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Diverse Secondary Metabolites Produced by Marine-Derived Fungus *Penicillium simplicissimum* Ch06 Through an OSMAC Approach

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Abstract— The high level of resistance to pathogenic bacteria causes the number of antibiotics to be limited. Therefore, searching for new antibiotics to kill these resistant bacteria is necessary. A new approach to obtaining new metabolic products with various structures is "One Strain Many Compounds" (OSMAC). This strategy can offer a quick, easy, and efficient way to increase the diversity of secondary metabolites by enhancing and activating "silent biogenetic gene clusters." Through this research, the secondary metabolites of *Penicillium simplicissimum* Ch06 in a solid rice medium were compared to the same medium with the addition of 3.5% MSG and 3.5% $NaNO_3$. The content of secondary metabolites was analyzed using LC-MS/MS method. The antibacterial activity of the fungus extracts was tested using the agar diffusion method. Adding MSG and $NaNO_3$ to rice media affected the formation of secondary metabolites from the fungus. The antibacterial activity of fungal extracts induced by adding MSG and $NaNO_3$ was greater than that of the control medium. The fungal metabolite profiles analyzed by liquid chromatography (LC) - mass spectrometry (MS) showed a clear difference of several peaks in the LC profile following the same trend with the variation of the antibacterial activity of the fungal extracts. Our results showed a clear difference in cultivation media with the addition of MSG / $NaNO_3$ and fungal bioactivity. This research will continue to determine the culture's mass and bioactive compounds' isolation from this fungus.

Keywords— Marine-derived fungus, *Penicillium simplicissimum*, One Strain Many Compounds (OSMAC), Antibacterial activity, LC-MS/MS

1. Introduction

Bacterial resistance is a global problem today. The World Health Organization (WHO) has reported several pathogenic bacteria resistant to antibiotics, posing a significant threat to global health. Due to the high level of resistance exhibited by these bacteria, treatment options are limited[1]. Consequently, there is a need to search for new bioactive ingredients that can effectively target groups of pathogenic bacteria and potentially serve as the next antibiotic candidate.

Standard cultivation methods have been widely used to extract bioactive compounds from fungi. However, studies on the fungal genome have revealed that cultivation methods frequently fail to show the full biosynthetic potential under standard laboratory conditions. This process becomes inefficient due to the high rediscovery of metabolites from the same microorganisms [2]. Several techniques have been demonstrated to be effective methods for activating the biosynthetic genes of microorganisms; a new approach is developing that has been limited by traditional methods for obtaining metabolic products with various structures. One Strain Many Compounds (OSMAC) is a simple and effective method for activating many metabolic pathways in microorganisms, such as "silent biogenetic gene clusters," to produce more natural products [3]. One of the strategies described by OSMAC is the variation of cultivation conditions. It has been reported that the ratio of the composition and conditions of the growth medium, salinity, and metal ions can regulate the degree and pattern of gene expression of secondary metabolites of microorganisms, resulting in the production of various secondary metabolites.
Gao reported 11 new secondary metabolites from the fungus *Aplosporellajaveedii* after it was cultivated on

rice media by adding sodium nitrate (NaNO3) or monosodium glutamate (MSG). The presence of 3.5% NaNO3 and 3.5% monosodium glutamate altered the metabolic pattern of fungi when compared to control fungi grown solely on rice [4]. Similarly, Zeeck et al. (2002) demonstrated that the OSMAC approach allowed the fungus *Aspergillus ocharaceus* to produce 15 additional metabolites previously thought to be produced only by aspinonene metabolites. Using the OSMAC principle, this research aims to activate a "silent" cluster of biosynthetic genes that act antibacterial agents[5]. OSMAC is very effective because, in addition to being more straightforward and more efficient, it does not require prior knowledge of the types of biosynthetic gene clusters or the regulatory processes that control gene expression.

2. MATERIALS AND METHODS

2.1 General

LC-MS analysis was performed on an XEVO G2-S Q-TOF instrument outfitted with a Waters Acquity QSM. For data processing, Mass Lynx NT software four is used. At 25˚C, the sample was injected at a 0.2 mL/min rate into column C18 UPLC. Silica gel 60 (Merck) for column chromatography (CC) was utilized. Thin-layer chromatography (TLC) Silica gel 60 F254 plates (Merck) were used to monitor the CC fractions. Spots on TLC were detected by UV light at 254 and 366 nm. Solvents were distilled before use.

