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Isolation and Identification of Phosphate Solublizing Bacteria from Tomato Fields

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Abstract

Microbes from soil were isolated and cultured using Pikovskaya's medium. The isolates were screened for phosphate activity and identified as a phosphate solubilizing bacteria (PSB). The Phaseolus radiatus (green gram) seeds were germinated using these PSB as test against deionized water as control by roll towel model. Seeds were grown well in the PSB and it was verified by the estimation of concentration of total protein and total phenol in the germinated plant root (i.e.), the protein and phenol concentration of the root extract in the PSB test was higher than that of control. Biochemical tests were performed and the species were identified as Pseudomonas cichorii. Antifungal tests were also performed to find out fungal activity of the isolates. This study mainly focused on P.cichorii which has the ability to solubilize inorganic phosphates present in the soil and can be used as a biofertilizer, which will give more yields.

Keywords: *Pseudomonas cichorii, Phaseolus radiates, Biofertilizer, PSB, pikovskaya's medium, phosphate activity, roll towel model, antifungal activity, inorganic phosphate, germinated plant root, biochemical test.*

1. Introduction

Phosphorus is considered as one of the major nutrients required for living organisms involved in most of the physiological processes (Dipak Paul and Sankar Narayan Sinha, 2013). It exists in nature in many organic and inorganic forms, majority either in insoluble form or in very poorly soluble form. In soil, insoluble phosphorous is found to be complexed with cations like iron, aluminium and calcium. It is a major growth limiting nutrient for plant after nitrogen (G. Prasada Babu *et al*, 2013). It plays a major role in virtually all the important metabolic processes that take place in the plant including respiration, macromolecular biosynthesis, photosynthesis, signal transduction, and energy transfer. Plant mineral nutrition depends mainly on the phosphorous content of the soil, which can be assimilated only as soluble phosphate present in the soil (M. S. Khan *et al*, 2010) and through nitrogen fixation in legumes (K. Saber *et al*, 2005). In nature, phosphorous enters into the soil during the decay of natural vegetation and animal excreta. Although phosphorous is abundant in the soil, it cannot be directly used by the plants because it is in an unavailable form for root uptake (Z. Rengel and P. Marschner, 2005). The deficiency of the phosphorous in the soil leads to browning of leaves accompanied by small leaves, weak stem and slow development of the plant as reported (Sonam Sharma and Ram Babu Tripathi, 2011).
Microorganisms are an integral component of the soil phosphorous cycle and are considered to be important for the transfer of phosphorous. Phosphate solubilizing microorganisms (PSM) through various mechanisms of solubilization and mineralization convert inorganic and organic soil phosphorus respectively into the bioavailable form facilitating the uptake by plant roots (A. A. Khan *et al*, 2009). This process not only compensates for higher cost of manufacturing fertilizers in industry but also reduces the need for the fertilizers (H. Rodriguez and R. Fraga, 1999). Other workers have reported an increase in the plant growth and nodulation in Green gram (A. Vikram and H. Hamzehzarghani, 2008), nutrient uptake and yield of soybean (A. R. Sandeep *et al*, 2008) by phosphate solubilizing microorganisms. In this regard, it is of great practical importance to study the combined effect of the phosphate solubilizing microorganisms on nodulation, plant growth and nutrition and legume crop yields. Adoption of such technologies by farmers will help in minimizing production costs (Y. G. M. Galal *et al*, 2002). In this present study, *Pseudomonas cichorii,* the phosphate solubilizing microorganisms were isolated from tomato fields and screened for phosphatase activity. The isolates were characterized by their cultural, biochemical tests and carbohydrate utilization tests.

2. Materials and methods

2.1 Sample collection

Three samples were collected from tomato fields of Hosur, Tamil Nadu (Fig.1) from different sites using randomized block design.

Fig.1

2.2 Isolation of microorganisms

Isolation of microorganisms was done by serial dilution. One gram of soil was diluted in 100 ml of distilled water. This gave a dilution of 10^{-2} dilution. Then this was serially diluted in three test tubes to give a final dilution of about 10^{-6} range. The final dilution of each sample was taken for culturing. The process is repeated for three other samples.

2.3 Culturing of isolates

The isolates were inoculated onto Pikovskaya's agar medium (PVK). The plates were incubated at 37ºC for 24 hours and results were observed.

