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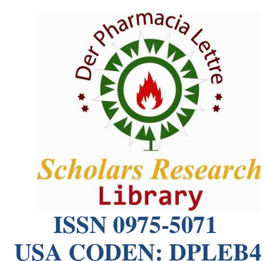

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Isolation and Identification of Polyhydroxyalkanoates Producing Bacteria from Soil Sample in Tropical Forest of Anai Valley, West Sumatra, Indonesia

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ABSTRACT

Isolation and identification of polyhydroxyalkanoates (PHA) producing bacteria from soil sample in tropical forest of Anai Valley, West Sumatra, Indonesia which grown in palm oil – Bacto Agar has been carried out. This study was conducted by making the soil sample which diluted until concentration 10^{-4}, and then it was inoculated into palm oil Bacto-Agar as the media. Palm oil was added about 2.3 grams in 500 mL bacto-agar and then incubated 30^oC for 48 hours. The screening of the bacteria indicated produced PHA in the cells after cultivation was using staining by 1% solution of Nile Blue-A. The positive result was indicated by appearance of bacterial colonies which showed orange fluorescence which looked under ultraviolet (UV) light at wavelength of 365 nm. Results showed that there were 26 colonies of bacteria producing PHA isolated. The identification of bacteria isolated were conducted by macroscopic, microscopic and biochemical reactions. It was obtained there were 7 isolates of bacteria which potential to produce biopolymers which it could classified into four genus i.e. Alkaligenes [$A_2S(1)$, $A_2S(16)$, $A_5S(7)$], Corynobacterium [$A_3S(1)$], Achromobacter [$A_5S(6)$]and Enterobacter [$A_5S(10)$, $B_4S(2)$].

Keywords: isolation, identification, polyhydroxyalkanoates, bacteria, palm oil, soil.

INTRODUCTION

Biopolymers are polymers that can be decomposed naturally by microorganisms such as bacteria and fungi. The advantage of biopolymer compounds compared with synthetic polymer compounds is biopolymer compounds can be broken down by microorganisms naturally in soil and water, so it does not damage the environment as caused by synthetic plastics [1,2,3]. Due to their characteristics, the biopolymers known as eco friendly polymers. The another advantage is biopolymer compounds can be produced from plant materials such as fats, oils and carbohydrates, which is their availability in the world are unlimited and can be updated throughout the time [4, 5].

Today has been commonly found microorganisms containing a biopolymer compound in its cell, the bacteria use the polymer as food reserves and energy which will be used in less favorable growth circumstances or run out of nutrient [6,7].

Based on this concept the researchers are vying for search and isolate various of microorganisms that potentially to save a biopolymer granules in their cells. Recently has been known more than 300 species of microorganisms can produce biopolymers PHA in the cell One example biopolymers that have been studied were poly (3-hydroxybutyrate) or P (3HB) and copolymers of poly (hydroxybutyrate-co-3-hydroxyvalerate) or P (3HB-co-3HV) [8].

Microorganisms which produce biopolymers can be isolated from soil and water. Soil is a dynamic place for biotic interactions and has five major components: water, minerals, air, nutrients, organic compound and living organisms in the soil such as actinomycetes bacteria, fungi, algae, and protozoa. In previous studies have been conducted screening bacteria producing bioplastics poly (3-hydroxybutyrate) from soil samples of biology forest education and research, Andalas University, Limau Manis, Padang. In the study identified forty bacteria isolates produce P (3HB). In this experiment soil samples taken from the soil at Anai Valley Forest. Anai Valley Forest is one of the tropical rain forest in West Sumatra, Indonesia. Therefore this place is a great potential for research because the place is a primary forest with moist air and is thought to contain a variety of nature soil microorganisms.

MATERIALS AND METHODS

Equipment
Petri dish (Iwaki), measuring cups (Iwaki), Erlenmeyer Flasks (Iwaki), Beakers (Iwaki), Dropper Pipets, stirring rod, needle ose, spirits light, hotplate (Cimarec 2), a test tube (Iwaki), laminar air flow , aseptic cabinets, sieve, digital scales (Ohaus), refrigerator (Sanyo), Incubator (Memmer), λ 365 nm UV light, autoclave (Automatic autoclave LS-2D Rexall Industries Co. Ltd serial number B0001107), aluminum foil and plate evaporator.

Materials
The materials used in this study are pure palm oil (PO) (PT Incasi Kingdom), crude palm oil (CPO) (PT Incasi Kingdom), soil from Anai Valley Forest, distilled water (Merck) , bacto agar (Merck), nile blue-A (Merck), ethanol 70% (Merck) and ethanol 96% (Merck).

