Impact of Different Bacterial Strains on the Production, Composition, and Properties of Novel Polyhydroxyalkanoates Using Crude Palm Oil as Substrate

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Polyhydroxyalkanoates (PHAs) are a group of biodegradable polymers produced from renewable sources by prokaryotic biocatalysts, accumulated intracellularly for energy and carbon storage. In the present study, production and characterization of PHAs synthetized by *Cupriavidus necator* (IPT 026 and IPT 027) and *Burkholderia cepacia* (IPT 119 and IPT 400) were evaluated using crude palm oil ($C_{16:0}$ = 26.44 %, $C_{18:1}$ = 54.50 %, $C_{18:2}$ = 13.41 %) as substrate (15 g L^{-1} crude palm oil, pH 7.0, 180 rpm, 72 h). All strains were able to synthesize novel PHA copolymers (0.10–1.45 g L^{-1}), and IPT 027 displayed the highest production. Copolymers monomeric composition (M_w = 173.78–389.30 kDa) was comprised mostly of hydroxyhexadecanoate (41.43–53.15 %) and hydroxy-9-octadecenoate (14.91–29.61 %). PHAs were predominantly amorphous, showed low polydispersity, and good thermal stability ($T_{onset} \geq 283$ °C), which increased proportionally to crystallinity. Crude palm oil constitutes an emerging alternative for PHAs production, and microorganism strains strongly affect polymer accumulation, monomeric composition, molar mass, and properties.

Key words:
Elaeis guineensis, bioconversion, PHA, characterization

Introduction

Polyhydroxyalkanoates (PHAs) are a group of bio-polyesters that have been largely studied due to their physicochemical characteristics and biodegradability. Their properties are comparable to those exhibited by traditional synthetic polyesters (petroleum based) and they are decomposed by naturally occurring prokaryotes, such as archaea and bacteria, into carbon dioxide and water[1,2,3].

The biopolymers synthesis occurs by numerous prokaryotes, intracellularly. PHAs yields and properties are very dependent on the synthesizer microbe, commonly, a feast/famine feed strategy is used to enrich PHA accumulation within the producer microorganism cells, stimulating growth phase (feast period) and then shifting to favor polymer accumulation (famine period), where limitation of at least one nutrient necessary for cell multiplication (such as N, P, Mg or Fe) is applied[4,5].

High costs of PHAs production is a limiting factor to their applications and competitiveness against synthetic plastics. Therefore, these expenses should be reduced to enhance economical sustainability. An approach to attenuate this problem is to use alternative and cheap substrates for the bioconversion, since customary ones such as sucrose, glucose and starch represent a major expense in the biomaterials production chain, among other problematic matters associated with their use[6,7].

Substrates such as sugarcane molasses, fermented cheese whey, hemicellulose hydrolysates, and industrial wastes are being reported as alternative low-cost carbon sources for PHA production[8,9,10]. Additionally, plant oil biomass and its related products have been reported to be very suitable carbon sources for PHA synthesis using a variety of microorganisms, revealing to be better substrates for the polymer production than sugars, carbon sources more traditionally used[11,12,13,14].

Plant oils contain higher carbon content per weight than sugars, suggesting a PHA yield of at least two times higher[15]. They are catabolized via β-oxidation cycle to produce polymers of different chain lengths[12]. The fatty acids are first converted to enoyl-CoA in the mentioned cycle, and then transformed to R-3-hydroxyacyl-CoA, polymerization precursor, by R-3-hydroxyacyl-CoA hydratase. Lastly, PHA polymerization is catalyzed by PHA synthase[16,17].

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Palm oil is one of the most important crops of agricultural oil production in the world, since its yield is about five times greater than that of oilseeds, such as soybean and rapeseed, per unit of planted area[18].

Although the palm oil industry causes some controversies due to its impact on the environment and ecosystems, the rising and unyielding pressure of population growth and the need for food, biofuels and bioplastics motivate an immediate step for good management practices of environmentally friendly operations (plantation and milling) while intensifying the oil yields for a more sustainable production and fulfilling the future demand[19,20].

In this context, the present study aimed to produce and characterize PHA using crude palm oil (*Elaeis guineensis*) as an alternative substrate for the submerged cultivation of whole cell biocatalysts *Burkholderia cepacia* (IPT 119 and IPT 400) and *Cupriavidus necator* (IPT 026 and IPT 027), intending to implement more viable biotechnological methods following market demand for biopolymers with suitable technological properties.

Materials and methods

Bacterial strains

Cupriavidus necator IPT 026 and IPT 027, and *Burkholderia cepacia* IPT 400 and IPT 119 were supplied by the Institute for Technological Research (IPT), in São Paulo, Brazil. The bacteria were grown at 33 °C in nutrient broth, maintained on nutrient agar (NA) at 4 °C, and transferred to new plates every 15 days.

