Investigation of Potential Marine Bacterial Isolates in Biodegradation Methods on Hydrocarbon Contamination

R Armus*, C Selry¹, I Marzuki², H Hasan³, Syamsia⁴, and A Sapar⁵

¹Environment Engineering Department, Nusantara Indonesia of College Technology, 90234, Makassar, Indonesia
²Chemical Engineering Department, Faculty of Engineering, Fajar University, Panakukang, 90231, Makassar, Indonesia
³Pharmacy Department, Faculty of Sports and Health, Gorontalo State University, 96112, Gorontalo, Indonesia
⁴Agrotechnology Department, Faculty of Agriculture, Muhammadiyah Makassar of University, 90222, Makassar, Indonesia
⁵Chemistry Department, Faculty of Mathematics and Natural Sciences, Tanjungpura University, 78124, Pontianak, Indonesia

*Email: rakhmadarmus@stiteknusindo.ac.id

Abstract, Hydrocarbon pollution, especially polycyclic aromatic hydrocarbons which are toxic, carcinogenic and mutagenic in the sea, has a negative impact on biota and must be controlled. Identification and isolation of bacteria that have the potential to degrade hydrocarbons in the sea need to be done to overcome the hydrocarbon pollution. The methods used include quantitative test for biodegradation capacity, identification of bacterial species, phenotype test for gram staining, biochemical test, and genotype test using PCR. The results of identification, selection and optimization of isolates, obtained seven types of consortium bacterial isolates with codes BI, BA, AB, BS, BP, BASA and BAPS which have the potential to degrade hydrocarbon contaminants. The phenotypic data showed that there were 3 isolates from the group of gram-positive Baccilus bacteria, while based on the genotype data, the three isolates were identified as Bacillus infantis and Acinetobacter baumannii. The results of the biodegradation activity test showed that the bacterial isolate was able to reduce the surface tension of aliphatic and aromatic hydrocarbons, so that the bacteria could act as a degrading hydrocarbon pollutant.

Keywords: Pollutants, Hydrocarbons, Bio-surfactants, Biodegradation, Bacterial Isolates

1. Introduction
The increasing development of the petroleum industry as an effort to meet energy needs can actually have an impact on the environment. Likewise, the increased activity above sea level as a means of traffic, shipping vessels and oil tankers, can have implications for the effects of pollution on the
marine environment [1]. Efforts to deal with oil spills (Oil Spill) that enter marine waters have been made both chemically and physically. However, these two methods, apart from requiring relatively large costs, also have ecological effects [2,3].

Pertamina Depot Makassar, which is located near the center of community activity and is one of the places for loading and unloading oil, is likely to have a number of oil spills that could contaminate the sea[4]. This in turn allows the growth of microbes capable of degrading hydrocarbons. Based on the foregoing, the author is interested in conducting research on a laboratory scale to find marine microbes that can effectively degrade hydrocarbon compounds in the marine environment as an initial step in overcoming pollution by hydrocarbons. Areas that are exposed to hydrocarbon contamination, hydrocarbon degrading bacterial populations can be found.

The negative impact that is most felt due to oil pollution in the sea is the deterioration of the quality of the environment or ecosystem and all aspects related to marine waters such as damage and deterioration of water quality. So that absolutely necessary efforts / actions to overcome these problems [5].

2. Material and methods

2.1. Sample collection

This research lasted for twelve months. This time period includes, research preparation, sampling, making culture and testing the capacity of bacteria to degrade hydrocarbons and data analysis. The research begins with sampling in the waters of the Port of Pertamina Depot Makassar. Figure 1. Sampling was carried out at three stations with a Cammerer Water Sampler and sediment trap. Samples were taken from certain sea water depths (5 and 10 meters). The sample taken was put into a sample bottle that had been sterilized with alcohol first, then placed in an ice box filled with ice in order to keep the sample durable (not damaged). Then the sample is taken to the laboratory for further treatment.

