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Research Article

Synthesis of Poly-(R-hydroxyalkanoates) by *Cupriavidus necator* ATCC 17699 Using Mexican Avocado *(Persea americana)* Oil as a Carbon Source

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Poly-R-hydroxyalkanoates (PHAs) are polymers produced by a vast number of bacterial species under stress conditions. PHAs exhibit different thermal and mechanical properties that depend on their molecular structure. In this work, PHAs were produced using avocado oil as the carbon source. *Cupriavidus necator* H16 was cultured in three-stage fermentation using fructose during the cell growth stages and avocado oil during the PHA synthesis stage. Different concentrations of avocado oil were used during the third stage to test the incorporation of various monomeric units into the PHAs. Biomass and PHA production were measured during the fermentation. DSC, FTIR, and gas chromatography analysis aided the PHA characterization. Different proportions of 3-hydroxyvalerate were present in the 3-hydroxybutyrate main chain depending on the concentration of avocado oil. The results suggest that avocado oil is a viable new substrate for PHA production.

1. Introduction

Poly-R-hydroxyalkanoates (PHAs) are polymers synthesized by a large number of bacterial species as a response to unbalanced nutritional conditions [1]. PHAs are thermoplastic polyesters of R-hydroxy alkanoic acids and accumulate intracellularly as granules that exhibit different properties depending on their chemical composition [2, 3]. A single monomer that forms the chain of PHAs typically contains from 3 to 15 carbon atoms [4], but the final chemical composition of PHAs is related to the synthesizer microorganism, the carbon source, the culture conditions, and the specificity of the PHA-synthase enzyme [5–7]. Homopolymers, copolymers, or terpolymers of PHAs can be obtained; for example, PHA copolymers can be synthesized from a combination of different substrates [8]. The thermal properties of PHAs, such as melting temperature and degree of crystallinity, depend on the length of the PHA monomeric units. Monomers containing more than five carbon atoms significantly decrease the polymer melting temperature, as well as the degree of crystallinity [9].

Many PHAs have main chains formed from monomers with different numbers of carbon atoms. Short-length-chain PHAs (PHA_{slc}) consist of monomers ranging from 3 to 5 carbons, whereas medium-length-chain PHAs (PHA_{mlc}) are formed from monomers containing 6 to 14 carbon atoms [4, 8]. One PHA_{slc}, poly(3-hydroxybutyrate) (PHB), is the most common PHA and was first identified by Maurice Lemoigne in 1926 [1, 4]. PHB biodegradability and biocompatibility make it an attractive material; however, its

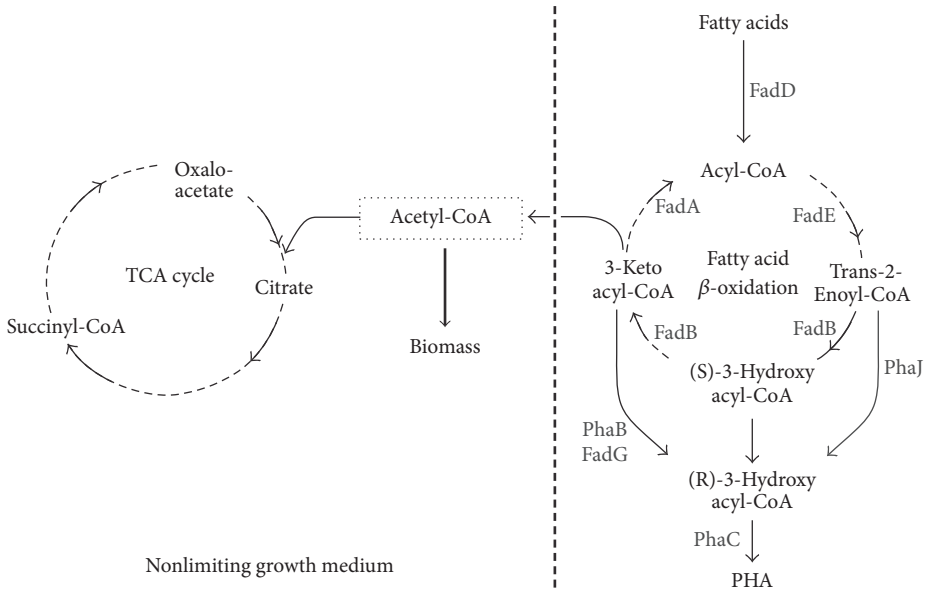

Figure 1: A simplified representation of fatty acid metabolism in *Cupriavidus necator*. In a nonlimiting growth media (left side), the tricarboxylic acid (TCA) cycle is active and the main product is biomass itself. Under unbalanced conditions (right side), caused by a noncarbon nutrient deficiency, C-source via fatty acid β-oxidation flows towards the poly-R-hydroxyalkanoate (PHA) biosynthesis pathway.

brittleness and limited degree of crystallinity have restricted its possible applications. The melting point of PHB (≈175°C) is also very close to its decomposition temperature (≈180°C), which creates challenges for thermal processing due to a narrow processing window [10]. Copolymers such as poly-(hydroxybutyrate-co-hydroxyvalerate) (PHBV) that contain (3)-hydroxybutyrate (3HB) and (3)-hydroxyvalerate (3HV) units in the chain show lower molecular weights and lower melting temperatures when compared to a PHB homopolymer. Babel and Steinbüchel [11] reported melting temperatures of 170, 162, 150, 145, and 137 when 3HV was in a 3, 9, 14, 20, and 25 mol% content, respectively.

