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DESIGN OF ESTIMATORS FOR SPECIFIC GROWTH RATE CONTROL IN A FED-BATCH *E. COLI* FERMENTATION

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Abstract. The specific growth rate is one of the most important process variables characterizing the state of microorganisms during fermentations mainly because the biosynthesis of many products of interest is often related with the values assumed by this parameter. In the particular case of the fed-batch operation of *Escherichia coli* for the production of recombinant proteins, it is important to maintain the specific growth rate below a certain threshold in order to avoid the accumulation of acetic acid throughout the fermentation and, additionally, it is often argued that both pre- and the post-induction specific growth rates should be closely controlled in order to achieve maximum productivities on the desired recombinant protein.
The main purpose of this work was to develop and implement reliable algorithms for the estimation of the specific growth rates in *E. coli* fermentation that can be used for the feedback control of this variable using a developed mathematical model of the process, together with common on-line measurements of dissolved and exhausted oxygen and carbon dioxide. For that purpose, a biomass asymptotic observer is derived for the on-line estimation of biomass. Using estimated biomass concentration values together with on-line process data, the determination of the specific growth rates is performed using observer-based estimators. Finally, a feedback-feedforward control algorithm was developed that uses estimated values of the specific growth rates and biomass concentration for the calculation of the feeding rate. The developed algorithms were validated by simulations where on-line variables used for the calculation of the estimators were corrupted with white noise.

1. Introduction

The specific growth rate is one of the most important process variables characterizing the state of microorganisms during fermentations mainly because the biosynthesis of many products of interest is often related with the values assumed by this variable. As opposed to batch fermentation, in fed-batch cultures it is possible to manipulate the specific growth rate at an appropriate value providing a desirable metabolic condition, resulting in maximum productivity.
Additionally, for certain types of measurements, of vital importance in the post-genomic era [4], like mRNA abundance or the analysis of the fluxome and proteome, microbial cultures have to be sampled at a pseudo steady-state condition that can be obtained by imposing a fixed specific growth rate, either in continuous or fed-batch cultures.
In the particular case of the fed-batch operation of *Escherichia coli* for the production of recombinant proteins, it is important to maintain the specific growth rate below a certain threshold in order to avoid the accumulation of acetic acid throughout the fermentation [7] and, additionally, it is often argued that both pre- and the post-induction specific growth rates should be closely controlled in order to achieve maximum productivities on the desired recombinant protein [5,22,11].
In order to keep the specific growth rate at a pre-determined value, the most common approach is to apply a feed-forward exponential feeding strategy, where the nutrients required by the culture for achieving the desired growth rate are pre-determined and satisfied at any moment [15,25]. However, the inherent features of a feed-forward method limit the application of this feeding scheme, due to the likely occurrence of external perturbations or variations on culture parameter.
Therefore, the development of reliable algorithms for the feedback automatic control of the specific growth rate in fed-batch systems is of paramount importance in fermentation technology. However, the performance of such algorithms is critically dependent on a reliable determination of the specific growth rate, which cannot be

obtained directly from common fermentation measurements mainly due to the lack of reliable sensors for the determination of biomass concentration. A combination of a reliable model of the process and on-line data is therefore often necessary for the estimation of both biomass concentration and specific growth rate.
Some algorithms used for on-line estimation of reaction rates using biomass concentration or other correlated variables measurements have been proposed. [18,19] proposed and validated experimentally an on-line estimation algorithm for multiple reaction rates. This procedure was applied to baker's yeast fermentation, and the algorithm required the on-line measurement of two or three state variables. Also, in [13] the author describes a methodology for the design of a new parameter estimator of biomass growth rate and yield coefficient for oxygen consumption on the basis of the theory of adaptive estimation, for a class of aerobic bioprocesses in fed-batch or continuous mode. In [12,14], the authors proposed an approach for on-line growth rate estimation for a class of aerobic batch processes with dissolved oxygen control in the culture medium. The only required on-line measurement is the oxygen consumption rate. An adaptive model-based algorithm for the on-line estimation of reaction rates is described by [16], considering the yield coefficients invariable and known, based on the approach of [3] to stirred tank reactors.
Regarding the development of control algorithms for the specific growth rate, there are very few examples where the closed loop control has been applied to *E. coli* or to other complex bioprocesses. In [9,10] the authors have developed a strategy for automatic control of the specific growth rate in fed-batch cultivation that requires the on-line monitoring of OUR or CER and feed rate and volume of the fermentation broth. This strategy was applied to fed-batch fermentation of *E. coli*.

