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Molecular Characterization and Antimicrobial Activity of Actinomycetes Isolated from Rhizosphere Soil

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ABSTRACT: Strains of Actinomycetes are isolated from soil samples obtained at different places, including Karimnagar and Warangal in Telangana. Chili plant rhizosphere soil samples were serially diluted and plated on starch-casein media for one to two weeks at 37°C. Using morphological, biochemical, and cultural characterization, the isolated cultures were studied for their morphology, substrate mycelial growth, and enzyme degradation. The experiments were conducted in compliance with the International Streptomyces Project (ISP). The strains KNR-1 and WGL-1 were characterized by biochemical procedures such as morphological, cultural, and 16S rRNA gene sequencing. Following characterization, the cultures were identified using 16S rDNA gene sequencing. *Streptomyces broussonetiae* KNR-1 (Accession No.OQ421513) and *Nocardioides* sp. WGL-1 (Accession No.OQ421515). Based on their appearance, the isolates were evaluated for antibacterial efficacy against pathogens. Antimicrobial activity was assessed in five clinical isolates. WGL-1 exhibited the highest antibacterial activity against *Escherichia coli* ANRPS-P5 (Accession No.OQ151987), *Staphylococcus aureus* ANRPS-P4 (Accession No.OQ151986), *Pseudomonas aeruginosa ANRPS-P3* (Accession No.OQ151984), *Salmonella enterica strain* -P2(Accession No.OQ151982), *and Klebsiella pneumoniae strain -P1* (Accession No *OQ151981)* while KNR-1 had the lowest. These isolated strains had antibacterial activity and could potentially be used in the research and development of novel antibiotics for use in the pharmaceutical or agricultural industries.

Keywords: Actinomycetes, Characterization, 16S rDNA, Antimicrobial activity.

INTRODUCTION

Actinomycetes are the most widespread category of microorganisms in the environment. The majority live as free-living saprophytes in soil, while others live as endophytic Actinomycetes in plants (Gupta, 2022). Actinomycetes heavily rely on soil for their existence (Trenozhnikova and Azizan 2018). The prospect that they may protect roots by limiting the growth of possible fungal diseases by releasing enzymes that destroy fungal cell walls and antifungal chemicals has been explored (Flores-Gallegos and Nava-Reyna 2019). Actinomycetes may enhance plant development by releasing plant growth promoters such as indole acetic acid (IAA) for root growth and siderophores for nutrient absorption (Djebaili *et al.,* 2020; Cao *et al.,* 2005; Hasegawa *et al.,* 1983). Actinomycetes heavily rely on soil for their existence (Trenozhnikova and Azizan 2018). The prospect that they may protect roots by limiting the growth of possible fungal diseases by releasing enzymes that destroy fungal cell walls and antifungal chemicals has been explored (Flores-Gallegos and Nava-Reyna 2019). Actinomycetes may enhance plant development by releasing plant growth promoters such as indole acetic acid (IAA) for root growth and siderophores for nutrient absorption (Djebaili *et al.,* 2020). They contributed several key bioactive chemicals with great economic potential and were constantly explored for novel bioactive compounds (Khanna *et al.,* 2011).

Streptomyces produce gram-positive antibiotics (Unaogu *et al.,* 1994). Although it has been suggested that the producing of antibiotics by soil actinomycetes is ecologically significant, there is just a smattering of evidence to support this assertion. manufacture in natural settings is not extensively researched. (Kumara Behera *et al*., 2017). It has been shown that Streptomyces is capable of producing antibiotics such as chloramphenicol, gentamycin, and thiostrepton (Schneider *et al*., 2022). These bacteria are free-living saprophytes that play an important role in the production of antibiotics, they play a key part in the recycling of organic matter as well as the generation of novel medicines, vitamins, enzymes, anticancer agents, enzyme inhibitors, immunological modulators, and nutritional components (Subathra *et al.,* 2022).

It is becoming more crucial to appropriately identify *Nocardioides* sp. in antibiotic susceptibility tests, as well as in clinical and epidemiological research. Molecular methods have shown a number of different taxonomic shifts within the genus *Nocardioides.* Wallace *et al*. (1988) found that different strains of *Nocardioides* sp. had different patterns of sensitivity to antimicrobial agents (Toyokawa *et al*., 2021). The

majority of *Nocardioides* sp. infections in people are caused by a bacterial group known as complex *Nocardioides* sp. *N. abscessus,N. asteroides, N. brevicatena paucivorans complex, N. nova* complex (which encompasses *N. nova, N. africana, and N. kruczakiae*), *N. transvalensis N. veterana,* complex, *N. farcinica,* and *N. cyriacigeorgica* were all subsequently determined to be separate species from the *N. asteroides* complex. The *N. cyriacigeorgica* species is home to the *N. asteroides* type VI drug pattern, which has been known for a long time to be a prevalent and notable pathogen in the United States (Han *et al.*, 2021). This has been the case for quite some time (Hashemzadeh *et al.,* 2021). Molecular investigations have demonstrated that *N. cyriacigeorgica* is the most widespread cause of *Nocardiosis* in both humans and animals in various regions of the globe (Azadi *et al*., 2020).

This article talks about how different Actinomycetes were found in a sample of rhizosphere soil. It then looks at how those Actinomycetes were put together. The 16S rDNA gene sequencing, in particular, was used to identify intra-microbial species differences. Its antibacterial activity in order to discover novel bioactive compounds against multidrug-resistant diseases.

