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Production and Characterization of Microbial Polyesters Poly (3-Hydroxybutyrat) From Oleic Acid as Sole Carbon Source.

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ABSTRACT

Production and characterization of microbial polyesters poly(3-hydroxybutyrate), P(3HB) from oleic acid as sole carbon source by Erwinia sp. USMI-20 have been carried out. The polymer granules accumulated in the cells of Erwinia sp. USMI-20 upon cultivation were identified by transmission electron microscopy (TEM). P(3HB) was extracted and characterized by using ^{1}H and ^{13}C nuclear magnetic resonance (NMR). The thermal properties including melting temperature (T_m) and glass transition temperature (T_g) for the polymer were analyzed by differential scanning calorimetry (DSC). The weigh-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity index (M_w/M_n) were determined by gel permeation chromatography (GPC). It was found that Erwinia sp. USMI-20 could produced P(3HB) with a maximum polymer content 55 % of the dry cell weight, an amount of polymer 1.9 g/l, a dry cell weight 3.5 g/l, a maximum specific growth rate at 0.30 /h, a maximum polymer production rate at 0.03/h, and $Y_{P(3HB)/C}$ 0.39 g/g , with the optimum fermentation time of 54 hours. The T_m and T_g of P(3HB) were 157 ^{o}C and 15 ^{o}C, respectively. The M_w was in the range of 500,000 to 820,000 Da, whereas the M_n was in the range of 235,000 to 340,000 Da with a M_w/M_n in the range of 2.1 to 2.5.

Keywords: production, characterization, microbial polyester, poly(3-hydroxybutyrate, oleic acid.

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INTRODUCTION

Polyhydroxyalkanoates, P(3HAs), are synthesized by a wide variety of microorganisms as intracellular carbon and energy storage materials [1,2,3]. P(3HAs) were accumulated in the cell under certain environmental conditions, such as nitrogen, oxygen and phosphate-limitation conditions. These biosynthetic polyesters have received attention and become a research interest among researchers, as they can be considered to be a source for developing novel biodegradable plastic materials [4,5,6]. 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) [7]. These polymers have been produced and marketed by Monsanto, who used fed-batch cultivation of Ralstonia eutropha. However, commercial applications of PHA have been limited by their high price (approximately US $ 16/kg).

The important field of investigation is the microbial production of PHAs based on inexpensive raw material or waste materials. Among these available substrates are carbon sources e.g. molasses [8], starch [9], whey from the dairy industry [10,11], surplus glycerol from biodiesel production [10,12], xylose [13,14] and plant oils [15,16,17], sources for nitrogen and phosphate: fish peptone [18], meat extract, casamino acids (CA), corn steep liquor (CSL), soybean hydrolysate and cotton seed hydrolysate [13].

Fats and oils are renewable and inexpensive agriculture co-product, however there have been only a few reports describing the use of fats and oil for PHA production. Pseudomonas strains produce PHA consisting of medium and long-chain-length 3-hydroxyalkanoate units from plant oil [19,20] and tallow [21]. Aeromonas caviae isolated from soil accumulates a random copolyester of 3-hydroxybutyrate and 3-hydroxyhexanoate, P(3HB-co-3HHx), from olive oil [22,23]. In those cases, the productivity of PHA on fats and oils are not high because of relatively low PHA contents in the cells (less than 30 wt. %). However, in another study, the wild type of R. eutropha has been observed to produce P(3HB) to as high as 80 wt. % upon growth on nutrient limitation condition from olive oil, corn oil and palm oil [15].

In a previous paper, we reported that the P(3HB) was produced by a new isolated microorganism identified as Erwinia sp. USMI-20 from palm oil or glucose as sole carbon source [16,17,24] . It was found that Erwinia sp. USMI-20 could produce P(3HB) up to 69 wt. % polymer content with a dry cell weight of 4.4 g/l from an initial amount of 14.5 g/l glucose followed by a feeding rate of glucose at 0.48 g/h. On the contrary, the microorganism can achieve 46 wt. % of P(3HB) and a dry cell weight of 3.6 g/l from a batch fermentation in a 10L fermentor from an initial concentration of of 4.6 g/l of palm oil. We also have reported that the biodegradation testing of the film polymer of P(3HB) produced under the tropical climate environment [17].

