Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Original article doi: 10.12980/jclm.4.2016J6-189

The antibacterial capacity of marine bacteria isolated from sponge *Acanthella cavernosa* collected from Lombok Island

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ARTICLE INFO

Article history:
Received 14 Sep 2016
Accepted 22 Sep 2016
Available online 27 Sep 2016

Keywords:
Antibacterial
Sponge-associated bacteria
Acanthella cavernosa
Phenolic compounds

ABSTRACT

Objective: To find a potent antibiotic producer from the sponge-associated bacteria as well as to profile the important substances.

Methods: Sponge collection, bacteria isolation, extraction and characterization of potent active compounds were carried out for this study.

Results: Approximately 59 single strains of bacteria were isolated from this sponge. Totally 40 strains showed activity against *Escerichia coli*, *Staphylococcus aureus* and *Vibrio eltor*. The chemical separation of the potent strain *Bacterium* sp. Lb.10%.2.1.1.b, using n-phase column chromatography revealed 7 active fractions (7, 8, 9, 10, 11, 14 and 15). The gas chromatography-mass spectrometer analysis of Fraction 7 indicated some phenolic compounds including 4-nonylphenol, methyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate, acetosyringone, 2,4-bis(1-phenylethyl)phenol, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester, tri(2-ethylhexyl) trimellitate and oleamide.

Conclusions: Indeed, this is a preliminary information in profiling chemical substances, produced by *Bacterium* sp. Lb.10%.2.1.1.b. Further purification and structural chemical determination were needed to find a comprehensive result.

1. Introduction

Indonesian is known for having rich marine biodiversity, such as sponges; about 850 species are found in eastern Indonesian seas[1]. Since sponges are becoming known as top bioactive producers, research investigating drugs from these marine biota is flourishing. The biggest problem in developing a drug based on a sponge's resources is the difficulty of improving the mass culture of sponge biomass. Their complicated and unstable chemical structure makes them difficult to synthesize. Several researchers reported a role of the relationship between the host and a symbiotic microorganism in producing a secondary metabolite through mediated mechanisms. Sponge-associated microorganisms play an important role in the biosynthesis of the host's secondary metabolite[2-4].

Investigating microorganisms for antibiotic producers is a sophisticated way to solve the problem in developing drugs from sponges. A study on *Acanthella cavernosa* (*A. cavernosa*) with its associated fungus *Fusarium* sp. reported that both of them produced succinic acid that is active against pathogenic bacterial and larval settlement[5]. This study showed the mutual relationship between the host and its symbiont in secondary metabolite production. Another study about the potency of *A. cavernosa* and the sponge's surface of associated microorganisms was the producing antiinfective and antiparasitic compound kalahinol. Changing the bacterial community on this sponge's surface has optimized the antiparasitic activity and increased the kalahinol production[6].

The capacity of Indonesian *A. cavernosa* and its microbial symbiont to produce antibacterial compounds was investigated to obtain the data and compare to the previous work in a different sampling location.

2. Materials and methods

Associated bacteria were isolated, antibacterial activity was screened, and selected bacterial extracts were fractionated. A separation technique using open column chromatography and gas chromatography-mass spectrometer (GC-MS) analysis were done to profile the secondary metabolite compounds.

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Foundation Project: Supported by the Riset Unggulan LIPI (Grant No. 3403.02.066A).
The journal implements double-blind peer review practiced by specially invited international editorial board members.

2.1. Isolation of sponge-associated bacterial sample

The sponge *A. cavernosa* voucher was collected from Teluk Kode, Lombok, Nusa Tenggara on 11 June 2014. Direct plating was applied for isolating the symbiotic bacteria[7]. Approximately 1 cm^3 was washed with sterile sea-water, macerated and stirred in 5 mL of sterile seawater. An aliquot (100 µL) sponge solution was diluted in 900 µL sterile seawater, transferred to the second tube until the dilution rate was 10^{-4}. Approximately 100 µL sponge solution at every concentration was poured into a 10% and a 100% marine agar plate and spread. After 1–2 weeks of incubation at room temperature (28–30 ˚C), the single colony was isolated and transferred to the 100% marine agar medium. The isolated bacterial strains were stored in 20% glycerol stock at –20 ˚C.

