Application of Dynamic Optimization Techniques for Poly(β-hydroxybutyrate) Production in a Fed-Batch Bioreactor

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Poly(β-hydroxybutyrate), PHB, is a microbial biopolymer produced by bacteria under unbalanced culture conditions. Such unbalanced conditions can be easily reached in a fed-batch bioreactor; therefore, it is important to know the system's behavior as well as its constraints to induce unbalanced conditions that allow the optimization of feeding profiles and, consequently, PHB productivity. In this work, dynamic optimization has been applied to maximize PHB productivity in a fed-batch bioreactor. Optimal feeding profiles of carbon and nitrogen sources, as well as their respective concentrations, were obtained using gPROMS (generalized process modeling system). When there is reliable bioreaction kinetics available, dynamic optimization becomes a very valuable tool for predicting optimal operating conditions to maximize PHB productivity, with a considerable reduction in time and experimental costs.

1. Introduction

At present, almost all plastics produced around the world are of petrochemical origin. They are nonbiodegradable polymers that remain on the Earth's surface for hundreds of years. The annual production of plastics in 2006 was estimated as 245 million tonnes,[1] and approximately 40% of this production is discarded into landfills after use. In addition, several hundred thousand tonnes of plastics are discarded every year into marine environments accumulating in oceanic regions.[2] Biodegradable polymers constitute a potential solution to handle environmental and solid waste management problems in the world.[2] These biodegradable plastic materials should retain the desired material properties of conventional synthetic plastics and degrade completely without leaving any undesirable residue when discarded.[3]

The most common biopolymers are polysaccharides (cellulose and starch), polyesters (poly(hydroxyalkanoate)s and poly(lactic acid)), protein-based polymers (silk and wool), and hydrocarbons (natural rubber). In recent years, both poly(hydroxyalkanoate)s (PHAs) and poly(lactic acid) have received much attention since they are biocompatible and biodegradable materials. Poly(lactic acid), an aliphatic polyester, is commonly produced by the polymerization of lactic acid obtained from bacterial fermentation. This biopolymer has several applications in biomedicine, food packaging, and compost bags, among others. On the other hand, among PHAs, poly(β-hydroxybutyrate) is the most commonly studied polymer. It is directly synthesized as an intracellular reserve material by a wide variety of organisms that belong to the genera *Alcaligenes*, *Azotobacter*, *Bacillus*, and *Pseudomonas*.[4] PHB is produced under unbalanced growth conditions, i.e., carbon source excess and nitrogen, phosphorus, sulfur, or oxygen limitations.[5] Microbial PHB is a highly crystalline thermoplastic polymer with a melting point of about 175 °C. Due to its properties, it is often compared to polypropylene because both polymers have similar melting points, crystallinity degrees, and glass transition temperatures. Therefore, this analogy is useful for visualizing the type of products that can be made from PHB.[6] In addition to the above-mentioned properties, PHB is a nonxenobiotic plastic, thus being fully degradable. PHB can be produced from renewable sources, such as starch, whey, and many other carbon sources such as glucose, sucrose, caproate, heptanoate, etc. Some of the characteristics of PHB are biocompatibility, high polymerization degree, insolubility in water, high crystallinity if extracted from its natural environment, and isotacticity—i.e., stereochemical regularity in its repeating units.[5] Although PHB offers attractive advantages, there are three main reasons for the present use of synthetic plastics. First, plant fibers and animal proteins are subject to dramatic changes in quality and availability. Second, the growth of the petrochemical industry has ensured that, at least in the developed world, oil-derived polymers are cheaper than their natural counterparts. Third, and the most important reason, manufacturing and processing synthetic plastics is easier than obtaining natural polymers.[6] However, such dominance is threatened because petrochemical reserves are getting smaller and environmental contamination caused by nondegradable plastics is increasing every day.

PHB production can be divided into two stages: cellular growth without nutrient limitation and nutrient limitation to improve PHB production. In the first stage, under balanced growth conditions and in the presence of an excess of oxygen, all nutrients are bound to the tricarboxylic acid (TCA) cycle, which is the cycle for energy generation and amino acid formation in microorganisms that use oxygen for breathing. In the second stage, just after maximum growth is reached, limiting conditions inhibit the TCA cycle and the PHB formation cycle begins.[4,7,8] Fed-batch fermentation is the most commonly used operation for producing PHB since this type of bioreactor allows achievement of high cell densities by manipulating feed streams and induces the desired nutrient limitations needed for obtaining high yields and productivity.

Many authors[9−13] have studied fed-batch fermentations to improve PHB yields. PHB production enhancement has been generally achieved by establishing different feeding policies. These policies have been developed on the basis of the authors' experimental experiences and trial and error procedures. The results obtained have shown a considerable increase in PHB productivity with regard to other previous works. However, trial and error procedures can easily result in under- and overfeeding of the substrate, which can lead to cell starvation, cell inhibition, and formation of undesirable products. In this way, more detailed

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studies are needed to promote an overcoming of undesired effects and provide better control of deviations in the microorganism's growth pattern. On the other hand, some authors have developed multiple fed-batch experiments to increase PHB productivity.[14−18] They have focused their attention on obtaining a very high cell density phase, followed by a high PHB production by means of a non-growth-associated mechanism. For example, Kim et al.[14] recommend that, to obtain an efficient PHB production by *Alcaligenes eutrophus*, the carbon source concentration (glucose) should be maintained at an optimum value. Therefore, they use two methods to measure and, consequently, maintain the glucose concentration at the optimum value: a CO_2 evolution rate by mass spectrometry to estimate glucose concentration and an online glucose analyzer. These online techniques allow glucose feeding to keep the concentration at the optimum value. The results obtained by Kim et al.[14] and other authors have been widely accepted compared to other yields reported in the open literature. They propose an online control for PHB production instead of an offline control.