2.2. Petroleum Characteristics

The Petroleum Refinery sample used consisted of two types of samples taken from Unit IV Pertamina, Jalan MT. Haryono No.77 Cilacap Regency, West Java and one type of BBMS fuel was taken from the Ujung Tanah gas station. The characteristics petroleum refinery sample are shown in the table 1, as follow:

| Table 1. The master sample specification used |
|-----------------------------------------------|
| Marine Fuel Oil-1 (MFO-1) | Industrial Diesel Fuel (IDF) | Fuel Oil (BBMS) |
| IL. No. Tank : 022/12 | IL. No. Tank: 127/12 | SPBU Pertamina 74.901.09 Ujung Tanah |
| Taken By : Lab.INSP/SHIFT B-1 | Taken By : Lab. INSP/ C-12 | |
| Batch Number : SE/Lab.23-R IV/200 | Batch Number : SE/Lab.23-R IV/200 | |

2.3 Method

In the direct plating method carried out in the field, the colonies that grew after incubation were observed for one to two weeks. The number of colonies was counted, then colonies with different shapes and colors were isolated. Each colony was purified, namely by rubbing 1 ose colony in a zigzag direction on a petri dish containing 100% marine media so that it was incubated at 30 °C for 1-2 days. After growing the new colonies were re-scratched on 100% marine media so that a single colony was obtained. The enrichment method was carried out by spreading 100% marine agar media culture liquid, carried out after two weeks of incubation, then such as the direct plating method, culture was
incubated at 30 °C for one week and the number of colonies was counted, isolated and purified. Storage of purification results is carried out using 25% glycerol in marine agar media [6].

Determination of isolate species using the Polymerase Chain Reaction (PCR) method with techniques through the DNA extraction stage of bacterial isolates, mixing PCR reducing components, then Running PCR, which is to multiply the DNA fragments through three cycles including denaturation of DNA, primary attachment and formation of hydrogen bridges in the print sequence area.

The absorbance value of the spectrophotometer measurements is plotted on semilogarithmic paper in the form of a growth curve. So that it can be seen the form of growth of each bacterial inoculum and the effectiveness of bacteria in degrading hydrocarbon compounds. The biodegradation percentage of hydrocarbons was obtained from the reduction of the substrate at the beginning of incubation and after the end of incubation through the extraction process.

![Sampling location map](image)

**Figure 1.** Sampling location, point A: station I, Point B: station II; Point C; station III.

### 3. Results and Discussion

Figure 1 is a 3 point sampling point. The potential for contamination of hydrocarbon waste from the BBM terminal of Makassar is a dense area of loading activities and community activities. The western area of the poatere port (MFO-1) is the outer zone which is the transportation route for tankers, while the inner zone (III) is the transportation route for cargo ships and passenger ships. The north side of the port coast while on the east side is a residential area and office center, and the navy (Lantamal) and there is a Pertamina BBG refinery. All activities on land lead directly to the sea. The data above is reinforced by the results of research which concludes that Soekarno Hatta port has been contaminated with hydrocarbons up to 200 mg / L of sea water. Hydrocarbon contamination will disrupt marine life. However, the fact that 4 types of bacteria were found in the area, shows that the bacteria can adapt to extreme environmental conditions, as well as the reason for choosing the Makassar BBM terminal port as the sampling location. Sampling was obtained at three location points with the coordinates of each type presented in table 2.

| Sampling point | Coordinate point |
|----------------|------------------|
|                | Latitude         | Longitude        |
| Station I      | 5° 11.24° 35’    | 119° 41.02° 46’ |
| Station II     | 5° 11.21° 77’    | 119° 41.05° 71’ |
| Station III    | 5° 11.24° 71’    | 119° 41.05° 44’ |

Sumber : Data Primer, Desember 2015
The data table 3. shows the environmental characteristics of the fuel unloading station at the Pertamina Depo Makassar where many marine bacteria live, especially conditions of temperature, pH, salinity and port depth. Marine bacterial growth is a standard environmental condition for the life of a particular bacterial species [7,8]. This means that the contaminated status of petroleum hydrocarbons is not contaminated due to the landing of BBM and BBG from a tanker to the Pertamina Depo Makassar.