2.2. Cultivation and antibacterial screening test

Each bacterial strain was cultured in 5 mL marine broth and incubated at 28 ˚C, 150 r/min. After 3 days, the bacterial broth was centrifuged at 6000 r/min to separate biomass and supernatant. The supernatant was extracted using ethyl acetate, while pellets were extracted using acetone. Organic solvent was removed using rotary evaporator. The dried extract was weight and stored in a refrigerator before being used for an antibacterial test.

The pellet and supernatant extract were diluted in methanol with a concentration of approximately 100 µL. The antibacterial test was carried out using the agar diffusion method[8]. Gram-positive bacteria used for bioindicators were *Staphylococcus aureus* ATCC 25923 (*S. aureus*) and *Bacillus subtilis* ATCC 6633 (*B. subtilis*), and wild strain *Escherichia coli* (*E. coli*) were purchased from the Microbiology Laboratory of the Indonesian University on 10 May 2014. Ampicillin and kanamicin were used for positive control. The potent strain that gave positive result to antibacterial test was sent to the Indonesian Culture Collection Laboratory for molecular characterization.

2.3. Mass cultivation of the potent bacterial strain and chemical separation

The strain with the highest antibacterial activity continued to be used for further analysis. About 10 L bacterial cultures were set up for antibacterial substance analysis. Approximately 10 L marine broth media was prepared with the pH adjusted to around 7.8. About 10 mL of the selected culture was poured into 10 L marine broth media, incubated in a rotary shaker incubator with a temperature of 28 ˚C at 110 r/min for 72 h. Bacterial broth was harvested and centrifuged at 6000 r/min at 4 ˚C for 15 min. The pellet and supernatant were separated and extracted using an organic solvent. The supernatant was extracted using ethyl acetate and evaporated to get the extract. The pellet was extracted using methanol and partitioned using ethyl acetate.

The ethyl acetate extract of pellet was chromatographed using the n-phase solvent system. *n*-Hexane-ethyl acetate and methanol was gradually applied to the silica gel column. Each fraction was collected and evaporated for antibacterial testing. The same method was applied to the supernatant extract.

2.4. GC-MS analysis

GC-MS analysis was conducted at the Regional Health Laboratory, Jakarta. The potent open-column fraction was injected into Agilent Technologies 7890A GC with autosampler and 5975 Mass Selective Detector and Chemstation Data System. This instrument was set to electron impact using ionization mode with electron energy 70 eV. The column used for analysis was a capillary column HP Ultra 2L, 30 m × 0.25 mm × 0.25 µm film thicknesses. The oven temperature was set initially at 70 ˚C, rising at 3 ˚C/min to 150 ˚C, held for 1 min and finally rose for 20 ˚C/min to 280 ˚C where it was held for 26 min. The injection port temperature was 250 ˚C, with an ion source temperature of 230 ˚C, at the interface temperature of 280 ˚C and the quadrupole temperature of 140 ˚C. The carrier gas was helium with a column flow of 1.0 µL.

3. Results

3.1. Isolation of potent bacteria

The isolation of *A. cavernosa*-associated bacteria has resulted in 59 bacterial strains. Only 45 strains could be recultured for antibacterial screening test. The nutrition that was contained in 10% marine agar broth was suggested as the minimum to get the bacterial strains with a high ability to inhibit pathogenic bacteria. Almost all of the bacterial extracts showed activity against *S. aureus*, *B subtilis* and *E. coli*. Table 1 shows the result of the antibacterial screening test.

Table 1
Result of antibacterial screening of bacterial extracts.

