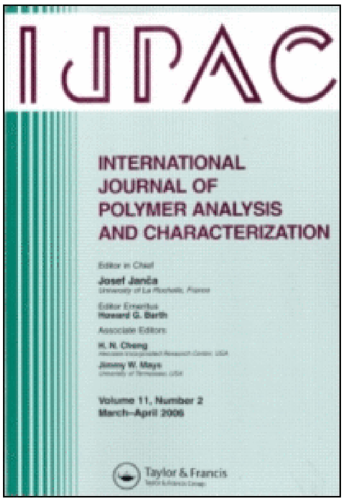

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Synthesis, Characterization, and Structural Properties of Intracellular Copolyester Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) Produced by *Comamonas* sp. EB 172 from Renewable Resource

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SYNTHESIS, CHARACTERIZATION, AND STRUCTURAL PROPERTIES OF INTRACELLULAR COPOLYESTER POLY(3-HYDROXYBUTYRATE-CO-3-HYDROXYVALERATE) PRODUCED BY *COMAMONAS* sp. EB 172 FROM RENEWABLE RESOURCE

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Microbial copolymer was produced by a local isolate,* Comamonas *sp. EB 172, using mixed organic acids such as acetic, propionic, and butyric acids as carbon sources in pH-stat fed-batch fermentation. Maximum polymer production (6.59 g/L) was achieved at 50 h of fermentation when 73.64 g/L mixed acids, generated from the acidogenic fermentation of palm oil mill wastewater, were used. Accumulation of polymer in the cell was 70% (wt/wt), which was observed under transmission electron microscope. The morphological, chemical, thermal, and mechanical properties of the solvent-extracted biopolymer were determined by various techniques (SEM, GC, ^{13}C NMR, FT-IR, TGA, and tensile testing). The copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) consisted of 87 mol% β-hydroxybutyric acid (HB) and 13 mol% β-hydroxyvaleric acid (HV). With chemical properties similar to commercial PHBV and mechanical strength of around 30 MPa and 8% elongation at break, the biopolymer offers potential for industrial applications.

Keywords: *Comamonas* sp. EB 172: characterization; Fed-batch fermentation; Mixed organic acids; PHBV

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INTRODUCTION

Accumulation of polyhydroxyalkanoates (PHAs) in bacteria, a natural biodegradable and biocompatible thermoplastic polymer, has been well documented throughout the years since its discovery in 1925 by Lemoigne. The polymer composition largely depends on the type of bacteria as well as the carbon sources used in the fermentation process. Thus, the PHA family represents an interesting group of biodegradable polymers that can be produced from renewable resources and have various applications, e.g., in packaging, agriculture, marine, and medical fields. Among the 150 different types of PHA produced in bacteria, homopolymer P(3HB) is the most studied PHA up to now. However, since it possesses comparatively high melting (T_m) and glass transition temperatures (T_g), as well as being both stiffer and more brittle than synthetic plastics, such as polypropylene, it is not well suited for thermoplastic applications.[1]

In order to improve the processability of PHB, many research efforts have been focused on the chemical modification of PHB by blending or co-grafting with other chemicals.[2–4] In contrast, the incorporation of 3-hydroxyvalerate (3HV, or abbreviated as V) monomer units into PHB polymer chains by feeding the bacteria with either propionic or valeric acid can dramatically change the properties and makes the polymer suitable for industrial use. The physical and thermal properties of PHBV copolymers can also be regulated by varying molecular structure, average comonomer composition, and comonomer composition distribution.[5] With increasing concentrations of HV units from 0 to 25%, the mechanical properties such as flexibility and toughness are greatly improved,[6] while a decrease in T_m and T_g offers good processability of the polymer.[7] However, the wide application of polyhydroxyalkanoates is restricted by the high production cost, compared to other petrochemical-derived plastics. Therefore, in order to use these polymers as commodity plastics, the cost has to be sufficiently lowered without affecting the useful properties of the original polymer.

