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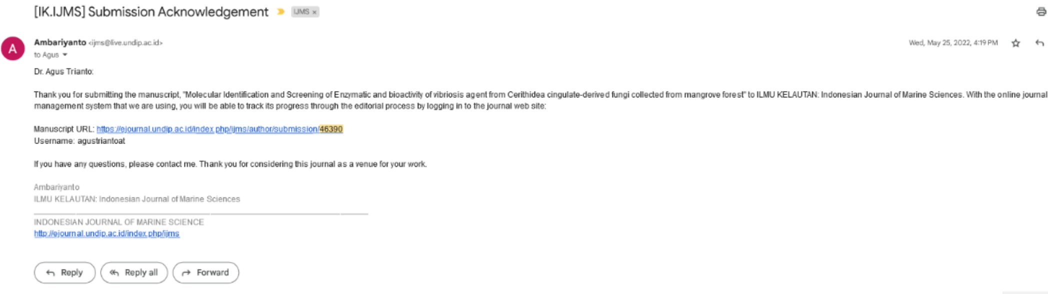

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Manuscript

Molecular Identification and Screening of Enzymatic and bioactivity of vibriosis agent from Cerithidea cingulate-derived fungi collected from mangrove forest

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Abstract

Mangrove ecosystems have a very broad biotechnological potential, including the microorganisms associated with them. This study aimed to explore the enzymatic and anti-vibrio activity of associated fungi from marine gastropods *C. cingulata*. The sample of mangroves was collected from the mangrove ecosystem (site sampling) in North Sulawesi, Indonesia. The fungi were inoculated on malt extract agar (MEA) using tapping method. The enzymatic and anti-vibrio assay were conducted by using the plug method that the activities were determined by the clear zone formed around the isolates. We had isolated a total 13 fungi from the gastropods *C. Cingulata* and *L. scabra.* Among them, the fungal isolate 19MB-C5-3 has the highest cellulolytic, proteolytic, and anti-vibrio activity (*Vibrio parahaemolyticus*, *V. harveyi* , *V. vulnificus*, and *V. alginolyticus*). Molecular identification by using universal primer ITS1 and ITS4 showed that isolate 19MB-C5-3 was closely related to *Aspergillus niger* with a similarity value was 99%..

Keywords: *C. cingulata*, Enzim, Fungi, Mangrove, Vibriosis

Introduction

Mangrove is a complex, unique, and dynamics ecosystem. The organisms living the ecosystem have special physiological adaptations to salinity (Gunawan et al., 2017). Having an open ecosystem system, mangroves have material arrangements that are influenced by marine physical factors such as tides and ocean currents, biological factors that are habitats such as shrimp and gastropods as well as chemical factors in controlling organic and inorganic materials (Sukardjo, 2004). Mangrove ecosystem have shown high biotechnological productivity and biological biodiversity both in tropical and sub-tropical areas (Kamruzzaman et al., 2017).
Gastropods are the dominant invertebrates in mangrove communities which also have an important role in ecological cycles and food webs by helping to provide nutrients for microbes and detrivores through mangrove litter (Kabir et al., 2014; Rahmawati et al., 2015). Gastropods have a wide distribution in several ecosystems in the indo-pacific including *C. cingulata*. There is a close relationship between marine organisms and symbiotic microorganisms in producing secondary metabolites. Proksch et al., (2003) stated the discovery of the same active compounds between marine invertebrates including gastropods and their symbiotic bacteria. Several studies have found benefits in organisms associated with gastropods such as antibacterial (Pringgenies, 2010), anti-drug-resistant bacteria (Bahry, Pringgenies & Trianto, 2017), and biofilm (Viju et al., 2020).
Mangrove fungi are known to be rich sources of enzymes and secondary metabolites such as antibacterial compounds that beneficial aquaculture. Aquaculture produce the organic and antibiotic waste that reducing the water quality and give a bad impact to the ecosystem (González-Gaya et al., 2021). Among the bacteria, genus Vibrio is main cause of various deceases on shrimp and fishes aquaculture over worldwide that causes considerable economic losses of US $ 9 billions annually (Novriadi, 2016). Application of microorganisms as biocontrol for reducing the pathogenic bacteria is an alternative methods as substitution of antibiotic. Proteases can be used to improve feed quality by reducing the size of protein and carbohydrate molecules to increase digestibility (Maytorena-Verdugo, Córdova-Murueta & García-Carreño, 2017).
The need for feed will continue to increase along with the increasing shrimp production through market demand. The biggest problem in the vannamei shrimp cultivation process is that 40-60% of the total production cost is allocated for feed, while the efficiency of feed absorption is not optimal (Olmos et al., 2011). This is due to the fact that vannamei shrimp is a carnivorous animal that does not easily digest vegetable protein, while the carbohydrates in feed are only absorbed 20% because it is not the main energy source (Kurniawan et al., 2019). Excess nutrition will cause problems because it requires more energy and a long digestion period to hydrolyze protein, fat and carbohydrate bonds (Rachmawati et al., 2020). On the other hand, improper pond management causes poor water quality that leads to vibriosis disease which can cause mass mortality in cultured shrimp and environmental pollution (Kusumaningrum & Zainuri, 2015; Sarjito et al., 2018)
In this paper we will discuss on the isolation of the fungi from gastropods, proteolytic, cellulolytic, and anti-vibrio activities screening, and the active fungus identification.

Materials and Methods

Gastropods collection and fungi isolation.

Gastropod samples were collected from three different mangrove ecosystem in North Sulawesi as shown in Figure 1. All laboratory activities are carried out in the marine natural product laboratory (MNP LAB) - Center of Research and Services (Cores-DU). The pathogenic bacteria in this study were a collection of MNP LAB.
The gastropod sample preparation method refers to Bahry et al, (2017) which has been modified by sterilizing the gastropod surface using 70% alcohol. The gastropod tissue rinsed with sterile seawater was then taped on malt extract agar (MEA) media (MERK, USA) in a Petri dish with three repetitions. Isolation media was supplemented with 100 mg/L of chloramphenicol to avoid bacterial contamination (Cristianawati et al., 2019). The petri dishes contain the samples were incubated at room temperature for seven days. All of the steps were conducted aseptically.

Fungal characterization and purification.
Fungi will be classified based on the characterization seen on the seventh day of incubation. Characterized macroscopically method Tarman, Safitri & Setyaningsih., (2013) was used to characterize fungal growth. Selected fungal isolates were purified on the sterile MEA and stored in an incubator for 7 days.

Screening anti-vibrio activity
The screening was carried out using the diffusion agar plug method based on Sibero et al., (2018). Vibrio strains of pathogenic bacteria (*V. harveyi*, *V. vulnificus,* and *V. parahaemolyticus*) were inoculated in nutrient broth media and then shaken. After incubation for 24 hours, the test bacteria were diluted to a turbidity of 0.5 McFarland and inoculated on trypticase soy agar (TSA) media. Mushrooms that were aged for 7 days on MEA media were placed on the surface of TSA media and incubated (24 hours, 32ºC). The clear zone formed indicates the presence of anti-vibriosis activity.

Screening of Protease activity
The activity of the protease enzyme was tested qualitatively based on the formation of a clear zone around the isolate grown on MEA media enriched with skim milk. The enzymatic index (EI) was determined by the relationship between the mean diameter of the clear zone and the mean diameter of the inoculated colonies (Parveen et al., 2017). The protease activity test was carried out using the spot inoculation/plate assay technique on MEA media enriched with skim milk. Mushrooms aged 5 days were cut into circles of 5 mm using a needle loop and inoculated on MEA media enriched with skim milk aseptically and wrapped around the edges of the petri dish with wrap. Mushrooms were incubated and observed at 1x 24 hours and 2x 24 hours. The results of protease activity appeared with clear zones between fungal inoculations (Cherkupally, Amballa & Bhoomi, 2017).

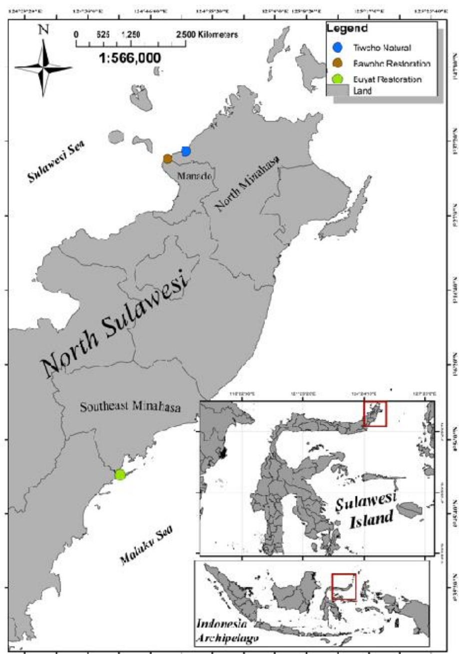

Figure 1. The gastropod samples collection sites in mangrove ecosystems in North Sulawesi, Indonesia.

Screening of cellulase Activity

The collection of pure fungal isolates was regrown on MEA media and incubated at room temperature (25ºC) for 7 days. After 7 days the fungal isolates were prepared for the screening process for the cellulolytic activity. The test medium, namely MEA enriched with Carboxymethyl Cellulose (CMC) as much as 1% fungal isolate, cut into rounds using a sterile blue tip then placed into the test medium, and incubated for 24 hours at 25ºC. The cellulolytic activity was observed by pouring Congo Red solution on the test culture until it was completely submerged for 30 minutes then rinsed with 1 M NaCl. The cellulolytic activity was indicated by the formation of a clear zone around the colony with a pink background (Lusi et al., 2017).

Molecular Identification

The 7-day-old fungal isolates were extracted using the Zymo Research Quick-DNA Fungal/Bacterial Miniprep Kit. Primers ITS 1(forward): 5 "-GGA AGT AAA AGTCGT AAC AAG G-3" and ITS 4 (reverse): 5 "-TCC TCC GCT TAT TGA TATGC-3" were used for DNA amplification (Suharna *et al.*, 2019). 2 l of template DNA was mixed with 2 l of ITS 1 10 M primer 2 l of ITS 4 10 M primer for 2 l, 25 l of PCR mix and 19 l of sterile aquabides (ddH2O) for a total volume of 24 l. The PCR setting refers to Suharna *et al.*, (2019) by preheating at 95ºC for 3 minutes. It was repeated in 34 cycles for denaturation at 95ºC for 1 minute, annealing at 56.1ºC for 1 minute, extension at 72ºC for 1 minute, followed by a final extension at 72ºC for 7 minutes. The Geldoc tool is used as a visualization of the PCR product. Sequence results were traced using the BLAST menu at NCBI, National Institute for Health, United States of America (www.ncbi.nlm.nih.gov). The search results were selected with the highest level of similarity tinggi (Mohan, Thangappanpillai & Ramasamy, 2016). The phylogenetic tree construction was carried out using the MEGA 7 application using the neighbor-joining tree method with 1000x bootstrap. (Kumar, Stecher & Tamura, 2016).

Result and Discussion

Gastropods Collection

Two gastropod (**Figure 2**) species were colected from three different mangrove ecosystem during this research. *Cerithidea cingulate* were collected at Buyat and Bawoho mangrove areas, while Lithorina scabra were collected at Tiwoho mangrove area.

