The existence of hydrocarbon contaminant decomposing bacteria in Ambon Bay

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Abstract The study was done on September until November 2018, aimed to identify colony morphology of the existence bacteria the decomposing of hydrocarbon contaminant on Ambon bay water. Sample Water and sediment were taken from 4 station : Port of P.T. Pelni Ambon (Station 1); Port of P.T. Pertamina Wayame (station 2); Port of Perikanan Nusantara Tantui (Station 3) and Port of P.T. Perikanan Nusantara Galala ( Station 4). Physical and chemical (pH, temperature, salinity, clarity and current flow) of water quality measured while sampling water and sediment were sampled. Isolation and colony morphology identification were done at Microbiology Laboratory of Deep Sea Study Centre –Indonesian Science Institute in Ambon. The bacteria was isolated in broth and solid media with carbon sources from kerosene. The result shows that : 3 different colonies on water sample and 5 different colonies on sediment from Station 1; 6 different colonies on sediment from Station 2; 5 different colonies on water sample from Station 3; 4 different colonies from water sample and 6 different colonies on sediment from Station 4.

Key words : Decomposing bacteria, Hydrokarbon, Colony morphology, Ambon Bay.

1. Introduction

Ambon Bay, located at 128°70 - 129°45 E dan 3°37 - 3°45 S, is one of the bays that play an important role in Eastern Indonesia. Ambon Bay consists of two regions, Outer Ambon Bay (OAB) and Inner Ambon Bay (IAB). These two regions are separated by a bottle neck sill with depth 12.8 m and width 7.8 m, which causes different characteristics between OAB and IAB. OAB’s current pattern is affected by Banda Sea and thus is more turbulent, while IAB has a more stable current.

The body of water that is included in the City of Ambon territory can be divided into 4 (four) ecological regions: Inner Ambon Bay with water body area of 11.03 km², Outer Ambon Bay with water body area of km², Baguala Bay with water body area of 58.48 km², and Ambon City Southern Coastal Area with water body area of 241.1 km². As a result of the Ministry of Marine Affairs and Fisheries policy of establishing responsible fishery management and of fighting against the Illegal, Unreported and Unregulated (IUU) Fishing in the Region of Fishery Management of the Republic of Indonesia, a regulation was rolled out, namely the Ministry of Marine Affairs and Fisheries Regulation Number : 56/PERMEN-KP/2014 about the Moratorium of Fishing Business License in the Region of Fishery Management of the Republic of Indonesia. This moratorium is applied to fishing boats that are made overseas.

Water contaminated with petroleum oil and its components especially hydrocarbons is common in the oil-producing and industrialized countries of the world and is a considerable threat to the environment and human health [1]

Various techniques have been used to prevent and tackling oil pollution in the waters, in physics, chemistry and biology. Accordingly biology, several types of marine bacteria are known has the ability to degrade oil. This is based on the fact that decomposition Petroleum components in the marine
environment are determined by the process of transformation and degradation through activity microbial. Oil spill incidents are not only caused by pipeline vandalisation and accidents such as collision or explosion of supertankers or oil well blowout. There have been cases of oil spills initiated by natural disasters as Hurricanes and due to wars. However, the largest and most damaging pollution events usually involve a spill of petroleum from a disabled tanker or drill platform at sea or to a lesser extent from a blowout or a broken pipeline on land [2;3].

Petroleum contamination is quite harmful for the higher organisms [4;5] but it is fortunate that microorganisms can thrive on it and assimilate [6;7]. Soon after major oil spill incident is reported, the efforts are concentrated on physical collection of the spilled oil with skimmers is the first response but this is rarely completely successful [8]. As per Office of Technology Assessment (OTA; USA), such mechanical methods are efficient at removing no more than 10-15 per cent of oil after a major spill [9]. Therefore, it is necessary to find potential oil decomposing bacteria that can be used in the management of oil spill that is effective and economics. From said microbe, aside from managing the oil spill, it is expected that it can also be used in an effort to restore oil-contaminated environments.

