Vol.62: e19170498, 2019
http://dx.doi.org/10.1590/1678-4324-2019170498
ISSN 1678-4324 Online Edition

Article - Agriculture, Agribusiness and Biotechnology

Microbial Synthesis and Characterization of Biodegradable Polyester Copolymers from *Burkholderia Cepacia* and *Cupriavidus Necator* Strains Using Crude Glycerol as Substrate

Plínio Ribeiro Rodrigues[1*]
https://orcid.org/0000-0002-6652-5470

Tatiane Aparecida Barroso Silvério[2]
https://orcid.org/0000-0001-7354-7646

Janice Izabel Druzian[3]
https://orcid.org/0000-0001-8940-6098

[1]University of Campinas, Graduate Program in Chemical Engineering, Campinas, SP, Brazil; [2]Federal University of Jequitinhonha and Mucuri Valleys, Institute of Science and Technology, Diamantina, MG, Brazil; [3]Federal University of Bahia, Graduate Program in Chemical Engineering, Salvador, BA, Brazil.

Received: 2017.08.08; Accepted: 2019.06.04.

* Correspondence: plinioeng@hotmail.com; Tel.: +55-19- 35213910 (P.R.R.)

HIGHLIGHTS

- PHAs copolymers were produced from crude glycerol by *B. cepacia* and *C. necator*.
- All PHAs revealed initial thermal degradation temperatures superior to 300°C.
- The strains used produced long chain length copolymers with low polydispersity.
- Five different monomers were identified in the PHA copolymers produced.

Abstract: Polymers are materials of pronounced importance in the modern world, since they are massively present in everyday life, especially in the form of packaging. However, most of these materials are derived from non-renewable sources and their disposal generates large volumes of waste, which is extremely damaging to natural environments. In this context, microbial biopolymers appear as a powerful alternative in the substitution of several applications of synthetic plastics, causing less harm to the environment, as they are

biodegradable and produced from renewable sources. In this study, evaluation and characterization of polyhydroxyalkanoates (PHAs) produced by *Cupriavidus necator* (IPT 026 and IPT 027) and *Burkholderia cepacia* (IPT 119 and IPT 400), using crude glycerol as substrate, were carried out (crude glycerol 15 g L^{-1}, pH 7.0, 150 rpm, 72h). The substrate chemical composition was determined and all microorganisms tested were able to utilize it to synthesize PHA. *C. necator* IPT 026 exhibited the highest polymer production (1.52 ± 0.03 g L^{-1}). *B. cepacia* strains produced low crystallinity PHA. All polyesters synthesized exhibited long chain length polymers with low polydispersity and initial thermal degradation temperatures superior to 300°C. The microorganism strains and the substrate composition highly affected PHAs synthesis, composition and thermochemical characteristics.

Keywords: Crude glycerol; biosynthesis; polyhydroxyalkanoates; characterization.

INTRODUCTION

Substitution of slow-degradable petroleum based plastics by biodegradable materials represents an important advantage in drastically reducing the time of plastics accumulation in natural environments and, at the same time, introducing a renewable and sustainable production route. In this sense, bacterial synthetized polyhydroxyalkanoates (PHAs) can be considered as a promising alternative for biodegradable polymers obtainment, reducing the negative impact cause by chemically produced plastics [1,2].

PHAs are a group of bio-polyesters completely synthesized by microorganisms, such as bacteria and archaea, with diverse structures. With more than 150 different monomers observed, the thermal and mechanical properties of PHAs have been studied and manipulated with the aid of genetic engineering. This monomeric diversity has allowed the development of several technological applications for these polymers, including the production of biodegradable plastics for packaging, bio-compatible implants and continuous drug delivery [3-5].

The biosynthesis of PHA is catalyzed by microorganisms, commonly in submerged conditions, in a medium containing a carbon source to be converted into product, such as starch, glucose, sucrose, fatty acids, and other micronutrients, depending on the demand of the microorganism used. More specifically, PHA production occurs intracellularly when there is excess carbon source and a limitation of at least one nutrient necessary for cell multiplication, such as N, P, Mg or Fe. The characteristics of the biopolymer produced will depend, among other factors, on the chemical nature of the carbon source supplied and on the synthesizer bacterium metabolic pathways [6,7].

The substrates commonly used in the industrial production of PHA are glucose and sucrose, however, one of the major barriers to increased polymer production is the expense with these conventional carbon sources. According with Pan *et al*. [8], the substrate cost can correspond to up to 50% of the total production expenses, a factor that is aggravated by the costs associated with the biomaterial extraction and recovering.

There is an effort to produce PHA from cheaper substrates, which would increase the commercial competitiveness of the product and provide a more complete culture medium, reducing the need for micronutrients supplementation [7,9,10]. Crude glycerol, byproduct of biodiesel production, has been emerging as a very promising carbon source due to its low cost and high abundance. This material is mainly composed of ethanol or residual methanol, glycerol and fatty acids, presenting favorable composition for its application as a fermentable substrate for PHA-producing bacteria [11-13].

In this context, this study aims to produce and characterize PHA using crude glycerol, from Santa Cruz State University (UESC) biodiesel pilot plant, as an alternative and complex substrate for the submerged cultivation of the bacterial strains *Burkholderia cepacia* (IPT 119 and IPT 400) and *Cupriavidus necator* (IPT 026 and IPT 027) towards the direction of implementing more viable biotechnological routes to supply the market demand for

environmentally friendly materials and to give sustainable destination for a waste byproduct regionally produced.

MATERIAL AND METHODS

Bacterial strains and substrate

Burkholderia cepacia IPT 400 and IPT 119, and *Cupriavidus necator* IPT 026 and IPT 027 were supplied by the Institute of Technological Research, in São Paulo, Brazil. The crude glycerol used as substrate was produced and donated by a biodiesel pilot plant in Santa Cruz State University (UESC), Ilhéus, Brazil.

Crude glycerol chemical composition

Crude glycerol chemical composition, after autoclaving, was determined in triplicate, total lipids content was defined according to Bligh and Dyer [14], crude protein, crude ash and moisture were determined by analysis methods published by the Association of Official Analytical Chemists [15]. Glycerol content was calculated by percent mass difference, according with Ribeiro *et al*. [12]. Total carbon (C) content was determined according to Nelson and Sommers [16] publication and total nitrogen (N) content was determined by the Kjehldal method [15].

