Biodegradation of Extractable Petroleum Hydrocarbons by Consortia *Bacillus cereus* and *Pseudomonas putida* in Petroleum Contaminated-Soil

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Received: March 8, 2018
Accepted: October 17, 2018

DOI: 10.22146/ijc.33765

Abstract: *Contamination of soil by the activities of exploration, production, and disposal of oil waste into the environment causes serious damage to the environmental ecosystem, the target of processing by the bacteria as a model for remediation of oil contaminated site. Thus, the study was focused on determining the biodegradation percentage of extractable petroleum hydrocarbons as a function of the oil concentration. This research was conducted in a slurry bioreactor with mixed contaminated soil to water ratio of 20:80 (wt.%). A consortium of* Bacillus cereus *and* Pseudomonas putida *bacteria 10% (v/v) and 15% (v/v) with the ratio of 2:3, 1:1, and 3:2 was inserted into the slurry bioreactor and a single reactor was used as a control. The result of identification with an initial concentration of extractable petroleum hydrocarbons of 299.53 ng/µL, after 49 days of incubation for bacterial consortium 10% (v/v), the concentration was reduced to 85.31; 32.43; 59.74; and 112.22 ng/µL respectively and the biodegradation percentage was 71.5; 89.17; 80.05; and 62.54%. As for the bacterial consortium concentration of 15% (v/v) with the same ratio and control, the effluent concentration was 12.48; 7.72; 18.93 ng/µL, respectively or the biodegradation percentage was 95.83; 97.42; 93.68%.*

Keywords: *biodegradation; extractable petroleum hydrocarbons; slurry bioreactor*

■ INTRODUCTION

Crude oil is a natural product, comprising of complex hydrocarbons mixture, created by the decomposition of plant remains from the Carboniferous period under high temperature and high pressure [1]. Extractable petroleum hydrocarbons (EPHs), polycyclic aromatic hydrocarbons or polyaromatic hydrocarbons (PAHs) compound, are generally used to identify various compounds of petroleum hydrocarbons in the range of C9-C36 [2]. EPHs or PAHs are the groups of compounds containing carbon and hydrogen, which consists of two or more integrated aromatic ring arranged in a linear, angular, and group configuration [2-3]. PAHs is a contaminant that is ubiquitous in the environment and has an enormous impact on the environment because of its potential toxicity, mutagenicity, and carcinogenicity [4-5]. The contamination of soil and groundwater by hazardous chemicals has become a major concern due to the associated risks to human health and the environment. Oil exploitation was first started in the 19th century to be used as a source of energy and later as a source of raw material. Crude oil is composed of hundreds of compounds [6].

There have been many remediation methods applied to polluted land, especially the petroleum-polluted land. Several oil companies in Indonesia, especially in the upstream sector, have used bioremediation method to treat the oil-polluted land, caused by blow out or problem during transportation, around their area of activity. Bioremediation method also widely used in cases of coast pollution caused by oil tanker carrier accident. The Indonesian government has emphasized on the importance of bioremediation method with the enactment of Minister of Environment decree [7], about Procedure and Technical Requirements of Petroleum Waste Processing and Biologically-Contaminated Soil by Petroleum. With the enactment of this law, several oil companies oil in Indonesia, e.g. UNOCAL in Borneo and CPI in Sumatera, were trying to use this method on their operational area [8].

Microbial bioremediation of hydrocarbon and water contaminated soil has emerged as a promising technology in recent years [9]. Several studies have shown that *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Acinetobacter spp*., *Flavobacterium spp*., *Yokenella spp*., *Alcaligenes spp*., *Roseomonas spp*., *Sphingobacterium spp*., *Capnocytophaga spp*., *Moraxella spp*., *Corynebacterium spp*., *Streptococcus spp*., *Providencia spp*., etc., as common hydrocarbon degraders [10-12]. Microorganisms have been employed for bioremediation of hydrocarbon-rich waste material products, along with their various recalcitrant noxious compounds, which are finally converted into environmentally friendly products. These microbes utilize waste material as carbon substrate, increase their population, and ultimately biodegrade hydrocarbon products to nontoxic products, such as H_2O and CO_2 [13].

