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PERLITE-IMMOBILIZED BACTERIAL CONSORTIUM ENHANCED DEGRADATION OF CRUDE OIL-CONTAMINATED MARINE SEDIMENT

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Abstract: The limit biodegradation of oil pollutant is their limited availability to microorganisms because application in the field uses liquid bacterial culture which makes the resistance and ability of bacteria become susceptible to being disturbed due to an inappropriate environment. Immobilization of cells is one way to overcome this problem and selecting suitable materials to protect bacteria is an important thing. The study aims to compare the crude oil biodegradation ability of bacterial consortium consisting of *Enterobacter cloacae, Bacillus licheniformis,* and *Bacillus* sp. in the form of free and immobilized cells using microcosms design. In this study, immobilized was used to immobilized bacteria on perlite as an inorganic carrier. Bacterial cells trapped in the perlite were counted to determine the entrapment capacity. The efficiency of biodegradation was evaluated every week for 28 days by measuring total petroleum hydrocarbon and another variable such as total number of bacteria and environmental changes. The results of crude oil biodegradation efficiency showed there was not significantly between immobilized cells (63.86% ± 2.29) that more efficient than the free cells (38.55% ± 0.34) after 28 days of incubation. Based on our result perlite can be use suitable carrier to immobilized cell.

Keywords: Bacterial consortium, biodegradation, crude oil, immobilized cells, perlite.

Introduction

Crude oil is a complex compound containing more than 85 % toxic hydrocarbons and several carcinogenic compounds for plants, animals, and humans. One of the techniques to eliminate and reduce contamination of crude oil is using bioremediation. Bioremediation is a type of biodegradation that depends on the metabolic activity of microbes (Nunal *et al.,* 2014).

An according to Damayanti *et al.* (2017), when oil spill occurs, petroleum products are mixed with water due to the actions of wind and waves which form oil mousses. The biodegradation rate of petroleum varies depending on the composition and chemical properties of the constituents. The process of degradation of petroleum components by microbes depend on the type of compound to be degraded, for example, the degradation of aliphatic hydrocarbons was better than aromatic. Hydrocarbons with high molecular weight have high boiling points so they are resistant to biodegradation and are toxic because their solubility in water is very low (Moore, 2015).

Biodegradation of petroleum in nature involves a series of consortium species so there is no strain of bacteria capable of degrading all components contained in petroleum (Zhu *et al.,* 2001). One of the keys to the success of bioremediation is keeping the cell count high. Oceans have highly mobile system and which have uncontrollable factors such as loss of an effective degrading bacterium (Chenn *et al.,* 2017). According to Bayat *et al.* (2015), the number of bacterial populations can be maintained by immobilizing them in a suitable carrier to avoid cell washout. Apart from maintaining cell count, immobilization also protects cells from organic pollutants present in the environment so that cells can degrade optimally (Dzionek *et al.,* 2016)

According to Dzionek *et al.* (2016), not all materials would be suitable to be used as carrier immobilization matrix. Selection of the carrier as a matrix proponents of cell immobilization are indispensable for the process of biodegradation by means of cell immobilization to proceed optimally for oil degradation. Materials used as the carrier must have high porosity so that it has large contact area for microbial adherence. Other than that, the carrier in the waste treatment process must have mechanical resistance to protect immobilized cells from various types of physical interference from outside (Martins *et al.,* 2013).

The types of carriers for cell immobilization are divided into two types namely organic and inorganic. Organic carriers have many functional groups that can stabilize biocatalysts and the price is relatively cheap. However, if applied in the biodegradation process it is very limited due to several weaknesses including resistance to biodegradation that is very low because it is biodegradable and the carrier has stability in a narrow pH range. On the other hand, inorganic carriers have very high physical resistance and can withstand extreme temperatures and pH, chemicals and microbial degradation. Examples of inorganic carrier types are, for example, magnetite, volcanic rock and silica-based materials (Dzionek *et al.,* 2016). Therefore, in this study, the researchers ~~is~~ used perlite as anorganic inorganic carriers to know their entrapment capability degrading bacteria cell and their ability in removal of petroleum contamination.

