Comparison of Viability and Efficacy of an Immobilized Bacterial Consortium in Four Different Carriers to Degrade Oil

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Abstract. This research aimed to evaluate the storage system’s effect on immobilizing bioremediation agents’ performance in four different carriers (Perlite, Silica, Vermiculite, and Zeolite). Measured parameters were the viability and efficacy of artificial bacterial consortium (Bacillus sp., B. licheniformis, and Enterobacter cloacae) immobilized in the four porous rock carriers by lyophilization. They were stored at room temperature (25-27°C) for three months. The experiment was taken place in a microcosm system with three replicates for 28 days. The results indicate that storage of immobilized bacteria for three months at room temperature will affect their viability. The efficacy of immobilized bacteria in Vermiculite and Perlite reached the highest rate until the first week, 61.44% and 60.18 %, respectively. However, the efficacy in decreasing oil concentration of immobilized cells in the four carriers was almost similar (90.53 – 91.63%), with no significant difference between each other in the late stage. This is very different with control, decreasing oil in only 10.85 - 13.57%, except in control with fertilizer (Cp) supplemented (90.63%). We conclude that bioremediation will improve oil removal. However, storage at room temperature for three months will decrease the performance of these immobilized cells.

1. Introduction

Indonesia is the largest archipelagic country in the world. Therefore, Indonesia can use its water area to transport goods, including crude oil. The high frequency of crude oil transportation in Indonesia’s water area increases the probability of an oil spill incident, either from underwater pipe leakage or oil tanker ships accident that releases crude oil to Indonesia’s open water or coastal areas. Based on Special Task Force for Upstream Oil and Gas Business Activities Republic of Indonesia (SKK Migas) data that Bisnis Indonesia reported, the volume of spilled oil from 2013 to 2016 is 3,025.6 barrels; 1,113.8 barrels; 226.4 barrels; and 787.2 barrels, respectively [1]. In May 2018, PT Pertamina’s underwater pipe leaked and polluter 12,000 ha of Balikpapan Bay [2]. Conventional methods, such as chemical and physical removal, are the first response option commonly used. However, they rarely achieve complete for cleaning up an oil spill. Bioremediation has emerged as one of the most promising secondary treatment options for oil removal to mitigate the impact of oil spill on Indonesia’s water and coastal areas.
Bioremediation is a process to treat the contaminated area by using living organisms or improving their efficiency to degrade pollutants. Two main approaches in the bioremediation technique are bioaugmentation and biostimulation. Bioaugmentation is the introduction of microorganisms culture that possesses the ability to degrade pollutants. In contrast, biostimulation is the modification of indigenous microorganisms environment by adding nutrients essential to their growth to improve pollutant degradation efficiency [3]. Bioremediation eliminates pollutants permanently, offers a cheaper, more environmentally friendly, and publicly more acceptable remediation technique compared to its counterparts [4].

Diaz et al. (2002) showed that immobilized hydrocarbonoclastic bacteria can survive better and has 4-7 times better crude oil degradation efficiency than the free cell form in high salinity seawater environment [5]. Furthermore, immobilized cells are recoverable and reusable, thus lowering the cost of remediation [6].

Porous rocks, such as perlite, silica, vermiculite, and zeolite, have been studied and successfully used as carriers for hydrocarbonoclastic bacteria. For instance, Acinetobacter junii cells were found in high concentration after being immobilized to perlite, especially onto the expanded form with a concentration of $12.65 \times 10^9$ CFU/g [7], while when immobilized onto Mg-exchanged zeolite, A. junii concentration reached with the same greater magnitude ($7.40 \times 10^9$ CFU/g) [8]. Acinetobacter sp. and Methylocaterium sp. immobilized to silica were able to continuously degrade 2,500 mg/L and 10,000 mg/L phenol at high rates for 55 days [9]. PAH degrading microorganisms, Bacillus sp. SB02 and Mucor sp. SF06 immobilized to vermiculite degraded 95.3% of 50 mg/kg benzo[a]pyrene at 25°C in 42 days, much faster than in their free cell forms [10].

Bacillus can form endospores as a response to high cell density or lack of nutrients, such as carbon and nitrogen [11]. Furthermore, Bacillus are able to form biofilm [12]. Biofilm provides protection from stressful environmental condition, such as the presence of antibiotic, predation or immune system recognition [13]. One of the Bacillus that can degrade crude oil is Bacillus licheniformis as it is capable of producing lipopeptide biosurfactant [14], surfactin [15], BL86 [16], and lichenysin [17].

