Research Article

Screening of different plant growth promoting traits from rhizobacteria.

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ABSTRACT

Use of chemical fertilizers for agriculture is all over in India, has resulted not only in the deterioration of soil health but also has led to some major environmental problems, such as soil and water pollution and other health related problems, besides increasing the input cost for crop production especially on the marginal farmers. So, there is an urgent need to recycle available organics and manipulate rhizospheric microflora in a more efficient way and improve and expand their usage. In our work we do screening of different microorganism which is promoting plant growth. In the present study, 88 isolates from the rhizospheric soil samples of different crops were screened initially on the basis of their plant growth promoting activity. These isolates were then tested in vitro for specific PGPR traits such as the production of phosphate solubilizing enzymes, Indole Acetic Acid (IAA), siderophores, Hydrocyanic acid (HCN), salicylic acid, hydrolytic enzymes and biosurfactants. Of the 88 isolates, four strain (8, 14, 18) number strain was found to be promising for all PGPR attributes.

KEYWORDS

Chemical fertilizer, Rhizobacteria, Soil sample.
1. INTRODUCTION
The use of chemical fertilizers has resulted not only in the deterioration of soil health but also has led to some major environmental problems, such as soil and water pollution and other health related problems, besides increasing the input cost for crop production especially on the marginal farmers. So, there is an urgent need to recycle available organics and manipulate rhizospheric microflora in a more efficient way and improve and expand their usage. Search for ecologically adaptable Plant growth promoting rhizobacteria with enhanced plant growth promotory properties and their use to enhance crop productivity can improve the socio-economic status of poor farmers. PGPR are indigenous to soil and are able to competitively colonize plant roots. An effective root colonist is a essential trait for PGPR in order to survive in the rhizosphere and rootsurface, and to begin and efficiently support host plant growth (Lugtenberg and Dekkers, 1999; Kamilova et al., 2005).

Originally, the definition of PGPR only referred to free-living beneficial rhizobacteria (Kloepper et al., 1989), but over the years the definition has been extended to any root colonizing bacteria including symbiotic rhizobacteria (Antoun and Prevost, 2006). A large number of bacteria including species of Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Rhizobium and Serratia have reported to enhance plant growth.

2. MATERIALS AND METHODS
2.1 Materials
2.1.1. Chemical
All chemicals were obtained from Hi-media and Qualigens, Mumbai (India) and were of analytical grade.

2.1.2. Organisms and Culture conditions
All the isolates were procured from the culture repository of Lab number 213, School of Life Sciences, North Maharashtra University, Jalgaon. Cultures were maintained on Nutrient agar at 4oC and periodically sub-cultured to maintain viability.

2.2 Methods
Screening of isolates for plant growth promoting traits
1. Phosphate solubilization
The test isolates were spot inoculated in Pikovskaya’s (PVK) agar and incubated for 5 days at 280C. Thereafter, the plates were observed for zone of clearance representing phosphate solubilisation.

2. Zinc Solubilization
The isolates were inoculated into nutrient agar medium (Bunt and Rovira, 1955) containing 0.1% of the insoluble zinc oxide (ZnO) incubated at 30oC for 36 to 96 h. Appearance of halo zone around the colonies indicated their ability to solubilize Zn.

3. Ammonia production
All the isolates were grown in test tubes containing peptone water: 10.0 g peptone; 5.0 g NaCl; 1000ml distilled water; pH 7.0 (Dye, 1962). The tubes were inoculated with 100 ml of 24h grown cultures in broth and incubated at 30oC for 4 days. The accumulation of ammonia was
detected by adding Nessler’s reagent (0.5 ml). A faint yellow colour indicated a small amount of ammonia, and deep yellow to brownish colour indicated maximum production of ammonia.

4. Hydrocyanic acid (HCN)
HCN production was evaluated by streaking the bacterial strains on Nutrient agar medium amended with 4.4 g of glycine per liter. Circular Whatman no. 1 filter paper soaked in picric acid (0.05% solution in 2% sodium carbonate) was placed in the lid of each Petri plate. The plates were then sealed airtight with Parafilm and incubated at 30°C for 48 h. Color change of the disc from yellow to reddish-brown was considered as an indication of HCN production.

