INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

BIOSYNTHESIS OF A POLYESTER POLY(3-HYDROXYBUTYRATE) FROM CRUDE PALM OIL (CPO) BY USING *Bacillus* sp. UAAC 21501

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Article Received on: 15/07/18 Approved for publication: 24/09/18

DOI: 10.7897/2230-8407.099194

ABSTRACT

A polyester poly(3-hydroxybutyrate) or P(3HB) is a natural polymer produced by certain bacteria that can accumulate P(3HB) granules in their cytoplasmic fluid. For these bacteria, the granule P(3HB) acts as a food reserve that can be used if the environmental conditions is not profitable for the growth. In this paper we describe the biosynthesis process of poly(3-hydroxybutyrate) polyester compounds from crude palm oil (CPO) using *Bacillus* sp. UAAC 21501 with fermentation techniques that have been carried out at the Laboratory of Biotan Sumatra, University of Andalas, Padang, West Sumatra, Indonesia. The fermentation process was carried out in a 250 ml conical flask using a rotary shaker incubator at a temperature of 50ºC, pH 7 and 200 rpm agitation with variations in fermentation time and CPO concentration. P(3HB) content accumulated in bacterial cells was determined by gas chromatography method. Then P(3HB) formed was extracted and methanolysis (esterification) to 3-hydroxybutyrate-methyl-ester. Results showed that the best CPO concentration in producing the amount of *Bacillus* sp. UAAC 21501 is at a concentration of 0.5 g/100 ml substrate with 48 hours of culture duration. In this condition, the amount of biomass obtained from *Bacillus* sp. UAAC 21501 was 164 mg/100 ml substrate, and the percentage of P(3HB) content in the cell was 0.133% w/w.

Keywords: polyester, crude palm oil, poly(3-hidroxybutyrate), *Bacillus* sp. UAAC 21501

INTRODUCTION

The use of synthetic polymers in the form of plastic in various life sectors since the last few decades has increased rapidly. This is due to the nature of the material in the form of plastic which has good impact resistance, good stretching power, water resistance and not easily decomposed[1]. Due to the increasing use of plastic, various problems arose in terms of environment and economy aspect. One of the environmental problems faced is the plastic accumulation in the ocean. For example, in a long-term study in the North Atlantic, one sample of seawater contained an equivalent of 580,000 pieces of plastic/km2. In addition to this condition, synthetic plastic cannot be decomposed and will survive in the environment for hundreds of years[2].

In recent years, the emergence of problems concerning synthetic plastics utilization has triggered experts to develops materials that are environmentally friendl, biodegradable and produced from renewable natural resources such as bioplastics. Poly(3-hydroxybutyrate) is a bioplastic from the poly-β-hydroxyalkanoate (PHA) group which is widely studied because it has the same physical and chemical properties as synthetic plastics such as polypropylene, but when discharged into the environment it will break down completely within a certain period of time[3].

One way to produce bioplastics that are widely studied is by fermentation using P(3HB)-producing bacteria using certain carbon substrates. But in producing P(3HB), the cost for carbon substrates contributes around 30%-50% of the total production costs[4]. Therefore, alternative steps such as use of cheap and renewable carbon substrate raw materials are needed to minimize the production cost of bioplastics.

Palm oil is potentially used as a carbon source to produce bioplastic P(3HB). Palm oil contains triglycerides which consist of saturated and unsaturated fatty acids which can be converted by bacteria into acetyl coA through the β-oxidation pathway[5].

In previous studies, P(3HB)- bioplastic producing bacteria was isolated and characterized by namely *Bacillus* sp. UAAC 21501 from Mount Talang hot springs, SolokDistrict, West Sumatra, Indonesia. To continue the research, a fermentation study was conducted to determine the ability of bacteria to produce bioplastics from different concentrations of CPO palm oil carbon sources and also to determine whether there was an effect of fermentation time on bioplastic production P(3HB).

MATERIALS AND METHOD

Materials and Equipments

The tools used were test tubes, ose needles, measuring cups, beaker glass, stirring rods, erlenmeyer flasks, spiritus lamps, spatel, aseptic cabinets, refrigerators, autoclaves, ovens, rotary shakers incubators, laminar air flow, incubators, centrifuges gas

chromatography, scissors, yarn, cotton, gauze and aluminum foil. The ingredients used were aquadest, nutrient agar, *Bacillus* sp. UAAC 21501, crude palm oil, barium chloride, diammonium phosphate, microelement solution, 0.1 M sodium hydroxide, phosphate buffer solution, 98% sulfuric acid, standard P(3HB) (Biopol®), chloroform and methanol[6].