Main carbon source

Crude palm oil (*Elaeis guineensis*) was acquired in the trade market of Salvador-BA, Brazil and stored at 4 °C, protected from light, for preservation.

Chemical composition of the substrate

Total carbon (C) content was determined according to Nelson and Sommers[21] publication. Fatty acid profile was determined by the capillary column gas chromatographic method according to Joseph and Ackman[22] and Nascimento *et al.*[23] Separation of the methyl esters in the fatty acids was performed using gas chromatography (Varian 3800) with a flame ionization detector (GC–FID) and a fused silica gas chromatography capillary column EliteWAX (30 m × 0.32 mm × 0.25 μm). Quantification of fatty acids, expressed in milligrams per 100 g sample, was performed by the addition of an internal standard (C23:0 Sigma®, USA) according to Joseph and Ackman[22] and calculated using Eq. (1).

$$\text{Concentration}_{(\text{mg/100 mg sample})} = \frac{A_{FA} \cdot M_{IS} \cdot F \cdot C_{TL}}{A_{IS} \cdot M \cdot F_{FA}} \cdot 1000 \quad (1)$$

where:

AFA = area of fatty acid methyl ester peak in the chromatogram of the sample;

MIS = weight (in milligrams) of the internal standard added to the sample;

F = correction factor of fatty acid methyl ester to fatty acid;

CTL = percentage composition of total lipids from the sample;

AIS = area of internal standard fatty acid methyl ester peak in the chromatogram of the sample;

M = sample mass (in milligrams);

FFA = correction factor response of each fatty acid methyl ester ionization detector, relative to C23:0.

PHA production

Culture media

Bacteria were stored at 4 °C in nutrient agar (NA) composed of 5.0 g L^{-1} meat peptone, 3.0 g L^{-1} beef extract, and 3.75 g L^{-1} agar. Inoculation was performed in nutrient broth (NB), composed of 5.0 g L^{-1} bacteriological peptone, 3.0 g L^{-1} beef extract, and distilled water, over a period of 24 h. PHA was produced using a two-stage cultivation strategy as described by Wang *et al.*[24] and Campos *et al.*[25]

Mineral media were used for the first culture (FC), with no nitrogen limitation, and as the second culture (SC), with nitrogen limitation. Both FC and SC were composed of nitrilotriacetic acid (0.1 g L^{-1}), ferrous ammonium citrate (0.04 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.1 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.004 g L^{-1}), $(NH_4)_2SO_4$ (nitrogen source, 0.625 g L^{-1} in FC and 0.361 g L^{-1} in SC); $Na_2HPO_4 \cdot 12H_2O$ (1.6 g L^{-1}), KH_2PO_4 (1.6 g L^{-1}) and the main carbon source (15 g L^{-1}). The pH of the media was adjusted to 7.0 with NaOH (10 mol L^{-1}) or HCl (10 mol L^{-1}).

Shaker flask cultivation

Tests were executed in triplicate in 250-mL flasks containing 50 mL of nutrient broth medium and 2 mL of pre-culture inoculum, which was incubated at 30 °C in a shaking flask without baffles for 24 h (best cell concentration, 1011 UFC mL^{-1}) at 150 rpm. FC cultivations were executed in flasks containing 80 mL nitrogen-non-limited mineral me-

dium with addition of 10 % v/v of the inoculum culture and incubated at 30 °C and 150 rpm in orbital shaker for 24 h. SCs were run in flasks containing 80 mL nitrogen-limited mineral medium with the addition of 10 % v/v of FC and incubated at 35 °C and 180 rpm in orbital shaker for 72 h.

PHA recovery and separation

Cell cultures were harvested by centrifugation at 15,700 ×g for 30 min at 5 °C (HITACHI, model CR 22G), washed twice with distilled water, transferred into round bottom flasks (50 mL), and frozen at –80 °C for subsequent lyophilization (LIOBRAS model L101) at –42 °C for 24 h. PHA extraction from the freeze-dried cells was performed using chloroform at 60 °C for 2 h with vigorous stirring on a magnetic stirrer plate with heating (model IKAHS 7)[25]. Biomass and PHA production (obtained after extraction) were calculated using a gravimetric method and expressed in g L^{-1}.

PHA characterization

Fourier transform infrared spectroscopy (FTIR)

PHA functional groups characterization was determined by FTIR spectroscopy (PerkinElmer Spectrum 100, Waltham, Massachusetts, USA) between the wave numbers of 4000 cm^{-1} and 400 cm^{-1} using a single-bounce attenuated total reflection (ATR) accessory with a Zinc selenide (ZnSe) crystal.

