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Malaysian Journal of Microbiology

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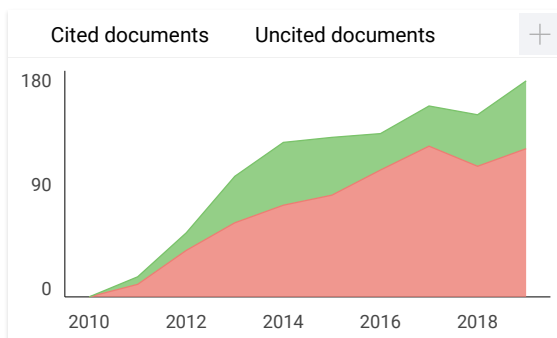
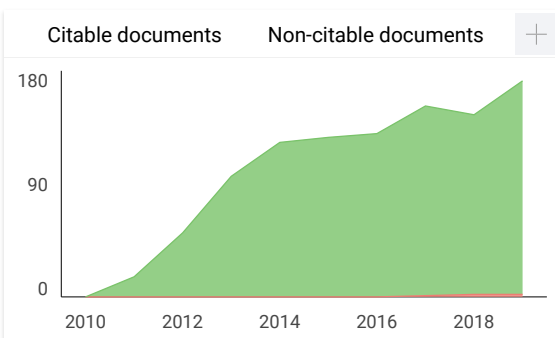
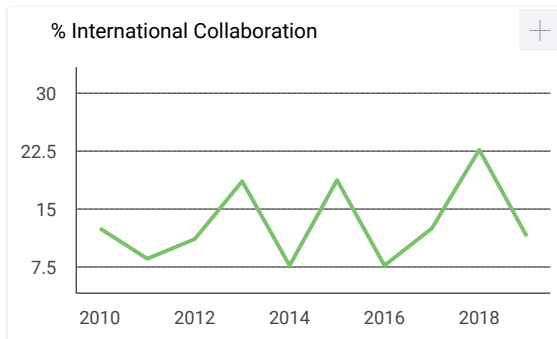
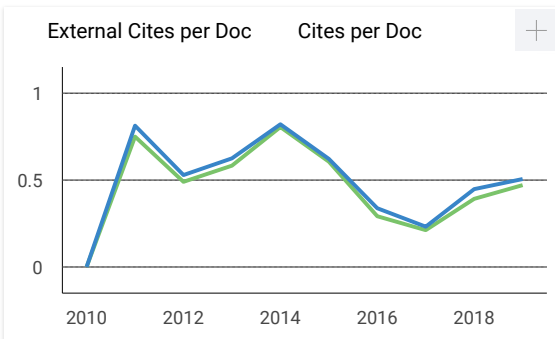
