Action Mechanism of Marine Endophytic Fungi *Aspergillus terreus* as Antibacterial Agent Against *Vibrio harveyi*

Ismi Isti'anah[1,2,3], *Muhammad Arief* Budiman[1], *Sugeng Heri* Suseno[1], *Roni* Nugraha[1], *Irzal* Effendi[2,4], and *Kustiariyah* Tarman[1,2,*]

[1]Department of Aquatic Product Technology, Faculty of Fisheries and Marine Science, IPB University, Jl. Agatis, 16680 Bogor, Indonesia
[2]Center for Coastal and Marine Resource Studies, IPB University, Jl. Pajajaran Raya, 16127 Bogor, Indonesia
[3]Department of Marine Biotechnology, Tual State Fisheries Polytechnic, Jl. Raya Langgur, 97611 Maluku, Indonesia
[4]Department of Aquaculture, Faculty of Fisheries and Marine Science, IPB University, Faculty of Fisheries and Marine Science, IPB University, Jl. Agatis, 16680 Bogor, Jawa Barat, Indonesia

Abstract. Mangrove ecosystems are among the most diverse marine environments, housing a variety of vegetation, such as shrubs, plants, trees, palms, and other adaptable species that thrive in both freshwater and saline conditions. These mangrove habitats serve as a rich source of secondary metabolites, contributing to various pharmacological activities and playing a crucial ecological role. Endophytic fungi constitute a substantial and measurable portion of fungal biodiversity, known to influence plant community structure and diversity. The purpose of this study is to evaluate the cytotoxic and antibacterial qualities of endophytic fungi that were isolated from Sonneratia alba mangrove leaves that were gathered in Buton, Southeast Sulawesi, Indonesia. The fungi were isolated using surface sterilization techniques and identified based on their morphological characteristics and ITS regions of rDNA. Isolate WB 1-2, obtained from the leaves, was identified as *Aspergillus terreus*. The marine endophytic isolate, *Aspergillus terreus*, exhibited antibacterial activity against *Vibrio harveyi*. Scanning Electron Microscope observations conducted after 24 hours revealed inhibited cell growth in *Vibrio harveyi* test cells, indicating damage to the cells caused by the hyphae of marine endophytic fungi.

1 Introduction

Mangroves are essential to preserving biodiversity and ecological equilibrium. These distinct ecosystems flourish in the wetlands that cross the border between freshwater and saltwater habitats along tropical and subtropical coastlines. Mangrove forests are globally significant because, remarkably, 24 percent of them are found in Indonesia [1]. Mangroves span 3,836.6

* Corresponding Author: kustiaz@apps.ipb.ac.id

hectares and are found throughout the coastal districts of the Buton Regency. Wabula is the district with the most area of mangrove forests—49.4 hectares—among these districts. Notably, four distinct mangrove species—*Rhizophora mucronata*, *Sonneratia alba*, *Rhizophora apiculata*, and *Avicennia* sp.—can be found in Buton's mangrove forests. These mangroves perform a number of vital tasks, including harboring marine life, storing carbon, and creating chances for ecotourism [2].

The antibacterial properties of *Sonneratia alba* are possible. According to a prior study, 69% of the isolated chemicals showed antibacterial activity against *Streptococcus*, *Candida albicans*, and other pathogenic bacteria and fungi. Accordingly, it is possible to utilize the endophytic fungus present in *Sonneratia alba* as a unique source of antibacterial, anticancer, and antibiotic chemicals [3]. *S. alba* extract is shown to be most efficient at 1% concentration when used to inactivate the White Spot Syndrome Virus (WSSV). The death rate of tiger shrimp is greatly decreased by this concentration [4]. The treatment with the highest mean survival rate for tiger shrimp was the one that used *S. alba* diethyl ether extract [5]. Compared to other therapies, the administration of 250 ppm of *Avicennia alba* leaf extract is more successful in treating vannamei shrimp infected with *V. harveyi [6].*

In order to survive in harsh circumstances, marine fungi produce secondary metabolites, which sets off a stress tolerance response [7]. The potential of naturally occurring biologically active chemicals has been thoroughly investigated, especially those that may interact with molecular targets in an efficient manner. Marine Natural Products (MNPs) have demonstrated several notable bioactivities, such as antimicrobial, antiviral, neuroprotective, anti-inflammatory, and anticancer properties [8]. The capacity of polysaccharides to alter the microbiota in the digestive system and impact the absorption of nutrients and chemicals makes them one of these substances that are regarded as nutraceuticals [9]. These processes are thought to be significantly influenced by marine microorganisms, including amino acid providers, bacteria, fungus, microalgae, and marine polysaccharides [10].

