strains

After gram staining of the bacterial strains from the pure culture slants and microscopic studies the bacterial strains which were identified to be phosphate solubilising bacteria like *Bacillus* spp strain and *Pseudomonas* species strain by studying the morphological structures were further confirmed by their ability to be grown on Pikovaskya agar media which is the most important test for the phosphate solubilising bacteria for both *Bacillus* spp and *Pseudomonas* spp.

The bacterial colonies were picked from the pure culture slants by the help of the inoculating needle and were streaked in the PKV agar media plates and were incubated at 37° Celsius overnight. In the next day the bacterial colonies showed a clear Halozone formation which confirmed them to be PSB bacterial species. For further studies these colonies were again grown in nutrient agar media and several Biochemical tests were performed.

Preparation of production media of phosphate solubilising bacteria as starter cultures (both *Bacillus* spp and *Pseudomonas* spp strains)

After the screening of the PSB bacterial strains like *Bacillus* spp and *Pseudomonas* spp from the pure culture slants the bacterial strains were transferred to the liquid broth which was also the production media and as well as the starter culture for the growth of cells. Production media is that media in which the number of viable bacterial cells of that particular bacteria increases because that bacteria is grown in that particular media only. Thus in phosphate solubilising bacteria both the *Bacillus* and *Pseudomonas* spp. strains were grown in Pikovaskyas

production media (Protocol followed for growth of PSB).

Thus a 100ml of two separate conical flasks were taken and PVK media was prepared after ph adjustments and autoclaved. Then inside the laminar airflow the pure cultures marked in the pure culture slants were transferred to the PVK production media conical flasks by the help of sterilized inoculating loop. Then the conical flasks were put in the rotary B.O.D shaker for 1 week or 7 days. The viable cell count in the production media or the liquid broth was found to come up to 10^9 Cfu/ml. Then for the mass production of PSB biofertilizer the inoculums from these starter cultures were transferred to larger flasks.

Mass production of PSB biofertilizer and preparation of inoculum

The Phosphate solubilising bacterial strains in the starter cultures were needed to be grown in large scale for which their mass production were required. So larger conical flasks of 1000 ml were taken and then again starter cultures were transferred to these larger conical flasks containing the appropriate growth media in aseptic conditions for small scale production and for large scale production again 1 litre of the starter cultures were put into the fermenter. Finally continuous agitation and proper aeration was done for about 1 week. The flasks were checked for time to time for the growth of the cell mass and that they were free of any contamination. After 1 week the cell population increased up to 10^9 cells/ml or 10^9 cfu/ml load in the larger conical flasks. Then the conical flasks were stored in cool temperatures so that they can be mixed with proper carrier materials. Moreover it is not advisable to keep the conical flasks for long time in storage because of the loss of cell load.

Carrier material preparation

The carrier should have the following characteristics a) It should have high organism matter content b) Low soluble salts less than 1% c) High moisture content capacity.

In this experiment for the inoculation to be made charcoal, cow compost and vermicompost was used as carrier material. There are many steps for preparation of the carrier material. The steps are discussed below-

First about 1 kg of dried cow dung and black coal was brought from different areas. Then by the help of mortar and pestle the entire coal was crushed to dried powdered form. After crushing also the remaining pieces were further powdered by the help of mixer and grinder. The dust form of coal as charcoal was made and to it 1% calcium carbonate and wooden charcoal or activated charcoal was mixed and neutralized so that no contaminants are present

A) Similarly the cow dung was also crushed and powdered with the help of mixer and grinder.

B) Some amount of vermicompost was also added as a carrier material.

Preparation of inoculum with carrier material (Mixing)

The mass produced bacterial cell cultures of both *Bacillus* spp and *Pseudomonas* spp were taken out of storage and then the cell cultures were mixed with the sterilized carrier materials in individual beakers. The mixing of the carrier materials and the production media were in the ratio 2:1 where 1 part of production media was mixed with 2 parts of carrier material or in other words 30:60 ratio of both. It was done manually and under aseptic conditions. The

cell count of that carrier mixed culture was found to be 10^8 CFU/gm. The biofertilizers were packed in polythene bags which are advised to be of 250 gm. Then the packets were left in room temperature for curing.

Storage of biofertilizers

The polythene packets containing biofertilizers were stored in cool place away from direct sunlight. The biofertilizers were then sent to the hilly regions for application on the proper fields of biofertilizer plots.

