Analysis of polycyclic aromatic hydrocarbons (PAHs) bioremediation by hydrocarbonoclastic degrading bacteria (Gordonia terrae)

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Abstract. One of the main focuses in developing bioremediation of Polycyclic Aromatic Hydrocarbons (PAHs) is to optimize the hydrocarbonoclastic degrading bacteria such as Gordonia terrae. However, the analysis of the bacteria’s capability to degrade PAHs in different concentrations is sparsely explored. This study aims to evaluate the remediation of PAHs by Gordonia terrae by analyzing bacterial activity, PAH degradation, pH, BOD, and COD. The initial PAH concentrations used were 15 ppm, 30 ppm, and 45 ppm for 14 days of incubation. The results show bacterial activity gradually increases in each concentration up to 10-day incubation and decreases in up to 14-day incubation. Moreover, the total PAHs were gradually decreased to 54%, 69%, and 77% in the 15 ppm, 30 ppm, and 45 ppm of initial concentrations, respectively. The final pH values were 6 for all concentrations. At the same time, the BOD and COD values of each concentration gradually decreased until the end of the experiments. This study shows that Gordonia terrae can degrade PAHs, which was achieved optimally after 10 days of incubation. Furthermore, this study indicates that PAH degradation is influenced by bacterial activity, pH, BOD, and COD.

1. Introduction
One of the main environmental problems today is the contamination of hydrocarbons from anthropogenic waste [1]. Increased human activities and industrial development have increased the threat of hydrocarbon pollution in the environment [2]. Polycyclic Aromatic Hydrocarbons (PAHs) are compounds consisting of carbon and hydrogen [3]. Pollution of PAHs has broad implications for terrestrial and aquatic ecosystems [4]. Various physical, chemical, and biological methods have been developed for cleaning the environment contaminated with Polycyclic Aromatic Hydrocarbons (PAHs). The biological method using aquatic microbes is one of the most efficient ways to degrade PAH waste. Biological methods using microbes are called bioremediation [5].

The fundamental basis of the development of bioremediation is knowledge of microbial ecology. Many bacterial species have been isolated and utilized as bioremediation agents for hydrocarbons [1, 6]. The use of bacteria to overcome environmental pollution is widely used because it is environmentally friendly and effective [7, 8]. Bacterial bioremediation agents have the enzymatic capacity to associate with hydrocarbons [4, 9].

Biological degradation has been widely accepted as an essential mechanism for removing organic pollutants from the environment [10]. One of the crucial processes in this biodegradation is the
enzymatic activity of bacteria. Several species of hydrocarbon-degrading bacteria have been developed to act as a hydrocarbon bioremediation agent [1]. One of the potential bacterial bioremediation agents is *Gordonia terrae*. However, analyses comparing *Gordonia terrae* bacteria’s ability at different hydrocarbon concentrations have not been widely reported. This study aimed to evaluate the ability of the bacteria *Gordonia terrae* to remediate Polycyclic Aromatic Hydrocarbons (PAHs) in varying concentrations. The results of this study provide essential information for utilizing bacteria such as *Gordonia terrae* in the bioremediation of aquatic environments.

2. Materials and Methods

2.1. Media preparation

The purified bacteria (*Gordonia terrae*) were taken 1% with a density of $10^8$ cells/ml, then added to the liquid Bushnell-Haas medium ($K_2HPO_4$ 1 g/L, $KH_2PO_4$ 1 g/L, $NH_4NO_3$ 1 g/L, $MgSO_4$ 0.2 g/L, $CaCl_2$ 0.02 g/L, and $FeCl_3$ 0.05 g/L) containing 15 ppm, 30 ppm, and 45 ppm of oil. The culture mediums were incubated at 36°C and shaken on a water bath shaker at a speed of 120 rpm, and then, analyzed for bacterial cell density, pH, decrease in total hydrocarbons, BOD and COD. The control treatments were media without the addition of bacteria.

2.2. Estimation of bacterial activity

Estimation of bacterial activity is done by observing the growth of bacterial cells. Bacterial density was calculated using a hemocytometer under a microscope with a magnification of 400 x. Count cells are colored for easy visualization and counting. Cell staining was performed using trypan blue (0.4% (w/v) in PBS). Trypan blue is a diazo dye that is very important in its use in staining dead cells. Sampling was carried out using the volumetric method, as much as 1 ml/experimental unit (x). The number of bacteria is calculated using the following formula:

$$\Sigma_{cell/ml} = \Sigma_{counted\, cells} \times \frac{selected\, box\, total}{\times\, dilution\, \times\, \Sigma_{sample}}$$

