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# Isolation, screening and characterization of plant growth promoting bacteria and their antifungal effect on *Rhizoctonia Solani* (J.G. Kühn 1858)

## Abstract

Rice is a major crop in developing world, where quantity and quality has been affected by many microbial diseases. The management of disease and enhance the production, many agrochemicals being used, but it is not economically affordable and also hazardous to environment. The identification of bio-control agents is necessary for agricultural practice. Utilization of plant growth promoting bacteria (PGPB) is to increase the productivity may be a viable alternative to inorganic fertilizers. The main goal of this study is to reduce the pollution and preserve the environment by ecofriendly agriculture practice. Plant growth promoting bacteria (PGPB) influence the plant growth by various ways viz., direct or indirect mechanisms. Keeping in this view, the present investigations were undertaken to screen the PGP isolates from various ecological niche in and around Tamil Nadu, India. Totally 32 bacteria were isolated from various environmental sources. The isolates were identified as *Pseudomonas aeruginosa* based on morphological and biochemical characters. The isolates exhibited antagonistic activity towards the fungal pathogen, *Rhizoctonia solani* causative agent of rice sheath blight disease. In this study 32 antagonistic isolates were tested for their PGP traits, extracellular cellular enzyme and siderophore production. Among the tested *Pseudomonas aeruginosa*, SS 14, 15 and 16 showed all the PGP activity except Phosphate (P)-solubilization and 3 strains also showed antifungal activity against fungal rice pathogen. The study, suggests that isolates used as inoculants/bio-fertilizers for rice cultivation; it enhance the growth of rice due to production of IAA, siderophore and also having antifungal activity against *R. solani* rice pathogen.

**Keywords:** PGP, antifungal activity, siderophores, *pseudomonas aeruginosa*, rhizoctonia solani

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## Introduction

The use of chemicals in controlling the plant pathogens resulted in accumulation of harmful chemical residues on environment and ill effect to non target organisms. Considering the effect of synthetic controllers in disease management has lead to the search of alternative agents. The control of plant pathogen by biological method was considered as a potential control agent in recent years. The most commonly used biocontrol agents are *Bacillus* and *Pseudomonas* genera which have important traits in plant growth promotion Santoyo et al.[1] *Pseudomonas* is a common inhabitant in a wide variety of environment; it has played a major role in the plant growth promotion, induced systemic resistance, biological control etc., Yuliar et al.,[2] Of these fluorescent *Pseudomonas* which have been studied for decades as model organisms of biological control to plant disease. Currently, there are three commercial formulations of *Pseudomonas* were registered. The biocontrol product from *Pseudomonas* sp., are relatively easy to apply, and they can be integrated with conventional products for disease control Stockwell et al.[3] Biocontrol bacterial species has an array of mechanisms such as antibiosis, competition, hydrogen cyanide and siderophore production, which is antagonistic to pathogens Singhet al.[4] Non-pathogenic *Pseudomonasstrains* have been used in biological control of different plant diseases. *Pseudomonas aeruginosa* used for control the Fusarium wilt disease in tomato by induction of systemic resistance Fatima et al.[5] Hence the present study was aimed to evaluate the production of IAA, HCN, Siderophore, Phosphate solubilization and antagonist activity of *Pseudomonas aeruginosa.*

## Materials and method

### Isolation and preliminary screening of the bacterial

Total 32 samples were collected from various locations in Tamil Nadu, out of 32 samples, 18 samples from oil contaminated soil, 3 samples from sea water, 2 samples from drinking water, 9 samples from paddy field (Table 1). The collected samples were processed within 24h for isolating the *Pseudomonas aeruginosa* by using serial dilution method on Cetrimide agar Medium plates (selective media). The plates were incubated at 35°C for 24-48 h, 32 isolates produced highly bluish green pigmented colonies were picked from these plates and maintained as pure culture at 4°C in nutrient agar slants.

