



BIOREMEDIATION OF KEROSENE CONTAMINATED SOIL WITH THE ADDITION OF BACILLUS CEREUS BACTERIA

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ABSTRACT:

The development of road construction in Indonesia is directly proportional to the need for human transportation, among the many types of road pavement, the use of asphalt as a road pavement material is the main choice. Asphalt Buton (Asbuton) is a mixture of asphalt with other mineral materials in the form of rock. To get pure asbuton, it is necessary to do extraction. Kerosene is widely used as a solvent in the manufacture of asphalt. This causes environmental pollution and causes loss of ecological functions and health problems. One method that is environmentally friendly, economical and effective for tackling oil pollution is to use bioremediation technology. Bacteria *Bacillus cereus* believed to be able to reduce the level of oil in the ground because *Bacillus cereus* belongs to the hydrocarbon clastic bacteria, besides that *Bacillus cereus* also able to produce enzymes that can hydrolyze proteins and complex polysaccharides and form endospores. In this study the soil samples used were *spiked soil* with a pollutant source in the form of kerosene with a concentration of 10% (w/w). Variables in this study include variations in the addition of bacterial inoculum and variations in the addition of nutrients. This research was conducted in a laboratory scale for 15 days. The analysis used in this study is *Total Petroleum Hydrocarbon* (TPH) using the gravimetric method to determine the oil content contained in the waste, and *Total Plate Count* (TPC) to determine the number of bacterial colonies, temperature, pH, and water content. The results showed that the best rate of reduction of kerosene concentration was

the addition of 10% inoculum concentration and with the addition of nutrients. *Bacillus cereus* capable of reducing kerosene levels up to 67,499 mg/kg with a degradation rate of 26.58%.

Keywords: Kerosene polluted soil, Bioremediation, *Bacillus cereus*, TPH

INTRODUCTION

Various activities such as exploration, exploitation, production, transportation, storage, processing and distribution of crude oil or refined oil often result in oil leaks and or spills into the environment (Rim-Rukeh, 2015). In the manufacture of asphalt originating from nature or what is often called asbuton to separate the bitumen from the minerals, so far extraction has been carried out using petroleum-based solvents (petroleum) which include the following: kerosene, premium, toluene, MTC, low n-pentane, naphtha, sulfuric acid (Kurniadji, 2014).

Kerosene is a liquid hydrocarbon fractional distillation of petroleum with a temperature of 150°C and 275 °C. Hydrocarbon compounds are oil content that is difficult to decompose. Based on its nature, according to Minister of Environment Decree No. 128 of 2003 states that soil contaminated with petroleum is included in the category of hazardous and toxic waste (B3) (Su-Ungkavatin et al., 2023). This causes soil contaminated with petroleum hydrocarbon compounds to become an

environmental problem which until now has always received serious attention.

To overcome the problem of oil pollution, cleaning efforts are carried out (*clean up*) or commonly known as remediation. Remediation of oil-contaminated soil can be done in several ways including by means of physics, chemistry and biology (Balba et al., 1998). In general, waste containing petroleum is treated physically by filtering, absorbing, burning or chemically using emulsifiers. This method can eliminate petroleum waste quickly but is not environmentally friendly and expensive (Okeke et al., 2022). Therefore we need a method of processing waste containing petroleum that is environmentally friendly and economical. One way is to use a biological agent called the bioremediation method (Zam, 2010).

Bioremediation is a process or effort to restore land contaminated with organic and inorganic waste using bacteria or microorganisms (living organisms) (Ali, 2019). Management using microorganisms is an alternative treatment for waste containing petroleum which is environmentally friendly, cheap, effective, and produces a stable and non-toxic final compound

(Pratiwi, 2012). According to Munawar et al. (2007), there are two approaches that can be used in oil spill bioremediation, namely biostimulation and bioaugmentation (Darmayati et al., 2015). Microorganisms that are known to have the ability to degrade hydrocarbon compounds, one of which is bacteria. These bacteria are called hydrocarbonclastic bacteria. In degrading hydrocarbon compounds, bacteria utilize these compounds as a source of carbon and energy needed for their growth (Bhaktinagara et al., 2015).

