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ISOLATION AND CHARACTERIZATION OF PLANT GROWTH PROMOTING ACTINOBACTERIA, *Amycolatopsis samaneae* (AM75), FROM THE RICE RHIZOSPHERE IN MIRZA AREA, ASSAM, INDIA

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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 samaneae*, was characterized as gram positive and non-motile. It exhibited varied colony characteristics on different selective media. The isolate producesseveral enzymesincluding urease, nitrate reductase, lipase, catalase, and amylase but did notdemonstrate cellulase or gelatinaseactivity. 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, though it did not produce hydrogen sulfide (H_2S) or solubilize phosphate. The optimal growth conditions for AM75 werepH 7.2, 30 ºC 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 isolatewas resistant to standard antibiotics, including Rifampcin, streptomycin, ampicillin, and nalidixic acid.

Keywords: Actinobacteria, plant growth promoting (PGP), *Amycolatopsis*, Rhizosphere, Rice.

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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 flavanoids, terpenoids, steroids, anthelminthic, antitumor agents, insecticides, and immunosuppressant (Berdy, 2005). The rhizosphere, which is 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 primaryand secondary metabolites, organic acids, and polysaccharides, attract numerous microbes and contribute to the unique characteristics of rhizospheric microbialcommunities (Ding et al., 2019).

Actinobacteria, which are gram positive, filamentous bacteria with high G+C content, can exhibitboth coccoid and rod shapes and produces both substrate and aerial mycelium (Pandey et al., 2004; Adegboye et al., 2012). These bacteria exhibitcharacteristics of bothfungi and bacteria (Das et al., 2008). Actinobacteria constitute a significant portion of rhizospheric microbiota in variousenvironments and have been isolated from the rhizosphere of a range crops, includingSoyabean, sugarcane, acacia, black rice, salt bush, and nutmeg (Rodrigues, 2018; Belgacem et al., 2023; Ningthoujam, 2016; Boukelloul et al., 2023; Mahulette et al., 2023). Approximately 45% of all known microbial bioactive compounds are produced by terrestrial actinobacteriaactinobacteria (Girao et al., 2019; Solecka et al., 2012), and about 70% of all known naturally occuring antibiotics are derived from actinobacteria,with 55% produced by *Streptomyces* and the reminder by other genera such as *Actinomaura*, *Actinoplanes*, *Nocardia*, *Micromonospora*, *Amycolatopsis*, *Stretosporangium,* and *Thermoactinomycetes* (Berdy, 2005). 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 *Nocardia* sp. and *Micromonospora* sp. in rhizospheric soils(Bian et al., 2012; Zhang et al., 2021; Raut et al., 2018). The present study investigates the plant growth promoting activities of *Amycolatopsis samaneae* isolated from rice rhizosphere in the Kamrup district of Assam, India.

MATERIALS AND METHODS

Soil samples for this present investigation were collected from the rhizosphere of Aijung variety rice in the Mirza area (26º05’35”N - 91º31’55”E), Kamrup district, Assam, India.The soil sample pH was initially measured using a 1:5 water-to-soilratio. The samples were then air dried, sieved, and stored at 4 ºC.Prior to isolating actinobacteria, the rhizospheric soil was incubatedwith $CaCO_3$ (10:1 ratio) at 28 ± 2 ºC for 24 hours to promote actinobacterial growth. From this mixture, 1 gm was used for serial dilution, and 250 µL of aliquots were spread on Starch casein agar media (SCA) under sterilized conditionsin a laminar air flow. The media were supplemented with antimicrobials Nalidixic acid (10µL/mL) and Amphotericin B (2.5 µL/mL). The experiment was conducted in triplicates, and the plates were incubated at 28 ± 2 ºC 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 ºC for subsequent experiments.

Gram positive isolates were streaked on SCA (de Olievera et al., 2010), SA, Actinomyces Agar (AA) and Tributyrin agar (TA) (Collins et al., 1995) to assess colony characteristicsincluding form, color, pigmentation and elevation (Shirling & Gottlieb, 1996). The isolate AM75 was subjected to growth testsundervarious pH conditions (5.2, 7.2 and 9.2) and temperature (20 ºC, 30 ºC and 40 ºC), as well assalt (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 atemperature of 28 ± 2 ºC.

