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Biodegradation of Crude Oil Spill Using *Bacillus Subtilis* and *Pseudomonas Putida* in Sequencing Method

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ABSTRACT

Crude oil, otherwise called petroleum, occurs naturally as a complex organic mixture underneath the subsurface. The activities related to its exploration, production, refining, storage and distribution are mostly accompanied with extreme pollution and other hazardous conditions. For these reasons, the need to critically devise the best possible solutions becomes paramount, particularly as regards oil spills. Therefore, the purpose of this research was to determine the efficiency of TPH removal in crude oil using *Bacillus Subtilis* and *Pseudomonas Putida*. The sequencing method was applied in a laboratory scale and under artificial seawater media conditions. The total petroleum hydrocarbon (TPH) serves as a significant parameter in detecting crude oil, although the extraction and analysis were conducted with the use of a separator funnel and gas chromatography mass spectrometry (GCMS), respectively. In addition, the simulated seawater media was described as the mineral salt medium (MSM), with 33% salinity. Moreover, five reactors were also employed, including K for control, B for *B. subtilis*, P for *P. putida,* BP for *B. subtilis* and *P. putida* sequence and PB for *P. putida* and *B. subtilis* sequence. The entire treatments obtained the access to two replicate reactors. Furthermore, the bacteria inoculum and crude oil concentration in each unit were estimated at 5% and 10% (v/v), respectively. The results achieved the maximum TPH removal at 66.29% in the PB reactor after 35 days. On the basis of ANOVA reports, no significant variation was observed between the sequential additions of a single bacterial treatment and consortium microbes. In summary, two bacterial species demonstrated high potential to degrade TPH, but predicted an increase in the break down time, as the nutrient or oxygen tends to accelerate the process.

Keywords: biodegradation, crude oil, seawater, sequence bacteria inoculum, single bacteria inoculum, TPH

INTRODUCTION

Petroleum is very useful in the performance of daily activities. Oil, diesel, gasoline, avtur, and liquefied petroleum gas (LPG) are the major products from the refining process. According to the Indonesian Decree of the Minister of Environment Number 128 in 2003, crude oil is the result of a natural process in the form of hydrocarbons. Under atmospheric pressure and temperature conditions, a liquid or solid phase is attained, including asphalt, mineral wax, or ozokerite, and bitumen during mining, but does not include coal or hydrocarbon deposits. Other solid forms are based on the events unrelated to oil and business operations.

The petroleum activities are not exempted from the impact on the immediate environment. One of the main prevailing problems is associated with crude oil spills. These events are also caused by tanker accidents, pipe leaks, as well as pressure anomalies during drilling and port operations. In addition, instances in ocean sources are referred as rapid spreads, due to the influence

of wind, waves and sea currents (EPA, 1999). The breeze blowing above the sea level is attributed to ocean currents (Hadi and Radjawane, 1999; Wibowo, 2018), and therefore shows the capacity to extend oil spills to shoreline areas. This also contributes to the negative impacts on marine ecosystems and coastal communities. Furthermore, TPH is commonly measured where hydrocarbon discharges are predominant (e.g., leaking gasoline, diesel, or fuel oil tanks and petroleum product spills). In most instances, the analytical approach does not provide specific information on the existing TPH fractions.

Majority of the efforts in dealing with oil spill effects have been conducted, using the physical and chemical methods. However, upon review, the techniques tend to leave behind a residue capable of sinking or simply migrating the oil to a different location, with the possibility of a lasting impact on the underwater ecosystem. Therefore, bioremediation offers a more effective waste treatment, with optimal cleaning potentials (Damayanti, 2013). Bioremediation refers to an economical and more effective technology for degradating hydrocarbons, due to operational ease and maintenance (Palanisamy et al., 2014). The main principle of this process is to explore the microbial metabolism (AlDisi et al., 2016).

Sudrajat et al. (2015) reported the ability of *Pseudomonas sp.* and *Bacillus sp.* to degrade TPH in the oil-contaminated soils by 67 and 61%, respectively, while Vinothini et al. (2015) revealed an estimate of 65% for *Pseudomonas putida* in crude oil category. Several bioremediation studies on petroleum-polluted soils and water have already been conducted. However, the effectiveness of crude oil degradation in seawater due to the sequencing method of bioremediation appears limited. Therefore, the purpose of this research is to determine the efficiency of TPH removal in crude oil in a laboratory scale by means of the sequencing method, using *Bacillus Subtilis* and *Pseudomonas Putida*.

