Modeling, Optimization, and Computer Control of the Cephalosporin C Fermentation Process

Wey-Bang Z. Chu and Alkis Constantinides
Department of Chemical & Biochemical Engineering, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08855

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In this study, a cephalosporin C producing strain, *Cephalosporium acremonium* (ATCC 36225), was chosen to determine the optimal conditions that maximize antibiotic production in a mixed substrate of glucose and sucrose. A model for cell growth and cephalosporin C production at different pH and temperature was developed and the associated parameters were evaluated experimentally. Pontryagin's maximum principle, in conjunction with the model, was used to predict the optimal temperature and pH control profiles to maximize the production of antibiotic.

INTRODUCTION

In batch fermentations for secondary metabolites, especially antibiotics, experimenters frequently find that the optimum conditions for unlimited growth of the microorganism differ substantially from the optimum conditions for the biosynthesis of the desired product.[1] Andreyeva[2] stated that the pH value should not be maintained constant during the batch fermentation of secondary metabolites, since the optimal pH values are different for growth and production of the secondary metabolite products. In the penicillin fermentation, the temperature for the maximum rate of antibiotic biosynthesis is different from that for the maximum rate of growth.[3,4] The implementation of temperature profiles has shown significant improvement in the penicillin production in the batch process.[4,5]

The objective of our work was to optimize the production of cephalosporin C in a batch fermentation process by controlling pH and temperature at different levels in different phases of fermentation. Cephalosporin C has been chosen for this study because it is a very important antibiotic. It is employed in the manufacture of 7-amino cephalosporinic acid (7-ACA) which is then reacylated to yield the commercially available synthetic cephalosporins. In 1985, annual sales of cephalosporins accounted for $1.2 billion of a total of $2.5 billion for all antibiotics in the U.S.[6]

The search for the optimal pH and temperature profiles for a fermentation process is a typical problem of optimization for which the use of kinetic models of the process is essential. For control purposes, the models must describe quantitatively the behavior of the fermentation process and show the effect of the changing of pH and temperature on the evolution of the fermentation states. Although it may be virtually impossible to understand the fermentation system entirely from the point of view of its cellular biology, a grasp of its essential mechanism should allow construction of a model that reflects at least several essential features of the process.

MATERIALS AND METHODS

Microorganism

Cephalosporium acremonium strain CW-19, ATCC 36225 (*Acremonium strictum*), was used for this study. The strain CW-19 is prototrophic and capable of producing ca. 1500 μg cephalosporin C/mL medium, when grown in a complex medium, or ca. 400 μg/mL, when grown in a defined medium.[7]

Equipment

Fermentations were carried out in a 2-L glass fermentor (Multigen model F-2000, New Brunswick Scientific Co.). This unit provided aeration, agitation, and temperature control. The pH of the fermentation medium was continuously monitored and controlled by a pH controller (model PH-122, New Brunswick Scientific Co.) using a steam sterilizable glass electrode (model 465-35-K9, Ingold Electrodes Inc., Andover, MA). An antifoam controller (New Brunswick Scientific Co.) was employed for the detection and control of foam within the fermentor vessel by adding antifoam solution (Antifoam B Emulsion, Sigma Chemical Co., St. Louis, MO) to the vessel. A dissolved oxygen analyzer (model DO-40, New Brunswick Scientific Co.) was used in conjunction with a steam sterilizable galvanic electrode (model M1016-0770, New Brunswick Scientific Co.) to ensure that the dissolved oxygen was above the critical level.

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Media and Culture Conditions

Cephalosporium cultures were maintained on agar slants. Cells from a slant were suspended in 6 mL sterilized water. One milliliter of this suspension was used to inoculate a seed medium. The slant medium and seed medium described by Matsumura et al. were used.[8] The medium used for the antibiotic production was a modification of the chemically defined medium No. 3 described by Demain.[9] A 2-L batch fermentor (working volume was 1.2 L) was used with 8% inoculum for the production of cephalosporin C. The fermentor was operated under the following experimental conditions: air flow rate of 0.6 vvm; agitation, 650 rpm; temperature of 24, 28, or 32°C; pH automatically controlled at 6.2, 6.6, 7.0, or 7.4 with 5*N* NaOH and 5*N* H_2SO_4 solution.