MATERIALS AND METHODS

A systematic investigation was conducted to isolate strains of *Streptomyces broussonetiae* and *Nocardioides* sp. from plant rhizosphere soil for molecular identification and antibacterial activity. The experiments were carried out in the laboratory at the Department of Biotechnology, Kakatiya University, Warangal, India.

Soil sampling and pretreatment. In the Indian state of Telangana, soil samples were collected from the chilli rhizosphere in the cities of Karimnagar and Warangal. Each sample was obtained at a depth of between 6 and 12 inches below the surface of the soil. These samples were immediately brought to the laboratory after being placed in sterile plastic bags, which were then carefully sealed. After being exposed to room temperature air for the appropriate amount of time, these soil samples were separated.

Isolation of Actinomycetes. At Kaminnagar and Warangal, samples of soil were taken at random from the rhizosphere of the chilli plant. To prepare the stock solution, one gram of soil from a rhizosphere that had been subjected to heat treatment was weighed and then suspended in 99 milliliters of sterile water from Millipore. In order to achieve a dilution with a factor of 10^{-1}, 1 mL of the stock solution was pipetted into a test tube, and then 9 mL of sterile distilled water was added to the tube. According to Kuester and Williams's (1964) research, one milliliter of a solution that was serially diluted starting with a 10^{-1} dilution generated 10^{-2} dilutions all the way up to 10^{-6} dilutions when it was placed in a test tube that contained nine milliliters of sterile distilled water. In order to identify actinomycetes, 10^{-1} diluted solutions were transferred from 10^{-4}, 10^{-5}, and 10^{-6} dilutions onto starch-casein agar medium. The appropriate amounts of rifampicin (30 g/ml), action (80 g/ml), and nystatin (50 g/ml) were also added to the medium at this time. After that, in order to prevent the growth of bacteria and fungi, the incubator was heated to 37 ºC for 7 to 14 days (Shirling & Gottlieb 1966) (Table 1 and Fig. 1).

Purification of Actinomycetes isolates. Single colonies of Actinomycetes isolates were isolated from a variety of colonies by using a sterile loop and an appropriate medium for the isolation of Actinomycetes called Actinomycetes isolation agar. Actinomycetes colonies that had been injected with fungus were moved to a separate sterile medium and subcultured so that pure colonies could be developed. They were cultivated on Actinomycetes the separation agar so that further research could be carried out (Fig. 2).

Morphological characterization of Actinomycetes. The isolates are examined under a microscope for morphology, i.e., shape, size, and color of the colonies.

Actinomycetes microscopic investigation

i) Lactophenol cotton blue staining (LPCB). The Actinomycetes can potentially be seen under a microscope due to this procedure's staining and maintenance of their structural integrity. A spotless tiny glass slide is used. a drop of 70% ethanol was inserted. The samples were each given a single drop of alcohol to study. Two drops of LPCB were added just before the alcohol was allowed to dry. The coverslip was positioned in-between the index and the middle fingertips of the hand. In order to prevent air bubbles from forming, the edge of the coverslip was brought into touch with one side of the drop of liquid, and then the drop was allowed to slowly drop. This preparation was first investigated with a low-power objective. Increasing the magnification to 40X allowed for a more detailed investigation of spores and other structures.

ii) Gram staining. The gram-positive or gram-negative status of an isolate may be ascertained using Cappuccino & Sherman (2004) method of identification.

iii) Coverslip method. Coverslip culture is a useful method for examining the micro-morphology of filamentous Actinomycetesbelow natural conditions. Spore chain, aerial mycelium, spore chain form and quantity, and so on may all be investigated. Actinomycetes isolation agar was used to cultivate and retain the isolates, while Bennett's agar medium was used to promote growth. After being disinfected with ethanol, a coverslip was positioned on a Petri plate at a 45 degree angle and incubated at 37 ºC. A high-power microscope and an oil immersion microscope were used to examine the plates on days 7, 14, and 21. ISP has been described in terms of the structure and morphology of its sporogenous hyphae, as well as the aerial and substrate mycelium morphologies (Duraipandiyan *et al.,* 2010; Nonomura, 1974).

Cultural characterization of Actinomycetes. The investigation of aerial mycelium's shape, structure, and growth, pigment synthesis, and Actinomycete characterization involves observing how different substrate mycelial growths respond to different kinds of culture mediums. The International Streptomyces

Project (ISP) found that incubation at 28 °C for 14 days yielded the best results, utilizing the techniques to analyze the cultures for morphological growth. Any diffusible colours, as well as aerial and substrate mycelial hyphae, were found (Manhas and Bala 2004). Yeast-malt extract agar, oatmeal agar, inorganic salt starch casein agar, glycerol asparagine agar, and tyrosine agar are all components of ISP medium 2 were employed as standard culture media (Table 1).

Plate inoculation for morphological investigations. A thin covering of sterile distilled water ranging from 3 to 5 milliliters was spread evenly over the bottom of each test tube. It was necessary to sterilize the wire loop by first heating it in a flame and then allowing it to cool under aseptic conditions before it could be used to transfer a loopful of culture into test tubes containing sterile distilled water and observe the process until a turbid suspension developed. This was done in order to be certain that there would be no contamination of any kind. A pool of inoculum formed after about 0.05 milliliter of the inoculum was dispersed over the surface of the agar on one side of the Petri dish. On the plate were formed five streaks that were uniformly spaced apart using a wire loop that had been treated with flame sterilization. Before putting the plates through their steps, they were given a 7-day, 14-day, and 21-day dark incubation at 25–28°C.

