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The influence of crude glycerin and nitrogen concentrations on the production of PHA by *Cupriavidus necator* using a response surface methodology and its characterizations

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ABSTRACT

The aim of this research was to evaluate the production and properties of polyhydroxyalcanoates (PHAs) obtained from crude glycerin (CG), a byproduct of the biodiesel industry, by *Cupriavidus necator* IPT 026. Experiments were carried out in shake flasks to determine the optimum CG (X_1) and nitrogen (X_2) concentrations to maximize biomass accumulation and biopolymer production. The highest PHA and biomass production (2.81 g L^{-1} and 4.34 g L^{-1}, respectively) occurred at 15 g L^{-1} of CG and 10 g L^{-1} of nitrogen with approximately 65% (w/v) cell accumulation (cell dry weight). Experiment 7 yielded the PHA with the optimum properties (15 g L^{-1} of CG and 3 g L^{-1} of nitrogen), which showed a melting temperature of 184.3 °C, crystallinity of 52.23%, thermal degradation occurring between 306.8 °C and 334.1 °C with a peak at 327.4 °C, and its molecular weight was 780 kDa. All experiments PHA production showed FTIR spectra similar to those reported in the literature. The monomeric profile of the produced PHAs showed a composition similar to that of the PHA standard, showing evidence of 3-hydroxybutyrate (3HB) monomer in the analyzed samples. Bacteria can use CG as an inexpensive substrate to produce value-added biodegradable products, such as PHA.

1. Introduction

Environmental pollution due to large-scale applications of synthetic polymers and their dependence on petroleum resources requires the development of alternative routes of polymer production that are environment friendly and renewable. Microbial biopolymer production can play an important role in the solution to this problem (Luengo et al., 2003; Koller et al., 2007). Polyhydroxyalkanoates (PHAs) are biodegradable polymers with material properties similar to those of petrochemical plastics (Khardenavis et al., 2007; Mizuno et al., 2010). These biopolymers can be produced by different bacterial and archaebacterial species as well as genetically modified plants (Sudesh et al., 2000). In bacteria, PHAs are produced under nutritional stress conditions, such as nitrogen or phosphate limitation, in conjunction with an excess carbon source in the cultivation broth. The type of PHA produced, such as homopolyesters, copolyesters, terpolyesters or polyester blends, depends on the carbon source. PHA is usually stored in the cell cytoplasm of bacteria as granules between 0.2 and 0.5 μm in diameter (Sudesh et al., 2000).

PHAs have a strong potential to replace petrochemical polymers in the future if their production costs decrease. The total PHA production cost depends on the microorganism (yield and productivity), the carbon and nitrogen sources (substrates), the fermentation conditions (temperature, aeration, pH-value) and the recovery and purification processes. The carbon source could account for 25–45% of the total production costs (Nath et al., 2008), highlighting the need to find cheaper carbon sources. Agro-industrial wastes are attractive candidates for this role because they have some of the desired characteristics, namely low prices and high availability. Moreover, using agro-industrial wastes as substrates to produce PHA avoids their final disposal, which solves an environmental problem. A wide variety of substrates, such as whey, lignocellulosic materials and CG (obtained from biodiesel production) have been used with different microorganisms to improve the yields and production of PHAs (Bosco and Chiampo, 2010; Nath et al., 2008; Koller et al., 2008; Pantazaki et al., 2009; Yu et al., 2006; Moralejo-garate et al., 2013; García et al., 2013).

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CG principally consists of residual ethanol or methanol, glycerol, fatty acid ethyl (or methyl) esters and residual fatty acids (Ashby et al., 2004). These carbon sources are direct precursors for the bacterial synthesis of biopolymers, such as polyhydroxyalkanoates (PHAs) or other products (Johnson and Taconi, 2007; André et al., 2010).

Cultivation strategies involving inexpensive, renewable carbon to reduce production costs and to obtain high productivity have been explored (Ojumu et al., 2004). An optimized medium might enhance the production of PHA. Parameters such as the carbon source, nitrogen sources and pH of the medium influence the metabolism of these bacteria and the accumulation of PHA. Statistical design experiments are used in many studies to reduce the number of experiments despite a large number of variables. Response surface methodology is an efficient tool used to study the interactive effect of parameters involved in fermentation processes seeking to optimize conditions for improved product yield (Pal et al., 2010). The application of statistical methods and response surface methodology has gained a lot of impetus for medium optimization and understanding the interactions among various physicochemical parameters involved in biopolymer production (Mu et al., 2009).

The present study aimed to produce and evaluate PHA obtained by *Cupriavidus necator* IPT 026. This study verified the influence of the CG and nitrogen on PHA production through media optimization using the response surface methodology. The properties of the produced PHA were also verified, in order to find the best.

2. Materials and methods

2.1. Microorganism

In this study, the microorganism *C. necator* IPT 026, provided by IPT (Institute for Technological Research, São Paulo, Brazil), was used. The strain was maintained on nutrient agar at 4 °C and subcultured monthly.

