**BIODIVERSITAS**
Volume 20, Number 11, November 2019
Pages: 3164-3169

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d201108

# Marine sponge-associated bacteria as biocontrol agents of vibriosis on whiteleg shrimp caused by *Vibrio parahaemolyticus*

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Manuscript received: 28 August 2019. Revision accepted: 10 October 2019.

**Abstract.** *Raharja NI, Widanarni, Wahyudi AT. 2019. Marine sponge-associated bacteria as biocontrol agents of vibriosis on whiteleg shrimp caused by* Vibrio parahaemolyticus. *Biodiversitas 20: 3164-3169.* Sponge-associated bacteria are commonly known as an excellent source of microbial's bioactive compounds attributed to biological properties, including antibacterial activity. In our previous study, 12 isolates from sponge *Aaptos* sp. and *Hyrtios* have been screened to have anti-*Vibrio* spp. activities *in vitro*. The objective of this study was to evaluate these bacterial isolates for controlling *Vibrio parahaemolyticus* infection *in vivo*, identify the most potential isolate, and analyze chemical composition of bacterial crude extract. As tested by pathogenicity assay on post-larvae of whiteleg shrimp stadia PL 10, all 12 isolates were not pathogenic bacteria as indicated by the survival rate of shrimps ranging from 80 ± 8.1 - 93 ± 4.7%. Conversely, more than 50% died after inoculated with pathogenic *V. parahaemolyticus*. Interestingly, all isolates could increase the survival rate of shrimps infected with *V. parahaemolyticus* ($10^5$ cells/mL) reach of 80 ± 0.8 - 91 ± 0.4%, nearly 40% higher than the positive control, as evaluated by challenge test. The best anti-*Vibrio* activity showed by D2.Z13 isolate which could improve the survival rate up to 91.11 ± 0.4%. 16S-rRNA-based identification showed that this isolate was closely related to *Pseudomonas aeruginosa* 1-4-b-9 strain. The extract derived from this isolate contained some major compounds such as 4,8-dihydroxy-2-2 (1'-hydroxyheptyl) and 1,2,4-oxadiazol which have been reported as antibacterial compounds. Our study indicates that these bacterial isolates could be developed as biocontrol agents of vibriosis in whiteleg shrimp.

**Keywords**: 16S-rRNA, bioactive compounds, GC-MS, sponge-associated bacteria, *Vibrio parahaemolyticus*, whiteleg shrimp

## INTRODUCTION

The demand for whiteleg shrimp (*Litopenaeus vannamei*) continues to increase every year. In 2014 whiteleg shrimp production in Indonesia reached 442,380 tons and increased in 2015 by 505,549 (DJPB 2016). However, the productivity of whiteleg shrimp was decreased by infectious diseases, one of the major diseases in shrimp cultured is vibriosis caused by some *Vibrio* species (Le et al. 2009). These bacteria could infect shrimp in all life stages, including larvae, protozoea and post-larva stages (Zheng et al. 2017) and causes mortality in shrimp reaching 80-85% of the total population (Aguirre-Guzman et al. 2013). Therefore, effective treatment for controlling vibriosis is necessary.

Currently, the farmer usually used antibiotics in shrimp feed to control the population of *Vibrio* sp. However, the use of antibiotics with inappropriate could cause *Vibrio* resistance (Chatterjee and Haldar 2012). For example, the *V. alginolyticus* isolated from seawater sediment showed the highest percentage of antibiotic-resistant to erythromycin E and penicillin P (100%) (Drais et al. 2018) and the vast majority of samples *V. parahaemolyticus* collected from the Eastern coast of Saudi Arabia exhibited high resistance to carbenicillin (98%), ampicillin (88%), and cephalothin (76%) (Ghenem and Elhadi 2018). Therefore, a new natural antibacterial compound is still needed to face resistance cases in *Vibrio*. Bacteria associated with sponge are known to have the ability to synthesis bioactive compounds attributed to biological activities including antibacterial (Wahyudi et al. 2018; Rini et al. 2017), antiaging (Prastya et al. 2019a), antiglycation and antioxidant (Prastya et al. 2019b), and anticancer (Priyanto et al. 2017) activities. Therefore, sponge-associated bacteria could be a promising source of bioactive compounds.

