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Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) production in a system with external cell recycle and limited nitrogen feeding during the production phase

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ABSTRACT

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) is a biodegradable, biocompatible and thermoplastic biopolymer produced by microorganisms. Considering their high current production cost, the use of wastes and culture strategies designed to increase the process productivity has been studied. The aim of the present work was to evaluate the P(3HB-*co*-3HV) production from *Cupriavidus necator* in a repeated fed-batch system with external cell recycle, maintaining a controlled residual cell growth during the production phase by nitrogen feeding strategy. The glucose, at the feeding culture medium, simulated the carbon concentration usually found in agro-industrial wastes. Two cultures were performed: one conducted with nitrogen exhaustion and another with nitrogen feeding in limited concentrations, during the production phase. Propionic acid was added to allow 3HV production. In both cultures 73 % of P(3HB-*co*-3HV) was produced, but at the culture with nitrogen feeding, during the production phase, productivity reached 1.24 $g_{P(3HB\text{-}co\text{-}3HV)}.L^{-1}.h^{-1}$ and $q_{P(3HB\text{-}co\text{-}3HV)}$ remain at the maximum level (0.13 $g_{P(3HB\text{-}co\text{-}3HV)}.g_{Xr}{}^{-1}.h^{-1}$) during more time. The 3HV percentage was similar at the end of both cultures. It can be concluded that the system with external cell recycle and nitrogen feeding, during the production phase, is a good alternative to utilize agro-industrial wastes with moderate carbon concentration and to increase productivity of P(3HB-*co*-3HV).

1. Introduction

Polyhydroxyalkanoates (PHAs) are a class of biodegradable, biocompatible, and non-toxic polyesters with similar material properties to petroleum-derived polymers. Among PHAs, the homopolymer poly(3-hydroxybutyrate) P(3HB) is the best-characterized [1], but it is highly crystalline and brittle, which restricts its use to a limited range of applications [2]. One possibility to avoid this problem is to incorporate 3-hydroxyvalerate units (3HV) to the homopolymer, thereby forming the copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) which presents reduction of the crystallinity and melting point, providing lower stiffness and greater toughness. Higher is the 3HV fraction in the polymer, better is the flexibility and resistance [3].

The PHAs are synthesized through a microbial pathway as a way for the cell to store carbon and energy, usually when the availability of non-carbon nutrients limits growth [1]. Many microorganisms are capable of producing PHAs, however, the bacteria *Cupriavidus necator* (formerly known as *Ralstonia eutropha*) is one of the most widely-studied, due to its ability to store up to 80% of its dry mass as polymer and to use different substrates as carbon source [4]. This strain can produce 3HV monomers from odd carbon-number substrates like propionic and valeric acids [5].

The high production cost of P(3HB-*co*-3HV), concerning feedstock, control of the process and downstream, make this biopolymer commercially uncompetitive against petroleum-derived polymers [6]. Some alternatives to solve these problems are the use of low cost substrates [7–9], strategies that increase the process productivity such as obtaining high cell density [10,11],

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genetic modifications [12], and further, the development of less costly extraction techniques [13].

Since that 40 to 48% of the total production cost can be ascribed to raw materials [14], the application of agro-industrial wastes, mostly containing moderate (e.g., citrus molasses (80 $g_{carbon}.L^{-1}$ [7]) and pineapple waste (105 $g_{cabon}.L^{-1}$ [9])) or low (e.g., whey (40 $g_{carbon}.L^{-1}$ [15])) carbon concentration, as carbon source could be an interesting approach. Therefore, feeding bioreactor strategies are needed to use these substrates when high cell densities are required (above 100 $g_{Xt}.L^{-1}$ (total dry cells (Xt)), for PHA production [1]), given the fact that its use leads to a dilution of the cells during fed-batch and continuous cultures. The highest values to cell concentration presented in literature, for PHA production, were obtained from high carbon concentration feeding strategies e.g., 221 $g_{Xt}.L^{-1}$ (800 $g_{glucose}.L^{-1}$ [16]), 208 $g_{Xt}.L^{-1}$ (700 $g_{glucose}.L^{-1}$ [17]), 81 $g_{Xt}.L^{-1}$ (500 $g_{glucose}.L^{-1}$ [18]) and 150 $g_{Xt}.L^{-1}$ (210 $g_{lactose}.L^{-1}$ (concentrated whey) [15]).

Fed-batch and continuous cultures conducted with cell recycle have been described in the literature to achieve high cell densities and high productivity for a wide range of products [19,20]. Ahn and coworkers [15] proposed a strategy with external cell recycle to produce P(3HB) by recombinant *Escherichia coli*, applying concentrated whey solution as carbon source (280 $g_{lactose}.L^{-1}$), and achieved 4.6 $g_{P(3HB)}.L^{-1}.h^{-1}$ of productivity. This is one of the highest productivities reported in literature for P(3HB) production. Ienczak et al. [21] developed a repeated fed-batch system with cell recycle that allows the use of substrates with moderate carbon source concentration (90 $g_{glucose/frutose}.L^{-1}$) to produce P(3HB), where the culture medium poor in nutrients can be removed from the bioreactor without remove the cells (recirculation).

Understanding the effect of process variables on the incorporation of 3HV units, facilitates the control of polymer production with specific desired physical characteristics. Aragão and coworkers [22] evaluated the effect of sustaining a limited nitrogen feeding during the P(3HB-*co*-3HV) production phase, at a fed-batch system using high carbon source concentration, and observed that the specific P(3HB-*co*-3HV) production rate was maintained in high values for extended periods, the incorporation of 3HV units was improved as well as the polymer productivity.

