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# Isolation, and Identification of Diesel Oil Degrading Bacteria in Water Contamination Site and Preliminary analysis with Potential Bacterial *Gordonia terrae*

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**KEYWORDS**

Diesel oil
Bioremediation
Bacillus cereus
*Gordonia terrae*

**Abstract** Bioremediation is one of kind method which use of bacteria to degrade of hydrocarbon to be another compound. This study aims to get isolation of indigenous bacteria and potential test with *Gordonia terrae* on diesel oil bioremediation. The water sample was taken from Tanjung Perak Port of Surabaya, Indonesia where contaminated with diesel oil. Analysis of in-situ water quality parameters were consist of Dissolved oxygen, temperature, pH, salinity. Strain isolate bacteria were isolated by Bushnell-Hass medium. The bacterial density result shows in IN6 shows that bacterial density increased up to 9 × $10^8$ cells/ml at day 2 incubation and decrease up to 1.79 × $10^8$ cells/ml at final incubation, IN8 increased up to 8.6 × $10^8$ cells/ml, then decrease up to 3.57 × $10^8$ cells/ml. While, EX8 increased up to 9.1 × $10^8$ cells/ml, and then decrease up to 3.06 × $10^8$ cells/ml. While, results of degradation diesel oil hydrocarbon show treatment IN6, TPH values showed a decrease in the initial hydrocarbon concentration of 30 ppm to 16 ppm. IN8 TPH value shows a decrease in the initial hydrocarbon concentration of 30 ppm to 16 ppm. EX8 TPH value shows a decrease in the initial hydrocarbon concentration of 30 ppm to 24 ppm. In this experiment was found that *Bacillus cereus* was effectively degrading of diesel oil and can be agent bacterial for bioremediation.

## Introduction

Total petroleum hydrocarbon is the measurable amount of petroleum-based hydrocarbon in environmental media. It is, thus, dependent on analysis of the medium in which it is found (Gustafson *et al*., 1997 in Drevininkas *et al*., 2005). Petroleum hydrocarbons are common site contaminants however, they are not generally regulated as hazardous wastes. Ship dismantling is a source of diesel pollution in coastal areas. The main activity is the dismantling and cutting apart of ships. Its activity has caused seawater and soil pollution as indicated by the seawater and soil having become muddy and black. Petroleum is a complex mixture of hydrocarbon and other organic compounds including some Organometallic constituents, most noticeably vanadium and nickel (Hamme *et al*., 2003). Petroleum hydrocarbon can be divided into four classes: saturates, aromatics, asphaltenes (phenols, fatty acid, ketones, esters and porphyrins) and resins (pyridines, quinolines, carbazoles, sulfoxides and amides) (Leahy *et al*., 1990 in Ibrahim, 2016).

Diesel is an engine fuel that contains rich hydrocarbons, ranging from $C^8$-$C^{26}$, and polyaromatic hydrocarbons (PAHs) and diesel oil have often been reported as one of the major hydrocarbon pollutants, as a result of spill incidents, storage tanks and leaking pipelines (Gallego *et al*., 2001). It consists of many

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components including aromatic hydrocarbons (23.9 %), cycloalkanes (33.4 %) and n-alkanes (42.7 %). Diesel is an environment contaminant (Palanisamy *et al*., 2014). Worldwide, about 1.7-8.8 million metric tons of diesel are released into the aquatic environment (Dadrasnia, 2013). Diesel spillage causes serious damage to the marine ecosystem. The components of diesel are toxic for the environment and potentially carcinogenic (Ramasamy *et al*., 2017). Pollution of diesel oil waste in the waters will have a long-term impact, especially for marine biota. When diesel oil in the sea can be consumed by marine biota, some hydrocarbons can be released together with their food, while others can accumulate in fat and protein compounds in the body of the organism. The nature of this accumulation can be transferred from one organism to another through the food chain. So, the accumulation of hydrocarbon waste in zooplankton can move to predatory fish. And so, on if the fish is eaten by larger fish, other marine animals and will even reach humans (Leppchen *et al*., 2006).