Bacteria associated with sponge are expected to be biocontrol agents of pathogenic bacteria, especially *Vibrio parahaemolyticus* in the cultivation of whiteleg shrimp. From our previous study (Rini et al. 2017), twelve isolates have successfully been isolated from sponge *Aaptos* sp. and *Hyrtios* sp. collected from Thousand Island, Jakarta-Indonesia. These isolates have been studied to have anti-*Vibrio* activity against *V. harveyi* both *in vitro* and *in vivo*, but the ability of that isolates to control *V. parahaemolyticus in vivo* has never been studied. Therefore, this study was aimed to evaluate the ability of these 12 bacterial isolates associated with sponges producing bioactive compounds in controlling vibriosis in whiteleg shrimp caused by *V. parahaemolyticus in vivo*, identify the selected isolate, and analyze the chemical composition of metabolites extracted from the most prospective isolate.

## MATERIALS AND METHODS

### Pathogenicity test of the potential bacterial isolate

The pathogenicity of twelve isolates from the previous study (Rini et al. 2017) was evaluated *in vivo* on *L. vannamei* post-larvae shrimp (10 days of age Post larva stage). This assay was carried out by inoculating the bacterial suspensions ($10^6$ cells/mL) to the aquarium (containing 3 L seawater, 15 individual shrimp). The inoculated aquarium was incubated for 7 days and feed using *Artemia salina* (5 individuals per mL) 4 times per day. Shrimp mortality was counted every day. The post-larvae infected with *V. parahaemolyticus* ($10^5$ cells/mL) and uninfected larvae were used as a positive and negative control, respectively. At the end of this experiment, the number of living post-larvae shrimp was counted and compared to the control.

### $LC_{50}$ value determination in *Vibrio parahaemolyticus*

Determination of $LC_{50}$ value was done by inoculating a suspension of *V. parahaemolyticus* bacteria with various cell concentrations ($10^7$, $10^6$, $10^5$ and $10^4$ cells/mL) to the investigated aquarium. Each treatment was repeated three times. Observations were made by counting the number of live and dead shrimp after 7 days of incubation. Determination of the $LC_{50}$ value is important to know the concentration of *V. parahaemolyticus* bacterial cells that can cause 50% of shrimp mortality. This value was then used for the challenge test.

### Challenge test on the post-larvae of *L. vannamei*

Twelve potential bacterial isolates were tested for their ability to control the infection of *V. parahaemolyticus* on shrimp. The concentration of *Vibrio* isolates used was based on its $LC_{50}$ value. The potential bacteria with a concentration of $10^6$ cells/mL were inoculated to the investigated aquarium. After 6 hours, the aquarium was inoculated with the *V. parahaemolyticus* culture ($10^5$ cells/mL). This experiment was carried out in three replications. The treated aquarium was incubated for 7 days and at the end of the tests period, the survival rate of shrimp was calculated by using the following formula Effendie (1997):

$$SR\ (\%) = \frac{Nt}{No}\ x\ 100$$

Where :

SR : Survival rate (%)

Nt : Number of shrimp live at the end of the experiment (individual)

No : Number of shrimp live at the beginning of the experiment (individual)