The utilization of marine fungus extracts containing natural antibacterial agents as immunostimulants during the fermentation process of aquaculture feed has the potential to improve disease resistance. Immunomodulators have been shown to have a significant therapeutic benefit [11]. Research efforts must be sustained in order to find safe, effective immunotherapy for a variety of diseases and to ensure that it can be scaled up within the best possible medication delivery system. The purpose of this research is to clarify the mode of action of the marine endophytic fungus *Aspergillus terreus* against the pathogenic bacteria *Vibrio harveyi* as an antibacterial agent.

2 Materials and methods

2.1 Isolation and selection of marine endophytic fungi samples

Mangrove leaf samples were collected from Buton Island, Southeast Sulawesi Province, labeled, and initially preserved by air-drying. These samples were subsequently transported to the laboratory for the isolation of endophytic fungi. A total of 120 samples were collected from this location, comprising both seaweed and mangrove samples. These samples were subsequently subjected to isolation to obtain marine endophytic fungi. The isolation of marine endophytic fungi employed surface sterilization methods. Surface sterilization of the samples was carried out using two methods: immersion in distilled water for 1 minute and treatment with 5% Sodium Hypochlorite (NaOCl) to remove adhering impurities. The samples were then aseptically placed on Potato Dextrose Agar (PDA) medium, with 4 to 5 pieces in each Petri dish. The Petri dishes containing the samples were incubated at room

temperature for approximately 3 to 7 days. Multiple transfers of mycelium to fresh growth media were conducted during the isolation process to obtain pure isolates. Morphological characteristics of the growing fungi colonies were observed. If the colonies were not pure, purification was conducted. Selected representative colonies were then transferred to slant agar media for storage [12].

2.2 Characterization of marine endophytic fungal samples

The isolated marine endophytic fungi were characterized based on macroscopic morphological features on PDA medium in Petri dishes. Macroscopic characteristics of the fungi included colony shape, colony color, and morphological characteristics of the colonies. Additionally, molecular methods, using the ITS region specific to marine endophytic fungi, were employed for identification at the IPB Culture Collection (IPBCC) Laboratory [13].

2.3 Antagonistic testing of marine endophytic fungi against pathogenic bacteria *Vibrio harveyi*

Antagonistic testing of selected fungal isolates against the pathogenic bacteria *Vibrio harveyi* was performed using the pour plate method. A 10 ml suspension of 24-hour-old *Vibrio harveyi* in NB medium was aseptically mixed with sterilized NA medium in 250 ml Erlenmeyer flasks. Subsequently, the NA medium mixed with *Vibrio harveyi* suspension was poured aseptically into Petri dishes. Marine endophytic fungi with a 1 cm diameter were prepared. Once the NA medium solidified in the Petri dishes, the marine endophytic fungi were placed on the surface of the medium, which had been previously inoculated with *Vibrio harveyi*. The test Petri dishes were then incubated at 37 °C for 24 hours, and the diameter of the clear zones formed was measured using calipers. The *Vibrio harveyi* inoculated into the NA medium served as a control, and the testing was conducted with three replications [14].

2.4 Action mechanism of marine endophytic fungi against pathogenic bacteria *Vibrio harveyi*

The determination of the mechanism of action of marine endophytic fungi against the pathogenic bacteria *Vibrio harveyi* was carried out through visualization using a Scanning Electron Microscope to study the morphological damage to *V. harveyi* cells caused by the selected immunostimulant candidate isolates. Suspension of bacteria was contacted with marine fungal isolates for 24 hours, followed by preparation for observation under the microscope [15].

3 Results and discussions

The sampling location was Buton Island, Southeast Sulawesi, chosen for its high biodiversity. In total, 160 pure isolates were obtained, categorized as 7 isolates with the code WB, 32 isolates with the code SM, 60 isolates with the code LB, 59 isolates with the code BM, and 2 isolates with the code KS (Table 1).