Perlite is a type of porous volcanic rock formed from cold lava from volcanic eruptions and is known as an inorganic carrier in degrading petroleum (Ivankovic *et al.,* 2010). In a previous study, Ivankovic *et al.* (2010) used perlite as carrier to the immobilization of *Acinetobacter junii* to remove phosphate (P) in wastewater. The perlite was successfully used as a support material or carrier to trap degraded bacteria and these bacteria proved to be more stable when immobilized on perlite during the degradation when compared to non-immobilized bacteria. However, ability of perlite to remove petroleum contamination using consortium bacteria is still not widely applied and information on petroleum biodegradation is still lacking, especially in Indonesia.

In addition, the use of perlite as a carrier material is also still rarely done. Many studies have used other inorganic carriers like zeolite, clay and others in wastewater treatment, such as the research by Wang and Peng (2010) which used natural zeolites as effective adsorbents in water and wastewater treatment. In another study, Wang *et al.* (2006) used diatomite, clinoptilolite, silk zeolite, and coal fly ash were chosen as carriers to degrade DBP (Di-n-Butyl Phthalate) under different primary concentration, vibration rate, pH, temperature in the presence of metal compound.

The method of immobilization of bacteria using a carrier to reduce oil spills is a new technique that has not been used in Indonesian marine waters because conventional biodegradation without immobilization is still being used. So in this study, we applied our novel method and used immobilization cell on perlite as carrier which consists of consortium bacteria for applying to oil removal in coastal Indonesian waters using microcosm design. According to Kangas (2005), the microcosm is a miniature artificial ecosystem, relatively small, closed or semi-closed and are used for experimental purposes. The microcosm design reflects a balance between three interrelated objectives including control, realism and generality to increase the probability of research success. This study was conducted to compare the bioremediation ability of petroleum degrading bacteria in the form of free cells and immobilized cells using perlite as a carrier in a microcosm design with used water and sediment from Ancol coastal area, North Jakarta, Indonesia, as media to stimulate biodegradation process which occurs in natural environment.

Materials and Methods

Preparation of the Oil-Degrading Consortium

The bacterial consortium used in this study refers to previous research done by Junusmin *et al.* (2019), and consists of *Enterobacter cloacae, Bacillus licheniformis,* and *Bacillus* sp., all bacteria known to have the ability to properly degrade crude oil. These three bacteria, isolated from Jakarta Bay in 2007, are a collection of bacteria from marine microbiology laboratory Research Center for Oceanography, Indonesia Institute of Sciences or *Lembaga Ilmu Pengetahuan* Indonesia (LIPI). The stock bacteria were prepared according to the method described by Alfiansyah *et al.* (2014), and each bacterial strain was sub-cultured separately in the marine broth (MB) medium because in the previous study by Alfiansyah *et al.* (2014), direct bacterial plating of water and sediment onto ONR7a medium seemed ineffective as a means to cultivate bacteria. It was indicated by the fact that no bacteria grew on ONR7a medium containing crude oil. Therefore, to cultivate or enrich bacteria using MB as an effective medium to growth medium for bacteria and the bacteria are grown for 24 hours. Equal volumes of suspension containing the different bacterial strains with the same cell density (10^8 CFU/mL) were mixed to form a consortium

Preparation of Immobilization Carrier and Immobilizing Bacterial Consortium

Perlite, as a carrier, was purchased from one of e-commerce marketplaces, Indonesia. Then, perlite was sieved to obtain a particle size of approximately 0.85 - 2 mm and the carriers were activated using hydrochloric acid (HCl) 5% and natrium hydroxide (NaOH) 5% for 2 hours. Next, perlite was washed several times with distilled water until it reached a neutral state (pH 7). The carriers were dried using an oven at 105°C and finally autoclaved at 121°C for 15 minutes.

The immobilization of the bacterial cells in perlite was done by using the method described by Wang *et al.* (2006). Bacterial consortium suspension (100 mL) was added to 100 g sterilized carriers (1:1) and shaken for 15 minutes. Then the immobilized cells were agitated at 30°C in a shaker incubator for 30 minutes. The immobilization step was completed by freeze-drying until the bacterial culture has been adsorbed completely in the carriers for 1 - 2 days.

