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Bioremediation of diesel oil: potential use of bacteria consortium *Lactobacillus fermentum* and *Clostridium beijerinckii* in degrading Total Petroleum Hydrocarbon (TPH)

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Abstract. Diesel oil is a major petroleum-refined product known as the primary environmental pollutant due to its toxic complex hydrocarbons and difficulty in decomposing naturally. This study aims to determine the efficiency of TPH (Total Petroleum Hydrocarbon) removal in the bioremediation process of diesel oil pollution by utilizing a bacteria consortium of *Lactobacillus fermentum* and *Clostridium beijerinckii* as biocatalysts. The diesel oil degradation test was conducted on a laboratory scale using Stone Mineral Salt Solution (SMSs) liquid media with a batch system (limited culture). The temperature was set at 30°C with a rotation speed of 150 rpm for 10 days. Different treatments of pH (5, 7, 9) and contact time (10, 20, 30 days) were applied to varied diesel oil concentrations (5, 10, 20%, v/v). The oil components were measured using Gas Chromatography-Flame Ionization Detector. Diesel oil samples were put into SMSs containing 10% bacterial consortium and 10% NPK fertilizer. The sensitivity test showed that the bacterial consortium was resistant, proven by the absence of an inhibition zone around the disc paper area containing diesel oil. The highest TPH removal efficiency found was 97.3% when 5% diesel oil was degraded by the bacterial consortium at pH 7 for 20 days. This study proves that the bacterial consortium can be recommended in bioremediation to alleviate diesel oil pollution in the environment.

Keywords: Diesel oil; Bioremediation; Total Petroleum Hydrocarbon (TPH); *Lactobacillus fermentum*; *Clostridium beijerinckii*

1. Introduction

Environmental pollution by petroleum and petroleum-related compounds, especially diesel oil, increases yearly. Diesel oil pollution is an ecological disaster affecting human social life and has biohazard effects on the surrounding environment [1]. Diesel oil is an engine fuel that contains hydrocarbons with C chains ranging from C8-C26 and polyaromatic hydrocarbons (PAHs) [2]. Diesel oil is a marine water and soil pollutant [3]. Soil contaminated with diesel oil spills affects the soil's ecological environment, impacting human activities. The environment affected by spills or contamination derived from diesel oil will experience changes in physical composition and chemical composition that affect the soil's texture and composition, thereby reducing soil porosity [4-5]. About 1.7 to 8.8 million metric tonnes of hydrocarbons, including diesel oil, are reported to pollute land and marine areas annually [6]. The pollution problem by diesel oil can be addressed in several ways, including using physical-chemical methods (e.g., in situ burning, adsorption, chemical dispersants, soil washing, and sorbents) and

biological methods. The current waste management technology for this problem is the bioremediation method [7]. Bioremediation is a detoxification method that aims to reduce pollution levels in recovering the environment affected by oil contamination [8-11]. In the petroleum bioremediation process, the use of bacterial consortium can affect the degradation process because each type of bacteria requires a specific substrate to degrade the petroleum components [12-14]. Some bacteria isolated from diesel oil-contaminated areas, e.g., *Lactobacillus* sp., *Acinetobacter* sp., *Vibrio* sp., *Moraxella* sp., and *Clostridium* sp., can reduce the content of diesel oil [15-18]. This study aims to determine the growth response of *Lactobacillus fermentum* and *Clostridium beijerinckii* bacterial consortium in environmental conditions polluted with diesel oil. The optimum condition will be tested using variations in pH and contact time that obtain the highest TPH (Total Petroleum Hydrocarbon) removal efficiency during the petroleum degradation process.

2. Materials and Methods

2.1. Diesel Oil Characterisation

The petroleum samples used were tested at the Lemigas ESDM Laboratory, Jakarta, using the gravimetric method to test Total Petroleum Hydrocarbon (TPH) concentration and Gas Chromatography-Flame Ionisation Detector (GC-FID).

