Article

Comparison of Biodegradation Performance of Marine Sponge Symbiont Bacteria Consortium against Anthracene and Pyrene

Ismail Marzuki [1,*], Ruzkiah Asaf [2], Mudian Paena [2], Admi Athirah [2], Khairun Nisaa [3], Rasheed Ahmad [4], Mudyawati Kamaruddin [5]

1 Department of Chemical Engineering, Fajar University, Makassar - South Sulawesi 90231, Indonesia; ismailmz@unifa.ac.id; ismailmz3773@gmail.com
2 Research Center for Brackish Aquaculture Fisheries and Extension Fisheries, Maros District - South Sulawesi 90512, Indonesia; qiaasaf@gmail.com; mudianpaena@yahoo.co.id; m.athirah@gmail.com
3 Fishery Faculty, Cokroaminoto University of Makassar, Makassar - South Sulawesi 90245, Indonesia; nisauicha27@gmail.com
4 Departement of Chemistry, Airlangga University, Mulyorejo-Surabaya, East Java,60115, Indonesi; rasheed.ahmad@fst.unair.ac.id
5. Postgraduate Program, Department of Medical Laboratory Science, Muhammadiyah Semarang University. Semarang Central Java, 50273, Indonesia; mudyawati@unimus.ac.id; ; mudya07@gmail.com

* Correspondence: ismailmz3773@gmail.com (I.M.); ismailmz@unifa.ac.id (I.M.)

Abstract: Every petroleum processing industry produces sewage sludge containing several types of polycyclic aromatic hydrocarbon (PAHs) components. The degradation of PAH components by physical, biological and chemical methods is not efficient. The use of marine sponge symbiont bacteria is considered an alternative method in the degradation and reduction of PAHs compared to the previous method. This study aims to explore the potential and performance of a consortium of sponge symbiont bacteria in degrading anthracene and pyrene. There are three types of bacteria (*Bacillus pumilus* strain GLB197, *Pseudomonas stutzeri* strain SLG510A3-8, *Acinetobacter calcoaceticus* strain SLCDA 976) were mixed to form a consortium. The interaction between the bacterial consortium suspension and PAH components was measured at 5-day intervals for 25 days. The biodegradation performance of bacteria on PAHs samples was determined based on five biodegradation parameters. The analysis results showed a decrease in the concentration of anthracene (21.89%) and pyrene (7.71%), equivalent to a ratio of 3: 1. The data was followed by a decrease in the abundance of anthracene (60.30%) and pyrene (27.52%), an equivalent ratio of 2: 1. The level of degradation of the pyrene component is lower than that of the anthracene component, presumably due to the higher toxicity of pyrene and the more stable molecular structure, making it difficult for bacterial cells to destroy it. The biodegradation products are organic compounds of alcohol, aldehyde, carboxylic acids and a small proportion of aromatic hydrocarbon components.

Keywords: performance; biodegradation; bacterial consortium; marine sponge; PAHs

1. Introduction

Anthracene and pyrene are a class of hydrocarbon aromatic polycyclic compounds. The molecular structure is stable by the ring's resonance ability of pi (π) bonds. The two types of PAHs were first isolated from coal tar, volatile compounds formed from incomplete combustion of organic compounds in protein-rich foods [1]. The primary sources of anthracene and pyrene are coal tar, condensation compounds of natural gas and petroleum distillation, and coal tar. Which is found in plants such as tobacco tar, can also be found in certain areas animals like deer and termites [2]. Anthracene is a moderately toxic and carcinogenic PAHs compound. Pyrene is a highly toxic PAHs group, carcinogenic

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and genotoxic or mutagenic [3-5]. Anthracene is widely used as an antiseptic and insecticide, while pyrene increases the octane number of fossil fuels [6,7]. The use of anthracene and pyrene in today's life is quite extensive so that they have the potential as components of pollutants in the air, soil and water [8, 9]. The character of PAHs, anthracene and pyrene, and other PAHs is difficult to decompose and accumulate in a material, including living organisms. Suppose these PAHs are released in the environment for a long time, followed by changes in weather, rain, fluctuating air temperatures. It is predicted that they can disrupt the ecological balance, change biogeochemical cycles, and even cause severe problems for the ecosystem, especially for the environment. -abiotic, biotic and environmental interactions [10 This condition can cause a negative chain effect because the PAHs component dissolves in water bodies, forming a flow from the high to the low, finally empties into the sea [11].

The sea is a giant container that can accommodate all the materials that go into it. There are thousands of types of biota interacting with each other in the sea, including the reversible relationship between all abiotic components and biotic components, both those already available in the sea and new materials that enter the marine environment [12,13]. This condition results in new adaptations in the sea, resulting in changes in new life patterns of marine ecosystems [14,15]. If the discharge of PAHs into the sea is not controlled, fish and other biota are potentially exposed to the carcinogenic components of anthracene and pyrene [16, 17]. The fish are caught by fishermen and eventually consumed by the community [18]. This process eventually forms the cycle and flow of the food chain; in the long run, it causes environmental quality to decline and human health levels to be lower [19].

The formation of interaction patterns and models, the order of a new ecosystem balance in the sea between abiotic and biotic components as a form of adaptation to the above conditions, causes many biotas to experience pressure regulating their lives. However, there is also biota that survives more with the new pattern [20, 21]. One of the biotas that survive with the new pattern is the sea sponge because it has a strong ability to adapt to changes in habitat [22]. There are four forms of adaptation of marine sponges: *first*, the ability to form a mutualism symbiosis with bacteria so that sponges can survive in habitats contaminated with waste types of PAHs and heavy metals [23]. *Second*, the nutritional pattern of sponges is a filter feeder with a body structure that has oscula, enabling it to obtain and absorbs food that is needed and not needed, disposed of by spraying it out [24]. *Third*, sponges can produce mucus substances that act as enzymes, which can be spread on the surface of their bodies as camouflage against predatory threats [25-26]; *Fourth*, the ability of sponges to absorb carbon components and convert them into energy for activities, including being able to absorb heavy metals [27 - 30]. Some scientific data show this adaptability because sponges are one of the marine biotas with an old civilization level. The DNA structure of sponges is easy to respond to habitat changes [31].

In addition to having the ability to degrade carbon components, especially PAHs, Sponges also have bioindicator and biomonitoring functions as well as bio-adsorption of heavy metals. It is due to their ability to produce enzymes (metabolic components) that can absorb and neutralize the toxic nature of the waste [32-34]. Sponges also have the function of biodegradation of aliphatic and aromatic hydrocarbon components, presumably because of the role of symbiotic bacteria they have or because of the metabolic components produced [35,36]. The function of biodegradation of PAHs and bio-adsorption of heavy metals has earned the marine sponge the nickname of biota with dual functions and significant benefits in reducing pollutants and maintaining the quality of the marine environment [37, 38].

