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Antimicrobial and Antioxidant Activities of Bacterial Extracts from Marine Bacteria Associated with Sponge *Stylotella* sp.

[1]Daning Yoghiapiscessa, [2]Irmanida Batubara and [1]Aris Tri Wahyudi

[1]Department of Biology, Faculty of Mathematics and Natural Sciences,
[2]Department of Chemistry, Faculty of Mathematics and Natural Sciences,
Bogor Agricultural University, Jl. Agatis, Dramaga Campus, Bogor 16680, Indonesia

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Corresponding Author:
Aris Tri Wahyudi
Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Indonesia
Phone/Fax: +62-251-8622833
Email: aristri2011@gmail.com

Abstract: Sponge-associated bacteria have great potential in developing marine pharmaceutical industry since they are capable of synthesizing numerous bioactive metabolite compounds. This study aimed to isolate, characterize and investigate potential antimicrobial, toxicity and antioxidant of bioactive compounds of bacteria associated with sponge *Stylotella* sp. A total of 138 bacteria were selected. During screening stage, 45 isolates (32%) produced a wide spectrum and species specific bioactive compound against microbial test strains (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Eschericia coli* ATCC 8739, *Enteropathogenic Escherichia coli* (*EPEC*) and *Candida albicans)*. Crude extract of five selected potential bacteria were extracted using ethyl acetate solvent. They were identified to have a wide spectrum inhibition capability against microbial test strains. The extracts toxicity were examined using the Brine Shrimp Lethality Test (BSLT) method. All extracts showed toxicity of LC_{50} in the range of 35.89-484.17 μg mL^{-1} against *Artemia salina*. By using Cupric Reducing Antioxidant Capacity (CUPRAC) radical reduction test revealed all the extracts showed potential antioxidant activites, ranging from 445.33 to 1610.00 μmol trolox/g extract. Based on 16S rRNA sequence analysis revealed that isolates STIL 33, STIL 37, STIL 44, STIL 55 and STIL 9 were closely related with *Pseudoalteromonas flavipulchra* strain NCIMB 2033, *Serratia marcescens* strain NBRC 102204, *Catenococcus thiocycli* strain TG 5-3, *Vibrio natriegens* strain ATCC 14048 and *Bacillus subtilis* strain JCM 1465, respectively.

Keywords: Antimicrobial, Toxicity, Antioxidant, CUPRAC, Marine Bacteria

Introduction

Sponges are sessile aquatic animal of the phylum Porifera which obtain nutrients through filtering the surrounding water column, allowing them to associate with various microorganisms. It is estimated that more than 50% sponge tissue are symbiont microorganisms (Taylor *et al*., 2007). Sponge symbiont microorganisms are known capable of synthesizing numerous bioactive compounds with unique, complex and different structure compared with terrestrial microorganisms. Secondary metabolites of sponge microbes are adaptive and play key roles in their host defense mechanism against pathogens, predators and competitors, in addition to in fouling process and competition for space and food resources. Nowadays, it is known that marine sponges and their associated microorganisms are economically important because they produce the most active bioactive compounds that are potentially useful for human in pharmaceutical industry sector, for instance as antimicrobial, antifungal, antioxidant, anticancer and anti-inflammatory agents (Jeganathan *et al*., 2013). Bioactive compounds mostly found in sponge symbiont microorganisms are generally derived from alkaloids, terpenes, terpenoids and peptides (Mehbub *et al*., 2014).

Numerous types of infectious diseases caused by pathogenic bacteria or yeast are one of basic problems in biomedical field that need to be seriously controlled. It

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requires continuous development to obtain various types of new antimicrobial compounds capable of acting as therapeutic agents for infections caused by certain pathogenic microorganisms (Penesyan *et al*., 2009). The arising problems drive the exploration of antimicrobial compound from microorganisms associated with sponge. These bacteria are widely reported to be highly potential as the producer of antimicrobial bioactive compound (Nazim *et al*., 2014).

Antioxidant compounds have an important role in improving human health. The compounds can decrease the risk of multiple chronic degenerative diseases, for instance gastrointestinal infection, cancer, Alzheimer, cataract and coronary heart disease. Nowadays, alternatives of natural antioxidant compounds are sought and developed to obtain compound which is specific and has better activity. Sponge symbiont microorganism is one of study objects in finding new antioxidant compounds (Pereira *et al*., 2015). This is because sponge is symbiotic with various autotrophic microorganisms which perform photosynthesis and exposed to numerous free radical sources such as toxic, waste material and continuous intensity of UV radiation, indicating antioxidant activity of sponge bacteria (Taylor *et al*., 2007). There are several reports on antioxidant compounds which were successfully isolated from sponge bacteria. Acyl-glycol carotenoid acid which is the red pigment of *Rubritalea squalenifasciens* associated with sponge *Halicondria okadai* was reported to be antioxidant against the formation of radical chain (Thomas *et al*., 2010). Antioxidant activities of bacterial extracts have also been reported from bacteria associated with sponge *Jaspis* sp. collected from Waigeo island, Raja Ampat Papua, Indonesia (Utami *et al*., 2014).

In the present study, the research focus was to screen marine bacteria associated with sponge *Stylotella* sp. collected from Kepulauan Seribu-Indonesia producing bioactive compounds and to determine potential bacterial isolates through the antimicrobial, toxicity and antioxidant activities of the bacterial extracts.

Materials and Methods

Sponge Sample Collection

The marine sponge *Sylotella* sp specimens used in this study were collected from Bira island of Kepulauan Seribu, Indonesia. The sponges were manually obtained from 1.5 to 3.0 m depth with the help of snorkeling mask. Sponge sampling was randomly carried out in the area prior to direct transfer using plastic bags with seawater to prevent sponge tissues contact with air. The samples were then taken onto laboratory for further analysis.

