Diversity of hydrocarbon-degrading bacteria in Pulau Pari and their potential roles for bioremediation

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Abstract. Oil spill accidents occur several times in the Indonesian sea, including Jakarta Bay. Studies on the application of oil spill bio-degradation techniques need to be developed but require baseline data on microbe species diversity and functions. We isolated several bacteria from Pulau Pari that can degrade hydrocarbons (hexadecane, phenanthrene, and dibenzothiophene) by using two step enrichment culture technique. The isolated microbes belong to several taxa, including α-subclass Proteobacteria, β-subclass Proteobacteria, γ-subclass Proteobacteria, the gram-positive high GC content (Actinobacteria), and Bacillus group. These marine bacteria degrade not only alkanes but also polyaromatic hydrocarbons (phenanthrene and dibenzothiophene). Alpha and gamma Proteobacteria were predominant alkanes and polyaromatic hydrocarbons-degrading bacteria. The ability of those bacteria to degrade both alkanes and polyaromatic hydrocarbon is a key-important trait for enhancing bioremediation of oil spills.

1. Introduction
Oil spill has occurred worldwide [1], affecting marine biota and reducing marine productivity [2]. Though the environmental hazard due to toxic waste produced from the oil spill, yet up to now, there is no efficient and effective technology for oil spill bioremediation [3]. Microbes play a significant role in oil chemical component bio-degradation. Information on species diversity and physiological properties of oil spill degrading microbes from tropical marine microbes is still limited. Particularly on hydrolyzing hexadecanes, polyaromatic hydrocarbon (phenanthrene and dibenzothiophene) is scarce [4]. Those substances are toxic to marine biota, commonly emitted during oil spill accidents. Earlier studies observed that several gram-negative bacteria grow on crude oil, and they assimilate oil components as sole carbon sources [5]. Those bacteria were generally isolated from oil-contaminated sites. The ecology of crude oil-degrading bacteria is not that clear [6]. Microbes assimilate hydrocarbon and use it as sole carbon sources are distributed widely on seawater, but not many reports on the isolation of hydrocarbon-degrading bacteria, especially alkanes and polyaromatic hydrocarbon from clean seawater [6]. Pulau Pari is located quite far from Jakarta Bay. The water quality of these areas is critical since Pulau Pari is one of the ecotourism areas; hence, it is vital to maintain good water quality [7]. Therefore, we conduct field observation with objective to isolate and identify bacteria assimilate hexadecane, phenanthrene, and dibenzothiophene which are some of the hydrocarbons that make up petroleum. A two-stage subsequent microbial enrichment technique was conducted to obtain
those alkanes and polyaromatic hydrocarbons (PAHs) degrading bacteria to verify species diversity of potential microbes for bioremediation of oil spill.

2. Materials and methods

2.1. Isolation PAHs degrading bacteria from the crude oil enrichment culture

Two-step culture enrichment techniques, was conducted to isolate alkanes and PAHs degrading microbes. Prior to PAHs enrichment, crude oil (2 % w/v) enrichment was performed on the seawater sample collected from the three sites. The crude oil enrichment media consist of a 190 ml seawater sample, 200 mg NH₄NO₃, 40 mg K₂HPO₄, 4 mg ferric citrate, 4 mg yeast extract, and 1 ml crude oil as the sole carbon source. Then sterile seawater was added up to 200 ml. The cultures were incubated for 14 days on a rotary shaker (150 rpm) at 30 °C. When there was solubilization of crude oil observed on the cultures, then those cultures (3% (v/v)) were sub-cultured into new crude oil enrichment media. The subcultures were incubated for seven days on a rotary shaker (150 rpm) at 30 °C. The subcultures obtained were serial diluted and transferred to solid media ONR7a containing PAHs.

