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Simultaneous Biosynthesis of Polyhydroxyalkanoates and Extracellular Polymeric Substance (EPS) from Crude Glycerol from Biodiesel Production by Different Bacterial Strains

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Abstract Simultaneous synthesis of polyhydroxyalkanoates (PHAs) and polyglutamic acid (PGA) was investigated in cultures of *Cupriavidus necator* IPT 026, *C. necator* IPT 027, *C. necator* IPT 029, and *Bacillus megaterium* INCQS 425 strains in a medium containing 2.0 % sucrose or crude glycerol from biodiesel (CGB), in an orbital shaker (35 °C, 180 rpm, 72 h). All the strains tested simultaneously produced PHA and PGA in a medium containing CGB. The *C. necator* IPT026 culture provided higher molecular mass PHA and PGA (1128.55 and 835.56 kDa, respectively). *B. megaterium* INCQS 425 promoted PGA production (1.90 g L^{-1}) with higher crystalline melting temperature (84.04 °C) and higher initial decomposition temperature (247.10 °C). Furthermore, the latter culture promoted the production of medium- and long-chain PHA (0.78 g L^{-1}) with high crystalline melting temperatures (~170 °C) and high initial decomposition temperature (307.53 °C) and low degree of crystallinity (20.2 %). These characteristics render these PHAs more appropriate and suitable for processes that require high temperatures, such as extrusion, increasing the possibility of industrial applications, especially in the packaging sector.

Keywords Polyhydroxyalkanoates · Polyglutamic acid · Crude glycerol · Simultaneous synthesis · Properties

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Introduction

Currently, there is a global trend to replace petroleum-based polymers with biopolymers, which are biodegradable and/or biocompatible. The concern about non-degradable waste in the environment, including some polymers, is growing every day. Therefore, it is necessary to develop, be aware of, and characterize alternative biodegradable polymers [1]. In this context, the use of industrial wastes in bioprocesses enables generating of low-cost products, adding value to the wastes, and minimizing environmental problems associated with their disposal.

Polyhydroxyalkanoates (PHAs) are natural polymers belonging to the group of polyesters, synthesized intracellularly by a variety of prokaryotes. They are mainly composed of carbon, oxygen, and hydrogen [1, 2]. In recent years, PHA have received much attention because of their thermoplasticity, low toxicity, good biodegradability, and environmental compatibility. These characteristics contribute to their wide use in the manufacture of biodegradable packaging to replace petroleum-based plastics [3, 4].

PHA polymers can be synthesized by some halophyte species of the genus *Haloferax*, *Halococcus*, *Natronobacterium*, and *Natronococcus* and by *Bacillus*, *Cupriavidus*, *Staphylococcus*, *Micrococcus*, *Methylobacterium*, *Paracoccus*, and *Rhodococcus* [5, 6]. Depending on the genus, some of these microorganisms are able to synthesize both PHAs and extracellular polymeric substances (EPSs) [6].

EPSs are hydrated biopolymers continuously produced during the growth and metabolism of the biomass [7]. These substances are typically secreted by microorganisms under stress, such as carbon-deprived environment, high mechanical shear stress, and high carbon-to-nitrogen ratio, and may be formed by proteins and polysaccharides of various sizes, depending on the degree of polymerization [8, 9].

Recently, the increased demand for natural polymers for pharmaceutical, food, and other industrial applications has led to a remarkable interest in polysaccharides produced by microorganisms. Indeed, a substantial interest has been aroused in the isolation and identification of new microbial polysaccharides that might have innovative applications as gelling, emulsifying, and stabilizing agents [10].

Discussions in the literature show that some bacteria are capable of producing PHAs and EPSs, such as polysaccharides [9], alginates [11], poly-γ-glutamic acid [12], and hyaluronic acid [13]. However, literature on the simultaneous synthesis of PHA and polyglutamic acid (PGA) is scarce.

PGA, or polyglutamate, is a polymer of D-glutamic acid and L-glutamic acid polymerized in the form of a capsule or extracellular viscous material [14]. Due to its non-toxicity, biocompatibility, and edibility, the application of this biopolymer is of great importance to various industrial applications, including its use as a cryoprotectant, controlled drug release material, biodegradable fiber, surgical adhesive, and thermoplastic [12, 14].

The cost in bioprocesses depends on the bacterial strains used, the process conditions, the purification steps, and especially, the origin of the primary substrate [1]. An important point for consideration for the increasing feasibility of PHA biosynthesis is the use of alternative carbon sources as a cost-reducing option of production [15]. Thus, industrial waste can be used in bioprocesses and contribute to minimizing environmental problems associated with its improper disposal.

Crude glycerol from biodiesel (CGB) is an important byproduct of biodiesel production process, comprising 10 % of the transesterification reaction product. It is estimated that by 2016, the world production of biodiesel should reach 37 billion gallons, which implies a production of ~4 billion gallons of crude glycerol [16].

The aim of this study is to investigate the simultaneous synthesis of PHAs and PGAs from batch cultures of different bacterial strains in a medium containing CGB and to characterize the obtained polymers.

Materials and Methods

Microorganism and CGB

Cupriavidus necator IPT 026, *C. necator* IPT 027 and *C. necator* IPT 029 and *Bacillus megaterium* INCQS 425 were provided by IPT (Institute for Technological Research, São Paulo, Brazil) and were maintained on nutrient agar at 4 °C.

CGB was supplied by Petrobrás (Candeias, BA, Brazil), where it has been generated during biodiesel production using castor bean oil and ethanol in the transesterification reaction.

Batch Cultures

Bacteria were cultured in three media [17]. For the initial culture, ~10-μL colonies were transferred into 50 mL of nutrient medium [3.0 g L^{-1} (*w*/*v*) peptone and 5.0 g L^{-1} (*w*/*v*) meat extract] and incubated in an orbital shaker (Tecnal, Mod. TE-424) at 150 rpm and 35 °C for 24 h. The culture was diluted 10× in a mineral medium containing nitrilotriacetic acid (1.9 g L^{-1}, *w*/*v*), ferrous ammonium citrate (1.0 g L^{-1}, *w*/*v*), $MgSO_4$ $7H_2O$ (0.5 g L^{-1}, *w*/*v*), $CaCl_2$ $2H_2O$ (3.0 g L^{-1}, *w*/*v*), and $(NH_4)_2SO_4$ (2.0 g L^{-1}, *w*/*v*) and incubated at 35 °C, 150 rpm, for 24 h. The culture was then diluted 10× in a limiting medium containing nitrilotriacetic acid (1.9 g L^{-1}, *w*/*v*), ferrous ammonium citrate (1.0 g L^{-1}, *w*/*v*), $MgSO_4$ $7H_2O$ (0.5 g L^{-1}, *w*/*v*), $CaCl_2$ $2H_2O$ (3.0 g L^{-1}, *w*/*v*), $(NH4)_2SO_4$ (0.5 g L^{-1}, *w*/*v*), Na_2HPO_4 $12H_2O$ (0.2 g L^{-1}, *w*/*v*), KH_2PO_4 (3.7 g L^{-1}, *w*/*v*), and a carbon source (CGB, 20.0 g L^{-1}, *w*/*v*). The pH was adjusted to 7.0 and the cultures were incubated at 35 °C, 180 rpm, for 72 h.

