Molekular Identification & Biotecnological Potential

by Rignolda Djamaluddin 22

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Molecular Identification and Biotechnological Potential of *Cerithidea cingulata*-and *Lottia scabra* Associated Fungi as Extracellular Enzyme Producer and Anti-Vibriosis Agent

Agus Trianto[1,2*], Ocky Karna Radjasa[1,3], Subagiyo[1], Erlita Octaviani[1], Armeilia Ardiva Isabella[1], Muhammad Syaifudien Bahry[2], Hartuti Purnaweni[4], Rignolda Djamaludin[5], Aiyen Tjoa[6], Ian Singleton[7], Darren Mark Evans[7], Karen Diele[8,9]

[1]Department Marine Science, Faculty of Fisheries and Marine Science, Universitas Diponegoro
[2]Marine Natural Product Laboratory, Centre for Research and Services, Universitas Diponegoro
[3]Tropical Marine Biodiversity Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Diponegoro
[4]Department Public Administration, Faculty of Social and Politic Science, Universitas Diponegoro
[5]Faculty of Fisheries and Marine Sciences, Universitas Sam Ratulangi
Jl. Prof. Soedarto, SH, Tembalang, Semarang, Central Java, Indonesia
[6]Agriculture Faculty, Univeristas Tadulako
Jl. Soekarno Hatta No.KM. 9, Mantikulore, Palu, Sulawesi Tengah 94148 Indonesia
[7]School of Natural and Environmental Sciences, New Castle University
Agriculture Building, King's Road, Newcastle upon Tyne NE1 7RU
[8]Centre for Conservation and Restoration Science, Edinburgh Napier University
[9]School of Applied Sciences, Edinburgh Napier University
Sighthill Campus, Sighthill Court Edinburgh, EH11 4BN.
Email: agustrianto.undip@gmail.com

Abstract

Mangroves are complex, unique, and dynamic ecosystems that host organisms that have special physiological adaptations to fluctuations in salinity, temperature, and pH. Gastropods have an important role in the mangrove ecosystem for food cycles and webs by helping to provide nutrients and micro-habitats for microbes. Micro-fungi isolated from mangrove ecosystems are productive sources of enzymes and bioactive compounds such as antibacterial and antifungal. In 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 meth. 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). Most fungal isolates have moderate growth rate. 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%). A. niger 19MB-C5-3 has potential as a bioremediation and anti-vibrio agent that can be applied in aquaculture.

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 detritivores through mangrove litter (Ka 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

*) Corresponding author

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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 *et al*., 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 environment pollution (Kusumaningrum & Zainuri, 2015; Sarjito *et al*., 2018). This paper discusses 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 (MER USA) with three repetitions. Isolation media was supplemented with 100 mg.L^{-1} 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 h, 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 h, 32°C). 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 d, 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 h and 2 x 24 h which was seen by the presence of a clear zone around the fungal disk (Cherkupally *et al*., 2017).

Screening of cellulase Activity

The fungal isolates were regrown on MEA media and incubated at room temperature (25°C) for

7 d. 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 h at 25°C. The cellulolytic activity was observed by pouring Congo Red solution on the test culture until it was completely submerged for 30 mins 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 (Sari *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 aquabidest (ddH2O) for a total volume of 24 l. The PCR setting refers to Suharna *et al*. (2019) by preheating 95°C for 3 mins. It was repeated in 34 cycles for denaturation at 95°C for 1 min, annealing at 56.1°C for 1 min, extension at 72°C for 1 min, followed by a final extension at 72°C for 7 mins. 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 *et al*., 2016). The phylogenetic tree construction was carried out using the MEGA 7 application using the neighbor-joining tree method with 1000x bootstrap. (Kumar *et al*., 2016).

