Research Article

Invitro Characterization of Plant Growth Promoting Phosphate Solubilizing Bacteria (PSB)

Madhavi Vedulla*, K. Laxmi Teja, A. Abhigna, Mohammad Ali, MD. Arshaduddin

Department of Microbiology Navaratnaa Crop Science PVT. LTD, IDA Cherlapally Medchal, Malkajgiri, Secunderabad, Telangana 500051, India

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Corresponding author:
Dr. Madhavi Vedulla

E-mail:
madhaviabf@gmail.com
yemurimadhavi@gmail.com

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) are the rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms like phosphate solubilization, siderophore production, biological nitrogen, IAA production, ammonia production, and HCN production. The potentiality of PGPR in agriculture is steadily increased as it offers an attractive way to replace the use of chemical fertilizers, pesticides, and other supplements. The strain Pseudomonas spp. was found to be efficient in terms of inducing growth, and the biochemical and physiological status of the seedling. The present study reports the potential of rhizobacteria in promoting rice growth. These microbes provide an excellent model for understanding the plant microbe interaction that can be subsequently engineered into crop plants to cope with climate induced stresses. Biochemical tests were carried out as per Bergey"s manual of systematic bacteriology. The morphological test was performed by visualizing the Gram-stained cells under a phase contrast microscope. In this study, we are going identify the Pseudomonas spp. and Streptomyces bacteria spp. On the base of molecular work using 16s rRNA and doing some activity like PGPR, and antifungal activity by doing this we can get the bacteria that are beneficial to plants and can go further for biofertilizers production.

Keywords: *Pseudomonas spp., rhizobacteria, 16s rRNA and Bio-fertilizers.*

1. INTRODUCTION

Over the last few decades, the agriculture policy in India has undergone a major change through diversification and emphasis on a sustainable production system. Rhizosphere researchers have been throwing up surprise and interesting ideas for research ever since the pleasant environment of microorganisms around plant roots is called the rhizosphere. The term rhizosphere was introduced for the first time for the first time by Hiltner [1]. The major influences that the rhizosphere microorganisms have on plants today become important tools to guard the health of plants in an eco-friendly manner. These microorganisms can effect effect plant growth often referred to as a plant growth promoter rhizobacteria. They are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turn;over and sustainable for crop production. In recent years considerable attention has been paid to PGPR to replace agrochemicals(fertilizers and pesticides) for the plant growth promotion by a variety of mechanisms that involve soil structure formation, decomposition of organic matter, recycling of essential elements, solubilization of mineral nutrients, producing numerous plant growth regulators, degrading organic pollutants, stimulation of root growth, crucial for soil fertility, biocontrol of soil and seed borne plant pathogens and in promoting changes in vegetation. An understanding of plant growth growth-promoting rhizobacteria and their interactions with biotic and abiotic factors is indispensable in bioremediation techniques. Energy generation process and in biotechnological industries such as pharmaceuticals, food, chemical, and mining. Furthermore, plant growth promoting rhizobacteria can reduce chemical fertilizers application economically, and environmentally beneficial for lower production cost as well as recognize the best soil and crop management practices to achieve more sustainable agriculture as well as fertility of soil.

Use of phosphate-solubilizing microorganisms is a potential area as these microorganisms contribute in to the soil p-cycle [2]. Microorganisms can enhance soil fertility through decomposition, mineralization, microbial storage, and release of nutrients in the soil. They may be considered inexpensive options for increasing P availability to plants [3]. Naturally occurring phosphate-solubilizing microbes have been reported since 1903. Phosphate-solubilizing microbes have been reported to have a direct relation with plant growth which provides a reason to select the most promising microbes for better crop growth and development[4]. soil microorganisms release low molecular weight organic acids that weather rock phosphate and tricalcium phosphate, and increase P availability for plant uptake [5][6].

PGPR (PLANT GROWTH PROMOTING RHIZOBACTERIA)

Chandler [7] concluded that different bacterial genera are vital components of soils. They are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turnover and sustainable for crop production.They stimulate

plant growth through mobilizing nutrients in soils, producing numerous plant growth regulators, protecting plants from phytopathogens by controlling or inhibiting them, improving soil structure, and bioremediating the polluted soils by sequestering toxic heavy metal species and degrading xenobiotic compounds (like pesticides).

