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# Isolation of Actinomycetes from rhizosphere soil of *Nephrolepis cordifolia* as a producer of antifungal compounds in the karst of Bantimurung

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**ABSTRACT**: *Actinomycetes* are anaerobic bacteria, either rod-shaped or filamentous, Gram-positi0ve, that produce antibiotic and antifungal compounds. This study aims to isolate and explore the potential of *actinomycetes* as producers of antifungal compounds from the rhizosphere of *Nephrolepis cordifolia* in the karst ecosystem of Bantimurung, South Sulawesi, Indonesia. Primary and secondary screenings were conducted against *Candida albicans* and *Aspergillus niger* using 17 *Actinomycetes* isolates from the rhizosphere of *Nephrolepis cordifolia*. All isolates were tested antagonistically, and two isolates that exhibited the highest antifungal activity were designated RKP-A.1-2 and RKP-B.1-1. These active isolates were then fermented for 16 days at a speed of 150 rpm. The fermentation products were then sonicated to separate the supernatant and biomass. The supernatant was extracted using ethyl acetate (1:1 v/v) and tested using the diffusion method. The test results indicated that the ethyl acetate extract from the fermentation of both isolates was able to inhibit the growth of *Candida albicans* up to a concentration of 0.5 mg/µ of extract from RKP-A.1-2 and RKP-B.1-1. The compound profiles of the secondary metabolites produced by *Actinomycetes* and extracts from karst and non-karst *Nephrolepis cordifolia* showed different compound profiles, as evidenced by distinct Rf values. Phylogenetic analysis of the 16S rRNA gene sequences revealed that RKP-A.1-2 and RKP-B.1-1 are closely related to *Streptomyces sp* . with a similarity value of 100.00%. From the results of this study, it can be concluded that *Actinomycetes* from the rhizosphere of *Nephrolepis cordifolia* in the karst ecosystem of Bantimurung have potential as producers of antifungal compounds that warrant further investigation. This research provides a significant contribution to the understanding and utilization of these microorganisms as potential sources of bioactive compounds.

**Keywords:** *Actinomycetes*, antifungal, karst, rhizosphere, *Nephrolepis cordifolia*, 16S rRNA gene sequence

## 1. INTRODUCTION

Antibiotic-resistant bacterial infections will still be on the rise in 2024. Data from studies and medical journals indicate that there are more than 2.8 million antibiotic-resistant illnesses worldwide. Around 4.95 million fatalities worldwide are thought to result from antibiotic resistance each year[1]. Therefore, the search for new antibiotic sources to combat infections caused by antibiotic resistance is crucial for the discovery of new antibiotics. These bioactive compounds are naturally produced by various species of fungi and bacteria, but the most intriguing class of microorganisms capable of producing these secondary metabolites is actinobacteria, particularly *actinomycetes*. *Actinomycetes* are a group of Gram-positive, aerobic bacteria that possess aerial hyphae similar to fungi [2, 3].

Some Actinomycetes have roles as antibiofilm, anticancer, antibiotic, antiviral, antiparasitic, and immunosuppressive agents, and they are involved in the production of approximately 23,000 bioactive secondary metabolite compounds [4,5]. Moreover, according to research by Suryaminarsih et al. (2020), *Actinomycetes* are capable of producing cell wall-degrading enzymes, such as chitinase and lipase, which can

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damage fungal cell walls [6]. The transitional forms of *Actinomycetes* enable these bacteria to serve as natural antifungal agents that are easily obtainable [5].

*Actinomycetes* inhabit various environments, including grasslands, mud, sediments, marine waters, soil, and the rhizosphere of plants and their surroundings [7]. Soil, in particular, serves as the primary habitat for *Actinomycete*s, offering optimal conditions for the growth and diversity of microorganisms [7]. It acts as the largest reservoir of biodiversity on Earth, hosting diverse microbial communities comprising bacteria, fungi, archaea, and viruses, all of which play essential roles in maintaining soil biodiversity [10]. Soil microorganisms contribute to ecosystem functions by providing fixed nitrogen sources for plants and regulating soil nutrients, thereby enhancing plant resilience [10].

The rhizosphere is the soil region surrounding plant roots where roots and soil meet, containing nutrients that produce secondary metabolites [8]. One of the microorganisms thriving in the mineral-rich and nutrient-abundant rhizosphere is microscopic fungi, commonly referred to as rhizosphere fungi. Rhizosphere fungi can be utilized as biocontrol agents, as they are reported to exhibit antagonistic activities against pathogenic fungi through mechanisms such as hyperparasitism and antibiosis. As a biotic factor, rhizosphere fungi have the ability to induce plant resistance to diseases and also contribute to plant fertility [9].

Karst ecosystems are a type of landscape typically characterized by high rock exposure, calcium-rich and nutrient-dense soil, and significant microbial activity [10]. These ecosystems exhibit a high diversity of microhabitats within karst regions [11]. According to previous research by Retnowati et al. (2024), isolating and identifying actinomycetes in the rhizosphere of plants in karst ecosystems in Gorontalo showed specific associations with various types of microbes [20].

*Nephrolepis cordifolia*, one of the recognized ferns, has a species count of 10,000. Common names for the fern *Nephrolepis cordifolia*, which is endemic to northern Australia and Asia, include fishbone fern, sword fern, ladder fern, and erect sword fern [12]. *Nephrolepis cordifolia* is a plant that grows best in forests. It has several chemical components, including terpenes, steroids, saponins, and alkaloids, which are used for their antibacterial, antiviral, antidiabetic, and anticancer effects. *Nephrolepis cordifolia* has been shown to have cytotoxic action and antibacterial and antifungal qualities in earlier studies by El-Tantawy et al. (2015) [13]. Renjan et al. (2021) state that *Nephrolepis cordifolia* exhibits considerable promise as a therapeutic plant [14].