Fatty acid profile on the crude glycerol lipids content was determined by a chromatographic method according to Joseph and Ackman [17] and Nascimento *et al*. [18]. The separation of the methyl esters in the fatty acids was performed using gas chromatography with a flame ionization detector and a fused silica gas chromatography capillary column EliteWAX (30 m × 0.32 mm × 0.25 µm). The quantification of fatty acids was executed by the addition of an internal standard (C23:0 Sigma®, USA) according to Joseph and Ackman [17].

Production of the polyesters

The biocatalysts (bacteria) were stored at 4°C in nutrient agar composed of 5.0 g L^{-1} meat peptone, 3.0 g L^{-1} beef extract, and 3.75 g L^{-1} agar. PHA production with crude glycerol was performed using two pre-cultures (inoculum and nitrogen-non-limited mineral medium) and one final culture (nitrogen-limited mineral medium) as described by Wang *et al* [19] and Campos *et al* [20].

Inoculation was performed in nutrient broth (inoculum medium) composed of 5.0 g L^{-1} bacteriological peptone, 3.0 g L^{-1} beef extract, and distilled water. The mineral medium used as second pre-culture (SP), with no nitrogen limitation, was 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}), 0.625 g L^{-1} $(NH_4)2SO_4$ (nitrogen source); $Na_2HPO_4 \cdot 12H_2O$ (1.6 g L^{-1}), KH_2PO_4 (1.6 g L^{-1}) and crude glycerol (15 g L^{-1}). Media pH was adjusted to 7.0 with NaOH (10 g L^{-1}) or HCl (10 g L^{-1}). The final culture (FC) mineral medium was prepared with the same composition used for SP except for the concentration of $(NH_4)2SO_4$, which was changed for 0.361 g L^{-1} for nitrogen limitation.

All tests were executed in triplicate in 250 mL Erlenmeyers, the inoculum flasks containing nutrient broth medium and the respective biocatalysts were incubated at 30°C in an orbital shaker for 24 h (best cell concentration, 1011 UFC/mL) at 150 rpm. SP cultivations were executed in flasks containing 80 mL nitrogen-non-limited mineral medium with addition of 10% v/v of the inoculum culture and incubated at 35°C and 180 rpm in orbital shaker for 24 h. FCs were ran in flasks containing 80 mL nitrogen-limited mineral medium with the addition of 10% v/v of SP and incubated at the same conditions used for SP for a period of 72 h. All media used in the bacterial cultivations were sterilized at 121°C for 20 minutes in autoclave.

Polyesters extraction and recovery

Cell cultures were harvested by centrifugation at 15,700 ×g for 30 min at 5°C, washed twice with distilled water, transferred into round bottom flasks and oven dried at 105°C until constant weight. PHA extraction from the oven-dried cells were performed using chloroform at 60°C for 2 h with vigorous agitation on a magnetic stirrer plate with heating. Subsequently, the solution (cells and chloroform) was filtered and stored in pre-weighed plates for 24 h to allow complete solvent evaporation, resulting in the recovery of polyester films.

Polyesters 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.

Determination of PHA composition

The composition of the polyesters was determined using gas chromatography–mass spectrometry (GC–MS; Clarus 500 PerkinElmer) with the TurboMass software version 4.5.0 and the NIST 98 library. Approximately 0.04 g of the dry PHA were subjected to methanolysis based on the method described by Braunegg *et al*. [21] with the modifications proposed by Brandl *et al.* [22]. The analysis conditions were established according to Campos *et al*. [20]. 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 spectra 98 NIST library, and the hydroxyalkanoates were quantified by area normalization.

X-ray diffraction analysis

Crystallinity of the PHA samples were measured by X-ray diffraction. The X-ray diffractograms 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. The percentage of crystallinity was calculated from the diffractograms according to Vonk's method [23].

Thermal characterization

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

Determination of PHA molar mass distribution

Molar masses, expressed by the weight average molar mass (Mw), the number average molar mass (Mn) and the polydispersity index (PDI = Mw/Mn), were obtained by size-exclusion chromatography (SEC) according to Campos *et al* 20 and Ribeiro *et al* [12].

High performance liquid chromatography (HPLC, PerkinElmer 200) with an auto-sampler and refractive index detector (PerkinElmer), a column Shodex KD 807 (30 cm × 78 mm × 5 µm) 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}. As the mobile phase, chloroform was employed at 1 mL min^{-1}. A standard curve (log Mw versus Retention time) 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).

Data treatment

Data treatment was performed using the tools available in the Origin software version 8.1 (OriginLab). Statistical significance was calculated by Tukey's test ($p<0.05$).

RESULTS

Crude glycerol composition

Crude glycerol is a byproduct of biodiesel production, the result of a transesterification reaction. In the processing of vegetable oils for biofuel production, a triacylglyceride reacts with an alcohol in the presence of a strong acid or alkali, producing a mixture of esters of fatty acids (biofuel) and glycerol [24].

Table 1 displays the chemical composition determined for the crude glycerol, after autoclaving, produced in UESC biodiesel pilot plant, used as substrate in the bio-catalyzed submerged synthesis of PHA. The values are disposed in percentage averages followed by their standard deviations. The carbon nitrogen ratio (C:N) present in the substrate and the fatty acids profile of the lipids portion are also displayed. It is possible to observe that the highest fraction in the byproduct is comprised of glycerol (48.01%). Crude protein (1.56%), lipids (1.48%) and ash/minerals (2.18%) were also identified. The presence of these components is associated with the residual oil used in the transesterification reaction [12,26].

Crude glycerol C:N was estimated in 118:1 (Table 1), this value contributes with the strategy used in this paper of reducing nitrogen supply to stimulate polymer production [20,27], furthermore, allowing to adjust the cultivation medium C:N to 39:1 with $(NH4)_2SO_4$ addition. This chosen value is within the C:N range used in literature for PHA production using the nitrogen limitation approach [27,28].

Table 1. Chemical composition of crude glycerol obtained as byproduct of biodiesel production.