Feeding bacteria with renewable resources such as mixed organic acids derived from the acidogenic fermentation of palm oil mill waste effluent provides a solution to this problem. The process integration approach towards "zero discharge" in palm oil industries in Malaysia as proposed by our research group can be well adapted to any industry.[8] While our earlier investigations were concerned with the suitability of POME-derived mixed organic acids as a substrate for PHA production[9,10] and the optimization of substrate generation;[11–13] currently we are trying to develop the fermentation process using locally isolated bacteria[14,15] and focusing on product characterization in order to address the market demand.[16] This article describes the microbial synthesis of the copolymer PHBV and examines morphological, chemical, and mechanical behavior of the solvent extracted copolymer by SEM, GC, FT-IR, NMR, and tensile testing.

MATERIALS AND METHODS

Materials

Comamonas sp. EB 172, a local PHA producer, isolated from an anaerobic digester treating palm oil mill effluent (POME), was used throughout this study.[14]

The culture was maintained on 20% glycerol stock in nutrient broth and was kept at −30°C. The carbon source used during copolymer production was composed of 34.50 g/L acetic acid, 15.75 g/L propionic acid, and 23.39 g/L n-butyric acid. This acid mixture is one of the several batches obtained during the acid recovery process in pilot scale.[17]

Biosynthesis of P(3HB-Co-3HV)

Comamonas sp. EB 172 was first cultivated overnight (12 h) in a 250 mL conical flask containing 50 mL nutrient-rich medium (8 g/L nutrient broth and 1 g/L yeast extract) at 30°C, pH 7.0, and 200 rpm. Then 10% v/v seed cultures were transferred into a 2 L fermenter (BIOSTAT A Plus, Germany) containing nutrient-rich media for cell mass production. Initially, pH of the growth medium, airflow rate, and agitation speed in the fermenter were 7.0, 1.0 vvm, and 200 rpm, respectively. pH was maintained at 7.5, and dissolved oxygen was controlled at 40% by regulating the stirrer speed by sequential cascade control. After 12 h of cultivation, mineral medium supplemented with 5 g/L sodium acetate was added to initiate polymer accumulation. The compositions of the mineral medium and trace elemental solution were the same as described by Hassan et al.[10] except that yeast extract was not added. pH stat feeding with the mixture of acids was started at 13 h and carried out over a 50 h period.

Analyses

Cell growth was monitored by measuring the optical density (OD) at 600 nm, and the reading was converted to the biomass by an appropriate calibration curve. PHB concentrations in the cell and organic acid in the solution were determined as described earlier.[9] The content of hydroxyvalerate comonomer units (HV) in the individual copolyesters was determined by gas chromatography using benzoic acid as an internal standard.[18] Standard PHBV with 8% HV used in this study for comparison was obtained from Aldrich Chemical Co. (USA).

Polymer Extraction and Precipitation

After cultivation, cells of *Comamonas* sp. EB 172 were separated by centrifugation (14,536 g, 10 min) and were washed with distilled water. After lyophilization, intracellular PHA copolymers were extracted by hot choloroform extraction at 50°C for 1 h, followed by filtration through a Whatman no. 1 paper filter to remove the debris. Polymer dissolved in chloroform was precipitated with twofold chilled hexane and was evaporated using a rotary evaporator at 55°C.[19] The collected polymer was dried at room temperature for 24 h.

Polymer Characterization

Polymer morphology. PHA granules obtained by solvent extraction were observed by using a JEOL JSM scanning electron microscope (SEM) for particle morphology. Powders were stuck onto the stub using double-sided tape and gold-coated in a sputter coater before viewing. Solution-cast film prepared for tensile testing was cut into 1 cm^2 shapes, fixed onto the stub, and viewed under SEM.

NMR. The structure and mole fractions of HB and HV units in the copolyester sample were investigated by the 100 MHz ^{1}H nuclear magnetic resonance (NMR) spectra recorded at 27°C on a $CDCl_3$ solution of polyester (5 mg/mL). Peak areas were determined by spectrometer integration by using tetramethylsilane (TMS) as an internal reference.

FT-IR analysis. The infrared spectra of the samples were recorded in the wave number range from 250 to 4000 cm^{-1} using a PerkinElmer Fourier transform-infrared (FT-IR) spectrophotometer (model 1725X) using a KBr disc.

