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THE OPTIMATION OF FERMENTATION FOR METABOLITE PRODUCTION BY SYMBIONT *Penicillium nalgiovense* FROM THE SPONGE *Gelliodes fibulata*

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ABSTRACT

The fungi are sponge symbionts. The fungus *Penicillium nalgiovense* acc MK087096 is a symbiont of the sponge *Gelliodes fibulata*. This symbiont has antibacterial activity, which supports the development of sponge-based drugs that are as effective as antibiotics. However, the primary challenge in developing marine resource-based medicine is the availability and sustainability of sponge raw materials. Fermentation biotechnology using sponge symbiont fumgi is an effective solution to address these challenges, as it allows the production of bioactive secondary metabolite compounds in large quantities, which can be used as raw materials for pharmaceutical preparation. The aim of this study was to determine the optimal medium and fermentation duration for producing secondary metabolites with antibiotic properties from the symbiotic fungus *Penicillium nalgiovense*, isolated from the sponge *Gelliodes fibulata.* This study was conducted naturally. The symbiotic fungus from the sponge *Gelliodes fibulata* was cultivated to facilitate growth. Fermentation was conducted with variations in secondary metabolite harvesting times of 2, 4, 6, 8, 10, and 12 days. Secondary metabolites were obstained using liquid-liquid extraction with ethyl acetate. The optimal medium and fermentation time were determined based on the yield percentage for each medium across the six time variations. The fermentation biotechnology of the symbiotic fungus *Penicillium nalgiovense* acc MK087096 from the spone *Gelliodes fibulata* was carried out on SDB, PDB, also coconut flake-enriched PDB media. The results showed the growth of the fungus and the production of bioactive secondary metabolites with antibiotic properties. The SDB medium was found to be optimal on the 10th day, with a yield value of 0.934% w/v. PDB medium was optimal on the 8th day, with a yield value of 0.087% w/v. Coconut flake-enriched PDB medium was optimal on the 6th day, with a yield value of 0.470% w/v. Therefore, SDB medium with a fermentation time of 10 days serves as the most effective solution to address the limitations and ensure the sustainable supply of active compounds derived from the sponge *Gelliodes fibulata* for pharmaceutical production.

Keywords: fermentation, microorganism, PDB, yield value, SDB

INTRODUCTION

Sponges, as one of the 830 species of marine biodiversity, are classified into three classes, namely *Calcarea*, *Demospongiae*, and *Hexactinellidae* (Marzuki, 2018). Sponges serve as hosts for various microorganisms such as fungi. They are reported to be rich in secondary metabolites that have a pharmacotherapeutic effects, making them valuable source of raw materials for drug development (Subramani *et al.*, 2013; Brinkmann, Marker and Kurtböke, 2017; Setyowati, Sudarsono and Murwanti, 2017; Mahfur *et al.*, 2022). The secondary metabolites produced are believed to originate from microorganisms attached to the sponge, resembling those produced by the host. This is because these microorganisms occupy about 60% of the sponge's body (Setyowati *et al.*, 2018).

Gelliodes sponges belong to the largest class, Demospongiae (Marzuki, 2018). The fungus *Penicillium nalgiovense*, which form a symbiotic relationship with the sponge, produce secondary metabolites that exhibit antibiotic activity (Mahfur *et al*., 2024). These metabolites have the potential to be developed into pharmaceutical products that are effective, safe, and convenient to consumers. However, the availability and sustainability of sponges remains a major challenge in the development of sponge-based metabolite drugs. Fermentation biotechnology of symbiotic sponge fungus offers an easy and effective alternative for producing high-quality secondary metabolites in large quantities within a short time. (Setyowati *et al*., 2018; Putu Oka et al., 2020; Mahfur *et al*., 2023).

Physicochemical properties of the fermentation process are crucial factors that have a significant influence. Physical factors include incubation time, temperature, osmotic salinity, and pH, while chemical factors involve carbon sources, nitrogen, and nutrients in the cultivation media (Pratiwi, 2008; Basu *et al*., 2015; Rendowaty, Djamaan and Handayani, 2017). Several studies have reported that sponge symbiotic fungi depend on carbon, nitrogen, and salt (salinity) sources. Simple carbon sources, such as glucose and dextrose in the media, significantly influence the biological activity of symbiotic fungi in producing secondary metabolites, both in terms of quantity and quality (Fuentes *et al*., 2015; Anuhya *et al*., 2017).