Actinomycetes biochemical characterization. In order to understand the physiological structure of actinomycete isolates, individual biochemical examination of each isolate is required. In the biochemical tests that was done for the purpose of characterisation, cappuccino had been used (Srinivasan and Laughlin 1982). The hydrolysis of starch, casein, and gelatin, the triple sugar iron test, carbohydrate fermentation, citrate fermentation, urease, catalase, hydrogen sulfide, the methyl red-vogues proskauer test, and other biochemical tests were used in the selection of high-quality culture isolates.

Antimicrobial activity. Both KNR-1 and WGL-1 were subjected to through a series of antimicrobial tests to see how effectively they performed against five different types of bacteria. These bacteria *were Klebsiella pneumoniae, Salmonella enterica, Pseudomonas aeruginosa, Staphylococcus aureus, and Escherichia* coli. At a temperature of 37ºC, the KNR-1 and WGL-1 strains were allowed for growth for seven days on starch-casein agar. Before being used for the purpose of swabbing test organisms onto nutrient agar plates, cotton swabs were sterilized. After swabbing the test organisms onto agar plates, covering them with KNR-1 and WGL-1 isolate agar discs, and then growing them for a whole day, the results were checked. The activity that was measured provides an indication of the diameter of inhibition on a millimeter-scale.

Phylogenetic study and molecular characterization of two Actinomycetes strains. For molecular analysis, each of the Actinomycetes strains with the highest antimicrobial capability following pathogen testing was selected.

Isolation of genomic DNA. On a nutrient agar plate, the pure Actinomycetes strains with the maximum antibacterial culture were cultured. Total genomic DNA was isolated from an overnight growing single colony using the Ultra Clean According to the manufacturer's instructions, a microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA) was utilized, and a 1% (w/v) agarose gel was used for every aspect of the quality assessment of DNA.

Sequencing and PCR amplification of the 16S rRNA gene. Using the 16S rRNA gene PCR and full-length sequencing analysis, the culture that was isolated was able to be identified down to the species level. In order to amplify the 16S rRNA gene sequencing PCR, the universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used. Both of these primers were designed to read 5'. After conducting an examination using agarose gel containing 1% weight-per-volume (w/v), the amplified PCR product was purified with the QIA quick purification kit in order to remove any impurities that could have been present. We were able to extract the sequencing of the 16S rDNA, which was 1500 base pairs long. The Sanger method is used inside Life Technologies' ABI 3500xL genetic analyzer in order to sequence purified amplicons (Life Technologies, USA). Following the modification of CHROMASLITE (version 1.5), the Basic Local Alignment Search Tool was used to do an analysis on the extra sequencing data. (BLAST). NCL in Pune, India, was the organization that completed the full-length gene sequencing for the strains.

Phylogenetic analysis. After then, the results from the sequencing were analyzed by utilizing a program called Basic Local Alignment Search Tool (BLAST), which could have been found on the website of the National Center for Biotechnology Information. BLAST algorithms may be used to discover members of a gene family as well as establish the evolutionary and functional relationships between sequences. In addition, these algorithms can be used to determine how sequences are related to one another. These two jobs are ones that can be completed successfully. A methodology referred to as neighbor-joining was used in order to reconstruct the evolutionary history in its entirety. In order to determine the relative amounts of evolutionary time that separate different species, the Jukes-Cantor method was used. Using Mega-X, we were able to successfully carry out a search into the process of evolution. The database of nucleotides that is maintained by the National Center for Biotechnology Information (NCBI) has been updated to include the nucleotides that were selected for inclusion in the database.

RESULTS

Collection and preparation of samples. Two Actinomycetes isolates, KNR-1and WGL-1, were found in pre-treated soil samples from the Rhizosphere of chilli plants in the Karimnagar and Warangal districts of Telangana state (Fig. 2). They were then cleaned up using the streak plate technique.

Characterization of physiological, biochemical, and antibacterial properties. In order to determine the cultural and physiological characteristics of the cultural isolates, the methods developed by the International Streptomyces Project (Shirling and Gottlieb 1996) were used. These methods were employed in order to determine the qualities of the cultural isolates. The presence of aerial mycelium, the color of the spore mass, the color that is characteristic of the reverse colony, the pigment that is diffusible, as well as the morphology of the sporophore and the spore chain, were all detected after 10 days of incubation on ISP-2 media.

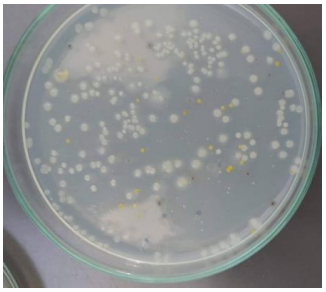

Fig. 1. Isolation of Actinomycetes.

Fig. 2. Purification of Actinomycetes via picking from a single colony.