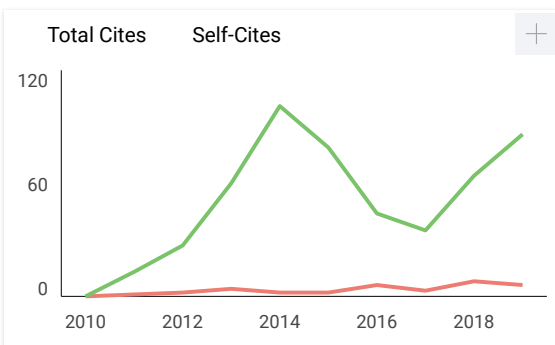
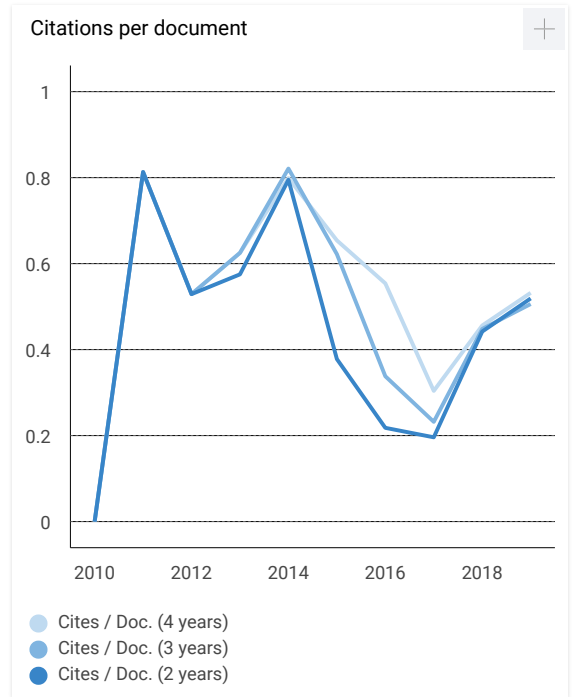
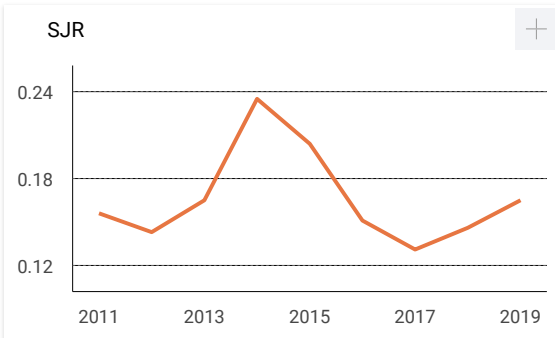
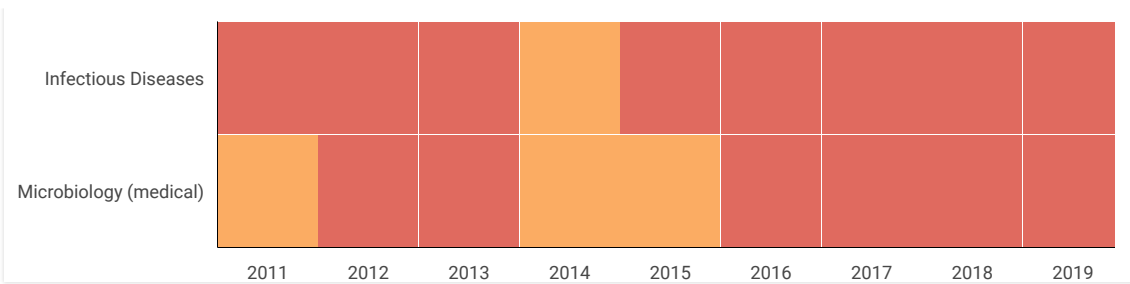
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Utilization of bacterial consortium for oil sludge biodegradation using bioslurry method