Composition	Average (%)	Fatty acid	Average (%)
Volatiles at 105°C	46.77± 0.14	C4:0	0.728
Total ash	2.18 ± 0.04	C8:0	0.081
Total lipid	1.48± 0.10	C11:0	0.092
Crude protein	1.56 ± 0.05	C16:0	7.816
Glycerol	48.01± 0.33	C18:0	3.626
		C18:1ω9c	16.634
C:N	118:1	C18:1ω9t	0.727
		C18:2ω6c	14.120
		C20:0	0.826
		C20:3ω6	55.350

Crude glycerol lipids fraction was composed of 13.17% saturated fatty acids (Butyric [C4:0], Caprylic [C8:0], Undecylic [C11:0], Palmitic [C16:0], Stearic [C18:0] and Arachidic [C20:0]), 17.36% monounsaturated fatty acids (Oleic [C18:1]), and 69.47% polyunsaturated fatty acids (Linoleic [C18:2] and Linolenic [C20:3]).

Crude glycerol bioconversion

The influence of four different biocatalysts, *Burkholderia cepacia* IPT 400 and IPT 119, and *Cupriavidus necator* IPT 026 and IPT 027, was tested for PHA production in submerged cultivations using crude glycerol as substrate, Table 2.

Although there are studies of PHA production by *Cupriavidus necator* IPT 026 and IPT 027 using crude glycerol as carbon source, originated in Comanche Biocombustíveis, Federal University of Bahia biodiesel pilot plant and Petrobras S.A. [12,13,20], it is relevant to evaluate the effect of a different crude glycerol source using biocatalysts more investigated in literature (*Cupriavidus necator* IPT 026 and IPT 027) to have a more complete understanding of their growth, polymer yield and characteristics in dissimilar cultivation mediums. Also, it is important to scrutinize how those microorganisms compare to novel strains (*Burkholderia cepacia* IPT 400 and ITP 119), since the substrate and the biocatalyst variables play an important role in the viability of PHA commercial scale up [32,33].

Table 2. PHA and biomass production, and PHA extraction in fermentation of 15 g L-1 of crude glycerol 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 027	1.50 ± 0.04[a]	2.44 ± 0.11[a]	61.39
C. necator IPT 026	1.52 ± 0.03[a]	2.77± 0.09[a]	54.99
B. cepacia IPT 400	1.17± 0.14[b]	2.04 ± 0.16[b]	57.35
B. cepacia IPT 119	1.04 ± 0.11[b]	1.70 ± 0.20[b]	61.37

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).

Thus, it was inferred that regarding polymer production *C. necator* IPT 026 presented the highest PHA performance, 1.52 g L^{-1} (Table 2). However, it was noted that *C. necator* IPT 026 and IPT 027 presented no statistical difference in polymer synthesis activity, as proven by Tukey's test (P<0.05), the same phenomenon was observed between *B. cepacia* IPT 400 and IPT 119.

Polyesters percentage extraction were in average very similar for *B. cepacia* and *C. necator*, revealing that the cultivation conditions established triggered polymer accumulation in a satisfactory manner for all strains studied (Table 2), according with standards reported in literature [34-36].

Polyhydroxyalkanoates characterization

All biomaterials produced by *C. necator* and *B. cepacia* strains in submerged cultivations with crude glycerol from vegetal oil transesterification were submitted to thermal, chemical and physical characterizations in order to have a better understanding of their properties and performances associated with their natural atomic structures.

Spectroscopy in the infrared spectra (FTIR) was executed to identify and characterize the samples functional groups, Figure 1. All polymers scanned showed the characteristic bands documented in the scientific literature related to PHAs structure, confirming the production of these polyesters.

The carbonyl of the ester group (C-C) transmittance band is noted at 972 cm^{-1} [37]. The 1048 cm^{-1} and 1290 cm^{-1} wave numbers, respectively, correspond to the asymmetric and symmetric stretching of the C–O–C group [38]. Wagging CH_3 is related to the wave number 1381 cm^{-1} [37].

The transmittance noted at 1728 cm^{-1} is attributed to the carbonyl group (C=O) axial deformation [39]. C-H carbon-hydrogen bond stretching of CH_3 and CH_2 groups were identified at 2993 cm^{-1} and 2938 cm^{-1}, correspondingly [39,40].

Figure 2 shows the chromatograms obtained by gas chromatography–mass spectrometry of the PHA samples produced in order to determine the polymers monomeric composition, Table 3. The mass spectrum of each peak was compared to the NIST library for polymeric units identification.

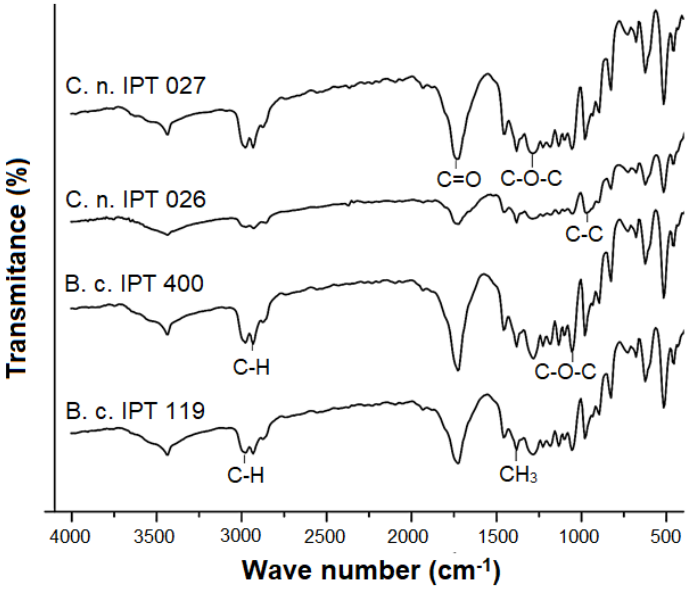

Figure 1. FTIR spectra of PHA produced by *C. necator* and *B. cepacia* strains in submerged cultivation with crude glycerol.

