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Exploration of metabolites of limestone Actinobacteria as fungal plant pathogen inhibitors and plant growth promoters

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Abstract

Agriculture is pivotal in India, yet the heavy use of chemical fertilizers adversely affects ecosystems. This study seeks eco-friendly alternatives to beneficial phyto micro-biomes. It involves isolating 200 actinobacterial strains from Andhra Pradesh's limestone quarries. These strains combat fungal pathogens: *Fusarium oxysporum*, *Rhizoctonia bataticola*, *Botrytis cinerea*, *Sclerotium rolfsii* (affecting Chickpea), and *Macrophomina phaseolina* (impacting sorghum). Initial screening revealed 10 actinobacterial isolates with fungal inhibition. Further testing, notably agar well diffusion, highlighted DRAH-24 as the most potent. Its metabolite strongly countered *Sclerotia rolfsii* (22 mm zone) and *Macrophomina phaseolina* (37 mm zone). Molecularly, DRAH-24 shares 99.80% homology with *Streptomyces rochei* OQ119704. DRAH-24's plant growth promotion and enzymatic prowess were also encouraging. This comprehensive research underscores *Streptomyces rochei* OQ119704's potential. It shows promising antagonistic traits and the ability to enhance plant growth and perform enzymatic functions. Consequently, it emerges as a viable candidate for integrating into agriculture—an eco-friendly substitute for chemical pesticides.

Key words : Streptomyces, Metabolite, Lime stone quarries, Fungal plant pathogen, Inhibition.

Actinobacteria are prokaryotic organisms belonging to the phylum of Gram-positive bacteria and the sub-class Actinobactereridae, order Actinomycetales. They are extensively disseminated in the natural landscape. Actinobacteria consist of high G+C content in their DNA and include mycelia-forming and spore-forming bacteria. These bacteria exhibit characteristics resembling both bacteria and fungi, yet remain distinct from them[62]. Among prokaryotes, actinobacteria represent one of the most primitive lineages[40]

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and are believed to have originated around 2.7 billion years ago[9]. Actinobacteria possess the ability to synthesize a diverse range of biologically active secondary metabolites, which hold significant commercial value such as pesticides, antibiotics, antifungals, antivirals, specialized metabolites, toxins, natural products, insecticides[11], and antiparasitic compounds. Their capacity to interact effectively with enzymes stems from their cell wall degrading ability, including enzymes like xylanase, cellulase, chitinase, and proteinase[7]. Streptomyces is the most prominent genus within actinobacteria, followed by *Saccharopolyspora*, *Nocardia*, *Frankia*, *Mycobacterium*, *Microbispora*, *Micromonospora*, *Actinomadura*, *Actinoplanes*, and *Amycolatopsis*. Around 50% of known microbial antibiotics are produced by Streptomyces species[45]. They exhibit a unique interaction with plants and have been abundant in the root-colonizing area of the rhizosphere for millions of years, coexisting with different species of Actinobacteria[12].

Streptomyces can produce numerous enzymes, antimicrobial, and antifungal compounds that function as protective agents for plant hosts against rhizosphere and soil pathogens, both in the endosphere and the soil[66]. While there is access to a tremendous number of clinical drugs, numerous pharmaceutical corporations and research laboratories are actively searching for new therapeutic medications to combat microbial pathogens. The persistent development of multi-drug-resistant pathogenic strains leads to severe disease outbreaks in various nations. To discover novel bioactive compounds with pharmaceutical and trade relevance, scientists are isolating actinobacteria from unexplored locations like wetlands[70], and marine environments[47], aiming to uncover new microorganisms and metabolites. Fungal pathogens are one of the major disease-causing agents, particularly in tropical and subtropical regions. They directly impact economically important plants and during storage[51]. While synthetic pesticides are effective in controlling plant pathogens, they also have detrimental effects on the environment, leading to significant consequences and the potential development of pathogen resistance[28]. Actinobacteria play a crucial role in promoting plant growth and serve as biological controls for cereal pathogens, insect pests, and grain legumes[31]. Consequently, biologists are increasingly interested in utilizing actinomycetes as agents to influence plant growth and provide biological control against soil-borne root diseases in crops. Numerous studies have demonstrated that actinobacteria protect various plants from soil-borne fungal pathogens, acting as fungus-antagonistic and root-colonizing microbes[53]. Therefore, this study aims to isolate actinobacterial strains from limestone quarries, focusing on their ability to inhibit fungal plant pathogens, promote plant growth, and exhibit enzymatic activity.