This research aims to contribute in the understanding of the existence of the hydrocarbon contaminant decomposing bacteria in Ambon Bay, so that it can be an additional reference to the research done before hand.

1.1. Research Objective

The objective of the research is: To know the bacteria isolates that can decompose the hydrocarbon contaminant in the waters of Ambon Bay.

1.2. Research Location and Time Frame

The research was done in the Research Center for Deep Sea (PPLD), Indonesian Institute of Sciences (LIPI), Ambon, the Province of Maluku, with sampling location at 4 (four) ports in the waters of Ambon Bay. The research took place on September 2017 - July 2018.

2. Materials and Methods

The equipment used for the analysis by a laboratory scale experiment are divided into 2 (two) types. Equipment for sample collection are Sample Bottle, Sample Spoon, Aluminium Foil, Rope, Speed Boat, Cool Box, Ice Pack, Sediment Grab, GPS, Notebook, Labels, Marker, Ballpoint, Scissors, Gloves, Plastic Bag, Rubber Band, Tissue, Face Mask, Autoclave, Oven, Refrigerator. While the equipment for testing and media production are Petri Dish, Test Tube, Serology Pipette, Micropipette, Analytic Scale (0.001), Spatula, Laminar Flow Cabinet, Bunsen Burner, Measuring Cup, Beaker, Cotton Wool, Refrigerator, Spreader, Incubator, Autoclave, Oven, Plastic Grab, Vortex Mixer, Label, Glove, Hot Plate, Magnetic Bar, Inoculating Loop, Microscope Slide, Cover Glass, and Tweezers. For the interview, the equipment used is the interview guide.

The materials used in this research are Sea Water Sample, Sediment Sample, Natural Sea Water, Kerosene as the source of carbon, Yeast Extract, Bacto Peptone, Bacto Agar, Distilled Water, and Crystal Purple Colouring.

2.1. Sampling Method

To decide on the sampling station, purposive sampling was used i.e., the observation station was decided by considering several factors and the condition of the sampling area. We collected the sediment and seawater samples from Ambon Bay. The decision on the location of sample collection was based on the ship traffic in the port as well as the condition of testing equipment. From the identification results, 4 (four) sample locations were determined to be Station I (PT. Pelni Port - Ambon), Station II (PT. Pertamina Port - Wayame), Station III (Perikanan Nusantara Port - Tantui), and Station IV (PT. Perikanan Nusantara Port - Galala). The water samples were collected using Sample Bottle at a depth of 1m, while the sediment samples were collected using Sediment Grab that was then placed into Sample Bottle and stored in Cool Box containing Ice Pack, which in turn was transported to PPLD LIPI Ambon for testing. Prior to sample collection, the physical condition of the area was measured, including the pH, Temperature, Salinity, Clarity, the time frame, the speed of the current, and the condition of the location of sample collection.
Figure 1. Map Location of Sampling Station

The production of bacteria to be isolated was done with solid medium in petri dish (pour plate method) in the medium *Marine agar* (MA). Dilution was done by taking 1 ml of the sample and placing it in the test tube containing 9 ml of NSW medium to get $10^{-1}$ dilution, and the process was continued until $10^{-3}$ dilution was obtained. 1 ml of each diluted series was then taken and placed in a petri dish containing the solid medium *Marine agar* (MA) equipped by filter paper soaked in Kerosene and had undergone sterilisation as the source of carbon. It was spread over evenly by making the figure 8 and then incubated for 3 days at temperature 35°C. The colonies that grew were observed microscopically for their shape, colour, margin, surface, texture, and colony elevation [10]. Pure isolates were then produced from the mixed cultures by inoculating 1 oz of each of colonies found to the petri dish containing the medium *Marine agar* (MA) and incubating it for 1 x 24 hours until the result gained was a single colonies/pure cultures.

| Material                      | Quantity |
|-------------------------------|----------|
| *Yeast Extract (Difco)*       | 1 gr/l   |
| *Bacto Peptone*               | 5 gr/l   |
| *Bacto Agar*                  | 15 gr/l  |