Thermal characterization

Thermogravimetric analysis (TGA) (PerkinElmer Model Pyris 1TGA Waltham, Massachusetts, USA) was performed to determine the initial degradation temperature (T_{onset}) and the maximum decomposition temperature (T_{max}). Five milligrams of PHA were placed in a platinum tray (cross-sectional area of $2.47 \cdot 10^{-5}$ m^2) and heated at the rate of 10 °C min^{-1} from 25 °C to 600 °C under a nitrogen flow rate of 40 mL min^{-1}.

X-ray diffraction analysis

Crystallinity and crystal peaks of the PHA sample were measured by X-ray diffraction. The X-ray diffractograms of the samples were obtained on a SHIMADZU (XRD-6000, USA) with graphite-filtered CuKa radiation (λ = 1.5433 Å) operated at 40 kV and 30 mA in the region from 5 to 80° (2θ) at a rate of 2° min^{-1}. The percentage of crystallinity was calculated from the diffracted intensity measured by XRD according to the Vonk's method[26].

Determination of PHA molar mass distribution

Molar masses, expressed by the weight average molar mass (M_w), the number average molar mass (M_n) and the polydispersity index (PDI = M_w/M_n), were obtained by size-exclusion chromatography (SEC) according to Campos *et al.*[25] and Ribeiro *et al.*[27]

High performance liquid chromatography (HPLC, PerkinElmer 200) with an autosampler and refractive index detector (PerkinElmer), a column Shodex KD 807 (30 cm × 78 mm × 5 μm) with an exclusion volume of 2×10^8 and an oven temperature of 35 °C were employed for separation. The polymer samples were dissolved in chloroform to a concentration of 7 mg mL^{-1}. In the mobile phase, chloroform was employed at 1 mL min^{-1}. A standard curve was created using polystyrene standards with a range size of 68–1,670,000 g mol^{-1} (Polystyrene High Mw Standards Kit Polymer Standards Service, USA).

PHA composition

The composition of the hydroxyalkanoates was determined using gas chromatography–mass spectrometry (GC–MS; Clarus 500 PerkinElmer) with the TurboMass software version 4.5.0 and the NIST 98 spectra library. Approximately 0.04 g of the dry PHA was subjected to methanolysis based on the method published by Braunegg *et al.*[28] with the modifications proposed by Brandl *et al.*[29] Analysis conditions were established according to Campos *et al.*[25] The hydroxyalkanoates were identified by comparison of retention time with poly(R)-3-hydroxybutyric acid (Sigma-Aldrich) as the control. The mass spectra were compared with the NIST 98 spectra library.

Data treatment

Data treatment was performed using the tools available in the software Statistica 8.0 (Statsoft Inc., Tulsa). Statistical significance was calculated by Tukey's test.

Results and discussion

Substrate chemical composition and medium carbon nitrogen ratio

Elaeis guineensis is a plant that has its origin in Africa and stands out for having high oil production per unit area, popularly known as oil palm or African oil palm, it is a monocotyledon species of the Arecales order that belongs to the subfamily *Arecoideae*[30]. Oil palm Southeast Asia plantations cover over 107,000 km^2, being a large-scale commer-

cial tree crop with fundamental economic importance[31].

Table 1 presents the chemical composition determined for the crude palm oil used as sole carbon source in the bio-catalyzed submerged cultivations for PHA synthesis studied in this paper. It is possible to observe that the highest fractions in the oil are comprised of Palmitic (C16:0) and Oleic (C18:1ω9) fatty acids. The substrate fatty acids composition is known to exert a major influence in PHA chain length and structure, since they may function as PHA copolymers[32,33].

Crude palm oil fatty acids profile was comprised of 31.28 % saturated fatty acids (Butyric [C4:0], Undecylic [C11:0], Lauric [C12:0], Myristic [C14:0], Palmitic [C16:0], Stearic [C18:0] and Arachidic [C20:0]), 54.92 % monounsaturated fatty acids (Palmitoleic [C16:1ω7], Oleic [C18:1ω9], and Gondoic [C20:1ω9]), and 13.41 % polyunsaturated fatty acid (Linoleic [C18:2 ω6]). Although biomass composition varies significantly depending on a variety of factors, such as plant development stage, growth location, and climate conditions[34], the substrate studied showed a similar composition to that reported by Mozzon *et al.* [35], displaying a high percentage of unsaturated fatty acids.