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ABSTRACT

Aims: Oil sludge is one of pollutant sources in the environment. Bacterial abundance, interaction, and compatibility of environmental factors ensure the success of biodegradation. The purpose of this study was to determine the effectiveness of bacterial consortium in degrading oil sludge using bioslurry method.

Methodology and results: The research design used was completely randomized design 4x5 with variation of bacterial consortium concentration and incubation time. Composition of contaminant and liquid phase in bioslurry method was 1:9 ratio with aeration, at room temperature. The liquid phase comprises distilled water with the addition of 2% (v/v) of molasses as nutrient for bacterial growth. Bacterial growth was evaluated using the Total Plate Count (TPC) method. Total Petroleum Hydrocarbon (TPH) measurements were evaluated using the gravimetric method while the oil sludge hydrocarbon component was evaluated by Gas Chromatography Mass Spectrophotometry (GCMS). The pH and temperature data were analyzed descriptively while TPC and TPH data were analyzed using Two Way ANOVA ($\alpha=0.05$). The bacterial consortium could grow on oil sludge hydrocarbon substrate with a range of temperature of 29 °C-32 °C and an optimum pH of 7. Biodegradation of TPH was 70.48% at consortium concentration of 15% in 14 days of incubation.

Conclusion, significance and impact of study: Biodegradation of oil sludge using a bacterial consortium by bioslurry method is one of the effective methods to reduce pollutants in the management of oil sludge.

Keywords: Bacterial consortium, biodegradation, bioslurry, molasses, oil sludge

INTRODUCTION

The amount of waste in the oil industry increases with the increase of oil industry activity. One of the industrial wastes is the oil sludge that settles at the bottom of the crude oil storage tanks. Periodically, the oil tank must be cleaned to accommodate new oils. Furthermore, the oil sludge at the base of the oil tanks becomes a potentially polluting material to the environment. The oil sludge contains the main compounds of aliphatic and aromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylene (BTEX), polycyclic aromatic hydrocarbons (PAHs), and heavy metal elements. Oil sludge is toxic, carcinogenic and mutagenic (Kriipsalu *et al.*, 2008; Liu *et al.*, 2012). Environmental contamination by the ingredients of the oil sludge can have an effect on the living organisms around it (Sari *et al.*, 2018). In addition, the exploited sludge in the environment can reach the groundwater, lakes or water sources that are the main source of clean water supply for both domestic and industrial needs (Atlas and Bartha, 1998).

Oil sludge hydrocarbon pollution treatments have been widely done physically, chemically, and biologically. Biodegradation is one of the most cost effective and cheaper ways than physical and chemical methods (Mansur *et al.*, 2015). Biodegradation of hydrocarbons utilizes the role of potential microbes in decomposing hydrocarbons. Previous study has revealed the ability of *Acinetobacter* sp. P2, *Bacillus subtilis* 3KP, (1), *Micrococcus* sp. LII 61, and *Pseudomonas putida* T1 (8) in oil removal and degradation of hydrocarbons in molasses substrates. The oil removal was supported by biosurfactant products which produced in molasses substrate (Ni'matuzahroh *et al.*, 2016a). Microbes can break down harmful compounds into harmless compounds by releasing their enzymes (Karigar and Rao, 2011). Oxygenase enzymes were produced by these aerobe hydrocarbonoclastic isolates on medium with aliphatic and polyaromatic hydrocarbon inductions (Sumarsih *et al.*, 2017).

Efforts are being made to accelerate the biodegradation of hydrocarbons by maintaining microbial survival, increasing microbial populations, and facilitating contact between contaminants and microbes through its biosurfactant product. Mix bacterial culture can increase biodegradation rate compared to pure culture (Cerqueira *et al.*, 2011). According to Leahy and Colwell (1990), single microbial species can only degrade certain compounds in the oil component, while using a microbial consortium can degrade hydrocarbon compounds that cannot be degraded by a single species because of the resulting enzyme capacity. Limitations of oxygen are one of the important factors that inhibit the biodegradation of hydrocarbons by aerobic hydrocarbonoclastic bacteria.

Bioslurry technology in biodegradation is effectively used to degrade hazardous contaminants present in sediments, solids or sludges in a reactor equipped with an agitator and aerator to supply oxygen during biodegradation (United States Environmental Protection Agency or USEPA, 1990). According to Gonzalez *et al.* (2008), the advantages possessed by bioslurry reactors in the biodegradation of contaminants include increasing mass transfer rate and contact between microorganisms, nutrients and pollutants. USEPA (2009) stated that the addition of microbes and aeration in the bioslurry reactor can be done to improve the performance of the reactor to the degradation of industrial waste. Sufficiency of macro and micro elements is needed to support microbial growth that has the potential to degrade hydrocarbons.

The incorporation of the biostimulation and bioaugmentation methods in the bioslurry reactor will optimize the biodegradation process of hydrocarbons in the oil sludge (Ni'matuzahroh *et al.*, 2016b). Utilization of molasses as biostimulation agents in the biodegradation of oil hydrocarbons began to receive attention (Ni'matuzahroh *et al.*, 2017). This study reported the influence of concentration variation of bacterial consortium on molasses medium and variation of incubation time on effectiveness of biodegradation of oil sludge using bioslurry method.