Optimization of growth conditions, including the incubation time and media, plays a significant role in increasing metabolite production. Optimization is the initial process of symbiotic fungal fermentation production of bioactive secondary metabolites (Molen *et al*., 2013; Aini, 2023). The aim of this study was to determine the optimal medium and incubation time for the fermentation of the symbiotic fungus *Penicillium nalgiovense* from the sponge *Gelliodes fibulata* in liquid media, including Sabouraud Dextrose Broth (SDB), Potato Dextrose Broth (PDB), and PDB enriched coconut flakes, to produce secondary metabolites with antibiotic properties. This study addresses the limitations and sustainability of raw material supply from *Gelliodes fibulata* sponges in the production process of pharmaceutical production processes.

RESEARCH METHODS

Equipment and Materials

Symbiont fungal *Penicillium nalgiovense* associated sponge *Gelliodes fibulata* from Gillilayar Lombok, Sabouraud Dextrose Agar (SDA Himedia ®) media, Sabouraud Dextrose Broth (SDB Himedia ®), Potato Dextorose Agar (PDA Himedia ®), Potatto Dextrose Broth (PDB Himedia ®), Aquadest, Natural Sea Salt (nucifera®), NaCl infusion, Ciprofloxacin injection (HJ®), 70% alcohol (Onemed ®), Ethyl acetate (Merck®)

Research Procedure

1. **Symbion fungus *Penicillium nalgiovense***
   The Penicillium nalgiovense acc MK087096 isolate from previous research (Mahfur et al., 2024)
2. **Cultivation of symbiont fungus**
   The *Penicillium nalgiovense* acc MK087096 isolate, stored at 4°C, was incubated at room temperature for 24 hours, then one ose of the *Penicillium nalgiovense* fungus was inoculated on a culture plate containing SDA media (Himedia®), PDA (Himedia®), dan PDA (Himedia®) enriched with coconut flakes, salinity was made the same as in its habitat, added ciprofloxacin injection (HJ). The culture was incubated at a temperature–25-28°C for 3-5 days.
3. **Fermentation of isolates, and harvesting time**
   Fungal isolates that had been rejuvenated on three different rejuvenation media were taken one - three ose each, and transferred into 200 mL of corresponding liquid media, namely Sabouraud Dextrose Broth. (SDB), Potato Dextrose Broth (PDB), dan PDB enriched with coconut flakes. Salinity was adjusted to match the habitat conditions, and ciprofloxacin injection (HJ) was added. The solution was homogenized and incubated

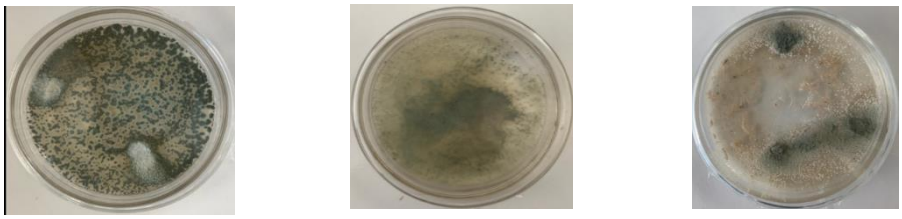
for 12 days. Shaking conditions at 120 rpm. Harvesting was conducted on the 2nd, 4th, 6th, 8th, 10th, and 12th days.

4. **Extraction**
   The fermentation supernatant at each fermentation time was extracted using the liquid-liquid extraction (LLE) method with ethyl acetate solvent at a ratio of 1:1, repeated three times, until the solvent phase was separated from the media. The extract was then concentrated using a rotary evaporator to obtain the fungal extract (Mahfur *et al*., 2024).
5. **Determination of media optimization and fermentation time for metabolite production**
   The optimal medium and fermentation time for fungal growth and secondary metabolite production were determined by calculating the percentage yield of the ethyl acetate extract obtained from the fungus and its supernatant on the 2nd, 4th, 6th, 8th, 10th, and 12th days of cultivation.