### Identification of the most potential bacterial isolate

The most potential bacterial isolate was cultured in *Luria Bertani* (LB) medium (Composition: yeast extract 5 g/L, tryptone 10 g/L, NaCl 10 g/L) and agitated in 120 rpm at 27 ºC for 24 hours. The culture was then used for bacterial DNA extraction. The DNA was extracted by using the Geneaid Genomic DNA Mini Kit (Blood/Cultured Cell) as following the manufacturer's instructions. DNA quality concentrations were quantified by using nanodrop devices (Thermo Scientific, USA). Amplification of the 16S-rRNA gene was performed using 63F primer (5'-CAG GCC TAA CAC ATG CAA GTC-3 ') and 1387R (5'-GGG CGG WGT CAA GTA CAA GGC-3') with targeted fragments of ± 1300 bp (Marchesi et al. 1998). The PCR reaction consisted of 25 µL GoTaq Green Mastermix 2x (Promega, Madison, USA), 5 µL for each primer (10 pmol), 2 µL DNA template (~ 100 ng/µL) and adjusted with nuclease-free water until 50 µL. Amplification was done using PCR T1-Thermocycler (Biometra, Goettingen, DE) with the temperature gradient used was pre-denaturation at 94 ºC for 5 minutes, denaturation at 94 ºC for 30 seconds, annealing at 55ºC for 45 seconds, elongation at 72 ºC for 1 minute 30 seconds. Denaturation, annealing, and elongation were repeated for 35 cycles. The process was ended with the final elongation at 72 ºC for 10 minutes and cooling C for 5 minutes. The PCR products were sequenced by using service provided by First Base, Malaysia through Genetika Sains Indonesia inc. The 16S-rRNA sequences were then aligned with the sequences from the GenBank database using the BLAST-N program in NCBI (www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic tree was constructed by using the Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 Neighbor-Joining method (Tamura et al. 2013).

### Extraction and identification of bioactive compounds from bacteria

Nearly 1% (v/v) of bacterial suspension was inoculated to 500 mL of Sea Water Complete (SWC) medium (composition: yeast extract 1 g/L, peptone 5 g/L, glycerol 85% 3.5 mL/L, seawater 750 mL/L, aquadest 246.5 mL/L) and agitated at 120 rpm in room temperature for 72 hours. The culture was then added with ethyl acetate in a ratio of 1: 1 (v/v) in separating funnel. The solvent layer was then evaporated at 40 ºC. The identification of bioactive compounds in the crude extract was conducted using GC-MS (Pyrolysis 5973 GC-MS, Agilent Technology). A total of 2 µL of extract solution was injected into a column with HP-5MS column type (length 30 m, diameter 0.5 mm, width 0.25 µm). Helium gas (99.999%) was used as a carrier gas with a flow rate of 104 µL/min, an operating time of 30 minutes at an oven temperature of 50 ºC, an injector temperature of 290 ºC, and an aux temperature of 290 ºC. The amount of compound obtained was reflected from the peaks in the chromatogram as analyzed by the GC-MS Pyrolysis program (WILLEY9THN 08. L).

### Data analysis

The data of this study were analyzed qualitatively and quantitatively. The data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using the one-way Analysis of Variance (ANOVA) then followed up with the Tuckey test using the SPSS (Statistical Program Software System) program version 16.0. Significant differences were those which $P < 0.05$ or $P < 0.01$.

## RESULTS AND DISCUSSION

### Non-pathogenicity of 12 potential isolates.

The pathogenicity test showed that all isolates tested were not a pathogen. The survival rate value of post-larvae whiteleg shrimp inoculated with the potential isolates was quite high ranging from 80 ± 8.1 - 93 ± 4.7%, while the positive control infected with the pathogenic *V. parahaemolyticus* bacteria was 46.67 ± 4.7% and the survival rate of the negative control (uninoculated with any bacterial isolate) was 93.3% (Figure 1).

### $LC_{50}$ value of *Vibrio parahaemolyticus* and *in vivo* anti-*Vibrio* activity of potential isolates

Based on the pathogenicity test, *V. parahaemolyticus* has the $LC_{50}$ value of $10^5$ cells/mL. The concentration was used in the challenge test. The best survival rate showed by shrimp inoculated with D2.Z13 isolate (91.11 ± 0.4%) (Figure 2). The survival rate of post-larvae whiteleg shrimp treated with potential bacterial isolates had a significant difference (P <0.05) compared to the positive control.

