Antibacterial Properties of Bacteria Associated with a Marine Sponge from Thousand Islands, Indonesia

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Abstract. Marine invertebrates, especially sponges, have been recognized as the source of marine natural products with various bioactivities. The problematic sustainability of the natural product research from sponges drives the research change from the sponge to the associated microbes. In the attempt to find new antibiotic candidates, we screened marine sponges for their bioactivities. A Lithistid sponge showed potential antibiotic activity. The difficulty in obtaining large amounts of Lithistid sponge samples hampered the development of potentially active compounds from this material. Several researches proved that sponge-associated microbial play an important role for producing active compounds. In this study, we focused on sponge-associated bacteria to find novel and potential candidates for antibiotics. In total, we isolated 16 strains of microbes from the Lithistid sponge. A preliminary study of antibacterial activities from the strains M4 SP3 121015 101F showed that a sample has a strong activity against *Bacillus subtilis*. Partial identification of the bacterium using 16S rRNA Sanger sequencing showed 100% sequence similarity to *Staphylococcus equorum*. The preliminary identification of substances contained in ethyl acetate extracts using GC-MS showed several substances such as fatty acid, phenol, sterol, and halogenated organic compound. Further chemical analysis of the active fraction using LC-MS/MS showed a putative compound with m/z of 443.07 as the major component and responsible for the bioactivity of the bacterium. The predictions of fragmentation suggested that the compounds were 5-Bromo-4-fluoro-2-methoxyphenyl)(5-iodo-3-thienyl)methanol. This study reported for the first time that the antibacterial activity against *Bacillus subtilis* showed by the substance produced by marine *Staphylococcus equorum* isolated from the Lithistid sponge. Nonetheless, further research for detailed characterization of potential compounds is indispensable.

Keywords: marine product · Lithistid sponge · antibiotics · GC-MS · 16S rRNA

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1 Introduction

Infectious diseases such as tuberculosis and diarrhea are the leading causes of death in many developing countries. Indonesia becomes the second highest cases of tuberculosis in the world (1). Other case is diarrhea; it's become a second killer to the children under five years old in Indonesia and fourth killer in the world (1). The other hand, some antibiotics are getting ineffective to combat anti-infective diseases. The research to find new antibiotic producers should be done continuously.

Currently, researchers give more attention to microorganism as a rich source of antibiotic compounds. Microorganism has been recognized for its potential producing unique secondary metabolism that very useful for the human. Many marine microorganisms were found in the symbiotic relationship with marine invertebrate and make collaboration to produce many bioactive compounds such as the antibiotic, antitumor, antioxidant, anti-inflammatory and anti-viral (2).

One of the mutual symbiotic microorganisms is sponge-associated bacteria. The bacterial play an important role in the host defense mechanism by colonizing mesohyl tissue as endosymbiotic bacteria. Sponge produces many bioactive compounds that mostly synthesized by its symbionts (2). There are more than 5,300 different bioactive compounds isolated from sponges and more than 200 additional new compounds from sponges are reported each year (2, 3). Since 1990–2009, new marine natural products were produced from 17 orders of Demospongiae, and about 89% of natural products were produced by 8 orders including Lithistida. There were 300 compounds were isolated from the ordo Listhistid (2, 4) successed to isolate theopapuamida an cytotoxic compound derived from the Papua New Guinea lithistid sponge *Theonella swinhoei*. Four depsipeptide mirabamida (A-D) reported derived from the sponge *Siliquariaspongia mirabilis* collected from Sulawesi that active anti-HIV (5). Antimicrobial compound polidiscamide A and discobahamin A-B was isolated from the *Discodermia* sp These compounds reported active against *B. subtilis and C. albicans* (6, 7). Most of the compounds derived from ordo Lithistid were difficult to synthesize, that was became one of the reasons why the microbiotics associate from this group should be explored.

The investigation of microorganisms associated with Lithistid sponge was very important to know the potency of its symbiotic bacteria. Bioactive compounds derived from this sponge was should be investigated to know the relationship with the secondary metabolite produce by the cultivable symbiont, such as microorganism associated with Litisthid sponge. In this study the several works such as bacterial isolation from Lithistid sponge, antibacterial screening of isolated strains, fractionation of potent substances and chemical constituent identification of the potent bacterial extract were done. The purpose of this works was to find the antimicrobial compounds derived from cultivable microbial isolated from the Litisthid sponge.

