

Isolation and characterization of plant growth promoting actinobacteria, *Amycolatopsis samanea* (AM75), from the rice rhizosphere in mirza area, Assam, India

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World Journal of Advanced Research and Reviews, 2025, 25(01), 1851-1858

Publication history: Received on 09 December 2024; revised on 21 January 2025; accepted on 24 January 2025

Article DOI: <https://doi.org/10.30574/wjarr.2025.25.1.0198>

Abstract

This study aimed to isolate plant growth-promoting actinobacteria from the rhizosphere of rice. Rhizospheric soil samples were collected from the Aijung variety of rice in the Mirza area, Kamrup district, Assam, India. The isolate AM75 identified as *Amycolatopsis samanea*, was characterized as gram-positive and non-motile. It exhibited varied colony characteristics on different selective media. The isolate produce several enzymes including urease, nitrate reductase, lipase, catalase, and amylase but did not demonstrate cellulase or gelatinase activity. Notably, isolate AM75 displayed various plant growth-promoting activities such as siderophore activity, hydrogen cyanide (HCN) production, indole-3-acetic acid (IAA) production, and zinc solubilization. However, it did not produce hydrogen sulfide (H₂S) or solubilize phosphate. The optimal growth conditions for AM75 were pH 7.2, 30°C temperature, and NaCl 2.5% concentration. Additionally, AM75 exhibited inhibitory activity against the plant pathogens *Erwinia chrysanthemi* ATCC11660, *Aspergillus niger* ATCC6275, and *Fusarium oxysporum* ATCC62506. The isolate was resistant to standard antibiotics, including rifampin, streptomycin, ampicillin, and nalidixic acid.

Keywords: Actinobacteria; Plant growth-promoting (PGP); *Amycolatopsis*; Rhizosphere; Rice

1. Introduction

Soil serves as a reservoir of natural products and its microbial diversity plays a crucial role in the production of secondary metabolites, such as flavonoids, terpenoids, steroids, anthelmintic, antitumor agents, insecticides, and immunosuppressants [1]. The rhizosphere, the soil volume adjacent to plant roots, is a zone where diverse microbial communities interact through physical, chemical and biological processes. Root exudates, including various primary and secondary metabolites, organic acids, and polysaccharides, attract numerous microbes and contribute to the unique characteristics of rhizospheric microbial communities [2].

Actinobacteria, which are gram positive, filamentous bacteria with high G+C content, can exhibit both coccoid and rod shaped and produces both substrate and aerial mycelium [3, 4]. These bacteria exhibit characteristics of both fungi and bacteria [5]. Actinobacteria constitute a significant portion of rhizospheric microbiota in various environments and have been isolated from the rhizosphere of a range crops, including Soybean, sugarcane, acacia, black rice, saltbush, and nutmeg [6, 7, 8, 9, 10]. Approximately 45% of all known microbial bioactive compounds are produced by terrestrial actinobacteria [11, 12] and about 70 % of all known naturally occurring antibiotics are derived from actinobacteria, with 55% produced by *Streptomyces* and the remainder by other genera such as *Actinomaura*, *Actinoplanes*, *Nocardia*, *Micromonospora*, *Amycolatopsis*, *Streptosporangium*, and *Thermoactinomyces* [1]. Additionally plant growth promoting actinobacteria protect plants from environmental stress and pathogens. Among these, *Streptomyces* species are the most prevalent plant growth promoting actinobacteria, followed by *Nocardiasp* and *Micromonosporasp* in rhizospheric

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soils [13, 14, 15]. The present study investigates the plant growth promoting activities of *Amycolatopsis samaneae* isolated from rice rhizosphere in the Kamrup district of Assam, India.