Table 1. Composition of medium *Marine agar* (MA) for 1 liter of NSW

Table 2. Composition of medium *Bacto agar* (BA) for 1 liter of NSW

| Material        | Quantity |
|-----------------|----------|
| *Bacto Agar*    | 15 gr/l  |

2.2. *Bacteria Isolation*

Bacteria isolation was done with dilution system whereby 1 ml of the sample was placed in test tube 1 containing 9 ml of medium NSW and mixed using a vortex mixer (10⁻¹ dilution). Then, 1 ml of mixture from test tube 1 was placed in test tube 2, also containing 9 ml of medium NSW (10⁻² dilution) and mixed further. This treatment was continuously done until $10^{-3}$ dilution was achieved. For the sediment sample, a 10⁻⁶ dilution was done at an early stage, by placing 1 gr of the sample into 5 ml of
NSW and then homogenizing the mixture. The result of the 10⁰ dilution was used for the next stages until 10⁻³ dilution was achieved. 1 ml of each of the dilution series was then poured into the prepared medium Marine agar, which in turn was spread evenly using a skewer with tip that had been covered with cotton and sterilized. A filter paper sterilized with kerosene as the source of carbon was placed on the cover of the petri dish, and then it was incubated in the incubator at temperature 35°C for 3 days (72 hours).

2.3. Colony Morphology Identification

The identification of colony morphology from the isolation result was done using a microscope. The items identified were the shape, margin, inside structure, elevation, texture, colour, quantity and diameter of the colony.

2.4. Purification

A purification of the colonies grown from several concentration was done after colony morphology identification from the isolation result. The purified colonies were the colonies that grows separately or the single colonies that were inoculated in a petri dish containing the medium Marine agar that were prepared according to the number of colonies that grow. There were approximately 1-2 oz of each colony that were inoculated in petri dish and incubated for 24 hours at temperature 35°C. The purification was done twice until it achieved a single colony (pure culture). After that, another inoculation was done in the medium Bacto agar to find out whether the colony obtained from purification is a facultative or obligate hydrocarbon decomposing bacteria.

2.5. Cell Morphology Identification

The identification of the cell morphology of all isolate resulting from the purification was done by taking 1 drop of sterile distilled water and placing it on a microscope slide added with 1 oz single colony from the purified product petri dish that was homogenized. Then, 1 drop of crystal purple colouring was added and the mix was further homogenized using inoculating loop, which was then covered with cover glass and fixed on a Bunsen burner. An observation of the shape of the bacteria cell was done using a microscope.

3. Result and Discussion

Microorganisms capable of degrading petroleum hydrocarbons and related compounds are ubiquitous in marine, freshwater, and soil habitats. Over 200 species of bacteria, yeasts and fungi have been shown to degrade hydrocarbons ranging from methane to compounds of over 40 carbon atoms [11]. In the marine environment, bacteria are considered to be the predominant hydrocarbon-degraders with a distribution range that even covers extreme cold Antarctic and Arctic environments [12; 13].

3.1. Colony Morphology

From the result of isolation, single colonies were obtained from each dilution stage for 7 types of sample from 4 different stations, but only 29 single colony isolates were taken and brought further into the purification stage because these 29 colony isolates were considered representative of all colonies, where after the process of colony morphology identification based on the observation of colony shape, margin, optical properties, elevation, texture, colour, and diameter, only different colonies were taken from each sample type. The identification of colony morphology was done by macroscopic and microscopic observations. From all observation results, it was found that there were 3 different types of colony in sample IA (Water Sample PT. Pelni Port), 5 different types of colony in sample IIIA (Water Sample Perikanan Nusantara Port), 4 different types of colony in sample IVA (Water Sample PT. Perikanan Nusantara Port), 5 different types of colony in sample IS (Sediment Sample PT. Pelni Port), 6 different types of colony in sample IIS (Sediment Sample PT. Pertamina Port), 6 different types of colony in sample IVS (Sediment Sample PT. Perikanan Nusantara Port). The shape of the colony morphology identification of every type of samples can be seen in the following figures:
Figure 2. Sample Colony Isolate IA

