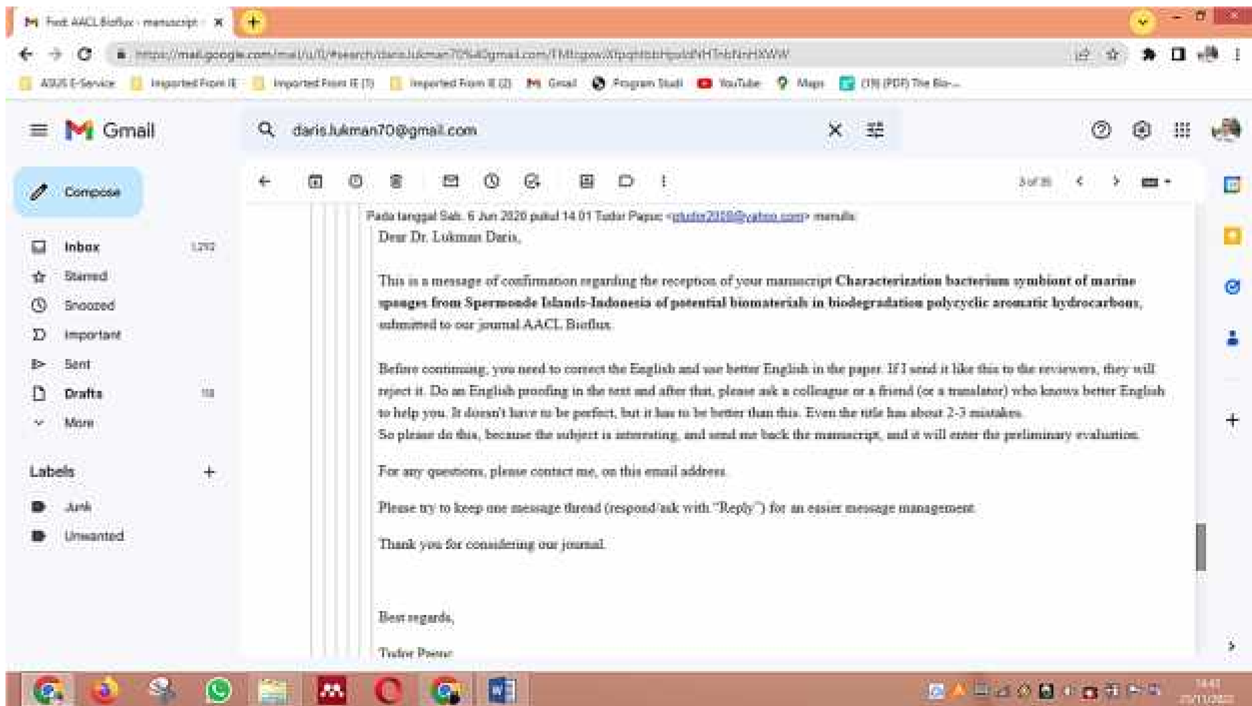
Balasan Editor (Tudor) Biofux, 6 Juni 2020



Pada tanggal Sab, 6 Jun 2020 pukul 14.01 Tudor Papuc <ptudor2008@yahoo.com> menulis:

Dear Dr. Lukman Daris,

This is a message of confirmation regarding the reception of your manuscript **Characterization bacterium symbiont of marine sponges from Spermonde Islands-Indonesia of potential biomaterials in biodegradation polycyclic aromatic hydrocarbons**, submitted to our journal AACL Bioflux.

Before continuing, you need to correct the English and use better English in the paper. If I send it like this to the reviewers, they will reject it. Do an English proofing in the text and after that, please ask a colleague or a friend (or a translator) who knows better English to help you. It doesn't have to be perfect, but it has to be better than this. Even the title has about 2-3 mistakes.
So please do this, because the subject is interesting, and send me back the manuscript, and it will enter the preliminary evaluation.

For any questions, please contact me, on this email address.

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Thank you for considering our journal.

Best regards,

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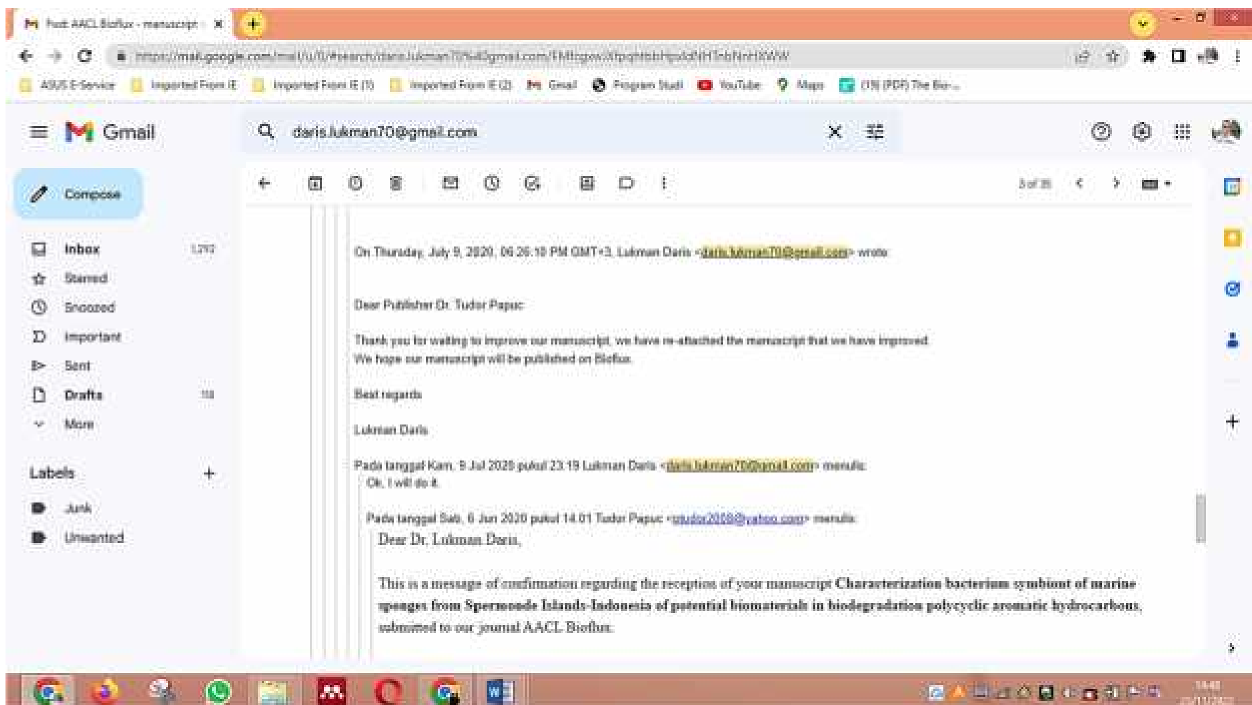
Balasan tanggal 9Juli 2020 (Dr Tudor)



On Thursday, July 9, 2020, 06:26:10 PM GMT+3, Lukman Daris <daris.lukman70@gmail.com> wrote:

Dear Publisher Dr. Tudor Papuc

Thank you for waiting to improve our manuscript, we have re-attached the manuscript that we have improved.
We hope our manuscript will be published on Bioflux.

Best regards

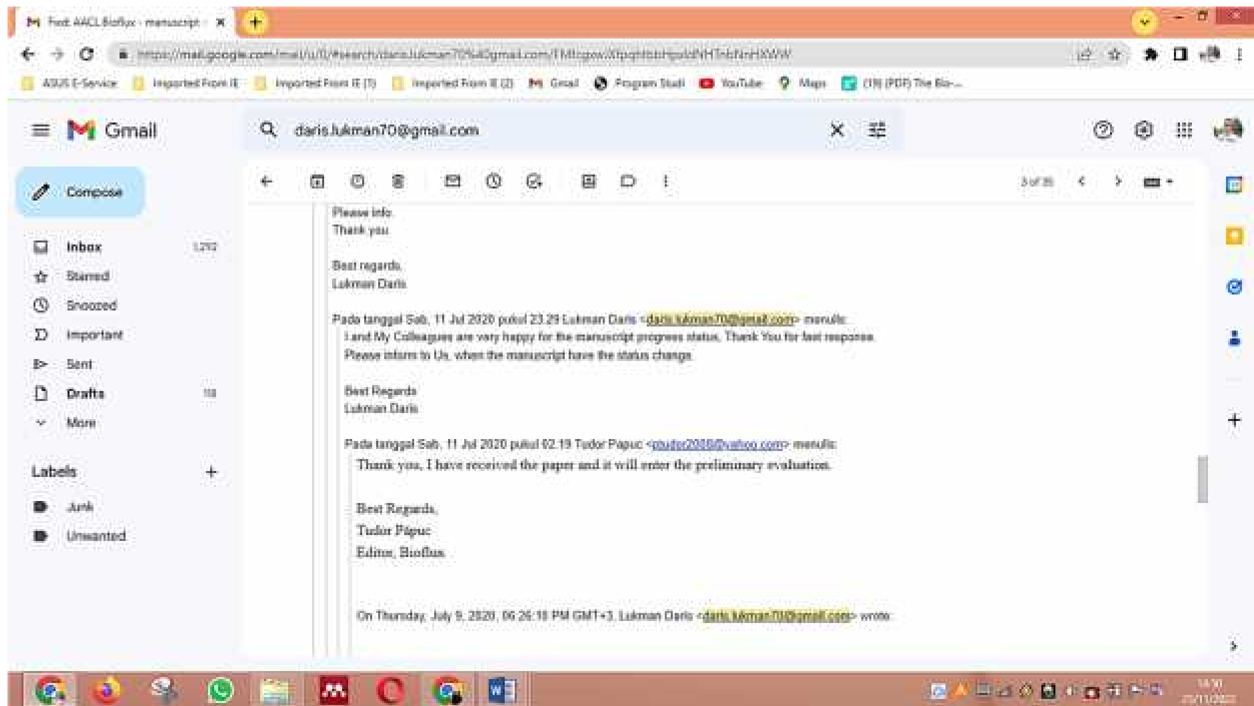
Lukman Daris

Pada tanggal Kam, 9 Jul 2020 pukul 23.19 Lukman Daris <daris.lukman70@gmail.com> menulis:
Ok, I will do it.

