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Identification bacteria associated with *Haliclona* sp. sponges from Enggano Island, Indonesia with antimicrobial activity against human pathogens

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ABSTRACT

Aims: Antimicrobial compounds are bioactive compounds that have ability to inhibit microbial growth activities. This study aimed to screen and identify bacteria associated with *Haliclona* sp. sponges from Enggano Island, Indonesia that had potential to produce antimicrobial compounds against *Escherichia coli*, *Candida albicans* and *Staphylococcus epidermidis*.
Methodology and results: The method used to screen and identify bacteria in this study including screening assay, morphological identification, Gram staining and spore staining method, biochemical tests and molecular identification based on 16S rRNA gene. This study resulted 16 isolates which were successfully isolated from *Haliclona* sp. According to screening assay, 5 isolates could potentially produce antimicrobial compounds coded as HEBS1, HEBS3, HEBS6, HEBB2 and HEBB3. Based on Gram staining, spore staining, biochemical test and molecular identification results, HEBS1 had proximity to *Brachybacterium paraconglomeratum*, HEBS3 had proximity to *Kocuria palustris*, HEBS6 had proximity to *Psychrobacter pasificensis*, HEBB2 had proximity to *Bacillus aryabhattai*, and HEBB3 had proximity to *Bacillus toyonensis*.
Conclusion, significance and impact of study: From 16 isolates that successfully isolated, there were 5 isolates that could potentially produce antimicrobial compounds against *Escherichia coli*, *Staphylococcus epidermidis* and *Candida albicans*. These isolates can be served as antimicrobial compounds producer. However, identification and purification of these antimicrobial compounds are needed to be done before applied it for medicine in the future.

Keywords: Antimicrobial compounds, associated bacteria, Enggano Island, *Haliclona* sp.

INTRODUCTION

Indonesia is an archipelago which most of its territory is an ocean and rich of natural resources. One of the province donating diverse natural resources in Indonesia is Bengkulu Province, which is famous with its tourist attraction like Enggano Island. Enggano Island is one of sub-districts in North Bengkulu Regency. The width of the area is 400,6 km^2, it has 6 villages located on the coastline, that is adjacent directly to the Indonesian ocean (North Bengkulu's Central Bureau of Statistics, 2017). This natural condition, rarely touched, makes the ecosystem in Enggano Island is still native, considered as Enggano's special attractiveness. One of the beauties of the sea in Enggano that can be enjoyed is sponge.

Sponge is an aquatic invertebrate, included as the oldest multicellular organism, that feeds itself by filter-feeding mechanism. Sponge belongs to Porifera phylum (Hentschel *et al.*, 2001). There are 3 main classes of sponges, those are Hexactinellida, Calcarea, and Demospongiae (Taylor *et al.*, 2007). Sponges are known to be able to produce bioactive compounds with anti-viral, anti-fungal, antibacterial, anti-inflammatory, and anti-tumor properties. Sponges that have been studied and known as the producers of bioactive compounds are *Aplysinella* sp., *Amphimedon viridis*, *Acanthella cavernosa*, *Craniella australiensis*, *Monanchora* sp., and *Haliclona* sp. (Joseph and Sujatha, 2011). Sponges also have mutual interactions with marine microbes like bacteria and fungus. Proksch *et al.* (2002) reported that marine microbes can produce secondary metabolites like the bioactive compounds to their hosts.

Microbes associated with sponges become popular in some studies in obtaining bioactive compounds. One group of sponges rich in bioactive sources is derived from *Haliclona* sp., which belongs to Chalinidae family. The

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microbes associated with *Haliclona* sp. sponges produces more antimicrobial compounds compared to the microorganisms associated with other sponges (Thomas *et al.*, 2010). This phenomenon underlies the selection of *Haliclona* sp. in this study. The metabolites successfully isolated from bacteria that are associated with *Haliclona* sp. are sterols, steroids, alkaloids, avarols, nucleosides, peptides, and polyketides (Blunt *et al.*, 2007).

Some studies show that the association of microbes and *Haliclona* sp. can produce antimicrobial compounds. Tokasaya (2010) has isolated some kinds of *Haliclona* sp.-associated bacteria from Raja Ampat ocean, Papua. According to the result of Minimum Inhibitor Concentration (MIC), isolates HAL-13, HAL-74 and HAA-01 had large spectrum antimicrobial activity against enteropathogenic *Escherichia coli* K1-1, *Vibrio harveyi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida tropicalis* and *Candida albicans*, and they could produce bactericidal compounds. According to the partial analysis of 16S ribosomal RNA encoding genes, those three isolates were identified as *Bacillus subtilis*. A study by Handayani *et al.* (2015) showed that there were 12 isolates of bacteria associated with *Haliclona fascigera* have activity against *methicillin-resistant Staphylococcus aureus* (MRSA).

The existence of sponges on Enggano Island is a good potential that introduce Enggano Island, so that it can become an important prospect in the health sector. Cahlia *et al.* (2018) has isolated some bacteria from *Haliclona* sp. from Enggano island and those isolates have activity against *Staphylococcus aureus*. Nowadays, the use of antibiotics in various treatments continuously causes microbial resistance. According to Allen *et al*. (2010), the continuity of antibiotics use in handling various diseases is a major challenge in the future therefore it is necessary to find new antimicrobial compounds to fight various infectious diseases and resistance to antibiotics.

Until now, the study of potential bacteria producing antimicrobial compounds associated with *Haliclona* sp. on Enggano Island has never been done and published yet. Thus, this study was conducted to screen the potential bacteria producing antimicrobial compounds associated with *Haliclona* sp. on Enggano Island and characterize the activity of the antimicrobial compounds.

MATERIALS AND METHODS

Collection of *Haliclona* sp. sponges

Haliclona sp. sponges were collected by purposive sampling technique. They were characterized by bright color, the body consisting of sponging fiber, and tubular body shape (Bell and Barnes, 2000). *Haliclona* sp. were collected on Banjar Sari beach (05°17.404'S 102°09.866'E) and Bak Blau beach, Meok village (05°19.141'S 102°13.470'E), Enggano sub-district, North Bengkulu Regency in depth 1-2 m with 25 °C to 27 °C sea water temperature by cutter representatively and put into a plastic bag filled with sea water (Cahlia *et al.*, 2018). Samples were treated and isolated near the collection place. Isolates were placed into a styrofoam box and brought to the laboratory by Navy's KRI Sibolga Bay Ship Republic of Indonesia.

