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PRODUCTION AND CHARACTERIZATION OF A BIODEGRADABLE POLYMER POLY(3-HYDROXYBUTYRIC ACID) FROM GLUCOSE USING *Erwinia* sp. USMI-20

PRODUKSI DAN KARAKTERISASI SENYAWA POLIMER POLI(3-HIDROKSI ASAM BUTIRAT) YANG MUDAH DIURAIKAN DARI GLUKOSE DENGAN *Erwinia* sp. USMI-20

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ABSTRACT

The production and characterization of a biodegradable polymer poly(3-hydroxybutyric acid), P(3HB) from glucose as sole carbon source by *Erwinia* sp. USMI-20 has been carried out.

Results showed that *Erwinia* sp. USMI-20 could produced P(3HB) with a maximum polymer content of 48 % of the dry cell weight, an amount of polymer of 2.8 g/l, a dry cell weight of 5.8 g/l, a maximum specific growth rate of 0.21 h^{-1}. a maximum polymer production rate of 0.02/h, yield $Y_{P(3HB)/}$C of 0.21 g/g, with the optimum fermentation time of 48 hours.

The melting temperature (T) and glass transition temperature (T_g) of the P(3HB) were 175 ˚C and 15 ˚C, respectively. The weigh-average molecular weight (M_w) was in the range of 1,000,000 to 1,120,000 D whereas the number-average molecular weight (M_n) was in the range of 420,000 to 580,000 D with the polydispersity index (M_w,M_n) in range of 1.9 to 2.4.

Key word: biodegradable polymer, glucose and *Erwina sp.*

ABSTRAK

Telah dilakukan produksi dan karakterisasi polimer mudah lupus (terurai) poli(asam 3-hidroksibutirat), P(3HB) dari glukosa sebagai sumber karbon tunggal menggunakan mikroba *Erwinia sp.* USMI-20.

Hasil kajian menunjukkan bahwa *Erwinia* sp. USMI-20 dapat memproduksi P(3HB) dengan kandungan polimer tertinggi 48% dari berat selnya, konsentrasi polimer 2,8 g/l, berat kering sel 5,8 g/l, koefisien pertumbuhan spesifik maksimum 0,21/j, laju penghasilan polimer maksimum 0,02/j, koefisien penghasilan polimer terhadap sumber karbon yang digunakan $Y_{P(3HB)/}$C adalah 0.21 g/g, dengan lamanya fermentasi optimum 48 jam.

Polimer (3HB) yang dihasilkan mempunyai suhu leleh T_m) dan suhu peralihan kaca (T_g) berturut-turut adalah 175 ˚C dan 15 ˚C. Purata berat berat-molekul (M_w) polimer yang dihasilkan adalah dalam kisaran 1.000.000 - 1.120.000 D, purata jumlah berat-molekul (M_n) 420.000 - 580.000 D dengan koefisien polidispersiti (M_W/M_n) 1,9 - 2.4

Kata kunci: polimer mudah terurai, glukosa dan *Erwina sp.*

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INTRODUCTION

A wide variety of bacteria synthesized of P(3HB) as an intracellular storage materials, and the P(3HB) accumulates as granules within the cytoplasm (Anderson and Dawes, 1990). The polymer P(3HB) is normally accumulated in response to the limitation of an essential nutrient and much of past work has concerned the control of the synthesis P(3HB) under imbalance growth conditions. This biosynthetic polyester has much received attention as they can be considered to be a source for developing novel biodegradable plastic materials (Doi, 1990). The isolated polymer is partially crystalline thermoplastic with biodegradable and biocompatible properties (Kusaka *et al.* 1997). The pathway and enzymes of P(3HB) synthesis have been studied extensively in *Ralstonia autropha (*Haywood *et al.*, 1988a: 1988b:1989).

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) (Yamane *et al.,* 1996). These polymers have been produced and marketed as Biopol (Zeneca bioProducts). In Japan and USA, Biopol bottles have been commercially available since 1991 and 1992, respectively. Biopol has also been tested for safety helmets, where it could reinforced with high performance knitted cellulosic fibers (Brandl *et al.,* 1995). This composite retains its mechanical properties in a normal outdoor environment, but completely degrades in soil after 40 days. P(3HB) also has potential to be used for pharmaceutical and medical purposes including controlled drug release, surgical sutures (Miller and Williams, 1987) tissue engineering (Williams *et al., 1*999).

In this paper, we report that the biosynthesis of P(3HB) by a soil isolated microorganism identified as *Erwinia sp*. USMI-20 from glucose. Further characterization of the isolated P(3HB) was carried out by using ^{1}H and ^{13}C NMR, DSC, and GPC (Gel permeation chromatograpghy).

