

Original Research Paper

Screening and Characterization of Sponge-Associated Bacteria Producing Bioactive Compounds Anti-*Vibrio* sp.

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Abstract: In Indonesia, vibriosis is the main disease in shrimp. This disease is caused by *Vibrio* sp that may decrease the productivity of shrimp cultivation. Thus, exploration for new bioactive compounds as vibriosis biocontrol agent is necessary. Marine sponge-associated bacteria is one of many sources for bioactive compounds. The aim of this study was to screen marine sponge-associated bacteria producing anti-*Vibrio* sp's bioactive compounds. Total 12 bacterial isolates (15%) of 80 isolates was isolated from marine sponges *Hyrtios* sp, *Verongula* sp. and *Smenospongia* sp. had anti-*Vibrio* sp activity in different spectra. The hemolytic assay showed that these 12 bacteria were not pathogen. Interestingly, 3 out of 4 potential isolates with the best anti-*Vibrio* activity have been confirmed to have genes involved in the synthesis of bioactive compounds, mainly Polyketide Synthase (PKS) and Non-Ribosomal Peptide Synthetase (NRPS) based on the occurrence of Ketosynthase (KS) and Adenilase (A) domain, respectively. Based on 16S rRNA gene, those four isolates were highly homolog to the *Bacillus* sp in different species and strains. Isolate coded as P2.24 was the only bacterium that had the widest spectrum of anti-*Vibrio* bioactive compounds against three *Vibrio* sp used i.e., *Vibrio harveyi*, *V. parahaemolyticus*, *V. vulnificus*. Consistently, an anti- *Vibrio* sp. activity of the P2.24 was also shown by antagonism assay using culture, supernatant and crude extract of the isolate. Our study indicates this bacterial isolate potentially to be further exploited for controlling vibriosis biologically and important for elucidation of bioactive compounds synthesized by this bacterium.

Keywords: Bioactive Compounds, Sponge-Associated Bacteria, PKS, NRPS, Vibriosis, 16S rRNA

Introduction

Vibrio sp. can cause vibriosis that is a common disease in marine aquaculture around the world. These bacteria can infect shrimp and result in mass death. One of the aquaculture animals that is often infected by *Vibrio* sp. is shrimp. Commonly, vibriosis affects shrimp in all stadia, including larvae, protozoa and post larvae and causes deaths of 80-85% of the total population (Chatterjee and Halder, 2012; Aguirre-Guzman *et al.*, 2013). The application of antibiotics for vibriosis treatment is indeed able to reduce the impact, but its use in the long term still remain negative impacts on the environment, including resistance case and accumulation of antibiotic residues on cultivated products. Therefore,

the discovery of new bioactive compounds as vibriosis biocontrol agent that more effective and environmental friendly is necessary.

The sponge has been known as the most potential source of marine natural products (Taylor *et al.*, 2007). Selvin and Lipton (2004) reported that secondary metabolites from marine sponge *Dendrilla nigra* are able to inhibit *Vibrio harveyi* and *V. alginolyticus* infecting *Panous monodon*. However, the development of sponge's bioactive compounds is hampered due to the limitation of sponge biomass in the environment. The concentration of bioactive compounds in sponge tissue can be very low. Consequently, the large amounts of sponge biomass are needed for large-scale production. For example, to get 18 g of bryostatin,

13.000 kg of sponge *Bryozoans neritina* biomass are needed (Taylor *et al.*, 2007).

In addition, the sponge has symbiosis interaction with marine microorganisms, especially bacteria. They are a potential source of bioactive compounds with various biological activities, including antibacterial (Yoghiapiscessa *et al.*, 2016), antifungal (Ozkaya *et al.*, 2014), antiviral (Hassan and Ibrahim, 2016) and other bioactivities. Rini *et al.* (2017) reported that some bacteria isolated from marine sponge *Aaptos* sp. and *Hyrtios* sp. were able to inhibit *B. subtilis*, *Escherichia coli*, *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*. Other marine bacteria associated with sponge *Jaspis* sp. also exhibited antibacterial properties against *Staphylococcus aureus*, *V. harveyi*, *E. coli*, *Pseudomonas aeruginosa*, EPEC K-11, yeast *Candida albicans* and *C. tropicalis* as reported by Abubakar *et al.* (2011). It is worth noting that some of the sponge-associated bacteria have been detected to have the genes encoding the enzyme that may correlate for bioactive compounds synthesis, including Non-Ribosomal Peptide Synthetase (NRPS) and Polyketide Synthase (PKS) (Brinkmann *et al.*, 2017; Zhang *et al.*, 2009a). Exploration of marine bacteria from Indonesian sea sponge has been rarely reported. Therefore, the aim of this study was to screen marine sponge-associated bacteria producing anti-*Vibrio* spp.'s bioactive compounds.

