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The optimation of fermentation time, antibacterial activity, and profiling secondary metabolite of symbiont fungi from Sponge *Gelliodes fibulata*

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ABSTRACT

Symbiont fungi are organisms that live in sponges tissue. Sponges are known to contain many metabolites which have the potential to be used as raw materials for medicine. Sponge *Gelliodes fibulata* belongs to the category demospongiae. The purpose of this study was to determine the optimal time to obtain the best secondary metabolite profile results in the sponge symbiont fungus *Gelliodes fibulata*. This research is included in experimental research. Beginning with the fungi culture of the sponge *Gelliodes fibulata*. Time variations 2, 4, 6, 8, 10, 12, and 14 are used to see differences in secondary metabolite production. A liquid extraction process is carried out to obtain secondary metabolites produced during fermentation. The final stage is to carry out qualitative analysis with TLC and antibacterial testing with the well-diffusion method. The results obtained indicate that the length of fermentation time influences the secondary metabolites obtained and automatically influences their antibacterial activity. The profile of secondary metabolites from TLC showed that the 10th day of fermentation had the secondary metabolites with high complexity and the highest yield 0.086%. The results of antibacterial activity showed that the 10th day of fermentation had the largest inhibition zone with 7.75 ± 0.44 mm compared to the other days of fermentation.

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Keywords: *Antibacterial, Gelliodes fibulata, fermentation, Sponge, Symbiont fungi*

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INTRODUCTION

Symbiont fungi are one of the microbes that live in sponge tissue. A symbiotic relationship of mutualism occurs between the symbiont fungus and its host, the sponge. This symbiotic mutualism occurs when the sponge obtains nutritional derivatives and active compounds from the symbiont fungus, while the fungus obtains nutrients from the metabolic products of the sponge (Leal et al., 2014). Symbiont fungi residing in the sponge can produce possible compounds that are the same or different from the original sponge, with varying properties (Webster & Thomas, 2016). The contribution of symbiont fungi in producing secondary metabolites in their host or sponge is very large, especially in producing compounds that have certain characteristics. These compounds have great potential as medicinal compounds, including alkaloids, terpenoids, steroids, quinones, and phenols (Freeman et al., 2020).

Sponges are one type of biological natural resource, their habitat in the sea reaches 830 species consisting of three classes, namely Calcarea, Demospongiae, and Hexactinellidae (Braekman & Daloze, 2004; Marzuki, 2018). Sponges are known to contain many metabolite compounds that have the potential as medicinal raw materials (Mahfur, Setyowati, et al., 2022; Mahfur, Wahyuono, et al., 2022). *Gelliodes fibulata* sponge is a sponge from the Demospongiae class, this class is the largest class that covers 90% of all sponge species (Setyowati et al., 2017). However, the *Gelliodes fibulata* sponge itself has not been widely studied and utilized and also for microorganisms associated with the sponge. Biotechnology is studying microorganisms that are widely used in fermentation.

A fermentation process is a form of biotechnology used to increase the production of secondary metabolites in microorganisms such as symbiont fungi (Mahfur et al., 2023; Samirana et al., 2021). Fermentation will produce secondary metabolite products in large quantities and have good quality. The process of forming fermentation products is influenced by many factors, one of which is the time or length of the fermentation process (Setyowati et al., 2018). Environmental factors in mushroom growth have an impact on the amount and variety of secondary metabolites produced, so it is necessary to optimize growing conditions. Optimization is the first step of fungus cultivation in producing bioactive secondary metabolite compounds (Freeman et al., 2020).

Research on the effect of fermentation time on secondary metabolite profiles and antibacterial activity in *Gelliodes fibulata* symbiont fungus has never been done before, so this research is very relevant. This study aims to determine the optimal time for fermenting the symbiont fungus from the sponge *Gelliodes fibulata*. The selection of optimal time is based on the metabolite profile produced, and antibacterial activity against *Escherichia coli*.

