PRODUCTION OF A BIOPOLYMER POLY(3-HYDROXYBUTYRATE) BY *Erwinia* sp. USMI-20 IN 10 L FERMENTOR

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INTISARI

Telah dilakukan percobaan produksi senyawa biopolimer poli(3-hidroksibutirat), P(3HB, menggunakan bakteri *Erwinia* sp. USMI-20 dari minyak kelapa sawit sebagai sumber karbon. Fermentasi dijalankan selama 66 jam dengan teknik pengkulturan sekelompok di dalam fermentor berkapasitas 10 L secara aerob, pada pH 7, suhu 30 °C, kecepatan aliran udara 1 vvm, kecepatan penggoncangan 200 rpm. Selama fermentasi berlangsung ditentukan kenaikan biomassa, kandungan polimer dan konsentrasi polimer yang dihasilkan. Hasil yang diperoleh menunjukkan bahwa bakteri *Erwinia* sp. USMI-20 mampu memproduksi P(3HB) mencapai 46 % b/b dengan berat sel kering sel bakteri 3,6 g/L dari 4.6 g/L minyak kelapa sawit yang diberikan ke dalam medium. Waktu fermentasi optimum untuk menghasilkan kandungan polimer tertinggi pada kajian ini adalah 48 jam.

Kata Kunci: poli(3-hidroksibutirat), minyak kelapa sawit, *Erwinia* sp. USMI-20 dan fermentor.

ABSTRACT

A batch culture of a locally soil isolated microorganism identified as *Erwinia* sp. USMI-20 was investigated for producing the intracellular bioplastic poly(3-hydroxybutyrate), P(3HB) from palm oil as a sole carbon source. A fermentation process was conducted through an single stage batch cultivation under aerobic condition at pH 7.0, incubation temperature of 30 °C, aeration rate 1 vvm, and agitation rate of 200 rpm for 66 hours in 10 L fermentor. The characterization of the polymer production was based on cell growth, polymer content and polymer concentration during fermentation. It was found that *Erwinia* sp. USMI-20 could produced P(3HB) up to 46 weight % of P(3HB) polymer content with a dry cell weight of 3.6 g/L from a batch fermentation in an 10 L fermentor from an initial concentration of 4.6 g/L of palm oil. The optimum fermentation time to obtain the high level of polymer production in this study was 48 hours.

Keywords: poly(3-hydroxybutyrate), palm oil, *Erwinia* sp. USMI-20, and fermentor.

INTRODUCTION

Poly(3-hydroxyalkanoate) (PHA) is an intracellular microbial thermoplastic that is widely produced by many bacteria. PHA are synthesized as carbon and energy reserve materials of the bacteria under certain environmental condition, such as nitrogen, oxygen and phosphate-limitation condition [1,2]. These polyester is accumulated as distinct granules in the cells, and it can be isolated from cells by means of solvent extraction [2, 3]. Biodegradable polymers offer an attractive alternative to tradisional petroleum-based non biodegradable polymers from an environmental perspective.

Among the PHAs which have been widely reported are poly(3-hydroxybutyrate), P(3HB), and its copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate, P(3HB-*co*-3HV)[4]. These polymers have been produced and marketed by Monsanto, who used fed-batch cultivation of *Alcaligenes eutrophus*. However, commercial applications of PHA have been limited by their high price (approximately US $ 16/kg).

One approach to reduce the cost of PHA production is to use inexpensive carbon sources for bacterial growth in PHA biosynthesis. PHAs can be produced from renewable resources and are biodegradable. Kato *et al.*, reported that a soil bacteria *Pseudomonas* sp 61-3 produced a mixture of P(3HB) homopolymer and a random

copolymer of 3-hydroxyalkanoate, 3HV units of C-4 to C-12 P(3HB-co-3HA) when alkanoic acid and plant oils were fed as the sole carbon source[5].

For this paper, we reported the production P(3HB) from palm oil as carbon sources by a locally soil isolated microorganism identified as *Erwinia* sp. USMI-20.

MATERIAL AND METHOD

Equipment

Fermentor 10 L (B Braun Biostat 10-E), Incubator Rotary Shaker (B. Braun Certomat RH), Gas Chromatography (Perkin Elmer Autosystem XL), 270 MHz ^{1}H and ^{13}C Nuclear Magnetic Resonance (Bruker), Freeze Drier (B Braun FD 5505P) and Rotary Vacum Evaporator (Eyela).