It was identified the presence of five different monomers constituting the structure of the copolymers produced in this study (Table 3). All samples evaluated revealed major presence of hydroxyhexadecanoate and hydroxy-9,11-octadecadienoate (Table 3), long chain length monomers [41], showing strong influence of the carbon source used in the polymers composition. Also, the copolymers synthetized by *B. cepacia* displayed a more diverse monomeric structure than what was observed for *C. cepacia* bacteria.

Figure 3 displays X-ray diffractograms for the PHAs produced with all bacterial strains investigated in this paper, along with their crystallinity index (I_c). It is possible to observe the diffraction peaks at 2θ for all samples evaluated at around 13.7°, 16.94°, 22.14° and 25.52°, reflecting semi-crystalline atomic arrangements in their structure [40]. However the picks intensity were largely different in response to the producer microorganism, indicating great effect of this variable in the property evaluated.

In general, the PHAs produced by *C. necator* strains displayed an I_c superior than PHAs produced by *B. cepacia*, with emphasis for *C. necator* IPT 026 and *B. cepacia* IPT 400, which displayed respectively the highest (50.36%) and the lowest (17.83%) I_c values.

PHAs and crude glycerol thermal behaviors are displayed in the thermogravimetric curves (TGA) and their associated derivatives (DTGA) presented in Figure 4. TGA measures the weight of a sample as a function of temperature, allowing to detect changes in its mass and determine temperatures that characterize a termal event in mass loss curves. Supplementary, the first derivative of the TGA curves (DTGA) is a direct indicative to the rate of decomposition of the sample [44].

In a decomposition process, chemical bonds break and polymers decompose to form gasous products and ashes/carbon black, therefore, the polymers habilite to endure high temperatures is a crucial aspect for its processing and industrial scale up [45].

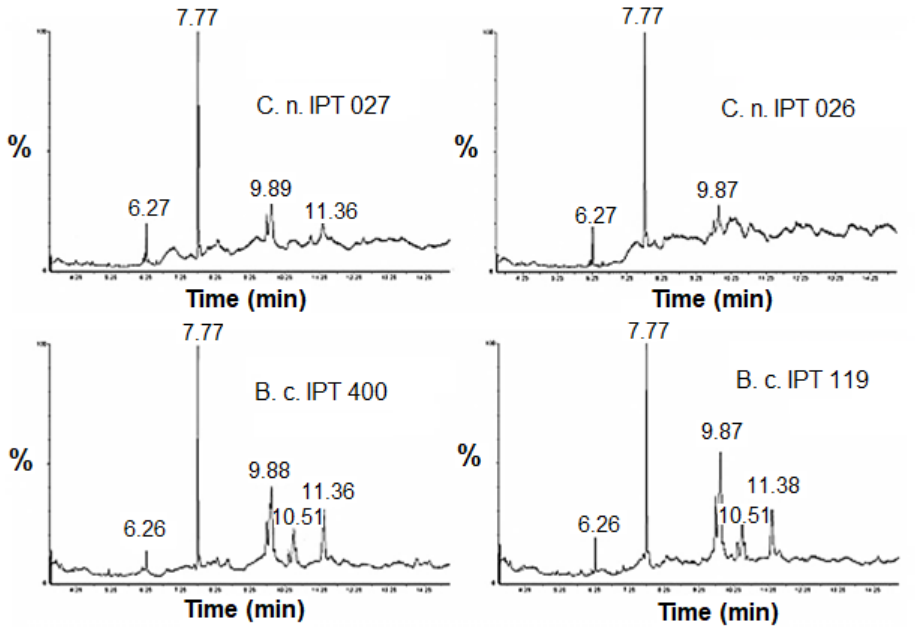

Figure 2. Chromatograms obtained by gas chromatography–mass spectrometry of PHA produced by *C. necator* and *B. cepacia* strains in submerged cultivation with crude glycerol.

Table 3. Monomeric profile of the PHAs produced by *C. necator* and *B. cepacia* bacteria obtained by gas chromatography–mass spectrometry in the NIST library.

Retention time (min)	Monomer	Composition on polymer sample (%)			
		IPT 027	IPT 026	IPT 400	IPT 119
6.27	Hydroxytetradecanoate	4.44	4.65	2.03	1.94
7.77	Hydroxyhexadecanoate	30.95	45.36	16.54	19.63
9.87	Hydroxy-9,11-octadecadienoate	38.31	33.84	43.79	45.23
10.51	Hydroxy-8,11-octadecadienoate	—	—	6.02	5.73
11.36	Hydroxy-7,10-octadecadienoate	15.72	—	13.28	12.08
-	NI	10.58	16.15	18.34	15.38

NI = Not Identified.

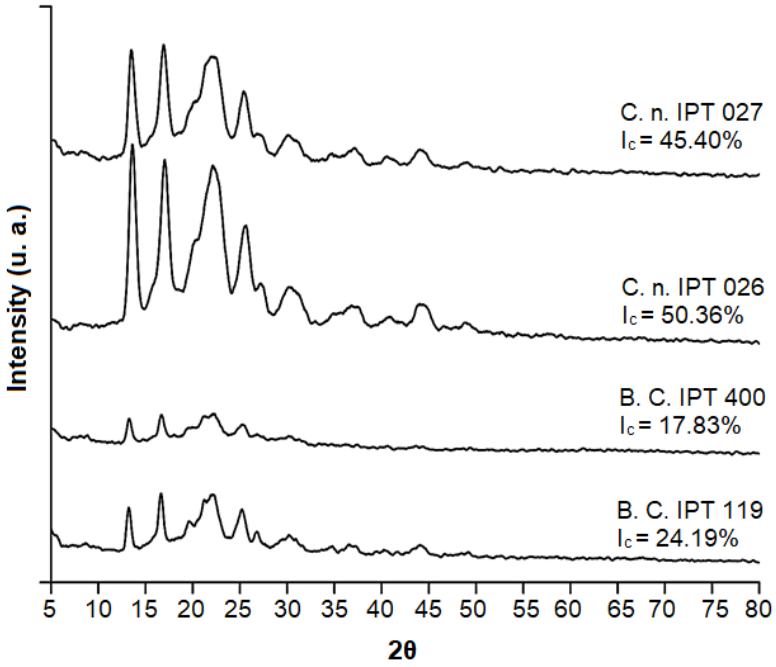

Figure 3. X-ray diffractograms of PHA produced by *C. necator* and *B. cepacia* strains in submerged cultivation with crude glycerol.