Dynamic optimization (DO) is a valuable tool for defining optimal operating conditions and maximizing PHB production in a fed-batch bioreactor, thus reducing experimental costs. At this point, it is important to note that a fed-batch operation does not directly imply an optimal metabolite production. Although this kind of bioreactor allows finding good conditions for primary and secondary metabolite production, the optimal operation conditions are really obtained by using an optimization technique applied to the bioreactor model. Thus, DO allows that nutrients fed into the bioreactor are those which microorganisms need in a particular fermentation time to grow or synthesize the desired metabolite. As a result, DO guarantees both an optimal cell growth and a metabolite biosynthesis, avoiding under- and overfeeding of the substrate.

Dynamic optimization for PHB production has not been studied extensively; however, this approach can be essential for maximizing polymer production in fed-batch bioreactors. Although many works have employed this optimization technique in different biotechnological processes,[19−26] dynamic optimization can be applied to PHB production in a similar way.

In this work, a dynamic optimization technique has been used to find optimal feed rate profiles for carbon and nitrogen sources to maximize PHB productivity in a fed-batch bioreactor. Optimal profiles were calculated on the basis of a control vector parametrization approach using generalized process modeling system (gPROMS) software. The main objective is to develop a useful predictive tool that will allow for a considerable reduction in time and experimentation costs. However, the precision of this methodology depends mainly on the accuracy of bioreaction kinetics. In addition, it is important to note that this is a theoretical work, and the results should be corroborated with experimental values.

2. Kinetics and Fed-Batch Model

In the present work, PHB production kinetics with *A. eutrophus* as obtained by Khanna and Srivastava[10] is employed. The specific growth rate (μ) is expressed as a function of the concentration of carbon and nitrogen sources: fructose (S_1) and urea (S_2), respectively. The specific growth rate containing two sigmoidal relations and two inhibition factors can be expressed as follows:

$$\mu = \mu_m \left[\frac{S_1^{n_1}}{S_1^{n_1} + k_{S_1}^{n_1}}\right]\left[\frac{S_2^{n_2}}{S_2^{n_2} + k_{S_2}^{n_2}}\right]\left[1 - \left(\frac{S_1}{S_{m_1}}\right)^{a_1}\right]\left[1 - \left(\frac{S_2}{S_{m_2}}\right)^{a_2}\right] \quad (1)$$

where μ_m is the maximum specific growth rate, S_{m_1} and S_{m_2} are the maximum values for substrate concentrations at which the specific growth rate is zero, and a_1, a_2, n_1, n_2, and k_s are kinetic parameters reported by Khanna and Srivastava.[10] The total biomass (X) is represented by two main components:

$$X = R + P \quad (2)$$

where R is the residual biomass that comprises the proteins, polysaccharides, nucleic acids, and lipids of the cell, and P represents PHB, which is an intracellular product. Mass balances for residual biomass (R) and PHB (P) are given by

$$\frac{dR}{dt} = \left(\mu - \frac{1}{V}\frac{dV}{dt}\right)R \quad (3)$$

$$\frac{dP}{dt} = (K_1\mu + K_2)R - \left(\frac{P}{V}\right)\frac{dV}{dt} \quad (4)$$

V is the reaction volume, t is the culture time, and K_1 and K_2 are growth- and non-growth-associated parameters, respectively. The product is assumed to be formed in both exponential and stationary phases as suggested by Khanna and Srivastava.[10]

Fructose and urea consumption rates are represented by

$$\frac{dS_1}{dt} = -(\alpha\mu + \gamma)R + \left[\frac{F_1 S_{o_1}}{V}\right] - \left(\frac{S_1}{V}\right)\frac{dV}{dt} \quad (5)$$

$$\frac{dS_2}{dt} = -\left[\frac{\mu}{Y_{X/S_2}} + m_{S_2}\right]R + \left[\frac{F_2 S_{o_2}}{V}\right] - \left(\frac{S_2}{V}\right)\frac{dV}{dt} \quad (6)$$

where α and γ are related to fructose consumption for cell growth and cellular maintenance, respectively, and F_1 and F_2 are the fructose and urea feed rates, respectively. In this work, these streams are assumed to be two independent streams fed with constant inlet concentrations (S_{o_1}, fructose; S_{o_2}, urea) to the bioreactor. Y_{X/S_2} is the yield of the total biomass with respect to urea, and m_{S_2} is a constant that represents urea consumption for cellular maintenance. All kinetic parameters employed in this work have been reported by Khanna and Srivastava[10] and are listed in Table 1.

