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Cover Page Footnote

We thank the Health, Safety, and Environment Department of PT. PERTAMINA RU V (Persero, for their kindness in giving us permission to collect the oil sludge as a sample for this research.

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Isolation of Asphaltene-Degrading Bacteria from Sludge Oil

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Abstract

Sludge oil contains 30%–50% hydrocarbon fractions that comprise saturated fractions, aromatics, resins, and asphaltene. Asphaltene fraction is the most persistent fraction. In this research, the indigenous bacteria that can degrade asphaltene fractions from a sludge oil sample from Balikpapan that was isolated using BHMS medium (*Bushnell-Hass Mineral Salt*) with 0.01% (w/v) yeast extract, 2% (w/v) asphaltene extract, and 2% (w/v) sludge oil. The ability of the four isolates to degrade asphaltene fractions was conducted by the biodegradation asphaltene fractions test using liquid cultures in a BHMS medium with 0.01% (w/v) yeast extract and 2% (w/v) asphaltene extract as a carbon source. The parameters measured during the process of biodegradation of asphaltene fractions include the quantification of Total Petroleum Hydrocarbon (g), log total number of bacteria (CFU/ml), and pH. There are four bacteria (isolates 1, 2, 3, and 4) that have been characterized to degrade asphaltic fraction and have been identified as *Bacillus* sp. *Lysinibacillus fusiformes*, *Acinetobacter* sp., and *Mycobacterium* sp., respectively. The results showed that the highest ability to degrade asphaltene fractions is that of *Bacillus* sp. (isolate 1) and *Lysinibacillus fusiformes* (Isolate 2), with biodegradation percentages of asphaltene fractions being 50% and 55%, respectively, and growth rate at the exponential phase is $7.17x10^7$ CFU/mL.days and $4.21x10^7$ CFU/mL.days, respectively.

Abstrak

Isolasi Bakteri Pendegradasi Fraksi Aspaltik dari Lumpur Minyak Bumi. Lumpur minyak bumi mengandung 30%-50% fraksi hidrokarbon yang terdiri dari fraksi jenuh, aromatik, resin, dan aspaltik. Fraksi aspaltik merupakan fraksi yang paling sulit didegradasi. Pada penelitian ini, bakteri pendegradasi fraksi aspaltik merupakan bakteri indigenos yang diisolasi dari sampel lumpur minyak bumi di Balikpapan dengan menggunakan media *Bushnell-Hass Mineral Salt* (BHMS) dengan 0.01% (b/v) ekstrak ragi, 2% (b/v) ekstrak fraksi aspaltik, dan 2% (b/v) lumpur minyak bumi. Kemampuan isolat mendegradasi fraksi aspaltik diuji menggunakan media BHMS yang ditambahkan 0.01% (b/v) ekstrak ragi dan 2% (b/v) ekstrak fraksi aspaltik sebagai sumber karbon. Selama uji biodegradasi dilakukan pengukuran parameter yaitu Total Petroleum Hydrocarbon (g), jumlah total bakteri (CFU/mL), dan pH. Empat isoat bakteri (isolat 1,2,3, dan 4) yang telah dikarakterisasi mampu mendegradasi fraksi aspaltik dan teridentifikasi secara berurutan sebagai, *Acinetobacter* sp., and *Mycobacterium* sp. Berdasarkan hasil penelitian, *Bacillus* sp. (isolat 1) dan *Lysinibacillus fusiformes* (Isolat 2) memiliki kemampuan terbaik dalam mendegradasi fraksi aspaltik, kemampuan biodegradasi fraksi aspaltik secara berurutan adalah 50% dan 55%, dan laju pertumbuhan pada fase eksponensial secara berurutan adalah $7.17x10^7$ CFU/mL.hari dan $4.21x10^7$ CFU/mL.hari.