The facts mentioned above prompted us to carry out mapping and a series of continuous research. It explores the function of sponges reducing PAHs and heavy metal pollutants in the marine environment through screening of symbiont bacteria. So that data, collections, and even formulas of marine sponge symbiont bacteria can be compiled with biodegradation and bio-adsorption, arranged in the form of a mobile crystalline bacterial

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consortium so that it is quickly mobilized to the pollution site [1,11,39]. Characterization of sponges and symbiotic bacteria, including analysis of the biodegradation potential of PAHs, is essential [40]. The application and increase in the biodegradation rate of symbiont bacteria to hydrocarbon pollutants, especially PAHs, is seen as a necessity [41,42]

Several species of sponge symbiont bacteria can also carry out the bio-adsorption function of several types of heavy metals, namely: Mercury (Hg), Chromium (Cr), Arsenic (As), Lead (Pb), Cadmium (Cd), Copper (Cu), Nickel (Ni), Zink (Zn) and Cobalt (Co) [43-46]. The development of the function and performance of biodegradation of PAHs and bio-adsorption of heavy metals by sponge symbiont bacteria in the form of a consortium of potential bacteria can be carried out efficiently and effectively manage polluted environments to reduce global trend waste pollutants [47]. Another effort is to carry out extreme environmental engineering contaminated with various PAHs and heavy metals [48]. The role and function of sponge symbiont biomass and bacteria in improving the quality and quality of the marine environment is enormous. It is especially in improving the biodegradation performance of PAHs through the role of the bacterial consortium formulation so that real work is realized to preserve the environment while keeping the marine sponge population growing and developing. The other is the cultivation of marine sponges using the trans-plantation method [49-51].

2. Results

An essential point of concern, observation and analysis in research comparing the degradation of the consortium of marine sponge symbiont bacteria to the anthracene and pyrene components consists of two main parts. These include: first, the preparation of the marine sponge symbiont bacteria consortium, which begins with the characteristics of the sampling point, Morphology analysis, bacterial isolation, phenotypic and genotypic analysis of bacterial isolates, selection of potential biodegradation bacteria isolated from marine sponges. Second, biodegradation parameters, an abundance of a substrate, percentage of degraded components, types of total components and functional groups, which are biodegradation products of the bacterial consortium.

2.1. Characteristics of seawater at the sampling location

Characterization of seawater at the point of acquisition of sponge samples is intended to investigate whether there is a relationship between the habitat of the sponge, the presence of hazardous and toxic contaminants and the potential symbiosis of microorganisms (bacteria) in the biodegradation of hydrocarbon components. The sampling location for sea sponges is in the waters around Kodingareng Keke Island, the administrative area of Makassar Metro City, South Sulawesi. The island is one of the Maritime Tourism Areas developed by the Makassar City government and is included in the Spermonde Archipelago Cluster.

Table 1. Characteristics of seawater sampling points of sponges around Kodingareng Keke Island

Sample code	Coordinate	Salinity (‰)	Temperature (°C)	pH	TDS (mg/L)	DHL (ds/m)	Depht dpl (m)	Distance from the beach (m)
Sp1	S 5°6′ 38, 12376′′ E 119° 17′70, 76544′′	28,3	29,4	7,47	7,41	14,46	± 3,20	± 200
Sp2	S 5°6′ 11, 62476′′ E 119° 17′60, 06228″	28,1	30,9	7,69	7,21	14,20	± 3,74	± 250
Sp3	S 5°6′ 23, 55372″ E 190° 20′27, 62376″	27,3	30,3	7,70	7,50	12,87	± 4,25	± 370

2.2. Analisis morfologi sampel spons laut

It is necessary to know the morphology of marine sponges as the source of bacterial isolates for the biodegradation of PAHs to trace the sponge body and cell structure. This bacterial symbiotic model has the potential for biodegradation of its main hydrocarbon components, PAHs. The morphological analysis of sponges also aims to make it easier and more efficient to determine the potential for degradation of sponge samples. It is considering that sponges are marine biotas with many functions but are vulnerable to threats of damage either due to predators, fishing activity disturbances, mainly due to the prolonged growth rate and development of sponges. It is necessary to understand whether there is a relationship between the body structure of marine sponges as a source of bacteria, living habitats, and slimy body surfaces with a bacterial symbiotic model that can biodegrade PAH-type hydrocarbon components. Species of marine sponges, based on the results of morphological analysis of sponge samples coded Sp1, Sp2 and Sp3, respectively, sponges of Niphates sp., Hyrtios erectus and Clathria (Thalysias) reinwardtii, respectively. The interesting thing about the samples of these three types of marine sponges is the consistency of their slimy surface. It is suspected that there is a relationship between the type and model of bacterial symbiosis that occurs and its relationship with PAHs biodegradation. [11,39].

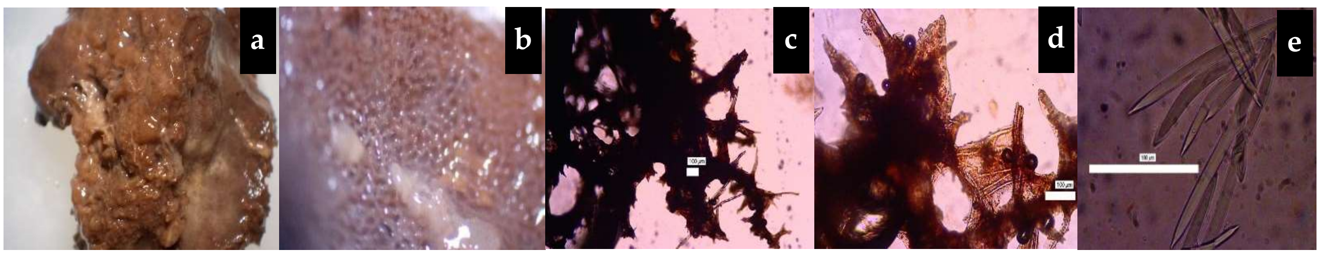

Figure 1. The morphology of the marine sponge Niphates sp. (Sp1). (a) Consistency: smooth, porous surface, covered by a muddy-like mucus, inelastic and brittle body; (b) Surface: granular spongy surface; (c) Skeleton: skeleton of spicules with echinating spicules; (d) Skeletal tract, high fibre spicular tract skeleton; and (e) Spicules: oxeal megasclera appear slight (40x).

