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STUDY ON THE ANTAGONISTIC ACTIVITY AGAINST TWO PATHOGENIC BACTERIA IN HUMANS OF *STREPTOMYCES MIP*_L26 ISOLATED FROM SOIL IN THE ROOT ZONE OF THE MAY CHANG TREE *(LITSEA CUBEBA)*

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ABSTRACT

Purpose: *Study on the antagonistic activity against two pathogenic bacteria in humans (Staphylococcus aureus and Streptococcus pneumoniae) of the Streptomyces MIP_L26 (Called MIP_L26), isolated from the soil in the root zone of the May Chang tree; to investigate the biological characteristics of MIP_L26*

Subjects and methods: *Experimental study, determine the antimicrobial activity against two pathogenic bacteria in humans (Staphylococcus aureus and Streptococcus pneumoniae), and some biological characteristics of MIP_L26.*

Results: *MIP_L26 was identified as having a strong antagonistic ability against the two bacteria causing Staphylococcus aureus and Streptococcus pneumoniae with an antibacterial ring diameter of 18 ± 5 mm and 13 ± 5 mm, respectively. Based on classification according to morphological, physiological, and biochemical characteristics, combined with the results of 16S rRNA gene sequencing, MIP_L26 was identified as Streptomyces gancidicus MIP_L26. The fermentation medium for the biosynthesis of antibacterial substances was ISP6 and the recovery time for antimicrobial substances was after 72 hours of fermentation.*

Keywords: Streptomyces MIP_L26, May Chang tree (Litsea cubeba), antagonistic activity, 16S rRNA.

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1. INTRODUCTIONS

Antibiotics remain an essential group of drugs in global healthcare. They directly eliminate or reduce the impact of disease-causing bacteria and help the human immune system in combating infections. However, antibiotic resistance in pathogenic bacteria has become an urgent problem, requiring research and finding intervention Antibiotic-resistant bacteria often cause hospital-acquired infections, and surgical wound infections and make antibiotic resistance worse [1]. Multi-drug resistant bacteria are appearing more and more, leading to a shortage of antibiotics for treatment. Therefore, finding new antibiotics is an extremely urgent requirement of medicine.

In the production of antibiotics from microbial sources, actinomycetes are considered the most potential source (with more than 75% of antibiotics originating from actinomycetes) [2].

In recent decades, the search for new sources of antibiotics sources has become increasingly difficult. Scientists have expanded their research to isolate actinomycetes from various sources. Actinomycetes from the soil in the root zone of medicinal plants can combat pathogenic microorganisms, produce Indole acetic acid (IAA), and produce extracellular enzymes [3], [4]. In Vietnam, endophytic actinomycetes from medicinal plants have been studied by scientists and have had initial success; however, there is limited research on actinomycetes from the soil in the root zone of medicinal plants.

With the orientation of using actinomycete strain MIP_L26 as a source of antibiotic production to

serve the health of soldiers and people, we carried out this project to study antagonistic activity against pathogenic bacteria in humans of the *Streptomyces* MIP_L26 isolated from the soil in the root zone of the May Chang tree against *Staphylococcus aureus* and *Streptococcus pneumoniae*. Additionally, we aim to explore the biological characteristics of MIP_L26. Survey the biological characteristics ofMIP_L26.

2. SUBJECTS AND METHODS

2.1. Subjects

- Microbial strain: strain *Streptomyces* MIP_L26 isolated from the soil in the root zone of the May Chang tree (*Litsea cubeba*) (harvested in the Bac Quang district, Ha Giang province).

- Tested microbial strains: *Staphylococcus aureus*, *Streptococcus pneumoniae* from the microbial strain collection at the Department of Microbiology, Military Institute of Preventive Medicine.

2.2. Methods

- Study Design: Experimental research.

Methods for determining antagonistic activity against human pathogens, specifically:

+ Diffusion method on agar plates [5], [6]: spread 50 µL of *S. aureus* and *S. pneumoniae* cultures on Mueller Hinton Blood agar MHBA . MIP_L26 was cultured on ISP6 liquid medium (g/l) Glucose: 10; Peptone: 5; Yeast extract: 3; MgSO4.7H2O: 1; FeCl2: 0.5, and trace element solution M).. M solution (%): $CuSO_4.5H_2O$: 0.64; $FeSO_4.7H_2O$: 0.11; $MnCl_2.4H_2O$: 0.79; $ZnSO_4.7H_2O$: 0.15, distilled water: 100 ml. After seven days, drop 100 µL of the cultured MIP_L26 solution, shaking at 200 rpm for 72 hours into the well with d= 8mm, incubate at 30ºC. After 24 hours observe the diameter of the antibacterial ring.

