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Selection and characterization of soil microorganisms in hydrocarbon biodegradation on crude oil contaminated media

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Abstract. *Fauzi M, Hariyadi HR, Setiawati MR, Wulandari AP, Suryatmana P. 2017. Selection and characterization of soil microorganisms in hydrocarbon biodegradation on crude oil contaminated media. Bioteknologi 14: 32-36.* This study aims to find the microorganisms that have the ability to degrade hydrocarbon in crude oil contaminated media. The crude oil was taken from PT, Pertamina RU VI, Balongan, Indramayu, West Java, Indonesia. This study was carried out from September to October 2016 in Laboratory of Soil Microbiology, Universitas Padjadjaran, Sumedang, West Java, Indonesia. The strains were from the same laboratory and as follows: *Azospirillum* sp., *Acinetobacter* sp., *Pseudomonas cepacia*, *Bacillus subtilis*, *Penicillium* sp., and *Aspergillus niger*. The selection of microbes is based on the characteristic of microorganisms on degrading hydrocarbon from crude oil on media containing crude oil (Total Plate Count = TPC), the capability on degrading the hydrocarbon of crude oil, and the capability of dissolving phosphorus microbes. The results showed that *Acinetobacter* sp. and *Azospirillum* sp. as nitrogen fixation bacteria, had higher cell population than *Azospirillum* sp. While *P. cepacia* and *Penicillium* sp., as phosphorus dissolving microbes group had higher cell population than *B. subtilis* and *A. niger*. On the process of biodegradation of hydrocarbon, *P. cepacia* had higher on efficiency of hydrocarbon than *B. subtilis, Penicillium* sp. was more efficient than *A. niger,* and *Acinetobacter* sp. was more efficient than *Azospirillum* sp. The characteristic capability of a microbe in dissolving phosphate substance in media was marked with *halo zone* form on Pikovskaya media in Petri dish, where *P. cepacia* had larger halo zone than *B. subtilis* and *Penicillium* sp. had larger halo zone than *A. niger*.

Keywords: Biodegradation, crude oil, hydrocarbon, microorganisms

INTRODUCTION

Oil spills are one of the major causes of soil damage in the environment. Leakage and oil spills happen during exploration activities, production, refinery, transportation, and petroleum storage (Das and Chandran 2011). Oil spill waste has been estimated as approximately 600,000 ton per year (Kvenvolden and Cooper 2003). WALHI (2007 in Ali 2009) reported that the case of contaminated soil was affected by hydrocarbon from big company activities. Contaminated soil by crude oil causes serious environmental problems, which demotes water and soil quality(DEQ 2011). So it is necessary to turn back soil fertility from toxic content frequently.

According to PP No. 18 1999, hydrocarbon substance of crude oil is classified into dangerous and toxic material (B3) in the soil. B3 poses physical and chemical substance potentially poisons living biology in the soil, and spoils food chain (Napoleon and Probowati 2014). Therefore, B3 is not allowed to be directly disposed into the environment before being neutralized in the refinery. The contaminated soil can be restored into healthy soil using environment friendly technology such as bioremediation. Bioremediation is one of the environment friendly technologies in landfarming treatment (Cookson 1996). A land farming treatment is an ex-situ process to remove pollution.

Crude oil contains inorganic and organic compounds with various form of hydrocarbon. Diversity of hydrocarbons that can contaminate the soil need various hydrocarbon-degrading microorganisms. Every single hydrocarbon-degrading microbe poses a different function. Bacterial groups can degrade aliphatic hydrocarbon groups. While fungi groups can degrade aromatic hydrocarbon groups. Therefore, application of microbe consortium can degrade various hydrocarbons in the soil.