2.2. Bacterial Cultivation and Growth Analysis

The bacterial consortium used in this study was *Lactobacillus fermentum* and *Clostridium beijerinckii*. They were cultivated in SMSs (Stone Mineral Salt Solution) growth media, a liquid media containing minerals and salts, i.e., 1 g $Na_2HPO_4.7H_2O$, 2.5 g NH_4NO_3, 0.5 g $CaCO_3$, 0.5 g $MgSO_4.7H_2O$, 0.2 g $MnCl_2.7H_2O$, and 0.5 g KH_2PO_4. The minerals and salts were dissolved in 1 liter of distilled water and then sterilized using an autoclave at 121°C for 30 minutes. The bacterial consortium act as a biocatalyst and was cultivated for 120 hours at 30°C using a shaker incubator with a rotational speed of 150 rpm. During cultivation, the bacterial consortium was given additional nitrogen source nutrients of 5% liquid NPK fertilizer. Growth curves were made using Total Plate Count (TPC) by counting the number of cells every 24 hours for 240 hours. Similarly, the ability of bacteria to reduce the levels of diesel oil compounds was indicated by the number of living bacteria from the beginning to the end of the study, which was calculated using the TPC method.

2.3. Sensitivity Test of Bacterial Consortium L. fermentum dan C. beijerinckii on Diesel Oil

The sensitivity test was performed to test the sensitivity or susceptibility of the bacterial consortium to the pollutant load, i.e., diesel oil. The bacterial consortium was placed in a petri dish containing liquid NA, then allowed to solidify. Paper discs exposed to diesel oil were placed on the solid NA and were incubated for 48 hours at 37°C.

2.4. Diesel Oil Degradation Test in SMSs Liquid Media

The diesel oil degradation test was done stepwise in a batch system with SMSs liquid media to find the optimum conditions for each parameter tested. The variations of each parameter were acidity value (pH) with variations of 5, 7, 9; contact time with variations of 10, 20, 30 days; and diesel oil concentration with variations of 5, 10, 20 % v/v. The composition used for pH and contact time optimization was 70% SMSs media (v/v), 10% NPK fertilizer (v/v), 10% bacterial consortium (v/v), and 10% diesel oil (v/v). Samples were replicated twice, and each replicate was incubated at 30°C using a shaker incubator with a rotational speed of 150 rpm. Each sample was tested by Lemigas ESDM Laboratory, Jakarta, to obtain Total Petroleum Hydrocarbon (TPH) levels. The lowest final TPH level from the variable results of pH, contact time, and diesel oil concentration will determine the optimum performance conditions of the bacterial consortium in degrading the TPH content in diesel oil. The formula used to calculate the TPH removal efficiency is as follows:

$$\%\ TPH\ Degradation = 100\% \times \frac{B - A}{B}$$

B is the initial TPH concentration before the degradation test (%), and A is the final TPH concentration after degradation (%).

3. Result and Discussion

The results of diesel oil characterization from the Lemigas ESDM Laboratory, Jakarta, show results that can be seen in the gas chromatography - flame ionization detector (GC-FID) analysis in Figure 1 and are classified based on specific gravity or API gravity were classified by specific gravity or API gravity (Table 1).