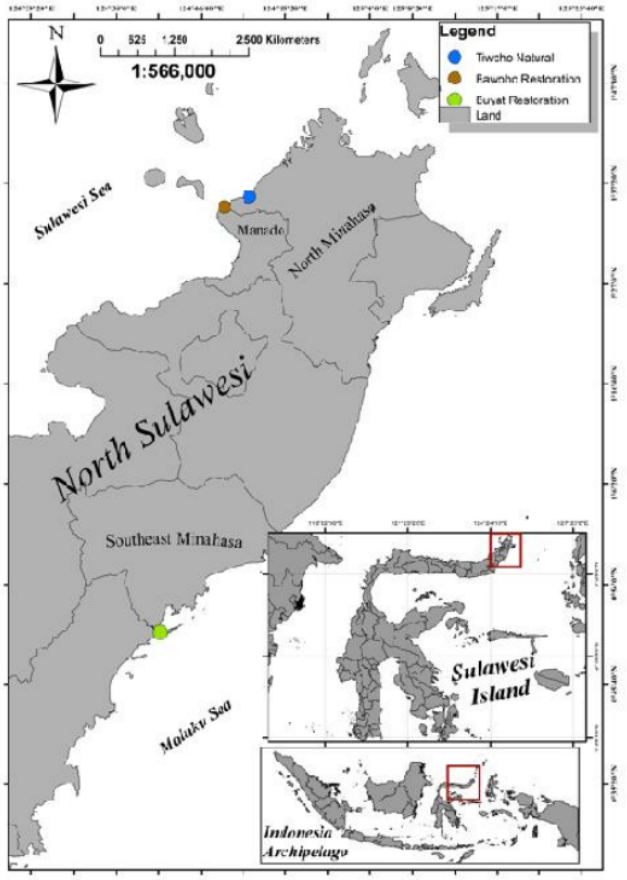


Figure 1. Site of gastropod sampling on mangrove ecosystems in three regions of North Sulawesi (Tiwoho, Bawoho, and Buyat)

a b

Figure 2. Gastropods a. *C. cingulata*., b. *L. scabra* collected from the mangrove ecosystem

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* is charaterized with sharp conical shell with many-whorled spire and rather small aperture that obviously can be seen. The key identification to this species is the 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 and Suryanti, 2019), Aceh besar (Irma and Sofyatuddin, 2012), Tanah besar (Nugroho *et al.,* 2019), and Cilacap (Sugiarto *et al*., 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.

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 and 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. 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 and 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 h 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 h or 48 h. 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 1. Morphology gastropods-associated fungal of *C. cingulata* and *L. scabra*.

Code	Color	Growth Pattern	Filament	Spore	Growing
19MB-C5-1	Light yellow	Spread	+	-	Medium
19MB-C5-2	Pale green	Swarm	-	-	Medium
19MB-C5-3	Black	Spread	+	+	Medium
19MB-C5-4	Pinkish white	Spread	+	-	Slow
19MB-C5-5	White	Spread	+	-	Medium
19 MT-07-1	Greyish brown	Spread	-	+	Medium
19 MT-07-2	Brown	Spread	-	+	Medium
19 MT-07-3	Orange white	Like cotton	+	-	Slow
19 MT-07-4	Yellowish green	Spread	-	+	Fast
19 MT-18-1	White	Spread	-	+	Medium
19 MT-18-2	Grey	Spread	+	-	Slow
19 MT-18-3	White	Spread	+	-	Fast
19 MT-18-4	White	Spread	+	-	Fast
19 Mba-A4-1	White	Spread	-	+	Fast
19 Mba-A4-2	White	Spread	+	-	Medium
19 Mba-A4-3	Grey	Spread	+	-	Medium

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

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 and 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 and 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 extracted 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.). 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 and 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 and 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 *et al*., 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 in 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 *et al*., 2021; Ooi *et al*., 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 *et al*., 1994). The isolate 19MB-C5-3 is the most potential because of it anti-vibriosis and enzymatic activities. The biotechnological potential (Figure 3.). Isolate 19MB-C5-3 was identified using molecular method. The quality of DNA isolation is visualized using geldoc as in Figure 4. 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%.