Keywords: asphaltic fraction, Bacillus, biodegradation, hydrocarbon, sludge oil

Introduction

In the petroleum industry, particularly exploration and refinery, organic and inorganic waste is produced, which might be harmful for the environment and exists as the pollutant [1]. Waste from activities related to the petroleum industry is also known as sludge oil. Sludge oil comprises oil, water, and mineral solids [2]. The oil components of the sludge oil comprise various types of hydrocarbons, including chains of saturated hydrocarbon, aromatics, resins, and asphaltene [3]. Asphaltene fractions of petroleum hydrocarbons is a mixture of compounds that have a complex chemical structure, which comprises polyaromatics, cycloalkanes,

and other elements such as N, S, and O. This complex structure of asphaltene made it difficult to degrade [4-5]. Basically, all petroleum hydrocarbons can be degraded by using micro-organisms, which is called the biodegradation process. Micro-organisms use hydrocarbons as their carbon source and produce nontoxic waste [6-7]. The process of biodegradation of petroleum hydrocarbon would be optimal by using indigenous micro-organisms. Indigenous micro-organisms are micro-organisms derived originally from sites that have already been contaminated by petroleum. However, the biodegradation of petroleum hydrocarbons by indigenous microorganisms on the contaminated site was less visible because of the lesser numbers of microorganisms relative to the high content of hydrocarbon fractions [4,6,7].

Asphaltene of sludge oil is one of the hydrocarbon fractions that are difficult to be degraded by microorganisms, and research on the existence of a microorganism that can specifically degrade asphaltene fraction has not been conducted thus far. This research was conducted to (a) isolate the microorganism, in this case bacteria, that can use asphaltene as a source of carbon from sludge oil, (b) determine the bacteria which are fastest in degrading asphaltene fraction based on its growth rate and biodegradation ability, and (c) identify the asphaltene degrading bacteria based on phenotypic and genotypic characters. Hopefully, from this research, the best degradation bacteria for asphaltene fraction will be isolated and subsequently be able to optimize the process of bioremediation on petroleum hydrocarbon pollutant with high asphaltene fraction.

Materials and Methods

Samples. Sludge oil sample was collected from sludge pond of PT. PERTAMINA RU-V, Balikpapan. A sludge pond is a place to collect all waste from refinery activity such as leakage of pipeline, residue of oil storage tanks, etc. The samples was taken using 20 L polytanks and stored at 4 °C for further use.

Extraction of hydrocarbon from sludge oil. Extraction of hydrocarbon was conducted to separate the hydrocarbons in oil sludge from other unwanted compounds such as heavy metals, soil, sand, etc. The extraction was done by dissolving oil sludge with diethyl ether and homogenized. The mixture of sludge oil and diethyl ether was then centrifuged at 120 rcf for 5 minutes. The supernatant that was formed was then separated and evaporated in the vial bottle, until all diethyl ether evaporated [7].

Fractionation hydrocarbon of oil sludge. The hydrocarbon extract that was obtained before was diluted with n-hexane. N-hexane was used as a solvent to separate the asphaltene fraction due to the character of asphaltene, which is that it is insoluble in n-alkane. The asphaltene fraction was obtained by filtering the hydrocarbon extract and n-hexane mixture with a vacuum pump. Thus, the solid state of asphaltene was obtained on the filter paper [8].

Isolation and selection of degrading-asphaltene fraction bacteria. The isolation of degrading asphaltene bacteria was done by using BHMS liquid medium (KH_2PO_4 1 g, K_2HPO_4 1 g, NH_4NO_3 1 g, $MgSO_4.7H_2O$ 0.2 g, $FeCl_3$ 0.05 g, $CaCl.2H_2O$ 0.02 g, 1 l distilled water [9]) into which 0.01% (w/v) yeast extract and 2% (w/v) asphaltene fraction was added. The addition of yeast extract is to use as a growth inducer to promote the initial growth of bacteria before the complex substrate was used. The isolation was done by adding 2% (w/v) of the oil sludge sample in a Erlenmeyer flask—which is already filled with BHMS medium + 0.01% (w/v) yeast extract + 2% (w/v) asphaltene fraction—and then by shaking it with a rotary shaker at 120 rpm on room temperature for 7 d or more. Positive result of biodegradation asphaltene fraction was characterized by degraded oil (formation of micelles) in media and the media become turbid.

