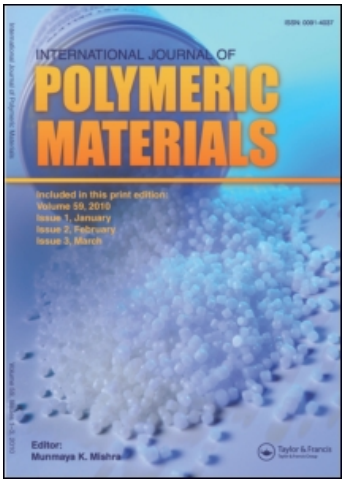

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Synthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV) copolymer produced in sequential feeding fed batch cultures of ralstonia eutropha

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SYNTHESIS AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-CO-3-HYDROXYVALERATE) (PHBHV) COPOLYMER PRODUCED IN SEQUENTIAL FEEDING FED BATCH CULTURES OF *RALSTONIA EUTROPHA*

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Alternative feeding of butyric and valeric acids to Nitrogen-depleted batch cultures of Ralstonia eutropha (reclassified from Alcaligenes eutrophus) produced poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV) with a low content of units HV (12%). FT-IR, NMR, and DSC techniques, were used for characterize the copolymers obtained. Sequential feeding of butyric and valeric acids is an alternative to control the synthesis of PHBHV with different percentages of units HV. When it was compared PHBHV obtained from two distinct carbon sources in the feeding was compared with a copolymer obtained from sequential feeding of butyric and valeric acids, the crystallinity of samples were similar in all cases.

Keywords: PHBHV copolymer, fed batch, characterization, sequential feeding, biodegradable polymer, crystallinity

INTRODUCTION

Poly(3-hydroxybutyrate) (PHB) and Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV), have attracted much attention in recent years as

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biocompatible and biodegradable polymers with potential applications in medical area [1]. In both polymers, their degradation products have shown to be non-toxic and biocompatible. The degradation takes place, in most cases, naturally in the body [2]. The range of pharmaceutical and biomedical uses based on these characteristics and their physicochemical properties of these materials is very extensive [2]. The properties of PHBHV varied with the percentage of HV units, the copolymer becoming less brittle when it contains a higher content of HV [2]. *Ralstonia eutropha* bacteria produces the copolyester with a composition varying from 0 to 47 mol% 3 HV units, depending on the carbon source supplied.

Doi *et al.* (1988) [3] reported that copolymer PHBHV, is produced by *R. eutropha* using butyric and valeric acid as carbon sources. Ishihara *et al.* (1996) [4] showed that a linear feeding (50 mL/h) with the proportion 1 : 1 wt, resulted in a maximum rate of copolyester production. The uptake of butyric acid and valeric acid are different in the metabolism of *R. eutropha* (Braunegg *et al.* 1995) [5] therefore, composition can be controlled by the feeding strategy of both substrates.

In this paper, the production of PHBHV obtained in fed batch using alternative feeding of butyric acid and valeric acids is reported and comparison with simultaneous feeding of both is presented as well as the differences on their physicochemical properties.

MATERIALS AND METHODS

Organism and Culture Conditions

Ralstonia eutropha ATCC 17699 was used in all experiments. The organism was precultured one day at 30°C, in a rotatory shaker at 200 rpm in a 500 ml Erlenmeyer flask containing 150 ml of growth medium proposed by Koyama *et al.* (1993) [6]. The biomass was centrifuged and used to inoculate, at 0.4 g weight humid of cells by liter of medium, the fermentor (5 L, BIOFLO II, New Brunswick company) equipped with two six-bladed disk turbine impellers, three baffles and dissolved oxygen control. The initial volume of culture was 3.5 L. Temperature was controlled at 30°C and pH at 7.0 with a 1 N HCl and 1 N NaOH solutions. The dissolved oxygen was maintained above 20% of saturation by manipulating the agitation speed and the aeration rate up to 550 rpm and 5 L/min respectively. Bacteria was grown in two phases; initially (in batch), in a medium without nitrogen limitation to reach the maximum quantity in biomass; and then in a second phase, with feeding of fresh limited medium nitrogen.

Batch phase medium contained in all cases (in g/l): butyric acid, 3; $(NH_4)_2SO_4$, 0.7; $Na_2HPO_4 \cdot 12H_2O$, 11.1; $MgSO_4$, 0.2; KH_2PO_4, 1.02; and 1 ml of solution of microelements was (in g/l): $FeCl_2$, 9.7; $CaCl_2$, 7.8; $CuSO_4 \cdot H_2O$, 0.156; $CoCl_3$, 0.119; NiCl $6H_2O$, 0.118; $CrCl_3$, 0.062 and

3.08 ml of HCl 35%. Composition of the feeding solution was the same, except for the carbon sources concentration and the ammonium concentration as specified.

