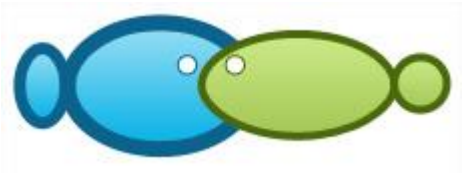

Characteristics of bioactive compounds of *Holothuria atra* (Jaeger, 1833) associated bacteria

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Abstract. Sea cucumbers have bioactive compounds with health benefits. These active compounds can be used as raw materials for marine aquaculture. This study aims to determine the potential of sea cucumber intestine-associated bacteria as a raw material for aquaculture through the investigation of its characteristics using the GC-MS method. *Holothuria atra* samples were collected from December 2018 to April 2019, from Bandengan, Jepara waters, Central Java, Indonesia. The solid-liquid method is used in the extraction process. Open Column Chromatography (OCC) is used in the fractionation process. Anti-bacterial sensitivity test was carried out using the Kirby-Bauer agar diffusion method. This study found 26 bacterial isolates resulting from the isolation of black sea cucumber (*H. atra*) symbionts. Of the 26 bacterial isolates, 9 bacterial isolates were found to have antibacterial activity against multi-drug resistant bacteria. 2 isolates were selected from the 9 test results, namely: T.1.2, which formed a 7.3 mm zone of inhibition against *Escherichia coli* and 7.8 mm against *Staphylococcus aureus*; and T.1.13, which was only active against *S. aureus* with an inhibition zone of 8.2 mm. Identification of selected isolates showed that isolate T.1.2 matched with *Bacillus manliponensis* species with 97% homology and that isolate T.4.1.25 matched with *Bacillus oceanisediminis*, with 95% homology. GC-MS analysis found methyl hexadecanoate/methyl palmitate, bis (2-ethylhexyl) -1,2-benzene dicarboxylate, 9-octadecenal, glycerol-1,3-dihexadecanoate and diisooctyl-1,2-benzene dicarboxylate in sea cucumber intestine-associated bacteria.

Key Words: culture, GCMS, Holothuroidea, intestines.

Introduction. Most sea cucumber species are found in shallow water ecosystems, up to a depth of 50 m, and are usually caught by fishermen by diving. This slow-moving animal does not have a special physical protection structure to defend itself from predator attacks. For survival and self defense, sea cucumbers use secondary metabolites by producing bioactive compounds such as holothurin or saponins and triterpene glycoside, which is a compound derived from saponins (Khotimchenko 2018; Bahrami et al 2018; Kamyab et al 2020). The saponin component has a similar structure to an active component from ginseng, ganoderma. These compounds present antibacterial, antifungal, antitumor, anticancer and antiseptic activities (Alfonso et al 2007; Farouk et al 2007; Adibpour et al 2014; Saad et al 2016; Ghadiri et al 2018).

Sea cucumbers also produce compounds that are antioxidants (Bordbar et al 2011; Nursid et al 2019), namely compounds that are adversarial to free radicals. One of the antioxidant compounds found in sea cucumbers is the SOD (superoxide dismutase) enzyme (Esmat et al 2013). The total antioxidant activity varies, depending on the species or type of sea cucumber (Oh et al 2017; Hou et al 2019). Other active compounds found in sea cucumbers are cell growth factors (CGF). CGF is responsible for stimulating the process of cell regeneration and accelerating the healing of physical wounds (Zang et al 2012; Wu et al 2014; Guo et al 2020). Sea cucumbers are known to have a variety of bioactive compounds useful as a basic material in fighting pathogenic bacteria in marine aquaculture. Therefore, the characteristics of some compounds from

sea cucumber-associated bacteria have significant real-world application potential. Based on this explanation, this study aims to determine the bacteria associated with sea cucumbers that produce bioactive compounds and to analyze the active compounds by the GC-MS method.

Material and Method

Sample collection. *Holothuria atra* samples were collected from December 2018 to April 2019 from Bandengan, Jepara waters, Central Java, Indonesia. 3 individuals with a weight of more than 300 g were selected, immediately put into a container previously prepared with sterile sea water, and stored in a cool box. The samples were then immediately transferred to the Laboratory of the Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, Semarang, Indonesia, for further analyses.