Table 1. Some parameters in sample of crude oil

No.	Parameter	Unit	Results
1	Total organic carbon	mg/L	134,.362
2	Total organic nitrogen	mg/L	141.96
3	Total phosphate	mg/L	27.08
4	Oil and grease	mg/L	118,400
5	pH	–	7.60
6	Temperature	°C	29

MATERIALS AND METHODS

Crude oil sampling

This process was conducted at one of PT Pertamina Hulu Energi West Madura Offshore's oil refineries by an in-house employee. The field is located in the middle of the ocean and therefore, is highly restricted to the personnel only.The crude oil was stored in a 500 mL sterile glass bottle with screw cap (Duran, Germany) and well packaged, using bubble wrap. Table 1 shows the value of certain parameters in the crude oil sample.

Bacterial preparation

This stage was achieved by inoculating the pure culture (*B. subtilis* and *P. putida*) on slanted NA media, using the scratch method. Subsequently, the inoculant bacteria were incubated at 37°C between 18–24 h before use.

Test of crude oil biodegradation

Prior to the biodegradation test, the range finding assessment on *B. subtilis* and *P. putida* was expected. On the basis of our previous study (2020), both bacteria were able to survive effectively at various concentrations of 5, 10 and 15% (v/v).

The independent variables include the bacteria type and inoculum percentage, termed 5% (v/v), while the dependent constraints were TPH, pH, temperature and CFU values. However, the research was conducted using a rotary shaker with 150 rpm speed at room temperature (Ibrahim, 2016) for 35 days. Moreover, the biodegradation test was performed in duplicates, while the sampling occurred at 3 intervals of days 0, 14 and 35. Furthermore, the addition of a bacterial inoculum for a single test reactor was initiated at day 0. Meanwhile, the bacterial inoculum was introduced in the sequence test up to 2.75 mL (2.5% of the amount of initial media) at the start of the first bacteria, followed by involving the second bacterial inoculum of 2.75 mL after the 7th day. Therefore, the number of added bacteria between the single test and similar sequence was 5.5 mL or 5% of the total media (MSM + Crude Oil).

MSM liquid or mineral salt media was prepared by dissolving various chemicals, with compositions, termed 1 g/L $NH_4.2SO_4$, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.2 g L $MgSO_4.7H_2O$, 0.05 g/L $FeCl_3$, 0.02 g/L $CaCl_2$, and 33.53 g/L NaCl, as referred by Ramasamy *et al.* (2017). These substances were weighed and dissolved in 1000 mL of distilled water in a volumetric flask. Subsequently, the mixture was transferred to several 250 mL Erlenmeyer flasks filled with 180 mL of MSM, prior to sterilizing with autoclave. The NaCl content of 33.53 g/L produced a salt concentration of 33 ‰ or 3.3%, as indicated in salt/sea water category. Moreover, the nitrogen source contained $NH_4.2SO_4$, while phosphorus incorporated KH_2PO_4 and K_2HPO_4. However, to achieve the C: N: P ratio of 100: 5: 1, there is need to involve nitrogen and phosphorus sources, based on appropriate calculations.

Analysis of total petroleum hydrocarbon

The TPH analysis was conducted using the gravimetric method, as modified by Imron (2018) and Nadhirawaty (2019). A sample of 15 mL (artificial media + crude oil + bacteria) was separated by applying a 4000 rpm micro-centrifuge for 15 minutes. Approximately, 10 mL of a distinct component was extracted twice with a separating funnel twice by adding 20 mL n-hexane solvent. This was then transferred to a beaker glass and allowed to stand until n-hexane evaporated. Subsequently, the resulting sample was conveyed to a small glass bottle weighed using an analytical balance and also allowed to stand until the entire n-hexane content was liberated, followed by the final weight determination. Furthermore, the mass difference of the extracted liquid in a small glass bottle before and after evaporation was recorded as a % reduction value with Formula (1):

$$\text{Removal} = \frac{(\text{weight of flask before extraction in gram} - \text{weight of empty flask in gram})}{\text{weight of initial sample in gram}} \times 100\% \quad (1)$$

The extraction results were analyzed using gas chromatography – mass spectrometry (GC – MS) (HP 6890, USA). Furthermore, the number of samples injected into GC – MS was 0.8 µL with the helium carrier gas. Injector and detector temperatures were specified at 310°C, while oven initial temperature was set to 50°C for 5 minutes, prior to subsequently increase by 5°C / minute to attained 300°C. This final point was then maintained for another 15 minutes.