PHA and PGA Extraction

Bacterial cultures containing EPS, the microbial cells, and PHA were centrifuged at 42,200×*g* for 30 min at 5 °C (HITACHI, model CR 22G). Extracellular PGA was extracted by the addition of absolute ethanol at 4 °C to the supernatant, in a ratio of 3:1 [18], and precipitated after stirring for 24 h. Pellets containing intracellular PHA were washed with distilled water and frozen at −80 °C for subsequent lyophilization (LIOBRAS, model L101) at −42 °C for 24 h [2]. After the lyophilization step, 0.5 g of cell material was suspended in 50-mL chloroform, in glass flasks, and incubated at 60 °C for 2 h, with vigorous agitation. After incubation, the samples were filtered under vacuum using 0.5-mm pore filters (Millipore, 47-mm diameter) to separate the cellular debris. The solvent was evaporated in an oven at 35 °C.

The production of intracellular PHA and extracellular PGA was calculated by gravimetric analysis and expressed as grams per liter.

Weighted Averaging MM

The molecular masses (MMs) of PHA and PGA were obtained by size-exclusion chromatography with HPLC (PerkinElmer Series 200) equipped with an autosampler and a refractive index (RI) detector (PerkinElmer).

For PHA separation, we used a Shodex KD 807 column (30 cm × 78 mm × 5 mm) at 30 °C. The polymers were dissolved in chloroform to a final concentration of 0.7 g L^{-1} and filtered (PTFE membrane, 0.22 μm) before separation. The mobile phase used was chloroform, at a flow rate of 1.0 mL min^{-1}. A standard curve (Log MM × retention time (RT)) was created using low polydispersity polystyrene standards (682–1,670,000 Da; (Polystyrene High Mw Standards Kit, Polymer Standards Service, USA).

To determine the molecular mass of PGA, HPLC-RI was used, with Shodex SB 804, 805, 806, and 807 columns in a series. The mobile phase was aqueous solution of $NaNO_3$ (0.05 M) at a flow rate of 1.0 mL min^{-1}. PGA was dissolved in the mobile phase to 0.3 % (*w*/*v*). A calibration curve (Log MM × RT) was constructed by separating 5.9 × 10^6 Da to 102,000 × 10^6 Da dextran standards (American Polymer Standards) under the same conditions.

FTIR Spectroscopy

PHA and PGA samples were qualitatively analyzed by Fourier transform infrared (FTIR) spectroscopy (PerkinElmer Model Spectrum 100) between 4000 and 400 cm^{-1} using KBr tablets.

DSC and TGA

Differential scanning calorimetry (DSC) (SHIMATZU DSC-50) was used to determine the crystalline melting temperatures (*Tm*) and crystalline melting enthalpies (ΔH_m) of PHA and PGA. Biopolymer samples were heated under inert atmosphere (N_2 20 mL min^{-1}) from 25 to 200 °C, with 10 °C min^{-1} heating rate. The degree of crystallinity of PHA (χ_c) was calculated as the ratio between ΔH_m of the obtained PHA and the crystalline melting enthalpy of 100 % crystalline biopolymers (ΔH^0_m) [19].

thermogravimetric analysis (TGA) (PerkinElmer Model Pyris 1 TGA) was used to determine the initial degradation temperatures (T_{onset}) and decomposition temperatures (T_{decomp}) of PHA and PGA samples. Samples (10 mg) were placed on a platinum tray and heated at a rate of 10 °C min^{-1} from 25 °C to a final temperature of 800 °C under inert atmosphere (N_2, 20 mL min^{-1}).

PHA and PGA Monomeric Composition

The identification and quantification of the constituent monomers of PHA were performed using gas chromatography/mass spectrometry (GC–MS) approach (Clarus 500 PerkinElmer) with TurboMass software v. 4.5.0 and NIST 98 library. PHA (~0.04 g) was subjected to methanolysis using methods described in the literature [20, 21], with some modifications [2]. The organic phase was separated after splitless injection into a capillary column DB-1 (30 m × 0.25 mm × 0.25 mm). Helium (flow rate 1.0 mL min^{-1}) was used as the carrier gas. The temperatures of the injector and detector were 250 and 240 °C, respectively. The mass spectrometer was programmed to scan between 50 and 550 m/z with a temperature program of 80–200 °C (20 °C min^{-1}). The mass spectra were compared with the spectra from 98 NIST library, and hydroxyalkanoate monomers were quantified by area normalization.

The PGA samples (10.0 mg) were initially hydrated in purified water (0.5 mL) over 12 h, hydrolyzed with 0.5-mL trifluoroacetic acid (1.0 M) for 10 h at 100 °C, and subsequently dried

under nitrogen gas, lyophilized (LIOBRAS L101), and dissolved in 1.0-mL ultrapure water. The hydrolyzed polymer solutions (10 μL) were separated by HPLC (PerkinElmer Series 200) using a Polypore H precolumn (4.6 mm × 30 mm × 10 mm) followed by a Polypore H column (220 mm × 4.6 mm × 10 mm) and detected with an ultraviolet (UV) detector operating at a 338-nm wavelength (PerkinElmer Series 200). The columns were placed in an oven at 50 °C. The mobile phase used was aqueous H_2SO_4 (pH 1.9) at a flow rate of 0.4 mL min^{-1}. Monomeric identification was carried out by comparing the retention times of the hydrolyzed polymers with the retention time of standard glutamic acid (Sigma-Aldrich).

Experimental results were analyzed using STATISTICA 7.0 software.

Results and Discussion

The CGB used in this study as a carbon and nutrient source for simultaneous PHA and PGA production was the same as the one used by Ribeiro and coworkers [22]. The authors showed that this CGB has a high content of minerals (especially Na and Zn), carbohydrates (43.11 ± 0.02 %), and total lipids (9.46 ± 0.02 %, of which 70.49 ± 0.04 % are saturated fatty acids and 29.51 ± 0.03 % are unsaturated fatty acids).

The fatty acid profile of CGB revealed the short- and medium-chain fatty acids, such as caproic (C6:0) and caprylic (C8:0) acids, and a significant long-chain fatty acid component, primarily palmitic (C16:0), stearic (C18:0), and oleic (C18:1ω9) acids [22]. The long-chain fatty acids arise during an incomplete chemical transesterification during fatty acid methyl ester generation [23]. According to Thompson and He (2006) [24], microorganisms use short-, medium-, and long-chain fatty acids as carbon sources to obtain polyunsaturated fatty acids that may function as copolymers of PHA.

Fatty acids can also be used as supplements in PHA formation. Fatty acid β-oxidation leads to an increase in acetyl-CoA concentration that, in turn, shifts the tricarboxylic acid cycle toward PHA synthesis [25]. Oleic and lauric acids have been extensively used as nutritional supplements for PHA production with *Aeromonas hydrophila* [26] and *Ralstonia eutropha* [27]. Supplementation of basal media with certain vegetable oils enhanced the exopolysaccharide production [28]. It has also been shown that plant and vegetable oils exert a stimulatory effect on PHA production and chain length [29].