Materials and Methods

Isolation of Sponge-Associated Bacteria

Sponges used in this study were *Hyrtios* sp., *Verongula* sp., *Smenospongia* sp. collected from Kepulauan Seribu Jakarta, Indonesia. Serial dilution method was applied in this study. One g of sponge biomass was washed by sterile seawater, then crushed aseptically. This materials were homogenized and serially diluted in 9 mL of 0.85% NaCl solution from 10^{-1} to 10^{-4} dilution. Nearly 100 μ L suspension of each serial dilution was plated into seawater complete (SWC) agar medium (1 g yeast extract, 3 mL glycerol, 5 g peptone, 250 mL distilled water and 750 mL seawater, 15 g agar) by spread plate technique, then incubated for 24-36 h at $\pm 27^{\circ}\text{C}$.

Screening of Bacteria Producing Bioactive Compounds Anti-*Vibrio* sp

Bacterial target used in this study were *Vibrio parahaemolyticus* ATCC 17802 and *Vibrio vulnificus* 195B (collection of standard of fish quarantine, quality control and fishery product safety, Jakarta, Indonesia) and *Vibrio harveyi* P-275 (collection of research and development center of brackish water aquaculture, Maros, Indonesia). Each bacterium was cultured in SWC medium and incubated for 24 h at 27°C , then used as a starter. About 500 μ L or 1% (v/v) of that bacterial

suspension were inoculated to 50 mL of melted SWC medium and poured into a sterile plate. After the medium containing the target bacteria was solid, each of 80 bacterial isolates was streaked onto the inoculated medium, then incubated for 24h at $\pm 27^{\circ}\text{C}$.

Hemolytic Test

Twelve bacterial isolates producing anti-*Vibrio* sp compounds were tested to know their hemolytic activity. Each of bacterial isolate was streaked onto blood agar medium and incubated for 24 h at $\pm 27^{\circ}\text{C}$.

Identification of NRPS-PKS Genes of the Potential Bacteria

Genomic DNA of bacterial cultures was extracted by using PrestoTM Mini gDNA bacteria Kit (Geneaid) according to the protocol's instructions. DNA quality and concentration were determined by using a NanodropTM 1000 Spectrophotometer. Adenylation (A) domain of NRPS gene and Ketosynthase (KS) domain of PKS gene from bacterial isolate were amplified by PCR using primer MTF/R (f: 5' AAR DSI GGI GSI GSI TAY BIC C-3); (r: 5-97 CKR WAI CCI CKI AIY TTI AYY TG-3) which targeted ± 1000 bp fragment and primer KSF/R (f: 5'-CSATGGAYCCSCARCRCGSV T-3'), (r: 5'-GTSCCSGTS CCRTGSSCYTCSAC-3') which targeted ± 700 bp fragment, respectively (Schirmer *et al.*, 2005). The mixture of PCR reaction consisted of 50 μ L PCR mix containing 25 μ L GoTaq Green[®] Master Mix 123 (Promega), 5 μ L of 10 pmol reverse primer, 5 μ L of 10 pmol forward primer, 11 μ L nuclease-free water and 4 μ L DNA template (100 ng/ μ L). PCR conditions were carried out in three steps including pre-denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, elongation at 72°C for 1 min and post PCR at 72°C for 10 min. The PCR products were sequenced in First Base, Malaysia. The sequences were then compared to the database of National Center for Biotechnology Information (NCBI) using the BlastX program. The phylogenetic tree was constructed by using Molecular Evolutionary Genetics Analysis (MEGA) 7.0 program with the neighbor-joining method in 1000x bootstrap replications.