Determination of colony forming unit (CFU)

The calculation of the number of bacterial colonies was performed at three consecutive intervals, termed days 0, 14 and 35, by the CFU test. This method involved a pour plate, based on Harley and Prescot (2002). First, the germ-free liquid NA medium with a temperature ranged of 42 – 45 °C was added to a sterile disposable petri dish, while a sample of 1 ml from each reactor, was diluted in a test tube containing 9 ml of sterile physiological NaCl solution. This step was conducted until the needed dilution level was attained. Subsequently, 0.1 mL sample was extracted, using a micropipette and the added to the already prepared NA media, followed by gradual agitation to achieve homogeneity. Furthermore, the entire petri dishes were incubated at 37°C for 24 h. The counting of bacterial colonies on each petri dish was conducted using a bacterial colony counter, and the number of colonies per Ml determined by the following formula:

$$\text{CFU/mL} = \frac{\text{number of bacterial colony per petri dish}}{\textit{sample per petri dish mL}} \times \frac{1}{\textit{dilution factor}} \quad (2)$$

Analysis of Statistic

The experimental data during biodegradation test were subjected to a two way analysis of variance (ANOVA), using Microsoft office excel. Statistical significance was defined as $p < 0.05$ for varying factors, including specie type, single bacterial inoculum – sequence bacterial inoculum, and sampling period with percentage response of TPH removal.

RESULTS AND DISCUSSIONS

Figure 1 shows the pH value during biodegradation at a range of 4.3–6.0, where a decline on days 0–14 was observed, followed by an increase on day 35. These fluctuations indicated the process of bacterial growth activity. However, the pH decrease suggested the crude oil degradation was due to the influence of acidic compounds. As

stated earlier, microorganisms utilize the hydrocarbons as carbon substrates to increase population, and also degrade the samples into harmless components, including CO_2 and H_2O. Furthermore, the crude oil content contains the following hydrocarbons, including paraffin (straight chain and branched chain), naphtha (alkyl cyclopentane and alkyl cyclohexane), and aromatics (alkyl benzene, aromatic fluorine naphtha, and aromatic polynuclear), dissolved gases i.e. nitrogen and carbon dioxide, sulfur compounds i.e. hydrogen sulfide and mercaptans, organic nitrogen compounds, organic oxygen compounds, organic metal compounds, colloidal particles: asphalt, resin and wax, water (BS & W) fresh or salty and solids matters i.e. sand, scale from pipes, impurities, and various corrosion products.

Aliphatic compounds comprise easily degradable long and short alkane chains (n-alkanes). In the petroleum decomposition process, the raw sample is converted into alcohol, using monooxygenase (enzyme). These alcohols are further converted to aldehydes and simple acids by dehydrogenation, causing a reduction in the pH value (Darsa et al., 2014; Nwinyi et al., 2014). Moreover, acid formation due to diesel fuel biodegradation, using *Bacillus subtilis* occurs after day 8 (Darsa et al., 2014). The first stage involves the breakdown of aromatic double bond to form aliphatic substances, including simple acids and aldehydes with easy access to metabolic processes, e.g. Krebs cycle.

According to Nwinyi et al. (2014), the decreased pH in the *Bacillus sp*. and *Pseudomonas sp.* application was observed after day 6. This was followed by a pH increase on day 7, as the alkalinity intensified with improved microbial activity (Nwinyi et al., 2014). According to Andriani et al., (2017), *B. subtilis* tolerance was possible at pH of 4 and 6, while a very drastic decline in the number of colonies at pH of 2 was reported. However, *P. putida* tends to survive between a pH of 6–8 (Elsayed and El-Nady, 2013). On the basis of Annadurai et al. (2008), Moore et al. (2006), *P. putida* was assumed to survive at an optimum pH of 4–8.

Figure 2 shows the temperature values in each reactor ranging between 30–31.5°C. This interval is equally important in maintaining similar condition to the pH, during the bacterial growth due to the significant influence of temperature on metabolic processes (Ibrahim, 2016). However, *B. subtilis* was tolerant at 40, 50, and 60°C (Andriani et al., 2017), while *P. putida* tends to develop optimally at 30°C and possibly extended to 40°C (Elsayed and El-Nady, 2013). According to Palleroni (2005), the maximum growth of *P. putida* occurred between 25–30°C. Moreover, the microbe existed as a mesophilic bacterium, with the possibility to survive between 15 – 35°C (Alagappan and Cowan, 2004).