METHODS

Microorganism:

Erwinia sp. USMI-20 was used as a strain in the present work. The bacteria was grown on nutrient agar slant at 30 ˚C for 30 hours, maintained at 5 ˚C, and subcultured monthly. The characteristics of this microorganism have been described by Majid *et al.* (Majid *et al*.,1999).

Medium:

Glucose was used as a carbon source for this experiment. The initial pH value of medium was adjusted at 7.0 by the addition of 0.1 N 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 $MgSO_4.7H_2O$ and 1.0 ml of microelement solution. The microelement solution contained 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 N HCl (Majid *et al.,* 1994).

Culture conditions*:*

In a preliminary experiment, the optimal condition for growth and polymer production were investigated by studying the eftect of various concentration of glucose as the sole carbon source on the production of P(3HB). The growth of Microorganism was carried out in a rotary shaker in a 500 ml conical flask containing 100 ml of a defined mineral medium together with the different concentrations of glucose. The concentrations used were 4.8, 9.7, 14.5, and 19.4 g/1. For this study, the cells were incubated for 48 hours at pH 7, temperature of 30 ˚C and agitation of 200 rpm. The production of P(3HB) were characterized based on cell growth, polymer content and amount of polymer.

Further production of P(3HB) by *Erwinia* sp. USMI-20 was carried out in mineral media containing 14.5 g/l glucose as the carbon source. The fermentation was conducted at 30 ˚C, pH 7.0 and agitation of 200

rpm for 66 hours. At regular intervals during the fermentation process, 100 ml of samples were collected, harvested, washed and lyophilized. Polyester were then extracted from the lyophilized cells with hot chloroform in a pressure tube and purified by reprecipitation with methanol.

Analytical procedures :

The polymer granules inside of *Erwinia* sp. USMI-20 was examined by transmission electron microscopy (TEM). 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% sulphuric 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, was added with 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 m 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, column temperature program following a 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 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 (Lee and Yoo, 1994) at the visible maximum absorbtion at wavelength of 625 nm. The colourimetric reaction was exhibited 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%) were added to the mixture. The resulting blue solution was determined against a standard calibration curve of known amounts of nitrogen.

The amount of glucose remaining in the medium was determined by a visible spectrophotometry method through an enzymatic reaction (bio Merieux PAP 1200, France) at the maximum absorbtion at wavelength of 505 nm.

The ^{1}H and ^{13}C NMR analyses of polyester sample was carried out on a Bruker NMR spectrometer. The 270 MHz ^{1}H and ^{13}C NMR spectra were recorded at 27 ˚C in a $CDCl_3$ solution of the polyester (-2 mg/ml). Tetramethylsilane (Me_4Si) was used as an internal chemical shift standard.

The glass transition and melting point of polyester were recorded by using a Perkin Elmer Model DSC-7 equipped with a cooling accessory under a nitrogen flow of 30 ml/min. Polyester sample of 2 mg was encapsulated in aluminium pans and heated at 10 ˚C/min from -10 to 200 ˚C. The melting point were determined from the DSC endotherm. For the measurement of the glass transition temperature, the sample was maintained at 200 ˚C for 1 min and then rapidly quenched to -100 ˚C . It was reheated form 100 to 200 ˚C at a heating rate 10 ˚C/min. The T_g was taken as the midpoint of the heatcapacity change.

Molecular weight data of the polyester sample were obtained by a gel permeation chromatography (GPC) using a Waters Model 600E Multi Solvent Delivery System equipped with a Water 410 Differential Refractometer detector and a PL gel 5μ MIXED column. Chloroform was used as an eluant at a flow rate of 0.8 ml/min. Polystyrene standard with a low polydispersity index were used to generate a calibration curve.

RESULTS AND DISCUSSION

In a preliminary experiment, the influence of the different concentrations of glucose on the growth and polymer production of bacteria were studied by increasing the concentration of glucose from 4.8 g/l to 19.4 g/1 with the concentration of ammonium phosphate fixed at 1.1 g/1. The results of this experiment is shown in Table I.

Figure 1. Time course for the bath production of P(3HB) by *Erwinia* sp. USMI-20[a] from glucose

Tabel I. Effect of different concentrations of glucose on the growth and P(3HB) production *by Erwinia* sp. USMI-20[a].

Glucose concentration (g/l)	Dry cell weigh (g/1)	P(3HB) content (wt. %)	Amount of P(3HB)(g/1)	Residual biomass (g/1)
4.8	5.0	0.7	12.2	4.3
9.7	5.5	1.5	22.3	4.0
14.5	5.9	2.8	46.1	3.1
19.4	5.5	1.7	25.4	3.8

[a]48 hours at 30 °C, pH 7.0, and 200 rpm.

It was found that the maximum amount of P(3HB) production was obtained by using 14.5 g/l glucose with equals to a carbon-nitrogen ratio of 15. On further increment of the concentration of the glucose higher than 14.5 g/1, there was no corresponding increase in the production of P(3HB) and the dry cell weight.

