Isolation and selection of Actinomycetes strains against *Phytophthora palmivora* from Durian soil in Dak Lak, Vietnam

Nha Nguyen Thi Minh, Ngo Le Van and Anh Đinh Thi Lan*
University of Science - Vietnam National University, Ho Chi Minh City, VIETNAM
*dtlanh@hcmus.edu.vn

Abstract

The present study focuses on selecting actinomycetes strains from Durian garden soil in Dak Lak province with strong antagonistic activity against the fungus Phytophthora palmivora (P. palmivora), which causes leaf blight and fruit rot disease in durian trees. A total of 117 actinomycetes strains were isolated from 15 soil samples, among which strain NMN64 exhibiting strong antifungal activity, was further investigated under suitable fermentation conditions. After 10 days of fermentation in PM43 medium (15 liters) at 35°C and pH 6.3, NMN64 showed strong antagonistic activity with 68% efficiency.

Initial isolation of the antifungal compound using Sephadex LH-20 gel filtration chromatography and HPLC analysis revealed that sub-fraction 3.2 was the most active one, achieving an efficiency of 63%. This result suggests that potential antifungal compound(s) against P. palmivora could be produced from strain NMN64, which could be used for controlling leaf blight and fruit rot in durian trees.

Keywords: Durian leaf blight, fruit rot disease, *Phytophthora palmivora*, antifungal activity, actinomycetes.

Introduction

Durian is a renowned tropical fruit tree with high economic value, originating from Southeast Asia. The global fresh durian market is estimated to reach USD 9.85 billion by 2024 and is projected to grow to USD 15.43 billion by 2029[7]. Dak Lak province has the largest durian cultivation area and production in Vietnam. The fungus *Phytophthora palmivora* is a primary and severe pathogen that can infect durian at all growth stages, impacting various parts of the tree and causing issues such as leaf blight, root rot, stem bleeding and fruit rot[9]. To address these issues, actinomycetes are considered a potential source due to their ability to produce potent, environmentally friendly antifungal compounds.

Actinomycetes are a group of Gram-positive, aerobic, filamentous bacteria with a high Guanine + Cytosine content in their DNA (above 55%). They develop a complex, highly branched filamentous network that lacks septa. This group is considered an important microbial source for the synthesis of natural compounds with biological activity[1,5]. Specifically, about 75% of antibiotics in use today are derived from actinomycetes, with the genus *Streptomyces* accounting for approximately 75% of antibiotics produced by actinomycetes, while the remainder comes from rare actinomycetes[8]. Around 70–80% of biologically active natural compounds applied in pharmaceuticals or agricultural chemicals, as well as other bioactive metabolites, are also produced by *Streptomyces*[4].

Material and Methods

Material: Actinomycetes strains were isolated from 15 soil samples collected from Durian orchards in Krong Nang district, Dak Lak province, Vietnam. Exclusion criteria included Durian orchards with high levels of fungal disease, poor yield, or recent use of fertilizers or pesticides around the time of sampling. The test fungal strains included *Phytophthora palmivora*, *Neoscytalidium dimidiatum*, *Corynespora cassiicola* and *Fusarium* sp., provided by the Research Center for Bioactive Natural Products (RCBNP), University of Science, VNU-HCM.

Methods: This study involved are as follows:

- Survey and record information on healthy durian orchards from farmers in the communes of Ea Toh, Ea Tan and DLie Ya, Krong Nang district, Dak Lak province.
- Methodology for sampling and processing durian orchard soil in Dak Lak province following Meenakshi et al[10].
- Isolation, purification and screening methods for actinomycetes, following El Karkouri et al[6].
- Preliminary assessment of the antagonistic potential of actinomycetes strains against *P. palmivora* using direct antagonism method.
- Examination of conditions affecting the production of bioactive compound of potential actinomycetes strains in small-scale fermentation (50 ml) and extraction of secondary metabolites based on the primary screening protocol (RCBNP_Primary Screening). Experimental setup details are provided in table 1.
- Examination of conditions affecting the bioactive compound production of potential actinomycetes strains in medium-scale fermentation (200 ml) and extraction of secondary metabolites based on the secondary screening protocol (RCBNP_Secondary Screening).
- Assessment of the bioactive compound production of potential actinomycetes strains under optimal conditions in large-scale fermentation (15 liters) and extraction of secondary metabolites based on the large-scale screening protocol (RCBNP_Large Scale Screening).