Isolation and purification of symbiotic bacteria. The samples were washed using clean, sterilized seawater to eliminate aquatic bacteria. Dissection from each sample was performed and the intestines were processed using a blender, and diluted in 5 mL of sterile seawater. The result of dilution was spread evenly on all media surfaces. To obtain pure isolates, the 26 isolates were stored on slanted agar media. Separation and purification of bacterial isolates was carried out by the streak method (Radjasa & Sabdono 2003).

Antibacterial activity test and molecular analysis of bacterial isolates. The diffusion method was used in the antibacterial activity test of sea cucumber isolates against MDR strains of *Staphylococcus aureus* and *Escherichia coli*. Antibacterial activity test was carried out by the diffusion test method. The isolate bacteria used were 2 days old, while the test bacteria was 1 day old (24 hours). The age of 1 day was chosen because the test bacteria were at their maximum growth. The initial preparation began with providing sterile petri dishes with 20 mL of Zobell 2216E media in each petri dish (Radjasa & Sabdono 2003). Molecular analysis of bacterial isolates conducted include DNA extraction (Walsh & Duffy 2013) and DNA amplification with 16S rDNA PCR (Radjasa & Sabdono 2003).

DNA extraction. DNA extraction was carried out by the Chelex method (Radjasa et al 2007; Lienhard & Schäffer 2019). Bacterial cells were dissolved in 50 µL of Aqua Bidest and 1 mL of 0.5% saponin in phosphate-buffered saline (PBS). The solution was allowed to sit for one night at 4^{o}C and was then centrifuged at 12000 rpm for 10 min. The supernatants from centrifugation were removed. 1 mL of PBS was added and the solution was then re-centrifuged. The supernatant from this process was removed to eliminate saponins. 100 µL of Aqua Bidest and 50 µL of 20% chelex were added to the solution. The solution was heated for 10 min and after that it was processed in a vortex machine for 5 min. The preparation solution was then rotated in a centrifuge at 12000 rpm for 10 min. The extract was stored in a freezer for 24 hours. After being taken out from the freezer, the quantity of DNA in the extract was determined using a nanodrop device.

PCR 16S rDNA amplification. DNA amplification was achieved using the 16s rDNA Polymerase Chain Reaction (PCR) method. 16S rDNA is a standard method for studying phylogenetic and diversity of marine microorganisms (Radjasa & Sabdono 2003). The primers used for 16S rDNA PCR in this study were universal primer 27F for bacteria (5'-AGAGTTTGATCMTGGCTCAG-3‘) and primer 1492R specific for eubacteria (5'-TACGGYTACCTTGTTACGACTT-3') (Isnansetyo & Kamei 2003). After the result of the DNA amplification was obtained, PCR product electrophoresis and visualization, PCR product purification, DNA sequencing, BLAST homology (Ramsay et al 2000) and phylogenetic analysis were carried out.

GC-MS analysis. The extracts were analyzed using Gas Chromatography-Mass Spectroscopy (GC-MS), with a Shimadzu QP 5000. A sample of 1 µL was injected into GC-MS, which was operated using a 25 m long glass column, 0.25 mm in diameter and 0.25 µm thickness, with a stationary phase of CP-Sil 5CB with a programmed oven temperature between 70-270°C with a rate of rise in temperature of 10°C min^{-1}, a helium carrier gas with a pressure of 12 kPa, and a total rate of 30 mL min^{-1} with a split ratio of 1:50.

Results and Discussion. Isolation of black sea cucumber intestine-associated bacteria obtained 26 isolates. Each isolate has a distinctive colony characteristic. Aspects that distinguish the characteristics of each of these isolates are form, texture, color and margin. Some similarities in the characteristics were observed in several different isolates, based on the morphology of the colonies. Based on their form, there were 2 isolates that were irregular, 3 isolates that were irregular (spindle), and 9 isolates that were circular. Two isolates with a nucleated circular form were also found. There were 4 isolates with spindle form and also nucleated irregular-circular and irregular-circular forms. There were 2 colonies with irregular-circular and 2 colonies with nucleated irregular-circular forms. It was also observed that there were irregular widening and irregular elongated shapes. Each colony observed had at least one of the colony shape characteristics previously described.

