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STUDY OF THE THERMAL PROPERTIES AND MOLECULAR MASS OF POLYHYDROXYBUTYRATE PRODUCED BY RHODOCOCCUS EQUI

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ABSTRACT

In the present study, the ability of Rhodococcus equi for polyhydroxyalkanoates (PHA) production was closely observed in the presence of crude palm kernel oil as a carbon source. Gas chromatography analysis proved that the biopolymer was a homopolymer that comprised of 38% polyhydroxybutyrate (PHB). The melting temperature (at 173°C) and glass transition temperature (at 2.79 °C) of PHA was determined using differential scanning calorimetry analysis, and the decomposition temperature (at 276°C) was determined by thermogravimetric analysis. The gel permeation chromatography revealed the average molecular weight ($\overline{M}_w$) to be at 6.42 KD, the number average weight ($\overline{M}_n$) at 373 KD, and the polydispersity ($\overline{M}_w/\overline{M}_n$) to be at 1.91. The present study demonstrated the use of an inexpensive substrate for PHB production by gram-positive bacteria that limited the study previously for the production of PHB polymer, which was analyzed by the polymer's thermal properties and molecular mass.

Keywords: *Rhodococcus Equi, Crude Palm Kernel Oil (CPKO), Polyhydroxybutyrate (PHB), Thermal Properties, Molecular Mass.*

1. INTRODUCTION

Many gram-positive and gram-negative bacteria biosynthesize polyhydroxyalkanoates (PHA) as intracellular carbon and energy storage materials and accumulate them under unbalanced environments such as nitrogen, phosphorous, or oxygen limitation with excess carbon sources [1, 2].

PHA is microbial polyester and is completely biodegradable in nature. It has received a considerable amount of interest from researchers because of its complete biodegradability and material properties that are similar to the conventional plastics [3]. Many types of PHAs with different properties have been reported based on the chemical structure formed by the organisms produced and their growth conditions (Figure 1). With over 150 different PHA monomers being reported, PHAs with flexible thermal and mechanical properties have been developed by utilizing renewable carbon sources [4]. Furthermore, PHAs have many applications in medicine, pharmacy, packages, etc. [5]. However, the most common forms of polymer found in microbial cells are poly-hydroxybutyrate (PHB) and polyhydroxy-valerate (PHV). They can be used in making plastic materials with properties that are similar to petrochemical plastics and can be used to replace these materials in many applications [7].

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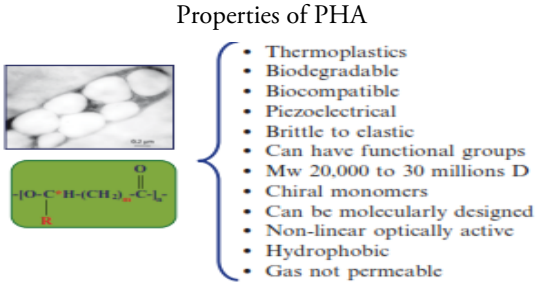

Fig.1. The main properties of PHA [6]

PHB has many valuable properties such as moisture resistance, water insolubility, and optical purity that distinguish it from other commonly available biodegradable plastics, which are either water soluble or moisture sensitive. It also shows good oxygen impermeability [8, 9]. PHB is a highly crystalline thermoplastic polymer with a relatively high melting temperature (170–180°C) and glass transition temperature (0–5°C) [10]. It can improve the thermal properties of PHB by grafting suitable monomers onto it without using the conventional stabilizers or antioxidants that are generally used for stabilizing commodity thermoplastics [11]. In a study, it was found that the thermal stability of PHB can be improved by grafting maleic acid (MA) onto PHB by different methods, resulting in improved crystallization and degradation behaviors of PHB [12]. Another study used pomace extract (EP) to improve the thermal stability and mechanical properties of PHB [13]. In the present study, the thermal properties and the molecular mass of PHB, produced by Rhodococcus equi (R. equi), were closely investigated using crude palm kernel oil (CPKO) as a carbon source.

