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Production of poly-β-hydroxybutyrate (PHB) by *Alcaligenes latus* from maple sap

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Abstract Maple sap, an abundant natural product especially in Canada, is rich in sucrose and thus may represent an ideal renewable feedstock for the production of a wide variety of value-added products. In the present study, maple sap or sucrose was employed as a carbon source to *Alcaligenes latus* for the production of poly-β-hydroxybutyrate (PHB). In shake flasks, the biomass obtained from both the sap and sucrose were 4.4±0.5 and 2.9±0.3 g/L, and the PHB contents were 77.6±1.5 and 74.1±2.0%, respectively. Subsequent batch fermentation (10 L sap) resulted in the formation of 4.2±0.3 g/L biomass and a PHB content of 77.0±2.6%. The number average molecular weights of the PHB produced by *A. latus* from maple sap and pure sucrose media were $300\pm66\times10^3$ and $313\pm104\times10^3$ g/mol, respectively. Near-infrared, ^{1}H magnetic resonance imaging (MRI), and ^{13}C-MRI spectra of the microbially produced PHB completely matched those obtained with a reference material of poly[(R)-3-hydroxybutyric acid]. The polymer was found to be optically active with $[\alpha]^{25}_{D}$ equaled to −7.87 in chloroform. The melting point (177.0°C) and enthalpy of fusion (77.2 J/g) of the polymer were also in line with those reported, i.e., 177°C and 81 J/g, respectively.

Keywords Maple sap · Sucrose · Biopolymer · Poly-β-hydroxybutyrate (PHB) · *Alcaligenes latus*

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Introduction

Presently, petroleum is considered as the principal source of most chemical industrial products including polymers and plastics. Nearly $24 billion (US) worth of hydrocarbon feedstocks are used annually in the chemical industry (Gavrilescu and Chisti 2005). Limited future availability, along with record high prices, of fossil fuel has incited various industrial and government agencies to search for renewable feedstocks to replace petroleum as sources of value-added chemicals. Maple sap is one of the most abundant and sucrose-rich renewable feedstocks, especially in Canada (Whitney and Upmeyer 2004). Maple sap has the consistency and clarity of water and contains between 10–30 g/L sucrose with trace amounts of glucose and fructose. It also contains nitrogenous and phenolic compounds, organic acids, and minerals (Morselli and Whalen 1996). These properties make the sap an ideal medium for bacterial growth and the useful biotransformations into other value-added products.

Maple sap has traditionally been used for the production of syrup, the most important nontimber forest product in Canada. Over the last 5 years, Canada has accounted for 84% of the world's production of maple syrup. As far as we are aware, apart from maple syrup and its coproducts, little work has been dedicated to the use of the sap as a renewable feedstock for the production of other chemicals. Woodward and Orr (1998) showed that maple sap has the potential to be converted into hydrogen using enzymes, and Morin et al. (1995) used low-grade maple sap as a raw material for exopolysaccharide production by *Enterobacter agglomerans*.

In the present study, we propose to use maple sap as a starting material for the production of poly-β-hydroxybutyrate (PHB). Recently, biodegradable plastics such as PHB have

received increased attention because of their thermoplastic or elastomeric properties that resemble those of petroleum-based plastics (Steinbuchel et al. 1992). Thus, in addition to being produced biologically, these alternative polymeric materials can be biodegraded into harmless products such as CO_2 and H_2O (Braunegg et al. 2004). To date, such biopolymers have been produced from raw materials such as sugar cane and molasses in Brazil, sugar beets in Europe, and corn in the USA.

In the present study, maple sap was used as the sole carbon source for *Alcaligenes latus* in shake flasks and in batch fermentor (10 L) for the production of PHB. This strain was chosen because it uses sucrose, the main sugar in maple sap, effectively for growth and for PHB production (Palleroni and Palleroni 1978; Wang and Lee 1997; Grothe et al. 1999), and it can be easily lysed to enhance the recovery of PHB (Byrom 1990).