Figure 2. Gastropods A. *C. cingulata.,* B. *L. scabra* collected from the mangrove ecosystem

Mangrove ecosystems can serve as a source of microbial diversity. The total number of sample collection and fungal isolates in different areas indicates that the area in Tiwoho Natural (MT) serves

more samples and related fungi than others (Figure 1). The Tiwoho Natural area shows good varieties according to the 20 hectares of native mangrove forests and biodiversity rehabilitated in 2004. More than 2000 hectares of mangrove growth is good and promotes natural generation (Brown & Djamaluddin, 2017). They are importantly involved in the life cycle of aquatic organisms. Previous research has reported a diversity of mangroves associated with fungi in various parts such as fruit, leaves, pneumatophores, and sediments. They report that the fruit and leaves are the highest amounts. The dominant fungi are Ascomycota and the dothideomycetes class (Lee et al., 2019).
Gastropod *C. Cingulata* has a very wide distribution and is almost found all over the world and distributed in tidal flat areas (Ge et al., 2015). Extensive previous research that *C. Cingulata* is found in mangrove habitats in various regions in Indonesia such as Pemalang (Puryono & Suryanti, 2019), Aceh besar (Irma & Sofyatuddin, 2012), Tanah besar (Nugroho, Soendjoto & Zaini, 2019), and Cilacap (Sugiarto, Suryono & Suprijanto, 2021). This distribution is strengthened by the results of research from Reid et al., (2013) the genus Cerithidea has undergone allopatric diversification in the western Indo-Pacific region.

Isolation dan charcterizition of the fungi
A total of 16 fungal isolates were isolated from mangrove gastropods using MEA media. Each fungus can be characterized based on color, pattern, growth speed, and appearance of filament and spore as stated in table 1.

Table 1. Morfologi gastropods-associated fungal

No	Code	Colour	Growth Pattern	Filament	Spore	Growing
1	19MB-C5-1	Light yellow	Spread	+	-	Medium
2	19MB-C5-2	Pale green	Swarm	-	-	Medium
3	19MB-C5-3	Black	Spread	+	+	Medium
4	19MB-C5-4	Pinkish white	Spread	+	-	Slow
5	19MB-C5-5	White	Spread	+	-	Medium
6	19 MT-07-1	Greyish brown	Spread	-	+	Medium
7	19 MT-07-2	Brown	Spread	-	+	Medium
8	19 MT-07-3	Orange white	Like cotton	+	-	Slow
9	19 MT-07-4	Yellowish green	Spread	-	+	Fast
10	19 MT-18-1	White	Spread	-	+	Medium
11	19 MT-18-2	Grey	Spread	+	-	Slow
12	19 MT-18-3	White	Spread	+	-	Fast
13	19 MT-18-4	White	Spread	+	-	Fast
14	19 Mba-A4-1	White	Spread	-	+	Fast
15	19 Mba-A4-2	White	Spread	+	-	Medium
16	19 Mba-A4-3	Grey	Spread	+	-	Medium

MEA media is the best medium for the fungal isolation process to produce secondary metabolites (Kossuga et al., 2012). Sixteen fungal isolates are found in the selection of this medium as which in table 1. This suggests there is a symbiotic interaction of nausea between fungi and gastropods. Previous research results from Silliman & Newell, (2003) It was discovered that Littorari marine gastropods do farming activities on marsh grass. This is done by eating grass tissue infected by fungi. It is suspected that the same interaction of Cingulata also occurs in mangroves, namely by eating litter from mangrove leaves.

Screening anti-vibrio agent

The screening of the fungi with potential antivibriosis agents were carried out against three Vibrio namely *V. harveyi*, *V. vulnificus*, and *V. parahaemolyticus* with the agar plug diffusion method. Observations for 2x24 hours showed that five isolates are shown in table 2. Among the active isolates, 19MB-C5-3 has the highest potential as anti-vibrio source as indicated by clear zone formed around the bacteria within 24 hours or 48 hours. The isolate 19Mba-A4-2 showed strong activity against *V. harveyi* but did not active against the other vibrio. While the other three isolates have weak activity against the vibrio.

Table 2. Screening of antibacterial activity from *C. cingulata* associated fungal

NO	Isolate Code	*V. harveyi*		*V. vulnificus*		*V. parahaemolyticus*	
		24 h	48 h	24 h	48 h	24 h	48 h
1	19MB-C5-1	+	+	-	-	-	-
2	19MB-C5-2	-	-	-	-	-	-
3	19MB-C5-3	++	++	++	++	++	++
4	19MB-C5-4	-	-	-	-	-	-
5	19MB-C5-5	+	-	-	-	-	-
6	19MT-07-1	-	-	-	-	-	-
7	19MT-07-2	-	-	-	-	-	-
8	19MT-07-3	-	-	-	-	-	-
9	19MT-07-4	-	-	-	-	-	-
10	19MT-18-1	-	-	-	-	-	-
11	19MT-18-2	-	-	-	-	-	-
12	19MT-18-3	+	+	-	-	+	+
13	19MT-18-4	-	-	-	-	-	-
14	19MBa-A4-1	-	-	-	-	-	-
15	19Mba-A4-2	++	++	-	-	-	-
16	19MBa-A4-3	-	-	-	-	-	-

Note: 19MB-C5= *C. cingulata;* 19MT-07= *L. scabra*; 19MT-18= *L. scabra;* 19MBa-A4= *C. cingulata.*

The potency of antibacterial compounds comes from secondary metabolites of microorganisms and is produced when the cell has reached a logarithmic phase that goes into a stationary phase. This phase is called idiophase which occurs due to the limitation of microbial growth nutrients (Okada & Seyedsayamdost, 2017). Antagonism of associated fungi to vibrio strain bacteria is one form of adaptation of other competitor microorganisms. In table 2, isolate 19MB-C5-1, 19MB-C5-3, and 19MB-C5-3 indicate activity on a strain of vibrio bacteria test. In extreme circumstances or pressured by other competitors in maintaining nutrients in the medium, the fungus secretes its secondary metabolites to perform self-defense (Pathak et al., 2012). Secondary metabolites of microorganisms can come from the conversion of primary metabolites designed as a form of chemical defense against other competitors' unfavorable microorganisms (Meenupriya & Thangaraj, 2010).
This is in accordance with Romanenko et al., (2008), That microorganisms that are symbiotic with marine invertebrates produce secondary metabolism which is a bioactive compound that has the potential to be a drug and pharmaceutical preparation. Strengthened by research by Kumar, (2015) found that extracts from C. Cingulata have a good antibacterial effect on human pathogens (*Bacillus cereus, Staphylococcus aureus, Vibrio cholerae, Enterobacter aerogenes,* and *Klebsiella pneumoniae*) and fish pathogen bacteria (*Proteus mirabilis, Aeromonas hydrophila, Serratia marcescens, Aeromonas formicans* and *Micrococcus* sp).

Screening of Enzymatic Activity

The cellulolytic and proteolytic assay showed four out of 13 isolates able to degraded both cellulose and protein. The active isolates are 19MB-C5-3, 19MT-18-1, 19MBa-A4-1, and 19MBa-A4-3. Among them isolate 19MB-C5-3 exhibited strong activity to the both cellulose and protein (Table 3).

Table 3. Screening of enzymatic activity of fungal isolates from *C. cingulata* and *L. scabra*.

NO	Isolate Code	Cellulolytic		Proteolytic	
		24 h	48 h	24 h	48 h
1	19MB-C5-1	-	-	-	+
2	19MB-C5-2	+	+	-	-
3	19MB-C5-3	+	++	++	++
4	19MB-C5-4	-	-	-	-
5	19MB-C5-5	-	-	+	+
6	19MT-07-1	-	-	-	-
7	19MT-07-2	-	-	-	-
8	19MT-07-3	-	-	-	-
9	19MT-07-4	-	-	-	-
10	19MT-18-1	+	+	+	-
11	19MT-18-2	-	-	-	-
12	19MT-18-3	++	+	-	-
13	19MT-18-4	-	+	-	-
14	19MBa-A4-1	++	++	+	+
15	19MBa-A4-2	-	-	-	-
16	19MBa-A4-3	+	++	+	+

Screening of cellulolytic activity is carried out on MEA media with a CMC substrate of 1%. CMC serves as a source of carbon in cellulase production (Utami et al., 2019). The source of carbon becomes the main factor affecting the production of cellulase in fungi. Cellulase itself is an enzyme that will be expressed by fungal cells in response to the source of carbon present in the medium (Ahmed dan Bibi, 2018). To carry out the production of fungal cellulase requires optimal conditions during the incubation period. Incubation time, temperature, and pH are significant factors in cellulase production by fungi (Ahmed *et al.*, 2018). Fungi use cellulase for their metabolic activity in breaking down cellulose. In addition, the cellulolytic activity produced by the fungus plays a major role in the bio decomposition process (Panchapakesan & Shankar, 2016). Arfi *et al.*, (2013) indicate that the halotolerant mushroom *Pestalotiopsis* sp. Can excrete cellulase even the presence of salt around the mangrove ecosystem can increase the number of cells produced. This is certainly a special attraction for fungi associated with mangroves to further research their potential in producing cellulose.

The presence of a clear zone around the fungal disk colony indicated by the notation (+) in table 3 indicates the occurrence of hydrolysis in the test media indicating the development of enzyme production while the area where the enzyme develops is called the hydrolysis zone (Kamath et al., 2010; Bahry, Radjasa & Trianto, 2021). Protease enzyme production itself is obtained from fungi that utilize nitrogen sources from proteins for their development by using different sources (peptone, tripton, casein, and yeast extracts) (Ahmed, 2018). The mechanism of proteolytic activity is due to the hydrolysis of protein bonds derived from skim milk agar (SMA) media into simpler amino acids. Protease activity can be optimized of them by enriching using MEA media as the results of research by Kamath et al., (2010).

Fungi that have proteolytic activity have the ability to produce protease enzymes secreted into their environment. Proteolytic enzymes serve to hydrolyze protein compounds into oligopeptides, short-chain peptides, and amino acids. The presence of this extracellular protease enzyme is essential for bacterial life because it provides for the need for nitrogen compounds that can be transported into cells. These types of fungi that have the ability to secrete protease enzymes have great potential to be used as a source of aquaculture probiotics, especially in shrimp farming (Bahry, Radjasa & Trianto, 2021; Ooi, Rasit & Abdullah, 2021). Likupang's restored mangroves are abandoned shrimp ponds rich in protein and cellulose from shrimp residues, and uneaten feed. In addition, shrimp farming is the disposal and deposition of shrimp pond waste left in the pond after harvest. It is a high content of uneaten food, rotting plankton, dirt, air debris, and microorganisms (Hopkins, Sandifer & Browdy, 1994)

The most potential isolate 19MB-C5-3 was identified using molecular method. The quality of DNA isolation is visualized using geldoc as in Figure 3.

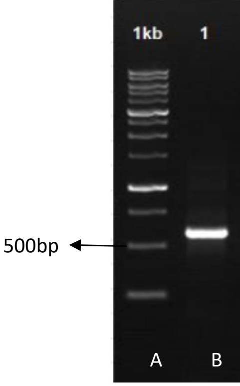

Figure 3. A. DNA ladder, B. DNA template of *C. cingulata* associated fungal isolate19MB-C5-3

The Blast analysis was used to determine the degree of similarity of isolates to other species in the NCBI genebank. The results of the blast analysis is shown in Table 4, isolate19MB-C5-3 of forward and reverse DNA have a sequence length of 954 base pairs and show similarity to Aspergillus niger isolate Strbr with accession number MK372989. The sample similarity rate was 99.40%.

