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Techneques for the Isolation and *In-vitro* Screening of Plant Growth Promoting Rhizobacteria (PGPR) from Rhizosphere

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Abstract: Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that can be found in the rhizosphere, in association with roots which can enhance the growth of plant directly or indirectly. This study encompasses the techniques used for the Isolation and *In-Vitro* Screening of Plant Growth Promoting Rhizobacteria (PGPR) from rhizosphere.

Key words: Bacterial diversity · Plant growth promoting rhizobacteria · Plant growth · Bio-Fertilizers

INTRODUCTION

Bacterial diversity is of particular importance in human sustenance since, these small creatures comprise the majority of earth's species diversity. Bacterial diversity is considered as one of the most useful resource with considerable significance in the global form of bioremediation and bio-prospecting [1]. Interaction between bacteria and roots of plants has been reported to be beneficial, detrimental or neutral and this delicate balance is a consequence of both soil and plant type [2]. Bacteria, beneficial to plants may be symbiotic or free living and are abundant near the roots. A group of bacterial population closely associated with rhizosphere region of soil is mainly involved in enhancing plant growth by several activities. Such beneficial free-living bacteria have been termed Plant Growth Promoting Rhizobacteria (PGPR) [3]. Current trends in agriculture are focused on the reduction of the use of pesticides and inorganic fertilizers, forcing the search for alternative ways to improve a more sustainable agriculture [4]. The use of plant growth promoting rhizobacterial (PGPR) inoculants as biofertilizers and/or antagonists of phytopathogens provide a promising alternative to chemical fertilizers and pesticides.

Mechanism of Action: The exact mechanism by which PGPR affect plant growth are not fully understood but are thought to include (i) the ability to produce or change the concentration of plant growth regulators like indole acetic acid, gibberellic acid, cytokinins and ethylene [5] (ii) asymbiotic N_2 fixation [6] (iii) antagonism against phytopathogenic microorganisms by production of siderophores [7], antibiotics and cyanide [8] (iv) solubilization of mineral phosphates and other nutrients [9]. Most popular bacteria studied and exploited as biocontrol agent includes the species of Pseudomonas flurosence and Bacillus spp. Some PGPR may promote plant growth indirectly by affecting symbiotic N_2 fixation, nodulation or nodule occupancy [10].

Techneques for the Isolation of Plant Growth Promoting Rhizobacteria (PGPR) from Rhizosphere: The soil samples that are used for bacterial isolation are collected from the root-free soil of rhizosphere about 10- 20cm deep in the soil under highly aseptic conditions using sterilized tool for diging the soil and then collect the soil in sterilized zip polythenes and store them in the -4°C till analysis.

Soil Ph: The pH of the soil is determined by taking 10gm of soil sample in 100ml distilled water at 1:2 soil water suspension with the help of combined electrode (glass and calomel) in digital pH meter [11].

Electrical Conductivity(E.C): Electrical conductivity of the soil sample is determined in the same ratio 1:2 soil water suspension used for measuring pH with the help of a Conductivity Meter [11]. It is expressed in deci Siemens per meter (dSm^{-1}) or µs/cm at 25°C.

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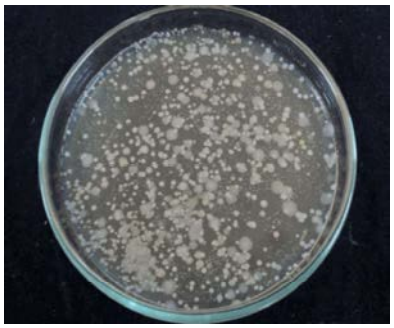
Serial Dilution/Microbial Count: The sample of each varietal rhizosphere soil was mixed thoroughly to make a composite soil. 10g of dry nd highly pulvirised soil sample is suspended in 90 ml of sterile distilled water considered as a stock solution then transferring 1ml of soil suspension into 9 ml sterile distilled water with the help of a sterile pipette to yield 10^{-1} dilution. Similarly, a series up to 10^{-7}dilution was prepared under aseptic condition. Bacteria are isolated by employing serial dilutionplate technique using nutrient agar (Peptone 5g/l; Beef extract 3g/l; NaCl 5g/l; Agar 15g/l) a general purpose medium.Then 0.1 ml soil suspension is introduced into sterilized nutrient agar media in Petri dishes and spread it thoroughly on the media incubated at 37°C for 24-48 hours and for each dilution the plates are taken in triplicates. After incubation period,visual morphological characterization of the bacterial colonies isolated on the agar petriplates is observed on the basis of colour, shape, size, elevation etc. of the bacrterialcolonies.Further the colonies are counted and calculated as per the relation given below. Colonies exhibiting prolific growth are selected for further streaking on fresh agar plates for purification and multiplication of the isolates is done by streak plate method.