Analysis

Cell Mass

Ten milliliters of culture was sampled to determine the cell mass. The mycelial mass was separated by filtration through a preweighed Whatman glass-fiber filter GF/B, washed twice with distilled water, and dried in an oven (95°C) to constant weight.

Cephalosporin C

Cephalosporin C titers were determined by an agar diffusion bioassay using *Alcaligenes faecalis* ATCC 8750. After being transferred from a freeze-dried storage, *Alcaligenes faecalis* was grown to a density of 200 klett units in heart infusion broth. Ten milliliters of this suspension were then used to seed 1 L heart infusion agar containing 0.01% triphenyl tetrazolium chloride and penicillinase. Ten thousand units of penicillinase were added per liter of medium to destroy penicillin N, although *Alcaligenes faecalis* was resistant to penicillin N and cephalosporin P.

Sugar Concentration

Glucose and sucrose were measured by an industrial analyzer, (YSI model 27, Yellow Spring Instrument Co.). In order to use the glucose analyzer to measure sucrose concentration, the sucrose's sample was converted first to glucose by invertase (No. I-4504, Sigma Chemical Company).

MATHEMATICAL MODELS

Cephalosporin C fermentation by *Cephalosporium acremonium* is characterized by morphological differentiation and catabolite repression by glucose. In submerged culture, *C. acremonium* shows three main morphological cell types during fermentation: hyphae, swollen hyphal fragments, and arthrospores. After being transferred into the fermentation medium, filamentous hyphae grow and differentiate into highly swollen fragments and arthrospores. The antibiotic is produced along with marked fragmentation.[10,11]

C. acremonium grows well in a medium with glucose as the major carbon source, but glucose represses the synthesis of cephalosporin C. Sucrose, a slowly metabolized carbon source, does not exhibit such a significant repression effect. In a chemically defined medium, with glucose and sucrose as major carbon sources, the production of cephalosporins is delayed until glucose is completely utilized. In the strain of *C. acremonium* CW-19, deacetoxycephalosporin C synthetase, the ring-expansion enzyme (expandase) does not appear as long as glucose is present. Afterwards, initiation of its formation is accompanied by the production of cephalosporins. Once expandase is formed, little inhibition of its activity by glucose is observed.[12]

Queener and Ellis[13] studied the different morphological forms under an electron microscope and found that the cross sections of these forms illustrated striking differences in cell wall thickness: unicellular arthrospores, ca. 250 ~ 350 nm; swollen hyphal fragment, 100–350 nm; and unswollen hyphal filament, 40–100 nm. The higher production of cephalosporin C by the thicker cell wall of the arthrospore and the swollen fragment,[10,11] indicated the possibility of some connection between cell wall synthesis and cephalosporin synthesis.[13]

For the modeling, it is postulated that the total biomass is composed of two cell types: thin hyphae and thicker cell wall forms. Thin hyphae represent newly formed hyphae which can be formed by both cell types. Thick wall cells represent fully swollen hyphae and arthrospores which are formed through morphological differentiation of thin hyphae and which are associated with cephalosporin C biosynthesis.

The equations describing the concentration of the two cell types and the total cell mass in a batch culture are

$$X = X_H + X_T \tag{1}$$

$$\frac{dX_H}{dt} = \mu_H X - \mu_T X_H - \delta_H X_H \tag{2}$$

$$\frac{dX_T}{dt} = \mu_T X_H - \delta_T X_T \tag{3}$$

where X is the concentration of total cell mass; μ_H and μ_T are the specific formation rates of thin hyphae (X_H) and thick wall cells (X_T), respectively; and δ_H and δ_T are the specific decay rates of thin hyphae and thick wall cells, respectively.