Isolation of Sponge-Associated Bacteria

Dilution method was applied for the isolation of sponge-associated bacteria. One gram sponge sample was cleaned using sterile sea water to remove the non-attached bacteria. This material was suspended and homogenized in 9 mL 0.85% NaCl (0.85 g NaCl, 100 mL distilled water) and mixed by vortexing for 10 min. The serial dilution method was then carried out using 0.85% NaCl to obtain concentration ranging from 10^{-1} to 10^{-5}, where 100 µL was aseptically drawn using pipette from the last four dilutions and spread onto Petri dish containing Sea Water Complete medium (SWC) (5 g bacto peptone, 1 g yeast extract, 3 mL glycerol, 750 mL seawater, 250 mL distilled water, 15 g agar), Starch Casein Agar (SCA) (1 g casein, 10 g soluble starch, 20 g NaCl, K_2HPO4 0.5 g, 20 g agar, 1 L distilled water), Nutrient Agar (NA) (13 g nutrient broth, 1 L distilled water, 20 g agar), Zobell Marine Agar (ZMA) (40.25 g zobell medium broth, 1 L distilled water, 20 g agar) and Marine Agar (MA) (0.5 g peptone, 0.1 g yeast extract, 1 L sea water, 20 g agar). After that, all plates were incubated at 27°C for 48 h. The growing colonies were selected based on the morphological identification (shape, elevation, margin, surface characteristics and color). Representative of each colonies morphology were serially streak-plated on SWC media until pure cultures were obtained.

Screening of Bacteria Producing Antimicrobial Compounds

Agar-Overlay method was applied for this test. Media used for preliminary screening were SWC for antibacterial test and Potato Dextrose Agar (PDA) (24 g potato dextrose broth, 1 L distilled water, 20 g agar) for anti-yeast test. One mL of liquid culture of tested microorganisms each of *P. aeruginosa, S. aureus* (collection of Biotechnology Laboratory, Bogor Agricultural University)*, B. subtilis, EPEC* (collection of Microbiology Laboratory, Bogor Agricultural University), *E. coli* ATCC 8739 and *C. albicans* (collection of Microbiology and Parasitology Laboratory, University of Indonesia-Jakarta), was incubated for 24 h prior to inoculation in 100 mL SWC agar medium (antibacterial test) or PDA (anti-yeast test) at ±48°C and pouring into Petri dish (1% (v/v) of microbial test strains (concentration 1×10^6 CFU/mL (Colony-Forming Units per milliliter), OD_{620} = 0.45). The plates were then allowed to set at room temperature and dried. Each of 138 bacterial isolates was streaked on the surface of agar media that have been seeded with above microbial test strains. Plates were incubated for 24 h at 37°C. The antimicrobial compound activity was indicated by clear zone formed around bacterial colonies.

Hemolytic Test

The bacterial isolates that produced antimicrobial compound were tested to know the possibility of the bacteria include in pathogene bacteria using hemolytic test. The bacterial isolates were streaked onto blood agar medium and incubated overnight (24 h) at room temperature. Clear zone formed around the colony indicates the positive to lysis blood cells and proves the isolate to be potential pathogens in human. There was no further testing to such isolates.

Extraction of Bioactive Compounds from Bacteria

The bacterial isolates were cultured in 1 L SWC broth medium. The cultures were incubated and shaked at 100 rpm for 72 h at 27°C. After incubation, bacteria cultures were mixed with ethyl acetate solvent 1: 0.75 (v/v) ratio in two replications. This was carried out to minimize the saturation level of the solvent to bind bioactive compounds. Mixed solvent and bacteria cultures were stirred at 250 rpm for 12 h at room temperature. These mixtures were separated and the ethyl acetate layers were evaporated using rotary evaporator at 40°C. The extracts were vacuumed and stored below 5°C for further test (Muller *et al*., 2004).

Antimicrobial Activity Test

Antimicrobial activities of crude extracts were tested using agar diffusion method on paper disc (6 mm in diameter). A total of 13 µL crude extracts (5 mg mL^{-1}) dissolved in ethyl acetate were dropped on sterilized paper disk prior to drying using hair dryer followed with oven at 37°C for 2 h. Each paper disc was put on semisolid agar medium which was already been inoculated by 1% (v/v) of test liquid culture (*P. aeruginosa, S. aureus, B. subtilis, E. coli* ATCC 8739*, EPEC and C. albicans)* in cell concentration of 1×10^6 cell/mL (OD_{620} 0.45). Positive control used was 5 mg mL^{-1} ampicillin while the negative control was ethyl acetate solvent soaked-peper disc. Each palte was then incubated at 37°C for 24 h. The clear zone formed around the paper disc indicated activity of the bacterial extract to inhibit microbial growth.

Toxicity Test of Bacterial Extracts

The toxicity of bioactive compound from bacterial extracts were tested using BSLT method in 50% lethal concentration (LC_{50}). *Artemia salina* eggs (0.1 g) were inoculated in 200 mL sea water. The eggs were hatched for 2 h using aeration system with 30°C light. After hatching, active brine shrimp larvae (nauplii) that were free from their egg shells were collected using pipette. Stock solution of 5000 ppm was prepared by dissolving the crude extract 0.05 g in 500 µL of Dimethyl Sulfoxide (DMSO) that added to 9.5 mL of sea water. The bacterial extracts were dissolved in sea water with different concentrations (0 µg mL^{-1} as the control, 25, 50, 100, 250, 500 and 1000 µg mL^{-1}) in triplicate. After that, twenty individual nauplii were introduced to each concentration (Meyer *et al*., 1982). The number of dead larvae was counted after 24 h exposure. The amount of mortality and logarithm concentration were presented in a linear regression curve. LC_{50} is the extract concentration that can kill 50% *A. salina* larvae and can be calculated following Probit scale analysis.