Simultaneous Production of PHA and PGA

Simultaneous production of intracellular PHA and extracellular PGA by *C. necator* IPT 026, *C. necator* IPT 027, and *C. necator* IPT 029 and *B. megaterium* INCQS 425 strains was conducted in the media containing sucrose (control) or CGB.

As shown in Table 1, most strains produced PHA and PGA when grown on a medium containing sucrose and CGB. Only *C. necator* IPT 027 did not convert sucrose to PGA. CGB promoted ~2-fold increase of PHA production by *Cupriavidus* strains compared with sucrose medium. The same was observed for *B. megaterium* INCQS 425 (0.78 g L^{-1}) but with production 3.1× higher than on sucrose medium (0.25 g L^{-1}) (Table 1). The results also revealed that CGB promoted 4.1-, 28-, and 2.4-fold increase in the production of PGA by *C. necator* IPT 026, *C. necator* IPT 029, and *B. megaterium* INCQS 425, respectively, compared with the sucrose medium culture (Table 1).

Table 1 Biomass, polyhydroxyalkanoates (PHA) and polyglutamic acid (PGA) produced by different strains grown in an orbital shaker (35 °C/180 rpm/72 h) in a medium containing 2.0 % (*w*/*v*) sucrose or CGB

Medium	Products	Strains			
		IPT 026	IPT 027	IPT 029	INCQS 425
Sucrose	Biomass (g L^{-1})	2.35 ± 0.17	2.56 ± 0.16	1.88 ± 0.09	3.16 ± 0.09
	PHA (g L^{-1})	0.12 ± 0.08a	0.14 ± 0.07a	0.09 ± 0.05a	0.25 ± 0.14b
	PGA (g L^{-1})	0.47 ± 0.15a	–	0.06 ± 0.02b	0.56 ± 0.03a
	PHA + PGA (g L^{-1})	0.59	–	0.15	0.78
CGB	Biomass (g L^{-1})	1.74 ± 0.10	1.96 ± 0.12	1.46 ± 0.13	2.81 ± 0.09
	PHA (g L^{-1})	0.28 ± 0.11a	0.33 ± 0.09a	0.16 ± 0.09a	0.78 ± 0.10b
	PGA (g L^{-1})	1.93 ± 0.13a	0.05 ± 0.02b	1.69 ± 0.11a	1.90 ± 0.17a
	PHA + PGA (g L^{-1})	2.21	0.38	1.85	2.68

Means followed by the same letters in the same line are not significantly different by Tukey's test ($P > 0.05$)

B. megaterium INCQS 425 was the best PHA producer, and the maximum PHA production from CGB, 0.78 g L^{-1}, was 4.8× higher than that of *C. necator* IPT 029 (0.16 g L^{-1}). This suggests that the production of PHA and PGA is strongly influenced by the bacterial strain used.

The highest biomass concentration from the CGB medium was observed for *B. megaterium* INCQS 425 (2.81 g L^{-1}) and was ~56 % higher than the biomass concentrations of *C. necator* IPT 026 (1.74 g L^{-1}) and *C. necator* IPT 027 (1.96 g L^{-1}) and ~92 % higher than biomass concentration of *C. necator* IPT 029 (1.46 g L^{-1}).

CGB (2.0 %) was used in PHA production by *Zobellella denitrificans* grown in an orbital shaker (200 rpm, 41 °C, 96 h), yielding 2.82 g L^{-1} [30], i.e., approximately four times more than the amount produced in this study by *B. megaterium* INCQS 425 (0.78 g L^{-1}; 180 rpm, 30 °C, 72 h, 2.0 % CGB). Campos and coworkers [2] used 1.5 % (*w*/*v*) CGB, which promoted accumulation of 2.8 g L^{-1} PHA by *C. necator* IPT 026 grown in a bioreactor (500 rpm, 35 ° C, 72 h), and this was about three times more than the maximum yield obtained in this study.

PGA synthesis is promoted by the presence of D- or L-glutamic acid in the culture medium [12]. *Bacillus subtilis* ZJU-7 grown in a 10-L bioreactor (450 rpm, 1.5 vvm, 37 °C, for 48 h) in a medium containing glutamic acid and glucose produced 101.1 g L^{-1} PGA [31], double of that obtained under the same conditions but with glucose and corn residues (46.4 g L^{-1}) [32]. In another study, *B. subtilis* BL62 was cultivated in a medium containing L-glutamic acid, citric acid, and glycerol, in an orbital shaker (180 rpm, 37 °C, for 72 h), generating 0.79 g L^{-1} PGA [33]. Tang and coworkers [34] produced PGA with *B. subtilis* NX-2 via a solid-state fermentation with dry mushroom residues, monosodium glutamate, and crude glycerol (35 °C, for 48 h) and obtained 115 g kg^{-1} solid substrates.

Taken together, the discrepancy in the PHA and PGA production may be associated with the bacterial species, origin of glycerol, and reaction conditions (time, agitation speed, and temperature). Considering the above results from the strains tested (Table 1), the highest simultaneous PHA and PGA production was obtained with *B. megaterium* INCQS 425 grown in the medium containing CGB.

The experimental results also revealed a linear correlation between specific PHA production from sucrose with both biomass (R^2 = 0.8) and PGA production (R^2 = 0.6). PHA production from CGB was linearly correlated with biomass (R^2 = 0.9) but not with PGA production.

Furthermore, for larger synthesis of biopolymers (PHA + PGA) (Table 1), PGA production exceeded PHA production. PHA and PGA can be produced simultaneously and serve as energy and carbon sources when the microorganisms are experiencing starvation conditions. In general, independently of the culture medium composition, PGA was the main energy storage compound under nitrogen-limited conditions for all the strains tested. Thus, CGB utilization contributes to a reduction of PHA production costs, and simultaneously, to PGA production and it is essential to understand the relationship between the endopolymers and exopolymers produced by a given microorganism.

An earlier study reported simultaneous production of PHA and EPS by *Bacillus* and *Bordetella* cultured in a bioreactor containing palm oil mill effluent [35]; however, the authors did not identify the composition of the obtained EPS. Some archaea, depending on the culture medium, are able to produce polymers, such as PHAs or exopolysaccharides, which usually consist of glucose, mannose, galactose, and uronic acids [9]. Other extracellular polymers, such as alginates [11], poly-γ-glutamic acid [12], and hyaluronic acid [13], can also be produced by bacteria capable of synthesizing PHAs.

EPS protects the cells from the harsh external environment and serves as an energy and carbon source when growth substrates are in short supply. The secreted PGA can be degraded to provide carbon during bacterial growth in carbon-limiting medium. Biosynthesis of PHA requires the involvement of such enzymes as PHA synthase, which catalyzes the polymerization of (*R*)-3-hydroxyacyl-CoA monomers and is a key enzyme in the PHA biosynthesis pathway [36]. Under nutrient-poor conditions, protein, and also PHA enzyme, synthesis is impaired, resulting in low PHA production. Under those circumstances, the decomposition of PGA may be used to provide the required nutrients for PHA synthesis. Thus, the decomposition of PGA can play an important role in PHA production, as it could supply nutrients to maintain the microbial metabolism. Additionally, it is known that glutamic acid enters the Krebs cycle as α-ketoglutaric acid and exits to form pyruvic acid, and then acetyl-CoA, which is the starting point of a polyhydroxybutirate production pathway [37]. However, the biosynthetic relationship between the two polymers requires further study.