The bioreactor volume varies over time according to the following expression:

Table 1. Model Parameters Reported by Khanna and Srivastava[10]

model param	value
μ_m	0.302
k_{S_1}	22.833
n_2	3.5938
k_{S_2}	0.234
n_2	2.213
α	0.48
γ	0.0348
Y_{X/S_2}	16.7
m_{S_2}	0.0000045
K_1	0.008
K_2	0.034
S_{m_1}	90.11
S_{m_2}	10.11
a_1	3.19
a_2	0.97

$$\frac{dV}{dt} = F_1 + F_2 \tag{7}$$

3. Dynamic Optimization

The fed-batch bioreactor is represented by a set of differential and algebraic equations (DAEs) with constraints and bounds on state variables solved using gPROMS. The optimization problem being considered in this work is the determination of optimal feed rate profiles for both substrates (F_1 and F_2) and their time-invariant concentrations (S_{o_1} and S_{o_2}) to maximize PHB production. The culture time is divided into a given number n of control intervals, and the optimization program allows optimization of F_1 and F_2 values for each time interval. S_{o_1} and S_{o_2} are also optimized, but are invariant over the complete operation time. State variables are the residual biomass, PHB, fructose, and urea concentrations and volume. The selected initial state $\mathbf{s}(0)$ is

$$\mathbf{s}(0) = [20.342.514]^T \tag{8}$$

This initial state for fructose and urea concentrations was obtained by maximizing the specific growth rate (μ) (see Figure 1). Additionally, the initial state for the inoculum was arbitrarily established, and it was assumed that it grew under favorable conditions. Therefore, the PHB concentration corresponds approximately to 15% of the inoculum concentration. The constraints on feed rates F_1 and F_2 and on their concentrations are

$$0 \le F_1, \quad F_2 \le 2 \tag{9}$$

$$0 \le S_{o_1} \le 700 \tag{10}$$

$$0 \le S_{o_2} \le 200 \tag{11}$$

These constraints are related to adequate values for an experimental application. Additionally, the values mentioned are not superior to the solubility of fructose and urea in water (3750 and 1080 g/L, respectively) at 30 °C (culture temperature). The volume is constrained by the size of the bioreactor (10 L):

$$V(t) \le 10 \tag{12}$$

Interior-point constraints are related to maximum fructose and urea concentrations at which complete inhibition occurs and to the maximum biomass concentration practically achievable in a bioreactor for *A. eutrophus*:[3]

$$0 \le S_1(t) \le 90.11 \tag{13}$$

$$0 \le S_2(t) \le 10.11 \tag{14}$$

$$0 \le X(t) \le 280 \tag{15}$$

The objective function selected to be maximized by choosing four control variables is the PHB total mass at the final time t_f:

$$\mathrm{OF} = P(t_f)\, V(t_f) \tag{16}$$

For the dynamic optimization of this problem gPROMS uses the control vector parametrization (CVP) approach.[27,28] The time horizon is divided into a number of elements, and control variables are approximated using a predefined basis function (piecewise-constant for F_1 and F_2, time-invariant for S_{o_1} and S_{o_2}). Parametrization transforms the original (infinite-dimensional) dynamic optimization problem into a nonlinear programming (NLP) problem where system dynamics should be integrated for each performance index evaluation (objective function denoted by eq 16). This approach is called the sequential direct strategy because the CVP approach has transformed the original problem into a master NLP with an inner initial value problem.[29]

4. Results and Discussion

All calculations were done on a Pentium IV computer, operating with a 3 GHz processor. In general terms, $3n$ algebraic and $5n$ differential equations were solved. A dynamic problem involving $n = 1$ time interval took 0.4 s, whereas dynamic problems with $n = 10$ and $n = 20$ took around 6.9 s. Additionally, the problem with $n = 1$ interval took 5 NLP iterations and 5 NLP line search steps, whereas the problems with $n = 10$ and $n = 20$ took 11 and 24 NLP iterations and 13 and 35 NLP line search steps, respectively.

PHB concentration maximization and the final culture time were optimized using the kinetic and bioreactor models together with the optimization process described in previous sections. For the dynamic optimization process, three different numbers of time intervals were established ($n = 1$, 10, and 20). To find the optimal fermentation time, an analysis of PHB productivity variation as a function of the final culture time was simultaneously developed. For this evaluation, a number of time intervals of 20 was established. As is shown in Figure 2, the final culture time that maximized PHB productivity was 49 h.

On the other hand, Figure 3 shows piecewise-constant optimal feed rates for control variables F_1 and F_2. Objective function values and optimal time-invariant concentrations S_{o_1} and S_{o_2} are presented in Table 2 for the different interval numbers selected. As can be seen in Table 2, the objective function is strongly improved when the optimization intervals are increased from 1 to 10 steps; in fact, the performance index is increased to about 100%. However, the use of a greater number of optimization stages does not enhance production significantly. A feed rate profile obtained by using a small number of intervals (e.g., the use of 10 steps instead of 20) may be viewed as a suboptimal result, although it may produce a performance index value very close to the absolute maximum. From a practical point of view, it will be more adequate to implement the culture at the experimental level with a limited number of partitions such as $n = 10$. The integral $\int_0^{49}(F_1 + F_2)\, dt$ (eq 7) is equal to 6 L for $n = 1$, 10, and 20; therefore, the final reactor volume resulting from the optimization reaches the upper constraint value defined for this variable (eq 10) for all cases.