Equation 1 Percentage mortality (%M) formula:

$$\%\ Mortality = \frac{\Sigma\ Nauplii\ Larvae\ Mortality - \Sigma\ Control\ Mortality}{\Sigma\ Initial\ Amount\ of\ Nauplii\ Larvae} \times 100\% \quad (1)$$

Determination of Antioxidant Activity

Antioxidant activity of each extract was determined using CUPRAC method. One mL of crude extract (500 µg mL^{-1}) in ethanol was added with 1 mL of 7.5×10^{-3} M Neucoprine, 1 mL of 10^{-2} M $CuCl_2$, 1 mL of 1 M NH_4Ac and 0.1 mL H_2O prior to incubation for 30 min. Standard curve was made using trolox with 50, 75, 100, 150, 200 and 400 µM in concentration. The sample absorbance and standard curve were then measured using ELISA reader at 450 nm wavelength. The Antioxidant capacity of crude extract was measured expressed in µmol trolox/g extract (Apak *et al*., 2008).

PCR Amplification of 16S rRNA gene and Sequence Analysis

Amplification of 16S rRNA genes was conducted using PCR using the universal primer for bacterial domain, 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al*., 1998) which targeted for conserved region and allowed the amplification of an approximately 1300-bp fragment. PCR was conducted in 25 cycles with the following conditions, pre-denaturation at 94°C for 2 min, denaturation at 92°C for 30 sec, annealing at 55°C for 30 sec, elongation at 72°C for 1 min and final elongation (post PCR) at 72°C for 7 min. The PCR products were purified and sequenced. The DNA sequences were analyzed using the BlastN program in National Center for Biotechnology Information (NCBI) GenBank database (http://ncbi.nlm.nih.gov/). The alignment of 16S rRNA genes was carried out using MEGA 5.0 software and the phylogenetic tree was constructed using neighbor-joining method with 1000x bootstrap replications.

Results

Isolation of Bacteria Associated with Sponge Stylotella sp

Total data calculation of sponge symbiont bacteria on various media showed that, based on total CFU of the highest dilution (30-300 colonies/dish), the highest number of total bacterial growth belonged to SWC, i.e., 6.6×10^6 CFU mL^{-1}. The second highest (6.0×10^6 CFU mL^{-1}) belonged to SCA, followed by NA with 3.3×10^6 CFU mL^{-1}, ZMA medium with 2.6×10^6 CFU mL^{-1} and the lowest, 1.4×10^6 CFU mL^{-1}, belonged to MA medium. The isolation of sponge *Stylotella* sp. symbiont bacteria was conducted using five series of dilution for each medium. A total of 138 types of bacteria based on morphological differences were found in the present study. The visual appearances of bacteria associated with sponge *Stylotella* sp. were mostly round-broken white colonies. There were few colonies which had pink, grey, orange and yellow in color pigmentation (data not shown).

Screening of Bacteria Producing Antimicrobial Compounds

The analysis result found potential antimicrobial compound activities during secondary screening, i.e., 45 (32%) out of 138 bacterial isolates with capability of inhibiting microbial test strains, or in another word, had antimicrobial compound activities in narrow to wide spectrum activity (Table 1). Twenty nine isolates were narrow spectrum activity and the other, 16 isolates were wide spectrum activity based on their capability of inhibiting more than one microbial test strain. Most of the isolates were capable of inhibiting the growth of *B. subtilis* (21 isolates), followed by inhibitory against *EPEC* growth (18 isolates), *P. aeruginosa* (17 isolates), *S. aureus* (11 isolates), *E. coli* (9 isolates) and the growth of *C. albicans* (8 isolates) (Fig. 1).

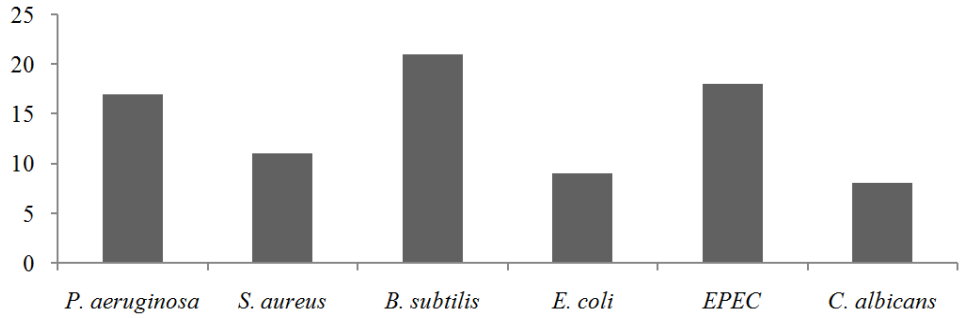

Fig. 1. Number of bacteria isolated from sponge *Stylotella* sp. which have antimicrobial activity against microbial test strains

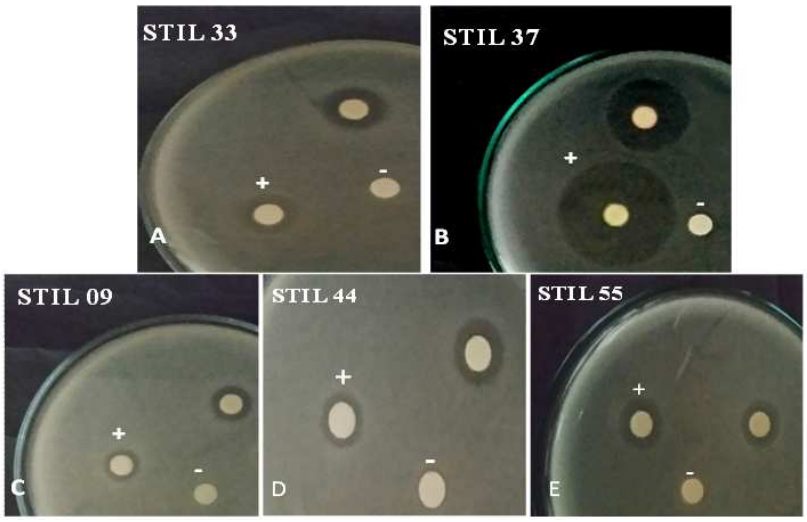

Fig. 2. Clear zones after 24 h incubation at 37°C formed around bacterial extracts. A. STIL 33 against *E. coli*, B. STIL 37 against *S. aureus,* C. STIL 09 against *C. albicans,* D. STIL 44 against *P. aeruginosa* and E. STIL 55 against *EPEC* (+ control = ampicillin 5 mg mL^{-1}, -control = ethyl acetate)