RESULTS AND DISCUSSION

Cultivation of symbiont fungus

The cultivation of the *Gelliodes fibulata* sponge symbiont fungus was carried out on SDA, PDA, and PDA media enriched coconut flakes, with conditions adjusted to match its natural habitat by adding natur sea salt, allowing the fungus to grow and produce optimal metabolites (Mahapatra and Banerjee, 2013; Cappuccino and Sherman, 2014; Putu Oka *et al*., 2020). Significant growth was observed in the three rejuvenation media used in this study, where growth conditions were made to match the fungus habitat. The cultivation results are shown in **Figure 1.**



(a) (b) (c)

Figure 1. ***Cultivation results of Penicillium nalgiovense fungus symbiont-associated sponge Gelliodes fibulata on three growth media: (a) SDA, (b) PDA, and (c) PDA enriched with coconut flakes.***

The growth data of the fungus on the three cultivation media **Figure 1** showed that macroscopically, the symbiont fungus growing on SDA, PDA, and PDA enriched with coconut flakes displayed predominantly green characteristics, indicating that the fungus could grow well as the nutrients required for growth ware present in the media. Thus, the cultivated *Penicillium nalgiovense* fungus grew well and exhibited consistent macroscopic characteristics.

The factors influencing fungal growth are the presence of nutrients in the media, including carbon sources from sugar, nitrogen, and salts (Cappuccino and Sherman, 2014; Anuhya *et al*., 2017). SDA and PDA are synthetic media with precisely measured compositions commonly used to isolate, cultivate, and maintain fungal microorganisms due to their characteristic low pH (4.5 to 5.6). This acidic environment inhibits the growth of bacteria, which typically require a neutral pH of 7.0, while the optimum temperature for fungal growth ranges between 25-30°C (Mahapatra and Banerjee, 2013; Cappuccino and Sherman, 2014). Thus, this condition selectively favors the growth of specific fungi such as *Penicillium nalgiovense.* The PDA media enriched coconut flakes is a modified nutrient medium developed in this study to enhance mycelium production and secondary metabolites

synthesis in the symbiotic fungus *Gelliodes fibulata* (Aini, 2023). This modified medium effectively supports the growth of *Penicillium nalgiovense* and facilitates the production of the secondary metabolite amphotericin B (Svahn, 2015).

Observations of fungal growth across all media showed visible culture growth within 24 hours, characterized by the formation of white mycelium. However, on SDA media, the mycelium appeared finer than on PDA media. White mycelium serves as an early indicator of fungal growth. Over the subsequent days, the fungal colonies expanded the adaptation phase of the fungus to new growth media. In this study, after 48 hours, conidia formation was observed, and the colonies turned dark green. The colonies exhibited a granular pattern, with the sporulation zone appearing narrower than the growth zone and concentric rings clearly visible. This is consistent with the characteristic growth of the fungus on the SDA and PDA media.

Fermentation of symbiont fungus

Liquid media, such as SDB and PDB, serve as fermentation media for the bioactive production of fungal secondary metabolites. The environment during fermentation significantly influences both fungal growth and the production of bioactive compounds (Gutiérrez *et al.*, 2020), Therefore, the media used during fermentation are the liquid forms of those used during the growth or cultivation stage, with identical nutrient compositions and pH levels. This consistency allows symbiotic fungus to adapt and grow directly. The shaker incubation method at a speed of 120 rpm was used in this study to accelarate fungal growth and promote the formation of secondary metabolites, the constant agitation ensures uniform nutrient distribution (Nursid. *et al*., 2015). The fermentation results from the three media (SDB, PDB, and PDB-enriched coconut flakes) harvested on day 10 are presented in **Table I**.

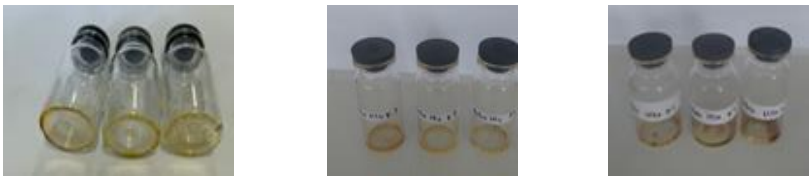
Table I. Macroscopic characteristics of *Penicillium nalgiovense*, fungal of symbiont associate sponge *Gelliodes fibulata*, on three fermentation media

Media	Fermentasi Results	Macroscopic characteristics
SDB		The SDB media is cloudy yellow, with mycelium that is bone white, and it is located at the bottom.
PDB		The media is clear with a yellowish-orange color, and the mycelium, which is bone white, increases in quantity and is located at the top and center of the media.