Antibacterial activity calculated by the difference between the diameter of the antibacterial ring and the diameter of the agar well (d = 8mm).

Control sample (Code: ĐC): drop 100 µL ISP6 medium.

+ Agar disk diffusion method [5], [6]: actinomycete strain MIP_L26 evenly spread on ISP6 agar at 30ºC. After seven days of cultivation, MIP_L26 agar placed on petri containing MHBA with the tested bacterial strains. The dishes were incubated at 4ºC for 4-5 hours to allow active compounds from the agar disk to diffuse into the medium. Subsequently, the dishes placed in the incubator at 30ºC, and results observed after 24 hours. The antibacterial activity determined by the diameter of the antibacterial ring and the diameter of the agar disk.

Two methods were tested simultaneously to evaluate the antagonistic ability of MIP_L26 in liquid and solid medium.- Studying biological characteristics of *Streptomyces* MIP_L26:

+ Morphological characteristics of MIP_L26 determined based on colony features, including the color of aerial mycelium, the color of substrate mycelium, and the ability to produce soluble pigments on ISP1-ISP7 media [7]. Spore chain and spore surface morphology observed under an electron microscope after seven days of cultivation.

- Evaluation of the ability to utilize carbon and nitrogen sources by MIP_L26:

+ Cultures of MIP_L26 were grown in triangular flasks containing 50 ml ISP6 medium supplemented with 1% different carbon sources (Glucose, Lactose, Maltose, Galactose, Cellulose) and 0.5% nitrogen sources Peptone, Beef extract, $(NH_4)_2SO_4$, KNO_3, and NH_4NO_3.

+ The ability to assimilate carbon and nitrogen sources of MIP_L26 determined by measuring the biomass formed after cultivation. Biomass was collected using filter paper, dried at 50ºC for 5 hours, weighed, biomass and evaluated.

- Study of the dynamics of the fermentation process:

+ The MIP_L26 cultured with shaking on a selected medium to determine the dynamics of the fermentation process. Parameters studied including biomass and antibiotic activity, determining the suitable time for antibiotic recovery [8].

- Identification MIP_L26 by 16S rRNA sequencing:

+ The MIP_L26 cultured on ISP6 liquid medium, and after 72 hours of centrifuge at 3,000 rpm/min in 10 minutes at 4ºC, the cells were harvested. Total DNA extracted, and the 16S rRNA region was amplified by PCR using the primer pair 27F (forward primer) 5’- AGAGTTTGATCMTGGCTCAG - 3’ and 1492R (reverse primer) 5’-TACGGYTACCTTGTTACGACTT - 3’ (Phu Sa). PCR was performed with the following thermal cycle: 94ºC for 5 minutes, 25 cycles (94ºC for 30s, 55ºC for 30s, 72ºC for 1 minute), and a final extension at 72ºC for 10 minutes. PCR products were checked by electrophoresis on a 0.8% agarose gel (Invitrogen). The size of the amplified DNA fragment after PCR reaction compared with a DNA ladder standard (10 Kb Plus DNA ladder Marker – Thermo Scientific). The PCR product was purified and sequenced at Apical Scientific Sequencing (Singapore).

Sequence comparison performed using the BLAST tool based on the Genbank database (www.ncbi.nih.gov). A phylogenetic tree constructed using MEGA6 software (Tamura, 2013) [9].

- Data analysis: Data was processed, and graphs generated using GraphPad Prism 9. The results of each experiment presented as the mean ± standard deviation (SD) from 3 random repetitions.

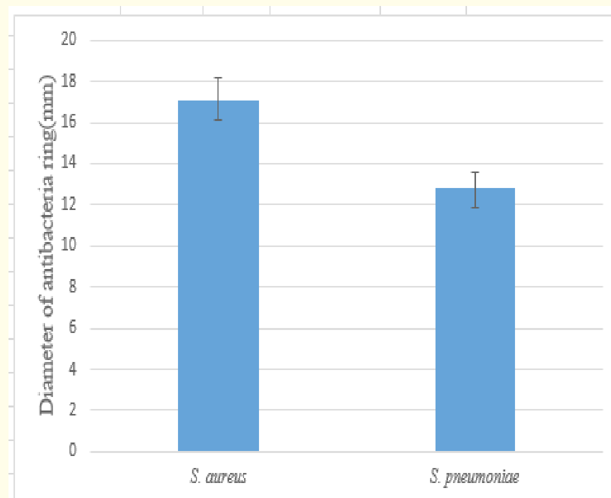
3. RESULTS AND DISCUSSIONS

3.1. Investigation of the antagonistic activity against human pathogens by *Streptomyces* MIP_L26

Preliminary assessment of the antagonistic ability against *S. aureus* and *S. pneumoniae* using the agar disk diffusion and agar well diffusion methods indicated that MIP_L26 exhibited strong antagonistic activity against both bacterial strains in humans. With the method of agar well diffusion, the diameter of the antibacterial ring against S. aureus and S. pneumoniae were at 18 ± 5 mm and 13 ± 5 mm, respectively (Figure 1).