Enterobacter cloacae are one of Enterobacter species that can potentially be utilized as a bioremediation agent as it can form biofilm [18] and biosurfactant [19]. Marine E. cloacae has shown the ability to produce exopolysaccharide that emulsifies aliphatic and aromatic hydrocarbon. E. cloacae was able to emulsify 25% of total hexane, 40% of total xylene, benzene, kerosene and paraffin in 9 days [19]. In another study, E. cloacae LG1 proved its ability as a potential crude oil bioremediation agent by degrading 92.91% of total chrysene; 92.21% of total anthracene, 77.19% of benzo(b)fluoranthene; and 52.33% of polyaromatic fractions [20].

The purpose of this experiment is to assess the viability and efficacy of hydrocarbonoclastic bacteria consortium Bacillus sp., Bacillus licheniformis, and Enterobacter cloacae immobilized to porous rock carriers (perlite, silica, vermiculite, and zeolite) after being stored at 25°C for three months, as well as to determine the ideal type of porous rock which can be used as carriers for Bacillus sp., B. licheniformis and Enterobacter cloacae. We can evaluate the effect of this storage system on the performance of immobilizing bioremediation agents in the four different carriers.

2. Materials and Method

2.1. Bacterial culture

Bacteria cultivation evaluates the effect of the storage system on immobilizing bioremediation agents’ performance in four different carriers (Perlite, Silica, Vermiculite and Zeolite). The cultures of hydrocarbonoclastic bacteria Bacillus sp., B. licheniformis and E. cloacae were provided by Microbiology Laboratory, Research Center for Oceanography-Indonesian Institute of Sciences (P2O-LIPI), Jakarta. The hydrocarbonoclastic bacteria were pre-grown separately in marine broth for 12 hours at 28°C. Cell density of each species of bacteria was determined using spectrophotometry ($\lambda = 600$ nm) and diluted based on the lowest concentration found.
2.2. Carriers
The carriers used in this experiments are perlite, silica, vermiculite and zeolite. The porous rocks were sieved through 850-2,000 µm sieve. Sieved rocks were activated using by soaking them in HCl 1% and agitated on a rotary shaker for 30 minutes [21]. Afterward, the acid was drained and the rocks were soaked in NaOH 1% and agitated on a rotary shaker for 30 minutes. The alkali was drained and the rocks were rinsed using distilled water. The rocks pH were adjusted to neutral. Activated porous rocks were dried using oven at 120°C for 4 hours.

2.3. Immobilization
Bacterial consortium culture was mixed to activated carriers in a 1:1 ratio in falcon tubes, incubated and agitated on a rotary shaker for 24 hours. Immobilized bacteria were preserved by lyophilization or freeze-drying at Biomaterial Laboratory, LIPI Cibinong, Bogor. Preserved immobilized bacteria were stored at 25-27°C for 3 months. Cell loading capacities in each carrier were performed by Total Plate Count method. One gram of immobilized bacteria was weighed aseptically and 9 mL of filtered marine water was added. The mixture was sonicated and vortexed for 10 minutes each. A serial dilution was then carried out and the last three dilutions were inoculated in Marine Agar using the pour plate method. For each replicate, 2 technical replications of 3 dilution factors were used to improve the accuracy of TPC. Bacterial culture was incubated at 28°C for 48 hours.

2.4. Mousse oil
Mousse oil was made by mixing Arabian Light crude oil (ALCO) and seawater in the ratio of 1:3 [22]. Mixing was done in 3 stages. Firstly, 250 g of seawater was added to 250 g of ALCO and mixed thoroughly. Afterwards, 250 g of seawater was added to the mixture and mixed. The latter step was repeated twice in order to produce 1 kg of mousse oil with ALCO to seawater ratio of 1:3 [23].

2.5. Experimental design
Figure 1 shows the design of the microcosm. PVC pipes (9 cm in diameter and 13 cm in height) were used as the microcosm containers. The microcosms were prepared by adding the components as stated in Table 1 into PVC pipes. One side of the pipes was sealed using 50-micron nylon filter mesh to keep the microcosms components inside while allowing seawater to diffuse in and out of the system. Glass tanks (20 x 20 x 15 cm³) were used as 2.2 L seawater containers. Aeration of each microcosm was maintained by the use of electric pump, connected to the microcosms using flexible tubes and pipettes. Each treatment had 3 replicates, and thus a total of 21 microcosms were constructed.