The nonlinear rate constants (μ_T, δ_H) associated with differentiation in eqs. (2) and (3) are formulated by assuming that glucose represses the formation of cephalosporin C by suppressing the rate of morphological differentiation:

$$\mu_T = \frac{\mu_T^m}{1 + I_1 S_1} \tag{4}$$

$$\delta_H = \frac{\delta_H^m}{1 + I_1 S_1} \tag{5}$$

where S_1 is the glucose concentration; I_1 is the repression constant of morphological differentiation by glucose; μ_T^m is

the maximum specific formation rate of thick wall cells; and is the maximum specific decay rate of thin hyphae related to cell differentiation. The decay of hyphae is due to the loss of cytoplasmic components. It has been observed that the density of hyphae changes from 0.35 to 0.20 g/cm^3 during the penicillin fermentation.[14] With the loss of cytoplasmic components, the rate of penicillin synthesis decreases.

In a chemically defined medium, with glucose and sucrose as major carbon sources, *C. acremonium* exhibits a biphasic growth due to successive utilization of the two carbon sources. Glucose is the preferred carbon source. The cells consume only the glucose as long as it is present in the system. After glucose is completely used, sucrose becomes the carbon source for cell growth, and maintenance. Sucrose is a slower metabolized substrate which does not suppress production of cephalosporin C. It is observed that the cell grows rapidly until the utilization of glucose is complete and then, after a lag period, a slower growth rate occurs, due to the utilization of the sucrose.

When both of these two carbon sources exist in a culture medium, the Monod expression is assumed to be applied to both substrates:

$$\mu_{S1} = \frac{\mu_{S1}^m S_1}{K_{S1} + S_1} \tag{6}$$

$$\mu_{S2}' = \frac{\mu_{S2}^m S_2}{K_{S2} + S_2} \tag{7}$$

where μ_{S1} and μ_{S2}' are the specific growth rates of thin hyphae on glucose and sucrose, respectively. Parameters μ_{S1}^m and μ_{S2}^m are the maximum specific growth rates of thin hyphae on glucose and sucrose, respectively; S_1 and S_2 are the concentrations of glucose and sucrose, respectively; and K_{S1} and K_{S2} are the saturation constants related to glucose and sucrose, respectively.

An intermittent lag period exists between complete exhaustion of the glucose and initiation of sucrose utilization. The length of the lag period (t_{lag}) reflects difficulties in production and/or derepression of the enzymes involved in the catabolism of the second carbon source. In our case, the intermittent lag is introduced into the growth rate expression of thin hyphae on sucrose through a ϕ_{lag} function:

$$\mu_{S2} = \mu_{S2}'\phi_{lag} \tag{8}$$

$$\phi_{lag} = \frac{\tan^{-1}\left[k_\phi\left(\frac{t - t_1}{t_{lag}} - 1\right)\right]}{\pi} + \frac{1}{2}; \qquad \text{if } t > t_1 \tag{9}$$

where t_1 is the time when glucose is used up; t_{lag} is the intermittent lag period reflecting the difficulties of adaptation of the microorganism in utilizing the second carbon source; and k_ϕ is a constant which is related to the steepness of the arctangent curve arising from zero. There are three parameters involved in this ϕ_{lag} function and only two of them (k_ϕ and t_{lag}) need to be defined. The glucose completion time, t_1, is set during the fermentation by the time when glucose is exhausted. The reason for using an arctangent function to simulate the intermittent lag of diauxic growth is its simplicity and flexibility. The arctangent function is basically a step function which rises from $-\pi/2$ to $\pi/2$. After being divided by π and added to 1/2, the arctangent function becomes ϕ_{lag} which rises from zero to one. The value of $t_1 + t_{lag}$ locates the place where the ϕ_{lag} function rises from zero and at which the slope of the function is $k_\phi/\pi t_{lag}$. The parameter k_ϕ is not sensitive in the prediction of the growth of hyphae. After k_ϕ is set to be 318.2 the value of ϕ_{lag} function is equal to 0.99 when $(t - t_1)/t_{lag}$ is 1.1, and is 0.01 when $(t - t_1)/t_{lag}$ is 0.9.