Table 1. Antimicrobial activity of sponge-associated bacteria against microbial test strains

No	Isolates Code	Tested Bacteria					Tested Yeast
		P. aeruginosa	*S. aureus*	*B. subtillis*	*E. coli*	*EPEC*	*C. albicans*
1	STIL 1	++	++	-	-	++	-
2	STIL 2	-	-	+	-	-	-
3	STIL 4	+	-	-	-	-	-
4	STIL 5	+	-	-	-	-	-
5	STIL 6	-	-	-	-	++	+
6	STIL 9	++	+++	++	++	+	+++
7	STIL 10	-	'+++	++	+++	++	+++
8	STIL 11	-	-	+	-	-	-
9	STIL 12	-	-	+ +	-	-	-
10	STIL 13	-	-	++	-	++	-
11	STIL 14	-	-	+	-	-	-
12	STIL 15	-	-	++	-	-	-
13	STIL 16	+	-	-	-	-	-
14	STIL 17	-	-	++	-	-	-
15	STIL 18	++	-	-	-	-	-
16	STIL 19	++	-	-	-	-	-
17	STIL 20	-	-	++	-	-	-
18	STIL 21	+	+	+	+	++	+
19	STIL 22	-	-	+	-	-	+
20	STIL 23	-	-	+	+	-	-
21	STIL 24	-	-	+	-	-	-
22	STIL 25	-	-	-	-	+	-
23	STIL 26	-	+	+	-	-	-
24	STIL 27	-	-	-	+	-	-
25	STIL 28	+	-	-	-	-	-
26	STIL 29	-	-	-	-	++	-
27	STIL 31	-	-	+	-	-	-
28	STIL 33	++	+	++	++	+	++
29	STIL 35	-	-	-	-	+	-
30	STIL 37	++	++	+	-	++	-
31	STIL 44	+	+	-	+	+	-
32	STIL 50	-	-	-	-	++	-
33	STIL 55	+	+	-	++	-	-
34	STIL 56	-	-	-	-	+	-
35	STIL 57	++	-	-	-	-	-
36	STIL 64	-	-	-	++	-	+
37	STIL 66	-	-	++	-	-	-
38	STIL 71	-	-	-	-	+	-
39	STIL 84	-	-	-	-	+	-
40	STIL 85	+	-	-	-	-	-
41	STIL 89	-	-	-	-	++	-
42	STIL 93	-	-	+	-	-	-
43	STIL 99	+	+	-	-	+++	+
44	STIL 104	-	-	++	-	-	-
45	STIL 106	++	++	-	-	-	-

Note: Weak +: 1-5 mm, medium ++: 6-10 mm, strong +++: 11-15 mm, -: no inhibitory activity, +: Positive inhibitory activity

Antimicrobial Activity Test

Five bacterial extracts with the best antimicrobial activity were extracted using ethyl acetate solvent (STIL 09, STIL 33, STIL 37, STIL 44 and STIL 55). The clear zone formed around paper disk indicated inhibitory activity toward bacterial or yeast test strains (Fig. 2), indicating that the sponge-associated bacteria bacteria produced bioactive compounds. The results showed that all extracts of bacteria associated with sponge were wide spectrum inhibitory activity, i.e., 5 isolates inhibited *P. aeruginosa, S. aureus and EPEC*; 4 isolates inhibited *E. coli*; and the other 3 isolates inhibited *B. subtilis*. Antimicrobial activity against yeast (*C. albicans*) was found in the two extracts, i.e., STIL 09 and STIL 33 (Table 2).

Hemolytic Test Analysis

The result showed that after 24 h incubation, 5 out of 45 isolates were positive capable of lysing blood

cells, indicated by the formation of clear zone around the colonies. The isolates were STIL 12, STIL 18, STIL 19, STIL 20 and STIL 93 (data not shown).

Toxicity of Bacterial Extracts

The toxicity of bacterial extracts were known through LC_{50} (Median Lethal Concentration). LC_{50} is the concentration in which 50% of larvae *A. salina* died after being given by extract treatment. Calculation results of the number of larvae *A. salina* mortality in comparison to the logarithm of the extract concentration were analyzed using Microsoft Excel program, which also gave the regression equations. The regression equations were used to calculate LC_{50} value. The Bioactive metabolite compound is toxic if it can kill 50% *A. salina* larvae less than 1000 µg mL^{-1} (LC_{50}) concentration. As shown in Table 3, five extracts from bacteria associated with sponge *Stylotella* sp (STIL 09, STIL 33, STIL 37, STIL 44 and STIL 55) were toxic against *A. salina*. The highest toxicity was performed by isolate STIL 37 with LC_{50} of 35.89 µg mL^{-1}, followed by STIL 09, STIL 33, STIL 44 and STIL 55 with LC_{50} of 69.50, 484.17, 82.79 and 156.68 µg mL^{-1}, respectively.

Antioxidant Activity of Bacterial Extracts

Antioxidant activity in reducing CUPRAC radical was successfully analyzed in five selected bacterial isolates. The activity was measured in antioxidant capacity unit (µmol trolox/g extract). Results revealed that the extracts from sponge symbiont bacteria had antioxidant capacity in the range of 445.33 to 1610.00 µmol trolox/g extract, with the highest capacity was performed by STIL 33 ethyl acetate crude extract (1610.00 µmol trolox/g extract) (Table 4).