2.4 Subculturing

All the four plates showed growth of different colonies. Four distinct colonies from each plate were identified and subcultured in PVK agar thereby totally 16 colonies were subcultured. The plates were incubated at 37^0C for 24 hours.

2.5 Screening

After 24 hours of incubation, the colonies showing zone of clearance were selected for screening and these were chosen as phosphate solubilizing bacteria (PSB). The bacterial isolates were screened by PVK and incubated at 37^0C. Gram staining was performed. These phosphate solubilizing bacterial isolates were subjected to biochemical tests viz., Methyl Red, Indole production, Urease test, Catalase test, Citrate Utilization, Starch hydrolysis, Nitrate Reduction, Oxidase, Vogas Proskauer, Triple Sugar Iron test.

2.6 Fungal activity test

PSB isolates were subjected to antifungal activity test. Potato Dextrose Agar (PDA) was used for this test.

2.6.1 Preparation of media as follows

20 g of peeled potato were cut into small pieces and added to 100 ml of distilled water. It was boiled and filtered.

To the filtered water, 2g of dextrose and 2g of agar were added. It was poured into petriplate and allowed to solidify.

The fungi were reinoculated in the centre of the new plates, along with the streaked PSB isolates in the top and bottom of the plate. It is then incubated for 24 hours at 37^0C.

2.7 Seed germination

Good quality *Phaseolus radiatus* (green gram) seeds were selected. Ten seeds were soaked in a control (deionized water) and another ten seeds were soaked in test solution for 1 hour (PSB isolate grown in a PVK broth). These seeds were germinated by roll towel model using blotting paper separately for 1 week (J. Daniel Hoy and E. Edwin Gamble, 1983).

2.8 Protein extract

One gram of root from both control and test plants were obtained and 1ml of PEB (Protein Extraction Buffer) was added. They were ground separately and filtered. Filtrate was collected and centrifuged for 5 mins at 10000 rpm. Supernatants were collected and stored for future use.

2.9 Total protein estimation

50 µl of protein extract from both the control and test plants were collected in separate test tubes. 950 µl of double distilled water and 3 ml of CBB (Coomassie Brilliant Blue) were added to each test tube. The mixture was incubated for 5 mins at room temperature and absorbance was read at 595 nm (Afsheen Mushtaque Shah *et al*, 2010).

2.10 Total phenol estimation

200 µl of protein extract from both the control and test plants were collected in separate test tubes. 1 ml of Folin-ciocalteau reagent (1:1 with water) and 1 ml of sodium carbonate (7.5%) was added to each test tube. The mixtures were vortexed and incubated for two hours at room temperature. Absorbance was read at 726 nm (A. Elizabeth Aninsworth and M. Kelly Gillespie, 2007).

3. Results and discussion

3.1 Biochemical tests

On gram staining, the isolates are confirmed as gram negative. The biochemical tests were performed to identify the isolates. The results of the biochemical tests are shown in Table.1.

3.1.1 Methyl Red (MR) Test

After 24 hrs of incubation, MR reagent is added and red colour appeared in the medium (Fig.2) and the isolates

are MR positive. It indicates that the bacterial isolates have ability to produce stable acids by the mechanism of mixed acid fermentation of glucose.

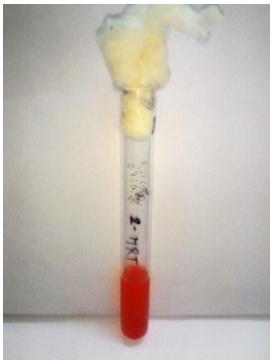

Fig.2

3.1.2 Indole production test

After 24 hrs of incubation, Kovases reagent was added and there was no colour change in the medium (Fig.3) and the isolates were indole negative. It indicates that the bacterial isolates do not have ability to convert tryptophan into indole.

Fig.3

3.1.3 Urease test

After 24 hrs incubation, the medium does not change its colour from yellowish orange to pink (Fig.4) indicating no urease activity. It indicates that the isolate does not have the ability to degrade the urea.

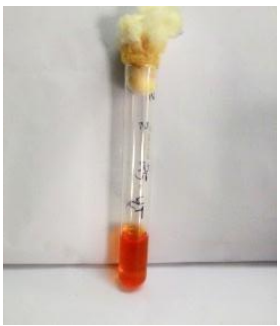

Fig.4

3.1.4 Catalase test

Hydrogen peroxide is added to smear of isolates on the slide. Bubbles are formed (Fig.5) indicating catalase activity. It indicates that the bacterial isolates have the ability to decompose hydrogen peroxide to water.

