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Screening of sponge-associated bacteria to control vibriosis in vannamei shrimp (*Litopenaeus vannamei*)

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Abstract. *Sarjito S, Amalia R, Sabdaningsih A. 2022. Screening of sponge-associated bacteria to control vibriosis in vannamei shrimp (*Litopenaeus vannamei*). Biodiversitas 23: 5333-5341.* An intensive shrimp culture with a high stocking density causes a higher chance of vannamei shrimp being infected with diseases. The most frequent disease is caused by *Vibrio* sp. Vibrio might cause a severe production loss in the shrimp culture, leading to larvae and mature stadium mortality at 50%. Therefore, a study on the alternative management of vibriosis disease using biocontrol agents is urgently needed. The sponge is a marine invertebrate that provides as a shelter for microbes to live and symbiosis with the animal. An evident ability of sponge-associated bacteria to produce antimicrobial compounds can be exploited as a biodegradable bio-agent, potentially as a solution to control vibriosis disease in a shrimp. This research aimed to isolate sponge-associated bacteria with vibriosis antibacterial potential. Sampling was done in Tulamben, Bali and Panjang island, Jepara, Indonesia with six sampling sites consisting of 15 sampling points. Moreover, vibriosis bacteria were provided by the laboratory of fish disease at Main Center of Brackish Water Aquaculture Jepara. Totally, 24 and 47 pure cultures were successfully isolated using Zobell 2216E agar medium from Tulamben, Bali and Panjang Island, Jepara respectively. The isolates were tested for antibacterial assay using plug agar diffusion methods. The top eight potential isolates were found in isolate B6.3C, B6.3D, B6.4E, B9.3C, PA.1, PA.4, PC.3, and PH.1 against *Vibrio vulnificus, V. anguillarum, V. alginolyticus, V. parahaemolyticus*, however there was no activity in *V. harveyi*. The molecular method was carried out to identify those potential isolates using universal primer 16S rRNA. The BLAST homology showed that all potential antibacterial isolates belong to the family Bacilllaceae, such as *Bacillus cereus*, *Virgibacillus salarius, B. aerius, B. paramycoides, B. thuringiensis*, and *B. altitudinis*. Further research will be carried out on microencapsulation and in vivo tests.

Keywords: Antibacterial, marine sponge, vannamei shrimp, vibriosis

INTRODUCTION

A demand for vannamei shrimp (*Litopenaeus vannamei*) on local and international scales affects the advancement technology of shrimp culture. It leads to a higher potential of diseases for the vannamei shrimp. Bacteria, viruses, and fungal-causing diseases might occur as a result of an imbalance between the host, the pathogen agent, and the environment (Raman et al. 2013; Sanguanrut et al. 2018). A frequent disease occurs in shrimp aquaculture caused by *Vibrio* sp. bacteria (Ruangpan and Kitao 1991; Rusmana et al. 2021). This type of bacteria can grow quickly in pond water accelerated by abundant organic materials. According to Singh (1986) and Zheng et al. (2017), if the *Vibrio* sp. population is higher than other bacteria, it might decrease the life level of isolate shrimp during the seeding and breeding period. Bacterial disease caused by the genus Vibrio has become a "big concern" for industrial shrimp farmers (Sarjito and Sabdono 2021). The paleness of hepatopancreas characterizes vibriosis infection in a shrimp; redness or paleness of the carapace of the body; redness of the uropod and telson; and the red antenna (Sarjito et al. 2018). Therefore, Vibrio can cause a severe production loss in shrimp aquaculture (Stalin and Srinivasan 2017). In larvae and mature stadiums, Vibriosis leads to death around 50% (Lightner 1996). *Vibrio* spp. has been found to infect shrimp, such as *V. harveyi, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus* (Chythanya et al. 2002; Kumar et al. 2014; Gopal et al. 2005; Chandrakala and Priya 2017; Sarjito and Sabdono 2021).

In recent years, a significant research result on the prevention and control of vibriosis disease has yet to be found. Therefore, alternative studies for control of vibriosis disease using biocontrol agents are urgently needed. A biocontrol agent is a disease control management strategy using micro and macroorganisms as control agents with antagonist's properties to the disease-causing agents. The working system of biocontrol agents in controlling the disease is by producing antimicrobial compounds to inhibit or kill the growth of pathogens (Teplitski and Ritchie 2009).