Marine endophytic fungi can be isolated from aquatic environments (the sea), but their life cycle is not dependent on aquatic environments, making them easily cultivable on a laboratory scale [16]. A total of 160 marine endophytic fungi isolates obtained from the isolation process then underwent the subsequent stage, which is the selection process. The selection process was carried out to ensure that the obtained isolates are the endophytic marine fungi are elite marine endophytic fungi with optimal antibacterial capabilities.

Moreover, the bioactivity of natural compounds derived from aquatic biomaterials is significantly higher compared to compounds produced by terrestrial organisms [17].

Table 1. The marine endophytic fungal isolates obtained from Buton Island, Southeast Sulawesi.

Isolat Code	Number of Samples
WB	7
SM	32
LB	60
BM	59
KS	2
Total	**160**

Marine endophytic fungi represent a recent source of bioactive compounds that can function as antimicrobials, antioxidants, anticancer agents, and participate in biotransformation processes [18]. The selection process was conducted on the 160 samples using selection and purification methods, resulting in 35 isolates that were subsequently subjected to the next stage because another isolates are not endophytic fungi which involved antagonistic testing against *Vibrio harveyi* bacteria. *Vibrio harveyi* is a common pathogen found in aquatic environments. The results of the antagonistic testing of selected marine endophytic fungal isolates can be seen in Figure 1.

Figure 1 presents the results of antibacterial activity testing for 35 selected endophytic fungal isolates, all of which exhibited antibacterial activity against *Vibrio harveyi* bacteria. The antibacterial activity was indicated by the presence of clear or hazy zones. The formation of clear zones indicated antibacterial activity, and the inhibitory zone categories were determined based on the diameter size of the clear zones formed. Hazy zones indicated antibacterial activity, but it was relatively weak. Isolates WB 1-2, WB 6-2, WB 7-1, SM 22-1, SM 27-2, BM-46, and LB 13-A showed activity against *Vibrio harveyi* bacteria with inhibitory zone diameters of 13.0 mm, 12.3 mm, 12.3 mm, 11 mm, 11 mm, and 11.3 mm, demonstrating strong antibacterial activity. These findings align with prior research, wherein the marine fungus *Nodulisporium* sp. KT29 was shown to enhance inhibition zones ranging from 9.3 to 13 mm [20].

The isolate with the highest inhibition zone is isolate WB 1-2. Isolate WB 1-2 was identified as the species *Aspergillus terreus* ATCC 1012 (Figure 2). Other studies have reported that the antagonistic effect of the marine endophytic fungi *Aspergillus terreus* SHE05 against the pathogenic bacterium *Aeromonas hydrophila* in fish yielded the highest antibacterial activity of approximately 14 mm [21]. The marine endophytic fungi species *L. pseudotheobromae* IBRL OS64 also exhibited antibacterial activity against pathogenic bacteria in aquaculture, namely *V. owensii* CCB-PG2 and *V. azureus* CCB-ST2H16, with inhibition zone diameters of less than 10 mm [22].

Observations regarding the mechanism of action of selected marine endophytic fungi were conducted at iLab National Research and Innovation Agency (BRIN), utilizing the Scanning Electron Microscope. The Scanning Electron Microscope was employed for observing the mechanism of action at varying magnifications, ranging up to 50000x. Control treatment observations revealed normal morphological conditions of *Vibrio harveyi* cells in culture, displaying oval-shaped and colonizing characteristics (Figure 3).

Test cultures from marine endophytic fungi isolates were used at concentrations of 1%, 3%, 5%, and 10%. Results indicated alterations in the morphology of test cells due to treatment with isolate WB 1-2 broth culture. Figure 3 showcases the morphology following treatment with WB 1-2 isolate culture 1% at a magnification of 2000x (Figure 4). Morphological observations after 24 hours revealed inhibited cell growth attributed to

damage to the *Vibrio harveyi* test cells. Figure 4 also illustrates *Vibrio harveyi* bacterial cells morphology had been changed.