Equipments Sterilization

The tools used in the study were first washed and dried. Glassware that has a mouth covered with cotton wrapped in gauze, then all the tools were wrapped using parchment paper, then sterilized in an autoclave at 121 ˚C, 15 lbs pressure, for 15 minutes. Spatel and ose needles are sterilized by means of flambier above the flame of a spiritus lamp for 20 seconds. Aseptic cabinets were cleaned from dust and sterilized by spraying 70% alcohol throughout the cabinet. All work is done in an aseptic technique[1].

Preparation of Medium

Nutrient Agar (NA) weighed as much as 20 g then dissolved in 1 liter of distilled water. Then, it was heated over the heater, then it was stirred until dissolves completely and transparent. Then it was covered with a cotton stopper wrapped in sterile gauze. Then it was sterilized with autoclave at 121˚C temperature, 15 lbs pressure, for 15 minutes [8,9,10]. A total of 4 ml of sterile NA media was put into a test tube, tilt it up to 10-20˚ on the mat, and left to solidify. All works was carried out aseptically in Laminar Air Flow[11,12].

Enrichment of *Bacillus* sp. UAAC 21501 Isolates

Bacillus sp. UAAC 21501 isolates was obtained from the UAAC Culture Center at the Biota Laboratory of Biota Sumatra, Andalas University, Padang, Indonesia. The culture was transferred with the help of Ose needle to the tilted agar medium in a new tube. The transfer work is carried out in an aseptic technique on Laminar Air Flow. Then the bacterial culture was incubated for 24 hours in an incubator temperature of 50 ˚C. After incubation, the culture is then stored in a refrigerator at 4 °C[13,14].

Preparation ofP(3HB)bioplastic-producing bacteria growth medium

The carbon source used in this study is crude palm oil with a concentration of 1 g/L, 5 g/L and 10 g/L. As a source of nitrogen, 1.1 g $(NH_4)_2HPO_4$ was used. Meanwhile, microelement solution was made by dissolving as much as 2.78 g $FeSO_4.7H_2O$; 1,98 g $MnCl_2.4H_2O$; 2,81 g $CoSO_4.7H_2O$; 1,67 g $CaCl_2.2H_2O$; 0,17 g $CuCl_2$; and 0.29 g of $ZnSO_4.7H_2O$ into 1 liter of 0.1 N. HCl. This microelement solution was sterilized by autoclave process at 121˚C, 15 lbs pressure, for 15 minutes, then it was added to the substrate as much as 2 ml for 1 L substrate volume [1,8] .

Preparation of *Bacillus* sp. UAAC 21501 bacterial suspension

As much as 1-2 ounces of bacterial colonies was suspended into 9 ml of 0.9% NaCl in a sterile test tube and then vortex. Turbidity of the bacterial suspension was compared with the McFarlandstandard 0.5 with a bacterial concentration of 3 x 10^8/ ml. McFarland standard solution was made by as much as 99.5 ml of 0.36 N H_2SO_4 solution mixed with $BaCl_2.2H_2O$ 1.175% solution as much as 0.5 ml in erlenmeyer. Then it was shaken until a cloudy solution was formed. This turbidity was used as a standard for bacterial suspension turbidity[11].

Preparation of *Bacillus* sp. UAAC 21501 bacterial inoculum

Bacillus sp. UAAC 21501 bacteria suspension was pipetted as much as 10 ml, then put into a conical flash 250 ml, then growth medium up to 100 ml was added to form a 10% inoculum.

Culture of *Bacillus* sp. UAAC 21501 in CPO medium

Culture of P(3HB) *Bacillus* sp. 21501-bioplastic producing bacteria was carried out at optimum conditions with 50 ˚C temperature and agitation 200 rpm. This culture was carried out at concentrations of 1g/L, 5g/L and 10 g/L of crude palm oil. Samples were taken at 36 hours, 42 hours and 48 hours. At each time period of sampling, as much as 100 ml sample was taken.

Separation of biomass and supernatant

The process of biomass and supernatant separation was carried out by centrifugation. As much as 100 ml of sample was centrifuged at 3000 rpm for 20 minutes. The clear layer of supernatant was separated from biomass deposits using pipette. The supernatant layer was used to determine pH, while dried biomass was used to determine dry weight and P(3HB) content[5].

Measurement of pH supernatant

PH measurement was done using a pH meter. The measurements of pH were carried out to determine the acidity level of the result from final fermentation process and observe theeffect on biopolymer production[14]

Determination of Biomass Dry Weight

Biomass dry weight was determined gravimetrically using analytical scales. Determination was done after the biomass separated from the supernatant, then washed with distilled water. Then the biomass cells were dried using an oven with temperature below 70˚C for 24 hours or until constant weight obtained. Then the weight of dry cells was determined by weighing.