Table 1
Conditions investigated in the small-scale fermentation process

Medium	Duration (Days)			
	0	**4**	**7**	**10**
PM3	P1_PM3_0D	P1_PM3_4D	P1_PM3_7D	P1_PM3_10D
PM39	P1_PM39_0D	P1_PM39_4D	P1_PM39_7D	P1_PM39_10D
PM43	P1_PM43_0D	P1_PM43_4D	P1_PM43_7D	P1_PM43_10D

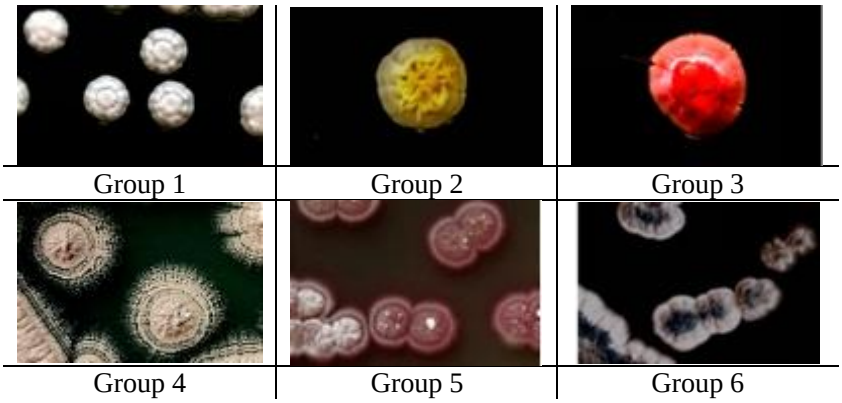


Figure 1: Colony morphology representing each group of actinomycetes

- Assessment of the biological activity of the extract after fermentation against *P. palmivora*, *Fusarium* sp., *N. dimidiatum* and *C. cassiicola* using the paper disk diffusion method[2].
- Initial isolation of bioactive compounds using Sephadex LH-20 gel filtration chromatography.
- Analysis of the extract using high-performance liquid chromatography (HPLC).

Identification: The DNA sequence encoding the 16S rRNA region was extracted, subjected to PCR amplification and underwent nucleotide sequencing. The sequence data was subsequently analyzed via comparative analysis against the NCBI genomic database.

Data Analysis Methods: Data from the experiments were processed using Excel and MCTATC software, following the LSD test for statistical analysis and data visualization. The results after three random repetitions are presented as mean ± standard deviation (SD).

Results and Discussion

The results of isolation and purification of actinomycetes from durian orchard soil: From the 15 soil samples collected from Durian orchards in Dak Lak province, the study successfully isolated 117 different actinomycetes strains, labeled NMN01 to NMN117. Based on the colony color, the isolated actinomycetes strains were classified into six main groups (Figure 1):

+ Group 1: White, 46 strains (39.31%),
+ Group 2: Yellow - Orange, 15 strains (12.82%),
+ Group 3: Pink - Red, 12 strains (10.26%),
+ Group 4: Brown - Gray, 36 strains (30.77%),
+ Group 5: Purple, 4 strains (3.42%),
+ Group 6: Green, 4 strains (3.42%).

Antagonistic activity results of Actinomycetes Strains against *P. palmivora*: Among the 117 isolated strains, four exhibited good ability to synthesize secondary antifungal compounds against *P. palmivora* including NMN07, NMN08, NMN64 and NMN101 with antagonistic efficacy of 70.00% or higher. These strains are considered as potential candidates for further experiments (Table 2).

Fermentation Results: Based on the recorded results from different fermentation media, the PM39 medium may not be suitable for the fermentation process of the potential actinomycetes strains. The biomass of the strains obtained in PM39 was low, showed no increase after times and there were no significant changes in the medium's color over the harvesting periods. In contrast, media PM3 and PM43 yielded more promising results. The biomass of the strains collected after fermentation days increased significantly and the color of the medium gradually changed over the harvesting periods.

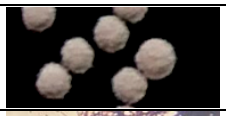
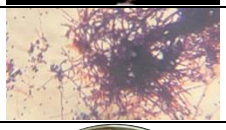
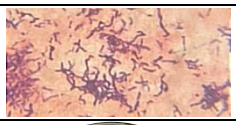
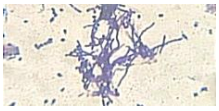
This suggests that the secondary compounds from the actinomycetes may have caused changes in the medium's color. Therefore, PM3 and PM43 are considered as potential fermentation media.