2.3. Analysis of total hydrocarbon reduction

Estimation of the total reduction of hydrocarbons is carried out using the gravimetric method. In order to calculate the amount of hydrocarbon content, Bushnell-Haas medium containing hydrocarbons were inserted into a separating funnel, added 5 ml of 3N HCl and 60 ml of purified n-hexane (by distillation at 60°C), followed by shaking for $\pm$ 15 minutes, and then, allowed to stand until n-hexane separate. After that, there will be three layers (used oil, n-hexane, and water). The water is then removed, then the layer of lubricating oil (oil) and n-hexane is filtered with filter paper smeared with $\pm$ 0.5 g Na2SO4 into a 100 ml beaker which has been weighed. The beaker is heated to a temperature until the n-hexane runs out, the water then evaporates, and what remains is only hydrocarbon oil (APHA, 1981). The beaker was removed and allowed to cool down and then weighed, and the weight was recorded. The gravimetric calculation formula is as follows:

$$\text{Hydrocarbon Weight (g)} = (W_2 - W_1)$$

Where $W_1$ is the weight of the dry beaker (g), and $W_2$ is the weight of the beaker with the used oil content obtained (g).

2.4. Analysis of BOD and COD

BOD measurement was carried out using the iodometric method according to SNI. 6869.72-2000. A 500 ml water sample was diluted in a beaker glass with distilled water that had been aerated for 2 hours so that the volume became 2000 ml. Samples were divided into 6 bottles of Winkler. Then 1 ml of MnSO4 and 1 ml of alkaline iodide were added into the Winkler BOD bottle on days 0 to 5. The solution was allowed to stand for 5-10 minutes until lumps formed, 5 ml of concentrated H2SO4 was added and
then homogenized until completely dissolved. A sample of 50 ml was dripped with starch indicator then titrated with Na$_2$S$_2$O$_3$ until the blue color disappeared and the titrant volume was recorded. The following formula calculates dissolved Oxygen (DO) and BOD:

$$\text{DO (mg/L)} = \frac{V \text{Thiosulfat} \times N \text{Thiosulfat} \times 1000 \times \text{BeO}_2 \times P}{V \text{Sample}}$$

$$\text{BOD : DO1} - \text{DO2}$$

Where DO1 is Dissolved Oxygen on day 0, DO2 is Dissolved Oxygen 5 days, BeO$_2$ is 8, and P is dilution.

COD measurements were carried out using spectrophotometry according to SNI. 6869.72-2009. The 100 ml sample was added with 4 N H$_2$SO$_4$ and 5 ml and 10 ml KMnO$_4$, respectively. The resulting solution was then heated to boiling, then added 10 ml of H$_2$C$_2$O$_4$. The resulting sample is then titrated using KMnO$_4$ in a hot state until it changes color to pink and the titrant volume is recorded. The following formula calculates COD:

$$\text{COD (mg/l)} = \frac{(A - B) \times N_{fas} \times 1000 \times \text{BeO}_2 \times P}{V \text{Sample}}$$

Where A is the volume (ml) of the blank titrant, B is the volume (ml) of the sample titration, N is the normality of FAS, BeO$_2$ is 8, and P is the dilution.

2.5. Data analysis
The data analysis used in this research is regression analysis. Regression analysis is a method used to obtain the relationship of a dependent variable (regressand) with a variable or more independent variables (regressors) [11]. In regression analysis, the relationship between factors is expressed in the form of a functional relationship expressed in an equation and is called a regression equation. The regression equation can be determined from the distribution of the observed data, and the shape is a straight line (linear) or in a non-linear (curved) form [12].

3. Results and Discussion
3.1 Bacterial activity during the bioremediation process
The activity of Gordonia terrae bacteria in the biodegradation process is closely related to the bacterial density value. Bacterial densities were observed at all hydrocarbon concentrations (15 ppm, 30 ppm, and 45 ppm) (Figure 1). On day 10, the total bacterial density at hydrocarbon concentrations of 15 ppm, 30 ppm, and 45 ppm gradually increased to 1.12×10^9 cells/ml, 1.10×10^9 cells/ml, and 1.26×10^9 cells/ml, respectively. However, the total bacterial density gradually decreased on day 14 to 4.25×10^8 cells/ml, 4.8×10^8 cells/ml, and 5.3 × 10^8 cells/ml for 15 ppm, 30 ppm, and 45 ppm, respectively.

In the lag phase, bacteria carry out the process of adaptation to environmental conditions such as pH, temperature, and nutrients [13]. This adaptation process may also occur because the bacterial isolates are synthesizing enzymes to degrade hydrocarbons [13,14]. The time in this phase is required for the cell to synthesize the enzymes needed for metabolic processes [14]. During the lag phase, bacterial isolates did not show any increase in the number of cells [15]. Then the bacteria undergo an exponential phase, with bacterial growth taking place very quickly. In this phase, microbes multiply by dividing themselves into two, and then each divides again into two so that in each generation resulting in twice the previous population [14, 15]. The growth of microorganisms is an indicator of the occurrence of the biodegradation process [16]. The growth of microbes will increase if they can live by utilizing the nutritional sources in their living media [16].
3.2 Hydrocarbon degradation by Gordonia terrae