The initial degradation temperatures (T_{in}) and the maximum decomposition temperatures (T_{maxdec}) of the thermogravimetric events obtained from the TGA/DTGA curves for the PHAs produced from crude glycerol are organized in Table 4. All PHAs synthesized exhibited initial thermal degradation above 300°C. The polymer synthetized by *C. necator* IPT 026 exhibited the best thermal stability with an initial degradation temperature of 314.39°C, a maximum decomposition temperature of 340.41°C and a total weight loss of 99.56%.

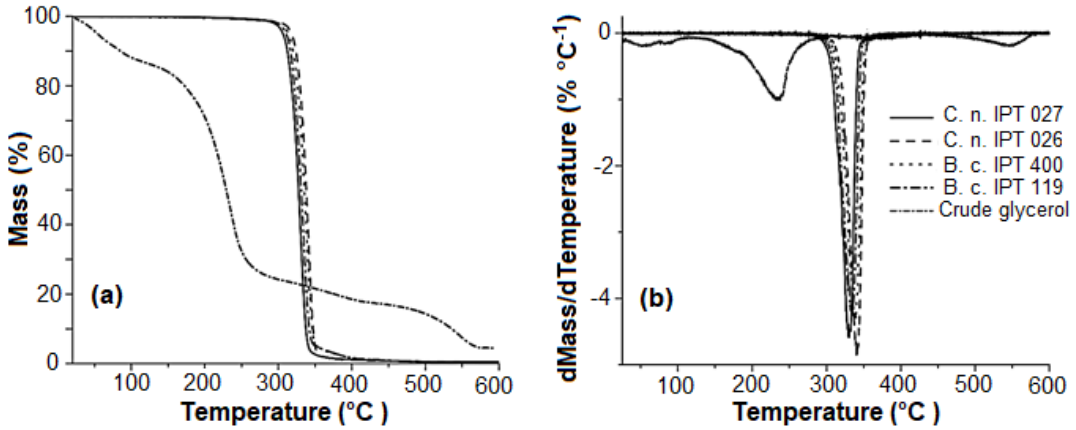

Figure 4. TGAs (a) and DTGs (b) of crude glycerol and PHAs produced by *C. necator* and *B. cepacia* strains in submerged cultivation.

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

Microorganism strain	T_{in} (°C)	T_{maxdec} (°C)	Total mass loss (%)
C. necator IPT 027	304.78	328.91	99.57
C. necator IPT 026	314.39	340.41	99.56
B. cepacia IPT 400	313.06	238.11	99.76
B. cepacia IPT 119	308.92	333.51	99.71

Figure 5 displays the HPLC-RI chromatograms of the biopolymers separation. Size-exclusion chromatography was used to obtain maximum, average and minimum molecular mass (Mw), number average molecular mass (Mn) and polydispersity (PDI) of the PHAs produced in this study, Table 5.

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

Microorganism	Mw (kDa)			Mn (kDa)	PDI
	Minimum	Medium	Maximum		
C. necator IPT 027	6.797	157.870	3353.883	106.294	1.485
C. necator IPT 026	10.383	164.051	4929.785	117.880	1.392
B. cepacia IPT 400	9.074	138.013	3553.366	93.023	1.484
B. cepacia IPT 119	8.241	143.416	3912.551	102.860	1.394

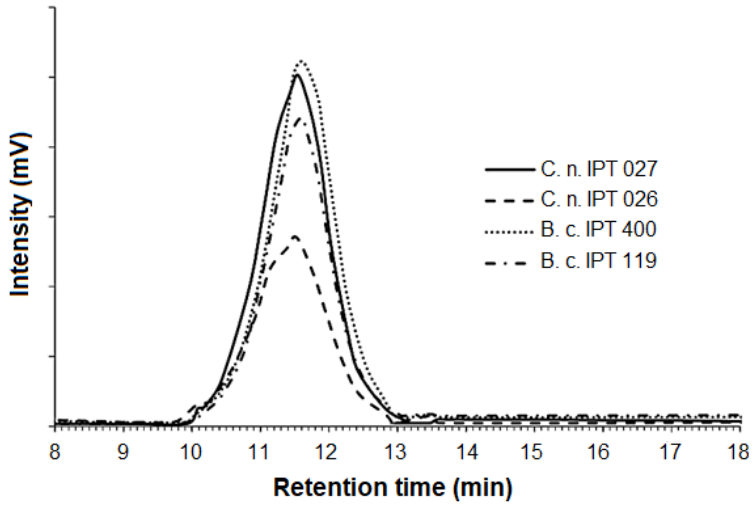

Figure 5. HPLC-RI chromatograms of PHAs produced by the *C. necator* and *B. cepacia* strains from crude glycerol in submerged cultivations.

In average, *C. necator* bacteria produced copolymers with the highest weight average molecular mass. *C. necator* IPT 027 PHA exhibited 157.870 kDa Mw and *C. necator* IPT 026 presented 164.051 kDa Mw. PHA polydispersity (Table 5) shows that *C. necator* and *B. cepacia* strains produced materials with good homogeneity of molecular masses, demonstrating good biosynthesis consistency regarding the bioconversion conditions offered.

DISCUSSION

Crude glycerol is a byproduct of biodiesel production highly available and underutilized. According to Fountoulakis and Manios [25], for every 9 kg of biodiesel produced by the transesterification of vegetable oils 1 kg of crude glycerol are generated, and the composition of this material varies largely depending on the process rout for the fuel production and on the origin of the lipids used for the transesterification.

Crude glycerol (produced in UESC biodiesel pilot plant**)** lipids fraction (Table 1) showed a high percentage of unsaturated fatty acids, what, according to Nwokola and Smartt [29], is usual for most oils originated from plants.

It is important to point that the presence of fatty acids in the fermentative medium is known to influence PHA chain length and structure. It has been reported 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 [30,31].

