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

Md. Salatul Islam Mozumder, Heleen De Wever, Eveline I.P. Volcke, Linsey Garcia-Gonzalez

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 polymer. 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...
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 (CO2) 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 CO2 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 (Oleone, 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(NH4)2SO4, 1.5 g/L KH2PO4, 4.47 g/L Na2HPO4•2H2O, 0.2 g/L MgSO4•7H2O, 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(NH4)2SO4•13.3 g/L KH2PO4, 1.2 g/L MgSO4•7H2O, 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 FeSO4•7H2O, 2.25 g/L ZnSO4•7H2O, 1 g/L CuSO4•5H2O, 0.5 g MnSO4•5H2O, 2 g/L CaCl2•2H2O, 0.23 g/L Na2B4O7•10H2O, 0.1 g/L (NH4)6Mo7O24, and 35% HCl 10 mL/L. The solution was filter sterilized through a 0.2-μm polyethersulfone (PES) filter (Whatman, UK). The carbon source and MgSO4•7H2O 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
analysis, as described in Section 2.8. All the shake flask experiments were conducted in duplicate to confirm the accuracy of the results.

2.6. Fed-batch experiments

Fed-batch experiments were performed in a 3-L bioreactor (Applichon Biotechnology, the Netherlands). The setup was equipped with on-line monitoring and an E-Z-control system (Applichon Biotechnology, the Netherlands) used to control the stirring speed, DO, foam formation, pH, and temperature. The DO concentration level was regulated at 55% of air saturation for phase 1 and 30% of air saturation for phase 2 using a cascade control strategy consisting of the agitation speed (850 up to 1000 rpm) and air and/or oxygen flow. Foaming was controlled using 30% antifoam C emulsion (Sigma–Aldrich Chemie, GmbH, Germany), and the pH was maintained at 6.80 by adding acid (2 M H_2SO_4) or base (5 M NaOH or 20% NH_4OH). The process temperature was fixed at 30 °C. A computer-based software program, BioXpert, was used to implement the developed feeding strategy for controlling the carbon source concentration in the fermentor at the desired level. Samples were collected at regular time intervals and analyzed according to Section 2.8.

2.7. Development of feeding strategy using glucose as the carbon source

Separate feeding strategies were developed for each phase of the two-phase fermentation process using glucose as the carbon source. Each feeding strategy was evaluated at least twice per substrate to confirm its applicability.

2.7.1. Phase 1: biomass growth

Two types of substrate feeding strategies, exponential feeding and two-stage feeding consisting of exponential feeding, followed by feeding based on alkali-addition monitoring, were developed to control the substrate concentration in phase 1 within an optimal range. In parallel, nitrogen was added using NH_4OH as a base to control the pH.

2.7.1.1. Exponential feeding. To maintain a maximal cell growth within the exponential growth phase, the substrate should be added according to its consumption. Thus, the concept of exponential feeding is based on the exponential growth of residual biomass (defined as the difference between the cell dry mass (CDM) and PHB concentration) (with concentration X in g/L):

$$\frac{dX}{dt} = \mu X$$

$$X = X_0e^{\mu(t-t_0)} = X_0e^{\mu\Delta t}$$

$$\Delta X = X_0(e^{\mu\Delta t} - 1)$$

where t denotes time (h), \(\mu\) is the specific biomass growth rate (1/h), and \(X_0\) represents the initial (at \(t=t_0\)) residual biomass concentration (g/L). During exponential feeding, the amount of feed solution needed to keep the substrate concentration constant is equal to the amount of substrate consumed ([1/\(Y_{XS}\)]\(\Delta X/V\)) (g substrate) divided by the substrate concentration in the feed solution \(S_{feed}\) (g/L) and is expressed as

$$\frac{\Delta F_1}{\Delta t} = \frac{1}{Y_{XS}} \frac{\Delta X}{\Delta t} V = \frac{1}{Y_{XS}} \frac{1}{\Delta t} \frac{X_0(e^{\mu\Delta t} - 1)V}{\Delta X}$$

where \(\Delta F_1\) is the volume (L) of feed solution fed to the fermentor for a period (\(\Delta t\)) at phase 1, \(V\) is the working volume of the fermentor medium (L), \(Y_{XS}\) is the biomass yield (g biomass/g substrate), and \(S_{feed}\) is the substrate concentration in the feeding solution (g/L). Given Eq. (3), it is clear that the accuracy of dosing depends on accurate knowledge of the microbial growth parameters \(\mu\) and \(Y_{XS}\) and the initial biomass concentration \(X_0\), in addition to the reactor volume and the feed concentration, which are known.