Burdman [8] investigated that plant growth-promoting rhizobacteria (PGPR) can greatly facilitate plant growth of many cereals and other important agriculture crops, different types of (PGPR) in soil suppress many plant pathogens and promote plant growth by different methods, such as direct and indirect production of different phytohormones, mineralization, and decomposition of organic matter and improve the bioavailability of different mineral nutrients like iron and phosphorus.

A plant growing under field conditions is not an individual; it is a complex community [9] with subtle and relatively constant partner relationships. A well-structured and regulated community of microorganisms is always associated with the plant [10] [11] [12]. This community is the Phyto microbiome [13] the Phyto microbiome plus the plant is the halobiont [13] [14]. Microbiome relationships exist with all multi-cellular organisms, and probably all eukaryotes. In fact, these probably predate the colonization of the land by plants [15]. This microbial community has been associated with terrestrial plants since their earliest evolution, to assist early land plants faced with challenges such as access to nutrients, novel and often-stressful conditions and pathogens [16].

RHIZOSPHERE

Walker [17] studied that the narrow zone of soil surrounding the root system is referred to as the rhizosphere. Rhizobacteria means a group of rhizosphere bacteria competent in colonizing the root system.

Hiltner [1] postulated that the rhizosphere as the area around a plant root that is inhabited by a unique population of microorganisms by the chemicals released from plant roots.

Schortemeyer [18] investigated that the ability of the rhizosphere to stimulate microbial activity has been long known. The rhizosphere contains significant amounts of plant-released carbonaceous substances, which in turn increase and diversify the microbial population around the root zone.

PHOSPHATE SOLUBILIZING BACTERIA

Eftekhari [19] studied that Phosphate solubilizing Rhizobacteria plays a vital role in accumulation and transformation of phosphate to plant roots. Mostly phosphorus is absorbed during vegetative growth and this absorbed form of phosphorus is re-translocated in seeds and fruits.

Liu [20] Analysed the phosphorus solubilizing microbes especially PSB are widely distributed in soils, freshwater, seawater, and sediments, and responsible for the cycling of insoluble P to soluble PO43– ion. Numerous types of research have concentrated on the screening of highly efficient PSB, and most PSB are Gram negative bacteria and belong to Pseudomonas, Acinetobacter, Pantoea, Enterobacter, and some PSB are Gram-positive bacteria belonging to Bacillus PSB have shown good performance for plant growth promotion.

2. MATERIALS AND METHODS

2.1 Collected soil sample

The Sample was collected from the rhizosphere soil of paddy at Acharya N.G Ranga University located at Rajendra Nagar in Hyderabad.

Media

Pikovskya's Agar

1. Pikovskya's agar - 3.13 gm
2. Sterile water - 100 ml

Peptone broth

Nutrient broth

Table 1: Components of PCR

Material	2X
Sterile water	20.8 µl
10X buffer	4 µl
2.5 mN dNTPs	4 µl
F - primer	2 µl
R - primer	2 µl
Taq polymerase	1.2 µl
Template	6 µl
Total Volume	**30 µl**

2.2 Research sample

Soil samples were collected from the paddy plants', rhizosphere. This sample was collected in the month of May from Hyderabad.

2.3 Isolation of Phosphate Solubilizing Bacteria spp.

The Phosphate solubilizing bacteria spp. was isolated from the rhizosphere soil, firstly by tenfold serial dilution of which 10-1 to 10-8 were spread over on Phosphate solubilizing specific media like Pikovskya's agar and incubated for 48 h at 30ºC. Bacterial colonies were repeated streaking on the same for obtaining the pure bacterial colonies. Identification of the isolates was made by a morphological test. The maximum number of colonies of phosphate solubilizing bacteria spp. are seen on 10-4, 10-5 and 10-6 plates.

2.4 Principle of Tertiary Streaking

Streaking is a technique used to isolate a pure strain from a single

Species of microorganisms, often bacteria. Samples can then be taken from the resulting colonies and a microbiological culture can be grown on a new plate so that the organism can be identified, studied, or tested.