To examine the entrapment capacity, an inoculated carrier with a weight of 1 g was added to 9 mL sterilized seawater. Then, the cultures were sonicated using Elmasonic S 300(H) (37 kHz, 10 minutes) and vortexed (50 Hz, 10 minutes) to release cells entrapped in the carriers. The number of cells was counted using the acridine orange direct count (AODC) method.

Microcosm Designs

The efficiency of crude oil degradation was evaluated by using a microcosm design on a laboratory scale. The microcosm design was created with a set of tools consisting of a 36 x 46 cm plastic container containing 6 liters seawater (non-sterilized) to support humidity in the research system. Three high PVC (polyvinyl chloride) pipes with diameters of 13 cm and 9 cm were placed inside the container as replications and marine sediment (sterilized) taken from Ancol coastal area in North Jakarta. The bottom of the pipe was made of a nylon membrane with a porosity of 0.22 µm. The top of the pipe was covered with a fiberglass disc in the middle that had been perforated for aeration by using a water pump and was delivered through assistance hose which ended with Pasteur pipette into the system microcosm (PVC pipe). The treatments used in the research are shown in Table 1. Sediment and water as medium simulation in microcosm were obtained from Ancol coastal area in order to make it like a natural environment for this study. To create simulation of an occurrence of oil spill reference was made to Damayanti *et al.* (2017). It was prepared by spiking oil mousse of Arabian Light Crude Oil (ALCO) into sediment and seawater because this type of oil often contaminates coastal area. Indonesian Oil moussed was prepared by mixing Ancol

seawater sterile with ALCO sterile in proportion 1:3 to achieve concentration of 100.000 ppm.

The experiments were conducted for 28 days with aeration. Measurements of all parameters were conducted in triplicates for each treatment at different sampling times (0, 7, 14, 21, 28 days). The first parameter was crude oil depletion based on total petroleum hydrocarbon, (TPH) analyzed using Fourier Transform Infrared Spectroscopy (FTIR) because when compared to using GC or GC-MS, the analysis using FTIR is simpler, easier to use and faster as this study only wanted to know the decrease in oil concentration without determining the amount and type of each compound separately. The second was based on total bacterial cells using the acridine orange direct count (AODC) method adapted from Hobbie *et al.* (1977), and the last was environmental factors, i.e., DO, pH, and salinity from pore water. The measurement of TPH and the bacterial count were performed on 0.5 g of oil-polluted sediment.

Total Petroleum Hydrocarbon (TPH) Measurement

Total petroleum hydrocarbon (TPH) measurements were carried out using Fourier transform infrared (FT-IR) spectroscopy (Thermo Scientific ™ Nicolet ™ iS ™ 5) with OMNIC software based on the Soxhlet Extraction 3540C (EPA, 1996) method which had been modified. The sediment was extracted first using a combination of 5 mL di-chloro-methane (DCM) and 5 mL n-hexane and then sonicated (37 kHz, 10 minutes). Petroleum extraction is repeated continuously with the DCM and n-hexane extraction solution alternately until the sample turns clear or about six times the extraction so that the petroleum contained in the sediment can be extracted completely. The results of oil extraction obtained are vortexed (50 Hz) until homogeneous. Furthermore, the petroleum extract is evaporated to form an oil crust. The evaporation results from the oil extract were then added with tetrachlorethylene as a solvent to measure TPH using FT-IR with OMNIC software (wavelength 4000-500 cm^{-1})

The results that will be displayed quantitatively by the software are the concentration of TPH (µg / mL) (Strother *et al.,* 2013). The effectiveness of petroleum degradation is calculated by using equation (1).

$$\text{Degradation effectivity (\%)} = \frac{C_0 - C_1}{C_0} \; x \; 100 \; \% \quad (1)$$

Noted:

C_0 : Oil concentration on day 0.

C_1 : The concentration of oil on the day to be calculated

Enumeration Bacteria

0.5 Sediment samples in the 15 mL propylene tube were added with 4.5 mL sterile sea water. Furthermore, the sample is diluted until 10^{-2} and 0.5 mL of the sample was added to the 4.5 mL acridine orange solution. After that, the samples were filtered using a milipore polycarbonate membrane filter (diameter 25 mm; porosity 0.22 µm) which has been immersed in a solution of sudan black for 24 hours to give a dark background so that bacteria can be counted clearly. Then, membrane filter was rinsed with distilled water sterile. The membrane filter was then observed using a microscope (magnification 1000x) and the number of bacterial cells was counted (Hobbie *et al.,* 1977).