KNR-1and WGL-1Actinomycetes isolates were isolated. The KNR-1and WGL-1 isolates were determined to be aerobic and gram-positive. Aerial mycelium with sporangium was seen on the slides. In isolate KNR-4, True mycelial structures were seen, including filamentous non-septate hyphae and substantial branching. Hyphae are included in the adaptable structure, which may produce spores. It was discovered that WGL-1 had aerial mycelium that had substantial branching, in addition to real mycelium and hyphae that did not septate. The colour of the aerial mycelium of thermophilic genera and species was used to identify them at first. The initial characterization was made possible by the production of diffusible pigments and the manner of spore formation (Ziyat *et al.*, 2019). Short and long-chain myceliums were found in the isolates. They were discovered to be spore-bearing and solitary with smooth conidia. KNR-1spore were smooth, whereas WGL-1 spore silhouettes were hairy (Fig. 3).

Fig. 3 demonstrates the coverslip culture method that was used on the KNR-1 and WGL-1 isolates. The aerial mycelium, the substrate mycelium, the organization of the sporogenous hyphae, and the morphology (which comprised straight, flexuous, and spiral-shaped forms) were all seen and documented by the International Streptomyces Project (ISP). It has been shown that there are plenty of variations in the morphology of spore chains.

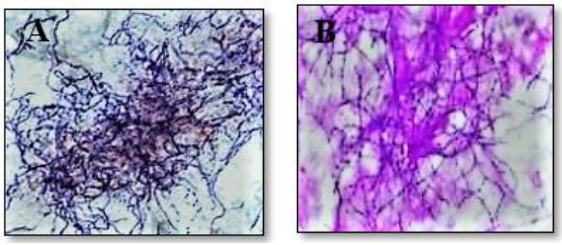

Fig. 3. Actinomycetes Coverslip culture method isolates KNR-1(A) and WGL-1 (B) using the Lactophenol cotton blue stains.

The two isolates were cultured in different environments to study the formation of hovering spore aggregates, reverse colony appearance, and morphology. A variety of hovering spore masses, hues, reverse colonies, and pigments was created by the aerial mycelium. The mycelium on the medium and spore mass surfaces was grey (KNR-2) and white (WGL-1). As aerial spores emerged, the colonies (KNR-1 and WGL-1) became leathery, powdery, and velvety (Fig. 4).

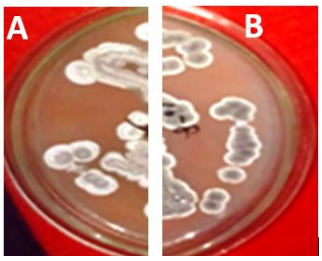

Fig. 4. The colour of the KNR-1 spore mass is leathery grey, whereas the colour of the WGL-1spore mass is chalky white, and concentric rings surround the colonies of KNR-1(A) andWGL-1(B).

Although the colonies appeared the same on the surface, they appeared different when seen from the back. This demonstrated a distinction in substrate hyphae. Grey (KNR-4) and white colony reverse isolates were used (WGL-1). Colony differentiation has been developed on top of this (Fig. 5).

As they grew, the colonies produced concentric rings with sharp edges at the extremities and a matte and prickly finish in the center (Fig. 4).

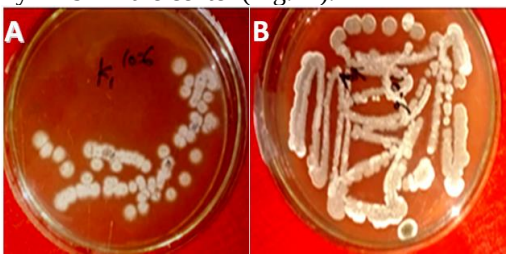

Fig. 5. Isolates showing different mycelial colors (A=KNR-1and B=WGL-1).

On various media to evaluate their growth characteristics, KNR-1and WGL-1 isolates were cultured on various media. The use of a sporulation-promoting culture medium is necessary for adequate

morphological characterisation of isolates generating catenulate spores. The rate of increase has also changed over time. The growth medium for two isolates was starch casein agar (ISP4). On glycerol asparagine agar (ISP5) and tyrosine asparagine agar (ISP7), the isolates grew thickly. This is consistent with previous studies (Bowden *et al.*, 1975). Other physiological characteristics include colony appearance, kind of aerial hyphae, and vegetative hyphal development replicating hyphae fragmentation pattern, and asexual reproduction, suggest that they are members of the Actinomycetes genus.

On the two isolates KNR-1 and WGL-1, enzymatic hydrolysis of starch, casein, gelatin, and urea was carried out, in addition to testing for the production of acids from various sugars, sulphur reduction, sugar fermentation, citrate utilization, indole testing, methyl red testing, the Voges-Proskauer test, and the catalase test. The results are broken down into their essential components and shown in Table 2.

Table 1: Growth rate of the isolates.

Growth rate in different media						
Isolates	**YEMEA**	**OMA**	**GAA**	**TAA**	**SCA**	**Soluble pigment**
KNR-1	++	+++	+++	+++	++	NSP
WGL-1	++	+++	+++	++	+++	NSP

+ indicates growth that is moderate; ++ indicates growth that is good; +++ indicates growth that is excellent; Starch casein agar is abbreviated as SCA. YEMEA stands for yeas, extract malt, and extract agar; OMA stands for oatmeal agar; GAA stands for glycerol asparagine agar; and TAA is for tyrosine asparagine agar. NSP: No-soluble pigment.

Table 2: Biochemical characterization of the isolates.