Results of Biological Activity Testing against *P. palmivora*: The results of the biological activity assessment of the extracts from selected actinomycetes against *P. palmivora* after small-scale fermentation are detailed in chart 1. The antagonistic efficiency of the actinomycetes is indicated by the size of the inhibition zone. The compounds produced by the actinomycetes have the ability to inhibit the growth of the pathogenic fungus, thereby preventing

mycelial growth and forming an inhibition zone. As the inhibition zone expands, the antagonistic efficiency increases.

The experiment identified two potential actinomycetes strains, NMN08 and NMN64, with high antifungal activity against *P. palmivora*, particularly in PM3 and PM43 media after fermentation for 7 and 10 days. The results indicate that PM3 is a suitable choice for NMN08, while PM3 and PM43 are suitable media for NMN64.

Table 2
Characteristics and antagonistic results of potential actinomycetes strains against *P. palmivora*

Characteristics	**Potential actinomycetes strains**			
Strains	NMN07	NMN08	NMN64	NMN101
Macroscopic morphology				
Microscopic morphology				
Antagonistic efficiency against *P. palmivora* (A%)	 A = 70,00%	 A = 71,67%	 A = 90,00%	 A = 75,00%

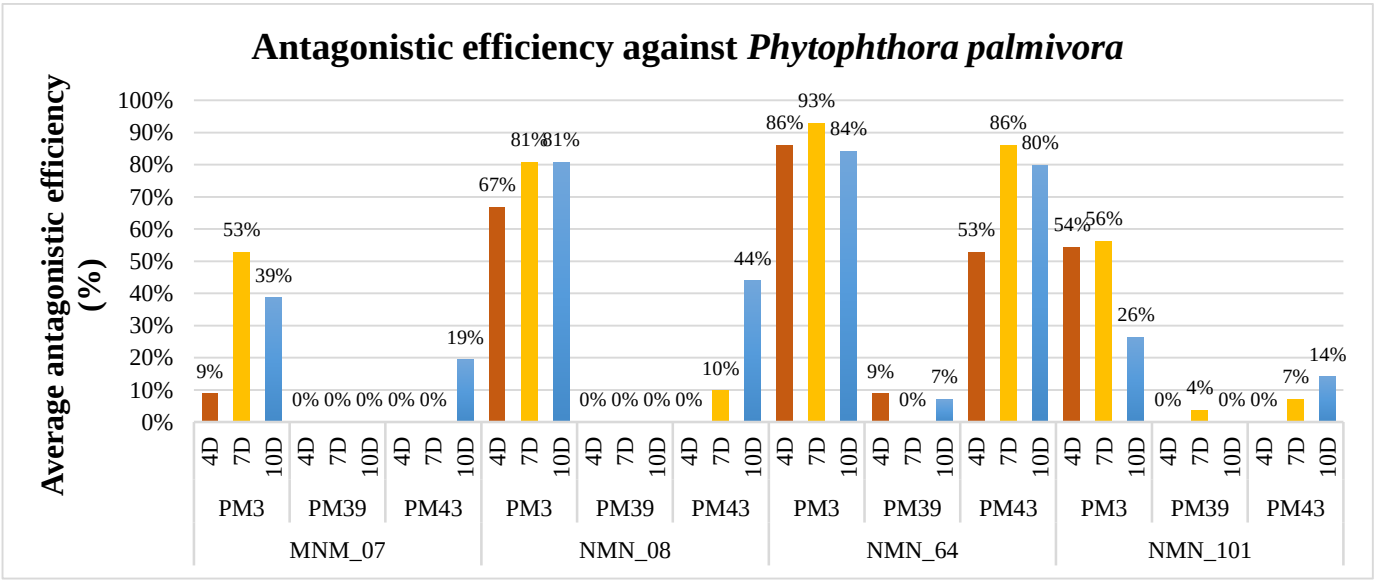


Chart 1: Results of the biological activity assessment of actinomycetes extracts against *P. palmivora* after small-scale fermentation

Table 3
Results of the antagonistic activity of the extract against *Fusarium* sp., *N. dimidiatum*, *C. cassiicola*

