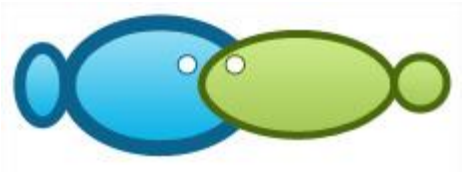

Selection and characterization of potential bacteria for polycyclic aromatic biodegradation of hydrocarbons in sea sponges from Spermonde Islands, Indonesia

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Abstract. Some mucous substances that cover the sponge body surface are related to the degradation ability of symbiotic bacteria of poly aromatic hydrocarbons (PAHs). This study aims to demonstrate the above statement through a morphological, phenotypical, and genotypic investigation of the bacterial symbionts. The following tracing methods were used in this study: morphological analysis, phenotype analysis (Gram staining and biochemical tests), microsymbiont genotyping using PCR (Polymerase chain reaction), and growth test activity of symbiont isolates on PAH contaminated solid and liquid media (anthracene, pyrene and mix 16 PAH ASTM standard). Three types of sponge symbiont isolate, *Pseudomonas stutzeri* RCH2, *Bacillus licheniformis* ATCC9789, and *Bacillus* sp. AB353 partially showed growth activity on both types of test media (solid and liquid media), whereas *Acinotobacter calcoaceticus* PHKDB14 showed no growth activity. These results indicate that there are morphological, Gram grouping, phenotypic characteristics, and bacterial symbiont genotypes correlation with PAH degradation ability.
Key Words: bacterial symbionts, degradation, PAH, sponge.

Introduction. Poly aromatic hydrocarbons (PAHs) are compounds that can significantly reduce the marine environment quality. PAHs can come from petroleum exploration activities, sea transportation, and ballast water of tank cleaning. These activities produce sludge waste containing PAHs. In the hydrological cycle, sludge waste will be carried by the current and end up at sea. Carcinogenic and mutagenic compounds in PAHs can threaten the life of marine biota including sponges (Zakaria et al 2009). Thus, the handling and management of PAH waste need to be seriously and intensively performed. Several physical, chemical, and biological methods applied to treat PAH waste have not yielded maximum results, as expected. It can be even be said that the methods are inefficient in reducing the toxicity of PAHs through degradation mechanisms (Yunker & Macdonald 1995).

Several studies have reported that some types of bacteria can degrade hydrocarbon components from PAH, both aliphatic and polyaromatic types (Samanta et al 2001; Rafin et al 2009; Sheikh & Pattabhiramaiah 2015). Furthermore, it is said that the source of bacteria that can reduce the toxicity of PAH is generally isolated from mud, mangroves, and seawater exposed to hydrocarbons and sponges (Marzuki et al 2015a). The bacteria are suspected to have specific characteristics that can degrade PAH.

Sponge bacterial symbionts that can degrade PAH are thought to originate from certain types of sponges that can produce substances with enzyme-like characteristics (Belila et al 2016; Liu et al 2017; Cita et al 2017). In general, these substances are found to cover most of the sponge body surface (De Rosa et al 2003). The substance is in the form of mucus produced by sponges to protect themselves against the

environmental changes (Ismet et al 2011; Marzuki et al 2016). The surface covering the sponge body is also intended as self-defense against predators and as an adaptation to extreme environmental changes (Cita et al 2017; Jesionowski et al 2018), which can be caused by contamination of toxic substances in the form of PAH hydrocarbons, heavy metals, plastic wastes, and strong underwater current pressures (Rao et al 2009; Zhang et al 2012; Bello-Akinosho et al 2015). Mucus produced by sponge bacterial symbionts has an enzyme-liked character assumed to correlate with the bacterial symbionts that can destroy several types of PAH (Akinde et al 2012; Jesionowski et al 2018; Kepel et al 2018). The overhaul of PAH chemical structure by bacterial symbionts can occur due to the bacterial capability of utilizing carbons in PAHs as an energy source to support their activities (Zhang et al 2012; Marzuki et al 2017; Cita et al 2017).

Specifically for bacteria isolated from sponges, mapping is needed because several types of bacteria are symbiotic with sponges and not all bacteria that are symbiotic with sponges can degrade PAHs (Akinde et al 2012; Muller et al 2014; Lavy et al 2014; Manzanera et al 2015; Marzuki et al 2020). Therefore, efforts should be made to identify sponges morphologically (growth forms, body surface consistency, skeletal structure, and spicular shape), phenotypes (gram groups of bacteria, cell color, dispersal, endospores, reactions with safranin reagents, and their solubility to KOH reagents) and genotype (base sequence and length of DNA sequences, species, and strains) (Marzuki et al 2015b; Liu et al 2017; Vaezzadeh et al 2017). The aim of this study is to report the analysis results of sponge morphology, phenotypes and genotypes of some sponge bacterial symbionts. The bacterial symbionts cell growth test results on PAH contaminated media are also reported to answer suspicions related to the ability of PAH degradation by sponge bacterial symbionts (Muller et al 2014; Marzuki et al 2014; Marzuki et al 2015a; Pawar 2017).