Based on its margin, filamentous, entire and erose forms were found in the bacterial colony in this study. Only one isolate had filamentous edges. 7 isolates were with erose margins and 18 other isolates were with entire margin. Based on texture, 19 isolates were convex and 9 were raised. The coloration of the bacterial colonies observed in this study ranged from off white, greenish yellow, milky yellow, yellow, yellowish white, orange, deep orange and clear white. The color characteristics of these symbiotic bacterial isolates have different hues in each isolate. White has 3 types of hue: off white in 2 isolates, yellowish white in 2 isolates, and clear white in 8 isolates. The orange color in the bacterial colonies observed showed 2 kinds of color variations, namely orange in one isolate and reddish orange in another isolate. The yellow color shows 3 color variations: yellow in 8 isolates, yellowish white in 2 isolates, and greenish yellow in 2 isolates.

Quantitative test of antibacterial activity on multi-drug resistant (MDR) strains. Quantitative test was carried out using agar diffusion methods with 6 mm paper discs. 26 bacterial isolates were tested against *S. aureus* and *E. coli*. Of all the isolates tested, 9 isolates were found to have antibacterial activity. 2 isolates were vetted for further testing based on their ability to inhibit the growth rate of the test bacteria, namely isolates T.1.13 and T.4.1.25. Isolate T.1.13 was chosen because it showed a small but clear zone of inhibition zone, with a size of 8.2 mm against *S. aureus.* The second isolate, coded T.4.1.25, was chosen because of its significant inhibition zone, which was 8.76 mm, although the isolate was only able to inhibit one type of test bacteria.

Identification of bacterial species. The results of DNA amplification from isolates T.1.13 and T.4.1.25 are presented in Figure 1. Isolates T.1.13 and T.4.1.25 produce a single band with a size of about 1500 bp (base pair) comparative to the DNA markers.

Molecular analysis of phylogenetics. The results of the 16S rDNA homology search results from isolates T.1.13 and T.4.1.25 with the DNA sequence of the Gene Bank database using the BLAST system are presented in Figures 1 and 2. The comparison of bacterial isolates with data records from the Gene Bank database is presented in Table 1.

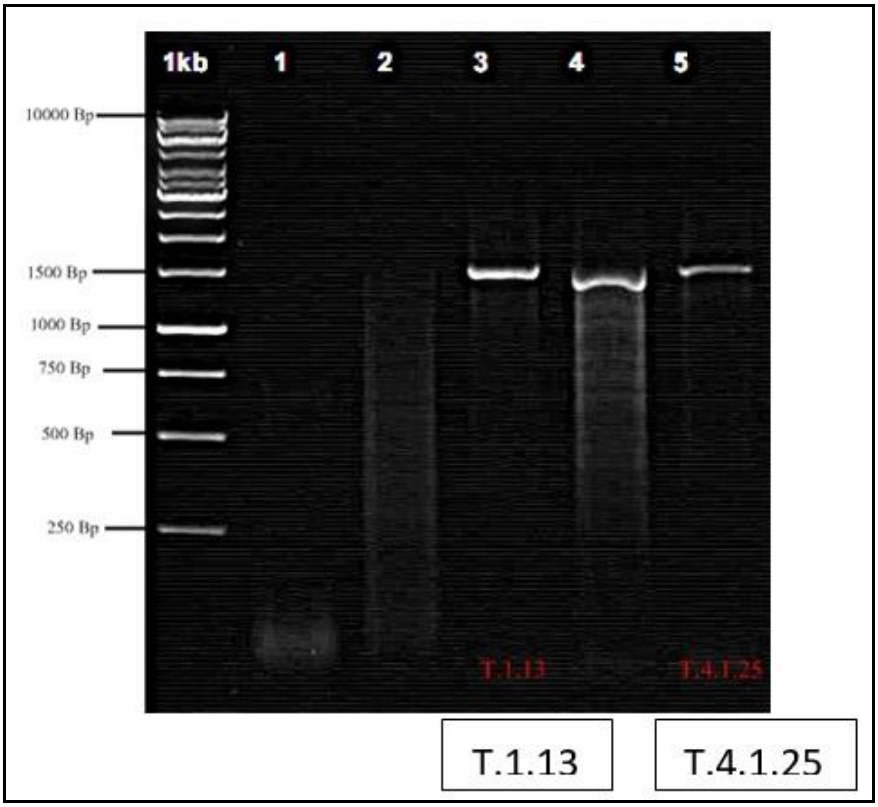

Figure 1. DNA amplification of T.1.13 and T.4.1.25.