Table 4. Identification of potential *C. cingulata* associated fungal based on BLAST analysis using the ITS region Sponge

Isolate	Sequence length (bp)	Next relative by GenBank alignment (AN, an organism)	Similarity (%)	Family
19MB-C5-3	954	MK372989 *Aspergillus niger* isolate Strbr	99.40	Trichocomaceae

Its primary use for DNA amplification because at ITS there are conservative areas for eukaryotic organisms such as fungi, namely the 18S, 5.8S, and 28S encoder genes (Calado et al., 2019). Eukaryotic organisms have 2 ITS regions with 3 genes that have a fairly high conservancy rate, namely ITS 1 which is located between 18S genes and 5.8S genes, while ITS 2 is located between 5.8S genes and 28S genes. The small subunit rDNA sequence of 18S genes develops relatively slowly making it suitable for use in the study of kinship relationships between species (Mulyatni *et al.*, 2016). ITS 1 primary as forward and ITS 4 as reverse was chosen because the primary was in

its conservative loci region for DNA amplification from fungi (Sibero et al., 2018b). The location of the primary conservative areas of ITS 1 and ITS 4 can be seen in Figure 6. then the amplification results will be visualized with electrophoresis to see the quality of amplification. This shows that its primer successfully amplified the fungal DNA isolate 19MB-C5-3 at a length of ± 500 base pairs for each primer. The combination of forward and reverse DNA results is used to find the best DNA quality.

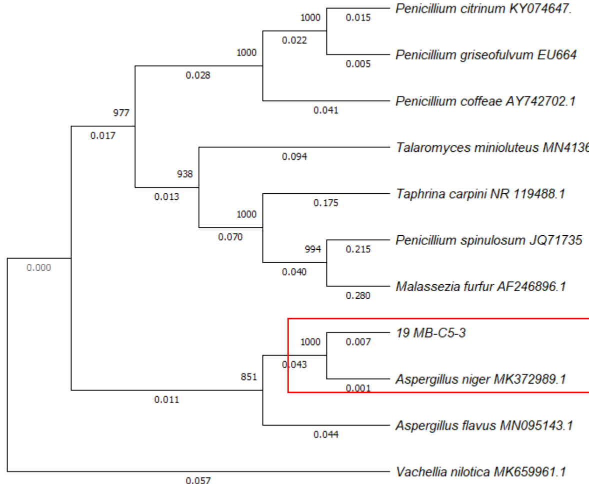

Figure 3. Phylogenetic tree based on the internal transcribed spacer (ITS) regions genes of *C. cingulata* associated fungal 19MB-C5-3

Phylogenetic trees reconstructed using MEGA 7 software using the neighbor-joining tree method with bootstrap 1000x show that C. cingulata associated fungal 19MB-C5-3 grouped one clade with *Aspergillus niger* as shown in figure 3.

The results of molecular identification of fungi that have cellulolytic activity with BLAST (Basic Local Alignment Search Tool) can be seen in Table 4 as well as phylogenetic trees created using ClustalX and Mega 7 software with the Neighbor-Joining Tree approach can be seen in Figure 3. It can be seen that Isolate 19MB-C5-3 has a degree of kinship with the fungus *Aspergillus niger* isolate Strbr MK372989. The number in the branch shows the bootstrap value of the Neighbor-Joining Tree analysis which means that after 1000 repetitions the confidence level of the branch forms a clade in a phylogenetic tree (Li & Wang, 2009).

Conclusion

The gastropods *C. cingulata* is a source of fungi that producing protease, cellulase, and anti-vibrio compounds. The *A. niger* 19MB-C5-3 has potential bioremediation agent in aquaculture application.

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Editor Decision

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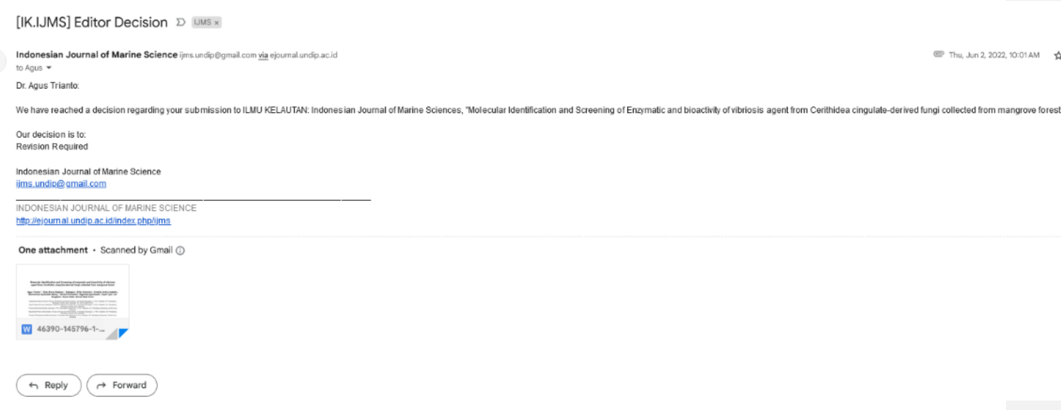
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to Agus

Dr. Agus Trianto:

We have reached a decision regarding your submission to ILMU KELAUTAN: Indonesian Journal of Marine Sciences, "Molecular Identification and Screening of Enzymatic and bioactivity of vibriosis agent from Cerithidea cingulate-derived fungi collected from mangrove forest".

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Reviewers' Comments

Molecular Identification and Screening of enzymatic and bioactivity of vibriosis agent from *Cerithidea cingulate*-derived fungi collected from mangrove forest

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Abstract

Mangrove ecosystem has a very broad biotechnological potential, including the microorganisms associated with them. This study aimed to explore the enzymatic and anti-vibrio activity of associated fungi from marine gastropods *C. cingulata*. The sample of mangroves was collected from the mangrove ecosystem (site sampling) in North Sulawesi, Indonesia. The fungi were inoculated on malt extract agar (MEA) using tapping method. The enzymatic and anti-vibrio assay were conducted by the plug method that. The positive results were determined by the clear zone formed around the isolates. We had isolated a total 13 fungi from the gastropods *C. Cingulata* and *L. scabra.* Among them, the fungal isolate 19MB-C5-3 had the highest cellulolytic, proteolytic, and anti-vibriosis activity (*Vibrio parahaemolyticus*, *V. harveyi* , *V. vulnificus*, and *V. alginolyticus*). Molecular identification by using universal primer ITS1 and ITS4 showed that isolate 19MB-C5-3 was closely related to *Aspergillus niger* with a similarity value was 99%..

Keywords: *C. cingulata*, Enzim, Fungi, Mangrove, Vibriosis

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Commented [A2]: Suggested title: ***Molecular identification and biotechnological potential of Cerithidea cingulata-associated fungi as extracellular enzyme producer and anti-vibriosis agent***

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Introduction

Mangrove is a complex, unique, and dynamics ecosystem. The organisms living the ecosystem have special physiological adaptations to salinity (Gunawan et al., 2017). Having an open ecosystem system, mangroves have material arrangements that are influenced by marine physical factors such as tides and ocean currents, biological factors that are habitats such as shrimp and gastropods as well as chemical factors in controlling organic and inorganic materials (Sukardjo, 2004). Mangrove ecosystem has shown high biotechnological productivity and biological biodiversity both in tropical and sub-tropical areas (Kamruzzaman et al., 2017).
Gastropods are the dominant invertebrates in mangrove communities which also have an important role in ecological cycles and food webs by helping to provide nutrients for microbes and detrivores through mangrove litter (Kabir et al., 2014; Rahmawati et al., 2015). Gastropods have a wide distribution in several ecosystems in the indo-pacific including *Cerithidea cingulata*. There is a close relationship between marine organisms and symbiotic microorganisms in producing secondary metabolites. Proksch et al., (2003) stated the discovery of the same active compounds between marine invertebrates including gastropods and their symbiotic bacteria. Several studies have found benefits in organisms associated with gastropods such as antibacterial (Pringgenies, 2010), anti-drug-resistant bacteria (Bahry, Pringgenies & Trianto, 2017), and biofilm (Viju et al., 2020).
Mangrove fungi are known to be rich sources of enzymes and secondary metabolites such as antibacterial compounds that beneficial aquaculture. Aquaculture produce the organic and antibiotic waste that reducing the water quality and give a bad impact to the ecosystem (González-Gaya et al., 2021). Among the bacteria, genus *Vibrio* is main cause of various deceases on shrimp and fishes aquaculture over worldwide that causes considerable economic losses of US $ 9 billions annually (Novriadi, 2016). Application of microorganisms as biocontrol for reducing the pathogenic bacteria is an alternative method as substitution of antibiotic. Proteases can be used to improve feed quality by reducing the size of protein and carbohydrate molecules to increase digestibility (Maytorena-Verdugo, Córdova-Murueta & García-Carreño, 2017).
The need for feed will continue to increase along with the increasing shrimp production through market demand. The biggest problem in the Vannamei shrimp cultivation process is that 40-60% of the total production cost is allocated for feed, while the efficiency of feed absorption is not optimal (Olmos et al., 2011). This is due to the fact that Vannamei shrimp is a carnivorous animal that does not easily digest vegetable protein, while the carbohydrates in feed are only absorbed 20% because it is not the main energy source (Kurniawan et al., 2019). Excess nutrition will cause problems because it requires more energy and a long digestion period to hydrolyze protein, fat and carbohydrate bonds (Rachmawati et al., 2020). On the other hand, improper pond management causes poor water quality that leads to vibriosis disease which can cause mass mortality in cultured shrimp and environmental pollution (Kusumaningrum & Zainuri, 2015; Sarjito et al., 2018)
In this paper we will discuss on the isolation of the fungi from gastropods, proteolytic, cellulolytic, and anti-vibrio activities screening, and the active fungus identification.

Materials and Methods

Gastropods collection and fungi isolation.

Gastropod samples were collected from three different mangrove ecosystem in North Sulawesi as shown in Figure 1. All laboratory activities are carried out in the marine natural product laboratory (MNP LAB) - Center of Research and Services (Cores-DU). The pathogenic bacteria in this study were the collection of MNP LAB.
The gastropod sample preparation method refers to Bahry et al, (2017) which has been modified by sterilizing the gastropod surface using 70% alcohol. The gastropod tissue was rinsed with sterile seawater, then taped on malt extract agar (MEA) (MERK, USA) with three repetitions. Isolation media was supplemented with 100 mg/L of chloramphenicol to avoid bacterial contamination

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The story line of your introduction is not strong enough.
Why do you use gastropods-associated fungi as the source of your isolates? Why specifically on *C. cingulata*?
In your methodology, your sample is not *C. cingulata* but why did you only mention this particular species in your introduction?

Commented [A6]: Mangrove communities or mangrove ecosystem?

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(Cristianawati et al., 2019). The petri dishes contain the samples were incubated at room temperature for seven days. All of the steps were conducted aseptically.