The growth rate of thin hyphae (μ_H) is formulated differently in the two phases of fermentation. In the first phase, only glucose is consumed so that the specific growth rate of thin hyphae is equal to the specific growth rate of thin hyphae grown on glucose (μ_{S1}). After glucose is exhausted (at t_1), sucrose replaces glucose as the major carbon source. The specific growth rate of thin hyphae is then expressed as the specific growth rate of thin hyphae grown on sucrose.

$$\mu_H = \begin{cases} \mu_{S1}; & t \le t_1 \\ \mu_{S2}; & t > t_1 \end{cases} \tag{10}$$

The cephalosporin C is synthesized by the cells in a complex enzymatic pathway. In order to model that process, it is postulated that the production rate of cephalosporin is proportional to the amount of the rate-limiting enzyme. The decomposition of cephalosporin C contributes a negative term to the rate of change of the concentration, given by eq. (11):

$$\frac{dP}{dt} = EX_T - \gamma P \tag{11}$$

where P is the cephalosporin C concentration; γ is the cephalosporin C decomposition rate constant; and E is the specific concentration of the rate-limiting enzyme inside the thick wall cells.

It is assumed that the concentration of the rate-limiting enzyme is growth-linked to the thick wall cell forms and the decomposition rate of the enzyme is linearly proportional to the enzyme concentration:

$$dE/dt = \alpha\mu_T X_H - \beta E \tag{12}$$

where α is the growth-link enzyme formation rate and β is the decomposition rate of the enzyme.

The rate of substrate utilization is related to the cell synthesis rate and maintenance of existing cells. The sucrose utilization rate is expressed accordingly:

$$\frac{dS_2}{dt} = -\frac{\mu_{S2} X}{Y_{S2}} - \frac{m_{S2} S_2 X}{K_{S2} + S_2 + I_2 S_1} \tag{13}$$

where Y_{S2} is the yield factor of cell from sucrose; m_{S2} is the maintenance coefficient; and I_2 is the inhibition constant of sucrose utilization by glucose. The maintenance term in eq. (13) is formed under the assumption that the sucrose-utilized maintenance is influenced by the glucose concentration in a manner analogous to competitive inhibition.

In the rapid growing period, maintenance can be neglected. All the glucose consumed is for cell production. The rate of glucose utilization becomes

$$-\frac{dS_1}{dt} = \frac{\mu_{S1} X}{Y_{S1}} \tag{14}$$

where Y_{S1} is the yield factor of glucose.

In the state equations [eqs. (2), (3), (11), (12), (13), and (14)], the control variables, temperature, and pH do not appear explicitly, so each parameter must be dependent on the pH and temperature. The complete global model was established in two steps: 1) estimation of the parameters of the state equations from experimental data under constant pH and temperature to form a local model, and 2) determination of regression functions for each parameter to correlate with pH and temperature from the results of the preceding estimation.

In this study, the software package NONLIN[15] was chosen to solve the nonlinear regression problem. The package is written in FORTRAN and is available on a AS/9000 computer at the Rutgers University computer center. For solving the differential equations, the fourth order Runge–Kutta method was used in the NONLIN program. In the proposed model there are six differential equations, six state variables, and sixteen parameters. Four of the six state variables are measured: total cell, glucose, sucrose, and cephalosporin C concentrations.

Examination of the NONLIN outputs revealed that the parameters I_1, I_2, and K_{S1} had extremely large confidence intervals and parameters Y_{S2}, and α were highly correlated with m_{S2} and γ, respectively. After analysis of the NONLIN output from our 15 sets of experimental data, the parameter values of I_1, I_2, and K_{S1} were set to 20, 300, and 0.1, respectively, because these values all fall within their corresponding confidence intervals in every experiment. The value of sucrose yield, Y_{S2}, was set to 0.4, and α, the formation rate constant of the key enzyme for cephalosporin C synthesis, was set to 9 in all experimental conditions. Since α is highly correlated with γ, any assignment to α will affect the estimation of γ, the rate constant of cephalosporin C decomposition. After α was set to 9 in all 15 regression analyses, the estimated values of the parameter γ were evaluated, and they were found to be close to 0.005 (h^{-1}). This also happens to be the non-enzymatic decomposition rate constant of cephalosporin C cited in the literature.[16]

Parameter identification was carried out using data from 15 experiments with constant pH and temperature in the ranges 6.2–7.4 and 24–32°C, respectively. One set of results is shown in Table I, which includes all the parameters for the state equations under pH 6.2 and 32°C. This set of parameters can be applied to the state equations confidently under that specific pH and temperature to form a local model.

