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ASSESSMENT OF POLYHYDROXYALKANOATE SYNTHESIS IN SUBMERGED CULTIVATION OF *Cupriavidus necator* AND *Burkholderia cepacia* STRAINS USING SOYBEAN AS SUBSTRATE

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Abstract - Polyhydroxyalkanoates (PHA) are biocompatible and biodegradable polyesters produced by prokaryotic microbes for energy storage and carbon reserve. These polymers are an option to diminish the massive impact caused by inadequate disposal of synthetic plastics. In this study, evaluation and characterization of PHA produced by *Cupriavidus necator* (IPT 026 and IPT 027) and *Burkholderia cepacia* (IPT 119 and IPT 400), using soybean as substrate, were carried out (soybean 15 g L^{-1}, pH 7.0, 150 rpm, 72 hours). The highest polymer production was achieved using IPT 027 (0.84 ± 0.07 g L^{-1}). All PHA produced showed the characteristic bands of polyester functional groups in the FTIR spectra. Polymers synthesized by *Cupriavidus necator* exhibited initial temperatures of degradation superior to 300oC and higher molecular weights than the ones produced by *Burkholderia cepacia*, which in turn, exhibited lower crystallinity (inferior to 30%), revealing high influence of the microorganism strain on PHA properties and production.
Keywords: Soybean; Biosynthesis; Biopolyester; Characterization.

INTRODUCTION

Petroleum based plastics are widely used in daily life due to their low cost, versatility, ease of processing and resistance, and their market share is gigantic and very profitable (Berto et al., 2017). However, the durability associated with the characteristics of non-biodegradability of these polymers compromise the world sustainability, since their accumulation in the environment entails great damages to the ecosystem.

As a result, the replacement of synthetic materials by biodegradable ones, at competitive costs, is an emerging need that must be fulfilled. A promising alternative to this demand is the production of bacterial biopolymers, especially the group formed by polyhydroxyalkanoates (PHA), since the referred materials can replace conventional thermoplastics (Ray et al., 2016; Faccin et al., 2013; Kourmentza et al. 2017; Koller et al., 2017).

PHA are a family of biocompatible and biodegradable polyesters with diverse structures and properties. The presence of 150 monomeric compositions has been reported, which grants them a broad set of applications. They can be intracellularly produced by numerous bacteria, being stored as an energy source, and also can be industrially biosynthesized from renewable raw materials, such as glucose and sucrose. However, these sugars represent a high cost in the production chain and therefore the use of cheaper carbon sources has been largely investigated and encouraged (HE et al.; 1999; Wu et al., 2000; Chen et al., 2016).

Yet, most studies are focused on the use of substrates predominantly constituted by carbohydrates (Wang et

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al., 2013; Carvalho et al., 2014; Ribeiro et al., 2016) or lipids (Altaee et al., 2016; Riedel et al., 2015; Morais et al., 2014; Shahzad et al., 2017), the use of complex substrates containing high fractions of both nutrients is an approach scarcely investigated in PHA synthesis (Koller et al., 2017).

Soybean plant (*Glycine max*) is one of the world's most economically important crops, cultured for the production of oleaginous grains used as feedstock in the manufacture of numerous products. This herbaceous vegetal has experienced the largest percentage increase in planted area since 1970, compared to any other major crop (Hartman et al., 2011; Figueiredo, 2016). United States and Brazil are, respectively, the first and second largest soybean producers in the world. In Brazil, world leader in productivity (kg/ha), the cost of production fell to about US-$ 6.30 per 60 kg/bag, which is around half the cost practiced in the United States (Figueiredo, 2016).

In this context, this study aims to evaluate and characterize PHA productions using soybean, largely available in Brazil, as an alternative and complex substrate for the submerged cultivation of the bacterial strains *Cupriavidus necator* IPT 026 and 027, and *Burkholderia cepacia* IPT 119 and 400. Since the use of low cost alternative substrates can enhance PHA production´s economic sustainability, this adds value to the raw material.