Table 2 shows the physical change results for individual reactor samples on days 0, 14 and 35. The illustration indicated similar conditions

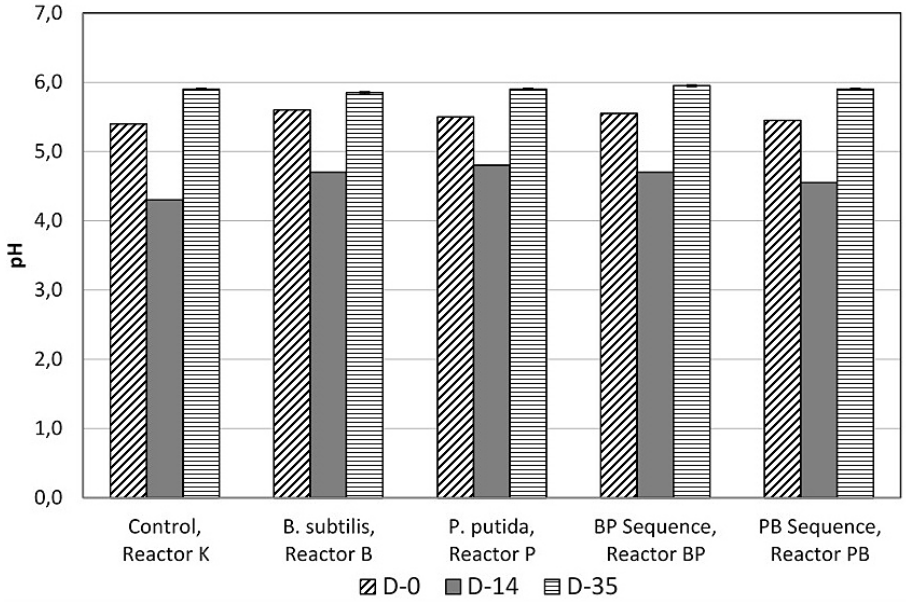


Figure 1. The pH value in each reactor

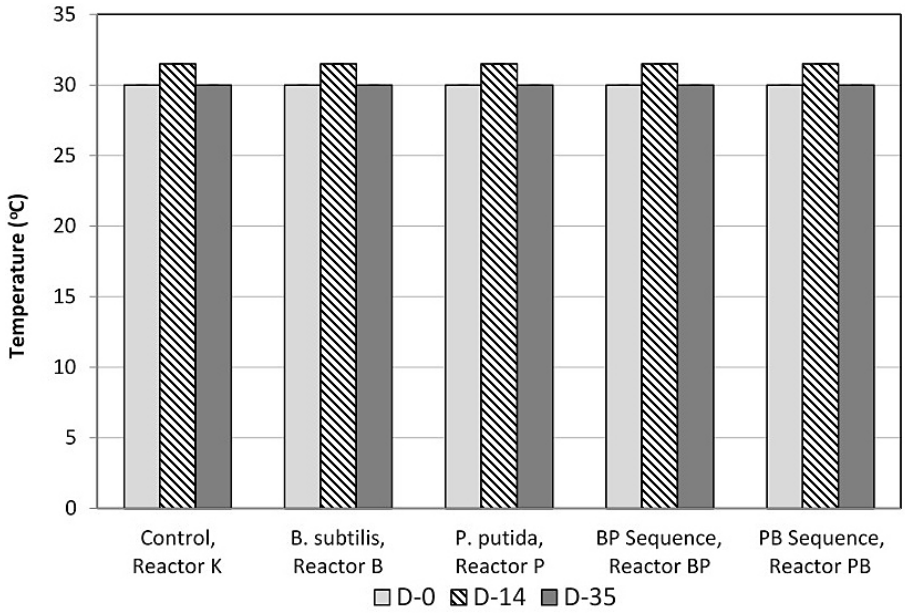
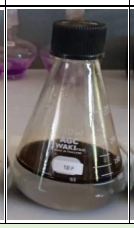
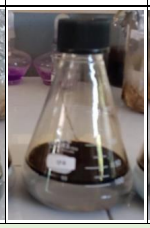
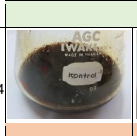


Figure 2. Temperature value at each reactor

on day 0, while based on the observation, a change was noticed in the treatment reactor on days 14 and 35. In addition, the crude oil in the control reactor showed equal circumstances to day 0. Meanwhile, a slight physical alteration was reported in comparison to the first day of the biodegradation test in the form of tiny oil granules in water.