Identification of the Most Potential Bacteria based on 16S rRNA Gene

16S rRNA gene was amplified using universal primer for bacteria, 63f (5'-CAG GCC TAA CAC ATG CAAGTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3' (Marchesi *et al.*, 1998) which targeted the conserved region approximately 1300-bp fragment. Fifty μ L PCR mix consisted of 25 μ L GoTaq Green[®] Master Mix 123 (Promega), 5 μ L of 10 pmol 1387r primer, 5 μ L of 10 pmol 63f primer, 11 μ L nuclease-free water and 4

μL DNA template (100 ng/ μL). PCR conditions were carried out in 35 cycles with pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, elongation at 72°C for 1 min 45 sec and post-elongation at 72°C for 10 min. The PCR products were sequenced in First Base, Malaysia. The sequences were compared by using the BlastN program in National Center for Biotechnology Information (NCBI) GenBank database. The sequences were deposited to NCBI GenBank (<https://www.ncbi.nlm.nih.gov>). Construction of phylogenetic tree was constructed using MEGA 7.0 software by neighbor-joining method with 1000x bootstrap replications.

Antagonism Assay for Culture and Supernatant of the Potential Bacterium

The potential bacterium with the widest spectrum in inhibiting three different *Vibrio* sp was used for this assay. Twenty four hours of bacterial suspension was counted by a direct-count method using hemocytometer. Nearly 1.5 mL of that suspension was centrifuged in 10.000 rpm for 5 min. The pellets and supernatants were separated into different tubes. The pellets were diluted with 150 μL of supernatants, so that the suspension contains ten times of cell number. Then, 20 μL of that concentrated culture was dropped onto the SWC agar medium containing the bacterial target. For the comparison, 20 μL of supernatants were also inoculated on that medium and the plates were incubated for 24 h at $\pm 27^\circ\text{C}$.

Antagonism Assay of Bacterial Crude Extract

Ten mL of 24 h bacterial suspension was used as a starter and inoculated into 1 L of liquid SWC medium. The culture was incubated and shaken at 120 rpm for 3 days at $\pm 27^\circ\text{C}$. The bacterial culture was then extracted by adding ethyl acetate solvent 1:1 (v/v) and shaken continuously for 20 min. The solvent layer was separated and evaporated using rotary evaporator at 42°C. The bacterial crude extracts obtained were diluted in DMSO. 20 μL of 1000 ppm crude extract was dropped into the steril paper disk and put on SWC medium plate containing the bacterial target. DMSO and 100 ppm of ampicillin were applied as a negative and positive control, respectively. The plates were incubated for 24 h at $\pm 27^\circ\text{C}$. This assay was conducted in triplicates.

Results

Bacteria Isolated from Sponges

Isolation of sponge-associated bacteria from 3 different sponges resulted 80 bacterial isolates which were morphologically different. Nine bacterial isolates were obtained from sponge *Hyrtios* sp., 20 bacteria

isolated from *Smenospongia* sp. and 51 bacteria isolated from *Verongula* sp.

Sponge-Associated Bacteria with Anti-Vibrio sp Properties

Of 80 bacterial isolates, 12 isolates (15%) were able to inhibit *Vibrio*'s growth indicated by clear zone formation around the bacterial colonies (Table 1). One bacterial isolate, coded as P2.24, could inhibit three *Vibrio* species, including *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus*. In addition, 10 bacterial isolate specifically only inhibit *V. harveyi* and 1 bacterial isolate coded as P3.310 could inhibit both *V. harveyi* and *V. parahaemolyticus*. Twelve bacterial isolates showed a diverse clear zone diameter, ranging from 0.1 mm to 5 mm. The best anti-*Vibrio* activity showed by P2.24 isolate due to its ability to inhibit three *Vibrio* species with different clear zone diameter. To confirm its anti-*Vibrio* spp activities, the concentrated culture, supernatants and metabolite extracts of the P2.24 isolate were tested in antagonism assay.

Hemolytic Reaction of the Potential Bacteria

Twelve bacterial isolates producing anti-*Vibrio* sp compounds were hemolytic negative. They did not lyse blood cells as indicated by no clear zone formed around the colony after 24h of incubation.

The Identity of NRPS-PKS Gene and 16S rRNA of the Selected Bacterial Isolates

Based on their ability in inhibiting *Vibrio*'s growth in wide spectra, three out of 4 potential bacterial isolates coded as P2.24, P3.310 and D4.13 have been identified to have both NRPS and PKS genes and one bacterial isolate coded as P2.211, did not have both genes. Adenylation (A) domain of NRPS and ketosynthase (KS) domain of PKS gene from these bacteria have been amplified by PCR method resulting ± 1000 bp and ± 700 bp DNA fragment, respectively (Fig. 1). Alignment using the BlastX revealed all NRPS and PKS genes were similar with NRPS and PKS genes of *Bacillus* spp in various strains (Table 2 and 3). In addition, amplification of 16S rRNA gene of four bacterial isolates resulted ± 1300 bp DNA fragment. All these isolates were homologous with *Bacillus* sp (Table 4).

