Production and Characterization of Polyhydroxybutyrate from Molasses and Corn Steep Liquor produced by *Bacillus megaterium* ATCC 6748

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ABSTRACT

The accumulation of polyhydroxybutyrate (PHB) granule in cells of *Bacillus megaterium* ATCC 6748 was significantly depended on the ratio of C-source and N-source in the medium culture. Sugarcane molasses (MOL) and corn steep liquor (CSL) were used as renewable raw materials, since they were rich in carbon and nitrogen respectively, leading to develop a low cost process of PHB production. The highest PHB production was observed after 45h of growth (43% w/w, dry matter) when 4% molasses and 4% CSL were used, whereas the highest biomass (7.2 g l^{-1}) was obtained at 4% molasses and 6% CSL. This indicated that bacterial growth increased as CSL concentration increased, whereas the PHB accumulation decreased. The formation rate of PHB up to 0.016 h^{-1} and specific growth rate up to 0.25 h^{-1} were observed during growth. The chemical structure and thermal properties of PHB produced from molasses and CSL were obtained the same properties as commercial PHB, except for the higher molecular mass (approx. 3.9 x 10^6 Da) and the lower degree of crystallinity (60% X_C). Thus, the present data indicate that molasses and CSL could be alternatively used for PHB production by this bacterium with high PHB content and adequate properties of biopolymer from a low cost process.

Keywords: Polyhydroxybutyrate, *Bacillus megaterium*, molasses, corn steep liquor, Thailand

1. INTRODUCTION

Polyhydroxybutyrate (PHB) is a biopolymer that can be used as a biodegradable thermoplastic material for waste management strategies and biocompatibility in the medical devices (Steinbüchel, 1995; Mona et al., 2001). The viability of microbial large scale production of PHB is dependent on the development of a low cost process that produces biodegradable plastics with properties similar or superior to petrochemical plastics (Doi and Steinbüchel, 2001; Sims, 2003; Apostolis et al., 2006).

The commercial production of PHB has been using relatively cheap substrates such as methanol (Suzuki et al., 1986), beet molasses (Page, 1992 a, b), ethanol (Alderet et al., 1993), starch and whey (Kim, 2000; Ghaly, 2003), cane molasses as a sole carbon source (Mona et. al., 2001), wheat hydrolysate and fungal extract (Apostolis et al., 2006) or soy cake (Fabiane et al., 2007). Various nitrogen-rich media, such as casein hydrolysate, yeast extract, typtone, casamino acids, corn steep liquor and collagen hydrolysate (Lee and Chang, 1995; Bormann et al., 1998; Khanna and Srivastava, 2005), have been used in PHB bioconversions using either *Cupriavidus necator* or recombinant *Escherichia coli* strains.

However, unrefined carbon sources such as corn syrup, cane molasses, beet molasses, or malt extract, also support PHB formation, obtaining yields of PHB comparable to, even better than the refined sugars. Beet molasses and malt extract promoted higher polymer production per

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liter (2.74 and 2.80 g l^{-1}, respectively) due to a growth stimulatory effect (Page and Knosp, 1989).

The metabolic pathway of PHB involved the regulation of its synthesis in the microbial cells (Anderson and Dawes, 1990). PHB was produced by a variety of microorganism under the environmental stresses such as nutrient limitation i.e. nitrogen, phosphorus or oxygen limitation (Steinbüchel and Füchtenbusch, 1998; Thakor, 2003). The microorganism and the strategy of production were affected on duration of fermentation, growth rate, carbon source concentration, etc. Chen and Page (1994) used the same strain of *Azotobactor vinelandii* growing on beet molasses to produce a 4-million Da P(3HB), it was the highest molecular mass so far. The effect of the substrate on the degree of polymerization was investigated on the influence of culture conditions on PHA molecular mass (Gerhart et al., 1998). Koyama and Doi (1995) reported that *Ralstonia eutropha* growing on fructose and pentanoic acid in a chemostat yielded P(3HB-*co*-3HV)s with molecular mass that increased along with the dilution rate of the cultures.