Under ideal conditions, the TPH concentration is expected to be environmentally non-toxic at ≤1% or around 1000 mg/kg in soil or sediment. The sample is a mixture of organic compounds consisting of hydrogen and carbon (C5–C36), as well as a petroleum derivative. Figure 3 shows the initial TPH concentration in each reactor under various conditions. In addition, the estimate was 2.637 mg/L for control reactor, while extensive values of 3.443 mg/L, 2.187 mg/L, 3.015 mg/L and 3.172 mg/L occurred at reactors B, P, BP and PB. Overall, high TPH values were observed. According to Medjor et al. (2018), the TPH in crude oil was approximately 3.000 mg/L. Furthermore, on day 14, the values of 2.469 mg/L, 1.776 mg/L, 2.163 mg/L, 2.568 mg/L and 1.873 mg/L, were

Table 2. Results of Physical Change of Samples at Each Reactor on Day 0, 14, and 35

Day	K	1B	2B	1P	2P	1BP	2BP	1PB	2PB
H-0									
All reactors showed the same physical characteristics: the bottom was clear and the top contains crude oil									
H-14									
	-	+	+	+	+	+	+	+	+
H-35									
	-	+	+	+	+	+	+	+	+

Explanation:
- = There was no change in physical characteristics, compared to H-0, but only a reduction in volume
+ = There was a slight physical change, compared to the previous day, but in the form of tiny oil granules in water.

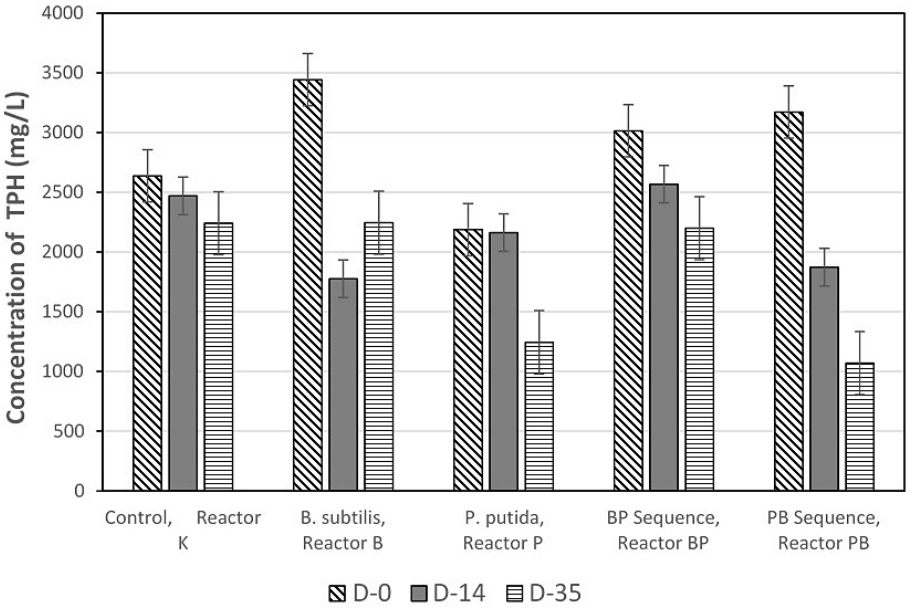


Figure 3. TPH concentration at each reactor

obtained for the reactors of control, B, P, BP and PB, respectively, while at day 35, the estimates extended to 2.241 mg/L, 2.245 mg/L, 1.245 mg/L, 2.199 mg/L and 1.069 mg/L, correspondingly.

On the basis of the ANOVA results, overall treatments in the reactor showed a P value > 0.05. This circumstance reported no significant differences in the TPH concentration values of control, with the additions of single and consortium bacteria in reactor and sequencing, respectively, during the 35 days biodegradation. However, the entire treatments, with different sampling periods, including days 14 and 35 demonstrated a P value < 0.05. The results indicated significant variation of the TPH concentration values on days 0, 14 and 15, in comparison to each reactor treatment.

Figure 4 shows the percentage of TPH removal for 35 days of biodegradation in a batch reactor. The TPH removal was maximum at 6.37% in the control reactor at similar duration, due to the contaminated nature of the added crude oil with bacteria. In addition, the PB reactor showed a more stable percentage removal of 40.96 and 66.29% at days 14 and 35, respectively. However, the entire treatments reactors, B, P, BP and PB were attained below 50%. Meanwhile, PB achieved 66.29% on the 35th day, probably due to the high initial TPH concentration of crude oil between 2.000 – 3.000 mg/L.

The environmental quality standard for seawater (maximum allowed concentration – MAC) of TPH in Romania was stipulated at 50.0 µg/L (Ţiganus et al., 2016). Moreover, TPH was assumed to instigate harmful effects on aquatic organisms above 50 µg/L in seawater. Gordon and Prouse reported that the oil concentrations in excess of 50.0 µg/L were possible to impede the phytoplankton photosynthesis. However, China and Russian Federation applied this concentration of 50 µg/L as seawater quality standard, based on China's Marine Monitoring Standards of GB 3097–1997 and Annex IV Sea Water Quality Standards in Russia. Furthermore, the European Union Environmental Protection Agency (EUEPA, 2009) acceptable standard limit for petroleum hydrocarbons was approximately 300 µg/L in river and basins (Inyang et al., 2018).