Materials and methods

Preculture preparation

A. latus (ATCC 29714) was acquired from the ATCC collection and maintained on agar slants and Petri dishes on the Mineral Medium 1018 (Grothe et al. 1999). The inocula were prepared in two steps: Several colonies of *A. latus* were used to inoculate a 50-mL Falcon tube containing 5 mL of sterilized nutrient broth medium (BD 234000, Difco) containing 5 g/L peptone and 3 g/L beef extract. After 24 h incubation at 33±1°C with an agitation rate of 200 rpm, the total volume was used to inoculate a 500-mL baffled shake flask containing 100 mL of sterile cultivation medium.

Cultivation medium

A. latus was cultivated in modified medium 3 as described by Grothe et al. (1999). The maple sap was collected from "Érablière Les Frères Beaulieu" (Ormstown, Quebec) and characterized as follows: sucrose (20 g/L), glucose (0.8 g/L), fructose (0.3 g/L), sodium (0.7 mg/L), potassium (68 mg/L), chloride (15.5 mg/L), phosphate (1.8 mg/L), sulfate (31.3 mg/Ll), pH 6.5–7; light yellow color. The maple sap was supplemented with: $(NH_4)_2SO_4$ (1.0 g/L), KH_2PO_4 (1.4 g/L), Na_2HPO_4 (1.8 g/L), $MgSO_4$ (0.2 g/L), and 1 mL/L of the trace element solution as described by Grothe et al (1999).

Fermentation procedure

Shake flask studies

A 5% (v/v) inoculum of the preculture was used to inoculate a 500-mL Erlenmeyer flask containing 100 mL sterilized maple sap or sucrose-based media. The flasks were then incubated at 33±1°C in an orbital shaking incubator (150 rpm) for 27 h. Cell growth was monitored by measuring the absorbance of the fermentation broth at 600 nm (A_{600}).

Fermentor studies

Fermentation was carried out in a 20-L stirred-tank bioreactor containing 10 L sap (Chemap AG, Männedorf, Switzerland) and equipped with accessories and automatic control systems for dissolve oxygen (DO), pH, antifoam, impeller speed, aeration rate, and temperature. The computer program allowed for automatic set-point control and registration of all stated parameters.

Maple sap was added to the fermentor and sterilized in situ at 121°C for 20 min. The reaction medium was subsequently cooled to 33°C and then inoculated (5% v/v) with a preculture of *A. latus*. The aeration and agitation rates were varied to maintain DO above 30% of relative saturation. Foaming during fermentation was controlled using both a mechanical foam breaker and a chemical antifoam agent (0.1 g/L aqueous emulsion of Biospumex 36 K; Cognis Chemical, Cincinnati, OH).

Cell growth was monitored by measuring absorbance at 600 nm (A_{600}). Samples were collected periodically and centrifuged (3,000×*g*) for 10 min. The supernatant was analyzed for sugars and ammonium nitrogen content. The biomass was washed twice with distilled water to remove residual culture medium, frozen, and then lyophilized for subsequent extraction and analysis of PHB.

Analytical procedures

Sugars and ammonia Sucrose, fructose, and glucose were measured using a Waters high-performance liquid chromatography (HPLC) system equipped with a pump (model 600) and auto-sampler (model 717 plus; Waters Chromatography Division, Milford, MA) and an ion-exchange column (Interaction ION-300, 300×7.8 mm, 7 μm; Interaction Chemicals, California, USA). Detection was carried out using a refractive index detector (model 2414). Ammonium was determined using an SP 8100 HPLC system equipped with a Waters 431 conductivity detector and a Hamilton PRP-X200 analytical cation-exchange column (250×4.1 mm, 10 μm; Chromatographic Specialties, Canada).

Biopolymer quantification The homopolymer (PHB) content produced by *A. latus* was determined as described by Comeau et al. (1988). Briefly, dry biomass was treated with acidified methanol in the presence of benzoic acid as an internal standard at 100°C for 3 h to convert 3-hydroxybutyric acid released from the polymer to its corresponding

methyl esters. The methyl esters were extracted in chloroform for subsequent analysis by a gas chromatographic system (Agilent 6890 GC-FID; Agilent Technologies, Wilmington, USA) equipped with a capillary column SPB-1 (15 m×530 μm, 0.15 μm; Agilent J&W GC Columns) connected to a flame ionization detector. The injector and detector temperatures were set at 265 and 275°C, respectively. The oven temperature was set at 50°C for 5 min and then increased at a rate of 30°C/min to 270°C. PHB content (%, w/w dry biomass) was expressed as percentage of polymer weight to lyophilized biomass weight. Poly[(R)-3-hydroxybutyric acid] (Fluka, Buchs, Switzerland) was used as reference standard, which was subjected to the same derivatization procedure described previously.

