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Bioremediation of Soil Contaminated Diesel Using Symbiotic Bacteria with Nutrient Variation Concentration

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Abstract**:** *Pollution of soil by hydrocarbon may be originated from exploration and exploitation activities of petroleum such as spills from the drilling process and leakage of pipes. The most widely used petroleum derivative products are diesel fuel. Diesel oil is one of the main products of petroleum refining and also one of the main source of pollution in the environment because its complex hydrocarbon structure. The low solubility of hydrocarbon in the soil cause low effectiveness of its biodegradation. This study was aimed to examine the effect of biostimulation combined with bioaugmentation method in degrading hydrocarbon compounds in the soil. In this study the variables used were symbiotic bacteria (Bacilus subtilis - Pseudomonas fluorescens, Bacillus subtilis - Pseudomonas putida, Pseudomonas putida - Pseudomonas fluorescens), concentration of inoculum addition (5% and 10% (w/w)), and concentration of inorganic fertilizers supplement in the form of urea and superphosphate (100: 10: 10 and 100: 5: 1). This research was conducted at 10% diesel fuel concentration (w/w). Bioremediation test of hydrocarbon-contaminated soil was quantified with parameters including Total Petroleum Hydrocarbon, temperature, pH, total microbial colonies and measurement of hydrocarbon components with Gas Chromatography-Mass Spectrometry. Resulst showed that the best result of TPH value was found from usage of P. fluorescens - P. putida mixture at 5.81% (H-15) with degradation rate of 62.35%. The mixture of symbiotic bacteria P. fluorescens - P. putida gave the best decline of TPH at 10% inoculum (w/w), ie 5,81% (H-15) with 62,35% degradation rate. Meanwhile, based on variation of ratio of inorganic fertilizer, P. fluorescens - P. putida also showed the highest decrease of TPH value at C: N: P ratio of 100: 5: 1, in which TPH lowered by 5,49% (H15) with degradation level of 64,04 %.*

Keywords: *Anorganic Fertilize, Bioaugmentation, Biostimulation, Hydrocarbon, Symbiotic Bacteria*

1. Introduction

The presence of petroleum in the water and the land is regarded as contaminating substance as it is not in its place [1]. Waste generated from petroleum processing are mainly hydrocarbon compounds. Hydrocarbon compound is the largest component of oil at about 90%, while the remaining constituent are non-hydrocarbon compounds [2]. The most widely used petroleum-derived products are gasoline and diesel fuel [3]. Diesel fuel is one of the main petroleum refining products that become main source of pollution in the environment because of its complex hydrocarbons structure. Hydrocarbon contaminants in the soil difficult to be degraded can alters physico-chemical and biological properties of soil because hydrocarbons are toxic for soil organisms and can disrupt plant growth [4].

Bioremediation is suggested for remediation of contaminated soil sites because of its low cost and ability to convert contaminants into harmless end products [5]. Biodegradation of hydrocarbon compounds is one of the most important processes involved in the weathering and eventual removal of oil from the environment [6]. The term "enhanced bioremediation" encompasses a broad continuum of technologies that differ with respect to their inputs. These technologies may involve the addition of electron acceptors or electron donors to stimulate naturally occurring microbial populations (biostimulation) or could be the introduction of specific

microorganisms to enhance the biodegradation of the target compound (bioaugmentation) [7]. Biostimulation is the use of nutrients for triggering biodegradation process of naturally-found indigenous microbes. Additional nutrients such as phosphorus and nitrogen are applied as growth trigger, even the smallest presence of contaminants can be used as trigger to activate enzymes [7]. Study of Wasify [8] described similar bacteria strains in degradation i.e. *Pseudomonas aeruginosa* (77.8%), *Bacillus subtillis* (76.7%), and *Acinetobacter iwolffii* (74.3%) had lower biodegradation capability by themselves compared to combination ability or consortium consisted of the three bacterial cultures at 88.5% degradation level.