Medium

Palm oil was used as a sole carbon sources in this fermentation. The pH value of medium was adjusted to 7.0 by the addition of NaOH. The composition of mineral salts medium was as follows (per liter of distilled water): 3.7 g of KH_2PO_4, 5.8 g of K_2HPO_4, 1.1 g of $(NH_4)_2HPO_4$, 10 ml of 1.0 M $MgSO_4$ and 1.0 ml of microelement solution. The microelement solution contain 2.78 g of $FeSO_4.7H_2O$, 1.98 g of $MnCl_2.4H_2O$, 2.81 g of $CoSO_4.7H_2O$, 1.67 g of $CaCl_2.2H_2O$, 0.17 g of $CuCl_2.2H_2O$ and 0.29 g of $ZnSO_4.7H_2O$ per liter of 0.1 M HCl [6].

Culture condition:

Erwinia sp. USMI-20 were cultivated in mineral media containing palm oil (4.62 g/L) as the carbon source in 10 L fermentor (B Braun Biostat E-10). The fermentation was conducted at 30° C, pH 7.0, aeration of 1 v/v/m, agitation of 200 rpm for 66 hours. At regular intervals during the fermentation process (0, 4, 8, 12, 18, 24, 30, 36, 42, 48, 54, 60 and 66 hours cultivation), 200 mL of samples were collected, harvested, washed and lyophilized.

Analytical procedure

The cellular polymer content was determined by using a capillary gas chromatography method. 20 mg of dried cells was subjected to methanolysis with a solution consisting of 1.7 mL methanol, 0.3 mL 98% sulfuric acid and 2.0 mL of chloroform at 100°C for a minimum of 4 hours to convert the constituents to their methyl esters. On completion of the methylation reaction, addition of 1 ml water to the reaction mixture would induce a phase separation. The lower chloroform layer was used for a gas chromatographic analysis with a Supelcowax column (30 mm by 0.32 mm) and a flame ionization detector. The operating condition for the GC equipment (Perkin Elmer Autosystem XL) was as follows: detector temperature at 250°C, injector temperature at 260°C will split injection, (1:100), the column temperature was held at 50°C for 5 minutes, then programmed to 220°C at 10°C/min.

The composition of the polymer samples were confirmed by analysis of 270 Mhz ^{1}H and ^{13}C NMR spectra. The NMR was recorded on a Bruker NMR and tetramethylsilane was used as an internal chemical shift standard. The biomass was determined by a gravimetric measurement of the dried cells. The sample was centrifuged at 10,000 rpm for 15 minutes and washed twice with distilled water to remove the remaining oil. The cells were then dried in a freeze drier (B Braun FD 5505P) for 24 hours. The amount of remaining nitrogen in the solution was determined by Berthelot reaction at wavelength of 625 nm[7]. The colorimetric reaction was formed by adding 5 uL of the aqueous solution with 2.5 mL of a solution containing phenol (1%) and sodium nitroprusside (0.006%). Subsequently, 2.5 mL of a second solution containing 0.5% sodium hydroxide, 5.4% sodium hydrogen phosphate and sodium hypochlorite (1%) was added to the mixture. The resulting blue solution was determined against a standard calibration curve of known amounts of nitrogen. The amount of remaining palm oil in the medium was determined by a gas chromatography method after the oil had been extracted with chloroform and converted to their respective methyl esters. The oil was determined indirectly from the amount of palmitic acid methyl ester from the extracted oil.

RESULT AND DISCUSSION

The microorganism used in this study was isolated from the soil in Universiti Sains Malaysia, Penang, Malaysia which coded as USMI-20. A specific isolation medium containing palm oil as the carbon source was used to screen PHA producers from the soil samples. Gram staining test on USMI-20 showed that the microorganism was a gram negative and short rod bacterium. Its colony on nutrient agar was smooth with a distinguished edge and yellowish in colour. The bacterium was able to grow on Mac Conkey medium. It also showed an obvious growth when inoculated on nutrient agar at 42°C for 24 hours.

Further identification of strain USMI-20 was done by applying a fast and easy identification kit, API 20E kit obtained from bioMerieux, France. This identification system involved 21 different tests which include

biochemical reaction with the various enzymes in the cell. A positive result would indicate that the strain under investigation contain these enzymes in its metabolism system. Otherwise, a negative reaction indicates the absence of such enzymes. In addition, the growth on the bacterium on some of the common carbon source are also tested in this identification test which include glucose, mannitol and sucrose. The identification of the strain was done by using API 20E kit with the result shown in Table 1. The genus of strain USMI-20 was confirmed by comparing the result from the API 20E kit with manual guide given with the identification kit test strip. Based on the this preliminary work, USMI-20 was identified as *Envinia* sp.