Several scientific studies worldwide have reported on the antagonistic activity of *Streptomyces* sp. against *S. aureus*. Research by Chang et al. (2021) revealed that *Streptomyces* YX44 (isolated from water pipes in Japan), exhibited strong activity against *S. aureus* [10]. Many strains of *S. aureus* produced penicillinase, an enzyme that destroys the cell wall of beta-lactam antibiotics, leading to antibiotic resistance, including multidrug-resistant strains. A scientific report by Sharma et al. (2019) demonstrated that *Streptomyces* M7 exhibited resistance against methicillin-resistant *S. aureus* [11]. This study also reported the purification results and determined the spectrum of antibiotics produced by the M7 strain. The complexity of mutations in *S. aureus* is increasing, leading to a rising antibiotic resistance (as reported by Bandar Ali Al et al., 2023 [12]). Therefore, the search for new antibiotic sources is necessary.

The preliminary test results regarding the antagonistic activity against *S. aureus* and *S. pneumoniae* were noteworthy, opening up potential applications in medicine for the active compounds derived from soil actinomycetes in the root zone of medicinal plants in general and the MIP_L26 in particular. Further in-depth studies are needed to understand the physiological and biochemical properties of the strain, optimizing conditions and fermentation media to obtain the highest antibacterial activity.



The diameter of the antibacterial zone (mm)

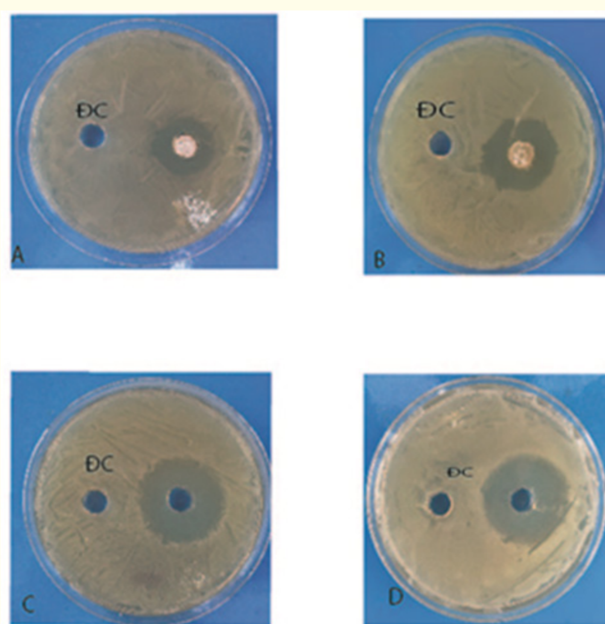


Figure 1. The Antagonistic activity test of Streptomyces MIP_L26 against S. aureus (fig 1A and 1C) and S. pneumoniae (fig 1B and 1D); ĐC: control

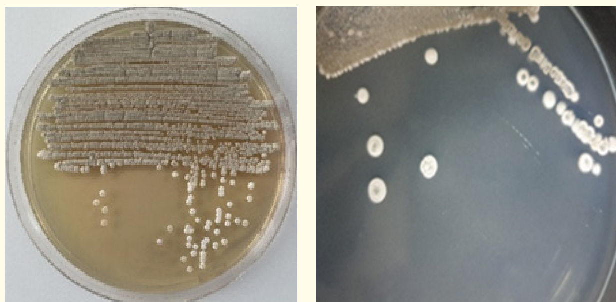
3.2. Morphological characteristics of *Streptomyces* MIP_L26

The color of the Streptomyces when cultured on ISP medium (according to the International Streptomyces Project ISP) is the first factor for classification of actinomycete strain, with Bergey's classification scheme (1963) [7]. The aerial and substrate mycelia of the Streptomyces in this study observed when cultured on ISP1 to ISP7 media, and along with the color of the colony, the formation of melanin is also one of the fundamental criteria for distinguishing between different Streptomyces strains.