The addition of Gordonia terrae bacteria to each treatment decreased the hydrocarbon concentration (Figure 2). The percentage reduction in hydrocarbon concentration at concentrations of 15 ppm, 30 ppm, and 45 ppm was 54%, 69%, and 77%, respectively. The high level of degradation of PAHs is influenced by the ability of added hydrocarbonoclastic bacteria to degrade hydrocarbon compounds [18]. The degradation conditions during this research were aerobic conditions, where hydrocarbons were the fastest degraded [16]. The mechanism of degradation by bacteria under aerobic conditions is alkane oxidation by the enzyme oxygenase [19]. The results of this study indicate that the highest concentration decrease occurred at a concentration of 45 ppm. A high concentration of hydrocarbons seems to increase the activity of bacteria in degrading hydrocarbons. The less waste oil added to the media, the less available nutrient sources so that the bacterial activity is low [19]. Among the hydrocarbon-degrading actinobacteria [21, 22] the genus Gordonia can absorb and consume aliphatic hydrocarbons [23, 24]. Gordonia sp. widely distributed in nature and can degrade, convert and synthesize organic compounds [25].

Figure 1. Abundance of Gordonia terrae during the bioremediation process

Figure 2. Degradation of PAHs by Gordonia terrae
3.3 pH, BOD and COD during Bioremediation Process

Measurements of pH were carried out to determine the degree of acidity of the treatment media before and after bioremediation with *Gordonia terrae* (Figure 3). During the bioremediation process until day 10, the pH value tends to be pH 7 to alkaline. Thus, it seems that bacteria can make homeostatic efforts to environmental acidity to the extent that they are still within their adaptive tolerance. The homeostatic efforts were made by exchanging K$^+$ cations from within the cell and exchanging them with H$^+$, which is abundant in the environment [26], that the acidity level of the environment can be reduced.

![Figure 3. pH values during the bioremediation process](image)

BOD and COD values decreased during the bioremediation process by the bacteria *Gordonia terrae*. The BOD value in each treatment decreased from the beginning of the treatment (68.05 mg/l to 83.75 mg/l) until the end of the treatment (5.67 mg/l to 7.0 mg/l). These results indicate that bacteria in aerobic conditions consume oxygen so that the number of bacteria will increase, and the degradation process will be faster. The indicator of organic contaminants in the waste that has been decomposed by degrading bacteria is the less amount of waste and oxygen. Hence, the BOD value is also small which indicates small BOD value indicates a low hydrocarbon residue[27].

![Figure 4. BOD during the bioremediation process](image)
The COD concentration in each treatment in this study decreased from the beginning of the treatment (166.3 mg/l to 173.4 mg/l) until the end of the study (8.30 mg/l to 9.55 mg/l). The decrease in COD concentration occurred due to the degradation of PAHs and other chemical compounds contained in the waste by hydrocarbonoclastic bacteria (x). COD is used to measure waste pollution by organic substances that can naturally be oxidized through biological processes (x). The higher the concentration of COD in waste, the higher the pollutants in the waste (x). The decrease in COD concentration was also due to competition due to the increased number of bacteria (x). Bacteria adapt to using substrates other than hydrocarbons, such as fatty acids and other compounds found in hydrocarbon wastes [28].

![Figure 5. COD during the bioremediation process](image)

### 3.4 Relationship of bacterial density with decrease in PAHs

In this study, the relationship between bacterial density (X) and a decrease in PAHs (Y) was analyzed. Regression analysis was conducted to determine the relationship between the independent variable (X) and the dependent variable (Y). The results of the regression of bacterial density and PAHs can be seen in Figure 5. The relationship between bacterial growth and PAHs reduction was linear, with the coefficient of determination $R^2 = 0.872$. These results indicate that the 87% decrease in TPH is influenced by bacterial density. Hence, changes in bacterial density will have a different effect on hydrocarbon degradation. It seems that hydrocarbons are the primary energy source for bacteria in this study [6].
The values of BOD, COD, and PAHs are fundamental indicators to determine the level of hydrocarbon pollution in the waters. The regression coefficients of BOD and COD for PAHs are presented in Figure 6. The relationship between BOD and COD parameters to the decrease in PAHs levels is linear with the coefficient of determination $R^2 = 0.994$. These results indicate that 99% of the decrease in PAH levels is influenced by BOD and COD values. The smaller the BOD, COD, and PAH numbers indicate that wastewater contamination is getting smaller [6]. The effect of BOD and COD values on decreasing PAH levels can be related to the availability of oxygen for the bioremediation process [29].
4. Conclusion
This study showed the effectiveness of bioremediation of Polycyclic Aromatic Hydrocarbons (PAHs) by Gordonia terrae at PAHS concentrations of 15 ppm, 30 ppm, and 45 ppm were 54%, 69%, and 77%, respectively. This study indicates that the decrease in the concentration of hydrocarbons is influenced by several factors such as pH, bacterial density, BOD, and COD. According to the result of this study, Gordonia terrae can be a bioremediation agent to restore the environment contaminated by hydrocarbon pollutants.

5. References
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