Figures 4 and 5 show the main process variables and rates as a function of time for the optimal solution obtained using $n = 10$. The total and residual biomass, PHB, fructose, and urea concentrations are shown in Figure 4. The volume dynamic evolution, specific growth rate, and PHB percentage inside the cell are presented in Figure 5.

During the first 10 h, fructose and urea mass flows are almost negligible (Figure 3b). This behavior, which results from the optimization process, is related to the initial values selected for urea and fructose concentrations inside the bioreactor (Figure 1). As was already mentioned, these concentrations were selected because they maximize the specific growth rate. At the beginning of the bioreaction the biomass concentration is very low; therefore, the specific growth rate is at its maximum value without the addition of significant amounts of substrates. Figure 5b shows that the specific growth rate is maintained almost at its maximum

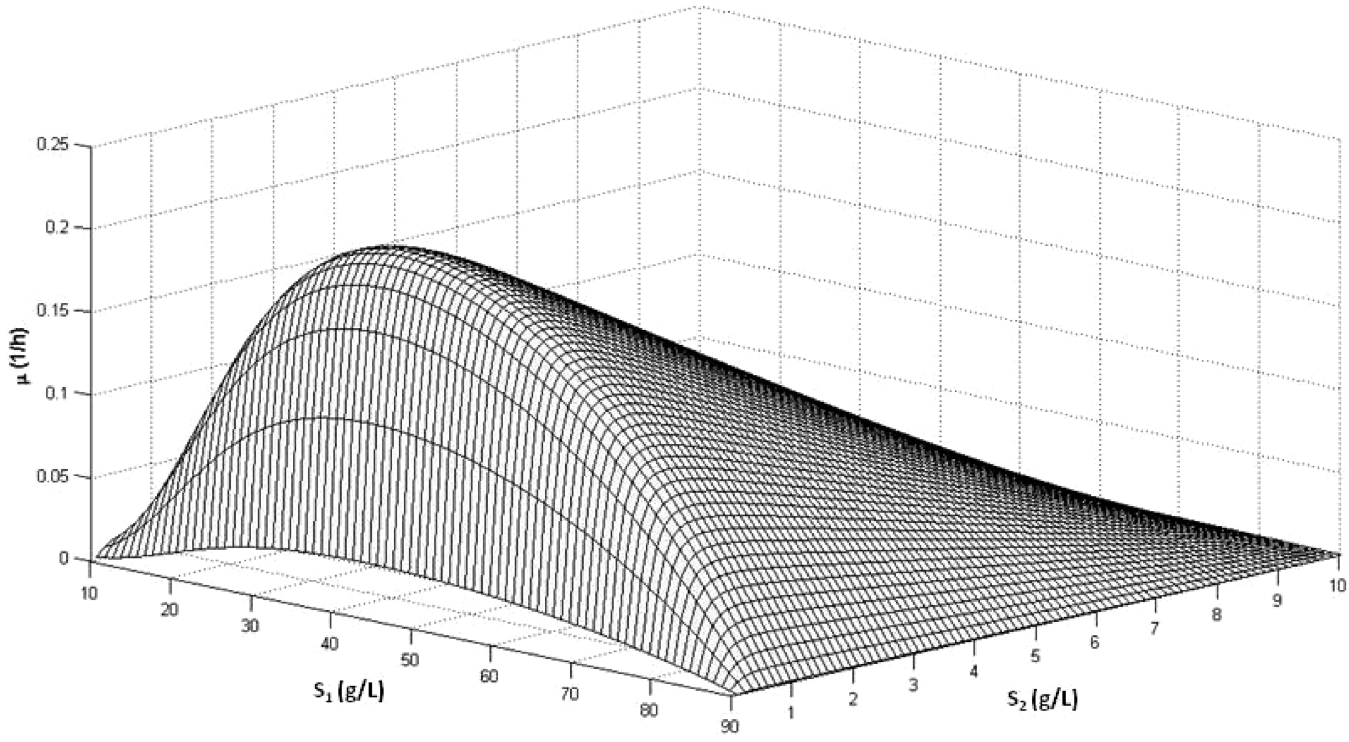

Figure 1. Specific growth rate (μ) as a function of the substrate concentration at the inoculum.