Table 1. Microcosm composition for oil degradation treatment

| Treatments                  | Component     | Sediments (g) | Mousse oil (g) | Gramafix (g) | Broth culture bacteria (mL) | Immobilized cell bacteria (g) in 4 different carrier |
|-----------------------------|---------------|----------------|----------------|---------------|-----------------------------|-----------------------------------------------------|
| Negative Control (Cn)       |               | 90             | 40             | -             | -                           |                                                     |
| Free Cell Control (Cb)      |               | 83             | 40             | -             | 7                           |                                                     |
| Fertilizer Control (Cp)     |               | 90             | 40             | 3.41          | -                           |                                                     |
| S                           |               | 83             | 40             | 3.41          | -                           | 7 (Silica)                                         |
| V                           |               | 83             | 40             | 3.41          | -                           | 7 (Vermiculite)                                    |
| Z                           |               | 83             | 40             | 3.41          | -                           | 7 (Zeolite)                                        |
| P                           |               | 83             | 40             | 3.41          | -                           | 7 (Perlite)                                        |
2.6. Scanning Electron Microscopy
Physical characteristics of porous rocks surface were observed by the help of scanning electron microscopy (SEM). SEM of porous rock carriers was performed in the Biomaterial Laboratory of LIPI Cibinong. Magnification of 60x and 1500x were used in the SEM analysis.

2.7. Enumeration
The density of microorganisms in each microcosm was determined by total plate count (TPC). TPC only estimated the number of culturable and aerobic bacteria. Serial dilution was made using 1 g of microcosm sediment. Marine agar (MA) media was inoculated with 100 µL of diluted microcosm sediment and spread thoroughly. Bacterial culture was incubated at 28°C for 48 hours before counting the colonies. For each replicate, 2 technical replications of 3 dilution factors were used to improve the accuracy of TPC.

Colonies were counted using a hand-tally counter. The normal range of colony numbers in one plate is between 25-250 colonies. The number of colonies out of the normal range was not considered valid and therefore, was not included in the enumeration calculation. The enumeration formula used in this experiment was based on Maturin and Peeler (2001) [24].

\[ N = \frac{\sum C}{V \times [(1 \times n_1) + (0.1 \times n_2)] \times d} \]

- \( N \) = number of colonies (CFU/g)
- \( \sum C \) = number of colonies on all plate
- \( V \) = volume added to each plate (0.1 mL)
- \( n_1 \) = number of plates counted of the first dilution
- \( n_2 \) = number of plates counted of the second dilution
- \( d \) = dilution from the first plates

2.8. Total Petroleum Hydrocarbon (TPH)
Analysis of TPH was used to determine crude oil degradation activity of immobilized bacteria. Fourier-transform infrared (FTIR) spectroscopy was used to analyze TPH. Crude oil was extracted from 0.5 g of microcosm sediment using 5 mL dichloromethane (DCM) and n-hexane, alternately. In
order to extract the crude oil from microcosm sediment completely, mixture of microcosm sediment and solvent was subjected to sonication for 10 minutes. The aqueous phase of the mixture was poured onto Na$_2$SO$_4$-added filter funnel. Na$_2$SO$_4$ powder was added to trap water molecules in the mixture, allowing only dissolved hydrocarbons to be transferred through the filter funnel into a test tube. The process was repeated using alternate solvents (i.e., DCM and n-hexane) until a clear aqueous extract was produced – usually to a total of 6 cycles. The solvent was allowed to evaporate completely, leaving a layer of crude oil at the bottom of the tube. For FTIR spectroscopy analysis, extracted crude oil was dissolved using 3 mL of tetrachloroethylene.