Table 1 – *Fatty acid profile of the crude palm oil (Elaeis guineensis) used in the submerged cultivation of B. cepacia and C. necator strains for polymer production*

Fatty acid	Average (%)
C4:0	0.23 ± 0.03
C11:0	0.05 ± 0.01
C12:0	0.10 ± 0.09
C14:0	0.33 ± 0.05
C16:0	26.44 ± 0.25
C16:1ω7	0.13 ± 0.02
C18:0	3.65 ± 0.09
C18:1ω9c	53.77 ± 0.46
C18:1ω9t	0.73 ± 0.02
C18:2ω6c	13.41 ± 0.28
C20:0	0.48 ± 0.05
C20:1ω9	0.30 ± 0.08

Table 2 – *PHA and biomass production, and PHA extraction in fermentation of 15 g L^{-1} of palm oil over 72 hours of incubation, pH of 7.0, and rotation of 150 rpm*

Microorganism	PHA (g L^{-1})	Biomass (g L^{-1})	PHA extraction (%)
C. necator IPT 026	1.20 ± 0.06^{b}	2.29± 0.08^{a}	52.55
C. necator IPT 027	1.45 ± 0.16^{a}	2.41 ± 0.14^{a}	60.03
B. cepacia IPT 119	0.43 ± 0.03^{c}	1.44 ± 0.04^{c}	29.63
B. cepacia IPT 400	0.10 ± 0.05^{d}	0.45± 0.09^{d}	22.96

Averages followed by different letters, in the upper right, show differences between strains in the same column, determined by Tukey's test (P<0.05).

For fermentation, the cultivation medium (SC) was supplemented with a limiting $(NH_4)_2SO_4$ supply to obtain a carbon-to-nitrogen ratio (C:N) approximately equal to 114:1. This value is within the C:N range documented in literature for PHA production using the nitrogen limitation approach. Reported C:N values range from 20:1 to 180:1, depending largely on the bacterial strain used for bioconversion[36,37,38].

Palm oil bioconversion

The effect of *Burkholderia cepacia* IPT 400 and IPT 119, and *Cupriavidus necator* IPT 026 and IPT 027, for PHA production was tested in submerged cultivations using crude palm oil as substrate in limiting nitrogen conditions, Table 2. Microorganism and substrate are variables of great importance in PHA bioconversion and their combined influence can largely influence the polymer production sustainability[9].

Thus, it was inferred that, regarding polymer synthesis, *C. necator* IPT 027 presented the highest PHA performance, 1.45 g L^{-1} (Table 2). In general, *C. necator* strains presented a better polymer synthesis activity compared to *B. cepacia*, with an average production 5 times higher. *C. necator* bacteria also presented the best biomass growth; these data reveal better adaptation of these strains to the palm oil substrate offered than that observed for *B. cepacia*.

Polyesters percentage extraction were significantly lower for *B. cepacia* bacteria, revealing that the cultivation conditions established triggered growth phase in a more effective manner than polymer accumulation (Table 2). This result suggests revaluation of the nitrogen limitation strategy (famine period) used for the mentioned strains in this study, indicating that a more severe nitrogen restriction must be set, since the limitation of this nutrient generates a cellular trauma that leads to PHA accumulation[39].

Variance analysis (ANOVA) showed that the different microorganisms studied presented greater impact on biomass production ($F = 273.1$, $p < 0.0001$) than on PHA synthesis ($F = 155.5$, $p < 0.0001$). This phenomenon reflects high variation in the strains development in the same medium and cultivation conditions, revealing very distinct growth behaviors.

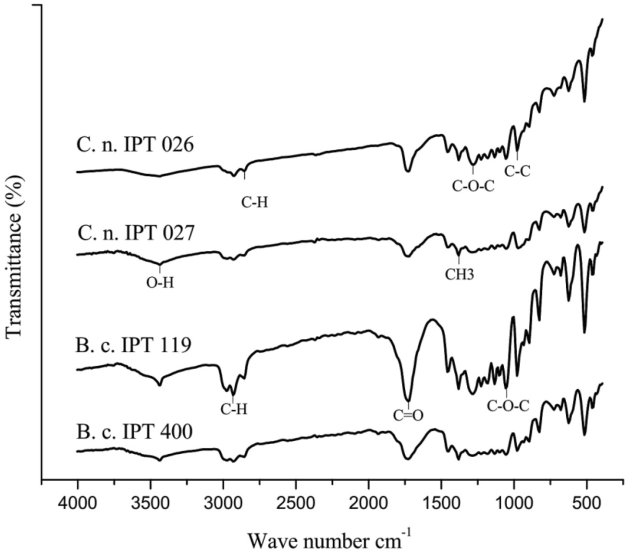

Fig. 1 – *FTIR spectra of PHA produced by C. necator and B. cepacia strains in submerged cultivation with crude palm oil*

Applying the same concentration of oil used in the present study, Wong *et al.*[24] described 2.60 g L^{-1} of PHA production by recombinant *C. necator* Re2160/pCB113 with crude palm kernel oil and 3.30 g L^{-1} with coconut oil (by the same strain). Altaee *et al.*[40] reported 0.54 g L^{-1} of PHA in their culture medium using palm oil as substrate and *Rhodococcus equi* as biocatalyst. Mozejko and Ciesielski[41] published 2.24 g L^{-1} PHA production with saponified waste palm oil and *Pseudomonas sp*. Gl01.