Bacillus megaterium grows in minimal medium without any added growth factors. The majority are mesophiles, with temperature optima between 30 and 45^{o}C (Kenneth, 2005). Indeed, the ability of *B. megaterium* to accumulate PHB is so dominant that the PHB content in the cells could reach up to 32% of the cell dry weight (Hori et al., 2002). PHB provides a reserve of carbon and energy, accumulated as intracellular granules which can be extracted from a wide range of bacteria. The average molecular mass of PHB is also affected on the method of extraction to cause severe damage to the granule, mostly an important loss of molecular mass of the polymer (Nuti et al., 1972; Senior and Dawes, 1973). In addition, the separation condition such as pH, temperature, duration and biomass to aqueous phase could reduce degradation (Berger et al., 1989). Chloroform was also used for recovery of PHB from *R. eutropha* (Hahn et al., 1994).

PHB is a highly crystalline thermoplastic polymer with a relatively high melting temperature (in the range of 170-180^{o}C) and a glass transition temperature in the range of 0-5^{o}C. It undergoes thermal degradation at temperature around the melting temperature (Ha and Cho, 2002; Marand et al., 2000).

This work, in order to study the effect of the ratio of molasses and corn steep liquor (CSL) in the medium on *Bacillus megaterium* growth and PHB accumulation and productivity were estimated by the time during growth. The evaluation of the PHB production from molasses and CSL as a low cost process affects the properties of the biopolymer synthesized by this bacterium, the chemical structure and the thermal properties of polyhydroxybutyrate (PHB) obtained from this fermentation were determined.

2. MATERIALS AND METHODS

2.1. Bacterial Stain and Culture Medium

Bacterial fermentation was carried out using *Bacillus megaterium* ATCC 6748. A lyophilized culture was reactivated at 30^{o}C for 24 h in a growth medium as DSMZ medium (DSMZ catalogue, 1993) containing 20 g l^{-1} fructose, 0.5 g l^{-1} NH_4Cl, 2.3 g l^{-1} KH_2PO, 2.3 g l^{-1} Na_2HPO_4, 0.5 g l^{-1} $MgSO_4$. $7H_2O$, 0.5 g l^{-1} $NaHCO_3$, 0.01 g l^{-1} $CaCl_2$, 0.5 g l^{-1} ferric citrate, and 5 ml trace elements solution. The solution of trace elements contains 0.01 g l^{-1} $ZnSO_4.7H_2O$, 0.003 g l^{-1} $MnCl_2.4H_2O$, 0.003 g l^{-1} H_3BO_4, 0.02 g l^{-1} $CoCl_2.7H_2O$, 0.001 g l^{-1} $CuCl_2.2H_2O$, 0.002 g l^{-1} $NiCl_2.6H_2O$ and 0.003 g l^{-1} $NaMO_4.2H_2O$. The nutrient components

as sugar, nitrogen, phosphate and trace elements were sterilized separately at 121°C for 20 min. After mixing all the ingredients together in a reservoir, the pH of the medium was adjusted to 7.0 using 0.1N NaOH. Bacterial culture grown on the exponential phase stored at 4°C in slope medium agar. However, bacterial cells were inoculated into liquid medium by using a wire loop.

In this study, molasses and corn steep liquor were used, instead of fructose and NH_4Cl in DSMZ medium. Molasses was supplied by the sugarcane industry (Kampangphet, Thailand) and pretreated with activated charcoal (1:1) for 2 h in order to remove colorants. Corn steep liquor was purchased from Sigma (Chemical Co., St. Louis, MO, USA).

2.2 Incubation and Cell Dry Weight Measurement

The batch culture was carried out in 100 ml of the medium volume, in 250 ml flask with 3 bottom baffles and incubated at 30°C for 50 h on a rotary shaker at 130 rpm. Bacterial cells were harvested by centrifugation at 10,000 rpm for 10 min at 10°C (Sorvall Rc-5C Plus, Germany) and the pellets were washed twice with distilled water. The cells were then dried by lyophilization in the freeze dryer (FTS system, USA).

2.3 Isolation of PHB

The dry cells were blended with chloroform using the high speed homogenizer (Ultra-Turrax T25, Canada) at 13,500 rpm for 10 min at room temperature. The supernatant was then filtered through the filter paper (Whatman No.4). The remaining solution was concentrated by rotary evaporator (Labo-Rota 300, Resona Technics, Switzerland). PHB was then precipitated by dropping the viscous solution into 10 volume of 95% ethanol. The white precipitate was formed at the interface of ethanol and then separated by centrifugation at 10,000 rpm for 20 min at 10°C, washed twice with ethanol and then air-dried at room temperature for 24 h.