Isolation of bacteria associated with *Haliclona* sp. sponges

A total of 1 g of sponge biomass was washed by sterile seawater, then crushed aseptically by using sterile mortar and pestle. These materials were homogenized and serially diluted in 9 mL of sterile distilled water with 10^{-1} to 10^{-3} dilution. Nearly 0.5 mL suspension of each serial dilution was plated into sea water complete (SWC) agar (contain 1 g yeast extract, 3 mL glycerol, 5 g peptone, 250 mL distilled water and 750 mL sea water, 15 g agar) by spread plate technique, then incubated for 24-36 h at ± 27 °C. The isolates grown on the medium were screened based on different characters, then purified to use in the next stage.

Screening of bacteria producing antimicrobial compounds against pathogenic microbes

In this study, bilayer method, dilution method, and the spotting method were applied to screen the potential bacteria in producing antimicrobial compounds using *Escherichia coli* and *Staphylococcus epidermidis* cultured in 25 mL nutrient broth (NB) medium, and *Candida albicans* cultured in 25 mL of PDB (potato dextrose broth) medium. At the same time, each isolate of bacteria associated with *Haliclona* sp. was cultured in 25 mL of liquid SWC medium. The culture was incubated in a shaker for 24 h at 1800 rpm at 27 °C to 30 °C. Each culture was added 20 µL into paper disc, then placed on medium containing pathogenic microbe and incubated for 48 h at 27 °C to 30 °C. The growth of pathogenic microbes would be inhibited by the potential isolates by the presence of clear zones around the paper disc.

Identification of selected potential antimicrobial isolates

The best potential isolates were selected based on the magnitude of the clear zone spectrum and observed by colony morphology, including the appearance of colony, colony surfaces, elevation, edges, and colors. Selected potential isolates were then identified by using Gram staining, spore staining, and biochemical tests consisting of sugar test, catalase test, motility test, citrate test, and urea test based on Lay (1994).

Gram staining was done by adding isolate on a sterile glass object, then tested by violet crystals reagent for 1 min. It was washed with aquades and pierced with solution of mordants (lugol) for 2 min and afterwards washed with aquades. Isolate was then administered with decolorization ethanol 96% and left for 1 min, and it was washed with aquades. The isolate was tested with Safranin. When bacteria able to maintain violet crystal reagent, bacteria are included into Gram-positive marked by purple color, whereas when bacteria are colorized by

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Safranin, bacteria are included into Gram-negative marked by red-pink color (Lay, 1994).

Spores staining was done by adding isolate on a sterile glass object, and then fixed over the Bunsen burner. Isolate were tested with malachite green and heated until steamy but not dry using hotplates for 15 min, then left for 1 min. Isolate was washed with aquades and treated with Safranin and left for 1 min. When bacteria produce spores, the spores will be observed in green under the microscope and the cells are pink (Lay, 1994).

The tests of sugar fermentation (glucose, maltose, lactose, and sucrose) were performed to see the ability of bacteria in fermenting sugar. The bacterial isolates were picked using the inoculum needle, isolates inserted into the medium to incubate at 37 °C for 48 h. Motility test was carried out by taking isolates by straight inoculum needle, it stabbed straight against the medium and incubated at 37 °C for 48 h. The motility test purposed to determine the presence/absence of motion tool in bacteria. The catalase test was using 3% of hydrogen peroxide (H_2O_2) reagent to determine the ability of bacteria in producing catalase enzyme. Isolates were taken using inoculum needle and placed on a sterile glass object, then administered with 3% of H_2O_2 reagent. A positive test is indicated by the formation of oxygen in isolates which characterized by the presence of bubbles.

The citric test was carried out by inoculated the bacteria with zig-zag form on tilt medium of Simon's citrate agar (SCA), then incubated at 37 °C for 48 h. Positive test results are indicated by the SCA medium color changed from green into blue. The urea test was carried out using the broth urea medium by taking isolate by inoculum needle and inserted into the medium, and then incubated at 37 °C for 48 h. Positive test result is indicated by the alteration of the medium color from yellow into purplish red (Lay, 1994).

Identification of the most potential bacteria based on 16S rRNA gene

The DNA of 5 potential bacteria was isolated based on Sambrook and Russell method with modification (Sambrook and Russell, 2001). The isolated DNA was amplified using the polymerase chain reaction (PCR) tools by using prokaryotic-specific primers namely: 63F forward primer (5A-CAG CAA GTC-3 GCC TAA CAC ATG) and reverse primer 1387R (5′-GGG CGG WGT GTA CAA GGC-3 ′) (Marchesi *et al.*, 1998). The composition of the PCR reaction consisted of La Taq DNA polymerase enzyme of 0.5 µL, 2× GC buffer of 25 µL, dNTPs (deoxynucleotide triphosphates) mixture of 8 µL, each primer (10 pmol) of 1.5 µL, ddH_2O (free water) of 9.5 µL, and template DNA of 4 µL. The PCR conditions were pre-denaturation (94 °C, 4 min), denaturation (94 °C, 45 sec), annealing (55 °C, 1 min), elongation (72 °C, 1 min 10 sec), and post PCR (72 °C, 7 min) with number of cycles was 30 cycles. The separation of PCR products was done on a mini-gel electrophoresis machine using 1% agarose at a 75 V for 45 min. DNA visualization was carried out in UV transluminator using ethidium bromide. The result was sent to PT. Genetika Sains Indonesia to analyze the DNA sequences. The result of the sequencing was then analyzed using the ChromasPro version 1.5 program and analyzed using the Mega 6.1 software (Tamura *et al.*, 2013) to perform the merge (contiq) forward and reverse. It compared with genomic data registered to the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool Nucleotide (BLASTn) on the site (http://www.ncbi.nlm.nih.gov/BLAST/). The homologous sequence obtained is aligned using the MEGA 6.1 software (Tamura *et al*., 2013) with bootstrap method (1000× bootstrap value). The phylogenetic tree was constructed to show the relationship of isolates with other species using the Neighbor Joining Tree method with 1000× bootstrap replications (Felsenstein, 1985).

