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Isolation and Characterization of Polyethylene-Degrading Marine Bacteria

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Abstract
Background: *The amount of plastic waste is currently increasing along with its wider use. Alot of environmental problem is occurred cause the accumulation of plastics in the oceans. One of the many efforts currently being developed is to look for bacteria from nature that can decompose these synthetic plastics. In this paper we reported that bacterial isolation from plastic waste taken from Indonesian Ocean seawater was carried out at the Biotechnology Laboratory of Sumatran Biota, Universitas Andalas, Padang, West Sumatra, Indonesia.*
Methods: *This research used descriptive methods included the sampling activities by purposive sampling and then bacteria isolation by streak plate methods. Then the bacteria were grown in Nutrient Agar media and specific media (Mineral Salt Agar added with polyethylene plastic as a nutrient source).*
Conclusion: *From the research was obtained 6 bacterial isolates and screening results by microscopic test and biochemical tests showed the genera of the bacteria namely Bacillus sp.*
Keywords*: isolation, bacteria, degradation, polyethylene, marine.*

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I. Introduction

The amount of plastic waste is currently increasing along with its wider use. The increase in the amount of landfill waste in Indonesia has reached 175,000 tons/day or the equivalent of 64 million tons/year. Meanwhile, the plastic waste management is still not on target, and tends to be not environmentally friendly. According to the Ministry of Environment, based on the results of studies conducted in several cities in 2012, the pattern of waste management in Indonesia is as follows: being transported and buried in landfills (69%), buried (10%), composted and recycled (7%), burned (5%) and the rest were not managed (7%). Currently, more than 90% of districts/ cities in Indonesia still use open dumping systems or burning waste. This certainly raises various problems, including disrupting the life of the surrounding organisms[1] .

The increase in the amount of plastic waste has emerged as a crisis in many areas due to reduced capacity of the Final Disposal Site (FDS), the increase in the amount of plastic waste in a sustainable manner and strict legislation. The second environmental problem is the accumulation of plastics in the oceans, for example in a long-term study in the North Atlantic, seawater samples taken contain the equivalent of 580,000 pieces of plastic per square kilometer. The next problem is non-degradability, plastic was not easily decomposed and will survive in the environment for hundreds of years[1].

Table 1. Several factors affecting the rate of degradation

Parameters	Factors
Physicochemical properties of ecosystems	Temperature, pH, oxygen, water, food sources, enzymatic reactions, presence of inhibitors, and redox reactions
Microorganisms in the ecosystem	The number of microorganisms, the micro-organism activity, the microorganisms adaptability and the distribution of micro-organisms
The properties of the material to be degraded	Polymer composition, molecular weight, molecular weight distribution, crystallinity, and hydrophobi-city.

One of the microbes that is known to help in the process of plastic degradation is bacteria. There are several bacteria that able to degrade plastic in vitro by utilizing plasticizers in plastics as a carbon source such as *Pseudomonas aeruginosa* and *Brevibacterium* sp. The genera *Brevibacillus*, *Pseudomonas* and *Rhodococcus* spp. has been able to degrade polyethylene through several treatments with a percentage of dry weight of 37.5% and 40.5% [2] .

The speed of biodegradation depends on several factors such as humidity, type of microorganism, temperature, pH, type of polymer, and thickness of the polymer. Biodegradation conditions which include pH, temperature, nutrients, minerals, oxygen, and humidity are adjusted to the type of microorganism used[3].

II. Material And Methods

Tools and Materials

The tools used in this research were erlenmeyer (Pyrex®), measuring cup (Pyrex®), reaction tube (Pyrex®) ,beaker (Pyrex®), stirrer bar, spatula, forceps, eye dropps, micropipette (Transferpette®), inoculation needle, digital scale d = 0,001 g (Metler Toledo®), spiritus lamp, object glass, autoclave (All american®), oven (Memmert®), laminar air flow (Elisa®), rotary shaker incubator (Bigger digital®), vortex (Digisystem®), mikroskop, magnetic stirrer hotplate, hot plate (Cimarec®), thermometer (Omron®) and pH meter (Hanna®).
The materials used in this research were Nutrient Agar Medium (NA) (Merck®), *Mc Farland* 0.5 standard solution, polyethylene plastic, 70% alcohol (Brataco®), aquades (Brataco®), spiritus (Brataco®), Potassium dihydrogen phosphate (Merck®), Dipotassium hydrogen phosphate (Merck®), Ammonium phosphate (Merck®), Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) (Merck®), Sodium chloride (Merck®), Ferro sulfate heptahydrate ($FeSO_4.7H_2O$) (Merck®), Calcium dichloride dihydrate ($CaCl_2.2H_2O$) (Merck®), Manganese sulfate hydrate ($MnSO_4.H_2O$) (Merck®), Copper sulphate pentahydrate ($CuSO_4.5H_2O$) (Merck®), Zinc sulphate pentahydrate ($ZnSO_4.7H_2O$) (Merck®), cotton, gauze, violet crystal, safranin and plastic wrap.