Streaking is rapid and ideally a simple process of isolation dilution. The technique is done by diluting a comparatively large concentration of bacteria to a smaller concentration. The decrease in bacteria should show that colonies are sufficiently spread apart to affect the separation of the different types of microbes. Streaking is done using a sterile tool, such as a cotton swab or commonly an inoculation loop. Aseptic techniques are used to maintain microbiological cultures and to prevent contamination of the growth medium. There are many different types of methods used to streak a plate. Picking a technique is a matter of individual preference and can also depend on how large the number of microbes the sample contains.

The three-phase streaking pattern is known as the T-Streak. The streaking is done using a sterile tool, such as a cotton swab or commonly an inoculation loop. The inoculation loop is first sterilized by passing it through a flame. When the loop is cool, it is dipped into an inoculum such as a broth or patient specimen containing many species of bacteria. The inoculation loop is

then dragged across the surface of the agar back and forth in a zigzag motion until approximately 30% of the plate has been covered. The loop then is re-sterilized and the plate is turned 90 degrees. Starting in the previously streaked section, the loop is dragged through it two to three times continuing the zigzag pattern. The procedure is then repeated once more being cautious to not touch the previously streaked sectors. Each time the loop gathers fewer and fewer bacteria until it gathers just single bacterial cells that can grow into a colony. The plate should show the heaviest growth in the first section. The second section will have less growth and a few isolated colonies, while the final section will have the least amount of growth and many isolated colonies.

2.5 Characterization of isolates

Isolates were characterized by the Gram staining method and morphological methods like (shape, color, margin, nature of colony, and texture) test.

2.6 PGPR Characteristics

• *Ammonia Production*

Ammonia is produced in large plants (1,000 to 1,500 t/day) by means of Haber-Bosch process [21]. Globally, more than 90% of ammonia is produced from fossil fuels through this method [22] [23] Test bacterial culture was inoculated in the 1% peptone water after 24hrs incubation at 30˚C, 0.5 ml of Nessler's reagent was added appearance of a yellowish-orange color indicates the production of ammonia.

• *Phosphate Solubilisation Activity*

All isolates were screened on Pikovskya's agar plates as described by Gaur [24] bacterial suspension of 10-8 gm/ml was spot inoculated plates were incubated for 3 days at 30°C, formation of halo zone around the bacterial spot indicates the phosphate solubilization activity.

The phosphate solubilization Index was measured by: - PSI = A/B*100

Where, A = colony + halo zone length (diameter) B = colony length (diameter)

Indole-3-Acetic Acid (IAA) production

PGPR (Plant Growth-Promoting Rhizobacteria) can produce Indole acetic acid (IAA) from the metabolism of L-tryptophan. IAA is one of the most physiologically active auxins. PGPR can influence plant growth by producing plant growth regulators like auxin, gibberellin, and ethylene [25]. IAA helps in the formation longer root with branched root hairs which are involved in nutrient uptake [26]. Bacteria synthesize auxins in order to control host physiological processes for their own benefit [27]. Bacteria have more than one pathway for the formation of IAA from tryptophan [28].

1. Test bacterial culture was inoculated in nutrient broth containing L- Tryptophane (5ug/ml) (could be also increased in the concentration of colony -100 ug/ml)
2. Incubated at 25+3°c for 5 days (45 hrs also used)
3. Centrifuged at 5000 rpm for 5 min
4. 2ml of supernatant pellet could be used for protein assay and was mixed with two drops of orthophosphoric acid and 4 ml of Salkowski's reagent
5. Appearance of the red-pink color indicates the IAA production.
6. OD value at 60c nm (bacterial).