2.2 Fungal Material and Fermentation

The fungal strain, *Penicillium simplicissimum* Ch06 was isolated from the sponge *Chelonaplysilla* sp. collected from Mandeh Island, West Sumatera, Indonesia, which was reported in our previous studies [6]. The fungus was cultured in 500 ml Erlenmeyer flasks containing 50 g of rice mixed with 65 mL of distilled water as a control medium (CM). The fungus was cut into pieces and inserted into a flask under sterile conditions. The Fermentation was maintained under static conditions at room temperature until the fungus completely overgrown the rice medium, which lasted for 3-4 weeks at 25 °C [7]. Following the same procedure, OSMAC cultivations were carried out by growing the fungus on a solid rice medium containing 3.5% MSG and 3.5% NaNO3 [4][8].

2.3 Extraction

After the fungus grows maximally on rice medium (CM) and OSMAC medium, each one is extracted with 200 ml of ethyl acetate, followed by evaporation to dryness to afford the crude extract. The secondary metabolite content of crude extracts was analyzed using LC-MS/MS.

2.4 Antibacterial Activity Test

S. aureus ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, methicillin-resistant *Staphylococcus aureus* (MRSA), and Multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA) were used as the pathogens for antibacterial activity test. 100 µL of the bacterial test suspension was poured into NA media. The antibacterial activity was evaluated using sterile disc paper dripped with 10 µL extract at a concentration of 5 % test compound in DMSO solvent and antibiotic disc chloramphenicol (30µg/disc) (Oxoid®) as a positive control placed on the medium's surface. The Petri dishes were then incubated for 24 hours at 37 °C. The clear zone around the paper disc indicated antibacterial activity. The experimental results were expressed as mean ± standard deviation of three replicates.

2.5 Preliminary Detection of bioactive compounds by LC-MS/MS

The extracts obtained were subjected to LC-MS/MS analysis to identify the bioactive compounds present in the fungal extracts. Each extract was injected into liquid chromatography with mass spectrometry using a volatile solvent mobile phase. The solvent was evaporated under a vacuum system and ionized the analyte in the gas phase, and the analyte was passed to Mass Spectrometry. LC-MS analysis was performed on a

XEVO G2-S Q-TOF instrument outfitted with a Waters Acquity QSM. For data processing, Mass Lynx NT software four was used. The sample was injected 5 µL into column C18 BEH 100. The identification of components was accomplished using computer searches in commercial libraries.

3. Result

Marine-derived fungi offer promising potential for discovering bioactive natural products that possess unique structures and potent pharmacological activities [9]. In our ongoing search for bioactive compounds from marine-derived fungi, a crude extract of *P. simplicissimum* Ch06, a fungus obtained from the marine sponge *Chelonaplysilla sp*, exhibited significant antibacterial activity. The antibacterial activity of the *P. simplicissimum*Ch06 ethyl acetate extract cultivated on CM was compared with fungal extracts grown on MSG and NaNO3 medium. Test results indicated an increased inhibition diameter against all pathogenic bacteria for the MSG and NaNO3 extracts compared to the CM extract (Table 1). The MSG extract showed growth inhibition of E. coli with an inhibitory diameter of 13.17±1.11, while the NaNO3 extract exhibited an inhibitory diameter of 12.29±1.64. However, the CM extract displayed no inhibitory activity (inactive).

Table 1. Antibacterial activity result of *P. simplicissimum* Ch06 extracts from rice/control medium and medium with the addition of 3.5% MSG and 3.5% NaNO3

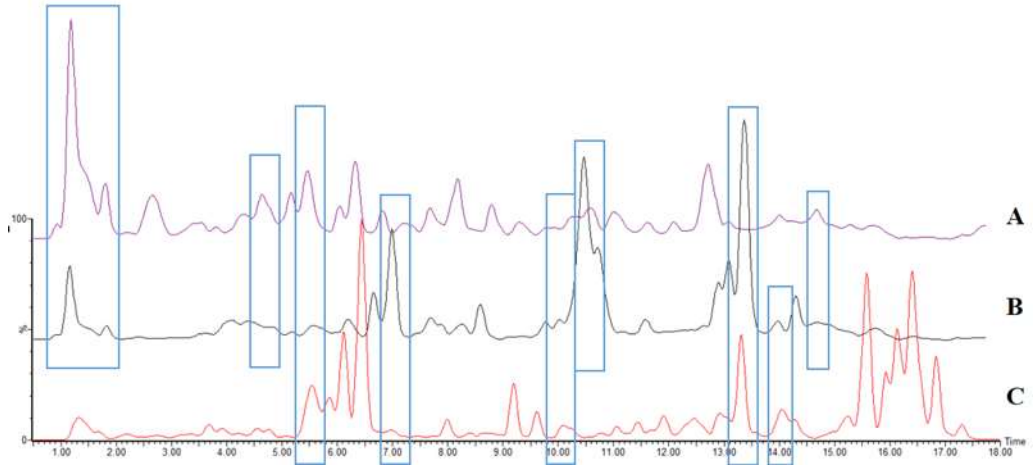
Tested Bacteria	Diameter inhibition zone (mm) ± Standard Deviation (SD)		
	Rice/control medium	MSG medium	$NaNO_3$ medium
E. coli	-	13.17±1.11	12.29±1.64
S. aureus	10.20±0.86	15.04±0.95	13.73±0.81
P.aeruginosa	8.26±0.73	14.05±0.88	14.27±1.76
MRSA	9.19±0.78	14.14±1.04	12.29±0.60
MDR-PA	8.19±0.97	11.60±1.34	11.00±0.98