Metanolysis Process

Type P(3HB) wasdetermined using standard P(3HB) which has undergone standardization tests. Standard P(3HB) was methanolized by adding 1.7 ml of methanol; 0.3 ml of 98% $H_2SO_4$and 2 ml of $CHCl_3$which were heated until 100˚C for 4 hours on the heater to convert P(3HB) to methyl ester group, which was then identified using Gas Chromatography[1,15].

Determination of P(3HB) Bioplastic using Gas Chromatography

P(3HB) contained in dry cells was determined by Gas Chromatography. After the methanolysis reaction was completed, as much as 1 ml of distilled water was added to the solution, to formed two layers. The chloroform layer was piped and as much as 5 µl was injected into a gas chromatography using the RPX-01 column and detected by Flame Ionization Detector (FID). The type of P(3HB) can be determined from the retention time of the peak appearance in standard P(3HB) chromatogram. The detector temperature was set to 250˚C, injector 260 ˚C and column 50 ˚C for 4 minutes and every 1 minute the temperature was increased by 10˚C until it reached 180 ˚C, then waiting time of 3 minutes was added. Biopolymer content can be analyzed from the area under the curve that was formed in the chromatogram.

RESULTS AND DISCUSSION

Bacillus sp. UAAC 21501 was obtained from UAAC Culture Center, Sumatran Biota Biotechnology Laboratory, Andalas University, Padang, Indonesia. We have isolated and purified this bacterium and identified biochemically, the results can be seen in Table 1. There is an indication that this bacterium was able to produce P(3HB) based on the test using Nile Blue A 1% reagent and incubation for 30 minutes, then viewed under UV light at 365 nm wave length, where bacterial colonies showed a yellowish fluorescent color, so this bacterium was determined asP(3HB)-producing bacteria[1,3.]

The formation of bioplastic P(3HB) occurs when bacteria was grown in a medium using a substrate containing excessive carbon sources and reduced other important elements, such as nitrogen. As a result, the bacterial cell growth will occur in unbalanced conditions, and that bacteria tend to consume carbon sources excessively and stores as much as possible for food reserve granules in its cells (Anderson).

Table 1: Characteristics of *Bacillus* sp. UAAC 21501 producing bioplastic[16]

Observation	Result
Macroscopic	
Colony form	Round
Colony coloration	White
Edge	Smooth
Elevation	Flat
Surface	Smooth
Microscopic	
Gram Stain	+
Cell form	Bacil
Size (length)	0,4 µm
Size (width)	0,1 µm
Endospore coloration	-
Biochemistry Test	
Motility Test	Motil
Catalase Test	+
Nutrient Agar Test	+
Aerob/Anaerob Test	+
TSIA Test	+
H2S Test	-
Oxydase Test	-
Indole Test	-
Urea Test	+
Citrate Test	-
Lactose Test	-
Glucose Test	-
Sucrose Test	-
Mannitol Test	-
Methyl Red Test	+
Voges Praskruer Test	+
Oxydase Fermentation Test	-
Arabinose Test	-
Xylose Test	-
Nitrate Test	-
Gelatine Test	+

Table 2 and Figure 1 shows the results of biosynthesis of P(3-HB) using CPO as carbon sources using *Bacillus subtilis* UAAC 21501. In most bacteria, P(3HB) is synthesized from acetyl-CoA through the work of three types of enzymes, namely ketothiolase, acetoacetyl-CoA reductase and P(3HB) synthase[17]. Crude palm oil (CPO) contains carbon sources in the form of fattyacids, including: palmitic acid (43.7%), oleic acid (39.9%), linoleic acid (10.3%), and stearic acid (4.4%). According to Majid et al (1993), to be able to produce P(3HB) bioplastic-producing bacteria will try to convert these fatty acids into acetyl-coA with the help of the β-oxidase enzyme.