Material and Method. Sponge samples, CH_3OH p.a., KOH p.a., 96% alcohol, sterile seawater, phosphate buffer saline (PBS), NA media, marine agar (MA), 25% glycerol, 0.9% NaCl solution, 2% formalin, Aqubides, MTT, KCl, $MgCl_2$, DMSO, bacterial biochemical test standards for 15 parameters were used. For the analysis of bacterial genotyping, 16S rRNA *E. coli* universal primers were used, namely forward primer FP-U1 (5'-CCAGCAGCCGCGGTAATACG-3') attached to the nucleotide base sequence 518-537, and reverse primer RP-U2 (5'-ATCGG (C)/T) TACCTTGTTACGACTTC-3') attached to the nucleotide base sequence 1.513-1.491. The DNA template, DNA polymerase Taq enzyme (Perkin-Elmer, Norwalk, Conn), reagents for PCR analysis, Triton X-100, EDTA, Tris-HCl, KCl, $MgCl_2$, paraffin, deoxynucleoside triphosphate, polyacrylamide gels, agarose, ethidium bromide p.a., anthracene cas. no: 120-12-7, pyrene cas. no: 129-00-0 and a mix 16 PAH ASTM standard (*Supelco*) were also used.

The tools employed included: scuba, underwater camera, GPS, scalpel, tweezers, jars, plastic bags, ice boxes, microscope phase contrast hemocytometer set, porcelain plates, mortar and pestle, blenders, glass sets, bunsen, analytical balance, hot plate, rubber suction, Whatman, oven, freezer, BOD bottle, thermometer, rounded Ose needle, test tube, 1.5 mL microtubes, Petri dish, vortex, centrifuge, magnetic stirrer, gel compacting container, universal paper, salinometer, stopwatch, laminar airflow (LAF), object-glass, autoclave, 0.2 µm filter, PCR machine (Biorad), BioEdit program, MAS-100 (Microbiology Air Sampler), and electrophoresis.

Sponge samples. Sponge samples were obtained from Kodingareng Keke Island (Makassar administrative area), Indonesia. Kodingareng Keke Island is part of Spermonde Islands. There were 4 types of sponge samples, named Sp1, Sp2, Sp3 and Sp4, obtained at 4 different sampling locations (Figure 1). Sample analysis was conducted from September 2018 to June 2019 at the Laboratory of Biochemistry, Faculty of Mathematics and Natural Sciences, Hasanuddin University. Parameters measured are listed in Table 1. The sampling point coordinates were $05^0$06'06.76"S, $119^0$17'10.66"E for Sp1, $05^0$06'06.87"S, $119^0$17'10.90"E for Sp2, $05^0$06'06.15"S, $119^0$17'10.18"E for Sp3, and $05^0$06'06.73"S, $119^0$17'10.34"E for Sp4.

Figure 1. Sponge sampling points (Kodingareng Keke Island), Spermonde Islands, South Sulawesi, Indonesia (source: google maps).

Morphological analysis of sponge. The sponge structure tracking applied the morphological analysis method to obtain information about the sponge cell structure, growth shape, body consistency, oscula size, skeleton and shape of spicules. Sea sponge samples were prepared in 70% methanol, selected on a cross-section of mesohyl that was cut transversely, then observed under a contrast phase microscope using a hemocytometer. The surface was smeared with 70% ethanol then observed again using a hemocytometer. The sponge pieces were sterilized using sterile seawater filtered with a 0.2 µm filter. Sponge samples were crushed with a blender to produce cell suspension, which was observed under a microscope using a hemocytometer to observe cell types, skeletons, and sponge species (Zhou et al 2016; Manzanera et al 2015; Marzuki et al 2016; Cita et al 2017).

Phenotypic analysis of sponge bacterial symbionts. The phenotypic analysis of sponge bacterial symbionts aims to analyze the relationship between sponge morphology, phenotypical characteristics (morphology, Gram test, and biochemical tests) to obtain a constructive figure of the ability of some sponger bacterial symbionts with growth activity on PAH contaminated media. Characterization of sponge symbionts was carried out by the Gram stain identification method and biochemical tests. The gram staining process was carried out by taking 1 g of bacterial gram dye on a pre-sterilized glass that has been previously sterilized from free fatty acids using 96% alcohol, which was then used as purple crystal drops (Gram A), Lugol drops (Gram B), drops of acetate alcohol (Gram C), and safranin test (Gram D). Tests were carried out with 1% KOH (Ismet et al 2011; Marzuki et al 2016; Vaezzadeh et al 2017). Biochemical test standards contained 15 parameter tests, namely: starch hydrolysis, casein hydrolysis, indole reaction, nitrite reduction, glucose fermentation, lactose fermentation, sucrose fermentation, citrate test, catalase test, urease test, H_2S test, methyl-red test, voges-proskauer, gelatin test and mannitol fermentation. Changes in color, turbidity, or sediment in the media were indicated by a reaction indicator (Marzuki et al 2016; Cita et al 2017).

