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Isolation and identification of Actinomycetes with antifungal activity from karts ecosystem in Maros-Pangkep, Indonesia

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Abstract. *Rante H, Manggau MA, Alam G, Pakki E, Erviani AE, Hafidah N, Abidin HL, Ali A. 2024. Isolation and identification of Actinomycetes with antifungal activity from karts ecosystem in Maros-Pangkep, Indonesia. Biodiversitas 25: 458-464.* Actinomycetes have yielded various biologically active secondary compounds with intriguing properties like antimicrobial, antiviral, and anticancer effects. This research aimed to isolate, identify, and screen the antifungi from soil environmental samples collected from the karst ecosystem in Maros-Pangkep, Indonesia. The active isolate is then fermented for the production of secondary metabolites. The fermentation process uses an M1 medium under agitated conditions at 150 rpm for 12 days. The isolate Actinomycetes were identified based on sequence gen 16S rRNA. Screening of antifungal activity was carried out against *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404 by antagonistic test. The diffusion method was applied using the paper disc to assess the antifungal activity. The result revealed that 8 isolates were purified from the soil samples collected. From the 8 isolates of Actinomycetes obtained, two Actinomycetes exhibited antifungal activities in the screening methods, namely isolates with code B11 and B 17. The crude extract of isolate B11 was active against *C. albicans* and *A. niger* at concentrations of 2 mg/paper disc, 1.5 mg/paper disc, and 0.75 mg/paper disc. Furthermore, isolate B17 was found to be only active against *C. albicans*. The phylogenetic analysis of the 16S rRNA gene sequences indicated that B11 showed the highest similarity to *Streptomyces tuirus* strain NBRC 15617.

Keywords: Actinomycetes, *Aspergillus niger*, *Candida albicans*, karst, *Streptomyces tuirus* strain NBRC 15617

INTRODUCTION

The discovery of new antibiotics has been enhanced through metabolite screening and chemical synthesis methods, leading to significant advancements in antibiotic compound production over the past decade. However, the issue of antibiotic resistance has concurrently escalated alongside the increased utilization of antibiotics (Selvin et al. 2009). Ongoing efforts involve the exploration of potential natural reservoirs, including plants, animals, and microorganisms, to identify lead compounds based on their functional molecular frameworks. The discovery of functional compounds for therapeutic purposes remains a major challenge for the industry, particularly concerning the rising prevalence of infectious diseases (Sharma et al. 2014). Furthermore, microbial infections will lead to around 10 million global deaths annually by 2050 (Mancuso et al. 2021). Hence, identifying novel therapeutic compounds from natural origins is crucial for advancing antimicrobial treatments (Roncarati et al. 2022). Actinomycetes represent a valuable reservoir of bioactive substances and enzymes (De Simeis and Serra 2021).

The abundant biodiversity in Indonesia, the world's second-largest mega biodiversity-rich tropical country, represents a prime asset for discovering chemical frameworks with immense potential for drug development. However, the wealth of biological resources remains underexplored, particularly concerning the pharmaceutical field. One promising source of bioactive compounds is microorganisms originating from specific ecological niches, such as karst formations. Karst regions play a crucial role for various organisms in sustaining their livelihoods through adaptation. A correlation exists between discovering novel compounds (new molecular frameworks) and organisms in regions exhibiting unique ecological characteristics (Lavoie et al. 2010).

Indonesia is a nation comprised of numerous Southeast Asian islands encompassing the largest karst region, covering an area of approximately 400,000 km^2 (Clements et al. 2006). The Maros-Pangkep Karst Region has been

internationally recognized since before World War II. It is renowned for its unique natural formations (geomorphology) that are unparalleled and not found elsewhere. The Maros-Pangkep Karst Region is characterized by its tower karst features, making it one of a kind in Indonesia. Furthermore, its breathtaking natural panoramas and abundant biodiversity potential add to its exceptional appeal (Ko 2001).

This research focuses primarily on sampling from the soil inside the cave in the Karst ecosystem. Cave environments are characterized by their lack of light, low nutrient levels, and high moisture content (Kováč 2018). In these specialized habitats, most microorganisms produce various biomolecules to compete for nutrients. As a result, microorganisms that have adapted to cave conditions have the potential to yield unique bioactive products, including antimicrobial compounds (Cheeptham et al. 2013). Among the microbial flora in cave ecosystems, Actinomycetes are the predominant members. Therefore, it is highly likely that Actinomycetes from caves are valuable resources for discovering novel antibiotics (Rangseekaew and Pathom-Aree 2019).