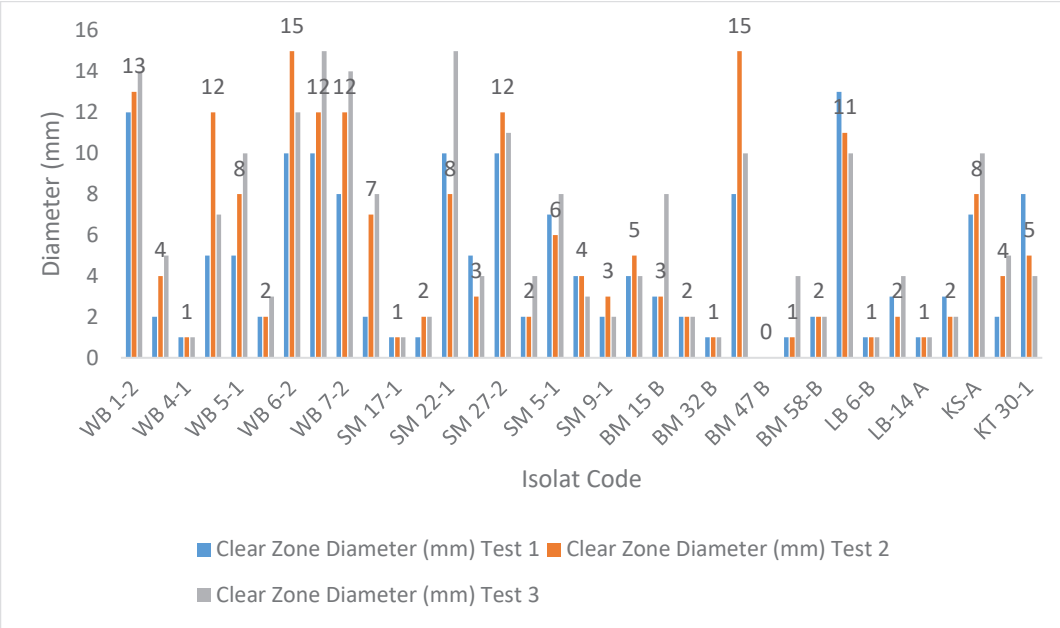

Fig. 1. Marine endophytic fungi antagonism test results with *Vibrio harveyi.*

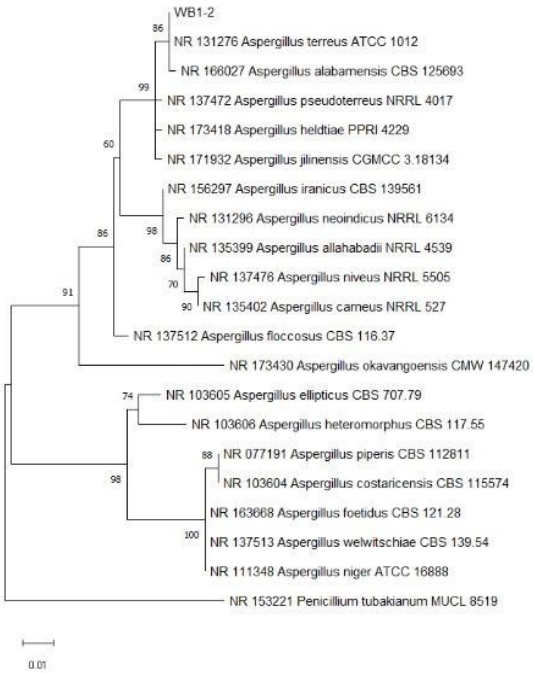

Fig. 2. The phylogenetic tree of WB 1-2 isolates as selected marine endophytic fungi.

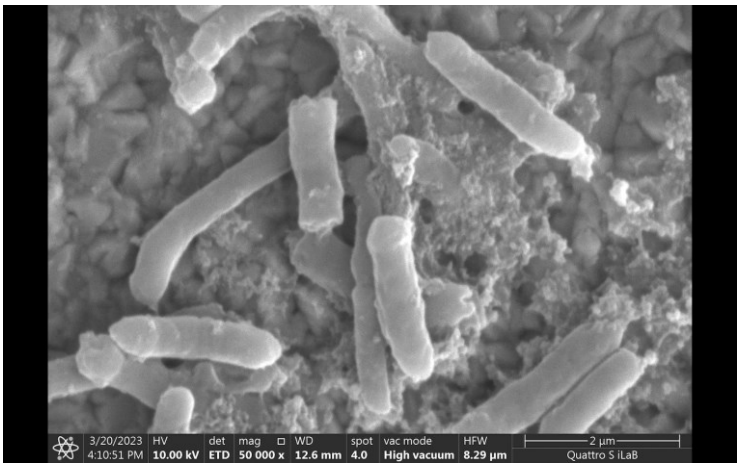

Fig. 3. Visualization of the control treatment (*Vibrio harveyi* colony).