2. Materials and methods

Soil samples for this present investigation were collected from the rhizosphere of Aijung variety rice in the Mirza area (26°05'35''N 91°31'55''E), Kamrup District, Assam, India. The soil sample pH was initially measured using a 1:5 water-to-soil ratio. The samples were then air dried, sieved, and stored at 4°C. Before isolating actinobacteria, the rhizospheric soil was incubated with CaCO₃ (10:1 ratio) at 28°C±2°C for 24 hours to promote actinobacterial growth. From this mixture, 1gm was used for serial dilution, and 250 µl of aliquots were spread on Starch casein agar media (SCA) under sterilized conditions in a laminar airflow. The media were supplemented with antimicrobials Nalidixic acid (10µl/ml) and Amphotericin B (2.5µl/ml). The experiment was conducted in triplicates, and the plates were incubated at 28°C±2°C for 7 to 14 days. Colonies with characteristics of actinobacterial morphology were sub-cultured on SCA plates and further purified on Streptomyces agar (SA) plates. Grown slants were stored in 4°C for subsequent experiments.

Gram-positive isolates were streaked on SCA [16], SA, Actinomyces Agar (AA) and Tributyrin agar (TA) [17] to assess colony characteristics including form, color, pigmentation and elevation [18]. The isolate AM75 was subjected to growth tests under various pH conditions (5.2, 7.2 and 9.2) and temperature (20°C, 30°C and in 40°C), as well as salt (NaCl) concentrations (2.5%, 5% and 10%). Carbon source utilization was evaluated using the Himedia rapid biochemical kit KB001, based on pH changes.

Enzymatic activities were assessed for isolate AM75 at the temperature 28±2°C. Catalase activity was determined on Trypticase Soy Agar (TSA) following Gerhardt et al. (1981) [19] and urease activity was tested on Urea agar slants (UA) with phenol red as a pH indicator (pH6.8). A positive urea test was indicated by the development of pink or red color [20]. Hydrogen sulfide (H₂S) production was evaluated in SIM agar deep tubes [21] with black precipitation signifying a positive result [20]. Lipase activity was tested on TA media, with clear halos around the colonies after a 4-day incubation confirming positive results. Gelatin hydrolysis was assessed in Alaksondrov media, substituting agar with 12% gelatin, and gelatin liquefaction was confirmed by refrigeration [22]. Nitrate reduction was tested in nitrate broth with 0.5% KNO₃, with pink to red coloration after adding sulfanilic acid and NN dimethyl -1-nathylamine indicating positive nitrate reduction [20]. Amylase activity was determined on Starch agar media, with clear halos around the colonies after adding iodine staining indicating positive results. Cellulase activity was assessed on Carboxymethyl Cellulose (CMC) media with clear zones around the colonies after congo red staining and washing with NaCl confirming activity.

Plant growth-promoting activities of isolate AM75 were evaluated using various assays. Siderophore production was assessed on chrome Azurol S (CAS) media, with orange to yellow halos indicating positive results [23]. HCN production was tested on Nutrient agar (NA) media amended with glycine. Phosphate solubilization was evaluated using Pikovskaya agar media [24] containing tricalcium phosphate. Zinc solubilization was assessed on Mineral salt agar containing insoluble 0.1% Zinc oxide [25]. Indole acetic acid (IAA) production was measured on YM Broth media containing 0.2% Tryptophan and incubated for 7 days at 28±2°C, with optical density reading at 660nm. Antimicrobial activity was tested against phytopathogens *Fusarium oxysporum* ATCC62506, *Aspergillus niger* ATCC6275, *Erwinia chrysanthemi* ATCC11660 and *Xanthomonas oryzae pv Oryzae* ATCC35933.