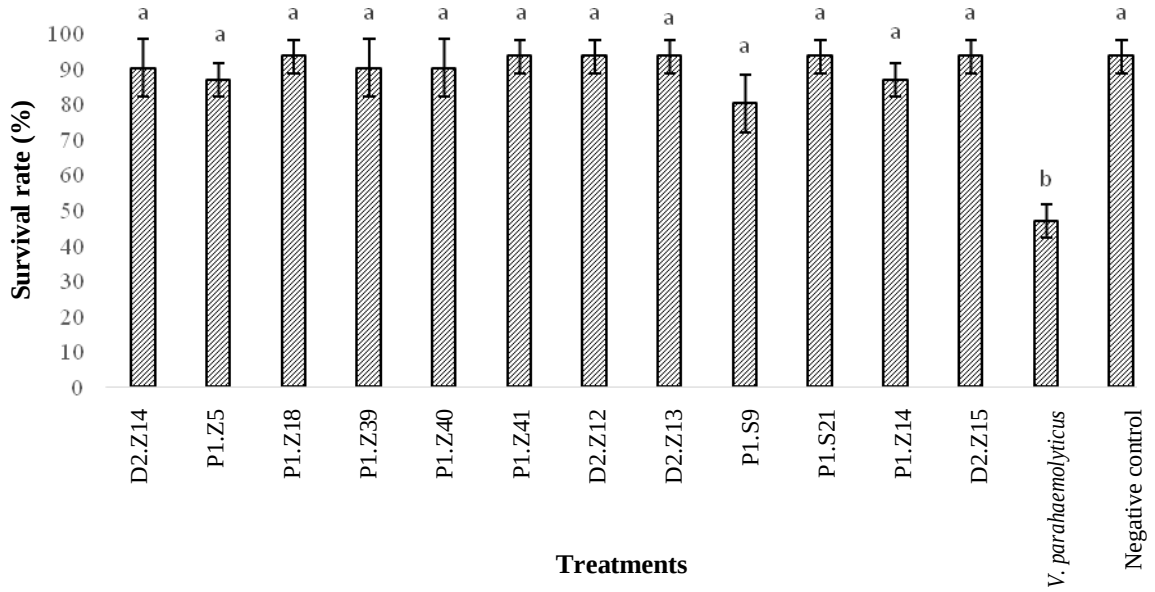


**Figure 1** The survival rate of whiteleg shrimp in 7 days after inoculated with sponge-associated bacteria and *V. parahaemolyticus* ($10^6$ cell / mL). The different letter above the bar represents that the data were significantly different based on the Tuckey test at P<0.05