Petroleum waste is a complex mixture containing alkanes, aromatics, nitrogen, sulfur, oxygen, and asphaltene fractions. Therefore it is difficult for single-species bacteria to biodegrade all components of oil, only degradation of certain types of petroleum compounds, but the population of the community microbial allows a higher degradation rate for some oil fractions. Moreover, some substances can be decomposed only by cometabolism [14-15]. Biodegradation of complex hydrocarbons, naphthalene and pyrene with the help of *Bacillus* spp., has been reported in many kinds of literature and the degradation was found to be ranging from 20 to 60% [16-18]. Unfortunately, no study has been reported regarding the possible role of a consortium of bacteria with different properties in degradation complex hydrocarbons with *Bacillus cereus* and *P. putida*. Therefore, the purpose of this research is to determine biodegradation level of EPHs in soil contaminated by polyaromatic hydrocarbons using a consortium of *B. cereus* and *P. putida* bacteria.

■ EXPERIMENTAL SECTION

Treatment Process

Polluted soil was obtained from oil drilling sites of Pertamina-Petrochina East Java (PPEJ), Tuban, Indonesia. Polluted soil was then separated from foliage, debris and other large objects and was moved from polluted location. Polluted soil was then mixed with water at 20:80 ratios to form slurry. Slurry with volume of 7 L was then inserted into slurry bioreactors. *Bacillus cereus* and *P. putida* with concentration of 10% and 15% (v/v) (bacteria ratio, 3:2; 1:1; 2:3) were then added to each bioreactor along with nutrition and aeration process. pH of operating condition was maintained on 4.5–8.8, temperature of 25–35 °C and stirring speed of 100 rpm was used throughout this process. EPHs analysis will be done every week, while temperature, pH and DO were monitored every day. The dissolved oxygen should be more than 2 mg O_2/L

Preparation of Medium and *Bacillus cereus* and *Pseudomonas putida* bacteria

Liquid medium was prepared by mixing 24 g of NBA with 1% glucose and 1% yeast extract into 1 L of distilled water. After sterilization by autoclave for 15 min at 121 °C, media was cooled until 28 °C. *B. cereus* and *P. putida* bacteria was taken from agar medium and dipped into liquid medium in laminar flow. The new medium was then incubated in an incubator shaker at 30 °C and 70 rpm. Bacteria were counted by Haemacytometer method.

Extraction and Analysis of Extractable Petroleum Hydrocarbons (EPHs)

EPHs degradation was confirmed by monitoring the disappearance of the 16 priority PAHs in sediment slurries. Sediment samples (10 g) were extracted in a Soxhlet apparatus for 16 h with 100 mL of *n*-hexane and 10 g of hydrated sodium sulfate to remove moisture (EPA Method 3540C), and the organic phase was concentrated to 1 mL by rotatory evaporation. Total and individual EPHs/PAHs in sediment samples were analyzed with a GCMS at Research Centre for Oceanography Indonesia Institut of Science, by GCMS Thermo Trace ISQ 1310 LT (Single Quadrupole Mass Spectrometer) method Supelco Standard QTM PAH Mix 47 930-U quantitatively. Systems are equipped with a Thermo TR-5 column (30 m length and 0.25 mm diameter). Helium was used as carrier gas with rate of 1.2 mL/min through column. The initial temperature

column was 50 °C and increased to 300 °C with rate of 62.5 C/min [19]. Biodegradation percentage of EPHs were calculated according to the following equation:

$$\text{Oil degradation } (\%) = \frac{[\text{EPH}]_0 - [\text{EPH}]_n}{[\text{EPH}]_0} \text{ x } 100$$