No.	Extract bacterial code	Diameter inhibition (mm)		
		S. aureus	*B. subtillis*	*E. coli*
1	S.Lb.10%.1.12.2	8.650	10.450	9.400
2	S.Lb.10%.1.3.10	9.075	11.415	8.350
3	S.Lb.10%.1.12.3	10.175	10.550	8.650
4	S.Lb.10%.1.12.5	10.250	9.775	10.300
5	S.Lb.10%.1.3.1	8.800	12.900	10.475
6	S.Lb.10%.2.1.1b	14.850	11.750	8.450
7	S.Lb.10%.1.12.10	10.200	10.250	10.100
8	S.Lb.10%.1.5.3	10.225	8.750	7.700
9	S.Lb.10%.1.12.8	9.200	11.575	9.800
10	S.Lb.10%.1.3.5	8.300	9.050	8.300
11	S.Lb.10%.2.1.6	10.325	9.850	7.200
12	S.Lb.10%.1.12.9	9.000	7.425	0.000
13	P.Lb.10%.1.3.10	9.750	10.050	9.250
14	P.Lb.10%.1.12.3	9.600	4.100	9.550
15	P.Lb.10%.1.3.1	7.300	10.000	7.600
16	P.Lb.10%.1.12.5	12.600	9.650	6.800
17	P.Lb.10%.1.5.3	8.625	9.400	8.550
18	P.Lb.10%.1.12.8	9.100	9.150	0.000
19	P.Lb.10%.2.1.6	7.600	9.775	0.000
20	P.Lb.10%.1.12.9	11.075	10.950	8.000
21	P.Lb.10%.1.3.5	11.250	9.100	7.800
22	P.Lb.10%.2.1.1b	9.225	12.200	8.500
23	P.Lb.10%.1.12.10	8.750	8.100	0.000
24	P.Lb.10%.1.12.2	10.450	10.300	9.325
25	S.Lb.100%.1.3.1	11.100	9.250	9.300
26	S.Lb.100%.1.5.4	8.450	10.525	8.900
27	S.Lb.100%.2.1.10	9.600	8.850	8.200
28	S.Lb.100%.2.1.9	9.500	11.225	0.000
29	S.Lb.100%.1.12.6	11.150	8.925	8.100
30	S.Lb.100%.1.12.3b	10.575	11.600	0.000
31	S.Lb.100%.1.12.1b	11.625	12.275	7.800
32	S.Lb.100%.1.12.3a	7.500	9.300	7.200
33	P.Lb.100%.1.3.1	7.425	9.875	8.700
34	P.Lb.100%.1.5.4	7.200	0.000	8.300
35	P.Lb.100%.2.1.10	9.750	0.000	0.000
36	P.Lb.100%.1.12.1b	9.375	11.025	8.900
37	P.Lb.100%.2.1.9	7.675	6.900	0.000
38	P.Lb.100%.1.12.3a	9.100	9.000	0.000
39	P.Lb.100%.1.12.6	10.250	8.100	0.000
40	P.Lb.100%.1.12.3b	8.575	10.350	0.000
	Ampicillin	14.650	16.600	37.500

S: Supernatant; P: Pellet.

Almost 90% bacterial extract actively inhibited Gram-positive and Gram-negative bacterial growth. A total of 24 extracts belonged to bacteria that were isolated from 10% marine agar, while 16 extracts were from 100% marine agar. The most potent extract from strain Lb.10%.2.1.1b was confirmed for further analysis. Molecular characterization of strain Lb.10%.2.1.1b using 16S rDNA partial gene sequencing showed it to be 99% similar to *Bacterium* FJAT-17799.

3.2. Separation of potential extract

The extraction of 10 L bacterial culture resulted in 1 996.0 mg dark brown supernatant extract and 1 250.0 mg pellet extract. The open column chromatography of the supernatant extract using the *n*-hexane-ethyl acetate gradient system revealed 15 fractions. Open column separation of the pellet extract using n-phase chromatography contained 6 fractions. All of open column fractions were tested for antibacterial activity, as described in Table 2.

The most potential anti-Gram positive bacteria from the supernatant separation were Fractions 7, 8, 9, 10, 11, 14 and 15, showed by the diameter inhibition range of 12–18 mm. The remaining fraction showed moderate anti-Gram positive bacteria against *S. aureus* and *B. subtillis*.