In this study, 14 distinct soil samples were collected from Andhra Pradesh state, covering areas like Betamcherla, Belam, and Ankireddypalli limestone quarries. The collected soil underwent cleaning to eliminate debris, followed by treatment with heat[13], calcium carbonate[52], and phenol[58]. For actinobacteria isolation, the treated samples underwent the serial dilution method[17,21] then followed by spread plate technique using Starch Casein Agar medium[41]. Incubation occurred

at 35°C for 5 days, colony growth was observed at 24-hour intervals for actinobacteria traits. Isolated colonies were maintained on SCA slants after incubation. Morphological, cultural, and physiological attributes were examined using standard procedures.

A total of 200 actinobacteria were isolated and tested for their antagonistic activity against fungal pathogens such as *Fusarium oxysporum*, *Rhizoctonia bataticola*, *Botrytis cinerea*, and *Sclerotium rolfsii* for Chickpea, as well as *Macrophomina phaseolina* for Sorghum. These fungal cultures were obtained from ICRISAT Patancheru, Hyderabad, Telangana. The test fungal cultures were maintained on potato dextrose agar, which was also used for subsequent studies. The screening employed the dual culture method, where four actinobacterial cultures were streaked at the corners of each Petri plate[32]. An 8mm disc of test fungal mycelium was placed in the centre of each plate and incubated at 28°C for 5 days. The zone of inhibition was observed and measured. Among the 200 isolates, only 10 exhibited inhibitory effects against the fungal plant pathogens. From these 10 isolates, DRAH-24 was selected for further investigation due to its effective inhibition compared to other isolates.

To study DRAH-24's antifungal metabolic activity against specific pathogens, a seed culture was generated and introduced into SC broth, incubated at 35°C (180 rpm) for 5 days. Post-incubation, the culture broth was centrifuged at 10,000 rpm for 20 minutes (4°C), yielding supernatant for antagonistic studies against fungal phytopathogens: *Fusarium oxysporum*, *Rhizoctonia bataticola*, *Botrytis cinerea*, *Sclerotium rolfsii* (Chickpea), and *Macrophomina phaseolina* (Sorghum). This validated metabolic activity using the agar well diffusion method[10]. For the fungal tests, spores were suspended in sterile saline (10ml). A 0.1ml diluted spore suspension was spread on potato dextrose agar. Wells (6mm) were made in the agar, and 200 µg of actinobacterial supernatant was added to the wells.

Chromosomal DNA was extracted using a spin column kit (HiMedia, India, or similar). The bacterial 16S rRNA gene (1500 bp)[16] was PCR-amplified, purified with Exonuclease I-Shrimp Alkaline Phosphatase (Exo-SAP)[18], and sequenced via the Sanger method on the ABI 3500xL genetic analyzer (Life Technologies, USA). Sequencing files (. ab1) were edited using CHROMASLITE (version 1.5) and analyzed using the Basic Local Alignment Search Tool (BLAST) to locate the nearest culture sequence in the NCBI database, deducing functional and evolutionary connections[3]. An initial BLASTN search found closely related type strain sequences, followed by pairwise alignment to calculate sequence similarity values[64]. The program conducts comparisons between nucleotide or protein sequences and sequence databases, subsequently determining the statistical significance of the identified matches[26]. MEGA 11 software analyzed the alignment using the neighbor-joining method for phylogenetic tree construction.

Plant growth promoting activity and enzymatic activities of DRAH-24 was carried out using standard protocols. Indole Acetic Acid (IAA) production[33], Hydrogen cyanide production[8], Ammonia production[14], Nitrate reduction[38], Amylase[65], Cellulase[56], Pectinase[37,56],