Biopolymer extraction The cells were first lyophilized and treated with chloroform for 12 h to extract the intracellular PHB. The mixture was filtered, and the filtrate was treated with methanol to precipitate the polymer. PHB was filtered off and dried at room temperature.

PHB characterization

Molecular weight determination The weight average molecular weight (M_w), number average molecular weight (M_n), and polydispersity index (M_w/M_n) of the biopolymers were determined by gel permeation chromatography, using a pump (Waters 510, Canada), an auto-sampler (Waters 715 Ultra WISP, Canada), a differential refractometer detector at 30°C (Waters 410, Canada), and a 60 cm PLgel 5 μL Mixed-C column with a linear range of molecular weight of 200–2,000,000 mol/g at room temperature. The mobile phase was chloroform ($CHCl_3$) with a flow rate of 1 mL/min. The dried PHB granules were dissolved in $CHCl_3$ and filtered through a 0.45-μm polytetrafluoroethylene filter. A 0.06-mL volume of the polymer solution was injected for each sample. Data collection and analysis were done with Waters Millennium software.

Thermal analysis The melting temperature (T_m) and crystallinity of PHB (3–10 mg in encapsulated aluminum pans) were measured using a Perkin Elmer Diamond differential scanning calorimeter (DSC). Each sample was first brought and maintained at −20°C, and then a temperature scan of 10°C/min from −20 to 220°C was made. The sample was maintained at this temperature for 5 min, and then a cooling scan at the same rate was performed from 220 to −20°C. A second run was recorded under the same conditions (isotherm, scanning rate, and temperature range). The first scan provides information on the sample's thermal history and the second, thermal information under the thermal program. In our investigation, T_m and the enthalpy of fusion (ΔH) were determined from the second scanned DSC thermograms. The crystallinity of PHB was estimated from the ΔH obtained by DSC. The fusion enthalpy of a theoretical 100% crystalline sample was assumed to be 146 J/g (Barham et al. 1984).

Thermal stability of PHB was determined by thermogravimetric analysis (TA Instruments TGA 2950) under nitrogen (flow rate of 60 mL/min) and a temperature-scanning rate of 50°C/min.

Spectroscopic measurements ^{1}H magnetic resonance imaging (MRI; at 500 MHz) and ^{13}C-MRI (at 125 MHz) spectra were obtained with Bruker Avance 500 in chloroform-*d* at 45°C at University de Montreal, Montreal, Canada. The near-infrared (IR) spectra of the solid polymer were collected by Nicolet Antaris Infrared Analyzer with white light source and InGaAs 2.6-μm detector. The spectra were recorded in the range of 1,000 to 2,500 nm (10,000 to 4,000 cm^{-1}). Specific rotation [α] was determined in chloroform by JACSO Model P1010 (sodium filter, cell length 50 mm) at 25°C.

Results

Table 1 summarizes the growth of *A. latus*, sucrose consumption, and PHB production in sucrose-based Grothe's medium and in a maple sap-based medium. After 27 h incubation, 2.9 and 4.4 g/L biomass were obtained in

Table 1 Comparison of PHB production between shake flasks and fermentor by *Alcaligenes latus*[a]

Scale	Substrate	Cell concentration (g/L)	PHB content (wt%)[b]	PHB concentration (g/L)[c]	PHB yield (g/g)[d]
Shake flask (100 mL)	Sucrose	2.9±0.3	74.1±2.0	2.15	0.23
	Maple sap	4.4±0.5	77.6±1.5	3.41	0.34
Fermentor (10 L)	Maple sap	4.2±0.3	77.0±2.6	3.26	0.32

[a] The initial sucrose concentration was 20 g/L; cultures were incubated for 27 h. Each value is an average of three determinations.
[b] Percentage of PHB to cell dry weight
[c] Gram PHB per liter of culture
[d] Gram PHB per gram of sucrose consumed

sucrose and maples sap, respectively. The PHB content reached 74.1 and 77.6 wt% in both sucrose and maple sap, respectively (Table 1). The overall PHB yield (gram PHB per gram sugar) was greater with maple sap than with sucrose, i.e., 0.34 and 0.23 g/g, respectively (Table 1).