Genotypic analysis of sponge microsymbiont by PCR method. Genetic analysis was carried out using the PCR method approach to determine species of bacteria, including the character/trait of bacteria. DNA extracted samples were filtered with a polycarbonate membrane filter (size 0.22 mm, Millipore, Bedford, MA, USA). Then, the membrane was transferred to sterile tubes separately and the cells were dissolved in the bead beater. Bacterial DNA extraction was carried out using the AxyPrep™ Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, Inc., CA, USA) extraction kit. Total DNA of extracted

symbionts was amplified with universal primers of 16S rRNA *E. coli*, namely forward primer FP-U1 (5'-CCAGCAGCCGCGGTAATACG-3') attached to the nucleotide base of 518-537, and reverse primer RP-U2 (5' -ATCGG (C/T) TACCTTGTTACGACTTC-3'), attached to the nucleotide base sequence 1.513-1.491. The total volume of PCR mix was 30 µL containing 19 µL DreamTaq buffer (Fermentas, MA), 6 µL dNTP (dATP, dTTP, dGTP, dCTP), 1.5 µL enzyme. 1.5 µL bacterial suspension, 1 µL primary DNA and 1 µL aquabedes. The PCR amplification process was carried out in 30 cycles at 95°C for 5 min, denaturation at 94°C for 1 min, annealing at 54°C for 1 min 20 seconds, extension at 72°C for 2 min, and elongation at 72°C for 10 min.

Sponge bacterial symbiont activity test. There were 2 test media used to determine the activity of sponge bacterial symbionts on PAH contaminated media, solid and liquid media. Each medium was contaminated with PAH (Anthracene, pyrene, type of mix 16 PAH ASTM standard). Observation of bacterial symbiont activity was carried out after 3 days in the incubator. The second test method used liquid media in the form of a bacterial symbiont suspension, which interacted with 3 solutions as each solution was contaminated with anthracene, pyrene, and a mix of 16 PAH ASTM standard in the reactor. The interaction reactor was aerated by a shaker at 100 rpm at room temperature for 5 days. The parameters of the bacterial symbiont fermentation reaction test against PAH were pH media and bubble occurrence (Syakti et al 2013; Marzuki et al 2015b; Abass et al 2017).

Results and Discussion. The water parameters and body surface aspect of sponges are presented in Table 1.

Table 1

Characteristics of sponge sampling points

Observation	*Sample code*			
	Sp1	Sp2	Sp3	Sp4
Depth from the surface (m)	3.2	3.6	3.3	4.8
Body surface consistency of sponges	slimy	slimy	slimy	rough
Salinity (‰)	29.3	29.8	29.3	29.5
pH	7	7	7	7
Temperature (^{0}C)	29	29	29	28

Several parameters measured at the sampling point (Table 1) show that the sponge samples live in a normal environment (depth, salinity, pH and temperature) referring to the Minister of Environment and Forestry Decree No. 51 of 2004, except for the surface consistency parameters of the sponge covered by mucus.

Morphological analysis of sponge. Mucus covering the sponge surface is thought to be produced by bacterial symbionts as a self-defense and response to environmental changes. Morphological identification results of sponge samples obtained from Kodingareng Keke Island are presented in Table 2.

Table 2

Sponge species identified in Kodingareng Keke Island, Indonesia

Sample code	*Species*	*Family*
Sp1	*Petrosia (Strongylophora) corticata*	Petrosiidae
Sp2	*Aulleta* sp.	Axinellidae
Sp3	*Neopetrosia* sp.	Petrosiidae
Sp4	*Callyspongia aerizusa*	Callyspongiiade

The morphology of samples are presented in Figures 2 to 5 as follows.