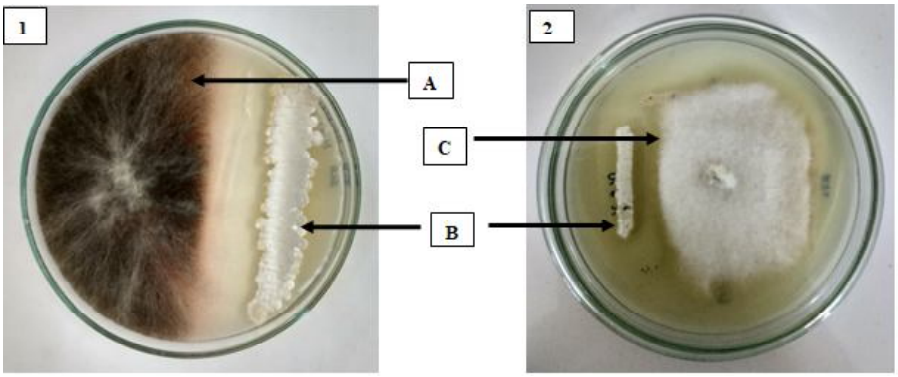
Caseinase[15], Protease[15], Chitinase[65], L-Asparaginase[20], Gelatinase[60,63].

Isolation, screening and characterization of potential actinobacterial strain :

In this current study, we collected 14 distinct soil samples from limestone quarries located in the Andhra Pradesh state. These samples were taken from the regions encompassing Betamcherla, Belam, and Ankireddypalli, with the primary goal of isolating potential actinobacterial strains. From this effort, a total of 200 actinobacterial strains were isolated and subjected to screening against various fungal plant pathogens. Specifically, *Fusarium oxysporum, Rhizoctonia bataticola, Botrytis cinerea,* and *Sclerotium rolfsii* were tested against Chickpea, while *Macrophomina phaseolina* was assessed for Sorghum. This assessment was conducted using the dual culture assay method detailed in Gopalakrishnan *et al*.,[32].

Out of the 200 isolates, only 10 actinobacterial strains demonstrated antagonistic activity against all five fungal plant pathogens. These strains were designated as DRBA-7, DRBA-38, DRBA-69, DRBE-14, DRBE-53, DRBE-60, DRBE-71, DRAH-13, DRAH-24, and DRAH-84. Among them, DRBA-7 and DRBA-69 displayed partial inhibition against *Fusarium oxysporum,* with no inhibition against the other pathogens. Similarly, DRBE-14, DRBE-53, and DRBE-60 partially inhibited *Rhizoctonia bataticola*, *Botrytis cinerea*, and *Sclerotium rolfsii* but showed no inhibition against the other pathogens. DRBA-38, DRBE-71, DRAH-13, and DRAH-84 exhibited mild inhibition only against *Botrytis cinerea*. Notably, DRAH-24 was the sole strain that demonstrated prominent inhibition against *Sclerotium rolfsii* and *Macrophomina phaseolina* (as observed in Plate-1). In contrast, all other tested actinobacterial strains only displayed very mild inhibition against the pathogens. Consequently, due to its significant inhibitory effect, DRAH-24 was selected for further analysis. Comparable findings have been reported previously in actinobacterial screening studies, as evidenced by the works of Arvind *et al*.,[6], Gottumukkala *et al.,*[34], and Gopalakrishnan *et al.,*[57].

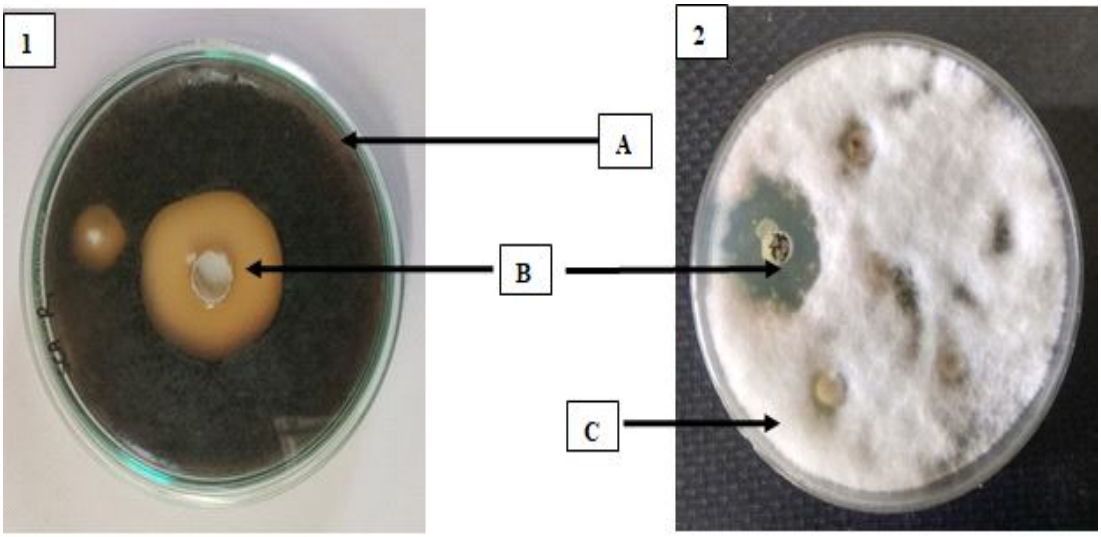
Plate 1- Screening of DRAH-24 against fungal phytopathogens :