One of the biocontrol agents' sources was derived from marine invertebrates. We previously investigated the potential of nudibranch-associated bacteria against *Vibrio harveyi* and *V. parahaemolyticus*. *Pseudoalteromonas piscicida* isolated from nudibranchs had inhibitory activity of 19.4 and 10.63 mm, respectively (Sarjito et al. 2020). In

the marine nutrient cycle, a sponge, which lives as a filter feeder, plays a zoobenthic roles, allowing marine microbes to easily infiltrate and reside in the sponge body. The flagellum cell is attached in the choanosome/middle part of the sponge's porous body, leading to a proper air and nutrient transfer, causing the marine sponge-associated microbes to be permanent. In this case, numerous bioactive compounds derived from sponge-associated microbes are evident (Sabdaningsih et al. 2020). A unique marine environment makes the production of the sponge's bioactive compound differs from the land's (Thakur et al. 2005). Several important bioactive compounds have been reported for antibacterial activities, including anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA), antituberculosis, antifungal, antiparasitic, antiviral, anticoagulant, anti-biofouling, anti-inflammation, UV protector, and cytotoxic potential as anti-cancer (Thakur and Müller 2004; Thakur et al. 2005; Anjum et al. 2016). However, the study on the biocontrol agents of vibriosis disease in vannamei shrimp derived from the sponge has yet to be reported. This research is urgently needed as an effort to control vibriosis disease in vannamei shrimp.

MATERIALS AND METHODS

Study area and specimen collection

A purposive random sampling technique was applied to collect various sponge species in Tulamben, Bali (Figure 1), on November 2020 and Panjang Island, Jepara, Indonesia, on October 2021 (Figure 2). Some of the sponge colonies were collected using a chisel and hammer. Samples were documented and labeled, then put into sterile plastic bags and stored in the cool box. The sponge identification was conducted according to morphological appearance compared to the identification book using references (Hooper et al. 2002; Hooper 2003; van Soest 1989; Colin and Arneson 199; Zea et al. 2014). Samples of the pathogenic bacteria *Vibrio* sp. were provided from vannamei shrimp of Main Center of Brackish Water Aquaculture Jepara. Namely *Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Vibrio vulnificus*, *Vibrio alginolyticus*, and *Vibrio harveyi*.

Isolation and purification

The sponge-associated bacteria were isolated from Tulamben, Bali, and Panjang island, Jepara. The bacteria were isolated using the Spread plate method (Brock and Madigan 1991) and cultivated on a ZoBell 2216E agar medium. Then, the petri dish containing samples was incubated for 48h at 37ºC (Sabdono and Radjasa 2006). Meanwhile, pathogen bacteria samples (*V. parahaemolyticus, V. harveyi, V. vulnificus, V. anguillarum, and V. alginolyticus*) were cultured for 24h.

The following step was observing the growing bacteria colonies and characterizing them based on the colony's morphology, including the shape, color, elevation, and edge (Cappuccino and Sherman 1987). Each colony with a different morphological appearance was purified using streak method to obtain pure isolate (Madigan et al. 2000). The obtained pure isolates were stored sideways in ZoBell 2216E medium in a test tube for further testing.

Screening of antibacterial activity

An antimicrobial activity screening aims to obtain candidate bacteria that capable of producing antibacterial compounds. The bacteria used in this study were five species of *Vibrio* from vannamei shrimp and sponge-associated bacteria. An antimicrobial activity test was carried out using the agar plug method. This method is usually used to demonstrate antagonism among microbes (Balouiri et al. 2016). The plug agar diffusion method was developed from various methods derived from various literature (Anteneh et al. 2021; Balouiri et al. 2016; Thakur and Müller 2004). This method was shown the ability to express antibacterial compounds on media without induction.