Crude oil hydrocarbons have both complex and simple forms in the soil. Composition of complex hydrocarbon can be degraded by giving the various species of microorganisms until the diversity of hydrocarbon optimally degraded. Whereas the only single microorganism applied into contaminated crude oil media limitedly degrades hydrocarbon (Ghazali et al. 2004). As we know, some microbe groups that degrade hydrocarbon substance are: *Azospirillum* sp. (Gałazka and Gałazka 2015), *Acinetobacter* sp. (Sihag et al. 2013), *Pseudomonas cepacia* and *Bacillus subtilis* (Ghazali et al. 2004), *Penicillium* sp. (Dhar et al. 2014), and *Aspergillus niger* (Flayyih and Jawhari 2014). The selection of microorganisms for applied bioremediation of hydrocarbons must be considered to be easy to be cultured and able to live in contaminated environments (Mrozik and Piotrowska-seget 2010). The way to identify the various microbes for bacteria or fungi is needed to find the microbe species dominantly degrading hydrocarbon compounds.

MATERIALS AND METHODS

Materials

Crude oil used for this research was from PT. Pertamina RU VI, Balongan, Indramayu, West Java, Indonesia (Figure 1).

Procedures

Media for production of biomass

The isolated soil microorganisms were from the Soil Biology Laboratory Collection, Department of Soil Science and Land Resource Management, Faculty of Agriculture, University of Padjadjaran. The media used to process the hydrocarbon degradation test were Potato Dextrose Agar (PDA)-liquid for *A. niger*; and *Penicillium* sp., Pikovskaya-liquid for *P. cepacia* and *B. subtilis*, and Natrium Agar (NA)-liquid for *Acinetobacter* sp. and *Azospirillum* sp. All microorganisms were grown previously on media-tilt for 3-4 days to pure the strain culture that would produce biomass and would be saved in incubator. Next, the six microorganisms produced their biomass in each liquid chosen media. Biomass was produced with 10 mL volume in 100 mL liquid media which were poured into Erlenmeyer glasses with a volume of 250 mL. These were kept for 7 days and centrifuged at 15 rpm. For ensuring the biomass uniformity morphology of microorganism, a gram staining test was carried out. Thus, the test for degradation process test on crude mixed oil media could be performed.

Preparation for degradation test

Before hydrocarbon degradation test was performed, a morphology test of microbe cell with gram staining method on microbe was done. This was to decide the characteristic of cell wall. Next, the test of hydrocarbon degradation process was performed in the following stages: each liquid media was prepared by mixing 1% of crude oil from the volume of media 100 mL then pour into Erlenmeyer glass 250 mL. The crude oil was from PT. Pertamina RU VI, Balongan, Indramayu, West Java, Indonesia. To culture microorganisms optimally, they were grown in media and centrifuging all inoculant at 150 rpm for a month (30 days). Cells of microbes were harvested to decide the research parameter. Then each bacterial group was compared based on the total population of microorganism, the capability to degrade the hydrocarbon and the capability of microbe in dissolving phosphorus substance (*P. cepacia, B. subtilis, Penicilium* sp., and *A. niger*). Determination on the population of microbe cell was conducted via a direct count method using hemacytometer to assess the chambers. This was carried out after the microbe culture was centrifuged for 30 days. The determination of total petroleum hydrocarbon (TPH) level used the gravimetric method. The phosphorus-dissolving test was carried out by growing the cell of microbes in a Petri dish using Pikovskaya media. Then, the halo zone was measured with a ruler.

Data analysis

The data was analyzed based on data scoring system.

