International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
(A Peer Reviewed, Referred, Indexed and Open Access Journal)
DOI: 10.22192/ijarbs Coden: IJARQG (USA) Volume 13, Issue 4-2026

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2026.13.04.008

Isolation and Characterization of *Streptomyces sp*., as Biocontrol agents against *Xanthomonas* and *Ralstonia* Plant Pathogens

Swamidurai, Arul Diana Christie[1]
Aththani Balamurugan, Annapoorani
Department of Microbiology
Sri Ramakrishna College of Arts and Science for Women Coimbatore
Corresponding Author: Dr. S. Arul Diana Christie
Department of Microbiology, 395, Sarojini Naidu Street, Avarampalayam
Coimbatore Tamil Nadu India.
Mail ID: *dianamicro@srcw.ac.in,* Phone: +91 7339088991
ORCID: 0000-0002-6764-4252

Abstract

The excessive use of chemical fertilizers in agriculture has headed to environmental deprivation and reduced crop production, demanding ecological alternatives. This study focuses on the isolation and characterization of soil-dwelling Streptomyces spp., along with Pseudomonas and Bacillus spp., as potential biocontrol agents against phytopathogens Xanthomonas spp. and Ralstonia spp. Rhizosphere soil samples were collected and subjected to serial dilution and followed by culturing. The isolates were identified through morphological, biochemical, and enzymatic profiling. Phytopathogens were isolated from infected citrus and potato samples and confirmed via selective media and biochemical tests. The soil isolates were evaluated for plant growth-promoting (PGP) traits such as phosphate and zinc solubilization, indole-3-acetic acid (IAA), siderophore, ammonia, and hydrogen cyanide production. Extracellular enzymatic activities including cellulase, catalase, and lipase were also assessed. Antagonistic assays demonstrated strong inhibitory effects of the isolates on the pathogens, particularly Bacillus sp. against Xanthomonas spp. Seed germination studies using groundnut and green gram revealed improved germination rates and vigor index following bacterial treatment, with Pseudomonas sp. showing the highest efficacy. These findings underscore the potential of Streptomyces, Pseudomonas, and Bacillus spp. as eco-friendly biocontrol agents and biofertilizers to enhance plant health and productivity.

Keywords: Biocontrol agents, Phytopathogens, PGPB , Biofertilizer.Streptomyces.

Introduction

The continuous growth in the human population increases the demand for food and living space (FAO, 2017). Human activities addressing food shortages and space constraints have negatively impacted the natural environment (Tilman *et al.,* 2011). Environmental issues like pollution, global warming, water scarcity, and the greenhouse effect have become widespread (IPCC, 2021). Plants, which serve as primary producers in food chains, are vulnerable to environmental changes, making them prone to diseases under stressful conditions (Boyer, 1982). To enhance crop yield and ensure a stable food supply, chemical fertilizers were introduced and widely adopted (Savci, 2012). However, these fertilizers pose risks to crops and contribute to environmental pollution (Savci, 2012).

The excessive use of chemical fertilizers has wedged soil properties, leading to acidification and imbalanced pH levels (Guo *et al.,* 2010). These changes have reduced crop productivity, resulting in stunted plant growth (Tilman *et al*., 2002). Consuming chemically fertilized crops has been associated with health issues in humans (Aktar *et al*., 2009). Scientific research has identified plant growth-promoting bacteria (PGPB), which serve as "biological fertilizers" and offer a safer approach to enhancing plant growth (Vessey, 2003). Plant Growth Promoting Streptomyces species (PGPS) promote plant growth while mitigating abiotic stress and plant diseases (Jog *et al*., 2014). Streptomyces sp. produce antibiotics that inhibit harmful pathogens (Barka *et al.,* 2016).

Beneficial bacteria support host plants by enhancing growth and providing protection against diseases (Glick, 2012). Actinobacteria—especially Streptomyces—are recognized for their contributions to the plant rhizosphere (Barka *et al*., 2016). These bacteria produce antimicrobial compounds and employ mechanisms including antibiotic production, fungal cell wall degradation, competition, and hyper parasitism, making them effective biocontrol agents (Vurukonda *et al*., 2018).

Streptomyces contribute to plant growth promotion by colonizing plant tissues without causing disease symptoms and producing phytohormones such as indole-3-acetic acid (IAA), which plays a role in root growth stimulation (Jog *et al.,* 2014).

Streptomyces produce antagonistic compounds like siderophores and secondary metabolites including antibiotics and antioxidants (Butt *et al.,* 2023). Microbial inoculants promote sustainable agriculture by improving plant health and nutrient availability (Qiu *et al.,* 2019).Plant diseases account for significant yield loss (Gohel *et al*., 2006). Chemical fungicides have led to resistant microorganisms; non-pathogenic microorganisms offer a promising solution (Heydary & Pessarakli, 2010). Actinobacteria, particularly Streptomyces, support plant growth and act as biological control agents (Kunova *et al*., 2016).

Streptomyces species produce antimicrobial compounds including toxins, VOCs, and antibiotics, protecting plants from pathogens (Vurukonda *et al*., 2018).

Interactions between Streptomyces and phytopathogens enhance plant defense mechanisms and productivity (Olanrewaju & Babalola, 2019). Streptomyces contribute to soil fertility (Vurukonda *et al.,* 2018). Despite their ability, commercialization remains limited, with only a few biopesticides available due to challenges in formulation and field performance (LeBlanc, 2022).

Materials and Methods

Collection of soil sample:

Rhizosphere soil samples were collected from various garden lands, without stones and hard materials in and around Thindal, Erode District Tamil Nadu India.

Serial dilution & Staining technique

Serial dilution for soil samples were prepared by adding 1 g of the soil sample to 9 ml of sterile water. Then serial dilution up to 10 -10 was done using sterile water. All the dilutions were spread on starch casein agar plates and Nutrient agar plate incubated at 30°C for 7 days. The isolated colonies were selected for the further processes. After incubation, macroscopic analysis was determined based on colony size, aerial mycelium, pigmentation and margin. (Hata *et al*., 2015) followed by gram staining technique.