Use of technology commonly on the remediation process includes mechanical the technology commonly used for the environmental remediation includes water and soil. However, these technologies are expensive and can lead to incomplete decomposition of contaminants. Therefore, one approach to remediate oh hydrocarbon is bioremediation process. Bioremediation process used to treat contaminated media, including media, water, soil and subsurface material by altering environmental conditions to stimulate growth of microorganism and degrade the target pollutants. Biological treatment is a similar approach used to treat wastes including wastewater, industrial waste and solid waste. Bioremediation depends on the ability of microorganisms to degrade diesel pollutants (Ibrahim, 2016). Some bacteria isolated from diesel contaminated areas have the ability to can produce enzymes for degrading and utilizing diesel as carbon and energy source (Patil *et al*., 2012). Furthermore, some microorganisms have the ability to can produce biosurfactant to increase the solubility of diesel. Bioremediation has many advantages over traditional clean-up methods of oil spills. Major advantages of bioremediation include cost saving and time amongst others. The recovery of environment after an oil spill depends on a number of factors including quantity spilled, chemical composition of the crude oil or petroleum product, and the biodegrading potential of the microbial population in the area affected (Sabate *et al*., 2004), these include temperature, pH, pollutant type and concentration, nutrients, oxygen availabilities and microorganism concentration on the impacted site. Bioremediation is a process of recovery (remediation) Environment that is contaminated with organic waste and inorganic waste by utilizing one of the bacteria. The ability of bacteria in hydrocarbon chains begins with the dissolution of hydrocarbons in the liquid phase by surfactants produced by the cell surface of the microorganism (Rosenberg, 2008).

Some studies have shown that *Rhodococcus* and *Gordonia* could degrade recalcitrant hydrocarbons such as polyaromatic hydrocarbon compounds (Bourguignon, 2014). Several bacterial genera with the ability to degrade petroleum hydrocarbons were isolated and identified from many environments in our previous study, including *Gordonia, Acinetobacter, Pseudomonas, and Bacillus* (Auffret *et al*., 2009). Some microorganisms such as *Rhodococcus* and *Gordonia* are extensively used for bioremediation of petroleum hydrocarbons (Hatayama *et al*, 2007, Lin *et al*, 2009). Degradation of car engine oil and c-alkane was degraded by isolated *Gordonia* (Koma *et al*., 2003). Therefore, it is necessary to isolate of indigenous bacteria from diesel oil


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contamination sites to get bacteria agent and its ability to degrade diesel oil and also to analyses of *Gordonia terrae* potential bacterial in water oil contamination base. Thus, this study aims to get isolation of indigenous bacteria and potential test with *Gordonia terrae* on diesel oil bioremediation.

## Materials and methods

*Sampling site*

This experiment was conducted in December 2018 until January 2019. The sampling location is located in Tanjung Perak Port Surabaya 112°43'22" East Longitude and 07°11'54" South Latitude, Surabaya Indonesia, Indonesia where indicated of oil contaminated site. This location is considered the second largest and busiest port in Indonesia and also is the center ship activity in Eastern Indonesia. This condition was indicated that diesel oil was contaminated by surrounding waters in Tanjung Perak Port. Water sampling was taken in most diesel contamination sites to get potential bacterial on it and analyzed in the Laboratory of Fishery Products Safety and Handling of Fisheries Products (Faculty of Fisheries and Marine Sciences, Brawijaya University, Indonesia). In this location in-situ and ex-situ parameter of water quality also be measured that consisted of temperature, pH, *dissolved oxygen* (DO), salinity, and total petroleum hydrocarbon. Analyze of Hydrocarbon was analyzed in Microbiology laboratory Faculty of Science, Islamic State University of Malang.