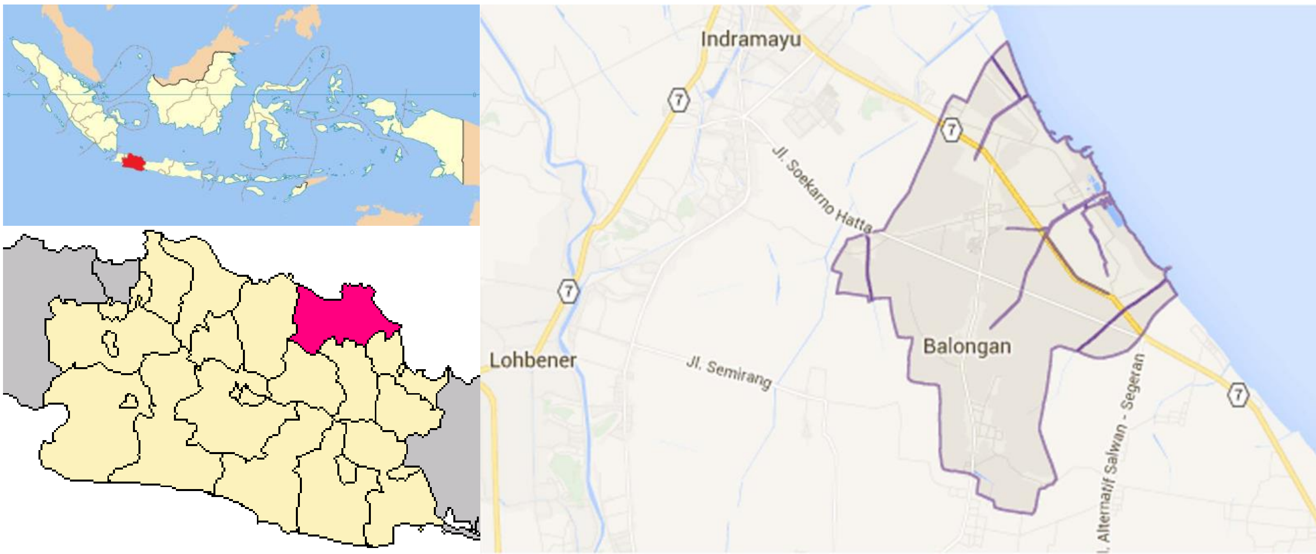

Figure 1. Location of Crude Oil Source from PT. Pertamina RU VI, Balongan-Indramayu, West Java, Indonesia.

RESULTS AND DISCUSSION

Total population of microbes cell

The result of this study showed that there is a difference in cell population level after the process of hydrocarbon degradation test for 30 days in different media with concentration of crude oil 1%. Additionally, each microbe from the 6 species were different from each other (Table 1). *Acinetobacter* sp. had a higher total population (34 x 107 and 140 x 108 CFU/gram) than *Azospirillum* sp. (166 x 105 and 167.5 x 106 CFU/gram). *B. subtilis* (49.5 x 107 and 115.7 x 108) had a higher cell population than *P. cepacia* (200 x 105 and 229 x 106 CFU/gram). *Penicillium* sp. had a higher cell population (378 x 103 and 115.8 x 104 CFU/gram) than *A. niger* (39.8 x103 and 56 x 104 CFU/gram). The determination for microbe cell population density was carried out with total plate count (TPC).

The efficiency of degradation of hydrocarbon

Based on the results of degradation of hydrocarbon on Table 2, the assertion of 6 microbes was carried out. It showed that *Acinetobacter* sp. (efficiency 93,40%) was more efficient than *Azospirillum* sp. (86,50%), then *P. cepacia* (99%) was more efficient than *B. subtilis* (84%), then *Penicillium* sp. (100%) was more efficient than *A. niger* (90,90%). The determination for hydrocarbon degradation measurement was conducted by determining hydrocarbon concentrate (TPH) for each treatment, then the value of TPH's efficient value was counted from each of TPH value.

Selection of microbes on the stage of hydrocarbon degradation test was carried out to know the capability of each microorganism in degrading crude oil hydrocarbon, until it became a comparison from microbe cell number in media test. Each microbe had a different capability in utilizing hydrocarbon as sole carbon source or substrate. The diversity of hydrocarbon forms, such as aliphatic and aromatic, can be degraded by specific microbes.