These data evidence and confirm high dependence of PHA production on the microorganism used for the bioconversion and the substrate offered, validating and encouraging the investigations on performance of new microorganisms and cheap carbon sources towards the sustainability of PHA production chain.

Biopolymers characterization

All biopolymers produced by *C. necator* and *B. cepacia* strains in submerged cultivations with crude palm oil were submitted to thermal, chemical and physical characterizations in order to evaluate the properties associated with their natural atomic structures and their capacity to attain standards that would endorse their commercial application.

The polymers functional groups characterization, presented in Fig. 1, was completed by spectroscopy in the infrared spectra (FTIR). All samples scanned showed the characteristic bands documented in the scientific literature related to PHAs structure, confirming the production of these polyesters.

The transmittance observed at 1728 cm^{-1} is associated with the axial deformation of C=O carbonyl group[42]. The C-H carbon-hydrogen bond stretching of CH_3 and CH_2 groups were present at the wave numbers 2931 cm^{-1} and 2854 cm^{-1}, in this order[42,43].

Infrared transmittance at 3440 cm^{-1} was assigned to the hydroxyl group of the polymer chain[45]. The vibration band of the carbonyl of the ester group (C–C) is noted at 972 cm^{-1} [45].

The bands 1048 cm^{-1} and 1288 cm^{-1} are associated, correspondingly, to the asymmetric and the symmetric stretching vibration of the C–O–C group[46]. Wagging CH_3 is observed at the wave number 1381 cm^{-1} [45].

In Fig. 2, it is possible to see the chromatograms obtained by gas chromatography–mass spectrometry of the PHA samples produced by all the strains assessed in this study to determine the polymers monomeric composition, displayed in Table 3. Mass spectra of each peak were compared against NIST library for polymeric units identification.

Identified was the presence of five different monomeric units composing the structure of all copolymers produced in this study (Table 3). All strains evaluated produced polymers with major presence of hydroxyhexadecanoate and hydroxy-9-octadecenoate. It is important to note that the major fractions of fatty acids in the palm oil substrate used in this study were composed of 16 and 18 carbons

Table 3 – *Monomeric profile of the PHAs produced by C. necator and B. cepacia bacteria, using crude palm oil as substrate, obtained by gas chromatography–mass spectrometry in the NIST library*

Retention time (min)	Monomer	Composition of polymer sample (%)			
		IPT 026	IPT 027	IPT 119	IPT 400
6.27	Hydroxytetradecanoate	1.86	4.69	3.61	4.92
7.78	Hydroxyhexadecanoate	47.51	53.15	50.34	41.43
9.75	Hydroxy-9,12-octadecadienoate	5.75	5.47	5.76	8.69
9.93	Hydroxy-9-octadecenoate	29.61	22.07	14.91	26.82
10.38	Hydroxyoctadecanoate	7.58	7.70	8.50	5.76
–	NI	7.69	6.92	16.88	12.38