Isolation of Phytopathogens from citrus canker:

Collection of Plant:

Citrus fruit and citrus leaf showed typical citrus canker infected leaves and fruit were collected from local places of Erode.

Processing of Sample:

Infected leaves were washed in tap water and rinsed with distilled water and blotted dry. Then lightly surface disinfected with 70% ethanol. Then small pieces of tissue bordering necrotic lesions were crushed in one or two drops of sterile saline (0.85% NaCl).

Isolation:

The suspension was streaked on non-selective media YDA(Yeast Dextrose Agar)and incubated at 250- 280°C for 2-5 days after incubation yellow color colonies appeared that was further purified by re streaked to maintain pure culture.

Isolation of pathogen from brown rot potato: (Ahmed et al, 2022)

Collection of sample:

Brown rot sample of potato was collected from, local market were washed in running spout water, surface disinfected by submersion in ethyl alcohol 95% and flamed. The infected potato oozed out was streaked on triphenyltetrazolium chloride medium, and incubated at 28+2°C for 24-48 hrs.

Characterization of the organism:

Identification of Rhizobacteria and Phytopathogens: Identification of isolates:

Basic identification was done using Gram staining techniques. And Biochemical analysis were performed using IMVic Tests.

Carbohydrate fermentation test:

The test cultures were inoculated into sterile carbohydrate fermentation broth with different carbon sources (Glucose, sucrose, Lactose, Mannitol) along with Durham's tube and incubated at 37°C for 24 hours. The color of the medium changes from red to yellow along with gas production indicated the positive result and the results were observed.

Catalase test:

A loopful of 48hr sold slant growth of the bacterium was smeared on a slide and was covered with few drops of hydrogen peroxide (H_2O_2). The reaction will be positive if gas bubbles are produced.

Urease Test:

Urease medium was prepared to added deionized water. The pH was adjusted to 6.7 and autoclaved at 121ºC for 20 minutes except urea. After autoclaved, cooled the media to 50 to 55ºC, then urea base was added into the media and mixed thoroughly. The tubes were slanted during cooling until solidified. The isolates were inoculated into the slant and incubated at 37ºC for 16 hours.

Starch hydrolysis:

The starch agar medium was used to carry out the starch hydrolysis. Potato starch (10 g) was added to 1000 ml of nutrient agar. 20 ml of sterilized cool medium was dispensed in to each of the Petri plates. After solidifying, starch agar plates were

spot inoculated with loopful culture of the bacterium and incubated for five days at 27±1°C. After incubation period is over, the plates were flooded with Lugol's iodine solution and observations were drawn for starch utilization by the bacterium.

Potassium hydroxide (KOH) solubility test:

A loop full of bacteria was aseptically removed from culture plates with tooth pick, placed on glass slide in a drop of KOH (3%) solution and stirred for ten second using a quick circular motion of hand, then the tooth pick was raised a few centimeter's above the slide and observed for the formation of viscid strand represent the bacterium as Gram-negative.

Fluorescence test:

Test bacterium was streaked on Petri plate containing Kings 1 medium (Protease peptone medium 20 g, Glycerol 10 ml, K_2HPO_4 1.5 g, $MgSO_4$ 1.5 g, Agar 15 g) and after two days incubation plates were observed under fluorescent microscope for further studies.

Levans test:

Levan is an extracellular bacterial polysaccharide (bête-2, 6-1 linked D-fructan), whose potential and actual uses are similar to those of dextran .Induction of the Levan was performed in NA medium containing 5% sucrose, Levan sucrase (E.C. 2.4.1.10), which catalyzes the synthesis of Levan form sucrose, is produced by a number of bacteria including R. solanacearum. The result showed that all group of R. solanacearum isolates were able to produce distinctive domed shaped or round colonies due to production of levan in sucrose containing NA medium (Table 3). When the bacteria were grown on a medium containing sucrose, the production of an extracellular enzyme (levan sucrase) was induced and sucrose was converted to levan and glucose. During the fermentation process, the bacteria also utilize sucrose for maintenance and growth. Levan production was also reported by soil-borne bacterium, Bacillus licheniformis by Ghaly et al. (2007).

Nitrate test:

The test cultures were inoculated into sterile nitrate broth and incubated at 37º C for 24 hours. After incubation, red color which appeared almost immediately after the addition of nitrate reagent A and B containing sulphanline and alpha – naphthalamine indicated positive result. The result were results.

Assessment of growth promoting characters of Rhizobacteria

Screening of Rhizobacteria for Siderophore production-Qualitative analysis (Yeole & Dube, 2000):

The cell free culture supernatant 0.5% was added to 0.5ml of 0.2% aqueous ferric chloride solution. Appearance of orange or reddish brown colour indicated the presence of siderophore.

Screening for plant growth promoting activity:

Phosphate Solubilization:

Pikovskya's Agar was inoculated with the isolates and incubated at 36±2°C for five days. Formation of halo zone indicated phosphate solubilization. It was calculated by following formula,

PSE (Phosphate Solubilization Efficiency) = Z C x 100 Z- Clearance zone including bacterial growth,C- Colony diameter Production

Production of Indole acetic acid (IAA) :

Bacterial cultures were grown for 72 h in King's B broth at 36±200 C. Fully grown culture was centrifuged at 3000 rpm for 30 min. The supernatant (2 mL) was mixed with two drops of Orthophosphoric acid and 4 mL of the Salkowaski reagent (50 mL 35% of perchloric acid, 1mL 0.5 M FeCl3 solution). The development of pink colour indicates IAA production.

EPS Production:

A qualitative assessment of the exopolysaccharide synthesis by a subset of bacterial isolates was conducted by Paulo et al. (2012). Each strain was inoculated onto 5-mm diameter paper discs in the medium (2 % yeast extract, 1.5 % K_2HPO_4, 0.02 % $MgSO_4$, 0.0015 % $MnSO_4$, .0015 % $FeSO_4$, 0.003 % $CaCl_2$, 0.0015 % NaCl, and 1.5 % agar) was adjusted by adding 10 % saccharose, resulting in a pH of 7.5. The production's standout elements were the size of the generated halo and its slime-like texture. EPS generation by bacterial isolates was verified by combining mucoid material with 2 mL of 100 % ethanol and observing precipitate formation.