Pada tanggal Sab, 6 Jun 2020 pukul 14.01 Tudor Papuc <ptudor2008@yahoo.com> menulis:
Dear Dr. Lukman Daris,

This is a message of confirmation regarding the reception of your manuscript **Characterization bacterium symbiont of marine sponges from Spermonde Islands-Indonesia of potential biomaterials in biodegradation polycyclic aromatic hydrocarbons**, submitted to our journal AACL Bioflux.

Email 11 Juli 2020



Please info.
Thank you

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Lukman Daris

Pada tanggal Sab, 11 Jul 2020 pukul 23.29 Lukman Daris <daris.lukman70@gmail.com> menulis:

I and My Colleagues are very happy for the manuscript progress status. Thank You for fast response.
Please inform to Us, when the manuscript have the status change.

Best Regards
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Pada tanggal Sab, 11 Jul 2020 pukul 02.19 Tudor Păpuc <ptudor2008@yahoo.com> menulis:

Thank you, I have received the paper and it will enter the preliminary evaluation.

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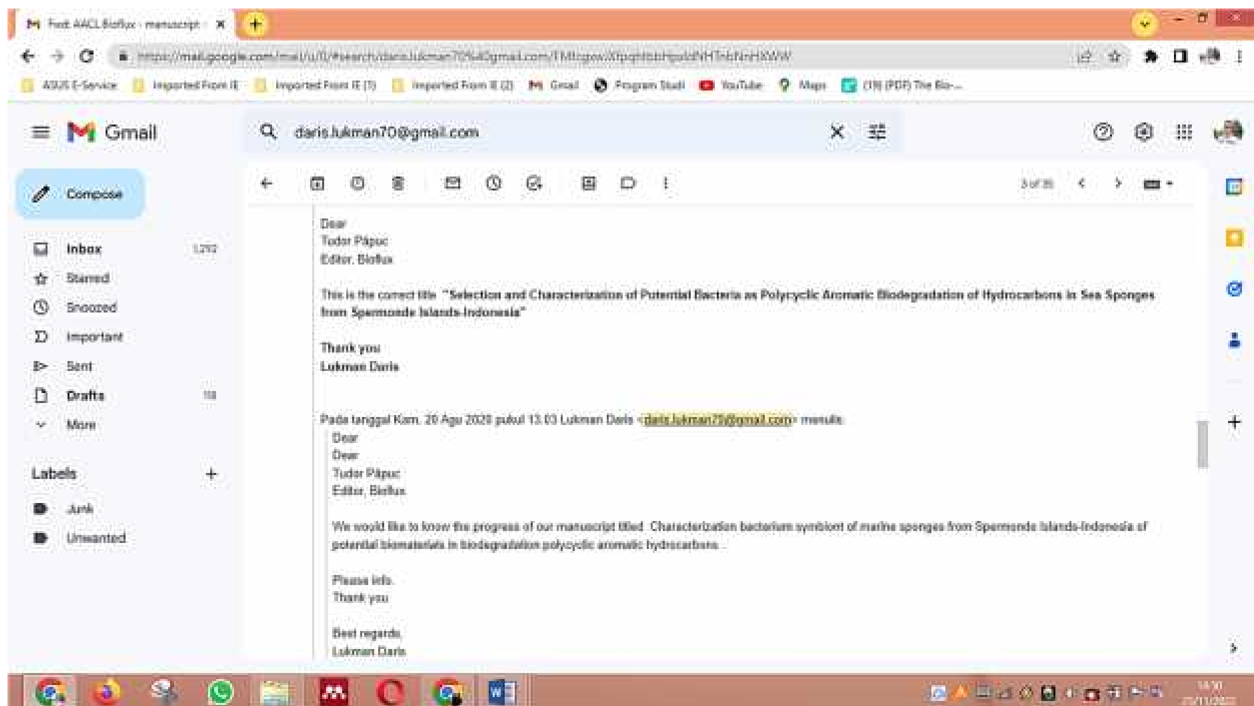
Tudor Păpuc
Editor, Bioflux

On Thursday, July 9, 2020, 06:26:18 PM GMT+3, Lukman Daris <daris.lukman70@gmail.com> wrote:

Email 20 Agustus 2020



Dear
Tudor Păpuc
Editor, Bioflux

This is the correct title **"Selection and Characterization of Potential Bacteria as Polycyclic Aromatic Biodegradation of Hydrocarbons in Sea Sponges from Spemonde Islands-Indonesia"**

Thank you
Lukman Daris

Pada tanggal Kam, 20 Agu 2020 pukul 13.03 Lukman Daris <daris.lukman70@gmail.com> menulis:

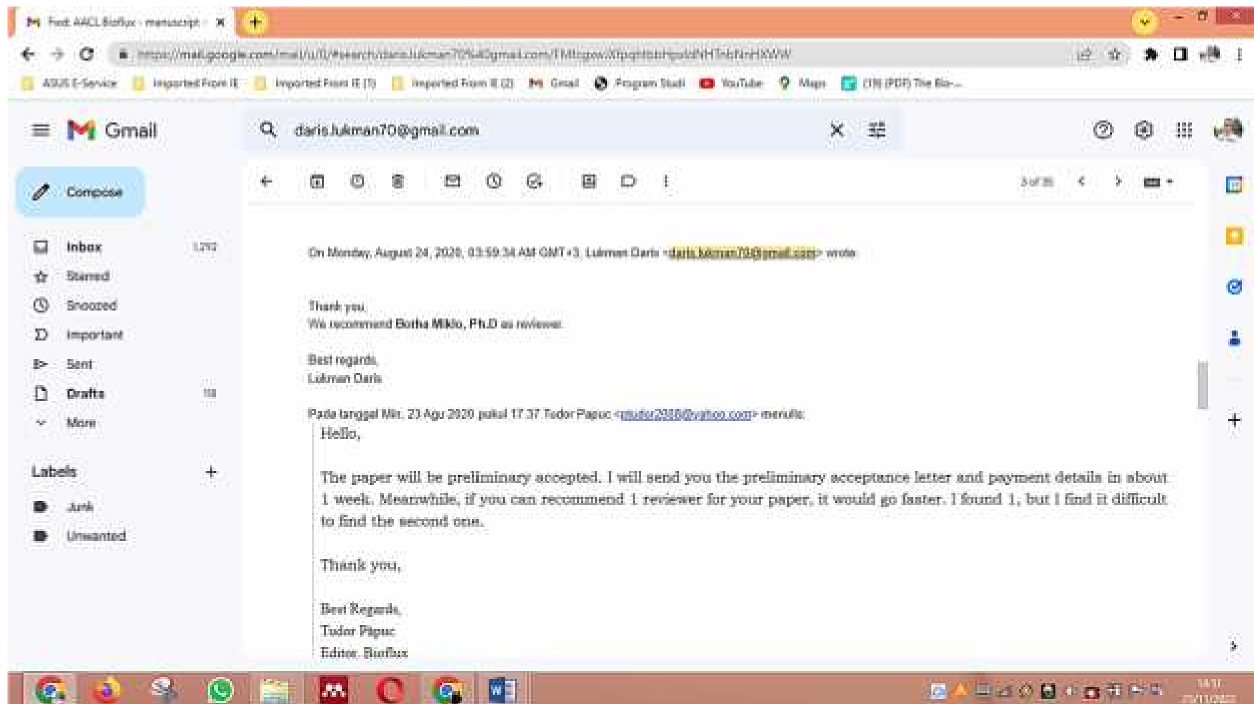
Dear
Dear
Tudor Păpuc
Editor, Bioflux

We would like to know the progress of our manuscript titled Characterization bacterium symbiont of marine sponges from Spemonde Islands-Indonesia of potential biomaterials in biodegradation polycyclic aromatic hydrocarbons .

Please info.
Thank you

Best regards,
Lukman Daris

Email 23 Agustus 2020



On Monday, August 24, 2020, 03:59:34 AM GMT+3, Lukman Daris <daris.lukman70@gmail.com> wrote:

Thank you,
We recommend **Botha Miklo, Ph.D** as reviewer.

Best regards,
Lukman Daris

Pada tanggal Min, 23 Agu 2020 pukul 17.37 Tudor Papuc <ptudor2008@yahoo.com> menulis:

Hello,

The paper will be preliminary accepted. I will send you the preliminary acceptance letter and payment details in about 1 week. Meanwhile, if you can recommend 1 reviewer for your paper, it would go faster. I found 1, but I find it difficult to find the second one.

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Best Regards,
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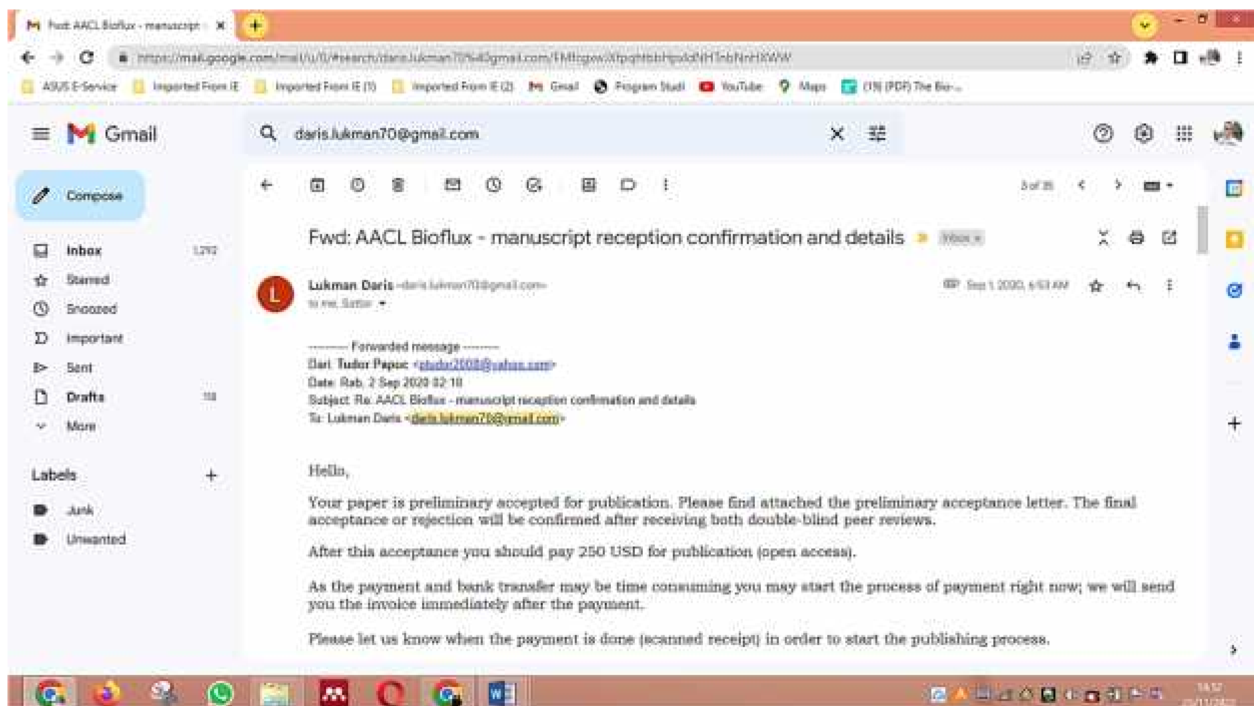
Email 2 September 2020. Accepted



Fwd: AACL Bioflux - manuscript reception confirmation and details

Lukman Daris <daris.lukman70@gmail.com>

Sep 1, 2020, 6:53 AM

---------- Forwarded message ---------
Dari: **Tudor Papuc** <ptudor2008@yahoo.com>
Date: Rab, 2 Sep 2020 02.10
Subject: Re: AACL Bioflux - manuscript reception confirmation and details
To: Lukman Daris <daris.lukman70@gmail.com>

Hello,

Your paper is preliminary accepted for publication. Please find attached the preliminary acceptance letter. The final acceptance or rejection will be confirmed after receiving both double-blind peer reviews.