Mass Spectrometry Liquid Chromatography (LC/MS-MS) is an analytical technique that combines the physical separation capabilities of liquid chromatography with the detection specificity of mass spectrometry. LC/MS-MS data can be used to determine the molecular mass, structure, identity, and quantity of compounds present in tested extract samples. One advantage of this method is its ability to analyze a broader range of components, including thermally labile compounds, highly polar or high-molecular-mass compounds, and even proteins. The ethyl acetate extracts from induced and standard cultivation treatments were analyzed using a Liquid Chromatography Mass Spectrometry instrument with the specifications UPLC-QToF-MS/MS (Ultra Performance Liquid Chromatography-Quadrupole Time of Flight-Mass Spectrometry). This instrument combines liquid chromatography, which separates the components of the sample, with mass spectrometry, which serves as a sensitive and selective detector for low-concentration samples.

The ethyl acetate extract of the *P. simplicissimum* Ch06 was analyzed as a spectrum using Masslynx version 4.1 and MZMine. Masslynx and MZMine are chromatograms and spectral analysis programs that provide information about retention times, molecular masses (m/z), and predictive formulas for these masses. The chemical formula of the compound being analyzed can be estimated using the chromatogram data, the intensity of the parent ion and the intensity of the daughter ions, and a linear combination of its atoms. The parent ion is determined using low energy (4 volts), while the daughter ion is determined using high energy (25-60 volts).

Compounds can be identified by examining the parent and fragment ions (daughter ions) and utilizing their molecular mass (m/z). Identification is achieved by comparing the molecular mass (m/z) [M+H]+ to previously reported databases, with the KEGG (Kyoto Encyclopedia of Genes and Genomes) database and previous journals on compounds from endophytic and symbiotic fungi being utilized. KEGG is a database collection encompassing genomes, biological pathways, diseases, drugs, and chemicals. It is commonly employed in bioinformatics research and education, including genomics, metagenomics, and metabolomics data analysis. The spectrum is fragmented using official websites such as MassBank, ChemSpider, and PubChem.

Figure 1 displays the LC/MS-MS profile of the ethyl acetate extract obtained from the fungus *P. simplicissimum* Ch06 cultivated on rice medium and medium supplemented with MSG and NaNO3. The ethyl acetate extract produced on rice medium exhibited 24 compound peaks, with one peak significantly dominating height. In the case of the ethyl acetate extract cultivated with the addition of 3.5% MSG, 21 peaks were observed, with five peaks showing higher dominance in height. Conversely, the ethyl acetate extract supplemented with 3.5% NaNO3 exhibited 24 peaks, out of which seven peaks were prominently higher in intensity. This indicates that adding MSG and NaNO3 inducers resulted in several compound peaks with significantly increased intensity compared to the original extract.



"Figure 1. LC Chromatogram of *P. simplicissimum* Ch06 extracts using UPLC-QToF-MS/MS (positive ion mode). A: Ethyl acetate extract from rice medium (CM). B: Ethyl acetate extract from the medium with the addition of 3.5% MSG. C: Ethyl acetate extract from the medium with the addition of 3.5% NaNO3.

Figure 1 displays three chromatograms featuring 30 peaks with the same molecular weight in the standard and induction extracts, albeit with varying area percentages. Some peaks demonstrate increased intensity, while others show decreased intensity following the application of an inducer during cultivation. Notably, new peaks with high intensities indicate the production of new compounds when the fungus *P. simplicissimum* Ch06 was induced in rice cultivation media by adding 3.5% MSG and 3.5% $NaNO_3$.
D-xylonate compounds were detected in the ethyl acetate extracts A and B with a retention time of 0.93 minutes, exhibiting m/z 167.0129 [M+H]+ and the formula $C_5H_{10}O_6$. However, there was a slight difference in the percentage area between the two extracts. Extract A represented 0.7 % of the total area, while Extract B represented 0.26%. D-xylonate has been previously isolated from *E. coli* and more recently produced by genetically engineered *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. In the presence of D-xylonate dehydratase, an essential enzyme in the biosynthesis of 1,2,4-butanetriol and other chemicals, D-xylonate can be converted into 2-keto-3-deoxy-D-xylonate (KDX) [10][11].