The aim of the present work was to evaluate the P(3HB-*co*-3HV) production by *C. necator*, in a system with external cell recycle and limited nitrogen feeding during the production phase, to achieve high cell density and polymer productivity. The feeding culture medium simulated the carbon concentration (glucose) verified in many agro-industrial wastes (90 $g_{glucose}.L^{-1}$). Propionic acid was added to induce the production of 3HV units.

2. Material and methods

2.1. Microorganism and culture media

The microorganism used at the present study was *C. necator* DSM 545 (Deutsche Sammlungvon Mikroorganismen und Zellkulturen, Braunschweig, Germany).

The strain was maintained in glycerol and nutrient broth (1:1) at −80 °C. Cultures were performed by using two seed cultures. The medium for the first seed culture was 5.0 $g.L^{-1}$ of meat peptone and 3.0 $g.L^{-1}$ of meat extract (Nutrient Broth, Difco™). The second seed culture was obtained in a mineral medium (MM) without nitrogen limitation, based on Aragão et al. [22], and was composed of (in $g.L^{-1}$): glucose 40.0; KH_2PO_4 4.4; ferric ammonium citrate 0.06; $CaCl_2.2H_2O$ 0.01; $MgSO_4.7H_2O$ 0.5; urea 2.15; nitrilotriacetic acid 0.19 and trace nutrients 1 $mL.L^{-1}$. The trace nutrient solution was composed of (in $g.L^{-1}$): H_3BO_3 0.3; $CoCl_2.6H_2O$ 0.2; $ZnSO_4.7H_2O$ 0.1; $MnCl_2.4H_2O$ 0.03; $Na_2MoO_4.2H_2O$ 0.03; $NiCl_2.6H_2O$ 0.02; and $CuSO_4.5H_2O$ 0.01. Both seed cultures were incubated in a rotary shaker (New Brunswick Sci. Company Inc., New Jersey, USA) at 35 °C and 150 rpm for 24 h. The second seed culture was used to inoculate the bioreactor for cultures with cell recycle strategies.

2.2. Bioreactor assembly and operation

The cultures were performed in a 5 L (working volume) bioreactor (BIOFLO 110, New Brunswick Sci. Company Inc., New Jersey, USA) with an initial volume of 4 L, equipped with pH, dissolved oxygen (DO (galvanic electrode, Mettler-Toledo GmbH, Switzerland)) and temperature controllers. The concentration of DO in the medium was not less than 30% (relative saturation with atmospheric air) by varying stirring speed and/or inlet air flow rate. The pH was kept constant at 7.0 by automatic supply of a 10% (v/v) HCl or 10% (w/v) NaOH solution. Temperature was maintained at 35 °C and propyleneglycol was used as anti-foaming agent and added when required.

2.3. Culture conditions

Two cultures were performed for P(3HB-*co*-3HV) production applying the same strategy developed by Ienczak et al. [21], one with total nitrogen depletion and one with limited nitrogen feeding, during the production phase.

For both cultures, the initial culture medium presented the same composition of the mineral medium described for the second seed culture (Section 2.1), except initial concentrations of glucose and urea that were 65 $g_{glucose}.L^{-1}$ and 8.6 $g_{urea}.L^{-1}$ (that represents an initial nitrogen concentration of 4 $g_{nitrogen}.L^{-1}$), respectively.

2.3.1. Feeding solutions

2.3.1.1. Culture medium. Two bottles of feeding culture medium were prepared, both with the same composition of the mineral medium described for the second seed culture (Section 2.1), except glucose and urea concentration. In the bottle used during the growth phase, glucose and urea concentration were, respectively, 90 $g_{glucose}.L^{-1}$ and 8.6 $g_{urea}.L^{-1}$ and, in the bottle used during the production phase, glucose concentration was 90 $g_{glucose}.L^{-1}$, without urea, aimed to induce nitrogen limitation/depletion. The glucose concentration, in the bottles, simulated the concentration usually presented in agro-industrial wastes with moderate carbon concentration.

2.3.1.2. Urea solution. At the culture that nitrogen was fed during the production phase, a third bottle was prepared containing only urea solution (6.45 $g_{urea}.L^{-1}$, that represents 3 $g_{nitrogen}.L^{-1}$).

2.3.1.3. Propionic acid solution. A propionic acid solution of 80 $g.L^{-1}$ was prepared in a fourth bottle, and fed to the bioreactor to induce 3HV production.

2.3.2. Cell recycle and feeding strategies

The cultures started as a batch. When the glucose concentration reached approximately 25 $g_{glucose}.L^{-1}$ (estimated by the linear correlation between optical density and cell concentration (Fig. 1) and the cell yield from glucose (0.5 $g_{Xt}.g_{glucose}^{-1}$)) the first feed using the external cell recycle was performed. During this process, initially one liter of the culture medium was removed from the bioreactor through the membranes, while the cells remained in the retentate flow. After that, one liter of the feeding culture medium was fed into the bioreactor through the microfiltration membranes. Proceeding the feed as described, the biomass that remained inside the membranes during removal of culture medium is loaded back to the bioreactor with the feeding culture medium, avoiding oxygen limitation to these cells. The system with cell recycle was applied