# Petroleum residues degradation in laboratory-scale by *rhizosphere* bacteria isolated from the mangrove ecosystem

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**Abstract.**This research is about petroleum bioremediation experiment to obtain bacterial isolate from mangrove ecosystem which potentially degrade petroleum. It was conducted in an Erlenmeyer batch system filled with growth medium of Stone Mineral Salt Solution (SMSS) plus petroleum residue, placed in an incubator shaker with a rotation speed of 120 rpm, temperature $30^0$C, for 14 research days. Indigenous bacteria that have been isolated and identified from the roots of mangrove plants are *Ochrobactrum anthropi* and *Bacillus* sp., *Ralstonia pickettii* and *Bacillus circulans*. Those bacteriain both monoculture and consortium form (mixed culture) are incorporated into erlenmeyer as remediator agents. All bacteria can utilize hydrocarbon compounds, but *Ralstonia pickettii* and *Bacillus circulans* reached exponential phase faster with more cell count than other bacteria. Compared to single cultures, petroleum degradation by a bacterial consortium provides a higher TPH reduction efficiency, i.e. at 5%, 10%, and 15% of initial TPH of 94.4%, 72%, and 80.3%, respectively. This study proved that all bacteria could optimize hydrocarbon compounds up to 15% TPH load.
**Keywords:** biodegradation, hydrocarbon, petroleum, mangrove, total petroleum hydrocarbon

## 1. Introduction

Petroleum contamination can occur in mangrove ecosystems due to oil spills occurring in the sea or coast which is swiped by tide to reach the mangrove ecosystem. Oil residues that pollute mangrove ecosystems can influence mangrove plants, especially on their root systems and on microorganisms that live in *rhizosphere*. If mangrove plants cannot adapt to residues, then the sustainability of mangrove forests can be threatened. The composition of residue is a hydrocarbon compound which can still be used by bacteria as a carbon source. Bacteria are groups of microorganisms that readily adapt to their environment, allowing them to use residues as a source of carbon and energy [1].

An effective petroleum removal strategy is required to prevent the destruction of mangrove ecosystems. Bioremediation is an effective and promising way to make quick, cheap remediation with minor health risks [2], [3], [4]. The breakdown of the hydrocarbon compounds by bacteria to become simpler compounds, causing changes in the physical and chemical properties of the compounds. Some of those properties that can be an indicator of the degradation process of hydrocarbon compounds include viscosity, specific gravity, density, water solubility, petroleum residual weight and surface tension [3]. The hydrocarbon splitting process and the breakdown of the residual constituent components can occur both non-biologically and microbiologically. Non-microbiologically for instance is due to evaporation, and dissolution, while microbiologically is due to the activity of microorganisms such as bacteria, yeasts, and fungi.

*Rhizosphere* of mangrove ecosystems that are covered by petroleum residues allegedly inhabited many bacteria that have been adapted to utilize the carbon source in the residue for its growth [5]. Based on these circumstances, this study was conducted to isolate bacteria from plant *rhizosphere* in the mangrove ecosystem and exploit its ability in the process of petroleum bioremediation.

## 2. Research Method

### *2.1. Isolation and purification of indigenous bacteria*

Petroleum comes from Kepulauan Seribu (Island's name in Jakarta region that means Thousand Island). The roots of mangrove plant originated from the conservation of the mangrove ecosystem of Pantai Indah Kapuk, Jakarta, Indonesia. Isolate bacteria grown in Erlenmeyer with batch culture containing SMSS (Stone Mineral Salt Solution) liquid medium. Degradation test was done by adding petroleum with initial concentration of Total Petroleum Hydrocarbon (TPH) that is 5%, 15%, 25%, and 50%. Samples in the form of *rhizosphere* were weighed 4.5 grams and inoculated into 45 mL of liquid SMSS medium, then incubated in an incubator shaker for five days at $30^0$C with 120 rpm shaking rate. Further diluted to $10^6$, and each dilution was taken as 1 mL mixed with SMSS medium until evenly distributed, then poured into a sterile petri dish. After the medium of SMSS in the sterilized petri dish solidified, the culture was incubated at a temperature of $30^0$C until bacterial colonies were found to grow.

### *2.2. Bacteria identification*

Identification stages include: morphological testing of colonies, cell morphology, Gram properties, presence or absence of endospores, biochemical testing. Furthermore, to determine the species of bacterial isolate obtained, the test results at the identification stage were matched with **Bergey's Manual of Determinative Bacteriology** [6]. The ability of isolates to degrade petroleum residue is studied by measuring viscosity, specific gravity, residual weight, observing a decrease in pH of the medium and calculating the total number of bacterial cells.