The selection of asphaltene degrading bacteria was conducted by using BHMS agar with addition of 0. 01% (w/v) yeast extract and 2% (w/v) asphaltene fraction and incubated at 25 °C. Colonies of bacteria that grew and then separated based on color, shape, and edge surfaces of the colony formed. Each colony of bacteria was inoculated on nutrient agar slant. Furthermore, bacteria were subjected to purification on nutrient agar plate using four-way streak methods. The single colony that grows on nutrient agar plate is purified asphaltene degrading bacteria [10]. Subsequently, the single colony obtained from purification was subjected to verification on BHMS liquid medium + 0.01% (w/v) yeast extract + 2% (w/v) asphaltene fraction. The verification of single colony bacteria was obtained to confirm that bacteria were asphaltene-degrading bacteria.

Asphaltene biodegradation assay. Bacterial isolates grown on BHMS liquid medium + 0.01% (w/v) yeast extract + 2% (w/v) asphaltene fraction was used as inoculums. Asphaltene biodegradation assay was done by using BHMS liquid medium + 0.01% (w/v) yeast extract + 2% (w/v) asphaltene fraction with addition of 10% inoculums. Furthermore, the culture of bacteria was shaken at 120 rpm, 25 °C for 13 d. Sampling was done every 24 h. All the parameters used in this assay are total petroleum hydrocarbon, total number of bacteria, and pH. Total petroleum hydrocarbon was measured instead of residual asphaltene fraction, because there may have been incomplete biodegradation of asphaltene fraction; thus, quantification of the entire fraction was necessary. Total petroleum hydrocarbon

was measured using gravimetric method of APHA [11] that has been modified, by extracting the total petroleum hydrocarbon in a medium with diethyl ether. The oil phase was separated from the water phase with liquid-liquid extraction method and then settled overnight to let the solvent completely evaporate in a vial. The total petroleum hydrocarbon (TPH), percentage of degraded asphaltene fraction, and biodegradation rate (BR) was done in accordance with the following equation:

TPH (g)= weight of (vial+hydrocarbon extract)(g)-weight of empty vial (g)

$$Degraded\ asphaltene\ fraction\ (\%) = ((b-a)/a)\,x100\%$$

Note: b = Final TPH (g); a = Initial TPH (g); t = time (day)

$$ABR\,(g/days) = \frac{Asphaltene\,at\,(t+1) - Asphaltene\,at\,(t)}{(t+1)-(t)}$$

Note: t = time (day)

The identification result based on phylogenetic character was matched with the identification result based on phenotype character, which has been done before.

The quantification of the number of bacteria was done using the total plate count method using nutrient agar plate [9]. Then, the bacterial growth rate (SGR) was calculated to indicate the number of new bacterial cells generated within a specific time frame (Moat *et al.*, 2002). This is given in the following equation (Moat *et al.*, 2002):

$$SGR\,(CFU/mL\,days) = (X' - X)/((t+1)-(t))$$

X = Cell density at (t) (CFU/mL); X'= Cell density at (t+1) (CFU/mL); t = time (day)

Identification based on phenotypic and phylogenetic characters. Phenotypic characterization of bacterial isolates was used as a reference strain bacterial for subsequent phenotypic identification. The phenotypic characters used were colonies bacterial morphology, bacterial cell morphology, and biochemical activities. The characters of colony morphology that were observed included shape, margin, elevation, color, and surface. In addition, the characters of bacterial cell morphology that have been observed included Gram staining, cell shape, and the characteristic of endospore. The result from this characterization was further used to identify the genus or species of bacterial isolates in Bergey's Manual of Determinative Bacteriology [12].