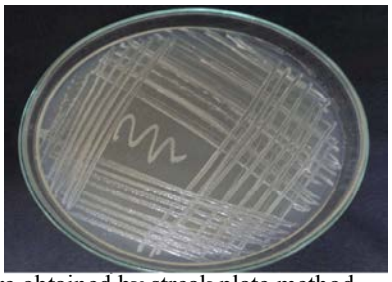
CFU/g = No. of colonies (average in triplicate) /Volume plated (ml) x Dilution Factor

Streak Plate Method: The streak-plate method offers a most practical method of obtaining discrete colonies and pure cultures. It was originally developed by two bacteriologists Loeffler and Gaffkey in the laboratory of Robert Koch [12]. The pure colonies which are of different size, shape and colour may be isolated/ transferred into test tubes/ petriplate culture media for sub-culturing or making pure cultures. In this method, the inoculating loop or transfer needle is sterilized on the flame till it becomes red hot, then cooled and is dipped in a diluted suspension of organisms or touched with a single bacterial colony which is then streaked on the surface of an already solidified agar plate to make a series of parallel, non overlapping streaks. After streaking the loop is again sterilized on red hot zone of flame. The petriplates are sealed with parafilm and incubated at 28-30°c for 24-48 hours in an inverted position.

Gram Staining: The Gram stain, a differential stain was developed by Dr. Hans Christian Gram, a Danish Physician, in 1984 that is why Gram's staining. It is a very useful method for identifying bacteria and classifying



Isolated bacterial colonies by serial dilution plate technique



Pure cultre obtained by streak plate method

bacteria into two major groups: the Gram positive and Gram negative. In this method a smear/thin film is made on a clean glass slide, air dry and heat fix the smear by passing through flame. The fixed bacterial smear is subjected to four different reagents in the order listed: crystal violet (primary stain), iodine solution (mordent), 80% alcohol (decolorizing agent) and safranin (counter stain). Cover the smear crystal violet for one to one and a half minute and wash the slide with distilled water and then cover with Gram's iodine for one to one and a half minute. Wash the slide with distilled water and decolorize by washing the slide with 80% alcohol, till the violet colour comes off the slide. Then immediately wash with distilled water and subsequently counter stain with safranin for 30s – one minute and again wash the slide with distilled water and examine under oil immersion lens of the microscope.

Composition of Stains/Reagents Used

Crystal Violet Solution: Solution A: 2.0 g of crystal violet is dissolved in 20 ml of 9o% ethanol.

Solution B: Dissolve 0.8 g ammonium oxalate in 80 ml distilled water. Mix solution A and B and store for 24 hours before use.

Gram's Iodine:10 g iodine crystals and 20 potassium iodide are dissolved in one litre distilled water(dry iodine and potassium iodide are ground in a mortar by adding a

few ml of water at a time until the iodine and iodide dissolve). Solution is filled in an amber glass bottle by rinsing the motor pestle with the remainder of the distilled water.

Stock Solution: Dissolve 2.5 grams of safranin in 95% ethanol and filter before use.

Working Solution: 10 ml of stock solution is mixed with 90 ml of distilled water

***In-vitro* Screening of Isolates for Their Plant Growth Promoting(PGP) Activities**

Phosphate Solubilizing Activity of Bacteria: The isolated bacterial strains are first screened for phosphate solubilization on a selective media i,e Pikovskaya's agar medium (PAM).Bacterial culture are inoculated on centre of plate containing Pikovskaya's agar media by inoculation loop under aseptic condition and incubated at 30±2°C for 5 days, a clear zones develops around the colonies showing phosphate solubilization activity of the bacteria [13].