Among 6 pellet fractions, high activity was shown by Fractions 1 and 3 and moderate by Fractions 2, 4, 5 and 6. Compared to the positive control kanamycin and ampicillin, all the fractions were weaker. The analysis of a single substance was needed to get a better conclusion.

Table 2
The antibacterial activity of column chromatography fractions.

Sample	Weight (mg)	Diameter inhibition in 100 µg (mm)	
		S. aureus	*B. subtillis*
Extract (supernatan)	1 996.0	12.000	11.450
Extract (pellet)	1 250.0	10.450	11.950
S. Fraction 1	179.6	9.800	12.050
S. Fraction 2	34.2	10.300	11.925
S. Fraction 3	1.1	11.225	10.200
S. Fraction 4	187.6	11.650	7.650
S. Fraction 5	50.5	10.050	7.975
S. Fraction 6	8.1	11.350	8.050
S. Fraction 7	11.9	18.850	18.350
S. Fraction 8	20.8	15.400	7.600
S. Fraction 9	13.5	12.725	8.150
S. Fraction 10	183.0	14.000	11.650
S. Fraction 11	20.7	13.725	13.875
S. Fraction 12	37.7	11.825	7.700
S. Fraction 13	162.7	10.650	7.500
S. Fraction 14	77.9	13.725	8.100
S. Fraction 15	700.3	15.350	15.150
P. Fraction 1	8.4	14.200	8.650
P. Fraction 2	23.5	10.750	8.750
P. Fraction 3	5.4	14.450	16.700
P. Fraction 4	6.6	11.300	15.775
P. Fraction 5	133.4	10.725	6.950
P. Fraction 6	16.6	10.825	6.950
Kanamycin		35.950	33.950
Ampicillin		44.450	33.600

3.3. GC-MS analysis of active fraction

Table 3 described the result of GC-MS analysis of Fraction 7. The highest quantity compound contained in Fraction 7 was 4-nonylphenol that appeared at retention time 29.592 min. The other compounds such as tri(2-ethylhexyl) trimellitate ($C_{33}H_{54}O_6$), 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester ($C_{16}H_{22}O_4$), phenol, 4-(1,1,3,3-tetramethylbutyl) ($C_{14}H_{22}O$) were quite amount in Fraction 7.

Table 3
Result of GC-MS analysis of S. Fraction 7.