In this study, we found that P(3HB) produced by Erwinia sp. USMI-20 from oleic acid, which one of a major free fatty acid in palm oil. Further characterization of the isolated P(3HB) was carried out by using ^{1}H and ^{13}C NMR, DSC, and GPC. In addition, the possible schematic pathway for the P(3HB) microbial synthesis by Erwinia sp. USMI-20 as discussed.

EXPERIMENTAL

Microorganism and medium

Erwinia sp. USMI-20 was used as a strain in this study. The characteristics of this microorganism have been described in previous studies [16, 24]. Oleic acid was used as the carbon sources for this experiment. The 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 [16].

Culture conditions

For the preliminary experiment, the optimal condition of the growth and polymer production were investigated by studying the effect of various concentration of oleic acid as 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 different concentrations of oleic acid: 5, 10, 15, and 20 g/l. as sole carbon source. For this study, the cells were incubated for 48 hours at pH 7, temperature of 30^{o}C and agitation at 200 rpm. The production of P(3HB) were characterized based on cell growth, polymer content, and amount of polymer.

The production of P(3HB) by Erwinia sp. USMI-20 was carried out in mineral media containing 5 g/l oleic acid as the carbon source. The fermentation was conducted at 30^{o} C, pH 7.0 and agitation at 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 re-precipitation with methanol. Further analyses on the samples were done as described in analytical procedures.

Analytical procedures

The polymer granules accumulated in the cells of Erwinia sp. USMI-20 upon cultivation was identified by transmission electron microscopy (TEM). 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% 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, 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 Auto-system 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 the program was run 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 the Berthelot reaction at the maximum visible wavelength of 625 nm. 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 [17].

The amount of remaining oleic acid in the medium was determined by a gas chromatography method after the acid had been extracted with chloroform and converted to their respective methyl esters. Caproic acid methyl ester (CAME) was used as the internal standard. 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 ^{o}C in a $CDCl_3$ solution of polyester (~2 mg/ml). Tetramethylsilane (Me_4Si) was used as the internal chemical shift standard.

The glass transition and melting temperatures 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 ^{o}C/min from -10 to 200 ^{o}C. The melting temperature were determined from the DSC endotherm. For measurement of the glass transition temperature, the sample was maintained at 200 ^{o}C for 1 min and then rapidly quenched at -100 ^{o}C . The sample then was heated form -100 to 200 ^{o}C at heating rate 10 ^{o}C/min. The T_g was taken as the midpoint of the heat-capacity change.

Molecular weight data of the polyester sample were obtained by gel 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 eluent at a flow rate of 0.8 ml/min, and the

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sample concentration used was 1 mg/ml. Polystyrene standard with a low polydispersity index were used to make a calibration curve.

RESULTS AND DISCUSSION

In preliminary experiment, the influence of the different concentration of oleic acid on the growth and polymer production of bacteria was observed by increasing the concentration of oleic acid 5, 10, 15 and 20 g/l with the ammonium phosphate remained fixed at 1.1 g/l. The results of this experiment is shown in Table 1. It was found that the maximum amount of P(3HB) production was obtained by using 5 g/l oleic acid with dry cell weight 4.1 g/l, PHB content 2.0 wt %, amount of P(3HB) 49.5 g/l. Further increase of the concentration of the oleic acid was higher than 5 g/l; no increase of the polymer content produced.

Table 1: Effect of different concentrations of oleic acid in mineral medium on the growth and P(3HB) production by *Erwinia* sp. USMI-20[a].