Sterilization Equipment
The tools used washed and dried. Glass tools that have a mouth covered with cotton wrapped in gauze, and all the tools wrapped in parchment paper, and then sterilized in an autoclave at 121°C, a pressure of 15 lbs, for 15 minutes. Spatula and ose needle sterilized by heated over spirit flame for 20 seconds. Aseptic closet is cleaned from dust and sterilized by spraying alcohol 70% throughout inside of the closet. All the work is done by aseptic technique.

Nile Blue-A solution of 1%
Weigh powder Nile Blue-A 50 mg and add into a 5 mL volumetric flask. Than dissolve with 96% ethanol and shake until Nile Blue-A powder dissolved and add up to the boundaries [1,5].

Preparation of Media
10 g bacto powder dissolve in 500 mL of distilled water, put in erlemeyer heated until boiling, stirring the solution until bacto powder completely dissolved and look clear. Then sterilized in an autoclave at a temperature of 121˚C with 15 lbs of pressure for 15 minutes. 2.3 g palm oil sterilized in an erlenmeyer which plugged wrapped cotton with gauze in the same way. Add the sterile palm oil into the sterile bakto solution. Then pour it into each petri dish as much as 15 mL.

Processing and Breeding Soil Sample
Weigh 10 g of sifted soil sample and dissolved in 100 mL of distilled water (10% suspension), then create a multilevel dilution until 10^{-4}. Then inoculated into refined palm agar media and CPO oil media as much as 1 mL. Then incubation at a temperature of 35-37 ° C for 24-48 hours. Count and record the number of bacteria growing. From each bacteria growing, select separate bacteria and move into two new Palm Olein and CPO media which has been given a number, and then incubation at 35-37˚C for 24-48 hours.

Detection of Bacteria Producing PHA
Each of the agar media that has grown colonies of bacteria, treated with Nile Blue-A 1% solution and incubated at room temperature for 30 minutes. Than see under UV light at a wavelength of 365 nm. When a bacterial colony reddish orange that indicates the bacteria are producing the granules P (3HB) in the cell. Nile Blue-A is soluble in lipid that binds to the P (3HB) compound in a bacterial cell. The granules P (3HB) contained in the body of the bacteria which produce P (3HB) will give orange fluorescences when treated with a Nile Blue-A solution seen under UV 365 nm wavelength [9].

Purification and Storage Stock Bacteria Produce PHA
Purification and storage of stock bacteria producing biopolymers do by inoculating colonies of bacteria that give positive results with Nile Blue-A 1% in the three oblique media. Then incubation for 24 hours at 35-37˚C. The first media used for the laboratory stock, the second media used for identification and third media used for backup[5].

Identification of Bacteria Producing PHA
Identification of the bacteria by using biochemical reaction methods was done at Balai Veteriner Bukittinggi, West Sumatra. Identification carried out by macroscopically and microscopically according to the Cowan end Steel's method (1974) and a number of biochemical reactions. Microscopic identification carried out on color and colonies form, while for microscopic identification carried out by gram staining techniques. Further, biochemical reactions testing include: the formation of hydrogen sulfide gas, the formation of indole, motiliti, fermentation of carbohydrates (lactose, glucose, sucrose and mannitol), methyl red testing, Voges Prokauer testing, catalase testing, the oxidation/fermentation testing, oxidase testing, the formation of urea and the use of citrate [10].

RESULTS AND DISCUSSION

The study was conducted to isolate the bacteria producing biopolymers PHA from soil samples Anai Valley Forest Area and identify the bacteria. Biopolymers are polymers that can be decomposed naturally by microorganisms such as bacteria and fungi. Today has been commonly found microorganisms containing a biopolymer compounds in his cell, the bacteria use the polymer as food reserves and energy which will be used in less favorable growth circumstances or run out of food [11].

In this experiment soil samples taken from the soil Anai Valley Forest. Anai Valley Forest is one of the tropical rain forest in West Sumatra, Indonesia. Therefore this place is a great potential for research because the place is a primary forest with moist air and is thought to contain a variety of nature soil microorganisms.

Samples were taken from two different places. The first sample was taken at the riverside (wet soil) labeled A and the second one was taken in the forest (moist soil) labeled B. For each places the samples were taken in five different points. Then the sample was sieved using a 40 mesh sieve, it means there was 40 holes in 1cm sieve. the purpose of sieving is to clean the sample from trash, leaves and gravel gravel.

Palm oil was used in this experiment as media,it was because palm oil containing a lot of saturated and unsaturated fatty acids, it can be broken down by the lipase (extracellular enzyme of bacteria) that can be used to produce biopolymers [5]. Amount of palm oil used is 4.6 g / L, this amount is based on previous studies that showing the number of palm oil to produce the highest P (3HB) is 4.6 g / L, with that amount of palm oil can be produced 66.7% w / w of P (3HB). As reported fatty acids from palm oil can be used in the production and characterization of microbial polyester P(3HB) [1,2,3].