### Isolation of *Rhizoctonia solani*

Diseased rice plants (sheath blight disease) were collected in Pattabiram, Tiruvalluar district of Tamil Nadu, India. The infected sheath was cut into small piece and surface sterilized in 0.1% mercuric chloride solution for few sec, washed in sterile distilled water repeatedly. Then plated into PDA medium in sterile Petri dishes. The plates were incubated at room temperature (27±1°C) for five days and observed for fungal growth. The fungus was subsequently purified, maintained on PDA slant separately.

**Table 1** Latitude and longitude of collected bacterial isolates

| Sample | Samples collected location | Latitude | Longitude | |
|---|---|---|---|---|
| SS1 | Thondiarpet | 13.1272 | 80.29003 | |
| SS2 | Aarani | 13.333 | 80.08562 | |
| SS3 | Periyapalayam | 13.3105 | 80.04703 | |
| SS4 | Vengal | 13.2516 | 80.01177 | |
| SS5 | Thamraipakkam | 13.2196 | 80.02974 | |
| SS6 | Pakkam | 13.1549 | 80.03323 | |
| SS7 | Thiruninravur | 13.1198 | 80.03072 | |
| SS8 | Avadi | 13.1067 | 80.09695 | |
| SS9 | Thiruvallur | 13.2544 | 80.00878 | |
| SS10 | Ponneri | 13.3378 | 80.1929 | |
| SS11 | Thandalam | 12.9995 | 80.11773 | |
| SS12 | Thirumulaivoiyal | 13.1387 | 80.13367 | |
| SS13 | Ambattur | 13.1143 | 80.14806 | |
| SS14 | Tiruttani | 13.1758 | 79.61091 | |
| SS15 | Thirumazhisai | 13.0486 | 80.06187 | |
| SS16 | Sevvapet | 13.125 | 79.96657 | |
| SS17 | Sekadu | 13.1081 | 80.08467 | |
| SS18 | Poonamalle | 13.0473 | 80.09453 | |
| Sea Water | SS19 | Nagapattinam | 10.7656 | 79.84239 |
| | SS20 | Pulicat | 13.4177 | 80.31853 |
| | SS21 | Thoothukudi | 8.76417 | 78.13484 |
| Normal Water | SS22 | Nagapattinam | 10.7656 | 79.84239 |
| | SS23 | Pulicat | 13.4177 | 80.31853 |
| | SS24 | Poonamalle | 13.0473 | 80.09453 |
| Rhizosphere Soil from Paddy Field | SS25 | Thiruvallur | 13.2544 | 80.00878 |
| | SS26 | Kallakuruchi | 11.7387 | 78.96091 |
| | SS27 | Salem | 11.6643 | 78.14601 |
| | SS28 | Cheyyar | 12.662 | 79.54348 |
| | SS29 | Thiruvannamalai | 12.2253 | 79.0747 |
| | SS30 | Thandurai | 13.1126 | 80.06048 |
| | SS31 | Redhills | 13.1992 | 80.19669 |
| | SS32 | Periyapalayam | 13.3105 | 80.04703 |

## Plant growth promoting mechanisms

Totally 32 *Pseudomonas aeruginosa* isolates were subjected to evaluate the ability to produce Indole acetic acid (IAA), phosphate solubilization, Hydrogen cyanide (HCN) and Siderophore.

## Indole acetic acid production

Thirty two *Pseudomonas aeruginosa* isolates were tested for IAA production by the methods of Bricket al.[6] The 32 isolates inoculated in sterilized nutrient broth supplemented with tryptophan (10µg/ml) and incubated at 37°C for 3days in shaker. The cultures were centrifuged at 10,000g for 10min. At 2 drops of orthophosphoric acid and 4ml of salkowski reagent (50ml. 35% of perchloric acid, 1ml of 0.5 M FeCl3 solution) was added to 2ml of supernatant and incubated for 20min. Development of pink to red color formation was considered as positive evidence for IAA production

## Phosphate solubilization

Thirty two *Pseudomonas aeruginosa* isolates were screened for their tri-calcium phosphate (TCP) solubilizing activity on Pikovskaya Agar plates. Isolates were spot inoculated on the agar plate aseptically. All the plates were incubated at 28±2°C for 5-days. A clear zone around a growing colony indicated phosphate solubilization activity by the isolate Singh et al.[7]