### *2.3. Residual viscosity measurement*

The measurement of the viscosity is carried out at a temperature of $30^0$C, which is started by separating the residual phase from the media (water) phase by passing the media phase to the container via a pipe connected to the suction pump. Once separated, the residue is then inserted into a cylindrical glass. A ball of metal material is inserted into a cylindrical glass containing residuals and the length of time the ball travels through the length of the residue in the glass [7]. Afterward, the determination of viscosity used the following equation:

$$\eta = \frac{2r^2 g\,(\rho - \rho o)\,t}{9d} \tag{1}$$

rho = dynamic viscosity coefficient (Pa.s), r = metal ball radius (m), g = earth gravity force ($m/s^2$), rho = metal ball density ($kg/m^3$) po, d = residual density ($kg/m^3$): the length of the residue through which the metal sphere (m), t = the travel time of the metal ball passes through the residue (s). The value of the viscosity obtained is converted:

$$v = \frac{\eta}{\rho o} \tag{2}$$

In units of $m^2/s$, then the kinematic viscosity unit used is mm/s equivalent to centistoke (cSt).

### *2.4. Specific gravitation measurement*

Specific gravity [7] was measured by comparing residual density ($kg/m^3$) with aquadest density ($kg/m^3$) at $30^0$C. Aquadest density at $30^0$C temperature measured 1012.2216 $kg/m^3$.

### *2.5. Measurement of final residual weight*

The final residual weight is measured by weighing the total residual at the end of the observation that

has been separated from the media (water) phase in grams.

*2.6. Calculation of the number of bacterial cells*

Calculation of bacterial cell count is done using total plate count method [2]. Before the number of bacterial cells is calculated, each culture in the Erlenmeyer is shaken first so that the residual phase with the media phase is listed perfectly (homogeneous). Sampling was performed daily for 14 days and the number of bacteria in the sample was calculated in units of cells/mL. Data on the number of bacterial cells obtained from each inoculum made the curve so that it could show the relationship between the number of cells with time.

$$r = \frac{3{,}32[\log x_t - \log x_o]}{t} \quad (3)$$

*Xt* = the number of bacterial cells at the end time ($t_x$), *X0* = the number of bacterial cells at the beginning ($t_x$), *T* = incubation time of $t_0$–$t_x$ so that it can be determined the increase in the number of cells per unit of time.

## 3. Results and Discussion

*3.1. Selection and identification of bacterial isolates*

Bacterial isolates obtained from *rhizosphere* of mangrove plants after purification were as many as 4 species. The results of the first stage selection showed that all the isolated *rhizosphere* bacteria isolates could grow on SMSS medium containing petroleum residue. The first stage selection was performed to determine the isolates of *rhizosphere* bacteria that survive and grow in environmental conditions containing petroleum residues, although the bacterial isolates may not be able to utilize petroleum residue as a source of carbon and energy.

The SMSS growth medium used in the first stage selection was a nutrient-rich medium, because it contained peptone (5 g/L) and yeast extract (1 g/L), in which the *rhizosphere* bacteria were resistant to residue but could not utilize the residue as a carbon sourcewhich allegedly still could grow and survive by utilizing the nutrients of the medium. In the first stage selection, the isolates were not resistant to the presence of petroleum residue and could not utilize the petroleum residue as a source of carbon and energy, the isolates would die and not grow. Selection phase II aimed at determining the isolates of bacteria that are resistant to the presence of petroleum residue and can use it as a source of carbon and energy. Therefore, the medium used is a poor medium of nutrition. The identification results showed that all the bacterial isolates obtained were Gram-negative, non-spore and one isolate was Gram positive, with spore. The complete identification results for each isolate are shown in Figure 1, Table 1 and Table 2.

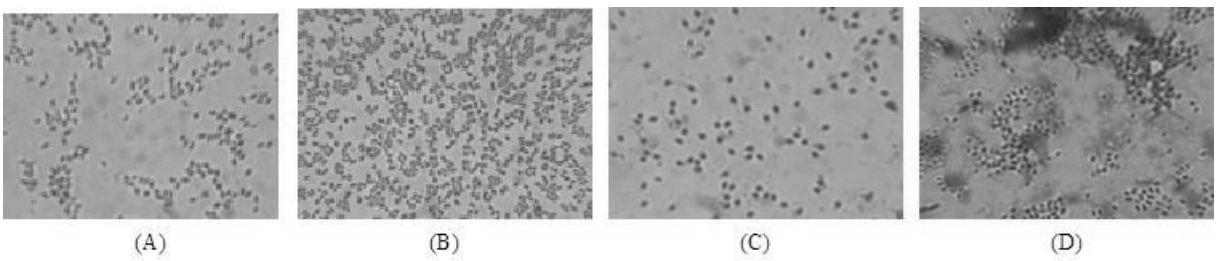


**Figure 1**. Identified bacterial isolates A) *Ralstonia picketii*, B) *Bacillus circulans* *C) Ochrobactrum anthropi*, D) *Bacillus sp.*