The addition of nutrients previously known to increase microorganism activity, including indigenous hydrocarbon-degrading organisms. The addition of nutrients on the two treatments (biostimulation and bioaugmentation-biostimulation combination) have contributed to increasing degradation level of hydrocarbon compound seen in the first three weeks of incubation [9]. Therefore, combination of bioaugmentation with biostimulation techniques is expected to accelerate biodegradation process of hydrocarbons and lower TPH (Total Petroleum Hydrocarbon) value. Ghazali *et al*. (2004) [10] study used a mix of *Bacillus subtilis* with *P. aeruginosa* bacteria consortium which was able to degrade hydrocarbon short and long chains of aliphatic n-alkane in petroleum diesel with C14 up to 74.5%. In addition, within the same genus, *Bacillus* spp. and *Pseudomonas* spp. Were found to be able to degrade diesel oil (C_{12}-C_{23}) and lower TPH about 75% with supplemented N and P nutrient elements [11].

Based on the background above, study was performed to analyze the effectiveness of mixed bacteria addition (*Pseudomonas fluorescens, Pseudomonas putida* and *Bacillus subtillis*), nutrients supplement in the form of inorganic fertilizers (urea and superphosphate) at varied level in the bioremediation process of hydrocarbon-contaminated soil. To determine reduce of contaminant concentrations, hydrocarbon type was specified as diesel fuel. Diesel fuel is hydrocarbon compound with number of C atom range from 12 to 20. Selection of hydrocarbon type was based on complexity level of the compound and the high level of environmental pollution caused by this hydrocarbon.

2. Materials and Methods

2.1. Bacterial Culture and Maintenance

Pure culture of *Bacillus subtilis, Pseudomonas putida* and *Pseudomonas fluorescens* isolates were sub-cultured on Nutrient Agar (NA) medium (Merck, USA). Each culture was streaked into agar and incubated in incubator (Ogawa Seiki, Japan) at 30°C temperature for 24 to 48 hours. Stock bacteria was then put into plastic case and stored in the refrigerator at 4° C. From stock, three oses of bacteria were transferred respectively to 250 mL erlenmeyer flask containing 100 mL of Nutrient Broth (Merck, USA) aseptically, then incubated in the shaker incubator (150 rpm) at room temperature for 18 hours [10]. Cell cultures were harvested in the middle of log-phase with OD 1.0 at $\lambda = 600$ nm. Bacteria was separated from medium by centrifugating at 4000 rpm for 30 minutes. Pellets obtained were rinsed twice using saline solution. Bioremediation test referred to [12], in which bacterial pellets or 10% inoculum (w/w) was suspended into 150 mL of sterile Mineral Salt Medium (MSM) composed of (NH4)2SO4 (3 g/L), KH_2PO_4 (4 g/L), Na_2HPO_4 (7 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), $CaCl_2 \cdot 2H_2O$ (0.001 g/ L), $FeSO_4 \cdot 7H_2O$ (0.001 g/L), Tween 80 (4.0 g/L) in 1000 mL of sterile distilled water with pH 7 (pH of MSM solution was neutralized using 10% NaOH or 5% HCl).

2.2. Bioremediation Experiments

Medium used in this study was sand (1.27 g/cm3 density). Sand had high porosity, thus increasing mobility of diesel oil in it [13]. Contaminants used was diesel fuel with density of 840 kg/m3 and specific gravity of 0.82 [13], which were expected to represent petroleum contamination in the soil. Hydrocarbon contamination in soil samples made deliberately using diesel fuel at 10% concentration (w/w) equivalent to 100 gram of its weight. In this case, 100 g of diesel fuel was found to be equal to 123 mL in volume. Sand used was prior dried under the sun for approximately 1 day. The process of drying was aimed to eliminate excess moisture content in the media. After dried out, sand was sifted through 10 mesh sieve.