Fungal characterization and purification.
Fungi will be classified based on the characterization seen on the seventh day of incubation. Characterized macroscopically method Tarman, Safitri & Setyaningsih., (2013) was used to characterize fungal growth. Selected fungal isolates were purified on the sterile MEA and stored in an incubator for 7 days.

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Screening anti-vibrio activity
The screening was carried out using the diffusion agar plug method based on Sibero et al., (2018). *Vibrio* strains of pathogenic bacteria (*V. harveyi*, *V. vulnificus,* and *V. parahaemolyticus*) were inoculated in nutrient broth and then shaken. After incubation for 24 hours, the test bacteria were diluted to a turbidity of 0.5 McFarland and inoculated on trypticase soy agar (TSA) media. Mushrooms that were aged for 7 days on MEA media were placed on the surface of TSA media and incubated (24 hours, 32ºC). The clear zone formed indicates the presence of anti-vibriosis activity.

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Screening of Protease activity
The activity of the protease enzyme was tested qualitatively based on the formation of a clear zone around the isolate grown on MEA enriched with skim milk. The enzymatic index (EI) was determined by the relationship between the mean diameter of the clear zone and the mean diameter of the inoculated colonies (Parveen et al., 2017). The protease activity test was carried out using the spot inoculation/plate assay technique on MEA media enriched with skim milk. Mushrooms aged 5 days were cut into circles of 5 mm using a needle loop and inoculated on MEA media enriched with skim milk aseptically and wrapped around the edges of the petri dish with wrap. Mushrooms were incubated and observed at 1x 24 hours and 2x 24 hours. The results of protease activity appeared with clear zones between fungal inoculations (Cherkupally, Amballa & Bhoomi, 2017).

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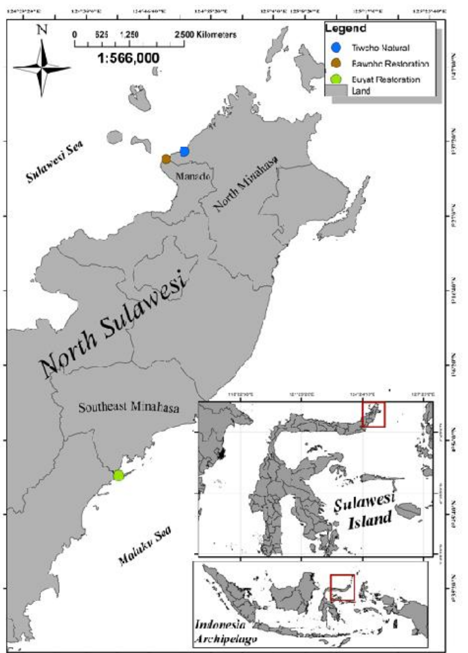

Figure 1. The gastropod samples collection sites in mangrove ecosystems in North Sulawesi, Indonesia.

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Screening of cellulase Activity

The fungal isolates were regrown on MEA media and incubated at room temperature (25ºC) for 7 days. Then, the fungal isolates were prepared for the screening of cellulolytic activity. The test medium, namely MEA enriched with Carboxymethyl Cellulose (CMC) as much as 1% fungal isolate, cut into rounds using a sterile blue tip then placed into the test medium, and incubated for 24 hours at 25ºC. The cellulolytic activity was observed by pouring Congo Red solution on the test culture until it was completely submerged for 30 minutes then rinsed with 1 M NaCl. The cellulolytic activity was indicated by the formation of a clear zone around the colony with a pink background (Lusi et al., 2017).

Molecular Identification

The 7-day-old fungal isolates were extracted using the Zymo Research Quick-DNA Fungal/Bacterial Miniprep Kit. Primers ITS 1(forward): 5 "-GGA AGT AAA AGTCGT AAC AAG G-3" and ITS 4 (reverse): 5 "-TCC TCC GCT TAT TGA TATGC-3" were used for DNA amplification (Suharna *et al.*, 2019). 2 l of template DNA was mixed with 2 l of ITS 1 10 M primer 2 l of ITS 4 10 M primer for 2 l, 25 l of PCR mix and 19 l of sterile aquabides (ddH2O) for a total volume of 24 l. The PCR setting refers to Suharna *et al.*, (2019) by preheating at 95ºC for 3 minutes. It was repeated in 34 cycles for denaturation at 95ºC for 1 minute, annealing at 56.1ºC for 1 minute, extension at 72ºC for 1 minute, followed by a final extension at 72ºC for 7 minutes. The Geldoc tool is used as a visualization of the PCR product. Sequence results were traced using the BLAST menu at NCBI, National Institute for Health, United States of America (www.ncbi.nlm.nih.gov). The search results were selected with the highest level of similarity tinggi (Mohan, Thangappanpillai & Ramasamy, 2016). The phylogenetic

tree construction was carried out using the MEGA 7 application using the neighbor-joining tree method with 1000x bootstrap. (Kumar, Stecher & Tamura, 2016).

Result and Discussion

Gastropods Collection

Two gastropod (**Figure 2**) species were colected from three different mangrove ecosystem during this research. *Cerithidea cingulate* were collected at Buyat and Bawoho mangrove areas, while *Lithorina scabra* were collected at Tiwoho mangrove area.

Commented [A20]: How do you know the species of these gastropods? Please provide the key identification of your samples. Why do you choose these two-particular species? Why do you choose these species as your fungal host, any specific reason? Please explain these question in your result and discussion section.

Figure 2. Gastropods A. *C. cingulata.,* B. *L. scabra* collected from the mangrove ecosystem

Mangrove ecosystems can serve as a source of microbial diversity. The total number of sample collection and fungal isolates in different areas indicates that the area in Tiwoho Natural (MT) serves more samples and related fungi than others (Figure 1). The Tiwoho Natural area shows good varieties according to the 20 hectares of native mangrove forests and biodiversity rehabilitated in 2004. More than 2000 hectares of mangrove growth is good and promotes natural generation (Brown & Djamaluddin, 2017). They are importantly involved in the life cycle of aquatic organisms. Previous research has reported a diversity of mangroves associated with fungi in various parts such as fruit, leaves, pneumatophores, and sediments. They report that the fruit and leaves are the highest amounts. The dominant fungi are Ascomycota and the dothideomycetes class (Lee et al., 2019). Gastropod *C. Cingulata* has a very wide distribution and is almost found all over the world and distributed in tidal flat areas (Ge et al., 2015). Extensive previous research that *C. Cingulata* is found in mangrove habitats in various regions in Indonesia such as Pemalang (Puryono & Suryanti, 2019), Aceh besar (Irma & Sofyatuddin, 2012), Tanah besar (Nugroho, Soendjoto & Zaini, 2019), and Cilacap (Sugiarto, Suryono & Suprijanto, 2021). This distribution is strengthened by the results of research from Reid et al., (2013) the genus Cerithidea has undergone allopatric diversification in the western Indo-Pacific region.

Commented [A21]: So, what is the correlation between mangrove-associated fungi and the gastropods-associated fungi? Please give more explanation with supporting reference.

Commented [A22]: Why do you only give discussion about *C. cingulata*, how about *L. scabra*? Both of them are your samples. Please give an equal discussion.

Isolation dan charcterizition of the fungi

A total of 16 fungal isolates were isolated from mangrove gastropods using MEA media. Each fungus can be characterized based on color, pattern, growth speed, and appearance of filament and spore as stated in table 1.

Table 1. Morfologi gastropods-associated fungal

Commented [A23]: What is the host for each fungus because you have two different gastropods species?

No	Code	Colour	Growth Pattern	Filament	Spore	Growing
1	19MB-C5-1	Light yellow	Spread	+	-	Medium
2	19MB-C5-2	Pale green	Swarm	-	-	Medium
3	19MB-C5-3	Black	Spread	+	+	Medium
4	19MB-C5-4	Pinkish white	Spread	+	-	Slow
5	19MB-C5-5	White	Spread	+	-	Medium
6	19 MT-07-1	Greyish brown	Spread	-	+	Medium
7	19 MT-07-2	Brown	Spread	-	+	Medium
8	19 MT-07-3	Orange white	Like cotton	+	-	Slow
9	19 MT-07-4	Yellowish green	Spread	-	+	Fast
10	19 MT-18-1	White	Spread	-	+	Medium
11	19 MT-18-2	Grey	Spread	+	-	Slow
12	19 MT-18-3	White	Spread	+	-	Fast
13	19 MT-18-4	White	Spread	+	-	Fast
14	19 Mba-A4-1	White	Spread	-	+	Fast
15	19 Mba-A4-2	White	Spread	+	-	Medium
16	19 Mba-A4-3	Grey	Spread	+	-	Medium

MEA media is the best medium for the fungal isolation process to produce secondary metabolites (Kossuga et al., 2012). Sixteen fungal isolates are found in the selection of this medium as which in table 1. This suggests there is a symbiotic interaction of nausea between fungi and gastropods. Previous research results from Silliman & Newell, (2003) It was discovered that Littorari marine gastropods do farming activities on marsh grass. This is done by eating grass tissue infected by fungi. It is suspected that the same interaction of Cingulata also occurs in mangroves, namely by eating litter from mangrove leaves.

Commented [A24]: You have mentioned this. Please do not repeat the same sentence.

Commented [A25]: What do you mean with this? Your data is not sufficient to give this statement. You didn't conduct any assay to test symbiotic interaction between the gastropods and their fungi. Please do not overclaim.

Screening anti-vibrio agent

Commented [A26]: Please provide the figure of this result.

The screening of the fungi with potential antivibriosis agents were carried out against three Vibrio namely *V. harveyi*, *V. vulnificus*, and *V. parahaemolyticus* with the agar plug diffusion method. Observations for 2x24 hours showed that five isolates are shown in table 2. Among the active isolates, 19MB-C5-3 has the highest potential as anti-vibrio source as indicated by clear zone formed around the bacteria within 24 hours or 48 hours. The isolate 19Mba-A4-2 showed strong activity against *V. harveyi* but did not active against the other vibrio. While the other three isolates have weak activity against the vibrio.

Table 2. Screening of antibacterial activity from *C. cingulata* associated fungal

Commented [A27]: What is your standard to give + and ++? Is there any minimum diameter to get + and ++, hence you have two different symbols?