Oleic acid concentration (g/l)	Dry cell weigh (g/l)	P(3HB) content (wt. %)	Amount of P(3HB) (g/l)	Residual biomass (g/l)
5	4.1	2.0	49	2.1
10	4.4	2.0	46	2.4
15	4.1	1.9	46	2.2
20	4.1	1.9	46	2.2

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

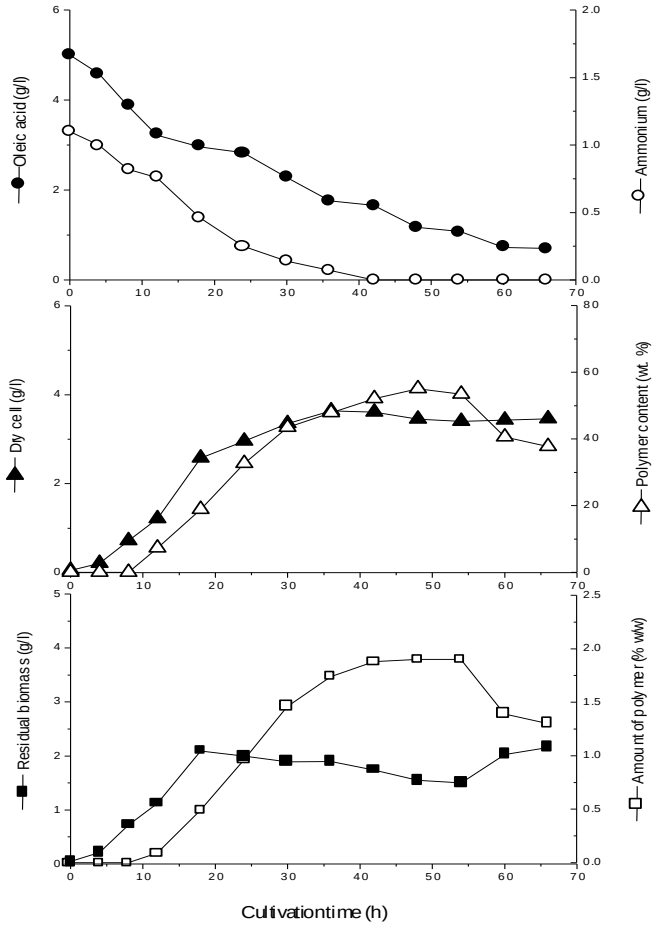

Figure 1: Time course for the batch production of P(3HB) from 5 g/l oleic acid by *Erwinia* sp. USMI-20.

Fig. 1 shows the time course for the production of P(3HB) from 5 g/l oleic acid as sole carbon source in a batch fermentation method. The appearance of the P(3HB) granules in the bacteria when grown in a mineral

medium containing oleic acid as carbon source is shown in Fig. 2. It was found that the number of granules accumulated ranging of 1 to 12 granules per-cell with diameters in the 100 to 350 nm range. The polymer produced was again extracted and characterized by Gas chromatography, ^{1}H and ^{13}C nuclear magnetic resonance techniques as shown in Fig. 3-4. Through these techniques, P(3HB) homo-polymer was confirmed to be synthesized by the microorganism.

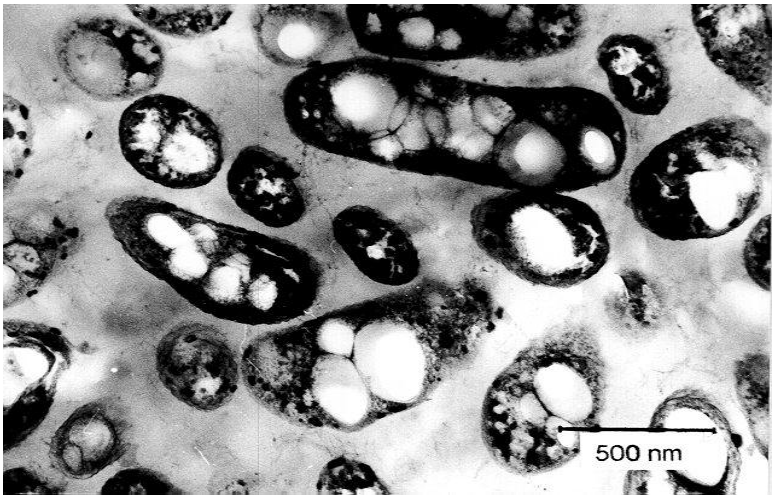

Figure 2: Electron micrograph of ultrathin section of *Erwinia* sp. USMI-20 which shows the P(3HB) granules (white fraction) after cultivation in mineral medium containing oleic acid as a sole carbon source.

