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A robust fed-batch feeding strategy independent of the carbon source for optimal polyhydroxybutyrate production

Md. Salatul Islam Mozumder[a,b,*], Heleen De Wever[a], Eveline I.P. Volcke[b], Linsey Garcia-Gonzalez[a]

[a] Flemish Institute for Technological Research (VITO), Business Unit Separation and Conversion Technology, Boeretang 200, 2400 Mol, Belgium
[b] Ghent University, Department of Biosystems Engineering, Coupure Links 653, 9000 Ghent, Belgium

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ABSTRACT

A three-stage control strategy independent of the organic substrate was developed for automated substrate feeding in a two-phase fed-batch culture of *Cupriavidus necator* DSM 545 for the production of the biopolymer polyhydroxybutyrate (PHB). The optimal feeding strategy was determined using glucose as the substrate. A combined substrate feeding strategy consisting of exponential feeding and a novel method based on alkali-addition monitoring resulted in a maximal cell concentration in the biomass growth phase. In the PHB accumulation phase, a constant substrate feeding strategy based on the estimated amount of biomass produced in the first phase and a specific PHB accumulation rate was implemented to induce PHB under limiting nitrogen at different biomass concentrations. Maximal cell and PHB concentrations of 164 and 125 g/L were obtained when nitrogen feeding was stopped at 56 g/L of residual biomass; the glucose concentration was maintained within its optimal range. The developed feeding strategy was validated using waste glycerol as the sole carbon source for PHB production, and the three-stage control strategy resulted in a PHB concentration of 65.6 g/L and PHB content of 62.7% while keeping the glycerol concentration constant. It can thus be concluded that the developed feeding strategy is sensitive, robust, inexpensive, and applicable to fed-batch culture for PHB production independent of the carbon source.

1. Introduction

Polyhydroxybutyrate (PHB) is an intracellular storage material that is synthesized by a number of microorganisms and has become of considerable industrial interest and of environmental importance as a biodegradable and biobased polyester. Although PHB is regarded as an effective substitute for conventional plastics for such applications as medical and agricultural uses [1] and food packaging [2], the full-scale commercialization of this biopolymer is hampered by its high production cost compared to other (bio)polymers [3]. The factors affecting the economics of PHB include the raw materials, process design, and downstream processing [4,5].

According to Shen et al. [6], 50% of the total production costs can be attributed to the raw materials of which the carbon source for growth and polymer accumulation accounts for 70–80%. Thus, to attain bulk commercial viability and to further improve the sustainability profile of PHB production by fermentation, it is desirable to use waste carbon sources instead of pure substrates. A wide spectrum of industrial by-products, such as whey, molasses, starch, and waste glycerol, have already been studied with regard to PHB production [7].

The production of biodiesel by the transesterification of oil with a short chain alcohol generates approximately 10% (w/w) glycerol as a co-product stream. Although pure glycerol is an important feedstock with applications found in the food, drug, and pharmaceutical industries, glycerol from biodiesel cannot be used in these applications due to the presence of impurities and requires further refinement prior to its use. As refining waste glycerol is expensive, it is important to search for alternative applications in which crude glycerol can be used as is with no refinement needed. Within this context, the biological conversion of crude glycerol to higher value chemicals, such as PHB, is an attractive alternative [8,9]. Indeed, utilizing crude glycerol as a cheap feedstock to produce PHB could increase the economic performance of both the biodiesel and biopolymer industries, though it should be noted that the presence of glycerol adversely affects the quality of the polymer by reducing its molecular mass [10–12]. Two prevalent cultivation methods are employed for PHB production, depending on the microorganism used. The more frequently applied method is a two-phase

* Corresponding author at: Boeretang 200, 2400 Mol, Belgium. Tel.: +32 14336903.
E-mail addresses: salatulislam.mozumder@vito.be, mdsalatulislam.mozumder@ugent.be (Md.S.I. Mozumder).

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fermentation process that consists of a cell-growth phase under favorable growth conditions to yield a high cell density, followed by a PHB production phase under imbalanced growth conditions by limiting a nutritional element, such as nitrogen, phosphate, or oxygen, to trigger PHB synthesis and accumulation [5,13,14]. The model organism for this cultivation process is *Cupriavidus necator* (formerly known as *Ralstonia eutropha*, *Alcaligenes eutrophus*, and *Wautersia eutropha*) [11,15,16]. For two-phase fermentation processes, the time at which nitrogen limitation is initiated, the choice of limiting nutrient, and the fermentation strategy are of utmost importance for maximizing PHB yield and productivity [5,17]. The second cultivation mode consists of a single-phase process with PHB accumulating in a growth-associated manner. Although PHB synthesis occurs under nutrient-sufficient conditions, it has been reported that applying nitrogen limitation enhances the final PHB content, making the recovery more economic. A well-known growth-associated PHB producer is *Alcaligenes latus* [17,18].