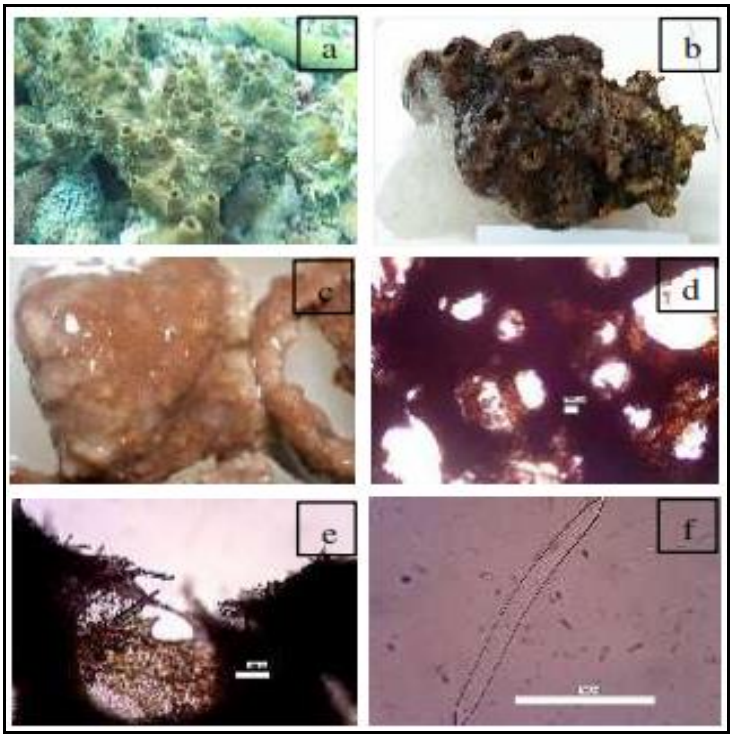

Figure 2. Morphology of *Petrosia corticata* (Sp1). a - growth form: sligthy globular with big size oscula; b - consistency: slippery surface covered with slimy mucus; inelastic and brittle body sponge; c - surface: granular; d - skeleton: spicule skeleton with echinating spicule; e - skeleton tract: paucispicular tract with high fibers; f - spicule: small megasclera oxea (magnification 40X).

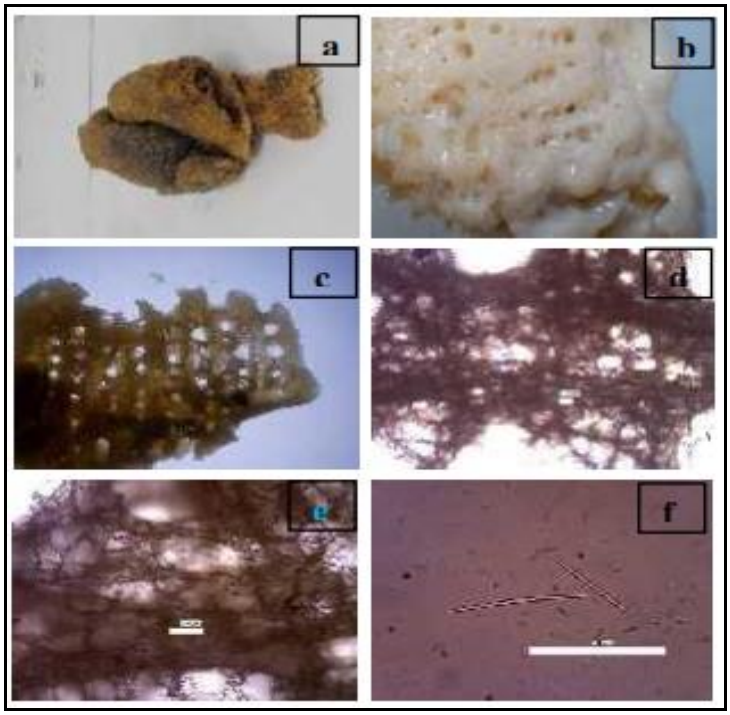

Figure 3. Morphology of *Auletta* sp. (Sp2). a - growth form: dark-yellow, slightly cylindrical; b - consistency: slippery surface, covered by mud-like slime, compressible, crumbly and fragile body; c - skeleton: pauci spicular, plumoreticulate; d - choanosome: anostoming choanosme skeleton; e - fiber: interconnected, f - spicule: slender oxea megasclere.