2 Material and Methods

Various type of media was prepared to find the marine bacteria potential strain. In this study, media used to bacterial isolation were M1, M2, M3 and actinomyces-agar media (M4). M1 medium consist of 16g agar (Himedia), 10 g starch (Merck), 4 g

yeast extract (Himedia), 2 g Peptone (Himedia), 1 L tropical sea water, 50 mg nystatine (AppliChem Panreac). M2 medium contained 16 g agar (Himedia), 3,75 g marine broth (ROTH), 1 L tropical sea water, 50 mg nystatine. The composition of M3 medium were 16 g agar, 1 L sea water and 50 mg nystatine. M4 medium was consist of 1 L of actinomycetes agar-seawater medium contained meat infusion 10 g, tryptose 10g, casein enzymic hydrolysate 4 g, yeast extract 5 g, dextrose 5 g, L-Cysteine hydrochloride 1 g, starch soluble 1 g, sodium chloride 5 g, monopotassium phosphate 15 g, ammonium sulphate 1 g, magnesium sulphate 0.2 g and calcium chloride 0.02 g, 16 g agar and 1 L tropical seawater. Solvent used for extraction were ethyl acetate(Merck) and acetone (Merck).

2.1 Bacterial Isolation

About 1 cm^3 of sponge SP3 (*Lithistid* sp) collected from Untung Jawa Seribu Islands waters was homogenized in 5 mL seawater sterile. 100 μL of the sponge solution was dropped into 900 μL sterile seawater in 1.5 mL microtube. Dilution method was applied until concentration 10^{-3}. Approximately, 100 μL of the solution in each concentration (10^{-1}, 10^{-2} and10^{-3}) was dropped and spread on to various media such as M1,M2, M3 and actinomycetes-seawater media. After incubation about 2 weeks, each colony was picked and isolated to get a single strain.

2.2 Anti-bacterial Screening Test

All of isolated sponge SP3 associated bacteria were cultured in 10 mL of the test tube and incubated during 1 week at 20 °C using shaking incubator. After harvested, the bacterial broth was extracted using ethyl acetate and applied for anti-bacterial assay. The tested microbial was *Vibrio eltor, E coli*, *S. aureus* and *B. subtillis*, purchased from Microbial Laboratory the University of Indonesia. Disk diffusion method was applied for antibacterial assay (8). Approximately 100 μL of each extract was dropped into the paper disk and placed on the surface of nutrient agar media containing pathogenic bacteria. 10 μg of ampicillin was used as positive control. After overnight incubation at 30 °C the zone of inhibition was measured. The most potential strain that showed the highest activity against tested pathogenic bacterial was chosen for the further experiment.

2.3 Cultivation of Selected Strain

Selected strain (M4 SP3 121015) was cultivated around 15 L for preliminary analysis of metabolite contained. The medium used for semi-large cultivation was same as the isolation media. Approximately 1.5 L of a pre-culture solution of selected strain was mixed to the 900 mL actinomycetes broth medium. Incubation was done during 72 h in shaker incubator, at 28 °C, 100 rpm. After harvested, the bacterial broth was centrifuged at 6000 rpm to separate pellet and supernatant. Both of them were extracted using an organic solvent (ethyl acetate for supernatant and acetone for pellet).

2.4 Fractionation of Active Substances

About 1.098 g of selected extract bacterial strain M4 SP3 121015 isolated from Untung Jawa Lithisthid sponge was fractionated using normal phase open column silica F254 chromatography (normal phase). The mobile phase was using gradient system with hexane -ethyl acetate solvent.

2.5 Liquid Chromatography-Mass Spectroscopy (LC-MS) Analysis

LC-MS analysis was done to preliminary identification of active fraction. Method used for LC were as flow rate 0.2 ml/min, mobile phase were water/methanol gradient with the ratio water/methanol of 90–75% (0–5 min), 75–50% (5–10min), 50–25% (10–15 min), 25–50% (15–20min), 50–75% (20–25 min). MS condition are, scan time 0.00 to 50.00 min, with range mass 50.00 to 1200.00, using ion mode positive ES+ and ES-with scan time of 5 s. inter scan time 0.1 s, cone voltage 25 V.

2.6 Bacterial Characterization

The selected of bacterial strain was characterized using 16S-rRNA gene. DNA was isolated and amplified using PCR (Polymerase Chain Reaction) with universal 27F primer: 5′-AGAGTTTGATCMTGGCTCAG and 1492R primer: 5′- TACGGYTAC-CTTGTTACGACTT. 16S rRNA gene was bi-directional sequenced and compared for homology with BLAST database. Phylogenetic analysis with maximum likelihood tree with bootstrap analysis was reconstructed using MEGA7.