Table 1

BLAST homology of bacterial isolates from black sea cucumber (*Holothuria atra*)

No	*Isolate*	*Relative match*	*Homology*	*Access record no*
1	T.1.13	*Alteromonas marina*	95%	NR0252601
2	T.4.1.25	*Bacillus oceanisediminis*	95%	NR117285.1

Correlation values between isolates T.1.13 with sequences in the database (>95%) and T.4.1.25 with sequences in the database (>95%) indicate that the matches in this molecular identification are at least at the genus level.

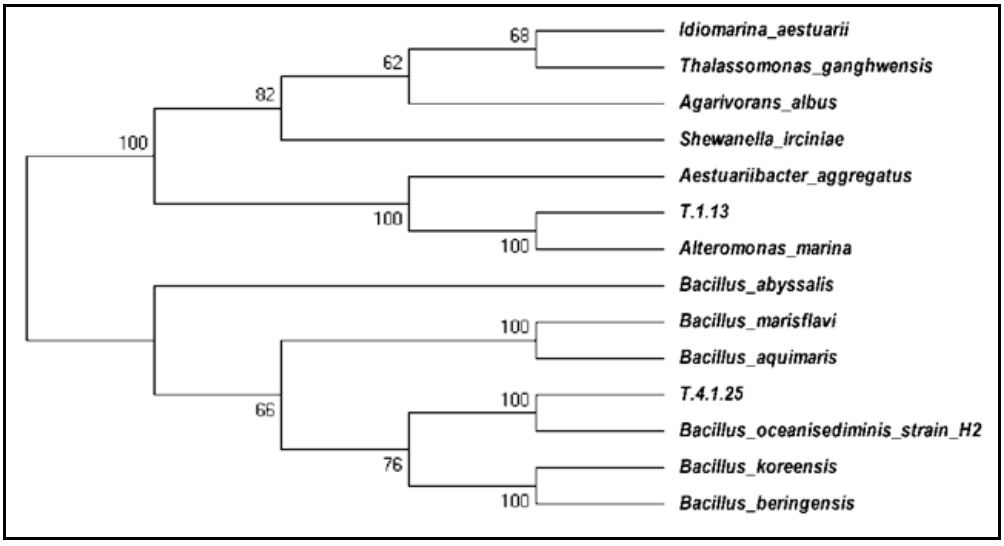

Figure 2. The phylogenetic tree of isolates.

Identification of black sea cucumber extract components was achieved by comparing the mass spectrum fragmentation pattern with the reference compound fragmentation pattern using WILLEY9THN 08.L databank. The results are presented in Tables 2 and 3.

Table 2

Identification of compounds using GC-MS

No peak	*Retention time (min)*	*Width of peak (% area)*	*Index of similarity*	*Name of compound*	*Molecular formula*
1	15.002	0.14	96	Methyl hexadecanoate / methyl palmitate	$C_{17}H_{34}O_2$
2	20.433	0.23	96	Bis (2-ethylhexyl) -1,2-benzene dicarboxylate	$C_{24}H_{38}O_4$
3	20.878	99.63	98	Diisooctyl-1,2-benzene carboxylate	$C_{24}H_{38}O_4$

The major component of the sea cucumber extract with isolate T.1.13 (retention time of 20.887 min) and T4.1.25 (retention time of 20.814 min) is diisooctyl-1,2-benzene dicarboxylate with a molecular mass (Mr) of 390 g mol^{-1}. Other components found are methyl hexadecanoate/methyl palmitate (Mr 270 g mol^{-1}) and glycerol-1,3-dihexadecanoate (Mr 569 g mol^{-1}). Both of these compounds are classified into lipids, as well as 9-octadecenal long chain aldehyde compounds (Mr 266 g mol^{-1}).