2.7 Isolation of genomic DNA from gram-negative bacteria

1. 15 ml overnight bacterial culture was (grown in LB medium) transferred to a 1.5 ml sterile Eppendorf tube and centrifuged at 5,000 rpm for 5 min to pellet the cells and discarded the supernatant
2. 1 ml of TE buffer (pH 8.0) was transferred and resuspended from the pellet by gentle mixing and centrifuged at 5,000rpm for 5 min to pellet the cells and discarded the supernatant and repeated those steps once again.
3. Resuspended the cell pellet in 200µl of lysis buffer and vortex to completely resuspend the pellet
4. Added 66µl of 5M NaCl solution (to remove the proteins and cell debris) and mixed well and centrifuged at 10,000 rpm for 10 min at 4'C
5. Carefully transferred the supernatant to a new tube and added an equal volume of chloroform and mixed well by inverting the tube until the phases are completely mixed which will form a milky solution.
6. The sample was incubated at 37°C for 5-10 minutes.
7. Spin at max speed (15,000 rpm) for 15 min at 4C, there is a white layer (protein layer) at the aqueous phenol: chloroform interface.
8. Carefully transfer the upper aqueous phase to a new tube using a 1 ml pipette.
9. To precipitate the DNA, added an equal volume of 100% ethanol and centrifuged at 5000 pm for 5 min.
10. Discarded the supernatant and rinsed the DNA pellet with 1 ml of 70% ethanol
11. Spun at 10,000 rpm for 5 min, carefully discarded the supernatant, and kept for air dry the DNA pellet.
12. Resuspended DNA in 25ul of sterile distilled water.

2.8 Agarose gel Electrophoresis.

1. Cleaned the gel casting tray and wrapped it with tape
2. Prepared 0.8 % gel and added 1.25µl of ethidium bromide in it and carefully poured it into the casting tray
3. Removed the tape attached to the casting tray and placed gel in the running tank filled with TAE buffer.
4. Removed the comb carefully without shearing the walls of the wells formed.
5. DNA samples were mixed with 6X loading dye on a strip of Parafilm and loaded into the wells.

6. Ladder DNA was loaded in the last well

7. Connected the electrodes to the power pack and the sample was run at 90 V for 30 minutes

8. After electrophoresis DNA was visualized using a UV trans illuminator

3. RESULTS

3.1 Bacteria isolated from the rhizosphere of the Paddy plant

1 gm of soil sample collected from rhizosphere soil of paddy was taken and added in 9 ml of saline blanks and did the serial dilution and then from dilution tubes of 10- 5, 10-6, 10-7 50µl was dropped on the pikovaskay's agar media and spread entirely on the plate with the help of glass spreader, the selected media was specific media for phosphate solubilizing bacteria, then grown bacterial colonies of PSB spp. from that colonies having a clear halo zone was selected which indicates good phosphate solubilizing zone and did tertiary streaking.