Renewable carbon sources, such as sucrose, cellulose, and triacylglycerol, have served as substrates for PHA synthesis. Extensive studies have been conducted on the use of inexpensive substrates, including starch, glycerol, soybean oil, sugar cane bagasse, molasses, and activated sludge, to reduce the production cost of PHB [5, 12–14]. Similarly, byproducts from the food and the agroalimentary industry, methane, mineral oil, and lignite have been used to synthesize PHBV copolymers [1, 4, 15, 16]. Therefore, the carbon source and the microorganism consumption affinity are of great importance for the production of specific PHAs.

One organism that has been extensively used in the synthesis of PHAs is *Cupriavidus necator* (formerly *Ralstonia eutropha*), due to its versatility to accumulate polymer in amounts as high as 90% of its dry cell weight (DCW) [6]. The ability of *C. necator* to synthesize PHB and PHBV, as well as other PHAs, has been previously reported [6, 20, 21]. The limiting productions costs had led to proposals for the use of cheaper carbon sources, such as organic debris, wastewater, or even vegetable oils [22]. The use of complex carbon sources could also extend the incorporation of 3HV units to the main chain or even the synthesis of PHA_{mlc} [8].

The use of fatty acids, such as those present in vegetable oils, as a carbon source drives the β- fatty acid oxidation metabolic pathway in *C. necator*. The PHA synthesis in *C. necator* is highly associated with growth conditions and is mediated by the acetyl-CoA precursor [23, 24]. In balanced nutritional environments, fatty acids provide precursors, free energy, and cofactors for cell growth and maintenance and for macromolecular synthesis. By contrast, noncarbon nutrient limitation leads to inhibition of the central enzymes of the tricarboxylic cycle (TCA cycle). Consequently, the acetyl-CoA is channelled towards the PHA synthesis [23–25], as depicted in Figure 1.

Mexican avocado *(Persea americana)* is a lipid-rich fruit that occupies a prominent place in the market [26]. In 2015, 51.4% of the globally commercialized avocado was produced in Mexico [26]. During its cultivation, a high amount of waste material is produced. For example, in Mexico, nearly 54% of the annual avocado production is considered as waste. Moreover, the farming, packing, transportation, and commercialization stages are also important sources of avocado wastes [27]. Furthermore, fruit peel and seed, representing 12 to 15% and 20 to 27% of fruit weight, respectively, are currently discarded. Only the fruit pulp is destined for human consumption [28].

The fatty acids composition in Mexican avocado mainly includes palmitic, stearic, oleic, linoleic, heptanoic, nonanoic, and heptadecanoic acids [26]. The high content of fatty acid and the amount of waste generated from its cultivation identify avocado as a possible and sustainable carbon source for the biosynthesis of PHAs. As a first attempt, and to

standardize the chemical composition of the substrate, the viability of PHA biosynthesis from avocado oil by *C. necator* was tested. The biopolymers synthesized from different oil contents were thermally and chemically characterized to demonstrate the feasibility of using this oil as an alternative substrate for PHA production.

2. Materials and Methods

2.1. Strain, Media, and Materials. C. necator H16 (ATCC 17699) was grown in mineral medium supplemented with fructose for 24 h, at 30°C and 200 rpm, for seed culture preparation. The medium contained, per litre of water, 10 g fructose, 3.70 g $(NH_4)_2SO_4$, 0.40 g $MgSO_4$, 6.36 g $Na_2HPO_4{\cdot}7H_2O$, 2.70 g KH_2PO_4, and 1.0 g nutrient broth.

The growth medium contained, per litre of water, 10 g fructose, 1.57 g NH_4SO_4, 5.66 g $NaH_2PO_4{\cdot}12H_2O$, 1.5 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 10 mg $CaCl_2{\cdot}2H_2O$, 20 mg $FeSO_4{\cdot}7H_2O$, and 1 mL of trace element solution (0.3 g H_3BO_3, 0.2 g $CoCl_2{\cdot}6H_2O$, 0.1 g $ZnSO_4{\cdot}7H_2O$, 30 mg $MnCl_2{\cdot}4H_2O$, 30 mg $Na_2MoO4{\cdot}2H_2O$, 20 mg $NiCl_2{\cdot}6H_2O$, and 10 mg $CuSO_4{\cdot}5H_2O$, in HCl 0.1 N solution); the pH was adjusted to 7.