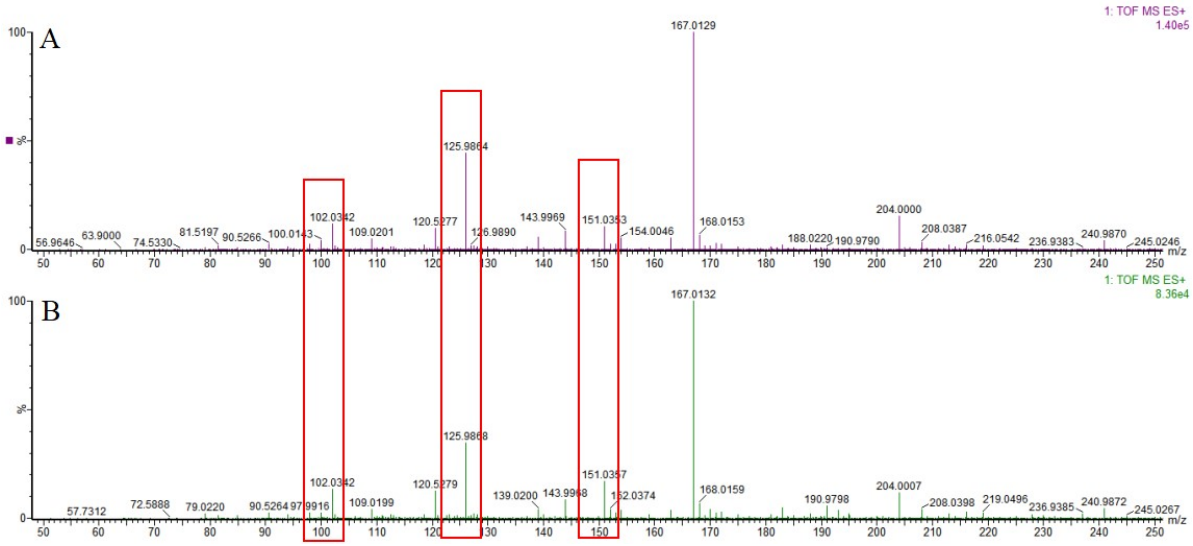


Figure 2 : The same fragmentation values of A: Ethyl acetate extract from rice medium (CM) B: Ethyl acetate extract from medium with the addition of 3.5% MSG.

Phenylacetic acid compounds were detected in the ethyl acetate extracts A and B, with respective retention times of 1.17 and 1.15 minutes and m/z 137.0632 [M+H]+, with the formula $C_8H_8O_2$. However, there was a difference in the percentage areas between the two extracts. Extract A represented 20.64 % of the total area, whereas Extract B represented 4.58 %. Previous research has isolated phenylacetic acid from *Streptomyces humidus* strain S5-55, which has demonstrated antifungal activity by inhibiting the growth of fungi such as *Pythium ultimum, Phytophthora capsici, Rhizoctonia solani, Saccharomyces cerevisiae,* and *Pseudomonas syringaepv*[12].

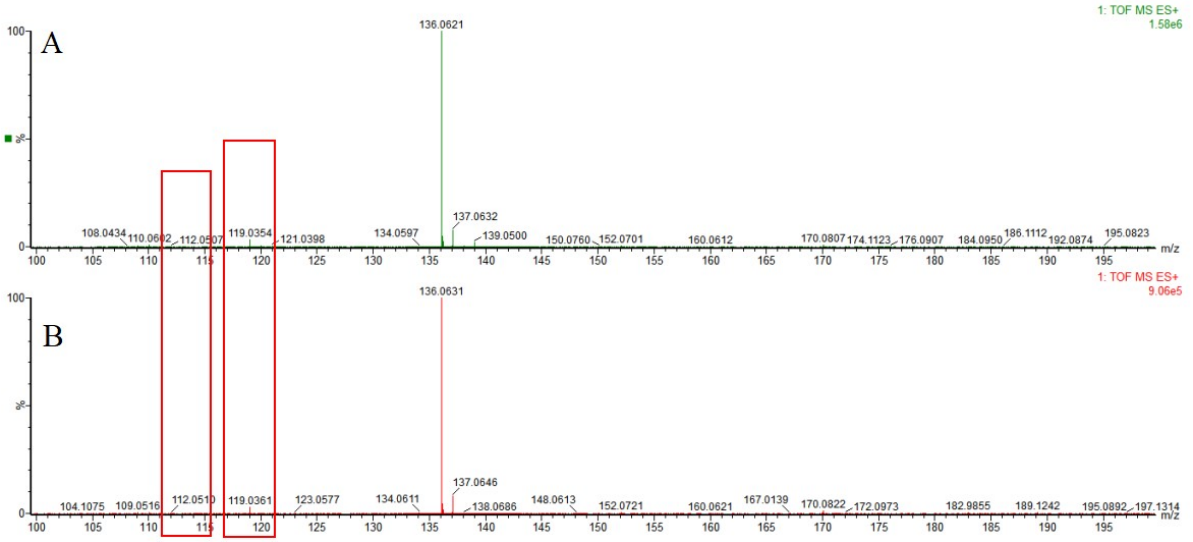


Figure 2 : The same fragmentation values of D-xylonate A: Ethyl acetate extract from rice medium (CM) B: Ethyl acetate extract from medium with the addition of 3.5 % MSG.
C: Ethyl acetate extract from medium with the addition of 3.5 % NaNO3.