In this study, bioaugmentation was carried out using bacteria *Bacillus cereus* result of isolation on *Asphalt Solid Waste* because according to (Rahayu & Mangkoedihardjo, 2022) the best approach to bioaugmentation is to use bacteria isolated from polluted locations because they are more likely to survive and reproduce when put back into a polluted environment. In addition, this study also applied a variety of biostimulation treatments to determine the effect in this kerosene bioremediation test. The type of stimulant used is urea and superphosphate as a source of nitrogen and phosphate. Contaminated soil samples were made using spiked soil by mixing sandy soil with kerosene.

RESEARCH METHODS

A. Preparation of Bacterial and Nutrient Inoculums

1. Bacterial Rejuvenation

Prepare bacterial isolates and propagate them by growing them on slanted agar containing NA (Nutrient Agar) (Merck, USA). With the scratch method and incubated in an incubator at 37°C for 24 hours (Lanyi, 1988).

2. Bacterial Cultures and Suspensions

Inoculate 1 bacterial colony aged 24 hours to *Nutrient Broth* (NB) aseptically then stirred using a shaker. Separation of bacterial cells from NB media was carried out by centrifugation at 4000 rpm for 30 minutes. The pellets formed were then rinsed using physiological water then the bacterial pellets were dissolved in NaCl solution according to the variation set and then homogenized. Cell cultures will be taken after obtaining an OD value of 1.0 on a spectrophotometer with a wavelength of 600 nm (Goegan et al., 1995).

3. Nutrient Preparation

Variations in the addition of nutrients to the soil using the ratio C:N:P 100:5:1. The N element is taken from urea fertilizer which contains 46% N and the P element is

taken from 36% superphosphate fertilizer (Patwardhan, 2017).

B. Contaminated Media Production (Spiked Soil)

The media used in this study was sandy soil with a density of 1.27 g/cm³. The selection of sand is used because of its high porosity which increases the mobility of kerosene in sand (McCarty et al., 2016). Contaminant used is kerosene with a specific gravity of 0.83 gr/cm³ (Pamukcu et al., 2014) which is expected to represent petroleum contamination in soil under actual conditions. Hydrocarbon

contamination in the soil sample will be created intentionally with a kerosene concentration of 10% w/w (Maiyoh et al., 2015).

C. Rate Analysis Total Petroleum Hydrocarbon (TPH)

TPH levels were measured using the gravimetric method to determine the level of TPH removal in kerosene-contaminated soils. Initially the heat-resistant container is weighed (A). A sample of polluted soil that has had its water content removed is weighed as much as 5 grams (B). The soil sample was mixed with 10 mL of n-Hexane in a centrifuge tube (C). Then stirred at 1000 – 2000 rpm for 30 minutes. Supernatant as much as 5 ml (D) and put into a heat-resistant container then evaporated with an

oven at 70°C until the solvent evaporates. Then it is cooled in a desiccator, then weigh the heat-resistant container containing the hydrocarbon residue (E). Calculation and analysis of TPH percentage calculation data using the following equation (Safdari et al., 2018):

1. $TPH = (AND - A) \times \left(\frac{C}{D}\right) \times \left(\frac{1000}{B}\right) \text{ (g/l)}$
 $TPH = y \times 1000 \text{ (mg/kg)}$
2. $\% TPH = \left(\frac{IT'S AT}{B}\right) \times 100\%$
3. $TPH \text{ removal efficiency} = \left(\frac{TPH0 - TPH15}{TPH0}\right) \times 100\%$

D. Analysis of the Number of Bacterial Colonies

Calculation of the number of microorganisms is an indicator of the process of biodegradation of hydrocarbons. The number of microorganisms will increase if they are able to live by utilizing the existing substrates in these hydrocarbon compounds. The basis for the calculation is by diluting the suspension with a certain dilution range in stages according to the method *Colony Forming Unit* (CFU). The CFU method uses the Pour Plate method (Shekhar et al., 2015). The analysis was carried out at (H-0), (H-3), (H-5), (H-10), and (H-15). 1 gram of soil sample was put into a test tube containing a sterile physiological solution (0.85% NaCl) with a volume of 9 ml then homogenized using a

vortex and labeled on the test tube 10^{-1} . Dilution test tube 10^{-1} Take 1 ml using a volume pipette and then put the solution into a test tube containing 9 ml of sterile NaCl solution and homogenize. This process was carried out until 10 dilutions were obtained⁵. After the dilution is complete, put 0.1 ml of the suspension into a sterile petri dish, then pour the NA media into the sterile petri dish and then homogenize it by rotating it to the right and left. Each sample was incubated in the incubator for 1x24 hours at 37° C. After the incubation period, the process of calculating the number of bacterial colonies was carried out using *colony counter*.