Table 3

Identification of compounds using GC-MS

No peak	*Retention time (min)*	*Width of peak (% area)*	*Index of similarity*	*Name of compound*	*Molecular formula*
1	20.188	6.61	87	9-octadecenal	$CH_3(CH_2)_7CH=CH(CH_2)_7CHO$
2	20.372	3.82	77	Glycerol-1,3-dihexadecanoate	$C_{35}H_{68}O_5$
3	20.814	89.57	95	Diisooctyl-1,2-benzene carboxylate	$C_{24}H_{38}O_4$

Isolation of bacteria associated with black sea cucumbers (*H. atra*) produces bacterial colonies with different shapes, textures and colors. There were 26 bacterial isolates which were successfully isolated from the black sea cucumber intestine. Bacterial morphology is influenced by the state and age of the medium. If bacteria are grown in a solid medium, a bacterial colony will be formed (Jeanson et al 2015; Chacón et al 2018). The difference in the shape, texture and color of bacterial colonies from the isolates indicate that there is a diversity of bacterial species symbiotic with black sea cucumber. Morphological differences and the number of colonies from bacterial isolates formed indicate that there are several species of bacteria that live in symbiosis with black sea cucumbers. Chacón et al (2018) state that the shape of the colony is different for each species, being characteristic for a particular species. Environmental parameters that affect bacterial abundance include specific isolation areas, temperature variations, pressure rises and, in some cases, salinity (Marietou & Bartlett 2014; Canfora et al 2014; Louca et al 2016).

H. atra is one of the marine invertebrates that produce secondary metabolites. A secondary metabolite is a compound produced by an organism that can be an indication of its response to the environment. Marine biota in the tropics face various challenges and compete for growth space, sunlight, food and avoid predators. Chemical compounds are useful in preventing and defending against predator attacks as a medium of competition, preventing bacterial infections, helping reproduction and preventing overexposure of ultra violet rays (Paul et al 2006; McClintock et al 2010).

Based on the qualitative tests, 9 isolates of symbiotic bacteria which indicated the ability to inhibit the development of the test bacteria were obtained. The isolates were

T.1.1, T.1.2, T.1.6, T.1.8, T.1.13, T.2.15, T.5.2.19, T.3.2.20 and T.4.1.25. Antibacterial activity is demonstrated by the formation of inhibition zones that appear as clear areas surrounding the paper disk. The inhibitory zones produced differ from one another because of the many types of bioactive compounds produced by these symbiotic bacteria. These results prove that these bacterial isolates have the potential to produce antibacterial compounds and inhibit the growth of test bacteria. The inhibition of the growth of the test bacteria can occur due to several factors, including the existence of competition as an effort to obtain space and nutrition between the isolates of the symbiotic bacteria and the test bacteria as well as the presence of a secondary metabolite excretion system in the symbiotic bacteria (Pringgenies & Dananjoyo 2011). Pringgenies & Renta (2014) concluded that if there are 2 competing species grown in the same environment, then one species will produce a compound that is toxic, thus disturbing the growth of rival species. Bacteria will develop self defense mechanisms to cope with threats to their survival.

The ability of each symbiotic bacterial isolate to inhibit the growth of test bacteria varies. Of the 26 symbiotic bacterial isolates, 1 isolate was found to be able to inhibit 2 test bacteria with code T.1.2. The remaining 8 isolates were only able to inhibit the growth of 1 type of test bacteria. This might be caused by a number of factors, including that *E. coli* and *S. aureus* are antibiotics-resistant species. According to Egorov et al (2018), these bacteria are able to produce enzymes that damage the structure, change the target site and block access to the target antibiotic site, so that secondary metabolite compounds from the bacterial isolates in this study may be unable to inhibit the growth of pathogenic bacteria. Bacteria develop different self-defense capacities. They develop a defense mechanism to counter threats to their survival. One such threat is environmental change and the presence of foreign substances, which can interfere with both intracellular and extracellular activities. Bacteria that are not able to neutralize these damaging substances will not be able to survive in the environment.

Environmental conditions can also affect the growth of bacteria. Less than ideal conditions can reduce or inhibit the ability of symbiotic bacteria to produce secondary metabolites. Conversely, favorable environmental conditions will positively influence the production of secondary metabolites. Verschuere et al (2000) showed that the difference in media resulted in differences in the number of bacterial isolates with antibacterial activity. The differences in the number of test bacteria that can be inhibited may be caused by differences in the components of the test bacterial cell walls (Pringgenies et al 2019; Sukmiwati et al 2019). Gram-negative bacteria have a more complex cell wall structure and contain more lipid components compared to gram-positive bacteria, which strengthen the cell walls of this type of bacteria.