NI = Not Identified

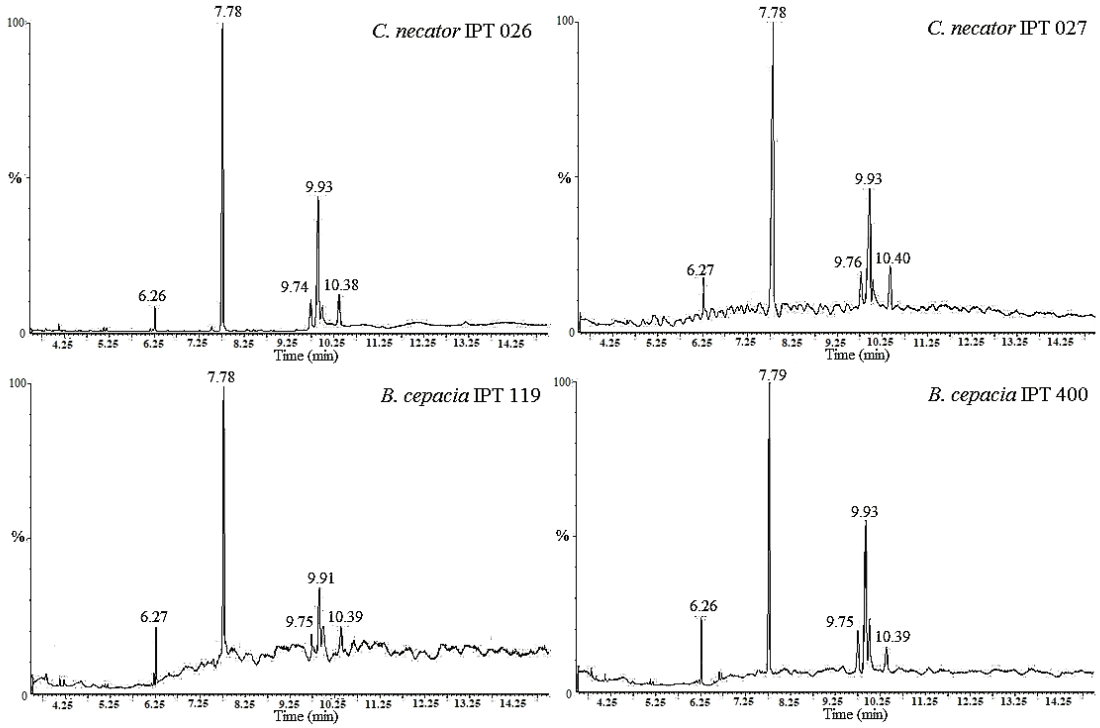

Fig. 2 – *Chromatograms obtained by gas chromatography–mass spectrometry of PHA produced by C. necator and B. cepacia strains in submerged cultivation with crude palm oil*

fatty acids (Table 1). These results indicate that the substrate offered for *C. necator* and *B. cepacia* played a key role in the PHAs compositions.

In concordance with the data exposed, Thompson and He[32], and Srivastava and Tripathi[33] reported that the presence of fatty acids in the fermentative medium is known to greatly influence PHA chain length and structure. It has been published that microorganisms use short, medium, and long chains of fatty acids as carbon sources to obtain polyunsaturated fatty acids, which may function as PHA copolymers.

X-ray diffractograms of the PHAs produced with *C. necator* and *B. cepacia* strains are displayed in Fig. 3, along with their crystallinity index (I_c), which is an indication of the percentage amount of polymer chains organized in the form of crystals in the material structure.

Crystallinity is an important property that largely influences polymer mechanical characteristics, such as yield stress, elastic modulus, and impact resistance. This parameter is also crucial for polymer processing and its desirable value must not be much higher than 50 %, or else brittleness and rigidity starts to cause complications related to the material applications[47,48,49].

It is possible to observe (Fig. 3) the diffraction peaks at 2θ for all polymer samples assessed, and their intensity variations due to the different producer microorganisms from which they originated. Despite that, all PHA presented peaks in 13.56°, 16.96°, 21.89° and 25.52°, typically associated with a semi-crystalline arrangement in polyesters[43].

I_c of the PHAs studied ranged from 48.47 % (*C. necator* IPT 026) to 27.80 % (*B. cepacia* IPT 400), demonstrating that the microorganism strain had significant impact in the polyesters structure arrangement. In general, the biomaterials produced by *C. necator* bacteria exhibited crystallinity indexes

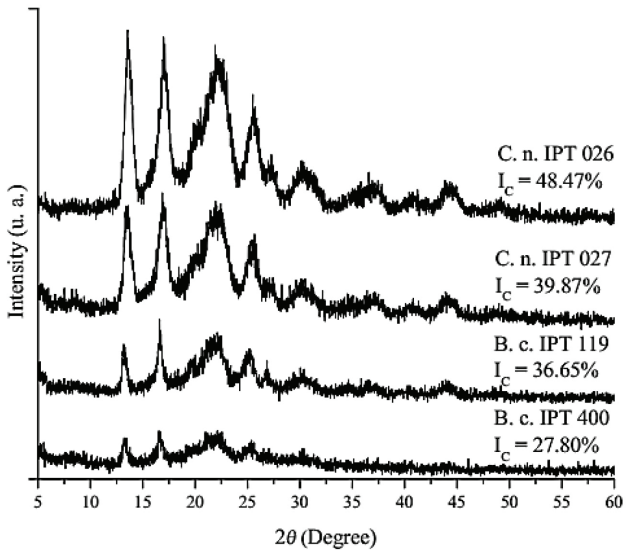

Fig. 3 – *X-ray diffractograms of PHA produced by C. necator and B. cepacia strains in submerged cultivation with crude palm oil*