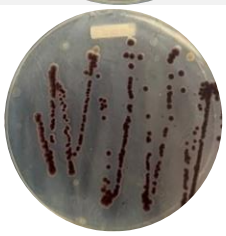
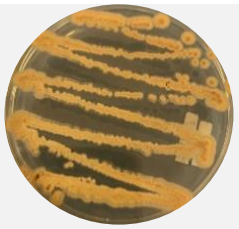
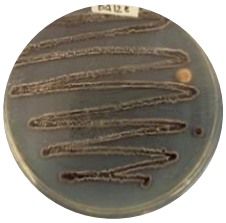
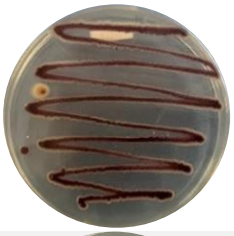
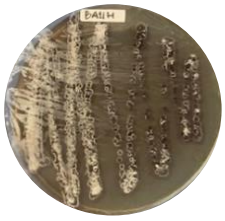
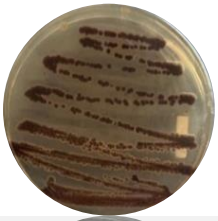
In previous research by Yu et al. (2020) [15], antifungal activity was identified in four compounds isolated from the rhizosphere soil. Sarika et al. (2021) [5] and Elshafie & Camel (2022) reported promising activities of *Actinomycetes* isolated from soil, indicating the production of bioactive metabolites with antibacterial and antifungal properties, particularly within the genus *Streptomyces* [16]. Additionally, research by Belyagoubi et al. (2018) in the karst ecosystem of Chaabe Cave, Algeria, demonstrated that *Actinomycetes* yielded a diversity of microorganisms capable of producing new antibiotics [17].

Based on the information above, the isolation and identification of *Actinomycete*s from the rhizosphere soil of *Nephrolepis cordifolia* as producers of antifungal compounds will be conducted in the karst of Bantimurung, South Sulawesi.

## 2. RESULTS AND DISCUSSİON

### 2.1 Isolation of Actinomycetes from the Rhizosphere Soil of Nephrolepis cordifolia in the Karst Ecosystem of Bantimurung

Soil sampling in the karst ecosystem was conducted at a depth of 10–15 cm. Soil samples were randomly collected near the roots of *Nephrolepis cordifolia* plants [18, 20]. Rhizosphere soil from *Nephrolepis cordifolia* ferns was weighed at 1 gram and subjected to dilution and isolation using the pour plate method on Inorganic Salt Starch Agar (ISP Medium No. 4) growth medium [19]. Isolates that grew on inorganic salt starch agar were then purified through three successive transfers on yeast malt agar (ISP Medium No. 2), resulting in a total of 17 pure isolates as shown in (Figure 1).

| Isolate Code | Aerial Mycelium | Substrate Mycelium | Isolate Code | Aerial Mycelium | Substrate Mycelium |
|---|---|---|---|---|---|
| RKP-A.1-2 |  |  | RKP-A.2-6 |  |  |
| RKP-A.1-3 |  |  | RKP-A.2-7 |  |  |
| RKP-A.1-5 |  |  | RKP-B.1-1 |  |  |
| RKP-A.1.6 |  |  | RKP-B.2-1 |  |  |
| RKP-A.1-7 |  |  | RKP-B.2-2 |  |  |
| RKP-A.1-8 |  |  | RKP-B.2-3 |  |  |
| RKP-A.1-9 |  |  | RKP-B.2.4 |  |  |

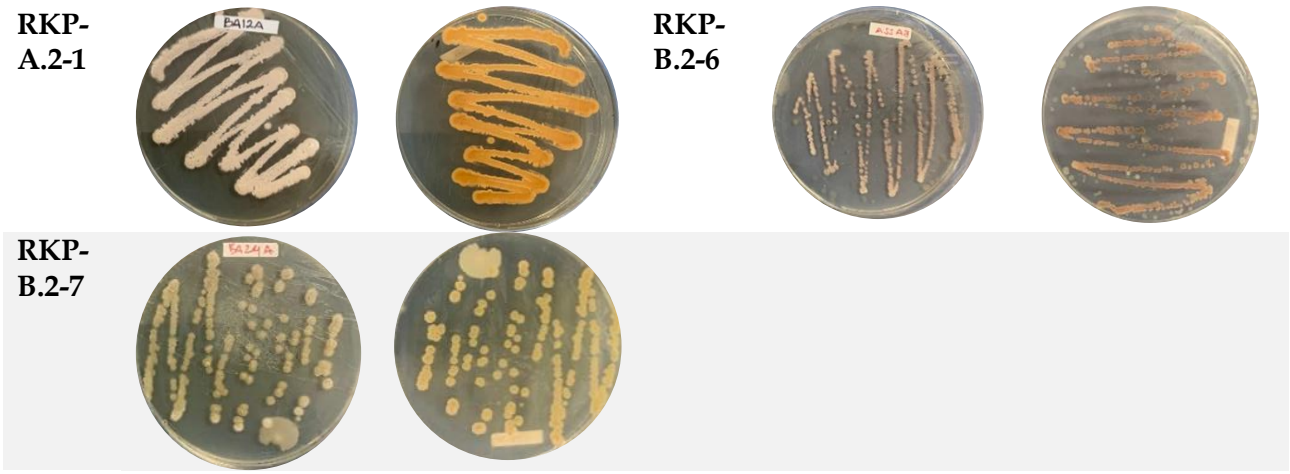



**Figure 1.** Characteristics *Actinomycetes* soil rhizosphere *Nephrolepis cordifolia* of the Karst Bantimurung ecosystem of southern Sulawesi.