Thompson and He [30], Ribeiro *et al*. [12], and Campos *et al.* [20] reported the characterization of crude glycerol from different sources, which exhibited higher lipid fractions than what was assessed in this paper. The referred authors also identified different fatty acids profiles than what was seen for the crude glycerol from UESC (Table 1). These dada reveals high diversity of compositions in crude glycerol based in its origin.

PHA production in submerged cultivations using crude glycerol as substrate (Table 2) showed that, in general, compared to *B. cepacia* biocatalysts*, C. necator* bacteria exhibited an average yield 37% higher. *C. necator* bacteria also presented the best biomass growth; what reveals better adaptation of these strains to the crude glycerol substrate offered than what was observed for *B. cepacia*.

In his studies, Ribeiro *et al*. [12] described 1.40 g L^{-1} of PHA production (150 rpm, 35ºC, 24 h) by *C. necator* IPT 027, using crude glycerol originated from Federal University of Bahia biodiesel Pilot plant, and a richer (and more expensive) micronutrients culture medium with lower nitrogen limitation than what was established in this paper. Figueiredo *et al*. [13] reported 0.16 g L^{-1} of PHA (150 rpm, 30ºC, 24 h) using the same strain and crude glycerol source from Comanche Biocombustíveis. Campos *et al* [20] reported 2.81 g L^{-1} of PHA (150 rpm, 35°C, 24 h) in his culture medium, using *C. necator* IPT 026 and crude glycerol also from Comanche Biocombustíveis. Interestingly, he used a excess nitrogen supply over carbon, with C:N of 0.6.

These data evidences high dependence of *C. necator* IPT 026 and IPT 027 on crude glycerol composition and on micronutrients supply. Therefore the use of crude glycerol with optimal and fixed composition for highest polymer conversions may be a challenging point for the industrial bio-recycling of the referred byproduct, when considered the use of *C. necator* IPT 026/027 as biocatalysts.

As for *B. cepacia*, Ribeiro *et al*. [12] reported 1.60 g L^{-1} PHA production (150 rpm, 35°C, 24 h) with the strain IPT 438, using crude glycerol originated from Petrobras S.A. A performance superior to what was found for all *B. cepacia* strains evaluated in this study.

The monomer composition (Table 3) revealed the production of copolymers with a rich composition. Ribeiro *et al*. [12] and Campos *et al.* [20] in their studies of PHA production with *C. necator* IPT 027 and *C. necator* IPT 026, respectively, using different sources of crude glycerol, reported a very distinct composition in their copolymers. Ribeiro noted a major presence of hydroxyhexadecanoate, hydroxytetradecanoate and hydroxybutyrate, while Campos identified mostly hydroxybutyrate, hydroxypentanoate and hydroxynonanoate. These data confirms that different compositions of the substrate offered for the bioconversion exerts enormous influence in PHA monomeric composition. Also it is noted that novel materials were produced in the present investigation using both *C. necator* and *B. cepacia* strains.

Figure 3 displays the crystallographic organization (crystallinity index - I_c) for the PHAs produced with all bacterial strains investigated in this paper. The I_c is an indicative of the percentage amount of crystallinity in the polymeric structure and its ideal value must not be much superior to 50%, or else polymer brittleness and rigidity starts to cause problems

related to the material applications and processing by extrusion [42,43]. Still, all I_c percentages found (Figure 3) were lower than what was reported by Campos *et al*. [20] and Figueiredo *et al*. [13] in PHAs productions with crude glycerol, therefore, revealing a better performance concerning their structural morphology in regard of the biosynthesis conditions set and the biocatalysts used in the present study.

As for the thermal behavior of the PHAs produced in this work, it is possible to infer (figure 4) that thermal degradation of all polymers occurred in just one mass loss event, this indicates the presence of no impurities remaining from the extraction and separation processes on those materials. Crude glycerol thermal behavior presented three mass loss events, which may be associated with its complex chemical composition (Table 1) [28].

In general, the thermal performances of all biopolymers synthesized in the present study were substantially superior to what was found in PHAs productions, using crude glycerol of different sources, reported by Ribeiro *et al*. [12,46] with *C. necator* IPT 027, *C. necator* IPT 029, *B. megaterium* IPT 429 and *B. cepacia* IPT 438, and by Campos *et al*. [20] with *C. necator* IPT 026.

All strains used in this study produced copolymers with expected values of molecular mass. Since average Mw has been reported to range between 50 and 3000 kDa depending on the biosynthesis conditions, on the biocatalyst and on the substrate used [47,48]. Hermann-Krauss *et al*. [4] reported comparable values of Mw in PHA bioconverted by archaea (Mw = 150 kDa and PDI = 2.1 using pure glycerol and Mw = 253 kDa and PDI = 2.7 using crude glycerol). The PDI values identified for the polymeric samples in this paper, ranging from 1.485 to 1.392, reflected the synthesis of materials far more uniforms than what was reported by Cha *et al*. [49] in his investigations with PHA production from wastewater sludge (PDI = 3.44).

CONCLUSION

All strains investigated were able to use the offered substrate to synthetize PHA. Concerning production, *C. necator* strains exhibited the highest performance in polymer synthesis. All microorganisms produced copolymers with high thermal stability, long chain length and low polydispersity. Biopolymers synthesized by *B. cepacia* revealed predominant amorphous molecular arrangements with the lowest crystallinity. *C. necator* and *B. cepacia* bacteria presented great effect in PHA production and characteristics, confirming the importance of the biocatalysts variable in the bioprocess. Scientific literature comparisons revealed that the use of crude glycerol of different sources and different cultivation conditions greatly affect the performance of *C. necator* ITP 027 and ITP 026 in their ability to produce PHAs, their compositions and properties.