Subsequently, batch cultures of *A. latus* were investigated and optimized for the production of PHB using maple sap as sole carbon source. Figure 1 summarizes the growth of *A. latus* on the maple sap-based medium and the accumulation of PHB within the cells in a 10-L working volume fermentor. PHB production was initially detected after 6 h of incubation, a period marked by a sharp decrease in ammonium concentration. The depletion of ammonium coincided with the bacteria entering into a stationary growth phase and an accelerated consumption of sucrose. The PHB content increased without increasing residual cell concentration (cell concentration minus PHB concentration), reaching a maximum yield of 77% (w/w) after 27 h of incubation. PHB accumulation was found to be associated with optical density and the consumption of sucrose.

^{13}C-MRI of PHB obtained from maple sap showed the following chemical shifts: 19.71 (CH_3), 40.84 (CH_2), 67.60 (CH), and 169.02 (CO), which were in full agreement with those obtained from a commercially available sample of poly[(R)-3-hydroxybutyric acid] (19.68, 40.83, 67.57, and 168.98, respectively) under the same conditions. Furthermore, the maple produced polymer showed the same near-IR spectrum, scanned between 1,000 and 2,500 nm, as the one obtained using poly[(R)-3-hydroxybutyric acid]. The near-IR spectra of both PHBs showed an almost perfect

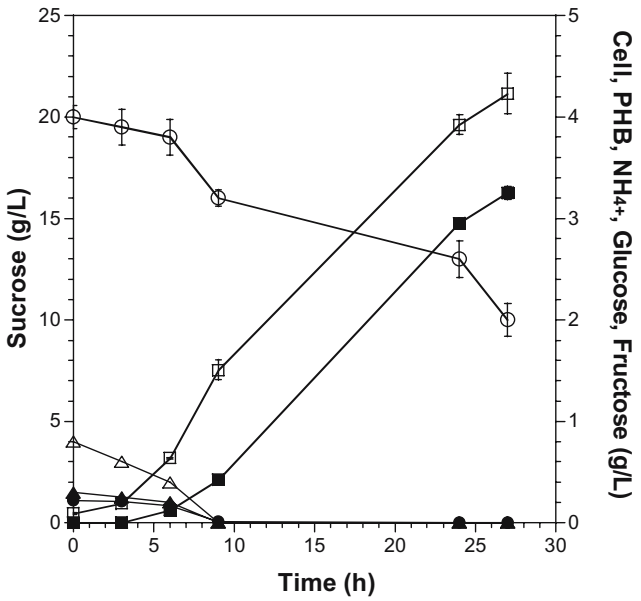

Fig. 1 Batch culture of *Alcaligenes latus* in 20 L fermentor using maple sap as sole carbon source. Symbols: *open circles*, sucrose; *open squares*, cell; *filled squares*, PHB; *filled triangles*, fructose; *open triangles*, glucose; and *filled circles*, ammonium

Table 2 Molecular weight, thermal analysis and specific rotation of purified PHB samples recovered after *A. latus* fermentation on maple sap (in fermentor) and sucrose (in shake flask)

Substrate	M_n[a] × 10^{-3}	M_w/M_n[b]	T_m[c] (°C)	ΔH[d] (J/g)	Crystallinity (%)	$[\alpha]^{25}_D$[e]
Maple sap	300±66	1.69	177.0	77.2	52.9	−7.87
Sucrose	313±104	1.42	176.6	81.0	55.5	n.d.[f]

[a] M_n: Number average molecular weight
[b] M_w/M_n: Polydispersity index
[c] T_m: Melting temperature
[d] ΔH: Enthalpy of fusion
[e] Specific rotation measured in chloroform (°)
[f] Not determined

overlap (96.75% similarity) among various absorptions obtained by their key functional groups –C=O, –C–OH, and –C–H. The polymer was found to be optically active with specific rotation $[\alpha]^{24}_D$ equaled to −7.87 (root square deviation [RSD] 8.7; n=5) as measured in chloroform (Table 2). Likewise, the specific rotation $[\alpha]^{25}_D$ for commercial PHB was −4.91 (RSD 13.9; n=8).