Sample Collection

Samples water from the sea (Indonesian Ocean, Padang, West Sumatra, Indonesia) were taken using purposive sampling technique, which were plastic food packaging that had been buried in the sea. Marine samples containing plastic sediment were taken as many as 10 plastic samples and each sample was repeated 2 times. The samples that have been taken were put into those plastics and the measurements of sea temperature and pH were measured directly.

Isolation dan Purification of Marine Bacteria

The seawater samples were weighed as much as 20 g, made up to 100 ml of sterile aquades in Erlenmeyer, then vortexed until homogeneous. The dilution was done up to 10^{-7}. Then from the dilution series as much as 1 ml was piped into a petridish and poured into NA medium (Merkc®) using the pour plate technique, then incubated at room temperature for 24 hours. The grown bacterial isolates were purified by the quadrant method. A grown single colony was inoculated on a sloping culture and labeled as a bacterial stock.

Plastic Biodegradation Test

Bacterial isolates were grown in the mineral agar medium added with plastic powder then inoculated into the mineral medium. A thin film of polyethylene plastic was added aseptically, and incubated at room temperature for 4 weeks. Then the plastic film was washed with 70% alcohol, rinsed with sterile aquades, and put in an oven at a temperature of 80^oC until it reached a constant weight. The final weight of plastic film was then measured. The reduction of plastic weight was calculated as a percentage using the following formula:

% degradation of file plastic = R1-R2/ R1 x 100% w/w[3]

Annotation:
R_1 = Initial Weight of Plastic Film
R_2 = The Final Weight of a Plastic Film

Characterization of bacterial isolates

Macroscopic Observation

Macroscopic observations were carried out by growing isolates on NA medium using the streak plate method. Then the shape, color, edge, texture, and surface of the colony was observed.

Microscopic Observation

Microscopic observations were done by cleaning the slide with alcohol and dried it on the spritus lamp. Then 1 ose of bacterial suspension was dropped aseptically on the slide, 1-2 drops of violet crystal was added for 1-2 minutes and washed with distilled water and then dried it in the air. Next, as much as 2-3 drops of lugol was added and waited for 1 minute, washed with distilled water and then air dried. The preparation slide was dropped with 96% alcohol drop by drop until it were clean and washed with distilled water then air dried. Next,

the preparations were dropped with safranin as much as 1-2 drops, left for 1-2 minutes then washed with running water and air dried. The cell shape was observed using a microscope with a magnification of 10x100[5].

Endospora Staining

The spore observation was carried out using the Schaeffer-Fulton method. The first stage of staining was to arranged the smear preparation. The liquid culture bacterial were applied to the cleaned object glass with 70% alcohol. Then it were spread using ose needle evenly to form a square. Furthermore, the preparation slide was covered with filter paper and 1-2 drops of Malachite Green solution was dropped evenly. Then, it was left for 1 minute. Then it was put in a water bath for 5 minutes. The filter paper was slowly removed and washed carefully using distilled water, then dried. Furthermore, the preparation slides were given safranin solution evenly and left for 30-60 seconds. The preparation slide was washed using distilled water and then dried. Next, the vegetative cells and spores (both inside and outside the vegetative cells) were observed under a microscope with magnification of 10x100. Bacterial spores were marked with green and vegetative cells with red[6].

Biochemical Identification

The bacterial identification using biochemical test was carried out at the Laboratory of Veterinary Disease Investigators (BPPV), Bukitinggi, West Sumatra, Indonesia. The tests carried out were: the formation of hydrogen sulfide and gas, the formation of indole, motility, fermentation of carbohydrates (lactose, glucose, sucrose and mannitol), red metal testing, Prokauer Voges test, catalase test, oxidation/ fermentation test, urea formation and use of citrate.