Crude oil degradation ability of immobilized bacteria was determined by the percentage of TPH (dTPH) decrease on each sampling time compared to the TPH on day-0. Therefore, dTPH was calculated based on the modified formula taken from Bishnoi, Kumar and Bishnoi (2008) [25].

\[
dTPH = \frac{TPH_n - TPH_0}{TPH_0} \times 100\%
\]

\[pTPH \quad = \text{percentage of the decrease of TPH on } n\text{th sampling day}\]
\[TPH_0 \quad = \text{concentration of TPH on 0th sampling day}\]
\[TPH_n \quad = \text{concentration of TPH on } n\text{th sampling day}\]

2.9. Environmental Parameters

Environmental parameters in microcosm study, such as pH, temperature, and salinity, were measured. To measure such parameters without adding risk of contamination to a microcosm, microcosm’s pore water was collected in a container that had been previously disinfected. Temperature and pH of pore water were measure using HORIBA Navi D-54 pH meter. Salinity was measured using hand refractometer. Environmental parameters were measured in 3 technical replications on each treatment replicate.

2.10. Data Analysis

Collected data were statistically analyzed using one-way analysis of variance (ANOVA) at 95% confidence level. Afterward, Tukey Honest Significant Difference (Tukey HSD) was performed to show which groups were significantly different to the others.

3. Result and Discussion

3.1 Porous rock carriers surface characteristics.

Perlite carrier (Figure 2A and 2B) was observed to have a highly porous and rough surface. Perlite’s pore sizes vary from 100 µm to 200 µm with irregular shapes. This observation aligned with the result of previous experiments where perlite showed a total porosity of 92% [26]. Such high porosity provided a relatively huge surface area as a support matrix to allow bacterial cell immobilization.

Silica carrier (Figure 2C and 2D) was a smooth surface with no visible pores. SEM analysis of 1500x magnification did not clearly show any porous structure on silica’s surface. The surface area’s values, total pore volume, and average pore radius of this carrier were the lowest than the other three materials studied. These were 12.865 m$^2$/g, 0.014 cc/g and 16.08 Å, respectively [21]. Low porosity and small pore indicated a relatively low surface area that potentially lowered bacterial cell immobilization efficiency and, consequently, the crude oil degradation activity.

Vermiculite carrier (Fig 2E and 2D), similar to silica carrier, had a smooth surface with no visible pores. Pores were not observed in its SEM analysis, and hence, its pore size was unable to be determined. However, SEM analysis showed a layered structure on the vermiculite surface. A chemically processed vermiculite had a total pore volume of 0.48 cm$^3$/g [27]. As a phyllosilicate, exfoliated vermiculite has layers. Between layers, interlayer spaces exist and increase the porosity of vermiculite. Therefore, interlayer spaces in vermiculite carriers provide areas for cell immobilization that are well-protected from external environment.
Lastly, the zeolite carrier (Figure 2G and 2F) a relatively rough surface. Small pores were observed on the surface of the zeolite carrier. However, the pore sizes could not be determined from SEM analysis of zeolite carrier as they were too small. Based on previous report by Jha and Singh (2011), zeolite of clinoptilolite type had pores that were 4-7 Å in diameter and 33% porosity [28]. Rough surface of zeolite carrier increases the surface area for bacterial cell immobilization.

![Figure 2. Visualization of mineral surface using SEM in two different magnification. a) Perlite in 60x; b) Perlite in 1500x; c) Silica in 60x; d) Silica in 1500x; e) Vermiculite in 60x; f) Vermiculite in 1500x; g) Zeolite in 60x; and h) Zeolite in 1500x; i) Perlite with Bacteria in 1500x; j) Silica with Bacteria in 1500x; k) Vermiculite with Bacteria in 1500x; l) Zeolite with Bacteria in 1500x](image)
3.2 Cell loading capacity

Cell loading capacities of porous rock carriers that had been inoculated with hydrocarbonoclastic bacteria *Bacillus* sp. PC-3, *B. licheniformis* and *E. cloacae* were analyzed. Table 2 shows that vermiculite had the highest cell loading capacity of $9.37 \times 10^6$ CFU/g, while silica carriers had the lowest cell loading capacity of $2.13 \times 10^5$ CFU/g. Variation in cell loading capacity as shown in Table 1 could be due to the difference in physical characteristics of porous rock carriers, such as porosity and surface area. This can be seen as highly porous rocks, such as perlite and vermiculite carriers, showed relatively high cell loading capacity, whereas silica carrier that was low in porosity and surface area had the lowest cell loading capacity of the four types of porous rock carriers.