Actinomycetes generate a wide range of bioactive metabolites, with many serving as significant starting points for therapeutics. Hence, investigating them offers a vast source of potentially potent compounds (De-Simeis and Serra 2021). Recently, there has been a notable increase in research endeavors aimed at isolating Actinomycetes from a wide range of cave environments, with a specific emphasis on investigating their potential for producing antimicrobial compounds (Belyagoubi et al. 2018; Hamedi et al. 2019; Long et al. 2019; Syiemiong and Jha 2019; Jaroszewicz et al. 2021). The Actinomycetes residing in karstic caves are expected to offer exciting prospects for uncovering new bioactive metabolites (Yusel and Yamac 2010). Fang et al. (2017) isolated *Nonomuraea cavernae* sp. nov, a novel actinobacterium, from a karst cave in Xingyi County, Guizhou Province, China. Actinomycetes were isolated from Karst Caves in the Eastern Black Sea Region of Türkiye. The Actinomycetes isolates are capable of inhibiting the growth of the test microorganisms. These findings indicate that karst caves offer promising prospects for isolating Actinomycetes capable of producing antimicrobial compounds (Tüfekci et al. 2023).

Furthermore, the environmental constraints within the caves create competitive conditions among cave microbes for resources, thereby increasing the likelihood of isolating novel microbial strains with the potential to produce beneficial substances for pharmaceutical applications. The objectives of this experiment were to isolate Actinomycetes from environmental samples collected from the karst ecosystem and characterize Actinomycetes strains using molecular methods.

MATERIALS AND METHODS

Isolation of Actinomycetes

The samples utilized in this study were obtained from the soil inside the cave in karst ecosystem of Bantimurung (inside Bantimurung-Bulusaraung National Park), South Sulawesi Province, Indonesia (Figure 1). The soil samples were carefully placed into sample pots and subsequently transported to the laboratory for analysis and investigation. The soil samples from the Karst ecosystem area were subjected to serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. From the last 3 dilutions, 0.1 mL was aseptically spread on the surface of International Streptomyces Project (ISP4) agar plates supplemented with 100 µg nystatin/mL of the medium. All agar plates were incubated at room temperature for 7 days until distinct colonies with distinguishable growth characteristics emerged. Subsequently, microbial isolates showing distinct colony morphology on each plate were purified repeatedly on ISP2 agar media until single colonies indicative of pure isolates were obtained. Once purified, the isolates were streaked onto agar plates for further research as stock cultures.

Antagonistic test of Actinomycetes

The Actinomycetes isolates were grown on ISP2 agar media, and then the agar block containing the cultures was cut using a stainless steel cork borer. Test fungi *Aspergillus niger* and *Candida albicans* were inoculated into Potato Dextrose Agar (PDA). The agar blocks were placed on the surface of the media where the fungi test were growing. The zone of inhibition surrounding the Actinomycetes agar block was observed and measured after 24 hours of incubation at 37°C (Rante et al. 2022).

Production of secondary metabolite

The active isolates were cultured in a 250 mL Erlenmeyer flask containing 100 mL of M1 medium (starch, yeast extract, and peptone) at room temperature for 3 days. Seed medium was transferred into 500 mL Erlenmeyer containing 100 mL M1 medium then the medium was adjusted at pH 7.4. Fermentation was conducted at room temperature for 12 days, with continuous shaking at a speed of 150 rpm. The fermentation product was sonicated for 1 hour and then filtered to separate the biomass and the fermented liquid. The fermented liquid was extracted with ethyl acetate (1:1 v/v) using a separating funnel, with each extraction lasting 20 minutes. Water phase the obtained extract was evaporated and stored in a desiccator for future use in subsequent tests (Rante et al. 2022).

Antifungi activity

The antifungal activity testing of water extract and ethyl acetate extract (B11 and B17) was conducted using the disc diffusion method on cultures of *C. albicans* and *A. niger*. *A. niger* ATCC 16404 and *C. albicans* ATCC 10231 suspensions were each taken in a quantity of 1 colony and spread on PDA medium in separate petri dishes. Ethyl acetate extract and water extract at concentrations of 2.5%, 5%, and 10% were pipetted onto disc paper (6 mm in diameter) with a volume of 20 µL. Nystatin (100 µg/disk) was used as a positive control. The petri dishes containing the cultures and various concentrations of extracts and the control were incubated for 3 x 24 hours at a temperature of 27±1°C. The inhibition zone formation parameter was determined by observing the clear zones around the disc paper on the PDA medium. The diameter of fungal growth inhibition was measured using a caliper (Keikha et al. 2015).