## Hydrogen cyanide (HCN) production

Hydrogen cyanide production of 32 *Pseudomonas aeruginosa*

isolates was evaluated by streaking the bacterial isolates on Nutrient agar medium amended with glycine. Whatman No.1filter paper soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed in the lid of each Petri plate. The plates were then sealed air-tight with Para film and incubated at 30°C for 48hrs. A color change of the filter paper from deep yellow to reddish-brown color was considered as an indication of HCN production Baker et al.[8]

### Siderophore production

The siderophore of 32 *Pseudomonas aeruginosa* isolates were studied on Chrome-azurol S-agar medium (CAS) Schwyn et al.[9] The 32 bacterial culture was spot inoculated on CAS agar medium and were incubated at 28°C upto 48h. The siderophore producing bacterial colonies were showed orange color around the colony.

### Antifungal activity

The antifungal activity of 32 isolated *Pseudomonas aeruginosa* (SS1-SS32) were studied against *R.solani*. *Pseudomonas aeruginosa* strain was spreaded in PDA plate. Mycelial plugs (8mm dia.) were taken from 5days old culture of *R.solani* and kept at the middle of fresh PDA plates. The zone of inhibition was measured after 5days by Nagarajkumar et al.[10]

Percent inhibition of mycelia growth was calculated using the following formula

$$\text{Percentage Growth Inhibition} = \frac{\text{Diameter of Control fungus} - \text{Diameter of treated fungus}}{\text{Diameter of Control fungus}} \times 100$$

### Screening of bacterial isolates for hydrolytic enzyme production

**Protease activity:** Thirty two *Pseudomonas aeruginosa* isolates were screened for their proteolytic activities i.e. caseinolytic activities.

**Casein hydrolysis:** The caseinolytic activity of 32 *Pseudosmonas aeruginosa* isolates were evaluated by using casein-agar plate (Composition of milk-agar base: 0.1% peptone, 2.0% agar with 10% of pasteurized skimmed milk was added after sterilization). A loopful of each test culture was streaked into the medium, incubated at 37°C for 48h. Zone of clearance around the colony indicates protease degradation activity Chaiharn.[11]

**Amylase activity:** Thirty two *Pseudomonas aeruginosa* isolates were spot inoculated on starch agar medium and incubated at 30°C for 48h. The plates were flooded with iodine solution and kept for a minute and poured off. Plates were visually screened for appearance of clear zones around the colonies Collins et al.[12]

**Cellulase activity:** Cellulase production was determined by using the method of Wood et al.[13] The 32 *Pseudomonas aeruginosa* isolates were inoculated into the medium containing carboxy methyl cellulose (CMC) and incubated for 2days at 37°C. After cell growth the presence of extracellular cellulase was detected by flooding the plates with 0.3% Congo red solution for 15minutes and plates were destained with 0.1% NaCl for 15minutes. The plates were visualized for halo zone indicating cellulase production.

**Pectinase activity:** The screening of Pectin degrading enzymes was done by inoculate the 32 *Pseudomonas aeruginosa* isolates were grown in pectin amended nutrient medium. The plates were incubated at 37°C for 2days. The incubated plates were flooded with Congo red solution and were washed with 0.1% of NaCl solution in order to remove excess and superficially adhered Congo red dye. The isolates producing Pectinase indicates the appearance of clear halo zone around the colonies Anand et al.[14]

**Lipase activity:** According to Kim et al.[15] lipase assay was done by supplementing the Nutrient agar plates with 1% Tween 80 (sorbitol monooleate; w/v). Thirty two *Pseudomonas aeruginosa* isolates were spot inoculated on to the agar plates and incubated at room temperature (28±2°C) for 24hrs. Appearance of dense opaque around the colony indicated positive for extracellular lipase production.