MATERIALS AND METHODS

Research sites

The research was conducted in an Ecology Laboratory, Environmental Laboratory and Microbiology Laboratory of Faculty of Science and Technology, Universitas Airlangga, Indonesia.

Material

Oil sludge came from Pertamina Balongan, Indonesia, in the form of liquid sludge simulated with the addition of sterile sand from Mojokerto, Indonesia, that has been sieved with mesh 200 with a composition of 30% (v/w). The bacterial consortium consists of four hydrocarbonoclastic bacteria, such as *Acinetobacter* sp. P2 (1), *B. subtilis* 3KP, *Micrococcus* sp. LII 61, and *P. putida* TI (8), which are collection cultures of Microbiology

Laboratory of Universitas Airlangga. The solvent used to extract hydrocarbons from the oil sludge was n-hexane. Media for culturing bacterial isolates include Nutrient Agar (NA) and Nutrient Broth (NB). Molasses was used as nutrients for bacterial growth.

Preparation for bioslurry reactor

Bioslurry reactors were used with glass and equipped with an aerator. The reactor has a capacity of 1.5 L, cylindrical, with a diameter of 0.1 m, and a height of 0.15 m (freeboard 0.02 m). The design of the reactor is illustrated in Figure 1.

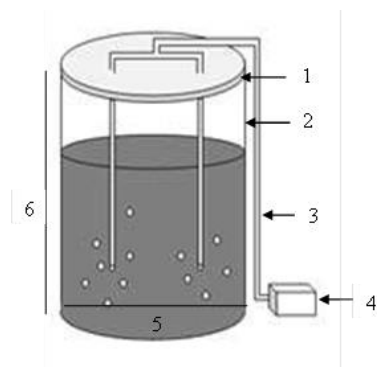


Figure 1: Bioslurry reactor design.

Information of Figure1:

1. Freeboard
2. Cylindrical glass
3. Pipe
4. Aerator
5. Diameter of reactor
6. Height of reactor

Preparation of bacterial isolate consortium

Each bacterial isolate was streaked on a slant NA medium, and then incubated for 24 h at 25 °C. The growing isolates were inoculated on NB medium and incubated for 24 h on the rotary shaker at 25 °C. Each culture was adjusted to optical density (λ 600 nm) up to 0.5. Consortium was composed with the same proportion of each bacterial culture and was applied in each bioslurry reactor.

Research design

This was a laboratory experimental study using a completely randomized design of 5x4 factorial pattern with three replications. This research consists of two factors, namely variation of concentration of microbial consortium and length of incubation time. The consortium concentration of microbes added consisted of 0% (control), 5%, 10% and 15% (v/v), while the incubation time was 0, 3, 7, 10 and 14 days. The study design is presented in Table 1.

Table 1: Treatment variation of microbial consortium concentration and incubation time.

Incubation time (Days)	Concentration of bacterial Consortium (% v/v)			
	V0	V1	V2	V3
H0	H0V0	H0V1	H0V2	H0V3
H1	H1V0	H1V1	H1V2	H1V3
H2	H2V0	H2V1	H2V2	H2V3
H3	H3V0	H3V1	H3V2	H3V3
H4	H4V0	H4V1	H4V2	H4V3

Note: V0: Concentration of microbial consortium 0% (v/v) as a control of treatments, V1: Concentration of microbial consortium 5% (v/v), V2: Concentration of microbial consortium 10% (v/v), V3: Concentration of microbial consortium 15% (v/v), H0: Day 0, H1: Day 3, H2: Day 7, H3: Day 10 and H4: Day 14.

Biodegradation test

The biodegradation test was performed by comparison between the contaminant and the liquid phase in the bioslurry treatment of 1:9. The bioslurry reactor was filled with 100 g of oil sludge, with consortium concentration at each reactor was 0%, 5%, 10% and 15% (v/v), and poured into it the distilled water with 2% molasses (v/v) up to the total volume of 1L aseptically.