**Table 1.** Characteristics of *Actinomycetes* soil rhizosphere *Nephrolepis cordifolia* of the Karst Bantimurung Ecosystem of Southern Sulawesi

| Isolate | Characteristic | | | | |
|---|---|---|---|---|---|
| | Meselium Aerial | Miselium Substrat | Colony Shape | Colony Edge | Colony Elevation |
| **RKP-A.1-2** | White | White Yellowish | Round | *Entire* | Umbonate |
| **RKP-A.1-3** | White | White Brownish-red | Round | *Entire* | Umbonate |
| **RKP-A.1-5** | Gray Whiteness | Gray Yellowish | Round | *Entire* | Umbonate |
| **RKP-A.1-6** | White Brownish-red | Brownish-red | Round | *Entire* | Umbonate |
| **RKP-A.1-7** | Gray White | Gray Yellowish | Round | *Entire* | Umbonate |
| **RKP-A.1-8** | Red White | Red | Round | *Entire* | Umbonate |
| **RKP-A.1-9** | Gray White | Gray | Round | *Entire* | Umbonate |
| **RKP-A.2-6** | Gray White | Gray Yellowish | Round | *Entire* | Umbonate |
| **RKP-A.2-7** | Red White | Red Brownish-red | Round | *Entire* | Umbonate |
| **RKP-B.1-1** | Brownish-red White | Red Brownish-red | Round | *Entire* | Umbonate |
| **RKP-B.2-2** | Gray | Gray Yellowish | Round | *Entire* | Umbonate |
| **RKP-B.2-3** | Gray | Gray Yellowish | Round | *Entire* | Umbonate |
| **RKP-B.2-4** | Gray | Gray Brown | Round | *Entire* | Umbonate |
| **RKP-B.2-1** | Red White | Red Brownish-red | Round | *Entire* | Umbonate |
| **RKP-B.3-3** | White Pink | White Yellowish | Round | *Entire* | Umbonate |

## 2.2 Antagonist Test of Actinomycetes Isolates

The initial screening of *Actinomycetes* isolates against the test fungi *Candida albicans* and *Aspergillus niger* was conducted using antagonism assays to assess antifungal activity, measured by inhibition zones on the test fungi. *Actinomycetes* grown on yeast malt agar and incubated for 7 days at 25 °C were sectioned using a stainless-steel cork borer and placed on petri dishes inoculated with the test microbes. Subsequently,

they were incubated at room temperature for 7 days, after which inhibition zones were observed and measured. The activity test results indicated that isolates RKP-A.1-2 and RKP-B.1-1 exhibited the highest activity in inhibiting the growth of *Candida albicans* and *Aspergillus niger*

**Table 2.** Testing activity of Actinomycetes isolate against fungi

| Isolate Code | Activity | |
|---|---|---|
| | **CA** | **AN** |
| RKP-A.1-6 | ++ | - |
| RKP-A.2-1 | +++ | - |
| RKP-A.1-2 | +++ | ++ |
| RKP-A.1-7 | + | - |
| RKP-A.1-1 | ++ | - |
| RKP-A.1-5 | - | - |
| RKP-A.1-8 | ++ | - |
| RKP-A.1-9 | - | - |
| RKP-A.2-7 | + | - |
| RKP-A.2-6 | - | - |
| RKP-B.1-1 | - | + |
| RKP-B.2-2 | - | - |
| RKP-B.2-3 | + | - |
| RKP-B.2-4 | + | + |
| RKP-B.2-1 | - | - |
| RKP-B.3-3 | - | - |
| RKP-B.3-1 | - | - |

Note: - (No inhibition zone), + (Weak:<5), ++ (Medium 5-10 nm), +++ (Strong: > 10 nm)

### 2.3 Fermentation and Extraction of Secondary Metabolite Compounds

In fermentation testing, it was found that the highest estimated production of secondary metabolites from *Actinomycetes* isolates with antifungal activity against *Candida albicans* occurred between days 14 and 16 (Figure 2). Secondary metabolites from *Actinomycetes* isolates were produced using M1 seed medium for starter cultures and M1 fermentation medium for fermentation. The ability of isolates to produce secondary metabolites was influenced by components of the fermentation medium, such as carbon, nitrogen, and minerals. The carbon and nitrogen sources in M1 seed and M1 fermentation media were soluble starch and KNO3, respectively. Minerals including magnesium, iron, potassium, and casein in the M1 fermentation medium also played a crucial role [21, 22]. Optimal production times for *Actinomycetes* RKP-A.1-2 and RKP-B.1-1 varied, indicating that the rate of carbon metabolism influenced biomass formation and metabolite production. Consequently, one of the most important elements in increasing the synthesis of secondary metabolites is the utilization of carbon-nitrogen sources [23]. *Actinomycetes* isolated in liquid media will develop thin layers on the surface without agitation, which can inhibit the optimal production of secondary metabolites. This is why mixing at 150 rpm is important. Additionally, agitation aids in the best possible use of medium nutrients by *Actinomycetes* isolates [21, 25].

Sampling was conducted on each isolate to examine antimicrobial activity, which indicates the secondary metabolite production process. Secondary metabolite extraction should ideally be performed during the stationary phase or at the peak production phase of secondary metabolites, as prolonged fermentation beyond these phases may not yield an increased abundance of secondary metabolites from the microorganism [24]. The sonication process was used to generate the supernatant. The obtained supernatant was then extracted using an ethyl acetate solvent (1:1 v/v) [25, 26].