Zn Solubilization:

The zinc solubilization test was determined by Saravanan et al. (2007). The isolates were grown on the medium containing dextrose: 10.0; $(NH_4)_2SO_4$: 1.0; KCl: 0.2; K_2HPO_4: 0.1; $MgSO_4$:0.2; pH: 7.0; insoluble Zinc compounds (i.e., $ZnCO_3$): 0.1 %; and agar after incubation at 30 ◦C for 48 h. The formation of the clearance zone around the colonies indicates Zinc solubilization.

Ammonia production:

Overnight grown bacterial cultures were inoculated in 10ml peptone broth and incubated at 30±0.1°C for 48hrs in incubator shaker. After 0.5ml of nessler's reagent was added. The development of faint yellow to dark brown colour indicated the ammonia production.

Determination of Nitrogen-Fixing Capacity, Potassium-Solubilizing capacity:

The strains were activated by streaking and inoculated into LB liquid medium for overnight incubation until they reached the logarithmic phase, and the bacterial suspensions were prepared. Afterward, 2.5 μL of the suspension was spotted on nitrogen-free Ashby medium and fermentation medium. The growth and size of the hydrolysis circle were observed to determine the strains' abilities in nitrogen fixation, potassium solubilization production. (Kizilkaya *et al*., 2008).

Screening for biocontrol activity

HCN production

Nutrient agar was amended with 4.4 g glycine liter1 and bacteria were streaked on modified nutrient agar plate. A Whatman filter paper no.1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with para film and incubated at 36±20°C for 4 days. Development of orange to red color indicates HCN production. (Castric & Castric, 1983)

Antagonistic activity of Rhizobacteria against phytopathogens. Antimicrobial activity assay:

Test organisms:

Antimicrobial activity of the rhizobaterias (Pseudomonas Streptomyces, Bacillus) were carried out against phytopathogen, Xanthomonas sp. and Ralstonia sp. The plates were incubated at 37°C for 24 hrs. In the case of bacterial pathogen they were observed for a zone of growth inhibition and the isolates which showed positive antimicrobial activities.

Screening for enzyme activity (Extracellular enzyme activity)

Amylase-Starch hydrolysis:

The sample was spot inoculated on starch agar medium plates and incubated at 30˚C for 48 h. At the end of incubation period, the plates were flooded with iodine solution, kept for a minute and then poured off. Iodine reacts with starch to form blue color compound. This blue colour fades rapidly. Hence the colorless zone surrounding colonies indicates the production of amylase.

Protease -Caseinase activity:

The qualitative assay of protease production was performed on sterile skim milk agar plates.

Sample was spot inoculated and followed by incubation at 30˚C and zone of clearance around the colony indicating the enzymatic degradation of protease.

Lipase activity:

The sample was inoculated on a tributytrin agar medium and incubated at 37℃ for 72h. The observation for a clear zone was examined around each isolates throughout the incubation period. The clear zones around the colonies indicated a positive result.

Catalase test:

Catalase test was performed by taking a 3- 4 drops of hydrogen peroxide (H_2O_2) was added to 48 h old bacterial colony which is grown on trypticase soya agar medium. The effervescence indicated catalase activity.

Cellulase activity:

Cellulase production was determined by using the method (Miller G.L 1959). M9 agar medium with yeast extract plates were inoculated with individual bacterial isolates and incubated for 3-5 d at 28℃. Bacterial growth surrounded by clear halos was considered as positive indication of cellulase production.

Phytotoxicity:

Effect of bacterial seed treatment on germination and vigor index in Groundnut and Greengram.

The effect of bacterial seed treatment on germination and vigor index in Groundnut and Green gram were determined slighty altered method of Priya *et al*., (2019) under laboratory .In laboratory condition, 7 for ground nut and 10 for green gram seeds inoculated with each antagonistic isolate were incubated in a sterile plastic container (30 cm diameter) impregnated with sterile moistened tissue paper (45cm×28cm). To maintain sufficient moisture, 5ml of sterile distilled water was added into the container daily to promote germination. The seeds were incubated at 28±2℃ in light incubator .seed treated with sterile distilled water was used to Control. The germination percentage, root, shoot length were determined after 10 days of growth condition.

Germination Rate % = [(Number of seed germinated)/(Total number of seeds)]×100

Vigor index = Germination rate × Total plant length

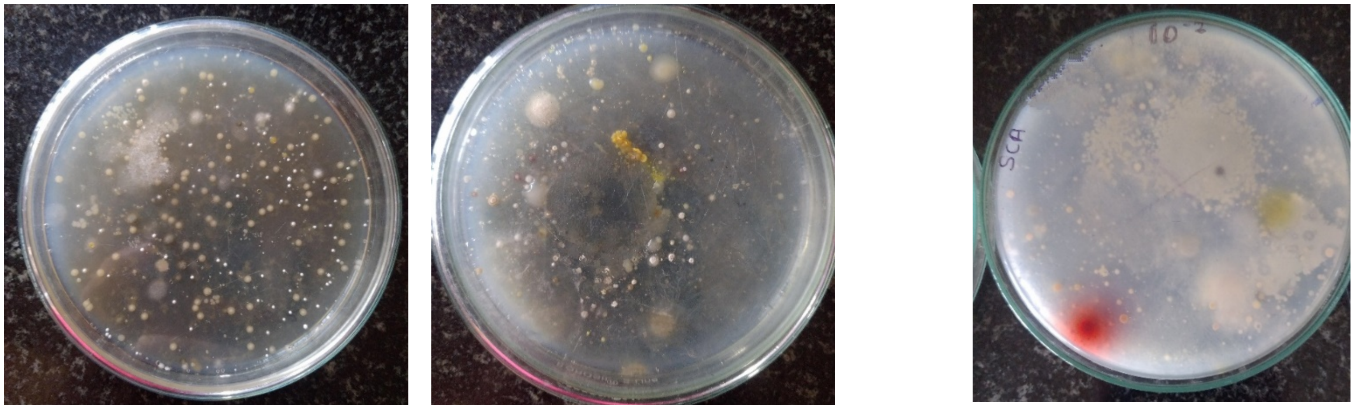
Results

Isolation of soil bacteria:

Phenotypic characterization:

Soil samples were collected from different locations and it was serially diluted and plated on starch casein agar and nutrient agar. After 7days incubation, Streptomyces was screened by morphological characterization based on colony size, aerial mycelium, substrate mycelium, pigmentation, and margin.