On the basis of the ANOVA results, overall reactor treatments showed a P value> 0.05, indicating the percentage of TPH removal in the control reactor with the additions of a single and consortium bacteria, did not provide any significant alteration during the 35 days biodegradation. However, the entire treatments in the reactors with different sampling times, including days 14 and 35, recorded a P value > 0.05, denoting the consistent percentage of TPH removal.

Figure 4 represents the optimal TPH removal in the use of consortium bacteria, compared to single unit. Several studies using single *B. subtilis* and *P. putida* for hydrocarbon biodegradation were conducted. A single isolate of *B. subtilis* was estimated to degrade TPH by approximately 60–80% within one month with a crude oil percentage of 2%. This situation, however, depends on other factors. Meanwhile, a single isolate of

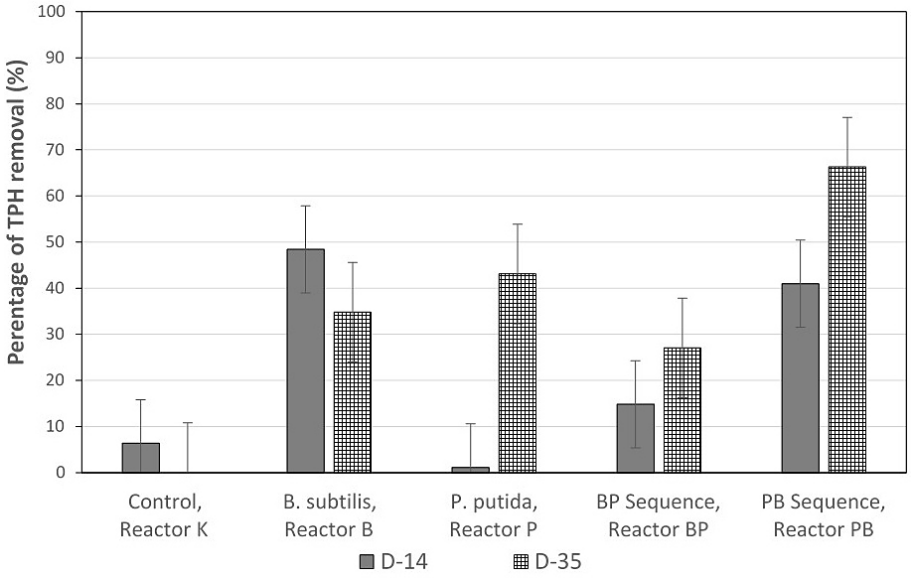


Figure 4. Percentage of TPH removal at each reactor

P. putida achieved approximately 60–70% under similar conditions. In addition, the related samples with high effectiveness attained 65% removal within 7 days. Furthermore, *Bacillus sp*. was the most widely applied in decomposing TPH in contaminated sea water. Moreover, the consortium of various Bacillus bacteria exhibited the greatest potential, extending to 95.5% (for 1% crude oil). The effectiveness level, based on certain papers, was not conclusive, due to the research data reflecting variations in bacterial inoculums, nutritional conditions, concentrations and petroleum types.

On the basis of Figure 4, the sequencing of *P. putida* and *B. subtilis* addition showed the highest TPH removal, compared to the sequencing *B. subtilis* and *P. putida* addition, probably due to the easy adaptation of *B. subtilis* in the media. Subsequently, the *B. subtilis* addition at day 7, and the TPH removal in the PB reactor increased significantly. The growth rates of certain bacteria rarely varied with minimal alterations in lag phase. Under particular stress regimes, microbial growth measures differed considerably, while lag phase remained relatively consistent (Hamill et al., 2020). However, an opposite situation occurred in BP reactor, immediately followed by gradual TPH removal after *P. putida* addition at day 7.

Microorganisms demonstrate the sufficient capacity to degrade crude oil compounds into carbon and nitrogen sources. Meanwhile, the time needed for bioremediation also varies. According to Azubuike et al. (2016), as well as Darmayanti and Afianti (2017), this interval depends on large pollutant concentration and crude oil type, extent and depth of the polluted area and environmental conditions, including temperature, nutrients and microbial populations. Several studies reported faster hydrocarbon biodegradation process of crude oil spills and more complete cleaning, as the applied microbes were not a single type, but a consortium (Nugroho, 2006). This ciricumstance occurred as the hydrocarbons served as a food source, and therefore, after complete breakdown, the microbes automatically expired. Furthermore, bioremediation was a more effective remediation process as no residue was formed. In general, the stages of the biodegradation process described by Sumarsono (2009) and Wenti (2012), are as follows: the sample contained a percentage of the microbial content of hydrocarbon degrading (hydrocarbonclastic) bacteria in the contaminated area; hydrocarbonoclastic bacteria utilize hydrocarbons as energy and carbon sources. Degrading microbes produce biosurfactants capable of dissolving the samples in the liquid phase, reducing surface tension, and increasing the accessibility of the degrading microorganisms to oil grains. Microbes transfer hydrocarbons into microbial cells by interacting with hydrocarbons in the liquid phase, through diffusion or active transport processes or with hydrocarbon droplets emulsified by bacterial cells. Lastly, character, number of cells and enzymes contained in microbes determine the degradation results. This instigated the need for several microbe types in directly