REFERENCES

1. Koller, M.; Maršálek, L.; Dias, M.M.S.; Braunegg, G. Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. *N. Biotechnol.* 2017, *37*, 24-38.
2. Mayeli, N.; Motamedi, H.; Heidarizadeh, F. Production of polyhydroxybutyrate by *Bacillus axaraqunsis* BIPC01 using petrochemical wastewater as carbon source. *Braz. arch. biol. technol.* 2015, *58*, 643-650.
3. Rodríguez-Contreras, A.; García, Y.; Manero, J.M.; Rupérez, E. Antibacterial PHAs coating for titanium implants. *Eur. Polym. J.* 2017, *90*, 66-78.
4. Hermann-Krauss, C.; Koller, M.; Muhr, A.; Fasl, H.; Stelzer, F.; Braunegg, G. Archaeal production of polyhydroxyalkanoate (PHA) co- and terpolyesters from biodiesel industry-derived by-products. *Archaea*. 2013, *2013*: ID 129268.
5. Goreva, A.; Shishatskaya, E.; Volova, T. Biodegradable microspheres based on polyhydroxyalkanoates (PHA) as drug carriers. *N. Biotechnol.* 2009, *25*: S37.
6. Arcos-Hernández, M.V.; Laycock, B.; Donose, B.C.; Pratt, S.; Halley, P.; Al-Luaibi, S.; Werker, A.; Lant, P.A. Physicochemical and mechanical properties of mixed culture polyhydroxyalkanoate (PHBV). *Eur. Polym. J.* 2013, *49*, 904–913.

7. Goma, E.Z. Production of polyhydroxyalkanoates (PHAs) by *Bacillus subtilis* and *Escherichia coli* grown on cane molasses fortified with ethanol. *Braz. arch. biol. technol.* 2014, *57*, 145-154.
8. Pan. W.; Perrotta, J.A.; Stipanovic, A.J.; Nomura, C.T.; Nakas, J.P.J. Production of polyhydroxyalkanoates by *Burkholderia cepacia* ATCC 17759 using a detoxified sugar maple hemicellulosic hydrolysate. *Ind. Microbiol. Biotechnol*. 2012, *39*, 459–469.
9. Bengtsson, S.; Karlsson, A.; Alexandersson, T.; Quadri, L.; Hjort, M.; Johansson, P.; Morgan-Sagastume, F.; Anterrieu, S.; Arcos-Hernandez, M.; Karabegovic, L.; Magnusson, P.; Werker, A. A process for polyhydroxyalkanoate (PHA) production from municipal wastewater treatment with biological carbon and nitrogen removal demonstrated at pilot-scale. *N. Biotechnol.* 2017, *35*, 42-53.
10. Gowda, V.; Shivakumar, S. Agrowaste-based Polyhydroxyalkanoate (PHA) production using hydrolytic potential of *Bacillus thuringiensis* IAM 12077. *Braz. arch. biol. technol.* 2014, *57*, 55-61.
11. Sindhu, R.; Ammu, B.; Binod, P.; Deepthi, S.K.; Ramachandran, K.B.; Soccol, C.R.; Pandey, A. Production and characterization of poly-3-hydroxybutyrate from crude glycerol by *Bacillus sphaericus* NII 0838 and improving its thermal properties by blending with other polymers. *Braz. arch. biol. technol.* 2011, *54*, 783-794.
12. Ribeiro, P.L.L.; Silva, A.C.M.S.; Filho, J.A.M.; Druzian, J.I. Impact of different by-products from the biodiesel industry and bacterial strains on the production, composition, and properties of novel polyhydroxyalkanoates containing achiral building blocks. *Ind. Crops Prod.* 2015, *69*, 212-223.
13. Figueiredo, T.V.B.; Campos, M.I.; Sousa, L.S.; Silva, J.R.; Druzian, J.I. Production and characterization of polyhydroxyalkanoates obtained by fermentation of residual crude glycerin from biodiesel. *Quim. Nova.* 2014, *37*, 1111-1117.
14. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 1959, *37*, 911-917.
15. AOAC (Association of Official Analytical Chemists). *Official Methods of Analysis of AOAC International*. 16th ed. Maryland: Gaithersburg, 1997.
16. Nelson, D.W.; Sommers, L.E. *Total carbon, organic carbon, and organic matter*. In: Page, A.L.; Miller, R.H.; Keeney, D.R., editors. *Methods of soil analysis. Part 2. Chemical and microbiological properties*, 2nd ed. American Society of Agronomy: Madison, United States, 1982; pp. 539-577.
17. Joseph, J.D.; Ackman, R.G. Capillary column gas chromatographic method for analysis of encapsulated fish oils and fish oil ethyl esters: collaborative study. *J. AOAC Int.* 1992, 75, 488-506.
18. Nascimento, I.A.; Marques, S.S.I.; Cabanelas, I.T.D.; Pereira, S.A.; Druzian, J.I. Screening microalgae strains for biodiesel production: lipid productivity and estimation of fuel quality based on fatty acids profiles as selective criteria. *Bioenergy Res.* 2013, *6*, 1-13.
19. Wang, B.; Sharma-Shivappa, R.R.; Olson, J.W.; Khan, S.A. Production of polyhydroxybutyrate (PHB) by *Alcaligenes latus* using sugarbeet juice. *Ind. Crops Prod.* 2013, *43*, 802-811.
20. Campos, M.I.; Figueiredo, T.V.B.; Sousa, L.S.; Druzian, J.I. The influence of crude glycerin and nitrogen concentrations on the production of PHA by *Cupriavidus necator* using a response surface methodology and its characterizations. *Ind. Crops Prod.* 2014, *52*, 338-346.
21. Braunegg, G.; Sonnleither, B.E.; Lafferty, R.M. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. *Eur. J. Appl. Microbiol. Biotechnol.* 1978, *6*, 29–37.
22. Brandl H, Gross RA, Lenz RW, Fuller RC. *Pseudomonas oleovorans* as a source of poly(-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Appl. Environ. Microbiol.* 1988, *54*, 1977–1982.
23. Vonk, C.G. Computerization of Ruland's X-ray method for determination of the crystallinity in polymers. *J. Appl. Cryst.* 1973, *6*, 148-152.
24. Geris, R.; Santos, N.A.C.; Amaral, B.A.; Maia, I.S.; Castro, V.D.; Carvalho, J.R.M. Soybean biodiesel - transesterification reaction to practical classes of organic chemistry. *Quim. Nova* 2007, *30*, 1369-1373.