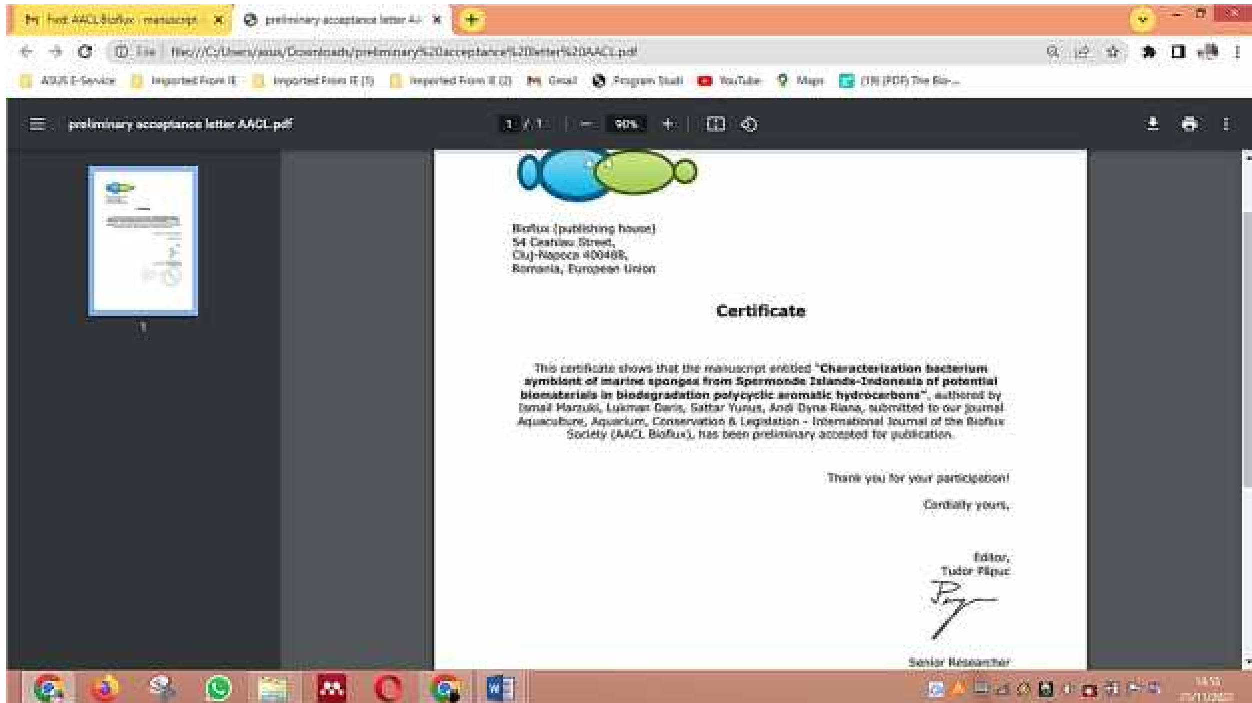
After this acceptance you should pay 250 USD for publication (open access).

As the payment and bank transfer may be time consuming you may start the process of payment right now; we will send you the invoice immediately after the payment.

Please let us know when the payment is done (scanned receipt) in order to start the publishing process.

Sertifikat Accepted, 2 September 2020

Dan Publish on line 31 Desember 2020, setelah dua kali verisi untuk setiap reviewer



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Certificate

This certificate shows that the manuscript entitled **"Characterization bacterium symbiont of marine sponges from Spermonde Islands-Indonesia of potential biomaterials in biodegradation polycyclic aromatic hydrocarbons"**, authored by Ismail Marzuki, Lukman Daris, Sattar Yunus, Andi Dyna Riana, submitted to our journal Aquaculture, Aquarium, Conservation & Legislation - International Journal of the Bioflux Society (AACL Bioflux), has been preliminary accepted for publication.

Thank you for your participation!

Cordially yours,

Editor,
Tudor Păpuc

Senior Researcher

Naskah Pertama

Characterization Bacterium Symbiont of Marine Sponges from Spermonde Islands-Indonesia of Potential Biomaterials in Biodegradation Polycyclic Aromatic hydrocarbons

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Abstract. The mucus substance that covers the surface of the sponge body has a relationship with the degradation potential of the symbiotic bacteria toward PAH. The truth of the statement is validated through investigation of morphology, phenotype and genotype of the symbiont bacteria. Search methods, namely morphological analysis, phenotype (gram and biochemical staining test), microsimbion genotype using PCR, and growth activity test for symbolic isolates in solid and liquid media contaminated with PAH (anthracene, Pyrenees and mix of 16 ASTM standard PAH). Three types of symbiotic isolate each sponge Pseudomonas stutzeri RCH2, Bacillus licheniformis strain ATCC9789 and Bacillus sp.AB353 partial, showed growth activity in both types of test media, whereas the bacterium Acinotobacter calcoaceticus strain PHKDB14, showed no activity. These results indicate that there is a morphological correlation, gram group of bacteria, phenotypic characteristics and symbionic bacterial genotypes against the degradation ability of PAH.
Key Words: Sponge, symbiont bacteria, biodegradation, spermonde Island, PAH

Introduction. The Aromatic Polycyclic Hydrocarbons (PAHs) contribute significantly to reduce the quality of the marine environment. PAH sources, such as petroleum exploration, maritime transportation, tanker washing ballast are potential activities to produce sludge waste containing PAH. The hydrological cycle naturally in the end the sludge waste will lead to the sea. The carcinogenic and mutagenic properties of PAH threaten the life of marine biota including sponges, so that the handling and management of this type of waste needs to be intensified. The treatment of waste containing PAH components using physical, chemical and biological methods is not as expected and declared inefficient and it is less effective in reducing PAH toxicity through a degradation mechanism.

Some scientific reports state that several types of bacteria are able to degrade hydrocarbon components, both aliphatic and poly aromatic. Sources of bacteria that can reduce the toxicity of PAH are generally isolated from oil, mud, mangroves, sea water exposed to hydrocarbons and sponges. This bacterium is thought to have specific characteristics, specific bacteria isolated from the sponges need to be mapped because the sponge Symbion types are diverse and not all sponge symbiotic bacteria can degrade PAH (Akinde et al 2012; Mu et al 2014; Lavy et al 2014,). The life pattern of the sponge fedeer filter, allows the degradation ability of the symbiotic bacterial PAH, in fact, not all sponge Symbiont bacteria have the degradation ability of the hydrocarbon component (Manzanera et al 2015; Marzuki et al 2020). The sponge symbiont bacteria capable in biodegrading PAHs are thought to originate from certain types of sponges that can produce substances characterized by enzymes (Liu et al 2017; Parama et al 2017; Belila et al 2016). Generally, these substances are found to cover most of the body surface sponge (Derosa at al 2003). Substances in the form of mucus are produced in response to sponges for changes in living habitat for self-protection (Meutia et al 2011; Marzuki et al 2016).The closure of the surface of the sponge body is also intended for self-defense against the threat of predators and adaptation to extreme environmental

changes (Muzaki et al 2017; Jesionowski et al 2018). Extreme sponge environment, due to contamination by toxic substances containing PAHs components, heavy metals, plastic waste, including sponges overcoming underwater currents (Bello et al 2015; Zhang et al 2012; Venkateswara et al 2009).

Exploration of various types of sponges is known not all sponges produce substances in the form of mucus. The production of mucus in a number of sponges is related to the dynamics of life they experience. Life pressure in sponge growth due to pollution interference, forcing sponges to produce certain substances in the form of mucus for protection in order to survive in extreme environments (Muzaki et al 2017; Marzuki et al 2015; Abdel et al 2013). Mucus is produced in a character like an enzyme. These substances are actually produced by bacteria that are symbiont with a sponge. A deeper investigation into mucus substances is characterized by an enzyme produced by sponge symbiont bacteria, specifically the presumption is that there is a correlation between symbiont bacteria and the ability to destroy several types of PAHs (Akinde et al 2012; Jesionowski et al 2018; Kepel et al 2018). The chemical structure destruction of PAHs by several types of sponge simbiont bacteria takes place through a specific mechanism that must be fulfilled so that the bacteria can work optimally. The reshuffle of the chemical structure of PAHs by symbiont bacteria takes place due to the ability of bacteria to make carbons as a nutrient to obtain energy to carry out its life activities (Parama et al 2017; Zhang et al 2012; Marzuki at al 2017). This biodegradation process can take place if it fulfils the condition that the bacteria can move. Temperature, pH, concentration and type of PAHs and the number of bacterial cells that accumulate in the biodegradation medium are factors that determine bacterial cell growth and development activity (Marzuki et al 2020; White et al 2012).

The character of the enzyme that appears in the mucus substance produced by the symbiotic bacteria is thought to be related to the ability of the degradation of the symbiont bacteria to several types of PAHs. This phenomenon directs attention to the morphological characteristics of the sponge (a form of growth, body surface consistency, structure and shape of spicules), phenotype (a gram group of bacteria, cell color, spread, endospores, reaction with safranin reagents, and solubility of KOH reagents) and symbiont bacterial genotypes sponge (arrangement and number of DNA, species and strains) (Liu et al 2017; Vaezzadeh et al 2017; Marzuki et al 2015). The characteristic characteristics of sponge symbiont bacteria are related to the biodegradation ability of PAHs, so it needs to be revealed through scientific facts. Morphological investigation, phenotype and genotype of sponge Symbiont bacteria, combined with symbiont bacterial cell growth test on PAHs contaminated media are needed to answer conjectures related to PAHs biodegradation by sponge Symbiont bacteria (Mu et al 2014; Marzuki et al., 2015a; Pawar et al 2017).

