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Isolation and Identification of Bioplastic Producing Bacteria from Soil at the Top of Marapi Volcano Mountain, West Sumatra, Indonesia

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ABSTRACT

The purpose of this research is to isolate and identify a bioplastic-producing bacteria namely P(3-hydroxybutyrate) [P(3HB)] from soil at the top of Marapi Volcano Mountain, in West Sumatra, Indonesia. Soil samples was taken by doing purposive sampling technique. Then, it was inoculated in oil palm-bacto-agar medium to obtained bacteria colony that has potential to produces bioplastic P(3HB) by using Nile Blue A as a stain reagent. Results showed there are 40 single colony isolated. Beyond them were 10 isolates that indicated as yellow fluorescent under ultraviolet light as indicated the bacteria accumulated P(3HB) granule in the cells. The 10 isolates then identified by macroscopic, microscopic, biochemistry test. The result were 6 bacteria genera, i.e Bacillus sp 1, Bacillus sp 2, Bacillus sp 3, Bacillus sp 4, Bacillus sp 5 and Bacillus sp 6.

Keywords: isolation, identification, bioplastic, nile blue A, Marapi Mountain

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INTRODUCTION

Plastic is one of chemical material that caused some environmental issues. Mostly, plastic made from synthetic polymer, which naturally has characteristics such easy to be formed, high chemical resistance and adjustable elasticity toward the needs. This characteristics made plastic become popular and mostly used as one-time-used material and wrapping material [1].

Sometimes plastic also burnt by peoples. Plastic combustion could release poisonous smoke like adipat and ftalat which has characteristic carsinogenic and in production process resulting pollution such as vinyl chloride in large amount. Compounds resulted from plastic combustion will caused large variety of human disease if sniffed, i.e triggering cancer, hepatitis, liver sirosis, nerve disorder and triggering depression. Besides, plastic wastes especially the plastic bag from supermarket which disposed careless also could clog in drainage channels and rivers. This condition caused isolation needs for environmental issues, especially problems because plastic wastes. Appropriate solution to solve this problems is with using bioplastic.

Bioplastic is plastic which could be degrade naturally by microorganisms such as bacteria and fungi [2,3,4]. The advantage of bioplastic compound compared to synthetic plastic compound based on petrochemical is because the characteristic that can be degrade naturally by microorganisms in soil and water. It makes bioplastic did not harmful to environment like what synthetic plastic done [5,6].

Microorganisms such as bacteria could accumulated some granule of bioplastic compound in their cell [7,8]. For the bacteria this polymer acts as food and energy reserves that will be used in less favorable growth condition or when they run out food sources [2,3].

Until now 300 species of microorganisms which could produced bioplastic in their cell already known. Beyond PHA that broadly studied, those are poly(3-hydroxybutyrate) or P(3HB) and its copolymer of poly(3-hydroxyibutyrate-co-3-hydroxyvalerate) or P(3HB-co-3HV) [6,9].

MATERIALS AND METHODS

Equipment

Equipmenta used in this research are autoclave, incubator, sterile bottle 100 ml, Erlenmenyer, reaction tube, pipette, spatel, beaker glass, Petri disk, micro pipette, aluminium foil, plastic wrap, cotton, gauze, spiritus lamp, Ose, ultra violet lamp, microscope, label, mask, gloves and other tools used in Microbiology Laboratory.

Materials

Materials used are soil samples taken from top of Marapi Mountain. Oil palm medium, bakto agar, Nile Blue A 1% and Ethanol pro-analysis.

Procedure

Sterilization

All tools were used firstly washed and dried, glass tools which has nozzle covered with cotton that wrapped in gauze. Then, all the tools wrapped with parchment paper, and sterilized with autoclave with 121^{o} C temperature, 15 lbs pressure for 15 minutes. Aseptic cabinet was cleaned from dust and sterilized by spraying alcohol 70% to all over insides part of the cabinet. All processes were done aseptically.

Making Palm Oil-Bacto Agar Medium

As much as 10 g of bacto agar powder were dissolved in 500 mL aquadest and put into Erlenmeyer flash, then it boiled while stirred until it was dissolved completely and clear. Then, the mixture was sterilized in autoclave with 121^{0}C temperature and pressure 15 lbs for 15 minute. Palm oil was weighed for 2.3 g and sterilized in Erlenmeyer with gauze-wrapped-cotton-plug, this process was done by same procedure with previous step. The sterilized palm oil was put into sterilized bacto agar solution. Then, this solution was poured into each Petri disk as much as 15 mL [5].