The fermentation results indicate that the highest estimated production of secondary metabolites from *Actinomycetes* isolates with antifungal activity against *Candida albicans* occurred on day 14 for isolate RKP-B.1-1 and on day 15 for isolate RKP-A.1-2 (Figure 2). The optimal times for Actinomycetes RKP-B.1-1 and RKP-A.1-2 vary because the general growth patterns of microorganisms are highly variable and influenced by genetic factors and nutrient sources. Extraction of secondary metabolites should be performed during the stationary phase or at the peak production phase of secondary metabolites, as prolonged

fermentation beyond these phases may not result in an increased abundance of secondary metabolites from the microorganism [24].

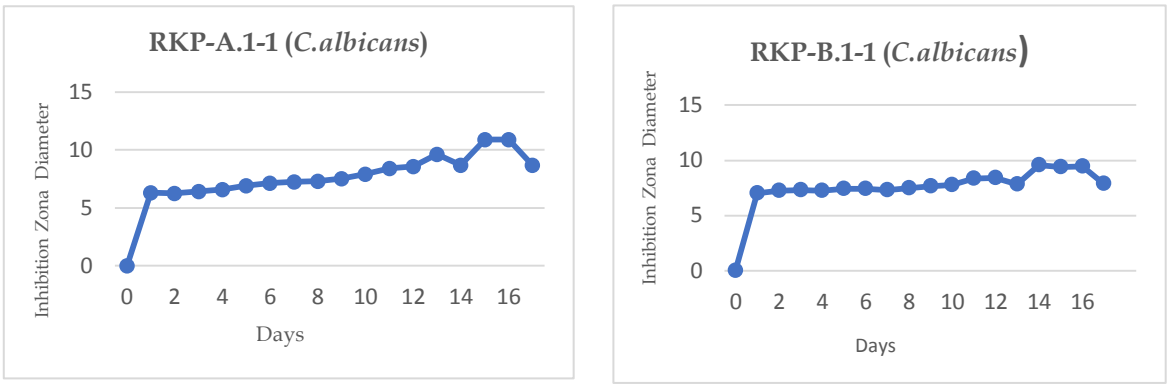
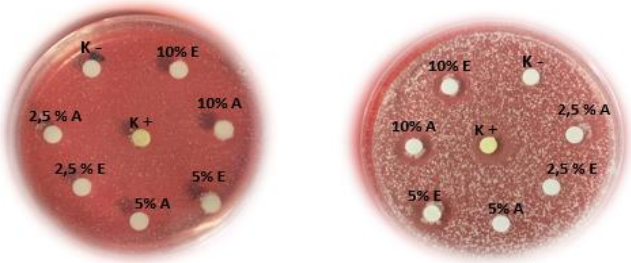



**Figure 2.** Growth curve for fermentation time on antifungal activity against the growth of *Candida albicans*

The sonication process was performed on the fermentation results to separate the supernatant and biomass. The supernatant obtained from this process was extracted using ethyl acetate solvent at a 1:1 v/v ratio [24]. The ethyl acetate solvent is capable of extracting secondary metabolites most effectively from the fermentation fluid compared to hexane or chloroform solvents [27].

## 2.4 Antifungal Activity Test

In antifungal activity testing, the inhibition zone diameter is categorized based on the antifungal activity of the test samples. The concentrations of the extracts used were 10%, 5%, and 2.5%, with positive controls using nystatin and negative controls using ethyl acetate solution. This indicates moderate antifungal activity, where no antifungal activity was observed against *Aspergillus niger*, while antifungal activity against *Candida albicans* was observed, albeit moderate or slight [21].




*C. albicans* *A. Niger*

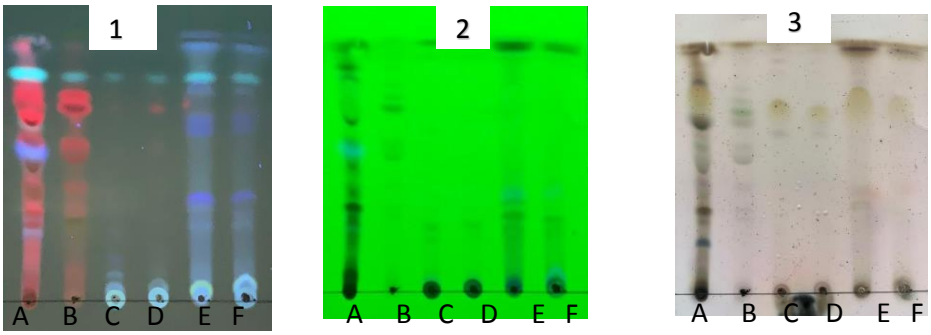
**Figure 3.** Results of antifungal activity test

**Table 3.** Results of mesuring the diameter of the inhibition zone for antifungal
Control - : Ethyl acetate, Control + (Bacteria) : Chloramphenicol, Control + (Fungi) : Nistatin, Paper disk : 6mm