The number average molecular weights of PHB produced from maple sap and pure sucrose media were 300±66×10^3 and 313±104×10^3 g/mol, respectively (Table 2). Table 2 shows that the polydispersity index (weight average molecular weight/number average molecular weight) of PHB obtained from maple sap-grown cells was slightly higher than the values obtained for the polymer from sucrose. Polydispersity values were lower than 2 under all tested conditions, indicative of the uniform formation of PHB within the cell cytoplasm. The melting temperature and enthalpy of fusion of PHB produced from maple sap (177.0°C and 77.2 J/g, respectively) perfectly matched results obtained from sucrose (176.6°C and 81.0 J/g, respectively). Thermogravimetric analyses of the purified PHB sample produced by *A. latus* in sucrose and maple sap are illustrated in Fig. 2. In maple sap, the recovered PHB showed a rapid thermal degradation between 288.0 and 319.4°C with a peak at 308.8°C (Fig. 2a). In sucrose-based media, the recovered PHB showed a rapid degradation between 286.5 and 317.7°C with a peak at 307.9°C (Fig. 2b).

Discussion

The maple sap that was used in this study contained sucrose (20 g/L), traces of several other monosaccharides, and minerals and had a neutral pH (pH 6.5), thereby allowing the sap to be used directly for bacterial growth. *A. latus* exhibits a remarkable capability of utilizing many organic

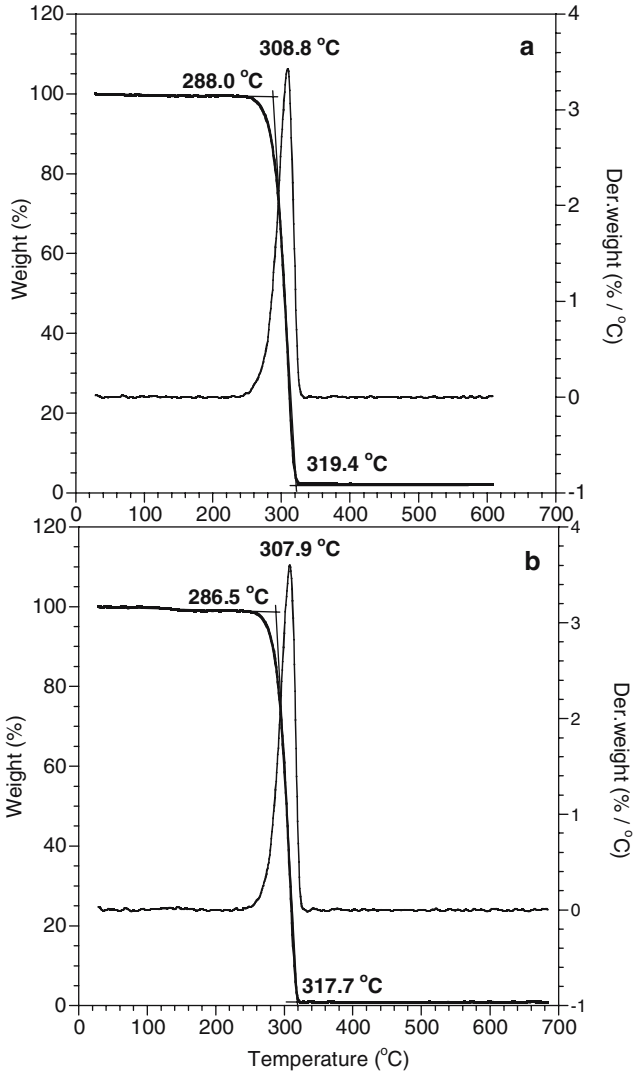

Fig. 2 Thermogravimetric analysis of the purified PHB sample produced in **a** maple sap solution and **b** sucrose

compounds including sugars, acids, alcohol, aromatic compounds, and amino acids as carbon and energy sources for growth and for production and accumulation of polyhydroxy alkanoates (Palleroni and Palleroni 1978). We found that the incubation of maple sap or sucrose with *A. latus* in shake flasks successfully produced PHB with higher amounts being obtained with sap (Table 1). The relative higher biomass obtained with maple sap compared to pure sucrose was attributed to the presence of the previously described organic compounds in the sap, which could be used by as supplementary growth substrates by the bacterium.