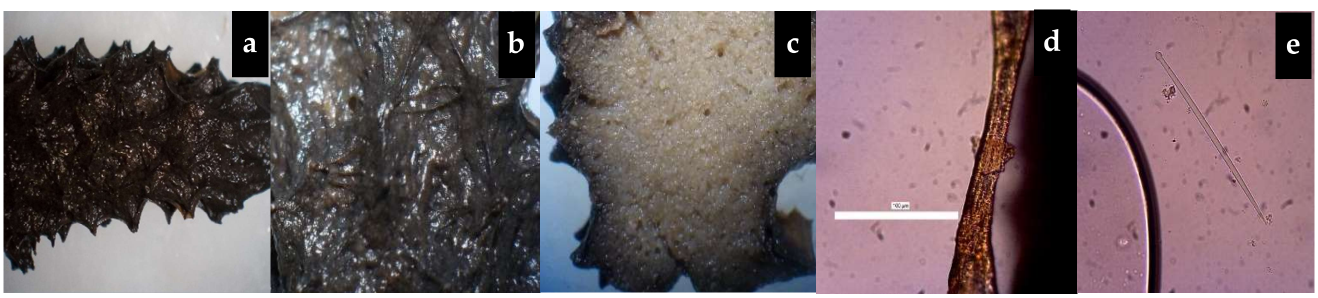

Figure 2. The morphology of the marine sponge Hyrtios erectus (Sp2). (a) Consistency: the surface of the body is smooth, dark in colour and covered by mucus substance, the ossicles are not identified, the characteristics of the sponge are fleshy, the body of the sponge is less elastic and looks brittle; (b) Surface: the surface looks like a pyramid-shaped ornament; (c) Choanosomes: body parts. Heavy fiber body sponge; (d) Skeleton: Fiber-character framework; and (e) Spicules, megacler sub stylostyle (10x magnification), no scleral microscopy.

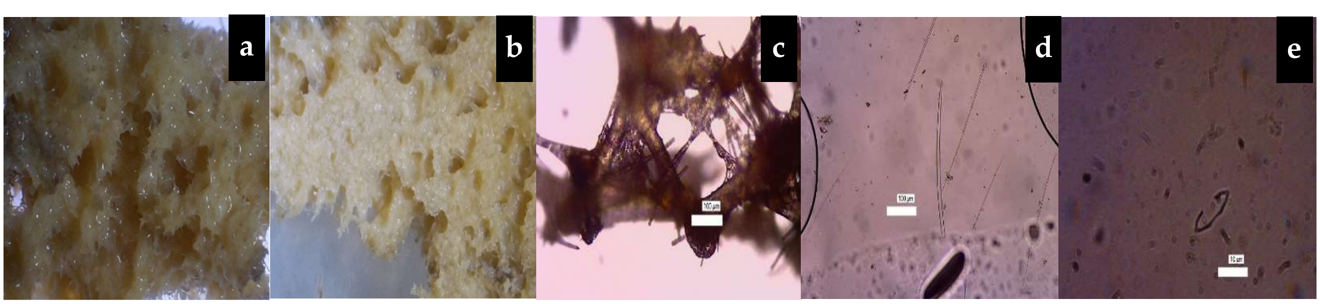

Figure 3. Morphology of the marine sponge Clathria (Thalysias) reinwardtii (Sp3). (a) Consistency: soft consistency, compressible spongy, slimy body surface but appear lighter; (b) Projection: sponge body brittle as it dries; (c) Choanosome: Anastoming reticulate choano-some skeleton; (d) Megasclere: megasclere appears elongated; and (e) Microsclera, Microsclera in the form of Chelae.

2.3. Isolation and phenotypic characteristics of marine sponge symbiont bacteria

Potential bacteria for biodegradation of PAHs can be obtained from marine sponges by isolation. Phenotypic characteristics characterized the isolates obtained to determine morphology, Gram group and the tendency of isolates' ability to several biochemical test reagents. This method is a preliminary test to predict marine sponge symbiont isolates' ability to biodegrade PAH components.

The results of the phenotypic characterization of 3 (three) bacterial isolates showed that these bacterial isolates had spores and were Gram-positive bacteria and generally reacted positively to 7 (seven) types of biochemical reagents that had been tested. Combining these three types of sponge symbiont bacterial isolates in the form of a bacterial consortium suspension was considered sufficient and fulfilled all the requirements needed to carry out the biodegradation process of PAH components [9,31]. Several conditions are needed so that bacteria can carry out the biodegradation reaction of hydrocarbon components as a substrate are to have enzymes capable of hydrolyzing amino acids as indicated by the indole test. In addition, it can also be able to carry out the fermentation reaction shown by the TSIA test, reducing nitrate to nitrile as shown by the nitrate test and breaking bonds. The urease test indicated carbon. A positive result of the citrate test showed that bacteria could reduce carbon, convert it into energy, and carry out the reaction of reducing carbohydrates to alcohol by the VP test and reducing carbohydrates to acid products by the Mr. test. The phenotypic analysis also gave clues that it could be concluded that the bacterial isolate coded Sp1, which was isolated from the sponge Niphates sp., belongs to the Bacillus group, Sp2 or sponge isolate *Hyrtios erectus*, is a bacterium from the Pseudomonas group. In contrast, the isolate coded Sp3 isolated from the sponge *Clathria* (*Thalysias*) *reinwardtii* is a bacterium belonging to the Acinetobacter group [22,24,29].

Table 2. Phenotypic characteristics of marine sponge symbiont bacterial isolates

Parameters	Marine sponge symbiont bacteria isolate		
	Sp1 (*Niphates* sp.)	**Sp2 (*Hyrtios erectus*)**	**Sp3 (*Clathria* (*Thalysias*) *reinwardtii*)**
Morphology	jagged stem shape, cream colour, clustered distribution, endospore is less clear	jagged stem shape, brown colour, separate distribution with endospores	round shape, bluish-cream colour, clustered distribution, endospores are less clear
Golongan Gram	reaction (-) with safranin reagent and (-) with 1% KOH alkaline solvent, Basil gram (+)	fixed colour with safranin reagent and (-) with 1% KOH alkaline solvent, Basil gram (+)	reaction (-) with safranin and (-) with 1% KOH alkaline solvent, Basil gram (+)
Indole test	-	-	+
Triple Sugar Iron Agar (TSIA)	+	+	-
Nitrate test	+	+	-
Simmone Citrate test	-	+	+
Methyl red (Mr) test	-	+	+
Voges – Proskauer (VP) test	+	-	+
Urease test	+	+	+
Provisional guess	Bacillus group	Pseudomonas group	Acinetobacter group

Note: - : not react; + : positive reaction

2.4. Genotypic analysis of marine sponge symbiont bacteria

t is necessary to know the bacterial genotype of marine sponge symbionts to ascertain the types and strains of bacteria used to degrade PAHs components. Based on the

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data on the grouping of marine sponge symbiont bacteria (Table 2), the three types of isolates were given a new code following the suspected bacterial class, Sp1-Bc, Sp2-Ps and Sp3-Ac, respectively.