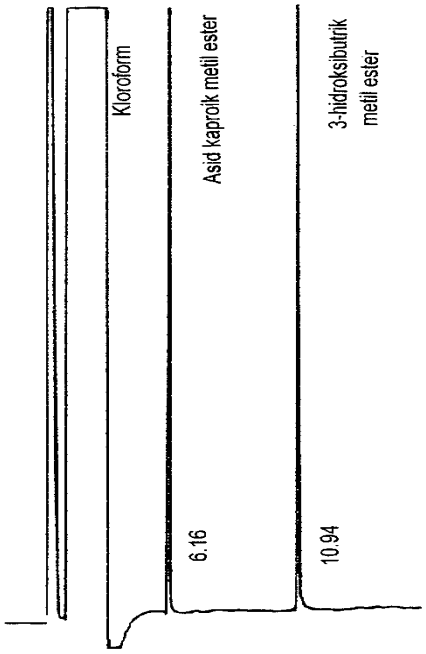

Figure 3: Gas chromatography analysis of the extracted P(3HB) produced from oleic acid by *Erwinia* sp. USMI-20 after conversion to their respective monomeric methyl esters.

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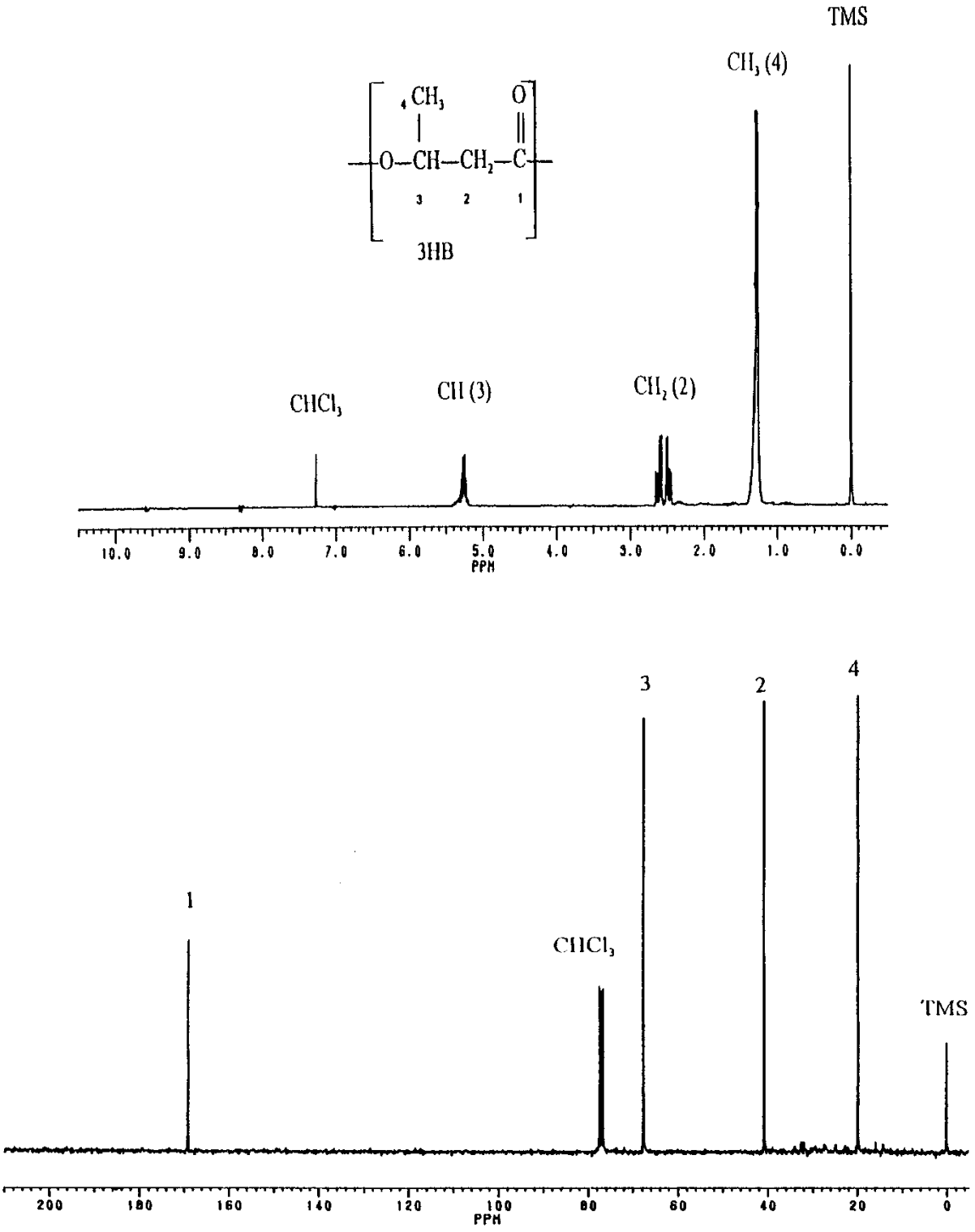

