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Modeling and Simulation of Cephalosporin C Production in a Fed-Batch Tower-Type Bioreactor

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Abstract

Immobilized cell utilization in tower-type bioreactor is one of the main alternatives being studied to improve the industrial bioprocess. Other alternatives for the production of β-lactam antibiotics, such as a cephalosporin C fed-batch process in an aerated stirred-tank bioreactor with free cells of *Cephalosporium acremonium*, or a tower-type bioreactor with immobilized cells of this fungus, have proven to be more efficient than the batch process. In the fed-batch process, it is possible to minimize the catabolite repression exerted by the rapidly utilization of carbon sources (such as glucose) in the synthesis of antibiotics by utilizing a suitable flow rate of supplementary medium. In this study, several runs for cephalosporin C production, each lasting 200 h, were conducted in a fed-batch tower-type bioreactor using different hydrolyzed sucrose concentrations. For this study's model, modifications were introduced to take into account the influence of supplementary medium flow rate. The balance equations considered the effect of oxygen limitation inside the bioparticles. In the Monod-type rate equations, cell concentrations, substrate concentrations, and dissolved oxygen were included as reactants affecting the bioreaction rate. The set of differential equations was solved by the numerical method, and the values of the parameters were estimated by the classic nonlinear regression method following Marquardt's procedure with a 95% confidence interval. The simulation results showed that the proposed model fit well with the experimental data, and based on the

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experimental data and the mathematical model, an optimal mass flow rate to maximize the bioprocess productivity could be proposed.

Index Entries: Cephalosporin C; tower-type bioreactor; fed-batch; modeling; simulation.

Introduction

The β-lactam family of antibiotics is the most important group among pharmaceutical products and makes up the largest part of the world's multibillion-dollars antibiotic market. Approximately 60% of the total worldwide production of antibiotics belongs to the β-lactam type *(1)*. Cephalosporin C is one of the most important antibiotics in this group. Its molecule is synthesized and produced as a secondary metabolite by strains of a strictly aerobic filamentous fungus, *Cephalosporium acremonium*. The cephalosporin C in its natural form has a relatively low antibiotic activity. However, its molecule can be modified through chemical or enzymatic methods to produce different semisynthetic cephalosporins, which have clinical use and a high market worth and are important to pharmaceutical industries *(2)*.

Industrial production of cephalosporin C is still carried out via conventional batch fermentation in aerated stirred-tank bioreactors utilizing submerged cultures of *C. acremonium*. The unfortunate feature of this fungus growth is the high viscosity of the culture growth, which results in an increase in energy costs in order to keep the dissolved oxygen concentration above the critical level. Tower-type bioreactor without mechanical agitation have appeared as an alternative process and are improving the production of cephalosporin C and reducing these costs *(3)*. Furthermore, there is a trend to use immobilized cells in beads of inert and biocompatible materials such as natural pellets in this type of bioreactor. Despite the mass transfer limitation, the whole-cell immobilization has the advantages of minimizing the broth viscosity and allowing greater cell longevity in continuous and semicontinuous processes such as the fed-batch process *(4)*.

The fed-batch process with a continuous or intermittent nutrient feed rate has been used to avoid high concentrations of some substrates that are inhibitors or cause undesirable precipitation. It is also used to regulate the metabolism of the microorganism, thus improving product formation. Regulatory mechanisms for cephalosporin C production in this process include catabolite repression of the β-synthetases exerted by rapidly utilized carbon sources such as glucose. These carbon sources are essential for cell growth but are prejudicial for antibiotic production *(5)*. The fed-batch process to produce cephalosporin C has been shown to be more efficient than the batch process that minimizes the repressive effects exerted by glucose and utilizes a suitable flow rate of supplementary medium.