NO	Isolate Code	*V. harveyi*		*V. vulnificus*		*V. parahaemolyticus*	
		24 h	48 h	24 h	48 h	24 h	48 h
1	19MB-C5-1	+	+	-	-	-	-
2	19MB-C5-2	-	-	-	-	-	-
3	19MB-C5-3	++	++	++	++	++	++
4	19MB-C5-4	-	-	-	-	-	-
5	19MB-C5-5	+	-	-	-	-	-
6	19MT-07-1	-	-	-	-	-	-
7	19MT-07-2	-	-	-	-	-	-

8	19MT-07-3	-	-	-	-	-	-
9	19MT-07-4	-	-	-	-	-	-
10	19MT-18-1	-	-	-	-	-	-
11	19MT-18-2	-	-	-	-	-	-
12	19MT-18-3	+	+	-	-	+	+
13	19MT-18-4	-	-	-	-	-	-
14	19MBa-A4-1	-	-	-	-	-	-
15	19Mba-A4-2	++	++	-	-	-	-
16	19MBa-A4-3	-	-	-	-	-	-

Note: 19MB-C5= *C. cingulata;* 19MT-07= *L. scabra*; 19MT-18= *L. scabra;* 19MBa-A4= *C. cingulata.*

The potency of antibacterial compounds comes from secondary metabolites of microorganisms and is produced when the cell has reached a logarithmic phase that goes into a stationary phase. This phase is called idiophase which occurs due to the limitation of microbial growth nutrients (Okada & Seyedsayamdost, 2017). Antagonism of associated fungi to vibrio strain bacteria is one form of adaptation of other competitor microorganisms. In table 2, isolate 19MB-C5-1, 19MB-C5-3, and 19MB-C5-3 indicate activity on a strain of vibrio bacteria test. In extreme circumstances or pressured by other competitors in maintaining nutrients in the medium, the fungus secretes its secondary metabolites to perform self-defense (Pathak et al., 2012). Secondary metabolites of microorganisms can come from the conversion of primary metabolites designed as a form of chemical defense against other competitors' unfavorable microorganisms (Meenupriya & Thangaraj, 2010).
This is in accordance with Romanenko et al., (2008), That microorganisms that are symbiotic with marine invertebrates produce secondary metabolism which is a bioactive compound that has the potential to be a drug and pharmaceutical preparation. Strengthened by research by Kumar, (2015) found that extracts from C. Cingulata have a good antibacterial effect on human pathogens (*Bacillus cereus, Staphylococcus aureus, Vibrio cholerae, Enterobacter aerogenes,* and *Klebsiella pneumoniae*) and fish pathogen bacteria (*Proteus mirabilis, Aeromonas hydrophila, Serratia marcescens, Aeromonas formicans* and *Micrococcus* sp).

Screening of Enzymatic Activity
The cellulolytic and proteolytic assay showed four out of 13 isolates able to degraded both cellulose and protein. The active isolates are 19MB-C5-3, 19MT-18-1, 19MBa-A4-1, and 19MBa-A4-3. Among them isolate 19MB-C5-3 exhibited strong activity to the both cellulose and protein (Table 3).

Table 3. Screening of enzymatic activity of fungal isolates from *C. cingulata* and *L. scabra.*

NO	Isolate Code	Cellulolytic		Proteolytic	
		24 h	48 h	24 h	48 h
1	19MB-C5-1	-	-	-	+
2	19MB-C5-2	+	+	-	-
3	19MB-C5-3	+	++	++	++
4	19MB-C5-4	-	-	-	-
5	19MB-C5-5	-	-	+	+
6	19MT-07-1	-	-	-	-
7	19MT-07-2	-	-	-	-
8	19MT-07-3	-	-	-	-
9	19MT-07-4	-	-	-	-
10	19MT-18-1	+	+	+	-

Commented [A28]: What do you mean with good? Just write the MIC value or diameter of inhibition zone of the extract.

Commented [A29]: 13 or 16?

Commented [A30]: What is your standard to give + and ++? Is there any minimum diameter to get + and ++, hence you have two different symbols?

11	19MT-18-2	-	-	-	-
12	19MT-18-3	++	+	-	-
13	19MT-18-4	-	+	-	-
14	19MBa-A4-1	++	++	+	+
15	19MBa-A4-2	-	-	-	-
16	19MBa-A4-3	+	++	+	+

Screening of cellulolytic activity is carried out on MEA media with a CMC substrate of 1%. CMC serves as a source of carbon in cellulase production (Utami et al., 2019). The source of carbon becomes the main factor affecting the production of cellulase in fungi. Cellulase itself is an enzyme that will be expressed by fungal cells in response to the source of carbon present in the medium (Ahmed dan Bibi, 2018). To carry out the production of fungal cellulase requires optimal conditions during the incubation period. Incubation time, temperature, and pH are significant factors in cellulase production by fungi (Ahmed *et al.*, 2018). Fungi use cellulase for their metabolic activity in breaking down cellulose. In addition, the cellulolytic activity produced by the fungus plays a major role in the bio decomposition process (Panchapakesan & Shankar, 2016). Arfi *et al.*, (2013) indicate that the halotolerant mushroom *Pestalotiopsis* sp. Can excrete cellulase even the presence of salt around the mangrove ecosystem can increase the number of cells produced. This is certainly a special attraction for fungi associated with mangroves to further research their potential in producing cellulose.

The presence of a clear zone around the fungal disk colony indicated by the notation (+) in table 3 indicates the occurrence of hydrolysis in the test media indicating the development of enzyme production while the area where the enzyme develops is called the hydrolysis zone (Kamath et al., 2010; Bahry, Radjasa & Trianto, 2021). Protease enzyme production itself is obtained from fungi that utilize nitrogen sources from proteins for their development by using different sources (peptone, tripton, casein, and yeast extracts) (Ahmed, 2018). The mechanism of proteolytic activity is due to the hydrolysis of protein bonds derived from skim milk agar (SMA) media into simpler amino acids. Protease activity can be optimized of them by enriching using MEA media as the results of research by Kamath et al., (2010).

Fungi that have proteolytic activity have the ability to produce protease enzymes secreted into their environment. Proteolytic enzymes serve to hydrolyze protein compounds into oligopeptides, short-chain peptides, and amino acids. The presence of this extracellular protease enzyme is essential for bacterial life because it provides for the need for nitrogen compounds that can be transported into cells. These types of fungi that have the ability to secrete protease enzymes have great potential to be used as a source of aquaculture probiotics, especially in shrimp farming (Bahry, Radjasa & Trianto, 2021; Ooi, Rasit & Abdullah, 2021). Likupang's restored mangroves are abandoned shrimp ponds rich in protein and cellulose from shrimp residues, and uneaten feed. In addition, shrimp farming is the disposal and deposition of shrimp pond waste left in the pond after harvest. It is a high content of uneaten food, rotting plankton, dirt, air debris, and microorganisms (Hopkins, Sandifer & Browdy, 1994)

Commented [A31]: How about fungi? Your material is fungi right?

The most potential isolate 19MB-C5-3 was identified using molecular method. The quality of DNA isolation is visualized using geldoc as in Figure 3.

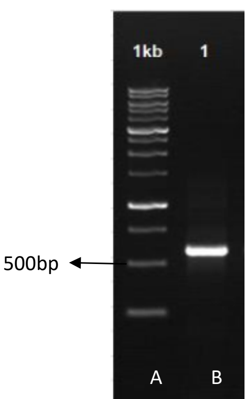

Figure 3. A. DNA ladder, B. DNA template of *C. cingulata* associated fungal isolate19MB-C5-3

The Blast analysis was used to determine the degree of similarity of isolates to other species in the NCBI genebank. The results of the blast analysis is shown in Table 4, isolate19MB-C5-3 of forward and reverse DNA have a sequence length of 954 base pairs and show similarity to Aspergillus niger isolate Strbr with accession number MK372989. The sample similarity rate was 99.40%.

Table 4. Identification of potential *C. cingulata* associated fungal based on BLAST analysis using the ITS region Sponge

Isolate	Sequence length (bp)	Next relative by GenBank alignment (AN, an organism)	Similarity (%)	Family
19MB-C5-3	954	MK372989 *Aspergillus niger* isolate Strbr	99.40	Trichocomaceae

Its primary use for DNA amplification because at ITS there are conservative areas for eukaryotic organisms such as fungi, namely the 18S, 5.8S, and 28S encoder genes (Calado et al., 2019). Eukaryotic organisms have 2 ITS regions with 3 genes that have a fairly high conservancy rate, namely ITS 1 which is located between 18S genes and 5.8S genes, while ITS 2 is located between 5.8S genes and 28S genes. The small subunit rDNA sequence of 18S genes develops relatively slowly making it suitable for use in the study of kinship relationships between species (Mulyatni *et al.*, 2016). ITS 1 primary as forward and ITS 4 as reverse was chosen because the primary was in its conservative loci region for DNA amplification from fungi (Sibero et al., 2018b). The location of the primary conservative areas of ITS 1 and ITS 4 can be seen in Figure 6. then the amplification results will be visualized with electrophoresis to see the quality of amplification. This shows that its primer successfully amplified the fungal DNA isolate 19MB-C5-3 at a length of ± 500 base pairs for each primer. The combination of forward and reverse DNA results is used to find the best DNA quality.

Commented [A32]: Please rewrite this part. Really confusing.

Commented [A33]: Where is Figure 6?

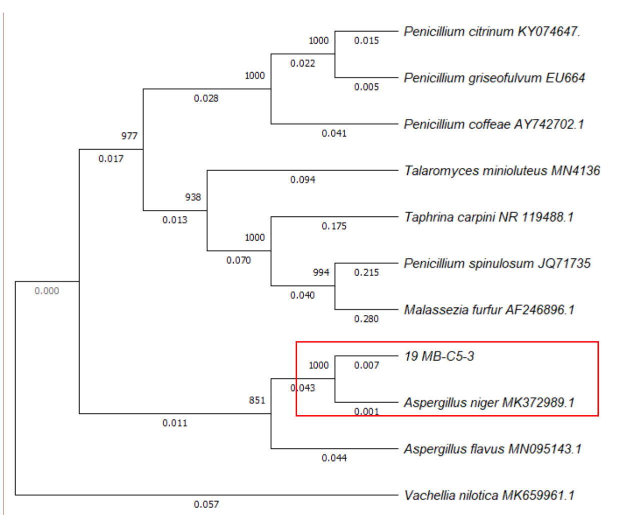

Figure 3. Phylogenetic tree based on the internal transcribed spacer (ITS) regions genes of *C. cingulata* associated fungal 19MB-C5-3

Commented [A34]: Please provide more *A. niger* in this phylo tree to confirm the clade.
How could *P. spinulosum* in one clade with *Malassezia furfur* and separated with other *Penicillium*?
Why do you use *Vachellia nilotica* as your out group?! Do you know it is plant and not fungi?

Phylogenetic trees reconstructed using MEGA 7 software using the neighbor-joining tree method with bootstrap 1000x show that C. cingulata associated fungal 19MB-C5-3 grouped one clade with *Aspergillus niger* as shown in figure 3.

The results of molecular identification of fungi that have cellulolytic activity with BLAST (Basic Local Alignment Search Tool) can be seen in Table 4 as well as phylogenetic trees created using ClustalX and Mega 7 software with the Neighbor-Joining Tree approach can be seen in Figure 3. It can be seen that Isolate 19MB-C5-3 has a degree of kinship with the fungus *Aspergillus niger* isolate Strbr MK372989. The number in the branch shows the bootstrap value of the Neighbor-Joining Tree analysis which means that after 1000 repetitions the confidence level of the branch forms a clade in a phylogenetic tree (Li & Wang, 2009).

Conclusion

The gastropods *C. cingulata* is a source of fungi that producing protease, cellulase, and anti-vibrio compounds. The *A. niger* 19MB-C5-3 has potential bioremediation agent in aquaculture application.

Acknowledgement

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Dr. Agus Trianto <agustrianto@lecturer.undip.ac.id>
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Wed, Mar 15, 3:20 PM

Indonesian Journal of Marine Science:

A revised version of "Molecular Identification and Screening of Enzymatic and bioactivity of vibriosis agent from Cerithidea cingulate-derived fungi collected from mangrove forest" has been uploaded by the author Dr. Agus Trianto.