A- (1) *Macrophomina phaseolina*; B- *Streptomyces rochei* DRAH-24; C- (2) *Sclerotium rolfsii*

To confirm the antagonistic activity of DRAH-24, the agar well diffusion method was employed against all five fungal plant pathogens. However, DRAH-24 exhibited notable inhibition specifically against *Sclerotium rolfsii* and *Macrophomina phaseolina* (as shown in Plate-2). These outcomes were compared with findings from related studies. In those studies, it was reported that metabolites produced by the actinobacteria could generate antifungal substances, which hinder the hyphal growth of fungal pathogens. Such similar findings were documented by Gopalakrishnan *et al.*,[32] and Kavitha *et al.*,[39].

Plate 2- Screening of DRAH-24 against fungal phytopathogens by agar well diffusion :



A- *(1) Macrophomina phaseolina; B- DRAH-24 broth culture; C- (2) Sclerotium rolfsii*

Molecular characterization of potential actinobacterial strain DRAH-24 :

The actinobacterial isolate DRAH-24 was identified to the species level using morphological, cultural, biochemical, physiological, and molecular characteristics. Identified as Streptomyces, DRAH-24's specific identification was established through 16S rRNA homology. The 16S rRNA (1500 bp) sequencing was conducted at NCIM, CSIR-NCL Pune, revealing a close association with *Streptomyces rochei* OQ119704 (DRAH-24). Phylogenetic allocation and 16S rRNA gene sequence identities are shown in Figure 3, with the tree constructed using MEGA11 software.