Material and Method. Sampel sponges, CH_3OH pa, KOH pa, alcohol 96%, Seawater steril, Phosphate Buffer Saline (PBS), Media NA, Marine Agar (MA), 25% gliserol, NaCl 0,9%, formalin 2%, Aqubides, reagent MTT, KCl, $MgCl_2$, DMSO, standard biochemistry ptest for bacteria 15 parameters, sequencing universal primary from gen 16S rRNA E.coli: FP-U1 (5′-CCAGCAGCCGCGGTAATACG-3′) on nucleotides 518-537, and RP-U2 (5′-ATCGG (C/T) TACCTTGTTACGACTTC-3′) correspond to nucleotides 1.513-1.491, DNA Template, DNA polymerase Taq (Perkin-Elmer, Norwalk, Conn) (Liu et al 2017; Marzuki et al 2015b; Abass et al 2017) reagent for analysis PCR, Triton X-100, EDTA, Tris-HCl, KCl, $MgCl_2$, parafin, deoxynucleoside triphosphate, gel polyacrylamide, agarose, Ethidium Bromide, Anthracene cas.no: 120-12-7, pyrene cas.no: 129-00-0 and mix 16 PAHs standard ASTM (Supelco).

The tools used include: Scuba, camera in water, GPS, scalpel, tweezers, jars, plastic bags, ice boxes, microscopes, phases, porcelain plates, mortar and pestle, blenders, glass sets, Bunsen, analytic balance, hot plate , rubber suction, Whatman paper, oven incubator, freezer, BOD bottle, thermometer, round ose, test tube, 1.5 mL effendorf, petri dish, vortex shaker, centrifuge, magnetic stirrer, container compacting gel, universal paper, salinometer, stop watch, haemocytometer, air flow laminary (LAF),

stainless steel, autoclove, 0.2 μm filter, PCR machine (biorad), BioEdit program, MAS-100 (Microbiology Air Sampler), electrophoresis.

Acquiring sponge samples. The Sponge samples were obtained from Kodingareng Keke Island (Makassar administrative region). Kodingareng Keke Island is part of the Spermonde Archipelago. There are four types of sponge samples obtained according to the sampling point at the coordinates, namely the code sample of Sp1 (S = 050 06 '06.76", E = 1190 17' 10.66"), Sp 2 (S = 050 06 '06.87", E = 1190 17 '10.90"), Sp 3 (S = 050 06' 06.15", E = 1190 17 '10.18"), and Sp 4 (S = 050 06' 06.73", E = 1190 17 '10.34"). The sampling location is clearer in accordance with Figure 1, as follows:

Figure 1. Location of Sponge Sampling Points (Kodingareng Keke Island), Spermonde Archipelago, South Sulawesi

Parameters measured at the sponge sampling point as stated in Table 1, below:

Table 1. Sponge Sampling Characteristics Points

Observation	Sample Code			
	Sp 1	Sp 2	Sp 3	Sp 4
Depth from the surface (m)	3.2	3.6	3.3	4.8
Consistency sponge surface	slippery	slippery	slippery	rough
Salinity (‰)	29.3	29.8	29.3	29.5
pH	7	7	7	7
Temperature (^{0}C)	29	29	29	28

Some parameters measured at the sampling point (Table 1) show samples of sponges living in a normal environment (depth, salinity, pH and temperature), except for the consistency parameters of the sponge tubular sponge covered with mucus. The mucus that covers the sponge body is thought to be produced by bacteria symbionts for the purpose of self-protection and sponge response to environmental changes. These environmental changes are caused by several things such as turbidity of the sea bed due to strong currents, the presence of pollution materials such as hydrocarbons, heavy metals, plastics (Taylor et al 2013; Marzuki et al 2015b; Wang et al 2017).

Analysis of Sponge Morphology. Sea sponge samples were prepared in 70% methanol, methyl parts were selected, cut transversely and observed with a phase contrast microscope haemocytometer, surface smeared with 70% ethanol, observed again with haemocytometer, sterilized sponge pieces using filtered sterile sea water with 0 filters, 2 μm. The sponge sample was crushed with a blender, then the cell suspension was observed with a microscope haemocytometer to see cell types, skeletons and sponge species (Manzanera et al 2015; Parama et al, 2017; Marzuki et al 2016; Zhou et al 2011)

Phenotype Analysis Bacteria Symbiont. Characterization of sponge symbiont bacteria using the method of identification of Gram staining and biochemical tests. The process is carried out by taking grams of bacterial dye 1 ose on the prefarat glass which has previously been released from free fatty acids with 96% alcohol, then used as purple

crystal drops (Gram A), drops of Lugol (Gram B), drops of acetate alcohol (Gram C) , and safranin test (Gram D), test with 1% KOH (Meutia et al 2011; Marzuki et al 2016; Vaezzadeh et al 2017).

The biochemical test standard consists of 15 test parameters on suitable media, namely: starch hydrolysis, casein hydrolyzed, indole reaction, nitrite reduction, glucose fermentation, lactose fermentation, sucrose fermentation, mannitol fermentation, citrate test, catalase test, urease test, H_2S test, methyl red test, Voges Proskauer test and gelatin test. Observable indicators based on the reactions that occur are observed visually by looking at changes in color, turbidity or sedimentation in the test media (Parama et al 2017; Marzuki et al 2016).

Genotypic analysis of spongy microsimbion using the PCR method. Single colonies were obtained from each of the samples isolates, heated to 95° C for 5 minutes. The 16S rRNA gene is amplified with 63f and universal bacterial primary. The PCR reaction has been formed in a 30-IL reaction volume containing 19 DreamTaq buffers (Fermentas, MA), 0.2 mM each dNTP (dATP, dTTP, dGTP, dCTP) 0.05 IL units, 1 DreamTaq polymers (Fermentas) mixed 1 M each is reversed and forward is primary. PCR progression is as follows: Symbiont bacteria are denatured for 5 minutes, 94° C, followed by 30 cycles of 1 minute denaturation at 94^0 C, 1 minute 20 seconds annealing at 54° C and 2 minute extension at 72° C, ending with elongation 10 minutes at 72° C. DNA extraction and PCR amplification. Production water samples were filtered through polycarbonate membrane filters (size 0.22 mm; Millipore, Bedford, MA, USA). Then, the membrane is transferred to a sterile tube individually and the cells are dissolved in a beaded bead. The total genomic DNA was then extracted using AxyPrep ™. Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, Inc., CA, USA). 16S rRNA gene symbiont bacteria samples, amplified using archival primer 109F/912R (Liu et al 2017; Bello et al 2015; Zhang et al 2012).

Activity of Sponge Symbionic Bacteria Activity. Two test methods were carried out to determine the activity of sponge Symbiont bacteria on PAH contaminated media, namely: first using solid media each contaminated with PAH (Antrachena, pyrene, mix 16 types of ASTM standard PAH). Observation of symbiont bacterial activity was carried out after 3 days of contact in the incubator. Both liquid media in the form of spongy symbiont bacterial suspension were interacted against 3 types of solution each contaminated with antrachena, pyrene, mix 16 types of ASTM standard PAH in the reactor. The interaction reactor is aerated with Shaker 100 rpm, room temperature, 5 day interaction period. Parameters of sponge symbiont bacterial fermentation reaction against PAHs, namely pH of the media and examination of air bubbles (Marzuki et al 2015b; Taylor et al 2013).

Results and Discussion

Analysis of Sponge Morphology. The results of identification of sponge sample morphology obtained from Kodingareng Keke Island are in accordance with Table 2, while the identification process with the target of calculating the number of symbionic bacterial cell DNA, determination of species and strains for each sponge sample is presented in Figures 2-5, following:

Table 2. Results of identification of Sponge sample species

Code of Sponge sample	Species	Family
Sp 1	Petrosia (Strongylophora) corticata	Petrosiidae
Sp 2	Aulleta sp.	Axinellidae
Sp 3	Neopetrosia sp	Petrosiidae
Sp 4	Callyspongia aerizusa	Callyspongiiade

The search for spongy structures using morphological analysis methods aims to obtain information about sponge cell structure, the shape of growth, the consistency of the body, the size of the oscula, the skeleton and the shape of sponge cell spicules, shown in Figure 2-5, below:

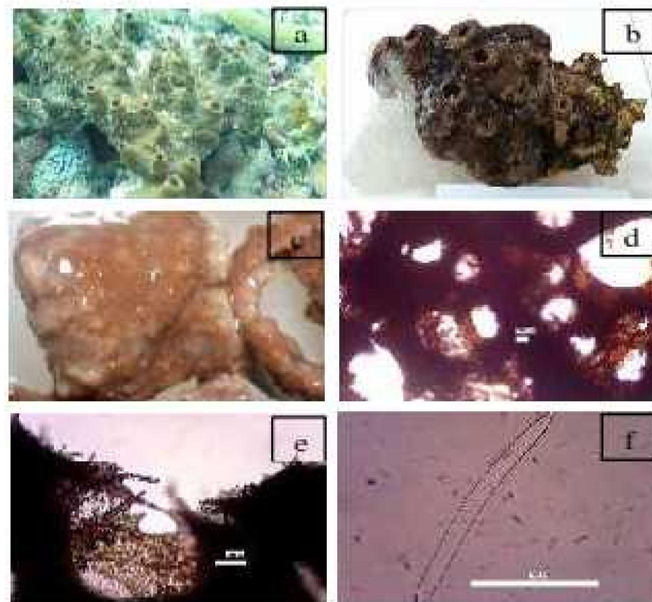

Figure 2. Morphology of sponge cell Petrosia (Strongylophora) corticata (Sp.1); (a) GROWTH FORM: Sligthy globular sponge, with big size oscula, (b) CONSISTENCY: Slippery surface sponge, covered by mud-like slime. Inelastic and brittle body sponge, (c) SURFACE: Granular sponge surface, (d) SKELETON: Spicule skeleton with echinating spicule, (e) SKELETON TRACT: Paucispicular tract skeleton with high fibre, (f) SPICULE: Small megasclera oxea (magnification 40x)