The potentiality to dissolve the phosphorus compounds

In addition, the cell population level and hydrocarbon degradation test needed to be tested. This was true especially of the phosphorus dissolving microbe groups, to know that they were able to dissolve the phosphorus substance in crude oil contaminated media, marked by a halo zone width in petri dish. Based on the results shown in Table 3, *P. cepacia* (0.16 mm) had a larger halo zone than *B. subtilis* (0.14 mm) for bacterial group. Then, for fungi group, *Penicillium* sp. (0.27 mm) had a larger halo zone than *A. niger* (0.18 mm).

Table 2. The efficiency of hydrocarbon degradation by soil microorganisms.

Microorganisms	**Efficiency of degradation Hydrocarbon (%)**
Nitrogen Fixation Microbes	
Acinetobacter sp.	93.40
Azospirillum sp.	86.50
Phosphorus Dissolved Bacteria	
Bacillus subtilis	84.00
Pseudomonas cepacia	99.00
Phosphorus Dissolved Fungi	
Aspergillus niger.	90.90
Penicillium sp.	100.00

Table 3. The result of phosphorus-dissolving microbes.

Microorganisms	**Halo zone Wide (mm)**
Phosphorus Dissolved Bacteria	
Bacillus subtilis	0.14
Pseudomonas cepacia	0.16
Phosphorus Dissolved Fungi	
Aspergillus niger	0.18
Penicillium sp.	0.27

Table 1. Total population of soil microorganisms.

Microorganisms	**TPC (CFU/gram)**					
	10^3	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**
Nitrogen Fixation Microbes						
Acinetobacter sp.	-	-	-	-	34	140
Azospirillum sp.			166	167.5	-	-
Phosphorus Dissolved Bacteria						
Pseudomonas cepacia	-	-	200	229	-	-
Bacillus subtilis	-	-	-	-	49.5	115.7
Phosphorus Dissolved Fungi						
Penicillium sp.	378.3	115.8	-	-	-	-
Aspergillus niger.	39.8	56	-	-	-	-

Discussion

The level of microbe cell population density is affected by microorganism enzymatic process in crude oil contaminated media, and the availability of sole carbon source also impresses the addition of microbe cells (Cookson 1995). Sufficient availability of a sole carbon source will increase the population cell. *Acinetobacter* sp. had higher total population than *Azospirillum* sp. It showed that *Acinetobacter* sp. had the best capability of metabolism. Furthermore, it has ability to survive in contaminated media. Jones et al (1983) reported that microbe is frequently found in site or in soil that is contaminated by dangerous and toxic waste, such as crude oil contamination (Jones et al 1983). According to Das and Chandran (2011), *Acinetobacter* sp. was known to be able to utilize the hydrocarbon of n-alkane, that had C10-C40 chain, as sole carbon source and energy. In literature, there was limited research discussing *Azospirillum* sp. in bioremediation process for crude oil. However, both *Acinetobacter* sp. and *Azospirillum* sp. were classified as plant growth-promoting rhizobacteria (PGPR) that was associated with plant root in phytoremediation process (Gałązka et al. 2012).

As phosphorus-dissolving microbes, *B. subtilis* had a higher population cell than *P. cepacia*. Then, *Penicillium* sp. had a higher population cell then *A. niger*. A previous study reported that *B. subtilis* could be found in some crude oil contamination sites and it still enhanced its population cell (Toledo et al 2006). In this study, *B. subtilis* was able to use the hydrocarbon of crude oil as a sole carbon source more than *Pseudomonas cepacea*.

Fungi is known to be the best degrading agent for hydrocarbon crude oil in the bioremediation process, compared to bacteria such *Penicillium* sp. and *A. niger*. Many studies have revealed that *A. niger* has higher activities in degrading hydrocarbon than *Penicillium* sp. (Al-Nasrawi 2012). However, this experiment showed that *Penicillium* sp. Had higher activities than *A. niger,* this can be seen from its high population.