Nicotinic acid compounds were detected in the ethyl acetate extracts A and B, with retention times of 1.79 and 1.83 minutes and m/z 124.0402 [M+H]+, respectively, and the formula $C_6H_5NO_2$. However, there was a difference in the percentage areas between the two extracts. Extract A represented 3.31 % of the total area, while extract B represented 0.77 %. Nicotinic acid was successfully isolated by Kim et al. from the fungus *Gibberella moniliformis* JS1055, obtained from the halophyte *Vitex rotundifolia*[13].

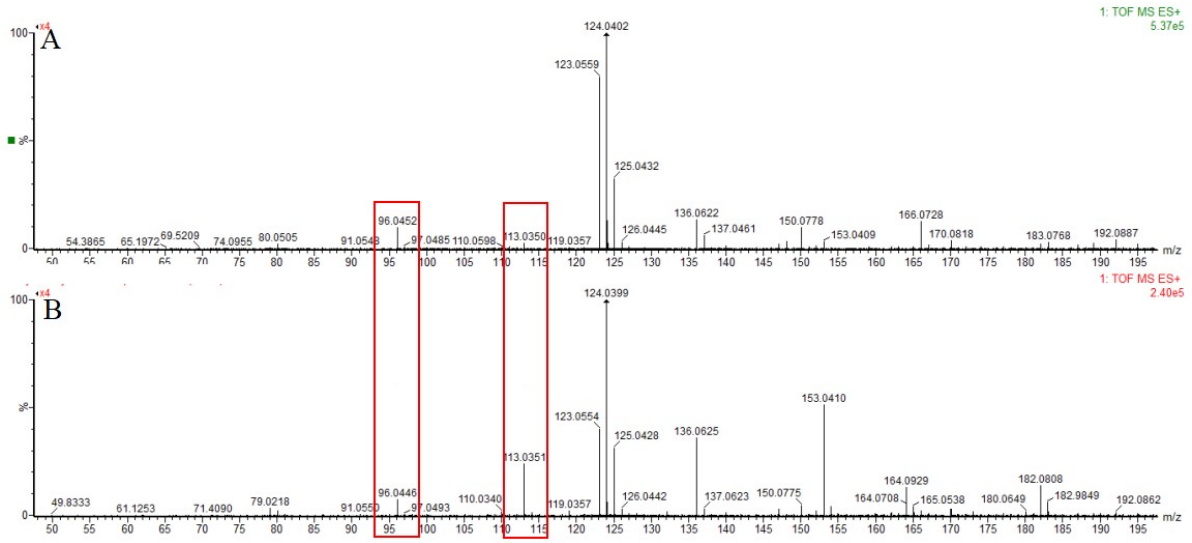


Figure 3 : The same fragmentation values of Nicotinic acid A: Ethyl acetate extract from rice medium (CM) B: Ethyl acetate extract from medium with the addition of 3.5% MSG.

Carlosic Acid compounds were detected in the ethyl acetate extracts A and B, with respective retention times of 3.57 and 3.52 minutes and m/z 228.0864 [M+H]+, with the formula $C_{10}H_{12}O_6$. However, there was a difference in the percentage areas between the two extracts. Extract A represented 1.48 % of the total area, while Extract B represented 0.59 %. Previous researchers isolatedCarlosic Acid from the fungus *Penicillium charlesii*NRRL 1887 by catalyzing the enzyme that converts dehydrocarolic acid to carolic acid [14].

Trichothosporon A compounds were detected in the ethyl acetate extracts A, B, and C, with respective retention times of 4.22, 4.22, and 4.20 minutes, exhibiting m/z 407.2391 [M+H]+ and the formula $C_{22}H_{30}O_7$, but with different percentage areas. Trichothosporon A (MSX 51320) is a sesquiterpenoid derived from Trichotheciumsp[15]. Based on the three LCMS profiles, it was found to have the same fragmentation values at 113, 245, and 265, indicating the presence of Trichothosporon A in all three extracts."