The genetic relationship for NRPS-PKS gene of the potential isolates was compared with their relative gene in GenBank NCBI database and some references by constructing the phylogenetic tree. The phylogenetic tree of A domain of NRPS gene and KS domain of PKS gene are shown in Fig. 2 and 3, respectively. The evolutionary relationship based on 16S rRNA of the potential bacterial isolate with their closest related strains is performed in Fig. 4.

Table 1: Growth inhibition of *Vibrio* sp by sponge-associated bacteria

Sponge	Isolate code	Growth inhibition of <i>Vibrio</i> species ¹⁾		
		<i>V. harveyi</i>	<i>V. parahaemolyticus</i>	<i>V. vulnificus</i>
<i>Hyrtios</i> sp.	D4.13	+++	-	-
	D4.15	+	-	-
	D4.19	+	-	-
	D4.110	+	-	-
<i>Verongula</i> sp.	P2.24	+	++	+
	P2.211	+++	-	-
	P2.212	+	-	-
	P2.34	+	-	-
<i>Smenospongia</i> sp.	P3.310	+	++	-
	P3.414	+	-	-
	P3.415	+	-	-
	P3.514	++	-	-

¹⁾ Clear zone diameter 0 mm: -; 0.1-2.5 mm: +; 2.5 -5 mm: ++; >5 mm: +++

Table 2: The identity of DNA fragment encoding A domain of NRPS gene from 3 bacterial isolates producing anti-*Vibrio* sp compounds using BlastX

Isolate code	Closest NRPS Relative	Query cover (%)	Similarity (%)	E-value	Accession
D4.13	Surfactin NRPS SrfA <i>Bacillus</i> sp. strain CMAA 1185	96	53	8e-64	WP_046160043.1
P3.310	Surfactin NRPS SrfA <i>Bacillus</i> sp. strain LM 4-2	94	56	6e-52	WP_046380780.1
P2.24	Non-ribosomal peptide synthetase <i>Bacillus amiloquifaciens</i>	85	42	2e-15	WP_021493393.1

Table 3: The identity of DNA fragment encoding KS domain of PKS gene from 3 bacterial isolates producing anti-*Vibrio* sp. compounds using BlastX

Isolate code	Closest PKS Relative	Query Cover (%)	Similarity (%)	E-value	Accession
D4.13	Ketosynthase; <i>Bacillus subtilis</i>	99	100	8e-159	SAJ35050.1
P3.310	Ketosynthase; <i>Bacillus subtilis</i> ,	99	99	7e-163	SAJ35050.1
P2.24	Ketosynthase; <i>Bacillus</i> sp. strain IX-119,	93	61	7e-48	AIU64905.1

Table 4: The identity of 4 bacterial isolates producing anti-*Vibrio* sp compounds based on 16S rRNA gene using BlastN

Isolate code (Accession No.)	Closest relative	Query Cover (%)	Similarity (%)	E-value	Accession
D4.13 (MH588269.1)	<i>Bacillus subtilis</i> strain PHYDG1	99	98	0.0	KY785660.1
P3.310 (MH588271.1)	<i>Bacillus subtilis</i> strain R242	100	98	0.0	KY515422.1
P2.24 (MH588270.1)	<i>Bacillus subtilis</i> strain Y10	100	97	0.0	KC708563.1
P2.211 (MH588272.1)	<i>Bacillus flexus</i> strain SML I301	100	99	0.0	MG937596.1