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Dr. Agus Trianto <agustrianto@lecturer.undip.ac.id>
to me

Wed, Mar 15, 3:21 PM

Reply Forward

Authors' Response/Comments

Dear Editors,

We have corrected the manuscript as suggested by the reviewers, even though, we cannot follow all of the suggestions. Below, are our responses to the reviewer comments and suggestions:

No	Sugesstion/Comment	Response
1	Suggested title: Molecular identification and biotechnological potential of Cerithidea cingulata-associated fungi as extracellular enzyme producer and anti-vibriosis agent	We can not follow the suggestion because we also work with other species
2	Please use proof reading service	The manuscript was revised by one of the author from UK.
3	Please check the correct scientific name because it known as *Cerithidea cingulata*	Already corrected, the miss-typing due to the auto correct by the computer
4	Font : Italic	Done
5	Which one is your sample? Is it mangrove or the gastropod?	The gastropods.
6	Inoculated or isolated? They are different meaning.	isolated
7	Please write your introduction part by inductively. The story line of your introduction is not strong enough. Why do you use gastropods-associated fungi as the source of your isolates? Why specifically on *C. cingulata*? In your methodology, your sample is not *C. cingulata* but why did you only mention this particular species in your introduction?	Already improved
8	Mangrove communities or mangrove ecosystem?	ecosystem
9	Bahry et al. 2017?	Bahry et al, 2017
10	Antibiofilm?	corrected
11	Font : Italic	corrected
12	Protease doesn't work on carbohydrate. Please check your reference.	corrected
13	Please rewrite this sentence.	corrected
14	This part is confusing in English. Please fix it.	Already re-write
15	What do you mean with tapped? Did you removed the tissue after tapping? Or do you let the tissue on the agar until the fungi grew on the tissue? This is very confusing.	Simply put the gastropods tissue into the media. This is a common terminology in this field

16	I am really sorry but I couldn't understand this part. Please rewrite this using good English.	corrected
17	Font : Italic	corrected
18	Mushroom?	Replaced with fungi
19	I am really sorry but I couldn't understand this part. Please rewrite this using good English.	corrected
20	You have mentioned it above!	corrected
21	Mushroom again?	
22	I am really sorry but I couldn't understand this part. Please rewrite this using good English.	corrected
23	Please rewrite this sentence	corrected
24	How do you know the species of these gastropods? Please provide the key identification of your samples. Why do you choose these two-particular species? Why do you choose these species as your fungal host, any specific reason? Please explain these question in your result and discussion section.	The key identification has already add in the manuscript
25	Font : Italic	corrected
26	So, what is the correlation between mangrove-associated fungi and the gastropods-associated fungi? Please give more explanation with supporting reference.	Done
27	Why do you only give discussion about *C. cingulata*, how about *L. scabra*? Both of them are your samples. Please give an equal discussion.	The discussion is more on the *C. cingulata* because this species give the active isolates.
28	What is the host for each fungus because you have two different gastropods species?	The explanation already add in the manuscript
29	You have mentioned this. Please do not repeat the same sentence.	corrected
30	What do you mean with this? Your data is not sufficient to give this statement. You didn't conduct any assay to test symbiotic interaction between the gastropods and their fungi. Please do not overclaim.	The statemen is base on the reference
31	What is your standard to give + and ++? Is there any minimum diameter to get + and ++, hence you have two different symbols?	The symbol "++" indicate a strong activity with inhibition zone more than 5 mm
32	What do you mean with good? Just write the MIC value or diameter of inhibition zone of the extract.	At this stage we use a qualitive test.
33	13 or 16?	Should be 16

34	What is your standard to give + and ++? Is there any minimum diameter to get + and ++, hence you have two different symbols?	"+" = the inhibition zone 0.1-0.5 mm, "++"= the inhibition zone more than 6 mm
35	How about fungi? Your material is fungi right?	yes
36	Please rewrite this part. Really confusing.	done
37	Where is Figure 6?	It should be figure 3
38	Please provide more *A. niger* in this phylo tree to confirm the clade. How could *P. spinulosum* in one clade with *Malassezia furfur* and separated with other *Penicillium*? Why do you use *Vachellia nilotica* as your out group?! Do you know it is plant and not fungi?	The outgroup has already corrected.

Final Revision

Molecular identification and biotechnological potential of *Cerithidea cingulata*-and *Lottia scabra* associated fungi as extracellular enzyme producer and anti-vibriosis agent

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Abstract

Mangrove ecosystems have a broad biotechnological potential, including microfungi associated with them. This study a explored the enzymatic and anti-vibrio activity of fungi associated with the marine gastropod *Cerithidea cingulata* and *Lottia scabra*. collected in three mangrove forests in North Sulawesi, Indonesia. The fungi associated with the specimens were inoculated on malt extract agar (MEA) using the tapping method. Enzymatic and anti-vibrio assays were conducted applying the plug method. Positive effects of the fungal compounds on Vibrio were indicated by clear zones formed around the isolates. A total of 13 fungal species were isolated from *C. cingulata* (19MB-C5 and 19MBa-A4) and *L. scabra* (19MT-07 and 19MT-18). The isolate with highest cellulolytic, proteolytic, and anti-vibriosis activity (*Vibrio parahaemolyticus*, *V. harveyi*, *V. vulnificus*, and *V. alginolyticus*), derived from *C. cingulata* 19MB-C5 was closely related to *Aspergillus niger, as revealed by molecular analysis* using the universal primers ITS1 and ITS4 (similarity value 99%).

Keywords: Enzyme, Fungi, Mangrove, Vibriosis

Introduction

Mangrove is a complex, unique, and dynamics ecosystem. The organisms living the ecosystem have special physiological adaptations to salinity (Gunawan et al., 2017). Having an open ecosystem system, mangroves have material arrangements that are influenced by marine physical factors such as tides and ocean currents, biological factors that are habitats such as shrimp and gastropods as well as chemical factors in controlling organic and inorganic materials (Sukardjo, 2004). Mangrove ecosystem has shown high biotechnological productivity and biological biodiversity both in tropical and sub-tropical areas (Kamruzzaman et al., 2017).
Gastropods are the dominant invertebrates in mangrove ecosystem which also have an important role in ecological cycles and food webs by helping to provide nutrients for microbes and detrivores through mangrove litter (Kabir et al., 2014; Rahmawati et al., 2015). Gastropods have a wide distribution in several ecosystems in the indo-pacific including *Cerithidea cingulata*. There is a close relationship between marine organisms and symbiotic microorganisms in producing secondary metabolites. Proksch et al., (2003) stated the discovery of the same active compounds between marine invertebrates including gastropods and their symbiotic bacteria. Several studies have found benefits in organisms associated with gastropods such as antibacterial (Pringgenies, 2010), anti-drug-resistant bacteria (Bahry et al., 2017), and antibiofilm (Viju et al., 2020).
Mangrove fungi are known to be rich sources of enzymes and secondary metabolites such as antibacterial compounds that beneficial aquaculture. Aquaculture produce the organic and antibiotic waste that reducing the water quality and give a bad impact to the ecosystem (González-Gaya et al., 2021). Among the bacteria, genus *Vibrio* is main cause of various deceases on shrimp and fishes aquaculture over worldwide that causes considerable economic losses of US $ 9 billion annually (Novriadi, 2016). Application of microorganisms as biocontrol for reducing the pathogenic bacteria is an alternative method as substitution of antibiotic. Proteases can be used to improve feed quality by reducing the size of protein molecules to increase digestibility (Maytorena-Verdugo, Córdova-Murueta & García-Carreño, 2017).
Along with the increasing market demand for vannamei shrimp, it encourages an increase in shrimp production and feed. The biggest problem in the Vannamei shrimp cultivation process is that 40-60% of the total production cost is allocated for feed, while the efficiency of feed absorption is not optimal (Olmos et al., 2011). This is due to the fact that Vannamei shrimp is a carnivorous animal that does not easily digest vegetable protein, while the carbohydrates in feed are only absorbed 20% because it is not the main energy source (Kurniawan et al., 2019). Excess nutrition will cause problems because it requires more energy and a long digestion period to hydrolyze protein, fat and carbohydrate bonds (Rachmawati et al., 2020). On the other hand, improper pond management causes poor water quality that leads to vibriosis disease which can cause mass mortality in cultured shrimp and environmental pollution (Kusumaningrum & Zainuri, 2015; Sarjito et al., 2018)
In this paper we will discuss on the isolation of the fungi from gastropods, proteolytic, cellulolytic, and anti-vibrio activities screening, and the active fungus identification.

Materials and Methods

Gastropods collection and fungi isolation.

Gastropod samples were collected from three different mangrove ecosystem in North Sulawesi as shown in Figure 1. All laboratory activities are carried out in the marine natural product laboratory (MNP LAB) - Center of Research and Services (Cores-DU). The pathogenic bacteria in this study were the collection of MNP LAB.
Samples of gastropods were put in a zip lock and stored in a cool box to avoid contaminants (Bahry et al,2017). Before the isolation, gastropods were sterilized 70% alcohol than the

gastropod tissue was rinsed with sterile seawater. The tapping method was used for the fungal isolation by tapping gastropod tissue on malt extract agar (MEA) media (MERK, USA) with three repetitions. Isolation media was supplemented with 100 mg/L of chloramphenicol to avoid bacterial contamination (Cristianawati et al., 2019). The petri dishes contain the samples were incubated at room temperature for seven days. All of the steps were conducted aseptically.

Fungal characterization and purification.

Fungi were classified based on the macroscopic characterization seen on the seventh day of growth (Tarman *et al*., 2013). Selected fungal isolates were purified on the sterile MEA and stored in an incubator for 7 days.

Screening anti-vibrio activity

The screening was carried out using the diffusion agar plug method based on Sibero et al., (2018). *Vibrio* strains of pathogenic bacteria (*V. harveyi*, *V. vulnificus,* and *V. parahaemolyticus*) were inoculated in nutrient broth and then shaken. After incubation for 24 hours, the test bacteria were diluted to a turbidity of 0.5 McFarland and inoculated on trypticase soy agar (TSA) media. The seven days aged of fungal isolates were overlaid on the surface of TSA media which grown by bacteria before than incubated (24 hours, 32ºC). The clear zone formed indicates the presence of anti-vibriosis activity.

Screening of Protease activity

The activity of the protease enzyme was tested qualitatively based on the formation of a clear zone around the isolate grown on MEA enriched with skim milk. The enzymatic index (EI) was determined by the relationship between the mean diameter of the clear zone and the mean diameter of the inoculated colonies (Parveen et al., 2017). After 5 days, the fungal isolates were cut in circles (fungal disks) and then placed on MEA media enriched with skim milk. Parafilm was used to avoid media contamination. Enzyme activity was observed at 1 x 24 hours and 2 x 24 hours which was seen by the presence of a clear zone around the fungal disk (Cherkupally, Amballa & Bhoomi, 2017).