3 Results

Choosing the method of sponge-associated bacterial isolation was the first strategy to screen potential marine microorganisms. Media selective and the treatment of how the bacterial was isolated became the important factors to find the potential strain. Actinomycete media was selected because in the previous research, this media produced the most strain that active against several pathogenic bacteria, such as *Staphylococcus aureus, Bacillus subtilis*, *S. typhimurium*, and *E.coli* (32). Actinomycete media was containing very rich nutrient, especially for carbon and nutrient source.

There were 56 strains that isolated from Lithistid sponge by using actinomycetes media. About 28.57% (16 strains) of isolated strains showed active against pathogenic bacteria, such as *V eltor, E. coli, S. aureus* and *B. subtilis*. The diameter of the inhibition data was displayed in (Table 2). However, another study by Anand et al. (2006) reported that about 21% of bacterial strains that were isolated from 5 specimens of sponges showed antibacterial activity (Table 1).

Among 56 isolated strains from Lithistid sponge, 16 strains showed antibacterial activity. M4 SP3 121015 101F showed the strongest antibacterial activity especially against *Bacillus subtilis and* moderate activity against *E coli*. Although Ampicilin showed higher activity, based to this data, this strain was selected for further analysis.

Table 1. The screening result of isolated microbial strains from Lithistid sponge (Sp3) on M4 medium

No	Strain code	Diameter of inhibition (mm)			
	M4 SP3 121015	*V. eltor*	*E. coli*	*S. aureus*	*B. subtilis*
1	101f	6.7	10.55	nd	14.7
2	102a	6.8	10.6	9.2	-
3	102b	8.75	10.75	9.6	6.9
4	104b	8.9	8.85	7.2	-
5	101-3	8.55	8.20	nd	8.05
6	101-7	7.80	9.35	nd	9.40
7	102-11	7.35	_	nd	8.45
8	102-13	8.00	8.30	nd	8.48
9	103-15	9.25	9.00	nd	9.50
10	103-17	8.40	9.80	nd	9.08
11	103-19	10.85	9.05	nd	10.98
12	104-28	9.40	9.15	nd	10.60
13	104-29	8.70	8.30	nd	8.70
14	104-32	10.50	8.50	nd	10.32
15	104-35	8.90	8.50	nd	9.90
16	104-37	9.05	8.30	nd	8.65
17	Ampicilin	nm	16.70	nm	32.20

nd: not detected, nm:not measured.

3.1 Extraction, Fractionation and Preliminary Active Compounds Determination

Semi-large fermentation, 15 L of bacterial cultivation, resulting 1.5 g of ethyl acetate extract. Normal phase open column chromatography of this extract resulting 26 fractions. Antibacterial evaluation of resulted fractions showed that Fraction 3 (FK3) was active against *Bacillus subtilis* (15.45 mm), *Staphylococcus aureus* (15,70 mm), *Vibrio eltor* (18,15 mm), *Escherichia coli* (16.30 mm). Fraction 11 (FK 11 against *Bacillus subtilis* (11.40 mm), *Staphylococcus aureus* (11.55 mm), *Vibrio eltor* (13.50 mm) and *Escherichia coli* (13.15 mm).

The preliminary constituent analysis using Gas Chromatography-Mass Spectroscopy spectral data of ethyl acetate extract contained compounds that listed in Table2.

GC-MS analysis resulted several compounds contained in ethyl acetate extract were fatty acids such as 1-hexadecanoic acid ethyl ester, hexadecanoic acid, hydrocarbon: 1-hexadecane, 1-Nonadecene, Octadecane, cyclohexane,(1,2-dimethyl butyl), cyclotetracosane, dodecane,4,6-dimethyl, 3-octadecene,(E)-, stigmasta-3,5-diene (sterol), 3-Benzylhexahydropyrrolo [1,2-A] pyrazine-1,4-dione (, Bis (2-ethylhexyl) phathalate and halogenate compound tetrapentacontane,1,54-dibromo. The major compound