RESULT AND DISCUSSION

Allowance *Total Petroleum Hydrocarbon* (TPH)

TPH parameter measurement is one of the important parameters in determining the success rate of the bioremediation process (Azubuike et al., 2016). In addition, this measurement aims to determine the ability of bacteria and variations in the addition of optimum bacterial inoculum in reducing the concentration of kerosene contained *inspiked soil*. Hydrocarbon content analysis was carried out on H-0, H-3, H-5, H-10, and H-15 using the gravimetric

method. The magnitude of the decrease in TPH values can be seen in Figure 1.

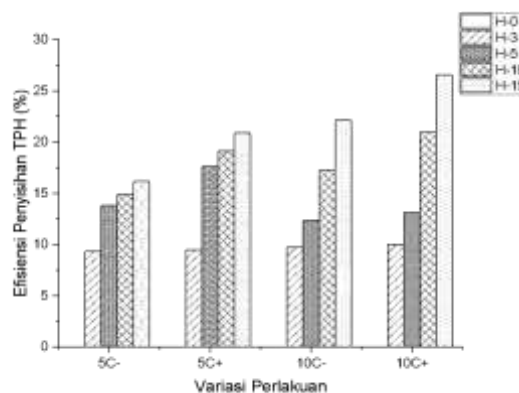


Figure 1. TPH Removal Efficiency

Information :

5C- : 5% Inoculum *Bacillus cereus* without added nutrients

5C+ : 5% Inoculum *Bacillus cereus* with the addition of nutrients

10C- : 10% Inoculum *Bacillus cereus* without added nutrients

10C+ : 10% Inoculum *Bacillus cereus* with the addition of nutrients

Based on the results of observations that have been made on TPH values in kerosene-contaminated soils, it shows that in general the TPH values in each treatment experience a decrease in TPH values. This is indicated by the increased efficiency of TPH removal (Figure 1). The decrease in TPH value is due to the presence of oil-degrading bacteria in the bioremediation process that are able to grow well in utilizing carbon sources for their survival. Hydrocarbon compounds are used by microbes as a source of

energy for metabolism and reproduction. The mechanism of the process of degradation of hydrocarbon compounds is based on the principle of bioremediation whereby groups of carbonclastic microbes carry out the process of overhauling hydrocarbon compounds with hydrocarbon oxidizing enzymes, so that microbes are able to degrade petroleum hydrocarbon compounds by cutting the hydrocarbon chains shorter. In addition, carbonclastic microbes have a mechanism to free themselves (desorption) from hydrocarbons. Hydrocarbon-oxidizing enzyme synthesis encoded by mutated microbial chromosomes and plasmids. Chromosomal and plasmid mutations affect the process of breaking down hydrocarbon molecules. Hydrocarbon compounds are natural organic compounds, therefore many types of microbes have evolved to use hydrocarbon compounds.

The best decrease in kerosene concentration (TPH) is the addition of 10% bacterial inoculum and the addition of nutrients. This can be attributed to research conducted by (Suja et al., 2014) which stated that the best inoculum concentration in degrading petroleum was 10%. If the concentration is below or more than 10%, it will give poor growth and degradation results. This is because if the concentration of bacterial inoculum is less, the degradation process will run

more slowly (Chen et al., 2008) and if the concentration is excessive, it can result in competition between bacteria, resulting in low growth and degradation processes (Zam, 2010). Therefore, the right amount of inoculum is needed because this is one of the factors to determine the success rate of bioremediation/degradation of petroleum hydrocarbons (Cohen, 2002). In addition, the addition of nutrients has a major effect on cell synthesis and growth as well as on the activity of enzymes produced by bacteria to degrade pollutants. This is because nitrogen is a basic element of protein and nucleic acids which play a role in growth, multiplication and formation of cell walls. Meanwhile, phosphorus is the main component of nucleic acids and lipid cell membranes which play a role in the transfer of biological energy (Maathuis, 2009). The more the number of bacteria, the faster the biodegradation process runs.