Determination of Total Petroleum Hydrocarbon (TPH) and percentage of biodegradation

The remaining TPH concentration during the treatment was measured by the modified gravimetric method of USEPA (1994) and Panda *et al.* (2013). The extraction was performed by sampling, 30 mL culture of each reactor at a predetermined observation point using n-hexane at a ratio of 1:1. The soluble hydrocarbons in n-hexane were evaporated to separate the hydrocarbon phase with the solvent. The extraction was done at a temperature of 60 °C. The hydrocarbons residues were weighed to obtain the weight of the remaining total petroleum hydrocarbons (TPH). Then, TPH was analyzed using GC-MS to identify content of the remaining hydrocarbon compounds. The value of TPH was used to determine the percentage of biodegradation using formula as below:

$$\text{eHydrocarbon biodegradation (\%)} = \frac{\text{Initial TPH} - \text{final TPH}}{\text{Initial TPH}} \times 100\%$$

Viability analysis of bacterial consortium

Counting the number of bacterial cells was performed at each observation of incubation time using pour plate method with a certain dilution series. Nutrient Agar (NA) medium was used to grow bacterial isolates that play a role in hydrocarbon biodegradation. Incubation was carried out for 24 h at 25 °C. TPC results were displayed with CFU/mL log values. At each point, measurements of temperature and pH were also made.

Data analysis

Bacterial growth was evaluated using the Total Plate Count (TPC) method. TPH measurements were conducted by the gravimetric method, evaluated by Gas Chromatography Mass Spectrophotometry (GCMS). The pH and temperature data were analyzed descriptively, while TPC and TPH data were analyzed using Two Ways ANOVA ($\alpha=0.05$) and continued with Duncan test.

RESULTS AND DISCUSSION

The success of oil sludge hydrocarbon biodegradation is indicated by parameters such as increasing of microbial number (CFU/mL); reduction of total residual weight of Petroleum Hydrocarbon (g/g) on slurry substrate; intermediate metabolite compound formation; pH and temperature change during treatment.

Table 2: Combination of consortium concentration and length of incubation time to total bacterial number (CFU/mL) and weight loss of oil sludge residue (g/g).

Treatments	Average of number of bacteria (log CFU/mL)	Average of oil sludge residue (g/g)
H0V0	16.549 ± 0.52 ^{abcde}	0.460 ± 0.009 ^k
H1V0	15.437 ± 0.43 ^a	0.456 ± 0.019 ^k
H2V0	17.000 ± 0.00 ^{bcdef}	0.458 ± 0.001 ^k
H3V0	17.765 ± 0.18 ^{fg}	0.462 ± 0.018 ^k
H4V0	17.537 ± 1.07 ^{efg}	0.431 ± 0.001 ⁱ
H0V1	15.945 ± 0.61 ^{ab}	0.388 ± 0.004 ⁱ
H1V1	16.049 ± 0.22 ^{ab}	0.373 ± 0.004 ^h
H2V1	17.347 ± 0.306 ^{defg}	0.359 ± 0.012 ^{hi}
H3V1	18.191 ± 0.19 ^g	0.385 ± 0.040 ^j
H4V1	19.759 ± 0.6 ^h	0.323 ± 0.004 ^g
H0V2	15.874 ± 0.98 ^{ab}	0.246 ± 0.007 ^e
H1V2	16.199 ± 0.23 ^{abc}	0.198 ± 0.007 ^{bc}
H2V2	17.347 ± 0.32 ^{defg}	0.375 ± 0.010 ^j
H3V2	19.7518 ± 0.63 ^h	0.269 ± 0.006 ^f
H4V2	19.422 ± 0.037 ^h	0.235 ± 0.008 ^f
H0V3	16.339 ± 0.66 ^{abcd}	0.179 ± 0.009 ^b
H1V3	17.212 ± 0.46 ^{cdefg}	0.322 ± 0.016 ^g
H2V3	18.293 ± 1.21 ^g	0.213 ± 0.009 ^{cd}
H3V3	19.531 ± 0.14 ^h	0.186 ± 0.010 ^b
H4V3	21.773 ± 1.22 ⁱ	0.142 ± 0.005 ^a