*Bacteria Isolation*

To isolate of specific hydrocarbon bacteria was used selective media Bushnell-Hass media Agar ($K_2HPO_4$ 1 gr / L; $KH_2PO_4$ 1 gr / L; $NH_4NO_3$ 1 gr / L; $MgSO_4$ 0,2 gr / L; 0,02 gr/L $CaCl_2$ and 0,05 gr/L $FeCl_3$ pH value of 7.0 + Agar) and 1 ml of diesel oil as carbon sources (Hassanshahian, 2013). The water sample diluted by $10^8$ dilutions and spread in media and incubated at 30° C for 1 week. Then, after incubation the single colony was taken and moving to Luria Broth medium (1 L: 1% peptone, 0.5% yeast extract, and 0.5% NaCl) in order to enrich of bacteria. Then, the bacteria culture was put in water bath shaker for 72 hours at 30° C, 170 rpm speed shaker. Bacterial identification is used by API 20E. Pure bacterial isolates grown on slant NA media were then incubated for 24 hours at 37° C.

*Bacterial Test for Diesel Oil Bioremediation*

The potential bacterial exogenous and isolated bacteria indegenous were prepared. Then, each bacteria was added by following amount treatment consisted; (IN6): Indigenous isolation bacteria ($1\times10^6$ cells/ml); (IN8): Indigenous isolation bacteria ($1\times10^8$ cells/ml); EX8: *Gordonia terrae* ($1\times10^8$ cells/ml) to LB broth medium and control treatment. After that, 30 ppm of diesel oil was added to all treatments and incubated in water bath shaker for 7 days at 32° C, 170 rpm speed shaker.

*Bacterial Cells Density*

In this study counting living bacteria using the hemocytometer method. Calculation of the number of bacteria indirectly is the number of bacteria counted that lives alone (Rohmah *et al*., 2018). The sample is diluted into eppendorf tube wich has been filled with sterile water, then the sample is mixed with trypan blue dye 0.4% (w/v) to distinguish between live bacterial cells and dead bacterial cells. Sampling was done using the volumetric method, as many as 1 ml/unit of the experiment.

$$\Sigma \text{ cells/ml} = \Sigma \text{ cell count} \times 250.000 \times n \times a$$

Information:

| | | |
|---|---|---|
| Σ cells/ml | : | Total bacteria (cells/ml) |
| *n* | : | dilution factor |
| *a* | : | trypan blue volume |

*Diesel Oil Degradation*

To analyses of diesel oil was used gravimetric method. Then, LB media containing


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the treated diesel oil put into a separating funnel, 5 ml of 3N HCl and 60 ml of n-hexane were added to the purification by multilevel distillation at 60° C, then shaken for ± 15 minutes then left until n-hexane separated (Yu *et al*., 2014). There are 3 layers, namely diesel oil, n-hexane and water. Water is removed, the layer of diesel oil and n-hexane is filtered with filter paper which has been smeared ± 0.5 g of $Na_2SO_4$ into the 100 ml Erlenmeyer which has been weighed. Erlenmeyer is heated at a temperature of 60° C (according to the boiling point of n-hexane) until n-hexane runs out, the water evaporates and what remains is only oil (Huang *et al*., 2005). The levels of used oil are calculated by:

*Level of used oil (g)* = W2 – W1

Information:
W1 : dry beaker (g)
W2 : beaker with the amount of used oil obtained (g)

**Results and discussions**

*Water Parameters Analysis*

The quality of seawater used for marine biota and other activities should ideally meet the standards, the value of sea water quality that exceeds the maximum threshold for its designation will be classified as polluted waters. The following results of water quality show in Table 1.

**Table 1. Result of water quality parameters**

| Parameter | Unit | Observation value |
|---|---|---|
| Dissolved Oxygen | mg/L | 4.5 |
| pH | - | 8 |
| Temperature | °C | 29.3 |
| Salinity | Ppt | 34 |