Table 1. Characteristics of *Envinia* sp. USMI-20 by API 20E kit (bioMerieux, France)

Reaction / Enzyme	Substrate	Result
1. Beta-galactosidase	Ortho-nitrofenyl-galactoside	+
2. Arginine dehydrolase	Argine	-
3. Lysine decarboksilase	Lysine	+
4. Ornitine decarboksilase	Ornitine	-
5. Citrate usage	Sodium citrate	+
6. Releasing of H_2S	Sodium thiosulphate	-
7. Urease	Urea	-
8. Tryptophane deseminase	Trytophane	+
9. Indol formation	Trytophane	-
10. Acetone formation	Sodium pyruvate	-
11. Gelatinase	Kohn's gelatine	-
12. Fermentation/oxidation	Glucose	+
13. Fermentation/oxidation	Manitol	-
14. Fermentation/oxidation	Inositol	-
15. Fermentation/oxidation	Sorbitol	-
16. Fermentation/oxidation	Rhamnose	-
17. Fermentation/oxidation	Sucrose	-
18. Fermentation/oxidation	Melibiose	-
19. Fermentation/oxidation	Amygdaline	-
20. Fermentation/oxidation	Arabinose	-
21. Sitocrome-oxidase		+

+ = positive reaction; - = negative reaction

In an preliminary experiment, the influence of the amount of palm oil on the growth of the bacteria was studied by increasing the amount of oil from 1.6 g/L to 7.7 g/L with the ammonium phosphate remained fixed at 1.1 g/L. It was found that the maximal amount of P(3HB) production was obtained by using 4.6 g/L of palm oil. On further increment of the amount of oil higher than 4.6 g/l, there was no corresponding increase in the production of P(3HB) or the dry cell weight (Table 2).

Table 2. The influence of the amount of palm oil on the growth of *Erwinia* sp. USMI-20 and polymer content [a].

Palm Oil g/l	Dried Cells (g/l)	Polymer Content [b] (% b/b)
1.6	2.4	54.2
3.0	4.4	53.7
4.6	5.6	66.7
6.2	5.5	57.3
7.7	5.5	51.9

[a]Cultivation time 48 h, temperature 30°C, pH 7.0 and agitation 200 rpm

[b]Polymer content in dried cells of *Erwinia* sp. USMI-20

Figure 1 shows the time course for the production of P(3HB) from 4.6 g/L of palm oil in a batch fermentation method carried out in 10 liter fermentor. Based on the figure, the bacteria was noted to initiate the polymer production under both nitrogen depletion as well as nitrogen limitation conditions. From the profile also observed, while fermentation running, the pH of medium was relatively stable around 6.5 to 7.0. The highest P(3HB) content was achieved at 48 hours of cultivation. At this time, the dry cell weight and P(3HB) content were of 3.6 g/l and 46 wt. % respectively.