Media and Reagents

The nutrient agar media for isolation and slant preparation was made. It includes peptone-5gm, beef extract-3gm, NaCl-5gm, agar-18gm and distilled water-1000ml. B) Pikovaskya agar media was prepared for *Bacillus* and *Pseudomonas* spp Halozone test for PSB bacteria only. In its composition it has glucose-10gm/ml, yeast extract- 0.5gm/ml, ammonium sulphate-0.5gm/ml, magnesium sulphate-0.1gm/ml, calcium phosphate- 5gm/ml, sodium chloride- 0.2gm/ml, potassium chloride-0.2gm/ml, manganese sulphate-0.002gm/ml, ferrous sulphate- 0.002gm/ml, agar- 1.8gm/ml and distilled water- 1000ml. C) Pikovaskya broth was prepared for production media and for mass production of PSB bacteria. In its composition it has glucose-10gm/ml, yeast extract- 0.5gm/ml, ammonium sulphate- 0.5gm/ml, magnesium sulphate-0.1gm/ml, calcium phosphate-5gm/ml, sodium chloride- 0.2gm/ml, potassium chloride- 0.2gm/ml, manganese sulphate- 0.002gm/ml, ferrous sulphate-0.002gm/ml and distilled water-1000ml.

The biochemical tests included methyl red test, Vogaeus Proskaur test, indole test, citrate test, catalase test, starch hydrolysis test and nitrate reduction test. In the gram

staining techniques crystal violet, Gram's iodine and safranin were used. Other reagents included Kovac's reagent, alpha naphthol, hydrogen sulphide and Gram's iodine.

Results and Discussion

In this research paper figure 1 shows the pure culture strain of the phosphate solubilising bacteria in nutrient agar slants. Figure 2 shows the gram staining of the PSB bacterial strains according to which the morphological identifications were made. Figure 3 shows the special experimental result of the halozone formation of PSB bacterial strains which were grown in Pikovaskyas agar media. Figure 4 shows the different production media which are actually the starter cultures which are further used in mass production of biofertilizers. In table 1 it shows the appearance of bacterial

strains in nutrient agar media plates. Table 2 shows the gram staining of the bacterial strains from the pure culture slants where the bacterial strains were identified and their morphology studied. In table 3 different biochemical tests were done for the PSB bacterial strains both for *Bacillus* spp and *Pseudomonas* spp and they showed either positive results or negative results.

These phosphate solubilising bacteria are very important in solubilization of insoluble phosphate to soluble phosphahate by release of organic acids. Thus the biofertilizers which are mentioned in this research paper after production through the selective and optimized media and mass production and then packed to send the hilly regions for their applications in various tea fileds. These bioinnoculants are ready to increase the nutrient supply of tea plants.

Table.1 Identification of bacterial strains on basis of external colony morphology an colour as appeared in nutrient agar plates

Plate	Colony/Strain	Form	Elevation	Margin	Colour	Transparency	Size
10 ⁻²	6	punctiform	raised	undulate	brownish	opaque	small
10 ⁻⁶	4	circular	flat	entire	greenish	translucent	moderate
	5	circular	punctiform	entire	offwhite	opaque	medium
10 ⁻⁸	1	circular	raised	entire	greenish tinch	opaque	entire
	2	rhizoid	raised	undulate	offwhite	opaque	medium
	3	punctiform	flat	curly	clear white	opaque	small

Table.2 Staining of bacterial strains for morphological identification from pure culture slants

Sl. no	Observation	+/-	Bacteria
Strain1	Rod shaped pink colour	Gram negative	<i>Pseudomonas spp</i>
Strain 2	Purple coloured chain structure	Gram positive	<i>Bacillus spp</i>
Strain 3	Rod shaped pink colour	Gram negative	<i>Pseudomonas spp</i>
Strain 4	Rod shaped pink colour	Gram negative	<i>Pseudomonas spp</i>
Strain 5	Rod shaped, Purple coloured,long chain forming,dispersed	Gram positive	<i>Bacillus spp</i>
Strain 6	Rod shaped pink colour	Gram negative	<i>Pseudomonas spp</i>

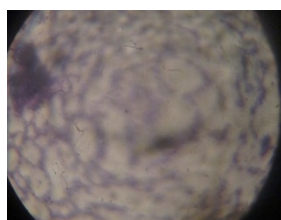
Table.3 Biochemical test of bacterial strains

Sl.no	Tests	<i>Bacillus</i>	<i>Pseudomonas</i>
1	Methyl red test	Positive	Positive
2	VP test	Negative	Negative
3	Starch Hydrolysis	Positive	Positive
4	Catalase test	Positive	Positive
5	Caesin test	Positive	Positive
6	Hydrogen sulphide	Negative	Negative
7	Indole	Negative	Negative

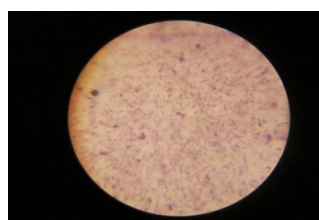
Fig.1 Pure culture slants of phosphate solubilising bacteria



Fig.2 Gram staining identification of both *Bacillus* spp and *Pseudomonas* spp strains



Bacillus spp



Pseudomonas spp

Fig.3 Halozone formation of PSB bacteria



Fig.4 Different type of production media of bacterial strains



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