PDB-enriched coconut flakes		The media is cloudy yellow with a thicker viscosity, containing bone-white mycelium that dissolves, and clumps of coconut milk are present on top of the media

Extraction of fungus symbiont

The extraction of *Penicillium nalgiovense* was performed using the liquid-liquid extraction (LLE) method with ethyl acetate as the solvent in a 1:1 ratio, and the process was repeated three times. Ethyl acetate, a semi-polar solvent, can extract both polar and non-polar compounds and has been used as a solvent for marine sponge extracts in previous studies. Ethyl acetate was chosen as the solvent because the sample used was a marine natural product-containing salt. Therefore, ethyl acetate serves to separate the salt and water, allowing the metabolites produced during the extraction process to be pure organic compounds free from unwanted substances (Stephenson, 2018). The extraction method using ethyl acetate also proved to extract bioactive compounds with potential as antibiotics (Tumiwa, Yudistira and Wewengkang, 2019; Paat, Wewengkang and Rotinsulu, 2020; Setyowati *et al.*, 2021; Mahfur *et al.*, 2023). The ethyl acetate extract of *Penicillium nalgiovense* contains a variety of compounds, such as alkaloids, flavonoids, terpenoids, and phenolic groups (Ludemann *et al.*, 2010; Shankar, K.P.G and Sathiavelu, 2022). The most abundant of these compounds is 2,3-Butanediol, which has a concentration of more than 85% of the secondary metabolites in the extract (Sabra *et al*., 2011; Mahfur *et al*., 2024). The ethyl acetate extract from the fungal symbiont sponge, which was measured as the yield value on day 10, can be seen in Error! Reference source not found.**.**



(a) (b) (c)

Figure 2. Dry extract of ethyl acetate fungal symbiont *Penicillium nalgiovense* (a) SDB, (b) PDB, and (c) PDB-enriched coconut flakes.

The data in **Figure 2** show that the characteristics of the dry ethyl acetate extract of the symbiotic fungus ranged from yellowish-brown to brown. To determine the effect of fermentation time on the production of bioactive compounds, percentage yield calculations were performed. The yield values for each fermentation time variable (days) and a graph showing the effect of time on yield growth are shown in **Figure 3.**

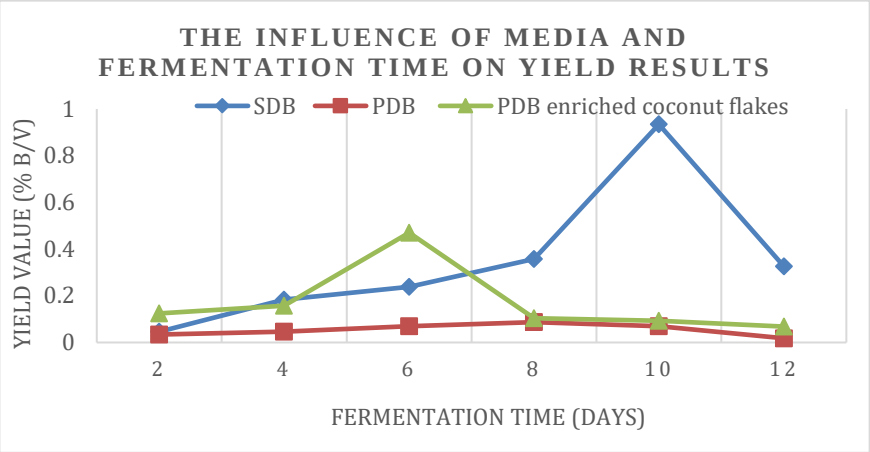


Figure 3. Graphic influence of media and fermentation time (days) one yield value (%b/v)

Based on **Figure 3** showing the effect of media and fermentation time (days) on yield **Figure 2**, differences in the results across the three media used were observed. These differences are attributed to the varying nutrient compositions of the two types of media: SDB and PDB. The SDB media uses peptone, while the PDB media uses potatoes, and the nutritional contents of these two substances differ significantly (Cappuccino and Sherman, 2014). The modified PDB media enriched with coconut flakes contained additional nutrients and vitamins derived from the coconut flakes, which promoted the growth of symbiotic fungus and the production of secondary metabolites.