Noted: Data labelled with different letters are statistically different ($\alpha = 0.05$; Duncan's test)

The result of combinations of consortium concentration variations and incubation time on total microbial count (CFU/mL) and weight loss of oil sludge residue (g/g) are presented in Table 2, while the percentage of biodegradation, pH, temperature measurement and the content of the hydrocarbon compounds resulting from biodegradation are shown in Figures 2, 3, 4 and 5.

Based on Table 2 (Duncan test results), the highest mean log of total microbial number (CFU/mL) in H4V3 treatment (15% bacterial consortium concentration at 14 days incubation time) was 21.7 ± 1.22 CFU/mL. It can be

seen that the residual oil sludge (g/g) decreases during incubation time. The average weight loss of the highest sludge (g/g) residue lies in the H4V3 treatment of 0.142 ± 0.0045 (g/g). The highest percentage of degradation was 70.48% with oil sludge concentration of 30% (0.48 g/g) for 14 days (Figure 2). Weight loss of oil sludge residue occurred significantly on H4V3 treatment (15% of increase in concentration in 14 days of incubation time). Increasing the number of bacteria and decreasing TPH are the result of metabolic activity of the microbial consortium present in the culture. Indigenous and exogenous bacteria used hydrocarbons in the oil sludge as a carbon source proven by the decreasing value of TPH in oil sludge. The consortium of exogenous bacteria also has a positive interaction with indigenous bacteria. The increasing number of microbial populations will increase the rate of degradation of hydrocarbon compounds (Wang *et al.*, 2011). With this bioslurry method, the percentage of TPH biodegradation up to 100% is predicted to be achieved at 48, 26, 22 and 18 days of incubation in control treatment and variation of bacterial consortium addition with concentration of 5%, 10%, and 15% respectively. The calculation analysis using the regression equation is not reported in this paper.

Molasses used as biostimulant agent in the oil sludge biodegradation contain simple sugars and minerals that are required for the growth of consortium-forming bacteria. The previous study also proved that molasses can be used as biosurfactant production media by exogenous bacterial consortium and indigenous bacteria from oil sludge (Ni'matuzahroh *et al.*, 2015; Ni'matuzahroh *et al.*, 2016a; Ni'matuzahroh *et al.*, 2017). Biosurfactant can increase the solubilization of hydrocarbon to enhance the bioavailability of hydrocarbon for hydrocarbonoclastic bacteria. Treatment with biostimulant using molasses was proven to achieve TPH biodegradation up to 33.01% in 14 days of incubation. The addition of a bacterial consortium 5, 10, and 15% significantly gave a difference in the percentage of TPH biodegradation compared to controls, which was 55.53%, 61.25% and 70.48% respectively. The effectiveness of bioaugmentation is influenced by the concentration of bacterial consortium and the interaction between bacteria. Utilization of bacterial consortium more than 15% is assumed to increase the percentage of hydrocarbon degradation, if the environmental conditions still support the bacterial growth.

The main constraint of the biodegradation process in closed systems is the limitations of oxygen used by aerobic bacteria degrading hydrocarbons. Increased incubation times generally have a growth inhibitory effect indicated by a slowing growth phase (Ni'matuzahroh *et al.*, 2016b). This study has successfully demonstrated that aerated supplying through bioslurry method can ensure the oxygen demand for aerobic microbes to break down hydrocarbons. This is evidenced by the ongoing exponential phase of bacteria at increasing incubation time in all treatments.