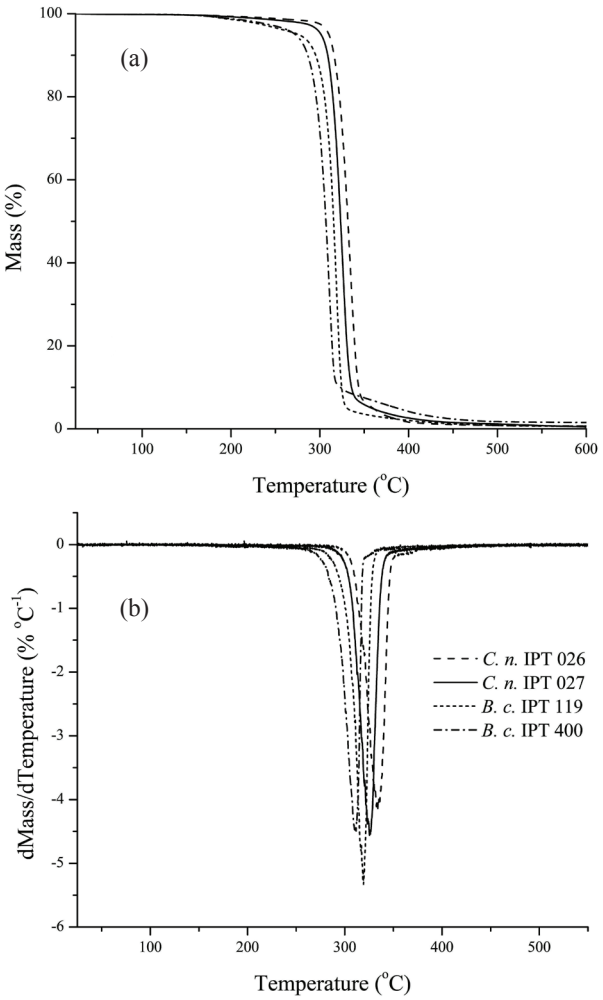

Fig. 4 – *TGAs (a) and DTGs (b) of crude palm oil and PHAs produced by C. necator and B. cepacia strains in submerged cultivation*

superior to those produced by *B. cepacia*, however, all polymers synthesized in this study showed predominant amorphous phase. This indicates good performance concerning their structural morphology in regard of the biosynthesis conditions set and the biocatalysts used[48,49].

The processing of biopolymers is also challenged by their often low thermal stability, since it restricts the temperatures and time by which these materials can be processed[50,51]. PHAs thermal behaviors are displayed in the thermogravimetric curves (TGA) and its associated derivatives (DTGA) in Fig. 4. TGA curves depict the mass variation of the polymer samples as a function of temperature increase, which is related to the loss of volatile components. DTGA curves, first derivatives of the TGA, show the rate of thermal decomposition of the sample.

Thermal degradation of all polymers studied occurred in only one mass loss event (Fig. 4), indicating the presence of no impurities remaining from the extraction and separation processes on those materials[36]. Initial degradation temperatures (T_{onset}) and maximum decomposition temperatures (T_{max}) of the thermogravimetric events obtained from TGA/DTGA curves for the PHAs produced from crude palm oil are presented in Table 4.

Table 4 – *Initial degradation (T_{onset}) and maximum decomposition temperatures (T_{max}), along with total weight loss of PHAs produced by C. necator and B. cepacia strains using crude palm oil*

Microorganism strain	T_{onset} (°C)	T_{max} (°C)	Total mass loss (%)
C. necator IPT 026	311.86	334.42	99.35
C. necator IPT 027	305.38	325.92	99.61
B. cepacia IPT 119	291.29	319.12	99.34
B. cepacia IPT 400	283.70	310.76	98.55

All polymers produced demonstrated initial thermal degradation above 283 °C, with emphasis on the PHA synthetized by *C. necator* IPT 026, which exhibited the best thermal stability with an initial degradation temperature of 311.86 °C, a maximum decomposition temperature of 334.42 °C and a total weight loss of 99.35 %. Similarly to what was found for the last strain, Ribeiro *et al.*[52] reported a PHA with initial degradation temperature of 316.7 °C in the bioconversion (*Burkholderia cepacia* IPT 438) of glycerol, and Campos *et al.*[25] described a T_{onset} of 306.8 °C for PHA produced by *C. necator* IPT 026 in consumption of crude glycerol.

The performances of all biopolymers synthesized in the present study were substantially superior to those found in PHAs produced by *Lysinibacillus* sp. (T_{onset} = 114.7 °C), reported by Mohapatra *et al.*[53], *Pseudomonas pseudoflava* (T_{onset} = 140–170 °C), reported by Reddy *et al.*[1] and *Bacillus megaterium* (T_{onset} = 192.23–281.83 °C), published by Ribeiro *et al.*[27]

Observed was a linear correlation between initial temperature of degradation and crystallinity index for the polymers evaluated (R^2 = 0.91). T_{onset} increases with the increase in I_c according to Eq. (2).

$$T_{onset} = 1.434\ I_c + 243.28 \quad R^2 = 0.9071 \qquad (2)$$

This correlation is reasonable and probably due to the ordered conformation of crystals in the polymer structure, maintained/linked by a group of chemical bonds stronger than those observed for amorphous conformations. Thus, an elevated amount of crystallinity would offer high resistance to thermal degradation once an elevated number of chemical bonds would have to be broken for the polymer to decompose[54].