Figure 4: 270 MHz ^{1}H and ^{13}C NMR of the extracted polymer indicating that the polymer produced from oleic acid by Erwinia sp USMI-20 was P(3HB)

The thermal properties including T_m and T_g of the P(3HB) extracted were of 157°C and 15 °C, respectively. The molecular weight (M_w) and molecular number (M_n) of P(3HB) produced by Erwinia sp. USMI-20 and the cultivation time showed in Table 2.

Table 1, showed that the cultivation data of Erwinia sp. USMI-20 in mineral medium containing oleic acid in different concentrations 5, 10, 15 and 20 g/l. The results showed, the optimum concentration of oleic acid to produce content P (3HB) level was 5.0 g/l. At this concentration, oleic acid in the medium produced P (3HB) about 49.5% w / w with the amount P (3HB) about 2.0 g/l and biomass residue was 2.1 g/l. The other concentrations of oleic acid obtained a lower polymer content, which was about 46.0 % w/w.

When the amount of oleic acid 5.0 g/l was transferred to the C / N ratio was about 20, which derived from oleic acid 5.0 g/l and ammonium dihydrogen phosphate 1.1 g/l. Both sources of carbon and nitrogen are two factors that can be manipulated effectively in producing P (3HB) [25]. Meanwhile, other researchers were using carbon sources and methods, but different bacterial species, namely R. eutropha H16 (ATCC 17 699) [15], to obtain impressive results which are obtained polymer content reached 82.0% w / w by weight dry cells. Thus, with further optimization of polymer content produced by Erwinia sp. USMI-20 still can be improved for production coefficient $Y_{P(3HB)/C}$ theory for oleic acid was 1.37 g /g [16].

Growth patterns showed that the use of nitrogen source with a high rate started from four to 36 hours. Average resource utilization of nitrogen in the time interval is 0.03 g / l / h. The source of nitrogen in the medium has been used up to 42 hours of culturing. Overall, during the growth phase of up to 36 hours to culture, the average utilization rate of oleic acid was 0.09 g/l / h. At rapid growth phase, oleic acid was used at the rate of 0:18 g/l/ h. After the fermentation process, which began at the 36th hour and ended at the 66th hour, the average utilization rate of oleic acid was 0.03 g/l/h. At the end of fermentation, oleic acid was detected in the medium with a relatively small amount of 0.7 g/l. In this experiment, the maximum specific growth rate was 0.3/h, which found to occur after 8 hours of culturing. Based on Fig. 1, the bacteria was noted to initiate the polymer production under both nitrogen depletion as well as nitrogen limitation. This situation was different from that shown by R eutropha only accumulate P (3HB) in the cell under nitrogen-limitation [4].

Production of P (3HB) started at the 12th hour culture, which was at 7.2% w / w and continued to the 54th hour and then decreased to the 66th hour of culturing. The highest polymer content that could be achieved was 55.0% w/w with 3.4 g cell dry weight/l and the amount of polymer and biomass residues successively 1.9 g/l and 1.5 g/l respectively. The highest P(3HB) content was achieved at 54 hours of cultivation. At this time, the dry cell weight and P(3HB) content were at 3.5 g/l and 55 wt. %, respectively. It was also observed that the maximum specific growth rate was 0.3/h, polymer production rate of 0.03/h, and yield of P(3HB) accumulated to carbon source used, $Y_{P(3HB)/C}$ was 0.39 g/g.