Besides the cell population, another criterion for measuring the microbes' capability in bioremediation system is the efficiency of hydrocarbon degradation. Bacteria of *P. cepacia* had the capability to degrade aromatic hydrocarbon, while *B. subtilis* was able to degrade aliphatic hydrocarbon (Ghazali et al. 2004). A characteristic of a carbon chain for aromatic species is recalcitrant in the environment, until the value of efficiency of *P. cepacia* was higher than *B. subtilis*. Therefore, it has been shown that *P. cepacia* was more efficient that other bacteria in bioremediation process.

Acinetobacter sp. and *Azospirillum* sp. were PGPR microbes (plant growth-promoting rhizobacteria). Research has revealed that both microbes have potential in bioremediation application (Huang et al. 2004). *Acinetobacter* sp. Had capability to degrade the hydrocarbon (Ellis 1994; Johnsen et al. 2005) and able to enhance rhizoremediation process in soil contamination (Bhattacharyya and Jha 2011; Pajuelo et al. 2011; Vershinina 2012). Both *Acinetobacter* sp. And *Azospirillum* sp. Were able to use the hydrocarbon as a sole carbon source and energy. However, in this study, *Acinetobacter* sp. had a higher level of efficiency than *Azospirillum* sp. in the hydrocarbon degradation process.

In relation to the efficiency of hydrocarbon degradation in fungi group, it showed that *Penicillium* sp. was more efficient than *A. niger*. Also, if compared to bacteria, the fungi group had a higher efficiency. The fungi were better than bacteria as a biological agent on hydrocarbon biodegradation process (Vanishree et al. 2014). Both of these fungi were known to be able to utilize crude oil as substrate affected from extracellular enzyme that were produced by them (Adekunle and Adebambo 2007). In addition, both had potential to act as hydrocarbon degrading agent of crude oil. However, the superiority of *Penicillium* sp. toward *A. niger* was supported by its capability to survive in a crude oil contamination situation, even in the saline soil that *Penicillium* sp. could survive while others could not (Vanishree et al. 2014). So, *Penicillium* sp. is more potential than *A. niger*.

The last criteria of the four microbes was their function as phosphorus dissolving microbes namely *P. cepacia* (*Burkholderia cepacia*) (Holmes et al. 1998), *B. subtilis*, *Penicillium* sp. and *A. niger*. The test on the capability of the microbes group in dissolving phosphorus substance on Pikovskaya aimed to know their other function, for example, as biofertilizer. In a bioremediation system, the nutrient is one of the most important factors needed to manage the continuity of bioremediation process. So, it can work better in the mineralization process by microbes. One of them is capable of dissolving phosphate substance. The availability of nutrients in the soil was a primary factor in bioremediation process (Alvarez and Illman 2006). Capability to dissolve the phosphate is also determined by carbon source and energy for hydrocarbon degradation process.

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REFERENCES

Adekunle AA, Adebambo OA. 2007. Petroleum hydrocarbon utilization by fungi isolated from Detarium senegalense (J. F. Gmelin) seeds. J Amer Sci 3 (1): 69-76.

Al-Hasrawi H. 2012. Biodegradation of Crude Oil by Fungi Isolated from Gulf of Mexico. J Bioremed Biodegrad 3 (4). www.omicsonline.org.

Ali M. 2009. Clearing of Hydrocarbon Spil Contaminated Land By Biopile Technique. UPN Veteran Jatim, Surabaya.

Alvarez PJJ, Illman WA. 2006. Bioremediation and Natural Attenuation: Process. Fundamentals and Mathematical Model. John Wiley & Sons Inc., New York.

Bhattacharyya PN, Jha DK. 2011. Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture. World J Microb Biotechnol 28 (4): 1327-1350.

Cookson Jr JT. 1995. Bioremediation Engineering: Design and Application. McGraw-Hill, Inc. USA.

Das N, Chandran P. 2011. Microbe Degradation of Petroleum Hydrocarbon Contaminants: An Overview. Biotechnology Research International 2011. https://www.hindawi.com.