■ RESULTS AND DISCUSSION

Morphology of Petroleum Contaminated Soil

Scanning Electron Microscope (SEM) used in this study to investigate the morphology of petroleum-contaminated soil. SEM is performed at 1000x magnification with a scale of 100 µm as shown in Fig. 2, visible surface and morphology of petroleum-contaminated soil was very dense brownish-shiny and covered by oil layer with pH 9.1 at temperature of 28 °C and the total concentration of EPHs/PAHs total 299.53 ng/µL. Base on this characteristic, polluted soil cannot be released directly into the environment because it does not meet the quality standard requirement according to the Indonesia Minister of Environment Decision No. 128 (2003), as shown in Table 1 and 2. Fig. 2 shows that oil has been absorbed into the soil and made changes in morphology and structure as well as decreased bioavailability of the soil. This has an impact on the difficulty of microorganisms doing a contaminant breakdown at the pollution site.

Decreasing the Concentration of EPHs in the Period of Time

Polluted soil by oil is complex issues, related to the movement of components poisonous in an environment such as naphthalene, fluorene, anthracene, fluoranthene, pyrene, and. EPHs is the compound which has more than one a ring on their the molecular structure and found at polluted site petroleum of high concentration (Fig. 3 and 4). Hydrocarbon components in polluted soil oil were identified using GCMS by comparison time retention GC and a mass spectrometry fragmentation with reference substance. The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions. Fig. 1 shows the main principle of aerobic degradation of hydrocarbons [20-21]. The initial intracellular attack of organic pollutants is an oxidative process, and the activation, as well as the incorporation of oxygen, is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, acetyl-CoA, succinate, pyruvate. Sugars required for various biosyntheses and growth are synthesized by gluconeogenesis.