Commercial Mexican avocado oil for PHA synthesis was obtained from a single batch (the same production number) (Ahuacatlán, Mexico) to ensure a homogeneous chemical composition of the substrate.

2.2. Fermentation Studies

2.2.1. Inoculum Preparation. Experiments were conducted in duplicate using 200 mL of growth media in 500 mL flasks at 30°C, pH 7.0, in an incubator with rotational agitation at 200 rpm (New Brunswick Innova 4300, USA). A 10% v/v of the seed culture was used to inoculate the growth medium to obtain 0.13 g L^{-1} (±0.1) of initial biomass (X) in the growth medium.

2.2.2. Fermentation. A fermentation procedure consisting of three different stages was carried out as follows.

Stage 1. Batch cultivation at an initial carbon/nitrogen (C/N) ratio of 14 using the growth medium: carbon depletion in the medium (3 g L^{-1} of fructose) determined the length of the stage.

Stage 2. A fed-batch stage to increase biomass density at C/N ratio of 6.5: two additions of fructose and ammonium were made. The time of addition was determined as the point when the fructose remaining in the media reached approximately 3 g L^{-1}.

Stage 3. PHA production under nitrogen limitation: avocado oil was added to the culture at the beginning of the stage, at 30 h. Different concentrations were tested: 5, 10, 15, 20, and 25% (v/v).

Control experiments consisted of additional flasks prepared using fructose as the carbon source for the three-stage fermentation.

2.2.3. Analytical Procedures. Fermentation samples were taken every two hours and immediately centrifuged at 10000 rpm for 10 min at 4°C. Fructose and ammonium were analysed in the supernatant, and the bottom pellet (biomass) was washed thoroughly with distilled water before lyophilizing for gravimetric estimation of the dry cell weights (DCW).

Fructose consumption in the fermentation media was quantified using a 3,5-dinitrosalicylic acid (DNS) method [29]. Ammonium consumption was analysed according to the protocol of Weatherburn [30].

The intracellular polymer was extracted from the lyophilized biomass using chloroform (1 g of biomass per 50 mL of solvent) at 60°C for 30 min with constant stirring. After incubation, PHA dissolved in the chloroform phase was filtered to eliminate cellular debris and then precipitated with hexane. The residual solvent in the polymer was removed by evaporation [31, 32].

Dimensionless biomass yield ($Y_{x/s}$) was estimated as the ratio of the amount of biomass produced to the amount of total substrate consumed. This was calculated at the end of Stages 1 and 2. Productivity was estimated at the end of the fermentation as the final PHA concentration achieved divided by the total cultivation time required to attain that concentration. Residual biomass was also calculated at the end of fermentation as final produced biomass (CDW) minus PHA concentration.

2.3. PHA Characterization

2.3.1. Gas Chromatography. Fatty acid methyl esters were derived from acid methanolysis of PHA at 100°C for 4 h by incubating 100 mg of PHA, 2 mL of chloroform, 2 mL of methanol (20% of HCl), and benzoic acid (as an internal standard) in borosilicate glass tubes with screw caps at 100°C for 4 h. After cooling, distilled water was added (1 mL), the tubes were vortexed for 60 s, and the lower phase containing the resulting methyl esters was recovered for analysis [33]. Fatty acid methyl esters (1 μL) were analysed on a gas chromatograph (SRI Instruments, Model 310, USA) equipped with a flame ionization detector (FID) and a 6 ft. × 1/8 in. silica gel column. Nitrogen at 30 mL min^{-1} was used as carrier gas and the injector and detector were set at 220 and 170°C, respectively. Reference standards were poly(hydroxybutyrate) and the copolymer poly(hydroxybutyrate-co-hydroxyvalerate) [12 mol% hydroxyvalerate] (Goodfellow, UK).

2.3.2. Fourier Transform Infrared (FTIR) Spectroscopy. FTIR was performed within wavenumber ranges from 600 to 4000 cm^{-1} (BUCK Scientific, model 530, USA). PHA was dissolved in chloroform before pouring the solution onto KBr plates to form the polymer films.