Weighted Average MM of PGA and PHA

PHA MM was obtained from a calibration curve of polystyrene standards (log MM = −0.095 × RT + 7.831, R^2 = 0.989). PGA MM was calculated using a calibration curve of dextran standards (log MM = 0.301 × RT + 14.666, R^2 = 0.99). Figure 1 shows the peaks corresponding to the produced PHA and PGA. MMs of the different PHA and PGA polymers produced in CGB cultures ranged from 384.84 to 835.56 kDa and from 968.75 to 1128.54 kDa, respectively, depending on the strains used (Table 2). Maximum PHA MM was obtained with *C. necator* IPT 026 (835.56 kDa), which corresponds to ~117.12 % molecular mass increase relative to PHA generated by *B. megaterium* INCQS 425 (384.84 kDa). The maximum PGA MM was also obtained with *C. necator* IPT 026 (1128.54 kDa) and was ~14.0 % greater than the MM of PGA obtained with *B. megaterium* INCQS 425 (989.13 kDa), a bacterium that we have shown to have a higher capacity to produce the biopolymer.

The obtained PHA polymer can have different MMs and, consequently, different properties (physical, mechanical, thermal, rheological, and others). Therefore, PHA properties depend on the size of the polymer chains, whose structural rearrangements may depend on the degree of polymerization.

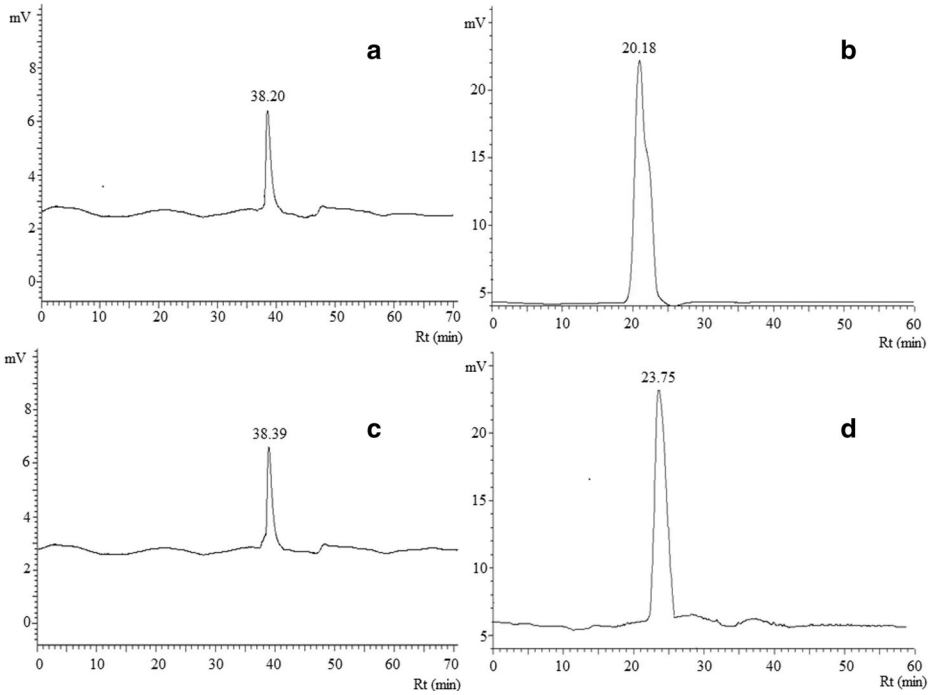

Fig. 1 HPLC-IR chromatograms for molecular mass of PGA (**a**) and PHA (**b**) obtained with *C. necator* IPT 026 and PGA (**c**) and PHA (**d**) obtained with *B. megaterium* INCQS 425 from CGB

C. necator produces high-MM PHA [38]. It is known that during the PHA accumulation phase in this bacterium, the components of the substrate used in the process can act as a chain transfer agent in the polymerization step [39]. Thus, depending on the substrates, different chain-length polymers can be obtained, and, consequently, polymers with different MM values. The use of glycerol as a substrate to grow *Burkholderia cepacia* (150 rpm, 30 °C, 96 h, 3.0 % glycerol) [40] and *Halomonas sp.* (150 rpm, 30 °C, 60 h, 3.0 % glycerol) [41], resulted in PHA MM of 304 and 140 kDa, respectively, which are lower than the lowest PHA MM obtained in the present study with *B. megaterium* INCQS 425 grown on 2.0 % CGB (384.84 kDa).

The molecular mass of PGA filaments may vary, depending on the microorganism and the process conditions, which may contribute to degradation of PGA by glutamyl hydrolases secreted into the culture medium in the late stationary phase [42, 43].

Table 2 Weighted average molecular masses of PHA and PGA simultaneously produced by different bacterial strains from CGB, in an orbital shaker (35 °C/180 rpm/72 h), as determined by HPLC-IR

Strains	PHA (kDa)	PGA (kDa)
Cupriavidus necator IPT 026	835.56 ± 0.03a	1128.54 ± 0.02a
Cupriavidus necator IPT 027	727.65 ± 0.03b	982.29 ± 0.03b
Cupriavidus necator IPT 029	459.90 ± 0.02c	968.75 ± 0.03c
Bacillus megaterium INCQS 425	384.84 ± 0.04d	989.13 ± 0.03d

Means followed by the same letters in the same column are not significantly different by Tukey's test ($P > 0.05$)

In general, MM PGA produced by *Bacillus* is on the order 10^5–10^8 Da [42]. Shimizu and coworkers [44] cultured *B. megaterium* WH320 in a medium containing glutamic acid (37 °C, 150 rpm, 120 h). The authors reported MM PGA ~2000 kDa. This is ~2× greater than the molecular mass of PGA produced by *B. megaterium* INCQS 425 (989.13 kDa) in a medium containing CGB as the only carbon source in this study.

FTIR Spectra of PHA and PGA

Figure 2 shows the FTIR spectra of the PHA and PGA samples from different bacterial strains growing in a CGB-containing medium. Two major bands are observed in the PHA spectrum (Fig. 2a): one ~1750 cm^{-1}, corresponding to the C=O group and another at 1300 cm^{-1}, related to the C–O group [45]. Peaks associated with the axial deformation of the carbonyl group (C=O; at 1750 cm^{-1}) and angular deformation in the plane of symmetry groups (CH_3; at 1380 cm^{-1}) were identified in the spectra of PHA produced from CGB, confirming therefore, that the obtained biopolymers are polyesters. Similar bands were seen in the spectra of PHA derived from glucose [46]. The occurrence of a band at 2920 cm^{-1} revealed the presence of unsaturated compounds that were probably derived from CGB [47].