RESULTS AND DISCUSSION

Collection of *Haliclona* sp. sponges

Sponges were collected from two different location as shown in Figure 1. The sponges were bright orange and they had tubular shape with spongin fiber. The map of Enggano Island was made based on RBI (Rupa Bumi Indonesia) map with shape file format in 1:50.000 scale and downloaded at https://tanahair.Indonesia.go.id/partal-web and constructed using Quantum Geographical Information System (QGIS) program/QGIS Project.

Isolation of bacteria associated with *Haliclona* sp. sponges

The isolation resulted 16 morphologically different bacterial isolates. There were 6 isolates coded as HEBS (isolated from Banjar Sari Beach) and 10 isolates were coded as HEBB (isolated from Bak Blau Beach, Meok).

Screening of bacteria producing antimicrobial compounds against pathogenic microbes

Sixteen isolates were used to screen the ability against pathogenic microbes by producing antimicrobial compounds through dilution method, bilayer method, and spotting method. The results of measurement of the clear zone are shown in Table 1. From this screening result, only 5 isolates ($HEBS_1$, $HEBS_3$, $HEBS_6$, $HEBB_2$, and $HEBB_3$) were chosen for subsequent experiment due to they are showing inhibitory activity towards different tested pathogens in this study.

The 5 potential isolates have different inhibitory activity against *E. coli*, *S. epidermidis* and *C, albicans* (Table 1). Each isolate had different inhibitory activity showed by the difference of clear zone diameter. $HEBS_1$ isolate had weak inhibitory activity against *Staphylococcus epidermidis*, indicated by the diameter of clear zone of 2 mm; $HEBS_3$ isolate had strong inhibitory activity against *Escherichia coli*, indicated by the diameter of clear zone of 44 mm; and $HEBB_3$ isolate had moderate inhibitory activity against *Candida albicans*, indicated by clear zone of 6 mm. The measurement of this clear zone was

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compared with positive control. The positive control used in this study was Chloramphenicol against *E. coli* and *S. epidermidis*, and Ketoconazole against *Candida albicans*. The inhibitory activity of these isolates was evaluate based on Davis and Stout (1971), 10-20 mm clear zone diameter has strong inhibitory activity, 5-10 mm clear zone diameter has moderate inhibitory activity and <5 mm clear zone diameter has weak inhibitory activity.

This screening shows the ability of potential isolates to produce antimicrobial compounds (Figure 2). According to Blunt *et al*. (2007), the secondary metabolites that can be isolated from bacteria associated with *Haliclona* sp. are sterols, steroids, alkaloids, avarols, nucleosides, peptides and polyketides, which most of these compounds have antimicrobial activity. The inhibition mechanism can occur in several ways, such as cell wall destruction and disruption of the protein synthesis process. According to Gale (1963), in bacteria, antimicrobial compounds can inhibit cell wall synthesis, damage cell membranes, and disrupt protein synthesis. In the inhibition of cell wall synthesis, antimicrobial compounds inhibit the binding of N-acetyl glucosamine and N-acetyl muramic acid, causing osmotic sensitivity of cell walls which leads to the cell wall destruction. While the mechanism of inhibition through protein synthesis occurs in ribosomes, precisely in large 50S subunits ribosomes and small 30S subunits ribosomes.