- *Dissolved Oxygen*
  The results of dissolved oxygen (DO) in the waters of the Port of Tanjung Perak amounted to 4.5 mg/L. This was alleged because the waters of the port of Tanjung Perak received more organic material. This indicated that addition of organic material from the port activities also originates from two rivers which lead to the waters of the port of Tanjung Perak. The condition of low DO concentrations in the waters of the port of Tanjung Perak shows that these waters in the condition of the contents are not suitable for the life of marine biota (Kordi dan Tancung, 2007 *in* Djoharam *et al*., 2018).
- *pH*
  pH result in Tanjung Perak Port water is 8. pH value is an important factor in waters because the pH value in water will determine whether will be acidic or basic which will affect biological life in the water. Changes in acidity of water, both in the direction of alkalis and acids, will greatly disrupt the lives of fish and other aquatic animals. The pH range that is suitable for aquatic organisms is not the same depending on the type of organism (Djoharam *et al*., 2018).
- *Temperature*
  The temperature value in the waters of Tanjung Perak port is 29.3° C. The water temperature exceeds the standard quality limit of a waters. The increase in water temperature in receiving water bodies, waterways, rivers, lakes etc. will have the following consequences: 1) The amount of dissolved oxygen in the water decreases; 2) The speed of chemical reactions increases; 3) Life of fish and other aquatic animals are disturbed. Increased temperature also


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causes an increase in the decomposotion of organic matter by microbes. Besides that, the temperature of river water is a limiting factor for aquatic organisms (Cech, 2005 in Djoharam *et al*., 2018).

*Bacterial Density*

Bacterial growth was observed by measuring the density of bacterial cells. The bacterial growth curve is used to study the bacterial response to diesel oil that is present in the growth medium (figure 1).

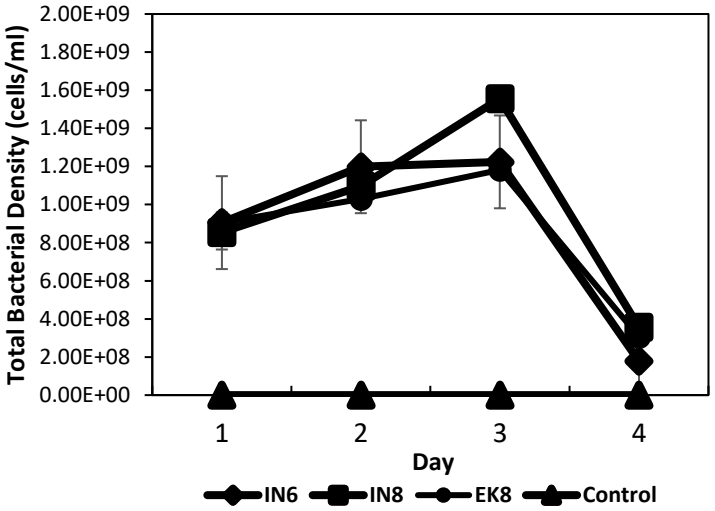


**Figure 1.** Results of Bacterial Density during incubation (n=2).

The result shows the density of indigenous bacteria and *Gordonia terrae* for 7 days incubation showed a difference. IN6 shows that bacterial density increased up to $9 \times 10^8$ cells/ml at day 2 incubation and decrease up to $1.79 \times 10^8$ cells/ml at final incubation. IN8 increased up to $8.6 \times 10^8$ cells/ml, then decrease up to $3.57 \times 10^8$ cells/ml. While, EX8 total bacteria density cell increased up to $9.1 \times 10^8$ cells/ml, and then decrease up to $3.06 \times 10^8$ cells/ml. this result was indicated that bacteria respond to the solar contaminant in media. According to Lestari *et al*. (2018), a decrease in the number of cells can be caused by nutrients which begin to decrease but the total number of bacterial cells is high which causes competition. In other hands, this result may cause of stationary level of bacteria towards hydrocarbon. According to Forget *et al*. (2011), during the stationary phase, bacteria will form biosurfactants which can help in degrading hydrocarbons. After the stationary phase is followed by a decrease because the number of bacteria is out of balance with the availability of nutrients. According to Nugroho (2012), hydrocarbon bacteria in their life activities require carbon molecules as a source of nutrition and energy to metabolize and reproduce. Another factor that affects the reduction in bacterial cell density is that there is a lack of nutrients such as N, P and C that are no longer suitable for growth.