For solving the second part of the problem, the STEPWISE procedure[17] in the SAS statistical library was adopted. The STEPWISE procedure uses a classical regression method to estimate the significance of each term in the correlation function, and uses several different techniques to decide which of the terms should be included. In this study, a backward elimination technique was used for selection of the significant terms. By backward elimination, the nonsignificant terms were deleted from the correlation function one by one until all the remaining variables in the function produce the F statistic significant at the 25% level. The resulting correlation function for parameter Y_{S1} is shown in Table II. A contour plot of parameter Y_{S1} is also plotted here to show the actual response of this parameter with respect to pH and temperature change (Fig. 1). The lighter symbols stand for the lower value of Y_{S1}; the darker symbols stand for the higher value of Y_{S1}. From the contour plot we know that the fermentation condition of pH 7.0 and 24°C gives the highest yield of cell from glucose.

The procedure described above has been carried out for all the parameters. The correlation functions for all the parameters in the kinetic model are shown in Table III in matrix form. After obtaining these correlation functions, the numerical values of the parameters of the model can be estimated in the whole ranges of pH 6.2–7.4, and 24–32°C.

Table I. Estimated values of parameters at pH 6.2 and 32°C.

	Estimate	Standard deviation
m_{S2}	0.02267	0.002635
μ_{S2}^m	0.02100	0.004156
μ_T^m	0.04526	0.003348
δ_T	0.00441	0.000237
δ_H^m	0.00668	0.001031
t_{lag}	34.7724	0.920983
β	2.18787	0.156140
γ	0.01076	0.001097
K_{S2}	10.1990	3.642970
Y_{S1}	0.46188	0.010310
μ_{S1}^m	0.04200	0.000964
K_{S1}	0.1	
I_1	20	
I_2	300	
Y_{S2}	0.4	
α	9	

Table II. Correlation function of parameter Y_{S1} with respect to pH and temperature.

	B value	Standard error	PROB > F
p_1	0.494498		
p_2	−0.015414	0.002490	0.0016
p_3	−0.193796	0.039682	0.0045
p_4	−0.056758	0.012334	0.0058
p_5	0.005535	0.003089	0.1331
p_6	0.039206	0.009462	0.0090
p_7	0.133917	0.035915	0.0136
p_8	−0.144083	0.051971	0.0393
p_9	−0.005569	0.002415	0.0692
p_{10}	−0.017634	0.009566	0.1246

Notes:

θ_1 = Temperature − 28.

θ_2 = pH − 6.8.

$$Y_{S1} = p_1 + p_2\theta_1 + p_3\theta_2^2 + p_4\theta_1 \cdot \theta_2 + p_5\theta_1^2 \cdot \theta_2 + p_6\theta_1 \cdot \theta_2^2 + p_7\theta_1 \cdot \theta_2^3 + p_8\theta_2^3 + p_9\theta_1^2 \cdot \theta_2^2 + p_{10}\theta_1^2 \cdot \theta_2^3.$$