Nile Blue A 1 % Solution

Nile Blue A ($C_4OH_4ON_6O_6S$) powder was weighed for 1 g and dissolved in ethanol absolute until the solution reached volume 100 mL [1].

Taking Soil Samples

Samples taken are top soil from Marapi Volcano Mountain, West Sumatra. Soils were taken from mountain top surface of Marapi Volcano Mountain as much as 10 spots. The soil samples were taken for 20 g with distance between one spot to another is 10 m.

Isolation of bacteria from Soil Samples

Soil samples were weighed for 10 g and made into suspension 10% in aquadest. The serial dilutions were done until 10^{-4}. The results were taken 1 ml and inoculated to oil palm bakto-agar medium in Petri disk. Next, it was incubated at 35^{o}-37^{o}C temperature for 24-48 hours. Amount of bacteria colony which grown was counted and noted. From each bacteria that had grown, then separated-bacteria moved into new oil palm medium [6].

Screening bioplastic-producing bacteria P(3HB)

Nile Blue A 1% solution was poured into oil palm-bacto-agar medium and incubated in room temperature for 30 minutes. After that, the result was observed under ultraviolet ligh with 365 m wave length. If bacteria colony produced yellow fluorescent, it means that bacteria is granul P(3HB) producer. Whereas, if colony has black coloration, it means that bacteria cell did not produce P(3HB). Then, the number of bacteria isolates which produces P(3HB) noted [6,8].

Purification and Storage from Stock of Bioplastic-Producing Bacteria

Pure stock was created by inoculating bacteria colony that showed positive result with Nile Blue A 1% ini palm oil and bacto-agar medium. Then it was incubated at room temperature. Each colony was inoculated at palm oil-bacto-

agar sideway medium with scratch technique. It was incubated at 35-37^{0}C for 24 hours and kept at 4^{0}C temperature and identified.

Polymer-Producing Bacteria Identification

Macroscopic

Identification was done macroscopically by observing colony coloration, form, edge, surface and elevation. The result was noted and showed in table form.

1. Gram Staining

Gram staining was done by preparing clean object glass and it was slide over flame of spiritus lamp. Then, aquadest sterile was dripped over the object glass. Bacteria inoculums which going to be investigated was taken aseptically and put over the aquadest drop and then slowly distributed evenly. Then, it was covered by cover glass by 45^0 angle and then let it dry. After that, violet crystal was dripped over it and let it stand for 1 minutes. The object glass then washed with water and iodine solution was dripped, let it stand for 2 minutes. Object glass rinsed with water and dripped by alcohol 95%, then let it stand for 1 minutes. Then, it rinsed with water and dripped by safranin, let it stand for 30 seconds. Rinsed it again with water, after that it was dried and observed with microscope. If bacteria cells has violet coloration then that bacteria belonging to Gram positive group. Besides, if bacteria cells showed red or light red coloration means those bacteria are Gram negative bacteria. With this coloration technique, bacteria cell form also could be observed [13].

2. Endospora Staining

This coloration was done by Klein coloration method. Dry and fat-free object glass was slide over flame. Then it dripped by inoculums and dried with spiritus lamp. After that, sulphate acid was dripped and let it stand for 3 seconds before washed under running water. Next, methylen blue was dripped and let it stand for 3 minutes before it washed under running water. Then it dried and observed under microscope [13].

3. Biochemistry Test

Bacteria identification was done by biochemistry test at Veteriner Laboratory, Bukittinggi, West Sumatra, Indonesia. The test done are hydrogen sulfide and gas forming, indol forming, motility, carbohydrate fermentation (lactose, glucose, sucrose and manitol), red methyl examination, Voges Prokauer test, catalase test, oxidase/fermentation test, urea forming and citrate usage [7,13].

RESULTS AND DISCUSSION

From isolation process of soil samples of Marapi Volcano Mountain from soil at the top area was obtained 40 different bacteria isolates. This isolates were inoculated using palm oil-bacto-agar medium. The filtering process of 40 bacteria was done by using Nile Blue-A solution, and was obtained 10 bacteria isolates which the colony showed yellow fluorescent and indicated accumulate P(3HB) in the cells. Those 40 bacteria isolates and producer of P(3HB) can be seen in Table 1.