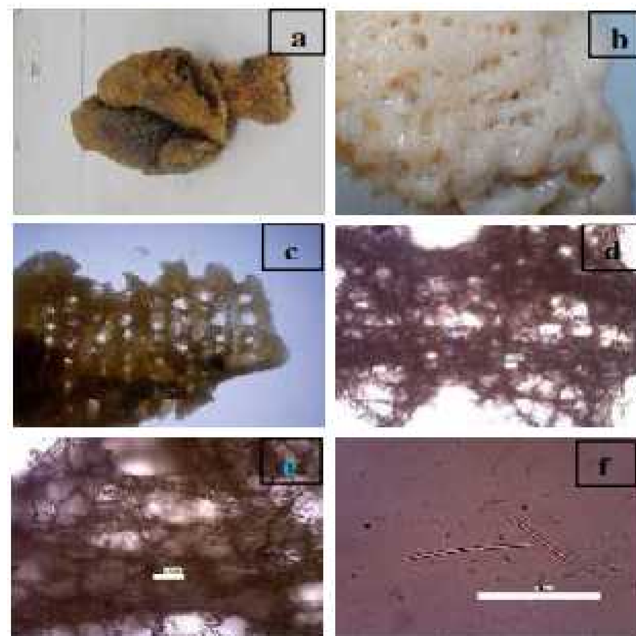

Figure 3. Morphology of sponge cell Auletta sp (Sp.2) (a) GROWTH FORM: Dark-yellow colored sponge, Slighty cylindrical shape, (b) CONCISTENCY: Slippery surface sponge, covered by mud-like slime, compressible, crumbly and fragile body, (c) SKELETON: Pauci spicular, plumoreticulate, (d) CHOANO SOME: Anostoming choanosme

skeleton, (e) FIBRE: Fibre interconnected, (f) SPICULE: Slender oxea megasclere. (magnification 40x)

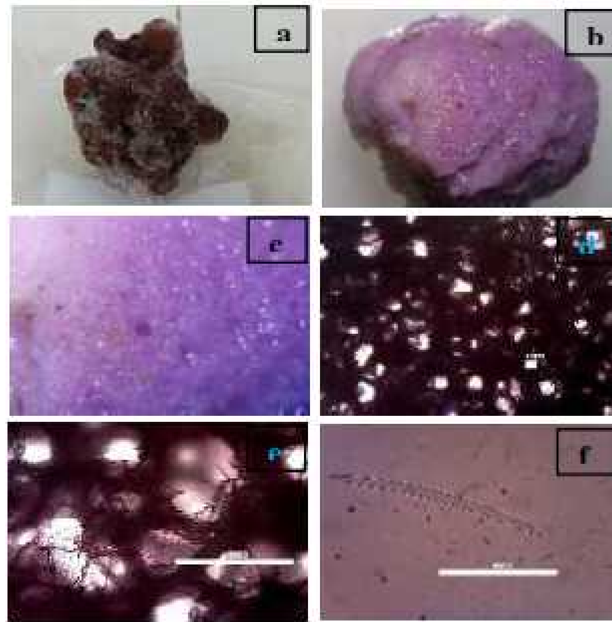

Figure 4. Morphology of sponge cell Neopetrosia sp (SP.3) (a) GROWTH FORM: Globular shape sponge. Purple colored, (b) CONSISTENCY: Hard and tough consistency, Slippery surface sponge, covered by mud-like slime, (c) SURFACE: Smooth sponge surface, (d) CHOANOSOME: Dense alveolate choanosome skeleton, (e) SKELETON: Multispicular skeleton, arranged round, (f) SPICULE: Oxea megasclere (magnification 10x), no Microsclere

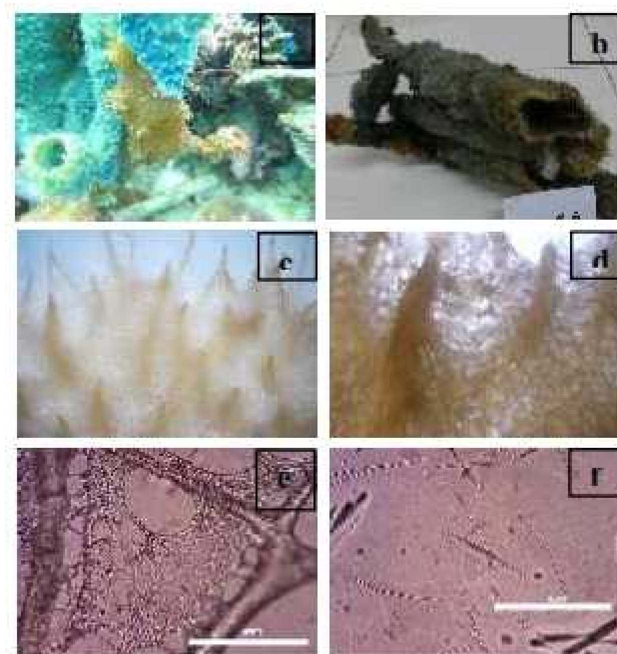

Figure 5. Morphology of sponge cell Callyspongia aerizusa (Sp.4). (a) GROWTH FORM: Cylindrical shape sponge, Blue and Yellow colored sponge, (b) COLOE IN ETOH: The color fades after being prepared with alcohol, (c) SURFACE: Pappilate surface sponge, (d) SKELETON: Primary and Secondary fibre. Interconnected tract, (e) FIBER: High density of fiber, (f) SPICULE: Slender oxea (magnification 40x), no microsclera.

The physical characteristics and body structure of the 4 types of sponge samples mentioned above are known as code samples Sp 1, Sp 2 and Sp 3, the surface of the body covered with mud resembling mucus reaches 80% - 86%, while sponge Sp 4, no mucus that closes parts of the body is found. It is thought that the sponge covered a large part of the body with mucus, indicating that the sponge's environment was disrupted, thus stimulating the sponge to symbiosis with bacteria capable of producing substances such as mucus, used to protect the body of the sponge. Another guess is that the symbiosis of bacteria with sponges has occurred earlier, because these bacteria need hosts so they are not tossed around by ocean currents. These symbionic bacteria, then produce mucous substances with enzyme characteristics, because the natural response of bacteria adapts to extreme changes in habitat growth due to PAHs contamination. The adaptation of the symbiont bacteria is beneficial not only to the symbionic bacteria but also to the main sponge (Lavy et al 2014; Marzuki et al 2016; Abdel et al 2013).

The four types of sponges have high fibrous skeletons, are interconnected with one another and dense, while the spicules of the four types of sponges generally expand and have small to medium size and shaped slinders. This sponge structure with skeleton and spicula does not describe the characteristic associated with sponge sensitivity against environmental changes. Skeletons and spicules function for nutrition and breathing, although skeletons with solid or interconnected fiber arrangements and small to moderate oxea are only a consequence of the life of the sponge Fedeer filter as an adjustment in the size of spray particles absorbed and sprayed with discharges (Meutia et al 2011; Abdel et al 2013; Marzuki et al 2015a).

The isolation of symbionic bacteria from the four sponge samples obtained a number of symbiont isolates, namely 2 isolates of Petrosia (Strongylophora) corticata, 3 isolates of Aulletta Sp, 3 sponges of Neopetrosia Sp, 2 isolates and Callyspongia aerizusa sponges. The total isolates obtained were 10 types, then 1 type of the isolate was chosen for each type of sample, so that 4 isolates were selected. Selected bacteria were coded following the sponge sample code and the number of isolates selected, (Table 3).

Phenotype Analysis of Sponge Symbiont bacteria. Phenotype analysis of spongy Symbiont bacteria aims to determine the biological characteristics of symbiont isolates. The target to be achieved in the phenotypic analysis of sponge Symbiont bacteria is to analyze the relationship between sponge morphology, phenotypic characteristics (symbiont morphology, Gram symbiont group and biochemical tests) to obtain a constructive picture of the ability of sponge symbiont bacteria to have growth activity on PAHs contaminated media. The results of morphological analysis and Gram staining test of spongy symbionts are presented in Table 3, follows:

Table 3. Results of Morphological Analysis and Gram Stain Test for Sponge Symbiont Bacteria

Sponge species	Symbion Bacteria Code	Baacteria Morphology	Bacteria Gram Type
Petrosia (Strongylo-phora) corticata	SP1. B2	round shape, bluish beige color, clustered spread, rod shape, color fixed with safranin, endospores less clear, insoluble with alkaline 1% KOH	Basil gram (+) + spora
Aulleta sp	SP2. B1	the shape of a serrated rod, brown color, separate spread of color remains with the safranin reagent, there is endospores, insoluble with 1% KOH alkali	Basil gram (+) + spora
Neopetrosia sp	SP3. B3	the shape of a serrated rod, brown color, separate spread of color remains with the safranin reagent, there is endospores, insoluble with 1% KOH alkali	Basil gram (+)

Callyspongia aerizusa	SP4. B1	round shape, brown color, separate spread, color changes with safranin reagent, no endospore, soluble with 1% KOH alkali	Basil gram (-)

Table 3 above shows that there are three gram-positive sponge Symbiont isolates from 3 different types of sponges. The morphological analysis of sponge simbion bacteria was intended to see whether there was a relationship between morphology (shape, color, cell distribution, endospore) and sponge symbionts with the ability of the symbiont bacteria to grow on PAHs contaminated media. The shape, color and distribution of symbiont bacterial cells cannot be used as a reference to suggest that the bacteria have a potential or not to PAHs biodegradation, while the reaction with safranin reagents and 1% KOH solution, to determine Gram class of test bacteria, Gram positive/negative bacteria can be mistaken one parameter to determine whether the sponge simbion bacteria can biodegrade PAHs (Nurhayati et al 2006; Marzuki et al 2016; White et al 2012). Determination of Gram group sponge symbiont bacteria to obtain an overview of whether there is a correlation between the Gram sponge symbionts group and the ability of bacterial activity on PAH (Parama et al 2017; Muzaki et al 2017; Marzuki et al 2014), contaminated media. The series of phenotypic analyzes is biochemical tests using specific reagents to determine the chemical processes that occur in isolated which imply the life dynamics experienced by sponges during their growth period. The results of the biochemical test are presented in Table 4, as follows:

Table 4. Characterization of Sponge Symbiont Bacteria Biochemical Test method

Reagent Biochemistry	Media	Sponge Symbion Bacteria			
		SP1.B2	SP2.B1	SP3.B3	SP4.B1
Starch hydrolysis	Starch gelatin	base	base	base	base
Hydrolysis of casein	Milk gelatin	acid	acid	acid	acid
Hidrolysis Gelatin	Nutrient gelatin	-	-	-	-
Reduction nitrate	Nitrate broth	-	-	-	-
Indol	Tryptone broth	-	-	-	-
H^2S	H_2S jelly	-	-	-	-
Red Metil	R-VP broth	+	+	+	+
VP	R-VP broth	+	+	+	-
Sitrat	S. Sitrat jelly	+	-	+	-
Urease	Urea broth	-	-	-	-
Glucose	Glucose broth	-	-	-	-
Lactose	Lactose broth	+	+	+	+
Sucrose	Sucrose broth	-	-	-	-
Mannitol	Mannitol broth	-	-	-	-
Catalase	NA tilted	+	+	+	-

The biochemical test results using the MR-VP reagent, showed that the isolate reacted positively with MR, meaning that the symbiont bacteria had the ability to induce glucose to produce stable acidic products causing the pH of the media to drop. Bacteria symbiont SP4. B1, does not react with VP, meaning that the fermented product of the isolate is not acetone, while the other three symbionts bacteria produce fermented products in the form of acetone. Sponge symbiont bacteria code of SP1. B2 and SP. B3, positive for citrate reagents shows that the two isolates can use fermented products as carbon sources. The lactose test shows that the four symbionts bacteria have a lactose enzyme capable of converting lactose to galactose and glucose (Muzaki et al 2017; Marzuki et al 2014). A positive reaction was also shown in the catalase test by isolating SP1 code. B2, SP2. B1 and SP3. B3, shows that the isolate is an aerobic group that can produce the enzyme catalase which is able to use H_2O_2 in the reaction. The positive reaction results in the casein hydrolysis of four symbionts bacteria, indicating that this bacterium reacts quickly by milk casein hydrolyzed by the enzyme renin takes place in an acidic atmosphere. The phenotypic characteristics of sponge symbionts isolates indicate that these isolates can live in a carbon-contaminated environment. This view is estimated that the growth activity of the four sponge symbiont bacteria that occur, generally follows the

relative mechanism of symbiont bacterial growth in hydrocarbon-contaminated media (Parama et al 2017; Marzuki et al., 2015b).

Genotype Analysis of Sponge Symbiont Isolates. The genotype analysis of four types of sponge symbionts bacteria to determine genetic properties, arrangement and number of DNA pairs, DNA arrangement quantity, and species of symbionts bacteria. Different species and strains show that the symbiont isolates have different characteristics from other bacteria, so it is estimated that the response revealed to a contaminant is forge certain mechanisms in carrying out fermentation reactions. The results of sponge symbiotic bacterial genotype analysis are shown in Table 5, as follows:

Table 5. BLAST (Basic Local Alignment Search Tool) Symbiont Sponge, Metode PCR

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
SP 2. B1	11-985 (974)	524.589-525.563 (974)	956/974 (98,15)	14/974 (0,01)	Bacillus licheniformis strain ATCC9789
SP 3. B3	15-975 (960)	574.123-575.089 (966)	932/960 (97,49)	16/960 (1,66)	Bacillus sp. AB353F.partial
SP 4. B1	21-934 (913)	574.323-575.258 (935)	906/935 (96,90)	12/935 (1,28)	Acinotobacter calcoaceticus strain PHKDB14

The phenotypic characteristics of sponge symbiont bacteria (Tables 3 and 4) are in accordance with the results of genotypic analysis (Table 5) which shows that the four spongy symbionts bacteria with different species and strains vary, meaning that the characters of the sponge symbiont isolates also dissociate from one another including the mechanism taken against the degradation of hydrocarbon components (Liu et al 2017; Lavy et al 2014). Different isolate characters show fermentation reactions that can occur in these different isolates including the response of these bacteria to PAHs contaminants. The combination of several specific characteristics seen in sponges, especially sponge morphology, phenotype (Gram bacterial group and biochemical test) and genotypes (species and strains) is strongly suspected that sponge symbiont bacteria can biodegrade PAHs or have growth activity in PAHs contaminated test media (Bello et al., 2015; Taylor et al 2013).

Results isolation of bacteria pf Sponge Symbiont. The isolate activity test carried out on solid media contaminated with PAH was aimed at tracing whether there was a relationship between living sponge dynamics (body covered with mucus), positive reactions on a number of specific reagents of biochemical tests, gram-positive isolates and strains with symbionts isolates on PAH exposed media. The results of the activity test for sponge symbiont isolates on solid media contaminated with PAH are presented in Table 6, as follows:

Table 6. Test of Sponge Symbiotic Isolates on Media Contaminated PAH

Sponge Symbion Bacteria	Type of PAH contaminated in solid media		
	Antrachena	Pyrene	Mix 16 PAH Std ASTM
SP1. B2	++	++	+
SP 2. B1	++	+	+
SP 3. B3	++	++	+
SP 4. B1	-	-	-

There are three types of symbionts bacteria (Table 6), each SP1. B2, SP2. B1 and SP3. B3, showed that there was growth activity in solid test media contaminated with antrachene, pyrene and mix 16 PAHs in ASTM. Observation of bacterial growth activity was carried out after incubation for 3 days at room temperature sponge symbionts bacteria (SP 4.B1) did not show cell growth activity. These results are the initial clues that there is a morphological correlation of sponges (body covered with mucus), symbiont bacterial phenotype (Gram positive group, reaction of several biochemical test reagents) and genotypes (Pseudomonas, Bacillus, strains) with bacterial growth activity on PAH exposed media. The activity of sponge symbiont bacteria in several types of PAHs showed that the type of symbionts bacteria could degrade PAH (Akinde et al 2012; Marzuki et al., 2015). Even so, it cannot be ascertained that the mechanism of biodegradation of the PAHs component by the symbionts bacteria runs with a certain pattern, but it is assumed that the symbionts bacteria forge a specific mechanism according to each character to the degradation of different PAHs. This assumption is based on different strains of bacterial isolates, although these symbiont bacteria have the same or different species (Liu et al 2017; Bello et al 2015), (Table 5). The other sponge symbionts bacterial activity test carried out was a symbionts bacterial growth activity test on liquid media contaminated with PAH with the test results presented in Table 7, follows:

Table 7. Activities of Symbiont Bacteria Sponges in PAHs contaminated Test

Sponge Symbiont Bacteria	Activity parameters	PAH type and contact periods (days)					
		Antracena		Pyrene		Mix 16 PAH Std ASTM	
SP1.B2	pH	7	6	6	6	7	6
	gas bubble	-		-		-	
SP 2. B1	pH	7	6	6	7	7	7
	gas bubble	-				-	
SP 3. B3	pH	7	6	7	6	7	6
	gas bubble	-		-		-	
SP 4. B1	pH	7	7	7	7	7	7
	gas bubble	-	-	-	-	-	-

The results shown (Table 7), in particular the three sponge symbiont bacteria (SP1. B2, SP2. B1 and SP3.B3) indicate growth activity or can carry out fermentation reactions on PAH contaminated media characterized by changes in pH 7 to pH 6 after the interaction period 10 days. Other parameters are gas bubbles formed at the 10 days contact period. The results shown (table 7) are suitable (Table 6), while SP4 4.B1 samples do not show growth activity in solid test media (Table 6). Liquid PAH contaminated test media by SP4 4.B1 Samples, also not visible, was indicated that there were no changes in degradation parameters (constant pH) and no air bubbles in liquid test media (Table 7). This parameter corresponds to the phenotypic character of the isolates in biochemical tests. The isolate fermentation reaction parameters were seen after a 10-day contact period, because bacteria experienced several phases in their activities, the adaptation phase in the growth environment generally lasts 3 to 5 days, then the development phase, namely the number of bacterial cells increases and cell size increases. Such conditions result in physical changes in bacterial cells (Marzuki et al 2017; 2015b; Bello et al 2015). The developmental phase generally lasts for 2-3 after the adaptation phase, or on days 6-8 the contact period for symbionts bacteria in liquid media exposed to PAHs. The next stage of bacterial activity is the stationary phase and death phase, usually traversed by bacteria in a short period of 2-3 days (Akinde et al 2012; Marzuki et al., 2015a).

Based on the phenotype data (Table 4), the sponge test Symbiont bacteria are aerobic bacteria capable of producing catalase enzymes to produce toxic gas peroxide (H_2O_2). Gas H_2O_2 if accumulated in the interactive medium at sufficient concentration, has the potential to kill off bacterial cells more quickly. Another factor that inhibits bacterial cells

from dividing further is the PAH degradation product. The contract period is 10 days, allowing degraded PAH components to produce simple and acidic organic compounds. Acidic pH of the interaction media causes symbionts bacterial cells to experience faster death (Bello et al 2015; Marzuki et al 2017). This is reinforced by fermentation reactions which are occupied by symbionts bacteria in an acidic atmosphere (Table 3) and decreasing interaction pH (Table 7).

The sponge morphology data (Table 2), (Figure 2-5), morphology of isolates and gram groups symbiont isolates (Table 3), sponge symbionts bacterial fermentation reactions (Table 4), genotypic characteristics (Table 5), when compared with test results sponge symbiont bacterial activity (Table 6 and Table 7), shows that there is a strong correlation of the ability of sponge symbionts bacteria activity in PAHs contaminated media with sponge life patterns and visible phenotypic and genotypic characteristics. This research identified two species of spongy symbiont bacteria having growth activity on PAHs contaminated media, namely Pseudomonas and Bacillus are not new results, because many previous research results concluded that Pseudomonas and Bacillus bacteria could degrade PAHs, even though the source of the bacteria was isolated from other materials such as mangrove, mud contaminated with sludge waste contains hydrocarbon components, sea water is polluted with PAHs (Manzanera et al 2015; Marzuki et al 2020).

The new results of this study, namely: first. The use of symbionts bacteria, isolated from the first sponge was carried out for PAHs biodegradation, secondly, it was found that the pattern of choosing potential sponges for PAHs biodegradation is a sponge whose surface is covered with mucus. This result leads us to a new understanding and ease of tracing or selecting sponges. The potential to degrade the type of PAHs is a sponge whose body surface is slippery or covered by mud resembling mucus, so sponge morphology analysis is not necessary. To be convincing in conducting development research, the isolates from sponge bacteria were tested for Gram group isolates and biochemical tests by only having Gram positive sponge group symbionts and positive reaction results on casein, lactose, citrate, VP. and MR test reagents. This information is important to be a guideline for conducting further research on the biodegradation method using sponge symbionts bacteria, considering the types of bacteria that may be symbiotic with sponges are quite a lot. If this is done, it can save analysis time and be able to do research more focused on the target .