Regarding modeling and simulating cephalosporin C process, some important studies should be mentioned. Matsumura et al. *(6)* developed a kinetic model of cephalosporin C production. The proposed model was based on morphologic differentiation of the fungus *C. acremonium*, on the

fact that methionine strongly stimulated cephalosporin C production, and on the catabolite repression exerted by glucose. The model proposed by Chu and Constantinides *(7)* for cephalosporin C production considered that antibiotic formation should be directly associated with the production of enzymes that affect the synthesis of this compound. Araujo et al. *(8)* proposed a kinetic model for cephalosporin C production with free and immobilized cells based on stoichiometric equations that represent the main phases of cell variation, substrate, and product concentrations. Araujo et al. *(9)* studied the effect of oxygen transfer on the effectiveness factor of the process rates of cephalosporin C production with immobilized cells. The study concluded that although oxygen limits the production rate, it is only slightly lower than the free-cell system. Cruz et al. *(10)* elaborated a mathematical model in fed-batch production of cephalosporin C that uses the main features of all the aforementioned models.

In general, the mathematical models used in bioprocesses are formed by a set of nonlinear differential equations with several unknown parameters. These parameters should be estimated using experimental results. Marquardt *(11)* developed the classic algorithm to estimate parameters by the nonlinear least-squares method. Nihtilä and Virkkunen *(12)*, using Marquardt's method, developed parameters for models that described the growth of and glucose consumption by *Trichoderma viride*. They observed difficulties in obtaining acceptable parametric values owing to the large number of parameters to be estimated in relation to the amount of experimental data. Matsumura et al. *(6)* estimated the kinetic parameters of a cephalosporin C production model. First, they estimated growth phase parameters and then the production phase parameters. Zangirolami et al. *(13)* formulated a structural model for fed-batch penicillin production. In each parametric estimate, the experimental data of the cell, penicillin, and substrate concentration were compared with the set of data obtained in the simulation.

In the present study, the model proposed by Araujo et al. *(8)* was modified to simulate cephalosporin C production in a fed-batch tower-type bioreactor with immobilized cells of *C. acremonium* and took into account the influence of supplementary medium flow rate. The balance equations described the reactions involved in this process, taking into consideration the effect of oxygen limitation inside the bioparticles. In the Monod-type rate equations, in addition to cell and substrate concentrations, dissolved oxygen was included as a reactant affecting the bioreaction rate. The set of differential equations was solved by the numerical method and the values of the parameters were estimated by the classic nonlinear regression method following Marquardt's procedure *(11)* with a 95% confidence interval. The simulation results show that the proposed model fit the experimental data, and based on the experimental data and the mathematical model, an optimal mass flow rate maximizing the bioprocess productivity could be proposed.

Table 1
Hydrolyzed Sucrose Concentrations

Run	Hydrolyzed sucrose (g/L)
4	115.2
5	86.4
6	144.0
7	107.5

Materials and Methods

Microorganism

C. acremonium ATCC 48272 (C-10) was used throughout this study.

Culture Media

Inoculum Preparation

A synthetic medium containing the following components was used: glucose (30.0 g/L); ammonium acetate (8.8 g/L); DL-methionine (5.0 g/L); oleic acid (1.5 g/L); $CaCO_3$ (2.0 g/L); KH_2PO_4 (2.0 g/L); and traces of inorganic salts $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.16 g/L), Na_2SO_4 (0.81 g/L), $MgSO_4 \cdot 7H_2O$ (0.384 g/L), $CaCl_2 \cdot 2H_2O$ (0.08 g/L), $MnSO_4 \cdot H_2O$ (0.032 g/L), $ZnSO_4 \cdot 7H_2O$ (0.032 g/L), $CuSO_4 \cdot 5H_2O$ (0.002 g/L). The pH was adjusted to 7.0 ± 0.1.

Main Fermentation

A synthetic medium defined by Demain et al. *(14)* and modified by Araujo et al. *(8)* containing the following components was used: glucose (27.0 g/L), DL-methionine (3.0 g/L), KH_2PO_4 (1.5 g/L), $CaCl_2$ (1.0 g/L), and other components used in the inoculum preparation. The pH was adjusted to 7.0 ± 0.1.

Supplementary Medium

Following glucose exhaustion, a supplementary medium containing hydrolyzed sucrose (glucose + fructose) and the same nutrients as the initial batch, except glucose, was added continuously. The sucrose was previously hydrolyzed at 40°C in 10^{-2} *M* sodium acetate buffer solution at a pH of 4.5 *(15)* and utilized invertase enzyme (Novo Ferment). The hydrolyzed sucrose concentration was different for each run. Table 1 gives the concentrations used.