Total bacterial cells were observed in 10 fields of view, with the number of bacteria observed around 10 - 100 cells. The number of cells is too much diluted with acridine orange solution as had been done at the previous stage. The number of cells obtained from observations are calculated and converted using equations (2).

$$\text{Total bacterial cells} = \text{n} \left(\frac{\frac{V_1 + V_2}{V_2}}{v_3} \right) x \left(\frac{\pi r^2}{A} \right) x \, DF \quad (2)$$

Noted:

n : average of bacterial cells in 10 fields of view

V1 : volume of acridine orange solution (mL)

V2 : volume of sample dissolved in Acridine Orange (mL)

V3 : volume of filtered sample (mL)

πr^2: area of the filter (r = the diameter of the filter surface covered by the sample, which is 20 mm)

A : the area of the microscope field of view (= 3.14 x (0.07) 2 = 0.0154 mm^2)

DF : $\frac{1}{\text{dilution factor}}$

Analytical Data

The experiment used a completely randomized design. First, the data were tabulated using Microsoft Excel 2007. Then the data were analyzed statistically by using the two-way Analysis of Variance (ANOVA) test two factorial with Tukey test (homogeneous sample) or Games-Howell (sample not homogeneous) post hoc if the ANOVA result shows significant ($p < 0.05$). The Kolmogorov-Smirnov test was used to test the normality of the data. Meanwhile, to test the heterogeneity of the data, the Levene Statistic was performed. Then the number of cells and degradation percentages were analyzed using Pearson correlation to find out the strength of the association between both. Every analysis was conducted using SPSS 20 software.

Results and Discussion

Viability and Capacity Entrapment

The results obtained from the bacterial entrapment capacity test for each carrier showed that perlite had an entrapment ability of 2.45 x 10^9 cells/g with a percentage of the number of living cells of 97.3% of the total immobilized bacterial cells of the carrier. According to Ivankovic *et al.* (2010), the high number of bacteria entrapped into perlite is due to the content of constitutive water, which is 2 - 5%, contained in perlite. This constitutive water was lost during the activation process, so it created a macropore structure in the carrier and provided a new surface area for bacterial colonization. Kaufhold *et al.* (2014), added that perlite has a low particle density so that it has a wide pore diameter, which is around 20 - 100 µm. The porosity of perlite after being activated will be significantly higher so that the number of cells trapped in the carrier will also increase. If the total number of bacterial cells trapped in the carrier increases, then more microbes will degrade crude oil in the microcosm system.

Oil Degrading Efficiency

The results showed that there were differences with significant ($p < 0.05$) between treatments affecting the efficiency of crude oil degradation (see Figure 1). The results of crude oil degradation in (NC, NF) treatment only showed an oil degradation efficiency of 38.55% ± 1.70 after 28 days of incubation. Compared to (IC, F) treatment as indicated by biostimulation, the ability of oil degradation efficiency was three times higher in percentage than that of (NC, F)

Table 1: Types of treatment in microcosm experiment (*free-cell bacterial density was 3 x 10^8 CFU/mL)

No.	Treatment (code)	Sediment (non-sterile)	Mousses Oil (sterile)	Immobilized Bacteria	Fertilizer	Bacterial Community
1	Uninoculated, no fertilizer (NC,NF)	90 g	40 g	-	-	Indigenous
2	Uninoculated, with fertilizer (NC,F)	90 g	40 g	-	3.41 g	Indigenous
3	Free-cell inoculated, no fertilizer (C,NF)	90 g	40 g	7 mL free cell	-	Indigenous & free cell
4	Immobilized,with fertilizer (IC,F)	83 g	40 g	7 g	3.41 g	Indigenous and Immobilized bacteria

treatment with the oil degradation efficiency of 63.86% ±2.29. However, (IC, F) treatment has an efficiency of oil-degrading that is not significant statistically ($p > 0.05$) with (C, NF) treatment as indicated in the bioaugmentation. This indicates that biostimulation and bioaugmentation produce the same ability efficiency of oil degradation. The (IC, F) treatment, as an indicated combination between biostimulation and bioaugmentation with immobilized bacteria, showed that its oil degradation percentage is significantly different from all treatments statistically. Moreover, the combination generates excellent degradation efficiency. Besides, the immobilization of the bacterial cell population can be maintained because the carrier is able to protect bacterial cells from any disturbances in the environment so that cells can degrade maximally (Dzionek *et al.,* 2016), which is also supported by the addition of fertilizer for nutrient supply to support bacterial growth.