In general, the four porous rock carriers in this experiment showed lower cell loading capacity compared to previous studies. Ivankovic, Hrenovic, and Sekovanic (2010) reported that perlite had a cell loading capacity of $1.68 \times 10^9$ CFU/g, far exceeded the one observed in this experiment, which was $5.37 \times 10^6$ CFU/g [7]. Khongkaem, Intasiri and Luepromchai (2011) reported that silica was able to hold up to $10^9$ CFU/g of the immobilized bacterial cell, while in this experiment, silica only carried $1.23 \times 10^5$ CFU/g [9]. Vermiculite was previously reported to be able to carry $10^{12}$ CFU/g of immobilized bacterial cell [10], while it only carried $9.37 \times 10^6$ CFU/g of immobilized bacterial cell in this experiment. Lastly, Hrenovic, Ivanovic and Tibljas (2009) reported that zeolite was able to carry $9.52 \times 10^9$ CFU/g of immobilized bacterial cell, while its cell loading capacity in this experiment was only $5.10 \times 10^6$ CFU/g [8]. Such low cell loading capacity might be due to lower concentration of inoculum culture ($4.98 \times 10^8$ CFU/g). The ones used in previous experiments had 2 digits higher ($5.27 \times 10^{10}$ CFU/g) inoculum concentration. The difference of incubation method that bioparticle in previous study were prepared by aerobically incubating might also contribute a good impact on bacterial growth.

![Table 2. Cell loading capacity of mineral carrier](image)

| Type of carrier | Cell Loading Capacity (CFU/g) |
|-----------------|--------------------------------|
| Silica          | $2.13 \times 10^6$             |
| Vermiculite     | $9.37 \times 10^6$             |
| Zeolite         | $5.10 \times 10^6$             |
| Perlite         | $5.37 \times 10^6$             |

3.3 Cell retention capability

Porous rock carriers could retain immobilized bacterial cells for 3 months at room temperature (25-27°C). The highest density of recoverable immobilized bacteria was $1.27 \times 10^4$ CFU/g which was observed in vermiculite carrier, while the lowest was silica with $1.80 \times 10^3$ CFU/g (Table 3).

However, regardless the type of porous rock carriers, decrease in immobilized bacteria density was observed after 3 months storage at room temperature (25-27°C). Decrease in immobilized bacteria density might be due to the incomplete elimination of water molecules in freeze-drying process. As a result, a portion of immobilized cells were not dormant and hence, undergo cell death as there was limited nutrient available.

Nonetheless, this result showed an improvement compared to the previous experiment by Wilson and Bradley (1996) whereby freeze-dried immobilized cells were not viable and cultivable beyond 2 months of storage at room temperature [29]. This showed that porous rock carriers were able to retain immobilized bacterial cells in a longer period compared to the ones used in the previous experiment. Unfortunately, the explanation of such observation in which different carriers show various cell retention capabilities has yet to be determined.
Table 3. Cell viability after storage at room temperature for 3 months.

| Type of carrier | Cell viability (CFU/g) |
|-----------------|------------------------|
| Silica          | 1.80 x 10^3            |
| Vermiculite     | 1.27 x 10^4            |
| Zeolite         | 8.43 x 10^3            |
| Perlite         | 1.06 x 10^4            |

3.4 Hydrocarbonoclastic bacteria density in microcosms

The density of hydrocarbonoclastic bacteria present in each microcosm was analyzed as one parameter that indicates crude oil degradation ability. The average of bacterial density during all immobilization cell treatment experiments was higher than control, except in fertilizer control (Cp) (Table 4). The highest bacterial density of every treatment was found after 7 or 14 days of incubation, and decreased thereafter (Table 4). This indicates that nutrients supplied by slow release fertilizer Gramafix were still available and other environment factors were still favorable for bacterial growth until 14 days of incubation. However, role of bacterial cell inside all carrier in enhancing oil degradation remains unclear. The low number of bacterial as an impact of storage may be the reason.

The highest bacterial average during experiment was observed in Perlite treatment, 8.37 x 10^8 CFU/gr (Table 4). The bacterial density dynamic in Perlite treatment was slightly different with other treatment. The highest density (1.94 x 10^9 CFU/gr) was achieved in the first week after incubation and the number during late stage (14 and 28 days after application) was higher than other, even when we compared with control fertilizer. Although, it was not significantly different. The second highest bacterial density was 1.93 x 10^8 CFU/mL which was observed in Vermiculite treatment. It was occurred after 14 days of incubation, and this result was significantly higher than Zeolite treatment, free cell control and negative control. However, this result was not significantly higher than fertilizer control and other porous rock carrier treatments. This observation may indicate that immobilizing hydrocarbonoclastic bacterial cells that had been stored for three months at room temperature had little to no significant effect on increasing the survival rate of cells. However, perlite and vermiculite has showed potential carrier to be used for the next experiment to find the right temperature for storage.