For DNA extraction, a mass culture of isolate AM75 was grown in 100 ml ISP2 broth in 500 ml conical flask at 28±2°C for 4 days. Biomass was collected by centrifugation at 8000 rpm, washed twice with sterilized double distilled water, 200 grams taken, and mixed with 800µl of aqueous lysis solution (100mM Tris HCL, pH 7, 20mM EDTA; 250mM NaCl; 2% SDS; 1mg/L Lysogyme). RNase (5µl 50mg/L) added and mixture was incubated at 37°C for 60 minutes. Proteinase K (10µl, 20mg/L) was subsequently added, and incubation continued at 65°C for 30 minutes. The lysate was extracted with equal volume of phenol and chloroform (50-50% v/v). Following the addition NaCl (159 mM final conc) and two volumes of chilled 95% ethanol, the DNA was precipitated and recovered by centrifugation. The precipitated DNA was cleaned with 70% (v/v) ethanol and centrifuged at 7000rpm for 10 minutes. The DNA was resuspended in 5µl TE buffer and stored at -20°C. DNA purity was assessed using a spectrophotometer at 260 nm and 280 nm, and the 16S rRNA gene was amplified using Taq DNA polymerase with primers 243F (GGATGACCCGCGCCTA) and A3R (CCAGCCCTTCGAC).

3. Results

Actinobacteria are ubiquitous microorganism found in diverse environments, including soil, water, and plant tissue. We isolated approximately 200 actinobacterial strains from kamrup district, Assam, India, which were subsequently

categorized into 82 distinct types. Catalase activity was observed in all but three of the isolates. Urease activity was present in 75% of the isolates, while 64% demonstrated gelatinase activity. Nitrate reductase activity was exhibited by 60% of the isolates, and 43% showed lipase activity. Amylase and cellulase activities were detected in 10 and 9 isolates, respectively. Notably 82% of the isolates displayed siderophore, 44% produced hydrogen cyanide (HCN) and 26% showed zinc solubilization. Indole acetic acid production was observed in only three isolates, while again eight isolates were able to solubilize phosphate, and six produced hydrogen sulfide (H₂S) gas.

In this study actinobacterial isolate AM75 obtained from Mirza area, Kamrup district, Assam, India, exhibited antimicrobial activity against both fungal and bacterial phytopathogens. Grams staining indicated that AM75 was a gram positive and non-motile. Colony morphology of AM75 characterized on various media including Streptomyces Agar (SA), Actinomyces agar (AA), Tributyrin agar (TA), and Starch casein agar media (SCA), with colonies ranging from irregular to entire, and from hard to powdery in texture. The colony color varied from white to cream to peanut brown across different media (Table 1, Fig 1). Biochemical and physiological assays for isolate AM75, detailed in Table 2, revealed positive results for urease, nitrate reduction, lipid hydrolysis, catalase, and amylase activities but negative results for gelatin hydrolysis and cellulase activity. Carbon assimilation test for AM75, yielded negative results for all the carbon resource tested. Optimum growth occurred at 30°C, with reduced growth at 40°C, and 20°C. AM75 also demonstrated higher growth at pH 7.2, compared to pH 9.2 and pH 5.2, and better growth at 2.5% NaCl as compared to 5% and 10% NaCl. Plant growth promoting activities such as siderophore production, hydrogen cyanide (HCN) production, zinc solubilization, indole acetic acid (IAA) production and citrate utilization were positive for AM75, while phosphate solubilization, H₂S production and methyl red-Vogesproskauer test were negative (Table 3).