MATERIALS AND METHOD

Materials

Gelliodes fibulata sponge from Gili layar, West Lombok, Nusa Tenggara Barat (NTB). *Escherichia coli* bacteria, Himedia brand Sabouraud Dextrose Agar (SDA) media, Himedia brand Sabouraud Dextrose Broth (SDB), Himedia brand Muller Hinton Agar (MHA), Aquadest, Natural Sea Salt, NaCl infusion, Ciprofloxacin injection (HJ), GF 254 silica gel plate (Merck), 70% alcohol (Onemed), Pro analysis Ethyl acetate (Merck), n-hexsan (Merck), Chloroform (Merck), Ethyl acetate (Merck) and Methanol (Merck).

Methods

Sponge identification.

Identification of sponge samples was carried out with the aim of confirm the correctness of the sponge sample. Identification was carried out at the Marine Natural Product Laboratory Diponegoro University, Semarang, Indonesia using reference (Uriz et al., 2003).

Symbiont fungus cultivation and purification

Gelliodes fibulata sponges were cleaned and sprayed on the surface with sterile sea salt water. Cultivation begins with the *Gelliodes fibulata* sponge cut using a sterile cutter longitudinally, then the inside of the sponge is sprayed with sterile sea salt water. The cut results were placed on a petri dish containing saline SDA media with the addition of chloramphenicol as much as 1 mL, with the inside of the sponge facing towards the media. Culture at 37°C storage for 5-7 days to obtain the growth of the sponge symbiont fungus *Gelliodes fibulata* (Purwantini et al., 2016). Cultivation will produce several fungi that grow in saline SDA media. The selected fungi are those that have the most dominant growth in the cultivation. The dominant fungal culture was cultured on saline SDA media with a sterile technique and incubated for 14 days.

Purification and identification of symbiont fungus

The purification procedure was performed according to each colony's unique morphological characteristics. The predominating fungi is called as isolate fungi and are cultured on the media to produce a pure isolate. The symbiotic fungus was examined under macro, micro, and molecular microscopes to identify it. The steps in molecular identification were DNA extraction, PCR process, and the last identification of phylogenetic trees were analyzed using MEGA 7.0 software, while statistical analysis used the Neighbor-Joining method with 1000 bootstrap replication fields (Mahfur et al, 2023).

Fermentation of symbiont fungus

Fermentation was carried out using saline SDB (Suboroud Dextrose Broth) media. Some of the predominating fungus was cultured into 200 mL of saline SDB media. Incubation time was carried out using a variety of days, it is 2, 4, 6, 8, 10, 12, and 14 days. Incubation was carried out using the shaker incubation method at 120 rpm at 37°C (Setyowati et al, 2017).

Extraction of secondary metabolites

Extraction began with the separation of fermentation products between mycelia and supernatant. The supernatant obtained was extracted by liquid-liquid extraction method using ethyl acetate solvent in a 1:1 ratio. The extraction results were evaporated using an evaporator at 60°C (Setyowati et al, 2018). The yield obtained was calculated.

Identification of metabolite profile

Identification of secondary metabolites was carried out using the thin-layer chromatography (TLC) method. The stationary phase used was silica GF_{254} and the mobile phase used was n-hexan: ethyl acetate (1:3). Identification of chemical compound groups using spray reagents. Dragendroff reagent was used for alkaloid identification, cytroborate reagent was used for flavonoid identification, the vanillin-sulfate reagent was used for steroid identification, and $FeCl_3$ reagent was used for phenolic identification (Mahfur et al, 2023; Wulansari et al, 2020).

Antibacterial test of extracts

The test was conducted on Mueller Hinton Agar (MHA) media using the well diffusion method. *E. coli* bacteria that had been inoculated were taken with an ose to make a bacterial suspension with a concentration similarity of 0.5% McFarland. The concentration of the extract used to test was 25 mg/mL with ethyl acetate solvent. Bacterial suspensions were then applied to MHA media. MHA media was prepared in as many as 2 petri dishes, each divided into 4 wells for testing extract samples resulting from variations in fermentation days. Incubation was carried out at 37°C for 24 hours. This bacterial test was repeated 3 times. The clear zone around the wells indicating the absence of bacterial growth was then measured with a caliper and counted (Cita et al., 2017).