No.	RT	% Area	Qual	Compound
1	3.452	0.31	95	Cyclohexanone ($C_6H_{10}O$)
2	20.725	0.85	94	2,6-Di(*tert*-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one ($C_{15}H_{22}O$)
3	28.124	0.99	72	Phenol, 4-(2,2,3,3-tetramethylbutyl) ($C_{14}H_{22}O$)
4	29.020	2.25	64	Phenol, *p*-nonyl ($C_{15}H_{24}O$)
5	29.241	7.05	87	Phenol, 4-(1,1,3,3-tetramethylbutyl) ($C_{14}H_{22}O$)
6	29.468	11.41	96	4-Nonylphenol ($C_{15}H_{24}O$)
7	29.592	6.89	96	4-Nonylphenol ($C_{15}H_{24}O$)
8	29.723	2.76	72	*p-t*-Amylphenol ($C_{11}H_{16}O$)
9	29.827	4.41	80	2-Methyl-6-t-butylanisole ($C_{12}H_{18}O$)
10	29.882	4.01	81	Phenol, *p*-nonyl ($C_{15}H_{24}O$)
11	29.944	2.71	72	9-Hydroxypyrimido[1,6-a]pyrimidin-4-one ($C_7H_5N_3O_2$)
12	30.054	8.25	83	Phenol, 4-(1,1-dimethylpropyl) ($C_{11}H_{16}O$)
13	30.158	5.81	60	Phenol, *p*-nonyl ($C_{15}H_{24}O$)
14	30.302	0.99	99	1-Octadecene ($C_{18}H_{36}$)
15	30.337	0.45	72	Phenol, 4-Octyl ($C_{14}H_{22}O$)
16	30.378	0.30	95	Octadecane ($C_{18}H_{38}$)
17	30.406	0.59	92	Phenol, *p*-nonyl ($C_{15}H_{24}O$)
18	30.468	0.73	72	Phenol, 4-(1,1-dimethylpropyl) ($C_{11}H_{16}O$)
19	30.530	0.74	64	Phenol, 4-(2,2,3,3-tetramethylbutyl) ($C_{14}H_{22}O$)
20	30.571	0.48	78	Phenol, 4-(1,1-dimethylpropyl) ($C_{11}H_{16}O$)
21	30.627	0.58	72	4-Nonylphenol ($C_{15}H_{24}O$)
22	30.682	0.49	74	Phenol, *p*-nonyl- ($C_{15}H_{24}O$)
23	31.054	0.37	72	1,2,4-Triazolo[4,3-b]pyridazine,6-chloro-8-(1-methylethenyl) ($C_8H_7ClN_4$)
24	31.330	0.30	43	(E)-2-Hydroxy-4'-cyano-stilbene ($C_{15}H_{11}NO$)
25	31.385	0.37	59	Benzoic acid, 2,3,4-trihydroxy-6-propyl, methyl ester ($C_{22}H_{26}O_8$)
26	31.633	0.33	98	Hexadecanoic acid, methyl ester ($C_{17}H_{34}O_2$)
27	31.750	0.47	90	2-*tert*-Butyl-3,3,7,7-tetramethyl-7H-tetrahydroindeno[bc]furan
28	31.792	0.37	99	Metyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate ($C_{19}H_{18}O_3$)
29	31.930	0.63	64	Cyclopentanecarboxylic acid, dodecyl ester ($C_{18}H_{34}O_2$)
30	31.957	0.55	96	Dibutyl phthalate ($C_{16}H_{22}O_4$)
31	32.137	1.40	97	3-Eicosene ($C_{20}H_{40}$)
32	32171	0.30	98	Icosane ($C_{20}H_{42}$)
33	32.233	0.44	38	9-[(2,2-dimethylpropanoyl)oxy]-9-borabicyclo[3.3.1]nonane ($C_{19}H_{28}BNO_2$)
34	32.316	0.58	38	Ethanone, 1-[1,1'-biphenyl]-4-yl ($C_{14}H_{12}O$)
35	32.392	3.61	91	1,4-Naphthalenedione, 4a,5,8,8a-tetrahydro-2-methoxy-4a, 8-dimethyl-, (4a.alpha.,8.beta.,8a.beta.) ($C_{13}H_{16}O_3$)
36	32.502	0.85	46	Benzaldehyde, 2-5-dimethoxy
37	32.763	0.97	99	E-15-heptadecenal ($C_{17}H_{32}O$)
38	32.854	0.55	35	Isolongifolene oxide ($C_{15}H_{24}O$)
39	32.950	0.31	35	Succinic acid, 3,4,5-trichlorophenyl undecyl ester ($C_{22}H_{29}CL_3O_4$)
40	33.033	0.19	87	1-Nonadecene ($C_{19}H_{38}$)
41	33.330	0.82	95	1-Docosene ($C_{22}H_{44}$)
42	33.695	0.38	86	Butyl citrate ($C_{18}H_{32}O_7$)
43	34.143	0.58	80	i-Propyl 11,12-methylene-octadecanoate ($C_{22}H_{44}O_2$)
44	34.274	0.59	99	Cyclotetracosane ($C_{24}H_{44}O_2$)
45	34.391	0.34	90	Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl) ($C_{10}H_{12}O_4$)
46	34.488	3.23	74	16,24-Cyclo-D(17a)-homo-21-nor-17a-oxachola-16, 20(22), 23-triene-4-carboxaldehyde, 23-hydroxy-4, 8-dimethyl-, (4.beta.,5. alpha.) ($C_{28}H_{40}O_2$)
47	34.557	0.60	87	Ethanone, 1-(4-hydroxy-3,5-dimthoxyphenyl) ($C_{10}H_{12}O_4$)
48	34.715	0.56	91	N-(2-(1,1,2,2,3,3,3-heptafluoropropyl)-4-methylene-1,3-oxazol-5(4h)-ylidene)2,6-dimethylaniline ($C_{15}H_{11}F_7N_2O$)
49	34.771	0.48	53	Dipropylene glycol dibenzoate ($C_{20}H_{22}O_5$)
50	34.895	0.30	93	Phenol, 2,4-bis(1-phenylethyl) ($C_{22}H_{22}O$)
51	35.074	4.26	91	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester ($C_{16}H_{22}O_4$)
52	35.205	0.28	99	9-Hexacosene ($C_{26}H_{52}$)
53	36.398	0.77	95	9-Octadecenamide ($C_{18}H_{35}NO$)
54	45.300	6.33	93	Tri(2-ethylhexyl) trimellitate ($C_{33}H_{54}O_6$)
55	45.410	4.82	70	Tri(2-ethylhexyl) trimellitate ($C_{33}H_{54}O_6$)