5. Nitrogen fixation
Nitrogen free LGI medium containing bromothymol blue (BTB; 0.025 mgL-1 ) as an indicator was used for the screening of nitrogen fixing microbes. Each plate after spot inoculation with the respective isolates was incubated at 370°C up to 24 h .The blue coloured producing isolates were marked as nitrogen fixers in the solid culture conditions. The colouring zone was calculated by deducting the colony diameter from the colouring zone diameter.

6. Siderophore detection
Qualitatively, siderophores were detected by assay using universal CAS assay (Schwyn and Neilands 1987). The strains were grown in sterile iron free succinic acid medium. Inoculated flasks were incubated for 48 hrs at 280°C on a rotary shaker (120 rpm). The cultures were centrifuged at 7000rpm for 15 min and 0.5 ml of supernatant was mixed with 0.5 ml of CAS reagent. The orange colour indicates the presence of siderophore.

7. Phenazine detection
Pigment Production Medium (PPMD) was used to assay presence of phenazine. Inoculated flasks were incubated for 72 hrs at 280°C on a rotary shaker (120 rpm). Five ml supernatant acidified upto PH 4. By addition of equal volume of benzene, shaking vigorously, the separated benzene and red acidic layers were collected individually. Benzene layer centrifuged at 5000 rpm for 10 min. the absorbance was determined at 367 nm .Red acidic layer neutralized ( PH 7.0). Chloroform was added and absorbance was measured at 690 nm wavelength

8. Antibiotic resistance/susceptible pattern
A total of three antibiotics viz. chloramphenicol, penicillin and tetracycline were studied for their resistance/susceptible pattern against the isolates. The required quantities of antibiotics were dissolved in sterilized Milli Q water and mixed into Nutrient agar just before pouring into the Petri plates. Upon solidification, the isolates were spot inoculated and incubated at 28°C for 72 hr.

9. Sulfur oxidation
Thiosulphate broth with initial pH 8.0 (adjusted by 1N NaOH) was prepared with glucose. Isolates were inoculated and incubated at 370°C at 120rpm. The reduction in pH was checked (upto 72 h) which indicated positive test for sulphur.

10. Indole-3-acetic acid (IAA) production
Production of indole-3-acetic acid (IAA) was spectrophotometrically determined as described by Fukuhara et. al. (1994). Isolates were grown in LGI medium supplemented with L-tryptophan (100 mg/L) at 28oC. The supernatant of the culture was obtained by centrifugation at 12,000 rpm for 15 min. IAA produced per ml culture was estimated by mixing drop of o-phosphoric acid and
5 ml Salkowsky reagent (0.01 M FeCl2 in HClO4), followed by measuring the color change at 530 nm (Costacurta et al., 2006). Pure Indole acetic acid was used as standard.

11. **Microtiter plate biofilm production assay**
Each selected strains were grown in 10 ml of LB medium at 28°C overnight. Biofilm formation assays were performed with LB medium. Overnight cultures in LB were transferred (0.1 ml) to 10 ml of LB and vortexed. After vortexing, 100 µl volumes were added to each well into microtiter plate for each strain and incubated overnight. The cell turbidity was monitored using a microtiter plate reader (Bio-Rad), at an optical density at 595 nm. After incubation period, medium was removed from wells and microtiter plate wells were washed five times with sterile distilled water to remove loosely associated bacteria. Plates were air dried for 45 min and each well was stained with 150 µl of 1% crystal violet solution in water for 45 min. After staining, plates were washed with sterile distilled water five times. At this point, biofilms were visible as purple rings formed on the side of each well. The quantitative analysis of biofilm production was performed by adding 200 µl of 95% ethanol to destain the wells. One hundred microliters from each well was transferred to a new microtiter plate and the level (OD) of the crystal violet present in the destaining solution was measured at 595 nm (O'Toole and Kolter, 1998).