The best asphaltene fraction degrading bacterial was further identified using phylogenic analysis. Phylogenic identification was done using DNA amplification and sequencing. DNA amplification of bacterial isolates was determined by direct sequencing of polymerase-Chain-Reaction (PCR) product using specific primer of 16S rRNA (*forward primer*: 5'-AGAGTTTGATCCTGGCTCAG-3'; *reverse primer*: 5'-GGTTACCTTGTTACGACTT-3'). PCR mixed consisted of 1.5µL forward primer, 1.5µL reverse primer, 4 µL DMSO, 2µL template, 25 µL Green Taq, and 16µL de-ion water. The following PCR profile was used: Pre-denaturation 95 °C (3 min), denaturation 95 °C (3 s), annealing 45 °C (30 min), elongation 72°C (2 min), final elongation 72 °C (7 min). The amplified DNA was then subjected to sequencing. The sequencing of the DNA was done by MACROGEN Company (South-Korea) using the same primer. The sequence DNA of bacterial isolates was subjected to similarities analysis with BLAST program from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/). The similarity result from the BLAST analysis was used as a source to construct a phylogeny tree by MEGA5 program [13].

Table 1. Colony and Bacterial Cell Characterization of Asphaltene Degrading Bacterial Isolates on Nutrient Agar Plate After 24 H

Isolates Code	Colony Characters					Bacterial Cell Characters		
	Shape	Margin	Elevation	Color	Surface	Gram stain	Shape	Endospore
Isolate 1	Round	Serrate	Raised	White	Rough, Shiny	Positive	Rod	Central spores, Ellipsoid
Isolate 2	Round	Entire	Convex	Off-white	Smooth, Shiny	Negative	Rod	Terminal spores, Circular
Isolate 3	Round	Entire	Convex	Yellowish Cream[a]	Smooth, Shiny	Negative	Cocobacillus	None
Isolate 4	Round	Entire	Convex	White transparent	Smooth, shiny	Negative	Coccus	None

[a]on 24 h bacterial isolate was cream, after 24 h become yellowish cream
[b]After 48 hour

Results and Discussion

Isolation and selection of degrading-asphaltene bacteria. Four asphaltene degrading bacteria were isolated and selected from oil sludge. The four were respectively referred to as isolate 1, isolate 2, isolate 3, and isolate 4. The four bacterial isolates that were suspected to use asphaltene fraction as source of carbon were separated based on morphology of colony on nutrient agar plate (Table 1).

Based on the verification test result, all four bacteria were verified to have the capability to grew in minimal salt medium with asphlatene as the only sole source of carbon and energy. The ability of bacterial degradation was revealed by the more degraded asphlatene fraction, visually, on a minimal salt medium compared to control. The degraded asphaltene fraction on medium by microbial acitivity was used as a positive indicator to prove not only the growth capabilities of bacteria, but also to use asphlatene fraction as the sole carbon and source of energy [4]. Consequently, all four bacteria were verified as asphaltene degrading bacteria.

Asphaltene biodegradation assay. The ability of four isolates bacteria to degrade asphaltene fraction were quantified in asphaltene biodegradation assay. The asphaltene biodegradation ability of the four bacterial isolates and control are presented in Figure 1. Control used in this research was BHMS medium + 0.01% (w/v) yeast extract + 2% (w/v) asphaltene fraction without addition of bacterial isolates.

Figure 1 reveals that all fourth bacterial isolates can grow on asphaltene as the sole carbon and energy source, but with different efficiencies. There was no significant loss of asphaltene fraction concentration on negative control, recording a ≈ 0.5% decrease after 13 days of incubation. The data also reveals that isolates 1 and 2 have better ability to degrade asphaltene fraction compared to isolates 3 and 4.

After 13 days of incubation, isolate 1 was able to degrade 50% asphaltene fraction with the highest biodegradation rate of 0.07 g/day on the fifth day of incubation. The consumption of substrate (asphaltene fraction) was used to enhance the growth of isolate 1. As is shown, that for the first three days the bacterial grew exponentially with an average growth rate of 7.17 × 10^7 CFU/mL.day.