The PGA spectra obtained in this study (Fig. 2b) showed characteristic carboxylic acid bands: band in the 1750 cm^{-1} region resulting from the axial deformation of the C=O bonds; bands in the 1620–1655 cm^{-1} region, related to the NH group (amide) bending; and a band in the 1090–1162 cm^{-1} region, resulting from the elongation of the C–N bond [48]. The spectra of PGA obtained with *C. necator* IPT 029, *C. necator* IPT 027, *C. necator* IPT 029, and *B. megaterium* INCQS 425 from a CGB-containing medium were similar to the spectra of γ-polyglutamic acid and its derivatives [48].

DSC and TGA

The obtained biopolymers were thermally evaluated with respect to the T_m, ΔH_m, T_{onset}, and T_{decomp} parameters because these properties influence the potential applications of these polymers [49].

Differential scanning calorimetry (DSC) curves of the obtained PHA (Fig. 3a; Table 3) showed endothermic peaks ranging from 158.41 to 171.30 °C, associated with crystalline

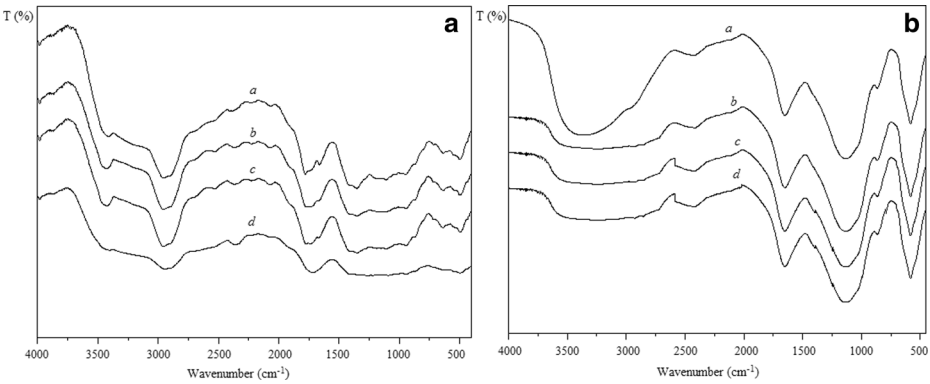

Fig. 2 FTIR spectra of PHA (**a**) and PGA (**b**) produced from CGB by *C. necator* IPT 027 (*a*), *B. megaterium* INCQS 425 (*b*), *C. necator* IPT 026 (*c*), and *C. necator* IPT 029 (*d*)

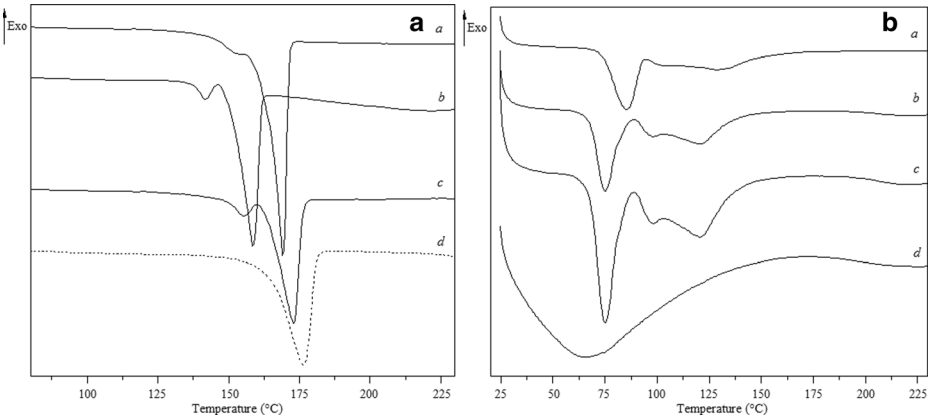

Fig. 3 DSC of PHA (**a**) obtained with *B. megaterium* INCQS 425 (*a*), *C. necator* IPT 029 (*b*), *C. necator* IPT 026 (*c*) from CGB medium and standard PHB (*d*). DSC of the PGA (**b**) obtained with *B. megaterium* INCQS 425 (*a*), *C. necator* IPT 029 (*b*), *C. necator* IPT 026 (*c*), and *C. necator* IPT 027 (d)

melting of these biopolymers. We noted that PHA produced by *B. megaterium* INCQS 425 and *C. necator* IPT 026 strains had the highest T_m (~170 °C) that was ~7.0 % higher than T_m of PHA obtained with *C. necator* IPT 029 (158.41 °C) and 3.4 % lower than standard PHB from Sigma-Aldrich (175.80 °C), a commercially used PHA. The similar data were obtained by Zhu and coworkers [40] who used orbital shaker cultures of *B. cepacia* strain and glycerol (150 rpm, pH 7.0, 30 °C, 96 h; T_m = 181.9 °C) and *C. necator* and CGB (250 rpm, pH 7, 30 °C, 120 h; T_m = 155 °C) [50].

PHA heteropolymer samples may exhibit more than one endothermic peak. The first melting endotherm of all PHA curves can be related to the melting of the unstable crystals

Table 3 Crystalline melting temperatures (T_m), crystalline melting enthalpies (ΔH_m), degree of crystallinity (χ_c), initial degradation temperatures (T_{onset}), and maximum thermal decompositions (T_{decomp}) of PHA and PGA simultaneously produced with different strains and CGB medium

Parameters		*C. necator* IPT 026	*C. necator* IPT 027	*C. necator* IPT 029	*B. megaterium* INCQS 425
PHA	T_m (°C)	171.30 ± 0.04a	167.40 ± 0.02b	158.41 ± 0.01c	170.22 ± 0.02a
	T_{onset} (°C)	236.10 ± 0.03a	259.91 ± 0.02b	225.83 ± 0.02c	270.43 ± 0.01d
	T_{decomp} (°C)	269.64 ± 0.02a	304.68 ± 0.02b	298.98 ± 0.03c	307.53 ± 0.01d
	ΔH_m (J g^{-1})	21.24 ± 0.04a	19.57 ± 0.06b	23.31 ± 0.04c	29.52 ± 0.03d
	χ_c (%)	14.55 ± 0.04a	13.40 ± 0.03b	15.97 ± 0.03c	20.22 ± 0.02d
	T_m (°C)	82.40 ± ±0.02a	65.80 ± 0.02b	75.06 ± 0.03c	84.04 ± 0.02d
PGA	T_{onset}[a](°C)	29.50 ± ±0.02a	24.15 ± 0.03b	31.18 ± 0.02c	35.18 ± 0.02d
	T_{decomp}[a] (°C)	68.25 ± 0.03a	52.16 ± 0.03b	61.32 ± 0.02c	68.10 ± 0.01d
	T_{onset} (°C)	237.47 ± 0.01a	188.51 ± 0.02b	192.13 ± 0.02c	247.10 ± 0.02d
	T_{decomp} (°C)	255.80 ± 0.02a	277.30 ± 0.01b	254.23 ± 0.01c	291.58 ± 0.02d

Means followed by the same letters in the same line are not significantly different by Tukey's test ($P > 0.05$)

[a] First thermal event

formed under DSC heating due to copolymers in the PHA. This endothermic peak was not observed in the standard PHB curve due to lack of copolymers in the polymer [51].