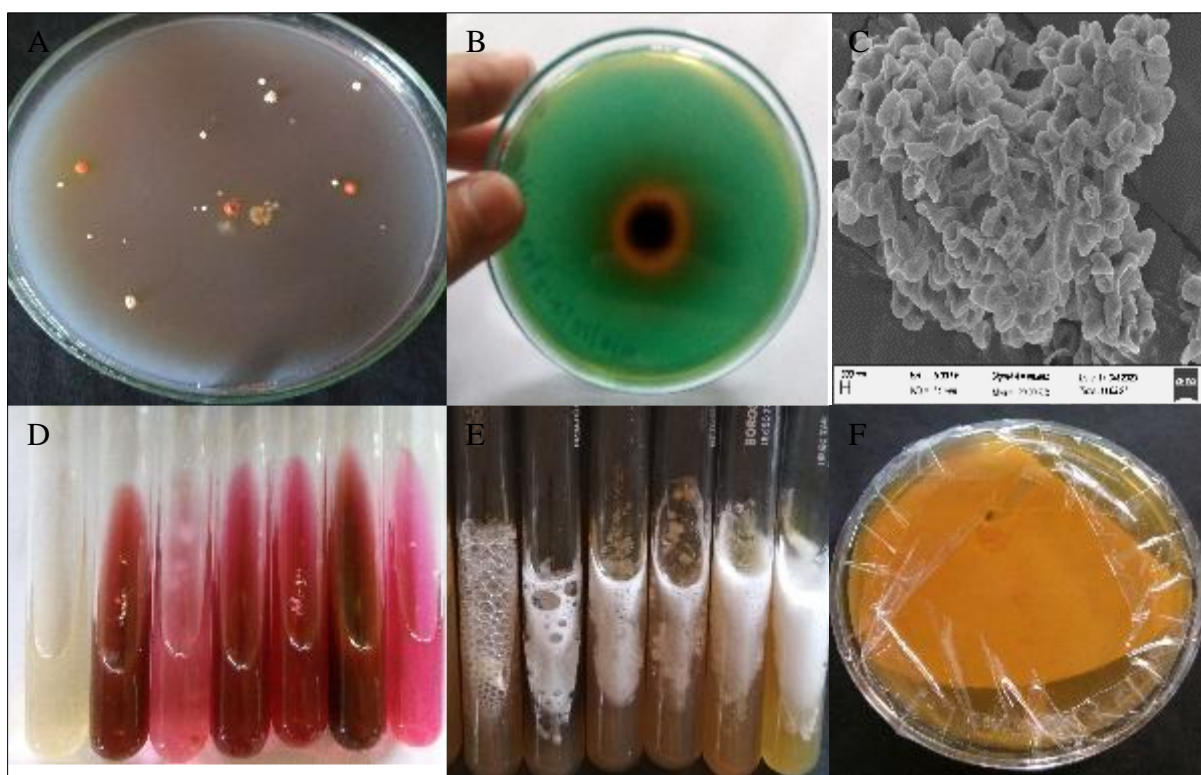


Figure 1 A) Mix culture B) siderophore C) SEM image D) Nitrate reduction, E) Catalase, F) HCN production of the strain AM75

Tables 1 Culture Characteristics of the strain AM75

Media	Form	size	Colour	colour	Elevatin	Margin	pigmentation	surface
SCA	Irregular	1-4	White	White	Undulate	Umbonate	No	Powdery
TA	Irregular	1	peanut	Dark brown	Undulate	Flate	No	Hard
SA	Round	3	Cream	Cream	Entire	Raised	No	Hard
AA	Round	2	Cream	Cream	Entire	Raised	No	Hard

Table 2 Biochemical and Physiological Characters of strain AM75

Culture	Growth characteristics
Motility	Non motile
Amylase	+
Lipase	+
Nitrate reductase	+
Cellulase	-
Gelatinase	-
Urease	+
Catalase	+
Optimum growth in Nacl	+
Optimum pH for growth	9.2
Optimum temperature for growth	40°C
Grams staining	+
Shape and growth	Irregular, hard powdery
Pigmentation	No

Table 3 Plant Growth Promoting Activities Of isolate AM75

PGP Activities	Results
Siderophore	+
HCN production	+
Zinc solubilization	+
Phosphate solubilization	+
IAA	+
H ₂ S	-
Methyl red	-
Vogespraueskaurer	-
Citrate utilization	+