Isolates	Starch	VP	Gelatin	Catalase	Citrate	Urea	Indole	MR	Casein	HS	Sugar Fermentation		
											S	L	D
KNR-1	+	-	NL	+	+	-	+	+	-	+	B	B	A
WGL-1	+	-	PL	+	-	-	-	+	-	+	B	B	B

ALK stands for alkaline, A for acid, and B for generation of both acid and gas. D = dextrose, L = lactose, S = sucrose; Liquefaction, PL: partial liquefaction, NL: No Liquidification; + Positive, - Negative,

In accordance with the Bergey's Manual, Part A (2001), two isolates, KNR-1 and WGL-1, tested positively for starch hydrolysis, suggesting that they may be able to break down starch through the synthesis of the enzyme amylase. These results are based on the fact that the isolates tested positive for starch hydrolysis. All five isolates were found to have negative casein hydrolysis since there was no opaque zone around their growth process. Isolate KNR-1 showed no evidence of gelatin hydrolysis, while isolate WGL-1 showed evidence of partial liquefaction. Urea hydrolysis was shown to be negative in KNR-1 and WGL-1 isolates.

Simultaneously, both isolates tested positive for H2S, indicating blackening of the flanks due to ferrous sulphide precipitation. The Voges-Proskauer test was negative for both isolates. When tested for antibiotic activity against using *Klebsiella pneumoniae, S. enatrica*, and isolate KNR-1, the maximum zone of clearance was around 20 mm, 17 mm against *S. enatrica*, and 15 mm against *Klebsiella pneumoniae.* The WGL-1 isolate has a moderate activity of 20 mm against *S. enterica* and a moderate activity of 20 mm against *Klebsiella pneumonia* (Fig. 6).

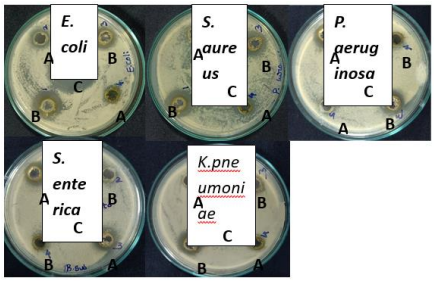

Fig. 6. Antimicrobial activity of crude extract (10 µl) from Actinomycetes (KNR-1and WGL-1), against Pathogenic bacterial strains (C) Control Streptomycin at 1mg/ml, A=KNR-1and B=WGL-1),

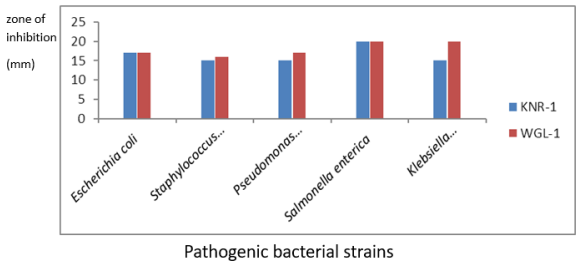

Fig. 7. Antimicrobial activity (in ZOI) against pathogenic bacterial strains shown by Actinomycetes isolates KNR-1, WGL-1.

Molecular Identification and Phylogenetic Analysis. The samples were for antimicrobial activity in addition to their molecular characterization. Because of its strong antibacterial and zone inhibition efficacy, the sample was chosen for molecular investigation. The technique outlined in the Methodology section was applied for the remaining steps of the molecular identification procedure. With consideration for ubiquity, adequate gene length, the conservation of evolutionary markers in bacterial species, and their ability to distinguish between closely related species, amplification of the 16S rRNA gene was investigated for molecular identification and as a specific marker (Arora *et al.*, 2016). The ability of the rRNA gene marker to distinguish across isolates has also been extensively studied (Abol Fotouh *et al.*, 2016). The relative mobility of the DNA sample in relation to the ladder is influenced by the genomic DNA (gDNA), 16S PCR amplicon, and ladder specification (Fig. 7). The results showed that the predicted length of the molecule was probably the same as the length of the sample sequence (1500bp).

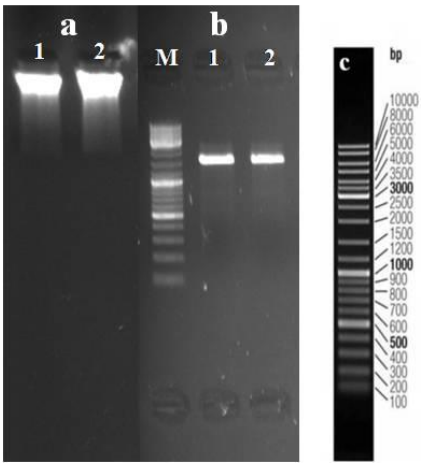

Fig. 8. Agarose gel electrophoresis. (a) DNA extracted from KNR-1and WGL-1, (b) PCR product of 16S rRNA gene of KNR-1and WGL-1, (c) Ladder specification (1500bp).

Results analysis for gDNA and the 16S Amplicon. Genome DNA was extracted from the KNR-1and WGL-1 samples and its molecular characterization is shown in Fig. 7. The first sample ladder is utilized for comparison, and the panel displays the genomic DNA sample's comparative location.