Extracts	**Inhibition Diameter (mm)***		
	N. dimidiatum	***C. cassiicola***	***Fusarium* sp.**
NMN08_P1_PM3_7D	$0,00 \pm 0,00^{b}$	$0,00 \pm 0,00^{c}$	$7,00 \pm 2,00^{a}$
NMN08_P1_PM43_7D	$0,00 \pm 0,00^{c}$	$0,00 \pm 0,00^{b}$	$10,67 \pm 0,58^{b}$
NMN64_P1_PM3_4D	$2,00 \pm 0,50^{b}$	$0,00 \pm 0,00^{b}$	$4,67 \pm 0,58^{c}$
NMN64_P1_PM3_7D	$4,00 \pm 0,50^{a}$	$2,17 \pm 0,29^{a}$	$12,67 \pm 0,58^{a}$

* In the same column, average values with the same superscript characters indicate no statistical difference ($P > 0.05$); a, b, c, d,… represent the ranking levels of the values.

Results of Biological Activity Testing against *Fusarium sp., N. dimidiatum, C. cassiicola*: The experiment was conducted with 36 different treatments to evaluate the antifungal activity of actinomycetes strains under appropriate environmental conditions and fermentation durations. Strain NMN08 fermented in PM43 and strain NMN64 fermented in PM3 after seven days showed good antifungal activity against *Fusarium* sp. (Table 3).

Accordingly, strain NMN08 is suitable for fermentation in PM3 at the 7-day and 10-day harvest periods and will continue to be studied in medium-scale fermentation. For strain NMN64, further investigation of HPLC analysis results is needed to select suitable fermentation conditions to enhance the production capacity of the desired antifungal compounds.

HPLC Analysis Results of NMN64: The HPLC results for strain NMN64 grown in PM3 and harvested after 7-day and 10-day fermentation, exhibited low absorbance intensity (AI < 0.20). Thus, PM3 is not suitable for obtaining secondary compounds from strain NMN64.

However, the HPLC analysis of the NMN64 extracts grown in PM43 harvested at day 4, 7 and 10 indicated potential peaks with similar retention times and similar UV absorption spectra. The absorbance intensity (AI) of these peaks increase from day 4 to day 10 (Table 4). This might correspond with the low antagonistic activity on day 4 (A = 53%) and will increase on days 7 and 10 with efficiencies of 86% and 80% respectively. Therefore, strain NMN64 fermented in PM43 and strain NMN08 in PM3 after 7 and 10 days was selected for further study in medium-scale fermentation.

Results of Biological Activity Testing against *P. palmivora*: The results indicate that the extract from the culture filtrate (CF) exhibited higher antifungal activity against *P. palmivora* compared to that from the biomass (MC). This suggests that a significant amount of antifungal secondary compounds from the two actinomycetes strains NMN08 and NMN64 is secreted into the fermentation medium. The CF extract after 10-day fermentation demonstrated the best antifungal efficacy (A = 70%), warranting further large-scale fermentation to obtain potential antifungal compounds (Chart 2).

HPLC Analysis Results: The potential peaks exhibited retention times and UV absorption maxima similar to those selected in the primary screening process. With high peak area percentages, these are considered promising peaks for the purification of antifungal compounds (Table 5).

Table 4
HPLC analysis results and maximum UV absorption spectra of the potential peaks from the two extracts NMN64_P1_PM43_7D and NMN64_P1_PM43_10D

NMN64_P1_PM43_7D				NMN64_P1_PM43_10D			
RT*	**UV max**	**AI**	**% peak**	**RT**	**UV max**	**AI**	**% peak**
6.40	265	1.08	21.54%	**6.64**	264	0.90	31.89%
7.12	258.1	0.18	12.61%	**7.47**	254.4	0.14	10.53%
9.41	223.1; 253.5; 306.7	0.30	16.06%	**9.63**	223.1; 252.6; 305	< 0.05	18.82%
13.20	260	0.66	16.20%	**13.41**	259	0.05	2.19%