2.7.1.2. Alkali-addition monitoring. An indirect feedback-control feeding strategy based on alkali-addition monitoring was developed from the balance of biomass growth. The stoichiometry of residual biomass growth (Eq. (4)) using glucose as the sole carbon source was determined given the residual biomass yield with VSE and the elementary biomass composition and by subsequently applying (elemental) balances for C, N, charge, H, and O. The composition of C. necator cells was taken from Ishizaki and Tanaka [28], and \(Y_{XS}\) was measured in a batch experiment.

$$C_6H_12O_6 + 1.97 O_2 + 0.72 NH_4^+ \rightarrow 3.79 CH_3_1.74 O_4.46 N_0.19 + 2.21 CO_2 + 0.72 H^+ + 3.78 H_2O$$

(4)

Hydrogen ions (H⁺) are produced during biomass growth, decreasing the pH of the mineral medium solution; thus, the medium needs to be neutralized using alkali to maintain the pH at the optimum level for the growth of C. necator. Based on the mass balance equation (Eq. (4)), the substrate feeding rate was estimated from the amount of alkali supplied to keep the pH constant. The substrate feeding rate based on the supplied alkali needed to keep the substrate concentration constant is equal to the sum of substrate consumed per hour ([1/\(Y_{XS}\)]\(C_b Q_b M_2\)) (g substrate/h) divided by the substrate concentration in the feed solution \(S_{feed}\) (g/L) and is expressed as

$$\frac{dF_2}{dt} = \frac{1}{Y_{XS}} \frac{1}{S_{feed}} C_b Q_b M_2$$

(5)

where \(C_b\) is the molar concentration of the base solution (mol/L), \(Q_b\) is the base flow rate (L/h), \(M_2\) is the molecular weight of the substrate, and \(Y_{XS}\) denotes the molar ratio between the ammonium and substrate consumption and is equivalent to the molar ratio between proton production and substrate consumption (\(Y_{XS,glucose}\) = 0.72).

2.7.1.3. Combined substrate feeding. A two-stage feeding strategy for biomass growth was developed, consisting of exponential feeding for the first 10 h, followed by feeding based on alkali-addition monitoring. This strategy was termed ‘combined substrate feeding’.

2.7.2. Phase 2: PHB accumulation

As imbalanced growth conditions by limiting a nutritional element (in this case nitrogen) triggers PHB synthesis, the feed should contain only the substrate and no nitrogen. The substrate feeding rate and substrate consumption rate are directly proportional to the PHB accumulation rate, which is in turn related to the residual biomass concentration (X) and specific PHB accumulation rate (\(\mu_p\), g PHB/g biomass/h). Therefore, the feeding rate of the feed solution in phase 2 needed to keep the substrate concentration constant is equal to the amount of substrate consumed per hour ([1/\(Y_{PS}\)]\(X_\mu_p V\)) (g substrate/h) divided by the substrate concentration in the feed solution \(S_{feed}\) (g/L) and is expressed as

$$\frac{dF_2}{dt} = \frac{1}{Y_{PS}} \frac{1}{S_{feed}} \frac{dP}{dt} = \frac{1}{Y_{PS}} \frac{1}{S_{feed}} X_\mu_p V$$

(6)

where \(P\) is the amount of PHB (g), \(F_2\) is the volume of feed solution fed to the fermentor during phase 2 (L), \(\mu_p\) is the specific PHB
accumulation rate (g PHB/g biomass/h), and \( Y_{PB} \) is the yield of PHB (g PHB/g substrate). Although the total amount of biomass remains the same during phase 2, the biomass concentration decreases due to dilution through the added feed volume and can be calculated as

\[
X = \frac{X_1 V_1}{V_1 + \frac{r^H dF}{2}}
\]

where \( X_1 \) denotes the biomass concentration (g/L) and \( V_1 \) is the working volume at the end of phase 1 (L).

