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Research Article

A Process Engineering Approach to Improve Production of P(3HB) by *Cupriavidus necator* from Used Cooking Oil

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Different feeding strategies, namely, exponential feeding and DO-stat mode, were implemented for the production of poly(3-hydroxybutyrate), P(3HB), by *Cupriavidus necator* DSM 428 with used cooking oil (UCO) as the sole carbon source. With the exponential feeding strategy, a cell dry mass of $21.3 \pm 0.9\,g\,L^{-1}$ was obtained, with a polymer content of 84.0 ± 4.5 wt.%, giving an overall volumetric productivity of $4.5 \pm 0.2\,g\,L^{-1}\,day^{-1}$. However, the highest P(3HB) volumetric productivity, $12.6 \pm 0.8\,g\,L^{-1}\,day^{-1}$, was obtained when the DO-stat mode was implemented together with the use of ammonium hydroxide for pH control, which served as an additional nitrogen source and allowed to reach higher cell dry mass ($7.8 \pm 0.6\,g\,L^{-1}$). The P(3HB) obtained in all experiments had a high molecular mass, ranging from 0.6×10^5 to $2.6 \times 10^5\,g\,mol^{-1}$, with low polydispersity indexes of 1.2-1.6. Melting and glass transition temperatures were also similar for the polymer produced with both cultivation strategy, 174°C and 3.0-4.0°C, respectively. The polymer exhibited a crystallinity ranging from 52 to 65%. The DO-stat strategy to feed oil containing substrates as the sole carbon sources was reported for the first time in this study, and the preliminary results obtained show that it is a promising strategy to improve P(3HB) production. Nevertheless, the process requires further optimization in order to make it economically viable.

1. Introduction

The cultivation strategy used for the production of polyhydroxyalkanoates (PHA) is an important factor to be taken into account for bioprocess optimization and improvement. Although the batch mode is the simplest and primary strategy for any bioprocess [1, 2], and it has been the most extensively used strategy for PHA production [3], the fed-batch mode is considered more efficient to achieve high cell density cultures with high volumetric productivities [1, 3]. However, the process set-up requires the selection of a suitable substrate feeding strategy to properly control the concentration of the carbon source throughout the fed-batch phase. Several strategies are available to feed the cultures and have been tested for PHA production from different substrates, including, among others, pulse feeding [4, 5], continuous feeding with defined feed rate [6, 7], exponential feeding [8, 9], control of nutrient feed through dissolved oxygen (DO) concentration (DO-stat mode) [10–12], and pH control (pH-stat mode) [13, 14].

The use of feeding profiles designed to match the maximum specific cell growth rate of the microorganism during the growth phase and linear or decaying linear feed rates during the nongrowth-associated production phase are often used [15]. The exponential feeding strategy has been successfully used for PHA production by *Cupriavidus necator* DSM 545 [9] and *Pseudomonas putida* KT2440 [15], using glucose as carbon source. Rathinasabapathy et al. [16] also tested an exponential feeding strategy for cultivation of *C. necator* in fructose and canola oil. However, adjusting the feeding

profile to the culture's needs in terms of substrate is a difficult task and the defined profile may result in over- or underfeeding [9].

Feeding may also be based on physiology as in pH-stat and DO-stat modes where it depends on acid production or oxygen utilization, respectively [15, 17]. Since this strategy is based on a parameter that is measured online (i.e., the DO concentration or the pH value), it allows for the substrate to be automatically fed to the culture. The DO-stat strategy has been tested for cultivation of different PHA producers, such as recombinant *Escherichia coli* strains [18], *Alcaligenes latus* DSM1123 [19], *Cupriavidus* sp. USMAA2-4 [20], and *Cupriavidus* sp. DSM19416 [11]. A strategy combining DO- and pH-stat modes was also reported to feed oleic acid to *Aeromonas hydrophila* [21] and *Pseudomonas putida* KT2442 [22] to overcome the fact that, for high cell densities, the DO concentration did not respond to substrate depletion. There are some reports on the cultivation of *C. necator* using the DO-stat mode, either alone or in combination with the pH-stat mode, to feed spent coffee grounds oil [10] or a mixture of acetic, propionic, and butyric acids [22] to the culture in the fed-batch phase. However, this strategy has never been reported for the cultivation of *C. necator* using used cooking oil (UCO) as sole substrate.