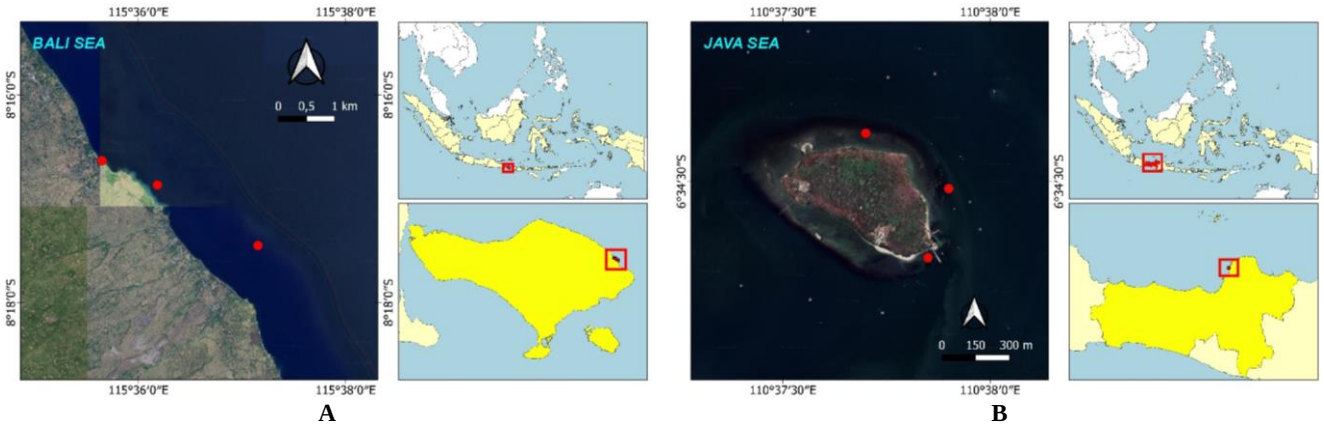

Figure 1. The sampling sites of marine sponges. Site 1 (08º16'38''S 115º35'22''E), site 2 (08º16'52''S 115º39'38''E), site 3 (08º17'27''S 115º37'9.2''E) at Tulamben, Bali Island (A) and site 1 (06º34'23''S 110º37'42''E), site 2 (06º34'31''S 110º37'54''E), site 3 (06º34'41''S 110º37'51''E) at Pulau Panjang, Java Sea, Central Java (B)

The first step was to inoculate the agar plate with sponge-associated bacteria (Balouiri et al. 2016). Then incubate the agar with the strain of bacteria looking for its antibacterial activity on Zobell 2216E medium (Thakur et al. 2004), using streak plate method (Balouiri et al. 2016). After incubation, the agar was cut into circles 6-8 mm in diameter. Additionally, it was placed in media previously inoculated with bacteria (Anteneh et al. 2021; Balouiri et al. 2016; Thakur and Anil 2000). The plate was then re-incubated for 48h at 37°C. The presence of antimicrobial activity is indicated by the formation of a clear zone around the agar plug (Thakur and Anil 2000; Balouiri et al. 2016; Sibero et al. 2018; Trianto et al. 2019). The clear zone that appeared was measured with a veneer caliper and recorded in millimeters in triplicates.

DNA extraction and amplification of 16S rRNA gene

Genomic DNA was extracted using Quick-DNA Miniprep Plus Kit Catalog Nos. D4068 and D4069 from Zymo Research follow the protocol for biological fluids and cells. The purity of the DNA was measured by Nanodrop (Thermo Scientific Nanodrop 2000 Uv-Vis Spectrophotometer) for the result of A260/280 (Susilowati et al. 2015). The bacterial DNA that was used for 16S rRNA gene sequencing was amplified with a polymerase chain reaction (PCR) using the universal primers 27F (5′AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′TACGGTTAACCTTG TTACGACTT-3′). PCR reaction with a total of 25µL consisted of 2.5 µL DNA template, each primer of 2.0 µL, ddH2O of 6 µL, and mix PCR (MyTaqTMRed Mix-Bioline) of 12.5 µL. The amplification reaction was carried out in a Thermal Cycler (BIO-RAD) T100 in accordance with Amalia et al. (2021), the specific steps are as follows: Initial denaturation at 95°C for 3 minutes; 30 cycles of denaturation at 95°C for 1 minute, then annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 7 minutes. The PCR products were examined using gel electrophoresis with agarose 1%, and the result was visualized by using UVIDoc HD5 (UVITEC Cambridge, UK). The amplicon with the desired size of 1500 bp was sent to 1st base, Malaysia for further sequencing analysis.

BLAST homology and phylogenetic tree

After the sequencing analysis was done, the resulting sequence was compared to other sequences from the GenBank database at the National Center for Biotechnology Information (NCBI), National Institute for Health (NIH), USA (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1997). BLASTn was used to identify similarity levels with other bacteria (Trianto et al. 2019).

Phylogenetic analysis of active bacterial isolates was constructed by MEGA X software. The Muscle algorithm was used to align the sequences and a neighbor-joining tree with a bootstrap method based on 1,000 replications was performed (Kumar et al. 2018) and feature neighbor-joining (Saitou and Nei 1978).

RESULTS AND DISCUSSION

The sponge is known to have a symbiosis interaction with diverse and complex prokaryotic communities (Thomas et al. 2016). They have an essential role in host defense against pathogenic microbes, likely due to their ability to synthesize bioactive compounds. This capability could be explored for controlling vibriosis. One of the most critical steps in screening for biocontrol agents is to evaluate the inhibitory activity between sponge-associated bacteria and vibriosis.