Different bacteria was found then filtered and inoculated into palm oil -bacto-agar medium. This process has purpose to move carbon sources from medium into bacteria cell, so carbon accumulation in cell could triggering P(3HB) production [7, 9]. Bacteria isolates which grew in palm oil-bacto-agar medium then detected under UV light 365nm, and intent on observing occurance of P(3HB)-producing bacteria. From 40 bacteria isolates grown, were obtained 10 bacteria isolates indicated as bioplastic/P(3HB)-producing bacteria. The isolates of bioplastic-producing bacteria proven to showed yellow fluorescent when colony observed under UV light 365 nm, as seen in Figure 1.

This condition supported by Djamaan *et al.* [4] premise, whom proposed that P(3HB) could be synthetic when bacteria inoculated in the medium which consist excessive carbon sources and other important sources, such as nitrogen, were reduced. This condition caused bacterial cell growth happened in unstable condition, so bacteria tends to consumed carbon sources excessively and save P(3HB) as much as they can inside the cell. The microorganisms could accumulating P(3HB) if bacterial cell growth limited and deficiency of important nutrition sources, such as nitrogen, in excessive carbon sources [6, 9].

Table 1. Isolates of P(3HB) producing bacteria from soil at the top of Marapi Volcano Mountain

Number	Spot	Sample Code	Isolates Code	P(3HB) Producer
1		T 1.1	TG 1	-
2		T 1.2	TG 2	-
3		T 1.3	TG 3	-
4	1	T 1.4	TG 4	-
5		T 2.1	TG 5	+
6		T 2.2	TG 6	-
7		T 2.3	TG 7	-
8	2	T 2.4	TG 8	-
9		T 3.1	TG 9	-
10		T 3.2	TG 10	+
11		T 3.3	TG 11	-
12	3	T 3.4	TG 12	+
13		T 4.1	TG 13	-
14		T 4.2	TG 14	-
15		T 4.3	TG 15	-
16	4	T 4.4	TG 16	-
17		T 5.1	TG 17	-
18		T 5.2	TG 18	-
19		T 5.3	TG 19	-
20	5	T 5.4	TG 20	+
21		T 6.1	TG 21	-
22		T 6.2	TG 22	
23		T 6.3	TG 23	-
24	6	T 6.4	TG 24	-
25		T 7.1	TG 25	+
26		T 7.2	TG 26	-
27		T 7.3	TG 27	-
28	7	T 7.4	TG 28	-
29		T 8.1	TG 29	-
30		T 8.2	TG 30	-
31		T 8.3	TG 31	-
32	8	T 8.4	TG 32	+
33		T 9.1	TG 33	+
34		T 9.2	TG 34	-
35		T 9.3	TG 35	+
36	9	T 9.4	TG 36	-
37		T 10.1	TG 37	-
38		T 10.2	TG 38	+
39		T 10.3	TG 39	+
40	10	T 10.4	TG 40	-

Explanation : + : colony with yellow fluorescent
- : colony without yellow fluorescent

From Figure 1, it can be seen that there is 10 bacteria with bacteria code TG 1, TG 2, TG 3, TG 4, TG 5, TG 6, TG 7, TG 8, TG 9, and TG 10 positive indicated as P(3HB) which produce yellow fluorescents. This condition happened because 10 bacteria isolates had p(3HB)-producing enzyme which expressed by gene phaA, phaB, and phaC so the bacteria had ability to forming P(3HB) granule inside cell that will be detected by resonance of fluorescent when Nile Blue A were added and observed under UV light. Ostle and Holt (1982), reported that Nile blue A solution was able to tied with P(3HB) compound inside bacteria cell. P(3HB) granule contained in bacteria cell will shows yellow fluorescent with the gave of Nile Blue A solution that observed under UV light 365 nm. Whereas 30 bacteria isolates did not indicated for produced p(3HB). This condition proved by occurance of dark spot for several colony. This happened because the gene which coded PHB-synthase enzyme did not expressed so the enzyme was not produced [12].