After realizing that it was possible to biotransform sugars in maple sap into PHB in shake flasks, we turned our attention to the production of the biopolymer in a batch fermentor with a 10-L working volume. Significant PHB accumulation was observed when the fermentation process was under nitrogen-limited conditions (Fig. 1). The biomass and PHB content obtained in the fermentor were comparable to those found in shake flasks. The batch fermentation results confirmed the operational conditions obtained in the preliminary studies in shake flasks (Table 1), indicating that maple sap could be used as a starting material for large-scale PHB production. However, the yield of PHB and biomass in our case using a batch reactor was inferior to those obtained by Yamane et al. (1996) who employed a fed-batch reactor using sucrose as a substrate.

The identity of the polymer was confirmed by ^{1}H-MRI, ^{13}C-MRI, near-IR, and optical rotation measurements and by comparison with an authentic reference material of poly [(R)-3-hydroxybutyric acid]. The results show that PHB produced from the maple sap was a highly pure and optically active homopolymer with great similarity to the commercially available poly[(R)-3-hydroxybutyric acid].

The molecular mass data of PHB obtained from maple sap corresponded well to those reported by Doi (1990) obtained with *Alcaligenes eutrophus* fermentation in glucose. Whereas Yamane et al. (1996) produced PHB with a M_n of 172×10^3 (M_w/M_n 1.84) in a high-cell-density fed-batch culture of *A. latus* using a sucrose-based medium. Discrepancy between M_n obtained in our study and those of Yamane et al. (1996) could be attributed to the differences in growth conditions, biopolymer extraction, purification techniques, or the purity of samples (Rehm 2003; Yezza et al. 2006). The molecular weight of polymers is believed to be one of the most important factors affecting their physicochemical and mechanical properties and thus their applications. The relatively high molecular weight measured for PHB suggested that the biopolymer had a degree of polymerization suitable for commercial utilization. Cox (1994) reported that the mechanical properties of PHB decrease significantly below a M_w of 400×10^3, and the material is quite brittle below 200×10^3.

The measured melting point and enthalpy of fusion for PHB obtained from the sap (T_m 177.0°C and ΔH 77.2 J/g) were comparable to those obtained using sucrose (T_m 176.6°C and ΔH 81.0 J/g) as the carbon substrate (Table 2). The present thermal properties of PHB were in line with the melting point 177°C and enthalpy of fusion 80 J/g reported for pure PHB homopolymer (Lee et al. 2002). The high enthalpy of fusion of the recovered PHB suggested a high crystalline nature, which was calculated to be 52.9 and 55.5% in maple sap and sucrose, respectively.

Thermogravimetric analyses of PHB produced by *A. latus* in either sucrose or maple sap showed that the polymer in both cases exhibited similar thermal stability with maximized degradation occurring at 307.9 and 308.8°C, respectively, which is above the melting point in each case (Fig. 2). The difference between the decomposition of the polymer and melting temperature of PHB produced in maple sap was high enough to permit processing of the biopolymer.

The high enthalpy of fusion and the relatively high M_w of the recovered PHB from maple sap suggested a high crystalline nature and a degree of polymerization suitable for commercial utilization.

The present work has clearly demonstrated the successful use of maple sap as a carbon source for *A. latus*, which in turn biotransforms excess carbon into PHB, known as a biodegradable plastic. Results obtained from maple sap are reproducible and are comparable to those obtained from pure sucrose. PHB obtained from maple sap exhibited similar properties (chemical, thermal, and spectroscopic) to that obtained from commercial sucrose. The present work may constitute the bases for the development of a process for the production of PHB from maple sap, providing that further reactor optimization is carried out using this renewable feedstock that is abundant in certain northern hemisphere countries such as Canada.

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