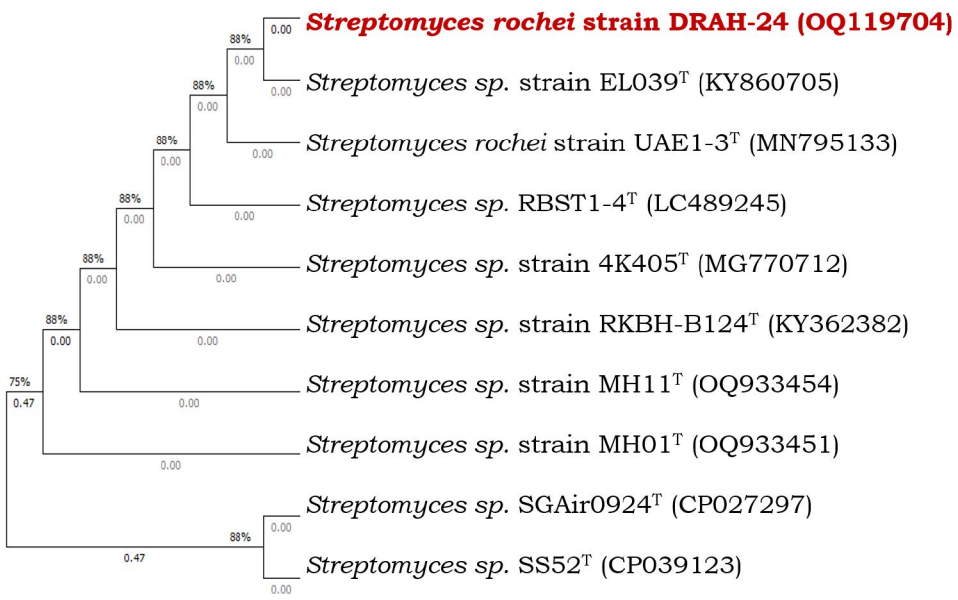


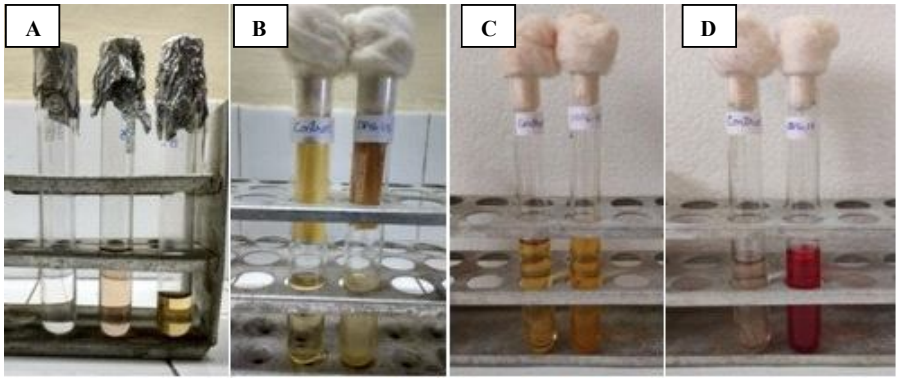
Figure 3. Phylogenetic analysis of Streptomyces sp. DRAH-24

Study of Plant growth promoting activity of DRAH-24 and enzymatic activity :

As actinobacteria are recognized for producing various plant growth-promoting hormones and hydrolytic enzymes, we further evaluated the potential isolate DRAH-24 *Streptomyces rochei,* which exhibited promising antagonism against *Macrophomina phaseolina* and *Sclerotium rolfsii*. Our screening revealed that DRAH-24 *Streptomyces rochei* demonstrated positive results for all the specified growth-promoting hormones and enzymes (Plate-3 and Plate-4).

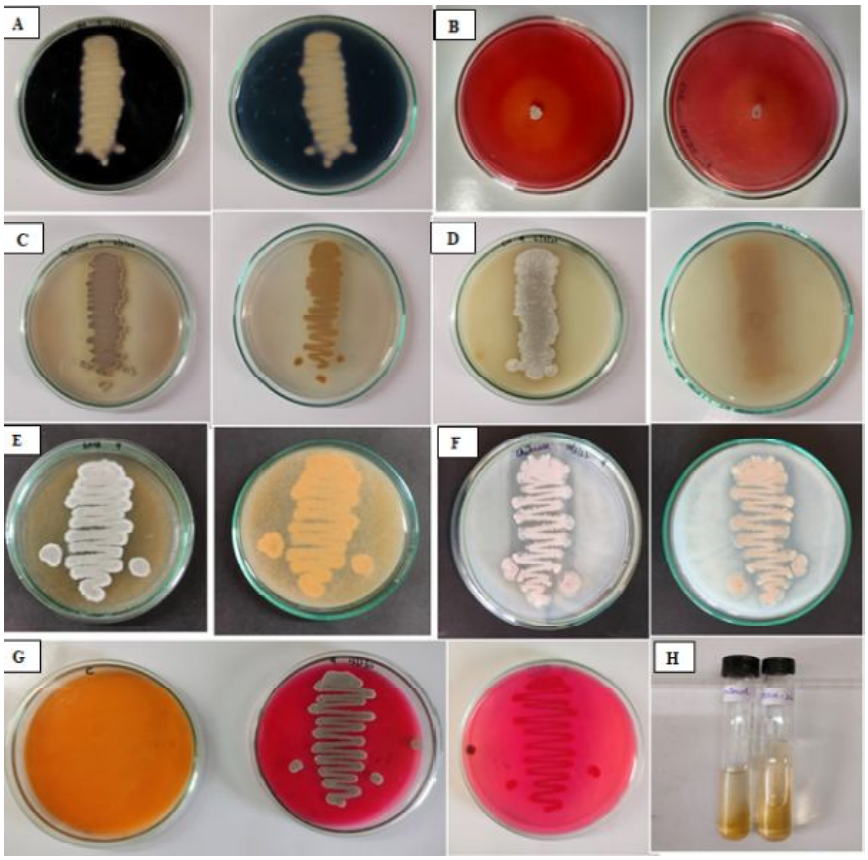
IAA-producing microorganisms contribute to plant growth and root elongation[55]. Those capable of generating phytohormones stimulate plant growth, boosting metabolite production, and aiding plant growth and seed germination[2]. HCN production is linked to disease suppression[67], as exemplified by Pseudomonas fluorescens strains suppressing tobacco black root rot[36]. Ammonia, a volatile compound, expands through soil pores, creating higher concentrations around bacterial colonies, aiding compound production and pathogen control[25,69]. Ammonia contributes to organic matter decomposition, soil structure improvement, enhanced plant nutrition, higher crop production, and greater phytoparasite tolerance[48]. Reports suggest that excessive ammonia from rhizobacteria can be harmful to plants if NO_2 transformation into NO_3 by nitrifying bacteria doesn't occur[68]. Nitrate reduction is employed to ascertain whether an organism possesses the ability to convert nitrate (NO_3^-) into nitrite (NO_2^-) or other nitrogen-containing compounds through the activity of the enzyme nitratase, also known as nitrate reductase[38].