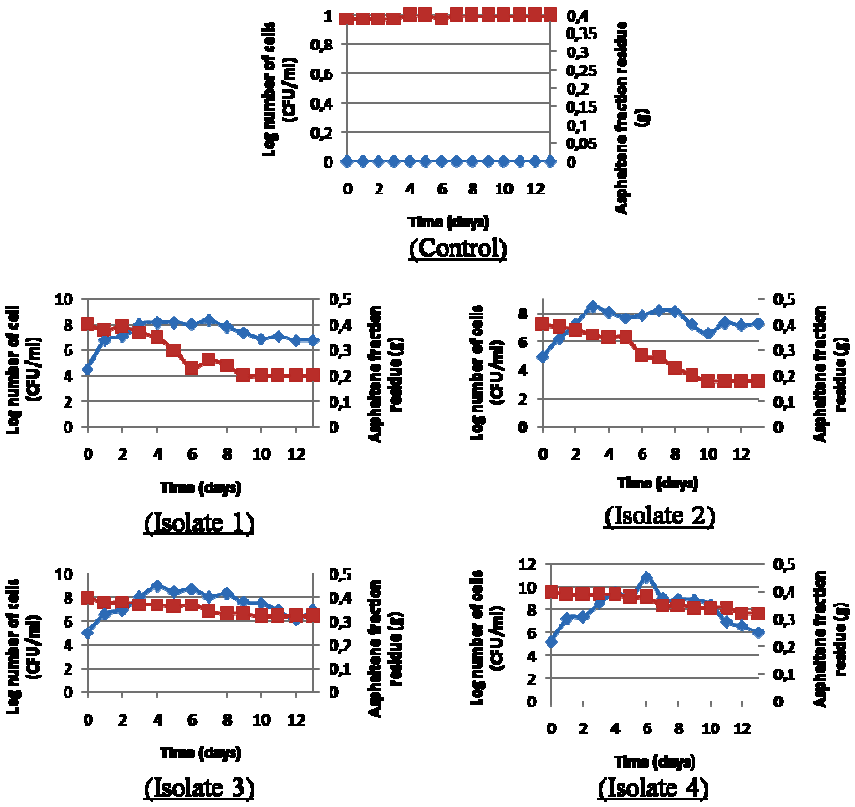

Figure 1. Asphaltene Biodegradation (■) and Bacterial Growth (♦) on BHMS Medium +0.01% (w/v) Yeast Extract + 2% (w/v) Asphaltene Fraction with Rotation at 120 rpm, 25 °C, for 13 Days

In addition, isolate 2 performed the greatest percentage of degraded asphaltene fraction that is 55% among all isolates. As in isolate 1, the bacterial growth rate of isolate 2 also reached the exponential phase for the first 3 days as the substrate (asphaltene fraction) was consumed rapidly with average growth rate of $4.21x10^7$CFU/ml.day. The highest biodegradation rate was achieved on the fifth day of the incubation period, which was 0.07 g/day.

Although slightly lower than the previous two isolates, isolates 3 and 4 have the same percentage of degraded asphaltene fraction, which was 20% with the highest biodegradation rate of 0.03 g/day on the sixth day of incubation. In contrast, the ability to grow on asphaltene solely as carbon and energy source of isolates 3 and 4 were different. Isolate 3 has exponential growth during the fourth day of incubation, while isolate 4 undergoes exponential growth until the sixth day of incubation. The average bacterial growth rate of isolates 3 and 4 is $4.17x10^6$ CFU/mL.day and $8.45x10^6$ CFU/ml.day, respectively.

In asphaltene biodegradation assay, changes in the medium pH were observed when witnessing the presence of acidic intermediate metabolite that was probably produced by the incomplete process of biodegradation. The pH changes in all treatments is shown in Figure 2. Based on the data, it is revealed that there are no significant pH changes in the control or treatment medium, which is in a neutral condition, except for isolate 4. During 13 days of incubation, the pH of isolate 4 decreased gradually and reach 5.9 on the 11th day of incubation and did not change until the end of the experiment. The changes in pH levels to acidic showed that during asphaltene biodegradation there were acidic metabolites that were produced and secreted into the medium. The presence of acidic metabolites (i.e., fatty acids) was used an evidence of incomplete hydrocarbon biodegradation. The acids that formed during biodegradation assay might be used for the bacteria to make the asphaltene fraction more soluble in water. This was evident from the color of media in isolate 4 turning brown (data not shown). Unfortunately, the composition of acid that was present in the medium did not take to further characterization.