MATERIALS AND METHODS

Bacterial strains

Cupriavidus necator IPT 026 and IPT 027, and *Burkholderia cepacia* IPT 400 and ITP 119 were supplied by the Institute of 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 subcultured every 15 days.

Main carbon source

Soybean was acquired in the trade market of Salvador-BA, Brazil, milled to a particle size of 20-40 mesh and stored at 4°C for preservation.

Chemical composition of carbon source

The soybean chemical composition was evaluated in triplicate, total lipids were analyzed according to Bligh and Dyer (1959), moisture, crude ash, and crude protein were determined by analysis methods published by the Association of Official Analytical Chemists (AOAC, 1997). Carbohydrate content was calculated by percent mass difference, according to Eq (1).

$$\text{Carbohydrate\%} = 100 - (\text{Ash\%} + \text{Protein\%} + \text{Moisture\%} + \text{Lipid\%}) \quad (1)$$

Total carbon (C) content was determined according to Nelson and Sommers (1982) and total nitrogen (N) content was determined by the Kjehldal method (AOAC, 1997).

The fatty acid profile was determined by a capillary column gas chromatographic method according to Joseph and Ackman (1992) and Nascimento et al. (2013). The 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): split injection (1:100), injector temperature at 250°C, detector temperature at 280°C, column temperature maintained at 150°C for 16 minutes and programmed to increase 2°C per minute until 180°C, remaining at this temperature for 25 minutes and then programmed to increase 5°C per minute until 210°C, remaining at this last temperature for 25 minutes.

The quantification of fatty acids, expressed in milligrams per 100 g sample, was executed by the addition of an internal standard (C23:0 Sigma®, USA) according to Joseph and Ackman (1992) and calculated using Eq. (2).

$$\text{Concentration}\ (\text{mg}/100\text{g sample}) = \frac{A_{FA} \times M_{SI} \times F \times C_{TL}}{A_{IS} \times M \times F_{FA}} \cdot 1000 \quad (2)$$

where A_{FA} = area of fatty acid methyl ester peak in the chromatogram of the sample; M_{IS} = weight (in milligrams) of the internal standard added to the sample; F = correction factor of fatty acid methyl ester to fatty acid; C_{TL} = percentage composition of total lipids from the sample; A_{IS} = area of internal standard fatty acid methyl ester peak in the chromatogram of the sample; M = sample mass (in milligrams); F_{FA} = correction factor response of each fatty acid methyl ester ionization detector, relative to C23:0.

PHA production

Culture media

The 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 by a two stage cultivation process**,** as described by Wang et al. (2013) and Campos et al. (2014).

Mineral media were used as 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 (whole ground soybean at 15 g L^{-1}). Media pH was adjusted to 7.0 with NaOH (10 mol L^{-1}) or HCl (10 mol L^{-1}).

Shake flask cultivation

A volume of 10 μL of the microorganisms studied were separately inoculated into 50 mL of NB medium in Erlenmeyer flasks, and incubated at 30°C, 150 rpm and 24h, in rotary orbital shaker (Tecnal, model TE-424). Subsequently, 10% v/v of inoculum was transferred to Erlenmeyer flasks containing 80 mL of FC medium, followed by further incubation at 35°C, 150 rpm for 24 h. Finally, 10% v/v of FC was transferred to 80 mL of SC and incubated at 35°C, 180 rpm and 72 h. All media used in the bacterial cultivations were sterilized at 121°C for 20 minutes in autoclave.

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 -8°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 2h with vigorous agitation on a magnetic stirrer plate with heating (model IKAHS 7), following the proportion of 0.5 g of cells per 50 mL of solvent (Campos et al., 2014). 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 PHA films. Cell dry mass (CDM) 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 group 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_{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}.

X-ray diffraction analysis

Crystallinity and crystal peaks 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 diffracted intensity measured by XRD according to Vonk's method (Vonk, 1973).

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. (2014) and Ribeiro et al. (2015).

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) 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}. As the mobile phase, chloroform was employed at 1 mL min^{-1}. A standard curve was created using monodisperse 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 Statistica 8.0 software (Statsoft Inc., Tulsa). Statistical significance was calculated by Tukey's test ($p<0.05$).