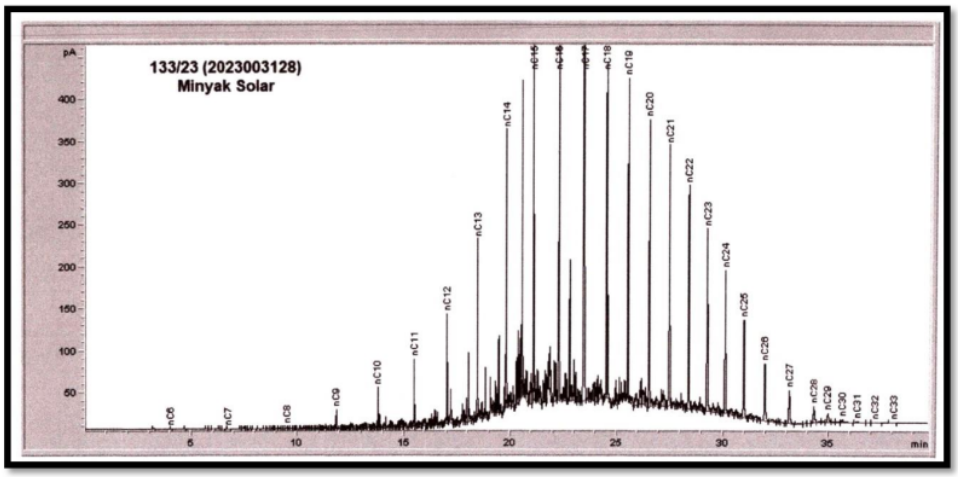

Figure 1. Initial Concentration of Diesel Oil

Based on Figure 1, that diesel oil has a chain of C15-C19 carbon atoms which can be seen from the peaks on the graph. It can also be seen from the graph that diesel oil is derived from the number of C33 carbon atoms which may have a low molecular weight that appears at the initial mooring time and is thought to be an isomer of branched alkanes. The mechanism for the formation of these compounds could not be explained in this study.

Table 1. Diesel Oil Samples Characteristics.

Parameters	Unit	Value	Methods
Density at 15°C	Kg/m^3	855,6	ASTM D 4052
API	-	33,8	ASTM D 4052
Spesific Gravity at 60/60 °F	-	0.8560	ASTM D 4052
TPH	%	87,27	Gravimetry

The specific gravity and API values based on preliminary characterization tests were 0.8610 and 32.85. These characteristics indicate that the diesel oil sample is a medium oil with a range of 31°-58° [19]. Total Petroleum Hydrocarbon (TPH) showed a value of 99.47%. The initial diesel oil characterization results were used as a reference for the initial TPH concentration before the bacterial consortium removed diesel oil TPH.

The growth of bacterial consortium *L. fermentum* and *C. beijerinckii* in SMSS media with and without the addition of diesel oil as a contaminant can be seen in Figure 2. The growth of the bacterial consortium without contaminants lasted for 240 hours. During cultivation, there were 3 growth phases: exponential, stationary, and death. Cultivation of bacteria without petroleum on SMSs media showed an exponential

phase that lasted from 0 hours to 144 hours. In this phase, the bacterial consortium began to adapt to the growth media and experienced a rapid increase in cell number. The bacterial consortium entered the stationary phase after 144 to 168 hours, characterized by a constant growth in the number of bacterial cells. After 168 hours, the death phase occurs, characterized by a decrease in the number of bacteria because the nutrients in the growth medium have begun to decrease; hence, bacterial cell metabolism also decreases.

Cultivation of bacterial consortium on SMSs media containing petroleum showed an exponential phase from 0 to 120 hours. The stationary phase occurs after 120 to 168 hours, indicated by the constant growth of the bacteria. The bacterial consortium began to enter the death phase with a decrease in the number of cells after 168 hours.

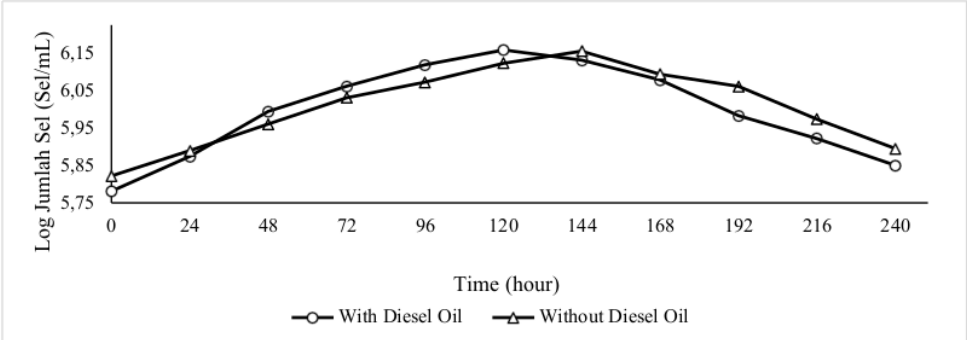

Figure 2. The Growth of Bacterial Consortium in SMSS Media With and Without Diesel Oil