2.4 Characterization of PHB

The chemical structure and the thermal properties of PHB were used as parameters for qualitative analysis of PHB. The investigations were followings:

Nuclear Magnetic Resonance (NMR) spectroscopy, the polymer was suspended in spectrochem grade deuterochloroform ($CDCl_3$). The ^1H NMR spectra of sample was obtained at 400 MHz using a model Bruker Advance 400 NMR spectrometer (Bruker BioSpin AG, Switzerland). The ^{13}C NMR spectral analysis was performed at 80 MHz. Samples were dissolved in chloroform (1 mg ml^{-1} solvent) that was employed for each analysis. The chemical shift scale was in parts per million (ppm).

Differential scanning calorimetry (DSC) obtained the thermal properties of samples by using a DSC-822 calorimeter (Mettler Toledo, Columbus, OH). The 30 mg sample was used for each thermal analysis in the range of -25°C to 190°C min^{-1} at a heating rate of 10°C min^{-1}. The first cooling was operated from 190°C to -25°C at a cooling rate of 190°C min^{-1} and held at -25°C for 5 min before second heating. The second cooling was operated from 190°C to -25°C at a cooling rate of 10°C min^{-1} (Fabiane et. al., 2007). This thermal analyzer was determined under N_2 flow of 20 ml min^{-1}. The temperature on heating was resulted glass transition temperature (T_g), melting temperature (T_m), and heating crystallization temperature (T_{hc}). The cooling crystallization temperature (T_{cc}) was obtained from the second cooling. The degree of crystallinity (X_C) was determined from the ratio of the melting enthalpy of the

sample (ΔH_m) and the melting enthalpy of pure crystalline PHB (ΔH_m^0 = 146 J/g) (Jianchun et al., 2003; Gogolewski et al., 1993)

Gel Permeation Chromatography (GPC) was used to determine the molecular mass of PHB sample using a GPC system (model 410E, Waters Corp., Milford, MA, USA). Two columns were in series (Waters, Styragel HT3 and HT5, 7.8 x 300 mm) and a refractive index detector, with chloroform as the elution solvent (flow rate, 0.6 ml min^{-1}) at 40°C. PHB dry samples were dissolved in chloroform (0.1% w/v), injection volume was 60 µl. Polystyrene standards dissolved in chloroform (0.1% w/v) were used to construct the calibration curve.

3. RESULTS AND DISCUSSION

3.1 PHB Production in Molasses and CSL Medium

PHB production was carried out in batch fermentation by *Bacillus megaterium* ATCC 6748. Molasses and CSL were employed to provide the desire concentrations of glucose and nitrogen in the fermentation medium. The optimum bacterial growth lead to optimum PHB accumulation is dependent on the composition of the fermentation medium. These medium were investigated two set of batch experiment, firstly using molasses at the level of 1-6% (w/w) and 0.5 g l^{-1} NH_4Cl, the second batch was using of CSL at the level of 0-6% (v/v) in stead of ammonium chloride and 4% (w/w) molasses at the optimum concentration for PHB content from the first batch. Note: 0% (v/v) CSL means using of 0.05% ammonium chloride in the medium.

The course of PHB concentration over time and the specific PHB formation rate are shown in Fig. 1 and 2 that the optimum value obtained when using of 4% (w/w) molasses and 4% (v/v) CSL in the medium. Although, using of ammonium chloride (0% (v/v) CSL) with 4% (w/w) molasses in the medium (Fig. 2) obtained higher specific PHB formation rate at the initial fermentation period but PHB concentration was lower (1.8 fold) at the late exponential phase. The results were calculated basis on the biomass and PHB content of biomass. The specific growth rates up to 0.16 h^{-1} were observed in the two sets of batch experiments. Similar value of μ_{max} (0.15-0.16 h^{-1}) has also been reported by Du et al. (2000). Maximum PHB productivity amounted to 0.056 g l^{-1} h^{-1} at PHB was about 40%.