RESULTS AND DISCUSSION

Substrate chemical composition

Soybean is a crop largely used as food for livestock and people, as it is an abundant source of protein and oil, and for the industrial manufacturing of diverse products. The nutritional content of the oleaginous grains is variable according to climate conditions, maturity stage, portion of the seed, and the plant variety (Genovese et al., 2006; Nwokola, 1996).

It is important to remark that although soybean is used for human consumption, the choice of a raw material for the industrial production of a specific bioproduct in a given country depends primarily on its availability and on its cost (Erickson et al., 2012). Thus in some cases, the use of edible substrates for the production of plastics or even biofuels is justified and overcomes the disputes with food production. Nevertheless, this analysis must be carried out very carefully, according to the reality of each market (Nonato et al., 2001; Dias et al., 2017).

Table 1 presents the chemical composition determined for the soybean seed used as substrate in the study of bacterial submerged cultivation for PHA production. The values are disposed as percentage averages followed by their standard deviations. The substrate carbon:nitrogen ratio (C:N) and the fatty acids profile of the lipid portion are also shown.

It is possible to observe that the highest fractions in the grain are constituted of proteins (33.62%), carbohydrates (32.16%), and fats (22.24%). These results were very similar to those reported by Silva et al. (2012) and Genovese et al. (2006).

However, Felberg et al. (2004) found a protein content fraction that is almost 10% higher than what was observed in this study. Nevertheless, it is expected that the composition of soybean may vary depending on a wide range of parameters of its cultivation.

Soybean lipid fraction was composed of 18.52% saturated fatty acids (lauric [C12:0], myristic [C14:0], palmitic [C16:0], stearic [C18:0] and arachidic [C20:0]), 22.99% monounsaturated fatty acids (oleic [C18:1]), and 58.49% polyunsaturated fatty acids (linoleic [C18:2] and linolenic [C18:3]). Commonly, most oils originated from plants display a high percentage of unsaturated fatty acids (Nwokola, 1996). Galão et al. (2014) found similar proportions assessing the fatty acid profile of the BRS245R soybean variety.

The presence of fatty acids in the substrate offered for PHA synthesis influences polymer chain length and structure (Muhr et al., 2013a; Muhr et al., 2013b). In their study of medium supplementation for PHA production, Srivastava and Tripathi (2013) found that palmitic acid supplemented media showed the presence of short chain length polyhydroxybutyrate.

Table 1. Chemical composition of whole soybean seed.

Composition	Avarege (%)	Fatty acid	Averege (%)
Moisture	7.72 ± 0.08	C12:0	0.07±0.02
Total ash	4.26 ± 0.10	C14:0	0.48±0.09
Total lipid	22.24± 0.09	C16:0	11.87±0.06
Crude protein	33.62 ± 0.12	C18:0	3.63±0.01
Carbohydrates	32.16± 0.39	C18:1ω9c	21.53±0.05
C:N*	9.5:1	C18:1ω9t	1.46±0.03
		C18:2ω6c	54.41±0.07
		C18:3ω6	4.08±0.02
		C:20	2.47±0.12

On the other hand, oleic acid and linoleic acid additions showed both saturated and unsaturated PHA of different chain lengths.

The carbon:nitrogen ratio (C:N) in the soybean is approximately 10:1, however, the nitrogen source in the substrate is composed of proteins, which are a non-readily biodegradable carbon source (Oliveira et al., 2016). Therefore, the medium was supplemented with $(NH_4)_2SO_4$ to a C:N of approximately 62:1 in the fermentation medium, considering no protein consumption, given the presence of a high fraction of carbon sources readily biodegradable in the substrate. The C:N values documented in the literature for PHA production are wide, ranging from 20:1 to 144:1, depending on the microorganism strain used (González-García et al., 2013; Kumar et al, 2004).

The chemical content found for soybean, rich in complex nutrients, suggests that this crop has a promising potential as carbon source/substrate for microorganism cultivations, since differences in trace elements and macronutrient concentrations can alter the growth and fermentation pattern in the synthesis of a desired product (Liu, 2017).