Thermogravimetric analysis. Thermogravimetric analysis (TGA) was performed on a Mettler instrument. The temperature was ramped at a heating rate of 10°C/min in a nitrogen atmosphere to a temperature well above the degradation temperature of the polymers (400°C).

Mechanical property test. The copolyester films were prepared by conventional solvent-cast techniques from chloroform solutions of polyesters at 1% (w/v) using a glass petri plate (Pyrex) as the casting surface. Films were cut into dog-bone shapes (20 mm span length and 3 mm width) using Rey-Ran test equipment. The mechanical property test was carried out at ambient temperature using a universal testing machine (Instron 5583; Instron, Norwood, Mass., USA) with a crosshead speed of 10 mm/min and 10 N load cell and accessorized with a 486 DX33 IBM-compatible personal computer. The mechanical properties tested were elongation (%), tensile strength (MPa), and Young's modulus (MPa).

RESULTS AND DISCUSSION

Biosynthesis of P(3HB-co-3HV)

Figure 1 shows the typical growth curve of *Comamonas* sp. EB 172 in a nutrient-rich medium. In a 2 L bioreactor, growth phase continued until the late log phase (12 h) at 30°C and pH 7.5 under aerobic conditions and was switched to

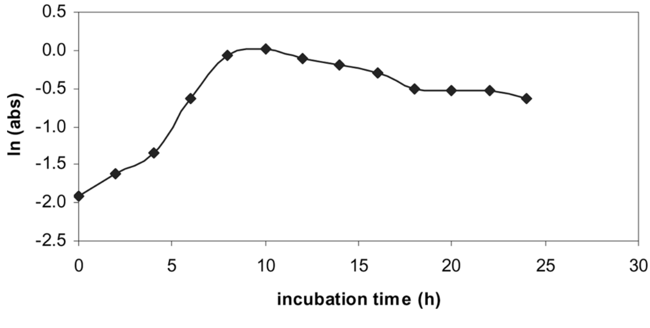
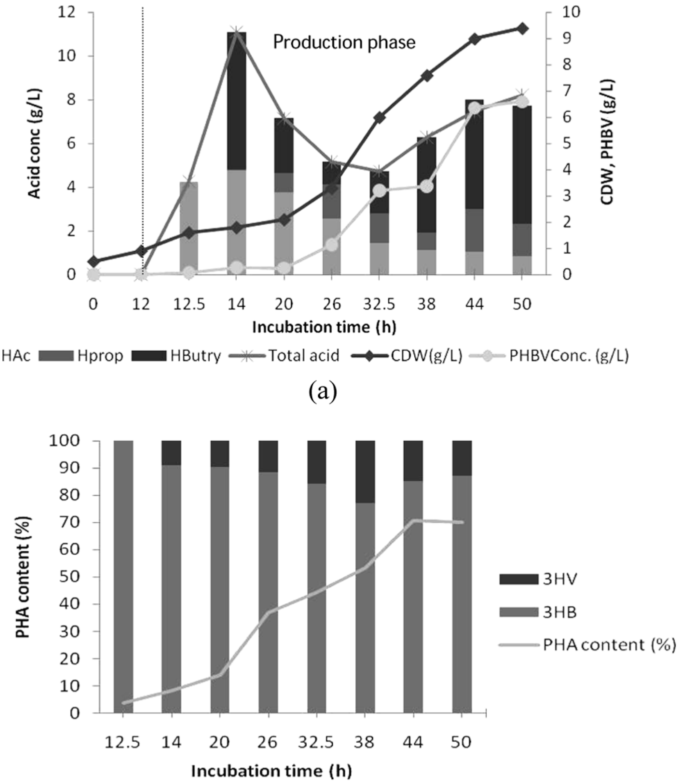


Figure 1. Natural logarithm of absorbance (cell mass) vs. incubation time; a typical growth curve of *Comamonas* sp. EB 172 in nutrient-rich medium.

accumulation phase by adding nitrogen-free mineral media with sodium acetate as the sole source of carbon.