Table 2: Data of biomass weight (dry cells) and pH of *Bacillus* sp. UAAC 21501 fermentation media

No.	CPO (g/100ml)	Time (hour)	Biomass (mg/100 ml)	pH
1	0.1	36	111	6.6
2		42	120	6.5
3		48	129	6.5
4	0.5	36	113	6.6
5		42	127	6.6
6		48	164	6.5
7	1.0	36	84	6.6
8		42	104	6.6
9		48	114	6.5

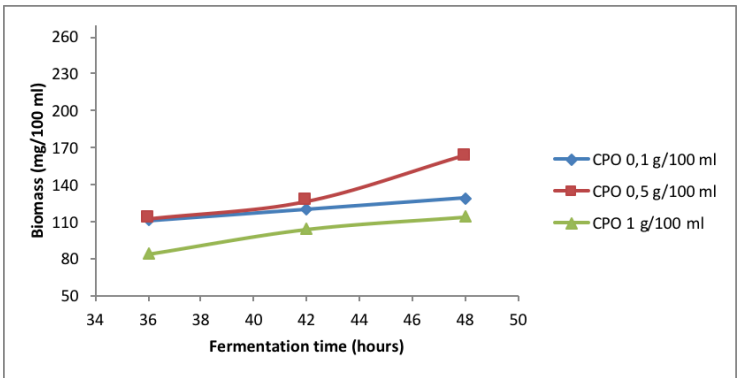

Figure 1: Profile of relationship between the amount of biomass and fermentation time at various concentrations of crude palm oil (CPO)

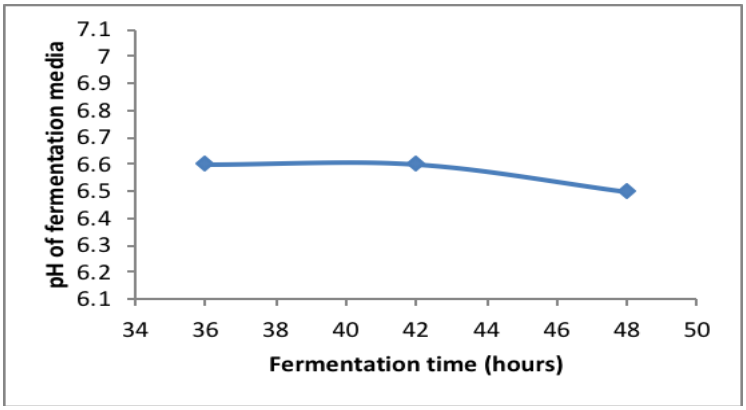

Figure 2: Profile of relationship between the pH of fermentation media and fermentation time at crude palm oil (CPO) concentrations of 0.5 g/100 ml

Profile of relationship between the pH of fermentation media and fermentation time at crude palm oil (CPO) concentrations of 0.5 g/100 ml showed in Figure 2. Changes in pH means it will reduce the production of P(3HB) in the bacterial cells[18]. Changes in pH during fermentation were caused by the formation of carboxylic acids as a by-product of fermentation. Therefore, to prevent a significant decrease in pH in the bacterial growth medium, phosphate buffer solution was added during the fermentation process.

Table 3 shows the results for determination of the amount of P(3HB) content that was carried out using a Gas Chromatography (GC) methods. In this experiment, the standard P(3HB) used was 10 mg Biopol® to ensure that the polymer accumulated in the *Bacillus* sp. UAAC 21501 bacterial cell was true P(3HB). Before it was injected into the GC device, the first sample was methanolysed, where as much as 20 mg of biomass was added to 1.7 ml of methanol, 0.3 ml of concentrated H_2SO_4 and 2 ml of chloroform, then it was heated in an oven at 100 ºC for 4 hours in a tightly closed test tube. This process aims to convert 3-hydroxybutyrate monomer into the 3-hydroxymethyl ester form so that it turned into a gas form and can be detected by GC devices[15].

After the reaction was completed, as much as 1 ml of distilled water was added to the solution and it was shake until two layers were formed. The chloroform layer located at the bottom was pipetted as much as 5 µl and injected into the GC device. The GC tool was operated with Helium as the mobile phase used, the column used was RPX-01 and the detector used was the Flame Ionization Detector (FID). Examples of chromatograms from P(3HB) which have been methanolysed (esterification) to 3-hydroxybutyrate methyl ester are shown in Figure 3. Without the methanolysis process, P(3HB) cannot be determined by gas chromatography.

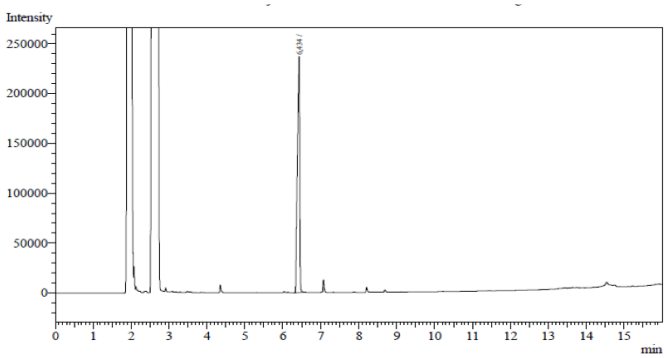

Figure 3: Profile of gas chromatography chromatogram of P(3HB) after methanolysis process into 3-hydroxybutyrate methyl ester