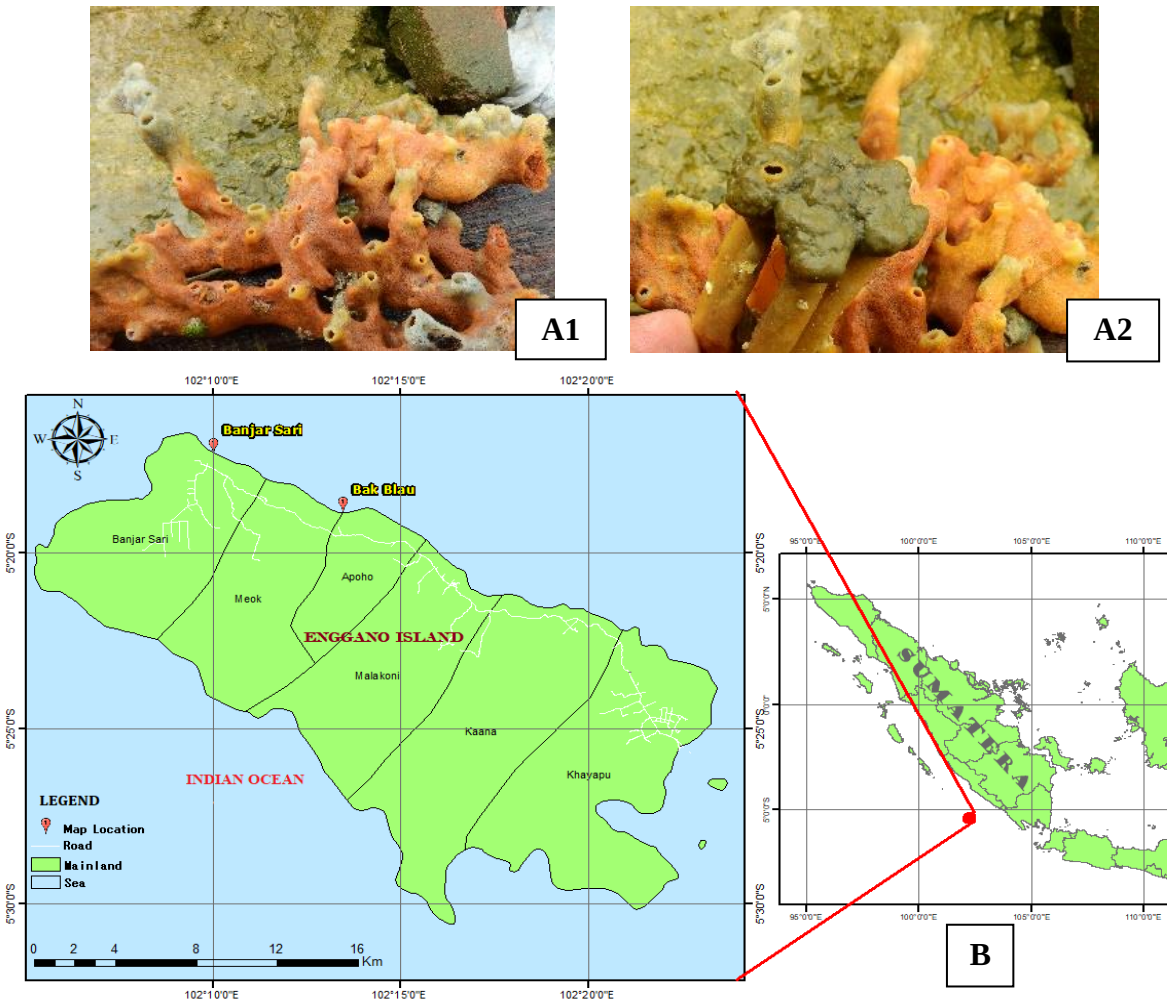

Figure 1: Sponges collection (A1) *Haliclona* sp. sponges collected from Banjar Sari beach and (A2) Bak Blau beach, Meok (Private Documentation) (B) Location of sponge samples collection, right side is Enggano Island's location seen from Sumatera Island as a whole and left side is Enggano Island. (map source: tanahair.indonesia.go.id)

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Table 1: Results of clear zones measurement and interpret based on Davis and Stout (1971) rating.

Potential isolates	Indicator pathogens	Diameter of clear zone (mm)	Inhibitory activity
$HEBS_1$	*E. coli*	0.50 ± 0.03	+
	S. epidermidis	2.00 ± 0.17	+
	C. albicans	0.00 ± 0.00	-
$HEBS_2$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	0.00 ± 0.00	-
$HEBS_3$	*E. coli*	44.00 ± 1.26	+++
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	0.00 ± 0.00	-
$HEBS_4$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	0.00 ± 0.00	-
$HEBS_5$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	0.00 ± 0.00	-
$HEBS_6$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	3.00 ± 0.26	+
$HEBB_1$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	0.00 ± 0.00	-
$HEBB_2$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	3.00 ± 0.17	+
$HEBB_3$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	6.00 ± 0.26	++
$HEBB_4$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	0.00 ± 0.00	-
$HEBB_5$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	0.00 ± 0.00	-
$HEBB_6$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	0.00 ± 0.00	-
$HEBB_7$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	0.00 ± 0.00	-
$HEBB_8$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	0.00 ± 0.00	-
$HEBB_9$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	0.00 ± 0.00	-
$HEBB_{10}$	*E. coli*	0.00 ± 0.00	-
	S. epidermidis	0.00 ± 0.00	-
	C. albicans	0.00 ± 0.00	-
Chloramphenicol	*E. coli*	27.00 ± 0.30	+++
	S. aureus	6.00 ± 0.20	++
	S. epidermidis	23.00 ± 0.44	+++
Ketoconazole	*C. albicans*	4.00 ± 0.10	+

- = none activity; + = weak; ++ = moderate; +++ = strong

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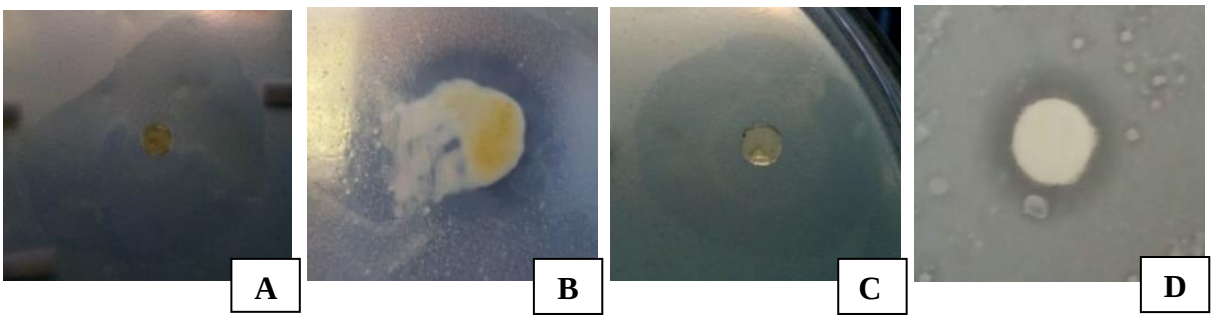

Figure 2: The inhibition zone produced by (A) $HEBS_3$ isolate against *Escherichia coli* and (B) $HEBB_3$ isolate against *Candida albicans*, (C) positive control (Chloramphenicol) against *Escherichia coli* (D) positive control (Ketoconazole) against *Candida albicans*.

Identification of selected potential antimicrobial isolates

Based on screening result, the 5 potential isolates' colonies were identified morphologically by observing several parameters (Table 2) and identified microscopically by Gram staining and spore staining method (Figure 3), then proceed with molecular identification.

Gram staining results revealed that $HEBS_1$, $HEBS_3$, $HEBB_2$, and $HEBB_3$ isolates were Gram-positive bacteria and $HEBS_6$ was Gram-negative bacteria (Figure 3). According to Pelczar and Chan (2007), bacteria are grouped into Gram-positive and Gram-negative bacteria based on their cell wall components. Gram-positive bacteria cell wall contains a thick peptidoglycan cell wall along with teichoic acid whereas Gram-negative bacteria cell contains a thin peptidoglycan cell wall with no teichoic acid, so it makes the color of the cell of both different (Panawala, 2017).

$HEBS_1$, $HEBS_3$, $HEBB_2$, and $HEBB_3$ isolates included into Gram-positive bacteria marked by purple color on the cell when observed under the microscope. The purple color indicates that the bacteria cell is able to retain the first color of the crystal violet even though it has been administered by decolorization alcohol 96% and Safranin as the second color substance. According to Pelczar and Chan (2007), Gram-positive bacteria cell wall was dehydrated when subjected to alcohol, so that pores shrink, absorption of cell wall and cell membrane were decreases. As such, the crystalline complex of violet with iodine could not come out of the cell, and Safranin could not fit into the cell wall and the cells remain purple.

Spore staining results revealed that all isolates did not produce spores (Figure 3). It shown by there was no green color appeared in the bacteria cells when observed under the microscope. Lay (1994) told that the bacterial cell appears pink and the spores will be green when it observed by microscope. The outer layer of the spore is a good barrier to chemicals, so the spores are difficult to be stained. Bacterial spores can be colored by heating because heating causes the outer layer of the spore to expand, so that malachite green can diffuse into the cell.