The bacterial consortium added with diesel oil enters the exponential phase faster than bacterial cultivation in SMSs media without contaminants (Figure 2). The exponential phase starts at hours 72 to 120. A rapid increase in the number of bacteria characterizes the exponential phase. It seems that the bacterial consortium of *L. fermentum* and *C. beijerinckii* could utilize the carbon element in petroleum as a nutrient source for cell metabolic activities, so bacterial cells divide faster. After hour 120, the bacteria entered the stationary phase until hour 168, characterized by a constant bacterial growth rate so that the number of bacterial cells decreased slightly. The availability of macronutrients and micronutrients that are complete, appropriate, and with the proper ratio is crucial to support bacterial growth. Bacterial cells' main constituents are carbon, oxygen, hydrogen, and nitrogen. These elements, primarily carbon, are crucial substrates to produce energy in bacteria so bacterial metabolism can occur properly [20-21].

Figure 3 shows the results of the sensitivity test of *L. fermentum* and *C. beijerinckii* bacteria to petroleum, where no inhibition zone formed around the disc paper containing petroleum. This result indicates that both bacteria are resistant or insensitive to petroleum and can grow in environments or media containing petroleum. Based on these results, the bacterial consortium showed the ability to degrade the TPH content in petroleum.

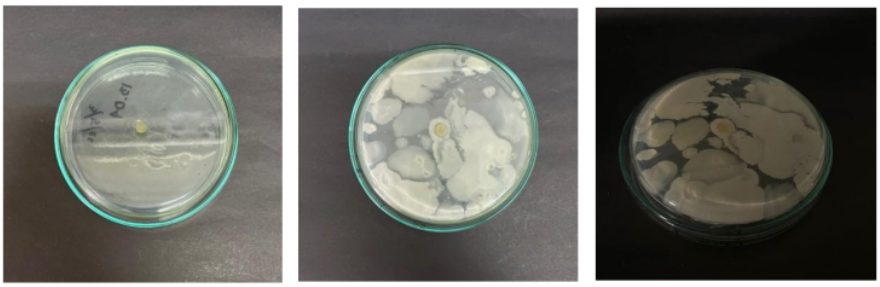

Figure 3. Result of Sensitivity Test of *L. fermentum* and *C. beijerinckii* Exposed to Diesel Oil (a) hour 0; (b) hour 24; and (c) hour 48

The resistant nature of *C. beijerinckii* to petroleum was shown by its ability to utilize hydrocarbons and cut long C chains to produce shorter C atoms [22]. Meanwhile, the resistant nature of *L. fermentum* to petroleum is shown by its ability to bind and detoxify xenobiotic compounds [23-26].

The degradation process lasted 10 days with pH variations of 5, 7, and 9 (Table 2). The lowest removal efficiency (94.87%) occurred in treatment with pH 5, while the highest removal efficiency (95.77%) occurred at pH 7. Based on these results, the optimum pH for the degradation process of diesel oil TPH by a consortium of bacteria *L. fermentum* and *C. beijerinckii* occurred at pH 7. This result is supported by [27], who mentioned that bacteria used for biodegradation generally occur at neutral pH.