Immobilization

The gel used for immobilization was composed of sodium alginate (20.0 g/L) and alumina (15.0 g/L); these components were mixed with the cells from the inoculum. Details of the preparation are found in Araujo et al. *(9)*.

Analysis

Glucose Concentration

Glucose concentration was measured using the enzymatic glucose oxidase method.

Cell Concentration

Free and immobilized cell-mass concentration was evaluated as dry weight (grams/liter) at 105°C for 24 h and as volatile suspended solids (grams/liter) at 600°C for 1.5 h.

Cephalosporin C Concentration

Cephalosporin C titers were determined by high-performance liquid chromatography.

Procedure

Fed-batch experiments were carried out in a 1.6-L tower bioreactor and lasted approx 200 h. Temperature was maintained at 26°C and air flow rate at 170 L/h (21.1°C and 1 atm). All runs started with 1.4 L of main fermentation culture medium. After glucose depletion, supplementary medium was added using a peristaltic pump at established flow rates. The same volumetric flow rate of 1.8 mL/h was maintained for all runs, and the invert sugar concentration varied for each run (Table 1). Samples were withdrawn periodically to determine pH, biomass, glucose, and antibiotic concentrations. Figure 1 shows a bioreactor during the fermentative process.

Mathematical Model

A kinetic model based on the model proposed by Araujo et al. *(8)* was used to describe the behavior of the fed-batch process. In this model, modifications were introduced to take into account the influence of supplementary medium flow rate. The stoichiometric equations developed in the present study are based on those proposed by Cruz et al. *(10)*. These equations were adapted to represent the process with immobilized cells, and they are composed of kinetic expressions of *C. acremonium* in this culture medium and consider the mass transfer through the gel particle. The balance equations also considered the effect of oxygen limitation inside the bioparticles. In the Monod-type rate equations, in addition to cell and substrate concentrations, dissolved oxygen was included as a reactant affecting the bioreaction rate.

In this model, it was assumed that during the growth phase and glucose consumption the cells under catabolite repression (cells X_1) were able to produce enzymes responsible for biomass formation. When glucose concentrations fall below a certain critical value (C_{s1c}), cells X_1 are transformed into derepressed cells (X_2). As pointed out by Chu and Constantinides *(7)*, these cells are able to produce large amounts of the enzymes. (E) responsible for cephalosporin C synthesis. Antibiotic formation is regulated by

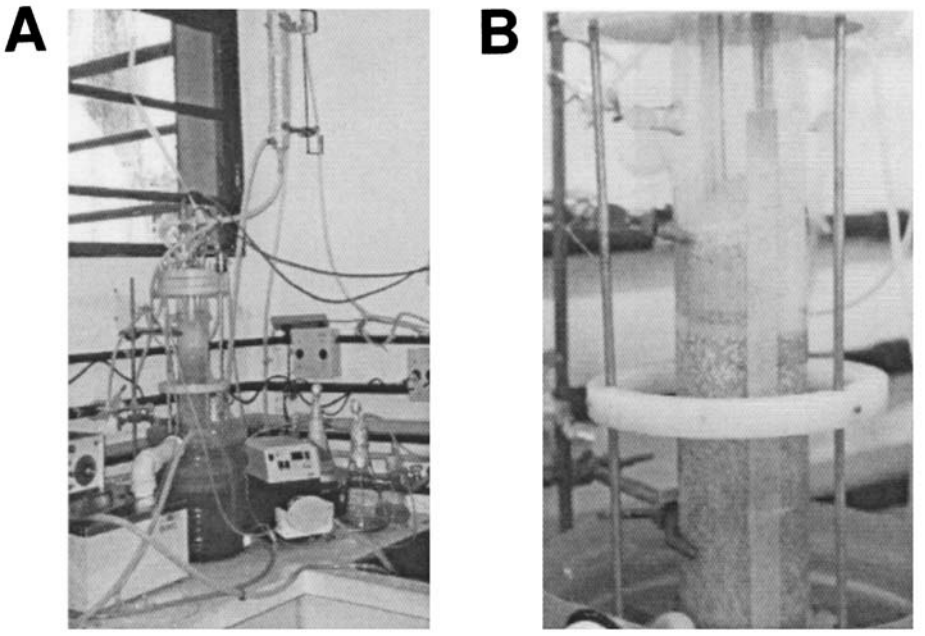

Fig. 1. **(A)** Bioreactor photograph during the fermentative process; **(B)** bioreactor detail.