The percentage of the oil degradation ability in immobilized cells continued to increase in each sampling period and is statistically significant ($p < 0.05$) with the overall treatment. The results of the time variation test determine how long the efficiency of petroleum degradation lasts for each treatment. The results also showed that (NC, NF) and (C, NF) treatments had undergone a significant increase in oil degradation on day 14 while the efficiency of oil degradation on day 21 and day 28 showed no significant increase. Unlike the (IC, F) treatment, the degradation process of petroleum continued to experience a significant increase from week to week. It means that the degradation process was still running efficiently until day 28. In addition, it proves that the combination of cell immobilization and the addition of fertilizers can maintain the bacterial metabolism properly in oil degradation. In this study, immobilized bacterial consortium performed better than free bacteria because the ability of oil degradation efficiency was significantly different ($p < 0.05$) with all treatments. The improvement ability of immobilized cells performed better than free bacteria, which was also found in a previous study by Chenn *et al.* (2017), with variations of environmental factors containing incubated temperature, initial pH and salinity of the medium. After 7d's biodegradation, the degradation efficiencies of crude oil (2%, w/v) of immobilized cells were more than 60% with temperature of 15 ± 30°C, initial pH of 6.0 ± 9.0, salinity of 15% ± 35%. A similar study was reported by Xue *et al.* (2017), that the biodegradation efficiency of immobilization was higher than that of free bacteria with use of natural organic as carrier. The degradation

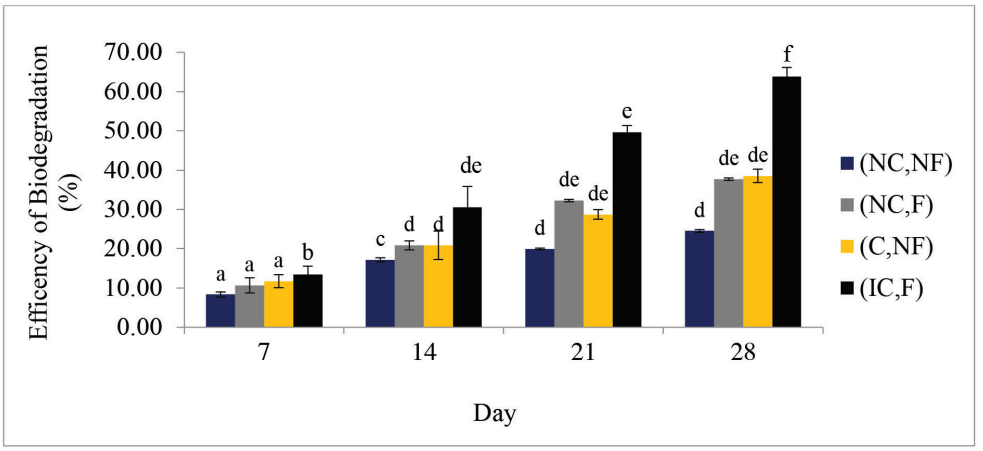

Figure 1: Percentages of oil degradation efficiency. The different (a-b) letters on the histogram showed significant differences between the Games-Howell tests (< 0.05). The results shown in the graph represent the average percentage of degradation as well as the standard deviation shown by error bars (n = 3)

efficiencies of diesel were in the order of immobilized bacteria by wood chips (73.39%), immobilized bacteria by maize straw (52.28%) and free bacteria (44.79%). But, novelty between this study and our work were from carrier used in the research. In this study, we use perlite which is nonorganic material to oil degradation while in the previous study, use of a bio-carrier of calcium alginate was as support matrix to immobilization. So, with this new finding we can know that a carrier for use for immobilization is not only from a bio-carrier or organic material but perlite can also to be the alternative carrier for immobilization bacteria with percentage degrading oil reaching 63.86% ± 2.29 in 28th day degradation.