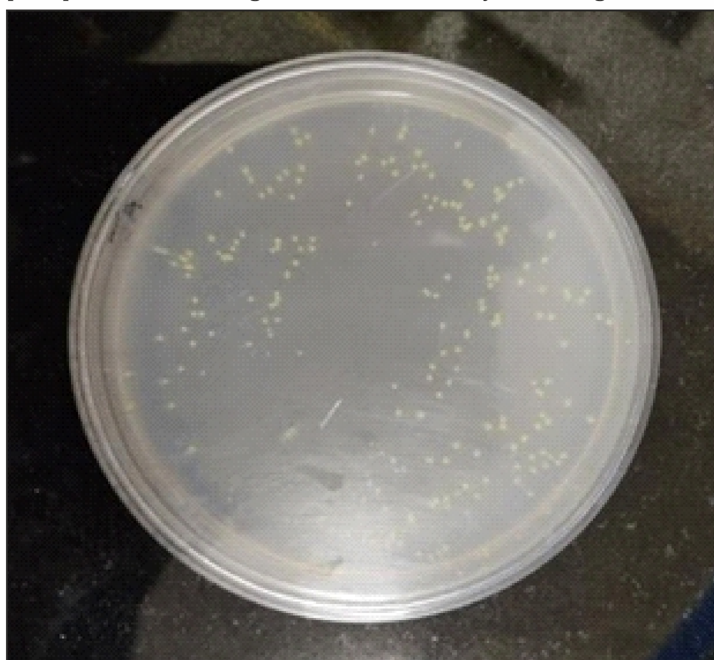


Fig 1: Phosphate solubilizing bacteria colonies grown on Pikovskya's agar media

3.2 Identification of Bacteria on the Bases of gram staining

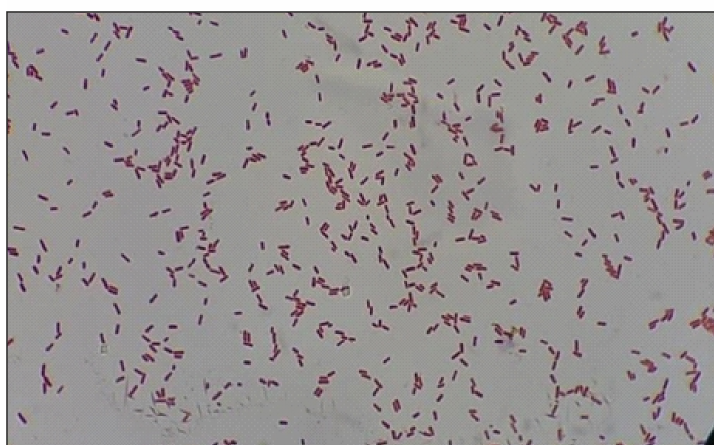


Fig 2: Gram staining of Phosphate Solubilizing bacteria showing that the bacteria are gram-negative.

Above fig show as the bacteria strain is in pink color which indicates that the isolated bacteria are gram-negative bacteria also looking at shapes i.e., rod shape hence looking at those images we can say that the isolated bacteria is Phosphate Solubilizing bacteria

Table 2: Morphological characteristics of Phosphate Solubilizing bacterial isolate

Characteristics	Paddv isolate
Colonv shape	Circular
Cell shape	Rod
Color of colonv	Yellowish
Transparencv	Opaque
Nature of colonv	Ung listening
Margin of colonv	Entire
Surface of colonv	Smooth

The grown colonies of phosphate solubilizing bacteria on Pikovskya's agar media were purified twice times by doing tertiary streaking and charcterizing those purified bacterial colonies and observing the shape, colony, transparency, surface, nature, and margin of those bacteria the grown bacteria were yellowish transparency was opaque and the nature was unlisting looking at those morphological characteristics it clearly shows that the bacteria grown on pikovskya's agar media was phosphate solubilizing bacteria.

Table 3: PGPR Characteristics

Strain	Ammonia production	Phosphate Solubilizing Activity	IAA (µg/ml)
Paddy/PSB	+	+	+

'+ 'positive, '- 'negative

The bacterium that was isolated was selected to observe the plant growth promoting rhizobacteria and some tests were performed like ammonia production, IAA production, and phosphate solubilizing activity.

Test bacterial culture was inoculated in the 1% peptone water, after 24hrs incubation at 30ºC, 0.5 ml of Nessler's reagent was added and there was an appearance of yellowish-orange color which indicates the positive result toward the production of ammonia therefore all the bacterial strain shows positive result.

For phosphate solubilizing bacteria the purified bacterial strain was screened on Pikovskya's agar media and kept for incubation for 4 days after incubation the bacterial colony was measured by calculating the bacterial size growth and halo zone the difference was calculated hence from the selected isolates of bacteria, phosphate solubilizing bacteria shows the good value.

For IAA production the bacteria were grown in nutrient broth containing L- Tryptophane was incubated for 5 days then 2ml of supernatant could be used for protein assay was mixed with two drops of orthophosphoric acid and 4 ml of Salkowski's reagent, there was the presence of red-pink color which indicates the positive result toward indole.

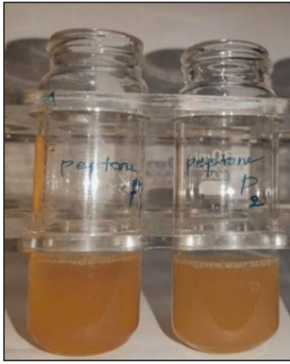


Fig 3: PSB strain showing positive results toward the ammonia production The appearance of yellowish-orange color indicates the production of ammonia.