Influence of bacterial strain on PHA production

In order to select the best biocatalyst for PHA production in submerged cultivation, the effects of different microorganisms on polymer accumulation were evaluated under the same cultivation parameters.

Table 2 shows the results obtained for PHA synthesis for each microorganism studied (ANOVA: $F = 221.5$; $p < 0.0001$), cell dry mass (CDM) production and polymer mass percentage in CDM are also displayed. *C. necator* IPT 027 showed the best performance in polymer production, yielding 0.84 gL^{-1} of polyesters in the fermentation media, followed by *C. necator* IPT 026 and *B. cepacia* IPT 400, which produced 0.54 gL^{-1} and 0.53 gL^{-1}, respectively. The last two strains did not present statistical differences in performance according to Tukey's test ($P<0.05$). *B. cepacia* IPT 119 produced 0.44 g L^{-1} of polymer, an amount that is 47.62% lower than what was achieved by the best performer bacteria. On average, *C. necator* strains production was 42.27% higher than what was observed for *B. cepacia.* These results show the great impact caused by the choice of the microorganism in the biosynthesis process.

Table 2. PHA, CDM and PHA mass percentage in CDM in fermentation of 15 g L^{-1} of soybean over 72 hours of incubation, pH of 7.0, and rotation of 180 rpm.

Microorganism	PHA	CDM	PHA in DCM (%)
	(g L^{-1})		
C. necator IPT 026	0.54 ± 0.014[a]	2.69± 0.090[a]	20.21 ± 0.16[a]
C. necator IPT 027	0.84 ± 0.021[b]	3.57 ± 0.050[b]	23.66 ±0.93[b]
B. cepacia IPT 119	0.44 ± 0.028[c]	5.93 ± 0.080[c]	7.38 ± 0.56[c]
B. cepacia IPT 400	0.53 ± 0.015[a]	5.33± 0.040[d]	10.05 ± 0.36[d]

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

Regarding bacterial cellular growth, *B. cepacia* IPT 119 exhibited the highest production, culminating with the lowest PHA accumulation (7.38%). All other bacteria exhibited the same trend of good cellular growth and low polymer accumulation.

In his studies, Ribeiro et al. (2015) assessed the production of PHA with *C. necator* IPT 027 using glucose as carbon source, reporting 2.41±0.10 g L^{-1} of polymer in the culture medium with 56.57±0.56% of cellular accumulation. This value is greatly superior to the one found using soybean as substrate with the same bacterial strain.

Possibly, this polymer production difference is partly due to the more complex composition of soybean, which would lead to a more difficult absorption of nutrients by the bacteria, different from glucose that would be more easily assimilated, given its structure.

Another point to consider is that, in addition to the carbon source, the supply of nitrogen is crucial for PHA biosynthesis and accumulation within the cell. The limitation of this nutrient generates a cellular stress that leads to energy savings in the form of PHA (Madison et al., 1999). Therefore, considering the high amount of proteins present in soybean, it is possible that the bacteria investigated might have been able to use the nitrogen present in this fraction to compensate for its limitation in the culture medium, compromising the accumulation of PHA within the cells and stimulating the growth phase.

The ratio of organic carbon available in the substrate by the nitrogen supplied in the culture medium was calculated as 62:1; however, if considered that the bacteria can access carbon and nitrogen present in the protein fraction of soybean, the carbon:nitrogen ratio decreases to approximately 9:1, lowering greatly nitrogen limitation in the medium.

Comparable observations were made by Ramadas et al. (2009) and Gowlda et al. (2014**),** who also reported good cellular growth but low PHA accumulation using carbon sources with high protein content.

Regarding the performance of different bacteria and substrates in PHA production, Pal et al. (2009) published a polymer yield ranging from 0.2 to 1.2 g L^{-1} using *Bacillus thuringiensis* and different synthetic media. Altaee et al. (2016) reported a PHA production of 0.54 g L^{-1} using *Rhodococcus equi* and crude palm kernel oil as substrate. This last value is similar to what was found for *C. necator* IPT 026 and *B. cepacia* IPT 400, and inferior to the result achieved by *C. necator* IPT 027 in the present study.