Both sets of batch experiments (Fig. 3 and 4) were found to increase molasses and CSL concentration led to higher microbial growth and less PHB accumulation. The highest growth (7.2 g l^{-1}) was obtained at 4% (w/w) molasses and 6% (v/v) CSL in the medium (Fig 4). This value was higher than the one (1.27 g l^{-1}) reported by Mona et al. (2001) for batch culture of *B. megaterium* on the medium of 3% cane molasses and ammonium chloride that were observed after 48 h incubation.

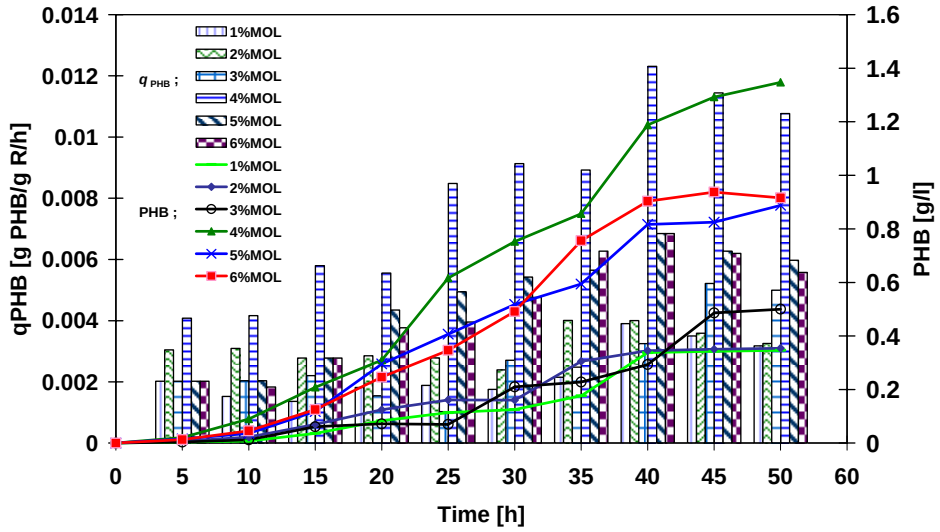

Fig. 1. The formation rate of PHB, q_{PHB} and **PHB** concentration during PHB accumulation (R = biomass – PHB, residual biomass) at the different molasses concentrations [1-6 % (w/w) MOL] and 0.5 g l^{-1} NH_4Cl in the medium.

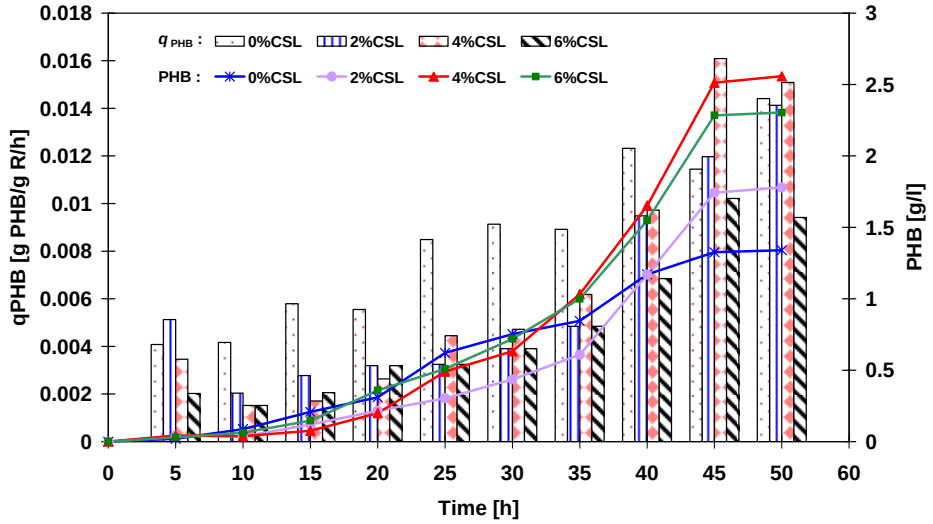

Fig. 2. The formation rate of PHB, q_{PHB} and **PHB** concentration during PHB accumulation (R = biomass – PHB, residual biomass) at the different corn steep liquor concentrations [0-6% (v/v) CSL] and 4% (w/w) molasses in the medium culture. Note: "0% CSL" means using of 0.5 g l^{-1} NH_4Cl in the medium.