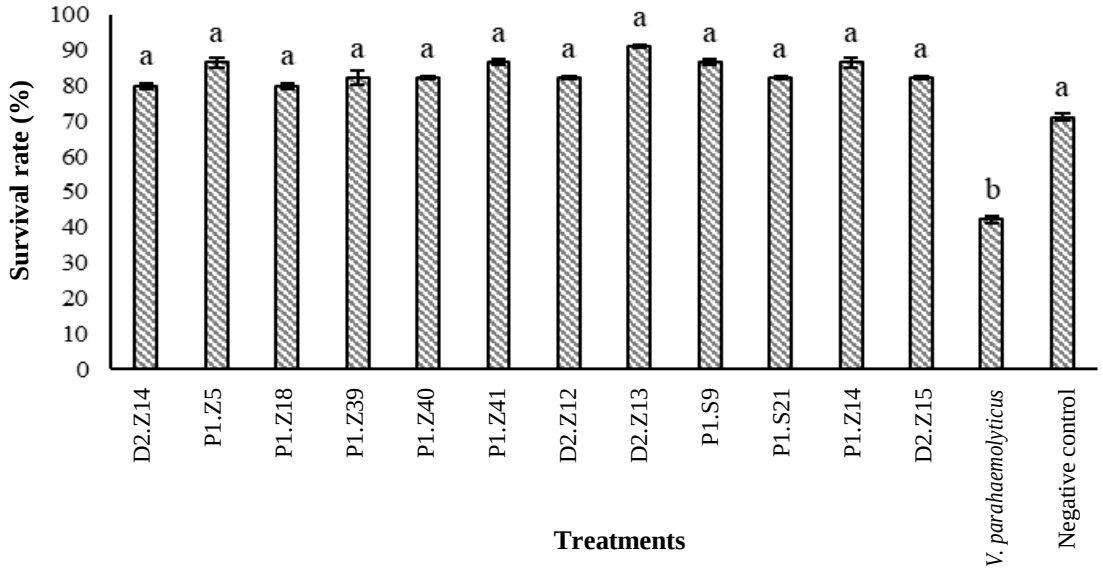


**Figure 2.** The survival rate of vanname shrimp inoculated with bacterial isolate and *V. parahaemolyticus*. The different letters show the significant value as analyzed by Tuckey test at P<0.05

**Chemical composition of a crude extract derived from D2.Z13 isolate**

Extraction of bioactive compounds resulted 0.98 g of crude extract. GC-MS based identification showed that D2.Z13-derived extract contained 9 major compounds in different in peak area and retention time as shown in Table 2. This extract contained some antibacterial compounds, including 4,8-dihydroxy-2- (1'-hydroxyheptyl) -3,4,5,6,7,8-hexahydro-2H- [1] -benzopyran-5 (conginginin D); 1,2,4-oxadiazol-5-one; pyrrolo [1,2-a] pyrazine -1,4- dione, hexahydro-3- (phenylmethyl)-, 3-benzyl -1,4- diaza- 2,5-dioxobicyclo[4.3.0] nonane; cyclo (pro-phe).

**The Identity of D2.Z13 isolate based on 16S-rRNA gene**

The 16S-rRNA gene amplification from the D2.Z13 isolate resulted in DNA fragment in size of ~1300 bp. Based on BLAST-N program, this isolate was highly similar to *Pseudomonas aeruginosa* strain 1-4-b-9 (identity: 98.75 %; acc. number KR149607.1). Consistently, this isolates also located in the same clade with its closest relative strain as shown in Figure 3.

**Discussion**

The sponge is known to have a symbiosis interaction with diverse and complex prokaryotic communities (Thomas et al. 2016). They have an important role for host defense against pathogenic microbes which is likely due to their ability to synthesis bioactive compounds. This capability could be explored for controlling vibriosis. One of the most important steps of a screening biocontrol agent is evaluating non-pathogenicity characters. The pathogenicity characters could be analyzed by the percentage of surviving larvae after inoculation of bacterial candidate suspension. The non-pathogenic bacteria is the bacteria that does not cause significant mortality compared to the survival rate of the control (Widanarni et al. 2008).

**Table 1**. Nine dominant compounds in D2.Z13-derived extract identified as antimicrobial agents

| Compound | Retention time (min) | Peak area (%) | Function | References |
|---|---|---|---|---|
| 4,8-dihydroxy-2- (1'-hydroxyheptyl)-3,4,5,6,7,8-hexahydro-2H-[1]-benzopyran-5 | 15.334 | 18.89 | Antibacterial, antifungal | Li et al. (2019) |
| 1,2,4-oxadiazol-5-one | 10.538 | 17.88 | Antibacterial | Cunha et al. (2018) |
| Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3- (phenylmethyl)- | 13.197 | 9.49 | Antibacterial, antioxidant | Tangjitjaroenkun (2018) |
| 3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane | 13.197 | 9.49 | Antibacterial | Gohar et al. (2010) |
| Cyclo (pro-phe) | 13.197 | 9.49 | Antibacterial | Song et al. (2018) |
| (3S-Trans)3-Benzylhexahydropyrrolo1,2-A)pyrazin | 13.197 | 9.49 | Antibacterial, antifungal | Li et al. (2008) |
| l-valine,n-propargyloxycarbonyl-, heptadecyl ester | 9.709 | 8.42 | Antifungal | Ireri et al. (2015) |
| l-proline, N-allyloxycarbonyl-, hexyl ester | 9.709 | 8.42 | Antifungal | Managumi et al. (2017) |
| 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0] nonane | 10.658 | 4.08 | Antibacterial, anticancer, antioxidant | Azman et al. (2017) |