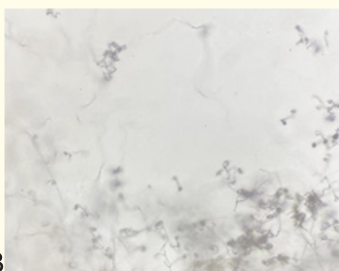
Table 1. The cultural characteristics of the MIP_L26 on ISP medium

Media	Mycelium		Pigment melanin
	Aerial mycelium	Substrate mycelium	
ISP1	Yellow	Yellow	-
ISP2	Yellow	White	-
ISP3	Yellow	White	-
ISP4	Yellow	White	-
ISP5	Gray	Yellow	-
ISP6	Gray	Yellow	-
ISP7	Gray	Yellow	-
Aerial mycelium, substrate mycelium, (-) Absent			

On the ISP6 medium, the aerial mycelium of MIP_L26 initially appeared white and opaque, later changing to a gray color. The substrate mycelium exhibited a white color initially, which turned yellow over time. No melanin pigment formation was observed (Table 1). The surface of the bacterial colony of MIP_L26 was raised, with a size of 2-3 cm. After 5-7 days of cultivation, MIP_L26 formed spores. Spore chains were produced from the aerial mycelium, and they exhibited a twisted-helical morphology (Figure 2). Therefore, the MIP_L26 displayed typical characteristics of *Streptomyces* according to Bergey's classification system (1963).



A

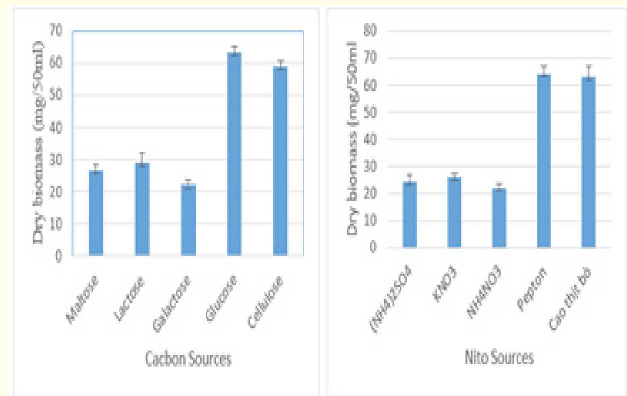


B

Figure 2. A. Morphology and color of the colony of *Streptomyces* MIP_L26 on ISP6; B: Spore formation structure under an electron microscope at a magnification of 400x.

- Investigation of the ability to use various carbon and nitrogen sources:

One of the important biochemical and physiological characteristics of the Streptomyces is its ability to assimilate different carbon and nitrogen sources. Simultaneously, this is also one of the crucial criteria for classifying streptomyces according to ISP. Investigating the ability to use different carbon and nitrogen sources is an initial basis for selecting the most suitable fermentation substrate for the growth of the MIP_L26.



(A) (B)

Figure 3. Growth of Streptomyces MIP_L26 on different carbon and nitrogen sources. (A): Carbon sources; (B): Nitrogen sources

The results presented in Figure 3 indicated that *Streptomyces* MIP_L26 has theability to grow on various tested carbon and nitrogen sources. However,MIP_L26 exhibits efficient utilization of glucose and cellulose as carbon sources and pepton and beef extract as nitrogen sources. This finding aligns with the studies by Silvia et al. (2016) and Schmidt et al. (2005), where organic nitrogen sources positively influenced antibiotic synthesis to form peptide chains, the backbone of antibiotics [13], [14].

Based on these results, the research team selected a cultivation medium with glucose as the carbon source and pepton as the nitrogen source for the fermentation medium to optimize the recovery time of the tested antimicrobial solution.

3.3. Determination of the fermentation process dynamics

On the chosen cultivation medium, the fermentation process for synthesizing antimicrobial substances by MIP_26 conducted to

monitor the variations in biomass production and antimicrobial activity. Simultaneously, the endpoint of fermentation for recovering the antimicrobial solution determined.

The research results (Figure 4) indicated that MIP_L26 started to grow and produced antimicrobial substances from the 24th hour. At the 72-hour fermentation mark, the strain exhibited the highest antimicrobial activity corresponding to the inhibition zone against the *S. aureus* . Therefore, it is suggested to conclude the fermentation process and recover the antimicrobial solution after 72 hours of cultivation. This observation aligns with studies by LinDa et al. (2017) and Firew Elias et al. (2022), where antibiotic activity was observed after 4-7 days of fermentation [15], [16]. Thus, the endpoint of fermentation for recovering the antimicrobial substance from MIP_L26 appeared to be relatively early.