Data Analysis

The results of the TLC analysis indicated a change in the color of the spots. The antibacterial assay was repeated three times, and the potential of the antibacterial was expressed as an average of three replications ± Standard Deviation (SD). The test results were statistically analyzed using one-way analysis of variance (ANOVA) SPSS program for Windows version 16.

RESULT AND DISCUSSION

Sponge identification and Cultivation of symbiont fungus

The identification results at the Marine Natural Product Laboratory of Diponegoro University are based on letter no. Idn.201101-Sp showed that the sponge used included the species *Gelliodes fibulata*. Cultivation was done by planting small parts of the sponge on saline SDA media. The cultivation results showed that 4 types of fungi grew in the process (Figure 1). The results of fungal growth were selected with the most dominating colony growth category in the media. then re-cultured into new saline SDA media for purification. The purpose of purification is to separate the dominant symbiont fungi from other fungi so that pure colonies are obtained in each medium (Ginting et al., 2019; Rosmania & Yanti, 2020). Green fungi are fungi that grow dominantly in the cultivation process and are selected to continue in the next test. After re-growing on the media for 14 x 24 hours, pure fungal isolates are obtained, it is fungi that contain one form of the same colony morphology. Pure fungal isolates obtained are continued to the fermentation stage, with to multiply symbiont fungi (Setyowati et al., 2018).

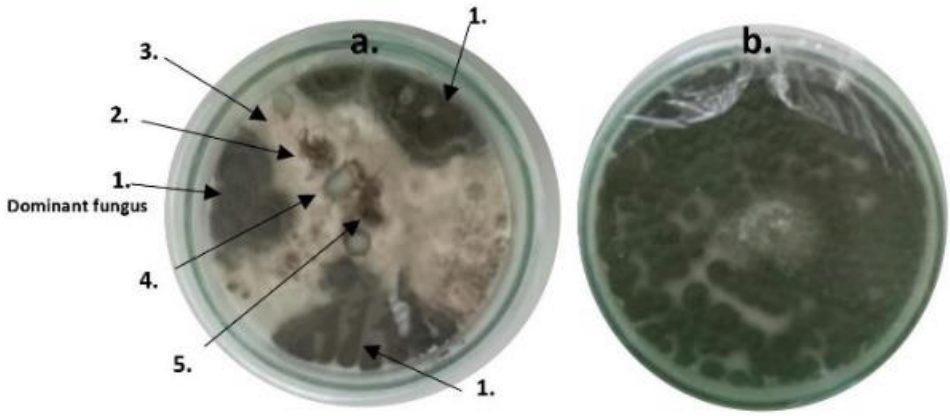

Figure 1. a.) Cultivation results of *Gelliodes fibulata* sponge. b.) symbiont dominant fungus culture. The number 1-5 indicate the types of fungi growing

Identification of fungus symbiont dominant

The aim of the purification procedure is to produce pure isolates according to their morphology. The green mycelium (Figure 1) was identified with micro, macroscopical, and molecular tests. The DNA sequencing was used to identify dominating fungal strains and registered in GenBank (accession numbers OQ451585, www.ncbi.nlm.nih.gov). The isolates showed 99.08% homology and 93% query cover to *Penicillium nalgiovense* (Figure 2). The isolates exhibit a high degree of similarity and are probably members of the same species because the maximum identity value is more than 97%.

Fermentation of symbiont fungi

Fermentation was carried out using saline SDB (Suboroud Dextrose Broth) media. A total of 5 parts of the cultured fungi plots were put into 200 mL of saline SDB media. The incubation time was carried out using a variety of days, it is 2, 4, 6, 8, 10, 12, and 14 days. Shaker incubation at 120 rpm was used at this fermentation stage. The fermentation process using liquid media will make the fungi produce