### 2.8. Analytical procedures

The growth of \( C. \) necator was roughly monitored by measuring the optical density (OD) using a spectrophotometer (UV–1800, Shimadzu, Japan) at 600 nm, with the sufficient dilution of the culture broth. The glucose concentration in the medium was determined off-line by the phenol-sulfuric acid method using glucose as a standard, as originally described by Dubois et al. \[27\]. The glycerol concentration was determined off-line through HPLC using MilliQ water as the mobile phase. The concentrations of ammonium (NH\(_4^+\)–N) were evaluated off-line colorimetrically with standard Hach Lange cuvette tests (Hach Lange GmbH, Germany). A gravimetric method was used to determine the cell concentration, which was expressed as cell dry mass (CDM). Culture broth (15–20 mL) was centrifuged (SORVALL RC+ centrifuge, Thermo Scientific, Clintonpark Keppelkouter, Belgium) at 7000 × g in pre-weighted screw-cap tubes for 30 min at 4 °C. The cell pellets were washed with distilled water, re-centrifuged, frozen at −20 °C, and lyophilized until a constant weight. CDM was determined as the weight difference between tubes containing the cell pellets and empty tubes. For the PHB analysis, dried samples and external standards (PHB, Biomer) were subjected to methanolysis in the presence of 50% (v/v) methanol and 50% (v/v) NaOH. The resulting 3-hydroxybutyric acids were analyzed by HPLC using 0.05% H\(_3\)PO\(_4\) as the mobile phase. The residual biomass was defined as the difference between CDM and the PHB concentration. The PHB content was calculated as the percentage of the ratio of the PHB concentration to the total cell concentration.

### 2.9. PHB extraction

After lyophilization, 1 g of dried cells was resuspended in 100 mL chloroform for 24 h with vigorous agitation at room temperature. After extraction, the cellular debris was separated by filtration (Whatman, Schleicher and Schuell, 75 mm radius). The chloroform fraction containing the solubilized polymer was poured into cold ethanol to precipitate the polymer. After filtration, PHB was resuspended into chloroform, and the precipitation procedure was repeated twice to further purify the polymer. The precipitated polymer was filtered and dried.

### 2.10. PHB characterization

The average molecular weight \( (M_w) \) was determined by gel permeation chromatography (GPC) using a Waters Breeze™ System with a combination of three column series (PSS SDV analytical 1000 Å, 5 μm, 300 × 8.00; PSS SDV analytical 100,000 Å, 5 μm, 300 × 8.00; PSS SDV analytical 1,000,000 Å, 5 μm, 300 × 8.00) and equipped with a 2414 differential refractive index detector. Chloroform was used as the eluent at 35 °C, and the applied flow rate was 1.0 mL/min. A calibration curve was obtained using narrow polystyrene standards (Polymer Laboratories) in the \( M_w \) range of 580–1,930,000 g/mol.

### 3. Results and discussion

#### 3.1. Development of a feeding strategy using glucose as the carbon source

##### 3.1.1. Phase 1: biomass growth

The development of a feeding strategy in a fed-batch culture for biomass growth to control the substrate concentration at its optimal level is essential to attain a maximal cell concentration and high biomass productivity. In addition, this approach affects the overall PHB productivity by preventing premature shifting to phase 2. In this study, two types of glucose feeding strategies, exponential feeding and combined substrate feeding, were evaluated to maintain the glucose concentration within an optimal range. First, a series of shake flask experiments were performed to determine the optimal glucose concentration (Fig 1). The results indicated that the initial concentration of glucose significantly affected the specific growth rate, which was found to be at a maximum at an initial glucose concentration ranging between 10 and 20 g/L, corresponding to previously reported values \[13,28\]. A decrease in growth rate was observed at higher glucose concentrations.