PHA Characterization

Thermochemical characterizations were executed for all PHA produced in order to evaluate their performance and capacity to attend standards that would permit their usage and commercial escalation.

PHA's functional group characterization, presented in Figure 1, was completed by spectroscopy in the infrared spectra (FTIR), the wave number (cm^{-1}) associated with each band corresponds to a particular functional group present in the polymer samples. Characteristic bands related to PHA structure documented in the scientific literature were observed in all samples scanned.

The band noted at 1728 cm^{-1} represents the axial deformation of a C=O carbonyl group. The presence of a carbonyl band at this wave number indicates strong presence of polyhydroxybutyrate in the material structure (Hong et al., 1999). C-H carbon-hydrogen bond stretchings of CH_3 and CH_2 groups were identified at the wave numbers 2931 cm^{-1} and 2854 cm^{-1}, respectively (Hong et al., 1999; Barud et al., 2011).

The band of the carbonyl of the ester group (C-C) is observed at 972 cm^{-1} (Xu et al., 2002). The bands at 1048 cm^{-1} and 1288 cm^{-1} are associated correspondingly to the asymmetric and the symmetric stretching vibration of the C–O–C group (Zhu et al., 2001). Wagging of CH_3 is related to the wave number 1381 cm^{-1} (Xu et al., 2002).

PHA and soybean thermal behaviors are displayed in the thermogravimetric curves (TGA) and their associated derivatives (DTGA) in Figure 2. It is possible to observe the mass variation of the samples with the temperature increase, which is related to the loss of volatile components. Thermal degradation of all polymers produced occurred in two mass loss events, which is normally associated with the presence of impurities remaining from the extraction and separation processes (González-García et al., 2013). The thermal events can be quickly identified by the inverted peaks displayed in the DTG curves.

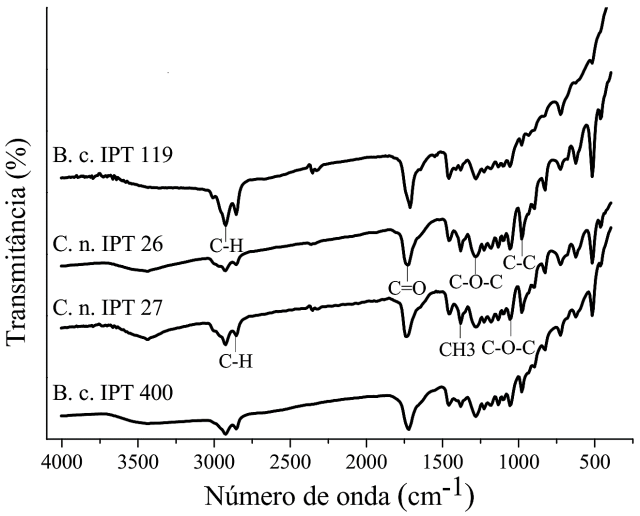

Figure 1. FTIR spectra of PHA produced by *C. necator*, and *B. cepacia* strains in submerged cultivation with soybean.