The main purpose of this work was to develop reliable algorithms for the estimation of the specific growth rates in *E. coli* fermentation that can be used for the feedback control of this parameter using a developed mathematical model of the process, together with common on-line measurements of both dissolved and exhausted oxygen and carbon dioxide. For that purpose, a biomass asymptotic observer is first derived for the on-line estimation of biomass. In a second stage, on-line process and observed data are used by estimators for the determination of the specific growth rates that can then be used for feedback control applications.

2. Mathematical Model of the Process

The development of the mathematical model for the fed-batch fermentation of recombinant *E. coli* was based on the assumption that the aerobic growth of the microorganism can follow three main different metabolic pathways [20]:

- oxidative growth on glucose, with a net reaction as follows:

$$S + O \xrightarrow{\mu_1} X + C \qquad \text{Equation 1}$$

where *S* states for glucose, *O* is the dissolved oxygen, *X* is the biomass and *C* means dissolved carbon dioxide.

- fermentative growth on glucose, where the global reaction can be described like:

$$S + O \xrightarrow{\mu_2} X + C + A \qquad \text{Equation 2}$$

where *A* means acetate.

- oxidative growth on acetate:

$$A + O \xrightarrow{\mu_3} X + C \qquad \text{Equation 3}$$

In the sequel the symbols *S*, *O*, *X*, *C*, and *A* mean concentrations.

The dynamics of a reaction network in a stirred tank bioreactor can be described by the following mass balance equations written in matrix form as [3]:

$$\frac{d\xi}{dt} = Yr(\xi, t) - D\xi + F - Q \qquad \text{Equation 4}$$

in which ξ is a vector representing the n state components concentrations ($\xi \in \Re^n$), r is the growth rate vector corresponding to m reactions ($r \in \Re^m$), Y is the matrix of yield coefficients ($Y \in \Re^{n \times m}$), F is the vector of feed rates and Q is the vector of gaseous outflow rates ($F, Q \in \Re^n$), and D is the dilution rate.

The associated dynamical model for *E. coli* fermentation can be represented as follows:

$$\frac{d}{dt}\begin{bmatrix} X \\ S \\ A \\ O \\ C \end{bmatrix} = \begin{bmatrix} 1 & 1 & 1 \\ -y_1 & -y_2 & 0 \\ 0 & y_3 & -y_4 \\ -y_5 & -y_6 & -y_7 \\ y_8 & y_9 & y_{10} \end{bmatrix} \begin{bmatrix} \mu_1 \\ \mu_2 \\ \mu_3 \end{bmatrix} X - D \begin{bmatrix} X \\ S \\ A \\ O \\ C \end{bmatrix} + \begin{bmatrix} 0 \\ \frac{F_{in}}{W} S_{in} \\ 0 \\ OTR \\ -CTR \end{bmatrix} \qquad \text{Equation 5}$$

where μ_1, μ_2, and μ_3 are the specific growth rates; y_i are the yield (stoichiometric) coefficients; F_{in} and S_{in} are the substrate feed rate and the influent glucose concentration, respectively; W is the culture medium weight. CTR is the carbon dioxide transfer rate from liquid to gas phase, and OTR is the oxygen transfer rate from gas to liquid phase.