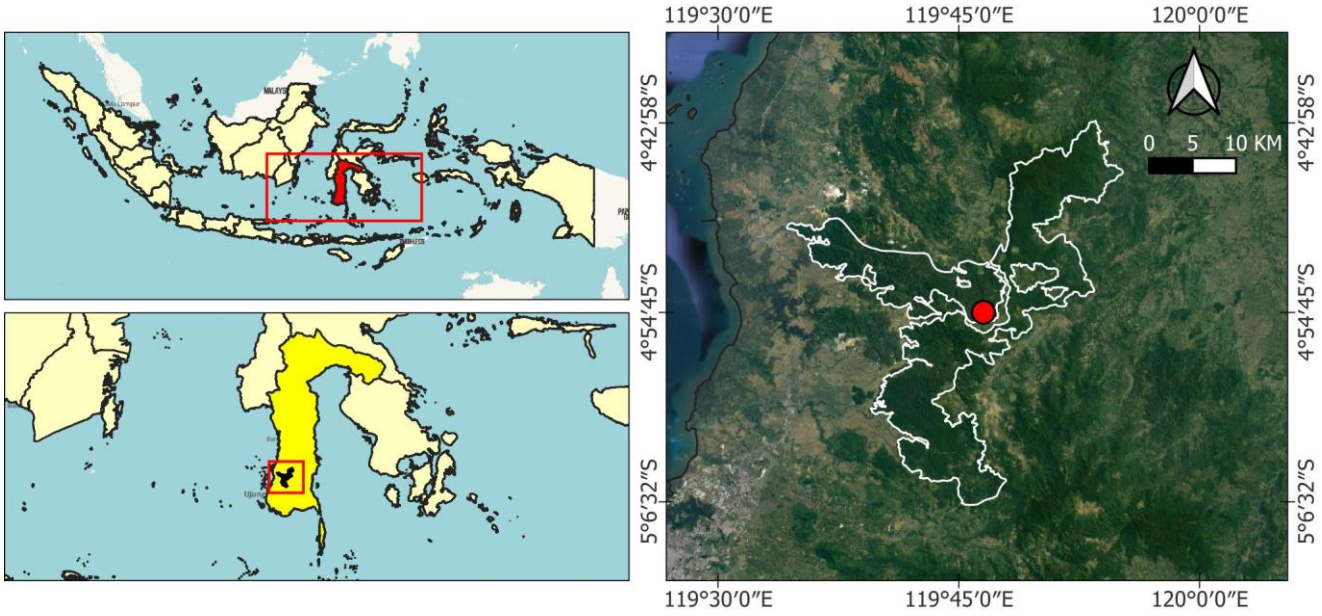

Figure 1. Map of research location in Bantimurung-Bulusaraung National Park, South Sulawesi, Indonesia

Molecular characterization of Actinomycetes isolates using the 16S-rRNA gene isolation of DNA

The DNA isolation process followed the method outlined by Song et al. (2004). Initially, 1 mL aliquot of a 7-day-old Actinomycetes culture was pipetted into a micro sterile tube. Next, the mixture was centrifuged at 13,000 rpm for 5 minutes, and the resulting supernatant was removed. The resulting pellets underwent a wash with 400 µL of TE (Tris-EDTA) and were subsequently centrifuged again at 13,000 rpm for 5 minutes. It was then resuspended in 400 µL of buffer SET (75 mM NaCl) and subjected to an incubation period at 37°C in a one-hour water bath. During this time, homogenization by inversion was performed every 15 minutes. Following this, 50 µL of SDS 10%, 20 µL of protease, and 50 µL of RNAase were added, and the mixture was incubated at 65°C in a water bath for 2 hours. Once this incubation period was completed, 167 µL of 5M NaCl was introduced to the pellets. Centrifuge at 5,000 rpm in a microcentrifuge for one minute to pellet the sample. Then, transfer the supernatant (~180 µL) to a new microcentrifuge tube. Save both the supernatant and pellet. Add 100 µl PBS (user-supplied) to the sample pellet and pipette mix until the pellet is visibly resuspended. Centrifuge at 5,000 rpm for 1 minute to pellet the sample. Add 1 mL PBS (user-supplied) to the new pellet and mix until the pellet is visibly resuspended. Centrifuge at 5,000 rpm in a microcentrifuge for 1 minute to pellet the sample and discard the supernatant. Add 100 µL TE Buffer and 25 µL lysozyme4 (100 mg/mL; user supplied) to the pellet. Pipette mix until pellet is visibly resuspended, then incubate at 55°C for 30 minutes. Add 20 µL 10% SDS (user provided) and 10 µL Proteinase K. Briefly pipette mix and incubate at 55°C for 10 minutes. Centrifuge 5,000 rpm in a microcentrifuge for 1 minute to pellet residual debris. Move the supernatant to a fresh microcentrifuge tube. Then, combine the sample with 800 µL (2 volumes) of Quick-DNA™ MagBinding Buffer and ensure thorough mixing. Next, purify the DNA using Quick-DNA™ MagBead Plus Kit; the purity of the DNA solution was checked by using a spectrophotometer at λ260 and λ 280nm, and the amount of DNA measured at λ260 nm (Zymo Research 2023).