Sponges collection

The sponges sampling at the six sites in Tulamben, Bali and Panjang island, Jepara identified as *Ulosa* sp. (B4), *Ircinia* sp. (B6), *Clathria reinwardtii* (B9), *Callyspongia* sp. (PA1 and PA4), *Haliclona* sp. (PB), *Amphimedon* sp. (PC), *Hippospongia* sp. (PE), *Gelliodes* sp. (PF), *Chalinula* sp. (PG), *Echinodyctium* sp. (PH), and *Hyrtios* sp. (PI). All of the specimens were identified morphologically (Hooper 2003). *Ulosa* sp. (B4) is digitate sponge with yellow color and sharp thorn, while B6 specimen has irregular round body-form, with cavities, green to pinkish color, with rough texture. Based on these characteristics, B6 might belong to *Ircinia* sp. (Colin and Arneson 1995). B9 specimen has orange to brownish color, soft to rough texture, and rod branching form. These morphological characteristics are similar to *Clathria reinwardtii* (Colin and Arneson 1995; van Soest 1989). The last PA1 and PA4 specimen have light green with fingers, has many grayish-white spines on its surface, and has a hard texture. Based on the sponge specimen data, it can be stated that the possible species of this specimen are *Callyspongia* sp., genus *Callyspongia*, and family Callyspongiidae. This is following the morphological descriptions found in various work of literatures (Colin and Arneson 1995; de Voogd 2005; Hooper et al. 2002; van Soest 1989, Zea et al. 2014). This genus is the most common genus found in all tropical regions in the world (Colin and Arneson, 1995). 45 species of *Callyspongia* have been described in Indonesia (de Voogd 2004). *Callyspongia* spp. generally grows in the form of tubes, but it can also grow in sheets or fingers (Colin and Arneson 1995). *Haliclona* sp. (PB) has a soft body, the spicules and fibres may reduce because very riable and easily torn (Hooper 2003). The other specimen is *Amphimedon* sp. (PC) which a member of Demosponge. *Hippospongia* sp. (D) is horny sponge, while *Gelliodes* sp. (PF) is encrusting sponge which has spongy texture, fibrous, elastic, and tough (Zea et al. 2014). The three others, *Chalinula* sp. (PG) is encrusting sponge with purple-brown color, lobate, soft sponge, then *Echinodyctium* sp. (PH) has honeycomb-like structure and the last *Hyrtios* sp. (PI) is greyish black in colour, and its skeleton consists largely of sand.

Isolation and screening of antibacterial activity

In In this study, 24 pure cultures from Tulamben, Bali from four sponges, and 47 pure cultures from Panjang island, Jepara were successfully isolated from eight sponges using Zobell 2216E agar medium. The specimens,

sponges as marine benthos have a great diversity microorganism in their mesophyl tissues, therefore, scientists investigated these tissues under transmission electron microscopy (Gloeckner et al. 2014) and classified into two term which are High Microbial Abundance (HMA) and Low Microbial Abundance (LMA) (Hentschel et al. 2003). One of the sponges from Tulamben that was used in isolation is *Ircinia* sp. (B6), a genus that falls into HMA category (Gloeckner et al. 2014; Hardoim and Costa, 2014; Kelly et al. 2021; Poppell et al. 2014), yielded the highest number of isolates, precisely 11 isolates. Compared with *C. reinwardti* (B9) specimen, a species and a genus that falls into LMA category (Turon et al. 2018; Turon and Uriz 2020), yielded the lowest number of isolates; only 3 isolates were obtained. The other sponge from Tulamben, *Ulosa* sp. (B4), according to Khan et al. (2011) is fall under the category of low microbial abundance (LMA) sponge, reflected by its lack of sponge-specific microbial communities. Despite being classified as LMA sponge, *Ulosa* sp. yielded more isolates (11 isolates) than *Ircinia* sp. (B6) and *Clathria reinwardtii* (B9) (10 and 3 isolates respectively). This anomaly might be explained by phenomenon called "The Great Plate Count Anomaly", where less than 1% bacteria that was observed using microscopic analysis in sponge tissue could be cultured in standard medium, and only small number of bacteria that could grow under laboratorium condition (Riyanti et al., 2020). *Callyspongia* sp. (PA.1 and PA.4), one of the specimens collected in Pulau Panjang also belongs to LMA classification and only have 6 isolates, 3 isolates for each specimens (Bayer et al. 2014; Giles et al. 2013; Gloeckner et al., 2014; Steinert et al. 2016). The other hand, the rest samples from Panjang Island were *Haliclona* sp. (PB and PD) (8 isolates from two sponges), *Amphimedon* sp. (PC) (3 isolates), *Hippospongia* sp. (PE) (6 isolates), *Gelliodes* sp. (PF) (5 isolates), *Chalinula* sp. (PG) (10 isolates), *Echinodictyum* sp. (PH) (4 isolates), and *Hyrtios* sp. (PI) (5 isolates). These eight sponges from Jepara, only three which are included in HMA category such as *Hippospongia* sp., *Chalinula* sp., and *Hyrtios* sp. (Gloeckner et al. 2014). From this result, obtained at least 5 pure isolates in Zobell 2216E in the sponge that categorized as HMA.