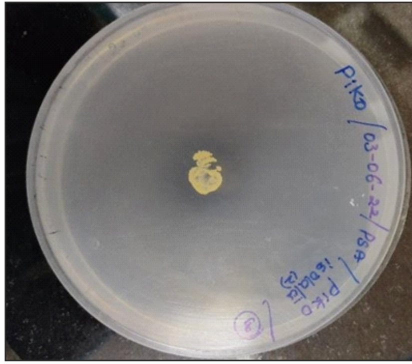
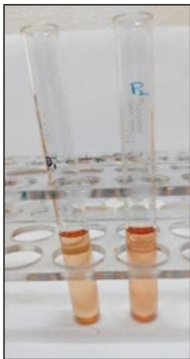


Fig 4: PSB strain showing a positive result for phosphate solubilizing activity The formation of a halo zone indicates the phosphate solubilizing activity of PSB.



After doing several tests, the PSB that was isolated from paddy shows good results and the bacteria were selected for DNA isolation. The overnight grown bacteria in nutrient broth were used for isolation, after that by using 16s rRNA primer did the PCR. Then the PCR product was run on 0.8% agarose gel and visualized at the gel documentation system and observed the successfully amplified bands were.

Fig 5: PSB strain showing a positive result for IAA production The appearance of red-pink color indicates the production of IAA by PSB

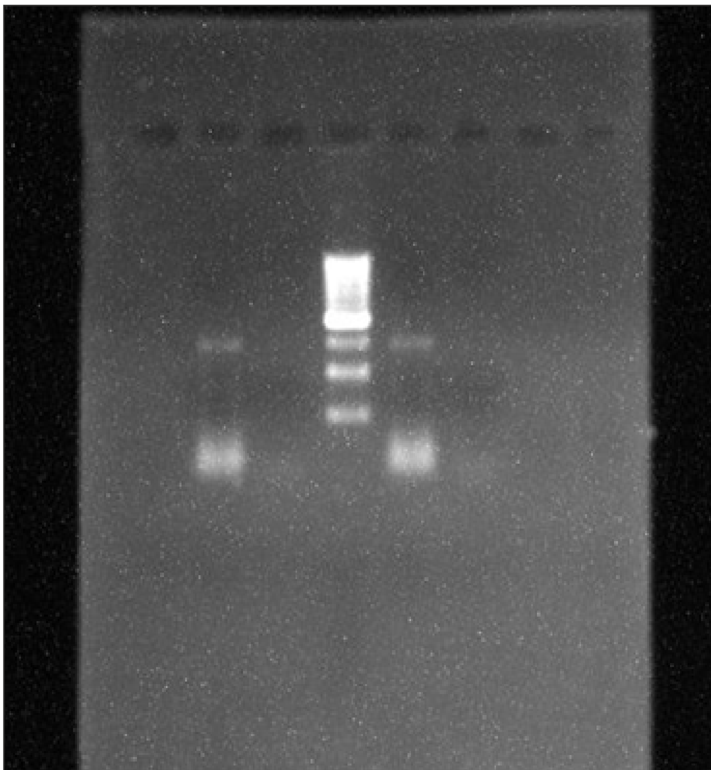


Fig 6: Successful amplification of bands using 16s rRNA primer by PCR.

4. DISCUSSION

In this study, the PSB bacteria were selected from rhizosphere soil from paddy fields at Acharya N.G. Ranga University situated at Rajendra Nagar in Hyderabad to check the different colonies and growth of bacteria, and the bacterial culture was inoculated on specific media pikovskya's agar for PSB.

Later gram staining is done and observed that the inoculated bacteria were gram-negative and the shape of those bacteria was rod-shaped. Afterward, the culture grown was streaked in freshly prepared specific media to obtain pure cultures.

Concerning PGPR characteristics IAA production, ammonia production, and phosphate solubilizing activity tests were done. Production of the red-pink color solution by adding orthophosphoric acid and Salkowski's reagent indicates the production of IAA. Production of the yellowish-orange color solution by adding Nessler's reagent indicates the production of ammonia. Formation of a halo zone around the bacterial spot when grown in specific Pikovskya's agar medium indicates the phosphate solubilizing activity of the bacteria.

5. CONCLUSION

1. Based on the morphological characteristics of the isolate, the culture was identified as phosphate solubilizing bacteria.

2. The PSB activities include Ammonia production, IAA production, and phosphate solubilizing activity.

3. The above isolated PSB had shown their product to growth promoting characteristics.

4. These characteristics would be considered potential biofertilizers.

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