It was found that the number of granules accumulated ranging between 1 to 12 granules per-cell with diameter in the range of 100 to 350 nm (see Fig.2). For many bacteria, P(3HB) functions either as carbon and/or energy reserve or as a sink for excess reducing equivalents [4]. This confirms that the P(3HB) granules consist of a hydrophobic core of amorphous that is surrounded by a membrane consisting of various catabolic and non-catabolic proteins [26].

Table 2: Molecular weight measurement of P(3HB) samples produced by *Erwinia* sp. USMI-20 from oleic acid[a].

Cultivation time (h)	P(3HB) content (wt. %)	M_w (Da)	M_n (Da)	M_w/M_n
24	32	500,000	240,000	2.1
36	47	740,000	320,000	2.3
48	52	820,000	330,000	2.5
60	55	820,000	340,000	2.4
66	38	720,000	290,000	2.5

[a] determined by GPC.

Table 2 showed the M_w and M_n of P(3HB) produced by Erwinia sp. USMI-20 from oleic acid were in the range of 500,000 to 820,000 Da and 240,000 to 340.000 Da, respectively. These differences that occurred depend strongly on the time of cultivation. It was also observed that the molecular weight distribution of the polymer produced were relative broad with their M_w/M_n were in the range of 2.1 to 2.5. In this study, the M_w and M_n of P(3HB) samples were calculated based on the calibration curve after correction by using Mark-Houwink parameters for both P(3HB) and polystyrene.

Molecular weight of a polymer is a critical feature for commercial applications of bio-plastic products, particularly in terms of the physical and the flexibility of processing. However, there is a significant weakness of the biosynthesis of the bacterial cell polymer compared with polymerization in chemical synthesis strategies, i.e there is no right strategy to control the molecular weight of the product and the ability to modify the structure of the end product. Polymerization processes in the cell are limited by an internal factor resulting molecular weight biopolymers that cannot be increased to a high value. These factors are known as the polymer chain transfer agent. For example, P (3HB) produced by bacteria using methanol as a carbon source has a molecular weight smaller than that using fructose or glucose [27].

In general, an increase in high molecular weight of a polymer, the more elastic properties will be acquired [28]. Various strategies have been used in the production of P (3HA) with high molecular weight. For example, P (3HB) with high molecular weight [ultra-high-molecular-weight P (3HB)] produced by recombinant E. coli bacteria [29], with a weight average molecular weight Mw 3-11 x 10^6 Da. Meanwhile, another study showed by inhibiting the enzyme P (3HA) depolimerase intra-cell genetically, the decomposition of biopolymers in the cell did not happen. A film of P (3HB) with high molecular weight, the elasticity of film can be increased up to 400%.

Based on this work, the ability of Erwinia sp USMI-20 to produce P(3HB) from oleic acid indicates that the bacteria utilizes the free fatty acid 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 R. eutropha is assumed [4]. Two acetyl-CoA molecules would be condensed to form acetoacetyl-CoA and then converted into 3-hydroxybutyryl-CoA before transform by P(3HB) synthase to P(3HB). However for Erwinia spUSMI-20, the polymer production from oleic acid occurs in both nitrogen depletion and limitation conditions. Thus, based on the result, USMI-20 provides the opportunity of investigating the role of β-oxidation in the production of P(3HB). Further investigation is now being carried out to observe this at the cellular level.

CONCLUSION

This study has showed that a soil isolated bacteria identified as Erwinia sp. USMI-20 can utilize oleic acid as sole carbon source to produce a homo polymer P(3HB). It was found that Erwinia sp. USMI-20 produced P(3HB) with a maximum polymer content of 55 % of the dry cell weight, an amount of polymer at 1.9 g/l, a dry cell weight was 3.5 g/l, a maximum specific growth rate was 0.30/h, a maximum polymer production rate at 0.03/h, $Y_{P(3HB)/C}$ was 0.49 g/l, with the optimum fermentation time about 54 hours. The T_m and T_g of the P(3HB) were 157 ^oC and 15 ^oC, respectively. The M_w was in the range of 500,000 to 820,000 Da whereas the M_n was in the range of 235,000 to 340,000 Da with a M_w/M_n in the range of 2.1 to 2.5.

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