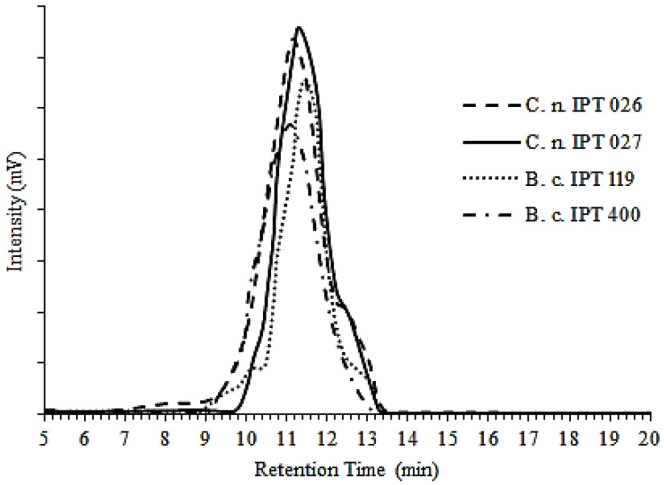

Fig. 5 – *HPLC-RI chromatograms of PHAs produced by the C. necator and B. cepacia strains from crude palm oil in submerged cultivations*

Fig. 5 displays the HPLC-RI chromatograms of the PHAs separation. Size-exclusion chromatography was used to obtain maximum, average and minimum molecular mass (M_w), number average molecular mass (M_n), and polydispersity (PDI) of the PHAs produced in this study, Table 5. A calibration curve was built using standard polystyrene with different values of M_w as a function of column retention time (t). This curve was used to calculate the samples M_w, and is presented in Eq. (3).

$$\log(M_w) = -0.8364t + 14.831 \quad R^2 = 0.9917 \quad (3)$$

The biocatalyst variable played an important role in the weight average molecular mass of the copolymers produced, since this response parameter varied largely depending on the bacterial strain. The highest (389.296 kDa) and the lowest (173.781 kDa) weight average molecular mass was observed for the PHAs produced by *B. cepacia* strains, IPT 400 and IPT 119, respectively.

Gumel *et al.*[55], studying the substrate effect in PHA production from *Pseudomonas putida*, found weight average molecular masses ranging from 55 to 77 kDa, while Ribeiro *et al.*[27], using different crude glycerol sources for PHA production, reported a range of 1400 to 3740 kDa, in submerged cultivations of *Bacillus megaterium*. Pan *et al.*[7] reported M_w of 450.8 kDa by *Burkholderia cepacia* using a detoxified sugar maple hemicellulosic hydrolysate, and Campos *et al.*[25] described a range of 510 kDa to 780 kDa, by *Cupriavidus necator* using crude glycerol. This illustrates the great variety of molecular masses reported in literature in response to the use of different biocatalysts, substrates and cultivation conditions.

Table 5 – *Molar mass of PHA produced by the C. necator and B. cepacia strains from crude palm oil*

Microorganism	M_w (kDa)			M_n (kDa)	PDI
	Minimum	Medium	Maximum		
C. necator IPT 026	5.61	321.28	10650.94	130.86	2.46
C. necator IPT 027	4.81	240.87	3553.37	141.83	1.70
B. cepacia IPT 119	5.09	173.78	9673.15	104.34	1.67
B. cepacia IPT 400	8.57	389.30	13164.13	190.71	2.04

Regarding polymer polydispersity (PDI) (Table 5), *C. necator* and *B. cepacia* bacteria produced materials with good homogeneity of molecular masses, representing good bacterial biosynthesis consistency. The PDI values identified for the polymeric samples in this study ranged from 1.67 to 2.46, revealing the production of materials far more uniform than those reported by Sang-Hyeop *et al.*[56] in their investigations with PHA production from wastewater sludge (PDI = 3.44), and Gahlawat and Soni[44], with their P(3HB-co-3HV) copolymer production by *C. necator* DSM 545 (PDI = 4.3) using waste glycerol.

Conclusions

The bacterial strains investigated were able to use crude palm oil as substrate to synthesize novel PHAs mostly constituted of long side chain monomers – C16 and C18, which is a rare feature in the polymers field. All copolymers produced presented variable monomeric composition and molar masses, with predominant amorphous molecular arrangements, high thermal stability, and low polydispersity. *C. necator* exhibited the highest performance in biosynthesis. *C. necator* and *B. cepacia* bacterial strains presented great effects in PHAs production and properties, confirming the importance of the biocatalysts variable in the bioprocess. Medium composition and fermentation conditions can be optimized to maximize biomass and biopolymer production. Additionally, the understanding of biochemical and cellular mechanisms of PHA production and accumulation, associated with new techniques of polymer extraction can provide valuable insights to overcome the hurdles in the utilization of these bioplastics.

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