III. Results and Discussions

The characteristics of bacteria isolated from the seawater can be observed macroscopically (Figure 1) and microscopically (Figure 2). These observations were carried out to identify the bacteria that were obtained. These results can be seen in Table.2. From the macroscopic and microscopic test results on 18 isolates, 13 Gram-positive bacteria and 5 Gram-negative bacteria were found. Of the 13 Gram-positive bacteria, 10 bacteria have endospores while three others do not. The 5 Gram-negative bacteria obtained had bacillus cell shape and yellowish color.

All of Gram positive and Gram negative bacteria have circular form and different margins. LNA 1-LNA 6 isolates had the same margin and elevation. The TN 1, TN 2, TM 1, LNA 1 isolates (Figure 1) had bacillus cell shape while the TM 2 isolate had coccus cell shape.

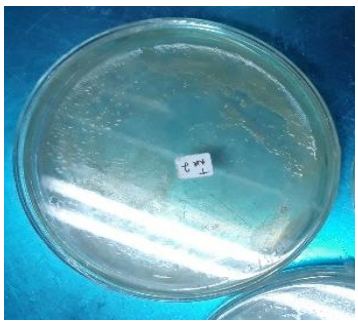

Fig. 1. Pure isolated bacteria for biochemical identification tests

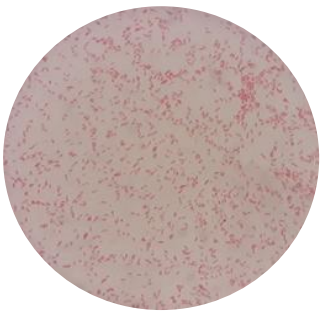

Fig. 2. Microscopic observation of polyethylene-degrading bacteria

Table 2. The results of macroscopic and microscopic tests of polyethylene-degrading bacteria isolates

No	Isolate Code	Macroscopic Characteristics of Bacterial Colonies				Microscopic Characteristics of Bacterial Colonies		
		Pigmentation	Form	Margin	Elevation	Gram	Cell Shape	Endospore
13	**LNA 1**	White	Circular	Serrate	Convex	Positive	Bacil	+
14	**LNA 2**	White	Circular	Serrate	Convex	Positive	Bacil	+
15	**LNA 3**	White	Circular	Serrate	Convex	Positive	Bacil	+
16	**LNA 4**	White	Circular	Serrate	Convex	Positive	Bacil	+

17	**LNA 5**	White	Circular	Serrate	Convex	Positive	Bacil	+
18	**LNA 6**	White	Circular	Serrate	Convex	Positive	Bacil	+

From the research, it was obtained 6 marine bacteria. The results of the biochemical testing which has been carried out in the Bukit tinggi, West Sumatra, Indonesia Veterinary Bacteriology Laboratory can be seen in Table 3. The biochemical test carried out was complete testing of Gram-positive and Gram-negative bacteria.

Table 3. Biochemical Test Results of Marine Bacteria Isolates

No	Treatments	Isolates					
		LNA 1	LNA 2	LNA 3	LNA 4	LNA 5	LNA 6
1	Gram	+	+	+	+	+	+
2	Aerob/anaerob	A	A	A	A	A	A
3	TSIA	K/K	K/K	K/K	K/K	K/K	K/K
4	Gas	-	-	-	-	-	-
5	H2S	-	-	-	-	-	-
6	Catalase	+	+	+	+	+	+
7	Oxidase	-	-	-	-	-	-
8	Mortility	+	+	+	+	+	+
9	Indol	-	-	-	-	-	-
10	Urea	-	-	+	-	-	-
11	Citrate	-	-	-	-	-	-
12	Lactose	-	-	-	-	-	-
13	Glucose	-	-	-	-	-	-
14	Sucrose	-	-	-	-	-	-
15	Mannitol	-	-	-	-	-	-
16	MR	+	+	+	+	+	+
17	VP	-	-	-	-	-	-
18	OF	-	-	-	-	-	-
19	Nitrate	-	-	-	-	-	-
20	Gelatin	+	+	+	+	+	+
21	Identification result	*Bacillus sp.1*	*Bacillus sp.1*	*Bacillus sp.2*	*Bacillus sp.1*	*Bacillus sp.1*	*Bacillus sp.1*

Table 3 showed the 6 bacteria isolates from seawater grown in the media added with the polyethylene synthetic polymers which were identified as *Bacillus* sp. [7,8,9].

IV. Conclusion

There are 6 bacteria isolates from seawater which have been able to degrade plastics and only able to live on mineral salt agar. All isolates obtained were identified as genus Bacillus sp. based on microscopic biochemical tests.

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