Table 3. Genotypic characteristics of marine sponge symbiont isolate that are potential as biodegradators of PAHs Anthracene and Pyrene components

Bacterial isolate	Sequence samples	Range sequence gen-Bank	Identities quality (%)	Gaps (%)	Species type
Sp1-Bc	15-967 (952)	710.748 – 711.700 (952)	922/956 (96,44)	6/956 0,63)	*Bacillus pumilus* strain GLB197
Sp2-Ps	14-975 (961)	3.666.632–3.667.587 (955)	890/974 (91,14)	30/974 (3,08)	*Pseudomonas stutzeri* strain SLG510A3-8
Sp3-Ac	9-961 (952)	12-964 (952)	951/954 (99,69)	2/954 (0,21)	*Acinetobacter calcoaceticus* strain SLCDA976

2.5. Biodegradation of PAHs. Components

2.5.1 Parameters of PAHs biodegradation by marine sponge symbiont consortium bacteria

The physical interaction media of the bacterial consortium suspension against the PAHs contaminants changed during the biodegradation process. Several general parameters can be observed as indicators of the ongoing biodegradation process, including media turbidity, fermentation odor, pH changes, and gas bubbles' formation. These parameters are characteristic of fermentation that is played by bacteria or enzymes on a substrate containing protein, glucose, including materials containing hydrocarbon components. Biodegradation that takes place follows the pattern of enzymatic reactions. The enzyme components are thought to be produced by bacteria in response to their habitat conditions contaminated with hydrocarbon components. The production of mucus with enzyme characteristics is produced as an effort for bacteria to defend themselves to survive in PAH contaminated media. Therefore, observation of physical changes in the interaction media is oriented towards the performance of enzymatic reactions with the appearance of fermentation odor parameters and abundance of gas bubbles [29-31].

The relative turbidity of the media increased with increasing interaction time, indicating that there was an increase in the size and number of bacterial cells. The increase in temperature, the relative increase in acidity of the interactive media, the emergence of gas bubbles and the smell of fermentation are strong indications of a biodegradation process occurring in the media. These parameters are characteristic of the fermentation process, which is thought to be played by enzymes produced by marine sponge symbiont bacteria as a response and adaptation of bacteria to survive in media contaminated with anthracene and pyrene components. These results indicate that a mix of three types of bacteria called the consortium of marine sponge symbiont bacteria can degrade anthracene and pyrene components [37,42]

Table 4. Biodegradation parameters between the suspension of a consortium of marine sponge symbiont bacteria and waste contaminated with Anthracene and Pyrene mix

Biodegradation Parameters	Interaction Periode (days)					
	0	5	10	15	20	25
Turbidity of interaction media (NTU)	1.01	6,56	12,82	17,62	24,83	26,43
Temperature (^{0}C)	29	29	30	30	30	29
pH	6,67	6,68	6,13	6,10	6,06	6,43

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Abundance of gas bubbles	nt	nt	+	++	++	+
Fermented smell	nt	nt	√	√√	√√	√√

nt : not detected
\+ : gas bubbles appear less abundant
++ : looks like a lot of gas bubbles
√ : weak fermentation smell
√√ : strong smell of fermentation

2.5.2 Analysis of the biodegradation performance of the marine sponge symbiont consortium on the PAHs component of Anthracene and Pyrene mix

Anthracene and pyrene act as substrates that are degraded by the bacterial consortium. The number of peaks detected by running GC-MS should only be 2 (two), identified with the anthracene and pyrene components, but this did not occur as expected, where there were new peaks. There is a tendency that the longer the interaction period between the suspensions of the marine sponge symbiont bacterial consortium, the more new peaks appear, indicating that these new peaks are components of biodegradation [11,42,52]. Based on the research results, it is known that the new peaks identified are generally organic compounds of alcohol, aldehyde, carboxylic acids and some ketones and aliphatic hydrocarbon compounds. However, it cannot be estimated with certainty because GC-MS records show the suitability or quality of the components below 90% (**Table 5**).

The peak of anthracene and pyrene components decreased with increasing interaction time. This result indicates that the medium's abundance of anthracene and pyrene components was getting smaller due to degradation. Under the same conditions, the number of new components tends to increase, and the total concentration of components of biodegradation products also increases.

Table 5. GC-MS chromatogram data on biodegradation of a consortium of marine sponge bacterial symbionts against

Peak number	**Retention time**	**Comp. peak height (10^6)**	**Quality (%)**	**Total Conc. (%)**	**Group of comp.**	**Approximate comp. on the Library/ ID**
Interaction time 5 days						
1	10.287	0.182	95	1.057	Aromatic	Naphthalene
2	17.468	0.120	47	0.424	Aldehyde	---
3	18,566	10.407	95	45,693	Aromatic **	Anthracene
4	21.819	10.902	96	51.914	Aromatic **	Pyrene
5	24.947	0.223	90	0,912	Alcohol	Phenol
Interaction time 10 days						
1	6.083	0.132	91	0.959	Methyl	Cyclotetrasiloxane
2	17.471	0.794	53	3.752	Aldehyde	---
3	18.559	8.568	95	40.589	Aromatic**	Anthracene
4	21.309	0.239	78	1.401	Aldehyde	---
5	21.809	9.113	96	50.686	Aromatic**	Pyrene
6	24.944	0.185	90	1.070	Alcohol	Phenol
7	26.824	0.240	62	1.543	carboxylic acid	---
Interaction time 15 days						
1	6.088	0.109	91	0.558	Organosilicon	Cyclotetrasiloxane
2	17.471	0.471	53	1.663	Carboxylate	---
3	18.564	7.233	96	38.311	Aromatic**	Anthracene
4	21.311	0.139	72	0.561	Indole, aldehyde	---
5	21.818	8.920	96	49.619	Aromatic**	Pyrene
6	22.085	0.080	93	0.384	Amide	Hexadecanamide
7	23.637	0.187	91	0.760	Amide	9-octadecetamide
8	23.685	0.123	93	1.084	Amide	9-octadecenamide