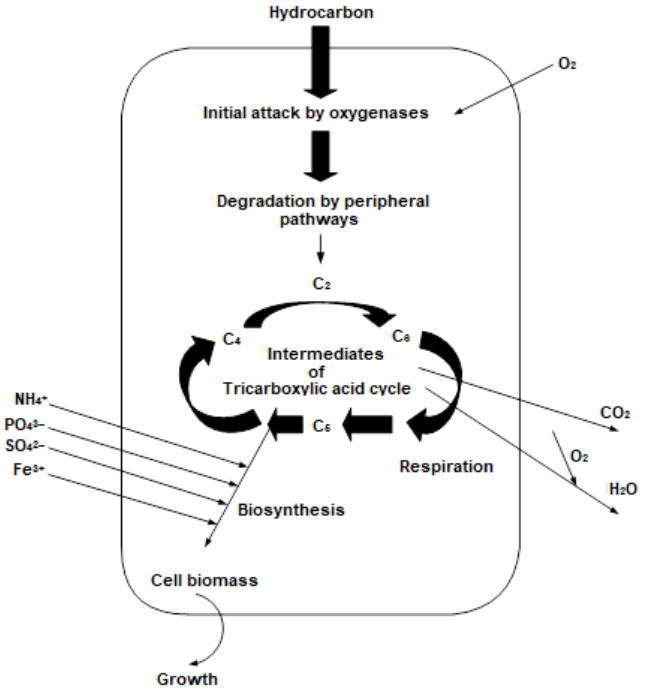

Fig 1. Main principle of aerobic degradation of hydrocarbons by microorganisms

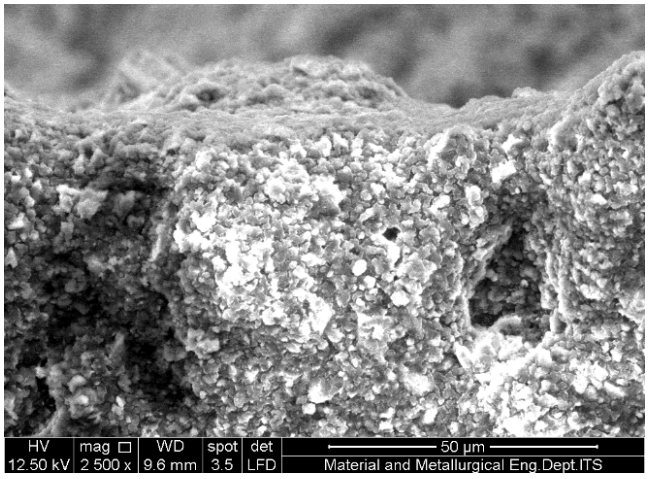

Fig 2. The form and morphology of petroleum contaminated-soil

EPHs in soil contaminated petroleum were degraded by a consortium of *Bacillus cereus* and *Pseudomonas putida* bacteria expressed in concentration. The combination of *B. cereus* and *P. putida* bacteria as hydrocarbon degrading bacteria works by fragmenting aliphatic, olefin, aromatic, and naphthalene hydrocarbon components. Hydrocarbons with aliphatic structures, such as alkanes, are more easily attacked by microbes than branched structures. Long chain hydrocarbons are more easily attacked by microbes than short chains, as well as unsaturated hydrocarbons more easily attacked by microbes. *B. cereus* bacteria used in this experiment as one of the bacterial consortiums provides good performance. Highly resistant to extreme concentrations of contaminants and limitations of oxygen and nutrients over time, in addition to being a facultative and gram-positive obligator making its response to degradation of contaminants enormous. These bacteria form spores (endospores) with thick spore walls and high levels of resistance to chemical and physical noxae. The heat resistance of these spores is the most important quality [22]. While the consortium of bacteria *P. putida*, including non-facultative obligator. Although this type of bacteria, growth is faster and resistant to hot and cold conditions [23], these bacteria are quite sensitive to extreme concentrations of contaminants and are susceptible to the limitations of oxygen and nutrients. Its nature as gram-negative makes at the time of biodegradation process, this bacteria tend to defend itself and slow in attacking EPHs/PAHs 3 and 4-rings, causing its biodegradation activity tend to be slow. From concentration of bacteria 10 and 15% at a ratio of bacteria 3:2; 1:1; 2:3 and control, best performance hydrocarbon degradation by a consortium of bacteria concentration 15% was at ratio of bacteria 1:1. The results of chromatogram shows that a consortium of these bacteria can give the reduction from 299.53 ng/µL until 7.72 ng/µL total EPHs after 49 days of the remediation period (Fig. 4). Meanwhile, at the same condition in concentration 10% (Fig. 3) at a ratio of bacteria 1:1, the degradation is from 299.53 ng/µL until 32.43 ng/µL EPHs in the polluted soil. This can be explained by the following arguments: at low temperatures, oil viscosity increased, poison evaporation, reduction of a short-chain alkane and the solubility of water decline, so that the biodegradation will slow down. This shows that the effects of the temperature on petroleum biodegradation occur not only on the physical composition and chemistry oil, but also on metabolic rate hydrocarbons by microorganisms and composition of microbial communities [8]. The condition is clearly

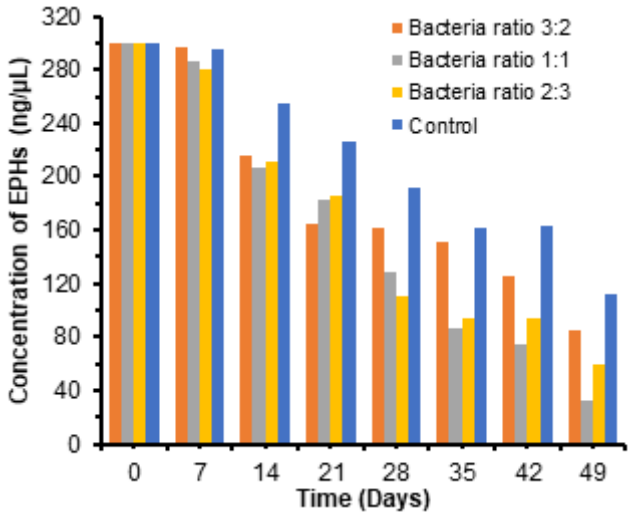

Fig 3. Quantitative degradation analysis of EPHs in slurry by concentration of microbial consortia *B. cereus* and *P. putida* 10% (v/v)