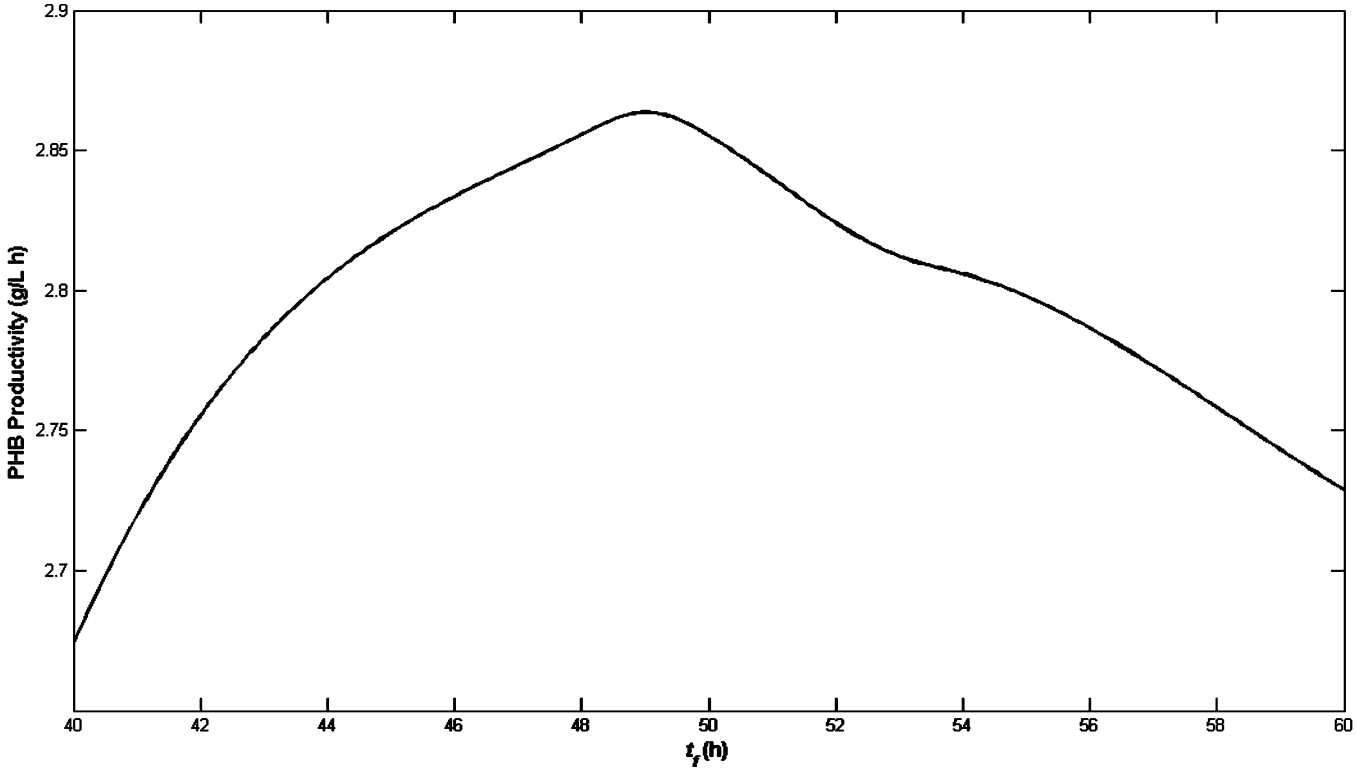

Figure 2. PHB productivity as a function of the fermentation final time.

value during the first 23 h. However, F_1 and F_2 should be increased to keep substrate concentrations at adequate levels to ensure a high cell production rate.

After approximately 25 h, the specific growth rate is forced to drop to zero (Figure 5b). The growth is limited by the nitrogen source, which is no longer fed to the bioreactor (Figures 3b and 4c). In the initial stage, the PHB production rate can be maximized by increasing the growth rate and/or the residual biomass concentration (eq 4). If there is no biomass, it is obvious that PHB production cannot occur; however, if there is enough concentration of bacteria in the medium, the question is for how long it would be necessary to maintain the growth rate at its maximum value. Since the kinetic constant related to PHB production in a stationary phase (K_2) quadruplicates the kinetic constant K_1, related to growth-associated PHB production, dynamic optimization suggests that it is an optimal strategy to