To determine the proximity of potential isolates to the genus level, a biochemical test was needed. The biochemical test results of potential isolates can be seen in Table 3. All isolates were positive on glucose and sucrose fermentation, and they were negative or not fermenting lactose. In addition, there were 2 isolates not fermenting maltose; $HEBS_6$ and $HEBB_3$. The motility test result showed that only $HEBB_3$ was motile. Catalase test results of all isolates were positive. Citrate test result showed that there were 3 isolates positive catalase; $HEBS_1$, $HEBS_6$, $HEBB_2$ and 2 isolates were negative catalase; $HEBS_3$ and $HEBB_3$. Urea test result shows only $HEBB_3$ was positive result.

Table 2: The colony morphology of the identified of bacterial isolates associated with *Haliclona* sp. sponges.

Isolate code	Appearance of colony	Colony surfaces	Elevation of colony	Edges of colony	Colony colors
$HEBS_1$	Circular	Smooth	Umbonate	Serrated	Cream
$HEBS_3$	Pint point	Smooth	Convex	Undulate	Pale Yellow
$HEBS_6$	Circular	Smooth	Convex	Entire	Peach
$HEBB_2$	Circular	Smooth	Raised	Entire	Cream
$HEBB_3$	Pint point	Smooth	Convex	Entire	Bright Yellow

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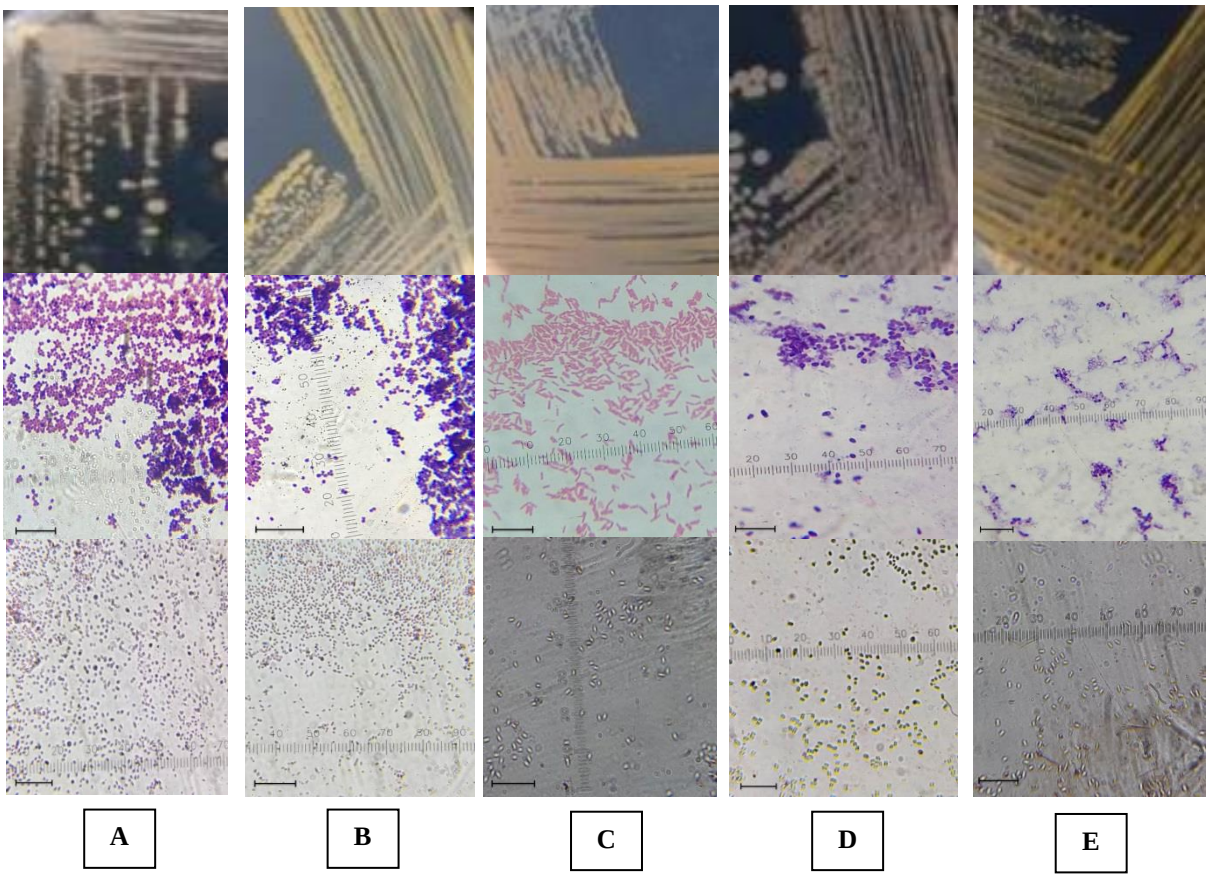

Figure 3: The results of morphological observation (upper), Gram staining observation (lower), and spore staining observation (lowest) of $HEBS_1$ (A), $HEBS_3$ (B), $HEBS_6$ (C), $HEBB_2$ (D), $HEBB_3$ (E) isolates (scale bar 10 µm).

Table 3: Biochemical test results of potential isolates associated with *Haliclona* sp. sponges.

Isolate code	Sugar test				Motility test	Catalase test	Citrate test	Urea test
	Glucose	Lactose	Maltose	Sucrose				
$HEBS_1$	+	-	+	+	-	+	+	-
$HEBS_3$	+	-	+	+	-	+	-	+
$HEBS_6$	+	-	-	+	-	+	+	-
$HEBB_2$	+	-	+	+	-	+	+	-
$HEBB_3$	+	-	-	+	+	+	-	+
Negative control	-	-	-	-	-	-	-	-

+ = positive result; - = negative result

Based on the results of Gram staining, spore staining and biochemical tests, each potential isolate could be suspected of being close to the bacterial genus as identified in the Bergey's Manual (Sneath *et al.*, 1994). The result showed that $HEBS_1$ isolate had proximity to *Staphylococcus*, $HEBS_3$ isolate had proximity to *Micrococcus*, $HEBS_6$ isolate had proximity to *Caryophanon*, $HEBB_2$ isolate had proximity to *Listeria*, and $HEBB_3$ isolate had proximity to *Marinococcus*. Molecular identification was proceeded to confirm the identification of the isolate due to it is more accurate than conventional method of macroscopic, microscopic, and biochemical tests. Moreover, identification through a sequence of 16S rRNA can identify bacteria to the species level. However, these two methods are complemented each other.