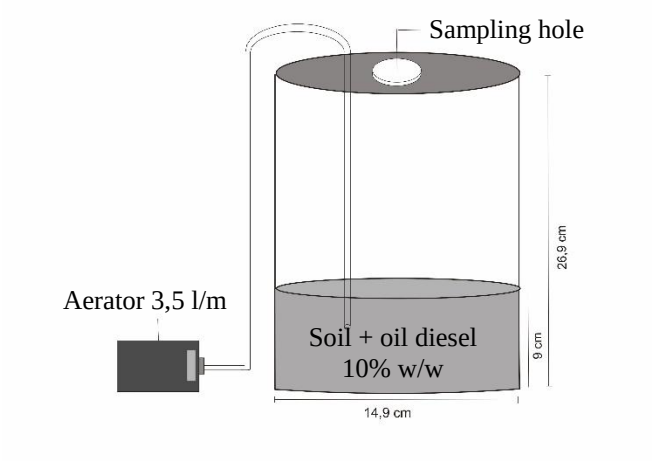

Fig. 1: Schematic diagram of the reactor used for bioremediation experiments.

Supplementation of inorganic fertilizer was designed to fulfil any essential nutrient requirements for microbes; carbon, nitrogen, and phosphorus. Carbon could be obtained from hydrocarbon contaminant in polluted soil samples, while nitrogen and phosphorus could be taken from NH_4NO_3 and superphosphate (SP36) fertilizer added. Before applied, urea and superphosphate was pre-treated by refined it into smooth powder using mortar, then sifted through 40 mesh sieve.

The medium was supplemented with 10% (w/w) diesel fuel waste as C source and combination of NH4NO3 and superphosphate fertilizer (SP36) as N and P source respectively that determined into two C:N:P combination ratios; 100:10:1 (100 g diesel oil waste, 26,08 g NH_4NO_3, 0,46 g SP36) and 100:5:1 (100 g diesel oil waste, 13,04 g NH4NO3, 0,46 g SP36). Examination of bioremediation process was conducted in a glass reactor with dimension of 149 x 269 mm (3.5 L volume) with assisted aeration using aerator (3.5 L/min speed measured with flowmeter) to help increasing oxygen level in the contaminated soil. Total of twelve reactors were used in this study. Variation of symbiotic bacteria mixture (*B. subtilis-P. fluorescens, B. subtilis-P. putida and P. fluorescens-P. putida*) at 10% (w/w) inoculum concentration combined with different C:N:P nutrient ratios (100:10:1 and 100:5:1) were added into them. Experiments were conducted with the following treatment combinations as presented in Tabel 1.

TABLE I: Treatments of Diesel Oil Hydrocarbon-Contaminated Soil

Treatment	C:N:P Ratio	Reactor Code
Soil + oil diesel	None	N0B0
Soil + oil diesel	C:N:P 100:10:1	N1B0
Soil + oil diesel	C:N:P 100:5:1	N2B0
Soil + oil diesel + mixed bacterial A	None	N1S10
Soil + oil diesel + mixed bacterial A	C:N:P 100:10:1	N2S10
Soil + oil diesel + mixed bacterial A	C:N:P 100:5:1	N0S10
Soil + oil diesel + mixed bacterial B	None	N1P10
Soil + oil diesel + mixed bacterial B	C:N:P 100:10:1	N2P10
Soil + oil diesel + mixed bacterial B	C:N:P 100:5:1	N0P10
Soil + oil diesel + mixed bacterial C	None	N1F10
Soil + oil diesel + mixed bacterial C	C:N:P 100:10:1	N2F10
Soil + oil diesel + mixed bacterial C	C:N:P 100:5:1	N0F10

2.3. Estimation of Total Petroleum Hydrocarbon

Measurement of diesel fuel hydrocarbon level from soil was performed as Total Petroleum Hydorcarbon (TPH) decline analysis using soxhlet extraction method continued with the gravimetric analysis. Extraction using soxhlet was conducted using liquid solvent, as it was one of the best method in separating bioactive compounds from nature. Soxhlet extraction was performed by means of heating and conducted repeatedly or continuously. Solvent used for extraction was alkane (C_6H_{14}) which was an n-hexane compound. The solvent had boiling point of 69°C, with standard condition of clear, colorless, and insoluble liquid. Test was conducted by initially weighing 5 g of soil samples from the reactor before dissolved with n-hexane solvent. The addition of n-hexane was aimed to bind oil contained in the soil samples. Measurement of TPH used gravimetric method (US EPA-821-R-1664 Metodhs 98-002, 1999).