Amplification of 16SrDNA sequences and phylogenetic tree analysis

The 16S rDNA sequencing was conducted through the PCR method using MyTaq HS Mix Red Protocol with the primers 27f (5'AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'GGTTACCTTGTTACGACTT-3'The thermal cycling process began with an initial denaturation of the target DNA at 95°C for 1 minute. This was followed by 25 cycles comprising denaturation at 95°C for 20 seconds, primary annealing at 55°C for 15 seconds, and primary extension at 72°C for 4 minutes. The process concluded with a cooling step at 4°C. Subsequently, the PCR amplification was verified through agarose electrophoresis gel and visualized using UV fluorescence after staining with ethidium bromide. The PCR products were then sequenced. The obtained sequencing results were compared with genomes in the bank database using NCBI BLAST. (www.ncbi.nih.gov/).

RESULTS AND DISCUSSION

The result revealed that 8 isolates were purified from the soil samples from the medium ISP 2. Amongst all isolates found, 2 isolates of Actinomycetes exhibited antifungal activities in the screening methods or antagonistic test using *A. niger* ATCC 16404 and *C. albicans* ATCC 10231; the isolates B11 and B17 also produce soluble pigment. Many genera belonging to the Actinomycetes group are known to produce pigments of different colors (Gupta et al. 2022). The samples utilized in this study were obtained from the soil inside the cave in Karst ecosystem Bantimurung (Figure 1).

Isolation of Actinomycetes using the pure plate methods with ISP 4 medium. It is necessary to characterize the morphological traits of the Actinomycetes to distinguish actinobacterial isolates from bacterial and fungal contaminants. Actinomycetes exhibit a branched mycelium structure comprising both aerial and vegetative mycelium. They typically produce spores, resulting in colonies that resemble a sandy surface (Lacey 1997). Furthermore, Actinomycetes colonies display a dry, hazy, granular appearance, powdery colonies with filamentous appearance grown on a medium, and their growth is not evenly distributed (Madigan et al. 2003). Moreover, numerous investigations have indicated that these pigments may possess bioactive attributes, including antibacterial, antifungal, antioxidant, and anticancer properties (Mesrian et al. 2021). The purpose of the antagonistic test is to assess the antifungal potential in the initial stages before fermentation is carried out. The active Actinomycetes namely isolates with code B11 and B 17 (Figure 2).

Fermentation and extraction of secondary metabolite isolate B.11 and B.17

Fermentation was conducted in an M1 medium composed of starch, yeast extract, and peptone for isolates exhibiting inhibition activity against the test fungi, namely Actinomycetes B11 and B.17. The fermentation process was conducted with agitation at 150 rpm and a temperature of 28°C. Agitation is necessary because Actinomycetes bacteria in unagitated liquid media form a thin layer on the media surface (Madigan et al. 2003), producing suboptimal secondary metabolite. Additionally, agitated conditions are intended to allow the Actinomycetes isolates to utilize the nutrients present in the media efficiently. The fermentation results revealed that the production of secondary metabolites against *C. albicans* was observed on the 8th day of fermentation for isolate B11 and on the 12th day for isolate B.17. For isolate B.11, antifungal activity started to decrease on the 9th day, while for isolate B.17, antifungal activity began to decrease on the 13th day (Figure 3).