reactions catalyzed by these enzymes. It was assumed that cephalosporin C and these enzymes suffer degradation. The stoichiometric equations and mass balance equations were described by Almeida et al. *(16)*. The differential equations were solved by numerical method. Regarding estimation of parameters, the classic nonlinear regression method following Marquardt's procedure *(11)* with 95% confidence interval was applied.

Results and Discussion

The model is composed of 6 differential equations, 6 state variables, and 16 parameters. Three of the five state variables were measured: total immobilized cell (C_x), glucose (C_{s1}) and cephalosporin C (C_p) concentrations. The growth phase and glucose consumption parameters were optimized for all fed-batch runs. The estimated parameters were μ_{max1}, k_x, Y_{xs}, k_{d2}, and m. Table 2 gives the parameters optimized in this process. Some parameters, such as k_{d3}, k_{d4}, and β, were determined by fitting to the experimental data. The parameters k_T and k_1 are related to morphologic differentiation present in the microorganism, and the adopted value was that used by Cruz et al. *(17)*. The parameters related to respiration and oxygen concentration were based on those estimated by Araujo et al. *(8)*. The coefficient α must be estimated empirically for each run because the relationship between the productivity and mass flow rate of glucose varied for each fed-batch run (Eq. 1). Table 3 gives estimated values of α.

Table 2
Estimated Parameters for Cephalosporin C Production Process in Fed-Batch Tower-Type Bioreactor with Immobilized Cells of *C. acremonium*

Cell, glucose, and product parameters	Symbols	Values
Maximum specific growth rate[a]	μ_{max1} (h^{-1})	0.04875 ± 0.0018
Contois constant[a]	k_{x1} (g S_1/g X_1)	0.04381 ± 0.0078
Yield coefficient[a]	Y_{xs1} (g X_1/g S_1)	0.5554 ± 0.0609
Death rate constant of cells X_1	k_{d1} (h^{-1})	0.0
Death rate constant of cells X_2[a]	k_{d2} (h^{-1})	0.0064 ± 0.0009
Kinetic constants	k_T (g X_1/L·h)	6.01
(morphologic differentiation rate constant)	K_1 (g X_1/L)	0.01
Decomposition rate of enzyme	k_{d3} (h^{-1})	0.002
Decomposition rate of cephalosporin C	k_{d4} (h^{-1})	0.002
Maintenance coefficient[a]	m (g S_1/g X_2·h)	0.0150 ± 0.0041
Empirical coefficient β	β (–)	0.010
Maximum specific respiration rate[b]	R_{max} (mmol O_2/g X·h)	1.055 ± 0.219
Kinetic constant (respiration rate)[b]	K_{O2} (mmol O_2/L)	0.00277 ± 0.00073
Volumetric mass transfer coefficient	K_La (h^{-1})	100
Oxygen concentration of saturation	C_L^a (mmol O_2/L)	0.22
Critical glucose concentration	C_{s1c} (g S_1/L)	0.8

[a]This parameter was estimated using nonlinear regression with 95% confidence limits.
[b]This parameter was estimated by Araujo et al. *(1)*.

Table 3
Values of Empirical Coefficient α

Run	Coefficient α
4	16.0
5	19.0
6	10.0
7	15.0

$$\varepsilon_{gel} \cdot \frac{\partial C_P}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r}\left(r^2 \cdot De_P \frac{\partial C_P}{\partial r}\right) + \beta \cdot C_{X2} \cdot \frac{R_{O_2}}{R_{max}} \cdot (\alpha \cdot \mu \cdot C_{X2} - k_{d3} \cdot C_E) - k_{d4} \cdot C_P \quad (1)$$

Several runs with different sugar mass feed rates were carried out according to the proposed model, and parameters estimate that it was possible to simulate the glucose consumed, cell growth, and product formation for all runs. The experimental data were compared with the simulated curves. Figure 2 shows the experimental results and the simulated curve of run 4 (115.2 g/L of hydrolyzed sucrose).