##### 3.1.1.1. Exponential feeding of glucose

A series of batch experiments at the bioreactor level were performed with \( C. \) necator DSM 545 to determine the initial residual biomass concentration \( (X_0) \), specific biomass growth rate \( (\mu) \), and biomass yield \( \left( Y_{XS} \right) \) (data not shown), estimated as \( X_0 = 0.4 \text{ (±0.03) g/L, } \mu = 0.149 \text{ (±0.012) h}^{-1}, \text{ and } Y_{XS} = 0.5 \text{ (±0.015) g biomass/g glucose, respectively.} \) These values were applied in an initial fed-batch fermentor experiment to evaluate the exponential feeding strategy (Fig 2a). The results showed that a biomass concentration of 24 g/L (CDM) was attained after 24 h and contained 8% PHB. The glucose concentration could be maintained within its optimal range (10–20 g/L) during the first 16 h of fermentation but then decreased from 15 to 2 g/L. The experiment was stopped after 24 h because glucose became limiting for biomass growth, and this decrease in glucose concentration indicated that \( X_0 \) and \( \mu \) were higher than the estimated values: \( X_0 = 0.438 \text{ g/L and } \mu = 0.157 \text{ h}^{-1}. \) Therefore, a second fed-batch experiment using these parameter values was set up (Fig 2b). The glucose concentration was again controlled within its optimal range during 17 h of fermentation but then gradually increased and reached 35.5 g/L after 32 h; at that point, growth ceased, and a maximal cell concentration of 42 g/L was obtained. The average specific growth rate \( \mu \) was calculated as 0.131 h\(^{-1}\), which was lower than the applied value, causing the overfeeding of glucose. Thus, the shake flask experiments confirmed the effect of glucose inhibition on the growth rate when the concentration was higher than 20 g/L (Fig 1).

The exponential feeding strategy has been developed to allow cells to grow at constant specific growth rates \[19\]. Nonetheless,
several authors have reported the necessity of feedback or feed-forward control to compensate for fluctuations due to process perturbations and parameter inaccuracies [29,30]. Our study confirmed that this simple feeding technique was ineffective to maintain the substrate concentration at the optimal level for C. necator DSM 545. Exponential feeding resulted in long-term over- or underfeeding due to deviations in the parameter values from the initially estimated values, resulting in growth repression or cell starvation. Moreover, the results indicated the need for a feeding strategy with a feedback control mechanism to compensate for fluctuations due to parameter inaccuracies.

3.1.1.2. Combined feeding of glucose. A feedback-control glucose feeding strategy based on alkali-addition monitoring was developed (Eq. (5)) and used in fed-batch culture in an effort to reach a high-density culture. As NH₄OH is volatile, part of the NH₄⁺ will be stripped as NH₃, which results in a higher need for the addition of base than is stoichiometrically needed according to Eq. (4). Consequently, by using 20% (NH₃-basis) NH₄OH as an alkali, glucose feeding based on Eq. (5) may result in overfeeding, causing the premature termination of biomass growth. Therefore, the proposed feeding strategy (Eq. (5)) was extended with a correction factor, η, resulting in the following control law (Eq. (8))

\[
\frac{dX}{dt} = \eta \frac{1}{Y_{NS}} \frac{1}{S_{feed}} C_0 Q_0 M_s \tag{8}
\]

There are a number of operating and process parameters, including temperature, air flow rate, flow pattern, stirring speed, method of NH₄OH dosing, that affect the NH₃ loss, and thus the correction factor value. As these parameters may vary with the experimental setup, it is very important to determine η for every experimental setup.

Experiments were performed to determine η from the correlation between the stoichiometrically needed and actually pumped amount of 20% (NH₃-basis) NH₄OH (Fig. 3). The results showed that an average of 25% more alkali was pumped; hence, the value of η was set to 0.75. Due to the buffering capacity of the mineral medium and low biomass production, it was observed that no alkali was added during the first 10–12 h of fermentation (Fig. 3). Although only a low amount of biomass was produced during that period, the substrate was consumed for biomass growth. As the objective of this study was to develop a feeding strategy independent of the carbon source used for controlling the carbon source at its optimal level, we decided to include an additional feeding strategy for the period that alkali was not added. As a result, combined feeding was applied during phase 1, which consisted of exponential feeding during the first 10 h using Eq. (3) with \( X_0 = 0.4 \) g/L and \( \mu = 0.1491 \) l/h, as determined from the batch experiments at the bioreactor level, followed by alkali-addition monitoring using Eq. (8). It should be noted that as an alternative for exponential feeding, higher initial substrate concentrations could be used. In this case, the feeding is only based on alkali-addition monitoring. The results showed that the cell concentration reached a maximal level of 73.5 g/L after 33 h, with \( \mu = 0.141 \) l/h (Fig. 4). PHB accumulation began after 25 h and increased to 6 g/L, corresponding to a final PHB content of 8%. Despite a small decrease in the glucose concentration at the end of the fermentation due to simultaneous biomass growth and PHB formation, glucose was overall properly controlled at its optimal level. Indeed, the feeding of glucose was based on the stoichiometry of residual biomass growth and thus the PHB production at the end of the biomass growth phase was not taken into account. By incorporating the PHB production in Eq. (4), the decrease of substrate concentration can be avoided.