From ten isolates which indicated as P(3HB)-producing bacteria, there is one isolates coded as TG9 which produced most sharp yellow fluorescent compared to another isolates (can be seen in Appendix 4). This condition occurred because each bacteria isolates has different ability to produces PHB-synthase enzyme which influenced by enzyme activity. Glazer and Nikaido (1995) reported that every organisms often produces same enzyme but with different enzyme activity because influenced by ability of the bacteria it self [10]. Other than that, enzyme biosynthesis regulation also affected the enzyme formation process. According to Madigan, *et al.* (2000), enzyme will synthesized if operator gen are free from resepsor, RNA polymerase could do the trancription of DNA to form mRNA [11]. Then, mRNA was experienced translation into enzymes. At no-inducer which will bind receptor and released it from operator of transcription process could happened and enzyme synthesis occurred. Kawaguchi and Doi (1992), reported that P(3HB) synthesized from acetyl KoA through 3 types of enzyme, i.e 3-ketothiolase, asetoasetil-KoA reduktase and P(3HB) synthase [12].

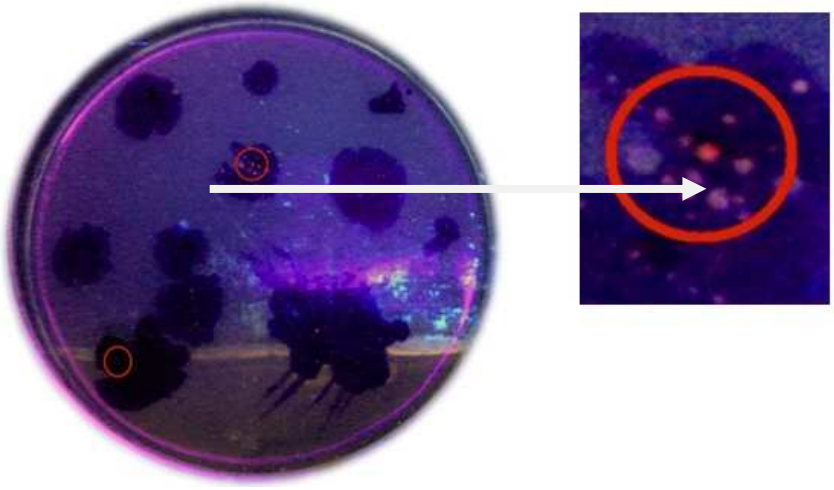

Figure 1. Detection of bioplastic-producing bacteria by Nile Blue A method, positive bacteria marked showed jingga fluorescent

Inside P(3HB)-producing bacteria cell, the biopolymer saved as granules of reserve food sources which scattered in the fluid of cytoplasm that will be reusable by the bacteria if environment condition less advantageous or run out of food sources. Agustien (2001) reported that strain AAP-17 could accumulating polymer granules of P(3HB) in their cell by 60%. *Alcaligenes eutropus* is one from the P(3HB)-producing bacteria which reported has ability to produces P(3HB) in their cell by 85% from all cell weight with usage of butyrate acid as the only carbon source [8].

Isolates Identification of Bioplastic-Producing Bacteria

Identification of bioplastic-producing bacteria isolates, including microscopic test, macroscopic test and biochemistry test.

Table. 2 Macroscopic observation of isolated bacteria from soil at the top of Marapi Volcano Mountain

Isolates Code	Macroscopic				
	Colour	Form	Edge	Surface	Elevation
TG 1	White	Round	Wavy	Smooth	Emerge
TG 2	White	Round	Flat	Rough	Emerge
TG 3	White	Round	Flat	Rough	Emerge
TG 4	White	Round	Flat	Rough	Emerge
TG 5	White	Round	Flat	Smooth	Emerge
TG 6	White	Round	Wavy	Rough	Emerge
TG 7	White	Round	Wavy	Rough	Emerge
TG 8	White	Round	Flat	Rough	Emerge
TG 9	White	Round	Flat	Rough	Emerge
TG 10	White	Round	Flat	Rough	Emerge

Table 2 showed characteristic from macroscopic observation of 10 bacteria isolates which able to produces P(3HB). The differences from each bacteria colony are characteristic for certain species. The microbe's colony differences are characteristic for one certain species. Colony form, coloration, surface are characteristic needed for species identification. Most of bacteria has whitish color, grayish, yellowish, until transparent, but for several species there is more distinct coloration pigment.

Table 3 showed microscopic characteristic of 10 isolates of P(3HB)-producing bacteria which observed under the microscope with 1.000 times magnification. The ten bacteria isolates are group of Gram positive bacteria and has bacil cell shape. The differences in the structure of each bacteria were different. Bacteria cell form could be cocci, bacil and spiral. Diplobacil formed bacteria are two bacteria cells which attached to each other. Diplobacil emerged from a couple of basil after cell fission and streptobacil appeared in form of chains [11,12].