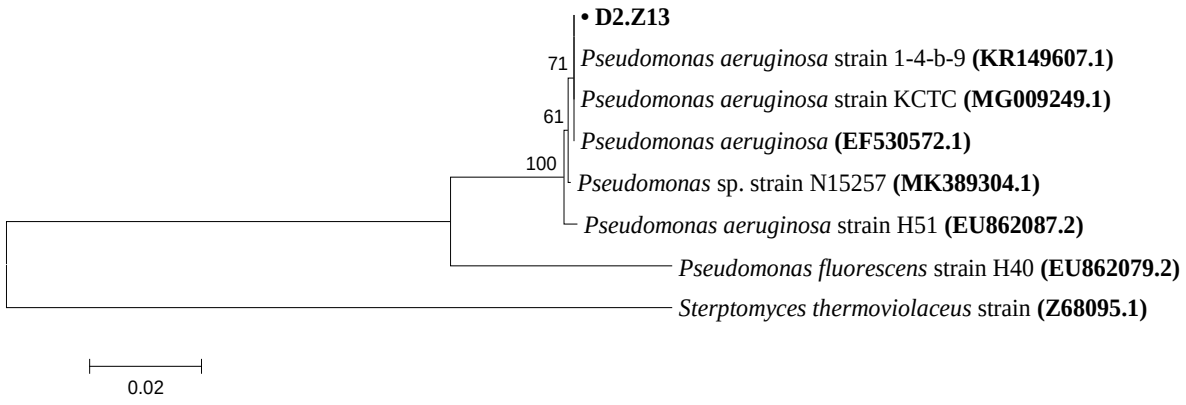


**Figure 3.** Phylogenetic tree of D2.Z13 isolate compared to its related strains based on 16S-rRNA sequences

In the present study, all 12 isolates were not pathogen to the post-larvae whiteleg shrimp, due to more than 80% of shrimp could survive after inoculated with $10^6$ cell/mL of each bacterial suspension. In contrast, more than 50% shrimp has died after inoculated with pathogenic *Vibrio* suspension. This result indicates that 12 sponge-associated bacteria did not interfere with the physiological processes of shrimps. The results of this study were also similar to some preceding studies. Rini et al. (2017), reported that the non-pathogenic marine bacteria exhibited a high survival rate on post-larvae pacific shrimp in range of 87.5-95.0% after inoculated with marine bacterial suspension. Supporting this result, Sasanti et al. 2010 concluded that some bacteria which showed a survival rate of shrimp approximately 80% could be developed as probiotics candidates.

Before conducting the challenge test for the potential bacterial isolates with *V. parahaemolyticus*, it is necessary to determine the number of *V. parahaemolyticus* concentrations needed to cause mortality of more than 50% postlarvae whiteleg shrimp ($LC_{50}$) (Cobo et al. 2012). The $LC_{50}$ value of *V. parahaemolyticus* obtained in this study was $10^5$ CFU's /mL. Supporting this result, the earlier study Joshi et al. (2014) also reported that *V. parahaemolyticus* isolated from Thailand shrimp farm has the $LC_{50}$ value around $10^5$ cell/mL. In this concentration, some *Vibrio* species are likely could infect shrimps and cause mass mortality through the quorum sensing mechanism (Gode-Potratz and McCarter 2011), therefore more than 50 % of shrimps has died after inoculated with $10^5$ CFU's/ mL of *V. parahaemolyticus*.