First step make sample dilution until 10^{-4}. Basically this dilution based on a principle of a cup count method, that assumes every living cell can develop into one colony and then the colony will be counted. So if there weren't dilution samples the colonies will grow very much and very difficult to quantify. Each diluted samples were taken 1 mL to be inoculated into media and then incubated in an incubator at a temperature of 35-37˚C for 24-48 hours. Temperature is an important factor affecting the growth. Low temperatures generally make a slow cellular metabolism whereas the higher temperatures increase the level of cell activity. 35-37°C is the optimum temperature for bacterial growth. During incubation the petri dish is placed upside-down to prevent water droplets from the condensation of water vapor on the surface of media order.

Calculation the number of bacterial colonies was made directly with the naked eye. The result of calculation the number of bacterial colonies growing as shown in Table 1. After the bacterial colonies counted, the bacteria immediately transferred to new media that has been given a number at the bottom. the purpose of numbering is to make it easier to determine positive bacteria after coloured.

Detection of bacteria producing biopolymers do by staining method with a Nile Blue-A solution. The purpose of using Nile Blue-A method because this method gives a fairly accurate and fast results with appearace a clear orange fluorescences in the bacteria colonies that produce P (3HB) after a few drops with a Nile Blue-A 1% solution in ethanol. According to Ostle & Hotl (1982) Nile Blue A solution is soluble compound in lipid that binds to P (3HB) in a bacterial cell. The PHA granules that contained in the body of the bacteria will be fluorescences (orange) when treated with a Nile Blue- A solution and saw under UV light at a wavelength of 365 nm [9].

Table 1. The number of bacteria colonies that grow after incubated in bacto-palm oil medium

No	Sample code	The number of bacteria colonies
1	A1C	15
2	A1S	8
3	A2C	16
4	A2S	16
5	A3C	26
6	A3S	16
7	A4C	28
8	A4S	13
9	A5C	12
10	A5S	11
11	B1C	4
12	B1S	20
13	B2C	16
14	B2S	4
15	B3C	3
16	B3S	15
17	B4C	6
18	B4S	4
19	B5C	6
20	B5S	4

A = Wet soil, C = CPO, B = Moist Soil, S = Palm olein (PO)
A1S = wet soil from first location with Palm Olein medium
A1C = wet soil from first location with CPO medium
B1S = wet soil from first location with Palm Olein medium
B1C = wet soil from first location with CPO medium
1,2,3,4,5 = samples taking place code

As shown in Table 2, there was 26 positive bacterial isolates that tearted with blue Nile-A. For sample A (wet soil samples/near the river) obtained 18 isolates and for sample B (dry soil) is obtained eight isolates. Positive bacterial isolates with Nile Blue-A of sample A was found in the samples with the code A2S (6 isolates), A3s (5 isolates), A4S (2 isolates) and A5S (5 isolates) and for sample B was found in a sample with the code B3S (5 isolates), B4S (1 isolate) and B5S (2 isolates).

Purification and storage of bacteria producing biopolymers. Purification and storage of stock bacteria producing biopolymers was done by inoculating colonies of bacteria that give positive results with Nile Blue-A 1% in the three oblique media. Purification of bacterial isolates aims to separate the product of inoculation that consists many different types of bacterial colonies in order to get pure bacterial colonies on each of bacteria. Storage stock of bacteria serve as a backup if there are bacteria do not grow and for further studies of the bacteria.

Table 2. The isolated bacteria producing PHA showed positive reactions with Nile Blue-A solution

No	Sample code	Positive isolates code Nile Blue-A	Information
1	A1C	_	do not fluorescence
2	A1S	_	do not fluorescence
3	A2C	_	do not fluorescence
4	A2S	6, 8, 12, 13, 14, 16	fluorescences
5	A3C	_	do not fluorescence
6	A3S	1, 3, 4, 5, 8	fluorescences
7	A4C	_	do not fluorescence
8	A4S	7, 10	fluorescences
9	A5C	_	do not fluorescence
10	A5S	6, 7, 8, 10, 11	fluorescences
n11	B1C	_	do not fluorescence
12	B1S	_	do not fluorescence
13	B2C	_	do not fluorescence
14	B2S	_	do not fluorescence
15	B3C	_	do not fluorescence
16	B3S	2, 7, 9, 12, 15	fluorescences
17	B4C	_	do not fluorescence
18	B4S	2	fluorescences
19	B5C	_	do not fluorescence
20	B5S	1, 4	fluorescences

whereas, 1,2,3 etc = number of bacteria on a petri dish that gave a positive reaction

To determine the type, genus and species of bacteria, identification is carried by macroscopic observation, microscopic observation and some biochemical reaction test. Macroscopic observation aims to see the colony, shape, and color properties of bacteria. Microscopic observation aims to see the morphology and determine the type of bacteria (Gram + or Gram -). Biochemical test aims to determine the genus and species of bacteria. Separation and identification of bacteria have been done in the Laboratory of the Veterinary Institute, from the identification of bacterial isolates obtained 7-producing biopolymers. The seven bacteria can be grouped into four genus, those are Alcaligenes (3 isolates), Achromobacter (1 isolate), Corynobacterium (1 isolate), Enterobacter (2 isolates) as shown in Table 3. In the year (2003), Djamaan also reported that Alcaligenes sp and Enterobacter sp potentially produce P (3HB). Genus grouping is based on the reaction of bacteria to chemical reagents [4].