bioactive compounds. Microorganism fermentation is influenced by physical and chemical factors. Physical factors that affect microorganisms include temperature, pH, and osmotic salinity, while chemical factors consist of carbon, nitrogen, and nutrient sources in the culture medium (Setyowati et al., 2018). Based on several studies, simple carbon sources such as glucose and dextrose greatly influence the growth of fungi associated with marine environments and can also influence the production of secondary metabolites that influence their biological activity (Anuhya et al., 2017; Mahapatra & Banerjee, 2013). SDB liquid media contains dextrose, carbohydrates, and nitrogen, and has a pH of 5-6, making it suitable for fungal growth media (Rendowaty et al., 2017). Fermentation time is an independent variable, it is days 2 to 14 with a difference of 2 days. The time variable is based on the growth phases that occur in microorganisms. The growth phase of microorganisms is divided into several parts, lag phase, log phase, stationary phase, and death phase. The lag phase is the growth phase of the fungi adapting to its environmental conditions. The log phase of fungi growth generally occurs from day 7th to day 14th, in the log phase there is an increase in the amount of biomass. In the next phase, if carbon as an important source of energy or nutrients has been used up, it does not mean that growth stops. The growth phase can continue due to the lysis of dead cells used as a source of nutrients (Carranza et al., 2017). This is the basis for the selection of fermentation time to see which time is the most optimum to get good results based on metabolites and antibacterial activity. The predominant fungi associated with fermented *Gelliodes fibulata* is *Penicillium nalgiovense* which has a growth phase where 150 hours is the most optimal growth time in the growth process (Papagianni & Sergelidis, 2014).

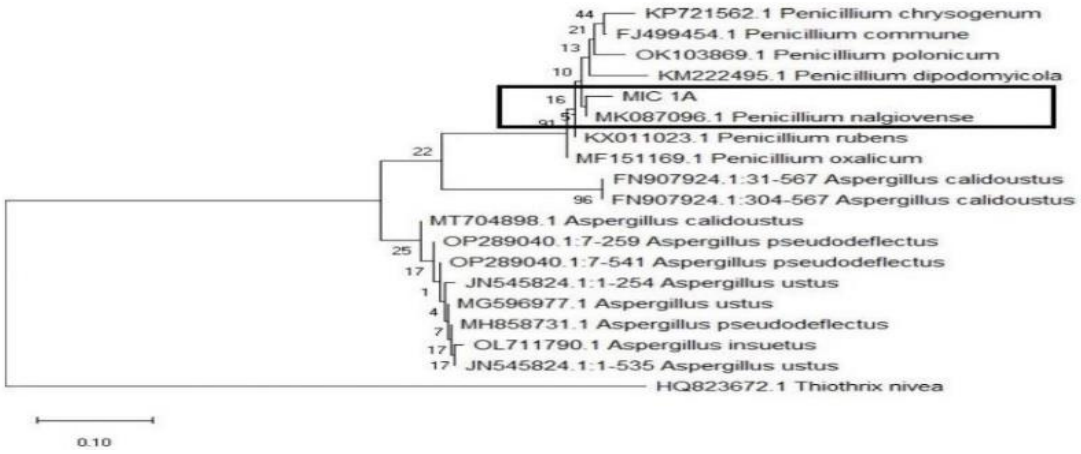

Figure 2. phylogenetic tree of predominating fungi on sponge *Gelliodes fibulata*

Extraction of secondary metabolites

The fermentation stage will obtain 2 parts, biomass (mycellium) and supernatant, a better extraction process is to use both parts. This is because the active compounds are not clearly known, whether they are inside the cells or outside the cells. therefore, the use of extraction processes for both parts results in optimal extraction (Samirana et al., 2021). In this study, the supernatant part was used for extraction like in some previous studies (Rendowaty et al., 2017). Extraction of symbiont fungal isolates from *Gelliodes fibulata* sponge aims to separate the active compounds present in the supernatant. Extraction is done by liquid-liquid extraction method from the supernatant of fermentation results. The extraction results obtained yields on each day of fermentation in succession are as follows, day 2 gets a yield of 0.018% b/v, day 4 as much as 0.041% b/v, day 6 as much as 0.041% b/v, day 8 as much as 0.059% b/v, day 10 as much as 0.086% b/v, day 12 as much as 0.071% b/v and day 14 as much as 0.046% b/v can seen Figure 3. The yield results obtained illustrate that fermentation time affects the amount of yield. This is clearly due to the growth phase of the fungi. The peak growth of the *Gelliodes fibulata* symbiont fungi

fermentation can be concluded to occur on day 10th when viewed from the resulting yield. The following days 12th and 14th, experienced a decrease in yield and it can be concluded that growth has entered the stationary phase and the death phase. The extraction yield is a description of the amount of metabolites produced, the more yield obtained, the more secondary metabolites contained in the extract (Samirana et al., 2021).