The feeding of mixed organic acids was initiated at 13 h by controlling pH at 7.0. Figure 2 shows the fermentation profile of PHBV biosynthesis in *Comamonas* sp. EB 172. Throughout the feeding period, the concentration of residual acid varied between 4 and 11 g/L. Acetic acid was consumed faster than propionic acid followed by n-butyric acid. The cell mass increased nearly twentyfold during the fermentation (Figure 2(a)). Little or no accumulation was observed during growth phase, and the copolyester content in the cells increased from around 4% to a maximum of 70% at 44 h (Figure 2(b)).

The composition of the copolymer was influenced by the concentration of mixed acids in the culture. 3HV fraction in the copolymer increased as the concentration of propionic acid in the broth decreased. Figure 3 shows gas chromatograms of standard PHBV and the copolymer biosynthesized by *Comamonas* sp. EB 172. GC analysis of dried cells revealed homopolymer of HB produced initially and HV content increased from 9 to 23% during fermentation (Figure 3(b)). This HV content



(a)

(b)

Figure 2. Biosynthesis of P(3HB-co-3HV) copolymer by *Comamonas* sp. EB 172 from mixtures of organic acids: (a) fermentation profile of cell mass, PHBV concentration, and residual acid concentration (g/L) and (b) PHA composition and percentage content throughout the fermentation. Values are average of two independent experiments.

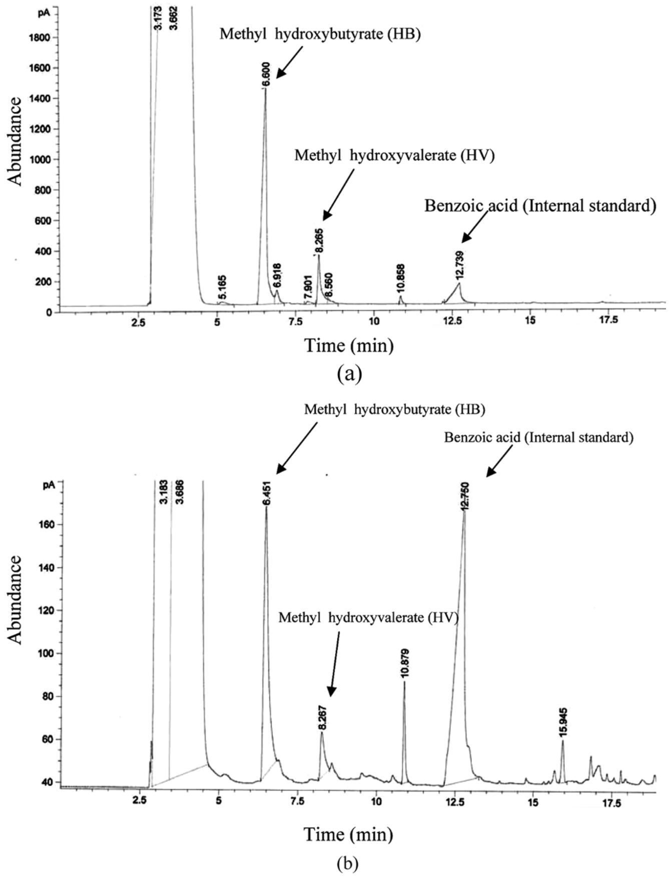


Figure 3. Gas chromatogram of (a) commercial PHBV from Aldrich with 8% HV content and (b) the copolymer P(87% HB-*co*-13% HV) obtained from *Comamonas* sp. EB 172.

may be the maximum as more of HB unit formed from the major fraction of the substrate (acetic and butyric acids). However, after 38 h, more HB was incorporated and HV content reduced to its final level of 13%. The PHA accumulation was further confirmed with phase contrast and transmission electron microscopy (Figure 4).

Polymer Morphology

Scanning electron microscopy (SEM) was used to compare the physical characteristics of the standard polymer with those of the biosynthesized copolymer. Significantly different particle morphology was observed. The standard PHB homopolymer and PHBV copolymer appeared as spherical particles with uniform diameter (Figure 5(a), 5(b)) whereas a flaky network structure was observed for our polymer (Figure 5(c)). The difference could be due to the extraction process employed. The surface topography of PHBV film was also examined using the micrograph (Figure 5(d)). Under SEM, the surface of PHBV film appeared smooth and homogenous.