## 2.5 Thin Layer Chromatography Profiles of Actinomycetes Isolate Extracts and Plant Extracts

| Treatment | | Inhibition Zone Diameter (mm) ± Standard Deviation | | | |
|---|---|---|---|---|---|
| | | RKP-A.1-2 | | RKP-B.1-1 | |
| | Concentration | *C. albicans* | *Aspergillus. n* | *C. albicans* | *Aspergillus. n* |
| **Ethyl Acetate Extract** | 10 % | 9.53 ± 0.40 | 6.00±0.00 | 8.3 ± 1.27 | 6.00±0.00 |
| | 5 % | 9.33 ± 0.11 | 6.00±0.00 | 7.93 ± 0.57 | 6.00±0.00 |
| | 2.5% | 8.53 ± 0.46 | 6.00±0.00 | 7.53 ± 0.75 | 6.00±0.00 |
| **Water Extract** | 10 % | 10.9 ± 0.63 | 6.00±0.00 | 10.23 ±0.92 | 6.00±0.00 |
| | 5 % | 7.96 ± 0.40 | 6.00±0.00 | 9.56 ± 0.11 | 6.00±0.00 |
| | 2.5 % | 6.26 ± 0.28 | 6.00±0.00 | 8 ±0.45 | 6.00±0.00 |
| **Control +** | 20 µl | 20.1 ±0.45 | 18.26±0.25 | 20.2 ± 0.17 | 18.5± 0.64 |
| **Control -** | 20 µl | 6.00±0.00 | 6.00±0.00 | 6.00±0.00 | 6.00±0.00 |

Secondary metabolites in plants can be obtained through extraction. One of the critical factors in the extraction process is the selection of a solvent that matches the polarity of the compound. Based on their polarity, solvents are categorized into three types: polar, semi-polar, and non-polar solvents [28]. This corresponds to the principle of "like dissolves like," where polar compounds dissolve in polar solvents and non-polar compounds dissolve in non-polar solvents [28]. Based on this, TLC (thin layer chromatography) and column techniques were used to do phytochemical screening. Spotted stains were created on the plates by TLC profiling with the eluent N-hexane: Ethyl acetate (3:1), which was then heated and sprayed with 10% $H_2SO_4$ to improve visibility. Based on the oxidizing qualities of sulfuric acid, which can damage the chromophore groups of active extract compounds and cause a shift towards longer wavelengths, the 10% $H_2SO_4$s pray is intended to make the spots more noticeable to the unaided eye. The research results revealed a profile of secondary metabolites produced by *Actinomycetes* and extracts from karst and non-karst Nephrolepis cordifolia plants, showing distinct compound profiles characterized by different Rf values. The extract from karst *Nephrolepis cordifolia* contained a greater diversity of compounds compared to the non-karst variety, attributed to specific soil conditions. Karst regions often have a rich mineral and nutrient content, which can influence the composition of compounds produced by plants.




1 : UV 254 nm
2 : UV 365 nm
3 : $H_2SO_4$ 10%

A : Plant extract *Nephrolepis cordifolia* from karst
B : Plant extract *Nephrolepis cordifolia* from Non-karst
C : Water extact of RKP-A.1-2
D : Water extact of RKP-B.1-1
E : Ethyl acetate extract of RKP-A.1-2
F : Ethyl acetate extract of RKP-B.1-1

**Figure 4.** Compound chromatogram profile extract ethyl acetate from *Nephrolepis cordifolia* and extract of the secondary metabolite *actinomicetes* using the motion phase N-Hexanel: Ethyl acetate (3: 1v/v)

### 2.6 Molecular identification of Actinomycetes using the 16S rRNA gene

Identification using molecular methods based on the 16S rRNA gene begins with sample preparation. Active isolates are grown for 7 days on ISP 2 media, followed by DNA extraction using the Geneaid PrestoTM Mini Bacterial gDNA Kit protocol. Approximately 1 x 10^9 isolates are taken and placed in 1.5 mL microcentrifuge tubes, to which Gram+ buffer is added to prevent DNA damage and lysosyme to lyse the DNA. Then, Proteinase K, a protease enzyme, is added to remove DNA from protein contaminants. Absolute ethanol is used for DNA precipitation. The obtained DNA is then amplified using PCR.The following thermal cycling conditions were used in the amplification of the actinobacteria 16S rRNA gene using PCR with Taq DNA polymerase and primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3'') and reverse primer 1492R ('-TACGGYTACCTTGTTACGACTT-3'): Taq DNA polymerase was triggered during the first denaturation stage, which was conducted for one minute at 95 °C. Single-stranded DNA is created by denaturing DNA. Primer annealing took place for 10 seconds at 52 °C, during which the primer bonded to the DNA template's corresponding wet base sequence. Starting from the primer site that had adhered to the target DNA's nucleotide base sequence and progressing from the 5' end to the 3' end of the single DNA strand, primer extension at 75 °C for 15 seconds aided in the elongation of a new strand of DNA. The amplification consisted of denaturation, primer annealing, and primer extension cycles totaling 35 cycles. A post-extension step at 72°C for 15 seconds was followed by cooling to 10°C. Subsequently, PCR products were obtained. The PCR products were then subjected to electrophoresis using a 0.8% agarose gel. The visualization of the electrophoresis results for isolates RKP-A.1-2 and RKP-B.1-1 showed single bands at approximately 1,500 bp, indicating successful amplification of the 16S rRNA gene, as depicted in Figure 5.