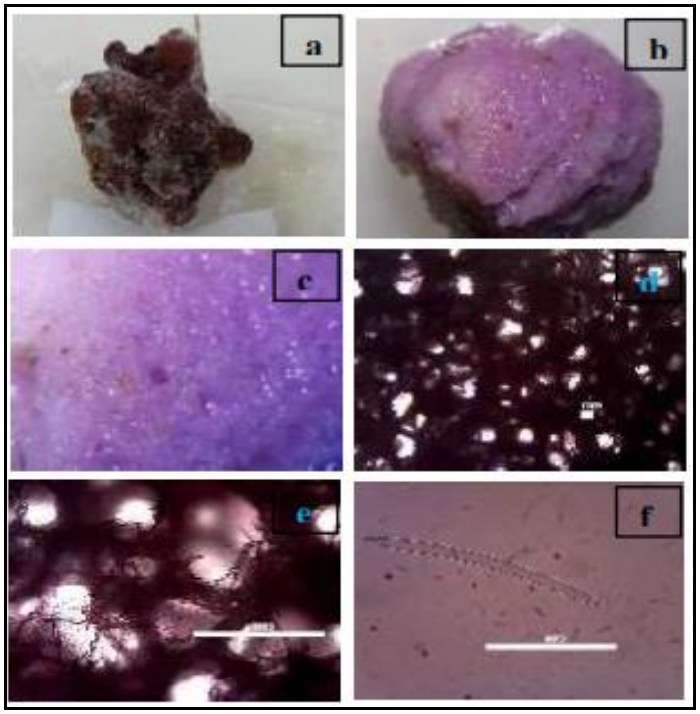

Figure 4. Morphology of *Neopetrosia* sp. (Sp3). a - growth form: globular shape sponge, purple colored; b - consistency: hard and tough consistency, slippery surface sponge, covered by mud-like slime; c - surface: smooth sponge surface; d - choanosome: dense alveolate choanosome skeleton; e - skeleton: multispicular, arranged round; f - spicule: oxea megasclere (magnification 10X), no microsclere.

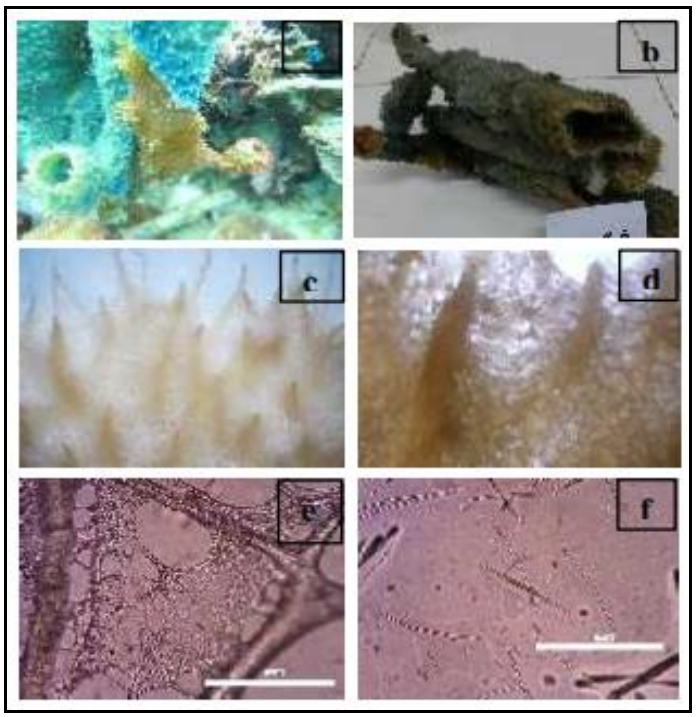

Figure 5. Morphology of *Callyspongia aerizusa* (Sp4). a - growth form: cylindrical shape, blue and yellow colored sponge; b - colorin ethol: the color fades after being prepared with alcohol; c - surface: pappilate surface sponge; d - skeleton: primary and secondary fiber, interconnected tract; e - fiber: high density of fiber; f - spicule: slender oxea (magnification 40X), no microsclera.

Based on observations, a substance in the form of mucus that covers the surface of the sponge with a covered area reaches 80-86% in sponges Sp1, Sp2, and Sp3. In sponge Sp4 there was no mucus covering the body. This was thought to be the response of Sp1, Sp2, and Sp3 in adjusting to the aquatic environment with an optimum condition. The situation triggers the sponge bacterial symbiont to produce mucous to protect the sponge body. Another allegation is that the bacterial symbiosis with a sponge has occurred earlier as bacteria need a host to be protected from ocean currents. These bacterial symbionts then produce enzymes characterized as natural responses to adapt to extreme changes due to PAH pollution. This adaptation process of the bacterial symbionts is not only beneficial to symbionts but also to the sponge as a host (Abdel-Monem et al 2013; Lavy et al 2014; Marzuki et al 2016).

The physical characteristics of the 4 types of sponges include the presence of high fibrous skeletons connected to one another, with a solid shape. The 4 sponge spicule types are expanded with small to medium-sized oxea and have a cylindrical shape. The structure of sponges with skeleton and spicules like this does not describe the typical characteristics related to sponge sensitivity to the environmental changes. Skeletons and spicules have roles in nutrition and respiration, although skeletons with a dense or interconnected fiber structure and small-to-medium oxea are only a consequence of sponge filter-feeding behavior by adjusting to mud particles, as mud particles are absorbed and discharged by spraying (Ismet et al 2011; Abdel-Monem et al 2013; Marzuki et al 2015c).

Isolation of bacterial symbionts in the 4 sponge samples resulted in 10 sample isolates, namely 2 isolates from *Petrosia corticata*, 3 isolates from *Auletta* sp., 3 isolates from *Neopetrosia* sp., and 2 isolates of *Callyspongia aerizusa*. One isolate was selected for each sample type, resulting 4 selected isolates. The selected bacterial isolates were encoded following the sponge sample code and selected isolate number (Table 3).