During the second phase, three different feed mediums were used, with butyric acid, valeric acid or both as carbon sources. In all cases the total concentration of carbon source in the feeding medium was 3 g/L, and the ammonia concentration was 0.01 g/l. The feeding of medium at 50 ml/h started when the ammonium was consumed. Two types of feeding strategies were used; a medium with both carbon source and a sequential addition of mediums with only one carbon source. When the feeding was by sequential pulses, the time of pulses was varied between fermentations as shown as in Table 1.

Measurements during fermentation, concentrations of butyric and valeric acids in the medium were determined by gas chromatography (CG Tracor, FID, AT-1000 column, injector temperature 160°C). The concentration of NH_4^+ was measured by the method of Weatherburn *et al.* (1967) [7]. The biomass was quantified by dry weight. PHA, precursor concentrations were determined as methyl esters by gas chromatography according with Braunegg's method [8].

Extraction and Purification of PHBHV

Lyophilized bacteria were stored in chloroform at 40°C during a night, and recovered by filtration (Whatman). The polymer was precipitated three times with Hexane.

Characterization of Copolyesters

FT-IR spectra (Perkin Elmer 1600) of samples was carried out on films of $2 \times 1\,cm^2$. Cast films were prepared by slow evaporation of chloroform from a PHBHV/chloroform solution. ^1H and ^{13}C nuclear resonance (NMR)

TABLE 1 Time of the experiments

PHBHV	*Feeding regime*[a] (*h*)
I[b]	V, Shaker flask
II[c]	B + V(30)
III	V(4)/B(8)/V(8)/B(8)/V(2)
IV	B(4)/V(24)/B(2)
V	B(4)/V(16)/B(10)
VI	V(30)

[a] B = butyric acid, V = valeric acid. Number in parenthesis indicates feeding period for substrate specified.
[b] Batch Experiment in a Shaker flask, 250 mL, 30°C.
[c] Simultaneous feeding of both carbon source.

analyses of the copolyester samples were performed on a Brocker DM × 500 MHz spectrometer, using $CDCl_3$ as solvent and TMS as internal reference. δ units were recorded.

Differential scanning calorimetry. The procedure was the following: the films obtained by casting were heated from −50 to 200°C (run I). The melting temperature was determinate from D.S.C. (Dupont 9100) endotherms after the samples were cooled down to −50°C. Finally the samples were heated up to 200°C (run II), for estimate Tg (onset). A scan rate of 10°C/min was used throughout.

Dynamic mechanical tests were carried out in the glass-rubber transition temperature range with a Perkin Elmer 7 series. The specimen was a thin film with dimensions of 15 mm × 5 mm × 0.2 mm. Measurements were performed at 1 Hz between −50 and 50°C. The heating rate was 1°C min^{-1}.

Results and Discussions

As it has been reported by Doi *et al.* (1988) when butyric acid and valeric acid are utilized as carbon sources, *R. eutropha* produces the copolymer PHBHV. We confirmed that the copolymer produced was PHBHV by the ^{1}H-NMR and FT-IR spectra. Both spectra showed good agreement with those reported by others authors [3, 9]. Table 1 shows the extension of the pulses tested. Synthesis of a polymer chain may commence during feeding of one substrate and be completed following the exchange of the carbon source.

When *R. eutropha* was grown in a shaker flask with valeric acid as the only carbon source, propionic acid was detected in the medium. This suggests that during cell growth, the c2- carbon fragments are cleaved from the initial carbon substrate by β-oxidation, which would provide energy for bacterial cell *via* the tricarboxylic acid (TCA), according with Zaidi *et al.* (1997) [10]. The PHBHV production is low because the medium was not nitrogen limited and *R. eutropha* accumulate small amounts of PHB during unrestricted growth [1]. It has been reported that during the production of Poly(3HB-4HB) in *R. eutropha*, the bacteria contained 9%(w/w) of PHB at the end of the initial growth phase [11]. Table 2 summarizes the results from a number of studies on the production of PHBHV by various culture methods. PHBHV has been produced by fed-batch, one stage or two stages chemostat culture. In the fed-batch experiments with alternate feeding of butyric acid and valeric acid we observed low productivity of the polyester compared with those reported using both carbon source in the feeding (see Tab. 1). Copolymers with a higher HV fraction (% 35 mol) could be obtained using the medium with both carbon sources. The total copolymer content diminishes as the time of addition of valeric acid feeding increases. When it was fed both carbon sources the proportion of incorporation of HV is different, according with Braunegg *et al.* (1995), who reported that the