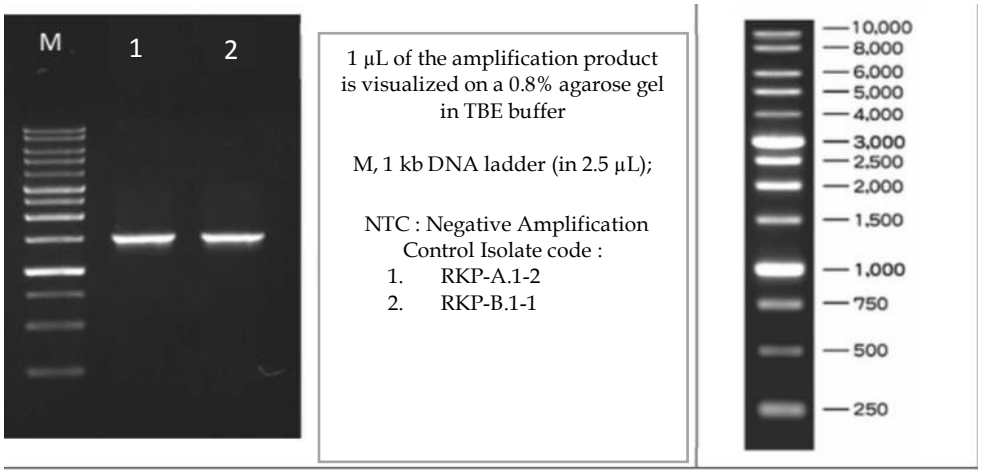



**Figure 5.** Electrophoresis of PCR Products from Amplification of 16S rRNA Gene of Isolates RKP-A.1-2 dan RKP-B.1-1

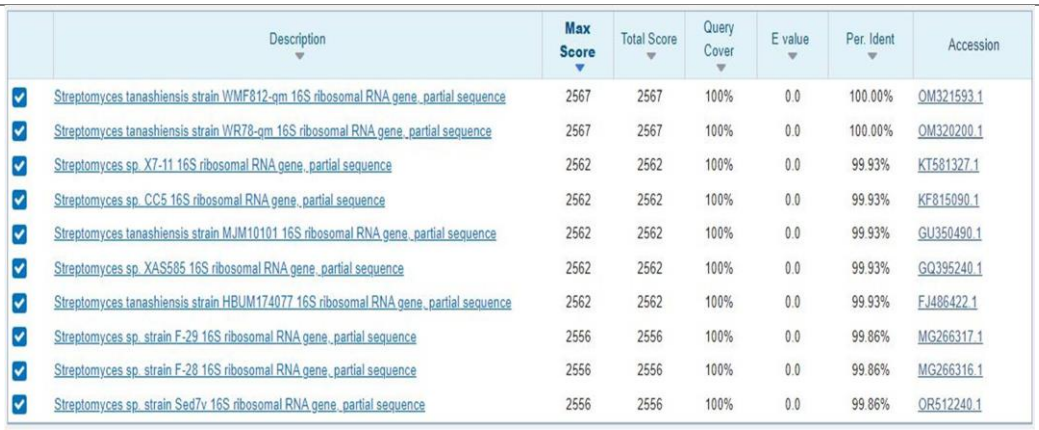
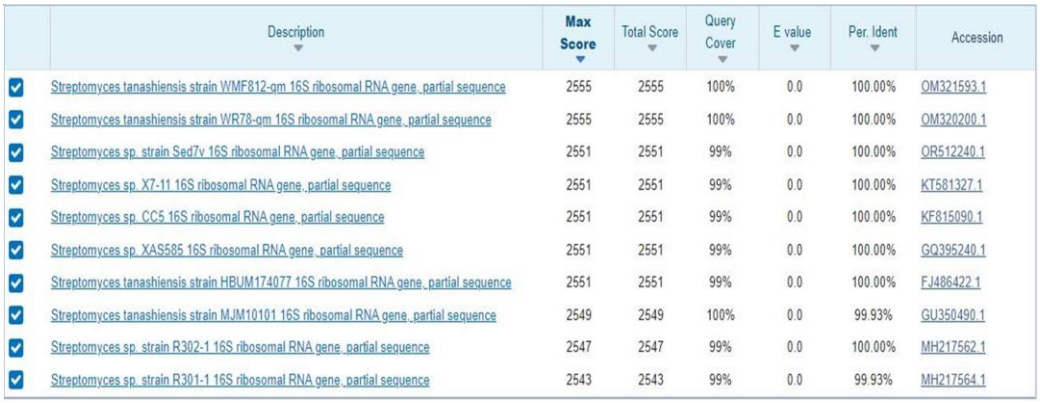
After the 16S rRNA gene was amplified, sequencing was performed to determine the nucleotide bases in the DNA sequence. The sequence is depicted as a string of alphabetical symbols representing the nucleotide bases that make up DNA: A (adenine), T (thymine), G (guanine), and C (cytosine).

The sequencing results were subsequently subjected to BLAST analysis to compare them with sequences of all bacteria available in the GenBank database using the BLAST program accessed via the website (*Basic Local Alignment Search Tool*) at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequence alignment was performed using the phylogenetic tree program Mega. The BLAST results on the phylogenetic tree (Figure 6) showed that isolates RKP-A.1-2 and RKP-B.1-1 are most closely related to strains of *Streptomyces sp*.*Streptomyces sp* is a gram-positive bacterial genus well-known for its ability to produce antibiotics. This bacterium belongs to the phylum Actinobacteria and is commonly found in soil and decaying vegetation, playing a role in biological control against soil-borne fungal pathogens. *Streptomyces sp* is classified within the order *Streptomycetales*, encompassing approximately 600 diverse and prolific antibiotic-producing species. They are abundant in various soil types, constituting around 10% of the total soil microbiome (Calvo-Peña et al., 2023) [29].*Streptomyces* is the genus most commonly found in soil, comprising approximately 70% to 95% of soil microbial populations. In a study conducted by Rante et al. (2024) [21], *actinomycetes* were isolated and identified from soil with antifungal activity in karst areas, where the produced *actinomycetes* belonged to the genus *Streptomyces sp*. *Streptomyces sp* is renowned for its ability to produce various bioactive compounds, including antifungal compounds such as Nystatin, a polyene antibiotic produced by *Streptomyces noursei*, and Amphotericin B, another polyene antibiotic produced by *Streptomyces nodosus* (Hyeon et al., 2023) [30].