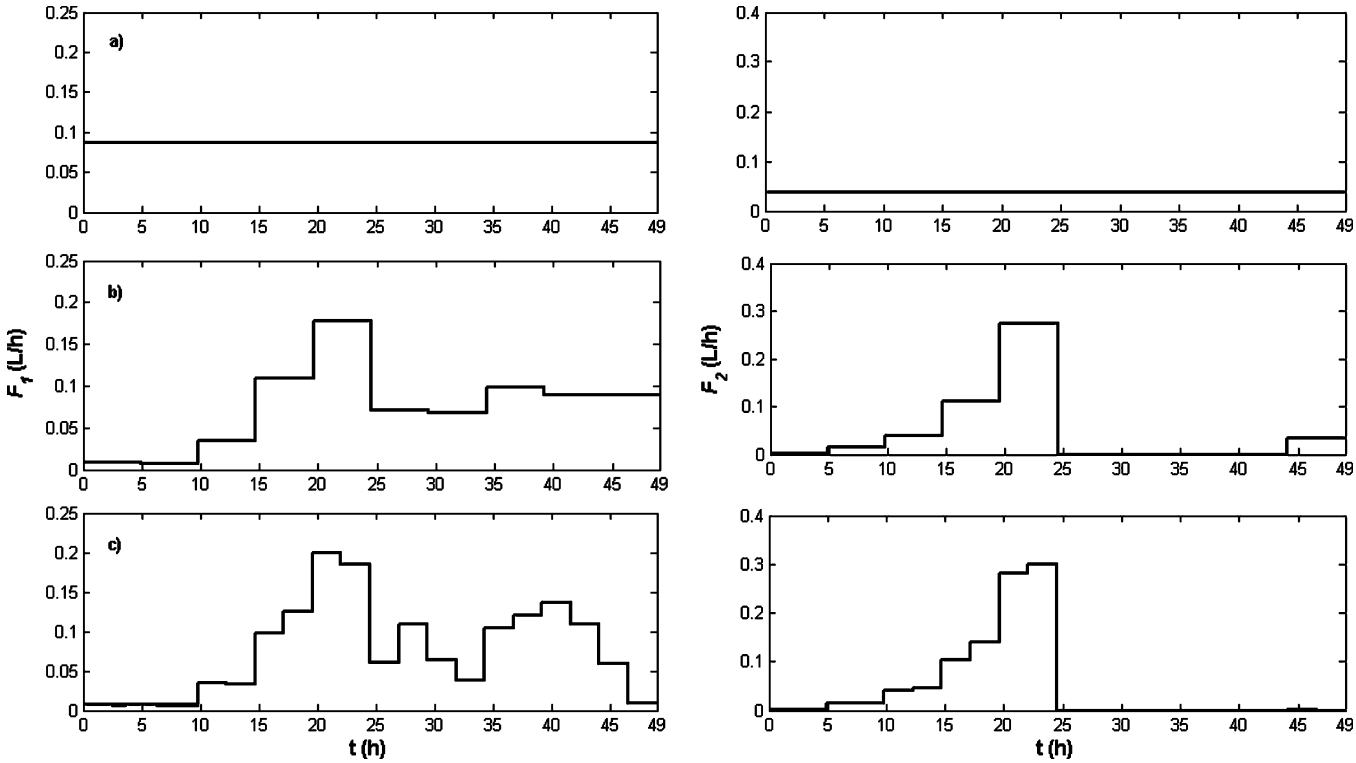

Figure 3. Piecewise-constant optimal feed rates as a function of the fermentation time. Figures on the left correspond to fructose (F_1) and figures on the right to urea (F_2). Number of optimization steps: (a) $n = 1$, (b) $n = 10$, and (c) $n = 20$.

Table 2. Results from the Dynamic Optimization for the Different Numbers of Stages Evaluated

no. of stages	objective function (performance index)	fructose concn, S_{o_1}, in F_1 (g/L)	urea concn, S_{o_2}, in F_2 (g/L)
1	698.60	223.8	32.7
10	1403.2	526.3	36.9
20	1405.8	529.8	34.4

produce sufficient residual biomass up to 25 h and, then, to allow PHB to increase by the non-growth-associated mechanism.

To produce biomass, both substrates are needed. However, once they are fed to the bioreactor, they dilute the reaction medium and, consequently, slow the biomass and PHB production rates (eqs 3 and 4). A balance among all the effects that occur simultaneously in the bioreactor has to be found to maximize the PHB final mass. To avoid a fast dilution of the bioreactor medium and to generate unbalanced conditions that maximize the PHB concentration, the urea mass flow is almost stopped after 25 h. This can be done because the nitrogen needed for cellular maintenance is extremely low ($m_{S_2} = 4.5 \times 10^{-6}$). Fructose concentration in the medium is required even in the stationary phase for two main reasons: cellular maintenance ($\gamma = 3.5 \times 10^{-2}$), which is 4 orders of magnitude higher than m_{S_2}, and inhibition of the TCA cycle and initiation of PHB formation cycle. Therefore, fructose has to be fed to the reactor during almost 90% of the process. PHB production under growth conditions is not an adequate operation mode since *A. eutrophus* can accumulate only 15 wt % PHB under these conditions.[30,31] Then, in this stationary stage, urea consumption is almost zero and all the fructose that enters into the cell is exclusively used for PHB formation. Any increase in the residual biomass production rate, as mentioned above, would enhance PHB production. This effect can be seen in Figure 4a; the increase in the total biomass is a consequence of PHB accumulation in the cytoplasm. The fructose concentration maintains almost constant values at the end of the bioreaction—during the last 15 h. Its concentration rate decreases at the same rate as it is consumed for both PHB production and cell maintenance. Then, during the last few hours, fructose feeding is selectively used for PHB accumulation.

Figure 4a indicates that a slight reduction in residual biomass concentration is observed after the stationary phase; such behavior is caused by the dilution effect.

As mentioned above, *A. eutrophus* can accumulate approximately 15 wt % PHB under balanced growth conditions. In spite of this, it is considered as a non-growth-associated PHB producer. The optimization study indicates that, during the growth phase, the PHB percentage remains almost constant at around 15% (Figure 5c). Due to the unfavorable conditions reached in the bioreactor after approximately 25 h, the percentage of PHB in the cells increases to 50%, which is a feasible value since it is known that *A. eutrophus* can accumulate PHB up to 90% of the dry cell weight.[31]

Optimization results are in agreement with the knowledge that PHB production is normally induced by limiting the cells with nitrogen availability in the presence of an excess of carbon source during the culture's stationary phase.[4,7] Consequently, the optimization strategy and the kinetics and kinetic parameters suggested by Khanna and Srivastava[11] are in agreement with the phases recognized to improve PHB production.

During the optimization procedure only the constraints defined by eqs 12 and 15 are active. Different optimizations were performed for different values of X at the final time. All the results obtained were quantitatively similar to those presented in this work.