Identification of Bacteria Based on 16S rRNA Gene

The 16S rRNA genes from five bacterial isolates were successfully amplified by PCR resulting 1300 bp DNA fragments. Those bacterial isolates (STIL 09, STIL 33, STIL 37, STIL 44 and STIL 55) were identified based on 16S rRNA gene sequence analysis. The BlastN results on NCBI Genbank revealed the bacteria associated with sponge *Stylotella* sp. belonged to the phylum Proteobacteria and phylum Firmicutes. There were 4 out of 5 bacterial isolates belonged to the phylum Proteobacteria, i.e., STIL 33 showed closely related with *Pseudoalteromonas flavipulchr*a NCIMB 2033, while isolate STIL 37 with *Serratia marcescens* strain NBRC 102204. Other classes were found in isolate STIL 44 showing closely related with *Catenococcus thiocycli* strain TG 5-3 and isolate STIL 55 closely related with *Vibrio natriegens* strain ATCC 14048. One isolate STIL 09 referred to the group from phylum Firmicutes, i.e., *Bacillus subtilis* strain JCM 1465 (Table 5).

Table 2. Antimicrobial activities of five selected bacterial extracts against microbial test strains

		Microbial Test Strain					
No	Isolates	*P. aeruginosa*	*S. aureus*	*B. subtillis*	*E. coli*	*EPEC*	*C. albicans*
1	STIL 09	++	++	++	+	++	++
2	STIL 33	++	++	++	++	+	+
3	STIL 37	++	+++	++	-	+++	-
4	STIL 44	++	++	-	+	++	-
5	STIL 55	++	++	-	+	++	-

Note: Weak +: 1-5 mm, medium ++: 6-10 mm, strong +++: 11-15 mm, -: No inhibitory activity, +: Positive inhibitory activity.

Table 3. LC_{50} values of the crude extracts of bacteria associated with sponge *Stylotella* sp

Isolates	LC_{50} (µg/mL)
STIL 09	69.50
STIL 33	484.17
STIL 37	35.89
STIL 44	82.79
STIL 55	156.68

Table 4. Antioxidant activity of bacterial crude extracts

Isolates	∑ Antioxidant Capacity (µmol trolox/g extract)
STIL 09	989.67
STIL 33	1610.00
STIL 37	1202.67
STIL 44	787.33
STIL 55	445.33

Table 5. Molecular identification of bacteria based on 16S rRNA gene

Isolates	Closest relative	Maximum identity	Accession No
STIL 09	*Bacillus subtilis* strain JCM 1465	100%	NR1184861
STIL 33	*Pseudoalteromonas flavipulchra* strain NCIMB 2033	100%	NR0251261
STIL 37	*Serratia marcescens* strain NBRC 102204	100%	NR1140431
STIL 44	*Catenococcus thiocycli* strain *TG 5-3*	100%	NR1048701
STIL 55	*Vibrio natriegens* strain ATCC 14048	100%	NR1178901

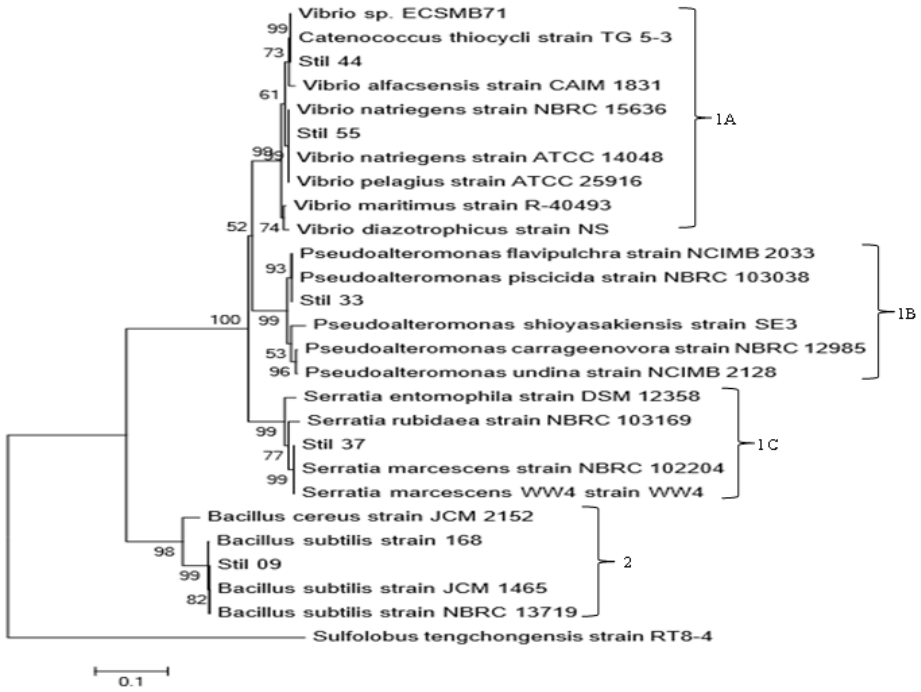

Fig. 3. Phyllogenetic tree of five bacterial isolates isolated from sponge *Stylotella* sp. compared with reference strains based on 16S rRNA gene sequences using Neighbor-joining method. The scale bar indicates 10% sequence divergence. Numbers at nodes represent bootstrap values in percentage based on 1000x replications. Clade 1 (Gram negative bacteria): Divided into three sub-clades, i.e., sub-clade 1A. Vibrionaceae (*Vibrio, Catenococcus*), sub-clade 1B. Pseudoalteromonadaceae (*Pseudoalteromonas*), sub-clade 1C. Enterobacteriaceae (*Serratia*); Clade 2 (Gram positive bacteria): Bacillaceae (*Bacillus*)

As shown in Fig. 3, there were two clades, clade 1 consisted of groups from gram-negative bacteria, while clade 2 of Bacillaceae group. Bacillaceae is gram-positive bacteria with bacil cell form, heterotrophic and capable of producing spores in stressed environmental condition. Clade 1 was consisted of three sub-clades, i.e., subs-clade 1a, 1b and 1c. Sub-clade 1a was consisted of family Vibrionaceae (genus *Vibrio*, genus *Catenococcus*), sub-clade 1b of family Pseudoaltero-monadaceae (genus *Pseudoalteromonas*) and sub-clade 1c of family Enterobacteriaceae (genus *Serratia*). Clade 2 was consisted of family Bacillaceae (genus *Bacillus*). In addition, *Sulfolobus tengchongensis* from Archaea strain RT8-4 was selected for out-group.