An additional equation was added to the model in order to account for weight variations. In small-scale and high-cell density reactors, the amount of culture removed or added during sampling, base and acid additions, evaporation and mass taken from the reactor due to gas exchanges can not be considered negligible [6]. Thus, the following equation for calculating weight variations was formulated:

$$\frac{dW}{dt} = F_{in,tot} = F_{in,S} + F_b + F_a - F_{evp} - F_{gas} - F_{smp} \qquad \text{Equation 6}$$

where F_b and F_a are the liquid mass flow of base and acid solution added to the bioreactor, and F_{evp}, F_{gas}, and F_{smp} are liquid mass flows evaporated from the bioreactor, and taken due to gas exchanges and to sampling, respectively.
For the kinetic model, the specific uptake rate of glucose (q_S) was found to be described by a Mond-type equation with non-competitive inhibition by acetate:

$$q_S = q_{S,\max} \frac{S}{S + K_S} \frac{K_{i,S}}{K_{i,S} + A} \qquad \text{Equation 7}$$

where $q_{S,max}$ is the maximum specific uptake rate, K_S is the Monod constant and $K_{i,S}$ represents the inhibition constant for acetate on glucose uptake.
The oxidative bottleneck exhibited by these microorganisms is accounted for by calculating an oxygen uptake rate, $q_{OS}=q_S \times k_{OS}$, where k_{OS} is the oxygen yield on glucose. The oxidative pathway for glucose is then the sole metabolic pathway while:

$$q_{OS} \le q_{O,\max} \frac{K_{i,O}}{A + K_{i,O}} \qquad \text{Equation 8}$$

where $q_{O,max}$ is the maximum oxygen uptake rate, and $K_{i,O}$ represents the inhibition constant for acetate on oxygen uptake.
After that threshold is reached, the microorganism will also follow the fermentative pathway. However, when the oxidative bottleneck is not fulfilled, and if acetate is present in the medium, it can be consumed, and the specific uptake rate of acetate (q_{AC}) under those circumstances can be described in a similar way as glucose uptake.
A full description of the mathematical model used for the fed-batch growth of *E. coli* can be found in [20].

As a consequence of the characteristics of the oxidative bottleneck, the three metabolic pathways represented in the mathematical model do not occur simultaneously in the cell, originating four partial models corresponding to different metabolic regimens:
- simultaneous oxidative and fermentative growth on glucose (μ_1, μ_2>0)
- oxidative growth on glucose (μ_1>0)
- oxidative growth on acetate and glucose simultaneously (μ_1, μ_3 >0)
- oxidative growth on acetate (μ_3>0)

3. Biomass Observer and Specific Growth Rate Estimators

Due to the inexistence of reliable sensors for the determination of biomass concentration for *E. coli*, the estimation of the specific growth rate necessarily begins with the observation of that state variable from available on-line data. Although in the authors' lab there exists the possibility of measuring on-line all the remaining state variables from equation 5 [20], for the sake of more general applicability, the algorithms described in this paper assume the availability of on-line data for dissolved oxygen and carbon dioxide, *CTR*, *OTR* and culture weight. Additionally, both the biomass observer and the specific growth rates estimators assume that the yield coefficient matrix *Y* is known.
Therefore, using the information available on-line and according to [24] it is possible to obtain biomass estimation using either Extended Kalman Observers (EKO) or Asymptotic Observers (AO) only if a partial model is considered. Taking into account that acetate consumption is very often negligible in these processes, the "simultaneous oxidative and fermentative growth on glucose" and the "oxidative growth on glucose" regimens are the only ones considered. Additionally, and although the performance of the EKO was shown to be superior for this process, the AO was selected for biomass estimation, as it requires no knowledge on the kinetic structure of the model and also due to the inexistence of tuning parameters.

The AO allows reconstructing the missing state variables even when the process is not exponentially observable and the kinetics are unknown [3]. Biomass estimation requires the integration of one differential equation associated with the auxiliary variable Z_1 where its dynamics is independent of growth rate vector $r(\xi,t)$:

$$\frac{d\hat{Z}_1}{dt} = -D\hat{Z} - \alpha_1 OTR - \alpha_2 CTR \qquad \text{Equation 9}$$

The variables α_i are a function of the yield coefficients matrix as follows:

$$\begin{bmatrix}\alpha_1 & \alpha_2\end{bmatrix} = \frac{1}{y_5 y_9 - y_6 y_8}\begin{bmatrix}-y_9 & -y_6\end{bmatrix} \qquad \text{Equation 10}$$