Isolation of PAHs degrading bacteria was performed following the method described by Mikolasch et al., 2016 [8]. Into a 190 ml sterilized seawater NH₄NO₃ 200 mg; K₂HPO₄ 40 mg; ferric citrate 4 mg; yeast extract 4 mg; 0.05 g CaCl₂, 24 mg SrCl₂-6H₂O, 0.1 mg ZnSO₄-7H₂O, 3.5 g MgSO₄-7H₂O, 0.01 g FeCl₃, 3 g sodium pyruvate, 3 g sodium citrate, 0.5 g yeast extract, 1 g tryptone, 1 ml crude oil, 15 g bacteriological agar and the volume was adjusted to 1 L with distilled water. The pH medium was adjusted to 8.0 before autoclaving. The ONR7a was supplemented with each of the tested PAHs to achieve final concentrations of 50 mg/l for dibenzothiophene (DBT), and 200 mg/l for phenanthrene (PHE) and hexadecane (HED). PAHs stock solutions were prepared in dimethylformamide (dimethyl sulfoxide at a final concentration of 0.5% was used as a co-solvent to normalize concentrations) with appropriate concentration. Cultures were incubated at 30 °C in the dark for ten days. The colonies that morphologically appeared varied on ONR7a agar were purified and then transferred to fresh medium ONR7a with PAHs or hexadecane. Only isolates with good growth on PAHs were cultured and preserved in media with glycerol at −20 °C for further physiological characterization.

2.2. Growth assays and degradation capacity of hexadecane, phenanthrene, dibenzothiophene

Purified bacteria isolates were grown on the Marine Agar for 48 hours at 30 °C. Bacteria cell was scrapped with a loop and then resuspended in ONR7a liquid media to get 1.2 OD at 600 nm. Cells suspension (1% (v/v)) was transferred into 100 ml ONR7a supplemented with each phenanthrene (PHE), dibenzothiophene (DBT), as the sole carbon source. Cultures were incubated at 30 °C in a rotary shaker with shaking at 150 rpm in 250-ml Erlenmeyer flasks for ten days in the dark. The control was prepared on a flask without bacterial inoculation, and all treatments were performed in triplicate.

Five milliliters of culture from each treatment were collected each day. Bacterial growth was determined by measuring culture density at 600 nm. Subsequently, the cells were harvested by centrifugation and resuspended in 100 ml ONR7a. The cell suspension was sonicated and centrifugated to remove precipitates. Three volumes of ethyl acetate were then added to the culture supernatants. Determination of residual PAHs in the lipid phase were analyzed by HPLC, as described previously [9]. All treatments were performed in triplicate.

2.3. Identification of hexadecane, PAHs degrading bacteria

Total genomic DNA was extracted using a modified method of Franco-Correa et al. [10]. The isolates used as DNA templates were prepared on Marine Agar plate for seven days. The biomass of bacteria was then harvested by scraping the cell from the colony on this media. The cell was then put into a microtube and incubated overnight at -20 °C. After overnight, the cells were cleaned three times with distilled water (500 μL) and the sample dispersed in the lysis solution. Furthermore, the partial 16S
rRNA gene sequence was determined from the PCR-amplification fragment. The fragment 16S rRNA gene sequence was amplified using universal primer 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGTACCTGTGTTACGACTT 3'). The PCR condition was set with denaturation at 94 °C for 1 minute and followed by 30 cycles amplification (denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds, and elongation at 72 °C for 1 minute 30 seconds). Then the 16S rRNA gene fragment determination was conducted by Macrogen®. The nucleotide sequence data obtained were analyzed using Cromaspro program version 1.6. The complete fragments of 16S rRNA gene sequenced were compared with other sequences in the Eztaxon-e server [11].