The optimum time for producing secondary metabolites in Actinomycetes B11 and B17 varies. This is because, in general, the growth patterns of microorganisms are highly diverse and influenced by genes and nutrient sources. The collection of secondary metabolites is best done in the stationary phase or during the highest secondary metabolite production phase. Prolonged fermentation beyond this phase does not increase the abundance of secondary metabolites from the microorganisms (Song et al. 2012). The fermentation results are then sonicated and separated into supernatant and biomass. The obtained supernatant is extracted using ethyl acetate solvent in a 1:1 v/v ratio to yield the ethyl acetate extract. At the same time, the aqueous layer is freeze-dried to obtain the water extract for further testing; ethyl acetate solvent is used to extract the fermentation liquid or supernatant. Sah et al. (2021) extracted antibacterial metabolites from the fermentation liquid using various organic solvents (n-butanol, chloroform, dichloromethane, ethyl acetate, and methanol), but only the ethyl acetate solvent was able to extract potent metabolites at a detectable level from the fermented broth. This could be attributed to the superior solubility of the metabolites in the ethyl acetate solvent (Sah and Lekhak 2017). The extracted ethyl acetate from Actinomycetes B.11 against *A. niger* and *C. albicans*, while the ethyl acetate extract from Actinomycetes B.17 only inhibits *C. albicans*. On the other hand, water extract does not exhibit inhibitory effects on the tested fungi (Table 1).

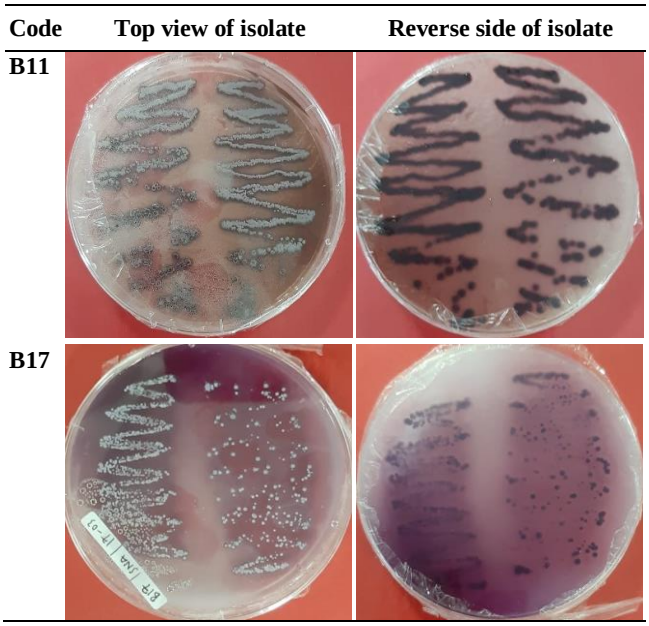

Figure 2. Actinomycetes isolated from karst Bantimurung-Bulusaraung National Park ecosystem, South Sulawesi, Indonesia

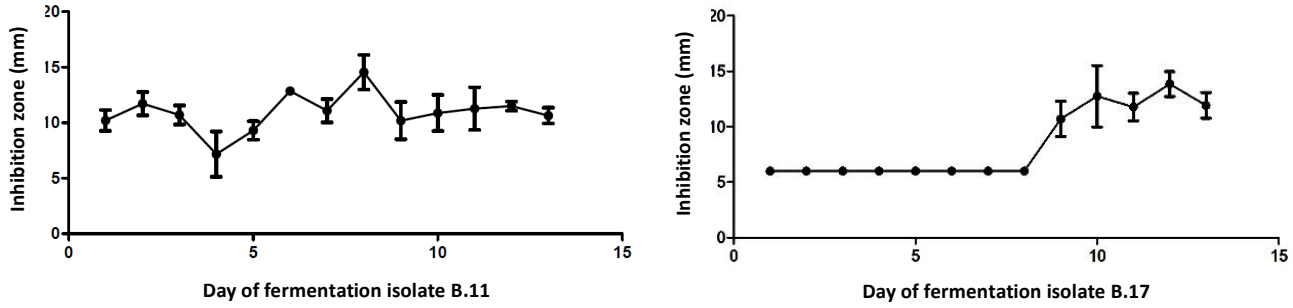

Figure 3. The curve depicts the relationship between the day of fermentation of isolates B11 and B17 and their antifungal activity (mm) against *Candida albicans*