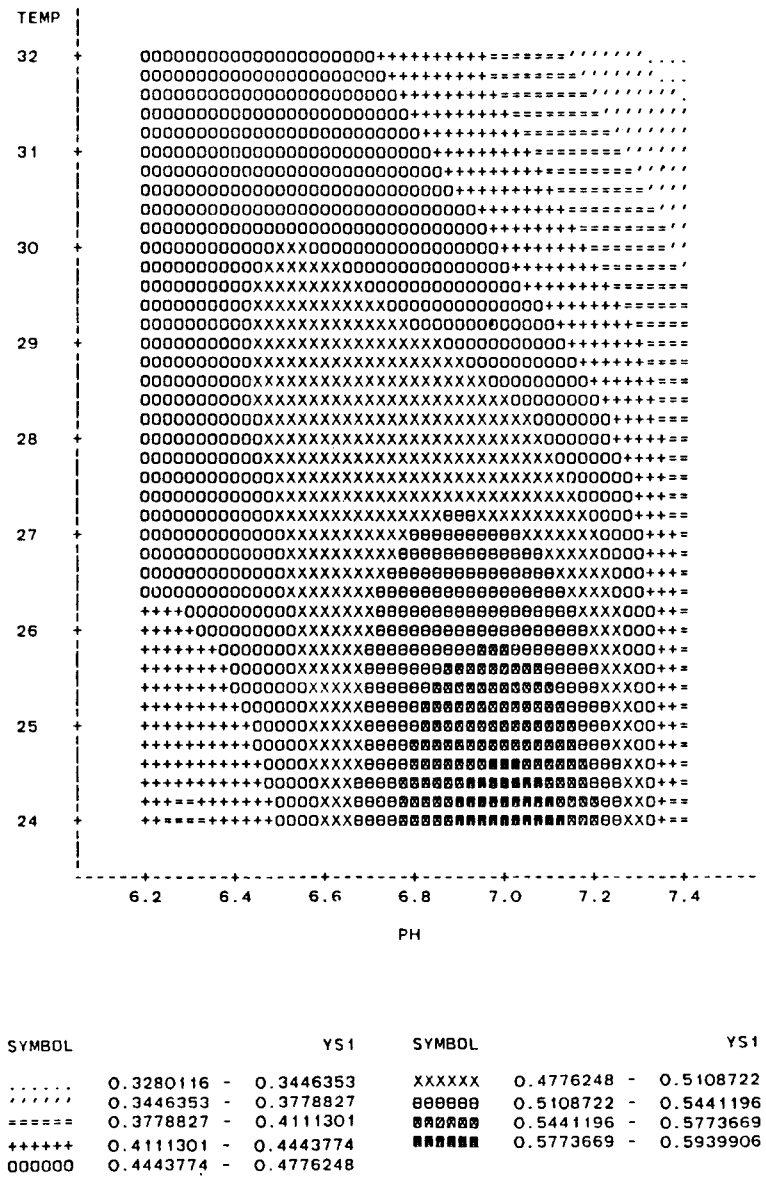


Figure 1. Contour plot of parameter Y_{S1} with respect to pH and temperature.

Table III. Correlation function of each parameter with respect to pH and temperature.

$$\begin{bmatrix} m_{S2} \\ \mu^m_{S2} \\ \mu^m_T \\ \delta_T \\ \delta^m_H \\ t_{lag} \\ \beta \\ \gamma \\ K_{S2} \\ Y_{S1} \\ \mu^m_{S1} \end{bmatrix} = \begin{bmatrix} 0.03681 & 0.00275 & 0.00000 & 0.00000 & -0.01665 & 0.01390 & 0.00000 & 0.00000 & -0.03375 & 0.07146 & 0.00000 & 0.00000 \\ 0.00961 & 0.00075 & -0.00263 & 0.00046 & 0.01963 & 0.00677 & 0.00000 & -0.00477 & -0.02148 & 0.00000 & -0.00155 & 0.00000 \\ 0.03441 & 0.00368 & -0.03230 & 0.00000 & 0.00000 & 0.00000 & 0.00161 & -0.00494 & -0.00530 & 0.06330 & -0.00107 & -0.00388 \\ 0.00408 & 0.00000 & 0.00440 & 0.00016 & 0.00770 & 0.00235 & 0.00000 & 0.00245 & -0.00278 & 0.00000 & -0.00036 & 0.00035 \\ 0.00406 & -0.00082 & 0.01669 & 0.00013 & 0.01202 & 0.00000 & -0.00062 & 0.00000 & 0.00000 & -0.05536 & 0.00000 & 0.00278 \\ 9.03318 & 0.06693 & 2.12029 & 0.00000 & 0.00000 & -0.63952 & -0.20039 & -0.60887 & 3.05159 & -5.52624 & 0.00000 & 0.00000 \\ 2.16216 & 0.08408 & -6.13806 & -0.01197 & 1.07091 & -0.50711 & 0.34845 & 0.23531 & 1.61583 & 12.70152 & -0.10083 & -0.68011 \\ 0.00964 & 0.00044 & 0.00735 & -0.00014 & -0.00866 & 0.00075 & -0.00022 & 0.00000 & -0.00424 & -0.01507 & 0.00026 & 0.00000 \\ 10.26915 & 0.00000 & -24.8557 & -0.13564 & 0.00000 & 0.00000 & 0.40604 & -1.45055 & 2.34382 & 41.20426 & 0.00000 & 0.00000 \\ 0.49450 & -0.01541 & 0.00000 & 0.00000 & -0.19380 & -0.05676 & 0.00554 & 0.03921 & 0.13392 & -0.14408 & -0.00557 & -0.01763 \\ 0.03717 & -0.00053 & -0.02067 & -0.00041 & -0.01423 & -0.01036 & 0.00000 & 0.00464 & 0.02179 & 0.04588 & 0.00000 & -0.00092 \end{bmatrix} \begin{bmatrix} 1 \\ \theta_1 \\ \theta_2 \\ \theta_1^2 \\ \theta_2^2 \\ \theta_1 \cdot \theta_2 \\ \theta_1^2 \cdot \theta_2 \\ \theta_1 \cdot \theta_2^2 \\ \theta_1 \cdot \theta_2^3 \\ \theta_2^3 \\ \theta_1^2 \cdot \theta_2^2 \\ \theta_1^2 \cdot \theta_2^3 \end{bmatrix}$$