The results obtained are similar to those reported by Kim et al.[14] In both works, approximately 50 h has been selected as the culture time. These authors reported a cell productivity of 3.28 g of cells/(h L), a PHB productivity of 2.42 g of PHB/(h L), and a concentration of accumulated PHB of 76 wt %, while

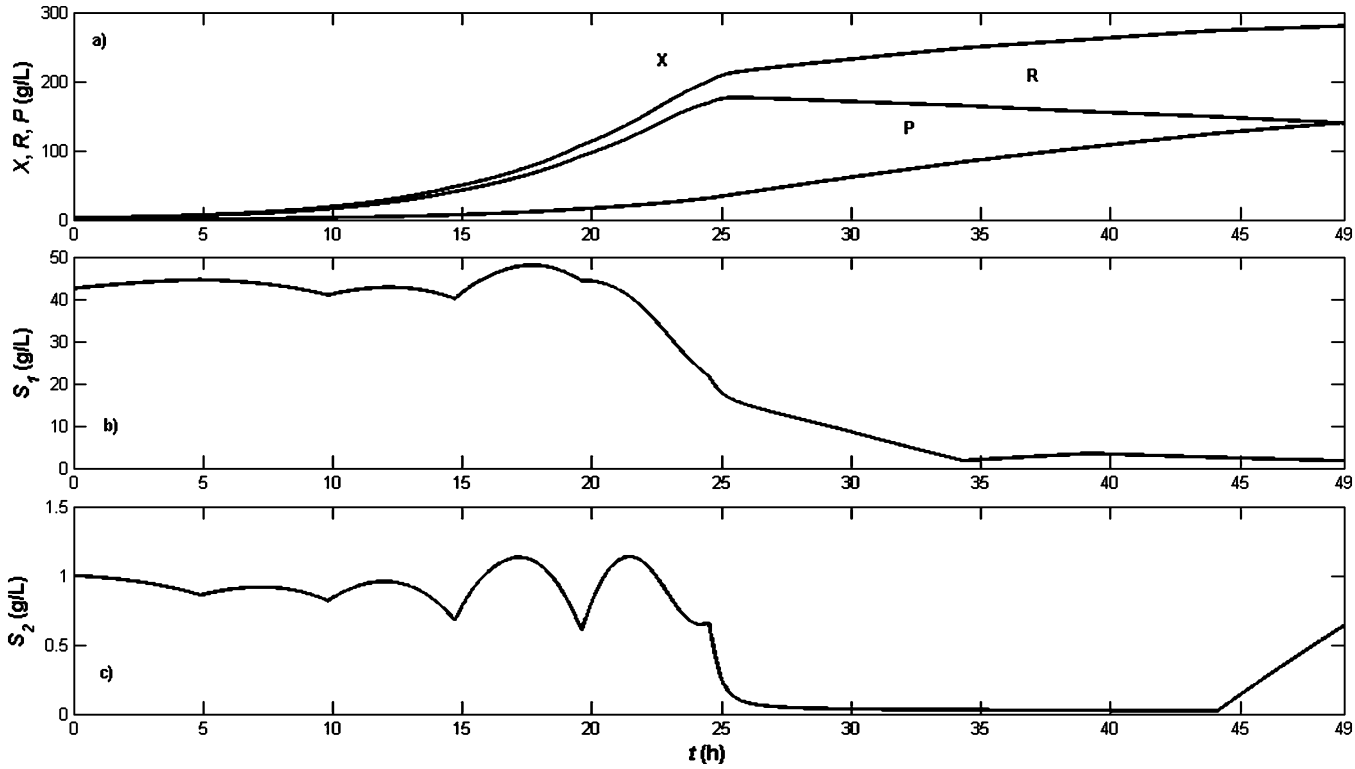

Figure 4. (a) Total (X) and residual (R) biomass and PHB (P) concentrations, (b) fructose concentration (S_1), and (c) urea concentration (S_2) as a function of the culture time. Optimal profiles were obtained by using 10 stages.