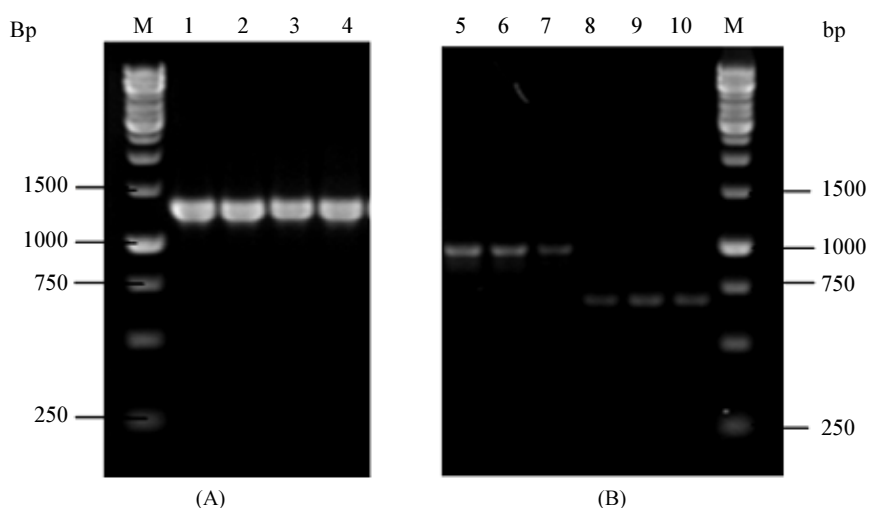


Fig. 1: Visualization by 1% agarose of (A) 16S rRNA gene and (B) NRPS-PKS gene. 16S rRNA gene (~1300 bp) (1) D4.13, (2) P3.310, (3) P2.24, (4) P2.211; A domain of NRPS gene (~1000 bp): (5) D4.13, (6) P3.310, (7) P2.24 and KS domain of PKS gene (~700 bp): (8) D4.13, (9) P3.310, (10) P2.24 and (M) Marker 1 kb ladder

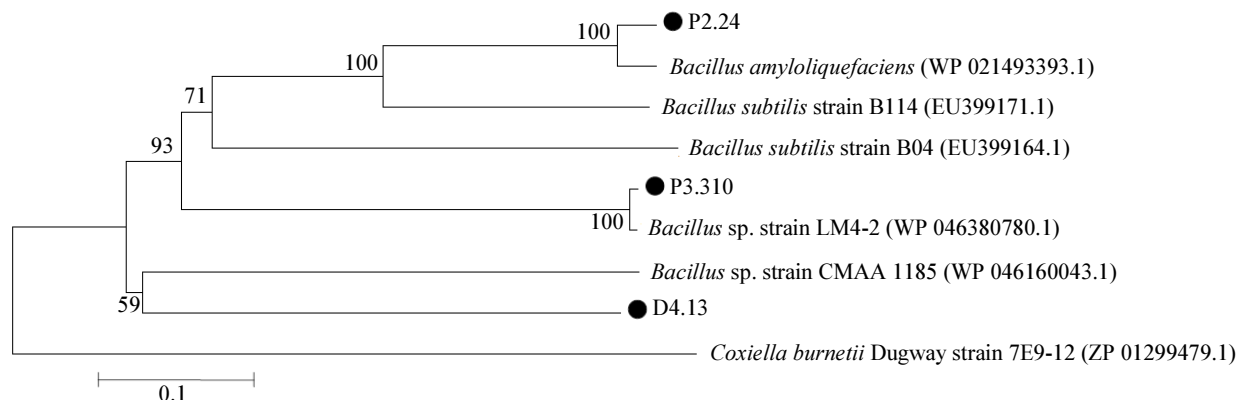


Fig. 2: The phylogenetic tree of A domain of NRPS gene of the bacterial isolates compared with their closest relative sequences in the Genbank database and some references sequences