Table 2. The compounds list detected using GC-MS

RT(min)	Compounds	Quantity	Molecular weight
19.367	Phenol, 2,4-bis (1,1 -dimethylethyl)	1.66	206.329
21.939	1-hexadecene	2.68	224.432
22.256	Hexadecane	2.84	226.442
27.020	1-Nonadecene	3.42	266.513
27.117	Octadecane	3.07	254.502
27.710	2-Hydroxy-3,5,5-trimethyl-cyclohex-2-enone	3.55	154.206
28.434	Dodecane, 4,6-dimethyl	1.13	198.388
28.496	Hexane,2,3,4-trimethyl-	2.09	128.255
28.648	Dibutyl phthalate	3.32	278.348
28.868	Hexadecanoic acid, ethyl ethyl ester	15.46	284.484
29.006	Hexadecanoic acid	4,63	257.422
29.765	3-octadecene, (E)-	2.42	252.486
30.034	Hexadecanoic acid, ethyl ester	19.19	284.484
30.544	Tetrapentacontane, 1,54-dibromo-	3.16	917.266
30.840	Cyclohexane, (1,2-dimethylbutyl)-	3.74	168.324
30.951	Cyclotetracosane	2.46	336.648
31.289	3- Benzylhexahydropyrrolo [1,2-A] pyrazine-1,4-dione	9.3	244.289
31.799	Bis (2-ethylhexyl) phathalate	12.93	390.564
36.067	Stigmasta-3,5-diene	1.23	396.691

Table 3. LC-MS/MS data of potential fraction FK11.

RT(min)	molecular weight (g/mol)
1.62	303.54
7.58	564.57
9.46	477.92
12.18	443.07
13.03	700.94
13.20	491.14
14.91	716.81
17.21	1082.14
17.80	852.51

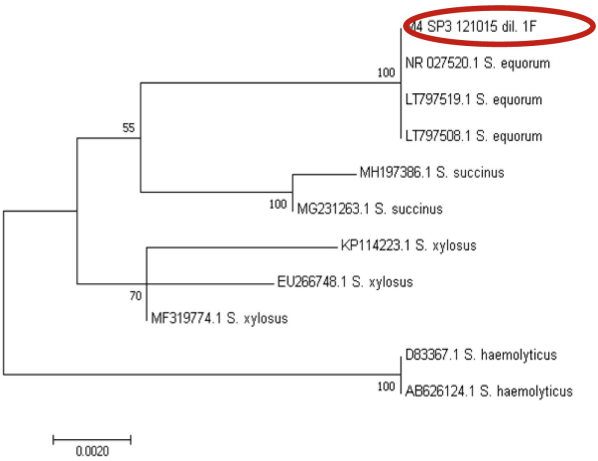

Fig. 1. Phylogenetic Analysis of 16S-rRNA of M4 SP3 121015 101F Gene

contained in the extract was hexadecanoic acid, ethyl ester. The interesting substance to be discuss were bis (2-ethylhexyl) phthalate and stigmasta-3,5-diene and benzylhexahydropyrrolo[1,2-A] pyrazine-1,4-dione (Table 3).

The major compounds showed at the retention time 12.18 min with the molecular weight 443.07. The fragmentation data of this peak were m/z: 443.07, 233.19, 211.05 and 21.76. This patern similar with the known MS spectrum of 5-Bromo-4-fluoro-2-methoxyphenyl)(5-iodo-3-thienyl)methanol ($C_{12}H_9BrFIO_2S$), describe in Fig. 1. While at the retention time 7.58 min was similar with the macrolactine B. ($C_{10}H_{44}O_{10}$).

3.2 Characterization of the Potential Strain

Morphological characterizations indicate that the strain M4 SP3 121015 101F were milky white with raised and entire shape. The microscopic analysis showed coccus and Gram negative bacteria. The band appears near the 1500 bp of marker was 16S-rRNA that used to specify of selected bacterial. Phylogenetic analysis of M4 SP3 121015 101F strain was closely related with *Staphylococcus equorum* with 100% homology (Fig. 1).

Picture at Fig. 1 showed that selected strain M4 SP3 121015 101F was closely identified as *Staphylococcus equorum* with 100% homology. S. *equorum* was known as bacteria that originally isolated from horse skin (9). Among the other strains that were isolated from Lithistid, *S equorum* showed the strongest activity against *Bacillus subtillis* (Tabel 2.).