DEQ [Departement of Environmental Quality]. 2011. Petroleum-Contaminated Soils Handling Options. Departement of Environmental Quality, Oregon, USA. www.oregon.gov/deq
Dhar K, Dutta S, Anwar M N. 2014. Biodegradation of Petroleum Hydrocarbon by Indigenous Fungi Isolated from Ship Breaking Yards of Bangladesh. Intl res J Biol Sci 3 (9): 22-30.
Flayyih I, Al-Jawhari H. 2014. Ability of some soil fungi in biodegradation of petroleum hydrocarbon. J Appl Environ Microbiol 2 (2): 46-52.
Gałazka A, Gałazka R. 2015. Phytoremediation of polycyclic aromatic hydrocarbons in soils artificially polluted using plant-associated-endophytic bacteria and Dactylis glomerata as the bioremediation plant. Polish J Microbiol 64 (3): 241-52.
Gałązka A, Król M, Perzyński A. 2012. The efficiency of rhizosphere bioremediation with Azospirillum sp. and Pseudomonas stutzeri in soils freshly contaminated with PAHs and diesel fuel. Pol J Environ Stud 21(2): 345-353.
Ghazali, Mohamad F, Abdul Rahman RNZ, Salleh AB, Basri M. 2004. Biodegradation of hydrocarbons in soil by microbe consortium. Intl Biodeterior Biodegrad 54 (1): 61-67.
Holmes A, Govan J, Goldstein R. 1998. Agricultural Use of Burkholderia (Pseudomonas) cepacia: A threat to human health? Emerg Infect Dis 4 (2): 221-227.
Huang XD, El Alawi Y, Penrose DM, Glick BR, Greenberg BM. 2004. A multiprocess phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. Environ Pollut 130: 465-476.
Johnsen AR, Wick LY, Harms H. 2005. Principles of microbe PAH degradation in soil. Environ Pollut 133: 71-84.
Kvenvolden KA, Cooper CK. 2003. Natural seepage of crude oil into the marine environment. Geo-Marine Letters 23 (3-4): 140-146.
Mrozik A, Piotrowska-seget Z. 2010. Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. Microb Res 165 (5): 363-75.
Napoleon A, Probowati D S J. 2014. Exploration of hydrocarbon degrading bacteria on soils contaminated by crude oil from South Sumatera. Degrade Min Land Manag 1 (4): 201-206.
Pajuelo E, Rodríguez-LLorente ID, Lafuente A, Caviedes MA. 2011. Legume-rhizobium symbioses as a tool for bioremediation of heavy metal polluted soils. In: Khan MS, Zaidi A, Goel R, Musarrat J (eds). Biomanagement of Metal Contaminated Soils, Environmental Pollution, Vol 20. Springer, Germany.
PP No. 18 1999. Peraturan Pemerintah Republik Indonesia. Nomor 18 Tahun 1999. Tentang. Pengelolaan Limbah Bahan Berbahaya Dan Beracun. [Indonesian]
Sihag S, Sharma S, Pathak H, Jaroli SDP. 2013. Biodegradation of engine oil by Acinetobacter calcoaceticus BD4, isolated from coastal area Mumbai. Intl J Biotechnol Bioeng Res 4 (3): 235-242.
Toledo FL, Calvo C, Rodelas B, Lopez JG. 2006. Selection and identication of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. Syst Appl Microbiol 2006; 29: 244{252.
Vanishree M, Thatheyus AJ, Ramya R. 2014. Biodegradation of petrol using the fungus *Penicillium* sp. Sci Intl 2: 26-31.
Vershinina ZR, Baymiev AK, Blagova DK, Chubukova OV, Baymiev AK, Chemeris AV. 2012. Artificial colonization of non-symbiotic plants roots with the use of lectins. Symbiosis 56 (1): 25-33.