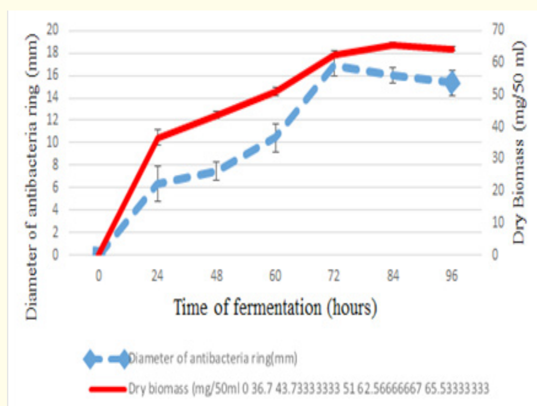


Figure 4. Dynamics of the fermentation process for synthesizing antimicrobial substances by the StreptomycesMIP_L26

3.4. Identification of MIP_L26 by 16S rRNA

To determine the biological safety and facilitate recognition of the Streptomyces for research and antibiotic production prevention trials, we conducted the identification of the aforementioned Streptomyces using 16S rRNA sequencing combined with morphological characteristics. The 16S rRNA sequence was compared with data on Genbank, and a phylogenetic tree constructed using the MEGA6 software (K. Tamura, 2013) [17]. The morphological, physiological, and biochemical characteristics of the *Streptomyces* MIP_L26 were compared to ISP and Bergey's classification [7] showed that MIP_L26 has numerous similarities with the *Streptomyces gancidicus* strain described by Suzuki in 1980. Therefore, the *Streptomyces* MIP_L26 identified as *Streptomyces gancidicus* MIP_L26 (Figure 5).

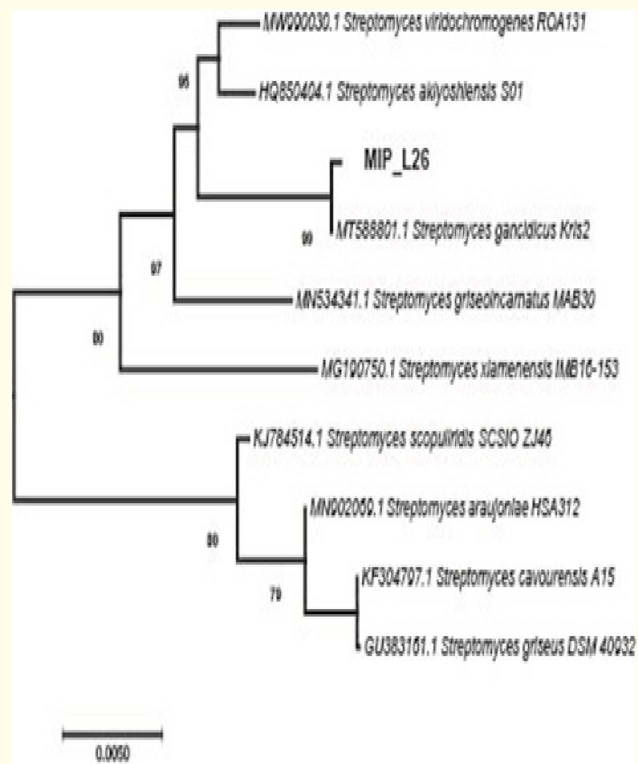


Figure 5. Phylogenetic tree based on 16S rRNA sequences

The study by Nayaka et al. (2020) isolated and identified the *Streptomyces* SN3 from cultivated soil in the Katamaka region, India.SN3 tested for resistance against various pathogenic bacteria and fungi. SN3 identified as belonging to the species *Streptomyces gancidicus.* Additionally, ongoing research is investigating the metabolic products of strain SN3 [18]. In 2020, Bhakyashree et al. isolated the strain VITBKA3 from Indian soil and tested its resistance against pathogenic bacteria such as *Staphylococcus aureus, Proteus vulgaris, Bacillus cereus, and Escherichia coli.* The strain VITBKA3 was sequenced and classified as *Streptomyces gancidicus* [19].

4. CONCLUSIONS

The research has determined that the *Streptomyces* MIP_L26 possessed strong antagonistic activity against two pathogenic bacteria in humans, *Staphylococcus aureus and Streptococcus pneumoniae,* with the diameter of

the antibacterial ring of 18 ± 5 mm and 13 ± 5 mm, respectively.

Based on the morphological, physiological, and biochemical characteristics, combined with the results of 16S rRNA gene sequencing, the *Streptomyces* MIP_L26 identified as *Streptomyces gancidicus* MIP_L26. The fermentation medium for biosynthesis of antibacterial substances is ISP6**.** Antibacterial recovery time is after 72 hours of fermentation.

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