Figure 3 illustrates the experimental results and the simulated curve of run 5 (86.4 g/L of hydrolyzed sucrose). Figures 4 and 5 present the

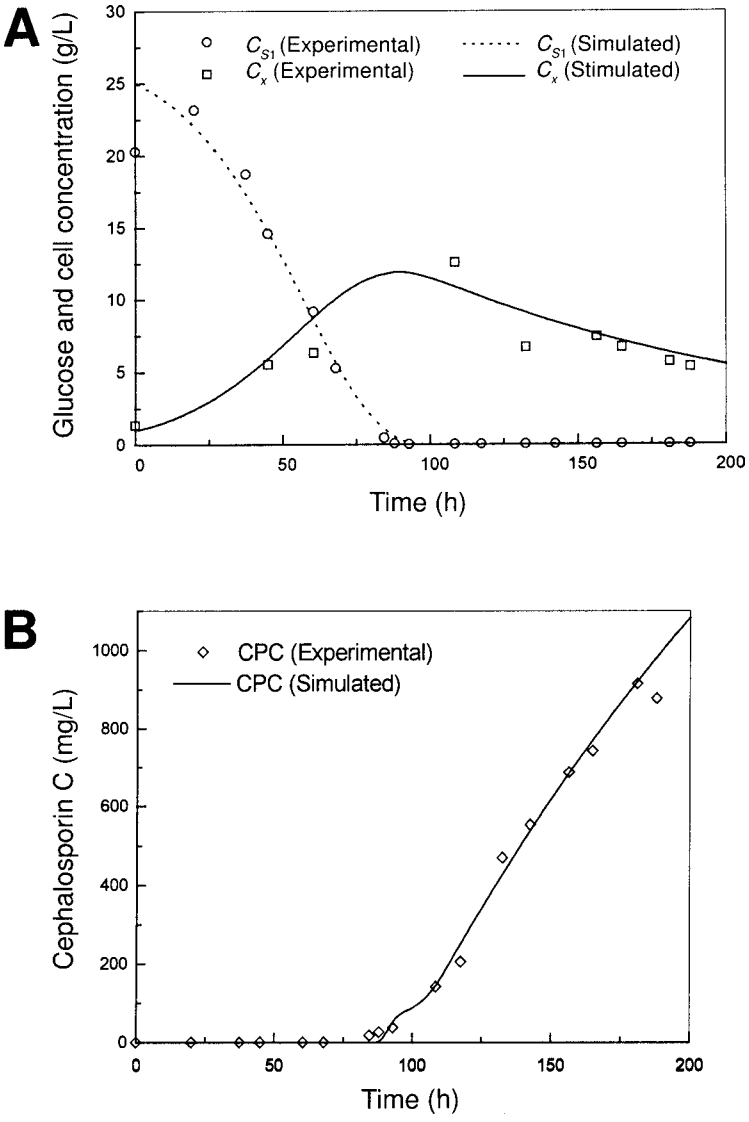

Fig. 2. Experimental data and simulation results of cephalosporin C fed-batch production: **(A)** glucose consumed and biomass formation; **(B)** product formation during run 4 (115.2 g/L of hydrolyzed sucrose). CPC, cephalosporin C.

experimental results and the simulated curve of run 6 (144.0 g/L of hydrolyzed sucrose) and run 7 (107.5 g/L of hydrolyzed sucrose), respectively.

The simulated results in Figs. 2–5 show that the proposed model fit well to the experimental data for glucose concentration, cell mass, and cephalosporin C concentration in the broth. It was observed that run 4, with 115.2 g/L of hydrolyzed sucrose and 1.8 mL/h of volumetric flow rate, led to the higher cephalosporin C production. In runs 5 and 7, when the hydrolyzed sucrose concentrations were 25 and 7% smaller than in run 4, respectively, the microorganism was able to produce antibiotic for 65 h after the