Plate :1 Screening of *strepyomyces sp* on Starch casein agar



The various pigment for the strains ranging from cream-yellow to brown, with creamy colonies were observed (table-1)(plate-1). Microscopic examination revealed that they are gram-positive, filamentous with branching mycelia.

Table :1 Screening of *Streptomyces sp* on Starch casein agar

S.NO	Enrichment soil sample	CFU/G
1	S1	To numerous to count
2	S2	To numerous to count
3	S3	To numerous to count

Two different bacteria were isolated from the soil by means serial dilution on nutrient agar medium, and pure colonies were obtained. One of the isolates showed gram-positive rods, while the other one was gram-negative rods.

Biochemical characteristics

The biochemical characterization of Streptomyces sp and other two bacterial isolates were shown in Table 2. All the three strains could reduce nitrate, utilize citrate, and hydrolyze casein, starch. Starch was hydrolysed by strains S_2 and S_3. Indole production was negative for all strains. The VP test was also negative. The strains fully utilize Glucose, Mannitol, and Sucrose. The S1 was identified as Pseudomonas sp, S2 was identified as Streptomyces sp and S3 was identified as Bacillus sp.(table -2) (plate-1)

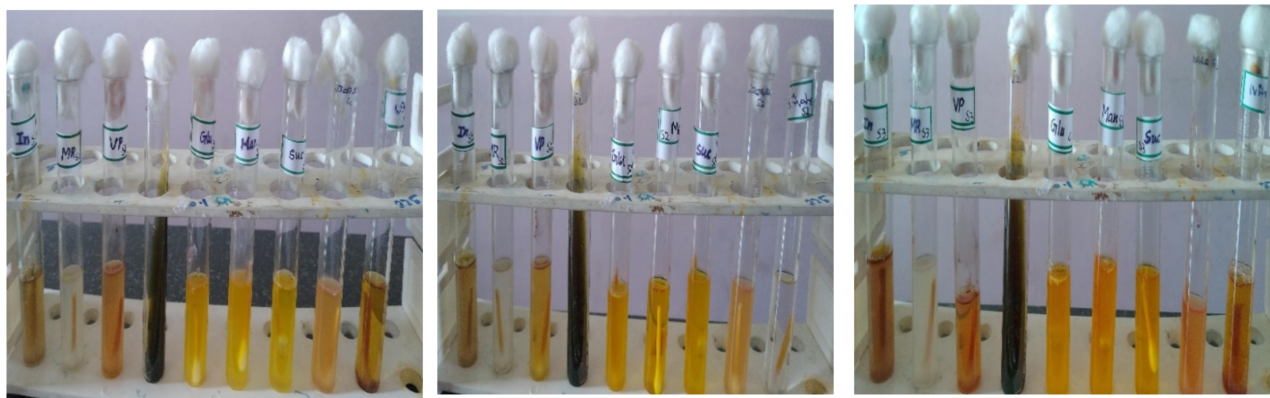


Table :2 Biochemical characterization of soil isolates

S.NO	Test parameters	S1	S2	S3
1	Indole	-	-	-
2	Methyl red	-	-	-
3	Voges Proskauer	-	-	-
4	Citrate utilization test	-	-	-
5	Urease	-	-	-
6	Glucose fermentation	+	+	+
7	Mannitol fermentation	+	-	+
8	Sucrose fermentation	+	+	+
9	Starch hydrolysis	-	-	-
10	Casein hydrolysis	+	+	+
11	Nitrate reduction test	+	-	+
12	Probable identify to genus level	*Pseudomonas sp*	*Streptomyces sp*	*Bacillus sp*

Isolation and identification of phytopathogens:

The pathogen Xanthomonas spp. was isolated from infected citrus leaf and citrus fruit (Picture 3.1). Infected parts were made into suspension and streaked on YDC (Yeast dextrose carbonate agar) agar medium. That showed pale yellow dry colony by (x1) and dark yellow mucoid colony (x2) on YDC agar (Table 3.2 Plate-3.2.1). The pathogen Ralstonia spp. was isolated from brown rot potato. The suspension was streaked on triphenyltetrazolium chloride medium that showed pink spots in the middle of the colony with a white edge (Plate 3.2.2).

Picture :3.1 Total number of isolates from each sample

Table:3.2 Morphological characterization of isolates

S.NO	Sample	No.of.isolates	Sample code
1	Lemon leaf	1	X1
2	Lemon fruit	1	X2
3	potato	1	X3

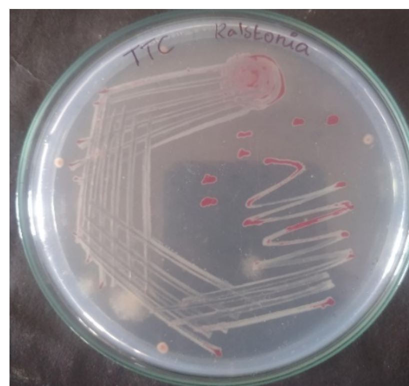
Table: 3.2 Morphological characterization of isolates

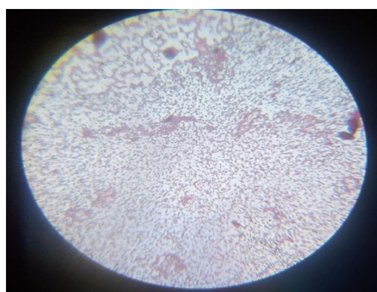
S.NO	Isolates	Colony characteristics	Gram's reaction
1	X1	Pale yellow, dry colonies(YDA)	Gram negative rod
2	X2	Dark yellow, mucoid colonies(YDA)	Gram negative rod
3	X3	Pink spot in the middle of the colony and a white edge(TTC)	Gram negative rod