Conclusion. Three types of sponge symbiont bacteria each Pseudomonas stutzeri RCH2, Bacillus licheniformis strain ATCC9789 and Bacillus sp.AB353.partial, isolated from successive sponges Petrosia (Strongylofora) corticata species, Aulleta sp and Neopetrosia sp, showed growth activity in the media solid interactions and liquid media contaminated with antrachene, Pyrenees and PAH mixes of standard ASTM. These findings indicate that there is a sponge morphology relationship, morphology of spongy Symbiont bacteria, gram group isolates and phenotypic characteristics and genotypes of spongy symbiont bacteria on the ability of bacterial growth activity in PAHs contaminated media. Investigation of potential sponges with the symbiosis of bacteria that can degrade poly aromatic hydrocarbons by having sponges that are covered by mucus, characteristic of phenotypes namely gram positive groups and reacts positively with MR-VP, citrate, calatase in biochemical tests.

The development of the next research was directed at more intensive potential and investigations to multiply the species of sponge symbiont bacteria as a new biodegradator material for PAHs by running a pattern of selecting sponges whose bodies were shrouded by mucus. The development of this research is in the future, continuous coverage of the performance aspects of sponge symbiont bacteria against PAHs by entering several degradation parameters besides pH and air bubble interaction media, for example investigating the maximum limit of PAHs concentrations biodegraded by bacteria using GC-MS, using a spectrophotometer to determine biodegradation media, fermentation odor that occurs, biodegradation product organic compounds using IR, including the potential to engineer extreme degradation media exposed to several types

of PAHs, the potential formation of PAH degradation bacteria consortium formulations and the potential use of symbionts bacteria to test heavy metal bio-absorption.

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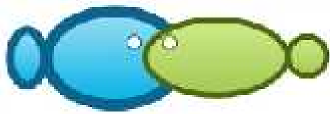

Naskah Revisi Pertama

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 genotypical 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, 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, 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.

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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. 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.

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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. The bacteria are suspected to have specific characteristics that can degrade PAH.

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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; Parama et al 2017). In general, these substances are found to cover most of the sponge body surface (De Rosa at 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; Parama 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 2015; 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).

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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). Parameters measured are listed in Table 1. The sampling point coordinates were S=05^{0}06′06.76″, E=119^{0}17′10.66″ for Sp1, S=05^{0}06′06.87″, E=119^{0}17′10.90″ for Sp2, S=05^{0}06′06.15″, E=119^{0}17′10.18″ for Sp3, and S=05^{0}06′06.73″, E=119^{0}17′10.34″ for Sp4.

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

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), except for the surface consistency parameters of the sponge covered by mucus. Mucus covering the sponge surface is thought to be produced by bacterial symbionts as a self-defense and response to environmental changes.

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Morphological analysis of sponge. 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; Parama et al 2017).

Phenotypic analysis of sponge bacterial symbionts. The ph enotypic 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, and Voges-Proskauer gelatin test. Changes in color, turbidity, or sediment in the media were indicated by a reaction indicator (Marzuki et al 2016; Parama et al 2017).

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Genotypic analysis of sponge microsymbiont by PCR method. The DNA extraction and PCR amplification, production water samples were filtered with a polycarbonate membrane filter (size 0.22 mm; Millipore, Bedford, MA, USA). 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), 0.2 µL dNTP (dATP, dTTP, dGTP, dCTP), 0.05 IL units, 1 DreamTaq polymerase (Fermentas), 1 µL dNTP (dATP, dTTP, dGTP, dCTP), 0.05 IL units, 1 DreamTaq polymerase (Fermentas), 1 µL forward and reverse primers. 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.

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Sponge bacterial symbiont activity test. There were 2 test methods carried out to determine the activity of sponge bacterial symbionts on PAH contaminated media, namely solid media as 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).

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Results and Discussion

Morphological analysis of sponge. 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 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. The morphology of samples are presented in Figures 2 to 5.

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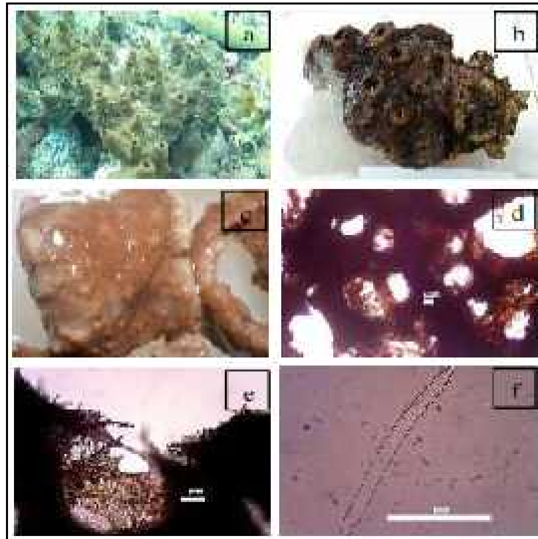

Figure 2. Morphology of Petrosia corticata (Sp1). a - growth form: sligthy globular with big size oscula; b - consistency: slippery surface covered with slimy mucus; inelastic and brittle body sponge; c - surface: granular; d - skeleton: spicule skeleton with echinating spicule; e - skeleton tract: paucispicular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

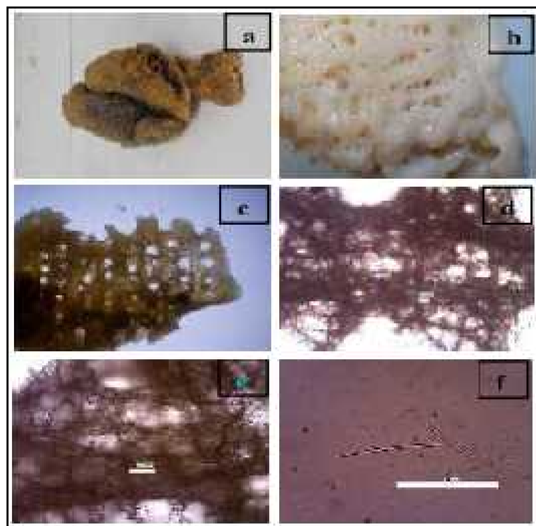

Figure 3. Morphology of Auletta sp. (Sp2). a - growth form: dark-yellow, slightly cylindrical; b - consistency: slippery surface, covered by mud-like slime, compressible, crumbly and fragile body; c - skeleton: pauci spicular, plumoreticulate; d - choanosome: anostoming choanosme skeleton; e - fiber: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

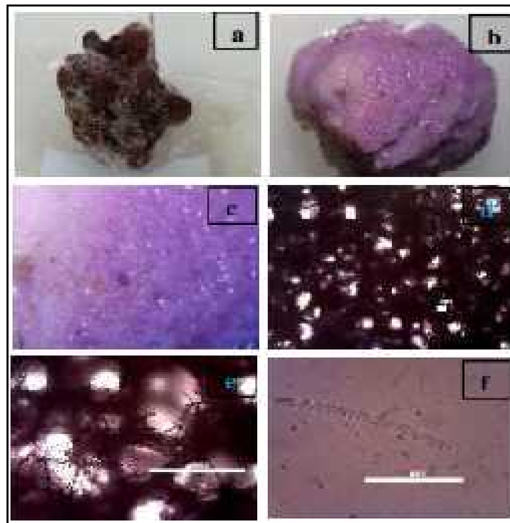

Figure 4. Morphology of Neopetrosia sp. (Sp3). a - growth form: globular shape sponge, purple colored; b - consistency: hard and tough consistency, slippery surface sponge, covered by mud-like slime; c - surface: smooth sponge surface; d - choanosome: dense alveolate choanosome skeleton; e - skeleton: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

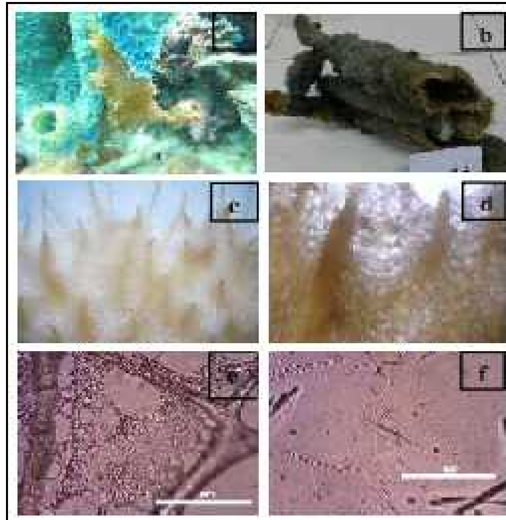

Figure 5. Morphology of Callyspongia aerizusa (Sp4). a - growth form: cylindrical shape, blue and yellow colored sponge; b - colorin ethol: the color fades after being prepared with alcohol; c - surface: pappilate surface sponge; d - skeleton: primary and secondary fiber, interconnected tract; e - fiber: 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, Sp 2, and Sp 3 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 2015).

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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 agar	-	-	-	-
Methyl red	R-VP broth	+	+	+	+
VP	R-VP broth	+	+	+	-
Citrates	S. citrate agar	+	-	+	-
Urease	Urea broth	-	-	-	-
Glucose	Glucose broth	-	-	-	-
Lactose	Lactose broth	+	+	+	+
Sukrose	Sucrose broth	-	-	-	-
Mannitol	Mannitol broth	-	-	-	-
Catalase	NA slant agar	+	+	+	-

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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. B1.SP4 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, B1.SP4 did not have this ability. The citrate test showed positive results on 2 isolates in SP1 isolates, B2 and SP3. The B1 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; Parama et al 2017).

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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 padat		
	Anthracene	Pyrene	Mix 16 PAH ASTM standard
SP1.B2	++	++	+
SP2.B1	++	+	+
SP3.B3	++	++	+
SP4.B1	-	-	-

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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.

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Table 7
Sponge bacterial symbiont activity in poly aromatic hydrocarbon (PAH) contaminated liquid media

Sponge bacterial symbiont	Parameter of Ac	Jenis PAH dan Waktu Kontak (hari)					
		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	-	-	-	-	-	-

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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 2015). 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.

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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). Test parameters during the isolation fermentation period were observed after 10 days of culture. This may be related to the pattern of bacterial growth which consists of several 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).