Figueiredo and coworkers [52] cultivated *C. necator* IPT 026 in a medium containing 1.5 % CGB (150 rpm, 72 h, 35 °C) and obtained PHA with *Tm* = 178 °C. Different T_m of PHA were found with *C. necator* IPT 026 cultivated in a bioreactor (500 rpm, 1.0 vvm, 72 h, 35 °C, CGB 1.5 %; T_m = 167.60 °C) [2] and cultivated in this study (2.0 % CGB, 180 rpm, 35 °C, 72 h, T_m = 171.30 °C). Apparently, the process conditions (temperature, stirring speed, and CGB concentration) cause influence on T_m of PHA.

Furthermore, based on ΔH_m, we note that the degrees of crystallinity of all the obtained PHA samples were <30 %. PHAs are semicrystalline polymers and their degree of crystallinity depends on their composition [53]. PHAs with crystallinity between 60 and 80 % are considered rigid. Flexible and more elastic PHAs have medium- (30–40 %) and short (< 30 %)-chain polymer lengths. By growing *C. necator* DMS 545 with CGB on an orbital shaker (250 rpm, 30 °C, 120 h), García and coworkers [50] obtained PHAs with 49 % crystallinity. Thus, the obtained low-crystallinity PHA raises expectation of industrial applications of these biopolymers, especially in the packaging sector.

The DSC curves of the obtained PGA (Fig. 3b) showed exothermic peaks with T_m values between 65.88 and 84.04 °C (Table 3). Maximum T_m was determined for PGA obtained by cultivating *B. megaterium* INCQS 425 (84.04 °C) and was 27.70 % higher than the T_m of PGA obtained with *C. necator* IPT 027 (65.80 °C).

TGA and dTGA curves showed that all PHA samples tested underwent only one thermal event, with T_{onset} ranging from 225.83 to 270.43 °C (Fig. 4, Table 3). PHA produced by *C. necator* IPT 029 underwent thermal degradation between 225.83 and 361.49 °C, maximum decomposition at 298.98 °C, and 96.5 % mass loss. PHAs produced by *B. megaterium* INCQS

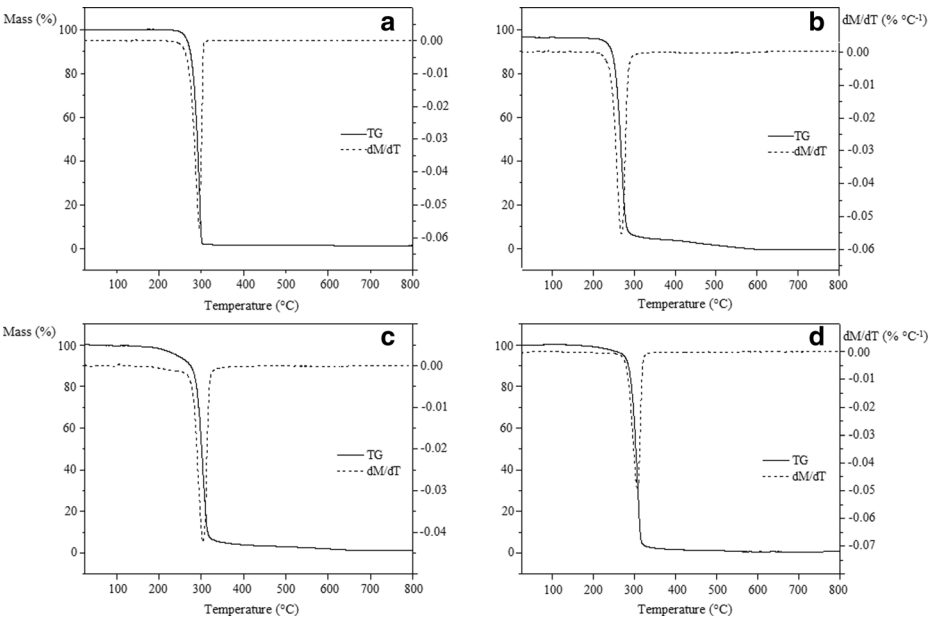

Fig. 4 Thermograms of standard PHB (**a**) and PHA obtained with *C. necator* IPT 026 (**b**), *C. necator* IPT 027 (**c**), and *B. megaterium* INCQS 425 (**d**) from CGB

425 underwent thermal degradation between 270.43 and 322.03 °C, maximum decomposition at 307.53 °C, and 96.34 % mass loss. This PHA had a greater thermal stability than Sigma PHB, which degraded between 258.40 and 301.83 °C, with 96.20 % mass loss and maximum decomposition at 294.80 °C.

T_{decomp} of the PHA produced by *B. megaterium* INCQS 425 grown on CGB (307.53 °C) was 9.3 % higher than that of the PHA produced from CGB by *B. cepacia* grown with orbital shaking (150 rpm, pH 7.0, 30 °C, 96 h; T_{decomp} = 281.50 °C) [40]; 13.5 % higher than the PHA produced by *C. necator* grown with orbital shaking (250 rpm, pH 7, 30 ° C, 120 h; T_{decomp} = 271 °C) [50]; and 12.3 % higher than the PHA produced from beet juice by *Alcaligenes latus* grown with orbital shaking (200 rpm, pH 7.0, 33 °C, 16 h; T_{decomp} = 273.86 °C) [19].

All the obtained PGA underwent two typical thermal events (Fig. 5, Table 3). The first event was associated with dehydration and the second was associated with decomposition of the biopolymer [54]. The PGA produced by *C. necator* IPT 027 underwent decomposition between 188.51 and 386.68 °C, with 9.5 % biopolymer mass loss and maximum degradation at 277.73 °C. On the other hand, the PGA produced by *B. megaterium* INCQS 425 underwent degradation between 247.10 and 351.71 °C, with 42.33 % biopolymer mass loss and maximum decomposition at 291.58 °C.

The PGA produced from CGB by *B. megaterium* INCQS 425 had T_{decomp} of 291.58 °C, 16.4 % lower than the T_{decomp} γ-glutamic acid (340 °C) obtained with *B. subtilis* grown in a medium supplemented with L-glutamate and glycerol in a bioreactor (350 rpm, 1.0 vvm, pH 7.3, 37 °C, 40 h) [48].