Sequencing and computational identification. The database maintained by the National Center for Biotechnology Information (NCBI), which conducts searches for regions of local similarity between sequences, was able to identify the culture sequence that was most like to the sequence of the purified amplicons. The statistical importance of matches is analyzed by the tool, which does so by comparing nucleotide or protein sequences to various sequence databases. BLAST algorithms may be used to locate members of a gene family as well as to investigate the evolutionary and functional relationships between sequences. (i) Pairwise alignment to get sequence similarity values between the query sequence and the sequences found in step I; (ii) BLASTN search to discover probable closely related type strain sequences (Altschul *et al.,* 1990). (i) Pairwise alignment to obtain sequence similarity values between the query sequence and the sequences identified in stage I. (ii) BLASTN search. (i) Pairwise alignment, with the goal of obtaining values indicating the degree to which the query sequence and the sequences discovered in stage I are comparable. (ii) a search using BLASTN. (States *et al*., 1991). As a direct result of this, isolate is connected to the top five to ten results found in the database. It is thus recommended to do further alignment and phylogenetic analysis of a large number of sequences. The findings collected suggest that the isolates KNR-1and WGL-1 are identical to Actinomycetes (Table 3 and 4) for the nearest and closest similarity index and sequence, for the accurate prediction of species and evolutionary connections (Karlin and Altschul 1990; Myers and Miller 1988). *Streptomyces broussonetiae* (KNR-1) 16s rRNA sequence (1500bp)

***Streptomyces broussonetiae*KNR-1 (Accession No.OQ421513)16s rRNA sequence (1500bp)**

>*Streptomyces broussonetiae* (KNR-1)

GAACGCTGGCGGCGTGCTTAACACATGCAAGT
CGAACGATGAAGCCTTTCGGGGTGGATTAGTG
GCGAACGGGTGAGTAACACGTGGGCAATCTGC
CCTTCACTCTGGGACAAGCCCTGGAAACGGGG
TCTAATACCGGATAACACTCTGTCCCGCATGGG
ACGGGGTTAAAAGCTCCGGCGGTGAAGGATGA
GCCCGCGGCCTATCAGCTTGTTGGTGGGGTAAT
GGCCTACCAAGGCGACGACGGGTAGCCGGCCT
GAGAGGGCGACCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGAAAGCCTGATGC
AGCGACGCCGCGTGAGGGATGACGGCCTTCGG
GTTGTAAACCTCTTTCAGCAGGGAAGAAGCGA
GAGTGACGGTACCTGCAGAAGAAGCGCCGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAG
GGCGCAAGCGTTGTCCGGAATTATTGGGCGTA
AAGAGCTCGTAGGCGGCTTGTCACGTCGGATG
TGAAAGCCCGGGGCTTAACCCCGGGTCTGCAT
TCGATACGGGCTAGCTAGAGTGTGGTAGGGGA
GATCGGAATTCCTGGTGTAGCGGTGAAATGCG
CAGATATCAGGAGGAACACCGGTGGCGAAGGC
GGATCTCTGGGCCATTACTGACGCTGAGGAGC
GAAAGCGTGGGGAGCGAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGTTGGGAACTA
GGTGTTGGCGACATTCCACGTCGTCGGTGCCGC
AGCTAACGCATTAAGTTCCCCGCCTGGGGAGT
ACGGCCGCAAGGCTAAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCAGCGGAGCATGTGGC
TTAATTCGACGCAACGCGAAGAACCTTACCAA
GGCTTGACATATACCGGAAAGCATCAGAGATG
GTGCCCCCCTTGTGGTCGGTATACAGGTGGTGC
ATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTC
TGTGTTGCCAGCATGCCCTTCGGGGTGATGGGG
ACTCACAGGAGACTGCCGGGGTCAACTCGGAG
GAAGGTGGGGACGACGTCAAGTCATCATGCCC
CTTATGTCTTGGGCTGCACACGTGCTACAATGG
CCGGTACAATGAGCTGCGATGCCGCGAGGCGG
AGCGAATCTCAAAAAGCCGGTCTCAGTTCGGA
TTGGGGTCTGCAACTCGACCCCATGAAGTCGG
AGTTGCTAGTAATCGCAGATCAGCATTGCTGCG
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CGTCACGTCACGAAAGTCGGTAACACCCGAAG
CCGGTGGCCCAACCCCTTGTGGGAGGGAGCTG
TCGAAGGTGGGACTGGCGATTGGGACGAAGTC
GTAACAAGGTAGCCG

***Nocardioides sp.*WGL-1(Accession No.OQ421515) 16s rRNA sequence (1500bp)**

>*Nocardioides sp.* (WGL-1)

CGAACGCTGGCGGCGTGCTTAACACATGCAAG
TCGAGCGGTAAGGCCCTTCGGGGTACACGAGC
GGCGAACGGGTGAGTAACACGTGGGTGATCTG
CCTCGTACTTCGGGATAAGCCTGGGAAACTGG
GTCTAATACCGGATATGACCTTCGGATGCATGT
CTGAGGGTGGAAAGATTTATCGGTACGAGATG
GGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAA
TGGCCTACCAAGGCGACGACGGGTAGCCGGCC
TGAGAGGGCGACCGGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGAAAGCCTGATG
CAGCGACGCCGCGTGAGGGATGACGGCCTTCG