3. Results

3.1. Diversity of PAHs hexadecane and PHAs degrading microbes
Using a two-step culture enrichment technique, 75 isolates of alkane and PAH degrading bacteria were obtained from seawater of Pulau Pari. The isolates mostly belonged to gram-negative reside within to alpha and gamma sub-class Proteobacteria. Less number of isolates resides under Betaproteobacteria, Actinobacteria, and Bacillus group (Table 1). Most isolates assimilate hexadecane as carbon source. Phenanthrene and dibenzothiophene hydrolyzing bacteria were less than hexadecane degrading bacteria, 42 isolates for phenanthrene and 35 isolates for dibenzothiophene. The number of species and species composition of each microbe quite varied.

| Table 1. Percentage of hydrocarbon-degrading bacteria. |
|-------------------------------------------------------|
|            | Hexadecane | Phenanthrene | Dibenzothiophene |
|            | Number of isolates | Percentage (%) | Number of isolates | Percentage (%) | Number of isolates | Percentage (%) |
| Alphaproteobacteria | 32 | 42.67 | 21 | 50.00 | 15 | 42.86 |
| Betaproteobacteria | 8 | 10.67 | 5 | 11.90 | 5 | 14.29 |
| Gammaproteobacteria | 26 | 34.67 | 10 | 23.81 | 11 | 31.43 |
| Actinobacteria (Gram positive High GC) | 6 | 8.00 | 4 | 9.52 | 3 | 8.57 |
| Bacillus sp. (Gram positive Low GC) | 3 | 4.00 | 2 | 4.76 | 1 | 2.86 |
| Total isolates | 75 | 42 | 35 |

3.2. Alpha subclass of Proteobacteria
Thirty-two isolates belonged to the alpha subclass Proteobacteria were hydrocarbon-degrading bacteria. Within these taxa, the species composition of hexadecane and PAHs degrading microbes quite diverse (Table 2). It comprises seven taxa (Citricella thioxidans, Novosphingobium spp., Porphyrobacter tepidarius, Sphingobium spp., Sphingomonas spp., Sphingopyxis sp., and Sinorhizobium sp.). All isolates assimilate hexadecane, about 66 % use phenanthrene as sole C-source and about 47 % degrade dibenzothiophene (Table 2).

3.3. Beta subclass of Proteobacteria
Eight isolates of the subclass Betaproteobacteria which are capable of degrading hydrocarbons were identified in the taxa Denitrobacter sp. and Nitrosovibrio tenuis. All isolates could use hexadecane as the sole source of C and five isolates each on phenanthrene and dibenzothiophene (Table 3).
Table 2. Hydrocarbon assimilation by isolates belonged to the Alpha sub class Proteobacteria.

| Species name            | Isolate code | Hexadecane | Phenanthrene | Dibenzothiophene |
|-------------------------|--------------|------------|--------------|------------------|
| *Citricella thiooxidans*| IMSB7        | +          | +            | +                |
|                         | IMSB8        | +          | -            | -                |
|                         | IMSB9        | +          | -            | -                |
|                         | IMSB10       | +          | -            | -                |
|                         | IMSB11       | +          | -            | -                |
|                         | IMSB12       | +          | +            | -                |
| *Novosphingobium spp.*  | IMSP2A       | +          | +            | +                |
|                         | IMSP2B       | +          | +            | +                |
|                         | IMSP2C       | +          | +            | -                |
|                         | IMSP3A       | +          | +            | +                |
|                         | IMSP4A       | +          | -            | -                |
| *Porphyrobacter tepidarius* | IMSP5A  | +          | +            | +                |
|                         | IMSP6A       | +          | -            | -                |
|                         | IMSP6B       | +          | +            | +                |
|                         | IMSP7A       | +          | +            | +                |
| *Sphingobium spp.*       | IMSP8A       | +          | +            | +                |
|                         | IMSP8B       | +          | +            | +                |
|                         | IMSP8C       | +          | -            | -                |
|                         | IMSP8D       | +          | +            | -                |
|                         | IMSP9A       | +          | -            | -                |
| *Sphingomonas spp.*      | IMSP10A      | +          | +            | +                |
|                         | IMSP11A      | +          | +            | -                |
|                         | IMSP12A      | +          | -            | -                |
|                         | IMSP13A      | +          | +            | +                |
|                         | IMSP14A      | +          | +            | -                |
| *Sphingopyxis sp.*       | IMSP21A      | +          | +            | +                |
|                         | IMSP22A      | +          | +            | -                |
|                         | IMSP23A      | +          | -            | -                |
| *Sinorhizobium sp.*      | IMSP31A      | +          | +            | +                |
|                         | IMSP32A      | +          | +            | +                |
|                         | IMSP33A      | +          | +            | +                |