In conclusion, we observed that from all the strains cultured in a medium containing CGB, *B. megaterium* INCQS 425 produced PHA and PGA of the highest thermal stability, indicating

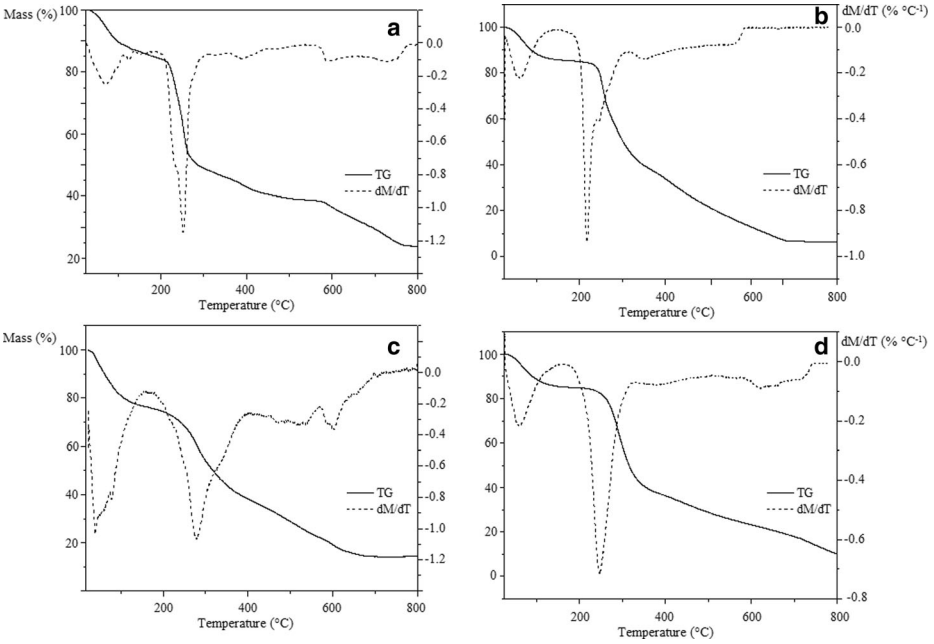

Fig. 5 Thermograms of PGA obtained with *C. necator* IPT 029 (**a**), *C. necator* IPT 026 (**b**), *C. necator* IPT 027 (**c**), and *B. megaterium* INCQS 425 (**d**) from CGB

greater application potential, especially in processes that demand high temperatures, such as extrusion.

PGA and PHA Composition

HPLC-RI chromatograms presented in Fig. 6 show that the hydrolyzed exopolymers obtained in this study contain L-glutamic acid. Therefore, although the method does not provide conclusive identification, the obtained exopolymers are formed at least by L-glutamic acid units, suggesting the presence of PGA.

Figure 7 shows GC-MS chromatograms of intracellular PHA produced from CGB simultaneously with PGA by *C. necator* IPT 026, *C. necator* IPT 027, *C. necator* IPT 029, and *B. megaterium* INCQS 425. The composition of the PHA polymer fraction is shown in Table 4. The majority of PHA polymers were composed of identifiable long carbon chain molecules. Furthermore, some unidentified peaks were observed, probably arising from methanolysis. *C. necator* IPT 026, *C. necator* IPT 027, and *C. necator* IPT 029 cultures generated PHA blocks consisting of long-chain copolymers, such as 11-hydroxyhexadecanoate (53.54–59.57 %), 12-hydroxy-9-octadecenoate (12.93–21.57 %), and 3-hydroxyoctadecanoate (12.70–29.93 %). The polymer produced by *B. megaterium* INCQS 425 also consisted of long-chain copolymer building blocks, such as 11-hydroxyhexadecanoate (55.59 %), 18-hydroxyoctadienoate (12.49 %), and 12-hydroxy-9-octadecenoate (20.07 %) and only 9.40 % of medium-chain copolymers, such as 4-hydroxynonanedioate. This composition can be related to the predominance of long-chain fatty acids (C16:0, C18:0, and C18:1ω9) in CGB used as the main carbon source.

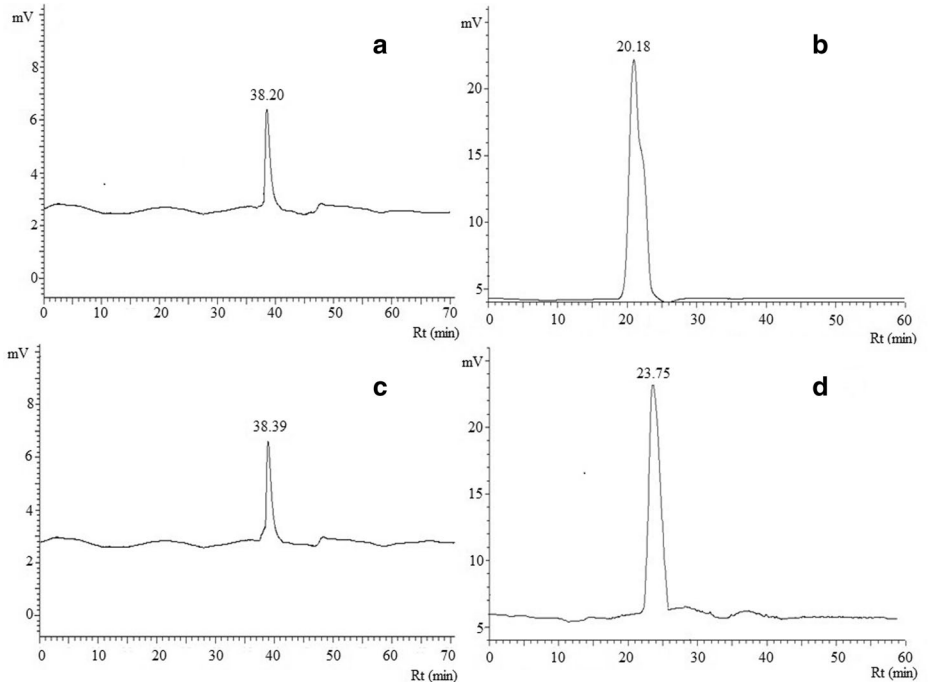

Fig. 6 HPLC-UV chromatogram of the standard L-glutamic acid (**a**), PGA obtained from CGB and *C. necator* TPI 026 (**b**), *C. necator* TPI 029 (**c**), and *B. megaterium* INCQS 425 (**c**)