2.3.3. Differential Scanning Calorimetry (DSC). DSC curves were obtained using a differential scanning calorimeter

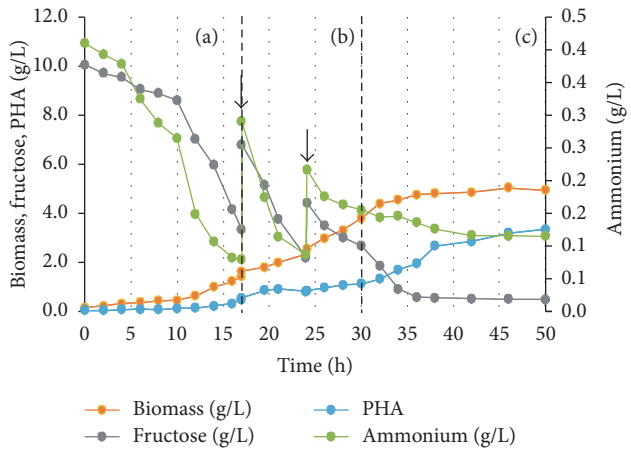

FIGURE 2: Profiles of fructose (grey circles), ammonium (green circles), biomass (orange circles), and poly-R-hydroxyalkanoate (PHA) (blue circles) production during the cultivation of *Cupriavidus necator* on fructose and avocado oil (20% v/v) in three-stage fermentation. Stage 1: batch cultivation (a), Stage 2: fed-batch stage (additions indicated by black arrows) (b), and Stage 3: PHA accumulation (c). Oil addition occurs at 30 h.

(Mettler Toledo DSC 823) according to López-Cuellar et al. [6]. Approximately 4 mg of the specimens was crimped in aluminium pans. The samples were evaluated under the following conditions: dynamic nitrogen atmosphere of 50 mL min^{-1}, a heating rate of 10°C min^{-1}, and an extended temperature range (−40 to 200°C). Two runs under same conditions were carried out; the first run erased the thermal history of sample. Thermograms obtained during the second run were analysed to determine the melting temperature (T_m) and melting enthalpy (ΔH_m) of the PHAs. Pure PHB (Goodfellow, UK) served as reference standard.

3. Results

3.1. Fermentation Studies. The representative profiles of the PHAs synthesized by *C. necator* H16 using fructose and avocado oil as carbon sources are depicted in Figure 2.

Stage 1 (Figure 2(a)), conducted as a batch cultivation, lasted 17 h and was initiated by adding 10 g L^{-1} of fructose and 0.42 g L^{-1} of ammonium to the medium. A lag phase of 10 h occurred. The concentration of substrate (i.e., fructose during Stage 1) decreased from 10 to 3.3 g L^{-1} (±0.10), whereas the biomass density, measured gravimetrically as DCW, increased from 0.13 to 1.44 g L^{-1} (±0.06) to achieve a growth yield ($Y_{x/s}$) of 0.19.

Stage 2 (Figure 2(b)), conducted as a fed-batch cultivation to increase cellular density, lasted about 12.5 h. Fructose as substrate was added on two different occasions: at 17.5 h and 24 h (Figure 2, black arrows). The first addition consisted of 3 g L^{-1} of fructose and 0.21 g L^{-1} of ammonium. For the second addition, the medium was supplemented with 2 g L^{-1} of fructose and 0.12 g L^{-1} of ammonium. During Stage 2, the biomass density increased from 1.44 (±0.06) to 3.79 g L^{-1} (±0.09), consuming about 5.63 g L^{-1} (±0.03) of fructose. At the end of Stage 2, an average $Y_{x/s}$ of 0.42 was achieved, matching the theoretical yield when simple sugars are used as carbon sources ($Y_{x/s}$ of 0.30–0.40) [8]. In addition, a slight accumulation of PHA was observed, but this only represented less than 30% of the DCW, in agreement with the balanced nutrient conditions.

Synthesis of PHAs (Stage 3) was first observed at 30 h (Figure 2(c)). The fructose remaining in the medium was about 1.9 g L^{-1} at the beginning of this stage. Avocado oil was added to the flasks in a single addition at the beginning of the stage to induce polymer synthesis. The avocado oil concentrations tested were 0 (used as a control), 5, 10, 15, 20, and 25% (v/v). Stage 3 lasted 20 h. During this time, nitrogen levels remained around 0.1 g L^{-1} to generate cellular stress and to promote polymer accumulation [6]. A rapid consumption of fructose was observed at the beginning of the stage and, by the end of the stage, fructose was barely detectable in the medium (<0.5 g L^{-1}).

The results of the 50 h, three-stage fermentation are summarized in Table 1. A significant amount of PHA accumulation was observed, ranging from 59 to 70% of the DCW. From the results, a positive trend for PHA accumulation was observed in flasks with avocado oil concentrations of 5, 10, 15, and 20% v/v. The highest PHA concentration was reached when the oil in the media was 20% (v/v), with PHA values of 3.48 g L^{-1} (±0.04) achieved, which represented 70.8% of the accumulated PHA in terms of DCW. However, flasks with and oil concentration of 25% (v/v) showed a decrease in PHA accumulation efficiency, reaching about 3.07 g L^{-1} (±0.02). The overall productivity of the experiments fluctuated between 0.053 and 0.070 g L^{-1} h^{-1}.