Figure 1 shows the profile of a time course for the production of P(3HB) from 14.5 g/1 glucose as the sole carbon source in a batch fermentation method. As shown in the figure, the lag phase for growth was relatively longer. When the growth of bacteria has occurred, both glucose and nitrogen were consumed simultaneously. It was observed that the average rate of nitrogen consumption was 0.04 g/1/h with the nitrogen source in the medium finished after 36 hours cultivation. Based on the figure, the bacteria was noted to initiate polymer production under both nitrogen depletion as well as nitrogen limitation. 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 5.8 g/1 and 48 wt. %, respectively. It was also determined that the maximum specific growth rate was of 0.21 h^{-1}, a polymer production rate of 0.02/h, and a yield of P(3HB), $Y_{P(3HB)}$/C of 0.21 9/9.

The appearance of the P(3HB) granules in the bacteria when grown in a mineral medium containing glucose as the carbon source is shown in Figure 2. It was found that the number of granules accumulated ranges from 2 to 20 granules per-cell with the diameter of the granules in the range of 100 to 300 nm.

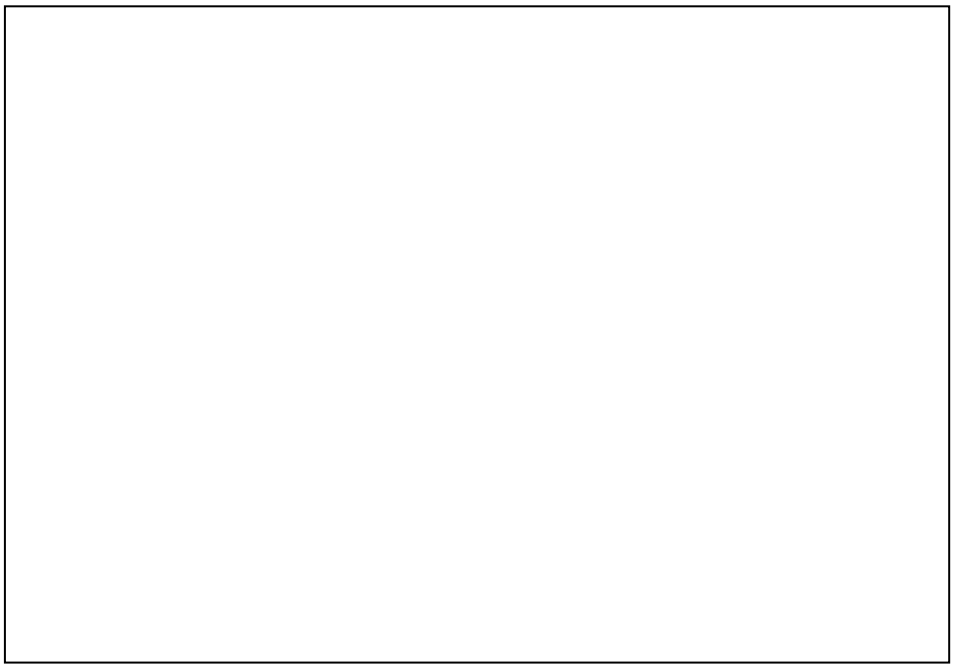

Figure 2**.** Electron micrograph of ultrathin section of *Erwinia* sp. USMI-20 showed the P(3HB) granules (white fraction) after cultivation in mineral medium containing glucose as carbon source.

The polymer produced by, *Erwinia* sp. USMI,-20 after cultivation was again extracted and characterized by ^{1}H and ^{13}C nuclear magnetic resonance techniques as shown in Figure 3 and 4. Through these techniques. P(3HB) homopolymer was confirmed to be synthesized by the microorganism.

Figure 3. 270 MHz ^{1}H NMR spectra of the extracted polymer indicating that the polymer is P(3HB)

Figure 4. 270 MHz ^{13}C NMR spectra of the extraced polymer indicating that the polymer is P(3HB).

The thermal properties T_m and T_g of the P(3HB) extracted were of 157 ˚C and 15 ˚C, respectively. These values were similar to the one reported for *R. eutropha (*Poirier *et al* **.,** 1995) as shown in Table II.

Table II. The melting point (T_m) glass transition temperature (T_g) of P(3HB) produced *by Erwinia* sp. USMI-20 from glucose compared to P(3HB) produced by *R. eutropha*[a].

P(3HB) samples	T_m (°c)	T_g (OC)
P(3 HB) *Erwinia* sp USMI-20.	175	15
P(3HB) R.,,.lropha [b]	175-180	4-15

Determined by DSC. [b] *Data from Doi (1 990) and Poirier el al. (* 1 995).