A total of 71 isolates bacteria from Bali and Jepara were tested for their antibacterial activity against pathogenic *Vibrio* spp. Antipathogenic activity against *Vibrio* spp. was seen in 6 isolates from Tulamben Bali and 19 isolates from Panjang island. After further confirmation testing, the number of active isolates decreased to eight. The eight isolates with the highest antipathogenic activity and had large inhibiting zone were B6.3C, B6.3D, B6.4E, B9.3C, PA.1, PA.4, PC.3, and PH.1. After screening with the plug agar method, they consistently inhibited *Vibrio* spp. and as shown in Table 1 and Figure 1. Isolates with the best antibacterial activity (Table 1) were chosen for further molecular identification.

The antipathogenic activity of those isolates varied against *V. vulnificus, V. anguillarum, V. alginolyticus*, and *V. parahaemolyticus*. The absent activity only showed against *V. harveyi* for all isolates. Table 1 indicates that each isolate has a specific activity to species of Vibrio. Isolate B6.3C and B6.3D can counter the growth of *V. vulnificus*, then isolate B6.4E and PH.1 inhibit only *V. alginolyticus*. Furthermore, isolate B9.3C has a clear zone to *V. parahaemolyticus*. The rest isolates, encoded by PA.1, PA.4, and PC.3 have an antibacterial activity to *V. anguillarum.*

Moreover, the most prominent isolate was B6.3D which has diameter inhibiting zone of 24.48 mm. This value was categorized as a solid inhibitory activity (> 20 mm), followed by B6.3C with a clear zone of 11.80 mm that was categorized as a strong inhibitory activity (10- 20 mm). Meanwhile, B9.3C, PC.3, PA.4, PH.1, B6.4E, PA.1 with a clear zone of 10.80 mm; 9.93 mm; 9.73 mm; 8.83 mm; 8.19 mm; 5.07 mm, respectively categorized as medium inhibitory activity (5-10 mm). Davis and Stout (1971) classified the zone of inhibition (ZOI) in four intensities corresponding to ZOI diameters: >20 mm, very strong; 10-20 mm, strong; 5-10 mm, medium; and <5 mm, no response. The differences in the ability to produce the clear zone were presumably dependent on the secondary metabolites that were produced by test isolates (Ouchari et al. 2019). According to El-Kholy et al. (2014), competition in nutrient absorption was a common phenomenon in natural habitats. Several bacterial species use the antagonistic activity or inhibition property as a weapon against their competitors. In general, bacteria produce several antimicrobial compounds or bacteriocins to inhibit or kill other competitor bacterial species (Benitez-chao et al. 2021; Simons et al. 2020). Nevertheless, the degree of inhibition depends on several other factors and may change in the body (Banerjee and Ray 2017).

Table 1. Selected isolates of antipathogenic assay from the plug agar method

Isolate code	Antipathogenic activity	Diameter of inhibiting Zone (mm)				
		V. harveyi	*V. vulnificus*	*V. anguillarum*	*V. alginolyticus*	*V. parahaemolyticus*
B6.3C	+	-	11.80	-	-	-
B6.3D	+	-	24.48	-	-	-
B6.4E	+	-	-	-	8.19	-
B9.3C	+	-	-	-	-	10.80
PA.1	+	-	-	5.07	-	-
PA.4	+	-	-	9.73	-	-
PC.3	+	-	-	9.93	-	-
PH.1	+	-	-	-	8.83	

Note: Isolate with numbers 1-4 from Tulamben, Bali; numbers 5-8 from Panjang Island, Jepara