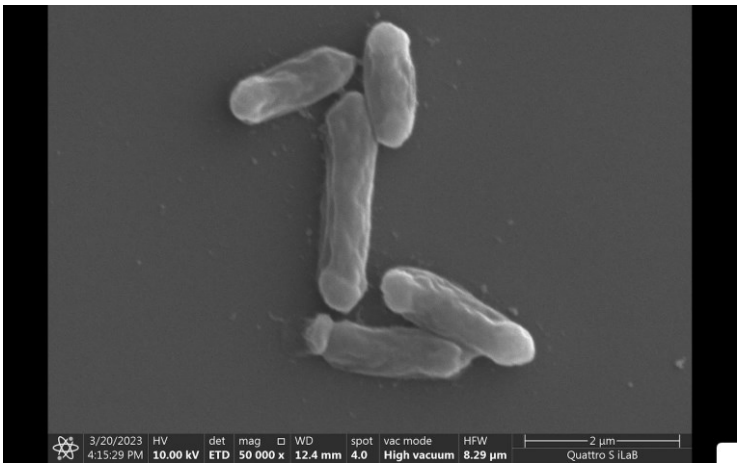

Fig. 4. Visualization of 1% culture treatment (cultural treatment of marine fungi isolates and *Vibrio harveyi* bacterial colonies).

Figure 5 illustrates changes in morphology and the reduction in the number of *Vibrio harveyi* cells treated with a 3% culture of marine endophytic fungi. Visualization of the mechanism of action indicated a decrease in the number of pathogenic *Vibrio* bacterial colonies. This was further corroborated by the significantly reduced luminescence of *Vibrio harveyi* compared to the control treatment.

At a 5% culture concentration, changes in test cell morphology became nearly imperceptible as they were enveloped by fungal culture (broth). Higher treatment concentrations with endophytic marine fungi isolate culture resulted in fewer visible normal bacterial cells (Figure 6). Based on bioactivity results, the culture exhibited cytostatic properties [26]. Culture broth derived from marine endophytic isolate samples led to a notable reduction in the number of test cells compared to control cells (Figure 6).

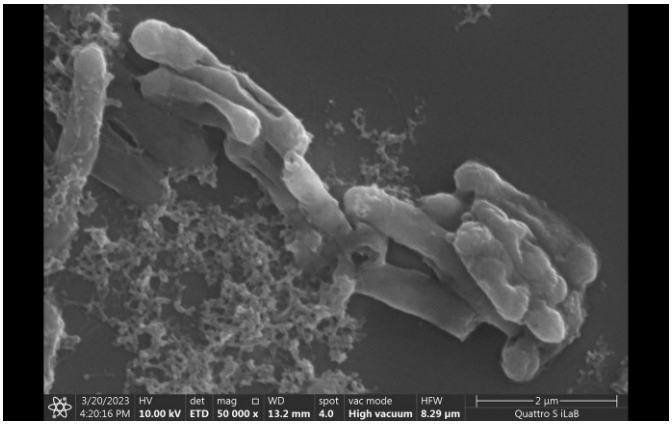

Fig. 5. Visualization of 3% culture treatment (cultural treatment of marine fungi isolates and *Vibrio harveyi* bacterial colonies).

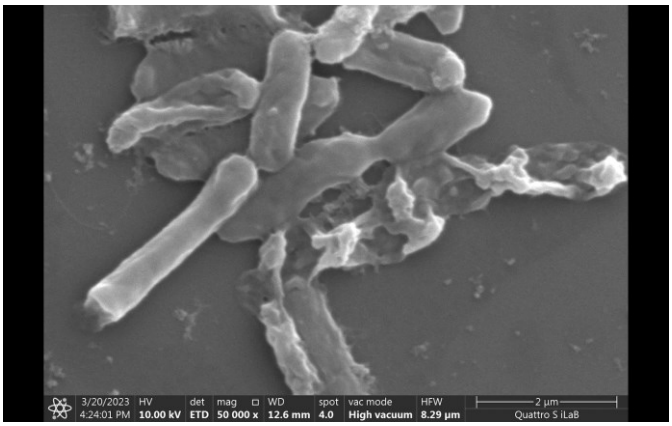

Fig. 6. Visualization of 5% culture treatment (cultural treatment of marine fungi isolates and *Vibrio harveyi* bacterial colonies).