Catalase activity was determined on Trypticase Soy Agar (TSA) following Gerhardt et al. (1981) and urease activity was tested on Urea agar slants (UA) with phenol red as a pH indicator (pH 6.8). A positive urea test was indicated by the development of pink or red color (Cappucino & Sherman, 1996). Hydrogen sulfide (H_2S) production was evaluated in SIM agar deep tubes (Harley & Prescot, 2002), with black precipitation signifying a positive result (Cappucino & Sherman, 1996). Lipase activity was testedon TA media, with clear halos around the colonies after a 4-day incubation confirming positive results. Gelatin hydrolysis was assessed in Alaksondrov media, agar with 12% gelatin, and gelatin liquefaction was confirmed by refrigeration (Blazevic & Ederer 1975). Nitrate reduction was testedin nitrate broth with 0.5% KNO_3, with pink to red coloration after adding sulfanilic acid and NN dimethyl-1-nathylamine indicating positive nitrate reduction (Cappucino & Sherman, 1996). Amylase activitywas determinedon Starch agar media, with clear halos around the colonies after adding iodine stainingindicating positive results. Cellulase activity was assessed on Carboxymethyl Cellulose (CMC) media withclear zones around the colonies after congo red staining and washing with NaCl confirming activity.

Plant growth promoting activities of isolate AM75 wereevaluated using various assays. Siderophore production was assessed on chrome Azurol S (CAS) media, with orange to yellow halos indicating positive results(Schwyn & Neilands, 1987). HCN production was testedon Nutrient agar (NA) media amended with glycine. Phosphate solubilization was evaluated using Pikovskaya agar media (Mehta & Nautiyal, 2001) containing tricalcium phosphate. Zinc solubilization was assessed on Mineral salt agar containing insoluble 0.1% Zinc oxide (Venkatakrishnan et al., 2003). Indole acetic acid (IAA) production was measured on YMBroth media containing 0.2% Tryptophan and incubated for 7 days at 28 ± 2 ^{o}C, with optical density reading at 660 nm. Antimicrobial activity was tested against phytopathogens *Fusarium oxysporum* ATCC62506, *Aspergillus niger* ATCC6275, *Erwinia chrysanthemi* ATCC11660 and *Xanthomonas oryzae pv Oryzae* 35933.

For DNA extraction, a mass culture of isolate AM75 was grown in 100 mL ISP2 Broth in a 500 mL conical flask at 28 ± 2 ºC for 4 days. Biomass was collected by centrifugation at 8,000 rpm, washed twice with sterilized double distilled water, 200 g taken, and mixed with 800 µL of aqueous lysis solution (100 mM Tris HCL, pH 7, 20 mM EDTA; 250 mM NaCl; 2% SDS; 1 mg/L Lysogyme). RNase (5µL, 50 mg/L) added and the mixture was incubated at 37 ºC for 60 minutes. Proteinase K (10 µL, 20 mg/L) was subsequently added, and incubation continued at 65 ºC for 30 minutes. The lysate was extracted with an equal volume of phenol and chloroform (50−50% v/v). Following the additionof 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 7,000 rpm for 10 minutes.The DNA was resuspended in 5 µL TE buffer and stored at -20 ºC. DNA purity was assessed usingaspectrophotometer at 260 nm and 280 nm, and the 16S rRNA gene was amplified using Taq DNA polymerase with primers 243F (GGATGACCCGCGGCCTA) and A3R (CCAGCCCTTCGAC).

RESULTS

Actinobacteria are ubiquitous microorganism found in diverse environments, including soil, water, and plant tissue. We isolated approximately 200 actinobacterial strains fromkamrup 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 shiderophore, 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 (H_2S) gas.

In this study actinobacterial isolate AM75 obtained from Mirza area, Kamrup district, Assam, India, exhibited antimicrobial activity against both fungal and bacterial phytopathogens. Gramsstaining indicated that AM75 was a gram positive and non-motile. Colony morphology of AM75 was 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).