In the previous work, UCO was demonstrated to be a suitable substrate for the cultivation of *C. necator* DSM 428 for production of P(3HB) [23, 24]. However, process optimization conditions were not explored. In this study, two different fed-batch strategies were tested for the first time, namely, exponential feeding and DO-stat mode, to feed UCO to the culture, aiming at improving polymer productivity and yield on a substrate basis. The impact of the tested cultivation strategies on the polymer's composition and molecular mass distribution was evaluated.

2. Material and Methods

2.1. Microorganism and Media. *C. necator* DSM 428 was reactivated from stock cultures kept at −80°C by inoculation in solid Luria Bertani (LB) medium (15 g L^{-1} agar), as described by Cruz et al. [10]. The mineral medium used for inoculum preparation and the bioreactor experiments had the following composition (per liter): $(NH_4)_2HPO_4$, 3.3 g; K_2HPO_4, 5.8 g; KH_2PO_4, 3.7 g; 10 mL of a 100 mM $MgSO_4$ solution; and 1 mL of a micronutrient solution. The micronutrient solution had the following composition (per liter of 1 N HCl): $FeSO_4{\cdot}7H_2O$, 2.78 g; $MnCl_2{\cdot}4H_2O$, 1.98 g; $CoSO_4{\cdot}7H_2O$, 2.81 g; $CaCl_2{\cdot}2H_2O$, 1.67 g; $CuCl_2{\cdot}2H_2O$, 0.17 g; and $ZnSO_4{\cdot}7H_2O$, 0.29 g [25]. The mineral medium was supplemented with 20 g L^{-1} UCO as sole carbon source. The UCO used in this study was supplied by the University canteen, and it was mainly composed of triglycerides (83.4 ± 9.1 wt.%), with minor amounts of di- and monoglycerides (6.7 ± 0.4 and 0.4 ± 0.1 wt.%, respectively). Oleic and linoleic acids were the major constituent fatty acids of UCO (37.5 ± 0.6 and 49.8 ± 0.5 wt.%, respectively), with minor contents of palmitic and stearic acids (9.0 ± 0.1 and 3.4 ± 0.6 wt.%, respectively) [26].

2.2. Bioreactor Cultivation. Bioreactor cultivation experiments were performed in 2 L bioreactors (BIOSTAT B-Plus, Sartorius, Germany), with an initial working volume of 1.5 L. The inoculum was 10% (*v*/*v*) of the initial reactor working volume. It was prepared by inoculating a single *C. necator* colony into the LB medium and incubation in an orbital shaker, at 30°C and 200 rpm, for 24 hours. The culture thus obtained was then transferred into the mineral medium supplemented with 20 g L^{-1} UCO as sole carbon source, further incubated for 48 hours, as described above, and used as inoculum for the bioreactor experiments.

In all experiments, the temperature was maintained at 30 ± 1°C and the pH was controlled at 6.8 ± 0.2 by the automatic addition of NaOH 2 M and/or 25% (*v*/*v*) NH_4OH. The dissolved oxygen concentration (DO) was maintained at 30% air saturation. The experiments comprised an initial batch phase (18-20 hours), followed by the fed-batch phase, wherein the UCO was supplied to the culture.

In experiment A (exponential feeding), UCO was fed according to the following profile:

$$F_s(t) = q_s \times X \times e^{\mu\left(t-t_f\right)}, \quad (1)$$

where $F_s(t)$ is the feeding rate (g UCO h^{-1} L^{-1}), q_s (g_s g_x^{-1} h^{-1}) is the specific substrate uptake rate, X (g L^{-1}) is the active biomass concentration at the end of the exponential phase, μ (h^{-1}) is the specific growth rate, t (h) is the initial time of feeding, and t_f (h) is the end of batch time, respectively. The pH was controlled by the addition of NaOH 2 M throughout the entire experiment.

In experiment B (DO-stat mode), the UCO feeding flow rate was automatically controlled as a function of DO concentration (under a constant stirring of 500 rpm). The pH was initially controlled by the addition of NH_4OH to prolong the exponential growth phase by serving also as a nitrogen source. Afterwards, the pH was controlled with NaOH 2 M to impose nitrogen-limiting conditions.