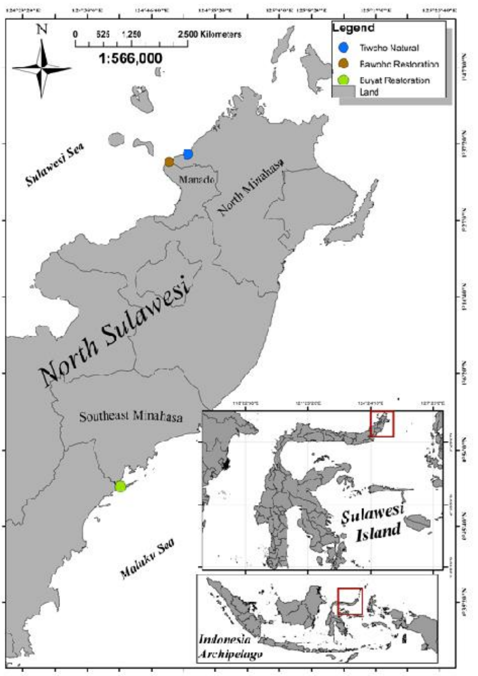

Figure 1. Site of gastropod sampling on mangrove ecosystems in three regions of North Sulawesi (Tiwoho, Bawoho, and Buyat).

Screening of cellulase Activity

The fungal isolates were regrown on MEA media and incubated at room temperature (25ºC) for 7 days. Then, the fungal isolates were prepared for the screening of cellulolytic activity. The test medium, namely MEA enriched with Carboxymethyl Cellulose (CMC) as much as 1% fungal isolate, cut into rounds using a sterile blue tip then placed into the test medium, and incubated for 24 hours at 25ºC. The cellulolytic activity was observed by pouring Congo Red solution on the test culture until it was completely submerged for 30 minutes then rinsed with 1 M NaCl. The cellulolytic activity was indicated by the formation of a clear zone around the colony with a pink background (Lusi et al., 2017).

Molecular Identification

The 7-day-old fungal isolates were extracted using the Zymo Research Quick-DNA Fungal/Bacterial Miniprep Kit. Primers ITS 1(forward): 5 "-GGA AGT AAA AGTCGT AAC AAG G-3" and ITS 4 (reverse): 5 "-TCC TCC GCT TAT TGA TATGC-3" were used for DNA amplification (Suharna *et al.*, 2019). 2 l of template DNA was mixed with 2 l of ITS 1 10 M primer 2 l of ITS 4 10 M primer for 2 l, 25 l of PCR mix and 19 l of sterile aquabides (ddH2O) for a total volume of 24 l. The PCR setting refers to Suharna *et al.*, (2019) by preheating at 95ºC for 3 minutes. It was repeated in 34 cycles for denaturation at 95ºC for 1 minute, annealing at 56.1ºC for 1 minute, extension at 72ºC for 1 minute, followed by a final extension at 72ºC for 7 minutes. The Geldoc

tool is used as a visualization of the PCR product. Sequence results were traced using the BLAST menu at NCBI, National Institute for Health, United States of America (www.ncbi.nlm.nih.gov). The search results were selected with the highest level of similarity (Mohan, Thangappanpillai & Ramasamy, 2016). The phylogenetic tree construction was carried out using the MEGA 7 application using the neighbor-joining tree method with 1000x bootstrap. (Kumar, Stecher & Tamura, 2016).

Result and Discussion

Gastropods Collection

Two gastropod (**Figure 2**) species were collected from three different mangrove ecosystem during this research. *Cerithidea cingulata* were collected at Buyat and Bawoho mangrove areas, while *Lithorina scabra* were collected at Tiwoho mangrove area. Identification of the gastropods were conducted base on FAO species identification guide for fishery purposes: The living marine resources of the western Central Pacific. Volume 1. Seaweeds, corals, bivalves and gastropods (FAO, 1998). The genus *C. cingulata* charaterized with sharp conical shell with many-whorled spire and rather small aperture that obviously can be seen. The key identification to this species isthe outer lip widens posteriorly in a wing-like spreading process. Whilst, the genus *L. scabra* has pale brown dense pattern of oblique with irregular dark stripes outer surface without nodulose sculpture. *C. cingulata* and *L. scabra* are the most common gastropods that can be found in the mangrove habitat in the North Sulawesi area. Gastropod *C. cingulata* has a very wide distribution and is almost found all over the world and distributed in tidal flat areas (Ge et al., 2015). Extensive previous research that *C. Cingulata* is found in mangrove habitats in various regions in Indonesia such as Pemalang (Puryono & Suryanti, 2019), Aceh besar (Irma & Sofyatuddin, 2012), Tanah besar (Nugroho, Soendjoto & Zaini, 2019), and Cilacap (Sugiarto, Suryono & Suprijanto, 2021). This distribution is strengthened by the results of research from Reid et al., (2013) the genus Cerithidea has undergone allopatric diversification in the western Indo-Pacific region.

Figure 2. Gastropods A. *C. cingulata.,* B. *L. scabra* collected from the mangrove ecosystem

Gastropods living in mangrove ecosystems can serve as a source of microbial diversity. The total number of sample collection and fungal isolates in different areas indicates that the area in Tiwoho Natural (MT) serves more samples and related fungi than others (Figure 1). The Tiwoho Natural area shows good varieties according to the 20 hectares of native mangrove forests and biodiversity rehabilitated in 2004. More than 2000 hectares of mangrove growth is good and promotes natural generation (Brown & Djamaluddin, 2017). They are importantly involved in the

life cycle of aquatic organisms. Previous research has reported a diversity of mangroves associated with fungi in various parts such as fruit, leaves, pneumatophores, and sediments. They report that the fruit and leaves are the highest amounts. The dominant fungi are Ascomycota and the dothideomycetes class (Lee et al., 2019).

Isolation and characterization of the fungi

A total of 16 fungal isolates were isolated from mangrove gastropods using MEA media. Each fungus can be characterized based on color, pattern, growth speed, and appearance of filament and spore as stated in table 1.

Table 1. Morphology gastropods-associated fungal of *C. Cingulata* and *L. scabra.*

No	Code	Color	Growth Pattern	Filament	Spore	Growing
1	19MB-C5-1	Light yellow	Spread	+	-	Medium
2	19MB-C5-2	Pale green	Swarm	-	-	Medium
3	19MB-C5-3	Black	Spread	+	+	Medium
4	19MB-C5-4	Pinkish white	Spread	+	-	Slow
5	19MB-C5-5	White	Spread	+	-	Medium
6	19 MT-07-1	Greyish brown	Spread	-	+	Medium
7	19 MT-07-2	Brown	Spread	-	+	Medium
8	19 MT-07-3	Orange white	Like cotton	+	-	Slow
9	19 MT-07-4	Yellowish green	Spread	-	+	Fast
10	19 MT-18-1	White	Spread	-	+	Medium
11	19 MT-18-2	Grey	Spread	+	-	Slow
12	19 MT-18-3	White	Spread	+	-	Fast
13	19 MT-18-4	White	Spread	+	-	Fast
14	19 Mba-A4-1	White	Spread	-	+	Fast
15	19 Mba-A4-2	White	Spread	+	-	Medium
16	19 Mba-A4-3	Grey	Spread	+	-	Medium

Note: 19MB-C5= *C. cingulata;* 19MT-07= *L. scabra*; 19MT-18= *L. scabra;* 19MBa-A4= *C. cingulata.*

MEA media is the best medium for the fungal isolation process to produce secondary metabolites (Kossuga et al., 2012). The fungal growth on MEA media from gastropod isolates shows that fungi live in gastropod tissues through a symbiotic mechanism. Previous research results from Silliman & Newell, (2003) It was discovered that Littorari marine gastropods do farming activities on marsh grass. This is done by eating grass tissue infected by fungi. It is suspected that the same interaction of Cingulata also occurs in mangroves by eating litter from mangrove leaves.

Screening anti-vibrio agent

The screening of the fungi with potential antivibriosis agents were carried out against three Vibrio namely *V. harveyi*, *V. vulnificus*, and *V. parahaemolyticus* with the agar plug diffusion method. Observations for 2x24 hours showed that five isolates are shown in table 2. Among the active isolates, 19MB-C5-3 has the highest potential as anti-vibrio source as indicated by clear zone formed around the bacteria within 24 hours or 48 hours. The isolate 19Mba-A4-2 showed strong

activity against *V. harveyi* but did not active against the other vibrio. While the other three isolates have weak activity against the vibrio.

Table 2. Screening of antibacterial activity from *C. Cingulata* and *L. scabra* associated fungal

NO	Isolate Code	*V. harveyi*		*V. vulnificus*		*V. parahaemolyticus*	
		24 h	48 h	24 h	48 h	24 h	48 h
1	19MB-C5-1	+	+	-	-	-	-
2	19MB-C5-2	-	-	-	-	-	-
3	19MB-C5-3	++	++	++	++	++	++
4	19MB-C5-4	-	-	-	-	-	-
5	19MB-C5-5	+	-	-	-	-	-
6	19MT-07-1	-	-	-	-	-	-
7	19MT-07-2	-	-	-	-	-	-
8	19MT-07-3	-	-	-	-	-	-
9	19MT-07-4	-	-	-	-	-	-
10	19MT-18-1	-	-	-	-	-	-
11	19MT-18-2	-	-	-	-	-	-
12	19MT-18-3	+	+	-	-	+	+
13	19MT-18-4	-	-	-	-	-	-
14	19MBa-A4-1	-	-	-	-	-	-
15	19Mba-A4-2	++	++	-	-	-	-
16	19MBa-A4-3	-	-	-	-	-	-

Note: 19MB-C5= *C. cingulata;* 19MT-07= *L. scabra*; 19MT-18= *L. scabra;* 19MBa-A4= *C. cingulata.*

The potency of antibacterial compounds comes from secondary metabolites of microorganisms and is produced when the cell has reached a logarithmic phase that goes into a stationary phase. This phase is called idiophase which occurs due to the limitation of microbial growth nutrients (Okada & Seyedsayamdost, 2017). Antagonism of associated fungi to vibrio strain bacteria is one form of adaptation of other competitor microorganisms. In table 2, isolate 19MB-C5-1, 19MB-C5-3, and 19MB-C5-3 indicate activity on a strain of vibrio bacteria test. In extreme circumstances or pressured by other competitors in maintaining nutrients in the medium, the fungus secretes its secondary metabolites to perform self-defense (Pathak et al., 2012). Secondary metabolites of microorganisms can come from the conversion of primary metabolites designed as a form of chemical defense against other competitors' unfavorable microorganisms (Meenupriya & Thangaraj, 2010).

This is in accordance with Romanenko et al., (2008), That microorganisms that are symbiotic with marine invertebrates produce secondary metabolism which is a bioactive compound that has the potential to be a drug and pharmaceutical preparation. Strengthened by research by Kumar, (2015) found that ethyl acetate fractions extracts from *C. cingulata* have 1 mg concentration in Minimal Inhibitory Concentration (MIC) for inhibiting both human pathogens (*Staphylococcus aureus,* and *Vibrio cholerae,*) and fish pathogen bacteria (*Serratia marcescens,* and *Aeromonas formicans*).

Screening of Enzymatic Activity

The cellulolytic and proteolytic assay showed four out of 16 isolates able to degraded both cellulose and protein. The active isolates are 19MB-C5-3, 19MT-18-1, 19MBa-A4-1, and 19MBa-

A4-3. Among them isolate 19MB-C5-3 exhibited strong activity to the both cellulose and protein (Table 3).

Table 3. Screening of enzymatic activity of fungal isolates from *C. cingulata* and *L. scabra*.