TABLE 2 Comparison between this study and others works

Author	*Strategy's production of PHBHV*	*Total cell dry wt (g)*	*PHBHV content (wt %)*	*Proportion of acids fed (wt) But-Val*	*Total fatty acids fed (g/L)*	*ρ* ($g_{PHBHV}/g_{r.b.}$ *h*)	*3HV mol %*
Kimm[a] [19]	Two-stage shake flask	2.2	46	1 : 1	–	n.r.	40
Doi[b] *et al.*	Two-stage shake flask	0.66	46	0 : 1	–	0.023	85
Ishihara[c]	Fed batch	7 g/L	n.r.	1.25 : 1	10.6	0.060	17
This study I[d]	One-stage shaker flask	1.60	< 3%	0 : 1	–	0.00005	48
II	Fed batch	1.54	55	1 : 1	4.5	0.022	35
III	Sequential	1.64	38	*	4.5	0.016	12
IV	Sequential	1.30	30	*	4.5	0.011	08
V	Sequential	1.84	35	*	4.5	0.014	06
VI	Sequential	1.70	12	*	4.5	0.003	07

[a] 200 mL of medium, valeric acid and glucose to 1:1 wt. 20 g/l total concentration of fatty acids.
[b] 200 mL of medium, valeric acid (20 g/l).
[c] Fed-batch, total concentration of fatty acids in the feeding 10.6 g/L, 30 h of fed.
[d] Shaker flask 250 mL of medium without Nitrogen limitation.
* See Table 1. N. R. = not reported.

uptake of butyric acid is bigger in comparison with valeric acid in *R. eutropha*. The content of polyesters in dried cells reported was in the range of 12–51%, depending on organic acid used in the feeding. For alternate substrate feeding, butyric and valeric acids were fed sequentially, as specified in Table 2, but the 30 h feeding period and the total amounts of each substrate fed to cultures were maintained unchanged. Doi *et al.* (1988) reported that when *R. eutropha was grown* in valeric acid (20 g/l) as only carbon source, PHB-85%HV was obtained, but we obtained only (PHB-48%HV) using 3 g/l of valeric acid. *R. eutropha* can synthesis HB units through two pathways [1, 3]. Madden *et al.* (1998) obtained a mixture of polymers (PHB homopolymer and PHB-HV copolymer) using on alternative feeding of glucose and propionic acid. He explains that probably the bacteria needs to consume the entire endogenous carbon source previously to consuming exogenous one. In contrast, our results show that only the copolymer was synthesized, using alternative feeding of butyric and valeric acids. Doi *et al.*, reported that the synthesis of HV units could be result of two pathways. These differences in the proportion of HV units can be explained in terms of the second pathway, which becomes active when the bacteria is subjected to metabolic changes under alternated pulses of different carbon sources are used. Two forms of both β-ketothiolase and acetoacetil-CoA reductase have been found in *R. eutropha*. The two ketothiolases have different substrate specifities [12].

The concentration of butyric acid used by Doi *et al.* (1988) of 20 g/L total fatty acid in the medium have been is reported as toxic for *R. eutropha* [2]. Moreover, it was previously reported [13] that when butyric acid concentration was higher than 3 g/L in PHB production, the specific production rate of PHB decreased due to substrate inhibition. Our results showed that the proportion of HV on the copolymer is favored by the butyric acid present in the medium. With this method we obtained PHBHV with a maximum value of HV units of 12%mol. It is evident that the content of HV units is low, for the following reasons: (a) when butyric acid is fed, only HB units are synthesized; (b) HB units is formed starting from butyric and valeric acids [3]. Madden *et al.* (1998) [14] reported that when *R. eutropha* is grown in a alternate feeding with glucose and propionic acid, the bacteria synthesized a mixture of polymers of PHB and PHBHV. The presence of two β-Ketothiolases in the PHBHV pathway in *R. eutropha* can explain the differences in the percentage of HV units present in the copolymer given the activity of these enzymes is different, depending of carbon source present in the medium. In *R. eutropha*, there are two ketothiolases, which together accept ketoacyl-CoAs with 4–10 atoms of carbon. Additionally acetoacetyl-CoA reductase is active with ketoacyl-CoAs of 4–6 carbons to generate R-(-)-3-hydroxyacyl-CoAs [12].