degrading the crude oil, as the enzymes of each species tend to complement each other, in terms of survival, using the available nutrient sources (Siahaan et al*.,* 2013).

Figure 5 shows the TPH content was composed of the C-chain hydrocarbons between C6–C24 and aromatic compounds of benzene and naptalene. The relative abundance represents the number (%) of sample constituents. Figure 5 (a) and (b) shows the TPC at control reactor in days 0 and 35, respectively. Moreover, the TPH reduction at the PB reactor were represented by Figure 5 (c) and (d). The results of GC analysis on days 0 and 35 showed the declining hydrocarbon concentrations. However, the total n-alkanes composition slightly increased, indicating a relatively high persistence of non-aliphatic hydrocarbons and possible conversion of aromatic hydrocarbons to n-alkanes. This was in accordance with the removal percentages, as represented in Figure 4, where the percentage removal at PB reactor were obtained at 40.96% and 66.29% on days 14 and 35, respectively. In addition, the results did not achieve significant proportions.

Crude oil is a combination of complex hydrocarbon compounds, as well as a mixture of

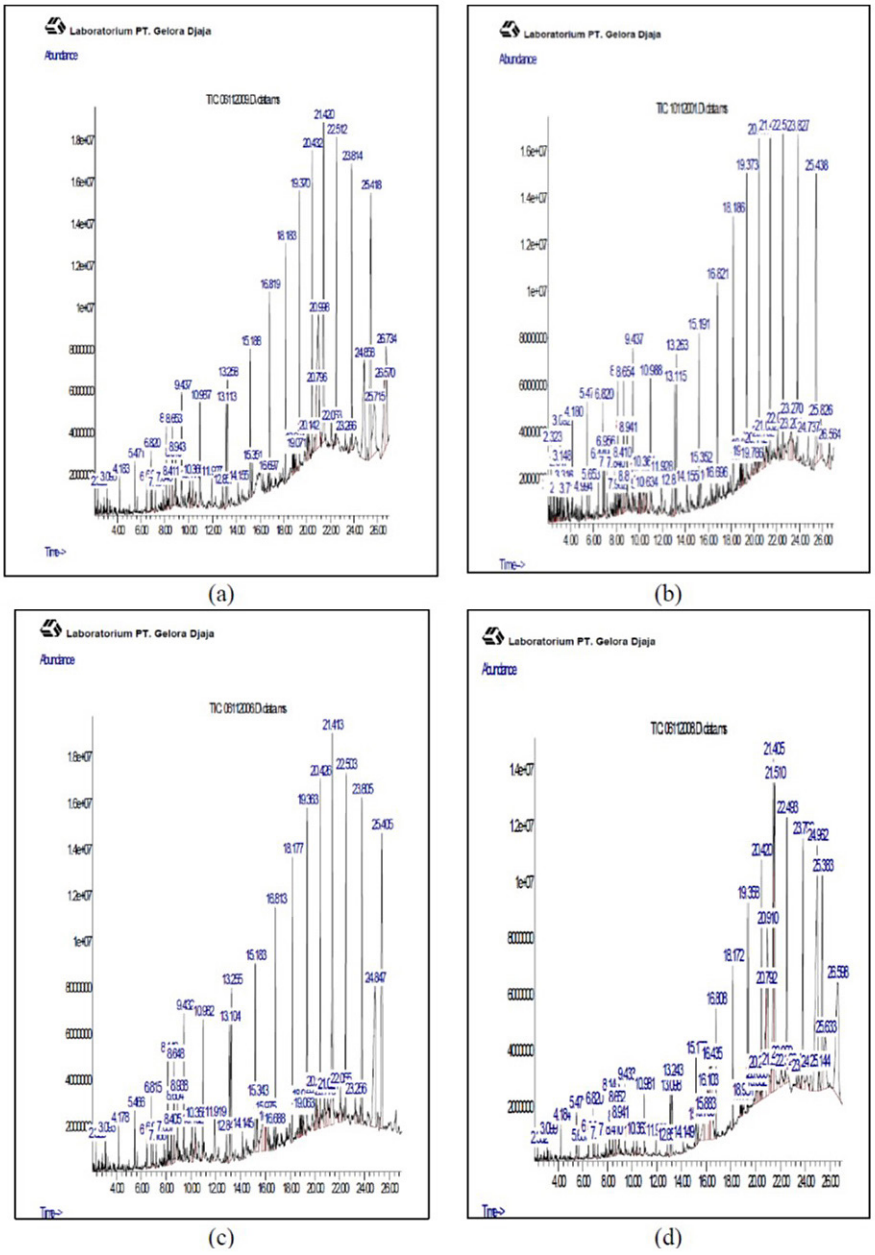


Figure 5. TPH degradation (a) Degradation of TPH at control reactor in day 0, (b) at control reactor in day 35, (c) at PB reactor in day 0, (d) at PB reactor in day 35

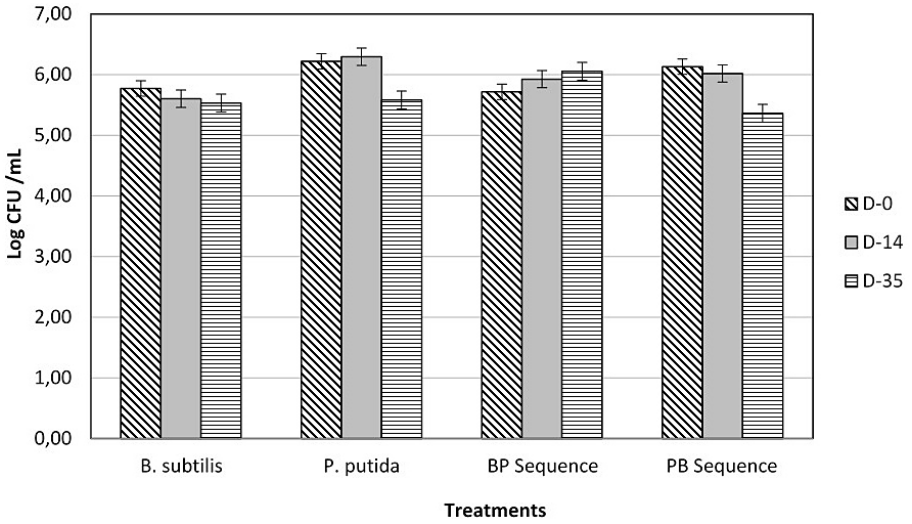


Figure 6. CFU at each reactor

aliphatic and aromatic substances. Imron (2018) described the aliphatic hydrocarbons as composed of long and short alkane chains. The alkanes are readily degradable hydrocarbons, with the simplest (single) bond, while total petroleum hydrocarbons (TPH) is a term used for any hydrocarbon blend in crude oil. However, several hundreds of these compounds presently exist, but do not completely appear in one sample. Furthermore, crude oil