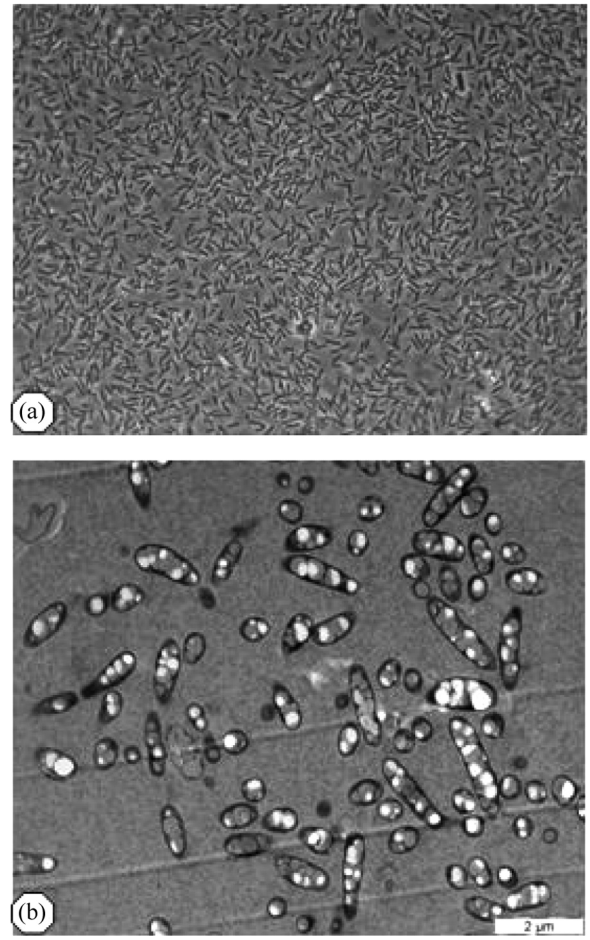


Figure 4. Cellular morphology of *Comamonas* sp. EB 172 under (a) phase contrast microscope (100×) and (b) transmission electron microscope showing refractile PHA granules (9,000×).

NMR

Figure 6 shows the ^{13}C NMR spectra of copolyester obtained from *Comamonas* sp. EB 172. The chemical shifts of the major signals assigned to the different types of carbon atoms in the PHBV structure (C=O, CH, CH_2 and CH_3) appear at almost identical chemical shifts and integration values, indicating that it is very similar in chemical composition to commercial PHBV (8% PHV) from Aldrich (USA) (spectra not shown). Furthermore, the spectra obtained in this work agree with those obtained by Doi[20] in a copolyester with 19% HV in *Alcaligenes eutrophus* (Table I).

FT-IR

The FT-IR spectra obtained for the sample confirmed the identical structure of the commercial PHBV and the biopolymer produced by *Comamonas* sp. EB 172