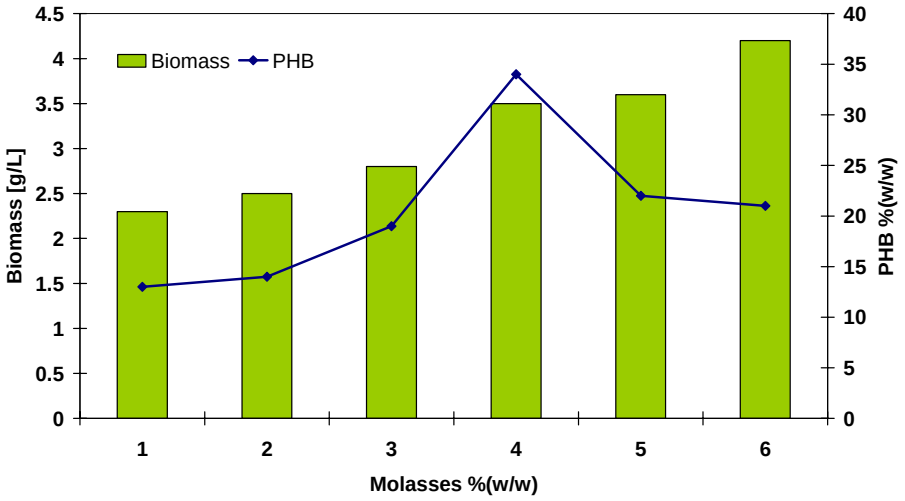

Fig. 3. Biomass and the PHB content of the biomass after 45 h of growth at the different molasses concentrations and 0.5 g l^{-1} NH_4Cl in the medium

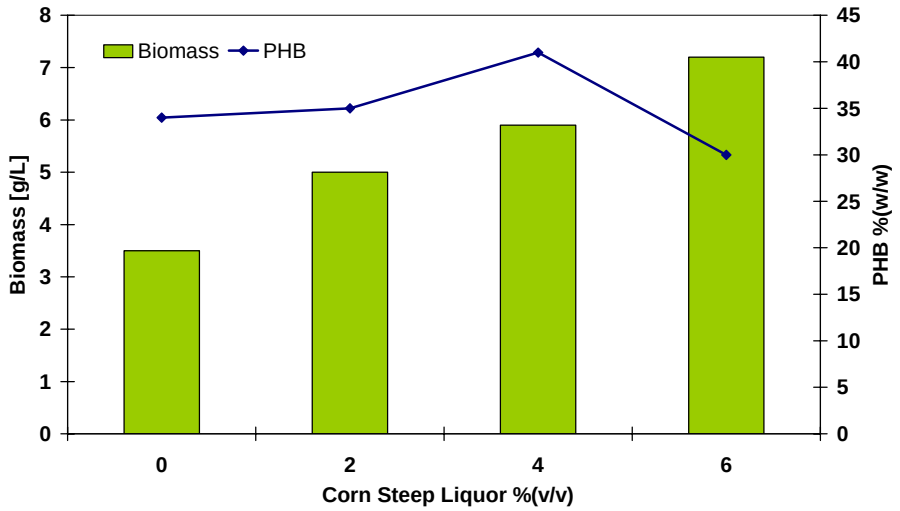

Fig. 4. Biomass and the PHB content of the biomass after 45h of growth at the different corn steep liquor concentrations and 4% molasses in the medium.

The PHB accumulation process of *B. megaterium* obtained PHB up to 43% (w/w, dry matter) when grown in 4% (w/w) molasses and 4% (v/v) CSL in the medium (Fig. 4). The highest PHB amount accumulated at the late exponential phase after 45 h of growth. Mona et al. (2001) reported the PHB production of 46% (w/w, dry matter) when 2% cane molasses were used with *B. megaterium*. However, Beaulieu et al. (1995) reported the production of PHB by *Alcaligenes eutrophus* DSM 545 obtained PHB from 17 up to 26% (w/w, dry matter) varied

among the different ammonium substrate and cane molasses concentrations, while in the first set of batch fermentation (Fig. 3) showed that higher amount of PHB up to 35% (w/w, dry matter) obtained when using of 4% molasses and 0.5 g l^{-1} NH_4Cl.