Conversely, control experiments (0% fed oil) produced 77% of the polymer, almost reaching the typical 80–85% PHA accumulation reported for the strain [34].

3.2. PHAs Composition Analysis through Gas Chromatography. From gas chromatography, the chemical composition of the PHAs was determined using benzoic acid (internal standard) as evaluation base. Representative chromatograms of the evaluated methyl esters are presented in Figure 3. The retention times for the reference standards and the samples were consistent (5.516 min for 3-hydroxybutyrate [3HB], 7.150 min for 3-hydroxyvalerate [3HV]).

The most abundant monomer detected in all samples was 3HB monomer ranging from 92.8 to 98.94% for samples fed with avocado oil, as summarized in Table 2. In all cases, 3HV monomeric units was also recognized, ranging from 1 to 7 mol%. The highest hydroxyvalerate amounts were found in the PHA synthesized from 20% (v/v) of oil, matching the biomass profiles. An unusual pattern was observed, wherein the accumulation of 3HV units was highly dependent on the avocado oil concentration, reaching a maximum value of 7% (Figure 2(c)). Other 3-hydroxy-acids containing more than five carbons were also identified in some PHAs, but in minimal quantities (i.e., less than 0.16 mol%). Thus, avocado oil could promote the synthesis of PHAs containing 3HB and significant fractions of 3HV monomers.

3.3. Functional Group Identification by Infrared Spectroscopy (FTIR). The spectra recorded from the PHAs, depicted in

Table 1: Final yields of poly-R-hydroxyalkanoates obtained from a three-stage fermentation of *Cupriavidus necator*.

Substrate	[a]Av. oil (% v/v)	[b]Biomass ($g\,L^{-1}$)	PHA ($g\,L^{-1}$)	[c]Residual biomass ($g\,L^{-1}$)	PHA (%)	Productivity ($g\,L^{-1}\,h^{-1}$)
Fructose	—	5.25 (±0.01)	4.04 (±0.02)	1.21 (±0.009)	76.87 (±1.03)	0.081
Fructose, avocado oil	5	4.45 (±0.02)	2.64 (±0.03)	1.82 (±0.012)	59.21 (±1.12)	0.053
	10	4.63 (±0.05)	3.14 (±0.04)	1.49 (±0.017)	67.69 (±1.38)	0.063
	15	4.74 (±0.04)	3.27 (±0.03)	1.47 (±0.013)	69.04 (±1.43)	0.065
	20	4.91 (±0.04)	3.48 (±0.04)	1.43 (±0.015)	70.83 (±1.31)	0.070
	25	4.61 (±0.03)	3.07 (±0.06)	1.54 (±0.011)	66.63 (±1.29)	0.061

[a]Av. oil: avocado oil concentrations (% v/v). [b]Biomass: measured gravimetrically. [c]Residual biomass: biomass concentration after PHA extraction.

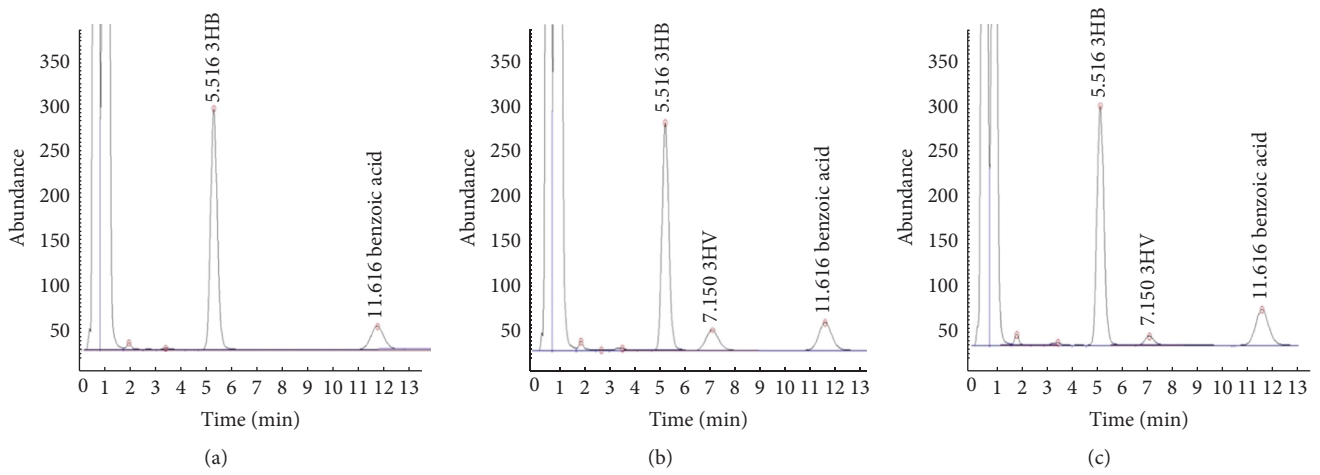

Figure 3: Chromatogram obtained from gas chromatography (GC) of (a) poly(3-hydroxybutyrate) (PHB) standard, (b) poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) standard with 12 mol% HV content, and (c) polymer produced from 20% v/v of avocado oil (7 mol% of HV). Benzoic acid was used as internal standard.