25. Fountoulakis, M.S.; Manios, T. Enhanced methane and hydrogen production from municipal solid waste and agro-industrial by-products co-digested with crude glycerol. *Bioresour. Technol.* 2009, *100*, 3043–3047.
26. García, I.L.; López, J.A.; Dorado, M.P.; Kopsahelis, N.; Alexandri, M.; Papanikolaou, S.; Villar, M.A.; Koutinas, A.A. Evaluation of by-products from the biodiesel industry as fermentation feedstock for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) production by *Cupriavidus necator*. *Bioresour. Technol.* 2013, *130*, 16–22.
27. Kumar, M.S.; Mudliar, S.N.; Reddy, K.M.K.; Chakrabarti, T. Production of biodegradable plastics from activated sludge generated from a food processing industrial wastewater treatment plant. *Bioresour. Technol.* 2004, *95*, 327-330.
28. González-García, Y.; Meza, J.C.C.; Reynoso, O.G.; López, J.A.C. Synthesis and biodegradation of polyhydroxyalkanoates: plastics of microbial origin. *Rev. Int. Contam. Ambient*. 2013, *29*, 77-115.
29. Nwokola, E.; Smartt, J. *Food and feed legumes and oilseeds*. 1st ed. Chapman & Hall: London, England, 1996.
30. Thompson, J.C.; He, B.B. Characterization of crude glycerol from biodiesel production from multiple feedstocks. *Appl. Eng. Agric.* 2006, *22*, 261–265.
31. Srivastava, S.K.; Tripathi, A.D. Effect of saturated and unsaturated fatty acid supplementation on bio-plastic production under submerged fermentation. *3 Biotech.* 2013, *3*, 389-397.
32. Ramadas, N.V.; Singh, S.K.; Soccol, C.R.; Pandey, A. Polyhydroxybutyrate production using agro-industrial residue as substrate by *Bacillus sphaericus* NCIM 5149. *Braz. arch. biol. technol.* 2009, *52*, 17-23.
33. Silva, L.F.; Gomez, J.G.C.; Rocha, R.C.S.; Taciro, M.K.; Pradella, J.G.C. Biotechnological production of polyhydroxyalkanoates for the generation of biodegradable polymers in Brazil. *Quim. Nova.* 2007, *30*, 1732-1743.
34. Aldor, I.; Keasling, J.D. Metabolic engineering of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) composition in recombinant *Salmonella enterica serovar typhimurium*. *Biotechnol. Bioeng*. 2001, *76*, 108-1014.
35. Almeida, A.D.; Nikel, P.I.; Giordano, A.M.; Pettinari, M.J. Effects of granule-associated protein phaP on glycerol-dependent growth and polymer production in poly(3-hydroxybutyrate)-producing *Escherichia coli*. *Appl. Environ. Microbiol.* 2007, *73*, 7912-7916.
36. Hassan, M.A.; Yee, L.N.; Yee, P.L.; Ariffin, H.; Raha, A.R.; Shirai, Y.; Sudesh, K. Sustainable production of polyhydroxyalkanoates from renewable oil-palm biomass. *Biomass Bioenergy*. 2013, *50*, 1-9.
37. Xu, J.; Guo, B.H.; Yang, R.; Wu, Q.; Chen, G.Q.; Zhang, Z.M. In situ FTIR study on melting and crystallization of polyhydroxyalkanoates. *Polymer*. 2002, *43*, 6893-6899.
38. Zhu, X.; Yan, D. In situ FTIR spectroscopy study on the melting process of isotactic Poly(Propylene). *Macromol. Chem. Phys*. 2001, *202*,1109-1113.
39. Hong, K.; Sun, S.; Tian, W.; Chen, G.Q.; Huang, W. A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. *Appl. Microbiol. Biotechnol*. 1999, *51*, 523-526.
40. Barud, H.S.; Souza, J.L.; Santos, D.B.; Crespi, M.S.; Ribeiro, C.A.; Messaddeq, Y.; *et al*. Bacterial cellulose/poly(3-hydroxybutyrate) composite membranes. *Carbohydr. Polym.* 2011; 83: 1279-1284.
41. Wong, Y.M.; Brigham, C.J.; Rha, C.; Sinskey, A.J.; Sudesh, K. Biosynthesis and characterization of polyhydroxyalkanoate containing high 3-hydroxyhexanoate monomer fraction from crude palm kernel oil by recombinant *Cupriavidus necator*. *Bioresour. Technol.* 2012, *121*, 320–327.
42. Laycock, B.; Halley, P.; Pratt, S.; Werker, A.; Lant, P. The chemomechanical properties of microbial polyhydroxyalkanoates. *Prog. Polym. Sci.* 2014, *39*, 397-442.
43. Rehm, B.H.A. Bacterial polymers: biosynthesis, modifications and applications. *Nat. Rev. Microbiol.* 2010, *8*, 578-592.

44. Hammer, A.; Giani, S.; Hempel, E.; Jing, N.; Nijman, M.; Riesen, R.; Schawe, J.; Schubnell, M. *Thermal analysis of polymers: Selected applications.* 1st ed. Mettler Toledo: Greifensee, Switzerland, 2013.
45. Bengtsson, S.; Pisco, A.R.; Johansson, P.; Lemos, P.C.; Reis, M.A.M. Molecular weight and thermal properties of polyhydroxyalkanoates produced from fermented sugar molasses by open mixed cultures. *J. Biotechnol.* 2010, *147*, 172-179.
46. Ribeiro, P.L.L.; Silva, G.S.; Druzian, J.I. Evaluation of the effects of crude glycerol on the production and properties of novel polyhydroxyalkanoate copolymers containing high 11-Hydroxyoctadecanoate by *Cupriavidus necator* IPT 029 and *Bacillus megaterium* IPT 429. *Polymer. Adv. Tech.* 2016, *27*, 542-549.
47. Madison, L.L.; Huisman, G.W. Metabolic engineering of Poly(3-hydroxyalkanoates): from DNA to plastic. *Microbiol. Mol. Biol. Rev.* 1999, *63*, 21-53.
48. Sudesh, K.; Abe, H.; Doi, Y. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog. Polym. Sci.* 2000, *25*, 1503-1555.
49. Cha, S.H.; Son, J.H.; Jamal, Y.; Zafar, M.; Park, H.S. Characterization of polyhydroxyalkanoates extracted from wastewater sludge under different environmental conditions. *Biochem. Eng. J.* 2016, *112*, 1-12.