Pikovskaya's agar media: grams per litre

Yeast extact	_	0.50 g
Ferrous sulphate	_	0.00001 g
Dextrose	_	10.0 g
Calcium phosphate	_	5.0 g
Ammonium sulphate	_	0.50 g
Potassium choloride	_	0.20 g
Magnesium sulphate	_	0.10 g
Manganese sulphate	_	0.0001 g
Agar	_	15.0 g
Distilled water	_	1000 ml

Quantitative Estimation of Phosphate:: Quantitative estimation of phosphate solubilization of those isolated bacteria which have already shown PSB activity on PA media, then these strains were introduced inPikovskaya's broth containing ($MgSO_4.H_2O$ (0.25 g/l), KCl (0.2 g /l), $(NH_4)_2SO_4$ (0.1g/l), $Ca_3(PO_4)_2$ (5g/l), glucose (10 g/l, yeast extract 0.50g/l, Ferrous sulphate 0.00001g/l, Manganese sulphate (0.0001g/l) and this test was carried out using Erlenmeyer flasks (250 ml) containing 100 ml medium was inoculated with 500 µl in triplicate with the bacterial strain. Un-inoculated medium served as control was also kept under similar conditions. The flasks were kept at shaker incubator at 120 rpm at 28°C- 30°C. The cultures were harvested by centrifugation at 10,000 rpm for 10 min. The phosphate in supernatant was estimated by vandate-molybdate reagent. The 1ml culture supernatant

Phosphate solublizing activity of bacteria

was mixed with 10ml vandate-molybdate reagent. The absorbance of the resultant yellow color is read at 470 nm in Spectrophotometer. The total soluble phosphate is calculated from the standard curve of stock solution of KH_2PO_4 containing 50 µg/ml and different aliquots are taken with difrrentcocentrations.The values of soluble phosphate liberated are expressed as µg/ml or µmol/

Production of IAA (Indoleacetic Acid): Fifty milliliter of Nutrient broth (NB) containing 0.1% DL-tryptophan is inoculated with 500 µl of 24 h old bacterial cultures and incubated at 28±2°C for 3 days. The bacterial cultures are centrifuged at 10,000 rpm for 10 min at 4°C[14]. The supernatant (2 ml) is mixed with two drops of orthophosphoric acid and 4ml of the Salkowski reagent (50 ml of 35% percholoricacid, 1ml 0.5M $FeCl_3$ solution). Development of pink colour indicates IAA production. Optical density is taken at 530nm with the help of spectrophotometer. Concentration of IAA produced by cultures is measured with the help of standard graph obtained from stock solution solution of IAA 5mg/ml and different aliquots are taken at different concentrations. The values of indole-3-acetic acid are expressed as µg/ml or µmol/ml [15; 16].

Production of NH_3: Bacterial isolates are tested for the production of ammonia in peptone water. Freshly grown cultures are inoculated in 10ml peptone water in each tube and incubated for 48–72 h at 30°C. Nessler's reagent


Production of siderophore

(0.5 ml) is added in each tube. Development of faint yellow to dark brown colour was a positive test for ammonia production [15].

Production of HCN: - Isolates were screened for the production of hydrogen cyanide, nutrient broth is amended with glycine (4.4 g/l) and bacteria are streaked on modified agar plate. A Whatmanfilter paper no.1 soaked in 2% sodium carbonate in 0.5% picric acid solution is placed in the top of the plate. Plates are sealed with parafilm and incubated at30°C for 4 days. Development of orange to red colour indicated HCN production [15].