In fed-batch cultivation, several directly or indirectly measured variables are used for control purposes. The directly measured variables include the pH, DO concentration, OD, substrate concentration, pressure, and gas outflow composition. The indirectly determined variables include the specific growth rate, cell concentration, oxygen uptake rate, CO₂ evolution rate, and respiratory quotient and are estimated or calculated from one or more of the directly measured variables [19]. Except for pH and DO, the determination of the variables requires dedicated sensors or analytical
equipment that are not commonly installed in bioreactors [27]. Furthermore, a substrate feeding strategy coupled with the measurement of pH (pH-stat) or DO (DO-stat) is also far from optimal, as the cells will be exposed to oscillations in substrate concentration. In this study, a feedback control strategy was developed for automated substrate feeding in fed-batch C. necator DSM 545 culture with the aim of maintaining the substrate concentration within its optimal range. The control strategy uses the online estimation of the cell concentration as a performance indicator of the fed-batch culture, an estimation that is based on the base consumption rate and the ratio between substrate conversion and acid production, as determined from the mass balance. A direct comparison of the results obtained using this organism with literature data is difficult, as the biomass growth phase is not represented as a separate phase in literature but as an integrated phase for PHB production. However, it can be concluded that the feeding strategy was efficient because glucose was controlled at its optimal level throughout the culture period, even at the highest cell density of 73.5 g/L. It should be noted that the cells had already accumulated a small amount of PHB during the growth phase, consistent with previous reports [13,31]. This shows that imbalanced growth conditions are a sufficient, but in no case a necessary condition for PHB accumulation.

The great advantage of this feeding strategy is that only online monitoring of the amount of base added during fermentation is required. As a consequence, the method is applicable to bioreactors equipped with standard pH probes, representing a low investment cost. In addition, the nutrient method can maintain the substrate concentration at its optimal value, thus allowing the cells to grow at a maximum specific growth rate. It should be noted however that the success of this feeding strategy mainly depends on the accuracy of the base dosing. Indeed, possible losses of base during dosing should be taken into account and need to be evaluated for every system, as this affects the feeding strategy and thus the substrate concentration.

3.1.2. Phase 2: PHB accumulation

After optimizing the feeding strategy for biomass growth, PHB synthesis was triggered by applying pyruvate concentration in the presence of glucose, which was achieved by replacing NH₄OH feeding with NaOH for pH control. The glucose concentration was maintained at the optimal level in phase 2 using the feeding strategy according to Eqs. (6) and (7). The value of $\mu_p$ was determined to be 0.09 g PHB/g biomass/h, and $Y_{PB}$ was taken from the literature as 0.30 g PHB/g glucose [32,33]. To determine the maximal PHB

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**Table 1**

Comparison of different substrate feeding strategy in a fed-batch process for PHB production using nitrogen limitation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Strain</th>
<th>pH-stat and continuous</th>
<th>DO-stat and continuous</th>
<th>Feeding strategy</th>
<th>CO₂ evolution rate (g CO₂/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Ac. eutrophus NCIMB 11599</td>
<td>Continuous</td>
<td>0.45</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td>Molasses</td>
<td>A. eutrophus NCIMB 11599</td>
<td>Continuous</td>
<td>0.83</td>
<td>0.83</td>
<td>0.83</td>
</tr>
<tr>
<td>glucose</td>
<td>Bacillus megaterium BA-019</td>
<td>Continuous</td>
<td>0.67</td>
<td>0.67</td>
<td>0.67</td>
</tr>
</tbody>
</table>

**Fig. 4.** Cell biomass production of C. necator DSM 545 in fed-batch cultivation using combined (exponential feeding for first 10h and then alkali addition monitoring) glucose feeding.
concentration and productivity, nitrogen limitation was applied when the residual biomass concentration reached 49, 56, and 62 g/L. The residual biomass was estimated based on the amount of total glucose pumped in phase 1, as described in Eq. (9).

\[ X = \frac{F_1 \times V_{XS}}{S_{\text{feed}}} \]  

(9)

The correctness of the estimated value was later confirmed by analytical results with less than 2% variation. The results are summarized in Table 1, and the overall results of PHB accumulation applying nitrogen limitation at 49 and 56 g/L residual biomass concentration are shown in Fig. 5.