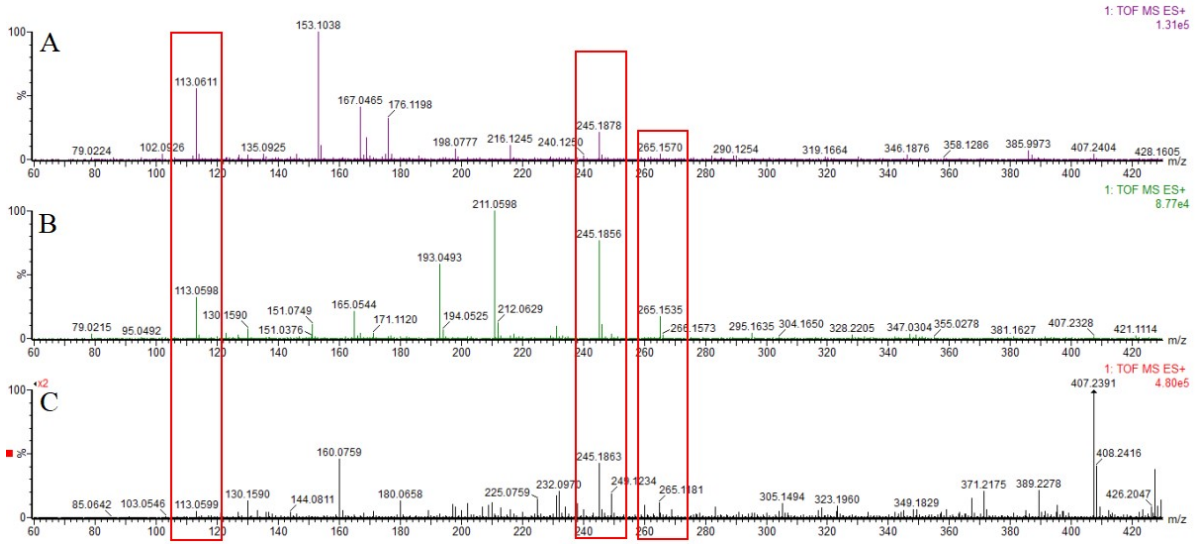


Figure 4 : The same fragmentation values of Trichothosporon A
A: Ethyl acetate extract from rice medium (CM)
B: Ethyl acetate extract from medium with the addition of 3.5 % MSG.
C: Ethyl acetate extract from medium with the addition of 3.5 % NaNO3.

The compound $C_{13}H_{27}N_2O_3$ was detected in the ethyl acetate extracts B and C, with a retention time of 5.56 minutes and m/z 259.2022 [M+H]+. However, there was a difference in the percentage of the area. Extract B represented 1.63% of the total area, while extract C represented 4.46 %.

The compound 5,8-Epidioxyergosta-6,9(11),22-trien-3-ol was detected in the ethyl acetate extracts B and C, with 6.99 and 6.93 minutes retention times, respectively. It exhibited m/z 427.2127 [M+H]+ and the formula $C_{28}H_{42}O_3$ but with different percentage areas. Extract B represented 7.31% of the total area, while Extract C represented 0.27 %. This compound was previously reported to be isolated from the fungus *Gibberella moniliformis* JS1055 [13]. Spectrum B and Spectrum C exhibited the same fragment values as reported [16].

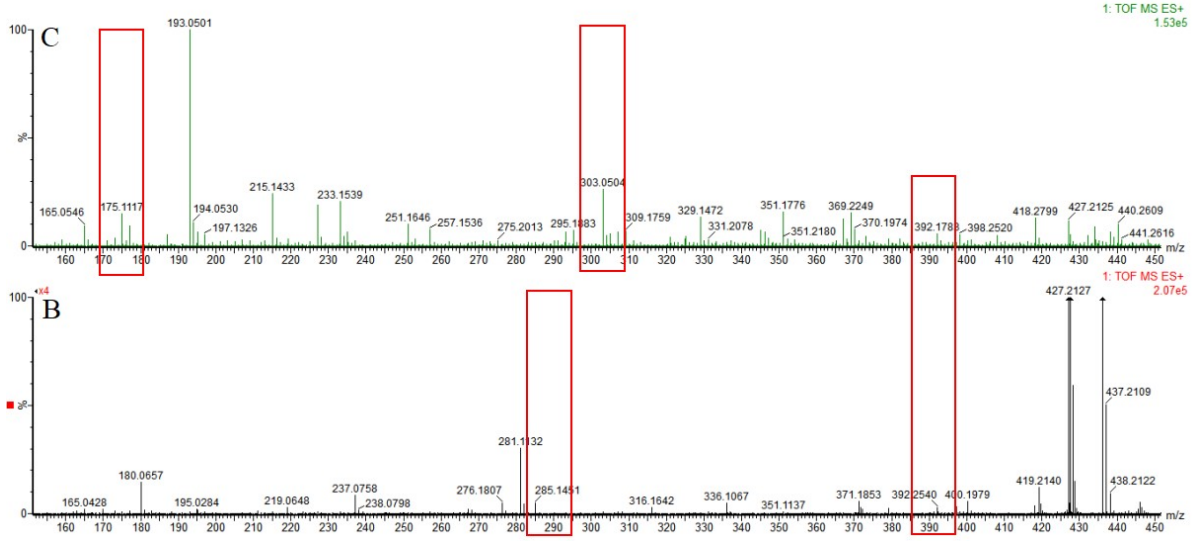


Figure 5 : The same fragmentation values of 5,8-Epidioxyergosta-6,9(11),22-trien-3-ol
B: Ethyl acetate extract from medium with the addition of 3.5% MSG.
C: Ethyl acetate extract from medium with the addition of 3.5% NaNO3.