Samples (15 ± 5 mL) were periodically withdrawn from the bioreactor for determination of the cell dry mass (CDM), UCO concentration, P(3HB) content in the biomass, and polymer composition.

2.3. Polymer Extraction and Purification. At the end of the cultivation runs, the broth (100 mL) was washed with n-hexane (1 : 1, *v*/*v*) to remove residual oil and centrifuged (7012 × *g*, 20 min). The biomass was further washed twice with deionized water (200 mL) and freeze-dried. The polymer was recovered from the dried biomass by Soxhlet extraction with chloroform (~10 g dry biomass extracted with 250 mL chloroform), at 70°C, for 24 hours. The solution thus obtained was filtered with 0.45 μm pore size filters (GxF, GHP membrane, PALL) to remove cell debris and precipitated in cold ethanol (1 : 10, *v*/*v*) under strong stirring. The polymer was collected by centrifugation (7012 × *g*, 20 min), dried at room temperature, and stored at 4°C.

2.4. Analytical Techniques. For CDM and residual oil quantification, 4-5 mL broth samples were mixed with *n*-hexane (1 : 1, *v*/*v*) and centrifuged (15,777 × *g*, 10 min). The biomass

pellet was collected, washed with deionized water, and lyophilized for the gravimetric CDM quantification. The upper hexane layer (2-3 mL) containing the residual UCO was transferred to preweighed tubes and placed in a fume hood at room temperature for 24 h, for solvent evaporation and oil quantification. All analyses were performed in duplicate.

Polymer content in the biomass was determined as described by Cruz et al. [26]. Briefly, 2-3 mg dried cells were hydrolyzed with 1 mL 20% (*v*/*v*) sulphuric acid in methanol and 1 mL benzoic acid (internal standard) in chloroform (1 g L^{-1}), at 100°C, during 3.5 hours. The resulting methyl esters were analyzed by gas chromatography (GC), as described by Cruz et al. [26]. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV), poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate), P(3HHx-*co*-HO), and poly(3-hydroxyocatonate-*co*-3-decanoate-*co*-3-dodecanoate), P(3HO-*co*-3HD-*co*-3HDd), in concentrations ranging from 0.325 to 5 mg mL^{-1} were used as standards.

2.5. Calculations. The active biomass was determined by

$$X_t = \mathrm{CDM}_t - \mathrm{P(3HB)}_t, \tag{2}$$

where CDM_t (g L^{-1}) and $\mathrm{P(3HB)}_t$ (g L^{-1}) are the cell dry mass and the concentration of polymer at time t (h). The overall volumetric productivity (r_p, g L^{-1} day^{-1}) was calculated by the following equation:

$$r_p = \frac{\Delta P}{\Delta t}, \tag{3}$$

where ΔP (g L^{-1}) is the polymer produced during cultivation time Δt (day). The growth ($Y_{x/s}$, g_x g_s^{-1}) and storage ($Y_{p/s}$, g_p g_s^{-1}) yields were calculated, respectively, by

$$Y_{x/s} = \frac{\Delta X}{\Delta S}, \tag{4}$$

$$Y_{p/s} = \frac{\Delta P}{\Delta S}, \tag{5}$$

where ΔX (g L^{-1}) and ΔP (g L^{-1}) are the active biomass and the polymer, respectively, produced during the run, and ΔS (g L^{-1}) is the UCO consumed for the same period of time.

2.6. Polymer Characterization. Polymer composition and purity were evaluated by GC analysis, as described above. Weight average ($\bar{M}_w$) and number average ($\bar{M}_n$) molecular mass were determined using a size exclusion chromatography (SEC) apparatus (Waters), equipped with a solvent delivery system composed of a model 510 pump, a Rheodyne injector, and a refractive index detector (Waters 2410), according to the procedure described by Cruz et al. [26]. The polydispersity index (PDI) was given by the ratio between ($\bar{M}_w$) and ($\bar{M}_n$).

The thermal properties of the polymers were determined by differential scanning calorimetry (DSC), as described by Morais et al. [27]. The glass transition temperatures (T_g, °C) were taken as the midpoint of the heat flux step transition; melting (T_m, °C) temperature and enthalpy (ΔH_m, J g^{-1}) were estimated, respectively, from the center and area of the endothermic peaks. The crystallinity (X_c, %) of the PHA samples was estimated as the ratio between ΔH_m associated with the detected melting peak and the melting enthalpy of 100% crystalline poly-3-hydroxybutyrate, P(3HB), estimated as 146 J g^{-1} [27].