When nitrogen was limited at the residual biomass concentration of 49 g/L, the nitrogen in the culture broth became depleted after a short time, thereby triggering PHB synthesis (Fig. 5a). The maximal biomass (CDM) and PHB concentration were 127.7 g/L and 97 g/L, respectively, after 56 h, resulting in a PHB content of 75.8% and PHB productivity of 1.74 g PHB/L/h. The glucose concentration in the medium fluctuated between 10 and 20 g/L; however, as this is considered to be the optimal range, it did not affect the cell growth and PHB production. In total, 438 g (673 mL of 65% glucose solution) glucose was fed during fermentation, resulting in \( Y_{PHB} = 0.22 \) g PHB/g glucose. When applying nitrogen limitation at a 56 g/L residual biomass concentration, a maximal biomass (CDM) concentration of 164 g/L and PHB concentration of 125 g/L were achieved after 62 h (Fig. 5b). The PHB content and productivity increased to 76.2% and 2.03 g PHB/L/h, and the glucose concentration (13–16 g/L) was properly maintained within its optimal range. In this experiment, 523 g of glucose (806 mL of 65% glucose solution) was added, which resulted in \( Y_{PHB} = 0.24 \) g PHB/g glucose. In both experiments, the PHB production reached its maximum concentration 26 h after shifting to phase 2. In order to totally consume the residual glucose, we advise to stop the glucose feeding 25 h after shifting to phase 2. When delaying nitrogen limitation until the residual biomass concentration reached 62 g/L, the process became unstable due to excessive foaming, which could not be controlled by the addition of antifoam. For all experiments, the DO concentration was regulated at 55% air saturation for phase 1 and 30% air saturation for phase 2 using a cascade control strategy; during the experiments, DO varied from 49 to 61% in phase 1 and 25 to 32% in phase 2.

A number of studies have focused on efficient process design for PHB production, and an overview of the final cell concentration, PHB concentration, PHB content, and productivity obtained from various cultures applying different feeding strategies, substrates, and microorganisms is given in Table 1. In the present study, the glucose concentration was maintained at its optimal level using a three-stage feeding strategy consisting of combined feeding at phase 1, followed by constant feeding at phase 2.

Our experimental results show that although this strain accumulated a low amount of PHB in the first phase (maximum 16%), imbalanced growth conditions by limiting a nutritional element such as nitrogen enhanced the PHB content (maximum 75.8%) and productivity. This is consistent with recently (and older) published studies where ammonium limitation was imposed to promote PHA synthesis by the same strain [34,35]. The latter study also confirms that an insufficient supply of nitrogen or phosphorus can be regarded as the main regulating factor for redirection of carbon flux from biomass to PHA synthesis for C. necator. Furthermore, it can be observed that applying nitrogen limitation at a higher cell concentration increased the final cell concentration, PHB concentration, and PHB productivity, though the PHB content stayed constant. The highest values were obtained when NH\(_4\)OH feeding was stopped at the residual biomass concentration of 56 g/L. Furthermore, the process became unstable in an attempt to further enhance fermentation performance by delaying nitrogen limitation at a residual biomass concentration of 62 g/L. The importance

![Fig. 5. Cell biomass and PHB production of C. necator DSM 545 in fed-batch cultivation using three-stage glucose feeding. Nitrogen limitation was applied at (a) 49 and (b) 56 g/L residual biomass concentration.](image-url)
of the timing of nitrogen limitation, the optimal residual biomass concentration for shifting to phase 2, and the instability of the culture at higher cell concentrations correspond to the findings of Kim et al. [13] (Table 1).

Based on the comparison of PHB production triggered by nitrogen limitation using various microorganisms, substrates, and feeding strategies (Table 1), the highest values reported to date were obtained by Kim et al. [13]: however, an online glucose analyzer was used to control the glucose concentration in that study. Although Kim et al. [13] were able to efficiently produce PHB, the use of an online glucose analyzer is limited to the substrate used and is expensive. Moreover, fluctuations in glucose concentration can occur due to delays in measurement time [19]. In the present study, comparable results were obtained using an inexpensive and robust feeding strategy that, importantly, can be applied for the production of PHB independent of the carbon source used.