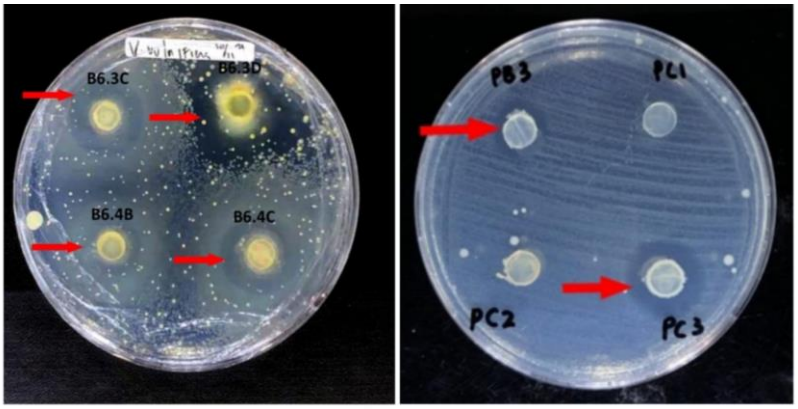

Figure 1. The plug agar result of bacterial isolates B6.3C, B6.3D, B6.4E, B9.3C, PA.1, PA.4, PC.3, and PH.1 against *Vibrio* spp. It showed that isolates B6.3C, B6.3D, and PC.3 (red arrow) had the strongest antipathogenic activity. The red arrow indicates the clear zone

Molecular identification of antibacterial activity

Isolates B6.3C, B6.3D, B6.4E, B9.3C, PA.1, PA.4, PC.3, and PH.1 were identified using a molecular method. The molecular identification was conducted by comparing the genetic features of the isolates to those of similar bacteria. As shown in Figure 2, the eight isolates have PCR products of approximately 1,500 bp in length. Further sequencing analysis indicated that B6.3C, B6.3D, B6.4E, B9.3C, PA.1, PA.4, PC.3, and PH.1 have 1,448 bp; 1,433 bp; 1,452 bp; 1,455 bp; 1,449 bp; 1,449 bp; 1455 bp; and 1,450 bp.in length, respectively (Table 2).

After sequencing the PCR amplification results of the 16S rRNA gene from potential sponges-associated bacteria, the results were edited to remove ambiguous nucleotides, moreover, the consensus sequence of DNA was compared to the BLAST database on NCBI (Table 3). The nucleotide length ranges from 1,433 to 1,455 bp, whereas the percentage of homology was from 98.25 to 100 %. Surprisingly, eight isolates are close relatives with Family Bacillaceae. There were *Bacillus* sp., *B. wiedmannii*, *B. cereus*, *Virgibacillus salaries*, *B. aerius*, *B. paramycoides*, *B. thuringiensis*, and *B. altitudinis* as performed in Table 2. The phylogenetic tree was made to understand the relationship between each other species. A total of 30 DNA sequences were aligned to construct the radial phylogenetic tree. it is proven that the isolates with the highest homology were always in the same clade (Figure 3).

Molecular identification was conducted for the most active sponge-associated bacteria (Table 1) by comparing 16S rRNA genes of the isolates with similar bacteria (Amalia et al. 2021). As demonstrated in Figure 2, all isolates have amplicons with a size of approximately 1,500 bp (Amalia et al. 2021), indicated by the bands in the 1,500 bp DNA ladder region. The BLAST homology of 16S rRNA sequence result revealed that eight isolates belong to *Bacillus* sp., *Bacillus wiedmannii*, *Bacillus cereus*, *Virgibacillus salarius*, *Bacillus aerius*, *B. paramycoides*, *B. thuringiensis*, and *B. altitudinis*. The result from Dat et al. (2021) showed that *Bacillus* was one of several genera that predominates cultivable isolates from marine sponges. *Bacillus* dominance in marine sponges might be due to their ubiquity in the marine environment because of their diverse metabolic and resilient spores, with the case of *B. cereus* (Liu et al. 2017a).

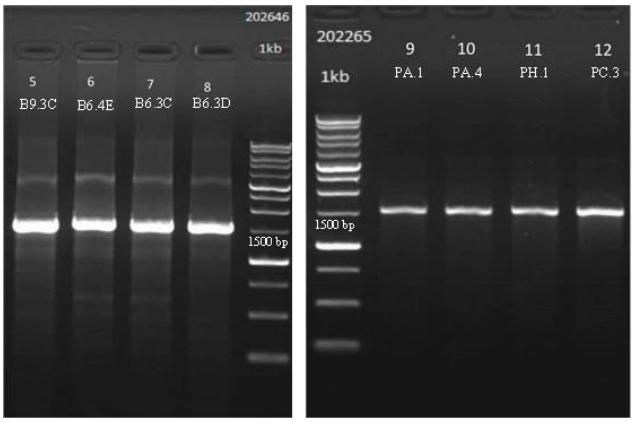

Figure 2. Evaluation of 16S rRNA gene amplification using electrophoresis gel; 1 kb: DNA markers