Analysis of the sponge bacterial symbionts. The phenotype analysis was performed to determine the biological characteristics of the bacterial symbiont isolates. The results of morphological analysis and Gram staining test for sponge symbiont isolates are presented in Table 3.

Table 3

Morphological analysis and Gram staining test of sponge bacterial symbionts

Sponge	*Sample code*	*Morphology*	*Gram bacterial group*
Petrosia corticata	SP1.B2	Rounded shape, cream bluish color, cluster distribution colony, rod shape, no color change after Safranin staining, less clear endospores, insoluble with 1% KOH	Bacillus, Gram (+) + spore
Auletta sp.	SP2.B1	Serrated rod shape, brown color, separated distribution, no color change after Safranin staining, no endospores, insoluble with 1% KOH	Bacillus, Gram (+) + spore
Neopetrosia sp.	SP3.B3	Serrated rod shape, brown color, separated distribution, no color change after Safranin staining, no endospores, insoluble with 1% KOH	Bacillus, Gram (+)
Callyspongia aerizusa	SP4.B1	Rounded shape, brown color, separated distribution, color change after Safranin staining, no endospores, soluble with 1% KOH	Bacillus, Gram (-)

The results indicate 3 isolates of sponge bacterial symbiont belonging to the Gram positive group. The 3 isolates are known to come from 3 different types of sponges, namely SP1.B2, SP2.B1 and SP3.B3. The morphological analysis of sponge bacterial

symbionts aimed to observe whether there was a relationship between morphology (shape, color, cell distribution, endospore) of sponge symbionts and the ability of bacterial symbiont to grow in PAH contaminated media. However, these results cannot be used as a reference in determining the PAH degradation potential. The reaction with Safranin reagent and 1% KOH solution was used to determine the Gram type of bacterial symbionts. Gram-positive/negative can be used as one of the parameters to determine the PAH degradation ability (Nurhayati et al 2006; Marzuki et al 2016; White et al 2012).

A series of phenotypic analyses, such as biochemical tests with specific reagents were performed to determine the chemical processes that occurred in isolates to draw the dynamics of sponges during their infancy. Biochemical test results are presented in the Table 4.

Table 4

Characterization of sponge bacterial symbiont biochemical test methods

Biochemical reagents	*Media*	*Sponge bacterial symbiont*			
		SP1.B2	*SP2.B1*	*SP3.B3*	*SP4.B1*
Starch hydrolysis	Starch agar	base	base	base	base
Casein hydrolysis	Milk agar	acid	acid	acid	acid
Gelatin hydrolysis	Gelatins	-	-	-	-
Nitrate reduction	Nitrate broth	-	-	-	-
Indole	Tryptone broth	-	-	-	-
H_2S	H_2S	-	-	-	-
Reagent Methyl Red	R-MR broth	+	+	+	+
Reagent-Voges Proskauer	R-VP broth	+	+	+	-
Citrates	Citrate	+	-	+	-
Urease	Urea broth	-	-	-	-
Glucose	Glucose broth	-	-	-	-
Lactose	Lactose broth	+	+	+	+
Sukrose	Sucrose broth	-	-	-	-
Mannitol	Mannitol broth	-	-	-	-
Catalase	Nutrient Agar Nutrient agar slant	+	+	+	-

Note: (+) - reaction; (-) - no reaction.

Biochemical tests to determine the physiological properties of bacteria showed mixed result. All isolates showed negative results on the gelatin hydrolysis, nitrate reduction, indole, H_2S, urease, glucose, sucrose, and mannitol tests. Negative results on the indole test showed that all isolates did not contain the tryptophanase enzyme. Negative results on the H_2S and glucose, sucrose, and mannitol tests indicated that all isolates were unable to ferment carbohydrates. Negative results also occurred in the urease test, indicating that the isolates were unable to convert urea to ammonia. Positive results shown by all isolates in the methyl red test indicated that all isolates could ferment the methylene glycone. A similar result appeared in the lactose test, indicating that all isolates had a lactase enzyme to convert lactose to galactose and glucose (Marzuki et al 2014; Cita et al 2017). The VP test aimed to determine the ability to form acetyl-methyl-carbitols (acetoin) from sugar fermentation (Ulfa et al 2016) showed positive results on 3 isolates. SP4.B1 showed a negative result. This was similar to the catalase test, as 3 isolates belonged to the aerobic group which could produce a catalase enzyme to hydrolyze H_2O_2 in the temporary reaction. However, SP4.B1 did not have this ability. The citrate test showed positive results on 2 isolates in SP1.B2 and SP3.B3, which means that both isolates could use citrate as carbon and energy source, while SP2.B1 and SP4.B1 showed the opposite results. The phenotypic characteristics of the sponge symbiont

isolates generally followed the growth mechanism of symbiotic bacteria on hydrocarbon-contaminated media (Marzuki et al 2015b; Cita et al 2017).