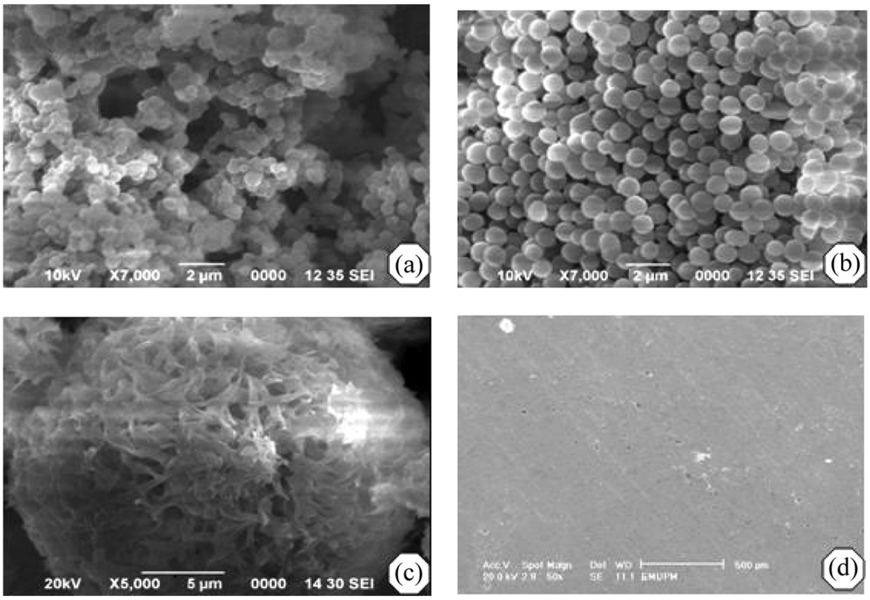


Figure 5. Scanning electron micrographs of (a) commercial PHB and (b) PHBV powder from Aldrich and PHBV produced by *Comamonas* sp. EB 172: (c) solvent-extracted polymer and (d) solution-cast film for mechanical testing.

(Figure 7). The band around 1384 cm^{-1} corresponds to the symmetrical wagging of CH_3 groups, while the band found at 1462 cm^{-1} corresponds to the asymmetrical deformation of the C–H bond in CH_2 groups, both of which are insensitive to crystallinity.[22] The band found at 1730 cm^{-1} was the C=O stretch of the ester group

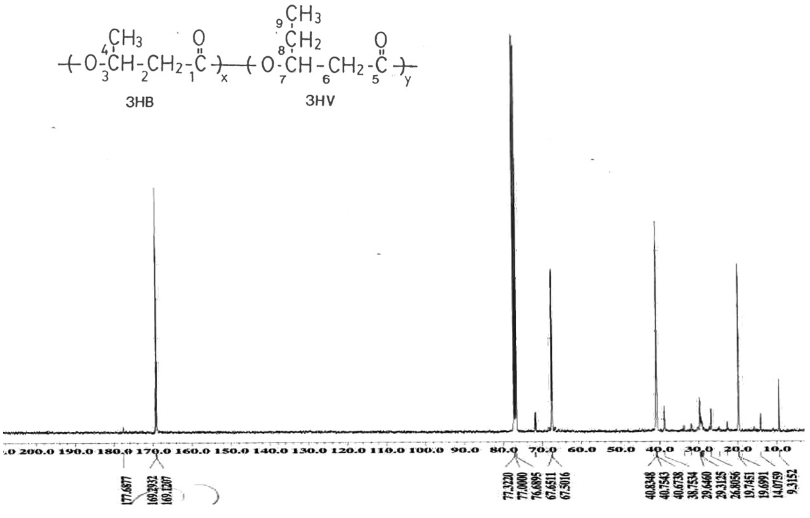


Figure 6. ^{13}C NMR of PHBV sample biosynthesized by *Comamonas* sp. EB 172. Deuterated chloroform was used as solvent.

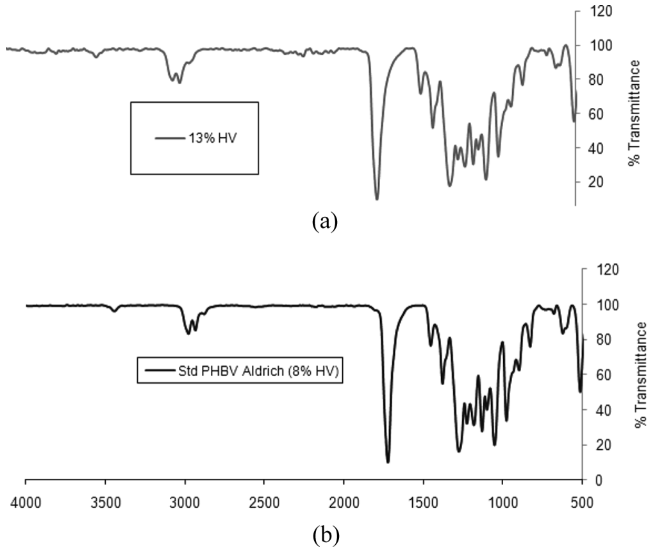
Table I. Chemical shift signals as obtained in ^{13}C NMR in this study with those reported in literature[20,21]