RT: Retention time.

4. Discussion

The most of potential active compounds were contained in supernatant extract. Adaptations to the marine environment such as high salinity, high pressure, low nutrient and low light scatter will trigger some marine bacteria producing secondary metabolite[9].

GC-MS analysis of active Fraction 7 indicated that compound 1 –

4-nonylphenol, appeared at a retention time of 29.468 min. It has the molecular weight of 202.36 g/mol. This compound is major containing in Fraction 7. The production of phenolic compounds in marine bacteria is strongly influenced by enzyme tyrosine-phenol-lyase[10-12]. The reported activity was anti-estrogenic and weevil pheromone[13]. Chalcone with nonylphenol chain was very promising as antifungal and antibacterial agent[14].

Compound 2 was methyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propionate with the retention time of 31.792 min and molecular weight of 292.34 g/mol. The previous study reported this compound confirmed for antibacterial and antioxidant with radical-scavenging activity mechanism. This compound is also found in the oil of the plant *Jatropa curcas*[15]. Compound 3 with retention time of 34.391 min, acetosyringone was widely used for analgesic, antipyretic, anti-inflammatory and anti-asthmatic drugs. This compound was also isolated in many plants including the seagrass *Posidonia oceanica*[16].

Compound 4 (retention time 34.895 min) was named phenol, 2,4-bis(1-phenylethyl) with the molecular weight of 302.41 g/mol. This compound was also isolated from the plant *Zanthoxylum integrifoliolum* and reported as having anti-inflammatory activity[17]. Compound 5 appeared at retention time of 35.074 min was 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester. This compound showed cytotoxic activity. This was previously isolated from marine *Streptomyces* sp. with the molecular weight of 278.34 g/mol[18]. Compound 6, namely wastri(2-ethylhexyl) trimellitate, with retention time of 45.3 min and molecular weight of 546.78 g/mol, showed anticancer activity[19]. Compound 7 (retention time 36.398 min) was oleamide, the fatty acid amide that showed activity as a hypnotic agent (modular neuro function)[20]. Some active fractions should be analyzed for characterizing their unknown active substances.

Investigation of *A. cavernosa*-associated bacteria, *Bacterium* FJAT-17799 showed potential antibacterial agents. Several active compounds detected by GC-MS spectrum were 4-nonylphenol, methyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate, acetosyringone, 2,4-bis(1-phenylethyl)phenol, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester, tri(2-ethylhexyl) trimellitate and oleamide. All of these compounds are reported to have biological activity and promising for the pharmaceutical industry.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors appreciate the Riset Unggulan LIPI for providing financial support (Grant No. 3403.02.066A). Great appreciation also goes to Mr. Dian Nurcahyo from Indonesian Culture Collection Laboratory for his help in bacterial characterization. We are also grateful to the laboratory staff of Laboratory Kesehatan Daerah DKI Jakarta for providing GC-MS instrument analysis.

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