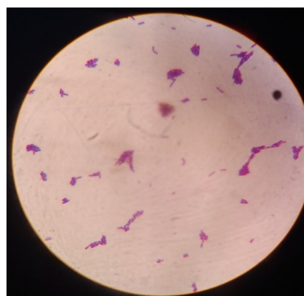
3.2.1 *Xanthomonas sp and Ralstonia sp*

Table 3.3 described the basic characterization of Xanthomonas spp.. It produced non-fluorescence on King's medium after incubation, and produced levan, which was confirmed by mucoid colony on nutrient medium with 5% sucrose. On KOH test, it produced stringy (or) slime-like thread which confirmed it as gram positive. The strains ferment glucose, sucrose, mannitol and able to hydrolyse starch. These all tests confirmed the Xanthomonas spp. (Plate-3.3). Table 3.4 explained the biochemical characterization of Xanthomonas spp. Based on Bergey's Manual of Systematic Bacteriology. The probable genus-level identification was confirmed for Xanthomonas spp.. The results showed citrate and catalase were positive, other were negative (Plate-3.4).

Table :3.3 Basic characterization of *Xanthomonas sp* isolates







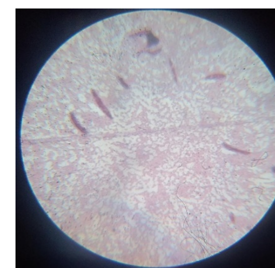
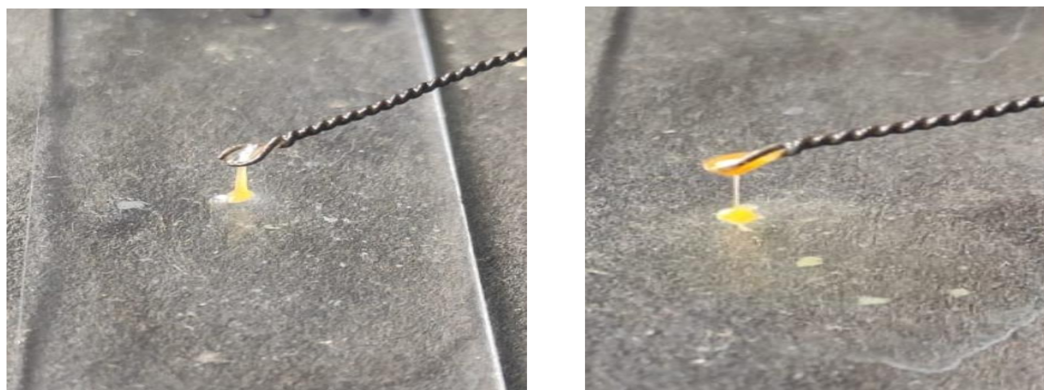


Table :3.3 Basic characterization of *Xanthomonas sp* isolates

S.No	Test parameters	Isolates	
		X1	X2
1	Non fluresence on king'B medium	-	-
2	Levan production	+	+
3	KOH test	+	+
4	Mannitol fermentation	+	+
5	Starch hydrolysis	-	-
6	Sucrose fermentation	+	+
7	Glucose fermentation	+	+

Plate 3.3 Non fluresence on king'B medium

3.3.1 Levan production Xanthomonas – Citrus Leaf   Xanthomonas – Citrus Fruit



3.3.2 KOH test

Table :3.4 Biochemical characterization of *Xanthomonas sp*

S.No	Biochemical test	Isolates	
		X1	X2
1	Indole	-	-
2	Methyl red	-	-
3	VogesProskauer test	-	-
4	Citrate utilization test	+	+
5	Urease test	-	-
6	Nitrate reduction test	-	-
7	Oxidase test	-	-
8	Catalase test	+	+
9	Probable identify to genus level	*Xanthomonas sp*	*Xanthomonas sp*

Ralstonia sp was obtained from brown rot potato sample. It produce pink spot in the middle of the colony and a white edge on TTC agar. The Gram stain showed Gram negative test of Ralstonia sp was also confirmed by potassium hydroxide solubility test. The positive test indicated by a elastic thread observed when loop raised from the bacterial solution by the tooth pick a few centimetre from glass slide. It indicated isolates are Gram negative. (Table -3.5) (Plate-3.5).

Plate: 3.5 Biochemical characterization of *Ralstonia sp*

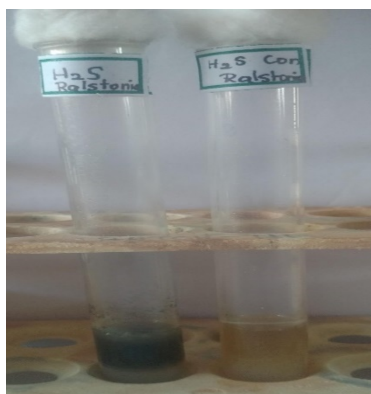
Nutrient agar

Levan test

KOH test

Starch hydrolysis test

H2S production



Glucose

Table: 3.5 Biochemical test of *Ralstonia sp*

S.NO	Test parameters	Result
1	Nutrient agar	Smooth and dirty white raised colonies
2	KOH	+
3	Starch	-
4	H_2s production	+
5	Glucose fermentation	+
6	Sucrose fermentation	+
7	Mannitol fermentation	+
8	Lactose fermentation	+
9	Probable identify to genus level	*Ralstoniasps*

Induction of the levan was performed in NA medium containing 5% sucrose, levan sucrase which catalyzes the synthesis of levan form sucrose. The result showed that Ralstonia sp isolates were able to produce distinctive domed shaped or round colonies due to production of levan in sucrose containing NA medium. When the bacteria were grown on a medium containing sucrose, the production of an extracellular enzyme (levan sucrase) was induced and sucrose was converted to levan and glucose. During the fermentation process, the bacteria also utilize sucrose for maintenance and growth.