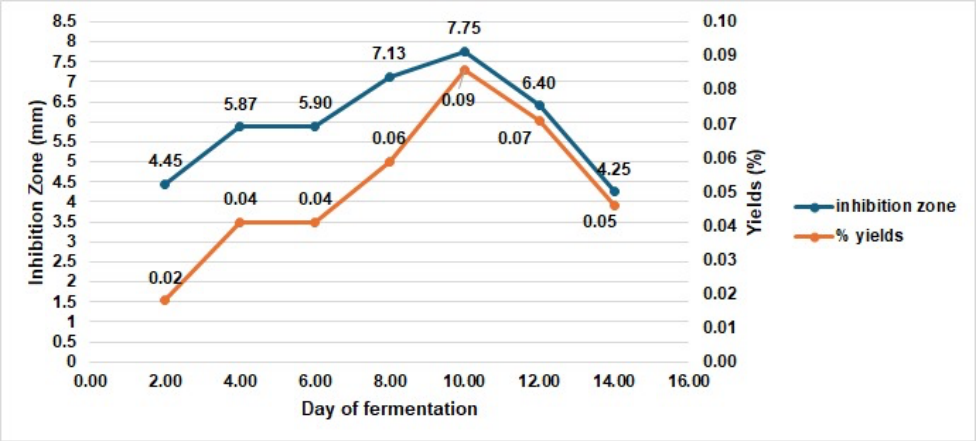

Figure 3. The curve of the effect of fermentation time with inhibition zone and % yield produced

Identification of metabolite profiles

The results of the identification of the secondary metabolite content of ethyl acetate extract of symbiont fungi show that the extract contains several classes of chemical compounds. The identification of chemical compound groups analyzed using spray reagents for each group of chemical compounds obtained results as in Table 1. The TLC profile of each extract from the fermentation time showed different results, which showed that fermentation time affected the content of secondary metabolites produced (Figure 4). The longer the fermentation time, the more the TLC profile shows the compound content. Starting from day 8th untile day 12th fermentation looks to have a more complex profile than other fermentation times. Clearly seen from the results of the TLC profile with vanillin sulfate reagent under visible light. On the day 8th and 10th fermentation had spots with blue-green color, and on day 12th fermentation the color of the spots became thinner, but other spots with purple appeared and then on the 14th day of fermentation all the spots were not visible. This indicates that the production of secondary metabolites in the fermentation phase occurs in accordance with the growth phase of the fungi.

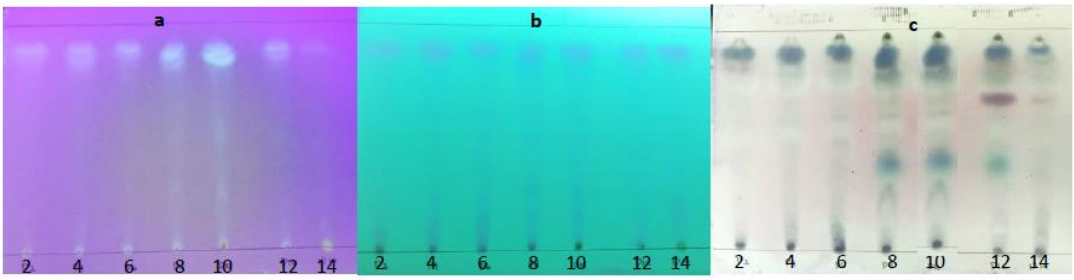

Figure 4. The TLC results of ethyl acetate extract of the sponge symbiont fungus *Gelliodes fibulata*. a) UV 366 b) UV 254 c) vanillin- Sulfate reagent under visible light