12. **Exopolysaccharide production (EPS):**
The EPS produced by the strains was determined as suggested by Mody et al. (1989). For this, the strains were grown in 100 ml of sterile nutrient broth. Inoculated flasks were incubated for 5 days at 280°C on rotary shaker (120 rpm). Culture broth was centrifuged (5,4339 g) for 30 min and EPS was extracted by adding three volumes of chilled acetone (to one volume of supernatant. The precipitated EPS was repeatedly washed three times alternately with distilled water and acetone. It was transferred to a filter paper and weighed after overnight drying at room temperature.

3. RESULTS AND DISCUSSION
1. **Phosphate solubilization**
Of the 88 isolates tested, fifty eight (65%) produced a halo-zone on Pikovskaya’s agar plate which demonstrated that the strains having phosphate solubilizing ability. It indicated that the isolated strains were able to solubilize phosphate either by producing several organic acids or enzyme phosphatases.

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**Plate 3.1:** Phosphate solubilization. a,b) shows zone of clearance around colony, c) control plate
2. Zinc solubilization
Solubilization of zinc can be accomplished by a range of mechanisms, which include excretion of metabolites such as organic acids, proton extrusion, or production of chelating agents (Sayerand and Gadd, 1997). In the present study, only nine isolates able to grow on medium embedded with zinc oxide.

![Photoplate 3.2: Zinc solubilization: a) zone of clearance around colony, b) control](image)

3. NH3 Production
Detection of ammonia production was done by recording the presence of the yellowish brown colour in some isolated strains. All the isolates have ability to eliminate ammonia. Out of 88, 39% isolates efficiently produce ammonia depending upon coloration. PAL plays important role in the biosynthesis of various defense chemicals by phenypropanoid metabolism. PAL catalyzes the elimination of ammonia from phenylalanine to produce trans-cinnamic acid which serves as substrate for the formation of phenolics like salicylic acid, phytoalexins and antibiotics.

![Photoplate 3.3 Detection of Ammonia production: a) yellow to brown color indicates presence of ammonia, b) control containing nutrient broth with reagent.](image)

4. Detection of Hydrogen cyanide
After 48 h incubation, isolated strain on Nutrient agar plate supplemented with 4.4% glycine, the colour of filter paper was not changed from yellow to reddish brown and all strains were not able to produce hydrogen cyanide only 213B strain was able to produce HCN. HCN is known to be intricately related to antifungal activity. HCN production has been shown to be both a beneficial
and a harmful property for plants (Cattelan et al. 1999). Blumer and Hass (2000) reported that HCN production has been proposed as a defense regulator against phytopathogens.

5. Nitrogen fixation
The nitrogen fixing bacteria were screened on nitrogen free LGI agar media containing BTB as an indicator. A total of 20 isolates showed blue colour zone on LGI-BTB agar which indicates the presence of ability to fix nitrogen. Isolate 14 have efficient nitrogen fixer as its zone of coloration was maximum as compared to other isolates.

![Nitrogen fixing bacteria](image)

Photoplate 3.5: Nitrogen fixing bacteria, a) Blue colour zone showed presence of nitrogen fixation.

6. Siderophore detection
Siderophore detection of different isolates were done by using CAS reagent, when CAS reagent was added in culture supernatant then colour of the supernatant was changes from blue to orange. It has been known for long time that plant associated microbes were able to produce siderophore.

![Siderophore detection](image)

A B

Photoplate 3.6 Siderophore detection a) orange color shows siderophore detection; b) Blue color shows absence of siderophore

The different results for the relevance of siderophore production for plant-associated bacteria undoubtedly reflect the natural gradient from iron sequestration by the host plant to leakage of iron from the plant tissue (Wensing et.al. 2010).
7. Phenazine detection
PCA was estimated in PPMD supernatants. In the same supernatant, PYC was estimated as given by Cox (1986). A total of 35% isolates showed presence of phenazine. This compound has broad-spectrum antibiotic activity and have role in antagonism towards fungal phytopathogens.