FT-IR Composition Analysis and Crystallinity

Based on the chemical structure of PHBHV, one can predict the FTIR bands will be sensitive to copolymer composition include the C—H bands and the C—C bands around 2900 and 977 cm^{-1}, respectively. Figure 3 shows two regions of the infrared spectra of the PHBHV copolymers. The considered bands were the C=O bond (1772 and 1740 cm^{-1}) and the C—O—C bond (1279, 1228 and 1185 cm^{-1}) [15, 16]. The band at 1185 cm^{-1} in the amorphous state is more intense [16], and the bands at 1279 and 1228 cm^{-1} are better defined in the amorphous state than in the crystalline state [15]. The most intense band in the crystalline state corresponds to the frequency at 1722 cm^{-1}, which correspond to ester carbonyl region, and is characteristic of the crystalline state. Band at 1740 cm^{-1} is a characteristic region of the amorphous state [15, 16].

The ratio of the peaks at 1185 cm^{-1} and 1382 defined the crystallinity index (Cl) [15]. The results show that all samples of PHBHV were semi-crystallines (See Tab. 4).

Our results don't showed differences in the crystallinity index. All samples had Cl in the range 52–62%. This is due maybe both comonomers are clearly chemically and geometrically similar.

TABLE 3 Summary of the thermal properties of obtained copolymers

Polymer	*Tg°C*	*Tm°C*
I	-8 ± 1	I. T.
II	-9 ± 1	120 ± 2
III	-7 ± 1	125 ± 2
IV	-4 ± 1	135 ± 2
V	-2 ± 1	140 ± 2
VI	-6 ± 1	130 ± 2

I.T. = Thermally unstable.

TABLE 4 Crystallinity index (Cl) of PHBHV samples

Sample	*Cl*
I	52
II	58
III	55
IV	53
V	60
VI	62

NMR

The ^{13}C NMR spectrum of PHB-%35HV is shown in Figure 2. The indicated peak assignments are straightforward with the chemical shifts for HB and HV units of the copolymer, in close agreement with values previously reported for the homopolymers PHB and PHV. The molar fraction was determinate of ^{1}H NMR spectrum, based on the intensity ratio of methyl HB and HV units. HB units can be synthesized from butyric and valeric acids, explaining the comonomers random distribution obtained. Blhum *et al.* (1986) studied the diads and triads from PHBHV. In his work, he demonstrated that the comonomer sequence distribution of PHBHV is a random. This conclusion was obtained based on the diads and triads analysis, using a Bernoullian model.

The ^{1}H NMR spectra of polymers obtained, show signs in parts for million (ppm) in: 0.9 assigned to the CH_2 (C4) of the HV, at 1.26 and 1.28 that are assigned to CH_3 (C4 and C5 of both monomers); in 2.49 and 2.62 assigned to CH_2 (C2); in 5.3 assigned to CH (C3). In the ^{13}C NMR the signs in the spectra of the polymers are in parts for million (ppm): in 19.7 assigned to the CH_2 (C4 HB), at 27 assigned to CH2 (C4 HV), at 38 assigned to CH_2 (C2 HV), in 41 that corresponds CH_2 (C2 HB), in 67 corresponding to CH (C3 HB), in 72 assigned to CH (C3 HV) and another in 169 corresponding to the C=O (C1, both monomers).

Thermal Properties

The thermal properties of PHBHV obtained by alternate feeding were investigated by differential scanning calorimetry (DSC). Our results showed that melting point of PHBHV diminishes as the percentage of units HV increases as previously described [1, 9, 17]. Onset of melting of the copolymers are shown in Table 3. Tg was inversely proportional to % HV higher content of HV. In the copolymers with a HV lower than 15% no significant differences was found as reported by Blhum *et al.* (1986) and Kunioka *et al.* (1989) [18]. In all cases an only melting point was observed (Fig. 1).

Blhum *et al.* (1986) and Bloembergen *et al.* (1986) reported that the decrease in the Tm is due to the unusual phenomenon of isodimorphism present in the copolymer PHBHV.

Dynamic Mechanical Measurement

The Figure 4 shows the dynamic storage modulus (E') and tan δ of a PHB-35HV sample as function of temperature.

The maximum E' value for PHB-35HV, around 3.04 Pa, was lower than this found by Cyras *et al.* (1999) of PHB-8HV in 3.5 Pa. The copolymer displayed typical behavior of semycrystalline polymers. At low temperature

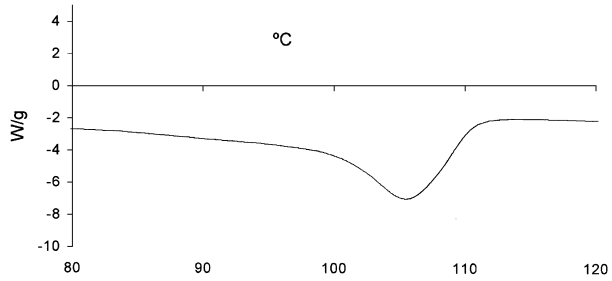

FIGURE 1 Thermogram of PHB-35%HV.