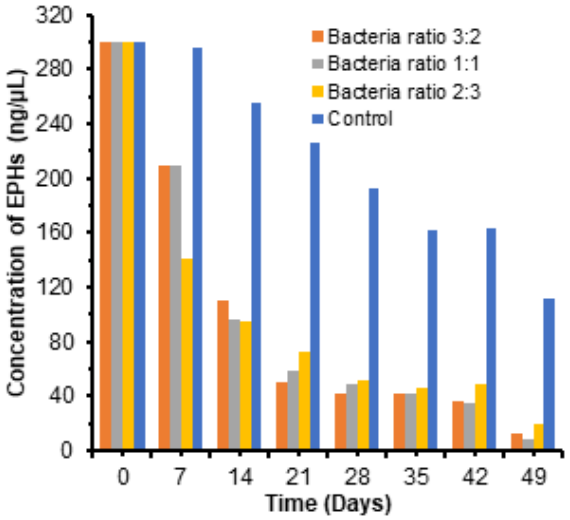

Fig 4. Quantitative degradation analysis of EPHs in slurry by concentration of microbial consortia *B. cereus* and *P. putida* 15% (v/v)

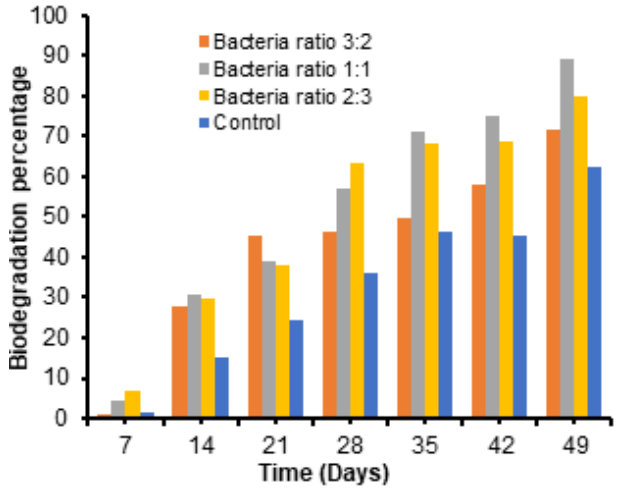

Fig 5. EPHs biodegradation in soil contaminated by bacteria concentration 10% (v/v)

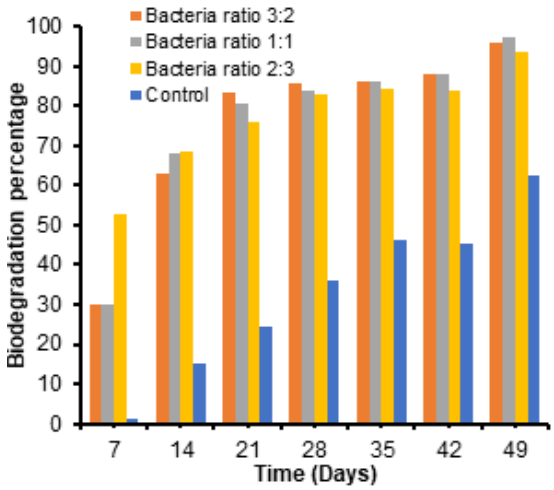

Fig 6. EPHs biodegradation in soil contaminated by bacteria concentration 15% (v/v)

Table 1. Degradation product of EPHs/PAHs with using consortia of *B. cereus* and *P. putida* bacteria 10% (v/v)