Table 3: Data of P(3HB) level and percentage in *Bacillus* sp. UAAC 21501 bacterial cell at CPO concentration of 0.5 g/100 ml

No.	Concentration of CPO	Time Variation (hour)	AUC	P(3HB) Level (mg/20mg)	P(3HB) Percentage (%)
1	0.5g/100ml	36	2677	0.0267	0.133
2		42	4376	0.0437	0.218
3		48	4879	0.0488	0.244

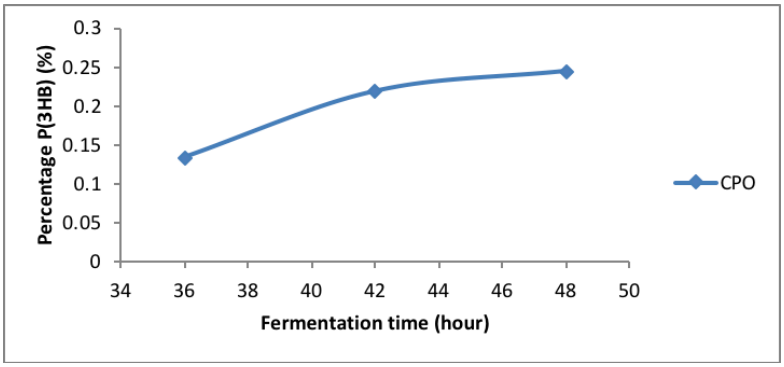

Figure 4: Graphic of the relationship between percentage of P(3HB) content with fermentation time in CPO concentration of 0.5 g/100 ml

Table 3 and Figure 4 showed that the relationship between percentage of P(3HB) content with fermentation time in CPO concentration of 0.5 g/100 ml. The maximum P(3HB) percentage accumulated in the cells is around of 0.244 % w/w detected after 48 hours of fermentation process. In previous study by Djamaan (2015)[1], oleic acid was used as a carbon source by using *Erwinia* sp. USMI-20 which able to produce P(3HB) of 55% b/ b. In addition, the use of glucose-peptone as a carbon source with *Bacillus mycoides* also produced P(3HB) with percentage of 35.60% b/b[19] and *Bacillus cereus* SPV with a carbon source of sugarcane molasses produced 61.07% b/b P(3HB)[20]. If the results of the previous study were compared with the results of this study with a P(3HB) obtained only 0.133%, this acquisition was still relatively low. However, if it is calculated from the conversion factor and the increase in added value of CPO, from only 0.5g / 100 ml it able to produce 0.133% P(3HB), which is quite significant. The acquisition of bioplastic P(3HB) can be influenced by the carbon source used and the factor of bacterial compatibility with the fermentation substrate can give an effect on the production of bioplastic P(3HB).

Based on these data, it can be seen that further optimization of carbon substrate is needed for the production of P(3HB) using thermophylic bacteria *Bacillus* sp. UAAC 21501, so the optimum conditions for bacteria to maximized and increased P(3HB) content obtained from fermentationcan be determined. According to Doi (1990)[21], a P(3HB) producing bacteriais able to accumulate P(3HB)polymer granules in their cells to reach 85% of the total cell weight. Our previous research using CPO in producing other types of polyester, namely poly (3-hydroxybutyrate-co-3-hydroxyvalerate). Akmal et al., (2015) has been able to achieve the polymer content in *Erwinia* sp. USMI-20 as much as 53% of the cell weight[1].

CONCLUSION

From the biosynthesis process of poly(3-hydroxybutyrate) polyester compounds from crude palm oil (CPO) using *Bacillus* sp. UAAC 21501 can be concluded that the best CPO concentration in producing biomass and P(3HB) polyester content was 0.5 g/100 ml with substrate biomass of 164 mg/100 ml and P(3HB) content of 0.133% b / b, with the fermentation duration of 48 hours.

AKNOWLEDGMENT

The author would like to say special thanks to The Dean of Faculty of Pharmacy, University of Andalas, Padang, Indonesia for her support this research under Lecturer Research Grant, Fiscal Year 2018, with Contract Number: 01/UN.16.10/DPPKM/FFARMASI/2018.

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Cite this article as:

Akmal Djamaan *et al*. Biosynthesis of a polyester poly(3-hydroxybutyrate) from crude palm oil (CPO) by using Bacillus sp. UAAC 21501. Int. Res. J. Pharm. 2018;9(9):93-98 http://dx.doi.org/10.7897/2230-8407.099194

Source of support: University of Andalas, Padang, Indonesia, Conflict of interest: None Declared