$$\% \text{ hydrocarbon oil degradation} = \frac{Amount\ of\ oil\ degraded}{Amount\ of\ oil\ added\ in\ the\ media} \text{ x } 100$$

3. Result and Discussion

3.1. pH Analysis

Microorganisms generally grew well at pH between 6.0-8.0, while optimum pH for hydrocarbon biodegradation in soil was 7.8 [15]. pH measurement was performed daily for 15 consecutive days using pH and moisture tester. Recording of pH from each bacteria combination was presented in Figure 2.

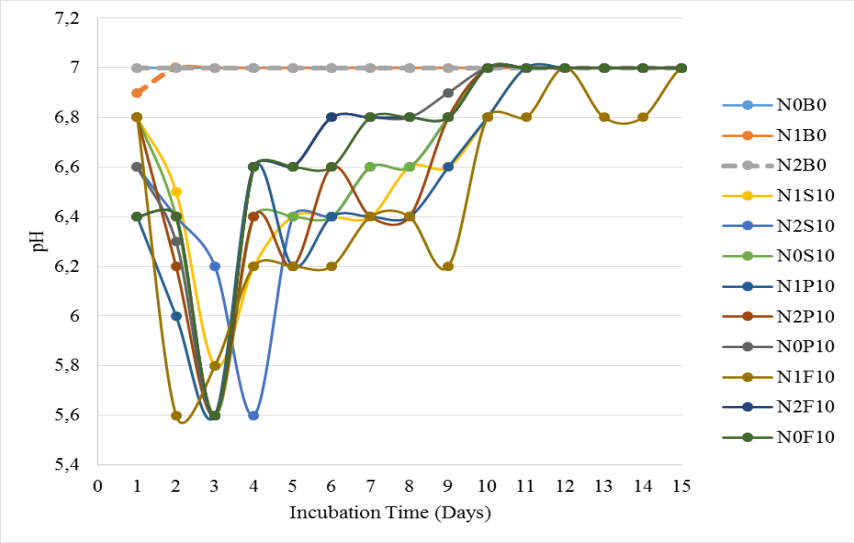

Fig. 2: pH Recording During Bioremediation Process.

The three control reactors, N0B0 (without both bacteria and inorganic fertilizers), NIB0 (100:10:1 fertilizer ratio-without bacteria), and N2B0 (100:5:1-fertilizer ratio without bacteria) showed a fairly stable pH range at 6.9 – 7.0. Reactor with addition of 10% inoculum had pH range at 5.6-6.8. Combination with fertilizer ratio of 100:10:1 (N1S10, N1P10 and N1F10) gave lower pH range compared to 100:5:1 ratio (N2S10, N2P10 and N2F10), while based on the bacteria used, *P. putida* - *P. fluorescens* (N1F10) gave lower pH range compared to other bacteria combinations. Lower pH suggested that medium had became increasingly acidic due to growing activity during diesel oil degradation. These activities was caused by various metabolites produced by cellular metabolism, such as organic acids and carbon dioxide during degradastion process. During its degradation, diesel fuel was broken into alcohol, then into aldehyde, and finally degraded to become carboxilic acid, all of which were acidic [16]. Increase in pH approaching neutral pH occurred on H-13 until 15, in which all reactors pH was recorded at range of 6.8-7.0. Rising pH was possibly an effect of organic materials decomposition [17].