+, almost more than 35 % of the substrate degraded, - no degradation. +, Biomass growth about 20 to 30 % of initial cell density measured at 600 nm.

Table 3. Hydrocarbon assimilation by isolated belonged to the Beta subclass Proteobacteria.

| Species name           | Isolate code | Hexadecane | Phenanthrene | Dibenzothiophene |
|------------------------|--------------|------------|--------------|------------------|
| *Denitrobacter sp.*    | IMSB27       | +          | -            | -                |
|                         | IMSB28       | +          | +            | +                |
|                         | IMSB29       | +          | +            | +                |
|                         | MSG30        | +          | -            | -                |
|                         | MSG31        | +          | +            | +                |
|                         | MSG32        | +          | +            | +                |
| *Nitrosovibrio tenuis* | IMSC26       | +          | -            | +                |
|                         | IMSC27       | +          | +            | -                |
3.4. Gamma subclass of Proteobacteria
Twenty-six isolates of alkanes and PAHs degrading bacteria belonged to the Gamma subclass of Proteobacteria isolated from seawater of Pulau Pari. These isolates were identified into ten taxa. All isolates showed the ability to degrade hexadecane, while for phenanthrene and dibenzothiophene there were only 10 (38%) and 11 isolates (42%), respectively (Table 4).

| Species name                | Isolate code | Hexadecane | Phenanthrene | Dibenzothiophene |
|-----------------------------|--------------|------------|--------------|------------------|
| *Alcanivorax dieselolei*    | IMSC19       | +          | +            | -                |
| *Escherichia coli*          | IMSC20       | +          | -            | +                |
| *Haemophilus* sp.           | IMSC11       | +          | -            | +                |
| *Haererehalobacter* sp.     | IMSG33       | +          | -            | -                |
| *Lysobacter concretionis*   | IMSC23       | +          | -            | -                |
| *Methylophaga thalassica*   | IMSC4        | +          | +            | -                |
| *Proteus mirabillis*        | IMSC5        | +          | -            | -                |
| *Pseudomonas* sp.           | IMSG7        | +          | -            | +                |
| *Stenotrophomonas maltophilia* | IMSB13   | +          | -            | +                |
| *Thialkalivibrio thiocyanodenitrificans* | IMSAC2   | +          | -            | -                |

3.5. Actinobacteria group
Six isolated bacteria belonged to the Actinobacteria group identified as *Brevibacterium casei*, *Cellulosimicrobium* sp., *Micrococcus luteus*, and *Promicromonaspora* sp. All of these isolates can utilize hexadecane as the sole carbon source. Meanwhile, four isolates belonging to all taxa obtained were able to degrade phenanthrene and three isolates could use dibenzothiophene as C-source (Table 5).

| Species name                | Isolate code | Hexadecane | Phenanthrene | Dibenzothiophene |
|-----------------------------|--------------|------------|--------------|------------------|
| *Brevibacterium casei*      | IMSAC3       | +          | -            | +                |
| *Cellulosimicrobium* sp.    | IMSAC5       | +          | +            | -                |
| *Micrococcus luteus*        | IMSAC4       | +          | +            | -                |
| *Promicromonaspora* sp.     | IMSAC2       | +          | +            | -                |

Table 4. Hydrocarbon assimilation by isolates belonged to Gamma subclass Proteobacteria.