Table 3. Characterization of isolated bacteria producing PHA

No	Treatment/ biochemical reactions	A2S(1)	A2S(16)	A3S(1)	A5S(6)	A5S(7)	A5S(10)	B4S(2)
1	Blood Agar	+	+	+	+	+	+	+
2	Colour of colony	Clear	Clear	White	White	White	White	Clear
3	Gram (Morfology)	- rod	- rod	+ rod	- coccus	-coccus	- rod	- rod
4	Aerob (A)/ Anaerob (AA)	A	A	A	A	A	A	A
5	TSIA	red	red	red	red	red	red	red
6	Gas	-	-	-	-	-	-	-
7	H_2S	-	-	-	-	-	-	-
8	Catalase	+	+	+	+	+	+	+
9	Oxydase	+	+	-	+	+	-	+
10	Motility	+	+	-	+	+	+	+
11	Indol	-	-	-	-	-	+	-
12	Urea	+	+	-	-	-	+	+
13	Citrate	+	+	+	-	+	+	+
14	Lactose	-	-	-	-	-	-	+
15	Glucose	-	-	-	+	-	+	+
16	Sucrosa	-	-	-	-	-	-	+
17	Mannitol	-	-	-	-	-	-	+
18	MR	-	-	-	-	-	-	+
19	VP	-	-	-	-	-	-	+
20	OF	-	-	-	-	-	+	+
21	KCN	+	+	+	-	+	+	+
22	Arginine	-	-	-	-	-	-	-
23	Lysine	+	+	+	-	+	+	+
24	Ornithin	-	-	-	-	-	+	-
25	Phenylalanine	-	-	-	-	-	-	-
26	Aesculin	-	-	-	-	-	+	+
27	Arabinose	-	-	-	-	-	-	+
28	Raffinose	-	-	-	-	-	-	+
29	Sorbitol	-	-	-	-	-	-	+
30	Trehalase	-	-	-	-	-	-	+
31	Xylose	-	-	-	-	-	-	+
32	Dulcitol	-	-	-	-	-	-	+
33	Malonat Broth	-	-	-	-	-	-	-
34	Nitrate	-	-	-	-	-	-	-
35	Gelatine	+	+	+	+	+	+	+
Conclusion		*Alcaligenes sp*	*Alcaligenes sp*	*Corynobacterium sp*	*Achromobacter sp*	*Alcaligenes sp*	*Enterobacter sp*	*Enterobacter aerogenes*

Alcaligenes including Gram-negative, rod-shaped cells, clear colony color. Specific reaction: Red TSIA indicates gas and H2S negative, the positive reaction of Catalase, Oxydase, Mortilitas. Negative reaction of Lactose and Glucose and negative OF reaction [9].

Corynobacterium including gram-positive bacteria, stem cell shape, white colonies, an Aerobic bacteria. Specific reaction: Red TSIA indicates H2S and Gas negative, positive Catalase, negative Mortility, Gelatin positive, and positive citrate. While achromobacter is a gram-negative bacteria, cell shape Coccus, white colonies, an Aerobic bacteria. Specific reaction: Positive reaction of Catalase, Oxydase, and Mortilitas, positive Glucose, negative reaction of Arginine, Lysine, Ornithine, and Phenylalanin. Enterobacter is a gram-negative bacteria, stem cells forms, colony is white, Catalase positive, Oxydase negative, Motility positive. For aerogenes, reactions are also shown in the reaction lactose, glucose, sucrose, mannitol gave a positive reaction. MR, VP, OF, KCN reactions are positive. Aesculin, Arabinose, raffinose, sorbitol, Trehalase, Xylose, Dulcitol are positive reactions [9].

CONCLUSION

The isolation and identification of PHA producing bacteria from soil sample in tropical forest of Anai Valley, West Sumatra, Indonesia is obtained 7 bacterial isolated potentially produce biopolymers. The 7 isolated bacteria can be

classified into four genus, those are Alcaligenes (3 isolates), Achromobacter (1 isolate), Corynobacterium (1 isolate) and Enterobacter (2 isolates).

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