Figure 4, were similar to the PHBV spectra reported in previous studies [35]. The PHAs exhibited the particular chemical bonds of PHAs and replicated the absorption spectra among the samples.

The most prominent peak, located around 1720 cm^{-1}, was related to the ester carbonyl group (C=O). The bands located in the region of 2800 to 3000 cm^{-1} corresponded to the methyl-methylene groups. The presence of these peaks was due to the symmetric and asymmetric stretching of the CH_3 and CH_2 groups and these peaks were related to the monomeric units in the lateral chain. Besides the C=O group, an asymmetrical C-H bending vibration in CH_3 group shows an absorption band at 1453 cm^{-1}, whereas C-O-H bond shows a peak at 1378 [36]. Other peaks were recorded in the 1100 to 1290 cm^{-1} region; bands found around 1176, 1221, and 1270 cm^{-1} were attributed to polymer crystalline structures, and their presence was related to the C-O-C functional group [24].

3.4. Thermal Properties by DSC. The recorded thermograms obtained during the second run of the DSC analysis of the PHAs are shown in Figure 5. The melting points of the synthesized PHAs ranged from 159 to 173°C, whereas the measured ΔH_m was between 51 and 57 $J\,g^{-1}$ (Table 2). A decreasing trend was observed for the T_m value with increasing oil concentration. The lowest T_m and ΔH_m values were reached at 20% v/v, at 159°C and 51.81 $J\,g^{-1}$, respectively. An oil concentration above 20% v/v caused an increase in T_m of the synthesized PHA. Traces of monomers with a higher number of carbons were detected in some fed oil samples; however, these monomers were not identified, as mentioned in Table 2.

The thermal properties of the control experiment (0% fed oil) were also estimated and compared against a PHB reference standard. T_m of the sample fed with 0% oil was 175.16°C and ΔH_m value was 67.05 $J\,g^{-1}$. T_m of the PHB reference standard was 176.3°C and ΔH_m value was 75.48 $J\,g^{-1}$, in agreement with previous reports [37, 38] and confirming the production of pure PHB in the controls.

4. Discussion

Different PHAs were synthesized by *C. necator* H16 in a flask system using fructose and avocado oil as substrates. Fermentation was conducted using a three-stage process in order to increase biomass densities and polymer accumulation, without triggering the substrate inhibition reported to occur at levels of fructose higher than 10 $g\,L^{-1}$ [39]. Different oil