Table 5. Hydrocarbon assimilation by isolates belonged to the Actinobacteria.
3.6. *Bacillus* group

Three isolates of hydrocarbon-degrading bacteria belonging to *Bacillus pumilus*, *Bacillus* sp., and *Oceanobacillus iheyensis*. All isolates can degrade hexadecane, two isolates degrade phenanthrene, and one degrade dibenzo thiophene.

| Table 6. Hydrocarbon assimilation by isolates belonged to Gram-positive low GC (Bacillus) |
|-----------------------------------------------|--------|--------|--------|--------|
| Species name                                    | Isolate code | Hexadecane | Phenanthrene | Dibenzothiophene |
| *Bacillus pumilus*                             | IMSB72     | +         | +         | -       |
| *Bacillus* sp.                                 | IMSB73     | +         | +         | -       |
| *Oceanobacillus iheyensis*                     | IMSB71     | +         | -         | +       |

4. Discussion

Our study is limited to the diversity of bacteria isolated from seawater on Pulau Pari, Indonesia that can grow on media contaminated with alkanes (hexadecane) and polyaromatic hydrocarbons (phenanthrene and dibenzo thiophene) as carbon source. Quite a diverse hydrocarbon-degrading bacteria succeeded to be isolated from seawater of Pulau Pari (Table 1), implying that these beneficial microbes are ubiquitous. The Alpha and Gamma subclass of Proteobacteria represent most of the community members, which may verify that the gram-negative bacteria significantly contribute to the bioremediation process of the oil spill in the marine environment. The isolation of the hydrocarbon-degrading microbes belonged to the Alpha subclass Proteobacteria re-emphasized the critical contribution of this bacterial group for bioremediation and hence contributed the maintaining the seawater quality. *Novosphingobium* and *Sphingopyxis* have been widely reported as a key actors in the biodegradation of oil spills. *Novosphingobium* has been shown in other study to contribute substantially to the abundance of many hydrocarbonoclastic genes [12]. *Sphingopyxis* is known to be associated with variations in hydrocarbon degradation and oxidation of reduced sulfur compounds which are present in crude oil [13]. This study also shows that many isolates belonged this group is able to use hydrocarbon (hexadecane, phenanthrene, and dibenzo thiophene) as the sole carbon sources (Table 2).

Several important members from the Gamma subclass of Proteobacteria were isolated and showing good ability to use all hydrocarbon tested in this study, except for *Haererehalobacter* sp., *Methylophaga thalassica*, and *Pseudomonas* sp. (Table 4), implies that gamma subclass Proteobacteria is another important actor in bioremediation of oil spill in the marine environment. Earlier studies observed that *Alcanivorax* groups are predominant when an oil spill has occurred. They can degrade oil spills under oxic and anoxic conditions [14], suggesting that the presence of this beneficial bacteria will speed up the bioremediation of oil spills. Though Actinobacteria are not predominate isolates (Table 5), isolation of this gram-positive high GC content microbial group enriched our information on bioremediation processes of an oil spill. Several gram-positive bacteria were also isolated (Table 6), indicating that microbial community structures of crude oil degrading bacteria in marine environments are diverse. Furthermore, *Bacillus* spp. were also found despite of their low abundance. The bacteria from this genus have been studied recently for oil spill bioremediation purposes due to their ability to produce biosurfactant [15] in broad range conditions including extreme temperature, level of acidity, and salinity [16]. Finally, enhancing and stimulating the activity of those bacteria on the assimilation and bioconversion of crude oil components is critical when oil spills occur, especially on marine areas that support ecotourism activities such as Pulau Pari.

5. Conclusion

The experiment on the isolation of hydrocarbon-degrading bacteria obtained diverse microorganisms belonging to alpha, beta, gamma subclass of Proteobacteria and the gram-positive Actinobacteria and *Bacillus* group. Stimulation on the growth of bacteria when an oil spill has occurred is beneficial for enhancing the bioremediation process. The enhanced biodegradation rate of crude oil components in
marine areas for ecotourism destinations is enhancing the economic value of hydrocarbon-degrading bacteria.

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