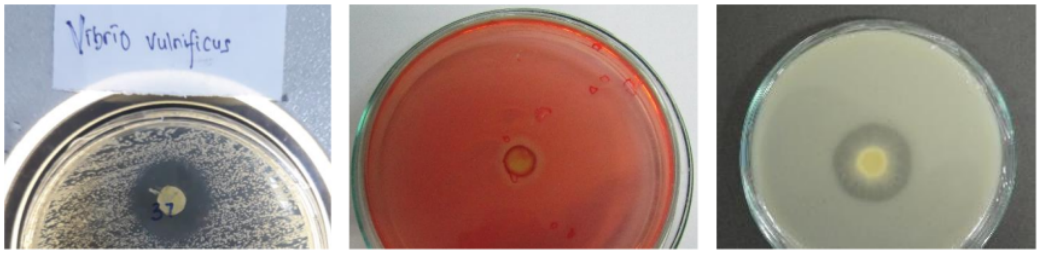
Table 3. Screening of enzymatic activity of fungal isolates from *C. cingulata* and *L. scabra*.

Isolate Code	Cellulolytic		Proteolytic	
	24 h	48 h	24 h	48 h
19MB-C5-1	-	-	-	+
19MB-C5-2	+	+	-	-
19MB-C5-3	+	++	++	++
19MB-C5-4	-	-	-	-
19MB-C5-5	-	-	+	+
19MT-07-1	-	-	-	-
19MT-07-2	-	-	-	-
19MT-07-3	-	-	-	-
19MT-07-4	-	-	-	-
19MT-18-1	+	+	+	-
19MT-18-2	-	-	-	-
19MT-18-3	++	+	-	-
19MT-18-4	-	+	-	-
19MBa-A4-1	++	++	+	+
19MBa-A4-2	-	-	-	-
19MBa-A4-3	+	++	+	+

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

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



a b c

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

The primers ITS1 and IT[26] 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*., 2011). 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.

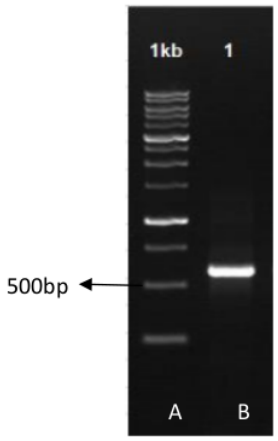


Figure 4. A. DNA ladder, B. DNA template of *C. cingulata* associated fungal isolate19MB-C5-3

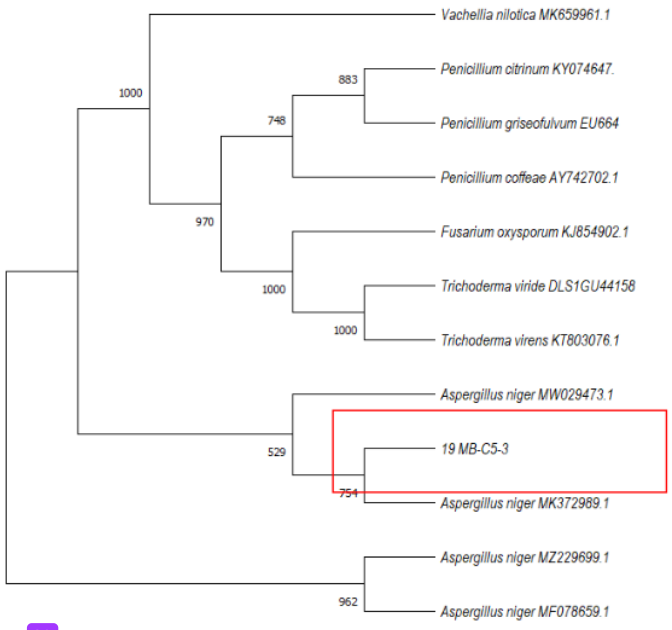


Figure 5. Phylogenetic tree based on the internal transcribed spacer (ITS) regions genes of *C. cingulata* associated fungal 19MB-C5-3

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 and 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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