3.2. Bacterial Colony

Total population of the three bacterial combinations (*B. subtilis - P. putida*, *B. subtilis - P. fluorescens* and *P. fluorescens - P. putida*) at 10% inoculum addition and both inorganic fertilizer ratios (100:10:1 and 100:5:1) during 15 days incubation period was presented in Fig 3. Population was found to be generally increasing in all reactors. On reactor with 10% inoculum (w/w) addition, N1F10 (100:10:1 fertilizer ratio, *P. fluorescens-P. Putida* combination) population had risen on H-5 onwards to H-15, from initial population of 1.26 x 10^{10} cfu/g into 1.45 x 10^{10} cfu/g. On the other hand, N2F10 (100:10:1 fertilizer ratio, *P. fluorescens-P. putida* combination) population experienced a decrease in H-10 to be 6.9 x 10^{9} cfu/g but had increased back in H-15 to be 9.78 x 10^{9} cfu/g. Rising bacterial population indicated the presence of bacterial metabolic activities. Increase of bacteria population occurred in logarithmic phase, because bacteria had adapted to oil content in the soil, thus it could utilize oil as carbon source for its growth. As it grew, cell division occurred and the number of bacteria colony elevated [18]. The greater rate the bacterial population increasing, biodegradation process of hydrocarbon in diesel oil would also occurred at faster rate [10].

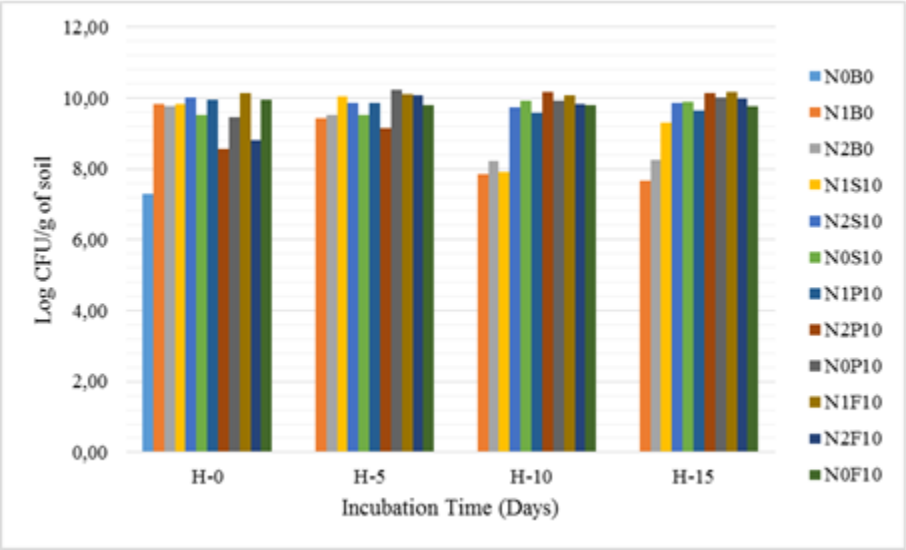

Fig. 3: Growth Mixed Culture Bacterial During Bioremediation Process.

The decline in bacterial population happened due decreased availability of substrate (nutrients) such as N, P, and C for growth and bacteria required some time for it to adapt to complex compounds including hydrocarbon. In the case of lowering nutrient availability at high number of bacteria population would induce competition among them. More intense competition in uptaking nutrients lead to limited and inoptimal nutrient absorption by the bacteria. Reduce in growth rate would ultimately inhibit the degradation process of hydrocarbon [10].

3.3. TPH Biodegradation Analysis

Bioremediation of hydrocarbon-contaminated soil using variation of symbiotic bacteria combined with different nutrient ratio for 15 days was aimed to determine the effect of various types of symbiotic bacteria combination, concentration inoculum addition, and optimum nutrient ratio required for degradation of hydrocarbon oil diesel. Analysis of hydrocarbon level was performed every five days on (H-0), (H-5), (H-10), and (H-15). Method used for TPH analysis was gravimetric method with soxhlet extraction. Resulting hydrocarbon level during bioremediation process was presented in Figure 4.