The Biomass estimate is then given by

$$\hat{X} = \hat{Z}_1 + \alpha_1 O + \alpha_2 C \qquad \text{Equation 11}$$

The design of specific growth rate estimators is based on the formulation proposed by [3] reformulated by [18] so that a decoupling of the dynamic model (equation 4) from the growth rate is achieved. The general equation for the estimator can be written as follows:

$$\frac{d\hat{\psi}}{dt} = \hat{\mu}\hat{X} - D\hat{\psi} + Y_a^{-1}(F_a - Q_a) - \Omega_1(\psi - \hat{\psi}) \qquad \text{Equation 12}$$
$$\frac{d\hat{\mu}}{dt} = \Omega_2(\psi - \hat{\psi})$$

ψ is obtained from the transformation $\psi = Y_a^{-1}\xi_a$ where ξ_a is the partition of the vector ξ that includes the measured state variables, and (Y_a, F_a, Q_a) the corresponding parts of (Y, Q, F):

$$\begin{bmatrix}\psi_1\\ \psi_2\end{bmatrix}=\begin{bmatrix}\alpha_1 & \alpha_2\\ \alpha_3 & \alpha_4\end{bmatrix}\begin{bmatrix}O\\ C\end{bmatrix} \qquad \text{Equation 13}$$

With:

$$\begin{bmatrix}\alpha_3 & \alpha_4\end{bmatrix}=\frac{1}{y_5y_9-y_6y_8}\begin{bmatrix}y_8 & y_5\end{bmatrix} \qquad \text{Equation 14}$$

Estimation of the specific growth rates corresponding to equations 1 and 2 will then come:

$$\begin{aligned}\frac{d\hat{\psi}_1}{dt}&=\hat{\mu}_1\hat{X}-D\hat{\psi}_1+\frac{1}{y_5y_9-y_6y_8}(y_9OTR+y_6CTR)+\omega_{11}(\psi_1-\hat{\psi}_1)\\ \frac{d\hat{\psi}_2}{dt}&=\hat{\mu}_2\hat{X}-D\hat{\psi}_2+\frac{1}{y_5y_9-y_6y_8}(y_8OTR-y_5CTR)+\omega_{12}(\psi_2-\hat{\psi}_2)\\ \frac{d\hat{\mu}_1}{dt}&=\omega_{21}(\psi_1-\hat{\psi}_1)\\ \frac{d\hat{\mu}_2}{dt}&=\omega_{22}(\psi_2-\hat{\psi}_2)\end{aligned} \qquad \text{Equation 15}$$

The calculation of the gains ω_{ij} for each instant *i* is made such that a second order dynamics [17] is obtained:

$$\begin{aligned}\omega_{11,i}&=2\zeta_1/\tau_1-\frac{X_i-X_{i-1}}{TX_i}\\ \omega_{12,i}&=2\zeta_2/\tau_2-\frac{X_i-X_{i-1}}{TX_i}\\ \omega_{21,i}&=(X_i\tau_1^{\,2})^{-1}\\ \omega_{22,i}&=(X_i\tau_2^{\,2})^{-1}\end{aligned} \qquad \text{Equation 16}$$

where *T* is the integration step. Therefore, the implementation of these algorithms requires the tuning of 4 parameters (ζ_1, τ_1, ζ_2, and τ_2).

4. Control Algorithm

Due to the growth of the microorganisms time-varying characteristics of the cultivation process occur and have to be taken into consideration if a controller is implemented. Using a feedforward–feedback controller these time-varying characteristics have to be kept in view in the feedforward contribution.