Table 2. BLAST homology analysis of potential sponges-associated bacteria

Isolate code	Length of nucleotide (bp)	Closest relatives	Homology	Accession number
B6.3C	1448	*Bacillus* sp.	100%	MN216227.1
B6.3D	1433	*Bacillus wiedmannii*	99.51%	ON231680.1
B6.4E	1452	*Bacillus cereus*	100%	MN216227.1
B9.3C	1455	*Virgibacillus salarius*	99.65%	MT299667.1
PA.1	1449	*Bacillus aerius*	99.59%	MZ502370.1
PA.4	1449	*Bacillus paramycoides*	98.25%	ON259746.1
PC.3	1455	*Bacillus thuringiensis*	99.65%	HM047298.1
PH.1	1450	*Bacillus altitudinis*	99.51%	MK521062.1

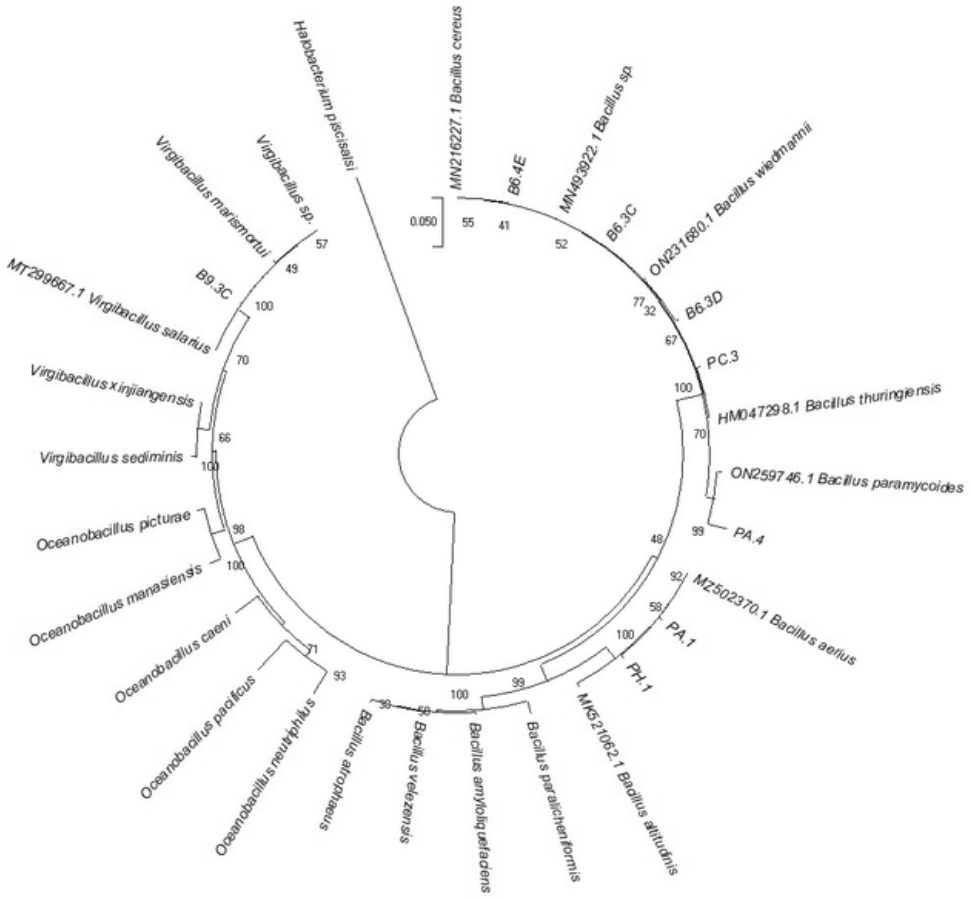

Figure 3. The radial phylogenetic tree based on 16S rRNA gene sequence analysis of antibacterial bacterial isolates B6.3C, B6.3D, B6.4E, B9.3C, PA.1, PA.4, PC.3, and PH.1 (blue circle) against *Vibrio* spp, using the Neighbor-Joining Approach. *Halobacterium piscisalsi* was used as an outgroup

Antibacterial activity

The eight potential isolates in antibacterial activity are Gram-positive bacteria. Bacillus was dominated that demonstrated antibacterial activity against *Vibrio* spp. (Table 2). This genus is known to have antimicrobial activity against many microorganisms (Sumi et al. 2015) and as probiotic in aquaculture (Kuebutornye et al. 2019). Most of the biologically active isolates in this study belong to *B. wiedmannii* isolated from Tulamben sponge specimens, *Ircinia* sp. (B6). Marine Bacillus species were known for their diversity of secondary metabolites such as lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, lipoamides, and isocoumarins that exhibited antimicrobial activity (Feliatra et al. 2017; Mondol et al. 2013).