The isolates of Ralstonia sp is able to oxidize the sugars which are indicated by colour change (reddish to yellow).the results of sugar fermentation test clearly showed that all groups of Ralstonia sp isolates obtained from the potato rot were able to oxidize the four basic sugars (Dextrose, sucrose, mannitol and lactose) by producing acid and gas. The acid production in sugar fermentation test by bacterial isolates was indicated by the colour change from reddish to yellow, gas production was noted by the appearance of gas bubbles in the inverted Durham’s tubes and the oxidation of sugar mannitol by the bacterial isolates indicated by the production of yellow to red colour.

Assessment of plant growth promoting characters of soil isolates:

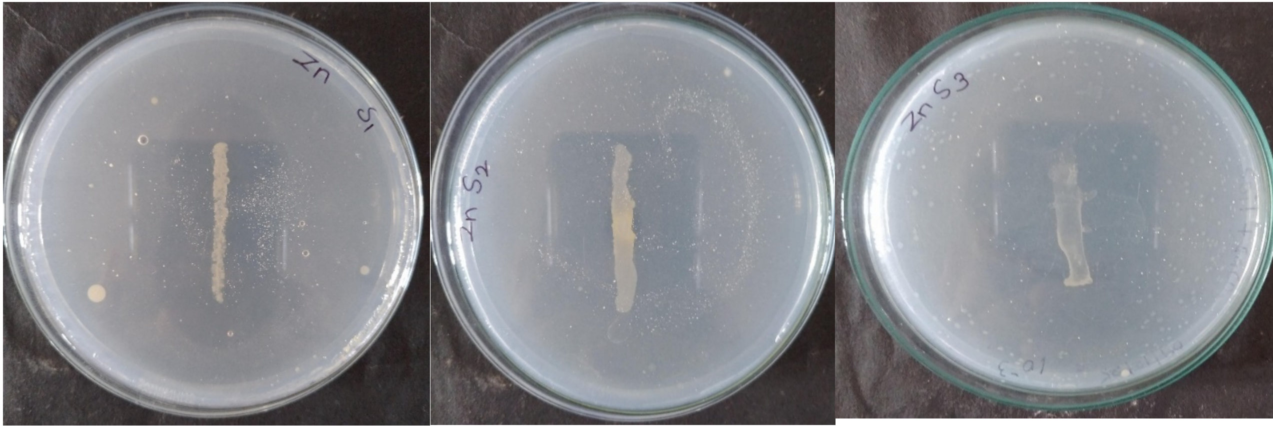
Table 3.6 revealed that isolate was positive for growth promoting characters. i.e., isolate produced clear zone on Pikovska's agar that confirmed phosphate solubilization character. On addition of Salkowski reagent into 72 hrs old king's broth culture its produce pink red colour indicated the production IAA and while adding Nessler's reagent into culture broth a development of Faint yellow to Dark brown present ammonium production. At last development of Orange to Red colour on Whats man Filter paper:1 that was placed on the lid of petriplate which was streaked by isolate as a positive result for HCN production (Plate 3.6).

Plate 3.6..Hydrogen Cyanide Test

Table: 3.6 Assessment of plant growth promoting characters of soil isolates

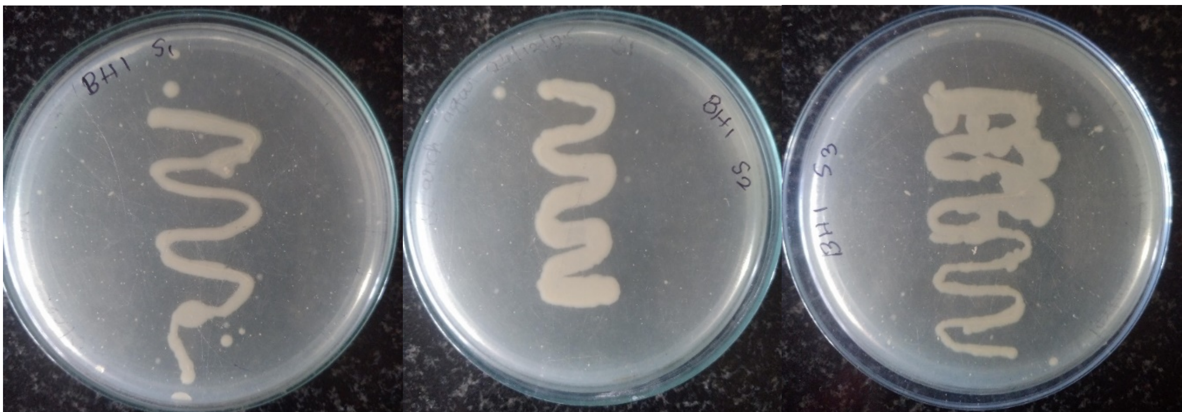
S.no	Test parameters	Colour appearance	*Pesudomonas*	*Streptomyces*	*Bacillus*
1	Indole acetic acid	Pink color	+	+	+
2	Nitrogen fixation test	Growth formation	-	-	-
3	Hydrognen cyanide test	Red colour	+	+	+
4	Ammonia production	Dark brown colour	+	+	+
5	Zinc solubilization	Zone formation	+	+	+
6	Phosphate solubilization	Zone formation	+	+	+
7	Potassium solubilization	Zone formation	-	-	-
8	Eps production	Zone formation	+	-	+
9	siderophore	Reddish brown	+	+	+

3.6.4 Zinc solubilization



3.6.5 Phosphate solubilization

3.6.6 EPS production



3.7. Siderophore production

Incubation of strains on nitrogen free Ash by medium for 4 days showed no growth that indicated, strain had no significant nitrogen fixation capacity. The Zinc solubilization test showed clear zone around colonies confirm the Zinc solubilization positive.EPS production was checked qualitatively by spot inoculation on BHI agar plate it showed positive for Pseudomonas sp, Bacillus sp by producing mucoid colonies.

Potassium solublization was confirmed by clear zone formation around colonied. The extracellular enzyme activity was depicted in table 3.7. Amylase activity was absent in three soil isolates. Cellulase and Catalase activity was positive for 3 isolates (Pesudomonas sp, Streptomyces sp, Bacillus sp). Bacillus sp produce lipase enzymes it produced zone around colonies (plate3.7).