The ten of bacteria isolates from Marapi Volcano Mountain top soil was identified by using biochemistry test, one of the process is catalase test. The result of catalase test are 10 bacteria isolates were positive contained catalase. This condition caused by the presences of buble produced by bacteria. According Cappucino and Sherman [13], buble-producing bacteria from catalase test are aerobic bacteria group. This bacteria was used oxygen to produces energy. H_2O_2 or hydrogen peroxide are toxic for aerobic bacteria. This type of bacteria will degrading that kind of toxic by using superoxide dismutase enzyme to breaking down superoxide toxic become water and oxygen. This condition caused the occurance of gas bubble in the examination if bacteria tested was aerobic bacteria.

Table 3. Microscopic observation and biochemical test of bioplastic-producing bacteria isolated from soil at the top of Marapi Volcano Mountain

Bacteria code	Nutrien Agar	Colony Coloration	Gram Staining	TSIA	Gas	H2S	katalase	Oksidase	Motilitas	Indol	Urea	Citrate	laktosa	glukosa	sukrosa	manitol	Methyl red (MR)	Voge Prekursor (VP)	OF	Identity
TG 1	+	White, bacil	+	m/k	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	*Bacillus* sp1
TG 2	+	White, bacil	+	m/k	-	-	+	-	+	-	-	-	-	+	-	-	-	+	-	*Bacillus* sp2
TG 3	+	White, bacil	+	m/m	-	-	+	-	+	-	-	-	-	+	+	+	-	+	-	*Bacillus* sp3
TG 4	+	White, bacil	+	k/k	-	-	+	-	+	-	-	-	-	-	+	-	-	+	-	*Bacillus* sp4
TG 5	+	White, bacil	+	k/k	-	-	+	-	+	-	-	-	-	-	+	-	-	+	-	*Bacillus* sp4
TG 6	+	White, bacil	+	m/m	-	-	+	-	+	-	-	-	-	-	+	-	+	+	-	*Bacillus* sp5
TG 7	+	White, bacil	+	k/k	-	-	+	-	+	-	+	-	-	-	-	-	-	+	-	*Bacillus* sp4
TG 8	+	White, bacil	+	m/k	-	-	+	-	+	-	-	-	+	-	-	-	+	+	-	*Bacillus* sp6
TG 9	+	White, bacil	+	m/k	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	*Bacillus* sp6
TG 10	+	White, bacil	+	m/k	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	*Bacillus* sp6

From mortility test can be seen that 10 bioplastic-producing bacteria isolates were positive motil. A bacteria was said as motil if that bacteria moves, grows and spreads troughout the media. Bacteria will classified as non-motil if it just grow around the inoculation area. Bacteria movement caused by flagella movement, which acted as locomotor tools for bacteria. The bacteria cell could moves because it has flagella (motil) and some bacteria could not moves because it has no flagella [13].

In order to determine type of P(3HB)-producing bacteria, then it was identification with several biochemistry reaction test as seen in Table 4. From the biochemistry test result, allegedly 10 isolates are from *Bacillus* genera which has different characteristic. The result showed that isolate TG 1 is *Bacillus* sp 1, TG 2 *Bacillus* sp 2, TG 3 *Bacillus* sp 3, TG 4, TG 5 and TG 7 is *Bacillus* sp 4, TG 6 *Bacillus* sp 5, dan TG 9 and TG 10 is *Bacillus* sp 6. Those bacteria were collected under UAAC (University Andalas Akmal Collection) names at Laboratory of Biota Sumatra, University of Andalas, Padang West Sumatra, Indonesia. Now the all bacteria are on going research to optimize the P(3HB) production by fermentation process from various carbon sources and also being characterized using the 16S rRNA analysis.

CONCLUSION

This research obtained 40 different bacteria isolates from soil samples at tha top of Marapi Volcano Mountain, West Sumatra, Indonesia and 10 beyond them produced P(3HB) with isolates code TG1, TG2, TG3, TG4, TG5, TG6, TG7, TG8, TG9, and TG10. Ten bacteria-producing P(3HB) isolates has characteristic such as round colony form, white until yellowish colony coloration, flat until serrated colony edge, smooth colony surface, gram positive bacteria, catalase positive and motil. Based on identification done, there is 6 bacteria species found, those are *Bacillus* sp 1, *Bacillus* sp 2, *Bacillus* sp 3, *Bacillus* sp 4, *Bacillu*s sp 5, and *Bacillus* sp 6.

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