The fermentation results on SDB media showed a short adaptation phase (lag phase) for the symbiotic fungus, lasting less than two days. By the second day, the symbiotic fungus began to grow (log phase) and produce metabolites, as evidenced by the increased yield. This rapid growth is attributed to the similarity between the new media and the previous one and the maturity of the fermented symbiotic fungus, which had already entered the log phase by the seventh day, allowing quicker adaptation. The log growth phase of the symbiotic fungus on this media occurred from the second to the sixth day. During this phase, secondary metabolite production increased, as indicated by the rising yield. The symbiotic fungus utilizes peptone and sugar nutrients in the media for growth. The stationary phase occurred from the sixth to the tenth day, during which the growth of the symbiotic fungus and the metabolites produced peaked and then remained relatively constant. During this phase, the nutrient sources in the media were nearly depleted, limiting further growth. However, growth did not immediately cease because cell lysis occurred in the dead symbiotic fungal cells, which served as an alternative nutrient source.

Fermentation entered the final phase of symbiotic fungal growth, the death phase, after the tenth day, as shown by the decrease in yield on the 12th day. The optimal fermentation time for the symbiotic fungus *Penicillium nalgiovense* in the SDB media occurred on the tenth day. During the stationary phase, the fermentation media appeared yellowish and cloudy, whereas the mycelium was bone-white and settled at the bottom of the media. The optimal yield percentage on the tenth day reached 0.934% b/v.

The growth of the symbiotic fungus on PDB media also experienced a lag phase lasting less than two days, with the log phase starting from the second day to the sixth day. However, the stationary phase for the symbiotic fungus on this media was shorter, occurring from the sixth to the eighth day, whereas the death phase began more quickly than on SDB media by the tenth day. This difference was attributed to the variation in carbon sources as nutrients. The carbon source in PDB media is potatoes, which contain polysaccharides that support fungal growth more readily than SDB media. The optimal fermentation time for the

symbiotic fungus *Penicillium nalgiovense* in PDB media was observed on the eighth day. During the stationary phase, the fermentation media appeared yellowish-brown, with bone-white mycelium located at the top and center of the media. The optimal yield percentage on the eighth day reached 0.087% b/v.

The modified PDB media enriched with coconut flakes showed a pattern nearly identical to that of the PDB media. The lag phase lasted for less than two days. However, the log, stationary, and death phases occurred more rapidly. The log phase occurred from the second to the fourth day, followed by the stationary phase from the fourth to the sixth day. The death phase began on the eighth day. This acceleration was due to the abundance of carbon sources as nutrients, which originated not only from the media but also from coconut flake modification. This indicates that the addition of coconut flakes provides extra nutrients and sugars for the growth of symbiotic fungus. Additionally, the abundance of nutrients impacts the faster growth and death phases. The optimal fermentation time for the symbiotic fungus in the modified PDB media enriched with coconut flakes occurred on the sixth day. During the stationary phase, the fermentation media appeared yellowish, cloudy, and thick, with bone-white mycelium that had begun to dissolve, and clumps of coconut milk formed at the surface of the media. The optimal yield percentage on the eighth day reached 0.47% b/v.

CONCLUSION

The fermentation biotechnology of the symbiotic fungus *Penicillium nalgiovense* acc MK087096, from *Gelliodes fibulata* sponge in SDB, PDB, and PDB-enriched coconut flake media, demonstrates the ability to grow and produce bioactive secondary metabolites. However, the optimal medium for the growth of the symbiotic fungus is SDB medium, while the optimal fermentation time for harvesting secondary metabolites is on the tenth day, with a yield percentage of 0.934% b/v. Therefore, SDB medium with a fermentation time of 10 days can be used to effectively grow the fungus and produce secondary metabolites with antibiotic properties to address the limitations and sustainability of the active ingredient supply derived from *Gelliodes fibulata* sponge in the production of pharmaceutical formulations.

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