Isolate AM75 displayed resistance to the antibiotics rifampcin, nalidixic acid, ampicillin, and streptomycin (Table 4) and demonstrated bacteriostatic activity against bacterial phytopathogen *Erwinia chrysanthemi* ATCC11660 (zone of inhibition with a diameter of 10mm and showed MIC 2µg/ml) but no activity against bacterial pathogen *Xanthomonas Oryzae Pv Oryzae* ATCC35933. Additionally AM75 exhibited antifungal activity against fungal phytopathogens *Aspergillus niger* ATCC6275 (500µg/ml MIC) and *Fusarium oxysporum* ATCC62506 (1000µg/ml MIC) (Table 5). Sequencing of isolate AM75 revealed it as *Amycolatopsis samaneae* (Accession number PP954879) through comparisons with homologous gene sequences in the NCBI database (Fig 2&3). This identification was supported by colony characteristics, biochemical and physiological tests, gram staining and 16S rRNA sequencing. The genus *Amycolatopsis samaneae* belongs to the Pseudonocardiaceae. This strain was employed for plant growth promoting activities in rice seed germination using filter paper bioassay method on petri plates. The results showed visible effect in the growth of both shoots and roots of germinated rice seeds as compared to those with controlled seed germination (Table 6).

Table 4 Antibiotic resistance by the isolate AM75

Standard Antibiotics	Sensitivity
Rifampcin	R
Nalidixic acid	R
Streptomycin	R
Ampicillin	R

Table 5 Minimum inhibitory concentration (MIC) of AM75

<i>Aspergillus niger</i> ATCC6275	<i>Fusarium oxysporum</i> ATCC62506	<i>Xanthomonas oryzae pv.</i> <i>Oryzae</i> ATCC35933	<i>Erwinia chrysanthemi</i> ATCC11660
500µg/ml	1000µg/ml	ND	2µg/ml
>500µg/ml	>1000µg/ml	ND	>2µg/ml

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CTTGTTGGTGGGGTAGTGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGCCACACTGGGACTGAGACAG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCGACCGCGGTGAGGGATGAC
GGCCTTCGGGTTGTAACCTCTTTCGCCAGGGACGAAGCGTAAGTGACGGTACCTGGATAAGAAGCACCGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGTGCAGCGTTGTCCGGATTTATTGGGCGTAAAGAGCTCGTAGGCGGTTGTCCGGTCCGGCT
GTGAAAACCTTGAGGCTTAACCTTGAGCTTGCAGTCGATACGGGCAGACTTGAGTTCCGGTAGGGGAGACTGGAATTCCTGGTGT
AGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCGATACTGACGCTGAGGAGCGAAAG
CGTGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCTGTAACGTTGGGCGCTAGGTGTGGGTGACATTCCACGTTGTC
CGTGCCGTAGCTAACGCATTAAGCGCCCCGCTGGGGAGTACGGCCGCAAGGCTAAAACCTCAAAGGAATTGACGGGGGCCGCA
CAAGCGGCGGAGCAATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGCTTGACATGCATCAGACAGCTGCAGAGATG
TGGTTCCCTTGTGGTTGGTGTACAGGTGGTGCATGGCTGTCGTGAGTCTGTCGTGAGATGTTGGGTTAAGTCCCAGCAACGA
GCCAACCCCTTATCCTATGTTGCCAGCGGTTATGGCGGGGACTCGTGGGAGACTGCCGGGTCAACTCGGAGGAAGGTGGGGG
ATGACGTCAAGTCATCATGCCCTTATGTCCAGGGCTTACACATGCTACAATGGCTGGTACAGAGGGCTGCCAAATCGTGAGG
TGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAGTCCGCTAGTAATCGCA
GATCAGCAACGCTCGGTTGAATACGTTCCCGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCCC
ATGGCC
    
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Figure 2 16S rRNA sequence of strain AM75 *Amycolatopsis samaneae* (Accession number PP954879)