* RT: Retention Time; AI: Absorbance Intensity; % Peak: % Peak Area

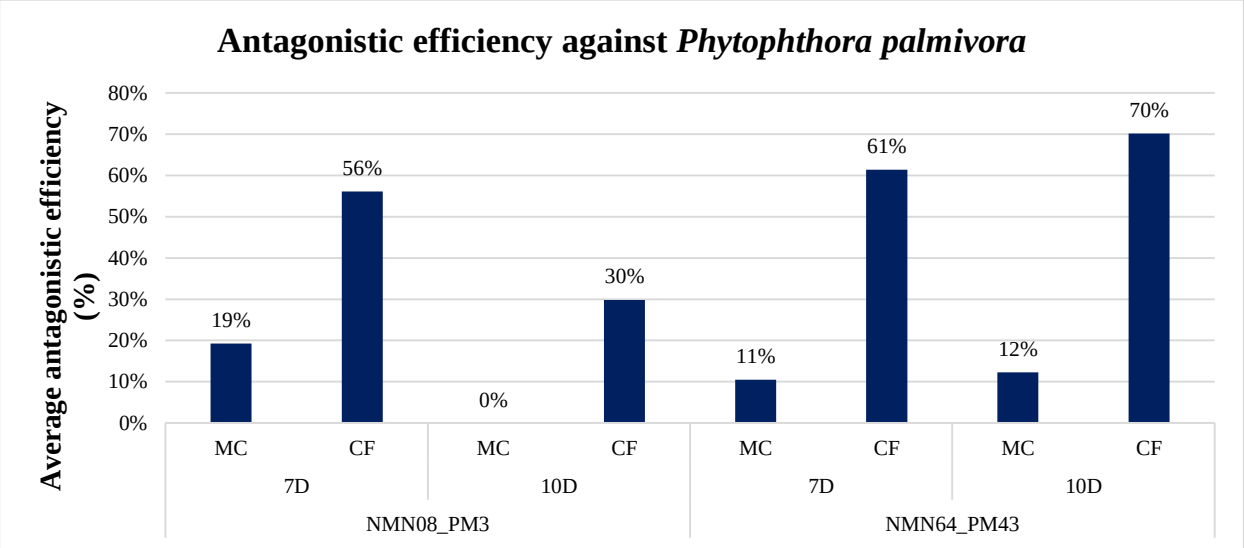


Chart 2: Results of the biological activity assessment of actinomycetes extracts against *P. palmivora* after medium-scale fermentation

Table 5
HPLC Analysis Results and Maximum UV Absorbance of Potential Peaks in the Treatment NMN64_S1_PM43_CF_10D

NMN64_S1_PM43_CF_10D			
RT*	**UV maximum absorbance**	**AI**	**% peak**
6.67	272.8	0.08	22.43%
7.47	254.4	0.23	20.96%

* RT: Retention Time (min); AI: Absorbance Intensity (AI); % Peak: % peak area

Based on the recorded results, strain NMN64 cultured in PM43 medium for 10 days shows potential for producing antifungal compounds and is therefore scaled up to 15 liters to obtain a larger quantity of the target compounds.

Secondary Metabolite Production of Potential Actinomycetes Strain under Optimal Fermentation Conditions in a 15-Liter Tank Fermentation: After processing the fermentation broth, five types of extracts were obtained as shown in table 6.

Table 6
Mass of Five Types of Extracts

Extracts	Mass (gram)
MCEA	1.0857
MCW	19.3180
MCPE	0.5517
CFEA_pH6	2.4679
CFEA_pH3	2.5329

Results of Biological Activity Testing against *P. palmivora*: Among the five extract samples tested for biological activity, only MCEA, CFEA_pH6 and CFEA_pH3 exhibited antifungal activity against *P. palmivora*. Specifically, the CFEA_pH6 extract showed strong antifungal efficiency, achieving 68%. The MCEA and CFEA_pH3 extracts demonstrated weaker antifungal effects against *P. palmivora*, with efficiencies of 26% and 12%, respectively (Table 7). This indicates that the potent antifungal compound secreted by strain NMN64 is released into the extracellular environment when cultured in PM43 medium for 10 days and is effectively soluble in ethyl acetate.