The deduction of the feed-forward component of the control equation is obtained from the mass balance for the substrate *S* of equation 5, when only the oxidative growth on glucose pathway is active:

$$\frac{dS}{dt}=-y_1\mu_1(t)X(t)-\frac{F(t)}{W(t)}S(t)+\frac{F_{in}(t)}{W(t)}S_{in} \qquad \text{Equation 17}$$

In the fed-batch phase the cultivation is operated under glucose limitation. Therefore, it can be assumed that *S=0* and *dS/dt=0* and the rearranging equation 17 gives:

$$F_{in} = \frac{y_1 \mu_{set}(t) X(t) W(t)}{S_{in}}$$ Equation 18

where μ_{set} is the desired growth rate that has necessarily to be smaller that the maximum value of μ_1.
The application of this equation usually implies that both biomass and culture weight are available. The most common approach is to estimate them based on the initial values of the feeding and assuming a pure exponential growth. However, external perturbations together with natural weight variations caused for example by evaporation are an important source of errors. Also, the application of this methodology may not adequately control bioprocesses because model parameters, such as the yield coefficients, must be given to compute the feeding rate and these values may change as the cultivation proceeds.
Therefore, the exponential feeding strategy is sometimes compensated for by incorporating some appropriate feedback control action. In [8], the authors developed a simple method to adjust feeding rate based on the calculation of the actual specific growth rate, and correcting the feed rate using a proportional action. A similar approach was proposed by [1]. In [2], a Proportional Integral action is used in a similar way.
The control equation for the proportional action obtained is very similar to the optimal adaptive control approach described by [23]:

$$F_{in} = \frac{y_1 \mu_{set} \hat{X}(t) W(t)}{S_{in}} - \tau_\mu \frac{\hat{\mu}(t) - \mu_{set}}{S_{in}} W(t)$$ Equation 19

For the application of this control law, y_1 and S_{in} are considered known and invariant, while W(t) is obtained on-line using a balance under the bioreactor. Estimates of $X(t)$ and $\mu(t)$ are obtained using equations 11 and 15, respectively.

5. Methods

The model simulations were performed by integrating equations 5 using the MATLAB version 7.1 subroutine ODE23s. The implementation of the observer and the estimators was conducted using the Euler integration method. Most of the mathematical operations behind the design of the observer and the estimators were performed using the Symbolic Math toolbox running in MATLAB 7.1.
For validating the developed algorithms “real” values of the state variables were obtained by integration of equation 5. The “real” values of the variables that can be obtained on-line, i. e., O, C, OTR, CTR and W were then corrupted with white noise, according to the standard deviations typically found in this process at the authors’ lab, originating “experimental” values. Then, the observer and estimator algorithms were used to obtain the “estimated” variables from the “experimental” data corresponding to the measured variables.

6. Results and Discussion

In a first stage, the developed algorithms for the observation of biomass and for the estimation of the specific growth rates were validated using the methodology described in the previous section. For that validation, several feeding profiles were used and it was verified that the approximation between simulated and “estimated” data was always satisfactory. This validation was performed after a trial-and-error approach for the determination of the best tuning parameters that were kept constant for all the simulations performed. An example of such experiments is shown in figure 1, where the results obtained using a constant feeding profile are presented.

After validation and tuning of the estimators, the control law from equation 19 was applied using the same methodology described in the previous section. Again, a trial and error tuning procedure for the definition of the parameter τ_μ was conducted, and the effects of the variation of that factor on the performance of the controller are shown in figure 2. It can be seen that an increase in the proportional factor of the controller increases the overshoot effect of the controller in the first hours of the fermentation.

Finally, the robustness of the controller to variations on model parameters and on the substrate concentration in the feeding solution was evaluated. It was found that after an appropriate selection of the tuning parameter, the controller has the capability to compensate for those deviations, keeping the controlled variable close to its

setpoint. Not surprisingly, the open-loop version of the controller, although maintaining the specific growth rate constant along the fermentation, is not able to compensate for these perturbations. Figure 3 shows an example of this behaviour for a change in the substrate concentration in the feeding solution.