**Table 1.** Visual observation results colonies of microorganisms.

| Bacteria | Reaction to Blood agar | Size | Shape | Elevation | Surface Condition | Colour |
|---|---|---|---|---|---|---|
| *Ralstonia pickettii* | Himolise | 2 mm | Irregular | Convex papillate | Rough | Green |
| *Bacillus circulans* | Non-himolise | 0.5 mm | Circullar | Low convex | Smooth | Grey |
| *Ochrobactrum anthropi* | Himolise | 1 mm | Circular | Low Convex | Smooth | Grey |
| *Bacillus sp.* | Himolise | 2 mm | Irregular | Convex papillate | Rough | Grey |

**Table 2.** biochemical test results.

| Bacteria | KIA | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|---|
| *Ralstonia pickettii* | K/A | + | + | - | + | - | - | + |
| *Bacillus circulans* | K/K | + | - | + | + | + | - | - |
| *Ochrobactrum anthropi* | K/K | - | - | - | + | - | - | + |
| *Bacillus sp.* | K/K | + | + | + | + | + | - | + |

A = Glucose; B = Maltose; C = Sucrose; D = Arabinose; E = Mannitol; F = Voges-Proskauer; G = Lactose

*3.2. Residue viscosity*

The residual viscosity of medium that was not given inoculum (control) reached 54.64 cSt. This value is significantly different from all other inoculum factors, either in the form of single culture or its mixtures. The lowest viscosity was shown in the mixed culture of 22.88 cSt (Figure 2).

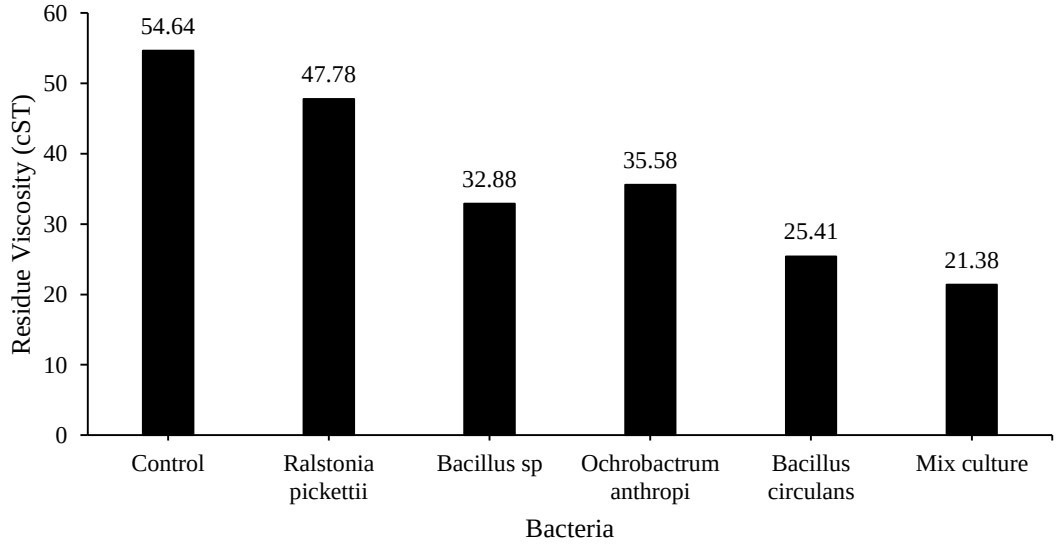


**Figure 2.** Residue Viscosity (cSt).

Each residual viscosity value due to the bioremediation agent in the form of single culture of *Ralstonia pickettii, Bacillus sp., Ochrobactrum anthropi* ranged between residual viscosity values due to its mixed culture and control. Bioremediation agent in the form of single culture *Bacillus circulans*, showed the lowest viscosity value (28.97 cSt), when compared with other single cultures. Provision of

bioremediation agent showed the smallest viscosity of 22.88 cSt. It was suspected that all isolates in the mixed culture worked to degrade residues by utilizing the corresponding residual components for each isolate, resulting in a much simpler fraction in large quantities. The number of these simple fractions causes a decrease in the value of viscosity.

*3.3. Specific residue gravitation*

The unallocated medium (control) of the residual-specific gravity value reaches 0.6911, the value is significantly different from all the specific gravity values of the residue on the medium given the level of other inoculum factors. The mixed inoculum shows the least specific residual gravity value of 0.6108 which means the highest degradability (Figure 3).

The specific gravity value is closely related to the viscosity value, the hydrocarbon compound having a low specific gravity value, the viscosity is also low. Hence the relationship between specific gravity and viscosity is directly proportional, which means the increase in specific gravity will be followed by increased viscosity and vice versa [3], [7].