Table 7
Antagonistic efficiency of extracts from actinomycetes strain NMN64 against *P. palmivora*

Extracts	Antagonistic efficiency (%)
MCEA	26%
MCW	0%
MCPE	0%
CFEA_pH6	68%
CFEA_pH3	12%

HPLC Analysis Results: The HPLC analysis results indicate that the CFEA_pH6 extract exhibits strongest antifungal activity. However, the HPLC chromatogram presents a mountain-like shape with low absorbance intensity (AI < 0.35). Furthermore, the peak at 19.25 minutes (with maximum UV absorbance at 226.7 and 276.3 nm) may represent a potential peak (Figure 2). Similarly, the CFEA_pH3 and MCEA extracts also possess antifungal activity, albeit weaker than that of CFEA_pH6. It is predicted that the active metabolites may not correspond to the larger peaks. Therefore, the potential antifungal compounds may possess strong biological activity at low concentrations. Additionally, all three extracts have a small mass, complicating the purification process. Consequently, it is necessary to combine the three biologically active extracts and to submit to the next separation step.

Preliminary Results of Isolation of Bioactive Secondary Compounds: The extracts MCEA, CFEA_pH6 and CFEA_pH3, which demonstrate significant biological activity, were combined to create a total extract. This extract initial separation using Sephadex LH-20 gel filtration chromatography, resulted in 45 smaller fractions, sequentially labeled from 2.1 to 2.45.

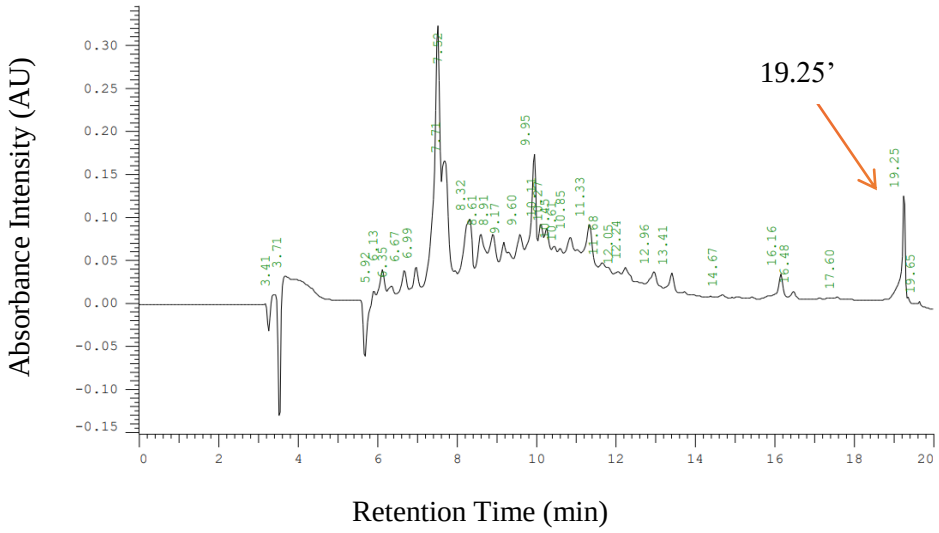


Figure 2: HPLC Results of Extract NMN64_L1_CFEA_pH6_10kppm

Fractions exhibiting similar HPLC profiles were combined into larger fractions, yielding six major fractions labeled from 3.1 to 3.6. Notably, fraction 3.2, which had a substantial mass of 3.6159 g, is initially regarded as a promising candidate for the extraction of target compounds.

Results of Biological Activity Testing against *P. palmivora*: Fraction 3.2 exhibited the highest antifungal efficacy among the six fractions, achieving a rate of 63%. In contrast, fractions 3.4 and 3.5 showed weak antifungal activity against *P. palmivora*, with efficacies of 11% and 26%, respectively (Table 8).

Table 8
Antagonistic efficiency of extracts in fraction 3 of actinomycetes strain NMN64 Against *P. palmivora* – A (%)

Fractions	Antagonistic efficiency (%)
3.1	0%
3.2	63%
3.3	0%
3.4	11%
3.5	26%
3.6	0%

Most natural compounds with antifungal activity tend to be large or medium-sized molecules. These compounds typically exhibit complex structures, comprising multiple aromatic rings, long carbon chains and functional groups such as hydroxyl, methoxy and amino groups, which enhance their interactions with key components of fungal cells, such as the cell membrane, cell wall and metabolic enzymes[3,11].

Fraction 3.2 exhibited good antifungal activity against *P. palmivora*, with a substantial mass and demonstrated the ability to inhibit three additional fungal species. Consequently, this fraction will undergo further HPLC analysis to identify potential peaks.