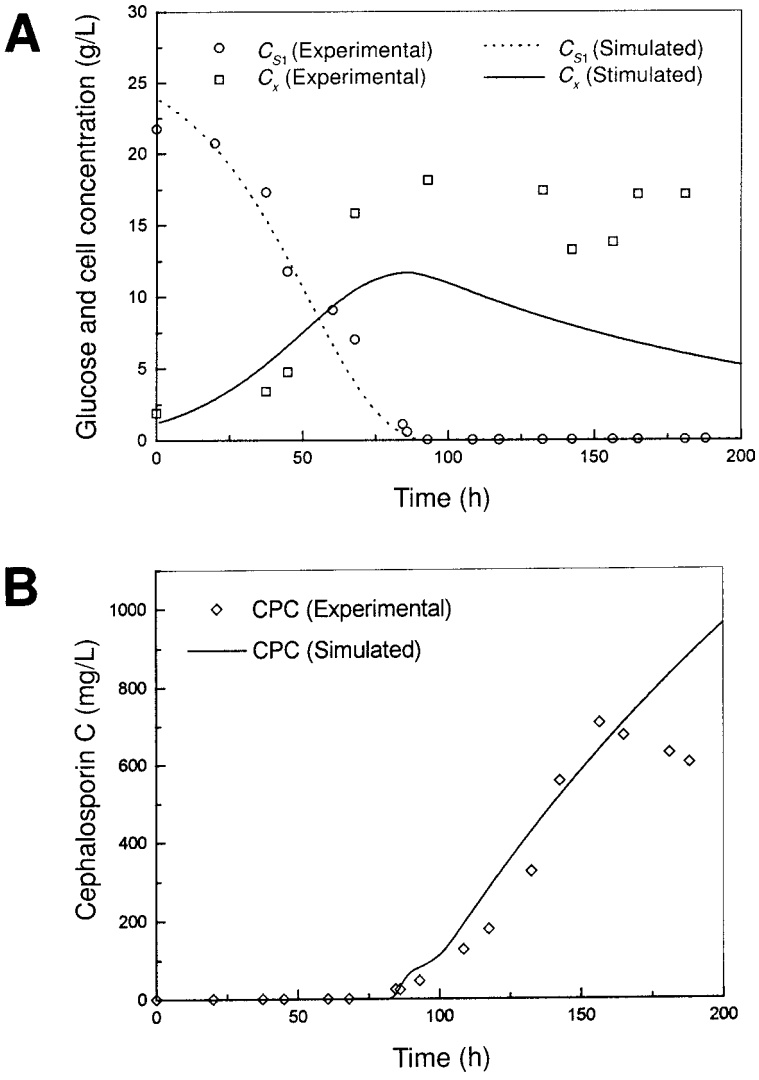

Fig. 3. Experimental data and simulation results of cephalosporin C fed-batch production: **(A)** glucose consumed and biomass formation; **(B)** product formation during run 5 (86.4 g/L of hydrolyzed sucrose). CPC, cephalosporin C.

beginning of the feed. Consequently, glucose concentration had little increase in these runs, as observed in Fig. 6.

Note the inflection that occurs in the product concentration curve at the beginning of the idiophase (*see* Fig. 2B at approx 90 h, Fig. 3B at approx 90 h, Fig. 4B at approx 100 h, and Fig. 5B at approx 75 h). This behavior is owing to the mass transfer diffusion intraparticle resistance. Glucose concentrations inside the gel particles are lower than in the broth, and this triggers the beginning of the production of antibiotic. When the supplementary feed is turned on, there is a dilution effect, which is over-