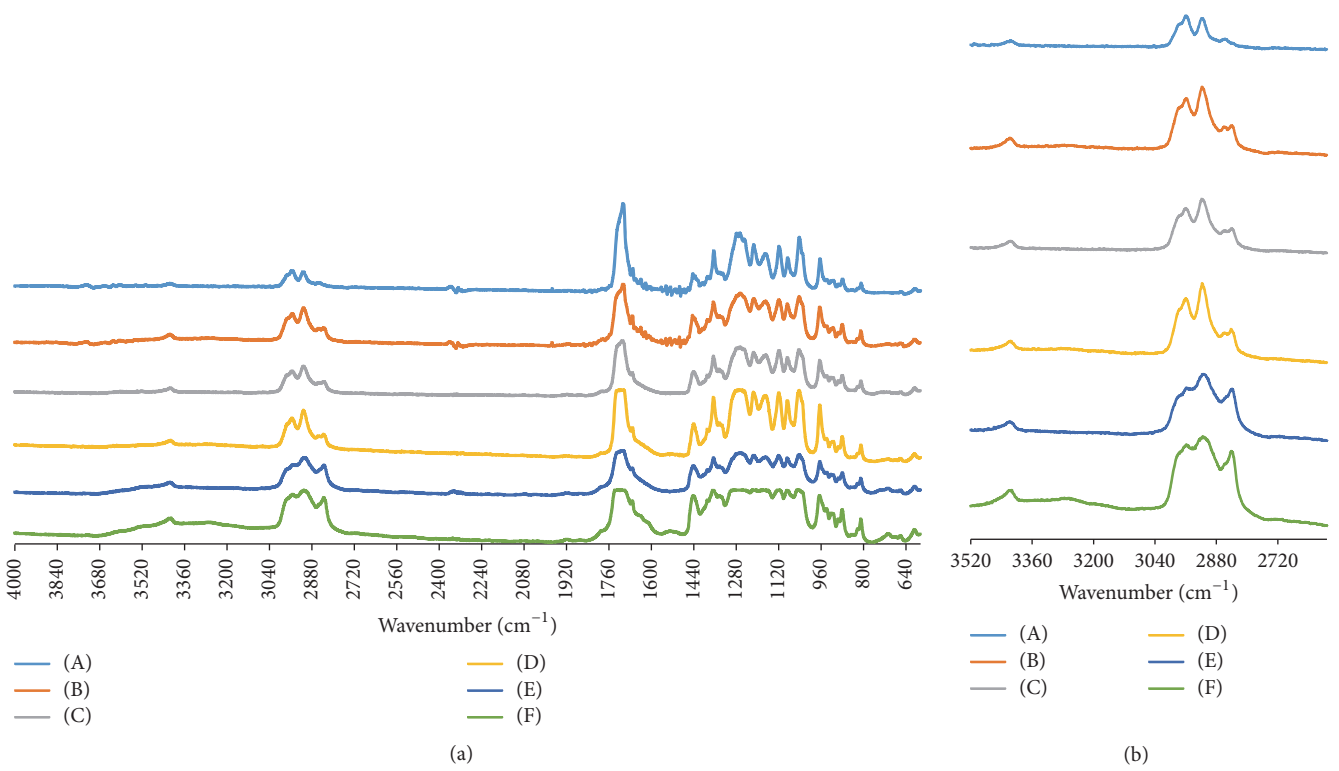

Figure 4: Fourier transform infrared spectroscopy (FTIR) spectra of the poly-R-hydroxyalkanoates (PHAs) produced by the addition of avocado oil: (A) 0 (control); (B) 5, (C) 10, (D) 15, (E) 20, and (F) 25 percent [v/v]. (a) 600 to 4000 cm^{-1}, (b) 2500 to 4000 cm^{-1}.

Table 2: Thermal properties and chemical composition of the poly-R-hydroxyalkanoates (PHAs) produced in *Cupriavidus necator*.

Substrate	[a]Av. oil (% v/v)	T_m (°C)	ΔH_m (J/g)	Monomeric composition of PHAs[b] (mol%)		
				3HB	3HV	3HA
Fructose	—	175.15	67.05	100.00	—	—
Fructose, avocado oil	5	173.22	56.68	98.94	1.06	
	10	169.71	55.32	97.15	2.77	0.08
	15	168.22	54.29	96.64	3.27	0.09
	20	159.57	51.81	92.83	7.01	0.16
	25	164.31	53.26	95.14	4.75	0.11

[a]Av. oil, avocado oil tested concentrations; [b]3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3HA, unidentified hydroxyl acid monomeric unit with more than 5 carbons.

concentrations (5, 10, 15, 20, and 25% v/v) were evaluated for PHA synthesis during Stage 3. The control experiments, using fructose as the carbon source during the overall process, complemented the studies.

Previous flask studies have typically been conducted in batch mode [12, 17–20], with overall productivities ranging from 0.025 to 0.08 $g\,L^{-1}\,h^{-1}$ (Table 3). In the current study, remarkable productivity was obtained, ranging from 0.05 to 0.07 $g\,L^{-1}\,h^{-1}$, for the evaluated oil concentrations (% v/v). Similar studies have achieved productivities between 0.025 and 0.05 $g\,L^{-1}\,h^{-1}$ [17, 19]. The highest productivity previously reported was reached using simple sugar as a carbon source [12] and resembled the productivity reported here for control samples (0.081 $g\,L^{-1}\,h^{-1}$). Hence, the feasibility of the three-stage fermentation was confirmed and suggested an interconnection with the operational mode of the system and the strain's affinity for the substrates [34].

A maximum biomass yield was reached with 20% v/v oil in the medium. Flasks with 25% v/v oil showed a decrease in cellular density and polymer accumulation. This yield decrease could be related to oxygen transfer limitations or a substrate inhibition [40]. Possibly, large oil amounts reduce

Table 3: Comparison of studies reporting PHAs production in *Cupriavidus necator*.

Strain	Substrate	Scale	Control strategy	PHA[1] produced	Biomass ($g\,L^{-1}$)	PHA ($g\,L^{-1}$)	PHA (%)	Productivity ($g\,L^{-1}\,h^{-1}$)	Reference
C. necator	Plant oils[2]	Flask	Batch	P(3HB)	3.6–4.3	2.9–3.4	79–81	0.04–0.05	Fukui and Doi [17]
C. necator (recombinant, *Aeromonas caviae*)	Plant oils[2]			P(3HB-co-3HHX)	3.5–3.6	2.7–2.9	76–81	0.04	
C. necator DSM 541	Fructose	Flask	Batch	P(3HB)	3.4	n.a	55	n.a	Dennis et al. [18]
	Palmitate			P(3HB-co-3HV-co-3HHX)	0.51	n.a	58	n.a	
	Oleate			P(3HB-co-3HHX)	1.44	n.a	57	n.a	
C. necator	Centrifuged fermented organic waste	Flask	Batch	P(3HB-co-3HV)	2.77	1.13	40.0	0.025	Ganzeveld et al., [19]
C. necator	Bagasse hydrolysate	Flask	Batch	n.a	6	3.9	65	0.08	Yu and Stahl [12]
C. necator	Palm oil	Flask	Batch	P(3HB-co-3HV)	3.6	2.66	74	n.a	Liu et al. [20]
C. necator	Fructose[3]	Flask	Fed batch	P(3PHB)	5,25	4.04	76.87	0.081	This study
	Fructose, avocado oil			P(3HB-co-3HV)	4.45–4.91	2.64–3.48	59–70	0.053–0.07	