**Statistical analysis:** The obtained data was subjected to one way ANOVA followed by Duncan's multiple range tests.

## Result and discussion

Since the *Pseudomonas aeruginosa* has been isolated from environmental sources it is not collected from pathogenic area and there are many reports for the use of *Pseudomonas aeruginosa* as biocontrol agent which have been isolated from various environmental sources Vives-Florez et al.[16] A total 32 *Pseudomonas aeruginosa* isolates were isolated from various environmental sources by serial dilution plate method. All 32 *Pseudomonas aeruginosa* isolateswere tested for plant growth promotion traits by qualitative determination of Indole Acetic Acid (Table 2). The maximum production was recorded in SS 14 (23.33mg/ml) followed by SS 16 (21.33mg/ml) and 20mg/ml in SS 15. The least production was recorded in SS 6. The *Pseudomonas aeruginosa* isolates were subjected to hydrogen cyanide production. Out of 32 isolates, 17 isolates produced the hydrogen cyanide. The maximum production was recorded in isolates SS 14, SS 15 and SS 16 (Table 3). Table 3 showed Siderophore production of *Pseudomonas aeruginosa*. Fourteen isolates produced the Siderophore. All the isolated *Pseudomonas aeruginosa* (SS1 to SS32) were studied for phosphate solubilization activity. Among the 32 isolates none of the isolates produced phosphate solubilization activity (Table 3). The cultural characteristic of *R. solani* causative agent of sheath blight was observed in which the colony colour was found to be dark brown to light brown with the sclerotia diameter of 1.5-2.7mm.

**Table 2** Quantification of IAA Production mg/ml from *Pseudomonas aeruginosa*

| Strain | |
|---|---|
| PAS1 | 14.00±2.00ab |
| PAS5 | 16.67±1.53bcd |
| PAS6 | 11.67±2.08a |
| PAS7 | 12.00±1.00a |
| PAS14 | 23.33±1.53f |
| PAS15 | 20.00±1.00de |
| PAS16 | 21.33±2.52ef |
| PAS17 | 17.00±2.00bcd |
| PAS23 | 18.00±1.73cd |
| PAS24 | 16.00±1.00bc |
| PAS25 | 19.00±2.00cde |
| PAS31 | 16.67±2.52bcd |
| F value | 11.248 |
| P value | < 0.001** |

Note ** denotes significant at 1% level; Different alphabet among strains denotes significant at 5% level using Duncan Multiple Range Test (DMRT)

All the 32 *Pseudomonas aeruginosa* isolates (SS1- SS32) were tested for antifungal activity against *R. solani* and zone of inhibition was taken as an indicator of antifungal property in dual culture plate method. Among the 32 isolates, only 15 *Pseudomonas aeruginosa* isolates were showed antagonistic to the test pathogen (Table 4). In which maximum antagonistic activity was recorded in SS14, SS15 and SS16 are belongs to *Pseudomonas aeruginosa* isolated from oil contaminated site. All the *Pseudomonas aeruginosa* isolates exhibited antagonistic activity of 45-75% against test pathogen. In the present study, Table 4 showed growth inhibition effect of different *Pseudomonas aeruginosa* isolates against *R. solani*. The maximum growth inhibition of 76.11% was recorded in SS14 followed by SS16 (75.27%) and 72.57% in SS15. The minimum growth of 40.44% was recorded in SS9. The growth inhibition effect of SS14 and SS16 were statistically similar. The production of lipase, amylase, pectinase, cellulose and protease of 32 *Pseudomonas aeruginosa* (SS 1 to SS32) were studied. Nearly 27 *Pseudomonas aeruginosa* isolates produced lipase, in which 9 isolates SS1, SS5, SS7, SS8, SS 13-SS17 showed maximum production. Nearly 18 of 27 *Pseudomonas aeruginosa* isolates produced amylase, maximum production was recorded in 8 isolates (SS1, SS5, SS7, SS8, SS13-SS16) (Table 5). 15 of 27 *Pseudomonas aeruginosa* produced pectinase and 8 isolates (SS1, SS5, SS7, SS8, and SS13-SS16) produced maximum quantity. Twenty seven *Pseudomonas aeruginosa* isolates produced cellulase, in which eight isolates (SS1, SS5, SS7, SS8, SS 13-SS16) produced maximum quantity. All the 32 *Pseudomonas aeruginosa* isolates produced protease of which 7 isolates (SS1, SS5, SS7, SS8, SS 13-SS15) produced a maximum quantity (Table 5). In the present result agreement with earlier reports. Indole acetic acid produced by more than 80% of bacteria, which have ability to regulate plant growth Jha.[17] However, phytohormones produced by microbes are more effective in plant growth due to their continuous and slow release Gupta.[18] The common PGP traits are considered to be IAA synthesis siderophore production, HCN production (Kamble and Naphade.[19]