Molecular characterization of Actinomycetes isolates using the 16S-rRNA gene

Molecular characterization of Actinomycetes B.17 was carried out in several stages: DNA isolation, amplification of 16S rRNA encoding genes by PCR, 16S rRNA gene sequencing, bioinformatics analysis, and phylogenetic tree analysis. In this study, molecular identification was only conducted on isolate B11 because it is active against *A. niger* and *C. albicans*. Isolation and amplification of 16SrRNA gene using PCR primer 27f and 1492r can be amplified around 1400 bp determined by electrophoresis gel agarose (Figure 4)

Characterization and molecular identification relied on examining 16S rRNA gene sequences using the universal primers 27f and 1492r (Zhang et al. 2009). The nucleotides resulting from the analysis of 16S rRNA gene sequences from Actinomycetes B11 were analyzed using BLAST (Basic Local Alignment Search Tool) via http://blast.ncbi.nlm.nih.gov/Blast.cgi (Table 2) to determine the relationships among reference strains. The 16S rRNA gene analysis results with reference strains are depicted as a phylogenetic tree (Figure 4).

Table 1. Antifungi activity test from secondary metabolite Actinomycetes B.11 dan B.17

Extract		Diameter of inhibition zone (mm)			
		B11		B17	
		C. albicans	*A. niger*	*C. albicans*	*A. niger*
Extract 10%	Ethyl acetate	9.75 ± 1.08	10.88 ± 1.55	12.66 ± 3.83	6.00 ± 0.00
	Water	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
Extract 5%	Ethyl acetate	9.34 ± 0.88	9.96 ± 0.93	9.78 ± 0.98	6.00 ± 0.00
	Water	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
Extract 2.5%	Ethyl acetate	9.08 ± 0.88	8.83 ± 0.36	9.29 ± 2.05	6.00 ± 0.00
	Water	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
Nystatin	-	24.82 ± 1.7	24.35 ± 3.29	24.19 ± 2.46	25.02 ± 0.99

Table 2. BLAST Nukleotida Actinomycetes B.11

Description	Max Score	Total Score	Query Cover	E Value	Per. Ident	Accession
Streptomyces tuirus JCM 4255 DNA, complete sequence	2545	15269	100%	0.0	99.93%	AP023439.1
Streptomyces tuirus strain NBRC 15617 16S ribososmal RNA, partial sequence	2545	2545	100%	0.0	99.93%	NR_041190.1
Streptomyces sp. ERI-CPDA-1 16S ribosomal RNA gene, partial sequence	2540	2540	99%	0.0	99.86%	HQ385919.1
Streptomyces tuirus strain ICSSB 1017 16S ribosomal RNA, partial sequence	2540	2540	99%	0.0	99.86%	NR_114666.1
Streptomyces violaceoruber strain NRRL B-3631 16S ribosomal RNA gene, partial sequence	2538	2538	99%	0.0	99.86%	AY999733.1
Streptomyces sp. NKS15 gene for 16S ribosomal RNA, partial sequence	2532	2532	99%	0.0	99.78%	LC489241.1
Streptomyces sparsus strain RHRM30 16S ribosomal RNA gene, partial sequence	2532	2532	99%	0.0	99.78%	MH209254.1
Streptomyces tuirus strain AR-7 16S ribosomal RNA gene, partial sequence	2532	2532	99%	0.0	99.78%	KX055838.1
Streptomyces tuirus strain PAS9 16S ribosomal RNA gene, partial sequence	2532	2532	99%	0.0	99.78%	KR296715.1
Streptomyces sp. SAS04 16S ribosomal RNA gene, partial sequence	2532	2532	99%	0.0	99.78%	KP986571.1

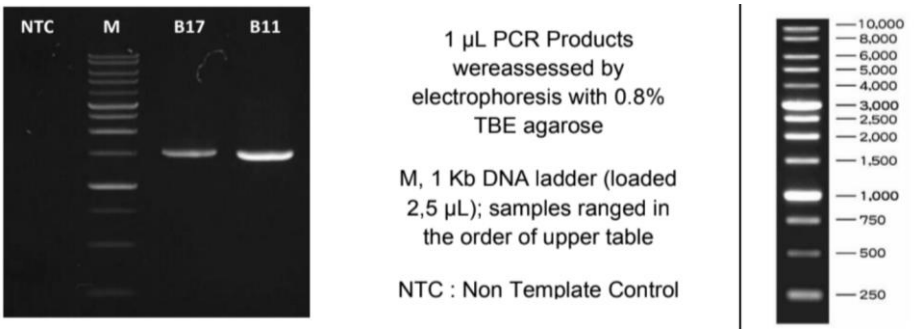

Figure 4. Electrophoresis of PCR Product from Amplification Gen 16S rRNA Isolate B.11