Discussion

In this study, bacteria associated with sponge *Stylotella* sp. producing bioactive compounds were isolated. A total of 138 bacterial isolates with different colony morphology were successfully isolated using five different media (SWC, SCA, NA, MA and ZMA).

High number of bacterial isolates obtained indicated that sponges widely associate with numerous microorganisms. Taylor *et al*. (2007) reported that 40-60% sponge tissues with density 10^9 cells mL^{-1} are symbiotic microorganisms.

The result of this study revealed that SWC media is relatively more effective as isolation media for sponge-associative bacteria than other media (SCA, NA, MA and ZMA), indicated with highest total bacteria (6.6×10^6 CFU mL^{-1}) growth in SWC. This was allegedly because complete nutrient composition was provided by SWC media. The media contains glycerol as carbon source and yeast extract as nitrogen source. Sea water composition in SWC media was allegedly responsible for significant increase in the growth of sponge-associated microorganisms. Sea water contains various dissolved

solid and gas contents, as well as various mineral salts such as calcium, magnesium, sodium, potassium, sulfate, bromide, bicarbonate and chloride. In addition, the salinity of sea water reaches up to 40‰, making it optimum for the growth of sea microorganisms (Sharqawy *et al*., 2010).

During screening stage, 32% (45 isolates) were found having potential antimicrobial compounds because they showed inhibitory activity toward tested microorganisms (Table 1). Several studies reported that 39 (31%) out of 125 bacteria isolated from sponge *Haliclona* sp collected from Raja Ampat Island, Indonesia show inhibitory activity toward at least one of tested microorganisms (Tokasaya, 2010). A total of 20 out of 68 isolates isolated from sponge *Jaspis* sp collected from Raja Ampat Island, Indonesia showed antimicrobial activity against microbial strains (pathogenic and non-pathogenic bacteria and yeast (Abubakar *et al*., 2011). Other researcher, Santos *et al*. (2010) have also reported that from 158 bacteria isolated from 9 types of sponge in Brazilian sea, 12 isolates (9%) of the total isolates had inhibitory activity toward at least one of the tested microorganisms. A total of 3 out 64 bacterial isolates from sponge *Aaptos* sp samples from Padang Panjang Island, Indonesia were reported capable of inhibiting the growth of Multi Drugs Resistant (MDR) strains (*E. coli* and *Proteus* sp) (Radjasa *et al*., 2007). In addition, Bharathiraja *et al*. (2014) reported that 14 bacterial isolates were isolated from sponge *Spirastrella inconstans*, 5 of them were pigmented and showed inhibitory activity against at least one indicator strain.

Five ethyl acetate extracts from bacterial isolates associated with sponge, which had wide spectrum inhibitory activity to the whole tested microorganisms were toxic, because it had range value of LC_{50} about 35.89-484.17 μg mL^{-1}. This showed that the extracts had ability, not only as a therapeutic agents to inhibit multiple tested microorganisms, but also as cytotoxic agents. In vitro cytotoxic activity test on *A. salina* was an initial screening to detect any potential anticancer and antitumor in tested extracts, because *A. salina* performed rapid cell division like cancer cells (Anderson *et al*., 1991).

Antimicrobial compounds with narrow spectrum activity were found in 29 bacterial isolates (Table 1), as they only were capable of inhibiting one specific tested microorganism. Further development, sponge associated bacteria with narrow spectrum activity were potential to be used in biomedical development as therapeutic agents to treat specific infectious disease after the causative organism is identified. Antimicrobial compounds with narrow spectrum have several advantageous compared with of wide spectrum ones. Species-specific antimicrobial spectrum will not inhibit the growth of normal bacterial flora and reduce the risk of bacterial resistance because it will only inhibit specific target bacteria.

The extracts from five bacterial isolates, i.e., STIL 09, STIL 33, STIL 37, STIL 44 and STIL 55 showed wide spectrum antimicrobial activity, they were able to inhibit many microbial test strains (Table 2 and Fig. 2). It indicated that the bacterial extracts contained bioactive compounds. Those isolates were to be the candidates for potential natural products producer for biomedical field development as chemotherapeutic agents to address various disease infections caused by Gram positive or Gram negative bacteria. In this study, isolates STIL 09 and STIL 33 were the candidate for potential chemotherapeutic agents producer against infectious diseases caused by pathogenic bacteria and yeast, while the others for development against infectious diseses caused by pathogenic bacteria.

BSLT using *A. salina* is a simple and fast bioassay method to test bioactivity of extract and has a positive correlation with potential antitumor and anticancer compounds (Anderson *et al*., 1991). BSLT method is represented with LC_{50} value (the concentration of tested extract which capable of killing 50% larvae *A. salina*). Bioactivity of the bacteria associated with sponge *Stylotella* sp showed that the toxicity was significant in the highest LC_{50} value of 35.89 μg mL^{-1} (Table 3). The results indicated that the fifth ethyl acetate extracts from bacteria were highly potential to be developed as anticancer agents. Ethyl acetate extracts of bacterial isolates associated with sponge *Stylotella* sp (STIL 09, STIL 37, STIL 44 and STIL 55). Based on the LC_{50} value, LC_{50} value of the bacterial extracts tended to be lower than LC_{50} of the fraction and hexane extract of *Typonium flagelliforme* with LC_{50} value ranged 381.07 to 762.08 μg mL^{-1} (Sianipar *et al*., 2013). Toxicity from marine bacteria have also reported by many researchers, such as Utami *et al*. (2014) said that extracts from marine bacteria associated with sponge *Jaspis* sp had a toxic effect with LC_{50} value in the range of 251.18 to 390.50 μg mL^{-1} which were performed by three bacterial isolates coded as HAL-74, HAA-01 and HAL-13 isolated from sponge *Haliclona* sp. at Waigeo Island, Raja Ampat District, Indonesia.