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9	24.944	0.506	93	1.824	Alcohol arimatic	Phenol
10	26.823	0,700	81	3.236	Carboxylate	---
Interaction time 20 days						
1	16.728	0.131	98	1.858	Alcohol	Benzenemethanol
2	17.467	0.139	53	0.982	Carboxylate	---
3	18.553	4.844	95	36.105	Aromatic**	Anthracene
4	20.329	0.141	46	0.946	Aliphatic	---
5	20.776	0.199	43	1.323	Aliphatic	---
6	21.803	7.980	96	48.603	Aromatic**	Pyrene
7	24.944	0.163	93	1.168	Alcohol aromatic	Phenol
8	26.823	0.219	49	2.016	Carboxylate	Tetraphtelic acid
Interaction time 25 days						
1	17.470	0.242	64	1.112	Methylene	---
2	18.560	4.132	95	35.297	Aromatic**	Anthracene
3	20.332	0.148	47	0.697	sulfurous acid	---
4	20.778	0.332	47	1.508	Aliphatic	---
5	21.140	0.178	47	1.253	Alcohol aromatic	---
6	21.811	7.902	96	47.959	Aromatic**	Pyrene
7	24.944	0.126	78	0.681	Alcohol aromatic	---
8	26.823	0.234	91	1.504	Carboxylate	Tetraphtelic acid

Note: --- : The compound cannot be determined with certainty because the quality is below 90% of the instrument library
** : Components of PAHs (anthracene, pyrene) as a substrate that are degraded

2.5.3 Abundance and biodegradation products of the marine sponge symbiont consortium against the PAHs component of Anthracene and Pyrene mix

The abundance of anthracene and pyrene components seems to decrease with increasing interaction time. The decrease in the abundance value of the component is identical to the decrease in the component peak. In the same condition, new peaks appear, which indicate new components of the biodegraded organic compound group. The number of new peaks formed is relatively more with increasing interaction time. These new peaks have different retention times, peak heights vary, indicating different types and abundances of these components [1,4,53]. Each new component that appears has its characteristics, namely functional groups, but as a whole is a group of organic compounds. Anthracene and pyrene components are shown in red numbers, while degradation products components are shown in green numbers (**Fig.4A-E**). Analysis of the degraded components on the chromatogram data (**Fig. 4A-E**) showed differences in the number and types of components. Based on the results of the analysis, it can be stated that : (1) the number of components resulting from biodegradation tends to increase with increasing interaction time; (2) The types of components of biodegradation products are different; and (3) All biodegradation products are organic compounds with dominant characteristics in the form of hydroxyl and carbonyl functional groups.

The maximum biodegradation process was characterized by a decrease in peak height or identical to a decrease in the abundance of the maximum test PAHs that occurred at the contact time between 15 – 20 days (**Fig. 4C-D**). The biodegradation process was relatively stagnant at the interaction period of 25 days (**Fig. 4E**)

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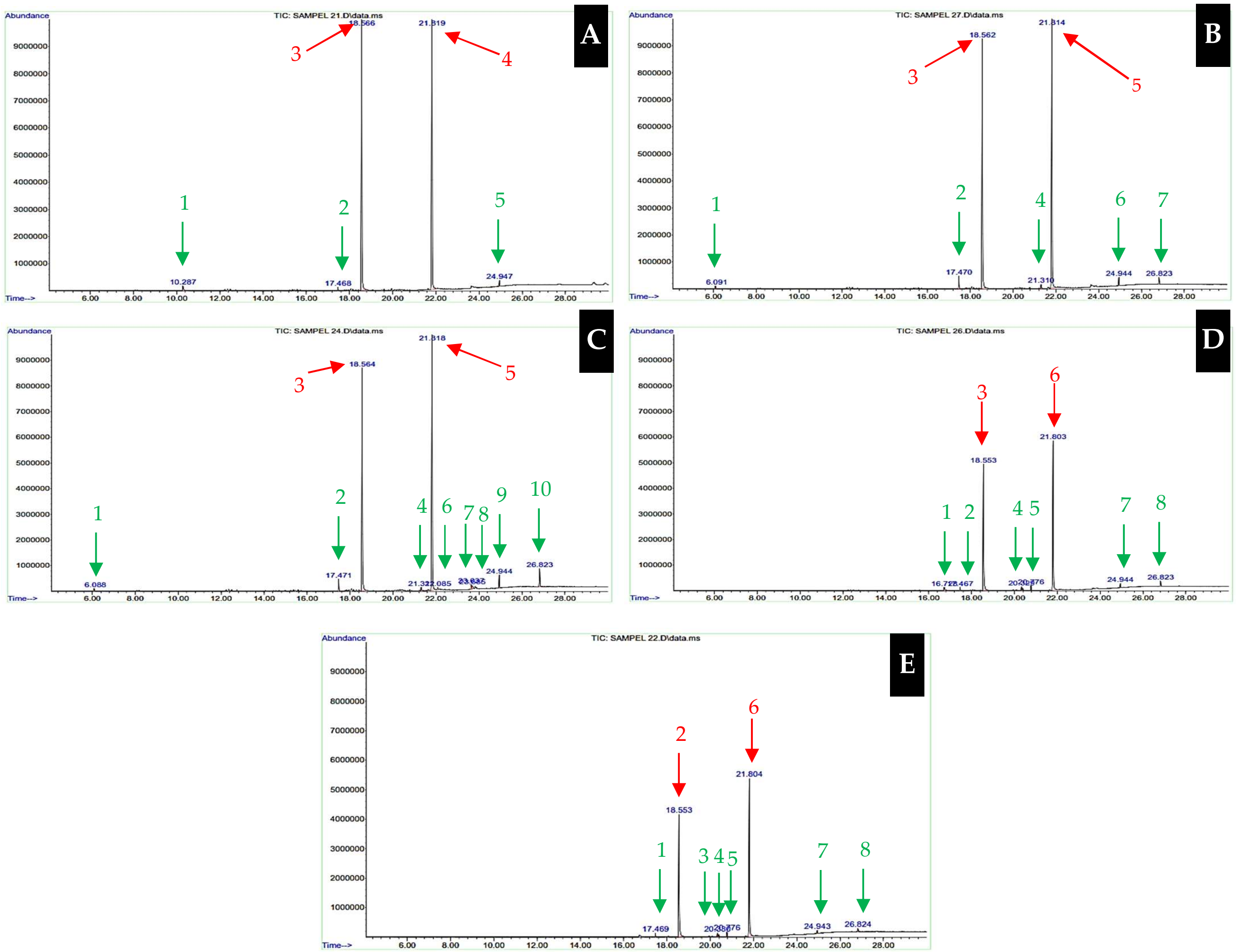

Figure 4. GC-MS chromatogram, showing the biodegradation performance of the marine sponge consortium bacterial suspension in the form of an abundance of Anthracene and Pyrene components and new peaks of biodegradation products based on interaction time. (a) the peak abundance of biodegradation at the 5-day interaction period; (b) 10 day interaction; (c) 15 day interaction; (d) 20 day interaction and (e) 25 day interaction.