Table : 3.7 Assessment of extracellular enzyme activity

S.NO	Test	Isolates		
		Pseudomonas	***Streptomyces***	***Bacillus***
1	Amylase activity	-	-	-
2	Cellulase activity	+	+	+
3	Lipase activity	-	-	+
4	Casinase activity	-	-	-
5	Catalase activity	+	+	+

The antagonistic activity of soil bacteria against phytopathogen was determined by well cut method (Table 4) (Plate-4). The maximum zone of inhibition was found against Xanthomonas sp 1 by Bacillus sp (15mm) followed by 14mm obtained to Xanthomonas sp 2 by Psedomonas sp.
.

Table: 4 Antagonistic activity of soil isolate against *Xanthomonas sp* and *Ralstonia sp*

S.No	Soil isolate	Zone of inhibition		
		***Xanthomonas sp* 1**	***Xanthomonas sp* 2**	***Ralstonia***
1	*Pseudomonas sp*	10mm	14mm	R
2	*Streptomyces sp*	R	13mm	11mm
3	*Bacillus sp*	15mm	10mm	R

R: Resistance

The effect in Groundnut and Green gram germination and vigor index using Steptomyces sps, Pseudomonas sp, Bacillus sp-treatments varied among the different isolates used. A significant effect on the vigor index (VI), germination rate (GR), Root Length (RL)and shoot length (SL) compared to the control were detected from all different treatments (table 5) (Plate 5) (Graph 5,Graph 5.1,5.2)

Table :5.5 Effect of bacterial seed treatment on germination and vigor index (Ground nut)

S.NO	Growth measurement	Bacteria used for seed treatment		
		Pseudomonas sps	***Streptomyces sps***	***Bacillus sp***
1	Growth rate(%)	100	100	100
2	Relative shoot length(%)	80.35	117.85	91.05
3	Relative root length(%)	86.43	77.32	95.54
4	Vigor index	124.45	84.85	109.28

The vigor index increased up to 124.45% and 139.62% in Pseudomonas sp treated, 84.85% and 101.37%in Streptomyces sp treated, 109.28%and 73.27% in Bacillus sp treated Ground nut and greengram under laboratory condition compared to control. Streptomyces sp, Pseudomonas sp, Bacillius sp treatment improved the growth rate of ground nut and green gram seed 100%under lab condition .

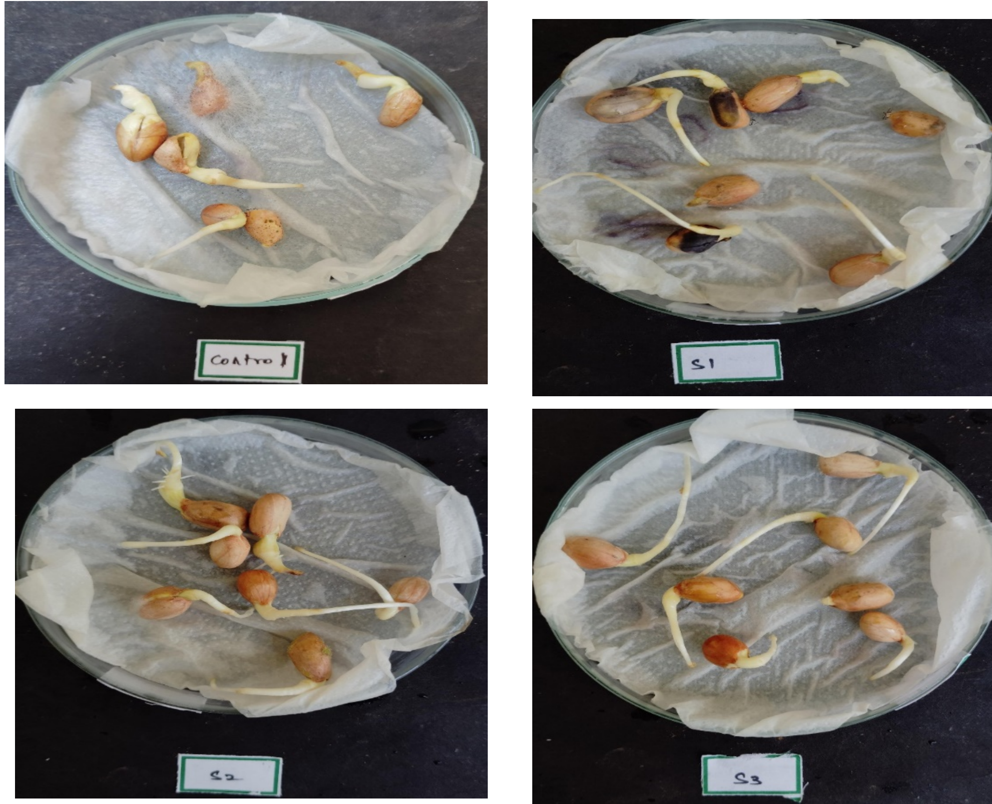


Plate: 5.1 Effect of bacterial seed treatment on germination and vigor index (Ground nut)