### 3.2. Validation of feeding strategy using waste glycerol as a carbon source

To demonstrate that the developed three-stage feeding strategy is independent from the carbon source used, the feeding strategy was validated using waste glycerol derived from a biodiesel production plant as the carbon source for PHB production. First, a series of shake flask experiments were performed to determine the optimal waste glycerol concentration, which was found to be 10–30 g/L (data not shown). For fed-batch culture, combined substrate feeding consisting of an initial 10 h of exponential feeding (Eq. (3)) followed by alkali addition monitoring (Eq. (8)) was used for the biomass growth phase using the parameter values (previously determined by a number of batch experiments at the bioreactor level) \( \mu_g = 0.4 \text{ g PHB/g biomass/h} \) and \( Y_{SP} = 0.48 \text{ g biomass/g glycerol} \), and \( Y_{NH4+} = 0.37 \text{ mole of NH}_4 \text{+/mole of glycerol} \). After 30 h, nitrogen limitation was initiated to trigger PHB biosynthesis. In the PHB accumulation phase, waste glycerol was added using the feeding strategy described by Eqs. (6) and (7) with parameter values (previously determined by a number of batch experiments at the bioreactor level) of \( \mu_g = 0.11 \text{ g PHB/g biomass/h} \) and \( Y_{SP} = 0.52 \text{ g PHB/g glycerol} \). As shown in Fig. 6, the biomass (CDM) and PHB concentrations obtained after 48 h were 104.7 g/L and 65.6 g/L, respectively, resulting in a PHB content of 62.7%. The maximum PHB productivity was as high as 1.36 g PHB/L/h, and the glycerol concentration was maintained at its optimal level using this three-stage feeding strategy. To ensure maximum PHB production and utilization of residual glycerol, we advise to stop the glycerol feeding 16 h after shifting to phase 2.

Table 2 compares the results from a number of studies on the production of PHB from pure and waste glycerol using various production strains. However, the substrate feeding strategy was not clearly described in these studies. Only Cavalheiro et al. [34] reported the pulse addition of waste glycerol for phase 1 and constant feeding of diluted waste glycerol for phase 2. From this overview, it can be concluded that the results obtained in the present study resulted in the highest reported values for PHB production from waste glycerol and even for pure glycerol as the carbon source. Only Koller et al. [12] and Ibrahim and Steinbuchel [38] reported a higher PHB content from waste glycerol and pure glycerol, respectively, whereas Cavalheiro et al. [11] achieved a higher PHB productivity using pure glycerol. Nevertheless, the CDM and PHB concentrations in these cases were much lower compared to the present study.

### 3.3. Product characterization

Based on the PHB characterization results, the average molecular weight of PHB decreased from 1.23 \( \times 10^6 \) Da to 6.24 \( \times 10^5 \) Da when waste glycerol was used as the carbon source instead of glucose, whereas the polydispersity index increased from 1.18 to 1.59. The molecular mass values in this study have the same order of magnitude as those obtained by other authors for PHB from glycerol [11] and from glucose [10]. Furthermore, the lower molecular weight in the presence of glycerol corresponds to previous reports [10–12]. It has been demonstrated by Madden et al. [10] that carbon sources present in the medium during the PHB accumulation phase by *C. necator* act as chain-transfer agents in the chain termination step of the polymerization process.

### 4. Conclusions

A new three-stage substrate feeding strategy for PHB production from the organic substrate was developed. The proposed feeding strategy consists of exponential feeding and feeding based on alkali-addition monitoring for biomass growth, followed by constant feeding for PHB production. The substrate concentration was controlled within its optimal range during the fed-batch culture, and using this feeding strategy and initiating nitrogen limitation at the optimal time resulted in maximal cell and PHB concentrations. This feeding strategy has the advantage of being sensitive, robust, inexpensive, and independent of the carbon source used, making the bioprocess more economical and enhancing the commercial viability of PHB as a biopolymer. In addition, this feeding strategy can be used for other types of fermentation processes that require pH control to achieve high cell density cultures.

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### References