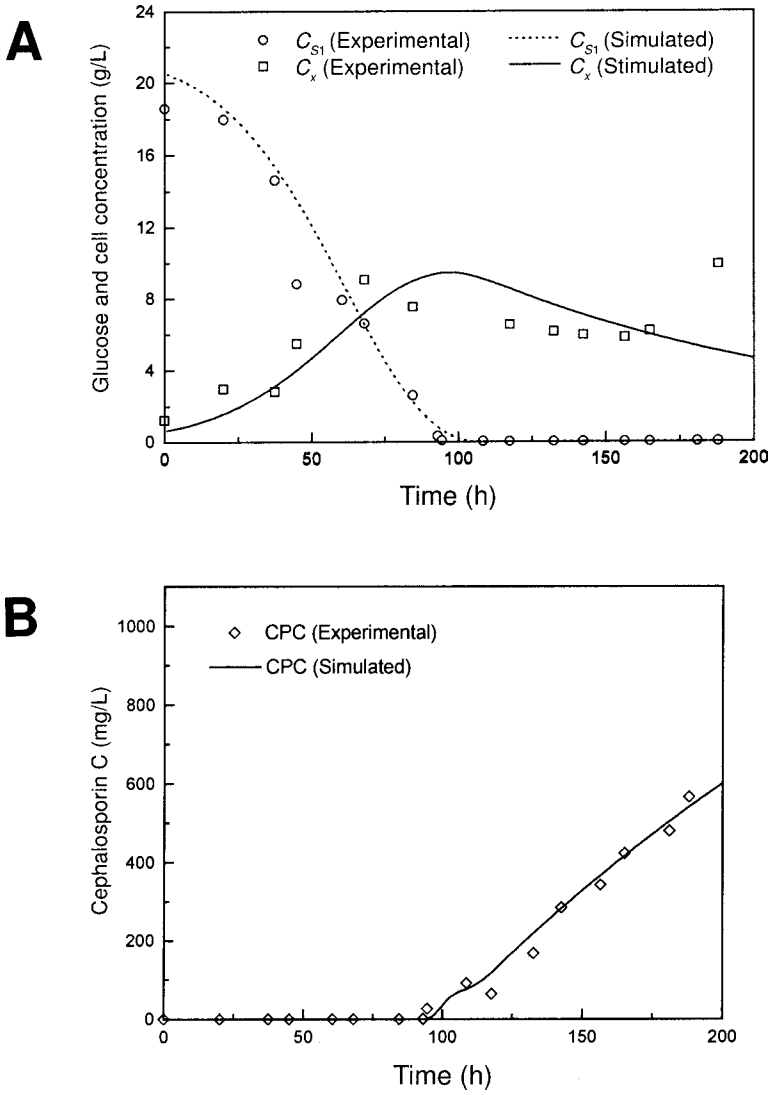

Fig. 4. Experimental data and simulation results of cephalosporin C fed-batch production: **(A)** glucose consumed and biomass formation; **(B)** product formation during run 6 (144.0 g/L of hydrolyzed sucrose). CPC, cephalosporin C.

come by the increasing rate of cephalosporin C production. The model has a response consistent with this phenomenon.

The model did not explain the decline in the production observed in runs 5 and 7. Probably, in these runs a shortage of carbon source occurred, causing metabolic damage in cells. Adding supplementary medium in a crescent flow rate would minimize these damages.

In run 6, when the hydrolyzed sucrose concentration used was 25% higher than for run 4, product degradation was not observed. However, a slightly lower production rate of cephalosporin C compared with run 4 was