Fed-batch operation is the most popular method utilized to achieve high cell densities, productivity, and yields of the desired products [19]. The main challenge in fed-batch fermentation is to control the substrate concentration within an optimal range, thereby avoiding limiting and inhibiting concentration levels. As a result, the substrate feeding strategy is crucial for successfully obtaining high cell density cultures. Several feeding strategies have been proposed to improve PHB productivity and yield, such as continuous feeding [20], pH stat [21,22], and dissolved oxygen (DO) stat [23–25], in addition to control strategies based on the carbon dioxide (CO_2) evolution rate or using a carbon source analyzer [13]. However, all the feeding strategies developed to date carry important drawbacks. Continuous feeding is a simple method without feedback mechanism, such that over- or underfeeding is likely to occur, thus affecting the metabolism of the microorganisms. Substrate feeding strategies with indirect feedback control, such as pH or DO stat, are based on the finding that DO or pH increases sharply upon the depletion of a carbon source. When the pH or DO becomes higher than its set point, the nutrient is added at a predetermined rate to the fermentor [19]. Due to the nature of this feeding method, the substrate concentration cannot be kept at the desired level and will oscillate from the set point value to zero. During the periods of carbon depletion, the biomass growth rate and thus the final productivity can be adversely affected. Furthermore, as no cell growth occurs during imbalanced growth conditions, no sharp DO or pH increase is expected upon carbon depletion, resulting in cell starvation due to substrate exhaustion resulting from the improper control of the substrate [13,18]. On-line monitoring systems are more efficient. The CO_2 evolution rate can be obtained from mass spectrometry measurements, allowing an estimation of the substrate requirement based on the conversion efficiency. The use of a carbon source analyzer allows the direct measurement of the substrate concentration in the reactor. However, such systems are expensive; moreover, an online substrate analyzer is limited to a specific type of (pure) substrate. As a consequence, it cannot be used when applying a waste stream as the carbon source.

The aim of this research work was to optimize the overall fermentation process for the production of PHB independent of the carbon source used. A sensitive, robust and inexpensive substrate control strategy independent of the carbon source used for a fed-batch fermentation process was developed to obtain a high cell density culture with high PHB productivity and content. To determine the optimal feeding strategy, glucose was used as a substrate and *Cupriavidus necator* DSM 545 as the model organism. To induce PHB biosynthesis and accumulation, imbalanced growth conditions were enforced through nitrogen limitation. The developed feeding strategy was then validated using waste glycerol as the sole carbon source.

2. Materials and methods

2.1. Organism

Cupriavidus necator DSM 545 was used as the microorganism. According to the DSMZ website (www.dsmz.de), this strain, a mutant of *C. necator* DSM 529, constitutively expresses glucose-6-phosphate dehydrogenase.

2.2. Carbon sources

The experiments were performed using either glucose (Merck, Germany, 650 g/L) or waste glycerol; the latter was kindly provided by a local biodiesel industry (Oleon, Belgium) and contained 85% (w/w) glycerol (see Section 2.8).

2.3. Culture media

Lennox broth (LB) medium (Invitrogen, Life Technologies Europe B.V., Belgium) was used as the seed medium for preculture 1 and was autoclaved at 121 °C for 20 min. The seed medium for preculture 2 contained 10 g/L carbon source, 3 g/L $(NH_4)_2SO_4$, 1.5 g/L KH_2PO_4, 4.47 g/L $Na_2HPO_4{\bullet}2H_2O$, 0.2 g/L $MgSO_4{\bullet}7H_2O$, and 1 mL/L trace element solution. For the fermentation culture, the initial medium consisted of 12 g/L glucose or 17 g/L waste glycerol, 4 g/L $(NH_4)_2SO_4$, 13.3 g/L KH_2PO_4, 1.2 g/L $MgSO_4{\bullet}7H_2O$, 1.87 g/L citric acid, and 10 mL/L trace element solution. The trace element solution of the mineral salt medium for preculture 2 and the fed-batch experiments had the following composition: 10 g/L $FeSO_4{\bullet}7H_2O$, 2.25 g/L $ZnSO_4{\bullet}7H_2O$, 1 g/L $CuSO_4{\bullet}5H_2O$, 0.5 g $MnSO_4{\bullet}5H_2O$, 2 g/L $CaC1_2{\bullet}2H_2O$, 0.23 g/L $Na_2B_4O_7{\bullet}10H_2O$, 0.1 g/L $(NH_4)_6Mo_7O_{24}$, and 35% HC1 10 mL/L. The solution was filter sterilized through a 0.2-μm polyethersulfone (PES) filter (Whatman, UK). The carbon source and $MgSO_4{\bullet}7H_2O$ were separately autoclaved at 121 °C for 20 min. All three solutions were aseptically added to the medium after cooling; the pH of the medium was adjusted to 6.80 with 5 M NaOH.

2.4. Inoculum preparation

Stock cultures of *C. necator* DSM 545 were stored at −20 °C in 2-mL cryovials containing 0.5 mL of 80% glycerol (Merck, Germany) and 1 mL of a late exponential-phase liquid culture in LB medium. These stock cultures were used to inoculate preculture 1 by transferring 200 μL to 5 mL of LB medium in 15-mL test tubes. The preculture was cultivated in an orbital shaker (Innova 42, Eppendorf, USA) for 24 h at 30 °C and 200 rpm. Subsequently, 2 mL of the strain was sub-cultured for 24 h at 30 °C and 180 rpm in 100 mL of preculture 2 seeding medium in 500-mL baffled flasks. When using waste glycerol as the carbon source, successive sub-culturing was performed five times to ensure a good adaptation of the microorganisms to the glycerol substrate. Lastly, the seed culture was used to inoculate baffled flasks (4% v/v inoculum, Section 2.5) or the bioreactor (12.5% v/v inoculum, Section 2.6).

2.5. Shake flask experiment

The effect of the glucose concentration on the growth of *C. necator* DSM 545 was investigated by monitoring the initial growth rate as previously described in literature [11]. Preculture 2 (4 mL) was inoculated into 100 mL of fermentation medium supplemented with glucose ranging from 5 to 60 g/L in 500-mL baffled flasks. The flasks were incubated at 180 rpm and 30 °C for 10 h to ensure favorable conditions for cell growth. Samples were then collected for