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Identification of 5 potential bacteria based on 16S rRNA gene

In this study, 16S rRNA gene amplification was done on 5 potential isolates based on the screening test conducted. The result of the amplification of 5 isolates showed that those isolates had a 16S rRNA gene, which was indicated by the presence of ± 1320 base pairs band on the results of electrophoresis visualization, as shown in Figure 4.

The 16S rRNA gene consists of 1540 base pairs which is very sustainable or conservative. The species having similarity of 70% or more DNA usually have sequential similarities of 97% or more, in which 3% is different and not evenly distributed along the primary structure, but it is concentrated in certain hyper variable regions. Thus, these different nucleotide sequences become a measure of phylogenetic distance between strains. So, the 16S rRNA gene is important in determining certain bacterial species and their kinship relationships (Stackebrandt and Goebel, 1994).

The results of sequencing then proceed with bioinformatics analysis using BLASTn. The results of BLASTn comparison of 16S rRNA sequences of those 5 isolates with other bacterial species at NCBI can be seen in Table 4, and their kinship relationships can be seen based on the analysis of the phylogenetic tree (Figure 5).

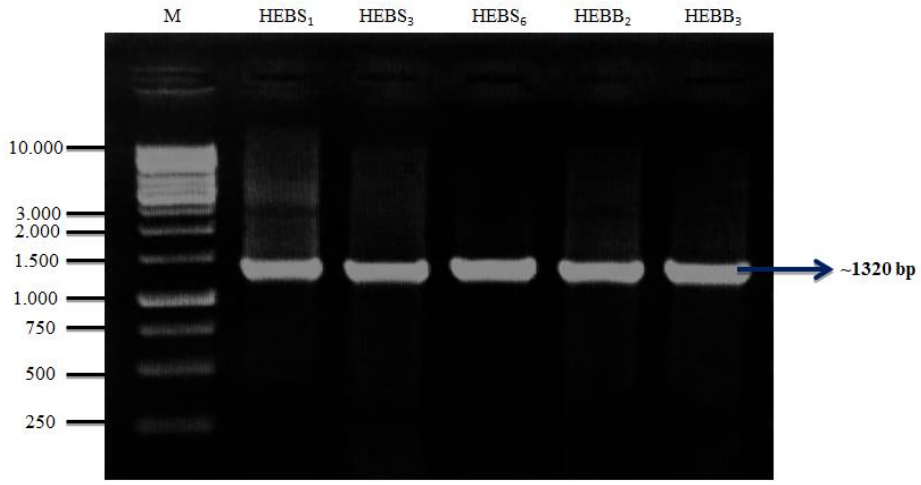

Figure 4: Visualization 16S rRNA gene of $HEBS_1$, $HEBS_3$, $HEBS_6$, $HEBB_2$, and HEBB in 1% agarose gel with approximate 1320 bp. Lane M: 1 kb ladder.

$HEBS_1$ colony is circular, smooth, umbonate, serrated and with cream color. $HEBS_1$ cell is coccus with staphylococcus arrangement. It is Gram-positive and non-spore forming bacteria. It is glucose, maltose, and sucrose positive, and lactose negative. It is non-motile, can reduce citrate and urea, and also catalase positive. BLASTn analysis results showed that $HEBS_1$ had 16S rRNA gene sequence, which was closest homology with *B. paraconglomeratum* strain ER41, with 99.92% of similarity value, and it is not a new species. While *B. conglomeratum* strain 0–2 and *Bacillus* sp. strain CW511 also have 99.92% of similarity value. From the characteristics that told by Takeuchi *et al.* (1995), the characteristics we found from *B. paraconglomeratum* and *B. conglomeratum* are the same. According to Sneath *et al.* (1994) on Bergey's Manual, the genus *Brachybacterium* grouped as irregular, non-spore forming, Gram-positive and rods bacteria, while the genus *Bacillus* grouped as endospore-forming, Gram-positive, rods and cocci bacteria. The molecular analysis showed that *B. paraconglomeratum*, *B. conglomeratum* and *Bacillus* sp. were in the same clade in phylogenetic tree construction. It showed that they have similarity in 16S rRNA, but they have some difference in biochemical behavior and appearance, for example, *Bacillus* sp. is spore-forming and motile, while *B. paraconglomeratum*, and *B. conglomeratum* are non-spore forming and non-motile. That's why they are separated. But, even *B. paraconglomeratum*, and *B. conglomeratum* are grouped into the same genus, they also have some differences, like different in tolerating pH and the colony color is also different. From that differences, we found that the characteristic of $HEBS_1$ was closest similar to *B. paraconglomeratum.*

$HEBS3_3$ colony is pint point, smooth, convex, undulate and with pale yellow color. $HEBS_3$ cell is coccus form with monococcus or diplococcus arrangement. It is Gram-positive and non-spore forming bacteria. It is glucose, maltose, and sucrose positive, and lactose negative. It is non-motile, can reduce urea, but citrate negative, and catalase positive. $HEBS_3$ had 16S rRNA gene sequence which was closest homology with *Kocuria palustris* strain VBW127, with the similarity value of 99.52%, and it is not a new species. $HEBS_3$ was closest to *Kocuria palustris* strain VBW127 because *K. palustris* strain VBW127 was located at the earliest position when it was analyzed. Furthermore, the construction of phylogenetic tree showing the kinship level of isolate with other bacterial

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Table 4: The identity of $HEBS_1$ $HEBS_3$ $HEBS_6$ $HEBB_2$ and $HEBB_3$ isolates based on 16S rRNA gene using BLASTn.