Additionally, damage to the cell plasma membrane (shrunken, ruptured, and grainy) was observed, along with changes in cell shape (rounded, ballooned). Visually, it can be deduced that the culture of marine endophytic fungi isolates effectively eliminated a majority of *Vibrio harveyi* cells at concentrations exceeding 5%. Figure 7 depicts increasingly elongated fungal hyphae with only a few remaining colonies of pathogenic bacteria *Vibrio harveyi*. Isolates of marine endophytic fungi are suggested to possess immunostimulating capabilities through their antibacterial compounds. This aligns with previous research on the antibacterial compound questin, isolated from the marine endophytic fungi *A. flavipes* strain HN4-13. Questin's antibacterial properties can also be employed against the aquatic pathogen *V. harveyi*. Mechanism of action studies revealed that questin affects the permeability and integrity of cell walls and membranes by binding to the cell surface, leading to bacterial cell wall and membrane damage, ultimately resulting in an unstable bacterial intracellular environment, cell lysis, and cell death [23].

One of the extracellular polysaccharide (EPS) substances secreted by certain fungi is β-glucan. These natural polymers play roles in host adhesion, cellular defense against external threats, and carbohydrate storage. EPS production may be linked to an organism's pathogenicity toward plant and animal hosts. Seaweed was used as the host tissue for the marine endophytic fungus in this study. Furthermore, chemically modified beta-glucans,

such as phosphorylated compounds, exhibit anti-inflammatory, anti-proliferative, and antiviral properties [24].

Hence, β-glucan could serve as a method for disrupting harmful bacteria cells, such as *Vibrio harveyi*. Research into EPS derivation, purification, and unique characterization is essential for comprehending their roles in various processes and applications in the food or pharmaceutical industries. In-depth data analysis concerning EPS derivation and application will facilitate the development and utilization of Ascomycota and Basidiomycota EPSs across various industrial, agricultural, and medical sectors [25].

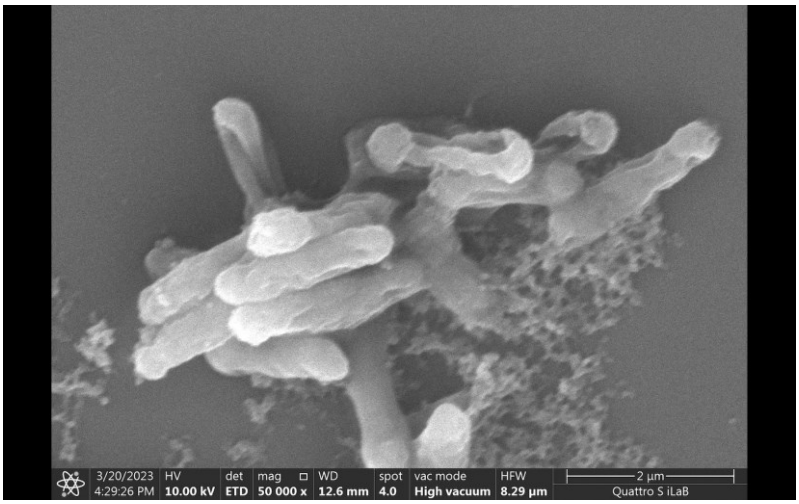

Fig. 7. Visualization of 10% culture treatment (culture treatment of marine endophytic fungi and *Vibrio harveyi* bacterial colonies).

4 Conclusions

Isolate WB 1-2, derived from *Sonneratia alba* mangrove leaves, was identified as *Aspergillus terreus*. The marine endophytic isolate *Aspergillus terreus* exhibited antibacterial activity against *Vibrio harveyi* to inhibit cell growth due to damage to the *Vibrio harveyi* test cells.

The authors would like to extend their gratitude to the Ministry of Education, Culture, Research, and Technology for the funding provided through the Doctoral Dissertation Research Scientific Research Program, in the name of Dr. Kustiariyah, S.Pi, M.Si.

