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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

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ABSTRACT

The potential of crude glycerol (CG) from different origins as carbon sources in the production of polyhydroxyalkanoate (PHA) copolymer using *Cupriavidus necator* IPT 027 and *Burkholderia cepacia* IPT 438 was investigated in this study. Different variables were kept constant during the subsequent microbial growth and PHA production. A maximum cell accumulation of 71.07% (w/v) was obtained when *C. necator* IPT 027 was cultivated with CG II (originated from the processing of biodiesel from residual fats and oils). The gas chromatography–mass spectrometry (GC–MS) analyses revealed novel PHA-constituents as building blocks of medium chains (3HTD) and long (15HPD and 11HHD) chains. Analyses of molar mass distribution revealed weight average molar masses (M_w) in the range of 552–8240 kDa and polydispersity indexes (PDIs) in the range of 1.6–2.2. The melting temperature ranged between 139.8 and 175.9 °C. The crystallinity was verified by X-ray diffraction (XRD) (35.92–66.07%) and differential scanning calorimetry (DSC) (33.30–57.80%). High decomposition temperatures (291.6–348.9 °C) were also observed. All PHAs presented Fourier transform infrared (FTIR) spectra that were similar to the FTIR spectra reported in the literature. The results obtained from this study indicate that *C. necator* IPT 027 and *B. cepacia* IPT 438 cultivated from different by-products from the biodiesel industry were capable of producing PHA copolymers that are suitable for industrial applications.

1. Introduction

Polyhydroxyalkanoates (PHAs) are biopolyesters that accumulate intracellularly in numerous microorganisms in limiting conditions of nitrogen (N), phosphorus (P), magnesium (Mg), oxygen (O), potassium (K), or sulfur (S) and in the presence of an excess carbon source (García et al., 2013). The most extensively investigated and representative form of PHA is poly(3-hydroxybutyrate) (PHB), which is a thermoplastic and biocompatible biopolymer with mechanical properties that are comparable to the mechanical properties of polypropylene and polyethylene, due to its high melting temperature, superior resistance to organic solvents, and excellent mechanical strength and modulus (Barud et al., 2011; Laycock et al., 2014). The main applications for PHB include medical devices and packaging and disposable items, such as glasses and diapers (Wang et al., 2013).

Biopolymers are gaining market share due to the environmental problems caused by synthetic polymers; however, the high cost of production inhibits the industrial application of these polymers (Khosravi-Darani et al., 2013; Posada et al., 2011). Studies have shown that the value of the carbon source can contribute 40–50% of the production costs (Koller et al., 2010; Posada et al., 2011; Khosravi-Darani et al., 2013). By-products from the biodiesel industry may be an efficient choice for use as carbon sources because they exhibit two highly desirable characteristics: low price and high availability (Naranjo et al., 2013).

Biodiesel based on lipid transesterification generates a crude glycerol (CG) stream that represents in weight 10% of the total biodiesel produced (Cavalheiro et al., 2012). Although glycerol is an important industrial feedstock, the glycerol-rich phase (GRP) from

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biodiesel has a relative low value due to the presence of impurities, such as methanol, salts, mono- and di-glycerides, and fatty acids (Cavalheiro et al., 2012; Spoljaric et al., 2013). However, this CG has a high potential for conversion into a variety of value-added products (Johnson and Taconi, 2007).

In order to decrease production costs and increase the market competitiveness of PHAs, the processability of the polymer must be improved. More than 150 monomers formed from hydroxyesters of fatty acids with carbon chains that contain from four to thousands of carbons can be combined within the family of PHAs (Fig. 1) to create polymers with extremely different properties for various applications (Khosravi-Darani et al., 2013). Considering the previously mentioned factors, this study evaluates the production, properties, and composition of novel PHAs using *Cupriavidus necator* IPT 027 and *Burkholderia cepacia* IPT 438 in culture with CG of different origins.

2. Materials and methods

2.1. Microorganisms

The bacterial strains *C. necator* IPT 027 and *B. cepacia* IPT 438 were provided by the Institute of Technological Research (IPT) in São Paulo, Brazil. The strains were maintained on nutrient agar at 4 °C and subcultured monthly.

2.2. Main carbon sources

D-Glucose monohydrate P.A. (Synth mark) and glycerol (Synth mark) were adopted as reference carbon sources for the production of PHAs. CG of different origins was donated by three different institutions: COMANCHE Biofuels of Bahia LTDA, Simões Filho, BA, Brazil (CG I); Pilot plant of Biodiesel, Federal University of Bahia, Salvador, BA, Brazil (CG II); and Petrobrás, Candeias, BA, Brazil (CG III). The CG was obtained from different respective raw materials: soybean, residual oils and fats, and castor beans.