The high PHB production achieved in this study was shown that molasses and CSL could be used as an alternative carbon and nitrogen source leading to improve the economics of the PHB process.

3.2 Chemical Structure

NMR analysis was used to determine quality of PHB structural composition. The ^{1}H and ^{13}C NMR spectra obtained from PHB samples produced from molasses and CSL, are shown in Fig. 5 and 6, respectively compared with the commercial PHB (Fluka, Sigma-Aldrich Chemicals, USA). Both spectra were found to match perfectly with each other. The peaks observed in the spectra coincide, corresponding to the different types of carbon atoms presented in the PHB structure, $\left[-O-CH-(CH_3)-CH_2-(C=O)-\right]_n$. The chemical shift signals of ^{13}C NMR spectrum obtained in the present work and the commercial PHB were agreed with those obtained by Fabiane et al. (2007) in Table 1.

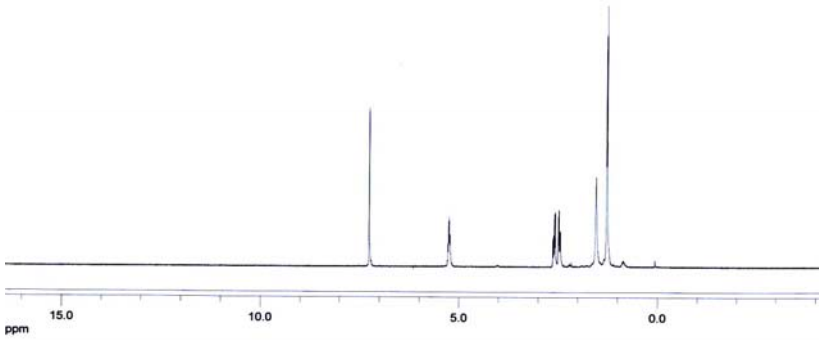

Fig. 5. ^{1}H NMR spectra of PHB produced from *Bacillus megaterium*

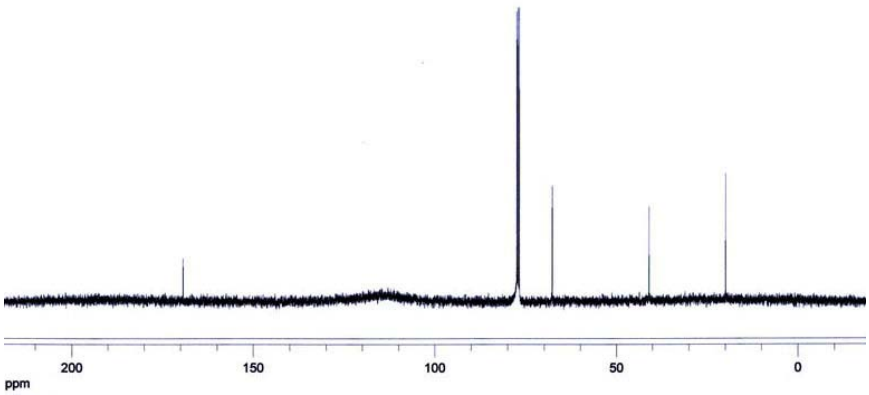

Fig. 6. ^{13}C NMR spectra of PHB produced from *Bacillus megaterium*

Table 1. The chemical shift signals obtained the ^{13}C NMR spectra for PHB sample and commercial PHB, compared to the results by Fabiane et al. (2007)

C atom	Chemical shift (ppm)		
	PHB_{sample}	Commercial PHB	PHB (Fabiane et al., 2007)
CH_3	19.92	19.81	19.65
CH_2	40.96	40.72	40.66
CH	67.77	67.34	67.48
C=O	169.29	169.48	169.03

3.3 Thermal Properties

The thermal properties of PHB samples and commercial PHB were investigated by differential scanning calorimetry (DSC). The data are shown in Table 2, the melting enthalpy led to calculate the degree of crystallinity (X_C), which is an important characteristic of a polymer in order to regulate the mechanical properties of the material (Kong and Hay, 2002). Since the melting temperature of PHB is around 170-180^{o}C (Matko et al., 2005), while that of the PHB sample is in the range (177^{o}C), which is closed to that of polypropylene. Highly crystalline polymers are usually stiff and brittle resulting in very poor mechanical properties with low extension at break (Savenkova et al., 2000), but they were very low resistance to thermal degradation. However, X_C of the PHB sample and the commercial PHB obtained 60% and 62%, respectively (Table 2), similar to the crystallinity of the PHB in the lyophilized cell powder was about 60% (Sei, 1994) and was in the usual range of crystallinity value.