NO	Isolate Code	Cellulolytic		Proteolytic	
		24 h	**48 h**	**24 h**	**48 h**
1	19MB-C5-1	-	-	-	+
2	19MB-C5-2	+	+	-	-
3	19MB-C5-3	+	++	++	++
4	19MB-C5-4	-	-	-	-
5	19MB-C5-5	-	-	+	+
6	19MT-07-1	-	-	-	-
7	19MT-07-2	-	-	-	-
8	19MT-07-3	-	-	-	-
9	19MT-07-4	-	-	-	-
10	19MT-18-1	+	+	+	-
11	19MT-18-2	-	-	-	-
12	19MT-18-3	++	+	-	-
13	19MT-18-4	-	+	-	-
14	19MBa-A4-1	++	++	+	+
15	19MBa-A4-2	-	-	-	-
16	19MBa-A4-3	+	++	+	+

Note: 19MB-C5= *C. cingulata;* 19MT-07= *L. scabra*; 19MT-18= *L. scabra;* 19MBa-A4= *C. cingulata.*

Screening of cellulolytic activity is carried out on MEA media with a CMC substrate of 1%. CMC serves as a source of carbon in cellulase production (Utami et al., 2019). The source of carbon becomes the main factor affecting the production of cellulase in fungi. Cellulase itself is an enzyme that will be expressed by fungal cells in response to the source of carbon present in the medium (Ahmed dan Bibi, 2018). To carry out the production of fungal cellulase requires optimal conditions during the incubation period. Incubation time, temperature, and pH are significant factors in cellulase production by fungi (Ahmed *et al*., 2018). Fungi use cellulase for their metabolic activity in breaking down cellulose. In addition, the cellulolytic activity produced by the fungus plays a major role in the bio decomposition process (Panchapakesan & Shankar, 2016). Arfi *et al*., (2013) indicate that the halotolerant fungus *Pestalotiopsis* sp. Can excrete cellulase even the presence of salt around the mangrove ecosystem can increase the number of cells produced. This is certainly a special attraction for fungi associated with mangroves to further research their potential in producing cellulose.

The presence of a clear zone around the fungal disk colony indicated by the notation (+) in table 3 indicates the occurrence of hydrolysis in the test media indicating the development of enzyme production while the area where the enzyme develops is called the hydrolysis zone (Kamath et al., 2010; Bahry, Radjasa & Trianto, 2021). Protease enzyme production itself is obtained from fungi that utilize nitrogen sources from proteins for their development by using different sources (peptone, tripton, casein, and yeast extracts) (Ahmed, 2018). The mechanism of proteolytic activity is due to the hydrolysis of protein bonds derived from skim milk agar (SMA) media into

simpler amino acids. Protease activity can be optimized of them by enriching using MEA media as the results of research by Kamath et al., (2010).

Fungi that have proteolytic activity have the ability to produce protease enzymes secreted into their environment. Proteolytic enzymes serve to hydrolyze protein compounds into oligopeptides, short-chain peptides, and amino acids. The presence of this extracellular protease enzyme is essential for microbial such us fungal and bacterial life because it provides for the need for nitrogen compounds that can be transported into cells. These types of fungi that have the ability to secrete protease enzymes have great potential to be used as a source of aquaculture probiotics, especially in shrimp farming (Bahry, Radjasa & Trianto, 2021; Ooi, Rasit & Abdullah, 2021). Likupang's restored mangroves are abandoned shrimp ponds rich in protein and cellulose from shrimp residues, and uneaten feed. In addition, shrimp farming is the disposal and deposition of shrimp pond waste left in the pond after harvest. It is a high content of uneaten food, rotting plankton, dirt, air debris, and microorganisms (Hopkins, Sandifer & Browdy, 1994)

The isolate 19MB-C5-3 is the most potential because of it anti-vibriosis and enzymatic activities. The biotechnological potential. (See Figure 3). Isolate 19MB-C5-3 was identified using molecular method. The quality of DNA isolation is visualized using geldoc as in Figure 4.

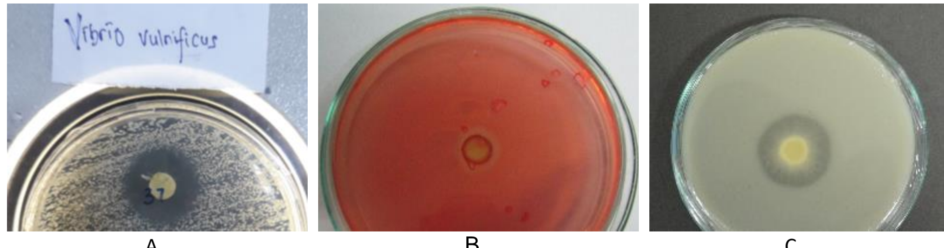

Figure 3. Biotechnological potential screening of isolate 19MB-C5-3, A. Activity against *V. vulnificus,* B. Activity of cellulolytic, C. Activity of proteolytic.

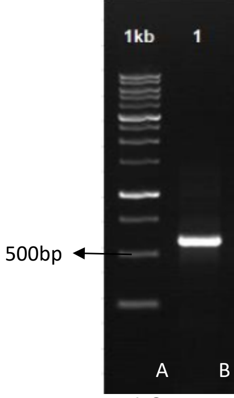

Figure 4. A. DNA ladder, B. DNA template of *C. cingulata* associated fungal isolate19MB-C5-3

The Blast analysis was used to determine the degree of similarity of isolates to other species in the NCBI genebank. The results of the blast analysis are shown in Table 4, isolate19MB-C5-3 of forward and reverse DNA have a sequence length of 954 base pairs and show similarity to *Aspergillus niger* isolate Strbr with accession number MK372989. The sample similarity rate was 99.40%.

Table 4. Identification of potential *C. cingulata* associated fungal based on BLAST analysis using the ITS region Sponge

Isolate	**Sequence length (bp)**	**Next relative by GenBank alignment (AN, an organism)**	**Similarity (%)**	**Family**
19MB-C5-3	954	MK372989 *Aspergillus niger* isolate Strbr	99.40	Trichocomaceae

The primers ITS1 and ITS4 were chosen because they can amplify the DNA sequences of 18S rRNA, 5.8S rRNA and 28S rRNA which are intended for eukaryotic organisms such as fungi. (Calado et al., 2019). Eukaryotic organisms have 2 ITS regions with 3 genes that have a fairly high conservancy rate, namely ITS 1 which is located between 18S genes and 5.8S genes, while ITS 2 is located between 5.8S genes and 28S genes. The small subunit rDNA sequence of 18S genes develops relatively slowly making it suitable for use in the study of kinship relationships between species (Mulyatni *et al.*, 2016). ITS 1 primary as forward and ITS 4 as reverse was chosen because the primary was in its conservative loci region for DNA amplification from fungi (Sibero et al., 2018b). The location of the primary conservative areas of ITS 1 and ITS 4 can be seen in Figure 5. then the amplification results will be visualized with electrophoresis to see the quality of amplification. This shows that its primer successfully amplified the fungal DNA isolate 19MB-C5-3 at a length of ± 500 base pairs for each primer. The combination of forward and reverse DNA results is used to find the best DNA quality.

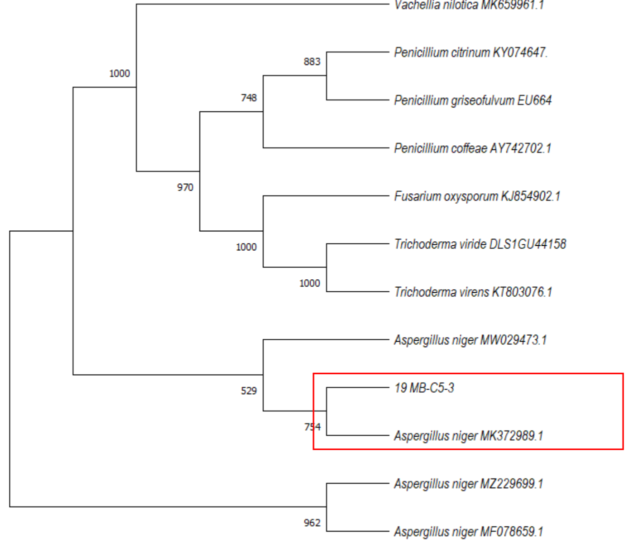

Figure 5. Phylogenetic tree based on the internal transcribed spacer (ITS) regions genes of *C. cingulata* associated fungal 19MB-C5-3

Phylogenetic trees reconstructed using MEGA 7 software using the neighbor-joining tree method with bootstrap 1000x show that *C. cingulata* associated fungal 19MB-C5-3 grouped one clade with *Aspergillus niger* as shown in figure 5.

The results of molecular identification of fungi that have cellulolytic activity with BLAST (Basic Local Alignment Search Tool) can be seen in Table 4 as well as phylogenetic trees created using ClustalX and Mega 7 software with the Neighbor-Joining Tree approach can be seen in Figure 6. It can be seen that Isolate 19MB-C5-3 has a degree of kinship with the fungus *Aspergillus niger* isolate Strbr MK372989. The number in the branch shows the bootstrap value of the Neighbor-Joining Tree analysis which means that after 1000 repetitions the confidence level of the branch forms a clade in a phylogenetic tree (Li & Wang, 2009).

Conclusion

The gastropods *C. cingulata* is a source of fungi that producing protease, cellulase, and anti-vibrio compounds. The *A. niger* 19MB-C5-3 has potential bioremediation agent in aquaculture application.

Acknowledgement

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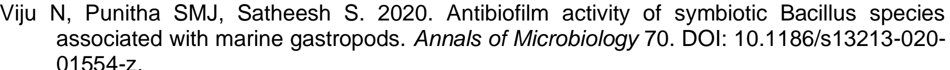

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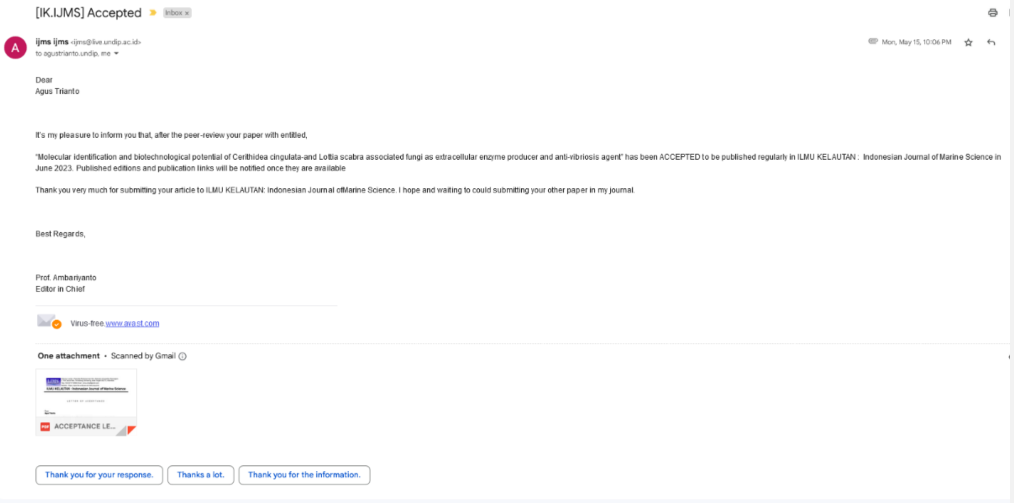

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