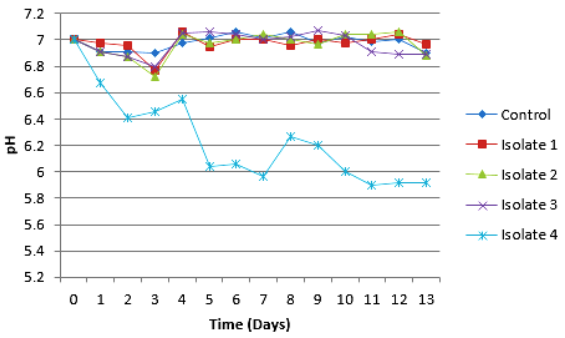

Figure 2. The pH Changes on BHMS Medium +0.01% (w/v) Yeast Extract + 2% (w/v) Asphaltene Fraction with Rotation at 120 rpm, 25°C, for 13 Days

Identification based on phenotypic and phylogenetic character. All four asphaltene degrading bacteria were subjected to identification based on phenotype characterization. Identification based on phenotype characterizations include morphology colony, morphology cells, and biochemical assay [9]. Based on colony and bacterial cell characters (Table 1), all four bacteria isolates have unique characteristics. Isolate 1 was observed as rod, Gram-positive, and spore-forming bacteria that is similar to isolate 2, but isolate 2 was identified as Gram-negative instead. Rod and spore-forming bacteria easily belong in the Bacillus genera, but to give complete determination, another characteristic was needed, such as spore shape and position within the cell (Table 1) and biochemical activity (Table 2). In contrast, isolates 3 and 4 were observed as Gram-negative and non-spore forming bacteria. Based on cell bacteria characteristic, the difference between isolates 3 and 4 was the shape of the cell—isolate 3 had a cocobacilli shape and isolate 4 had a coccus shape; this further lead them to be identified as having different genera.

Further, to provide a more comprehensive analysis to classify the bacteria isolate, a biochemical test was conducted (Table 2). Based on these phenotypic data, asphaltene-degrading bacterial isolates were identified using *Bergey's Manual of Determinative Bacteriology* [12]. Furthermore, bacteria isolates 1, 2, 3, and 4 were identified respectively as *Bacillus* sp., *Bacillus sphaericus*, *Acinetobacter sp.*, and *Methylococcus* sp.

Table 2. Biochemical Activities of Asphaltene-degrading Bacterial Isolates

Character	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Citrate utility	-	-	+	-
Lactose fermentation			-	-
Glucose fermentation	+[a]	+[a]	-	+[a]
Urase test	-	-	-	-
Gelatin hydrolysis	-	-	-	-
Starch hydrolysis	-	+	-	-
Casein hydrolysis	+	-	+	+
Lipid hydrolysis	+	-	-	+
Indole test	-	-	-	-
Methyl-red test	-	-	-	-
Voges-Proskauer test	-	-	-	-
Motility	+	-	-	+
Sulfide production	-	-	-	-
Nitrate Reduction test	+	-	+	-
Catalase activity	+	+	+	+

*no gas production

Classification of isolate 2 as *Bacillus sphaericus* was done as this species has a special characteristic that was unique among another species in the *Bacillus* genera, which is circular spores in the terminal position of the bacteria cell [12].

Further identification was done by genotypic characteristic based on DNA sequence data. Unfortunately, only isolates 1 and 2, which have better asphaltene-degradation ability were subjected to sequence. Molecular identification was based on 16S rDNA sequence analysis. The results of BLAST analysis reveal that bacterial isolate 1 had the highest similarity 92.1% with *Bacillus* sp. enrichment culture clone HSL48A. Further, bacterial isolate 2 had the most similarities 93.7% with *Lysinibacillus fusiformes* strain Y11. *Lysinibacillus fusiformes* was also known as *B. sphaericus* or *B. fusiformes* [23]. These data was also supported by phylogenetic analysis that was constructed using neighbor-joining method for isolates 1 (Figure 1) and 2 (Figure 2). The identification result based on genotypic character was supported by the results of phenotypic characterization, which was done earlier.