Hygromycin B compounds were detected in the ethyl acetate extracts A and B, with retention times of 10.00 minutes each and m/z 527.2764 [M+H]+, with the chemical formula $C_{20}H_{30}N_3O_{13}$. However, the percentage areas differed between the two extracts. Hygromycin B is an antibiotic compound that has been found in the bacterium *Streptomyces hygroscopicus*. It is widely used in industry and biological research as an aminoglycoside antibiotic [17].

Phytosphingosine compounds were detected in the ethyl acetate extracts A and B, with 10.53 and 10.44 minutes retention times, respectively. It exhibited m/z 318.3031 [M+H]+ and the chemical formula $C_{18}H_{39}NO_3$ but with different percentage areas. Extract B showed a higher percentage, with 17.28% of the total area. Nocardicin A, a novel monocyclic β-lactam antibiotic, was detected in ethyl acetate extracts B and C, with 13.36 and 13.32 minutes retention times, respectively. It had m/z 498.3801 [M+H]+ and the chemical formula $C_{23}H_{24}N_4O_9$[18].

Aflatrem compound was detected in the ethyl acetate extracts B and C, with retention times of 14.29 and 14.27 minutes, respectively, and m/z 501.3957 [M+H]+ with the chemical formula $C_{32}H_{39}NO_4$. However, the percentage areas differed, with B representing 8.16% and C representing 3.67%. Aflatrem is a mycotoxin compound isolated from the fungus *Aspergillus flavus*. It contributes to the toxicity of *A. flavus*-infected plants [19]. Spectrum B and C exhibit the same fragment value at 502, as reported by Nielsen [20].

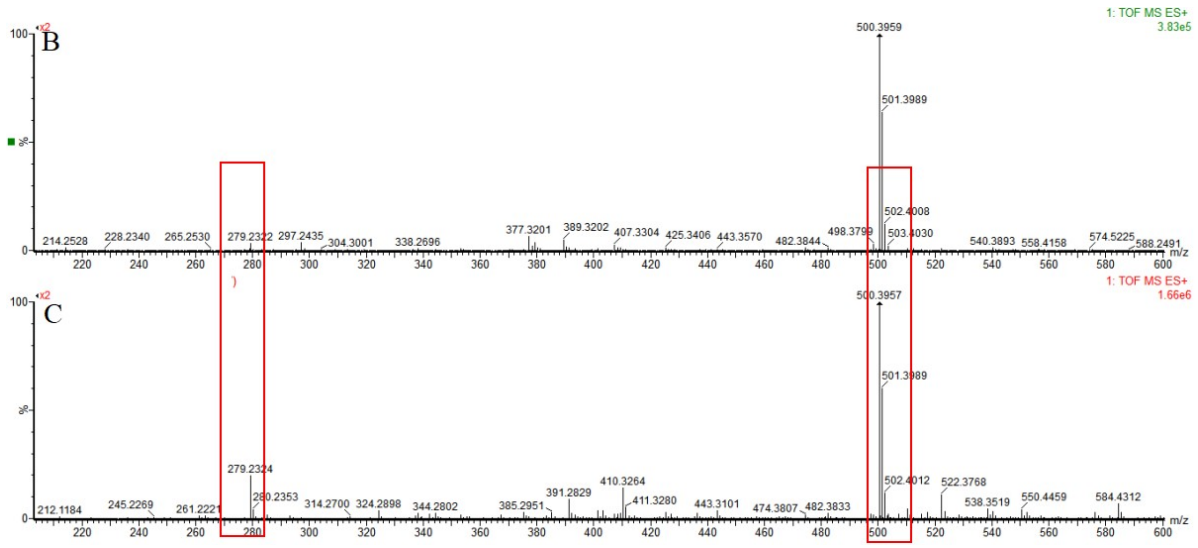


Figure 6 : The same fragmentation values of Aflatrem B: Ethyl acetate extract from medium with the addition of 3.5 % MSG. C: Ethyl acetate extract from medium with the addition of 3.5 % NaNO3.