Addition of slow release fertilizer Gramafix might potentially be an important factor in improving remained hydrocarbonoclastic bacterial cells survival rate. This might be due to the supplementation of nitrogen and phosphate released by Gramafix that previously acted as limiting factors to hydrocarbonoclastic bacteria metabolism after the addition of crude oil in microcosms. As a result, more hydrocarbonoclastic bacteria – indigenous, free-cell or immobilized – were able to undergo metabolism that degraded crude oil in the process. Previous studies has proved that application of this fertilizer in the right amount will positively enhance the growth of oil degrading bacteria and increase the rate of oil degradation [21,30,31].

Table 4. The number of bacteria in each treatment during experiment

| Treatment | Total Plate Count (CFU/mL) |
|-----------|----------------------------|
| t         | 0 day | 7 days | 14 days | 21 days | 28 days |
| Cn        | 1.98 x 10^7 | 1.18 x 10^7 | 2.31 x 10^7 | 2.10 x 10^7 | 1.61 x 10^7 |
| Cb        | 1.48 x 10^7 | 5.28 x 10^7 | 1.20 x 10^7 | 6.67 x 10^7 | 2.07 x 10^7 |
| Cp        | 6.35 x 10^8 | 1.53 x 10^8 | 2.30 x 10^8 | 3.85 x 10^8 | 5.24 x 10^8 |
| S         | 1.49 x 10^7 | 6.07 x 10^8 | 1.73 x 10^8 | 3.92 x 10^8 | 4.82 x 10^8 |
| V         | 2.66 x 10^7 | 1.11 x 10^8 | 1.93 x 10^8 | 3.97 x 10^8 | 1.63 x 10^8 |
| Z         | 1.83 x 10^7 | 6.22 x 10^7 | 4.71 x 10^7 | 6.49 x 10^7 | 1.63 x 10^8 |
| P         | 1.47 x 10^7 | 1.94 x 10^7 | 7.83 x 10^7 | 9.12 x 10^7 | 5.35 x 10^7 |

Note: Values incorporated in the same subset (a, b, c, d) on the same sampling day do not have significant differences and vice versa. Cn = Negative Control; Cb = Free Cell Control; Cp = Fertilizer Control; S = Silica; V = Vermiculite; Z = Zeolite; and P = Perlite
3.5 Crude oil degradation
The ability to degrade crude oil was analyzed based on the percentage of TPH decrease. Based on Table 5 the highest percentage of TPC decrease was observed in Zeolite treatment, which amounted to 91.72% after 28 days of incubation. On the other hand, the lowest percentage of TPH decrease was observed in negative control microcosm, which only amounted to 10.85% after 28 days of incubation.

Table 5. Oil Degradation Percentage in each treatment during 28 days experiment.

| Treatment | 7 days | 14 days | 21 days | 28 days |
|-----------|--------|---------|---------|---------|
| Cn        | 2.55\(^a\) | 3.83\(^a\) | 5.98\(^a\) | 10.85\(^a\) |
| Cb        | 3.35\(^a\) | 4.77\(^a\) | 8.11\(^a\) | 13.57\(^a\) |
| Cp        | 55.74\(^b\) | 88.21\(^b\) | 89.61\(^b\) | 90.63\(^b\) |
| S         | 56.52\(^b\) | 89.27\(^b\) | 90.17\(^b\) | 90.74\(^b\) |
| V         | 61.44\(^b\) | 90.35\(^b\) | 91.1\(^b\) | 91.63\(^b\) |
| Z         | 58.59\(^b\) | 89.81\(^b\) | 90.85\(^b\) | 91.72\(^b\) |
| P         | 60.81\(^b\) | 88.95\(^b\) | 89.86\(^b\) | 90.53\(^b\) |

Note: Values incorporated in the same subset (a, b) on the same sampling day do not have significant differences and vice versa. Cn = Negative Control; Cb = Free Cell Control; Cp = Fertilizer Control; S = Silica; V = Vermiculite; Z = Zeolite; and P = Perlite