Supporting data measured at the Pertamina Makassar BBM station sampling location is presented in Table 3, as follows:

| Sampling Kode | Temp. (°C) | pH | Salinity | Dept (m) |
|---------------|------------|----|----------|----------|
| Station I     | 29         | 7  | 29.5 ‰  | ± 15 dpl |
| Station II    | 27         | 7  | 29.3 ‰  | ± 5 dpl  |
| Station III   | 28         | 7  | 29.5 ‰  | ± 10 dpl |

Source : Data Primer, Desember 2015

3.1. Isolation and Purification of marine bacteria

Microscopic analysis of marine bacteria isolation results, namely color, texture, cell size and colony growth, is an observational indicator in determining the miksymbionts species. Bacterial colonies taken for isolation are colonies of the same shape and size and grow dominantly. The two colonies were identified to grow well at 10-2-10-4 dilution, from the dilution series growth was carried out on marine media for bacterial growth because it was neutral and contained many nutrients, namely yeast / extract as a protein source, while peptone as a source amino acid. The isolation results are shown in Figure 2.

Figure 2. Results of isolation and morphological analysis, results of colony growth after incubation of 1x24 hours on marine agar media.

3.2. Isolation of industrial oil and fuel oil degrading bacteria

For bacterial isolation, the samples that have been taken are then taken to the laboratory for further research on industrial oil degrading bacteria. Initially, synthetic seawater (ALS) media was made, which is media made using sterile distilled water with the addition of synthetic materials or certain chemicals that have properties or contents similar to natural seawater. The composition of ALS is NaCl, KCl, MgSO₄, MgCl₂.6H₂O, Tris (Hydroxy methyl-Aminomethane), aquades, CaCl₂.2H₂O, NH₄Cl, K₂HPO₄, FeSO₄. After the material is dissolved in distilled water, it is followed by making a growth medium with a composition of 2 ml FeSO₄, 4 ml K₂HPO₄, and 1 ml petroleum (a source of nutrition) suspended into 9 ml of ALS each. After that take 10 ml of sea sediment and water samples. the surface sea is then transferred to the growth medium. At this stage, the incubation is carried out for 4 to 6 days. Observe the changes. Before incubation, 2 layers were seen on the Erlenmeyer flask. The top layer is petroleum while the bottom layer is ALS. This occurs because of the difference in density of the two types of solutions.
After incubation in the shaker for 4-6 days, the results obtained in Erlenmeyer 1 petroleum looks sticky on the wall and the color change at this stage indicates that the bacteria that live in the media are bacteria that can degrade industrial oil. When the industrial oil appears to have decreased by about 50%, it is transferred to another medium to enter the culture stage.

From the results of measurements of the bacterial inoculum using the spectrophotometer method, a growth curve was obtained that was not much different for each type of petroleum substrate. This is because the bacterial population at each station has similar characteristics in degrading hydrocarbons. Observation of the growth curve shape of the culture with bacterial samples from station I on three types of petroleum substrates can be seen in Figure 2.

It was observed that the growth curves tended to be the same for the three types of petroleum substrates used. Where the form of growth in the industrial oil substrate type MFO-1 and industrial oil type IDF follow the ideal growth curve, namely the form of sigmoid growth. The shape of the curve that is formed only has 3 types of growth phases, namely the exponential phase, the deceleration phase, and the stationary phase. Meanwhile, the substrate type of diesel fuel (BBMS) when it reaches the slowing phase of bacterial growth tends to still increase.

During bacterial growth, the industrial oil substrate type MFO-1 obtained optical density values which were only close to 2 and the optical density values on the IDF type industrial oil substrate exceeded 2, while the optical density values on the BBMS oil substrate reached 3. The difference in optical density values from the three This type of petroleum is caused by differences in the level of growth activity of the bacterial communities that live in each culture medium. Bacteria on the BBMS oil substrate appear to have more active growth. The BBMS oil used has a thicker physical form compared to the other two petroleum, so that microorganisms need greater activity to remodel the petroleum so that it can be used as a nutrient to support its life.

**Figure 3.** Bacterial growth at station I, II, and III; sample specification Marine Fuel Oil-1 (MFO-1), Industrial Diesel Fuel (IDF), Fuel Oil (BBMS)
From the results of measurements of the bacterial inoculum using the spectrophotometer method, a growth curve was obtained that was not much different for each type of petroleum substrate. This is because the bacterial population at each station has similar characteristics in degrading hydrocarbons. Observation of the growth curve shape of the culture with bacterial samples from station II on three types of petroleum substrates can be seen in Figure 3.