GGTTGTAAACCTCTTTCGACAGGGACGAAGCG
CAAGTGACGGTACCTGTAGAAGAAGCACCGGC
CAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGGTGCGAGCGTTGTCCGGAATTACTGGGCGT
AAAGAGCTTGTAGGCGGTTCGTCGCGTCGTTCG
TGAAAACTTGGGGCTCAACCCCAAGCTTGCGG
GCGATACGGGCGGACTAGAGTACTTCAGGGGA
GACTGGAATTCCTGGTGTAGCGGTGAAATGCG
CAGATATCAGGAGGAACACCGGTGGCGAAGGC
GGGTCTCTGGGAAGTAACTGACGCTGAGAAGC
GAAAGCGTGGGTAGCGAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGGTGGGTACTA
GGTGTGGGTTTCCTTCCACGGGATCCGTGCCGT
AGCTAACGCATTAAGTACCCCGCCTGGGGAGT
ACGGCCGCAAGGCTAAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGCGGAGCATGTGGA
TTAATTCGATGCAACGCGAAGAACCTTACCTGG
GTTTGACATACACCGGAAACCTGCAGAGATGT
AGGCCCCCTTGTGGTCGGTGTACAGGTGGTGCA
TGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTATCTT
ATGTTGCCAGCGCGTAATGGCGGGGACTCGTG
AGAGACTGCCGGGGTCAACTCGGAGGAAGGTG
GGGACGACGTCAAGTCATCATGCCCCTTATGTC
CAGGGCTTCACACATGCTACAATGGCCGGTAC
AGAGGGCTGCGATACCGTGAGGTGGAGCGAAT
CCCTTAAAGCCGGTCTCAGTTCGGATCGGGGTC
TGCAACTCGACCCCGTGAAGTTGGAGTCGCTA
GTAATCGCAGATCAGCAACGCTGCGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACG
TCATGAAAGTCGGTAACACCCGAAGCCGGTGG
CCTAACCCTTGTGGAGGGAGCCGTCGAAGGTG
GGATCGGCGATTGGG

Phylogenetic analysis and sequence alignment. Only high-quality sequencing data was used for comparative and phylogenetic analyses (Saitou and Nei 1987; Jukes and Cantor 1969; Koichiro *et al.,* 2013; Felsenstein, 1985; Saitou and Nei 1987; Jukes and Cantor 1969). The neighbor-joining approach, which was developed by Saitou and Nei (1987), was used to infer the history of evolution. A total branch length value of 0.26091989 (best tree) indicates that the tree in question is the best one (8) and (9). According to the results of the bootstrap test (1000 repetitions), which indicates the taxa that collected together close to the branches (Felsenstein, 1985), the proportion of trees that included duplicates. The phylogenetic tree is shown proportionally when the lengths of the branching structures are measured using the same units as the tree itself. After using the Jukes-Cantor technique (Jukes and Cantor 1996), the evolutionary distances were computed based on the number of base substitutions that occurred at each site. We looked at the sequences of nine different nucleotides. There were no blank spots, and the data points were not missing any information. The completed dataset had a total of 599 sites. MEGAX was responsible for doing research on evolutionary processes (Koichiro *et al*., 2013).

Table 3: Sequence producing significant alignments in BLAST analysis.

Sr. No.	Description	Max Score	Total Score	Query Cover	Per. ident	Accession
1.	***Nocardioides* sp. strain KNR-1**	**1991**	**1991**	**100%**	**100**	**OQ421515.1**
2.	Nocardioides alcanivorans strain NGK65	1954	1954	100%	99.26	NR_181938.1
3.	Nocardioidaceae bacterium	1954	1954	100%	99.26	OU764944.1
4.	Nocardioidaceae bacterium WCKl	1832	1832	97%	97.93	HM277865.1
5.	Nocardioides daejeonensis strain MJ31	1814	1814	98%	97.39	NR_109431.1

Fig. 9. Phylogenetic tree illustrating the relationships between other species and *Streptomyces broussonetiae* (KNR-1).

Table 4: Sequence producing significant alignments in BLAST analysis.

Sr. No.	Description	Max Score	Total Score	Query Cover	Per. ident	Accession
1.	Streptomyces broussonetiae strain WGL-1	**1836**	**1836**	**100%**	**100**	OQ421513.1
2.	Streptomyces broussonetiae strain T44	1799	10798	100%	99.2	CP047020.1
3.	Streptomyces broussonetiae strain T44 16S ribosomal RNA gene	1799	1799	100%	99.2	MN826232.1
4.	Streptomyces broussonetiae strain T44 16S	1799	1799	100%	99.2	NR_181957.1
5.	Streptomyces broussonetiae strain T44 16S ribosomal RNA	1799	1799	100%	99.2	NR_181006.1

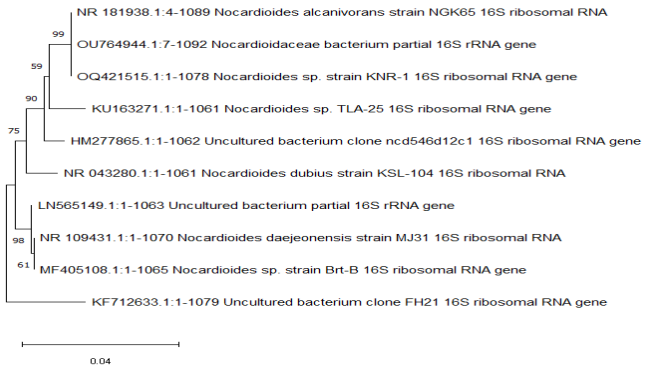

Fig. 10. Phylogenetic tree showing the relation of *Nocardioides* sp. *(*WGL-1) with other species.