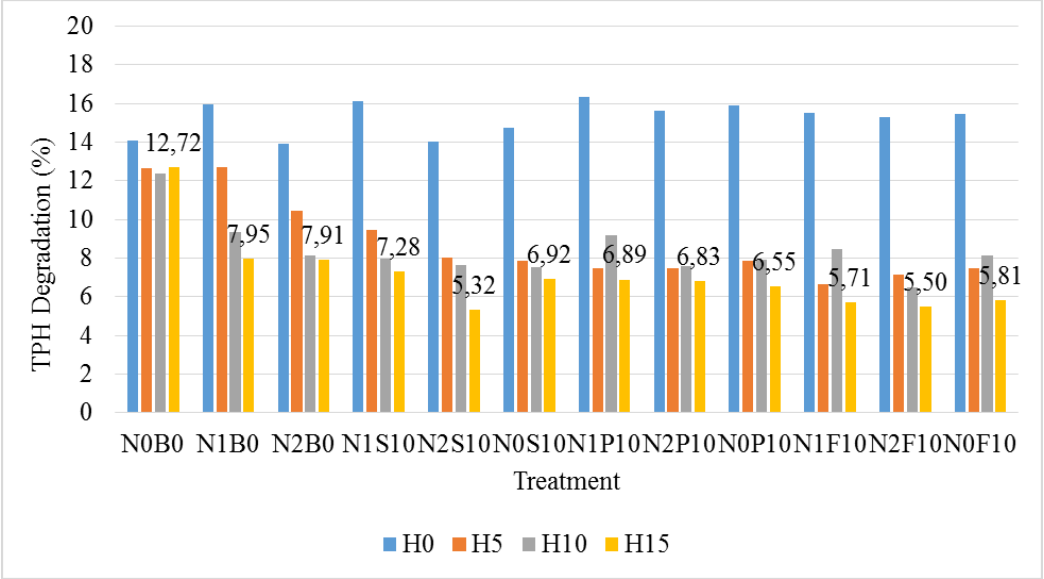

Fig. 4: Total Petroleum Hydrocarbon (TPH) of Each Reactor During Bioremediation Process.

The highest reduce of TPH was found from 10% inokulum in reactor N0F10 (without fertilizer, *P. fluorescens-P. Putida* combination), from 15.44% (H0) to 5.81% (H-15) with 62.35% degradation level and N1F10 reactors (100:10:1 fertilizer ratio, *P. fluorescens-P. putida*) which lower from 15.52% (H-0) to 5.71% (H-15) and 63.22% degradation level. In the other hand, addition of bacteria combination to reactor (*B. subtilis* - *P. putida*, *B. subtilis* - *P. fluorescens* and *P. fluorescens* - *P. putida*) at 10% inoculum concentration and inorganic fertilizer ratio of 100:5:1 showed the best resulting TPH decrease was from N2F10 (100:5:1 fertilizer ratio, 10% inoculum of *P. fluorescens-P. putida*) from 15.28% (H-0) to 5.49% (H-15) with degradation level of 64.04%. Result indicated that bioaugmentation (inoculum of symbiotic bacteria mixture) was able to increase effectiveness of petroleum degradation compared to treatment without bacteria inoculation and in the absence of bacteria. As according [19], inoculation of bacteria (bioaugmentation) could accelerate degradation process of petroleum hydrocarbon compared to if bacteria was not inoculated. Based on addition of inorganic fertilizers, decreasing TPH with the use of various bacterial mixture supplemented with inorganic fertilizers provided better reduce of hydrocarbon content. This could be attributed to microorganism requirement of carbon as energy source for its activities, while nitrogen and phosphorus were important cellular composition compounds decisive for microorganism growth. This three elements should presented in the right ratio to achieve optimum bacterial growth, and also to accelerate hydrocarbon degradation rate of contaminating diesel oil.

4. Conclusion

Based on variation of symbiotic bacteria used, mixtures of *B. subtilis* - *P. putida*, *B. subtilis* - *P. fluorescens*, and *P. fluorescens* - *P. putida* affected degradation rate of petroleum hydrocarbon diesel fuel during 15 days incubation period, with best result of TPH decrease was from 15.44% (H-0) to 5.81% (H-15) and degradation rate of 62.35%. Highest decline of TPH was from supplemented C:N: P at 100:5:1 ratio in mixture of *P. fluorescens-P. putida* which showed TPH of 5.49% (H-15) and 64.04% degradation level.

5. Acknowledgements

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