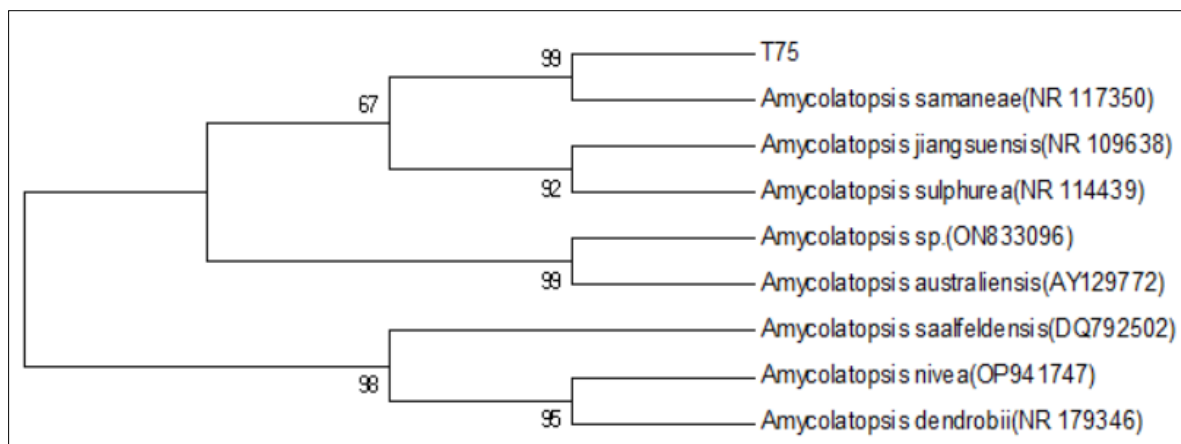


Figure 3 Phylogenetic tree of the strain AM75 *Amycolatopsis samanae* (Accession number PP954879)

Table 6 Effect of strain AM75 on rice seed germination

	Strain AM75	Control
FRESH WEIGHT ROOT (cm)	0.0152±0.006*	0.009±0.005*
FRESH WEIGHT STEM (cm)	0.0511±0.033	0.0744±0.078
DRY WEIGHT ROOT (mg)	0.0025±0.001*	0.0024±0.003*
DRY WEIGHT STEM (mg)	0.0157±0.002	0.022±0.004*
ROOT LENGTH (cm)	8.6±0.59*	7.08±0.31
SHOOT LENGTH (cm)	14.98±0.49	13.48±0.07

*significance

4. Discussion

Previous studies have highlighted various applications of *Amycolatopsis*. Use of *Amycolatopsis* sp as Charcoal rot disease control was reported by Gopalkrishnan et al [26]. Additionally *Amycolatopsis* sp having siderophore, IAA, and phosphate solubilizing activities obtained from rhizosphere of Maize was also reported Alipour Kafi et al [27]. Hydrolytic enzyme activity against *Cercospora* sp by *Amycolatopsis* sp was also documented [28]. Additionally, it has been reported to present in rice rhizosphere [29]. Alekhya and Gopalakrishnan (2016) [26] observed that this genus has protease, pectinase and glucanase activities. *Amycolatopsis* species were first noted for producing Vancomycin, a glycopeptides antibiotic in 1950 [30]. Furthermore it was also reported to have chromium biodegrading capacity [31]

5. Conclusion

The filamentous bacterium *Amycolatopsis samanae* (Strain AM75, Accession number PP954879) isolated from rice rhizosphere soil in the Kamrup district of Assam, India, demonstrated antimicrobial activity against plant pathogen *Erwinia chrysanthemi* ATCC11660, *Aspergillus niger* ATCC6275, and *Fusarium oxysporum* ATCC62506. Identification via 16S rRNA sequencing confirmed and indicated positive biochemical and enzymatic activities. Besides its antimicrobial activities this strain also promotes plant growth, highlighting its potential as eco-friendly agent for agricultural use. Further research is required to assess its effectiveness in field conditions.

Compliance with ethical standards

Acknowledgments

The authors are grateful to the Department of Botany, Cotton University for providing facilities for our research work.

Disclosure of conflict of interest

All the authors have no conflict of interest.

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