2.3. Composition of carbon sources

2.3.1. Chemical composition of carbon sources

The reference carbon sources (glucose and glycerol) and CGs I–III were evaluated in triplicate, in terms of total lipids (Bligh and Dyer, 1959), volatiles at 105 °C (AOAC, 1997), crude ash (AOAC, 1997), and residual crude protein (Kjeldahl method, AOAC, 1997). The carbohydrate content was calculated by difference [100 − (ash + protein + volatile + lipid) percentages]. The concentration of the carbon on the samples of glucose, glycerol, and CGs I–III was determined based on the percentage of carbon contained in the sample (Chemical composition, Table 1) and the amount added to the fermentative medium (Campos et al., 2014). Nitrogen contained was defined to according with the ammonium sulfate concentration and protein of main carbon source. The C/N ratio was subsequently calculated.

The fatty acid profile was determined by the 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 (GC) capillary column Elite-WAX (30 m × 0.32 mm × 0.25 μm). The quantification of fatty acids, which is expressed in milligrams per 100 g sample, was performed by the addition of an internal standard (C23:0 Sigma®, USA) according to Joseph and Ackman (1992) using Eq. (1).

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

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.

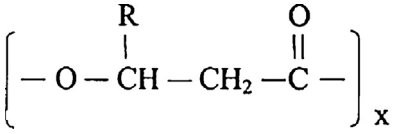

Fig. 1. Chemical structure of PHA produced by bacteria. PHA are commonly composed of (*R*)-β-hydroxy fatty acids, where the '*R*' group varies from methyl (C1) to tridecyl (C13) (Madison and Huisman, 1999).

2.3.2. Determination of heavy metals, semimetals, and minerals of the main sources of carbon and PHAs

The determination of heavy metals, such as cadmium (Cd) and chromium (Cr), and semimetals, such as arsenic (As), in the samples of glucose; glycerol; CGs I–III; and PHA copolymers, was performed via atomic absorption spectroscopy in a graphite furnace (Spectrometer Spectra AA 240Z) with an autosampler (PSD-120, Varian, USA) to determine the toxicity of the samples. Measurements of sodium (Na), copper (Cu), and zinc (Zn) were performed in an atomic absorption spectrometer in flame mode, and background correction was performed using a deuterium arc lamp (AA-55B, Varian, USA).

The samples were gravimetrically weighed and digested according to Menezes-Filho et al. (2009). All samples were analyzed in triplicate and were always injected in duplicate into the spectrometer; differences lower than 10% were considered to be acceptable. The results were expressed in $\mu g\,g^{-1}$ of dry weight. Certified reference materials, linear ranges (LR) and limits of detection (LoD) were used to validate the developed methods and to evaluate the homogeneity of small quantities of samples. Samples of bovine liver National Institute of Standards and Technology (NIST) 1577b (Gaithersburg, MD, USA) were used to detect the following elements: Na (LR: 2180–2660 $\mu g\,g^{-1}$; LoD: 10 $\mu g\,g^{-1}$), cadmium (Cd) (LR: 0.44–0.56 $\mu g\,g^{-1}$; LoD: 0.25 $\mu g\,g^{-1}$), Cu (LR: 144–176 $\mu g\,g^{-1}$; LoD: 1.50 $\mu g\,g^{-1}$), Zn (LR: 76–88 $\mu g\,g^{-1}$; LoD: 1.50 $\mu g\,g^{-1}$), chromium (Cr) (LR: 0.76–1.67 $\mu g\,g^{-1}$; LoD: 0.20 $\mu g\,g^{-1}$), and arsenic (As) (LR: 0.90–12.10 $\mu g\,g^{-1}$; LoD: 0.50 $\mu g\,g^{-1}$).

2.4. Culture media

The microorganisms were stored below 5 °C in nutrient agar (NA) that was composed of 5.0 g L^{-1} meat peptone, 3.0 g L^{-1} beef extract, and 3.75 g L^{-1} agar. Periodic replating was performed every 15 days, and inoculation was performed in nutrient broth (NB, 5.0 g L^{-1} bacteriological peptone, 3.0 g L^{-1} beef extract, and distilled water) over a 24 h period. PHA was produced using two culture as reported and described by Wang et al. (2013) and Campos et al. (2014). A mineral media, used as the first culture (FC), without nitrogen limitation, and a second culture (SC), similar a mineral media with nitrogen limitation, which were composed of nitrilotriacetic acid (19.1 g L^{-1}), ferrous ammonium citrate (10 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (50 g L^{-1}), $CaCl_2{\cdot}2H_2O$ (5 g L^{-1}), $(NH_4)_2SO_4$ (nitrogen source, 200 g L^{-1} in FC and 50 g L^{-1} in SC); $Na_2HPO_4{\cdot}12H_2O$