2. EXPERIMENTAL

2.1. PRODUCTION AND EXTRACTION OF PHB

Rhodococcus equi isolated from fertile soil sampled at Gunung Lang, Perak, Malaysia, was selected for PHB production by using the minimal salts medium as described by Kahar et al. [14]. It was supplemented with a trace element as described by Dio et al. [15] and 1% CPKO as a substrate for production. The biosynthesis experiment was carried out using one-stage cultivation method in Erlenmeyer flasks. The pre-culture was prepared by inoculating three loops full of fresh bacterial cells into 50 ml Tryptic soy broth (TSB) medium and incubated at 30 °C on a rotary shaker at a speed of 200 rpm for 24 hours. Approximately 3% (v/v) of the bacterial broth was used to inoculate the TSB medium in the flask, and the substrates were added separately under sterile conditions. The mixture was incubated for 48 hours at 30 °C under aerobic conditions in a shaker incubator at 200 rpm. The bacterial cells were harvested by centrifugation followed by washing with hexane to remove the residual oil and then with water to remove the remaining hexane and lyophilized. The PHB was extracted by stirring the lyophilized cells for five days in chloroform with a 1 g: 100 ml ratio of cells to chloroform [16]. The stirred cells were filtered using Whatman ® No. 1 filter paper to remove the cell debris and collect the chloroform extract. The filtrate was then concentrated to about 20 ml by using Eyela N-1000 rotary evaporator (Eyela, Japan). The concentrated polymer solution was precipitated by drop-wise addition to vigorously-stirred chilled methanol. The precipitated and purified polymer was later collected and air-dried.

2.2. DETERMINATION OF PHB CONTENT BY GAS CHROMATOGRAPHY (GC)

The PHB content was determined by GC-2010 (Shimadzu, Japan). Approximately 15-25 mg of freeze-dried cells were methanolyzed by adding 2 ml of methanol solution and 2 ml of chloroform. This mixture was then heated at 100 °C for 140 minutes with gentle

shaking for every 30 minutes. The reaction was allowed to cool, and 1 ml of water was added followed by vortexing the mixture for 1 minute so as to allow it to separate to form two layers. The lower chloroform layer was first recovered into a new tube containing anhydrous sodium sulfate to remove the moisture. About 0.5 ml of chloroform solution was mixed with 0.5 ml of caprylate methyl ester in gas chromatography (GC) tubes. The samples were then analyzed by using GC method equipped with a fused silica column and nitrogen as a gas carrier [17]. Sample solutions of converted hydroxyacyl methyl ester (50 µL) were injected at an injector temperature of 270 °C. The monomers of PHA were identified depending on the peak areas at certain retention time in GC in terms of weight (%) of the cell dry weight (CDW).

2.3. Differential Scanning Calorimetry analysis (DSC)

The thermal properties of the polymer were measured by using Pyris 1 (Perkin Elmer, USA) instrument equipped with the cooling accessory. The instrument was first equilibrated at room temperature before cooling at –40 °C and a heating rate of 5 °C/min. The 5 mg of polymer was encapsulated in an aluminum pan and maintained at about –40 °C/min, followed by heating up to 200 °C at a heating rate of 5 °C/min. The second phase of cooling and heating was performed in the same manner except for the heating part, which started from –40 °C to 200 °C. The glass transition temperature (T_g) and melting temperature (T_m) were determined from the DSC thermogram.

2.4. Thermogravimetric analysis (TGA)

The thermal stability of PHB polymer was determined by using STA 6000 instrument (Perkin Elmer, USA). Approximately 10 mg of the polymer sample was loaded in the aluminum pan, and the heating temperature started from 30 °C to 920 °C at a heating rate of 10 °C/min nitrogen atmosphere. This analysis was used to measure the decomposition temperature (T_d), which defined the temperature for 5% weight loss of the polymer sample.

2.5. Gel Permeation Chromatography analysis (GPC)

The molecular weights of the purified polymer were determined by Agilent 1200 gel permeation chromatography (GPC) system that was connected to a refractive index detector with Shodex K-806 (Agilent, United States) columns. Polymers got dissolved in HPLC grade chloroform at a concentration of 1 mg/ml and were later filtered through 0.45 µm PTFE membrane. Chloroform was used as the eluent with a flow rate of 0.8 ml/min at 40 °C. Universal calibration was generated by using a narrow polydispersity polystyrene standard (Agilent, United States).