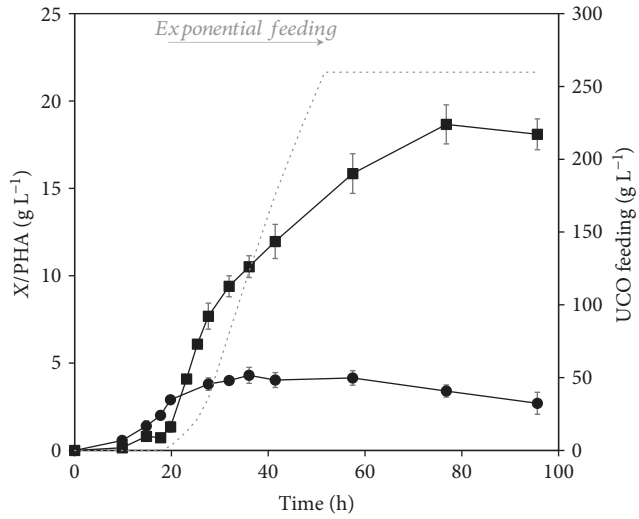

Figure 1: Production of PHA (■) and active biomass (X) (●) by *C. necator* cultivated in fed-batch mode with an exponential feeding profile (UCO feeding).

3. Results and Discussion

3.1. Fed-Batch Cultivation with Exponential Feeding Profile. The results obtained in experiment A, wherein the exponential feeding strategy was tested, are presented in Figure 1 and Table 1. During the initial batch phase of the experiment, the culture grew with a maximum specific cell growth rate of $0.14 \pm 0.02\ h^{-1}$, which is similar to the values obtained in previous studies for cultivation of *C. necator* under batch mode, 0.12-0.14 h^{-1} [23, 24].

The fed-batch phase was initiated after 20 hours of cultivation (Figure 1) by supplying the culture with UCO under the defined exponential profile. The exponential feeding profile was designed aiming at maintaining the specific cell growth rate observed during the batch phase ($0.14 \pm 0.02\ h^{-1}$) for an extended period of time during the fed-batch phase, thus increasing the overall biomass production. However, the oil feeding was stopped at 55 hours of cultivation since accumulation of unconsumed UCO was noticed. The experiment was further prolonged up to 96 hours to allow the culture to use the accumulated UCO. This UCO accumulation in the bioreactor might have been caused by an overestimation of the feeding profile that was based on data obtained in previous batch experiments [23]. This dependency of the designed feeding profile on previously determined data is a disadvantage of this strategy since it is more prone to result in inadequate feeding of the culture.

Table 1: Parameters obtained for cultivation of *C. necator* DSM 428 under fed-batch mode using an exponential feeding profile (experiment A) and the DO-stat mode (experiment B) to feed UCO to the culture. μ_{max}: maximum specific cell growth rate; X: active biomass; CDM: cell dry mass; P(3HB) content: polymer content in the biomass; P(3HB): polymer concentration; r_p: volumetric productivity; $Y_{x/s}$: growth yield; $Y_{p/s}$: storage yield.

Exp.	Feeding strategy	μ_{max} (h^{-1})	X ($g\,L^{-1}$)	CDM ($g\,L^{-1}$)	P(3HB) content (wt%)	P(3HB) ($g\,L^{-1}$)	r_p ($g\,L^{-1}\,day^{-1}$)	$Y_{x/s}$ ($g_x\,g_s^{-1}$)	$Y_{p/s}$ ($g_p\,g_s^{-1}$)
A	Exponential profile	0.14 ± 0.02	3.4 ± 0.4	21.3 ± 0.9	84.0 ± 4.5	17.9 ± 0.9	4.5 ± 0.2	0.11 ± 0.01	0.65 ± 0.03
B	DO-stat	0.21 ± 0.01	7.8 ± 0.6	27.2 ± 0.5	77.0 ± 5.7	19.8 ± 1.8	12.6 ± 0.8	0.21 ± 0.02	0.52 ± 0.07