2.5.4 Functional groups of biodegradation products

The FTIR chromatogram (Fig. 5) presents the components of biodegradation products in the form of organic compounds. Each of these is characterized by functional groups identified according to the range of wavenumbers that are identical to a particular functional group. Overall, it can be stated that the longer the interaction time, the more the number of components and the types of components identified with various variations based on the interaction time (Fig. 5A-E), although all components that appear to be dominated by organic compounds belonging to the alcohol group, aldehydes, ketones, carboxylic acids and some aromatic components [5,8,11].

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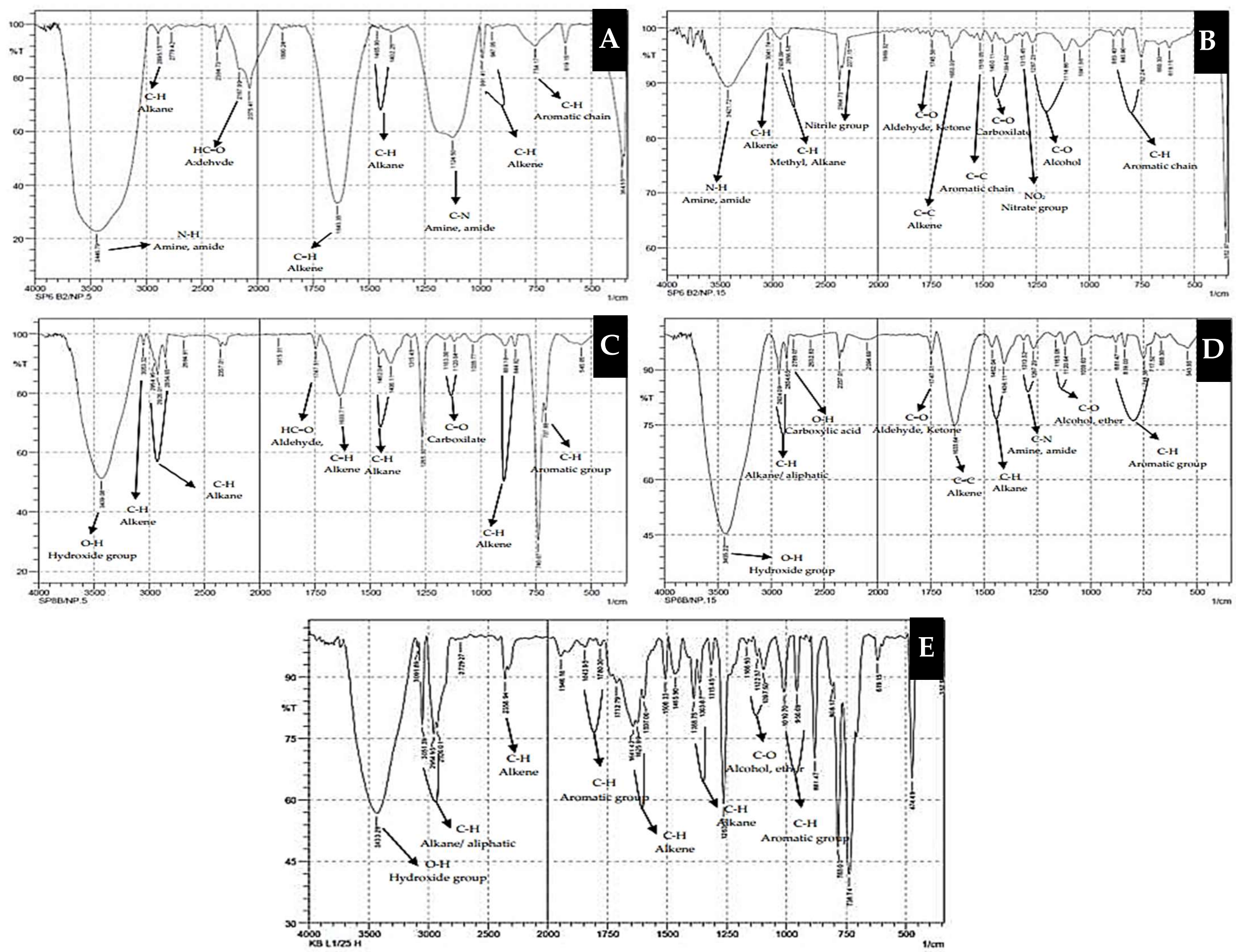

Figure 5. The FTIR chromatogram displays the functional groups of organic compounds of biodegradation products based on the interaction time. (a) 5 day interaction; (b) 10 day interaction; (c) 15 day interaction; (d) 20 day interaction and (e) 25 day interaction.

2.6. Comparison of the biodegradation of the marine sponge symbiont consortium bacteria against the components of An-trasena and Pyrena

The performance and level of biodegradation of the marine sponge symbiotic bacterial consortium against anthracene were different from that of pyrene. Comparison of the biodegradation of the bacterial consortium between anthracene and pyrene based on the abundance of components with an interaction time of 25 days (Fig.6A), the decrease in the abundance of anthracene components reached 60.30%, while pyrene was only 27.52%, or equivalent to a ratio of 1:2. The comparison of the bio-degradation of the bacterial consortium based on the decrease in concentration between anthracene and pyrene (Fig. 6B) reached a ratio of 1:3, i.e. the percentage decrease in the concentration of anthracene reached 21.89%, while the concentration of pyrene was only 7.71%. The ratio of the total concentration of PAHs (Ant. + Pyr.) that was degraded by the bacterial consortium was 17.23 % (Fig. 6C), while the total concentration of the biodegradation product components formed was 17.67 %. These results indicate that a consortium of marine sponge symbiotic bacteria more easily degrades the anthracene component than the pyrene component [2,11,54].