Total Bacteria (*Total Plate count*)

The bacterial population is an indicator of how the bioremediation process can run and how many bacteria play a role in the bioremediation process. Calculation of total bacterial colonies used the plate count method which was diluted to 10^{-5} . According to (Tomasiewicz et al., 1980), in the plate count method the best number of colonies that can be counted ranges from 30-300 microbes per mL or per gram of sample. Data from TPC

analysis during the study can be seen in Figure 2.

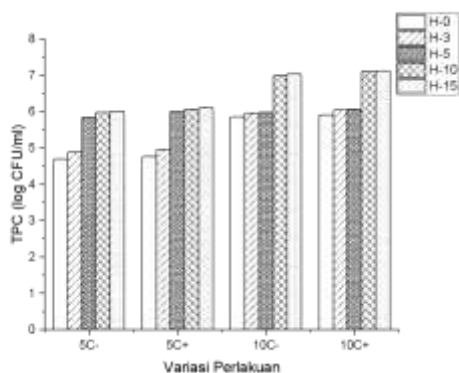


Figure 2. Number of Bacterial Colonies Information :

5C- : 5% Inoculum *Bacillus cereus* without added nutrients

5C+ : 5% Inoculum *Bacillus cereus* with the addition of nutrients

10C- : 10% Inoculum *Bacillus cereus* without added nutrients

10C+ : 10% Inoculum *Bacillus cereus* with the addition of nutrients

Based on Figure 2, it can be seen that there was an increase in the number of colonies until the last day, indicating metabolic activity. The increase in the number of bacterial colonies that occurs is a logarithmic phase because the bacteria have adapted to oil so that they can use the main carbon source, namely kerosene, for their growth. Therefore, bacteria can reproduce cells so that the total number of bacterial cells increases (Schnurr et al., 2013). The greater the rate of increase in the number of bacterial colonies, the faster the process of biodegradation of hydrocarbons in oil.

Degree of Acidity (pH)

The degree of acidity (pH) is a form of measurement used to indicate a substance, solution, or object is acidic or basic. pH has an important role because biological and microbiological activities require a certain pH range in order to obtain optimal results. Based on the results of pH analysis on day 0, the pH value was quite stable in the range of 6.9 – 7.0. After the third day, the pH value fluctuated in each reactor, this could be due to bacterial activity in degrading kerosene as a food source. The decrease in pH indicates that microbial activity forms acid metabolites to adapt to new environmental conditions. Another possibility is that the decrease in pH occurs due to the accumulation of organic acids (especially gluconic, pyruvate, citric and succinic acids) which are formed from organic metabolism (Tsao et al., 1999). The reactor with the addition of nutrients has a lower pH compared to the reactor without the addition of nutrients, this is because the 10% S contained in the fertilizer will react with water, oxygen and CO molecules.² in the soil will produce sulfate ions and a number of H ions⁺. This was also clarified by (Behera et al., 2022) stating that fertilization can lower soil pH because fertilizers contain sulfur and ammonium which will hydrolyze to produce H⁺ ions, causing soil pH to decrease. Meanwhile, an increase in pH

can occur due to the influence of the decomposition process of organic matter (Averill & Waring, 2018). However, all reactors tend to be in the pH range of 6-9 so that bacteria can still grow and reproduce well (Meel et al., 2020).

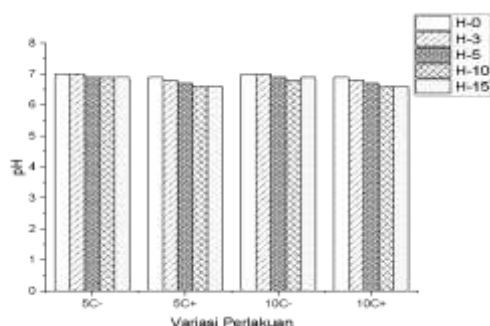


Figure 3. pH

Information :