Production of extracellular enzymes by microorganisms has an important role in the management of plant pathogens. All the plant growth promoting bacteria exhibit a wide variety of mechanisms, the exact mechanism is not clearly known. The mechanisms such as production of phytohormones, suppression of deleterious organisms and promotion of the mineral nutrient uptake are usually believe to be involved in plant growth promotion Lalande et al.;[20] Glick.[21] Blumer et al.,[22] reported that IAA production promotes plant growth. Siderophores is one of the bio-control mechanisms belonging to PGPB groups under iron limiting condition. PGPB produces a range of siderophore which have a very high affinity for iron, the availability of iron in the environment would suppress the growth of pathogenic organisms including plant pathogenic fungi Whipps.[23]

The bacterial strains produce different hydrolytic enzymes was analyzed because of its important mechanism of fungal inhibition Malleswari et al.[24] HCN production of the bacteria has been postulated to play an important role in the biological control of pathogens Meena et al.[25] Production of hydrolytic enzymes involved in pectin degradation, are the component involved in degradation of fungal cell wall which was observed in the isolates. Proteolytic enzyme production was detected as formation of clear zone around the bacteria. Amylase was also observed in most of the isolates. Similarly, PGP activities among the bacterial strains have been reported by some other workers Djuric et al.;[26] Kumar et al.;[27] Ashokvardhan et al.[28]

**Table 3** Quantification of HCN, Siderophore, Phosphate solubilization from *Pseudomonas aeruginosa*

| S. No. | HCN production | Siderophore production | Phosphate solubilization |
|---|---|---|---|
| PAS 1 | - | + | - |
| PAS 2 | - | - | - |
| PAS 3 | + | + | - |
| PAS 4 | + | + | - |
| PAS 5 | + | - | - |
| PAS 6 | - | - | - |
| PAS 7 | + | - | - |
| PAS 8 | - | - | - |
| PAS 9 | - | - | - |
| PAS 10 | + | - | - |
| PAS 11 | + | - | - |
| PAS 12 | + | - | - |
| PAS 13 | + | - | - |
| PAS 14 | ++ | ++ | - |
| PAS 15 | ++ | ++ | - |
| PAS 16 | ++ | ++ | - |
| PAS 17 | + | - | - |
| PAS 18 | + | - | - |
| PAS 19 | + | - | - |
| PAS 20 | + | + | - |
| PAS 21 | - | + | - |
| PAS 22 | - | - | - |
| PAS 23 | - | + | - |
| PAS 24 | - | + | - |
| PAS 25 | - | + | - |
| PAS 26 | + | - | - |
| PAS 27 | - | + | - |
| PAS 28 | - | - | - |
| PAS 29 | - | - | - |
| PAS 30 | - | + | - |
| PAS 31 | - | - | - |
| PAS 32 | + | + | - |

+: Production;

++: High production;

-: No production.

**Table 4** Per cent growth inhibition of *Rhizoctonia solani* by plant growth promoting *Pseudomonas aeruginosas* bacteria in Dual culture method