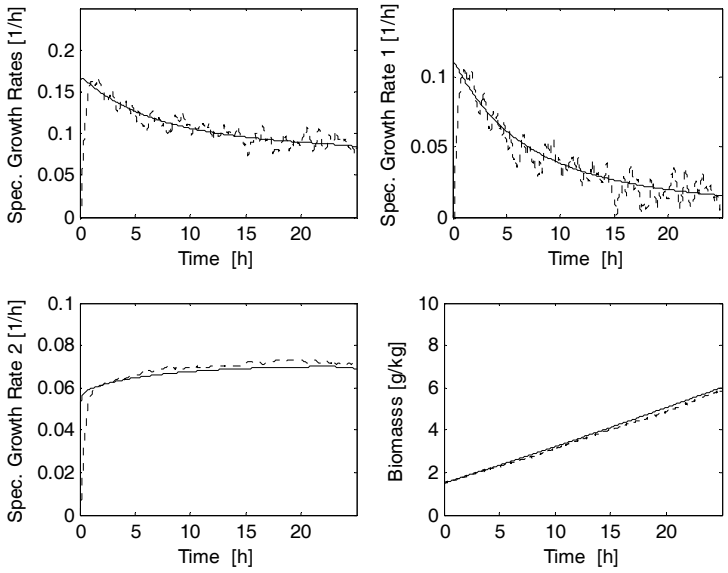

Figure 1 Validation of the observer and estimator algorithms using a constant feeding profile. Dotted lines correspond to "estimated" values while full lines are the simulated data. The first chart corresponds to the total specific growth rate values, i. e., the sum of μ_1 and μ_2.

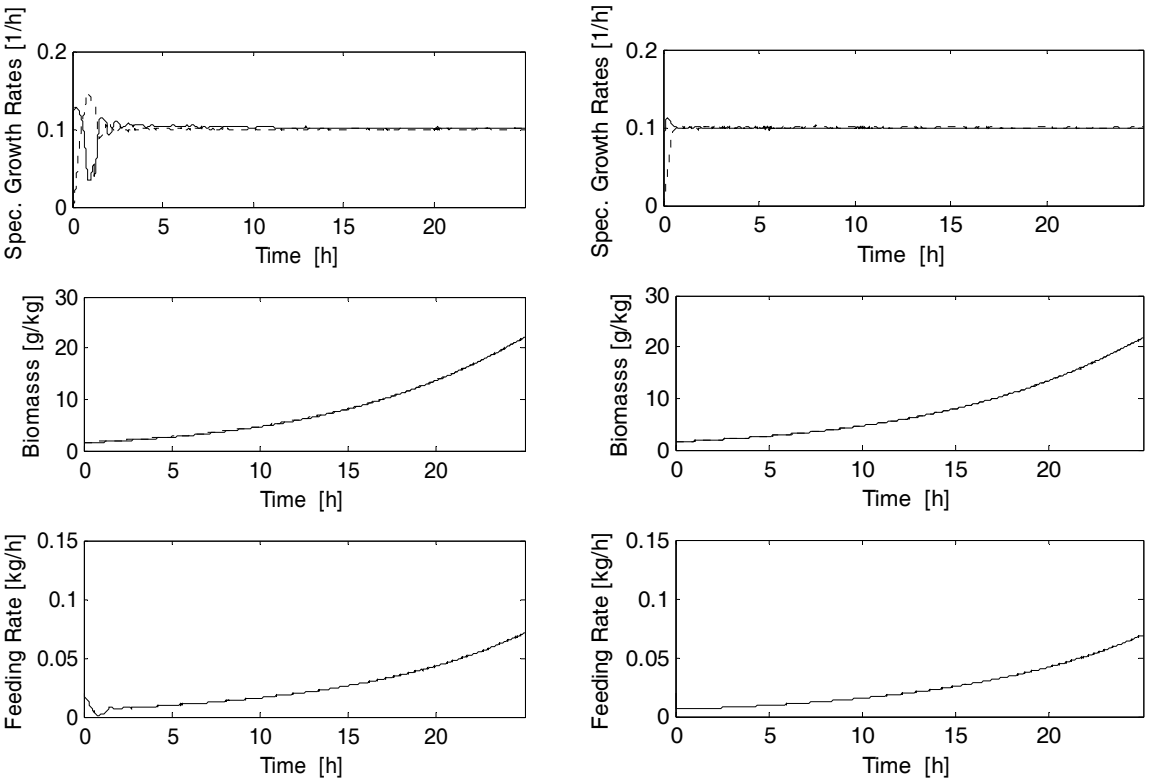

Figure 2 Effects of the variation of the tuning parameter τ_μ on the controller's performance for a setpoint of μ=1 h^{-1}. The charts on the right correspond to τ_μ=*1*, and charts on the left were obtained with τ_μ=*10*. Dotted lines correspond to "estimated" values while full lines are the simulated data.