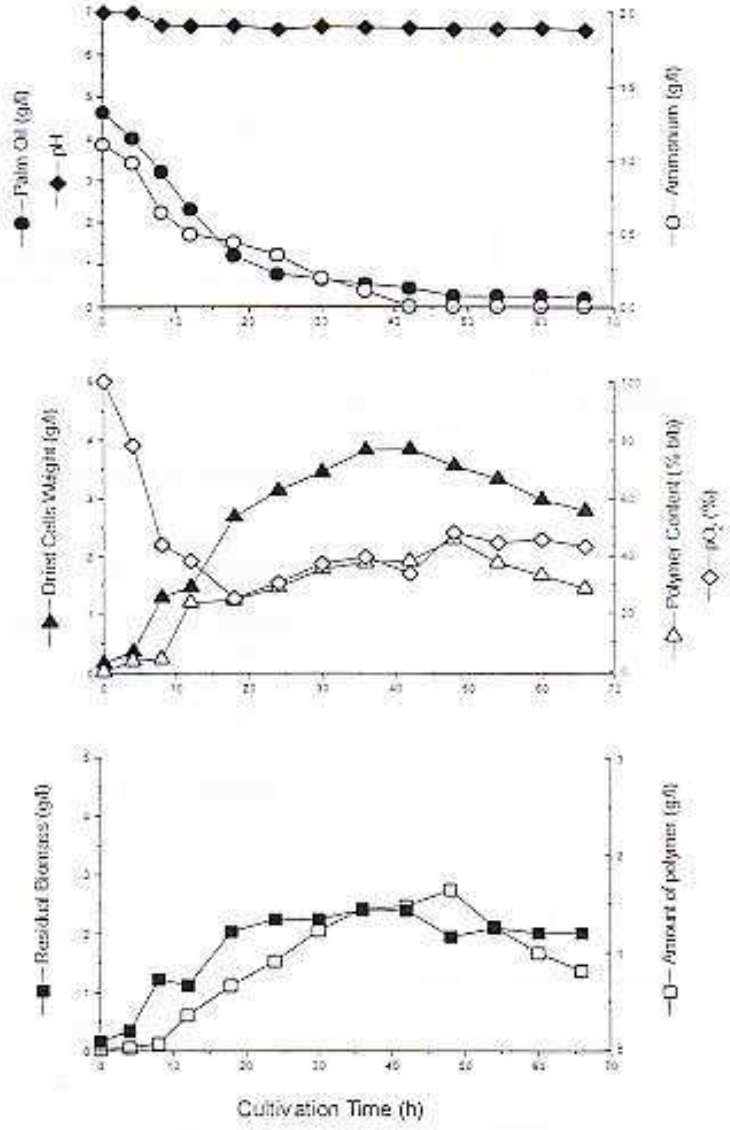

Figure 1. Time course for the batch production of P(3HB) in an 10 liter fermentor from palm oil by *Erwinia* sp. USMI-20.

The polymer was again extracted and characterized by the gas chromatography and ^{1}H and ^{13}C nuclear magnetic spectroscopy techniques (Figure 2 and 3). Through these two techniques with comparison to P(3HB) standard spectra, P(3HB) was confirmed to be synthesized by the microorganism [2,4].

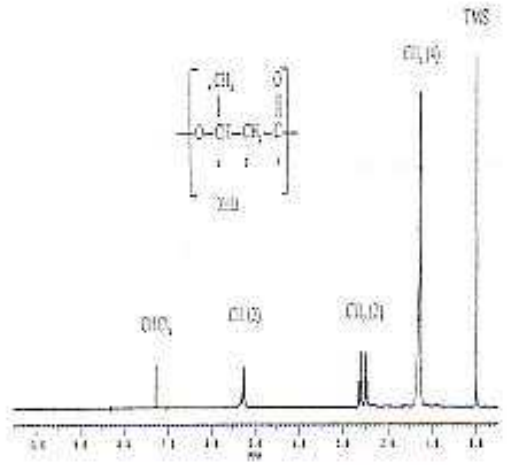

Figure 2. The 270 MHz ^{1}H NMR spectra of extracted polymer from *Erwinia* sp. USMI-20 cells after cultivation indicated that the polymer produced was P(3HB).

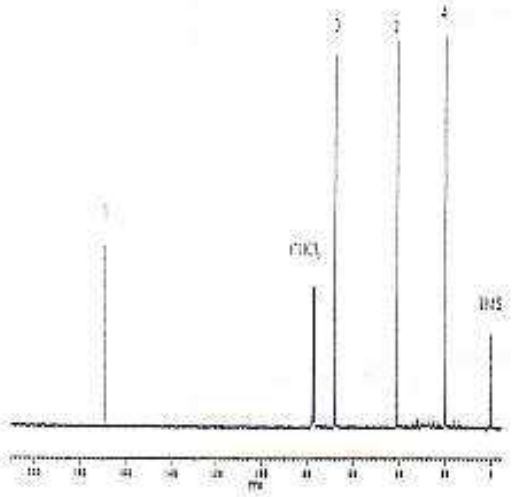

Figure 3. The 270 MHz ^{13}C NMR spectra of extracted polymer from *Erwinia* sp. USMI-20 cells after cultivation indicated that the polymer produced was P(3HB).

Based on this work, the ability of *Erwinia* sp. USMI-20 to produce P(3HB) from palm oil indicates that the bacteria utilize the oil by breaking it down through the β-oxidation cycle to form acetyl-CoA, the starting unit for the polymer production. Once the acetyl-CoA is generated, the normal pathway for the production of P(3HB) as in *Alcaligenes eutrophus* is assumed [1,2]. However for *Erwinia* sp. USMI-20, the polymer production from palm oil occurs in both nitrogen depletion and limitation conditions. Thus, based on the results, *Erwinia* sp. USMI-20 provides the opportunity of investigating the role of β-oxidation in the production of P(3HB) from palm oil. Further investigation is now being studied to observe this at the cellular level. Besides this, the processes for the fermentative P(3HB) are also being developed by fed-batch technique today.

CONCLUSIONS

The *Erwinia* sp. USMI-20 could produced P(3HB) up to 46 weight % of P(3HB) polymer content with a dry cell weight of 3.6 g/L from a batch fermentation in a 10 liter fermentor from an initial concentration of 4.6 g/L of palm oil. The optimum fermentation time to obtain the high level of polymer production in this study was 48 hours.

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