Antioxidant capacity of CUPRAC radical in this study was an equivalent ratio, expressed as Trolox equivalent Antioxidant Capacity (TEAC) (Apak *et al*., 2008). Antioxidant activity was derived from regression linier standard trolox curve analysis ($y = 0.0029x+0.0275$ $R^2 = 0.9963$). Out of fifth ethyl acetate extracts of bacterial isolates associated with sponge *Stylotella* sp, isolate STIL 33 showed the highest

antioxidant capacity, i.e., 1610.00 µmol trolox/g extract, while isolate STIL 37 also showed relative good antioxidant capacity in inhibiting CUPRAC radical with the value of 1202.67 µmol trolox/g extract (Table 4). Interestingly, both isolates contained bright pigment color on their morphologies phenotype, in which isolate STIL 33 produced orange color pigment, while isolate STIL 37 produced pink pigment. Isolates with no pigment in this study tended to show lower antioxidant activity. Several pigments have been reported to significantly contribute to the increasing of antioxidant activity. Yellow carotenoid pigment has an important role in photosynthetic aquatic microorganisms and photoprotection against UV radiation in sea water with its high antioxidant activity and capability of inhibiting the formation of cancer cell (Shindo *et al*., 2008). Acyl-glycol carotenoid acid, the red pigment of *Rubritalea squalenifasciens* associated with sponge *Halicondria okadai,* also had antioxidant activity, i.e., capable of inhibiting the formation of radical chain (Thomas *et al*., 2010). The antioxidant capacity of extract from bacteria associated with sponge *Stylotella* sp, STIL 09, STIL 33, STIL 37, STIL 44 and STIL 55 were 989.67 µmol trolox/g extract, 1610.00 µmol trolox/g extract, 1202.67 µmol trolox/g extract, 787.33 µmol trolox/g extract and 445.33 µmol trolox/g extract, respectively. Therefore, compared to bacterial isolates from sponges *Haliclona* and *Jaspis* sp symbiont which have antioxidant capacity of 649.92 µmol trolox/g extract (Utami *et al*., 2014), ethyl acetate extract of bacterial isolates in this study showed better antioxidant capacity. Bacteria isolated from sponge *Stylotella* sp also showed better activity compared to several Chokeberry extracts which also had potential antioxidant and cytotoxic. *Aronia prunifolia* cultivar had antioxidant activity was 232.30 µmol trolox/g extract, cultivar Viking of *A. Melanocarpa* was 206.20 µmol trolox/g extract and cultivar Aron of *A. Melanocarpa* was 177.80 µmol trolox/g extract (Rugina *et al*., 2012).

Antioxidant activity from marine bacteria has also reported from many researchers, such as Pereira *et al*. (2015) reported that bacteria associated with some marine sponge collected from the subtidal marine regions of India had the potential as antioxidant compound. The highest radical reduction activity was found in methanol extract of isolate GUVFCFM-3 with 67.83% scavenging of DPPH (2,2-diphenyl-2-picrylhydrazyl) free radicals and 65.87% scavenging of superoxide free radicals. Three bacterial isolates associated with sponge *Sarcotragus spinolosus* and *Crambe crambe* showed DPPH radical reduction activity more than 40% with the lowest IC_{50} values of 851 ppm (Perino *et al*., 2013). Other marine bacteria such as *Bacillus* sp. group, isolated from Agatti Island sponge, Lakshadweep archipelago, Maldives, showed potential antioxidant activity against free radicals. IC_{50} value of the active compound for DPPH activity, H_2O_2 scavenging activity, nitric oxide scavenging activity were 15.025, 23.730 and 41.700 µg mL^{-1}, respectively (Gopi *et al*., 2014).

Bacterial extracts extracted from bacteria associated with the marine sponge *Stylotella* sp showed significantly activity in reducing CUPRAC radical. Therefore, all extracts from five bacterial isolates associated with sponge *Stylotella* sp were potential to be developed as natural antioxidant products from marine bacteria.

Conclusion

Forty five isolates out of 138 bacteria (32%) isolated from sponge *Stylotella* sp produced bioactive compounds with antimicrobial activities. The best antimicrobial activity of ethyl acetate extract was performed by STIL 33. Five potential bacterial extracts, i.e., STIL 09, STIL 33, STIL 37, STIL 44 and STIL 55 showed potential antioxidant and toxicity activities with STIL 33 as the highest antioxidant activity (1610.00 µmol trolox/g extract) and STIL 37 showed the strongest toxicity (LC_{50} = 35.89 µg mL^{-1}). Those bacterial extract can be developed as antimicrobial and antioxidant agents.

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Author's Contributions

Daning Yoghiapiscessa: Took part in all experiment process as well as data analysis and manuscript preparation.

Irmanida Batubara: Involved in study design, result verification and scientific discussion.

Aris Tri Wahyudi: Coordinated the study**,** integrated all data and involved in manuscript writing.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

References

Abubakar, H., A.T. Wahyudi and M. Yuhana, 2011. Skrining bakteri yang berasosiasi dengan spons *Jaspis* sp. sebagai penghasil senyawa antimikroba. Ilmu Kelautan, 16: 35-40.

Anderson, J.E., C.M. Goetz., J.L. McLaughlin and M. Suffness, 1991. A blind comparison of simple bench-top bioassay and human tumour cell cytotoxicities as antitumor prescreens. Phytochem. Anal, 2: 107-111. DOI: 10.1002/pca.2800020303

Apak, R., K. Guclu, M. Ozyurek and S.E. Celik, 2008. Mechanism of antioxidant capacity assays and the Cupric ion Reducing Antioxidant Capacity (CUPRAC) assay. Microchim. Acta, 160: 413-419. DOI: 10.1007/s00604-007-0777-0

Bharathiraja, S., R. Rajasekaran and J. Suriya, 2014. Antibacterial activity of marine bacteria isolated from sponge *Spirastrella inconstans*. J. Chem. Pharm. Res., 6: 395-398.