A final polymer production of $17.9 \pm 0.9\,g\,L^{-1}$ was obtained, corresponding to an overall volumetric productivity of $4.5 \pm 0.2\,g\,L^{-1}\,day^{-1}$ (Table 1). These values are considerably higher than the ones obtained under batch cultivation of *C. necator* with UCO, namely, a polymer production of 3.8-7.4 $g\,L^{-1}$ and a volumetric productivity of 3.4-3.6 $g\,L^{-1}\,day^{-1}$ [23, 24]. The improved polymer production and productivity were a result of the higher polymer content in the biomass that reached 84.0 ± 4.5 wt.% at the end of the cultivation, while under the batch mode it had reached only 38-63 wt% [23, 24]. The polymer content in the biomass obtained in experiment A is also slightly higher than the values (72-81 wt.%) reported for the fed-batch cultivation of *C. necator* using soybean oil, with pulse feeding [5, 28].

There was an overall oil consumption of $28.0 \pm 1.3\,g\,L^{-1}$ during the 96 hours of experiment A. The growth and storage yields were $0.11 \pm 0.01\,g_x\,g_s^{-1}$ and $0.65 \pm 0.03\,g_p\,g_s^{-1}$, respectively. The storage yield obtained in experiment A was within the values obtained under batch mode (0.29-0.70 $g_p\,g_s^{-1}$) [23, 24] and close to the values reported for the fed-batch cultivation of *C. necator* with pulse feeding of soybean oil (0.72-0.85 $g_p\,g_s^{-1}$) [5, 28].

The results obtained with experiment A show that the exponential feeding strategy tested for the fed-batch cultivation of *C. necator* with UCO allowed for improvement of P(3HB) production compared to the batch mode operation. However, it was difficult to adjust the feeding profile to the actual culture's substrate consumption and an overfeeding resulted in UCO accumulation in the bioreactor. Mozumder et al. [9] also reported the difficulty in maintaining the exponential glucose profile feeding adequate to the culture's needs in terms of substrate, and the defined profile resulted in over- or underfeeding. Nevertheless, the exponential feeding strategy might be more successful when used in combination with other feeding strategies, for example, based on the monitoring of other parameters, such as suggested by Mozumder et al. [9].

3.2. Fed-Batch Cultivation under a DO-Stat Mode. In an attempt to have a feeding strategy that more accurately matched the culture's requirements for cell growth and polymer synthesis, the DO-stat mode was tested in experiment B. This strategy is based on the online measurement of the DO concentration, which tends to increase upon substrate depletion, thus signaling the automatic feeding of UCO to the culture. Figure 2 presents the results obtained for the fed-batch cultivation of *C. necator* under the DO-stat mode. The culture grew with a specific growth rate of $0.21 \pm 0.01\,h^{-1}$ and reached an active biomass of $5.9 \pm 1.1\,g\,L^{-1}$ within 7 hours of cultivation. The higher cell growth rate compared to experiment A was due to the fact that the pH was controlled with ammonium hydroxide, which served as an additional nitrogen source. The nitrogen availability during the initial batch phase changed the carbon-to-nitrogen ratio and promoted a faster cell growth.

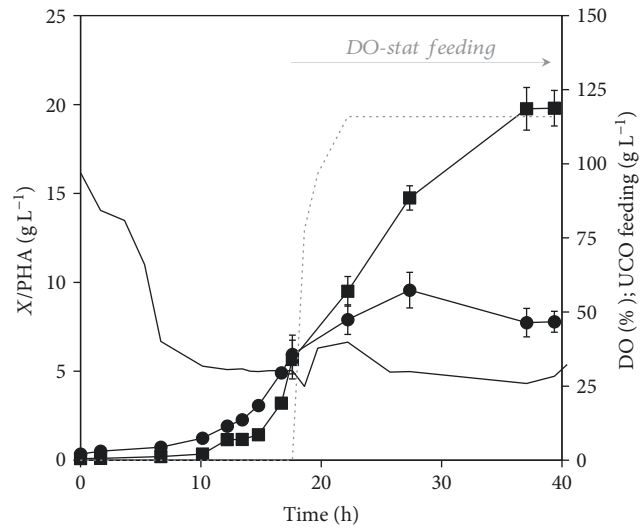

Figure 2: Production of PHA (■) and active biomass (●) production by *C. necator* cultivated under DO-stat mode (experiment B), in which UCO was automatically fed (….) as a function of the DO concentration (——) that was kept at 30% air saturation.