Table 2. Thermal properties of PHB

Sample	T_g (^{o}C)	T_{hc} (^{o}C)	T_m (^{o}C)	T_{cc} (^{o}C)	ΔH_m (J g^{-1})	X_C (%)
PHB_{sample}	-1.0	40.32	177.2	113.8	87.7	60
Commercial PHB	1.01	44.9	172.1	99.4	90.2	62

T_g: glass transition temperature, T_{hc}: crystallization temperature on heating, T_m: melting temperature, T_{cc}: crystallization temperature on cooling, ΔH_m: melting enthalpy of the sample, X_C: degree of crystallinity.

3.4 Molecular Mass

Gel permeation chromatography (GPC) was used to estimate the molecular mass of PHB sample used to, since molecular mass is an important factor to determine physical properties of polymers. However, high molecular weight is a high quality of PHB, and has a less limited industrial application. Chen and Page (1994) reported substrates and culture conditions which affected the molecular weight of PHB, including the method of PHB isolation which cause severe damage of granules and led to loss of molecular mass of polymer (Senior and Dawes, 1973). For example, Fabiane et al. (2007) extracted PHB from bacterial pellets using Soxhlet extractor and treat for 48h with chloroform, their molecular weight was about 5.2x10^5 Da. While, Chen and Page (1994) extracted PHB from the biomass using commercial bleach (30% Na_2CO_3, pH 10.0) at room temperature for 10 min and gained very high molecular weight (approx. 4x10^6 Da). However, PHB in this work were extracted by blending cell with chloroform with high speed homogenizer for 10 min and obtained very high molecular mass (approx. 3.9x10^6 Da) and lower polydispersity as well.

The present data obtained for the PHB sample and other polymers reported in literatures are shown in Table 3.

Table 3. The molecular weight of PHB; weight average ($\overline{M_w}$), number average ($\overline{M_n}$) and polydispersity ($\overline{M_w}/\overline{M_n}$) determined by GPC.

Polymer	$\overline{M_w}$ (kDa)	$\overline{M_n}$ (kDa)	$\overline{M_w}/\overline{M_n}$	Reference
PHB_{sample}	3900	2651	1.47	
Commercial PHB	275	168	1.64	
PHB	4100	3300	1.24	Chen and Page (1994)
PHB_{SOY}	790	348.8	2.26	Fabiane et al. (2007)
PHB	177	91	1.95	Galego et al. (2000)

4. CONCLUSION

The proportion of carbon to nitrogen in the initial medium was affected on amount of bacterial growth and PHB accumulation. In addition, a novel strategy for the maximum production of a biodegradable polymer, polyhydroxybutyrate could be developed, based on the kinetic parameters obtained from batch culture experiments such as the specific growth rate, μ [h^{-1}], the specific PHB formation rate, q_{PHB} [g PHB/g R/h] (R=biomass-PHB, residual biomass) and PHB productivity, r_{PHB} [g PHB/l/h].

In the present work, a low cost of raw material as sugarcane molasses and CSL could improve the economics of the process and obtained high PHB production when 4% (w/w) molasses and 4% (v/v) CSL were used. Thus, substrates from renewable resources with low cost for commercial PHB production was a target to verify the chemical structure and thermal properties of polymers from fermentation processes.

The method of PHB isolation was also influence the quality of polymer. Therefore, bacterial cells were blended with chloroform using high speed homogenizer for a short time to cause lower damage of PHB the molecular weight. The identical PHB sample was verified to commercial PHB and other PHB data that reported in literatures. Thus, sugarcane molasses and CSL could be used as an alternative carbon and nitrogen source, respectively for the PHB production. The obtained PHB were the same thermal properties as commercial PHB with higher molecular mass (approx. 3.9 x 10^6 Da) and lower degree of crystallinity.

6. ACKNOWLEDGEMENTS

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