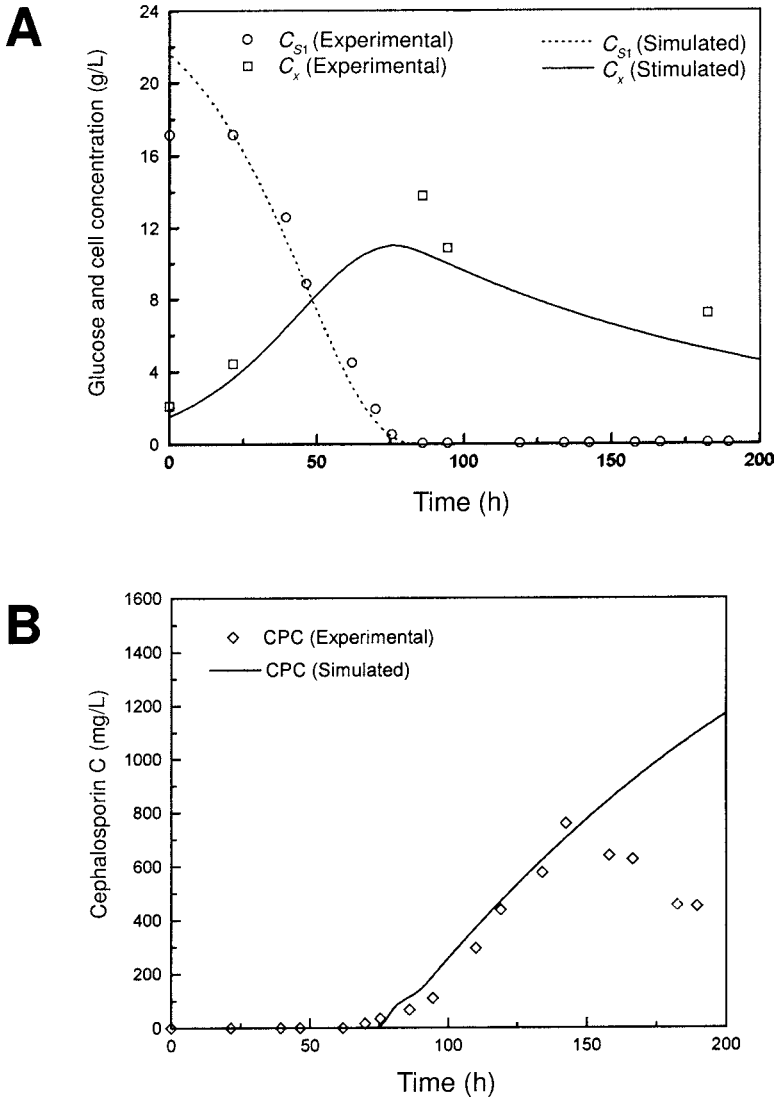

Fig. 5. Experimental data and simulation results of cephalosporin C fed-batch production: **(A)** glucose consumed and biomass formation; **(B)** product formation during run 7 (107.5 g/L of hydrolyzed sucrose). CPC, cephalosporin C.

observed, indicating that catabolite repression begins to exert its influence on the process.

Conclusion

It was evident from the results that the model proposed to simulate the fed-batch process of cephalosporin C production in a tower-type bioreactor fit the experimental data satisfactorily and providing valuable information for further process optimization.

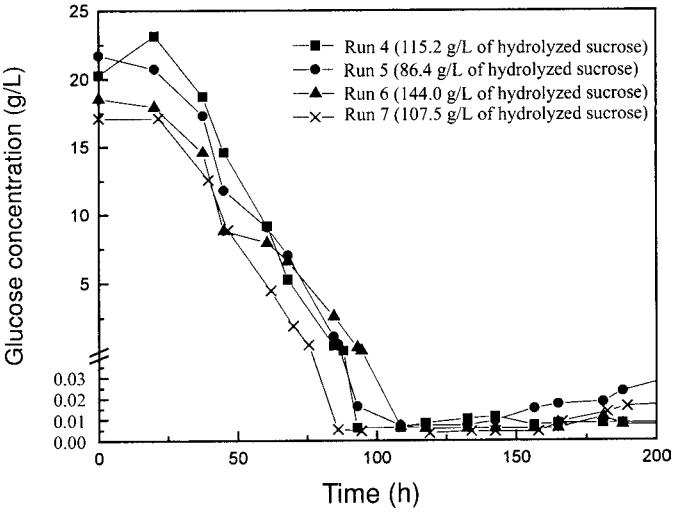

Fig. 6. Experimental results of glucose concentration in runs 4, 5, 6, and 7 with 115.2, 86.4, 144.0, and 107.5 g/L of hydrolyzed sucrose, respectively.

In runs 5 and 7 it was observed that the low sugar concentration in the supplementary medium minimized the repressive effect exerted by glucose, resulting in a high production of antibiotic in the beginning of the feed. After a certain interval of time, however, there was a shortage of carbon source, and the microorganism was no longer able to synthesize the antibiotic. To overcome this problem, the use of a crescent flow rate of supplementary medium or a small increase in sugar concentration is suggested.

The model was a good fit for the concentration profiles of cephalosporin C for runs 4 and 6 but could not predict the decline observed at the end of runs 5 and 7. We are presently working on a hybrid neural network algorithm to infer on-line the concentration of the antibiotic, with the purpose of optimizing fermentation time and interrupting the process when the maximum yield is achieved.

Acknowledgments

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