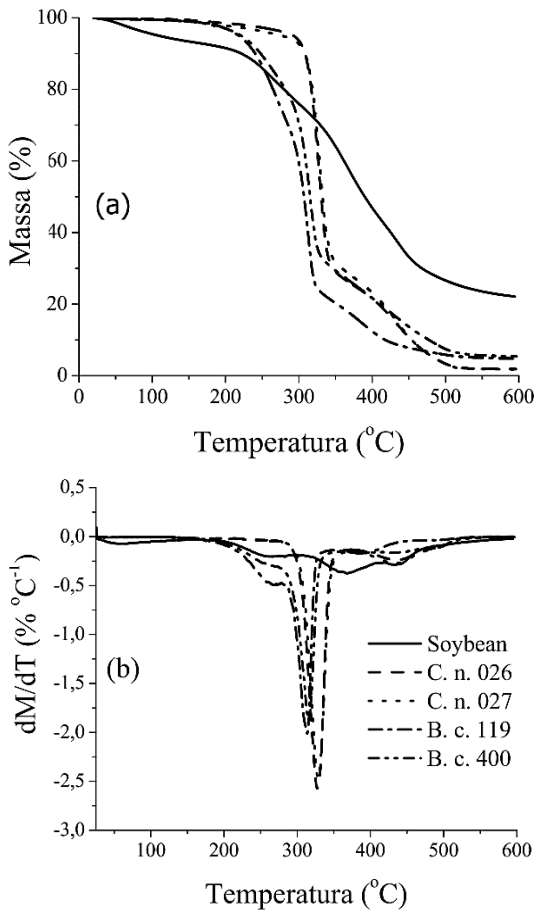

Figure 2. TGAs (a) and DTGs (b) of soybean seed and PHA produced by *C.necator* and *B. cepacia* strains in submerged cultivation.

Soybean thermal degradation occurred in three mass loss events with a total weight loss of 77.88%. It is possible to observe that the first event displayed by the substrate is also seen in PHA produced by *B. cepacia* strains, and the third event existent in soybean is present in PHA produced by *C. necator*. The second event in soybean thermogravimetric curves was not detected in any of the polymer samples. It is possible to infer that all PHA produced presented substrate traces in its composition, remaining from the polymer extraction process.

The first thermal event seen in PHA produced by *C. necator* and the second event seen in PHA produced by *B. cepacia* were the most significant part of their mass loss behavior with temperature changes. They were different from each other and were not noted in soybean TGA/DTGA curves, being related to the characteristics of the polyesters produced.

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 PHA produced and for soybean are organized in Table 3.

The polymer synthetized by *C. necator* IPT 027 exhibited the best thermal stability with an initial degradation temperature of 307.03°C, a maximum decomposition temperature of 328.41°C and a total weight loss of 98.26% (first event). This performance was superior to that found by Ribeiro et al. (2015) in his studies of PHA production with glucose (257.6°C), glycerol (286.8°C) and crude glycerol (267.9°C) using *C. necator* IPT 027, indicating great influence of the substrate and the biosynthesis conditions on thermal behavior. The same author also reported a T_{in} of 316.7°C for a PHA produced by *B. cepacia* IPT 438 using glycerol as substrate, a result superior to those achieved by all *B. cepacia* strains used in this paper.

Nevertheless, the polyesters produced by *B. cepacia* IPT 119 and 400 displayed thermal performances superior to what was observed in the results reported by Ribeiro et al. (2016) and Ray et al. (2016) for PHA synthetized by *Bacillus megaterium* and *Pannonibacter phragmitetus*, respectively.

It is important to remark that high decomposition temperature is a crucial factor for polymer processing in industry, since the material has to be able to resist, with no structural degradation, the required temperatures for extrusion and injection molding to manufacture biodegradable films and molded pieces (Bengtsson et al., 2010).

Diffractograms for PHA produced with all bacterial strains studied and their degree of crystallinity (I_c) are shown in Figure 3. It is possible to observe the

Table 3. Initial degradation (T_{in}) and maximum decomposition temperatures (T_{maxdec}), along with total weight loss of soybean seed and PHA produced by *C. necator* and *B. cepacia* strains.

Microorganism strain	First event		Second event		Total weight loss (%)
	T_{in} (°C)	T_{maxdec} (°C)	T_{in} (°C)	T_{maxdec} (°C)	
C. necator IPT 026	304.35	327.21	411.51	432.77	98.16
C. necator IPT 027	307.03	328.41	410.44	433.16	98.26
B. cepacia IPT 119	206.09	263.63	296.87	312.94	95.23
B. cepacia IPT 400	207.15	262.48	298.73	315.93	94.61

Soybean seed						
First event		Second event		Third event		Total weight loss (%)
T_{in} (°C)	T_{maxde} (°C)	T_{in} (°C)	T_{maxdec} (°C)	T_{in} (°C)	T_{maxdec} (°C)	
205.59	261.18	340.99	367.89	408.79	434.30	77.88%