Table 2. Degradation of Diesel Oil TPH with Varying pH in SMSs Liquid Media

pH variation	Initial TPH concentration (%; w/w)	Final TPH Concentration (%; w/w)	Removal efficiency (%)
5	87.27	5.10	94.16%
7	87.27	8.18	90.63%
9	87.27	7.98	90.86%
Kontrol	87.27	1.85	97.88%

All pH variations resulted in a removal efficiency higher than 94% (Table 2). A pH value of 8-9.5 can cause microorganisms to die. Bacteria can grow and develop well in neutral pH conditions. Therefore, pH will affect the rate of biodegradation reactions influenced by changes in the ion structure on the active side of the bacterial enzyme work. The acidic pH value can be adjusted to neutral with the addition of lime to increase the decomposition or degradation of diesel oil twice. This pH adjustment can change the solubility, bioavailability, form of chemical compounds, and macro and micronutrients.

At t_0, the initial TPH concentration was 87.27% (w/w), while on days 10, 20, and 30, the TPH concentration dropped to 5.1% (w/w), 6.1% (w/w) and 7.2% (w/w) respectively (Figure 4). TPH removal increased with increasing contact time until day 20 and no additional removal until day 30.

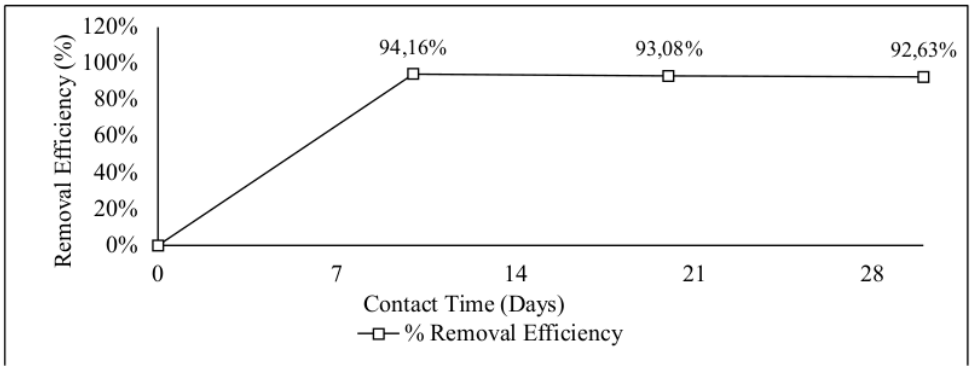

Figure 4. Removal Efficiency of Diesel Oil TPH with Varying Contact Time in SMSs Liquid Media

The removal of diesel oil TPH directly increased with the length of contact time (Figure 4). This result is also supported by the bacterial growth curve that shows bacterial consortium reached the exponential phase faster on SMSs media with diesel oil. The nutrients available in the sample at this stage are complete and suitable to support the growth of the bacterial consortium. The highest removal efficiency reached 95.48% for 20 days. The research results [28] showed that biostimulation by adding 100 grams of organic matter resulted in a TPH removal efficiency of 54.43% by a consortium of *Pseudomonas* sp. and *Bacillus* sp. in 14 days. Another study by [29] showed a TPH removal efficiency of 52.9% in 42 days by utilizing a mixed culture of *Xylocarpus granatum* mangrove sediment bacteria from the Lagoi area, Riau Islands. Compared to this study's results, using a consortium of *Lactobacillus fermentum* and *Clostridium beijerinckii* bacteria resulted in better TPH removal efficiency of 94.16% in only 10 days.

4. Conclusions

The growth response of the bacterial consortium of *L. fermentum* and *C. beijerinckii* showed that the bacterial culture could grow well in SMSs liquid media containing diesel oil contaminants under controlled environmental conditions in the pH range of 5 to 7, temperature range of 27-30°C, within 10 to 30 days. Total petroleum hydrocarbon (TPH) removal efficiency reached 94.16% at 30°C and pH 7, for 10 days. TPH removal progressed along with the consortium's growth by utilizing the carbon source in the diesel oil sample. The bacterial consortium has the potential as a biodegradator of diesel oil pollution in the environment.

Acknowledgments

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