[1]3PHB, 3 hydroxybutyrate; 3HV, 3 hydroxyvalerate; 3HHX, 3-hydroxyhexanoate. [2]Olive oil, corn oil and palm oil tested once at a time. [3]Control experiments. n.a: Not available.

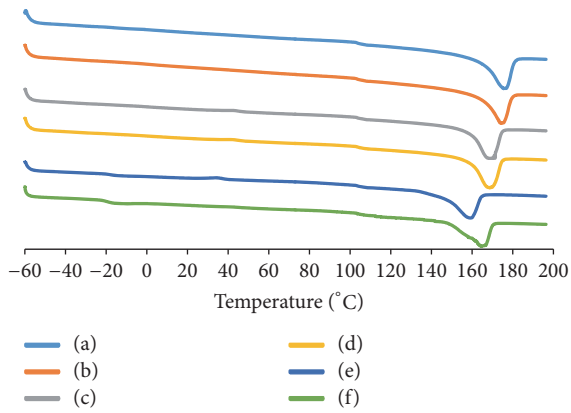

Figure 5: Thermograms obtained during the second run of differential scanning calorimetry (DSC) of the poly-R-hydroxyalkanoates (PHAs) produced by the addition of avocado oil at (a) 0 (control), (b) 5, (c) 10, (d) 15, (e) 20, and (f) 25 percent [v/v].

oxygen transfer to the cells, thereby delaying synthesis and accumulation of polymers. However, further studies on larger scales are required to analyse the oxygen transfer dynamics.

C. necator H16 is well known to exhibit a preference for synthesizing PHAs containing 3HB units as the most abundant monomer regardless of the carbon source, even from vegetable oils. Some studies have confirmed the synthesis of pure PHB when *C. necator* is grown in vegetable oils [41]. Conversely, Du et al. [42] achieved the production of PHBV using fatty acids from food scraps, whereas Dennis et al. [18] demonstrated that the *C. necator* synthase could accept C6 substrates (Table 3). A few studies have also identified larger monomeric units when using vegetable oils as carbon sources [6, 21].

In the present study, PHAs were composed mainly of 3HB monomers, followed by 3HV ranging from 1 to 7 mol%, and small quantities of 3HA (more than 5 carbons). Interestingly, all samples fed with avocado oil contained identifiable 3HV monomers and the presence of these monomers was highly correlated with the oil concentration in the medium. In some manner, the particular fatty acid composition of the avocado oil seems to promote the formation of 3HV precursors. Although Ganzeveld et al. [19] used centrifuged fermented organic waste and Liu et al. [20] employed palm oil to produce the same copolymer reported in this work (PHBV), the incorporation of 3HV monomers has not been sufficiently investigated when vegetable oils are used as the substrate (Table 3).

The thermal properties of the PHAs were enhanced by the presence of 3HV monomers in the polymer. As reported by Babel and Steinbüchel [11], T_m depends on the percentage of 3HV incorporated into the polymer. The melting temperature of the synthesized PHAs ranged from 159 to 173°C, in accordance with the 3HV mol% content (1 to 7 mol%). The presence of 3HV units in the polymer and even the small quantities of other 3HA improved the general thermal properties of PHA. Consequently, avocado oil as the substrate for PHA synthesis promoted the production of a more versatile material than what was obtained with pure PHB [43, 44], as demonstrated by DSC, FTIR, and GC analysis.

5. Conclusions

C. necator H16 is capable of incorporating small quantities of 3HV units into a PHA copolymer when avocado oil is used as substrate for PHA synthesis. The PHAs exhibited different monomeric compositions and properties, depending on the concentration of added oil. However, the highest yield, with a greater incorporation of larger monomer units (HV and more), was obtained when 20% (v/v) oil was added. Incorporation ranging from 1 to 7 mol% of 3HV monomeric units into the polymeric main chain was demonstrated. The results confirmed the feasibility of using avocado oil as a renewable carbon source for PHA production processes.

Disclosure

Partial results of this manuscript were presented as an abstract at the 9th Congress of FEBiotec (Annual Congress of Biotechnology).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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