Questinol was detected in the ethyl acetate extracts A and B, with retention times of 15.29 and 15.25 minutes, respectively, and m/z 300.2910 [M+H]+, with the chemical formula $C_{16}H_{12}O_6$. It was isolated for the first time from an extract of the marine-derived fungus*Eurotiumamstelodami*. Questinol is an anthraquinone derivative that has shown promise in preventing and treating inflammation [21]. Spectrum A and B represent questinols, which exhibit a fragment at 301, identical to the report by Nielsen [20].

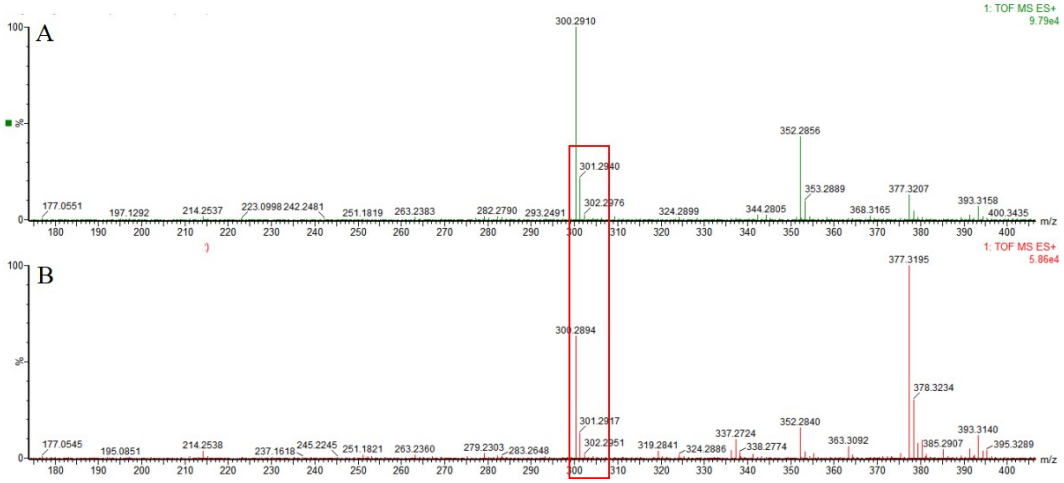


Figure 7 : The same fragmentation values of Questinol A: Ethyl acetate extract from rice medium (CM) B: Ethyl acetate extract from medium with the addition of 3.5% MSG.

Many researchers adopt a more objective approach to harnessing the potential of microbes, employing techniques such as introducing heterologous compounds, promoter regulation, mutation induction, or altering cultivation conditions to stimulate secondary metabolites [22]. Introducing an inducer factor, namely 3.5 % NaNO3 and 3.5 % MSG, into the cultivation media makes it evident that there are notable differences in the metabolites produced. It is widely recognized that nitrogen sources are essential for synthesizing crucial proteins, nucleic acids, and nitrogen-containing secondary metabolites. Amino acid-based nitrogen sources play a significant structural role as amino, and amide groups are integral to secondary metabolite structures. Fungi grown on different media exhibit distinct metabolic profiles, which can impact the formation and diversity of secondary metabolites [23][24].

Tudzynski has stated that all environmental factors, including the quality and quantity of nitrogen sources used in growth media, can impact the growth, differentiation, and biosynthesis of metabolites in fungi [25]. Nitrogen metabolism is selectively regulated through the use of nitrogen sources that are easily assimilated under specific conditions. This regulatory mechanism ensures the transcriptional activation of structural genes encoding enzymes and permeases necessary for the energy-dependent uptake and degradation of less favorable nitrogen sources. Following the assimilation of nitrogen sources such as nitrate, nitrite, urea, or amino acids, NH4+ is formed, which is then converted to glutamate through glutamate dehydrogenase (GdhA) and further to glutamine via glutamine synthetase (GS). Glutamine is a significant effector of nitrogen metabolite repression, facilitating the utilization of reduced nitrogen sources such as ammonium and glutamine when multiple nitrogen sources are available [26]. This regulatory mechanism acts as a positive regulator, activating the expression of nitrogen source genes when glutamine or ammonia levels are low [21]. The table below presents the interpretation of LC/MS-MS data obtained from the ethyl acetate extract of *P. simplicissimum* Ch06 in different cultivation media. The OSMAC concept explores alterations in microbial metabolite profiles influenced by changes in fermentation conditions, such as variations in media, temperature regimes, culture vessels, and other factors [27].

4. Conclusion

The addition of MSG and NaNO3 to the rice medium affected the formation of secondary metabolites of the fungus *P. simplicissimum* Ch06. The secondary metabolite profile analysis results using the LCMS/MS method showed an increase in peak metabolites and area compared to the control medium, which increased antibacterial activity.

5. Acknowledgment

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Conflict of interest: There is no conflict of interest.

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