5C- : 5% Inoculum *Bacillus cereus* without added nutrients

5C+ : 5% Inoculum *Bacillus cereus* with the addition of nutrients

10C- : 10% Inoculum *Bacillus cereus* without added nutrients

10C+ : 10% Inoculum *Bacillus cereus* with the addition of nutrients

Temperature

Temperature is also one of the factors that affect the growth of bacteria. Temperature can have a direct effect due to enzymatic chemical reactions that have an impact on the ability to biodegrade. Based on the results of temperature analysis, the temperature range measured in all reactors is still relatively stable, namely around 28°C - 31°C which range is still in the mesophilic temperature range needed for bacteria to

grow, which is between 20°C - 40°C. This is reinforced by the statement of (Gloria, 2019), that the biodegradation process of kerosene which is carried out biologically is in mesophilic conditions. The increase in temperature is caused by metabolic reactions that occur in decomposing bacteria. Decomposing bacteria need energy to metabolize chemical compounds, including pollutant compounds that are broken down during the bioremediation process. When these bacteria metabolize pollutants, they release energy in the form of heat as a by-product. This can cause an increase in temperature in the surrounding environment.

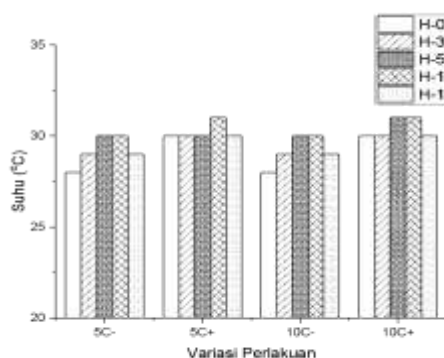


Figure 4. Temperature

Information :

5C- : 5% Inoculum *Bacillus cereus* without added nutrients

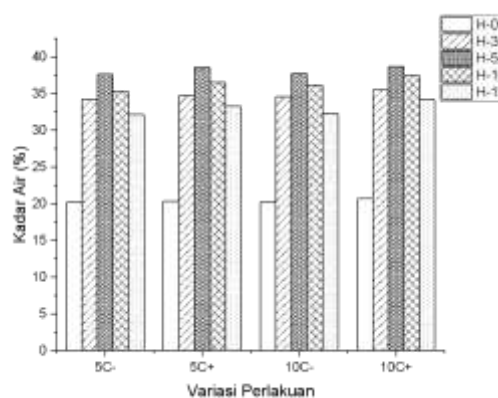
5C+ : 5% Inoculum *Bacillus cereus* with the addition of nutrients

10C- : 10% Inoculum *Bacillus cereus* without added nutrients

10C+ : 10% Inoculum *Bacillus cereus* with the addition of nutrients

Water Rate

Testing the parameter for water content aims to control the amount of water present in the reactor. Because water can affect the presence of contaminants, gas transfer, the level of toxicity of contaminants, the movement and growth of microorganisms. Water content that is too low can limit the movement of microbes and inhibit the metabolism of microbial cells. Based on the results of observations and tests, the value of the water content in all reactors ranged from 20-39%. This value is still in the range of good water content for the bioremediation process, namely 20-80% (Anasonye et al., 2015). The reactor with the addition of nutrients has a higher water content because the addition of nutrients can increase the porosity of the (Maestre et al., 2007). This condition will also affect the level of soil aeration and the status of soil water content. However, on the 10th to 15th day the water content decreased, this decrease in water content could be due to the aeration/stirring process which was carried out every day during the study so that it could cause evaporation. In addition, it can be caused by the absorption of H₂O by hydrocarbon degrading microorganisms.



Picture 5. Water Level

Information :

5C- : 5% Inoculum *Bacillus cereus* without added nutrients

5C+ : 5% Inoculum *Bacillus cereus* with the addition of nutrients

10C- : 10% Inoculum *Bacillus cereus* without added nutrients

10C+ : 10% Inoculum *Bacillus cereus* with added nutrients

CONCLUSION

Based on the results of research that has been carried out on kerosene polluted soil using bacteria *Bacillus cereus* shows that Bacteria *Bacillus cereus* has the potential to reduce kerosene levels in polluted soil. Judging from the variation in bacterial inoculum and the variation in the addition of nutrients, the reactor that had the highest TPH removal efficiency was the reactor with the addition of 10% bacterial inoculum and with the addition of nutrients with a TPH removal efficiency of 26.58% (1.50,E+06 CFU/ ml).

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