Bacillus wiedmannii is spore-forming Bacillus strain, facultatively anaerobic, and previously categorized in *B. cereus* group before being assigned as a novel species (Miller et al. 2016). Some reports showed that members of *Bacillus* genus were able to be isolated from *Ircinia* genus (Prokof'eva et al. 1999; Thakur and Müller, 2004; Thakur et al. 2005). Furthermore, several studies have reported its antibacterial activity against aquaculture pathogenic bacteria, especially *Vibrio* spp. (Ravi et al. 2007). In this study, each isolate is capable of inhibiting specific *Vibrio* spp. due to every species having different mechanism to counter non favourable environment through secreting various proteinase and antimicrobial compounds against pathogens (Sumi et al. 2015). The other mechanism of bacteria to survive is producing bacteriocin, from group of *Bacillus* sp. (Motta et al. 2008; Sumi et al. 2015) also producing secondary metabolites such as alkaloids, flavonoids, and saponins that are capable of inhibit the growth and reproduction of pathogens (Feliatra et al. 2021). Possibly one or several combinations of these mechanisms was responsible for these results. Several studies showed that *B. cereus* is capable of inhibiting *V. alginolyticus* (Feliatra et al. 2021; Setiaji et al. 2020), by producing secondary metabolites compounds i.e., alkaloid, flavonoid, and saponin (Feliatra et al. 2021). These secondary metabolites compounds are capable to disrupt the peptidoglycan, one of the major constituents of bacterial cell wall, that causes lysis in cells (Feliatra et al. 2021).

PA.1 and PH.1 have high similarities with *B. aerius and B. altitudinis*, where *B. aerius* and *B. altitudinis* were proposed as new species that isolated from high altitudes

(Shivaji et al. 2006). *B. aerius* was selected as probiotic from intestine of healthy catfish and the study found this application could improve growth performance, innate immunity, and disease resistance of *Pangasius bocourti* (Meidong et al. 2017). Moreover, *B. altitudinis* was reported as antibacterial against *Pseudomonas aeruginosa* and *Escherichia coli* (Hwang et al. 2016) as well as antifungal on *Alternaria alternata* (Sun et al. 2021). It is also employed as spore supplementation on pig growth; however, the application did not enhance the growth performance (Crespo-Piazuelo et al. 2021). and the lowest similarity was performed in PA.4 which is closely related to *B. paramycoides.* This species was previously part of marine *B. cereus* group which then was assigned and described as new species by Liu et al. (2017b). *Bacillus paramycoides* is a Gram-positive, facultative anaerobic, non-motile, rod-shaped bacteria (Liu et al. 2017b). Trianto et al. (2019) reported antibacterial activity of *B. paramycoides* that was isolated from marine sponges against several human pathogenic bacteria. Isolate PC.3 was similar with *B. thuringiensis*, this species was well studied by many researchers (Jung et al. 2008; Salazar-Marroquin et al. 2016), the ability is not only to inhibit the growth of pathogens but also has characteristic as bactericidal against Gram-negative bacteria (Cahan et al. 2008).

The other genus from Family Bacillaceae that was found in this study is *Virgibacillus salarius.* This species has previously been isolated from soft coral (Sulistiyani et al. 2010) and hard coral (Ayuningrum et al. 2020), appointed as anti Multi Drug Resistant pathogenic bacteria. Moreover, Santos et al. (2010) isolated *Virgibacillus* genus, more exactly *V. pantothenticus*, from marine sponge, *Haliclona* sp. in Brazil and showed antimicrobial activity against *Corynebacterium fim.*

This study indicated sponge associated bacteria could be developed as antivibriosis and to alleviate the problems of antimicrobial resistance in aquaculture. Several publications showed *B. cereus* could be employed as probiotics in aquaculture, especially in shrimp farming (Barman et al. 2018; Ravi et al. 2007). According to research from Barman et al. (2017) and Roets-Dlamini et al. (2022), *B. cereus* has capability in nitrogen cycle which are nitrification and denitrification, therefore, this species is feasible become probiotics as well as bioflocs application in aquaculture. Although *B. cereus* is a pathogenic bacterium for humans and some animals (Bottone 2010), this seems not the case for many aquatic organisms such as shrimp (Ravi et al. 2007). *B. cereus* is also recommended by the Council of the European Union in Council Directive 70/524/EEC. Members of *Virgibacillus* genus is also capable of being employed as probiotics in aquaculture, such as the use of *V. proomii* as probiotics in sea bass larviculture (Hamza et al. 2016); the consortium of several bacteria, including *Virgibacillus* sp., in biofloc system in Indian white shrimp (*Penaeus indicus*), with improved growth and immunity (Panigrahi et al. 2020). Ultimately, further research is needed to better understand antimicrobial mechanisms and to isolate antimicrobial compounds from sponge associated bacteria.

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