Isolation and selection of degrading-asphaltene bacteria. The isolation seed that was used in this experiment is sludge oil stored in sludge ponds of an oil refinery plant for a long period of time. Therefore, all the isolate bacteria have the ability to degrade asphaltene fraction, which known to be available in relatively high concentration. Colwell [15] revealed that sludge oil from refinery activities of the petroleum industry has a high concentration of asphaltene fraction compared to oil exploration activity. In this experiment, verification assay was needed to prove that all bacteria isolates have the exact ability to grow and utilize asphaltene as the carbon source. It was difficult to specifically find asphaltene-degrading bacteria in large amounts, because of the complex chemical structure of asphaltene fraction. Otherwise, according to Leahy and Colwell [14], bacteria that have the ability to degrade persistent fraction hydrocarbon can be found on contaminated areas that have been exposed to petroleum hydrocarbons for a long period of time.

Biodegradation of aspalthene assay. Based on asphaltene biodegradation data, there was a common phenomenon when there was an increasing rate of asphaltene biodegradation along with an increasing rate of bacterial growth (exponential phase), and this happened until a constant rate of bacterial growth (stationary phase) was attained. Thus, the correlation of asphaltene biodegradation rate with bacterial growth rate reveals that asphaltene fraction was used as the carbon source [16].

Complex hydrocarbon compounds of asphaltene fraction will be used by bacteria as the carbon sole for

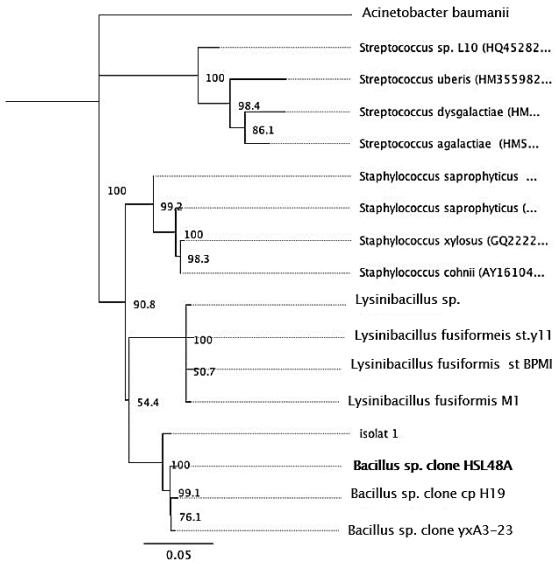

Figure 3. Phylogenetic Position of Isolate 1. The Tree was Constructed based on of Approximately1500 Bp 16S rRNA Gene Sequences using Neighbor-Joining Method. Moreover, 100 Bootstrap Replications were performed.

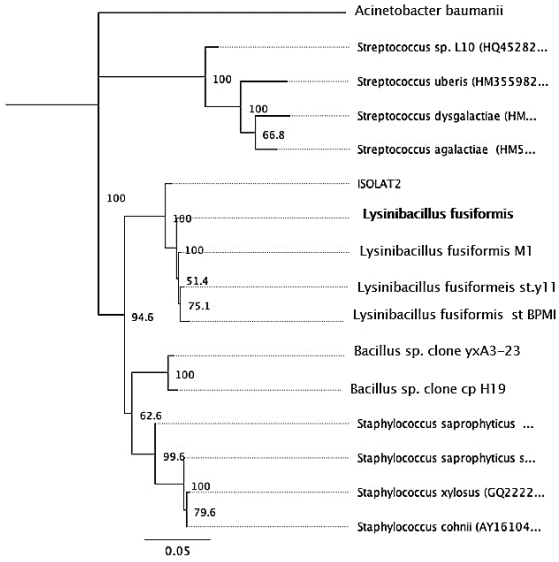

Figure 4. Phylogenetic Position of Isolate 2. The Tree was Constructed based on of Approximately1500 Bp 16S rRNA Gene Sequences Using Neighbor-Joining Method. Moreover, 100 Bootstrap Replications were Performed