Isolate	Description	E-value	Percentage identity	Accession number
$HEBS_1$	*Brachybacterium paraconglomeratum* strain ER41 16S ribosomal RNA gene	0.0	99.92%	MT124566.1
	Brachybacterium conglomeratum strain 0–2 16S ribosomal RNA gene	0.0	99.92%	MN061004.1
	Bacillus sp. (in: Bacteria) strain CW511 16S ribosomal RNA gene	0.0	99.92%	MK034257.1
$HEBS_3$	*Kocuria palustris* strain VBW127 16S ribosomal RNA gene	0.0	99.52%	KC534322.1
	Kocuria palustris strain APP63 16S ribosomal RNA gene	0.0	99.52%	MT534060.1
	Kocuria assamensis strain ADL041 16S ribosomal RNA gene	0.0	99.52%	MK818797.1
$HEBS_6$	*Psychrobacter pacificensis* strain LPB0279 16S ribosomal RNA gene	0.0	99.58%	MN577288.1
	Psychrobacter faecalis strain hyss43 16S ribosomal RNA gene	0.0	99.58%	FJ613305.1
	Psychrobacter piscidermidis strain MON006 16S ribosomal RNA gene	0.0	99.42%	KF042028.1
$HEBB_2$	*Bacillus aryabhattai* strain ZJJH-1 16S ribosomal RNA gene	0.0	99.84%	MT605510.1
	Bacillus aryabhattai strain ZJJH-2 16S ribosomal RNA gene	0.0	99.84%	MT605509.1
	Bacillus aryabhattai strain QZTU 16S ribosomal RNA gene	0.0	99.84%	MT605459.1
$HEBB_3$	*Bacillus toyonensis* strain J5BS2 16S ribosomal RNA gene	0.0	99.69%	MT415973.1
	Bacillus thuringiensis strain JB10 16S ribosomal RNA gene	0.0	99.69%	MT193295.1
	Bacillus cereus strain DOS-CTC-1 16S ribosomal RNA gene	0.0	99.69%	MF076224.1

species by the Neighborhood Joining Tree method with 1000× repetition bootstrap value, as shown in Figure 5, in which *K. palustris* strain VBW127 is the closest to $HEBS_3$ isolate, although *K. palustris* strain APP63 and *K. assamensis* strain ADL041 also have 99.52% of similarity value.

Kocuria palustris and *K. assamensis* are grouped into the same genus; *Kocuria*. The genus *Kocuria* was proposed by Stackebrandt *et al.* (1995) to accommodate phylogenetically distinct *Actinobacteria* formerly classified in the genus *Micrococcus*. Before $HEBS_3$ was identified as *Kocuria palustris*, we found that the characteristic of it was quite similar to the genus *Micrococcus* when compared in Bergey's Manual. According to Kovacs *et al.* (1999), *K. palustris* is round, in which the cell arrangement is in pairs or tetrads, Gram-positive, non-motile. The colonies are pale yellow with a colony diameter of 2-3 mm, the surface of the colony is slippery with irregular edges. *Kocuria palustris* is an aerobic microbe and grows in medium with a range of 7% NaCl. It shows positive result on catalase and urea test, and negative on citrate test. These characteristics are the same as the results of this study on macroscopical, microscopical, and biochemical tests.

If we compared the characteristics of *K. assamensis* (Kaur *et al.*, 2011) and *K. palustris*, they are quite different in appearance and biochemical behavior even they were in the same clade on phylogenetic tree construction (Figure 5), the differences showed by the color of colony and also from Gram staining characteristic. So, $HEBS_3$ is closer to *K. palustris* than *K. assamensis*.

$HEBS_6$ colony is circular, smooth, convex, entire and with peach color. $HEBS_6$ cell is short basil shape with diplobasil arrangement. It is Gram-negative and non-spore forming bacteria. It is glucose, and sucrose positive, but lactose and maltose negative. It is non-motile, cannot reduce urea, citrate and catalase positive. $HEBS_6$ had 16S rRNA gene sequence, which was closest homology with *Psychrobacter pasificensis* strain LPB0279, with 99.58% of similarity value, and it is not a new species. While *P. faecalis* strain hys43 also has 99.58% of similarity value and *P. piscidermidis* strain MON006 has 99. 42 % of similarity value. *Psychrobacter* cell is

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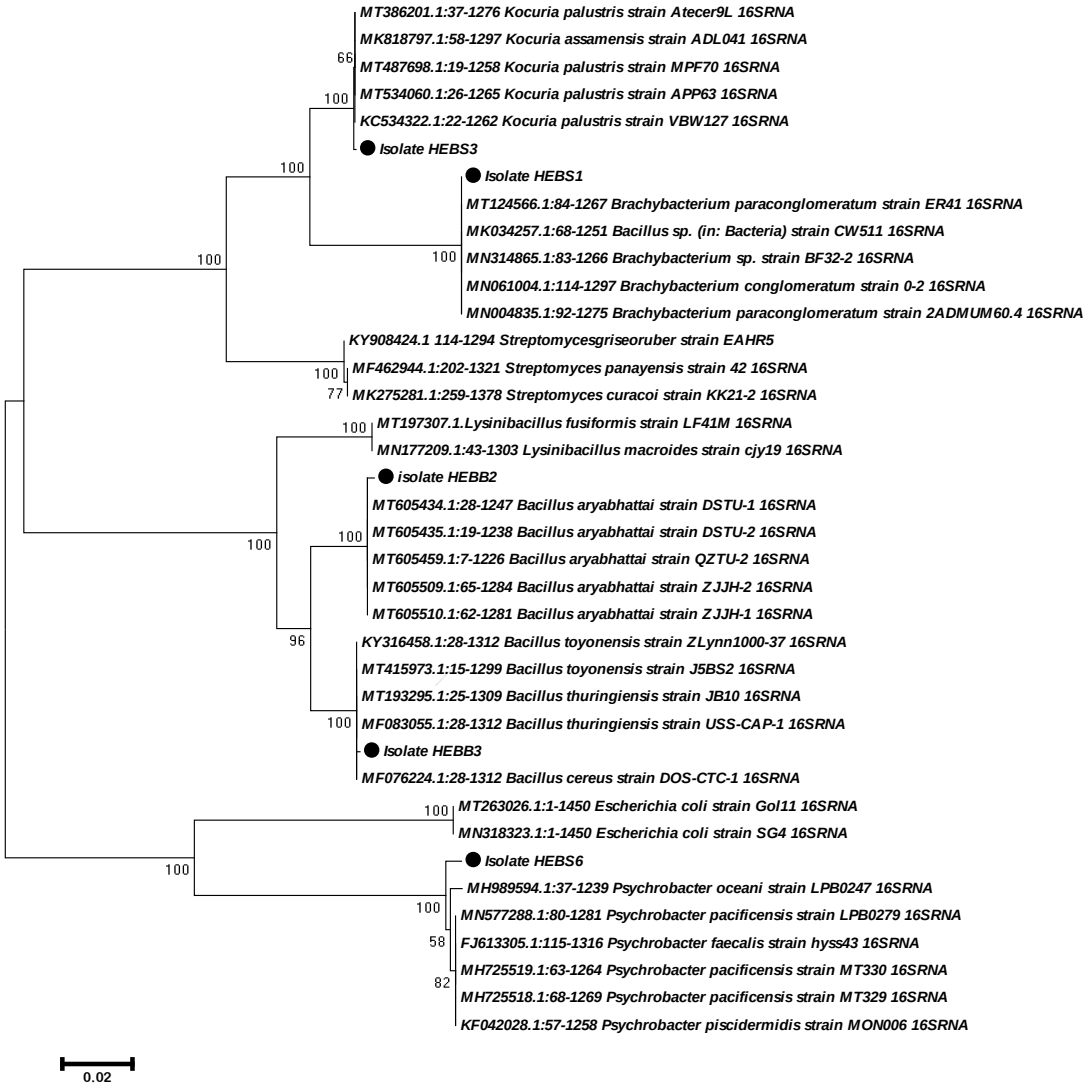