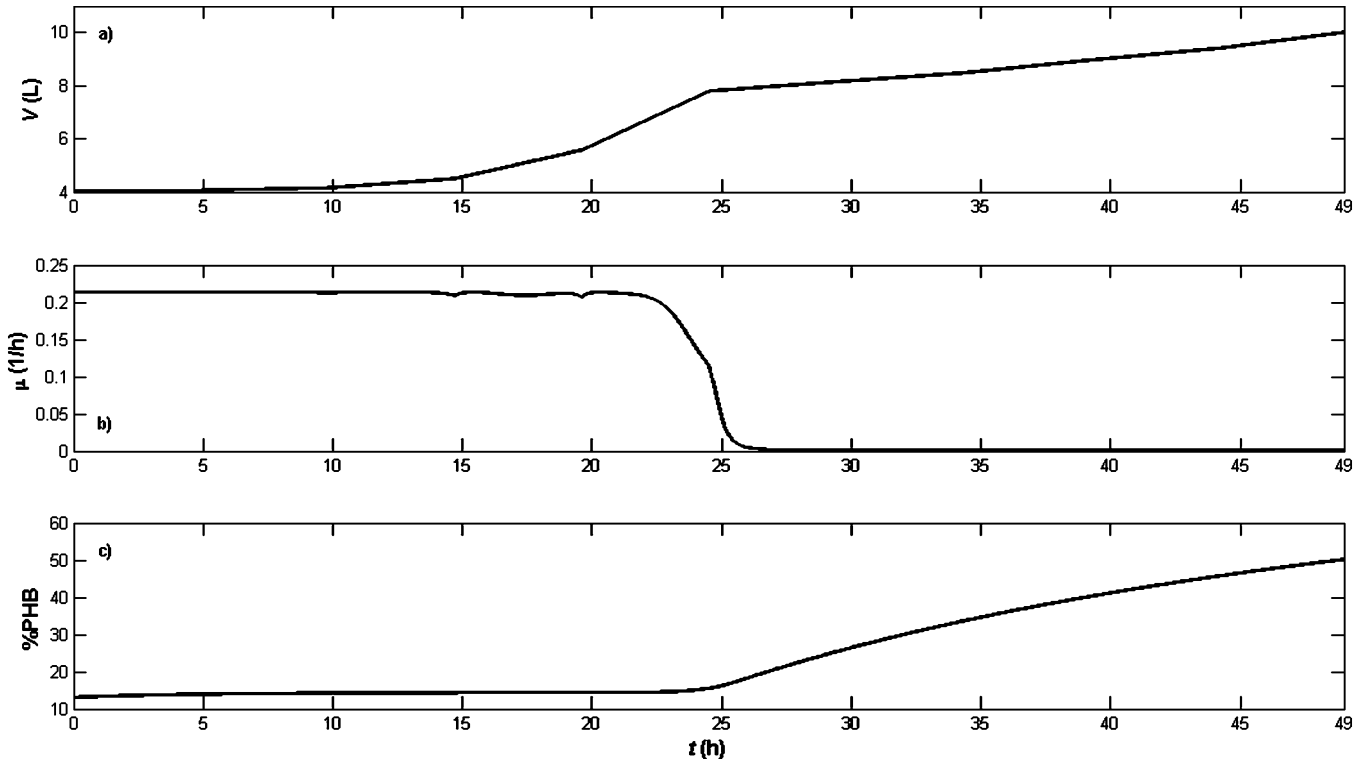

Figure 5. (a) Total volume (V), (b) specific growth rate (μ), and (c) percentage of PHB inside the cell as a function of the culture time. Optimal profiles were obtained by using 10 stages.

in this work the values mentioned are 5.71 g of cells/(h L), 2.87 g of PHB/(h L), and 51 wt %, respectively. Kim et al.[14] controlled the carbon source between 10 and 20 g/L for all the culture time, while in this work this value was controlled between 40 and 50 g/L and only in the cell growth stage. Kim et al.[14] also reported the two phases found in this work.

5. Conclusions

Optimal feed profiles for fructose and urea streams and optimal time-invariant inlet concentrations of the substrates were obtained using dynamic optimization applied to a fed-batch bioreactor for poly(β-hydroxybutyrate) production. For

an operation of 49 h, fructose and urea feed mass flows should be adapted at least every 4.9 h to obtain a maximum PHB production. The optimal feed profiles are completely feasible by using simple laboratory pumps and controllers; therefore, the proposed dynamic optimization can be seen as a useful tool to improve PHB generation.

In the theoretical study developed, it was confirmed that two different phases are present: cellular growth followed by PHB formation without residual biomass production for improving PHB yields.

At the beginning, knowing appropriate kinetic and bioreactor models and system constraints, the outcome of the dynamic optimization process becomes a predictive control tool which allows for a considerable reduction in time and experimentation costs. As the kinetics and kinetic parameters used in the optimization process become more accurate, the dynamic optimization can constitute an indispensable tool to define the proper operating conditions that ensure higher PHB levels.

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Nomenclature

a_1, a_2 = model coefficients in eq 1
F = volumetric flow (L/h)
k_s = saturation constant (g/L)
K_1 = growth-associated parameter (g of PHB/g of cells)
K_2 = non-growth-associated parameter (g of PHB/(g of cells h))
m_{S_2} = constant that involved urea consumption for cellular maintenance (g of urea/(g of cells L))
n = number of control intervals
n_1, n_2 = model coefficients in eq 1
OF = objective function or performance index
P = poly(β-hydroxybutyrate) concentration (g/L)
R = residual biomass concentration (g/L)
s = state variable vector
S = substrate concentration (g/L)
S_m = substrate concentration at which total inhibition occurs (g/L)
S_o = inlet substrate concentration in separated streams (g/L)
t = time (h)
V = volume (L)
X = total biomass concentration (g/L)
Y_{X/S_2} = yield of total biomass with respect to urea (g of cells/g of urea)

Greek Letters

α = constant related to cell growth (g of fructose/g of cells)
γ = constant related to cellular maintenance (g of fructose/(g of cells h))
μ = specific growth rate (h^{-1})

Subscripts

1 = fructose
2 = urea
f = final
m = maximum

Superscripts

T = transposed

Acronyms

CVP = control vector parametrization
DAEs = differential and algebraic equations
gPROMS = generalized process modeling system
NLP = nonlinear programming
PHA = poly(hydroxyalkanoate)
PHB = poly(β-hydroxybutyrate)
TCA = tricarboxylic acid

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