	Chemical shift (ppm)			
Carbon atom	This work	Kemavongse et al.[21]	Doi[20]	Peak number
C=O (HB)	169.12	169	169.03	1,5
C=O (HV)	169.29	169	169.5	
CH_2 (HB)	40.75	40	40.8	2
CH_2 (HV)	38.75, 26.8	38, 27	38.8, 26.8	6,8
CH (HB)	67.4	72	67.40	7
CH_3 (HB)	19.74	19.66	19.76	4
CH_3 (HV)	9.31	9	9.3	9

present in the molecular chain of highly ordered crystalline structure, and the band at 1286 cm^{-1} corresponds to the stretching of the C–O bond. Characteristic peaks of intense bands located at 800–1200 cm^{-1} correspond to the stretching of the C–O bond of the ester group. All these bands in the IR spectra of the biopolymer obtained from *Comamonas* sp. EB 172 coincided well with those of the commercial PHBV.

Mechanical Properties

PHBV, by virtue of its improved mechanical performance and biodegradability over PHB, has been the focus of characterization studies by many researchers.[23–25] In this study, solvent-cast films with hydroxyvalerate content of 13% were subjected to tensile strength, percentage elongation at break, and Young's modulus



(a)

(b)

Figure 7. FT-IR spectra of PHBV biosynthesized by *Comamonas* sp. EB 172 (a) and the commercial PHBV from Aldrich (b).

Table II. Comparison of mechanical properties of biosynthesized P(3HB-co-3HV)

Copolymer composition	Tensile strength (MPa)	Elongation at break (%)	Young's modulus (MPa)	Reference
PHBV (13% HV)	29.51 ± 0.92	8.63 ± 0.28	703.5 ± 8.9	This study
PHBV (14% HV)	35	N.d.	1500	Doi[20]
Biopol® PHBV chips	31.3 ± 0.5	5.2 ± 4.3	1315 ± 5.9	Luo and Netravali[25]
PHBV (20%)	38	15	4700	Muhamad et al.[26]
PHBV (1.13% HV)	19.7	0.17	Nd	Wang et al.[24]

measurement ((Table II). These three basic mechanical properties are important to compare the usefulness of a polymer for a particular commodity application. In general, the results for tensile strength and elongation required for breaking correlated well with those reported in the literature.[24–26] Comparatively lower Young's modulus in our sample could be associated with the low molecular weight, as reported by Luo and his associates.[23]

TGA Analysis

Thermal stability of the biopolymer produced was studied using TGA. Results showed that the copolymer possessed a higher thermo-degradation temperature (281.93°C) than commercial PHB (260.39°C) and PHBV sample (261.47°C) (thermograms not shown). The higher thermal stability of the extracted polymer seemed to be due to the crystalline morphology of PHBV.

CONCLUSION

In the current study, a rather complete characterization of the morphological, chemical, structure and mechanical properties of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer produced by *Comamonas* sp. EB 172 using organic acid mixtures from POME was carried out. Generally, thermal and mechanical properties and molecular weights of biopolymers varied with HV content, type of microorganism, and mode of fermentation. The feeding strategy described herein has been modified from our previous reports where carbon and nitrogen during the fermentation were maintained at different ratios during growth as well as in the production phase.[15,16] In this study, in order to make it simple and suitable for scaling up, feeding only with carbon source has been applied and polymer accumulation was triggered by adding mineral media into the bioreactor during active growth phase. With similar initial cell concentration (0.3–0.8 g/L),[15] the polymer accumulation and productivity were improved from 59% to 70% and from 0.096 to 0.1318 g/L/h, respectively, by this method.

Based on the characterization of the PHBV produced by *Comamonas* sp. EB 172, it was observed that the copolymer obtained from *Comamonas* sp. EB 172 has properties similar to those of the commercial PHBV (Aldrich). The ^{13}C NMR and the FT-IR analyses not only showed similarities with the commercially available PHBV powder, but also revealed a substantial degree of purity for the sample produced from waste carbon source (POME). Since these properties enable a broader range of applications for the PHBV produced, the fermentation process described herein represents an interesting alternative for the production of poly(hydroxyalkanoates) from renewable resources.

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