In the observation of the growth curve, there was no visible difference in shape between the three types of petroleum substrates that were observed. Where the form of growth in the industrial oil substrate type MFO-1 and industrial oil type Y follow the ideal growth curve, namely the form of sigmoid growth. The shape of the curve that is formed only has 3 types of growth phases, namely the exponential phase, the deceleration phase, and the stationary phase. Meanwhile, when the type of BBMS substrate reaches the slowing phase of bacterial growth, it tends to increase. Based on the results of previous research, this form tends to be known as the linear phase [9]. It's just that the growth speed between the three in reaching the deceleration phase is somewhat different.

During bacterial growth, the optical density value of the MFO-1 type industrial oil substrate was only close to 1.5 and the optical density value of the Y type industrial oil substrate exceeded 2, while the optical density value of the BBMS oil substrate reached 2.5. The difference in the optical density values of the three types of petroleum was caused by differences in the level of growth activity of the bacterial community that lived on each culture medium. Bacteria on the BBMS oil substrate appear to have more active growth. The BBMS oil used has a thicker physical form compared to the other two petroleum, so that microorganisms need greater activity to remodel the petroleum so that it can be used as a nutrient to support its life.

The difference in the color change of the bacterial culture from station III in Figure 3 of the types of petroleum during use as a growth substrate can be seen in table 3 below. The difference in the color of the culture obtained was caused by the difference in the basic color of each petroleum that was tried, also due to differences in the composition of the three types of petroleum. The characteristics of bacterial growth at station III are as follows:

In observing the growth curve, there was no visible difference in shape between the three types of petroleum substrates that were observed. Where the form of growth in the industrial oil substrate type MFO-1 and industrial oil type IDF follow the ideal growth curve, namely the form of sigmoid growth. The shape of the curve that is formed only has three types of growth phases, namely the exponential phase, the deceleration phase and the stationary phase. Meanwhile, when the type of BBMS substrate reaches the slowing phase of bacterial growth, it tends to increase.

During bacterial growth, the industrial oil substrate type MFO-1 obtained optical density values which were only close to 2.5 and the optical density values on the IDF type industrial oil substrate exceeded 2.5 while the optical density values on the BBMS oil substrate reached 3. The difference in density values The optic of the three types of petroleum was caused by the difference in the level of growth activity of the bacterial community that lived on each culture medium.

Based on the results of growth curve analysis using semilogarithmic paper, the generation time (cleavage time) of bacteria from each type of petroleum is as follows: industrial oil type MFO-1 generation time obtained is 28 hours, industrial oil type IDF generation time is 33 hours while BBMS oil has a generation time of 36 hours.

3.3. Phenotype analysis through characterization of the rRNA gene molecule of microsymbiont isolates
The results of genotype analysis through the characterization of the 16S rRNA gene molecule of Callyspogia sp can be done by identifying the sequence of DNA gene pairs through the polymerase chain reaction (PCR) method. The results obtained from DNA gene sequencing are raw data in the form of a chromatogram, then the gas molecules are sorted and stretched. Table 3 is the result of isolation and amplification of DNA 1 and 2. The chromatograms obtained were processed using BioEdit software version 7.2.5. The data that has been processed by the BioEdit program is used as the
basis for reprocessing the multiple alignment of the isolate gene molecule sequences against the DNA molecule database in GenBank, where the 16S rRNA gene molecule is universal in bacteria, in general it can be compared with the sample RNA sequence [10,11].

Denaturation of enzyme proteins changes the composition of the amino acids on the active site, changes their shape, and causes the enzyme to lose its catalytic ability. If the denaturation continues until the enzyme loses solubility and solidifies / coagulates / thickens, the enzyme can no longer return to normal. This enzyme denaturation causes the metabolism of the bacteria to be inhibited and causes the bacteria to die.

The results of the isolation sequencing were opened through the BioEdit program, then the DNA sequence of the sample bacteria was entered into the BLAST program, the sequence was identified using the DNA database at the site.[12] The results of alignment of the sample sequence with the GenBank sequence show a high homologous similarity, can be seen in the following table.