As shown in Table III, the M_w and M_n, of P(3HB) being produced by *Erwinia sp.* USMI-20 from glucose were in the range of 1,000,000 to 1,120,000 D and 420,000 to 520.000 D, respectively. It was observed that the molecular weight distribution of the polymer produced were relatively broad with the M_w/M_n, in the range of 1.9 to 2.4. For this study, the M_w and M_n, of P(3HB) samples were calculated based on the calibration curve by using Mark-Houwink parameters for both P(3HB) and polystyrene.

Table Ill. Molecular weight measurement of P(3HB) samples produced by *Erwinia sp.* USMI-20 from glucose[a] .

Cultivation time(h)	P(3HB) content (wt. %)	M_w (D)	M_n (D)	M_w/M_n
24	20	1,070,000	490,000	2.2
36	41	1,000,000	420,000	2.4
48	48	1,090,000	590,000	1.9
60	26	1,120,000	520,000	2.2
66	13	1,120,000	520,000	2.1

[a] Determined by GPC.

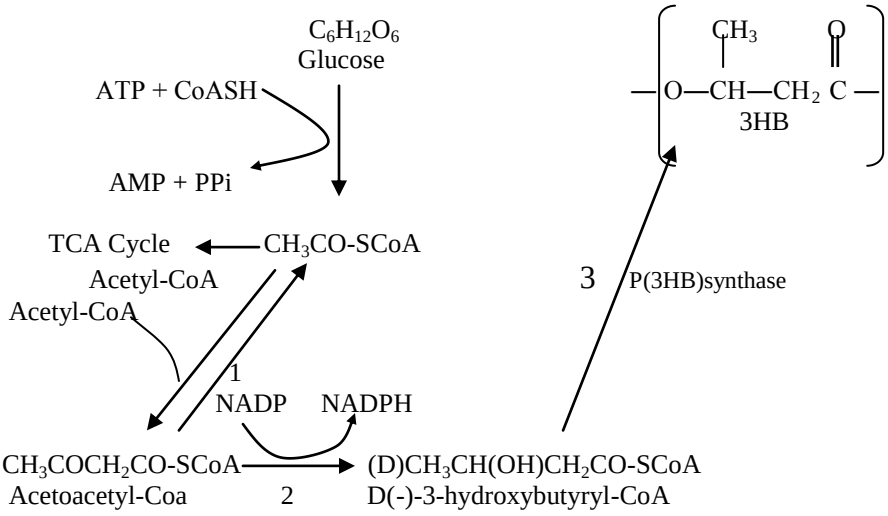

Figure 5. The possible schematic pathway for the production of P(3HB) from glucose by *Erwinia* sp. USMI-20. 1. β-Ketothiolase, 2. Acetoacetyl-CoA reductue and 3. P(3HB) synthase.

Based on this work, the ability of USMI-20 to produce P(3HB) from glucose indicates that the bacteria can utilizes glucose as a sole carbon source. The possible pathway for the production of P(3HB) by *Erwinia* sp. USMI-20 from glucose (Figure 5). Firstly, glucose should be broken down to be acetyl-CoA as the starting unit for the polymer production. Once the acetyl-CoA is generated, the normal pathway for the production of P(3HB) as in *R. eutropha* (Doi, 1990). P(3HB) is through to be synthesized by a sequence of three enzymatic reactions; enzyme 3-ketothiolase catalyses a the reversible condensation reaction of two acetyl-CoA molecules to be acetoacetyl-CoA, an intermediate which is reduced to (*R*)-3-hydroxybutyI-CoA by NADPH-linked acetoacetyl-CoA reductase. P(3HB) is then produced by the polymerization of (*R*)-3-hydroxybutyryl-CoA with P(3HB) synthase. In *R. eutropha*, the genes for all three enzymes have been cloned and analysed, which revealed that a biosynthetic operon containing the *phb-A-phbC* genes coding for the synthasethiolase-reductase, respectively (Steinbuchel, 1996) For *Erwinia* sp. USMI-20, further investigation is now being carried out to observe this at the cellular level.

CONCLUSIONS

The result of the present studies showed that a soil isolated bacteria identified as *Erwinia* sp. USMI-20 can utilize glucose as the sole carbon source to produce P(3HB) homopolymer.

It was found that the maximum polymer content of 48 % of the dry cell weight, an amount of polymer of 2.8 g/1, a dry cell weight of 5.8 g/1, a maximum specific growth rate of 0.21 h^{-1}, a maximum polymer production rate of 0.02/h, $Y_{P(3HB)/C}$ of 0.21 g/1, with the optimum fermentation time of 48 hours.

The T_m and T_g of the P(3HB) were 175 ˚C and 15 ˚C, respectively. The M_w was in the range of 1,000,000 to 1, 1 20,000 Da whereas the M_n was in the range of 420,000 to 580.000 Da with the M_w/M_n in range of 1.9 to 2.4.

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hydroxybutyryl –CoA, Tabel I