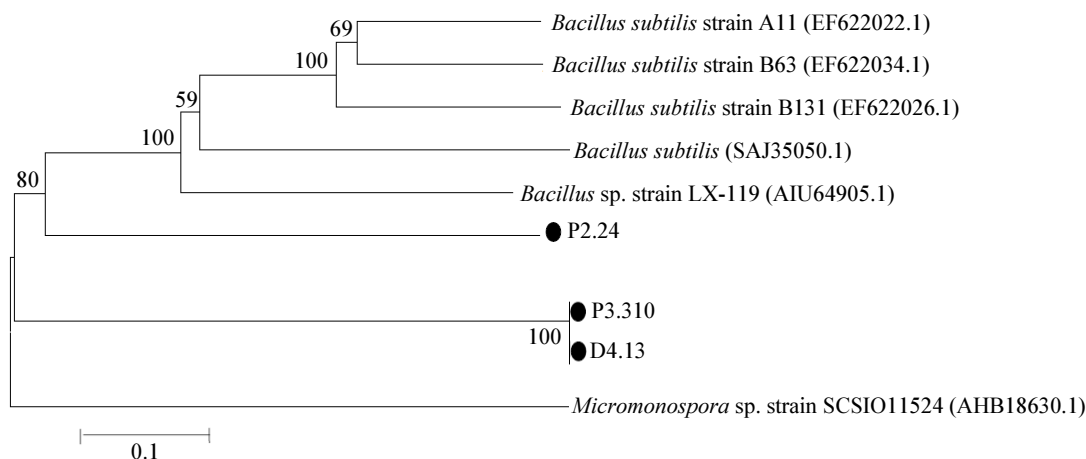


Fig. 3: The phylogenetic tree of KS domain of PKS gene of the bacterial isolates compared with their related species

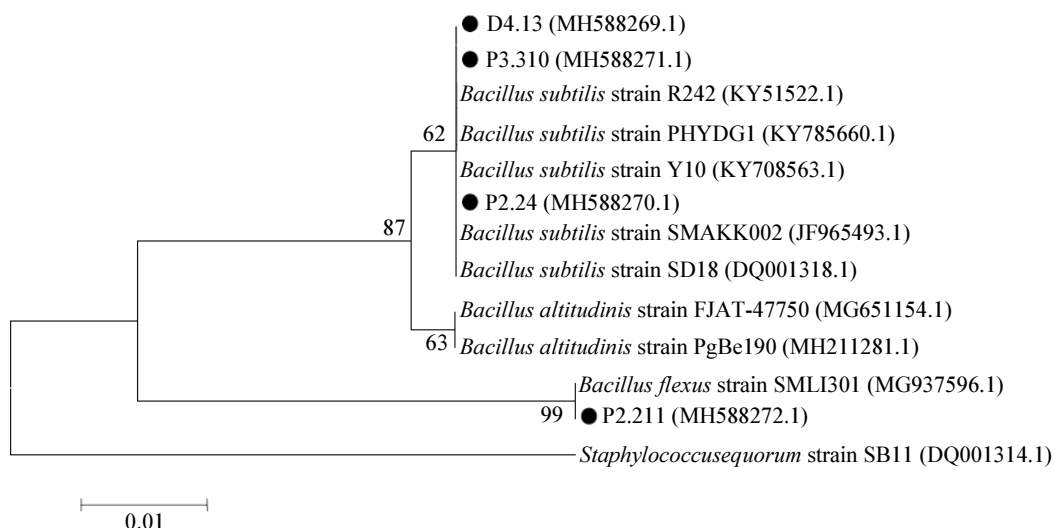


Fig. 4: The phylogenetic tree of the bacterial isolates based on 16S rRNA gene compared with their closest related species