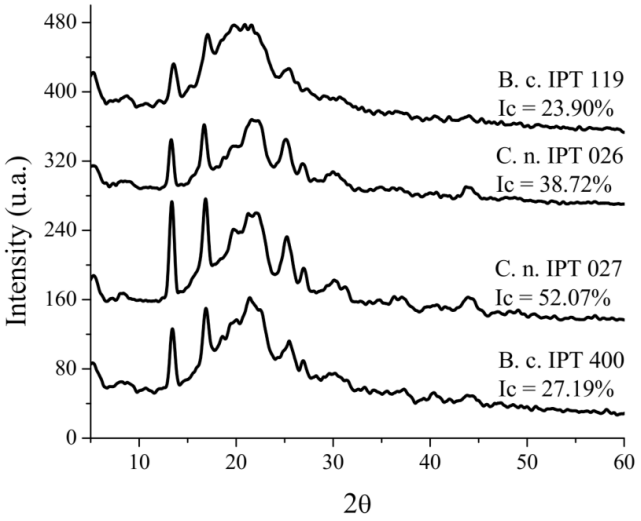

Figure 3. X-ray diffractograms of PHA produced by *C. necator* and *B. cepacia* strains in submerged cultivation with soybean.

diffraction peaks at 2θ for all samples evaluated, reflecting the atomic arrangement in their structure. There were discrete variations in the curves due to the different producer microorganisms. Even so, all PHA presented peaks at 13.34°, 16.85°, 21.70° and 25.30°, which are typical of semi-crystalline polyesters (Barud et al., 2011).

Degree of crystallinity (I_c) is an important factor for mechanical performance and processability since it affects the molecular conformation and intermolecular packing of the atoms of the material. The I_c ideal value must not be much superior to 50%, otherwise, problems related with brittleness and rigidity starts to emerge, making polymer processing more challenging and requiring corrective actions that can result in an operation price rise (Laycock et al., 2013; Rehm, 2010).

I_c of the polymers studied ranged from 52.0% to 23.9%, demonstrating that the microorganism strain had significant impact on the polyester structural arrangement. PHA produced from *C. necator* IPT 027 exhibited the highest I_c, showing the predominant crystalline structure of this biomaterial. In contrast, all other polyesters assessed exhibited a predominant amorphous structure, that can be comparatively detected by the intensity of the diffraction peaks in the 2θ range (Figure 3).

On average, the materials produced by *C. necator* strains displayed an I_c of 45.4%, superior to *B. cepacia*, with an I_c of 25.5%, revealing that, in regard to this property, the last strains produced better polymers, which is associated with their specific bioconversion ability.

PHA degrees of crystallinity reported in the literature (Campos et al., 2014; Ribeiro et al., 2015; Ribeiro et al., 2016; Bengtsson et al., 2010) are highly dependable on the substrate and on the producer microorganism, with values ranging from 66.7% to 35.3%. Comparatively, the polymers produced in this paper exhibited low crystallinity and great potential for industrial processing and applications based on their molecular arrangement.

Weight-average molecular mass (Mw), number-average molecular mass (Mn) and polydispersity (PDI) data, obtained from size-exclusion chromatography, are organized in Table 4 for the polymers produced from soybean. Figure 4 illustrates the biopolymer separation chromatograms. The calibration curve of standard polystyrene with different values of Mw as a function of column retention time (t), used to calculate the samples Mw, and its coefficient of determination, are displayed in Eq (3).

$$\log(\mathrm{Mw}) = -0.8364\mathrm{t} + 14.831 \quad R^2 = 0.9917 \tag{3}$$

The microorganisms that produced the highest weight-average molecular mass were *C. necator,* with an average Mw of 216.081 kDa, against an Mw average value of 124.323 kDa observed for *B. Cepacia* strains, showing the impact of different synthesizer microorganisms on polymer size, even when offered the same substrate and growth conditions. These data are coherent with the literature, since PHA Mw has been reported to range between 50 and 3,000 kDa depending on the biosynthesis conditions (Madison et al., 1999; Sudesh et al., 2000). PHA polydispersity, which measures the heterogeneity of polymer molecular sizes, shows that *C. necator* IPT 026 produced a material that is more homogenous, with a PDI of 1.81. In contrast, *B. cepacia* IPT 119 exhibited the highest dispersion in polymer size with a PDI of 2.83, what can be confirmed by Figure 4. Yet, in his investigations with PHA production from wastewater sludge, Sang-Hyeop et al. (2016) reported values of PDI that extended to numbers as high as 3.44.