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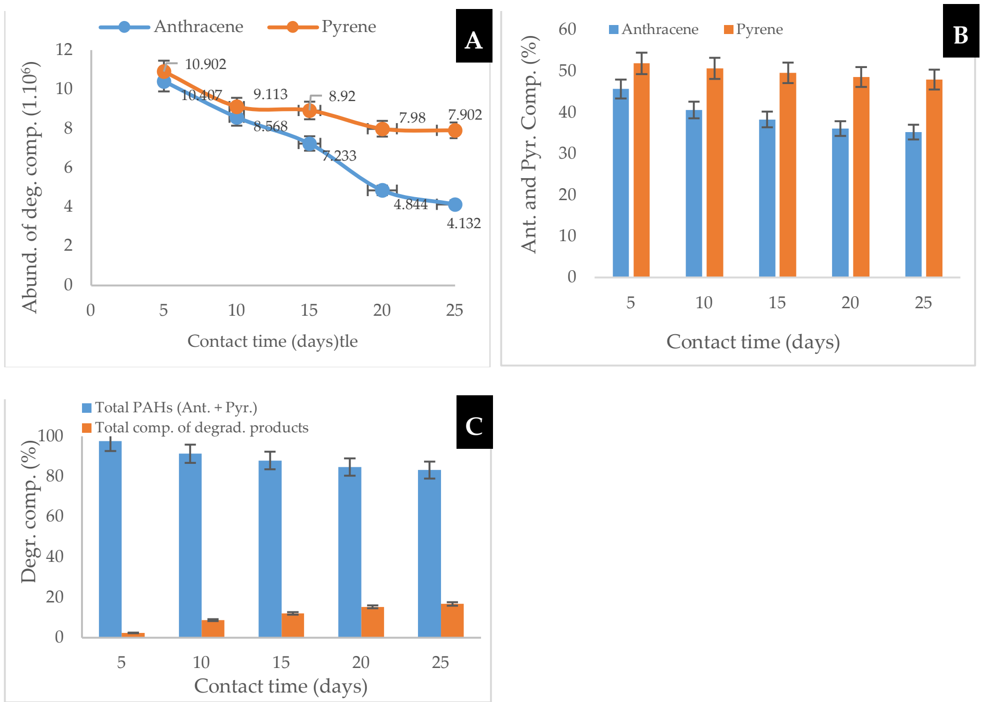

Figure 6. Biodegradation performance of a consortium of marine sponge symbiotic bacteria against Anthracene and Pyrene based on contact time. Fig. 6A. Decreased abundance of Anthracene and Pyrene components; Fig. 6B. Reduction of Anthracene and Pyrene components; and Fig. 6C. Increase in components of biodegradation products and decrease in total PAHs (Anthracene and Pyrene)

3. Discussion

The seawater conditions at the sponge sampling point showed good quality. This is based on data on salinity, pH, electrical conductivity, total dissolved solids (TDS) (Table 1), but it does not mean that the seawater is free from contamination with hydrocarbon components. The results of the morphological analysis of marine sponges showed that the three types of marine sponges that were the source of bacterial isolates had a characteristic slimy body surface (Fig. 1-3). The mucus on the surface of the sponge body is thought to be a substance produced by symbiotic bacteria for self-protection and adaptation to the dynamics that occur in the sponge's growth habitat [5,15,54]. The results of the phenotypic analysis (Table 2) indicated that the mix of the three isolates in the form of a bacterial consortium was estimated to meet the requirements for carrying out the PAH biodegradation process through the mechanism of destruction of the molecular structure of the aromatic ring. Genotypic analysis was carried out to ascertain the character of the three types of isolates used in carrying out the biodegradation function of PAHs so that data on the strains of bacterial symbiont isolates were obtained (Table 3).

The combination of three types of analyzes carried out on both marine sponges and isolated bacteria (Figs 1-3; Tables 2 and 3) indicates the presence of a common thread or strong relationship indicating that marine sponge symbiont bacteria that have the ability to carry out PAH biodegradation functions are bacteria which is obtained from a type of sponge whose body is slimy or the surface of the body of a sponge is dark in colour. One of the novelties of this research is that it provides an understanding and can be used as a method or guideline in tracing potential bacteria from marine sponges that can carry out the biodegradation function of hydrocarbon components, especially PAHs, making it easier for us to compile data and collections of PAH biodegradative bacteria [11,37,39].

The reference state that the biodegradation process of PAHs components (anthracene and pyrene) by a consortium of marine sponge symbiont bacteria has been running, it can be seen from the data and observes several indicators, including turbidity values, changes in temperature, pH, gas bubbles and the smell of the interaction media fermentation (Table 4) [56]. Another analysis that can be used as a reference is the degradation of anthracene and pyrene by a consortium of sponge symbiont bacteria by observing the decrease in the abundance of anthracene and pyrene substrates in the media after the interaction lasted several days (Table 5). The abundances referred to are peak height (Fig. 4A-E), change in the percentage of a substrate as a degraded component (Table 5), the formation of new peaks as a component of biodegradation products (Fig. 4) and the percentage of total components of biodegradation products (Table 5). The new peaks formed are simple organic compounds in the form of alcohols, aldehydes, ketones, carboxylic acids, aromatic components with their respective characteristics (Table 4; Fig. 5) [2,40,57].

The anthracene component is more easily degraded by a consortium of marine sponge bacterial symbionts than pyrene (Fig. 6). Theoretically, this can be accepted with several assumptions: (1) the toxicity level of pyrene is higher than anthracene, so that bacterial cells are more difficult to last longer in the biodegradation process compared to anthracene; (2) the structure of the pyrene molecule is more stable than that of anthracene so that bacteria are more difficult to destroy the pyrene molecule than the anthracene molecule, (3) the number of pyrene aromatic rings is more, and it forms a compact structure, the resonance can run longer than the number of rings and resonance anthracene so that bacterial cells need a relatively long time to degrade the pyrene molecule or aromatic ring compared to anthracene, (4) Acid compounds, degradation products that are formed as well as limiting substances for the biodegradation process. In this condition, bacterial cells can experience mass and sudden death, or bacterial cells have difficulty dividing themselves further [7,16,37,58]

4. Materials and Methods

4.1 Materials

The materials used include three types of marine sponges coded Sp1 (Niphates sp.), Sp2 Hyrtios erectus), Sp3 (Clathria (Thalysias) reinwardtii, (Fig. 1-3), which are collected by researchers [11,29,,42]. Marine sponge symbiont bacteria code Sp1-Bc, Sp2-Ps, Sp3-Ac, (Table 3), methanol pa, n-hexane for GC, anthracene cas number 000120-12-7and pyrene cas number 000129-00-0 (Supelco), Na2SO¬4 pa, aquabides, physiological 0.9% NaCl, (commercially obtained), set of materials for sponge morphology analysis, set of materials for standard biochemical tests following the guidelines in Microbiology: A Laboratory Manual [59] and set of materials for genotypic analysis of bacterial isolates. Bacterial isolates of marine sponge symbionts are the research stock, part of a previous publication [11, 29, 32, 37]

4.2 Sampling point characteristics

Parameters observed and measured were related to seawater conditions at the sponge sampling point, including coordinates, salinity, pH, TDS, HDL, the distance from the sampling point to the nearest shoreline and the sampling depth from sea level (Table 1). All data displayed has not been previously published. Determination of sampling points and sampling techniques are guided by fisheries and marine experts who are members of a team of Non-Governmental Organizations concerned with marine life.