As shown on Table 5 microcosms of immobilized cell treatments showed higher percentage of TPH decrease compared to negative control and bacterial control in a duration of 28 days. The percentage of TPH decrease in microcosms of immobilized cell treatments surpassed was over 90%, significantly higher than those observed in microcosms of negative control and bacterial control that only experienced 10.85% 13.57% of TPH decrease respectively. However, nitrogen and phosphorus control such high percentages of TPH decrease observed in microcosms of immobilized cell treatments were not significantly different from that of nitrogen and phosphorus control. This shows that immobilization of hydrocarbonoclastic bacterial cells to porous rock carriers had not improved the ability of crude oil degradation in polluted microcosms in this experiment. Moreover, the percentage of TPH decrease observed in nitrogen and phosphorus control microcosm might suggest that supplementation of nitrogen and phosphorus previously were the limiting factors in crude oil metabolism was a more important factor in increasing the efficacy of bioremediation compared to cell immobilization to porous rock carriers.

High rate of crude oil degradation was observed on the first and second weeks of incubation and slowed down thereafter. This can be seen as the crude oil degradation in microcosms supplemented with slow release fertilizer Gramafix (immobilized cells treatments, and nitrogen and phosphorus control) were around 60% in the first 7 days and substantially increased further as much as about 30% in the next 7 days (day-7 to day-14). Afterwards, the rate of crude oil degradation slowed down as the increase in percentage of TPH decrease was only about 1% per 7 days of incubation. This might be due to the complete release of nutrients by Gramafix in 14 days after application [32]. As most nitrogen and phosphorus had been utilized and depleted in the first 14 days, those nutrients became limiting factors in crude oil metabolism by hydrocarbonoclastic bacteria and therefore, inhibited further crude oil degradation [33].
3.6 Microcosms environmental parameters

3.6.1. pH. Generally, pH of all microcosms experience an increase throughout the incubation. Addition of nitrogen and phosphorus source in the form of slow release fertilizer Gramafix lowered the pH in a microcosm environment. pH value of microcosms added with Gramafix were around pH 6, lower than those of negative control and free-cell control which were pH 6.7 and 6.8 respectively on the first sampling time (day-0).

A variation in microcosm pH increase was observed. This might be due to the production of crude oil degradation side products, such as phthalic acid and benzene acetic acid that were produced as byproducts of anthracene degradation by *B. licheniformis* [34]. Moreover, fatty acids were required in the production of biosurfactants [35].

3.6.2. Temperature. In general, all microcosms experienced a slight temperature decrease throughout 28 days of incubation. The microcosms temperature ranged between 22.5°C to 24.5°C. However, the difference between each microcosm temperature was not significant. Moreover, the temperatures were still in the range of optimum temperatures for hydrocarbon degradation, which is 20-35°C [36].

3.6.3. Salinity. Generally, all microcosms experience an increase in salinity throughout 28 days of incubation. Microcosms salinity ranged from 32‰ to 62‰. There was a variation in the initial salinity whereby microcosms that were supplemented with nitrogen and phosphorus source had higher salinity of about 40‰, while negative control and free-cell control only had 32‰ and 33‰ salinity, respectively.

In the first 14 days of incubation, salinity increased due to the release of nutrients by the slow-release fertilizer Gramafix. Gramafix was reported to release its nutrients completely in 14 days [32]. Salts might get concentrated due to the decrease of seawater volume in microcosms, thus increasing salinity beyond 14 days of incubation [37]. Evaporation might also reduce the salinity. In a previous study using a closed reactor at a similar nutrient level (fertilizer), the salinity range during the experiment was observed at 45-50 ‰ [23].

4. Conclusion

Based on the results, it can be concluded that the introduction of bioremediation has increased oil degradation. Storage for three months at a temperature of 25-27 °C still allows the immobile bacteria in porous rock to be viable, but the number has been decreased. The difference in efficacy between carrier treatments was not significant due to low in cell number, and biased by the impact of fertilizer application in this experiment. It is important to note that crude oil degradation via bioremediation can be assumed to be very effective during the first 14 days of incubation. Vermiculite, Zeolite and Perlite are a good candidate carrier, although the right immobilization technique for long storage need to be improved.

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