Table 4. Molar mass of PHA produced by the strains *C. necator* IPT 026 and IPT 027, and *B. cepacia* IPT 119 and IPT 400 from soybean.

Microorganism	Mw (kDa)			Mn (kDa)	PDI
	Minimum	Medium	Maximum		
C. necator IPT 026	4.624	240.868	2661.828	132.938	1.81
C. necator IPT 027	4.624	191.293	2195.533	77.968	2.45
B. cepacia IPT 119	9.430	130.287	977.804	45.907	2.83
B. cepacia IPT 400	4.199	118.359	888.038	47.604	2.49

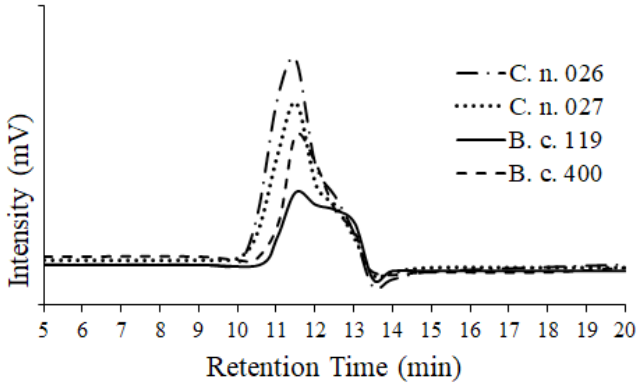

Figure 4. Chromatograms of PHAs produced by the *C. necator*, and *B. cepacia* strains from soybean.

Higher PDI values are often related to either chain transfer reactions or depolymerase activity during granule formation within the cells or to random decay of active synthase molecules, which is an undesirable phenomenon in the biosynthesis (Laycock et al., 2013).

CONCLUSIONS

All strains studied were able to use the offered substrate to synthetize PHA. Regarding production, *C. necator* strains exhibited the highest performance in polymer accumulation. These microorganisms also produced polyesters with the highest thermal stability and weight-average molecular masses. Polyesters synthesized by *B. cepacia* revealed predominant amorphous molecular arrangements with the lowest crystallinity. *C. necator* and *B. cepacia* influenced importantly PHA production and characteristics, confirming that the biocatalyst variable is of critical significance in the process. The relationship between PHA accumulation, cellular production and substrate protein content must be closely investigated in order to support more evidence and trigger higher PHA production. Lastly, medium composition and fermentation conditions can be optimized to maximize biopolymer production.

NOMENCLATURE

A_{FA}	Area of fatty acid methyl ester peak in the chromatogram of the lipid fraction of soybean (m^2).
A_{IS}	Area of internal standard fatty acid methyl ester peak in the chromatogram of the lipid fraction of soybean (m^2).
C	Total carbon content on soybean (g).
C_{TL}	Percentage composition of total lipids from the sample (%).
F	Correction factor of fatty acid methyl ester to fatty acid (Dimensionless).
F_{FA}	Correction factor response of each fatty acid methyl ester ionization detector, relative to C23:0 (Dimensionless).
I_c	Degree of crystallinity (Dimensionless).
M	Lipid sample mass (mg).
M_{IS}	Weight of the internal standard added to the soybean lipid sample (mg).
Mn	Number-average molecular mass (g mol^{-1}).
Mw	Weight-average molecular mass (g mol^{-1}).
N	Total carbon content on soybean (g).
PDI	Polydispersity (Dimensionless).
t	Time (s).
T_{in}	Initial degradation temperature (°C).
T_{maxdec}	Maximum decomposition temperature (°C).

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