Antibacterial test of extracts

Antimicrobial activity test against *E. coli* ATCC 25922 was conducted using the well diffusion method. The diffusion method was chosen because it has several advantages compared to other methods including the amount of substance used can be adjusted, the time is shorter, easy to apply, and simpler

(Tumiwa et al., 2019). Antimicrobial testing aims to determine the activity of inhibiting bacterial growth from extracts of symbiont fungi associated with the sponge *Gelliodes fibulata*. Antibacterial activity arises because the ethyl acetate extract of symbiont fungi contains alkaloid, terpenoid, phenolic, and flavonoid compounds (Table 1). Terpenoid compounds are able to damage porins due to disruption of membrane and cell wall formation in bacteria (Wulansari et al., 2020). Flavonoid compounds have a mechanism of inhibition of DNA and RNA formation, cell membrane function, and oxygen utilization by bacteria. Alkaloids have a mechanism of action by disrupting the constituent components of peptidoglycan so that the cell wall layer is not formed intact and causes cell death (Amalia et al., 2017). While phenol compounds are bactericidal, the antibacterial activity of phenol compounds plays a role in damaging the cell wall and enzymes in the plasma membrane of microorganisms (Bouarab-Chibane et al., 2019).

Table 1. Identification results of secondary metabolite compound classes of ethyl acetate extract of the sponge symbiont fungus *Gelliodes fibulata*

Name of compound	Reagent	Observation result	appearance
Alkaloid	Dragendrof	+	orange red spot
Flavonoid	Cytroborate	+	Yellow spot
Steroid/Triterpenoid	Vanilin-sulfate	+	Blue green spot
Phenolic	$FeCl_3$	+	purple spot

+ showed has chemical compound class

The results obtained in antibacterial testing against *E-coli* are indicated by the creation of clear zones around the wells. This indicates that there is growth inhibitory activity on *E-coli* bacteria from fungi extracts isolated from sponges. The results of antibacterial testing of ethyl acetate extract from day 2 to day 14 of fermentation successively showed an inhibition zone of 4.45 ± 0.38 mm, 5.87 ± 0.32 mm, 5.90 ± 0.53 mm, 7.13 ± 0.35 mm, 7.75 ± 0.44 mm, 6.40 ± 0.60 mm, 4.25 ± 0.22 mm (Table 2). The criteria for antibacterial activity based on the inhibition zone follows the previous classification, where the classification criteria are divided into 4 criteria, namely weak, moderate, strong, and very strong (Tumiwa et al., 2019). The results of antibacterial testing of ethyl acetate extract of symbiont fungi showed 2 criteria, namely moderate criteria and weak criteria. The moderate criteria were the results of fermentation days 4, 6, 8, 10, and 12, while the weak criteria were the results of fermentation days 2, and 14. The 10^{th} day's fermentation has a better inhibition zone compared to other days because fermentation on that day produces complex metabolites. Supported by TLC profile data which shows that the compounds that contribute to increasing activity are terpenoids. The presence of terpenoid compounds can be seen in the results of analysis using vanillin-sulfate reagent with the appearance of a blue green-color spots on TLC which appears on days 8^{th}, 10^{th}, and 12^{th} of fermentation.

Table 2. Antibacterial testing results of ethyl acetate extract of the sponge symbiont fungus *Gelliodes fibulata* against *E-coli*

Fermentation Duration (day)	Inhibition Zone (mm)	Criteria
2	4.45±0.38	Weak
4	5.87±0.32	Moderate
6	5.90±0.53	Moderate
8	7.13±0.35	Moderate
10	7.75±0.44	Moderate
12	6.40±0.60	Moderate
14	4.25±0.22	Weak

Data are presented as mean±SD.

CONCLUSION

From the results, it can be concluded that fermentation time affects the yield of extracts produced and also affects their antibacterial activity. Day 10th fermentation has a complex metabolite profile and more yield and has a better inhibition zone compared to other days.

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