The DO-stat mode was implemented at 17 hours of cultivation by starting the substrate feeding as a function of the DO concentration that was set at 30% of air saturation. At that time, NH_4OH was changed to NaOH for pH control so that nitrogen-limiting conditions were imposed. The culture continued to grow, though at a lower rate, and reached a maximum active biomass of $9.6\,g\,L^{-1}$, at 27 hours of cultivation (Figure 2). No further cell growth was noticed afterwards as a result of nitrogen exhaustion.

The automatic substrate feeding rate was very high during the first 5 hours after initiating the DO-stat control mode, as a response to the rise in DO concentration (Figure 2). Overall, $116\,g\,L^{-1}$ of UCO entered the reactor. Afterwards, the DO concentration remained rather constant (26-30%) and no further substrate feeding occurred.

Table 2: Physical-chemical and thermal characterization of P(3HB) produced by *C. necator* from UCO and comparison to P(3HB) produced from other oil-containing substrates.

Cultivation mode	Feeding strategy	Substrate	M w ($g\,mol^{-1}$) $\times 10^5$	PDI	T_m (°C)	T_g (°C)	ΔH_m ($J\,g^{-1}$)	X_c (%)	References
Batch	—	UCO	2.6	1.6	172	3.0	n.a.	n.a.	[24]
		SCG oil	4.3-4.7	2.2-2.5	n.a.	n.a.	n.a.	n.a.	[33]
		Margarine waste	n.a.	n.a.	173	7.9	83	57	[27]
Fed-batch	Exponential feeding	UCO	0.6	1.2	174	3.0	95	65	This study Experiment A
	DO-stat mode	UCO	2.6	1.6	174	4.0	75	52	This study Experiment B
		SCG oil	2.3	1.2	172	8.4	n.a.	58	[10]

n.d.: not detected; n.a.: data not available.

Polymer accumulation was initiated during the batch phase, but increased production occurred during the fed-batch phase (Figure 2). Maximum polymer concentration ($19.8 \pm 1.8\,g\,L^{-1}$) was reached at 37 hours of cultivation, corresponding to an overall volumetric productivity of $12.6 \pm 0.8\,g\,L^{-1}\,day^{-1}$ (Table 1). This value is considerably higher than that obtained in experiment A ($4.5 \pm 0.2\,g\,L^{-1}\,day^{-1}$) and within the wide range of values (6.3-25 $g\,L^{-1}\,day^{-1}$) reported for fed-batch cultivations of the same strain with pulse feeding of soybean oil [28] or jatropha oil [29].

A lower volumetric productivity ($4.7\,g\,L^{-1}\,day^{-1}$) was reported in a previous study, in which *C. necator* was cultivated using spent coffee grounds (SCG) oil as substrate, under the DO-stat mode [10]. The observed differences are probably related to the substrate used in each study. In fact, although both oils were rich in linoleic acid (49.8 and 38.4 wt.%, respectively), UCO had a higher content of oleic acid (37.5 wt.%) [26], while SCG oil was richer in palmitic acid (39.4 wt.%) [10]. Cell growth and polymer synthesis may have been stimulated in experiment B by the presence of higher contents of linoleic and oleic acids in UCO, since both fatty acids are known to be preferred carbon sources for *C. necator* cultivation [10, 28, 29]. Moreover, the presence of other components in SCG oil, such as sterols, tocopherols, and esters, may have also impacted on the culture's performance.

Although the overall consumption of UCO in experiment B ($38.0 \pm 2.0\,g\,L^{-1}$) was higher than that observed for experiment A ($28.0 \pm 1.3\,g\,L^{-1}$), a lower storage yield was observed ($0.52 \pm 0.07\,g\,g^{-1}$) (Table 1). It is likely that more carbon has been deviated for cell growth and maintenance, which is in accordance with the higher growth yield ($0.21 \pm 0.02\,g\,g^{-1}$) obtained in experiment B. In fact, by controlling the pH with ammonium hydroxide, cell growth was faster and a higher cell density was reached, which required more substrate to fulfill the cell metabolism needs.