4.3 Marine sponge morphology

Morphology, cell structure, and types of sea sponge samples in the study were analyzed at the Microbiology Laboratory of Sebelas Maret University, Surakarta, Central Java. Sponge types and morphological data are part of the researcher's stock from previous publications [11,29,32,37,42].

4.4 Isolation and phenotypic analysis of isolates of marine sponge symbiont bacteria

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Isolation of bacteria using Swab method, purification by a direct plating method, morphological analysis by direct observation, Gram staining, phenotypic analysis and determination of isolates of marine sponge symbiont bacteria following the Laboratory Manual procedure [59]. The data showed (Table 2) is the researcher's stock, part of previous publications [29,32,37].

4.5 Isolate genotypic analysis

Character and species of isolates of marine sponge symbiont bacteria were determined through genotypic analysis to see the sequence and sequence of nitrogen base pairs using the PCR method (Table 3). The data displayed is the researcher's stock and is part of the previous publication [11,29,32,37,42]

4.6 Biodegradation interactions and processes

4.6.1 Interaction of biodegradation components

Selected bacterial isolates (Sp1-Bc, Sp2-Ps, Sp3-Ac) from the research stock [11,29,37], were cultured respectively. Change the culture results in the form of a bacterial suspension. Each type of bacterial suspension, pipette 10 mL and put in an Erlenmeyer flask, diluted to 100 mL, homogenized and adapted for 1 x 24 hours in an incubator. Mix the bacterial suspension (consortium), 5 mL pipette and put it in a row of 15 different test tubes, then incubate for 1 x 2 hours in an incubator: prepared anthracene and pyrene 1000 ppm each, both mixed together. A total of 2 mL of mixed PAHs (Ant + Pyr) were put in each test tube which had previously been filled with bacterial suspension. Interaction between bacterial suspension and PAHs aerated on a shaker incubator at 200 rpm. The interaction time is 25 days, and every five days observations are made, biodegradation parameters are measured [4,11,58].

4.6.2 Parameters of biodegradation of PAHs by bacteria

Measurement of the parameters of the biodegradation of bacterial suspensions against PAHs was carried out every five days of interaction, using appropriate measuring instruments and also direct observations for the parameters of the abundance of gas bubbles formed and the odor of fermentation (Table 4) [11,12]

4.6.3 Biodegradation performance of PAHs by bacteria

Measurement of abundance and components of biodegradation products was carried out every five days of contact, carried out by extracting components of anthracene and pyrene that were not degraded, as well as components of biodegradation products using n-hexane. The n-hexane extract was water-free using Na_2SO_4 and ready to run using GC-MS (Table 5, Fig. 4A-E) [1,37]

4.6.4 Functional groups for biodegradation of PAHs by bacteria

Aqueous-free n-hexane extract was partially used for the determination of the functional groups of each biodegradation product using the FTIR instrument (Fig 5A-E)

4.7 Comparison of biodegradation rates of components between sene and pyrene

The variables reviewed in comparing the biodegradation level of the bacterial consortium against anthracene and pyrene, including the analysis of the decrease in peak height of the degraded components based on the interaction time according to the equation:

$$Abund.of\ degr.comp.(\%) = \frac{(peak\ height)no - (peak\ height)nt}{(peak\ height)no}\ x\ 100\ \%, \quad (1)$$

where: no and nt represent the initial and final peak heights of the biodegradation process [7,14] respectively. The decrease in the concentration of the substrate component (anthracene or pyrene) that underwent biodegradation was determined using the equation:

$$Conc.of\ degr.comp.\mathrm{X}\ (\%) = \frac{(initial\ conc.of\ comp.X) - (final\ conc.\ of\ comp.X)}{(Total\ conc.\ of\ comp.)}\ x\ 100\ \%, \quad (2)$$

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where: X is the component of anthracene or pyrene PAHs that undergoes degradation based on an interaction time of 25 days [31]. Determination of the concentration comparison of the components of the biodegradation product of the sponge symbiont bacteria consortium against all identified components, using the equation:

$$Cerc. of\ comp. biodegr.\ products\ (\%) = \frac{Total\ comp.\ of\ biodegr.\ products}{Total\ conc.\ of\ comp.}\ x\ 100\ \%, \quad (3)$$

Equation (1) was used to calculate the degradation rate of anthracene and pyrene by a consortium of bacteria based on the recorded peak height data (Fig. 6A; Fig. 4A-E and Table 5). Equation (2) was used to determine the concentration of the degraded anthracene and pyrene components (Fig. 6B) and equation (3) to determine the total concentration of the components of the biodegradation product (Fig. 6C). Each result obtained is compared between the two to get the value of the biodegradation ratio.

5. Conclusions

Based on the results obtained and the analysis that has been carried out, several things can be stated: The bacterial consortium succeeded in degrading 21.89% of the anthracene component and 7.71% of the pyrene component. The total concentration of biodegradation product components reached 17.67 %. Several types of organic compounds from biodegradation products found in this study include alcohol, aldehydes, carboxylic acids, ketones and a small number of aromatic components. The use of a bacterial consortium was low successful in increasing the level of biodegradation of the tested PAHs, compared to the use of single bacteria, presumably due to competition between bacterial cells in the media. Acid components of biodegradation products have the effect of decreasing the strength of bacterial cells in continuing the biodegradation process. The flow of the research carried out indicated that the search for sponge symbiont bacteria has the potential for biodegradation of PAHs by selecting sponges whose body surface is covered with mucus.

6. Patents

There are three patent works that are part of this manuscript, in the public testing process stage at the Ministry of Law and Human Rights of the Republic of Indonesia, each with the title: (1) Performance of microsymbionts from marine sponge culture as a biodegradator of polycyclic aromatic hydrocarbons (PAH), code registration: P15202008653; (2) Tracing method for bacterial isolates isolated from marine sponges as biodegradator material for polycyclic aromatic hydrocarbons (PAH), registration code: S15201907661 and (3) New metal-lo-clastic bacterial species cultured from marine sponges as biomaterials -adsorbent of several kinds of heavy metals, registration code: P15202008653.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, I.M., K.N., and M.K..; methodology; I.M., R.A., and K.N.; validation, Rh.A., R.A. and M.P.; formal analysis, I.M. and A.A.; investigation, I.M. and M.P.; resources, I.M. and M.K.; data curation, K.N.; writing—original draft preparation, I.M.; writing—review and editing, K.N. and Rh.A.; visualization, I.M.; supervision, R.A.; project administration, M.P. and A.A.. All authors have read and agreed to the published version of the manuscript."

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Conflicts of Interest: All authors declare that they have no conflicts of interest in the preparation and publishing of this paper.

Sample Availability: The sample is provided by the author. There are materials provided by the author, especially materials that are not available in the laboratory. Samples of the compounds ... are available from the authors.

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