## 3. CONCLUSION

Out of 17 isolated *Actinomycetes* isolated from tanah near from soil rhizosphere of *Nephrolepis cordifolia* , two isolats are capable of producing a secondary metabolite that can inhibit *Candida albicans* growth up to a concentration of 0.5 mg/µ. Based on the sequence of 16S rRNA, molecular fingerprinting indicates that RKP-A.1-1 and RKP-B.1-1 have a strong phenotypic similarity of 100.00% with *Streptomyces sp*.

**Table 4.** Top 10 BLAST hits results against NCBI, Excluding uncultured sample sequences

| No. Sample Code | Link to the results |
|---|---|
| **G-3231-3 RKP-A.1-2** |  https://www.ncbi.nlm.nih.gov/nuccore/OM321593.1,OM320200.1,KT581327.1,KF815090.1,GU350490.1,GQ395240.1,FJ486422.1,MG266317.1,MG266316.1,OR512240.1 |
| **G-3231-4 RKP-B.1-1** |  https://www.ncbi.nlm.nih.gov/nuccore/OM321593.1,OM320200.1,OR512240.1,KT581327.1,KF815090.1,GQ395240.1,FJ486422.1,GU350490.1,MH217562.1,MH217564.1 |

| | Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|---|
| ☑ | Streptomyces tanashiensis strain WMF812-gm 16S ribosomal RNA gene, partial sequence | 2567 | 2567 | 100% | 0.0 | 100.00% | OM321593.1 |
| ☑ | Streptomyces tanashiensis strain WR78-gm 16S ribosomal RNA gene, partial sequence | 2567 | 2567 | 100% | 0.0 | 100.00% | OM320200.1 |
| ☑ | Streptomyces sp. X7-11 16S ribosomal RNA gene, partial sequence | 2562 | 2562 | 100% | 0.0 | 99.93% | KT581327.1 |
| ☑ | Streptomyces sp. CC5 16S ribosomal RNA gene, partial sequence | 2562 | 2562 | 100% | 0.0 | 99.93% | KF815090.1 |
| ☑ | Streptomyces tanashiensis strain MJM10101 16S ribosomal RNA gene, partial sequence | 2562 | 2562 | 100% | 0.0 | 99.93% | GU350490.1 |
| ☑ | Streptomyces sp. XAS585 16S ribosomal RNA gene, partial sequence | 2562 | 2562 | 100% | 0.0 | 99.93% | GQ395240.1 |
| ☑ | Streptomyces tanashiensis strain HBUM174077 16S ribosomal RNA gene, partial sequence | 2562 | 2562 | 100% | 0.0 | 99.93% | FJ486422.1 |
| ☑ | Streptomyces sp. strain F-29 16S ribosomal RNA gene, partial sequence | 2556 | 2556 | 100% | 0.0 | 99.86% | MG266317.1 |
| ☑ | Streptomyces sp. strain F-28 16S ribosomal RNA gene, partial sequence | 2556 | 2556 | 100% | 0.0 | 99.86% | MG266316.1 |
| ☑ | Streptomyces sp. strain Sed7v 16S ribosomal RNA gene, partial sequence | 2556 | 2556 | 100% | 0.0 | 99.86% | OR512240.1 |

| | Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|---|
| ☑ | Streptomyces tanashiensis strain WMF812-gm 16S ribosomal RNA gene, partial sequence | 2555 | 2555 | 100% | 0.0 | 100.00% | OM321593.1 |
| ☑ | Streptomyces tanashiensis strain WR78-gm 16S ribosomal RNA gene, partial sequence | 2555 | 2555 | 100% | 0.0 | 100.00% | OM320200.1 |
| ☑ | Streptomyces sp. strain Sed7v 16S ribosomal RNA gene, partial sequence | 2551 | 2551 | 99% | 0.0 | 100.00% | OR512240.1 |
| ☑ | Streptomyces sp. X7-11 16S ribosomal RNA gene, partial sequence | 2551 | 2551 | 99% | 0.0 | 100.00% | KT581327.1 |
| ☑ | Streptomyces sp. CC5 16S ribosomal RNA gene, partial sequence | 2551 | 2551 | 99% | 0.0 | 100.00% | KF815090.1 |
| ☑ | Streptomyces sp. XAS585 16S ribosomal RNA gene, partial sequence | 2551 | 2551 | 99% | 0.0 | 100.00% | GQ395240.1 |
| ☑ | Streptomyces tanashiensis strain HBUM174077 16S ribosomal RNA gene, partial sequence | 2551 | 2551 | 99% | 0.0 | 100.00% | FJ486422.1 |
| ☑ | Streptomyces tanashiensis strain MJM10101 16S ribosomal RNA gene, partial sequence | 2549 | 2549 | 100% | 0.0 | 99.93% | GU350490.1 |
| ☑ | Streptomyces sp. strain R302-1 16S ribosomal RNA gene, partial sequence | 2547 | 2547 | 99% | 0.0 | 100.00% | MH217562.1 |
| ☑ | Streptomyces sp. strain R301-1 16S ribosomal RNA gene, partial sequence | 2543 | 2543 | 99% | 0.0 | 99.93% | MH217564.1 |