Genotype analysis of sponge symbiont isolates. Genotype analysis of 4 types of sponge bacterial symbiont isolates was performed to determine the genetic characteristics of DNA sequence and number, while BLAST was used to observe the similarity level and DNA sequence difference from other bacteria registered at GenBank (www.ncbi.nlm.nih.gov). The results of the BLAST sequencing analysis showed that the 4 symbiont isolates obtained consisted of several species (Table 5). Isolate SP1.B2 had a 98.85% similarity level to *Pseudomonas stutzeri* RCH2 with a 0.4% difference. This indicated that SP1.B2 is of *P. stutzeri*. SP2.B1 had a 98.15% similarity level to *Bacillus licheniformis* ATCC9789 with a 0.01% difference. SP3.B3 had a partial similar sequence to *Bacillus* sp. AB353F with 97.49% similarity level and 1.66% difference, thus SP3.B3 could be classified as *Bacillus* sp. SP4.B1 had a similar sequence with *Acinetobacter calcoaceticus* PHKDB14 with 96.9% and 1.28% difference. Thus, SP4.B1 was included in *A. calcoaceticus* species.

Table 5

BLAST analysis of sponge symbionts

Symbiont code	*Sample Sequence*	*Sequence Gen Bank*	*Quantity (%)*	*Difference (%)*	*Species*
SP1.B2	17-972 (955)	608.723-609.690 (967)	944/955 (98.85)	4/955 (0.42)	*Pseudomonas stutzeri* RCH2
SP2.B1	11-985 (974)	524.589-525.563 (974)	956/974 (98.15)	14/974 (0.01)	*Bacillus licheniformis* strain ATCC9789
SP3.B3	15-975 (960)	574.123-575.089 (966)	932/960 (97.49)	16/960 (1.66)	*Bacillus* sp. AB353F partial
SP4.B1	21-934 (913)	574.323-575.258 (935)	906/935 (96.9)	12/935 (1.28)	*Acinotobacter calcoaceticus* strain PHKDB14

The genotype analysis results showed the differences of sponge bacterial symbiont characteristics based on the phenotype analysis (Tables 3 and 4). Different responses of each isolate on the media tests indicated that each isolate had certain mechanisms to carry out fermentation and reactions including possible different responses against PAH contaminants. SP2.B1 and SP3.B3 showed similar results in almost all tests, most likely because both come from the same bacterial genus, *Bacillus*.

Sponge symbiont isolates activity in PAHs exposed media. The isolate activity test carried out on PAH contaminated solid media aimed to explore whether there was a relationship among sponge dynamics (body covered with mucus), biochemical tests, Gram groups and strains with the fermentation activity of symbiont isolates on PAH-exposed media. The test results of sponge symbiont isolate activity on PAH contaminated solid media are presented in Table 6 and on liquid media in Table 7.

Table 6

Sponge symbiont isolates activity on solid media contaminated by poly aromatic hydrocarbons (PAH)

Sponge bacterial symbiont	*Type of PAH contamination on media (solid)*		
	Anthracene	*Pyrene*	*Mix 16 poly aromatic hydrocarbons ASTM standard*
SP1.B2	++	++	+
SP2.B1	++	+	+
SP3.B3	++	++	+
SP4.B1	-	-	-

A bioremediation technology to degrade PAH contaminants has been recommended as an efficient and economical psychological treatment (Margesin & Schinner 1997). SP1.B2, SP2.B1, and SP3.B3 showed growth in solid media contaminated with anthracene, pyrene, and a mix of 16 PAH ASTM standard after incubated for 3 days at room temperature. These results were in line with the results obtained on bacterial isolates cultured on liquid media, as they performed fermentation reactions characterized by changes in pH from 7 to 6 after 10 days of interaction. Another observed parameter consisted in the bubbles that formed on the 10th day of interaction.

Table 7

Sponge bacterial symbiont activity in poly aromatic hydrocarbon (PAH) contaminated liquid media

Sponge bacterial symbiont	*Parameters*	*Type of poly aromatic hydrocarbon and contact time (day)*					
		Anthracene		*Pyrene*		*Mix 16 PAH ASTM Standard*	
		5	10	5	10	5	10
SP1.B2	pH	7	6	6	6	7	6
	air bubble	-	√	-	√	-	√
SP2.B1	pH	7	6	6	7	7	7
	air bubble	-	√	√	√	-	√
SP3.B3	pH	7	6	7	6	7	6
	air bubble	-	√	-	√	-	√
SP4.B1	pH	7	7	7	7	7	7
	air bubble	-	-	-	-	-	-

The presence of sponge bacterial symbiont activity on several types of PAH indicated that the bacterial symbionts could degrade PAH (Akinde et al 2012; Marzuki et al 2015a). Some studies also reported that *Pseudomonas* (Harayama 1997; Samanta et al 2001; Guo et al 2010) and *Bacillus* (Samanta et al 2001; Toledo et al 2006; Guo et al 2010) could degrade PAH contaminants. In the case of SP4.B1, symbiotic bacteria that did not show cell growth activity in neither liquid nor solid media. Different results were reported by Rafin et al (2009), who stated that *Acinetobacter* could degrade PAH, but it remained unknown if the strains tested were similar to the observed isolates or not.