The final residual weight at the inoculum level of single culture *Bacillus sp* reached 22.65 g and was not significantly different from the residual weight of the medium without inoculum (control) reaching 22.83 g, but the other single culture inoculum levels and mixtures were all significantly different with control. The final residual weight of the medium inoculated with the smallest mixed culture was 16.88 g compared with the weight of the final residue on the inoculated medium with other control factors. Mixed cultures were the best inoculum factor in reducing the final residual weight compared with all the inoculum factors tested, since the medium inoculated with mixed culture showed the least final residual weight of 16.88 g.

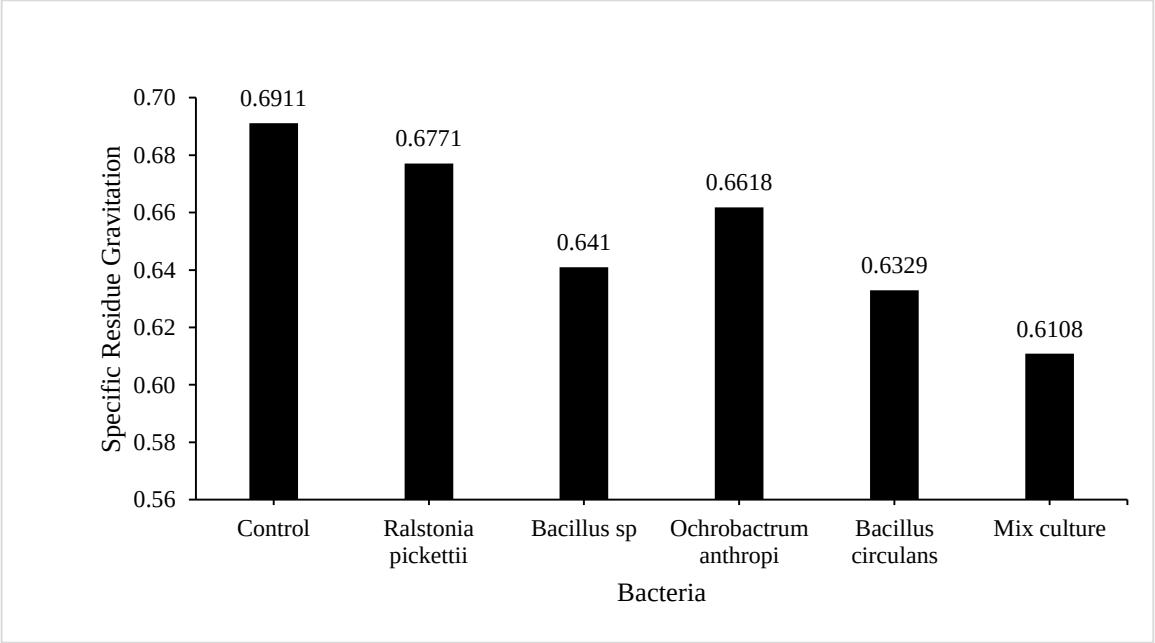


**Figure 3.** Spesific residue gravitation.

The use of nutrients by bacteria will be directly proportional to the population of growing bacteria, if the number of bacterial populations is high then the nutrients used are also numerous. Residue degradation by bacteria is the process of using residues as nutrients by utilizing the hydrocarbon compounds which contained therein, so that the reduction of residuals used as nutrients will be proportional to the addition of a growing population of bacteria. Thus, there is a relationship between degradation rate of petroleum residue with growth rate of degrading bacteria.

The degradation process was discontinued on day 14 although the degradation process was still going on, but the degradation rate was very slow. Based on the k values of each level of inoculum factor, it

can also be explained that single culture of *Bacillus circulans* has the highest degradation rate, followed by mixed cultures, single cultures of *Ralstonia pickettii, Bacillus sp., Ochrobactrum anthropic*. The results also showed that the residual degradation process could proceed well, in the form of a single culture and mixed culture. Although mixed cultures consisting of four isolates, residual degradation processes indicated by viscosity, specific gravity, and the weight of the final residue and decreased pH of the medium, all indicated a strong degradation ability.

## 4. Conclusion

Isolates of *rhizosphere* bacteria in the form of mixed cultures have a higher ability than isolates in the form of a single culture in degrading petroleum residue as indicated by changes in viscosity, specific gravity and residual weight. While in the form of a single culture, each isolate has a specific ability to degrade petroleum residues such as *Bacillus circulans* has the highest ability to decrease viscosity and decreases specific gravity, *Ralstonia pickettii* vesicular has the highest ability to decrease the pH of the medium, and *Bacillus* sp. has the highest ability to reduce the weight of the final residue.

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