*EPHs/PAHs	Concentrations				
	Initial	Final			
		**RB 3:2	RB 1:1	RB 2:3	Control
Naphthalene	115.65	33.76	0.77	10.28	23.88
Acenaphthylene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
2-Bromonaphtalene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Acenaphthene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Fluorene	30.27	7.93	4.18	6.88	15.23
Anthracene	101.18	22.26	8.40	19.87	38.84
Phenanthrene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Fluoranthene	9.69	5.96	5.356	6.11	8.42
Pyrene	18.26	7.32	6.15	7.92	12.81
Benzo_(a)_anthracene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Chrysene	24.48	8.07	7.56	8.68	13.03
Benzo_(b)_fluoranthene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Benzo_(a)_pyrene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Dibenzo (ah) anthracene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Indeno_(123-cd)_pyrene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Total	299.53	85.31	32.42	59.74	112.22

Note: < 0.01 = Limit of Detection (LOD) Instrument, Units: ng/µL

*EPHs degradation analysis with gas chromatography-mass spectrometry

**RB : Ratio of bacteria (*B. cereus* and *P. putida*)

very consistent with the fact that hydrocarbons degradation is greatly influenced by time, where the long remediation period, the concentration of EPHs/PAHs degradation is greater. Overall, point to the different level of degradation between each bioreactor. Fig. 4 also shows that 49 days of remediation period, the concentration EPHs tend to be constant, because it reaches the equilibrium stage. The results above shows that EPHs were vanished from the bioreactor was probably because of the mineralization of EPHs into CO_2 and H_2O (Fig. 1). In this study, the CO_2 value was not analyzed, as it focused more on bacterial performance in degrading EPHs during the remediation period.

Table 2. Degradation product of EPHs/PAHs with using consortia of *B. cereus* and *P. putida* bacteria 15% (v/v)

*EPHs/PAHs	Concentrations				
	Initial	Final			
		**RB 3:2	RB 1:1	RB 2:3	Control
Naphthalene	115.65	0.31	0.19	5.12	23.88
Acenaphthylene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
2-Bromonaphtalene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Acenaphthene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Fluorene	30.27	1.77	1.17	1.88	15.23
Anthracene	101.18	4.98	2.11	5.15	38.84
Phenanthrene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Fluoranthene	9.69	1.51	1.32	1.65	8.42
Pyrene	18.26	1.89	1.37	2.53	12.81
Benzo_(a)_anthracene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Chrysene	24.48	2.02	1.57	2.59	13.03
Benzo_(b)_fluoranthene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Benzo_(a)_pyrene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Dibenzo (ah) anthracene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Indeno_(123-cd)_pyrene	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Total	299.53	12.48	7.72	18.93	112.22

Note: < 0.01 = Limit of Detection (LOD) Instrument, Units: ng/µL

*EPHs degradation analysis with gas chromatography-mass spectrometry

**RB : Ratio of bacteria (*B. cereus* and *P. putida*)

Biodegradation Percentage of EPHs in the Period of Time

Biodegradation EPHs depends on the complexity of the chemical structure, type and position in group substitution and the level adaptation enzymatic. To soil contaminated petroleum, a consortium of bacteria demonstrating ability good to degradation EPHs 2, 3 and 4-ring and increase biodegradation percentage. On the other hand, percent biodegradation EPHs of soil contaminated oil after 49 days remediation period was ranged between 29.9 to 97.42% (Fig. 6) to concentration 15% in ratio of bacteria 1:1, compared with the ratio of bacteria 3:2; 2:3 and control, each percent biodegradation was 30.03 to 95.83%; 52.73 to 93.68% and 1.29 to 62.54%. Compared to control, bioreactor with the ratio of bacteria 1:1 resulted in biodegradation percentage 1.56 times greater. As for bacteria concentration 10% (Fig. 5) with ratio of bacteria 1:1, biodegradation percentage range from 4.4 to 89.2% after 49 days remediation period, while to the ratio of bacteria 3:2; 2:3 and control, biodegradation percentage was each 0.96 to 71.52%; 6.64 to 80.05% and 1.30 to 62.54%, respectively. To the ratio of bacteria 1:1, biodegradation percentage 1.42 times greater than compared to control. It is suspected that this because the group bacteria of polluted soil are indigenous bacteria that cannot utilization a hydrocarbon oil for growth, so the rate of degradation to be slow [24]. The condition is consistent with the fact that hydrocarbons degradation is strongly influenced by time, where the longer remediation period caused a greater reduction concentration of EPHs.