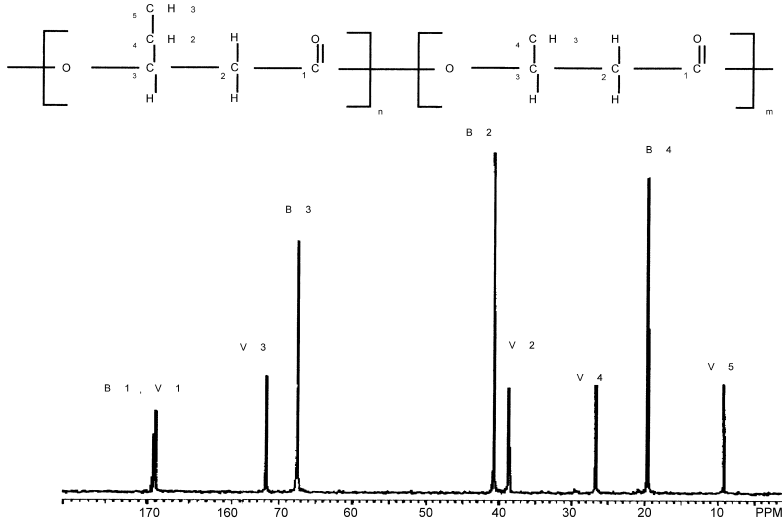

FIGURE 2 Structure of PHBHV and ^{13}C NMR spectrum of bacterial PHBHV in $CDCL_3$. B and V refer to hydroxybutyrate and hydroxyvalerate units, respectively.

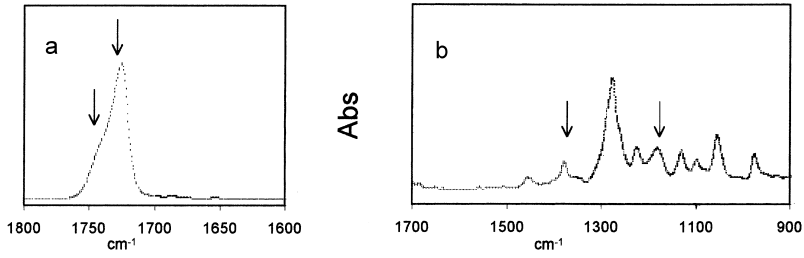

FIGURE 3 FTIR Spectra of the peak of PHB-35HV copolymer: (a) bands at C═O bond, (b) the bands the C—O—C bond.

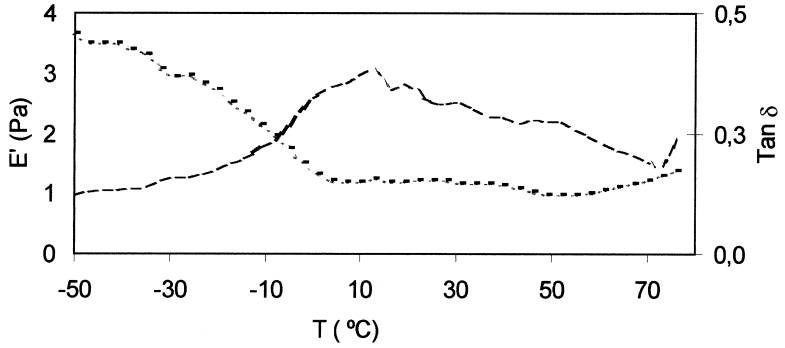

FIGURE 4 Dynamic storage modulus and tan δ of PHB-35HV samples as temperature function (1 Hz).

the amorphous part of the polymer is in the glassy state, and the modulus slightly increase. The loss modulus decreases in the range -50 at -10 C, and was roughly constant.

CONCLUSIONS

Use of sequential pulses of butyric and valeric acid was tested for the synthesis of PHBHV. No significant differences in physicochemical properties were found between copolymers synthesized by linear feeding with both carbon source and those synthesized by alternate pulses. The copolymers synthesized by *R. eutropha* produce a high crystallinity, when butyric and valeric acid were alternate in the feeding. When the valeric acid is present in the medium, the total productivity of PHBHV decreases. The adequate control in a time of pulses allows the control in units HV incorporation into the copolymer. However the productivity of PHBHV is minor when alternate feeding of butyric and valeric acid is used to produce the copolymer.

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