2.2. Crude glycerin characterization

Crude glycerin (CG) was acquired from Comanche Biocombustíveis, Bahia, Brazil. The CG was generated in a plant that produces biodiesel from refined vegetable oils and waste oils using sodium methoxide as a catalyst. According to the manufacturer, the CG contains 82% (w/w) glycerol, 6.5% (w/w) Na^+, 0.09% (w/w) methanol and less than 1.0% (w/w) monoacylglycerides. The CG was analyzed in triplicate for the following properties: acidity (pH), volatile content at 105 °C, residual crude protein content (Kjeldahl method), ash content (AOAC, 1997) and total lipids content (Bligh and Dyer, 1959). The carbohydrate content was calculated by difference [100 − (ash + protein + volatiles + lipid) percentages].

2.3. Culture media

A variety of media types were used at various stages in this study. The media to prepare the inoculum was a nutrient broth containing 5 g L^{-1} peptone and 3 g L^{-1} beef extract. PHA was produced using two media (Aragão et al., 1996), a mineral media (without nitrogen limitation) consisting of 19.1 g L^{-1} nitrilotriacetic acid, 10 g L^{-1} ferrous ammonium citrate, 50 g L^{-1} $MgSO_4.7H_2O$, 5 g L^{-1} $CaCl_2{\cdot}2H_2O$, 200 g L^{-1} $(NH_4)_2SO_4$ and 223.8 g L^{-1} $Na_2HPO_4{\cdot}12H_2O$ and a second culture (with nitrogen limitation) similar a mineral media with concentration of substrate (CG) and nitrogen ($(NH_4)_2SO_4$) supplemented according to the experimental design. The pH of the media was adjusted to 7.0 with 10 mol L^{-1} NaOH or 10 mol L^{-1} HCl.

2.4. Shaker flask cultivation

Cell growth was monitored spectrophotometrically (PerkinElmer mod. Lambda 20) by measuring the optical density at 620 nm after 48 h of incubation to determine the optimal cell concentration. Experiments were performed in 250 mL flasks containing 50 mL of nutrient broth medium and 2 mL of a pre-culture inoculums, incubated at 30 °C in shaker flask for 24 h (best cell concentration, 10^{11} UFC mL^{-1}) at 150 rpm. The two following cultures were performed in 250 mL shake flasks containing 80 mL of mineral medium and 10% (v/v) of previous culture, with and without nitrogen limitation at 35 °C for 72 h at 150 rpm.

To evaluate the effects of the initial CG and nitrogen (ammonium sulfate) concentrations on cell production and polymer accumulation, the shake flask cultivations were carried out according to a central composite design of experiments (DOE) with three replications at the central point. The levels of the coded (X_1 and X_2) and uncoded (CG and nitrogen, respectively) variables are presented in Table 1. Experimental results were analyzed using the STATISTICA 7.0 software (Statsoft).

Mathematical models were fitted to the experimental points. An analysis of variance (ANOVA) model tested the predictive power using a Fischer (F) test for the regression. The model fit was tested by the F test for lack of fit and the determination coefficient (R^2) (Barros Neto et al., 2010). Fit-testing and prediction of the model were performed at a significance level of 5% ($P \leq 0.05$).

To find the levels that maximize or minimize each estimated response, we calculated the stationary point of the surface related to the optimal combination of X_1 and X_2. Furthermore, the nature of the surface was evaluated based on the signals from the characteristic roots of the quadratic equations (λ_1 and λ_2) from the surface of the second order.

2.5. Biomass

The culture was collected and centrifuged at 42.200 × g for 30 min at 5 °C (HITACHI, model CR 22G). Pellets were washed twice with distilled water, transferred to 50 mL glass round bottom flasks and frozen at −80 °C for subsequent lyophilization (LIOBRAS, model L101) at −42 °C for 24 h. Biomass production was calculated using a gravimetric method and was expressed as g L^{-1}.

2.6. Polyhydroxyalkanoate extraction

After lyophilization, 0.5 g of cells was re-suspended in 50 mL of chloroform in glass flasks and incubated at 60 °C for 2 h with vigorous agitation. After incubation, the samples were filtered under a vacuum using Millipore filters (0.5 mm pore, 47 mm diameter) to separate the cellular debris. The solubilized polymer in chloroform was placed in an oven to evaporate the solvent. The production of PHA was calculated using a gravimetric method for the polymer obtained after extraction. The production (Y_1) was expressed as g L^{-1}.

2.7. PHA characterization

2.7.1. Fourier Transform Infrared Spectroscopy (FTIR)

PHA samples were analyzed qualitatively by FTIR spectroscopy (PerkinElmer Model Spectrum 100, PerkinElmer, Waltham, Mass., USA) between 4000 cm^{-1} and 600 cm^{-1} using a single-bounce ATR accessory with a Zinc selenide crystal.

2.7.2. Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TG)

Thermogravimetric Analysis (PerkinElmer Model Pyris 1 TGA Waltham, Mass., USA) was used to determine the on-set