Fig.5

3.1.5 Citrate utilization test

After 24 hrs of incubation, the bromothymol blue reagent is added and the slant media gives blue colour (Fig.6) and shows citrate activity. It indicates that the bacterial isolates have the ability to use citrate.

Fig.6

3.1.6 Starch hydrolysis test

After 24 hrs of incubation, iodine was spread over the plate giving a violet colour over the medium (Fig.7) indicate starch hydrolysis.

Fig.7

3.1.7 Gram staining test

On staining, the isolates were gram negative.

3.1.8 Nitrate reduction test

After 48 hrs incubation, the two nitrate solution are added. The broth does not turn into deep red (Fig.8) and shows no nitrate reductase activity.

Fig.8

3.1.9 Oxidase test

After 24 hrs incubation, the oxidase solution is added. The slant change colours from pale yellow to dark brown colour (Fig.9) indicating oxidase activity. It indicates that the bacterial isolates have the ability to produce certain cytochrome c oxidase.

Fig.9

3.1.10 Voges Proskauer (VP) test

After 24 hrs incubation, VP reagent A and B are added. The medium appeared as cherry red colours (Fig.10) indicate VP activity. It indicates that the bacterial isolates have the ability to produce acetone.

Fig.10

3.1.11 Triple Sugar Iron (TSI) test

After 24 hrs of incubation, the slant appeared yellow and gas is produced at the bottom (Fig.11) which indicates that the bacterial isolates have the ability to produce glucose, lactose, sucrose fermentatively and produce gas also.

Fig.11

Table.1: Results of biochemical tests

Test name	Result
Gram staining	Gram negative
Oxidase	Positive
Catalase	Positive
Voges Proskauer	Positive
Indole production	Negative
Methyl Red	Positive
Citrate utilization	Positive
Urease	Negative
Triple Sugar Iron	Positive
Starch hydrolysis	Positive
Nitrate reduction	Negative

Results of these biochemical tests confirmed that the isolates are *Pseudomonas cichorii*.

3.2 Anti-fungal activity

After 24 hrs of incubation, the plates are examined, there were no zones of clearance around the bacteria (Fig.12) and did not show any antifungal activity.

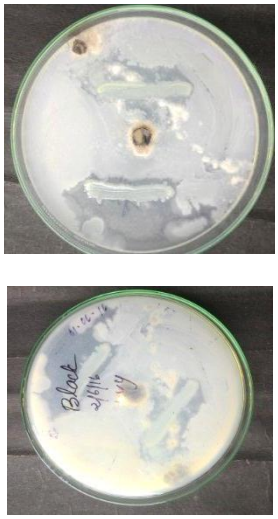

Fig.12

3.3 Seed germination

After one week, seeds germinated in test samples grew well than the control (Fig.13) as they were soaked in PSB for 1 hour.

Fig.13 (Left-test: Right-control)

Plants were removed safely from the test sample and the control to examine root development (Fig.14).

Fig.14 (Left-test: Right-control)

The roots grew better in the test sample than the control sample.

3.4 Estimation of total protein

The 50 µl supernatant was diluted in 950 µl of double distilled water and 3 ml CBB reagent was added. It gave light blue colour (Fig.15) and the results were read at 595 nm. The results were not in accordance with previous report (Afsheen Mushtaque Shah *et al*, 2010).

Fig.15

Table.2: Total protein estimation

	Test (PSB)	Control (water)
Protein concentration (mg/g of fresh root)	3.04	2.48

3.5 Estimation of total phenol

The total phenol content was estimated using Folin-ciocalteau reagent and sodium carbonate and it was found to be 20.66 mg/g of fresh root in test sample while it was just 11.7 mg /g of fresh root in control sample. The results were not in accordance with previous report (Memnume Sengul *et al*, 2009).

Phosphate solubilizing bacteria, *Pseudomonas cichorii* were isolated from tomato fields in Hosur and the isolates were screened for antifungal activity. The PSB isolates were tested with seeds of *Phaseolus radiatus* for germination and protein and phenol concentration in the roots were estimated. The results indicated that these PSB can be effectively used as biofertilizers in agriculture.

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