*Total Petroleum Hydrocarbon (TPH)*

TPH is the number of petroleum hydrocarbons measured from environmental media. TPH is an organic compound consisting of hydrogen and carbon, for example benzene, toluene, ethylbenzene and xylene isomers. Petroleum hydrocarbons are various types of hydrocarbons found in petroleum. The percentage of degradation is the total amount of


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hydrocarbons that have been degraded by hydrocarbonoclastic bacteria. Thus, TPH is defined as the analytical method used to measure the amount of petroleum hydrocarbons in a medium (Tang *et al*., 2011). After, treatment shows removal of diesel oil degradation in figure 2.

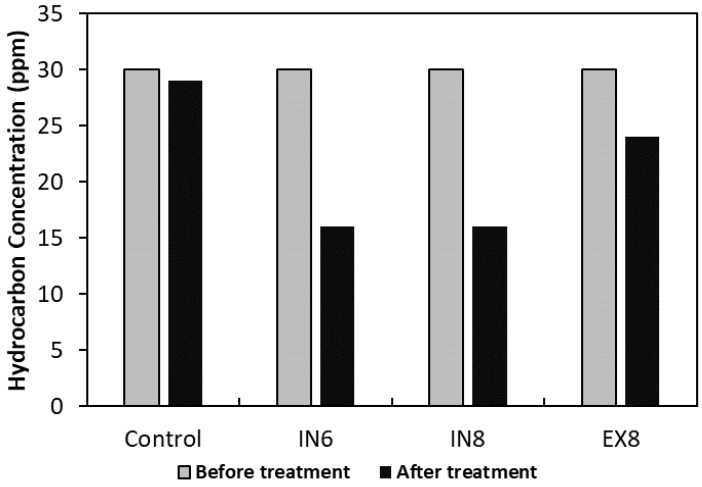


**Figure 2.** Result of Diesel Oil Degradation

In treatment IN6, TPH values showed a decrease in the initial hydrocarbon concentration of 30 ppm to 16 ppm. IN8 TPH value shows a decrease in the initial hydrocarbon concentration of 30 ppm to 16 ppm. EX8 TPH value shows a decrease in the initial hydrocarbon concentration of 30 ppm to 24 ppm. This result indicated that the decrease in the best hydrocarbons concentration from indigenous bacterial isolates with initial densities of IN6 and IN8. Biodegradation test results on diesel oil showed that all bacterial isolates have the ability to degrade oil indicated by a decrease in oil content after testing. Each bacterial isolate has a different ability to degrade diesel oil. The ability of bacteria to degrade diesel oil is caused by bacteria producing enzymes that are able to break down complex organic compounds into simpler compounds (Drevininkas *et al*., 2005).

*Identification of Indigenous Bacteria*

Bacterial identification was carried out to find out the type / indigenous bacterial species that had been isolated from the aquatic environment contaminated with diesel oil hydrocarbon waste. This indigenous bacterium will be used as a bioremediation agent to reduce or even eliminate the pollution of diesel oil hydrocarbon waste. The Identification of indigenous bacteria was carried out using the KIT API 20 E V5.0 method.

Based on the results of the identification it can be seen that indigenous bacterial isolates are able to grow alive at 30 ppm diesel oil concentration is a bacterium species of *Bacillus cereus.* Indigenous bacterial isolates have a yellowish pigmented colony, irregular in shape, with a high edge (lobate), high elevation (raised), and rough and regular internal structures. This result was similar to Janaki *et al*. (2016), mentioned that *Bacillus cereus* has a high potential for hydrocarbon degradation by producing biosurfactant.

**Conclusions and suggestion**

Bacterial play *an* important role as bioremediation agent in Environment. This preliminary research was found that bacteria *Bacillus cereus* that isolated from water contaminated site was successfully degrade of diesel oil compare to *Gordonia terrae*. In the


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stationary level of bacteria were response at 5 days incubation which after that the density of bacteria decreased. The total removal of diesel oil in Bacillus cereus was from 30 ppm to 16 ppm in both concentration application rate $1\times10^6$ and $1\times10^8$ cells/ml. Characteristic of biosurfactant produced by *Bacillus cereus* can be optimized as candidate bacteria for removal hydrocarbon for future research.

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