Tables 1. Culture characteristics of the strain AM75

Media	Form	Size	Color	Color	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

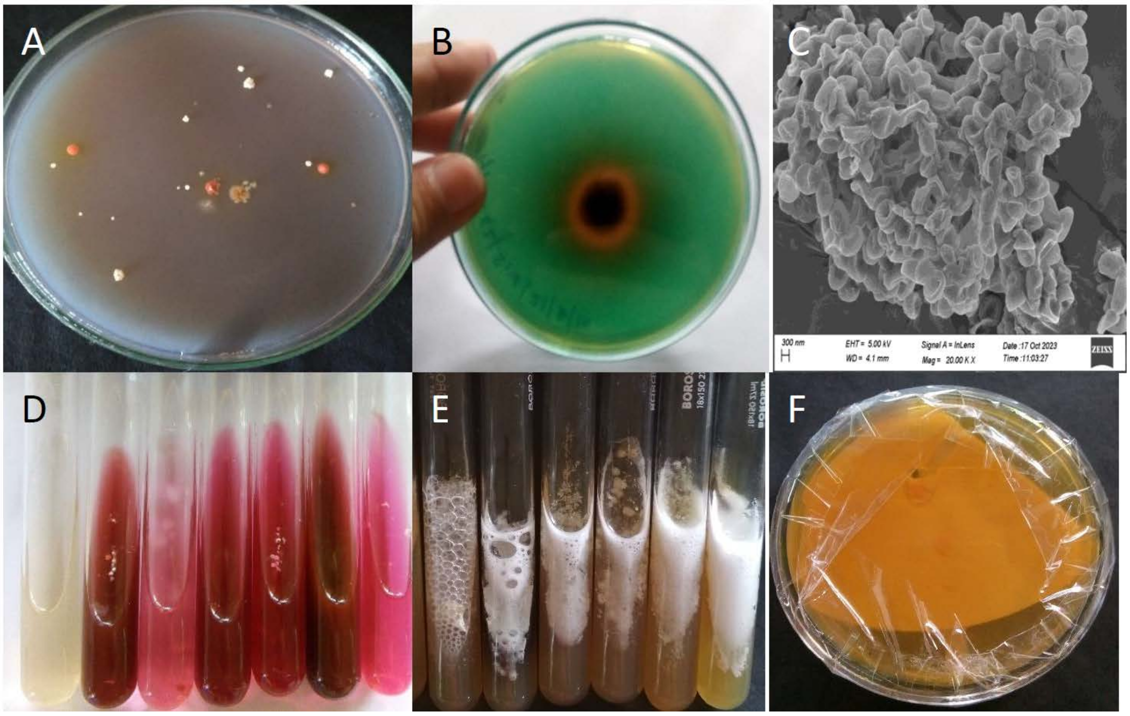

Figure 1. A) Mix culture, B) siderophore, C) SEM image, D) Nitrate reduction, E) Catalase, F) HCN production of the strain AM75

Biochemical and physiological assays forisolate AM75, detailed in Table 2, revealed positive resultsfor urease, nitrate reduction, lipid hydrolysis, catalase, and amylase activitiesbutnegative 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 ºC, with reduced growth at 40 ºC and 20 ºC. 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, H_2S production and methyl red-Voges proskauer test were negative (Table 3).

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	40ºC
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_2S	-
Methyl red	-
Voges praueskaurer	-
Citrate utilization	+

Isolate AM75 displayed resistance to the antibiotics rifampcin, nalidixic acid, amphicillin, and streptomycin (Table 4) and demonstrated bacteriostatic activity against bacterial phytopathogen *Erwinia chrysanthemi* ATCC11660 (zone of inhibition with a diameter of 10 mm and showed MIC 2 µg/mL) but no activity against bacterial pathogen *Xanthomonas Oryzae Pv Oryzae*ATCC35933. Additionally AM75 exhibited antifungalactivitiy against fungal phytopathogens *Aspergillus niger* ATCC6275 (500 µg/mL MIC) and *Fusrium oxysporum* ATCC62506 (1,000 µg/mL MIC) (Table 5). Sequencing of isolate AM75 revealed it as*A.samaneae* (Accession number PP954879) through comparisons with homologous gene sequences in the NCBI database (Appendix 1, Fig. 2).This identification was supported by colony characteristics, biochemicaland physiological tests, grams staining and 16S rRNA sequencing. The genus *A. samaneae* belongs to thefamily Pseudonocardiaceae.