Plate 3- Study of different Plant growth promoting activity of DRAH-24



A- IAA Production; B- HCN production; C-Ammonia production; D-Nitrate reduction

Plate 4- Study of different enzymatic activities by DRAH-24



A-Amylase; B- Cellulase; C- Pectinase; D- Caseinase; E- Protease;
F- Chitinase; G- L-asperginase; H- Gelatinase

Actinomycetes exhibit antifungal properties against pathogenic fungi due to various factors like antibiosis and parasitism. Numerous Streptomyces species are known to degrade fungal cell walls made of chitins[22,23,27,30]. Chitinase aids in this degradation process[59]. The production and function of lytic enzymes in breaking down fungal pathogenic cell walls have been reported by Lima *et al*.,[44], Singh *et al*.,[61], Gupta *et al.,*[35], Gomes *et al.,*[29], and Mohan and Singara Charya[49]. Derived from milk, casein is a phosphoprotein substrate with an exceptionally high molecular weight. It can be enzymatically degraded by various proteases, including both endo- and exoproteases, resulting in the formation of peptide chains and amino acids[19,43]. Previous reports have indicated that microorganisms producing protease and chitinase can serve as effective biocontrol agents against protein cell wall-bearing pathogens like *Phytophthora and Pythium*[44].

In a study by Gopalakrishnan *et al.,* (2011)[32], it was noted that two out of five FOC antagonistic actinomycetes produced cellulase (KAI-32 and KAI-90) and protease (CAI-24 and CAI-127). Cellulose, a major polysaccharide (20-50%) in plant biomass, can be broken down by microbial enzymes, including cellulase[4,5,46,]. Protease and cellulase-producing microorganisms play crucial roles in organic matter decomposition, nutrient mineralization, and promoting plant growth. Protease activity in actinobacteria isolates was reported by[54], and gelatinase activity was reported by Shejul[60]. The production of α-amylase plays a crucial role in transforming starches into oligosaccharides, as documented by Abraham and Herr[1] and T. Ashokvardhan *et al.,*[65] through their documentation of amylase activity. L-Asparagenase, an amidohydrolase enzyme, contributes to organic matter decomposition and plays a significant role in the nitrogen cycle. Enhancing its activity in the soil results in heightened nutrient availability. L-asparagine is a key player in the soil's nitrogen cycle. L-glutaminase breaks down L-glutamine into L-glutamic acid and ammonia. Likewise, L-asparaginase breaks down L-asparagine into L-aspartic acid and ammonia[42,50].

In this investigation, we isolated 200 actinobacteria from 14 soil samples within Andhra Pradesh's limestone quarries—encompassing Betamcherla, Belam, and Ankireddypalli. These isolates were screened for antagonistic activity against Chickpea's *Fusarium oxysporum*, *Rhizoctonia bataticola*, *Botrytis cinerea*, and Sorghum's *Macrophomina phaseolina.* DRAH-24 consistently displayed antimicrobial efficacy in dual culture assays and agar well diffusion against *Sclerotium rolfsii* (Chickpea) and *Macrophomina phaseolina (Sorghum)*. Considering DRAH-24's versatile antifungal capabilities, we sequenced its 16S rRNA gene @ NCIM, CSIR-NCL, revealing 99.80% similarity to *Streptomyces rochei* OQ119704. Subsequently, DRAH-24 underwent screening for various action mechanisms (IAA, HCN, Ammonia production, Nitrate reduction and cell wall-degrading enzymes; plate 3 and 4, indicating potential involvement of multiple antifungal metabolites.

Hence, DRAH-24 *Streptomyces rochei* OQ119704 holds promise as an actinobacterial strain for discovering novel secondary metabolites, making it an environmentally friendly bio-fungicide for diverse biocontrol applications.

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