Figure 5: The phylogenetic tree of $HEBS_1$, $HEBS_3$, $HEBS_6$, $HEBB_2$ and $HEBB_3$ isolates based on 16S rRNA gene compared with its closely related species. Construction based on Neighbor Joining Tree method with bootstrap 1000× value.

coccobacilli vary in length from extremely short to relatively long. Gram-negative, non-motile, aerobic, non-pigmented, and non-spore forming, catalase and oxydase positive, most of them are psycotrophic (5-20 °C) but some of them able grow in 35-37 °C too. Colony is smooth and opaque, sometimes found associated with fish. It can ferment glucose and unable to form acid from fructose or sucrose or to hydrolyze starch (Sneath *et al.*, 1994). Although *P. faecificensis* and *P. faecalis* were in the same clade from phylogenetic tree construction and have similarity in 16S rRNA gene, but they have difference in biochemical behavior and appearance, for example *P. faecificensis* able to produce acid from glucose (Maruyama *et al.*, 2000) while *P. faecalis* cannot produce acid from glucose (Kampfer *et al.*, 2002), and they are different in colony color. So, the characteristic that closest to $HEBS_6$ was *P. faecificensis.*

$HEBB_2$ colony is circular, smooth, raised, entire and with cream color. $HEBB_2$ cell is short basil with streptobasil arrangement. It is Gram-positive and non-spore forming bacteria. It is glucose, maltose, and sucrose positive, but lactose negative. It is non-motile, cannot reduce urea, citrate and catalase positive. $HEBB_2$ had 16S rRNA gene sequence which was closest homology with *Bacillus aryabhattai* strain ZJJH-1, with the similarity value of 99.84%, and it is not a new species. While

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B. aryabhattai strain ZJJH-2 and *B. aryabhattai* strain QZTU also have 99.84% of similarity value. *Bacillus aryabhattai* colonies are peach-colored, entire, round, and flat. Cells produce ellipsoidal and central endospores and are motile. It grows at 10-37 °C and pH 6-10. It does not grow at 42 °C or at pH 4 or 11. It can tolerate up to 11.6 % NaCl and resistant to UV radiation. It grows on peptone. It produces acid from various carbon sources and utilizes a number of sugars, amino acids and other carbon compounds as sole carbon sources. Oxidase positive, urease positive, can reduce Nitrate to Nitrite. Glucose, fructose, maltose and lactose are positive (Shivaji *et al.*, 2009). Those characteristics are the same with characteristics that $HEBB_2$ has and it was reinforced by phylogenetic tree construction result where they are in the same clade (Figure 5).

$HEBB_3$ colony is pint point, smooth, convex, entire and with bright yellow color. $HEBB_3$ cell is basil with monobasil arrangement. It is Gram-positive and non-spore forming bacteria. It is glucose and sucrose positive, but lactose and maltose negative. It is motile, urea and catalase positive, but cannot reduce citrate. $HEBB_3$ had 16S rRNA gene sequence, which was closest homology with *Bacillus toyonensis* strain J5BS2, with 99.69% of similarity value, and it is not a new species. While *B. thuringiensis* strain JB10 and *B. cereus* strain DOS-CTC-1 also have 99.69% of similarity value. The characteristics of *B. toyonensis* (Jiménez *et al.*, 2013) and *B. thuringiensis* (Federici *et al.*, 2010) is quite different in biochemical characteristic and appearance. So, even *B. toyonensis* and *B. thuringiensis* are quite similar in 16S rRNA gene and were grouped into the same genus, the characteristics comparison result showed that $HEBB_3$ has closer characteristics to *B. toyonensis* than *B. thuringiensis.*

From conventional identification method that we used, some of potential bacteria's characteristics are not matches with known descriptions from some literatures. It caused the difference result from genus identification and species identification. It might be happened because bacteria adapted with the environment they lived. According to Ochman and Santos (2005), physical and biochemical characters are characters that are not static and change due to stress and evolution, so this also affects the outcome of identification. So, molecular identification is the better way to make sure the accuracy of conventional identification.

CONCLUSION

This study introduced the nature potential owned by Enggano Island and the discovery of potential bacteria producing antimicrobial compounds. Total of bacteria associated with *Haliclona* sp. successfully isolated were 16 isolates. From those 16 isolates, there were 5 isolates that could potentially produce antimicrobial compounds against *E. coli*, *S. epidermidis* and *C. albicans*. Identification result based on 16S rRNA gene analysis and phylogenetic tree analysis using Neighbor Joining Tree method with 1000x repetition bootstrap values revealed that $HEBS_1$ had proximity to *Brachybacterium paraconglomeratum*, $HEBS_3$ had proximity to *Kocuria palustris*, $HEBS_6$ had proximity to *Psychrobacter pasificensis,* $HEBB_2$ had proximity to *Bacillus aryabhattai*, and $HEBB_3$ had proximity to *Bacillus toyonensis*. The results of this study can be further reviewed to identify the pure antimicrobial compounds produced by potential isolates and can be alternative way to be developed and applied in the health field for future.

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