| Strain | Mycelial growth (cm) | Percent growth inhibition |
|---|---|---|
| Control 1 | 6.00±0.50$^{h}$ | 0.00±0.00$^{a}$ |
| Control 2 | 7.20±0.30$^{i}$ | 0.00±0.00$^{a}$ |
| SS3 | 1.80±0.10$^{a}$ | 75.24±2.02$^{j}$ |
| SS4 | 2.55±0.05$^{d}$ | 64.71±1.10$^{f}$ |
| SS5 | 3.00±0.10$^{e}$ | 56.91±1.23$^{d}$ |
| SS7 | 2.15±0.05$^{bc}$ | 68.60±1.47$^{gh}$ |
| SS9 | 4.30±0.20$^{g}$ | 40.44±0.81$^{b}$ |
| SS10 | 1.72±0.05$^{ab}$ | 70.71±4.63$^{hi}$ |
| SS11 | 2.20±0.20$^{c}$ | 68.64±0.96$^{gh}$ |
| SS12 | 1.85±0.05$^{f}$ | 64.71±1.10$^{f}$ |
| SS13 | 2.90±0.00$^{e}$ | 60.37±0.96$^{e}$ |
| SS14 | 1.50±0.00$^{a}$ | 76.11±0.24j |
| SS15 | 1.65±0.15$^{a}$ | 72.57±1.50$^{i}$ |
| SS16 | 1.55±0.05$^{ab}$ | 75.27±0.44$^{j}$ |
| SS18 | 2.15±0.15$^{bc}$ | 69.49±0.68$^{gh}$ |
| SS19 | 2.25±0.05$^{c}$ | 67.82±1.98$^{g}$ |
| SS20 | 4.07±0.15$^{fg}$ | 46.00±0.76$^{c}$ |
| F value | 24.93 | 717.086 |
| P value | < 0.001** | < 0.001** |

** denotes significant at 1% level; Different alphabet among strains denotes significant at 5% level using Duncan Multiple Range Test (DMRT)

**Table 5** Production of extracellular enzymes by different isolates of *Pseudomonas aeruginosa.*

| S No | Strain Number | Lipase | Amylase | Pectinase | Cellulase | Protease |
|---|---|---|---|---|---|---|
| 1 | S1 | ++ | ++ | ++ | ++ | ++ |
| 2 | S2 | + | + | + | + | + |
| 3 | S3 | - | + | - | + | + |
| 4 | S4 | + | + | - | + | + |
| 5 | S5 | ++ | ++ | ++ | ++ | ++ |
| 6 | S6 | - | + | - | + | + |
| 7 | S7 | ++ | ++ | ++ | ++ | ++ |
| 8 | S8 | ++ | ++ | ++ | ++ | ++ |
| 9 | S9 | + | + | + | + | + |
| 10 | S10 | + | + | - | + | + |
| 11 | S11 | + | + | - | + | + |
| 12 | S12 | + | - | + | + | + |
| 13 | S13 | ++ | ++ | ++ | ++ | ++ |
| 14 | S14 | +++ | ++ | ++ | ++ | ++ |
| 15 | S15 | ++ | ++ | ++ | ++ | ++ |
| 16 | S16 | ++ | ++ | ++ | + | + |
| 17 | S17 | ++ | + | + | + | + |

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Table Continued..

| S No | Strain Number | Lipase | Amylase | Pectinase | Cellulase | Protease |
|---|---|---|---|---|---|---|
| 18 | S18 | - | - | - | + | + |
| 19 | S19 | - | - | - | + | + |
| 20 | S20 | - | - | - | + | + |
| 21 | S21 | - | - | + | + | + |
| 22 | S22 | + | + | + | + | + |
| 23 | S23 | + | + | + | + | + |
| 24 | S24 | + | - | - | + | + |
| 25 | S25 | + | - | - | + | + |
| 26 | S26 | + | - | - | + | + |
| 27 | S27 | + | - | - | + | + |
| 28 | S28 | + | - | - | + | + |
| 29 | S29 | + | - | - | + | + |
| 30 | S30 | + | - | - | + | + |
| 31 | S31 | + | - | - | + | + |
| 32 | S32 | + | - | - | + | + |

+: Production;
++: High production;
-: No production.

## Conclusion

Our results, 32 isolates were screened for the PGP traits. Among them 14 showed better production of PGP traits and antifungal activity against *R. solani*. Based on the results, the PGPB as used for bio-fertilizer/biopesticides for alternative to chemical fertilizers. Further detail study need to develop a formulation for large scale field application.

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## Conflict of interest

The author declares no conflict of interest.

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