Production of Siderophore: Production of siderophore by antagonistic bacteria is assayed by plate assay. The tertiary complex Chrome Azural S (CAS) / Fe^{+3} / hexadecyltrimethyl ammonium bromide served as an indicator. To prepare one liter of blue/green agar, 60.5 mg of CAS is dissolved in 50 ml of distilled water and mixed with 10ml of iron (III) solution (1mM FeCl.6H O in 10 mMHCl)[16]. While constantly stirring, this solution is slowly added to 72.9 mg of hexadecyltrimethyl ammonium bromide (HDTMA) dissolved in 40ml of water. The resultant dark blue/greenliquid is added in nutrient agar to make Chrome Azural S (CAS). Spot inoculation of bacterial isolates is done on CAS agar andincubated at 30°C for 48–72 h. Development of yellow–orange halo around the growth is considered as positive for siderophore production. [17, 18].

Estimation of Siderophore: The quantitative estimation of siderophoreis done by CAS-shuttle assay, in which the strains are grown on Succinate medium and incubated for 24-30 hrs at 28°C with constant shaking at 120 rpm on shaking incubator separately. During incubation, every 20 min 5 ml broth is centrifuged at 10,000 rpm at 4°C in cooling centrifuge for 10 minute and cell free supernatant is mixed with 0.5 ml CAS solution. The color obtained is measured using the spectrophotometer at 630 nm with reference containing 0.5ml uninoculated succinate medium and 0.5 ml CAS solution. The percentage of siderophore units is estimated as the proportion of CAS color shifted using the formula:

% Siderophore units = (Ar - As)/Ar x 100,

where"Ar" is the absorbance at 630nm of reference (CAS assay solution+ uninoculated media) and "As" is the absorbance at 630nm of the sample (CAS assay solution + supernatant) [19].

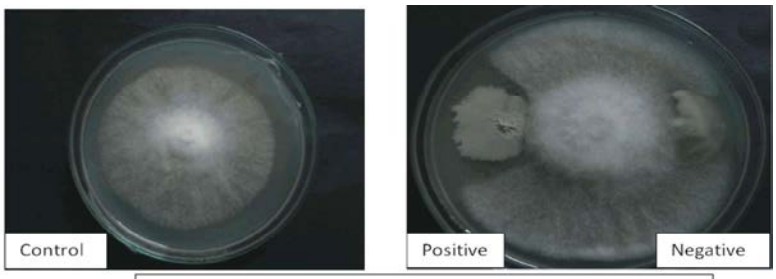
Characterization of Siderophore:

Hydroxamate Type of Siderophore

Tetrazolium Test: This test is based on the capacity of hydroxamic acids to reduce tetrazolium salt by hydrolysis of hydroxamate groups using a strong alkali. The reduction and the release of alkali show red color. To a pinch of tetrazolium salt, added 1-2 drops of 2N NaOH and 0.1 ml of the test sample. Instant appearance of a deep red color indicated the presence of hydroxamatesiderophore [19].

Catecholate Type of Siderophore

Arnow's Test: To 1 ml of cell-free supernatant is added 1 ml of 0.5 N HCl and 1 ml of nitrite- molybdate reagent, the catechols produced yellow color, immediately 1 ml NaOH solution is added which resulted in red color formation. The color was stable for 1 hour and the absorbance was measured at 510 nm using a UV-vis spectrophotometer [19].



Antifungal potential of plant growth promoting rhizobacteria

Antifungal Assay: Bacterial isolates are assayed for antagonist activity against fungi by using dual culture technique. Isolated bacteria are spot inoculated away from the the centre on nutrient agar plates/ PDA plates and also the 4mm disc of fungi is introduced at the centre of the plate. The plates are incubated for 5–6 days at 28°C. Observe the bacteria showing antagonist activity against fungi, to be used as a potential in the biological control of the fungi and calculate the percentage inhibition of fungal colony growthby the given formula viz:

Percentage inhibition (I) = **C-T/C x 100**

Where C- mycellal growth of pathogen in control
T- mycellial growth of pathogen in dual plate.

CONCLUSION

This study illustrates the significance of the techneques for the Isolation and In-Vitro Screening of Plant Growth Promoting Rhizobacteria (PGPR) from rhizosphere. Such type of study is necessary as it advocates that use of PGPR as inoculants or biofertilizers is an proficient approach to replace chemical fertilizers and PGPR isolates may be used as biofertilizers to improve the growth and productivity of commercially grown medicinal and aromatic plants under local agro-climatic conditions.

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