These results demonstrate that the DO-stat strategy was successful for feeding UCO to the culture, resulting in improved volumetric productivity. A huge advantage of this strategy is that it allows for the substrate to be automatically fed to the culture as a function of parameter that is measured online (i.e., the DO concentration). This strategy has been reported in some studies for the production of different PHA polymers. Faezah et al. [11] reported the use of the DO-stat mode to feed oleic acid and/or γ-butyrolactone to *Cupriavidus* sp. USMAA1020, as a strategy to regulate the molar fraction of 4-hydroxybutyrate in the P(3HB-*co*-4HB) polymer. The same strategy was tested by Kim et al. [12] to feed fructose and/or γ-butyrolactone to *C. necator* ATCC 17699. A combination of pH-stat with DO-stat strategies was also proposed by Huschner et al. [30] for feeding a mixture of sodium salts of acetic, propionic, and butyric acids to *C. necator* H16. There are no reports on the use of the DO-stat strategy to feed UCO or other oils containing substrates as the sole carbon sources for the fed-batch cultivation of *C. necator*.

3.3. PHA Characterization. The polymer produced from UCO in this study was a 3-hydroxybutyrate homopolymer, poly(3-hydroxybutyrate), P(3HB) (Table 2), which is in accordance with the literature reports for polymers produced by *C. necator* when cultivated in oil-containing substrates as sole carbon sources, under different cultivation modes [5, 27–29, 31].

Fed-batch cultivation under the DO-stat mode (experiment B) resulted in a polymer with a molecular weight of $2.6 \times 10^5\,g\,mol^{-1}$ (Table 2). Values of the same order of magnitude were reported for P(3HB) synthesized by *C. necator* from UCO under batch conditions ($2.6 \times 10^5\,g\,mol^{-1}$) [24] or pulse feeding (2.0×10^5–$20 \times 10^5\,g\,mol^{-1}$) [32]. The values are also similar to the polymer produced by the same culture from SCG oil under pulse feeding (2.3×10^5–$4.7 \times 10^5\,g\,mol^{-1}$) [33] and with a DO-stat mode [10].

A lower molecular weight value ($0.6 \times 10^5\,g\,mol^{-1}$) was obtained for the P(3HB) produced in experiment A, with UCO exponential feeding (Table 2). This low value might be related to the prolonged cultivation time (96 hours). According to Budde et al. [34], PHA is continuously turned over by *C. necator*, which is accompanied by a decrease in average polymer molecular mass. This might justify the observed decrease in the average molecular weight given the long cultivation time of the experiment. On the other hand, the polydispersity index (PDI) of the polymers was found to be lower (1.2-1.6) than those reported for P(3HB) obtained from SCG oil (2.2-2.5) [33], meaning that the P(3HB) produced in this study was highly homogeneous.

Thermal properties were also assessed after polymer extraction and purification. A melting temperature of 174°C was determined for the polymers produced in experiments A and B (Table 2). Similar values (172-173°C) were reported for P(3HB) produced from UCO [24], margarine waste [27], and SCG oil [10]. The glass transition temperatures (3.0-4.0°C) were also within the range of values reported for P(3HB), 3.0-8.4°C. According to Laycock et al. [35], melting and glass transition temperatures of P(3HB) typically range between 162-181 and −4 and 18°C, respectively, depending on the bacterial strain, carbon source, polymer extraction and purification procedures, etc.

The polymer's crystallinity was 65 and 52% for experiments A and B, respectively. The simultaneous detection of a glass transition and melting, which are thermal events associated, respectively, with the amorphous and crystalline regions in the polymer, shows that the P(3HB) produced in both experiments is a semicrystalline material. The crystallinity of the polymer depends on many factors, including its chemical structure, intermolecular interactions, and processing conditions, but it is commonly between 55 and 80% [35], roughly including the crystalline degrees determined for the produced P(3HB).

4. Conclusions

Improved P(3HB) volumetric productivity was obtained with the implementation of a DO-stat strategy to feed UCO to *C. necator* DSM 428 during the fed-batch phase of a bioreactor cultivation. This strategy was tested for the first time, and it resulted in adequate substrate feeding for cell growth and polymer synthesis. A high molecular weight homogeneous homopolymer, with thermal properties similar to the P(3HB) synthesized by *C. necator* under different cultivation conditions and substrates, was obtained. The results of these preliminary studies show that the DO concentration can be used as a reliable online parameter for the automatic feeding of UCO to *C. necator* for P(3HB) production and can be used to improve the overall economic viability of the process.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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