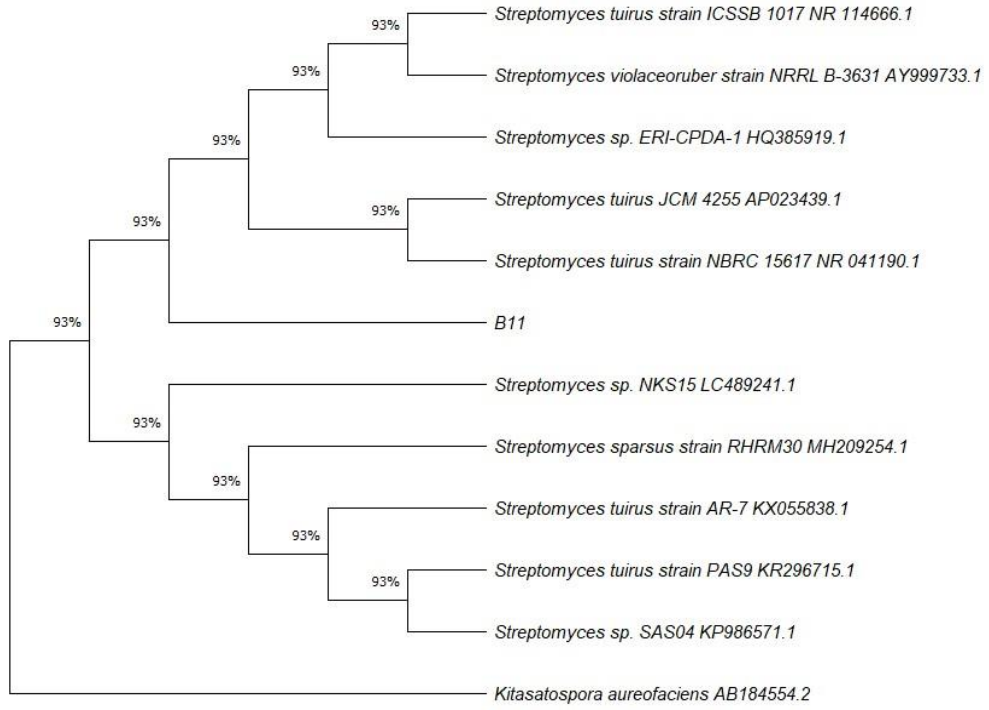

Figure 5. The phylogenetic tree was constructed using the neighbor-joining tree method

The 16S rRNA sequences of the isolates were compared to reference sequences in GenBank and revealed that the isolates belong to the *Streptomyces* with a percentage identity of 99.93 (Table 2). Based on the phylogenetic tree, it is shown that isolate B11 is most closely related to *Streptomyces tuirus* strain NBRC 15617 (Figure 5). Among the different general of Actinomycetes, Streptomyces is the most recognized genus and is widely distributed in soils (Dhanasekaran et al. 2008). Most recent antifungal metabolites were identified from *Streptomyces*.

Streptomyces have been widely studied for their production of medically important secondary metabolites. Over 95% of the Actinomycetes strains were obtained from soil, with Streptomyces sp. being the predominant genus (Barka et al. 2016). Antibiotics derived from *Streptomyces* species play a pivotal role in the realm of medicine, constituting roughly half of the therapeutically significant antibiotics. Additionally, studies have shown that, on average, individual strains of Streptomyces can generate more than 30 secondary metabolites (Lee et al. 2020).

Conclusion, among the eight Actinomycetes isolates obtained from soil in the karst ecosystem, 2 isolates of Actinomycetes exhibited antifungal activities against *A. niger* ATCC 10231 and *C. albicans* ATCC 10231 with codes B11 and B 17. The ethyl acetate extract from Actinomycetes B11 was able to inhibit *C. albicans* and *A. niger*, while the ethyl acetate extract from Actinomycetes B17 only inhibited *C. albicans.* Phylogenetic analysis of the 16S rRNA gene sequences revealed that isolate Actinomycetes B.11 was most closely related to *S. tuirus* strain NBRC 15617 with a similarity value of 99.93 %.

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