This strain was employed for plant growth promoting activities in rice seed germination using the filter paper bioassay method on petri plates. The results showed a 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
Amphicillin	R

Table 5. Minimum inhibitory concentration (MIC) of AM75

Aspergillus niger ATCC6275	*Fusarium oxysporum* ATCC62506	*Xanthomonas oryzae* pv. Oryzae ATCC35933	*Erwinia chrysanthemi* ATCC11660
500 µg/mL	1,000 µg/mL	ND	2 µg/mL
> 500 µg/mL	> 1,000 µg/mL	ND	> 2µg/mL

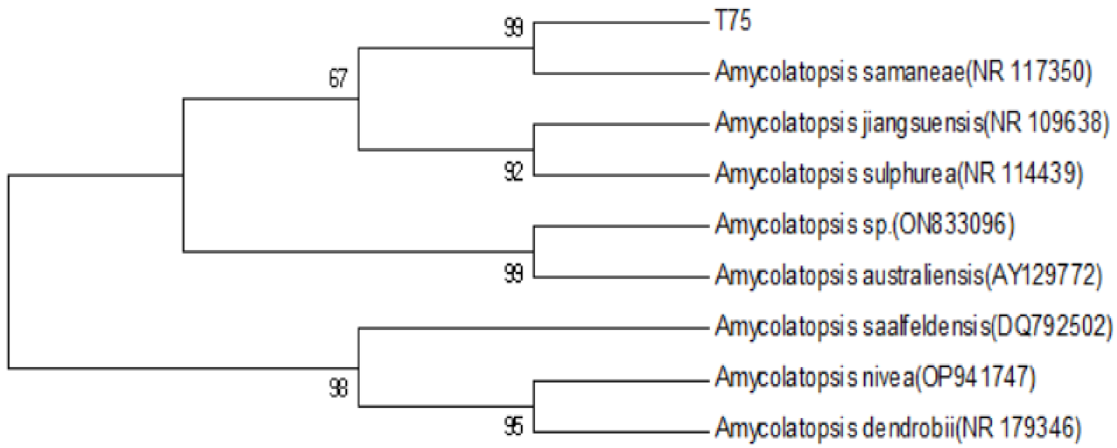

Figure 2. Phylogenetic tree of the strain AM75 *Amycolatopsis samaneae* (Accession number PP954879)

Table 6. Effect of strain AM75 on rice seed germination

	Strain AM75	Control
FRESH WEIGHT ROOT (cm)	0.0152 ± 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

Note: *: significance.

DISCUSSION

Previous studies have highlighted various applications of *Amycolatopsis.* Use of *Amycolatopsis* sp. as Charcoal rot disease control was reported by Gopalkrishnan et al. (2019). Additionally, *Amycolatopsis* sp. having siderophore, IAA, and phosphate solubilizing activitiesobtained from the rhizosphere of Maize was also reported by Alipour Kafi et al. (2021). Hydrolytic enzyme activity against *Cercospora* sp. by

Amycolatopsis sp. was also documented (Dhanyakumara et al., 2022). Additionally, it has been reported to be present in rice rhizosphere (Ningthaujam et al., 2016). Alekhya & Gopalakrishnan (2016) observed that this genus has protease, pectinase and glucanase activities. *Amycolatopsis* species were first noted for producing Vancomycin, a glycopeptides antibiotic in 1950 (McCormick, 1956). Furthermore, it was also reported to have chromium biodegrading capacity (Camargo et al., 2004).

CONCLUSION

The filamentous bacterium *A. samaneae* (Strain AM75, Accesion 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 an eco-friendly agent for agricultural use. Further research is required to assess its effectiveness in field conditions.

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Appendix 1. 16S rRNA sequence of strain AM75 *Amycolatopsis samaneae* (Accession number PP954879)

CTTGTTGGTGGGGTAGTGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGG
GTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAGGGAT
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GTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAA
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