Number of Bacteria During Incubation

The process of crude oil degradation in each treatment also involves indigenous microbes found in sediments and seawater used in the bioremediation microcosm system. The data results of bacteria cells during incubation from each sampling time are presented in Figure 2. The lowest number of bacterial cells belongs to the (NC, NF) treatment, while the (IC, F) treatment shows a very high bacterial cell density in every sampling with 9.05 ± 0,00 log 10 cells/mL on day 21. The number of bacterial cells in immobilized cells seems to have decreased on day seven. The decrease occurred because on day seven the bacteria had not been able to adapt well to the environment with a high-concentrated oil so that the bacteria required an acclimatization process to be conducted on the same day. Total bacterial cells in immobilized treatment on day 14 of bioremediation also increased. It was stable the next day with several bacterial cells that were not significantly different from day 21 until the end of bioremediation (day 28), as can be seen in Figure 2. It proves that the cell immobilization in the carrier can maintain the bacterial cell properly. Bayat *et al.* (2015), stated that cell immobilization is a limitation of cell movement in the supporting matrix, which provides benefits for bacterial cells such as protecting cells from various disturbances originating from the outside including toxic chemicals and heavy metals. The carrier can keep the bacteria inside the supporting matrix as well as increase the work efficiency of microbes to obtain maximum results of oil degradation.

The results of the correlation test (Pearson correlation) showed that total bacterial cells had a significant positive correlation with the percentage of degradation efficiency (Sig. 0.22 $p > 0.05$) although the correlation coefficient obtained ($R = 0.250$) indicated that both had a weak correlation. This implies that the effectiveness of crude oil degradation is

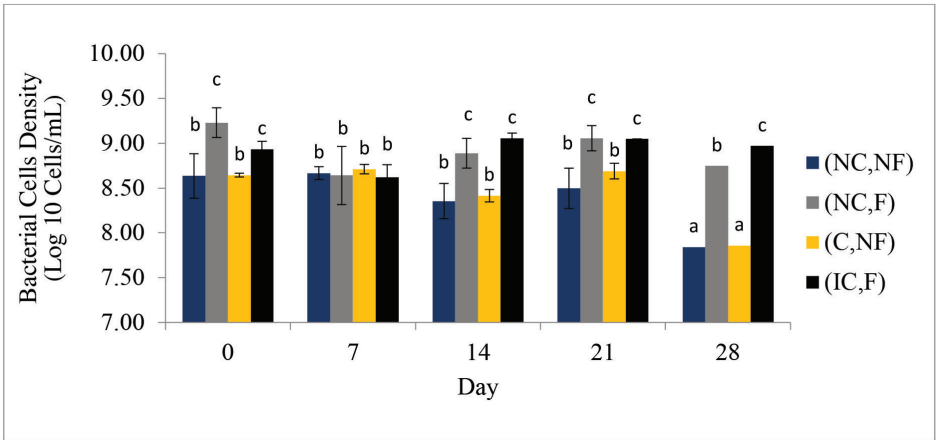

Figure 2: Bacterial cell density in each sampling time. The different letters (a-b) on the histogram showed significant differences between the Games-Howell test (<0.05). The results on the graph represent the average percentage of degradation as well as the standard deviation shown by error bars (n = 3)

strongly influenced by the total bacterial cells in each treatment so that the increase in the number of bacterial cells causes an increase in the percentage of the oil degradation efficiency.

Environmental Condition

Environmental conditions, such as pH, salinity, DO, and temperature of the contaminated sites, can also affect crude oil biodegradation. This research was conducted at 21 - 27°C. All treatments showed a decrease in temperature on day seven but the decrease that occurred was not significant with the temperature on day 0 of bioremediation. Zhu *et al.* (2001), stated that the environment temperature affects the nature of the oil spill, the population, and the microbial activity so when the environmental temperature decreases, the population of microbial will also decrease. Generally, the rate of oil biodegradation decreases along with the decreasing temperature. Biodegradation of hydrocarbons can occur at various temperature variations with the optimal temperature for the oil degradation process ranging from 20 - 30 °C (Das & Chandran, 2011).