4 Discussion

Isolation of the marine microorganism with the target of antibiotic producer need several strategies such as optimizing the nutrient (carbon & nitrogen) source (10). Besides the special primary target of actinomycete strain, other bacterial also grow easily by using dilution method for bacterial isolation. The potential antibacterial producer strains

isolated from Lithisthid sponge was promissing. This study was become important for investigating the source of antibiotics producer. *S equorum* M4 SP3 121015 101F was one of the isolated potential strains isolated from Lithistid sponge that produced active antibacterial compounds. In the food fermentation industry, *S. equorum* is a common microbiota that was used for fermented meat and cheese production (11,12) and high salted fermented seafood (13). *S. equorum* have been isolated from many different sources such as human tissue and body fluid (14), ostrich feces (15) seabed sediment (16), fermentation food (14, 31) and sponge (15). A few researchers have isolated *S. equorum* from marine invertebrate. Firstly, Anand group have reported *S. equorum* was isolated from *Spongia* sp (19). *S. equorum* contained two unique orthologs which encode potassium voltage-gated channel and a protein in ball domain, these genes contribute to high salt tolerance of *S. equorum* (13).

Among those known application of this bacteria, this study has revealed another potency of *S. equorum* to produce antibacterial compound against *B. subtilis*, *S. aureus, E. coli* and *V. eltor*. The data resulted from this study showed that besides being used for food processing, the *S. equorum* strain was very potential as antibiotics producer. While, many pharmacological activities such as antibacterial activity against *Listeria monocytogens* (12), antifungal against *Candida albicans* (14) and anti-parasite against *Plasmodium falciparum* was reported before (20).

The activity of another sponge associated bacteria was previously reported that several sponge associated bacteria producing the polyhydroxy alkanoate (18). GC-MS analysis indicated that the ethyl acetate extract of this strain contained several active compounds such as Stigmasta-3,5-diene, Bis (2-ethylhexyl) phthalate and3-Benzylhexahydropyrrolo [1,2-A] pyrazine-1,4-dione. Stigmasta-3,5-diene previously was reported contained in antibacterial extract of *Cordia rothii* (21). Bis(2-ethylhexyl) phthalate also reported active against *Candida albican* and several pathogenic bacteria (22, 23, 25, 17, 26, 27, 28). The 3-Benzylhexahydropyrrolo [1,2-A] pyrazine-1,4-dione isolated from Streptomyces sp. was a very potential candidate of antifungal compounds that very moderate cytotoxic against normal cell line RAW 264 (29).

LC-MS analysis of active fraction FK11 showed $[M]^+$: 443.07 g/mol. Compare to MS spectral data base, the molecular weight and fragmentation was similar to the compound 5-Bromo-4-fluoro-2-methoxyphenyl) (5-iodo-3-thienyl) methanol. The MS spectra showed at m/z 233.19 was the 5-Bromo-4fluoro-2 methoxyphenyl group and m/z 211.05 was 5-iodo-3-thienyl group ion molecule (Fig. 2). There was not bioactivity data reported before, regarding this compound. Another peak at retention time 7.58 min was predicted as macrolactin B. ($C_{10}H_{44}O_{10}$) with molecular weight 564,57 g/mol. This antimicrobial compound previously reported derived from *Staphyloccocus* sp. isolated from *Corallina officinalis* L. Macrolactin B was reported active against *Escherichia coli, Staphylococcus aureus subsp. aureus*, and *Pseudomonas aeruginosa* with MIC 100 μg/mL (30).

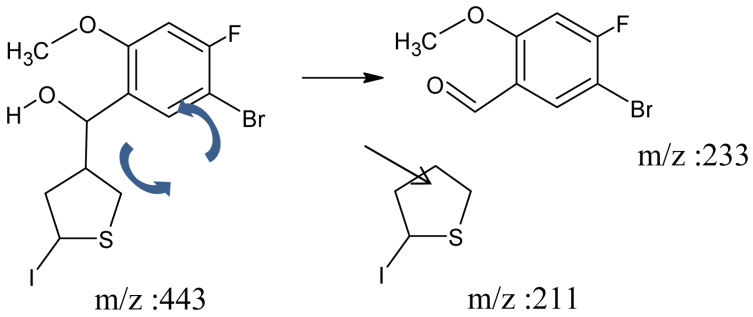

Fig. 2. The ion molecule fragmentation of major compound in fraction FK11.

Otherwise the fingerprint of several compounds on ethyl acetate extract and active fraction of *S.equorum*, already detected by using GC-MS & LC-MS, some unknown compounds need further investigation. Further purification and characterization of unknown compounds should be done to get comprehensive information about how the value of this strain.

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