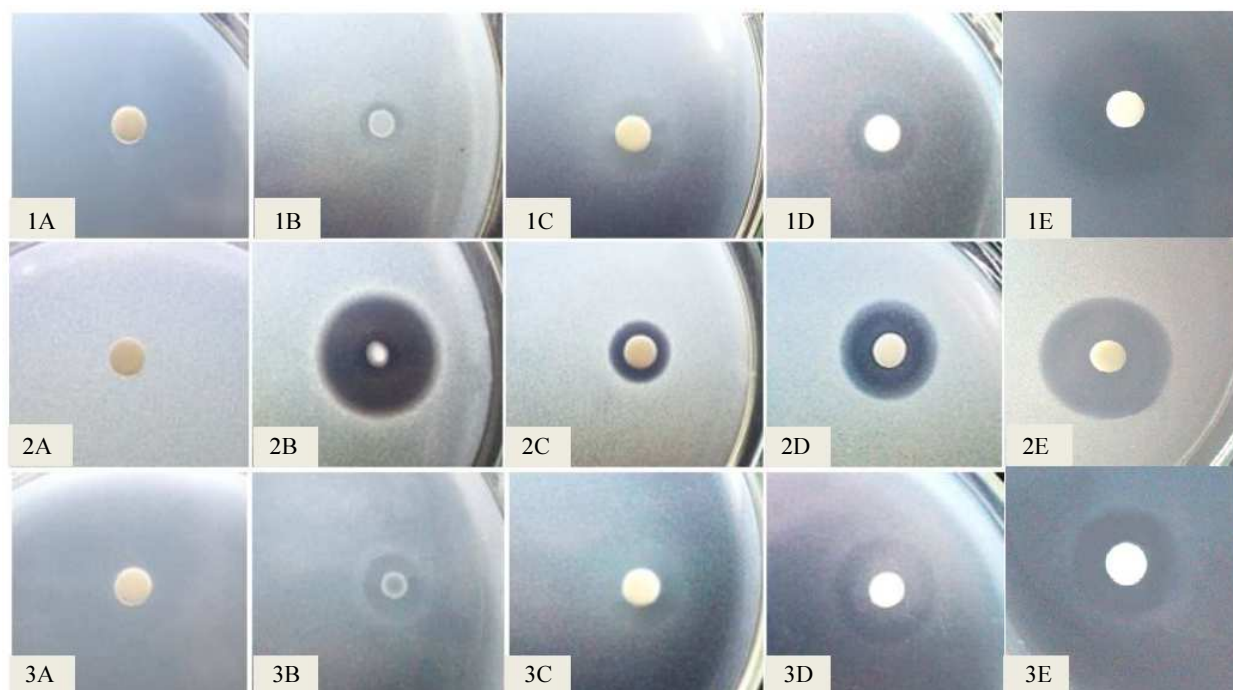


Fig. 5: Anti-*Vibrio* sp activities of the bacterial isolate P2.24 confirmed by its concentrated culture, supernatants and metabolite extracts, against three *Vibrio* species: *V. harveyi* (1), *V. parahaemolyticus* (2), *V. vulnificus* (3). Negative control (DMSO) (A), concentrated culture (B), supernatant (C), 1000 ppm of crude extracts of bacterial metabolites (D) and 100 ppm of ampicillin as positive control (E)

Antibacterial Activity of Culture, Supernatant and Extract of P2.24

Bacterial culture used in this assay contains approximately 7.8×10^7 cell/mL medium. Culture, supernatant, as well as the extract of the isolate P2.24 consistently exhibited anti-*Vibrio* sp activities as indicated by clear zone formation around paper disk (Fig. 5). The strongest inhibitory effect was shown by the concentrated culture of the isolate P2.24 against *V. parahaemolyticus*. The clear zone was also exhibited by ampicillin as positive control. There was no clear zone obtained from DMSO as negative control.

Discussion

In this study, twelve out of 80 bacterial isolates or about 15% bacteria isolated from three sponges exhibited their ability to inhibit *Vibrio* species growth. The ability of these bacteria to produce anti-*Vibrio* compounds indicated by clear zone formation around bacterial colonies. The bioactive compounds produced by these bacteria may inhibit the growth of *Vibrio* bacteria. The synthesis of bioactive compounds is an important defense mechanism as a protection from environmental stress and pathogen colonization, both for the bacteria itself and for its host (Lee *et al.*, 2001). Twelve bacterial isolates showed various spectra in inhibiting *Vibrio* spp.

The different anti-*Vibrio* spectra indicating the diversity of antimicrobial compounds produced by these sponge-associated bacteria. Different compounds may have different mechanisms in inhibiting *Vibrio* colonization. The resistance capability of each *Vibrio* species also might influence the effectivity of bioactive compounds work. Twelve bacterial strains also showed a non-hemolytic reaction on blood agar suggesting that these bacteria were not the pathogen.

An indication of capability in producing anti-*Vibrio* sp compounds of the potential bacterial isolate has been confirmed by molecular evidence. The Adenylation (A) domain of the NRPS gene and Ketosynthase (KS) domain of the PKS gene are the most conserved domain compared to other domains (Schirmer *et al.*, 2005). Both A domain and KS have been identified in three potential bacterial genome. The enzyme polyketide synthase and non-ribosomal peptide synthetase are multidomain enzyme that responsible for biosynthesis of bioactive compounds in many microorganisms, including bacteria (Su *et al.*, 2014), actinomycetes (Ziemert *et al.*, 2014; Xi *et al.*, 2012) and fungi (Boettger and Hertweek, 2013). Those three bacterial isolates coded as P2.24, P3.310 and D4.13 with NRPS and PKS gene allowed the possibility to synthesize bioactive compounds by hybrid NRPS-PKS pathway. According to Zhu *et al.* (2009), Hybrid NRPS-PKS gene may occur with a combination

of domains from both genes in an open reading frame. The hybrid NRPS-PKS has potential to synthesis novel bioactive compounds with hybrid structure. Leinamycin is an antitumor compound derived from *Streptomyces atroolivaceus* which synthesis of that compound by hybrid NRPS-PKS gene (Tang *et al.*, 2004).