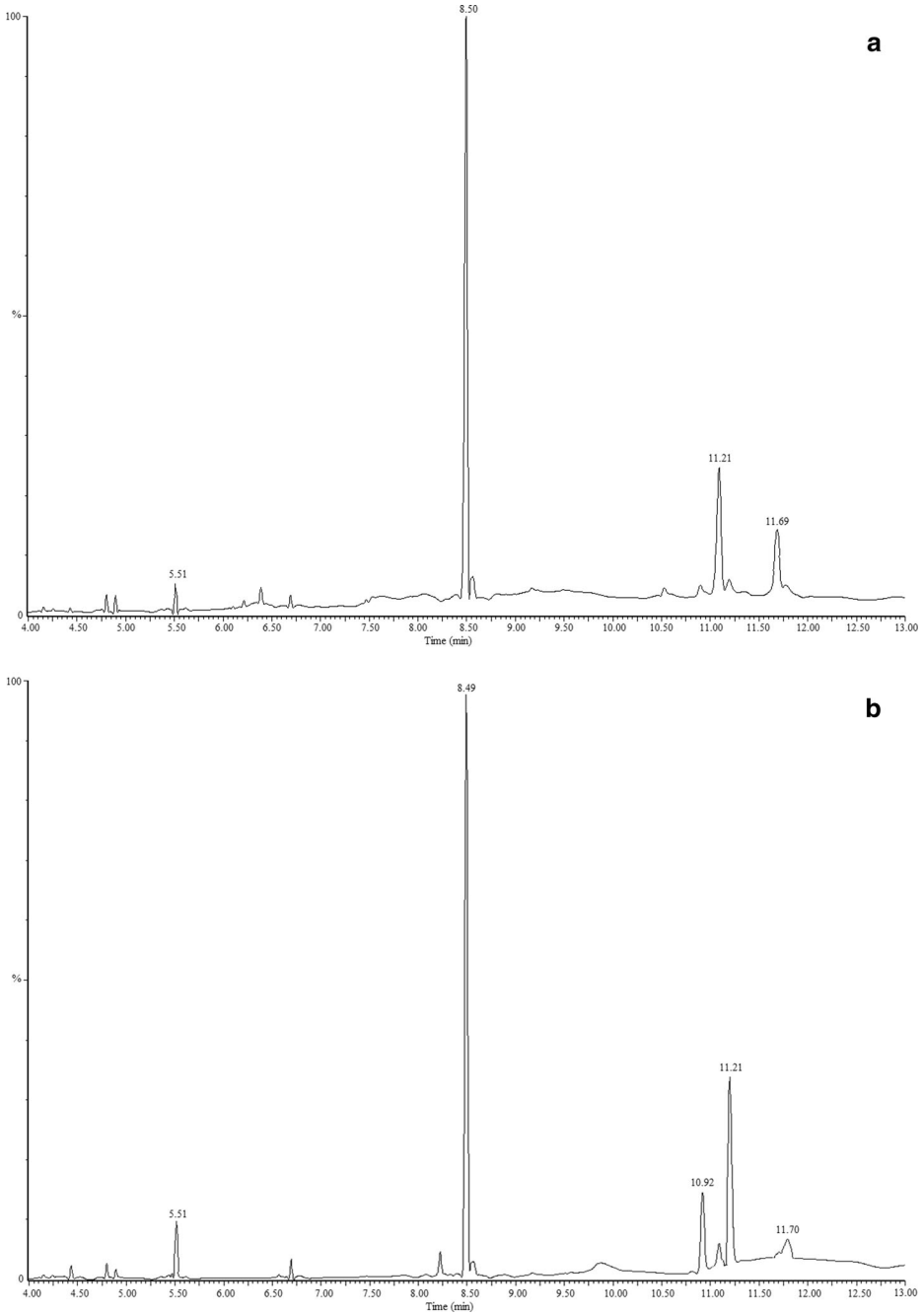

Fig. 7 GC-MS chromatogram of PHA obtained from CGB and *C. necator* TPI 027 (**a**) and *B. megaterium* INCQS 425 (**b**)

Sravastava and Tripathi [25] studied the effect of saturated and unsaturated fatty acid substrates on PHA composition produced by *Alcaligenes sp*. NCIM 5085 and observed that palmitic acid (16:0) resulted in short-chain PHA (3-hydroxybutyrate) and medium-chain PHA

Table 4 Composition (%) of PHA produced by *C. necator* IPT026, *C. necator* IPT 027, *C. necator* IPT 029, and *B. megaterium* INCQS 425 from 2.0 % CGB (180 rpm, 35 °C, 72 h), as determined by GC-MS

Retention time (min)	Identification	Strains			
		C. necator IPT 026	*C. necator* IPT 027	*C. necator* IPT 029	*B. megaterium* INCQS 425
5.5	4-hydroxynonanedioate	ND	ND	ND	9.40 ± 0.05
8.5	11-hydroxyhexadecanoate	55.50 ± 0.03	59.47 ± 0.04	53.54 ± 0.02	55.59 ± 0.04
10.9	18-hydroxyoctadienoate	ND	ND	ND	12.49 ± 0.03
11.2	12-hydroxy-9-octadecenoate	19.53 ± 0.05	21.57 ± 0.04	12.93 ± 0.02	20.07 ± 0.04
11.7	3-hydroxyoctadecanoate	12.70 ± 0.04	14.54 ± 0.04	29.93 ± 0.02	ND
Other peaks	Unidentified	12.27 ± 0.04	4.42 ± 0.02	3.60 ± 0.02	2.45 ± 0.05

ND not detected

(3-hydroxydodecanoate, 12 carbon 3-hydroxyalkanoate, and 14 carbon 3-hydroxyalkanoate). The addition of oleic acid (18:1) that resulted in medium-chain saturated PHA (3-hydroxyvalerate) and linoleic acid (18:2) led to a wide range of medium-chain saturated, ringed, and unsaturated hydroxyalkanoates with 6–20 carbon chain lengths. Dalal and co-workers [55] produced PHA from oleic acid by *Pseudomonas aeruginosa* and reported PHA blocks composed mainly of 3-hydroxyoctanoate, 3-hydroxydecanoate, and unsaturated monomer chains. The formation of monomers of different lengths on the same substrate indicates that a common fatty acid metabolism intermediate serves as a precursor in the synthesis of PHA monomers. Acetyl-CoA is a likely candidate because of its central role in carbohydrate metabolism [56].

Biopolyesters produced by *C. necator* IPT 026 from 1.5 % crude glycerol (150 rpm, 35 °C, 72 h) were primarily identified as short-chain PHA building blocks, such as 3-hydroxybutyrate (56.05 %) and 3-hydroxypentanoate (7.85 %), and medium-chain blocks, such as 8-hydroxynonanoate (27.82 %) [2]. Ribeiro and coworkers [22] cultivated *C. necator* IPT 027 under the same conditions, with 1.5 % CGB from processing waste fats and oils, and obtained short-chain PHA copolymers, e.g., 3-polyhydroxybutyrate (9.3 %), and long chain copolymers, e.g., 3-hydroxytetradecanoate (12.11 %), 15-hydroxypentadecanoate (4.98 %), and 11-hydroxyhexadecanoate (60.02 %). The chemical composition of PHA blocks is dependent on the carbon source used during the PHA accumulation stage [57]. Therefore, the difference between the PHA composition in this study and the PHA composition found by other authors [2, 22] could be related to the source and concentration of CGB.

The complex organization of medium- and long-chain building blocks of PHA may have prevented the well-defined formation and distribution of crystalline structure in the polymeric material, contributing to their low crystallinity (Table 3) and improvement of mechanical properties [58].

Conclusion

The use of CGB for cultivation of *C. necator* IPT 026, *C. necator* IPT 027, *C. necator* IPT 029, and *B. megaterium* INCQS 425 promoted the simultaneous biosynthesis of PHA and PGA. Maximum

PHA and PGA production was obtained with *B. megaterium* INCQS 425. The thermal stability and low degree of crystallinity of PHA obtained with *B. megaterium* INCQS 425 are more appropriate and suitable for use in processes that require high temperatures, such as extrusion, increasing the possibility of industrial application, especially in the packaging sector. Considering the vast range of applications, biodegradability, and eco-friendly manufacturing processes, PHA and PGA biopolymers are two alternatives for a more sustainable future, but their production cost has been a drawback. The utilization of the cheapest waste for simultaneous production of PHA and PGA can be a cost-reducing option. However, the optimization of process parameters as a strategy for simultaneous maximum bioproduct production is necessary.

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