8. Antibiotic resistance pattern
Productions of antibiotics which are low molecular weight organic compounds are deleterious to the growth or other metabolic activities of other microorganisms (Fravel, 1988). The antibiotic production is an important mechanism of biological control where it inhibits cell wall synthesis of bacteria, thus inhibiting their growth (Subba Rao, 1999). Resistant of PGPR to several antibiotics might have an ecological advantage of survival in the rhizosphere when they are introduced as inoculum. Survival of inoculant in soil leads to bacterial colonization on roots and expression of their beneficial effect.

**Table 3.** Antibiotic resistance pattern. (Chl.: Chloramphenicol, Tetra.: Tetracycline, Strepto.: Streptomycin)

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<tr>
<th>Strains</th>
<th>Chl. (30 µg)</th>
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9. Sulfur reduction
Detection of sulphur oxidation was evaluated by pH reduction in medium. Almost all the isolate able to reduce the pH and out of 88 isolates 30% isolate showed significant reduction up to pH 5.0 (ref. fig). The reduction in the pH of the growth medium is due to the production of sulfuric acid (Kelly and Wood, 2000). Inadequate S supply limits N2-fixation and eventually the N supply of legumes and reduces the plant growth. Application of S increased shoot length and yield (Scherer, 2001).

10. Indole Acetic Acid production
The isolated strains were able to produce IAA using tryptophan as precursor and some isolated strains were not able to produce IAA. A total 16 selected isolates were tested for quantitative estimation of IAA in the presence of tryptophan.

IAA, a member of the group of phytohormones, is generally considered to be the most important native auxin. IAA may function as important signal molecule in the regulation of plant development. All ten isolates were positive for IAA production. Among them, four isolates 12,15,21 and 81 were highest producers of IAA (Ref. Fig). It has been reported that IAA
production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability.

11. Exopolysaccaride production
Among all the isolates 68% isolates were able to secrete EPS. Higher EPS producing nature of some isolates may lead to better soil aggregation and increased soil aggregate stability thereby helping the plants to take higher volume of water and nutrients from rhizosphere soil resulting in better growth of plants. Similar observations were reported by Miller and Wood (1996).

12 Biofilm formation
Among all the isolates 14% strains produce highest amount as compare to others. The Pseudomonas putida can respond rapidly to the present of root exudates in soil, converging at root colonization sites and establishing stable biofilm (Espinosa-Urgel et al., 2002). The bacteria colonize root elongation zones and root hairs, forming dense biofilms (Assmus et al., 1995).

Fig 3.1: Sulfur oxidation by pH reduction method of strains a)1 to 44, b) 45 to 88
4. CONCLUSION

Plant growth promoting rhizobacteria (PGPR) are commonly used as inoculants for improving the growth and yield of agricultural crops. Development of an effective PGPR inoculant necessitates the presence of a diverse set of traits that can help in colonization of the rhizosphere and survival under varying environmental conditions. Thus the present study focuses on the screening of effective PGPR isolates with multiple traits related to biocontrol of phytopathogenic fungi. In the present study, 88 isolates from the rhizospheric soil samples of different crops were screened initially on the basis of their plant growth promoting activity. These isolates were then tested in vitro for specific PGPR traits such as the production of phosphate solubilizing enzymes, Indole Acetic Acid (IAA), siderophores, Hydrocyanic acid (HCN), salicylic acid, hydrolytic enzymes and biosurfactants. Of the 88 isolates, four strain (8,14,18 and ) number strain was found to be promising for all PGPR attributes. The intrinsic antibiotic test showed that most of the isolate was resistant to tetracycline (30µg/ml), chloramphenicol (30µg/ml). One secondary metabolite phenazine was identified through spectrophotometry.

5. REFERENCES


strains that produce exopolysaccharide during growth on and detoxification of olive mill wastewaters. *Bioresour Technol.*, 99, 5640–5644.