References

1. S.H. Andarias, L. Aba, F.I. Taharu, F.I. Jumiati, J. Yanti, Y. Badaraf, J. Jamaludin, J. Masy. *Mandiri* **7**, 4, (2023).
2. T. Mustari, A. Tenri, S. Ramadan, L. O. A. Munafi, N. Al-Kutub Indones, **60** (2023).
3. D. Handayani, H. Rivai, R. Mulyana, N. Suharti, R. Rasyid, & T. Hertiani. J. of Appl. Pharm. Sci., **8**, 2, (2018).
4. Muliani et al IOP Conf. Ser.: Earth Environ. Sci. 1119 012042 (2022).
5. Muliani et al IOP Conf. Ser.: Earth Environ. Sci. 564 012054 (2020).
6. D. Rusadi, W. Wardiyanto, R. Diantari, e-J. Rek. dan Tek. Bud. Perairan, **8**, 1, (2019).
7. S. Sahoo, K. Subban, J. Chelliah J, Front. Microbiol. **12**, 650177, (2021).

8. A. Karthikeyan, A. Joseph, B.G. Nair, J. of Gen. Eng.. and Biotechnol**. 20**(14), 1-38 (2022).
9. Y. Tang, Y. Cui, A.D. Agostini, L. Zhang, Prog. in Mol. Biol. and Trans. Sci. **163,** 445–469, (2019).
10. I. Ahmed, M. Asgher, F. Sher, S.M. Hussain, N. Nazish, N. Joshi, A. Sharma, R. Parra-Saldívar, M. Bilal, H.M.N. Iqbal, Mar. Drugs. **20**, 208, (2022).
11. I. Bricknell I, R.A. Dalmo. Fish & Shellfish Immunol. **19**, 457-472, (2005).
12. Sopalun, K., Laosripaiboon, W., Wachirachaikarn, A., & Iamtham, S. South African J. of Bot., **141**, 66-76, (2021).
13. Noverita, D. Fitria, E. Sinaga. *Jurnal Farmasi Indonesia*. **4**, 4, 171- 176 (2009).
14. Vurukonda, S. S. K. P., Giovanardi, D., & Stefani, E, *Int. J. of Mol. Sci*, **19**, 4, 952. (2018).
15. Wahjuningrum, D., Hariati, S., Yuhana, M., Effendi, I., Citarasu, T., Utami, D. A. S., & Tarman, K, Front. Microbiol. **13**, 837586, (2022).
16. B. Ahmad, M. Shah, S. Choi. Mar. Drugs. **17**, 282, 1-37, (2019).
17. A.M. Abdel-Azeem, F.M. Salem, M.A. Abdel-Azeem, N.A. Nafady, M.T. Mohesien, E.A. Soliman, New and future developments in microbial biotechnology and bioengineering (Elsevier BV, Amsterdam, 2016).
18. S.H. Wahyuni, Hasanuddin, E. Purba. J. Per. Trop., **3**, 1, 31-42, (2016).
19. D. Wahjuningrum, T.R. Efianda, K. Tarman, M. Yuhana, I. Effendi, & F. Saputra. J. Akua. Indones., **19**, 2, 95-105, (2020).
20. H.H.A El-latif, S.W.M. Hassan, E.A. Beltagy J. Pure Appl. Microbiol. **15**, 4, 2367-2381, (2021)
21. M.T.M. Jalil, N.A. Zakaria, N.S.M.Suhaimi, & D. Ibrahim. Iranian J. of Microbiol, **14**, 3, 383-394 (2022).
22. L. Guo, F. Zhang, X. Wang, H. Chen, Q. Wang, G. Qianqian, C. Jiacai, W. Xi, L. Wang, Biotech*,* **9**, 1, 14*,* (2019).
23. Philippini, R. R., Martiniano, S. E., dos Santos, J. C., da Silva, S. S., & Chandel, A. K, *Bioprocessing for biomolecules production* (Wiley Online Library, New Jersey, 2019).
24. M. Osińska-Jaroszuk, A. Jarosz-Wilkołazka, J. Jaroszuk-Ściseł, K. Szałapata, A. Nowak, M. Jaszek, E. Ozimek, M. Majewska, World J. of Microbiol. and Biotechnol., **31**, 12, 1823–1844, (2015).
25. Kumari M, Taritla S, Sharma A, Jayabaskaran C*,* Front. in Microbiol, **9**, 1777, (2018).