Other important factors that affect the biodegradation of petroleum are pH and salinity. The results of pH measurements in each treatment ranged from pH 6 - 7. Almost all treatments showed a statistically significant increase of pH each day except for the control treatment of fertilizers, which had a stable pH. Hassanshahian and Cappello (2013) argued that most species of petroleum-degrading bacteria can degrade crude oil optimally at pH = 6 - 8. It means that the pH obtained in the present study is the optimum condition for the microbial degradation of crude oil.

Hassanshahian and Cappello (2013) stated that the decrease in the hydrocarbon metabolism rate by bacteria is due to the increased salinity from the effect of negative ions on bacterial cell metabolism, especially for microbes that cannot survive at high salinity. There was a decrease or an increase in extreme salinity which may interfere with microbial growth so that the degradation process continues. The results of the salinity measurement in this study ranged from 35 to 55 ppt. There was no significant effect ($p > 0.05$) on salinity in the whole treatment which indicates that each treatment has a salinity that is not too significant or relatively stable from time to time. Hence, this stable salinity condition is one of the things that supports the process of oil degradation running properly.

In this study, DO in treatment was around 0.34 - 3.74 mg/L. It showed that it fluctuated from day to day (Figure 3). The fluctuation

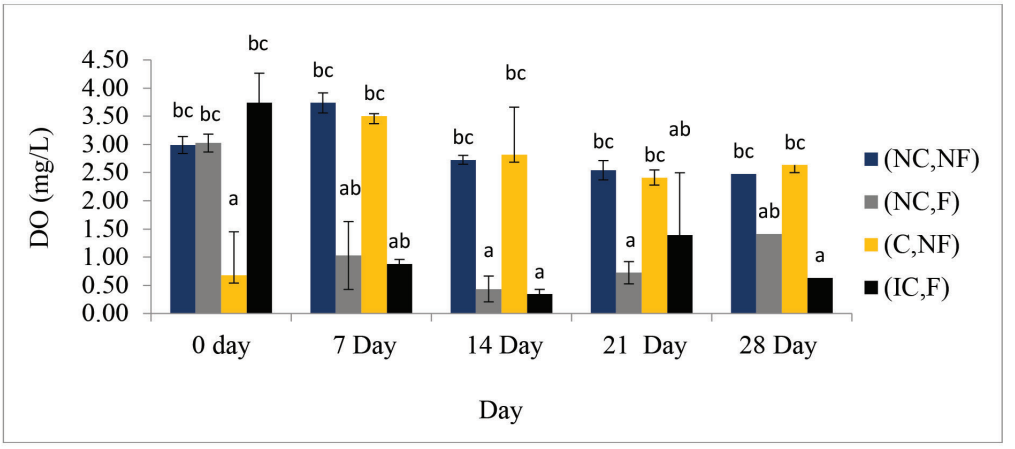

Figure 3: Dissolved oxygens in each sampling time. The different letters (a-b) on the histogram show significant differences between the Games-Howell tests (< 0.05). The results on the graph represent the average percentage of degradation as well as the standard deviation shown by error bars (n = 3)

that occurs is assumed to be due to the inhomogeneity of aeration to the treatment. According to Zhu *et al.* (2001), oxygen is one of the most important requirements in the process of petroleum degradation by microbes. Microbes require oxygen for the initial breakdown of hydrocarbons. Based on this statement, the decrease in DO is assumed to be due to microbial activity that utilizes oxygen to aid in petroleum degradation. DO content in (C, NF) and (NC, NF) treatments was higher than that of other treatments. It points out that the activity of petroleum degradation by microbes in both treatments was lower when compared to the immobilized treatment (IC, F).

Conclusion

Immobilized bacterial cells on perlite can degrade crude oil better than free living bacteria with an efficiency of 63.86% ± 2.29 after 28 days incubation. The degradation of crude oil-contaminated marine sediment by immobilization of cells increases two times that of bacterial free cells. This implies immobilization affect the ability of cells in degrading crude oil because the carrier can maintain the total cell bacteria properly. Based on our results, perlite as an inorganic material can be used as a suitable carrier for immobilized cell to enhance degrading crude oil for bioremediation.

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