HPLC Analysis Results: A similarity was observed between the maximum UV absorbance and retention time of the extracts CFEA_pH6 and fraction 3.2. Furthermore, when comparing the absorbance intensity and biological activity of these two extracts, the results showed a positive correlation (Table 9). The peak at 19.28 minutes may represent a potential peak with antifungal activity. For the HPLC results of the extract from fraction 3.2 at a concentration of 50 mg/ml, the peak at 19.28 minutes is relatively pure, with a high absorbance intensity (AI = 1.402) and the substantial extract mass of fraction 3.2 (3.6159 g) facilitates the purification of secondary compounds with antifungal potential in subsequent experiments (Figure 3).

Strain identification result of actinomycetes NMN64: Identification of the strain NMN64 was performed through analysis of the 16S rRNA gene sequence. The genomic DNA encoding the 16S rRNA region was extracted, subjected to PCR amplification and underwent nucleotide sequencing.

Table 9
HPLC Results and Antagonistic Efficiency Against *P. palmivora* of Extracts CFEA_pH6 and 3.2

Extracts	RT*	UV max	AU	A (%)
CFEA_pH6 10mg/ml	19.25	226.7; 276.3	0.120	68%
3.2 50mg/ml	19.28	237.2; 277.5	1.480	63%

* RT: Retention Time (min); AI: Absorbance Intensity (AU); A (%): Antagonistic efficiency (%)

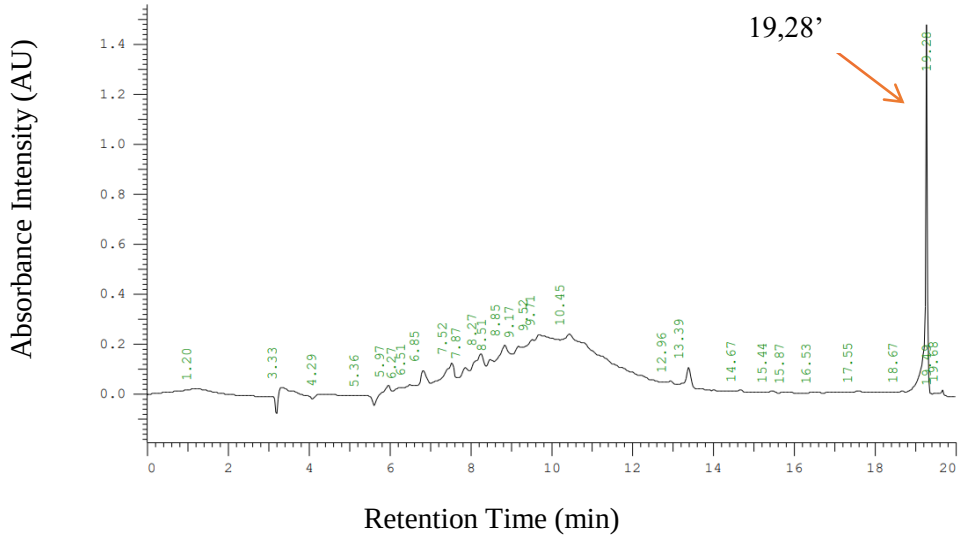


Figure 3: HPLC Results of Extract 3.2_50mg/ml

The obtained sequence data was analyzed through comparative analysis against the NCBI nucleotide database utilizing the BLAST algorithm. Sequence analysis revealed that the isolate exhibited 99.865% sequence homology with *Streptomyces* sp., indicating its taxonomic position within the *Streptomyces* genus.

Conclusion

In conclusion, a total of 117 strains of actinomycetes were isolated from 15 soil samples collected from Durian orchards in Dak Lak Province. Among these, 4 out of 117 strains, specifically NMN07, NMN08, NMN64 and NMN101 exhibited strong antagonistic activity against *P. palmivora*, with an antagonistic efficiency exceeding 70%. At a 50ml scale, the PM3 medium was optimal for strain NMN08, while the PM43 medium was most suitable for strain NMN64, yielding antagonistic efficiencies of 80% or higher. At a 200ml scale, strain NMN64 cultured in PM43 medium demonstrated the highest antifungal efficiency after a 10-day fermentation period.

At a 15 liters fermentation scale, fraction 3.2 derived from the active extracts of the strain NMN64 was found to possess the highest antifungal activity, reaching 63% antagonistic efficiency, laying a foundation for further studies aimed at discovering compounds that are both highly effective against fungal pathogens and environmentally friendly.

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