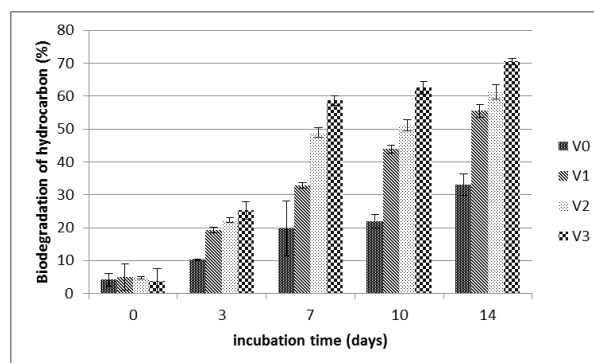


Figure 2: The percentage of hydrocarbon biodegradation at various concentrations of bacterial consortium and time of incubation. V0 is concentration of bacterial consortium 0% (v/v) as a control of treatments, V1 is concentration of bacterial consortium of 5% (v/v), V2 is concentration of bacterial consortium of 10% (v/v), and V3 is concentration of bacterial of consortium 15% (v/v).

The complexity of the compounds contained in the oil sludge and the high residual weight are thought to be the causes of the difficulty of the microbes used in the study to degrade the recalcitrant compounds. This can be seen in the increase in the average value of log results in the total number of microbes (CFU/mL) that are not so high. The substrate attached to the bottom of the bioslurry reactor and adhering to the sand soil during the study was also suspected to be the cause of lack of availability of hydrocarbon sources for the microbial consortium used. The statement is supported by Goswami and Singh (1990) which states that the physical and chemical properties of hydrocarbon such as form, structure, solubility and hydrocarbon toxicity become one of the determinants of accessibility of hydrocarbon compounds by microbes and may affect the use of substrate for bacterial growth.

Based on Figure 3, the pH value at various treatment variations increased and decreased within the time range of day 0 to day-14. According to Zhu *et al.* (2001) the biodegradation process will take place optimally if it occurs in sufficient conditions of nutrients, oxygen and pH ranges from 6-9. Charlena *et al.* (2010) states that the increasing activity of microbes in the biodegradation of hydrocarbons, will also increase the organic acids produced. Temperature is an environmental factor that affects the biodegradation of hydrocarbons especially on metabolic processes and growth of microorganisms (Silvia, 2010).

Based on Figure 4, it can be seen that the temperature on days 0 and 3 on various concentrations of microbial consortium tends to remain unchanged, but on the 7th, 10th and 14th days it tends to increase. The increase in temperature is likely due to an increase in ambient temperature. Biodegradation of petroleum takes place over a wide temperature range but is not always a major factor limiting biodegradation if other environmental factors exist under optimal conditions (Atlas, 1981).

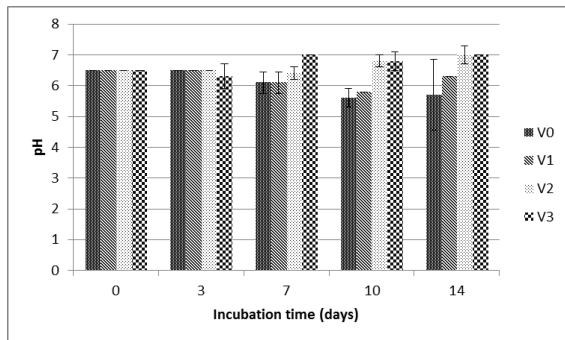


Figure 3: pH in all treatments in bioslurry reactor. V0 is concentration of bacterial consortium of 0% (v/v) served as a control of treatments, V1 is concentration of bacterial consortium of 5% (v/v), V2 is concentration of bacterial consortium of 10% (v/v), and V3 is concentration of bacterial consortium of 15% (v/v).