Sponge bacterial symbiont isolates degrade PAH compounds into oxygen or aerobic atmosphere. Aerobic bacteria use oxygen as an electron receiver to break down organic and inorganic materials into smaller compounds, usually in the form of carbon dioxide and water as the final product (Habe & Omori 2003). The test parameters isolated during the fermentation period were observed after 10 days of culture, because it is related to the growth pattern of bacteria, which consists of several growth phases. The bacterial growth phase starts from the adaptation phase in the growth environment, which generally lasts for 3-5 days. Then, the bacteria enter the development phase as the number of bacterial cells increase, and cell size increases, resulting in physical

changes (Marzuki et al 2015b, 2017; Bello-Akinosho et al 2015). This phase generally takes place 2-3 days after the adaptation phase or in 6-8 days after incubation in media exposed to PAH. The next stage is the stationary and death phase, which is commonly finished in 2-3 days (Akinde et al 2012; Marzuki et al 2015a).

The results of the bacterial isolate activity test on PAH media were relevant to the results obtained in biochemical tests. SP1.B2, SP2.B1, and SP3.B3 showed similar results on all test parameters. This was due to the fact that bacteria SP1.B2, SP2.B1, and SP3.B3 belonged to Gram-positive bacteria, while SP4.B1 was Gram-negative bacteria.

The determination of the maximum limit of bacterial culture in PAH media was performed by considering the type of bacterial isolate as aerobic bacteria that could produce a catalase enzyme and peroxide toxic gas (H_2O_2). The high concentration of H_2O_2 gas in the media could quickly kill bacteria. Another factor of bacterial death is a decrease in PAH which inhibits bacterial cell division. A 10-day culture period allows the PAH component to degrade into simple and acidic organic compounds. Acidic pH in culture media will cause a faster death of bacterial cells (Bello-Akinosho et al 2015; Abass et al 2017; Marzuki et al 2017).

The results obtained showed a possible relationship among sponge bacterial symbiont activity on PAH contaminated media and sponge dynamics, as well as apparent phenotypic and genotypic characteristics. In this study, 2 sponge bacterial symbionts were identified to grow on the PAH contaminated media, namely *Pseudomonas* and *Bacillus*. Both bacteria were not newly reported to degrade PAH, although the bacterial source was isolated from other materials, such as mangroves, contaminated sludge containing hydrocarbon components, or seawater contaminated by PAHs (Manzanera et al 2015; Marzuki et al 2020).

Conclusions. 3 sponge bacterial symbionts, *Pseudomonas stutzeri* RCH2, *Bacillus licheniformis* ATCC9789, and *Bacillus* sp. AB353 isolated from *Petrosia corticata*, *Aulleta* sp., and *Neopetrosia* sp., respectively. Bacterial isolates showed growth activities on solid and liquid media contaminated with anthracene, pyrene, and a mix of 16 PAH ASTM standard as related to the Gram test, phenotypic, and genotypic characteristics. Investigations of sponges with potential to form a symbiosis with bacteria that can degrade polyaromatic hydrocarbons showed that bacteria select sponges with a body surfaces covered with mucous. The phenotypic characteristics were Gram-positive and positively reactive to MR-VP, citrate, and catalase enzyme in biochemical tests. For further research, it is necessary to conduct more intensive investigations to multiply these sponge bacterial symbiont species as PAH degrading bacteria by adopting a sponge selection pattern based on the mucus covered body surface.

Acknowledgements. Authors would like to thank the Directorate of Research and Community Service, Directorate General of Research and Development, Indonesian Ministry of Research and Technological Affairs (SK.Ristekdikti No.0045/E5/LL/2018 and Contract Agreement No.11/A/LPPM-UNIFA/2018), which has provided a funding to conduct this study.

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Received: 09 July 2020. Accepted: 01 September 2020. Published online: 02 December 2020.
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How to cite this article:
Marzuki I., Daris L., Yunus S., Riana D. A., 2020 Selection and characterization of potential bacteria for polycyclic aromatic biodegradation of hydrocarbons in sea sponges from Spermonde Islands, Indonesia. AACL Bioflux 13(6):3493-3506.