| Table 4. Results of BLAST (Basic Local Alignment Search Tool) bacterial isolate at a dept of 5 m. |
| Sample B1 | Sample B2 | Sample B3 |
| Bacillus pumilus | Bacillus anthracis str. Ames | Bacillus anthracis partial sequence |
| Bacillus safensis | Bacillus anthracis | Bacillus anthracis str. Ames strain |
| Bacillus humilus | Bacillus cereus | Bacillus cereus |
| Bacillus safensis | Bacillus cereus | Bacillus cereus |
| Bacillus humilus | Bacillus cereus | Bacillus cereus |

| Table 5. Results of BLAST (Basic Local Alignment Search Tool) bacterial isolates at a depth of 10 m |
| Sample A1 | Sample A2 | Sample A3 | Sample A4 |
| Bacillus infantis | Bacillus amyloparentes | Acinetobacter baumannii | Bacillus subtilis |
| Bacillus drentensis | Bacillus methyloparphic | Acinetobacter baumannii | Bacillus subtilis |
| Bacillus infantis | Bacillus amyloparentes | Acinetobacter baumannii | Bacillus subtilis |
| Bacillus oceanisediminis | Bacillus amyloparentes | Acinetobacter baumannii | Bacillus subtilis |
| Bacillus firmus | Bacillus amyloparentes subsp. plantarum | Acinetobacter baumannii | Bacillus subtilis |

4. Conclusion

Based on the results of the research and discussion, it can be concluded that gram-positive bacteria groups of Bacillus and Acinobacter groups were identified that can live in areas contaminated with oil spills in the waters of the pertamina depot, Makassar.

Reference
[1] Marzuki I, Chaerul M, Bachtian E, Asmeati and Paserangi I 2020 Biodegradation of aliphatic waste components of oil sludge used micro symbiont of Sponge Niphates sp. IOP Conf. Ser. Earth Environ. Sci. 429 012056
[2] Riani E, Cordova M R and Arifin Z 2018 Heavy metal pollution and its relation to the malformation of green mussels cultured in Muara Kamal waters, Jakarta Bay, Indonesia Mar. Pollut. Bull. 133 664–70
[3] Armus R, Noor A, Ahmad A and Lukman M 2019 Plankton Abundance Between Dry And Rainy Season In Tallo Freshwater And Pangkep River Hasanuddin Univ. Vol 20, No 2 (2019)

[4] Dahuri R, Harim A and G Bengen D 1995 Struktur Ikan Karang Dan Interaksinya Dengan Komponen Lifeform Karang Penyusun Terumbu Karang Pulau Hoga Dan Karang Karedupa Di Kepulauan Tukang Besi, Kabupaten Buton Propinsi Sulawesi Tenggara Inst. Pertan. Bogor Vol. 3 No. 2 (1995): Desember 1995

[5] Hughes T M C, Weaver A J and Godfrey J S 1992 Thermohaline forcing of the Indian Ocean by the Pacific Ocean Deep Sea Res. Part Oceanogr. Res. Pap. 39 965–95

[6] Yümün Z Ü and Önce M 2017 Monitoring heavy metal pollution in foraminifera from the Gulf of Edremit (northeastern Aegean Sea) between Izmir, Balıkesir and Çanakkale (Turkey) J. Afr. Earth Sci. 130 110–24

[7] Rahmatullah, Ali M S and Karina S 2016 Keanekaragaman Dan Dominansi Plankton Di Estuari Kuala Rigaih Kecamatan Setia Bakti Kabupaten Aceh Jaya J. Ilm. Mhs. Kelaut. Dan Perikan. 1 325–30

[8] Armus R, Noor A, Lukman M and Ahmad A 2019 Relationship between distribution of dimethyl sulfide (DMS) and plankton community structure in Spermonde, Indonesia IOP Publ. 1341

[9] Sulfahri, Mushlihah S, Husain D R, Langford A and Tassakka A C M A R 2020 Fungal pretreatment as a sustainable and low cost option for bioethanol production from marine algae J. Clean. Prod. 265 121763

[10] Huang L, Jian W, Song X, Huang X, Liu S, Qian P, Yin K and Wu M 2004 Species diversity and distribution for phytoplankton of the Pearl River estuary during rainy and dry seasons Mar. Pollut. Bull. 49 588–96

[11] Giannetti B F, Marcilio M D F D F B, Coscieme L, Agostinho F, Liu G and Almeida C M V B 2019 Howard Odum’s “Self-organization, transformity and information”: Three decades of empirical evidence Ecol. Model. 407 108717

[12] Baum A, Rixen T and Samiaji J 2007 Relevance of peat draining rivers in central Sumatra for the riverine input of dissolved organic carbon into the ocean Estuar. Coast. Shelf Sci. 73 563–70