generated several petroleum products with a very high contribution to environmental pollution. As a result of numerous chemicals in crude oil and other petroleum products, individual measurement becomes unrealistic, but evaluating the TPH quantity appears very useful. The TPH chemical content included hexane, benzene, toluene, xylene, naphthalene, fluorene, other gasoline constituents, jet fuel, mineral oil, and other petroleum products. A range of petroleum hydrocarbons was monitored at various levels depending on the state and test locations (Das, and Chandran, 2011). On the basis of the molecular weight, n-alkanes are randomly divided into three fractions, termed alkanes with low molecular weight (C9–C10), violation of alkanes of medium molecular weight (C16–C22); and the molecular weight of the alkanes (C23 and C24) [35].

Figure 6 shows the number of bacteria in each reactor during biodegradation testing. On the basis of this figure, the number of living bacteria appeared constantly stable. The number of bacterial colonies attained log 5.4 CFU/mL – log 6.29 CFU/mL, indicating sufficient survival during the biodegradation test.

CONCLUSIONS

On the basis of the results and discussion, the biodegradation of initial TPH concentration (3.172 mg/L) achieved 66.26% for 35 days at 35‰ salinity, using the sequence method of 5% (v/v) *P. putida – B. subtilis* inoculum. However, the TPH concentration was estimated at 1.069 mg/L, but subsequently improved up to the standard, as maximum allowed concentration – MAC – was specified at 50.0 μg/L. In summary, *P. putida* and *B. subtilis* showed a great potential application in crude oil bioremediation of contaminated seawater in addition to certain important factors, including nutrient and surfactant properties. This is a significant effort in accelerating the degradation efficiency.

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