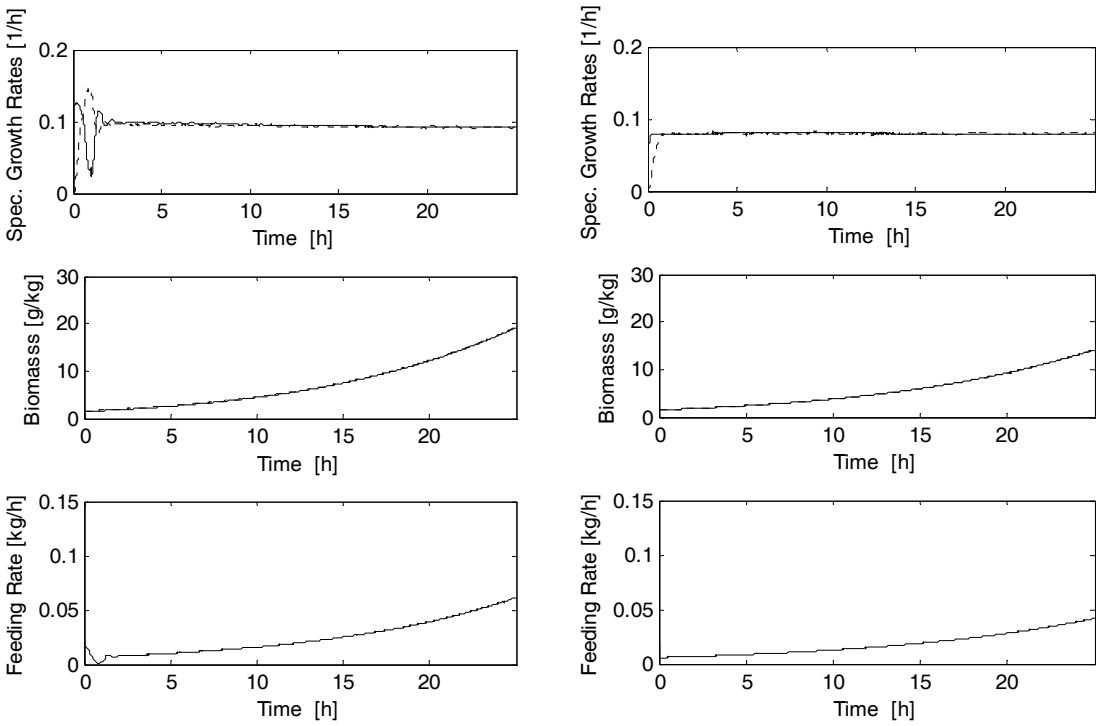

Figure 3 Effects of a 25% variation on the substrate concentration on the feeding solution on the controller's performance. The charts on the left correspond to the results obtained with the open-loop version of the controller, while charts on the right were obtained with the feedback-feedforward controller with $\tau_\mu=10$. Dotted lines correspond to "estimated" values while full lines are the simulated data.

7. Conclusions and Future Work

With the developed algorithms, it was possible to estimate with great accuracy the specific growth rates and biomass concentration during a recombinant *E. coli* fermentation using on-line data from dissolved oxygen and carbon dioxide, oxygen and carbon dioxide transfer rates and culture weights. The estimated data were then used for the implementation of a feedback-feedforward controller that is able to keep the specific growth rate at the desired setpoint even in the presence of perturbations both in the model parameters and in the concentration of substrate in the feeding.

Future work involves the implementation of a more rational tuning procedure, including the optimization of tuning parameters by minimizing the differences between the desired setpoint and the real specific growth rates and also the experimental validation of the algorithms.

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