Based on qualitative tests, the isolates with the best inhibitory ability and the size of inhibition zone formed against pathogenic bacteria were selected for further testing. Two symbiotic isolates of black sea cucumber bacteria were selected in this study. The first isolate was T.1.1.3, with an inhibition zone of 8.2 mm against *E. coli* test bacteria. The second chosen isolate is T.4.1.25. This isolate was chosen because of its ability to inhibit the growth of *S. aureus* and because its diameter of the inhibitory zone is the most sizable, at 8.76 mm, between isolates with the same activity. The antibacterial activity of black sea cucumbers against pathogenic bacteria shows its potential as a material for antibacterial agent products. The use of sea cucumbers as a source of antibacterial ingredients can increase the preservation and commercial value of this species (Bordbar et al 2011).

Electrophoresis results from 16S rDNA gene amplification showed that isolates T.1.13 and T.4.1.25 had a single band that was about 1500 bp compared to the DNA marker used. According to Radjasa & Sabdono (2003), the size of 1500 bp corresponds to the expected size of the 16S rDNA gene of bacteria, which are commonly between 1500-1600 bp. The DNA replication of the bacterial isolates that obtained single bands indicated that the primers used were specific primers to amplify the 16S rDNA gene in bacteria. Likewise, the ones used in the amplification reaction had already been in the right conditions. Amplification of 16S rDNA has become a standard for studying the phylogenetic and diversity of marine microorganisms (Radjasa & Sabdono 2003).

Sequencing using BLAST searching indicated that the symbiotic isolates of black sea cucumber symbiotic bacteria with code T.1.13 had a homology of 95% with *Alteromonas marine*. The process also indicated a homology match between isolate T.4.1.25 with *B. oceanisediminis*, with a value of 95%. According to Hagström et al (2002), the results of 16S rDNA sequencing with a homology value above 97% can represent the same species. While the compatibility of the sequence with a value between 93-97% can identify bacterial taxon at the genus level.

Some bacteria of the genus *Bacillus* are malicious pathogens for humans and animals. *B. manliponensis* and *B. oceanissediminis* are gram-positive, spore forming, and rod-shaped species. *B. manliponensis* is an anaerobic species, while *B. oceanissediminis* is an aerobic species. *Bacillus* is naturally found in various environments, including those that live freely or are pathogenic. Some *Bacillus* species produce extracellular enzymes such as protease, lipase, amylase, and cellulase that play an important role in the digestive system of animals (Raveendran et al 2018). This genus is also included in five commercial probiotic products consisting of bacterial spores that have potential for colonization, immunostimulation, and as antibacterial agent (Duc et al 2004).

Conclusions. The isolation process resulted in 26 isolates of black sea cucumber-associated bacteria. Of the 26 bacterial isolates from *H. atra*, 9 bacterial isolates had antibacterial activity against multi-drug resistant bacteria. Two isolates were selected from the 9 test results, namely T.1.2, which formed a 7.3 mm zone of inhibition against *E. coli* and 7.8 mm against *S. aureus*, and T.1.13, which was only active against *S. aureus* with an inhibition zone of 8.2 mm. Identification of selected isolates showed that isolate T.1.2 matched with *B. manliponensis* with 97% homology and that isolate T.4.1.25 matched with *B. oceanisediminis* with 95% homology. GC-MS analysis of sea cucumber intestine-associated bacteria found methyl hexadecanoate/methyl palmitate, bis (2-ethylhexyl) -1,2-benzene dicarboxylate, 9-octadecenal, glycerol-1,3-dihexadecanoate and diisooctyl-1,2-benzene dicarboxylate.

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Received: 12 June 2020. Accepted: 03 July 2020. Published online: 21 August 2020.
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How to cite this article:
Santosa G. W., Djunaedi A., Susanto A., Pringgenies D., Ariyanto D., 2020 Characteristics of bioactive compounds of *Holothuria atra* (Jaeger, 1833) associated bacteria. AACL Bioflux 13(4):2161-2169.