A concentration of 10% bacteria, although the pollutant reduction is quite good, the percentage of biodegradation relatively significant, possibly because the bioavailability EPHs is more complicated when interacting with nonaqueous phase liquid (NAPL) and soil colloids, resulting in less or not at all available for microorganisms. This can occur at a greater rate, so desorption becomes very slow, limits the flux of contaminants into the water phase, and the contaminant is biologically ready. The combination of the above phenomena causes different distribution and partition of

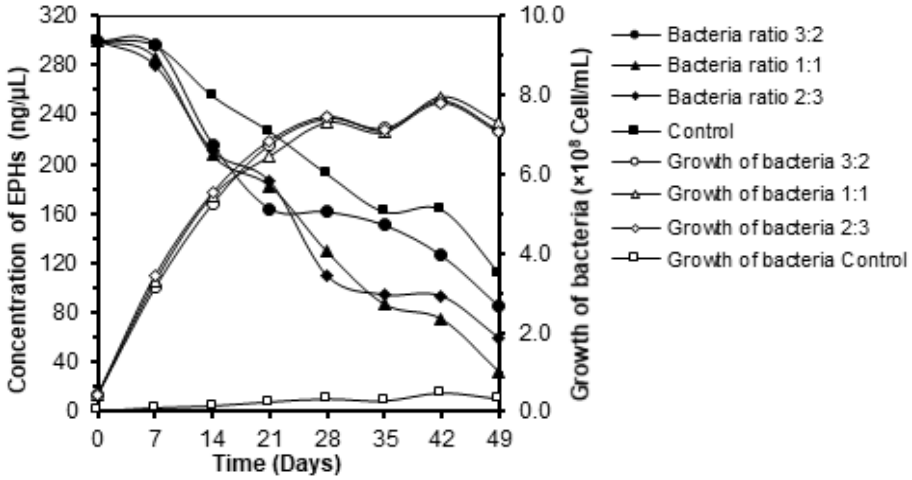

Fig 7. Growth profile of microbial consortia in the soil contaminated-oil with bacteria concentration 10% (v/v)

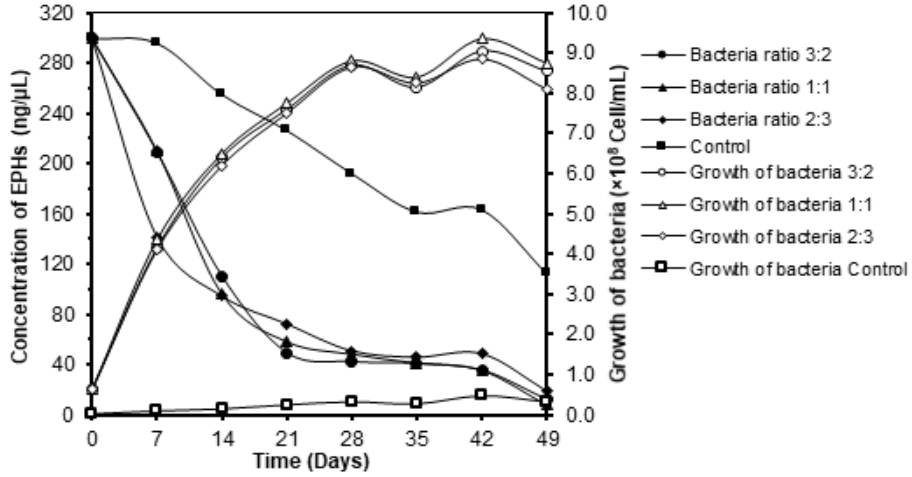

Fig 8. Growth profile of microbial consortia in the soil contaminated-oil with bacteria concentration 15% (v/v)

the polluter in the soil which make biologically less susceptible, resistant to biodegradation, and more persistent in the soil [25].