The KS sequence alignment in amino acid level showed that all KS sequences were similar with type I PKS gene of *Bacillus* spp in a various percentage of similarity, while A sequences of three bacterial isolates were similar to NRPS gene of *Bacillus* spp. The low similarity value of the genes may indicate the unavailability of data in the Genbank NCBI database for the same genus or bacterial species. The results also allow for the novelty of the genes involved in synthesis of new bioactive compounds (Zhang *et al.*, 2009a).

Based on 16S rRNA gene, bacterial isolate P2.24, the widest anti-*vibrio* spectra producer, was highly homolog with *Bacillus subtilis* strain Y10 with 97% similarity, while three other strains were also identified as *Bacillus* sp with high similarity. Marine *Bacillus* is one of the biggest bioactive compounds producers group. Some previous studies reported that some *Bacillus* strains isolated from South China Sea sponge exhibited an excellent antimicrobial activity against *Staphylococcus aureus*, *Aspergillus niger*, *Candida albican*, *Pseudomonas fluorescens*, *B. subtilis* and *E.coli* (Zhang *et al.*, 2009b). Supporting the study, Manikandan *et al.* (2014) also reported *Bacillus subtilis* strain SMAKK002 isolated from marine sponge exhibited the strong antimicrobial activity against some multidrug resistance human pathogens.

The bacterial isolate P2.24 was the only one bacterium that had the widest spectra of anti-*Vibrio*'s bioactive compounds against all three *Vibrio* species, including *Vibrio harveyi*, *V. parahaemolyticus*, *V. vulnificus*. The consistent anti-*Vibrio* activity was also performed by concentrated culture, supernatants and metabolites extracts of that bacterium. The inhibitory zone formation around the bacterial colony indicates the diffusion of bioactive compounds synthesized by the bacterium on the growth medium so that targeted bacteria growth around diffusion area was inhibited. The bioactive compounds produced by P2.24 isolate was an extracellular compound, as it indicated by the inhibition of *Vibrio*'s growth when supernatant was tested through antagonism assay.

In addition, the anti-*Vibrio* activity of P2.24 isolate was also confirmed by its metabolites crude extract. However, it still crude extract. It would be important to purify, separate and identify the crude extract compounds. In some previous studies, anti-*Vibrio* activity of crude extracts derived from sponge-associated bacteria has also been reported, but the activity of these

extracts only inhibited one *Vibrio* species. As examples, bioactive compounds from *Flavobacterium* sp isolated from *Acropora muricata* sponge only inhibited the growth of *V. harveyi* (Ginting *et al.*, 2010). Crude extracts from bacteria isolated from *Acanthos-trongylophora* sp only inhibited *V. cholerae* as reported by Legina (2016). The anti-*Vibrio* activities of the P2.24 have the potency to be further developed to control vibriosis disease in aquaculture since their broader anti-*Vibrio* spectrum properties in inhibiting more than one *Vibrio* species. On the other hand, to elaborate this result, it is also necessary to purify the bioactive compound so that the kind of compound that plays a role in the inhibition of *Vibrio* sp can be known.

Conclusion

Of 80 isolates isolated from marine sponges *Hyrtios* sp, *Verongula* sp., and *Smenospongia* sp, 12 isolates (15%) had anti-*Vibrio* activities in different spectra. Of 4 bacterial isolates with the best anti-*vibrio* activity, three isolates have been identified to have PKS and NRPS genes. Based on 16S rRNA gene, those 4 isolates were highly homolog to *Bacillus* genera. One bacterial isolate coded as P2.24 was the only bacterium that had the widest spectra of anti-*Vibrio*'s bioactive compounds as tested by antagonism assay using culture, supernatant and crude extract of the bacterium. This P2.24 is important to be further exploited for controlling vibriosis biologically and essential for the elucidation of bioactive compounds synthesized by this bacterium.

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Author's Contributions

Aris Tri Wahyudi: Has guided the project, determined the research framework, data analysis and involved in paper writing.

Jepri Agung Priyanto: Has contributed to the experimental works, data analysis and paper writing.

Wenang Maharsiwi: Has contributed in the experimental works and data analysis.

Rika Indri Astuti: Has contributed to the interpretation of data and paper writing.

Ethics

This article is original from the authors works. The corresponding author ensures that all of the other authors have read and recognized the manuscript.

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