3. RESULTS AND DISCUSSION

3.1. Gas chromatography (GC)

The composition and the content of biopolymer were quantified by a GC machine. The bacterial strain R. equi had the ability to produce PHB by using CPKO as a carbon source and yield 1.43 (g/l) as dry biomass comprising of up to 38% of PHB. This result was similar to the result of another experiment in which Rhodococcus rubber used fructose as a carbon source [18].

3.2. Thermal properties

The results presented in Figures (2) and (3) show the thermal properties of PHB that were investigated by DSC and TGA analysis. The results listed in Table (1) show that the melting temperature (T_m) of PHB extracted

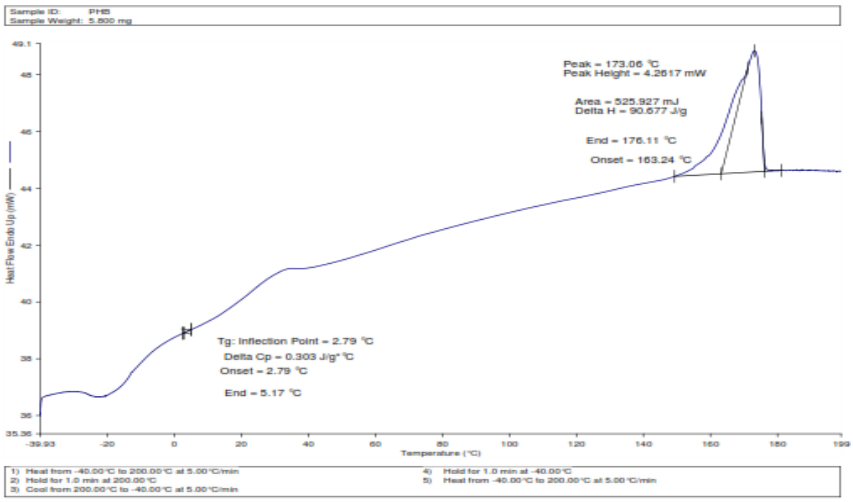

Fig.2. Melting temperature (T_m) and glass transition temperature (T_g) of PHB produced by R. equi using CPKO as carbon source.

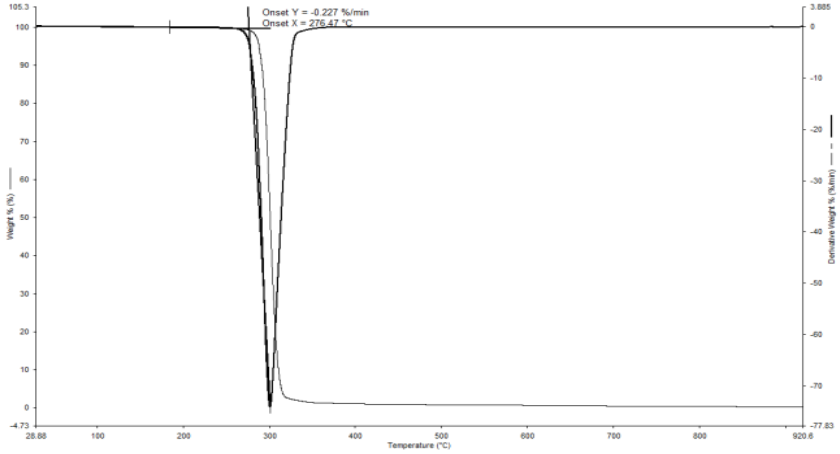

Fig. 3. Decomposition temperature (T_d) of PHB produced by R. equi using CPKO as carbon source.

by using CPKO as a carbon source was 173 °C, which was supported by previous findings [19, 20]. On the other hand, the glass transition temperature (T_g), 2.79 °C, was in the range mentioned in a previous study [21]. The decomposition temperature (T_d) was approximately 276 °C, which was similar to the results of PHB produced by Bacillus sphaericus NII 0838 [22], which is mainly associated with the ester cleavage of the PHB component by ß-elimination reaction [23].