Figure 3. Sample Colony Isolate IIIA

Figure 4. Sample Colony Isolate IV
Figure 5. Sample Colony Isolate IS

Figure 6. Sample Colony Isolate IIS
Figure 7. Sample Colony Isolate I

Legend:

1. Roman numerals I = PT. Pelni Port, II = PT. Pertamina Port, III = Perikanan Nusantara Port, IV = PT. Perikanan Nusantara Port
2. A = Air (Water), S = Sediment
3. 10^1, 10^2, 10^3 = Dilution
4. Numbers 1-19 = colony numbers

3.2. Pure Isolate

From the result of colony morphology identification, bacteria purification was done. The 29 colonies resulting from the identification that grew on early-stage isolate was purified by being inoculated in the medium Marine agar and incubated for 24 hours at temperature 35°C, where the incubation produced 28 single colonies that grew in said medium. Stage II purification was then done by taking the isolates resulting from stage I purification, inoculating them in the medium Marine agar, and then incubating them for 24 hours at temperature 35°C, the incubation produce 28 isolates that grew in said medium.

The purification was done in order to obtain pure isolates from the colonies grown from several dilution concentrations or from the mixed cultures. The colonies that grew at the purification stage underwent another inoculation in the medium Bacto agar with the purpose of finding out whether those colonies are facultative or obligate hydrocarbon decomposing bacteria. If the bacteria grew in the medium Bacto agar, that means the bacteria is an obligate hydrocarbon decomposing bacteria, because the bacteria can use only the kerosene as the source of carbon. If the bacteria did not grow, that means it is facultative because it tends to use other sources of carbon found in Marine agar before using the hydrocarbon in the form of kerosene from the filter paper. From the result of the 7-day incubation at temperature 35°C in the medium Bacto agar, 18 isolates were found to have grown and the incubation was continued for 13 days at temperature 35°C. All sample codes that grew counted 28 isolates have the tendency to be active in the oil environment and only make use of the hydrocarbon in the form of kerosene for its activity as the only source of carbon in its metabolism process. From the identification results, it was observed that the speed of growth of the isolates in the medium of Bacto agar were much slower at 7-13 days compared to the 24-hour growth in the medium Marine agar. Additionally, there was a change in the colony morphology in the form of shape, margin and diameter of the colonies, which was caused by the environmental adjustment as well as the source of carbon in the two media.

3.3. Bacteria Cell Morphology

Cell morphology identification was done by observing the shape of bacteria cells from isolates at the stage II purification. There were 15 isolates with general round shape, 9 isolates were shaped as bacil, while the remaining 4 had the shape of ovoid.

In this research, only the colony morphology and cell morphology identifications were done, while the type was not identified. Based on the sources of previous research, it is known that the dominant bacteria in decomposing oil are from the genus Hydrocarbon degrading bacteria and fungi are widely distributed in marine, freshwater, and soil habitats. Similarly, hydrocarbon degrading cyanobacteria have been reported [14;15], although contrasting reports indicated that growth of mats built by cyanobacteria in the Saudi coast led to preservation of oil residues [16]. Typical bacterial groups already known for their capacity to degrade hydrocarbons include Pseudomonas, Marinobacter, Alcanivorax, Microbulbifer, Sphingomonas, Micrococcus, Cellulomonas, Dietzia, and Gordonia groups [17].
4. Conclusion

There are 28 isolates that were indicated to be able to decompose hydrocarbon, proven by the capability of those isolates to grow in the medium of Bacto agar which used only kerosene as the source of carbon for its metabolism process.

Recommendation

There is a need to do a research further identifying the specific types of hydrocarbon contaminant decomposing bacteria found in Ambon Bay waters along with their capabilities in decomposing oil spill.

5. References

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