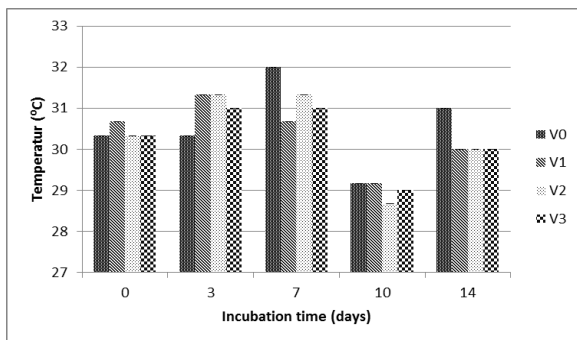


Figure 4: Temperature in all treatments in bioslurry reactor. V0 is concentration of bacterial consortium of 0% (v/v) served as a control of treatments, V1 is concentration of bacterial consortium of 5% (v/v), V2 is concentration of bacterial consortium of 10% (v/v), and V3 is concentration of bacterial consortium of 15% (v/v).

Overall concentration of microbial consortium, incubation time and other factors such as pH and ambient temperature will affect the total microbial count and percentage of oil sludge degradation. In the combination treatment, it was found that the best treatment was found in H4V3 (15% of inoculum with 14 days incubation).

The result of GCMS analysis showed that variation of bacterial inoculum concentration also gave difference in the percentage of hydrocarbon component composition at the end of incubation time (Figure 5). Aliphatic components are degraded faster than aromatic and polyaromatic components. The accumulation of aromatic compounds in culture with the addition of 10% and 15% is a bit of bacterial growth inhibitor.

The use of bioslurry reactors is considered effective when used in biodegradation of organic compounds such as hydrocarbons. This is evidenced by a short time of 14 days that can degrade oil sludge by 55.06% (initial TPH 30%). Cassidy (2001), states that the weakness possessed by the bioslurry reactor in the process of

biodegradation of hydrocarbon compounds is the formation of unpredictable foams. The production of this foam can occur rapidly during the stirring process especially when the speed of stirring and aeration are quite high.

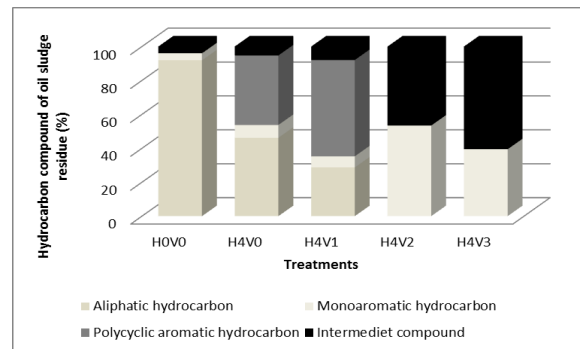


Figure 5: The remaining hydrocarbon components in all treatments at 14 days of incubation. H0V0 is control of treatments in 0 day incubation, H4V0 is control of treatments in 14 days incubation, H4V1 is concentration of bacterial consortium of 5% (v/v) in 14 days incubation, H4V2 is concentration of bacterial consortium of 10% (v/v) in 14 days incubation, and H4V3 is concentration of bacterial consortium of 15% (v/v) in 14 days incubation

Two percent of molasses can be used as biostimulant in the biodegradation of oil sludge hydrocarbons. Determination of minimum level of nutrients in molasses that was needed for metabolism of hydrocarbon degrading bacteria becomes research targets that has prospective to be developed for efficient waste treatment. The use of molasses in the handling or treatment of hydrocarbon waste can also support the utilization of organic waste in the environment.

CONCLUSION

Variations in the concentration of microbial consortium and length of incubation time using bioslurry reactor have an effect on total microbial number (CFU/mL) and weight loss of oil sludge residue (g/g). The best combination was found in the H4V3 treatment (15% of consortium, 14 days incubation) with a mean log value of total microbial cells of 21.77 ± 1.22 CFU/mL and the mean percentage of oil sludge biodegradation of 78.04%. The combination of biostimulation, bioaugmentation and bioventing methods using bioslurry reactor is highly prospective to be used in the processing of oil sludge.

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