subsequent entry to bacterial metabolic pathways for generating energy. This energy will be used by bacteria to promote growth or proliferation of cells and other cellular activities [17]. According to Rontani *et al.*, in Flores *et al*. [18], asphaltene degradation by bacteria was influenced by bacterial ability to degrade various types of hydrocarbon structures on asphaltene fraction. Asphaltene-degrading bacteria generally begins with

the most susceptible hydrocarbon structure, which is straight-aliphatic chains, then continues to the aromatics structure, and then ultimately forms a polycondensed structure that is known as the most difficult to degrade [18].

On the other hand, there are differences in biodegradation of the asphaltene capability of each bacterial isolate. The reason for this phenomenon is the decomposition process of complex hydrocarbon compounds such as asphaltene fraction requiring a complex enzymatic reaction [3]. For example, the complexity of enzyme produced by bacteria will become a limiting factor for biodegradation of asphaltene, and there will be limited availability of hydrocarbon in asphaltene fraction in a medium that can be used by bacteria that can cause the death of bacteria [20].

In this experiment, the efficiency of asphaltene biodegradation by bacteria was analyzed indirectly, and the changes of pH in the medium were studied [18]. The principle of measurement of the pH level of the medium is based on the complete process of hydrocarbon biodegradation that converts hydrocarbon into CO_2 and water by using enzymatic reactions [20]. Otherwise, if the bacterial enzyme was not sufficiently complete to conduct complete biodegradation, the bacteria will produce organic acid, a simpler form of the hydrocarbon compound, and a lesser amount of CO_2 and water [21]. However, isolate 4 that caused a medium pH level to have an acidic tendency was because it produced organic acid through metabolism. We assumed that these organic acids were further used to dissolve the asphaltene fraction to a greater extent in the water phase (as shown during the experiment); thus, another bacteria that includes the novel essential enzyme in asphaltene degradation could easily use the fraction. Thus, the bacteria that has the ability to degrade complex hydrocarbon fraction tend to exist as consortia to promote complete hydrocarbon degradation, as demonstrated by Flores *et al*. [18].

Identification Based on Phenotypic and Genotypic Characters. The bacterial identification revealed that all bacterial isolates were able to use hydrocarbon as the carbon and energy sources [3,18,20,22]. *Bacillus* sp.—widely known as bacteria that can use various types of hydrocarbon fraction as carbon and energy sources [3,18,20]—include asphaltene fraction [18,22]. Similar to *Bacillus* sp., *Acinetobacter* sp. and *Methylococcus* sp. are known as hydrocarbon degrading bacteria [20]. Various species and strains of *Acinetobacter* were found to be use asphaltene fraction as the carbon and energy sources [4,20]. However, asphaltene degradation of *Methylococcus* sp. is not known yet. According to Das and Chandran [20] *Methylococcus* sp. are known as bacteria that can use hydrocarbon with carbon $C_1 - C_8$, and it is possible that these bacteria degraded the straight aliphatic chain in an asphaltene structure.

Four asphaltene-degrading bacteria were obtained from the oil sludge sample, referred to as isolates 1, 2, 3, and 4, which were identified as *Bacillus* sp., *Lysinibacillus fusiformes*, *Acinetobacter* sp., and *Methylococcus* sp., respectively. Among these four asphaltene-degrading bacteria, *Bacillus* sp. and *Lysinibacillus fusiformis* have the best ability to degrade bacteria with the percentage of asphaltene biodegradation being 50% and 55%, respectively with growth rate $7.17x10^7$ CFU/mL.days and $4.21x10^7$ CFU/mL.days, respectively.

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We thank the Health, Safety, and Environment Department of PT. PERTAMINA RU V (Persero, for their kindness in giving us permission to collect the oil sludge as a sample for this research.

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