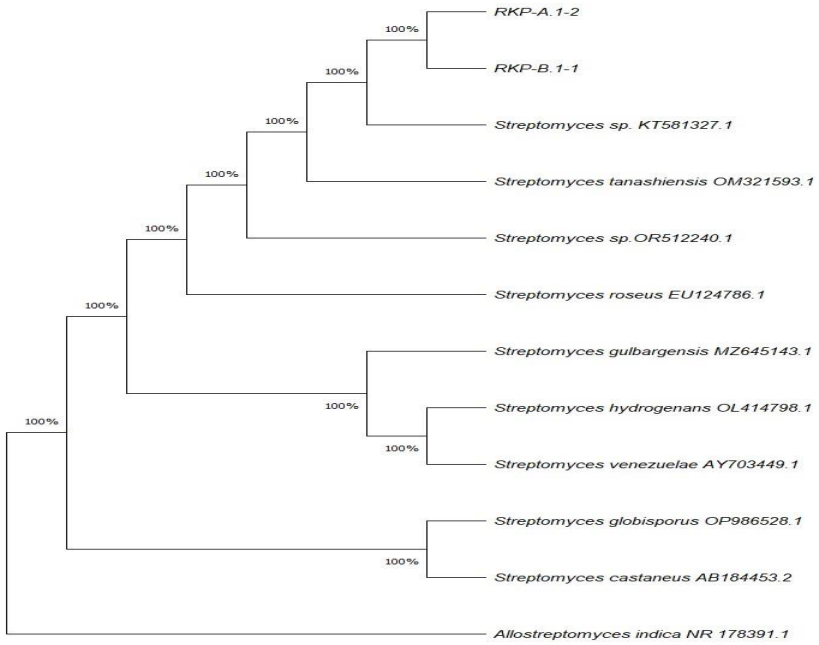



**Figure 6.** Phylogenetic tree of Actinomycetes isolates RKP-A.1-2 dan RKP-B.1-1

## 4. MATERIALS AND METHODS

### 4.1. Sampling

Soil samples were taken from around the roots of fern plants in Maros, South Sulawesi, approximately 1-5 cm from the soil surface. The soil was placed in a sterile container and tightly sealed to prevent contamination with other microorganisms.


**Figure 7.** Map of sampling locations in Leang-Leang Village, Kec. Bantimurung Regency Maros

### 4.2 Sample Preparation and Isolation of Actinomycetes

The samples that have been collected are then taken to the *Actinomycetes* isolation laboratory. The sample was then crushed until smooth and homogeneous, then sieved and weighed 1 gram, then heated for 1 hour at 60 °C for 15 minutes. The sample was then diluted $10^{-1}$ to $10^{-5}$. Soil samples that have been diluted are then planted in petri dishes containing Inoganic Salt Starch Agar solid media. Then the petri dish was incubated at room conditions for 5-7 days. After that, the petri dish containing the isolated sample was observed. Candidate isolates were selected which were considered *Actinomycetes* candidates and then purified on Yeast malt agar media. Then it is multiplied to be used as stock and stored in the refrigerator.

### 4.3 Antagonist Test of Actinomycetes Isolates

This test was carried out by growing each isolate again on Yeast malt agar medium for 7 days. *Actinomycetes* isolates were cut using a stainless steel cort borer then placed in a petri dish containing the test microbes, then incubated for 1x24 hours. After that, observations were carried out by measuring the zone of inhibition using a caliper.

### 4.4 Fermentation and Extraction of Secondary Metabolite Compounds

The selected isolate is continued with a fermentation test. The first fermentation was made as a Starter with 120 ml of M1 Seed medium for each isolate. The starter was kept in a shaker for 5 days at a speed of 120 rpm. Then, StarterI is added to the fermentation media, each amounting to 10% of the total fermentation volume. The *fermentation* media used was 1.2 L of M1 f*ermentation* for each isolate, which was divided into 6 Erlenmeyer flasks filled with 200 ml in a 500 ml Erlenmeyer flask. The Erlemeyer to which the starter has been added is then shaker for 16 days at a speed of 120 rpm.

The fermentation had been shekered for 16 days and then sonicated for 1 hour. Next, it is fermented with ethyl acetate solution. The extraction process is carried out by inserting ethyl acetate and fermentation media into a volumetric flask with a ratio of (1:1 v/v), then mixing and shaking for approximately ± 20 minutes so that 2 phases are formed, after which the extract is separated and dried.

### 4.5 Antifungal Activity Test

Antifungal activity tests were carried out using the Kirby-Bauer method in *Maltyeart Agar* media. The test microbes used were Candida albicans and Aspergillus niger and the positive test used Nystatin. Three concentrations were made, namely (10%, 5% and 2.5%). Each paper disc was added with 20 µl *Actinomycetes* extract/disc. Next, the inhibition zone was observed and measured for 1x24 hours.

### 4.6 Thin Layer Chromotagraphy

Ethyl acetate extract from the fern *Nephrolepis cordifolia* which has been obtained from Karst and non-Karst areas is then continued with TLC testing. The chamber contains the eluent N-Hexane and Etik acetate (4:1 v/v). After that, it is observed under a UV 256 nm and UV 366 nm lamp, then it is sprayed using 10% $H_2SO_4$ reagent then the plate that has been sprayed is heated again.

### 4.7 Molecular Identification of Actinomycetes

Molecular identification of Actinomycetes was performed based on the 16S rRNA gene. DNA extraction was carried out using the Quick-DNA Magbead Plus Kit. Subsequently, amplification of the 16S rRNA gene was 2 conducted using the MyTaq HS Red Mix kit with Taq DNA Polymerase and primers 63f (5'- 293 CAGGCCTAACACATGCAAGTC-3') and reverse primer 1387r (5'-GGGCGGTGTGTACAAGGC-3'). Following this, electrophoresis of the amplified 16S gene products was performed, followed by bidirectional sequencing using the Sanger DNA Sequencing method with Capillary Electrophoresis and Bioinformatics 29Analysis of the Sanger Sequencing results. Additionally, sample sequences and databases were aligned using the CLUSTAL W method in the phylogenetic tree program MEGA6.

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