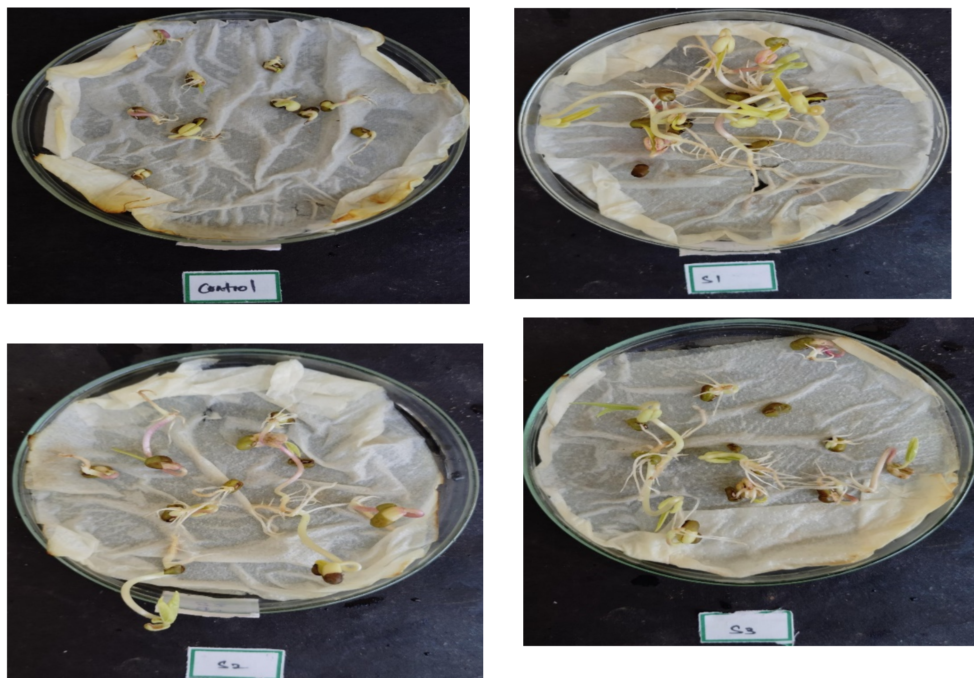


Table :5.6 Effect of bacterial seed treatment on germination and vigor index (Green gram)

S.NO	Growth measurement	Bacteria used for seed treatment		
		Pseudomonas sps	*Streptomyces sps*	*Bacillus sp*
1	Growth rate(%)	100	100	100
2	Relative shoot length(%)	71.62	98.64	136.48
3	Relative root length(%)	80	93.33	86.66
4	Vigor index	139.62	101.37	73.27

The improvement of RL using Streptomyces sp., Pseudomonas spp. and Bacillus spp. showed an overall increment of more than 77.32% (2.0cm) in ground nut, 80%(1.5cm) in green gram and followed by SL improvement showed more than 71.62% (4.5cm) in green gram, 117.85%(9.0 cm) in ground nut. Table 5. These results suggested that treatments with bacterial rhizobacteria could improve the germination & vigor of Groundnut & Green gram.

Discussion

Phenotypic and Microscopic Characterization:

The morphological characters such as aerial and substrate mycelia, pigmentation, and colony margins facilitated in the identification of Streptomyces species, which are well-known for their filamentous structures and spore formation. The additional two isolates Pseudomonas sp. (Gram-negative rods) and Bacillus sp. (Gram-positive rods) revealed typical colony morphology consistent with their respective genera.

Biochemical Characterization:

All three isolates displayed versatility against the substrates used in biochemistry by being able to casein and starch hydrolyze, and they also demonstrated positive nitrate reduction and citrate utilization (metabolic versatility) as outlined in Biochemical Assays (Table 2). The identification of Streptomyces, Bacillus, and Pseudomonas spp. was justified by standard biochemical profiles as aligned with Bergey's Manual of Systematic Bacteriology. Also, the lack of positive results for indole and Voges- Proskauer (VP) tests was useful in restricting genus level identification of such isolates.

Isolation and Identification of a Pathogen

The isolation of Xanthomonas spp. from citrus and Ralstonia spp. from potato rot was sufficient to diagnose important phytopathogens within the diseased plant tissue. Pathogens from these genera were identified based on colony morphology on selective media (YDC and TTC), a series of biochemical tests (levan production, oxidase test, and sugar fermentation), and KOH string tests. It should be noted that the string test that misidentified Xanthomonas as Gram-positive would ordinarily be correct since Xanthomonas is Gram-negative, as KOH test results would suggest.

Characteristics of Plant Growth-Promoting Agents

Soil bacterial isolates exhibited several plant growth-promoting traits (Table 3.6) such as phosphate and zinc solubilization, IAA (Indole acetic acid) production, ammonia, and HCN (hydrogen cyanide) production. These properties contribute positively towards the enhancement of nutrient accessibility and limitation of soil-borne pathogens. All of the isolates did not demonstrate any ability of nitrogen fixation which shows that

the PGP effect of such isolates was most probably because of different approaches. EPS (Extracellular polysaccharide) production by Pseudomonas and Bacillus sp. indicates their ability to form biofilm which increases the chances of bacterial colonization in the rhizosphere region.

Mendes R (2024) studied the role of exopolymeric genes in modulating the tomato rhizosphere microbiome. The research highlighted that EPS production is crucial for root colonization and persistence in the rhizosphere, emphasizing the importance of biofilm formation in plant–microbe interactions. Ma *et al.,* (2022) focuses on the regulation of biofilm exopolysaccharide biosynthesis and degradation in *Pseudomonas aeruginosa*. While primarily about a pathogenic strain, the insights into EPS production and biofilm formation are relevant to understanding similar processes in beneficial PGPB.

Conclusion

This study, focused on the in vitro activities of a potential PGP of Streptomyces sp, Pseudomonas sp, Bacillus sp from Rhizosphere soil a safe potential biocontrol agent against Xanthomonas sp and Ralstonia sp. These analyses facilitated the best selection of the PGP Streptomyces candidates. Plant growth promotion assays also demonstrated the growth-promoting effects of seeds treated with the Streptomyces spp. Pseudomonas sp, Bacillus sp, and treatments were supported by their enzymatic activities and PGP traits. Results have indicated the potential of Streptomyces sp, Pseudomonas sp, Bacillus sp as a biocontrol agent against Xanthomonas sp, Ralstonia sp and plant growth-promotors in Green gram and Ground nut offering the applicability of Streptomyces spp. in improving plant production.

Acknowledgments

The authors would like to thank AWE biocare Private Limited, Erode for providing constant support and technical assistant for this work.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

Dr.S.Arul Diana Christie to the literature search for related studies involved in the literature review, in the writing of the manuscript, in the design of the study and in the interpretation of the data obtained from the literature. Ms.A.B. Annapoorani was involved in data analysis and interpretation

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How to cite this article:
Swamidurai, Arul Diana Christie, Aththani Balamurugan, Annapoorani. (2026). Isolation and Characterization of *Streptomyces sp*. as Biocontrol agents against *Xanthomonas* and *Ralstonia* Plant Pathogens. Int. J. Adv. Res. Biol. Sci. 13(4): 85-105.
DOI: http://dx.doi.org/10.22192/ijarbs.2026.13.04.008