The Growth of Bacteria in the Biodegradation of EPHs in the Period of Time

Increases in population bacteria degrade EPHs in all ratio of bacteria aeration system correlate with the rate of rapidly decreasing of EPHs. This is different at control bioreactor, that shows a significant slowdown of biodegradation. That biodegradation can effectively, environmental conditions necessary to support growth and development microbes to be in accordance. And as long as the increase of bacterial population does not necessarily influence the bacterial ratio because the use of the bacterial ratio of *B. cereus* and *P. putida* is constant.

Biodegradation EPHs/PAHs by a consortium of bacteria 10% (Fig. 7) shows the growth of bacteria increasing every week, good bacteria to the ratio of 2:3; 1:1; 3:2 even controls on the 49 days remediation period. Visible constant degradation, as between contaminants and products tending to equilibrium, where the substrate has declined and relatively constant, and at the same time a population of bacteria increased toward the stationary phase growth. The decrease in EPHs content in the course of the cultivation of the microorganisms in liquid culture is presented in Fig. 7 and 8. It was found that the reduction of hydrocarbons in medium with acidic conditions after 7 days was barely, which to some extent probably reflected reduced volatility under low pH conditions. The bacteria are growing significant but

not accompanied by a reduction of contaminant. It could be because solubility compound 3 and 4-ring high enough, so relatively inhibiting the rate of degradation. Aliphatic hydrocarbons structure, as alkanes easier attacked by microbes from the branching-structure. Hydrocarbons long-chain is more vulnerable by microbes of a short-chain, as well as unsaturated hydrocarbons having a bond that is more vulnerable by microbes [26-27]. Meanwhile, degradation of EPHs is presented in Fig. 8 show the growth of bacteria are very significant and at the same time EPHs significantly degraded. This shows that, the substrate has been reduced and tend to constant. According to Atlas (1981), the utilization of hydrocarbon microbes is highly dependent on the chemical composition of components in a mixture of petroleum and environment the best. In addition, a consortium of bacteria can use some EPHs as the only source of carbon to grow in the polluted soil, and the number of nutrients may limit the degradation of EPHs in the natural environment [28]. When the addition of nutrients, microorganisms will be rapidly growing, and degradation of EPHs increase especially on the metabolism process and the growth of bacteria. In order biodegradation can occur effectively, required environmental conditions suitable to support growth and microbes development.

■ CONCLUSION

The potential of extractable petroleum hydrocarbons (EPHs) biodegradation using oil-contaminated soil and a consortium of exogenous bacteria were evaluated. The study was done using slurry bioreactor with a consortium of bacteria B. cereus, and P. putida successfully degrades most fraction of recalcitrant oil sludge. Based on the results obtained from this study, it can be concluded as follows; the concentrations of reduced EPHs and biodegradation percentage can reach 7.72 ng/μL and 97.42% in 49 days of remediation period in slurry bioreactor with concentration of consortia *B. cereus* and *P. putida* 15% (v/v) at bacteria ratio of 1:1; while 32.43 ng/μL and 89.17%; in the remediation period and the same bacterial ratio for bacterial concentration 10% (v/v). From this result can be used to develop a safe and economical large-scale treatment technology for petroleum contaminated-soils.

■ ACKNOWLEDGMENTS

This study was financially supported by the Directorate General of Higher Education-Ministry of Research Technology and Higher Education Republic of Indonesia (No. 1335.6/E4.4/2014).

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