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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 (H2O2). The high concentration of H_2O_2 gas accumulated in the media could kill mass bacteria in a faster period. Another factor is that PAH degradation products inhibit bacterial cell division. A 10-day culture period allows the PAH component to degrade into simple and acidic organic compounds.

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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 correlation among sponge bacterial symbiont activity on PAH contaminated media and sponge dynamics, as well as apparent phenotypic and genotypic characteristics. In this study, 2 species of 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).

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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. The future development of this research is carried out on aspects of the degradation performance of sponge bacterial symbiont on PAH by adding several degradation parameters besides pH and air bubble interaction media, such as investigating the maximum limit of PAH concentrations degraded by bacteria using GC-MS and spectrophotometer to determine ontological densities of degraded media, the odor of occurred fermentation, types of organic compounds degradation products using FTIR/IR, including the potential to engineer extreme degradation media exposed to several types of PAH, the potential formation of a consortium formulation of PAH degrading bacteria groups and the potential use of bacterial symbionts for the absorption of heavy metals.

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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, which has provided a funding to conduct this study.

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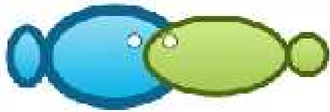

Selection and characterization of potential bacteria for polycyclic aromatic biodegradation of hydrocarbons in sea sponges from Spermonde Islands, Indonesia

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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 genotypical 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.

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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).

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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.

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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).

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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 Laboratory of Biochemistry, Faculty of Mathematics and Natural Sciences, Hasanuddin University. Parameters measured are listed in Table 1. The sampling point coordinates were S=05^{0}06′06.76″, E=119^{0}17′10.66″ for Sp1, S=05^{0}06′06.87″, E=119^{0}17′10.90″ for Sp2, S=05^{0}06′06.15″, E=119^{0}17′10.18″ for Sp3, and S=05^{0}06′06.73″, E=119^{0}17′10.34″ for Sp4.

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

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. ~~Mucus covering the sponge surface is thought to be produced by bacterial symbionts as a self-defense and response to environmental changes.~~

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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 ph enotypic 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; 1) starch hydrolysis, 2) casein hydrolysis, 3) indole reaction, 4) nitrite reduction, 5) glucose fermentation, 6) lactose fermentation, 7) sucrose fermentation, 8) citrate test, 9) catalase test, 10) urease test, 11) H_2S test, 12) methyl-red test, 13) voges-proskauer, 14) gelatin test and 15) 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).

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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 DNA extraction and PCR amplification, production water samples were filtered~~ 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. ~~0.05 μL IL units, 1 DreamTaq polymerase (Fermentas), 1 μL dNTP (dATP, dTTP, dGTP, dCTP), 0.05 μL units, 1 DreamTaq polymerase (Fermentas), 1 μL forward and reverse primers.~~ 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.

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Sponge bacterial symbiont activity test. There are 2 test media carried out to determine the activity of sponge bacterial symbionts on PAH contaminated media, namely; solid and liquid media. ~~There were 2 test methods carried out to determine the activity of sponge bacterial symbionts on PAH contaminated media, namely; solid and liquid media.~~ As 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).

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Results and Discussion

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

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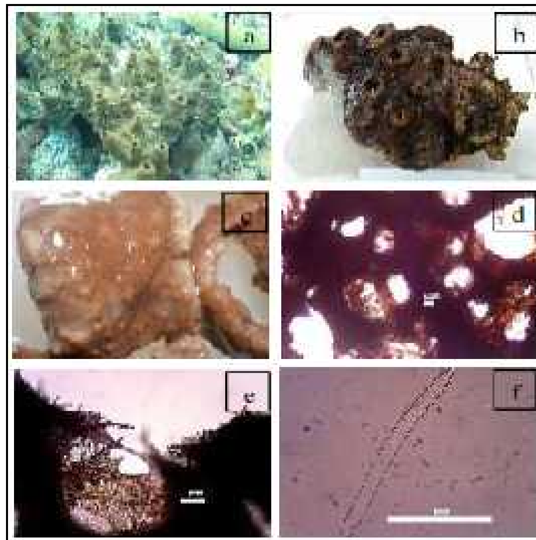

Figure 2. Morphology of Petrosia corticata (Sp1). a - growth form: sligthy globular with big size oscula; b - consistency: slippery surface covered with slimy mucus; inelastic and brittle body sponge; c - surface: granular; d - skeleton: spicule skeleton with echinating spicule; e - skeleton tract: paucispicular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

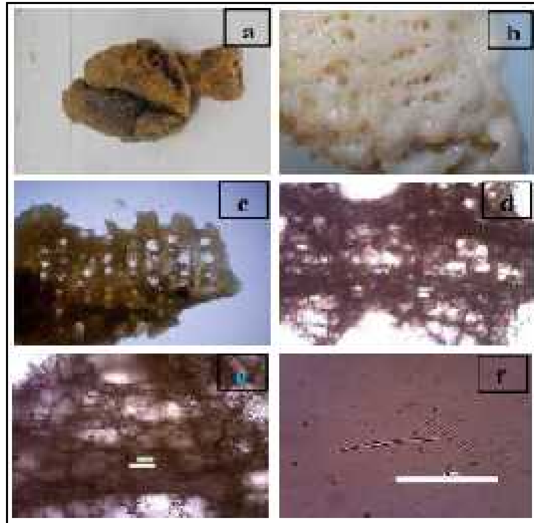

Figure 3. Morphology of Auletta sp. (Sp2). a - growth form: dark-yellow, slightly cylindrical; b - consistency: slippery surface, covered by mud-like slime, compressible, crumbly and fragile body; c - skeleton: pauci spicular, plumoreticulate; d - choanosome: anostoming choanosme skeleton; e - fiber: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

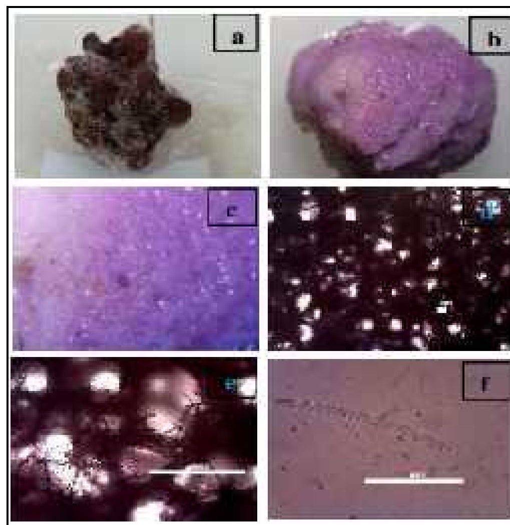

Figure 4. Morphology of Neopetrosia sp. (Sp3). a - growth form: globular shape sponge, purple colored; b - consistency: hard and tough consistency, slippery surface sponge, covered by mud-like slime; c - surface: smooth sponge surface; d - choanosome: dense alveolate choanosome skeleton; e - skeleton: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

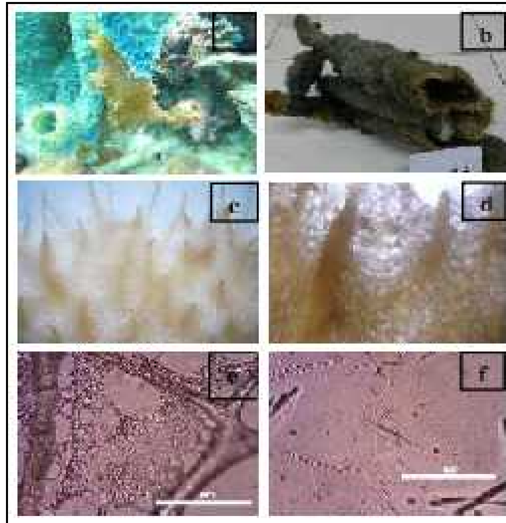

Figure 5. Morphology of Callyspongia aerizusa (Sp4). a - growth form: cylindrical shape, blue and yellow colored sponge; b - colorin ethol: the color fades after being prepared with alcohol; c - surface: pappilate surface sponge; d - skeleton: primary and secondary fiber, interconnected tract; e - fiber: 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, Sp 2, and Sp 3 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).

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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 ~~R-VP~~ broth	+	+	+	+
Reagent- Voges Proskauer	R-VP broth	+	+	+	-
Citrates	~~S.~~ citrate	+	-	+	-

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Urease	Urea broth	-	-	-	-
Glucose	Glucose broth	-	-	-	-
Lactose	Lactose broth	+	+	+	+
Sukrose	Sucrose broth	-	-	-	-
Mannitol	Mannitol broth	-	-	-	-
Catalase	~~NA~~ Nutrient Agar (NA) 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. ~~B1.SP4~~ 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, ~~B1.SP4~~ 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

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Symbiont code	Sample Sequence	Sequence Gen Bank	Quantity (%)	Difference (%)	Species
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) ~~padat~~		
	Anthracene	Pyrene	Mix 16 PAH ASTM standard
SP1.B2	++	++	+
SP2.B1	++	+	+
SP3.B3	++	++	+
SP4.B1	-	-	-

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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.

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Table 7

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

Sponge bacterial symbiont	Parameters of Ac	Type of PAH and Contact Time (day) ~~Jenis PAH dan Waktu Kontak (hari)~~					
		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

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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. This is because it is related to the growth pattern of bacteria which consists of several growth phases. ~~Test parameters during the isolation fermentation period were observed after 10 days of culture. This may be related to the pattern of bacterial growth which consists of several 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 accumulated in the media could kill mass bacteria in a faster period. Another factor is that PAH degradation products inhibit bacterial cell division.~~ The high concentration of H_2O_2 gas in the media could kill bacteria in a fast time. 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 ~~correlation~~ among sponge bacterial symbiont activity on PAH contaminated media and sponge dynamics, as well as apparent phenotypic and genotypic characteristics. In this study, 2 species of 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

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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. ~~The future development of this research is carried out on aspects of the degradation performance of sponge bacterial symbiont on PAH by adding several degradation parameters besides pH and air bubble interaction media, such as investigating the maximum limit of PAH concentrations degraded by bacteria using GC-MS and spectrophotometer to determine ontological densities of degraded media, the odor of occurred fermentation, types of organic compounds degradation products using FTIR/IR, including the potential to engineer extreme degradation media exposed to several types of PAH, the potential formation of a consortium formulation of PAH degrading bacteria groups and the potential use of bacterial symbionts for the absorption of heavy metals.~~

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