DISCUSSION

Actinomycetes strains were isolated and heat-treated from chilli plant rhizosphere soil and evaluated morphologically, culturally, and biochemically. Isolates KNR-1 and WGL-1 displayed substantial branching and true mycelial structures composed of filamentous non-septate hyphae. The hyphae that carry the spores are designed with a flexible structural framework. The genus its description as aerobic Actinomycetes with a high GC content. These actinomycetes have a greater ability to create acid from carbohydrates, and they also grow enormous monopodial and aerial mycelia, respectively (Sharma *et al.,* 2014.). The KNR-1 strain displayed a flexible spore chain morphology, and then the WGL-1 strain arrived with its open loops in the retinaculum. Both of these morphologies may be seen in the image below. According to H.D. Tresne's 1961 taxonomy of actinomycetes, the chromogenicity of aerial mycelium is considered to be a significant component in the classification of actinomycetes. Actinomycetes have significant tools, such as spore morphology and spore surface decoration (El-Naggar and El-Shweihy 2020). KNR-1colonies were grey-puffed and leathery with elevated powdered centres and granulated edges. WGL-1 colonies were white chalky-circular with elevated smooth centres and broken borders. The cover slip approach (Khan and Saha 2008) and the slide culture technique (Okami and Umezawa 1968) are two methods that may be used in order to detect spore-bearing hyphae and spore chains when seen via a light microscope. The isolates grew and sporulated normally. Oatmeal agar (ISP3) aided in the rapid development of the isolates. The aerial mycelium development that occurred on yeast extract-malt extract agar (ISP2) must be ascribed to the nutrients that were present in the medium. It was shown in prior studies (Marroquin, 1962; Kirk *et al.,* 2002) that the medium composition facilitated the formation of both aerial and substrate mycelium. Both isolates showed signs of starch hydrolysis, catalase activity, methyl red presence, and hydrogen sulfide production. These enzymes are the most prevalent kind used in production. Pramanik *et al.* (2020), and they are widely used in the food processing, medicinal, soap, and detergent industries. Actinomycetes may degrade simple and complex compounds in their environment (Devanshi *et al*., 2021). As well as their composite components and genetic composition (Jagannathan *et al.,* 2021).

Tested for antibacterial activity were KNR-1and WGL-1 isolates. It was formerly thought that distinguishing active secondary from Actinomycetes would be exceedingly difficult. However, with recent breakthroughs in next-generation sequencing (Manteca and Yagüe 2019). The discovery of new isolates paves the path for the investigation and identification of novel bioactive substances with exceptional therapeutic promise. *Streptomycetes* make antibacterial substances, although only 3% of them have been noticed. Actinomycetes have received a lot of interest due to the worrying increase in multidrug-resistant infections and novel phytopathogens. Actinomycetes have been isolated from previously unknown locations by scientists.

The analysis's results are shown in (Table 3). The same table shows the relative abundance of the four nucleotides in the first, second, and third codon positions as well as a comparison of the closely related Actinomycetes species. The Actinomycete strain KNR-

1 has been identified as *Streptomyces broussonetiae*, while WGL-1 has been identified as *Nocardioides sp.*. As a result, the strains were classified as Actinomycetes members. The fact that there is little data on the enzyme activities of the uncommon Actinomycetes that have been discovered suggests that they may be capable of producing hydrolytic enzymes that may be exploited in industry.

CONCLUSIONS

The KNR-1 and WGL-1 Actinomycetes that have been obtained were isolated from the indigenous flora of soil resources by utilizing starch casein agar, which is used for isolating bacteria, and Actinomycetes isolation agar medium, which is used for isolating yeast. This resulted in smooth, leathery, matte colonies that were cultivated using the spread plate method. When a soil sample taken from the chili rhizosphere is heated, the formation of Actinomycetes is enhanced while the growth of other organisms is inhibited. In addition, the fertility of the soil provides evidence that actinomycetes are present. Actinomycetes are well-known for their ability to produce soluble inorganic materials via the breakdown of chemical compounds that originate from organic forms. This process occurs when Actinomycetes feed on organic matter. Isolate yields were all over the place, despite the fact that KNR-1 and WGL-1 showed dominating growth with better sporulation. From the rhizopheric soil of chilli plants, *Streptomyces broussonetiae* and *Nocardioides sp*. were able to be isolated, and their cultural, morphological, and biochemical characteristics were investigated. The KNR-1 and WGL-1 Actinomycetes isolate shown antibacterial activity against *Klebsiella pneumonia, Salmonella enterica, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Escherichia*. However, these Actinomycetes isolates had a lower level of activity against *Klebsiella pneumoniae* in comparison to other test pathogens. The collected DNA was used as a template in PCR reactions, and the 16S rDNA gene was sequenced to identify the various isolates. The size of the *Streptomyces broussonetiae* amiplicons was 1500 base pairs, while the size of the *Nocardioides sp.* amiplicons was also 1500 base pairs. Actinomycetes are the genus from which the isolates KNR-1 and WGL-1 originate. These isolates have antibacterial activity and can be used in the research and development of novel antibiotics for either the pharmaceutical or agriculture industries.

FUTURE SCOPE

Based on the results of this study, it is possible to conclude that Actinomycetes, *Nocardioides* sp. WGL-1 and Streptomyces broussonetiae KNR-1 produce a diverse range of compounds with diverse biological properties. Among the isolates obtained from Karimnagar and Warangal rhizospearsoils of various plants, the strains demonstrated high antibacterial activity. As a result, this strain's antibacterial compounds could be used to treat diseases caused by drug-resistant organisms. This is suggested, but more research and studies are required to identify the active compound responsible for the antibacterial activity and to establish it.

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Conflict of interest. None.

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