3.3. Molecular Mass

The GPC was used for estimating the molecular mass of PHB sample since the molecular mass is an important factor in determining the physical properties of polymers [24]. GPC was used for determining the average molecular weight ($\overline{M}_w$), number-average molecular weight ($\overline{M}_n$), and polydispersity index ($\overline{M}_w / \overline{M}_n$) of polymer. The results of GPC for PHB of R. equi is listed in Table (2), which also includes the results of other studies. The results collectively indicated that the molecular weight of PHB produced by R. equi is greatly affected by the adverse environmental conditions, type of microbes, nature of carbon sources, and methods of biopolymer extraction; this is in agreement with the findings of Tan and his co-workers [25].

4. CONCLUSION

R. equi is a bacterial candidate that can produce the homopolymer PHB, which is considered as a substitute for petroleum-based plastics. The homopolymer can be used in numerous applications and can be produced by using CPKO as a potential substrate for PHB biosynthesis. The GC analysis evaluating the PHB content revealed that CPKO is rich in carbon content. The thermal properties of PHB polymer produced from R. equi were also characterized and compared with the results of previous studies. The PHB sample obtained from this study was found to have high molecular weight with low polydispersity index value. Thus, it can be utilized by the pharmaceutical industries as a substitute for synthetic polymers in the preparation of biodegradable films.

TABLE 1: THERMAL PROPERTIES OF PHB PRODUCED BY CPKO 1% AS CARBON SOURCE.

Term	Value
T_m	173 °C
T_g	2.79 °C
T_d	276 °C

TABLE 2: THE MOLECULAR WEIGHT OF EXTRACTED PHB IN COMPARISON WITH THE RESULTS OF OTHER REPORTS; WEIGHT AVERAGE ($\overline{M}_w$), NUMBER AVERAGE ($\overline{M}_n$) AND POLYDISPERSITY ($\overline{M}_w / \overline{M}_n$) DETERMINED BY GPC.

Polymer	Producing bacteria	$\overline{M}_w$ (KD)	$\overline{M}_n$ (KD)	$\overline{M}_w / \overline{M}_n$	References
PHB sample	R. equi	642	373	1.91	
PHB (soy)	Cupriavidis necator	790	348.8	2.26	(26)
PHB (molasses and corn steep liquor)	Bacillus megaterium ATCC 6748	3900	2651	1.47	(27)
PHB (pulp industry waste)	Bacillus subtilis MSBN17	640	380	1.68	(28)
PHB-co-PHAV(bagasse extract)	Halomonas campisalis	1394	838.5	1.66	(29)

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دراسة الخصائص الحرارية والكتلة الجزيئية للبولي هايدروكسيبيوتيريت المنتج بواسطة بكتريا الرودوكوكاس ايكوي

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الملخص:

قد لوحظ في الدراسة الحالية قدرة بكتريا الرودوكوكاس ايكوي لإنتاج البوليمر الحيوي بولي هايدروكسيالكونيت بوجود زيت لب النخيل الخام كمصدر كاربوني. وقد اثبتت الكروماتوكرافيا الغازية تركيب البوليمر الحيوي الذي كان من نوع البوليمر المتجانس بوليهايدروكسيبيوتيريت، ومحتوي البوليمر الحيوي المنتج الذي وصل الى 38% . كما تم وصف الخصائص الحرارية للبوليهايدروكسيبيوتيريت والتي تضمنت درجة حرارة الانصهار ودرجة حرارة التحول الحراري والتي بلغت 173 م° و 2.79 م° على التوالي والذي تم قياسها باستخدام تحليل الفرق الكالوري المسح الضوئي، في حين كانت درجة حرارة التحلل 276 م° والتي تم قياسها باستخدام التحليل الحراري الوزني . كما كانت قيمة متوسط الوزن الجزيئي هو 6.42 كيلودالتون، وقيمة عدد وزن متوسط الوزن الجزيئي هو 373 كيلو دالتون، وكانت قيمة التشتت المتعدد هو 1.91 والتي تم قياسهم باستخدام كروماتوكرافيا عبور الهلام. اوضحت الدراسة الحالية استخدام مصدر كاربوني غير مكلف لانتاج البوليهايدروكسيبيوتيريت من بكتريا موجبة لصبغة كرام حيث ان الدراسات المسبقة حول انتاج هذه البكتريا للبوليمر الحيوي محدودة جدا مع دراسة الخواص الحرارية والكتلة الجزيئية للبوليمر المنتج لما لهما من تاثير كبير عند استخدام هذا البوليمر الحيوي في مجالات تطبيقية متعددة.