Gopi, M., N.B. Dhayanithi, K.N. Devi and T.T.A. Kumar, 2014. Marine natural product, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (C7H10N2O2) of antioxidant properties from *Bacillus* species at Lakshadweep archipelago. J. Coast. Life Med., 2: 632-637. DOI: 10.12980/JCLM.2.201414J40

Jeganathan, P., K.M. Rajasekaran, A.N.K. Devi and S. Karuppusamy, 2013. Antimicrobial activity and characterization of marine bacteria. Ind. J. Pharm. Biol. Res., 1: 38-44.

Marchesi, J.R., T. Sato, A.J. Weightman, A.T. Martin and J.C. Fry *et al*., 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Applied Environ. Microbiol., 64: 795-799. PMID: 9464425

Mehbub, M.F., J. Lei, C. Franco and W. Zhang, 2014. Marine sponge derived natural products between 2001 and 2010: trends and opportunities for discovery of bioactives. Mar. Drugs, 12: 4539-4577. DOI: 10.3390/md12084539

Meyer, B.N., R.N. Ferrigini., L.B. Jacobsen, D.E. Nicholas and J.L. Laughin, 1982. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Med., 45: 31-35. DOI: 10.1055/s-2007-971236

Muller, W.E.G., V.A. Grebenjuk, N.L. Thakur, A.N. Thakur and R. Batel *et al*., 2004. Oxygen-controlled bacterial growth in the spons *Suberites domuncula*: Toward a molecular understanding of the symbiotic relationships between spons and bacteria. J. Applied Environ. Microbiol., 70: 2332-2341.

Nazim, K., S.K. Sherwani, M.U. Khan, R. Kausar and G. Rizvi, 2014. Antibacterial activity of marine sponge collected from Sunhari Beach. FUUAST. J. Biol., 4: 233-236.

Penesyan, A., Z.M. Jones, C. Holmstrom, S. Kjelleberg and S. Egan, 2009. Antimicrobial activity observed among cultured marine epiphytic bacteria reflects their potential as asource of new drugs. FEMS. Microbiol. Ecol., 69: 113-124. DOI: 10.1111/j.1574-6941.2009.00688

Pereira, S.V., P. Parvatkar and I.J. Furtado, 2015. Evaluation of antioxidant producing potential of halophilic bacterial bionts from marine invertebrates. Ind. J. Pharm. Sci., 77: 183-189. DOI: 10.4103/0250-474X.156572

Perino, E., A. Horta, R. Manconi, R. Pronzato and R. Pedrosa, 2013. Antioxidant activities in *Sarcotragus spinolosus* and *Crambe crambe* (porifera, demospongiae) associated bacteria. Rapp. Comm. Int. Mer Medit. DOI: 10.1016/j.copbio.2013.05.119

Radjasa, O.K., D.S. Kencana, A. Sabdono, R.A. Hutagalung and E.S. Lestari, 2007. Antibacterial activity of marine bacteria associated with sponge *Aaptos* sp. against Multi Drugs Resistant (MDR) strains. J. Mate. Dan Sains, 12: 4147-152.

Rugina, D., Z. Sconta, L. Leopold, A. Pintea and A. Bunea *et al*., 2012. Antioxidant activities of chokeberry extracts and the cytotoxic action of their anthocyanin fraction on HeLa human cervical tumor cells. J. Med. Food, 15: 700-706. DOI: 10.1089/jmf.2011.0246

Santos, O.C.S., P.V.M.L. Pontes, J.F.M. Santos, G. Muricy and M. Giambiagi-deMarval *et al*., 2010. Isolation, characterization and phylogeny of sponge-associated bacteria with antimicrobial activities from Brazil. J. Res. Microbiol., 161: 604-612. DOI: 10.1016/j.resmic.2010.05.013

Sharqawy, M.H., J.H. Lienhard and S.M. Zubair, 2010. Thermophysical properties of seawater: A review of existing correlations and data. Desalinat. Water Treatment, 16: 354-380. DOI: 10.5004/dwt.2010.1079

Shindo, K., E. Asagi, A. Sano, E. Hotta and N. Minemura *et al*., 2008. Diapolycopenedioic acid xylosyl esters a, b and c, novel antioxidative glycoc30-carotenoic acids produced by a new marine bacterium *Rubritalea squalenifaciens.* J. Antibiot., 61:185-191. PMID: 18503197

Sianipar, N.F., W. Maarisit and A. Valencia, 2013. Toxic activities of hexane extract and column chromatography fraction of rodent tuber plant (*Typhonium flagelliforme* Lodd) on *Artemia salina.* Indones. J. Agric. Sci., 14: 1-6.

Taylor, M.W., R. Radax, D. Steger and M. Wagner, 2007. Spons-associated microorganisms: Evolution, ecology and biotechnological potential. J. Microbiol. Mol. Biol. Rev., 71: 295-347. DOI: 10.1128/MMBR.00040-06

Thomas, T.R.A., D.P. Kavlekar and P.A. Lokabharathi, 2010. Marine drugs from sponge-microbe association-a review. Mar. Drugs, 8: 1417-1468. DOI: 10.3390/md8041417

Tokasaya, P., 2010. Spons-associated bacteria producing antimicrobial compounds and their genetic diversity analysis. Unpublished thesis in partial fulfillment of the requirements for the degree of undergraduate of Microbiology, Bogor Agricultural University, Bogor, West Java, Indonesia

Utami, A.W.A., A.T. Wahyudi and I. Batubara, 2014. Toxicity, anticancer and antioxidant activity of extracts from marine bacteria associated with sponge *Jaspis* sp. Int. J. Pharma. Bio. Sci., 5: 917-923.