Twelve non-pathogenic isolates have been challenged to *V. parahaemolyticus in vivo*. The challenging test was conducted to evaluate the ability of bacterial isolates to repress the shrimp mortality caused by the infection of *V. parahaemolyticus*. Surprisingly, the inoculation of each isolate candidate reduced shrimp mortality up to 90%, or nearly 40 % higher than noninoculated treatments. Reduction of shrimp mortality may cause by the inhibition of *V. parahaemolyticus* growth and infection. All isolates exhibited different effects on shrimp survival rate. These isolate probably synthesis different anti-*Vibrio* compounds which give different effects on shrimp survival rate, as well as indicating a high diversity of anti-*Vibrio* compounds produced by these sponge-associated bacteria. Supporting this result, the shrimp fed with probiotics *Bacillus cereus* exhibited a higher survival rate (51.48 ± 16.51%) compared to untreated shrimps (53.01 ± 8.48%), as reported by Vidal et al. (2018). Another study also reported that sponge-associated bacteria could enhance shrimps survival rate ranging 70-90%, higher than the positive control which is only inoculated with *V. harve*yi (38.3±2.9%) (Rini et al. 2017). The tendency of survival values is also influenced by the density of infected bacteria (Wang et al. 2015) as well as the shrimp stage. Shrimp larvae in the zoea stage are more susceptible than mysis and post-larvae (Soto-Rodriguez et al. 2015).

D2.Z13 isolate, the most prospective isolate, showed the highest survival rate of 91.11%. The anti-*Vibrio* activity of this isolate possibly influenced by its capability in synthesizing bioactive compounds. The crude extract derived from this isolate identified to have some antimicrobial compounds which could be classified into benzene compounds, alcohols, esters, fatty acids, and amino acids. The most dominant compounds from D2.Z13-derived extract were 4,8-dihydroxy-2- (1'-hydroxyheptyl) -3,4,5,6,7,8-hexahydro-2H- [1] -benzopyran-5 (conginginin D). These compounds have been reported to have strong antimicrobial activity against *Acinetobacter baumanii* and *Staphylococcus aureus* (Li et al. 2019). In addition, some compounds including alkaloids, phenols, and flavonoids have been widely reported to have potential antibacterials (Nazim et al. 2014). According to the data, this sponge-associated bacteria strain could provide new sources of antimicrobial substances.

16S-rRNA based identification exhibited that D2.Z13 isolate was highly homolog to *Pseudomonas aeruginosa* 1-4-b-9 strain (similarity: 99%). Genus of *Pseudomonas* is well studied as one of the most promising sources of microbial bioactive compounds. *Pseudomonas aeruginosa* isolated from Antarctica which is known to be able to produce six dicopiperazines and two phenazine alkaloids. Phenazine pigment is known to have antibacterial activity and two phenazine alkaloids are known to play an active role in inhibiting the growth of Gram-positive bacteria such as *B. cereus*, *Micrococcus luteus* and *S. Aureus* (Saha et al. 2008). In addition, *P. Aeruginosa* strain H51 and *P. fluorescens* strain H40 isolated from sponges *Haliclona sp.* are known to have antibacterial activity against 36 Gram-positive and Gram-negative test bacteria (Santos et al. 2010).

In conclusion, based on the pathogenicity test and the challenge test of *V. parahaemolyticus in vivo*, it was known that twelve isolates of bacteria associated with sponge producing anti-*Vibrio* compounds were able to increase the survival rate of whiteleg shrimp. D2.Z13 as the most prospective isolate has been identified as *Pseudomonas aeruginosa* strain 1-4-b-9. The most dominant compounds in D2.Z13-derived extract were 4,8-dihydroxy-2- (1'-hydroxyheptyl) and 1,2,4-oxadiazol which are biologically active. This study suggested that these sponge-associated bacteria could be a good alternative for searching and developing of anti-Vibriosis agent.

## ACKNOWLEDGEMENTS

The authors appreciate the Ministry of Research, Technology and Higher Education of Indonesia that has promoted this project through "*Penelitian Dasar*" (Basic Research) 2019 (Grant No: 3/E1/KP.PTNBH/2019) awarded to Aris Tri Wahyudi. The authors also thank Jepri Agung Priyanto, who has given valuable advice and constructive criticism of this manuscript.

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