Notes:

θ_1 = Temperature − 28.

θ_2 = pH − 6.8.

The kinetic model, together with the correlation functions for all the parameters, is called a global kinetic model which could be used to predict the evolution of fermentation states in a range of pH and temperature. The kinetic model, with a specific set of parameters estimated regressively from the experimental data, is called a local kinetic model. A local kinetic model can only predict fermentation states at the specific pH and temperature under which the experiment has been carried out. As an example, a simulated result of the global kinetic model (solid line), the local kinetic model (broken line), and the experimental data at pH 6.2 and 32°C are shown on Figure 2.

CONTINUOUS MAXIMUM PRINCIPLE

The basic algorithm of Pontryagin's Maximum Principle includes the state equations, the objective function, the Hamiltonian function, and the adjoint equations. The state equations, representing the kinetics of the fermentation [eqs. (2), (3), (12), (11), (13), and (14)], can be concisely written as:

$$d\mathbf{y}/dt = \mathbf{f}'(\mathbf{b}[\boldsymbol{\theta}(t)], \mathbf{y}); \qquad t_0 \leq t \leq t_f \tag{15}$$

where $\mathbf{y}$ is a six-dimensional vector of state variables (X_H, X_T, S_1, S_2, P, and E); $d\mathbf{y}/dt$ and $\mathbf{f}'$ are six-dimensional vectors of the rate of change of state variables; $\mathbf{b}$ is an 11-dimensional vector of the parameters; and $\boldsymbol{\theta}(t)$ is a two-dimensional vector of control variables at time t.

The above equations have known initial conditions represented by the vector:

$$\mathbf{y}(t_0) = \mathbf{y}_0 \tag{16}$$

The objective function is usually defined as a linear combination of the final values of the state variables:

$$O_{bj} = \mathbf{c}^T\mathbf{y}(t_f) \tag{17}$$

where $\mathbf{c}^T$ is the transpose of a vector of constants, $\mathbf{c}$. In our case, the objective function is defined as the final value of the concentration of cephalosporin C which needs to be maximized:

$$O_{bj} = P(t_f) \tag{18}$$

where $P(t_f)$ is the final concentration of cephalosporin C.

The Hamiltonian function is defined as the product between the transpose of the vector of adjoint variables, $\mathbf{z}^T$, and the vector of functions, $\mathbf{f}'$:

$$H = \mathbf{z}^T\mathbf{f}' \tag{19}$$

The adjoint variables are given by

$$d\mathbf{z}/dt = -\partial H/\partial \mathbf{y} \tag{20}$$

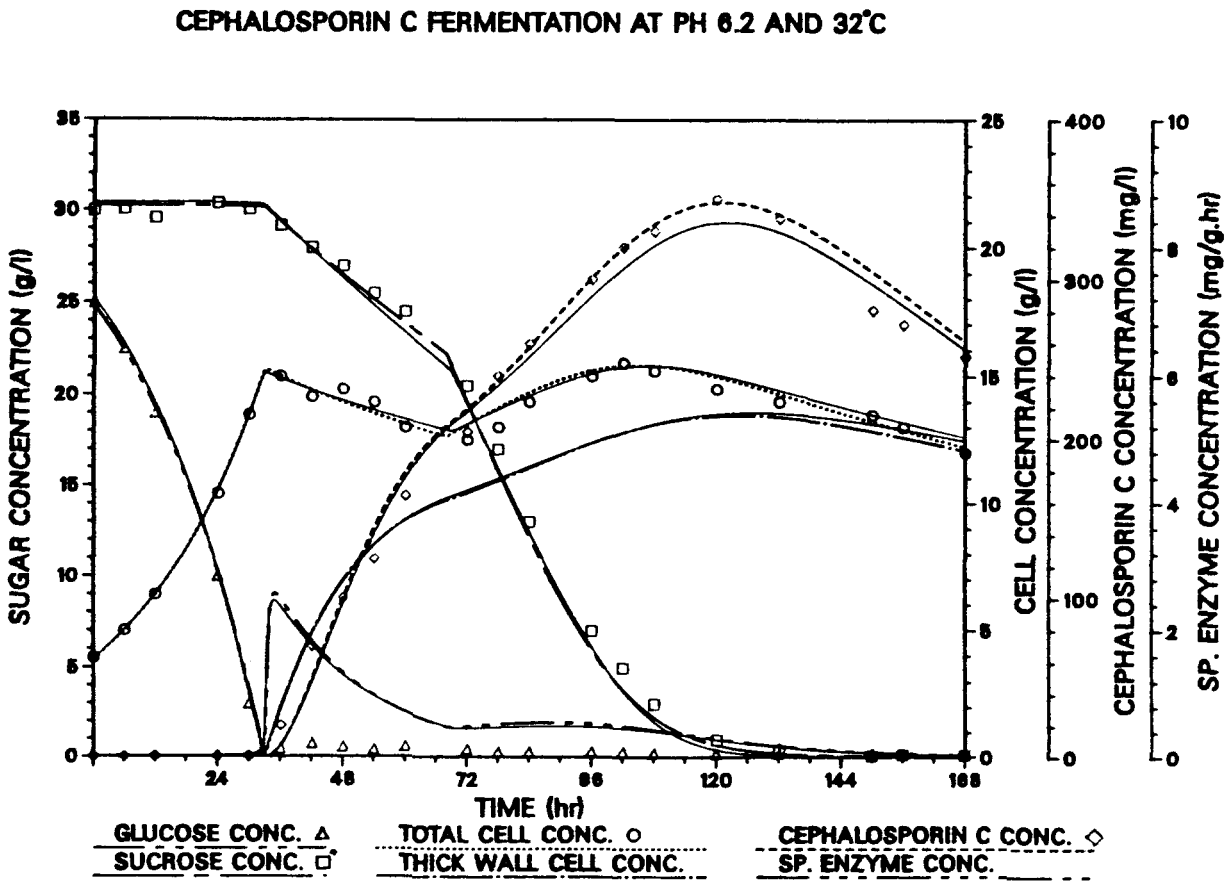


Figure 2. Simulation results of global kinetic model (solid line), local kinetic model (broken line), and experimental data of cephalosporin C fermentation at pH 6.2 and 32°C.

with the boundary conditions

$$\mathbf{z}(t_f) = \mathbf{c} \tag{21}$$

The continuous maximum principle can incorporate inequality constraints on the control variable:

$$G[\boldsymbol{\theta}(t)] \leq 0 \tag{22}$$

In our study such constraints are:

$$\begin{aligned} 24 \leq \text{temperature} \leq 32 \\ 6.2 \leq \text{pH} \leq 7.4 \end{aligned} \tag{23}$$

i.e.

$$\begin{aligned} |\theta_1| - 4.0 \leq 0 \\ |\theta_2| - 0.6 \leq 0 \end{aligned} \tag{24}$$

where θ_1 represents temperature at 28°C and θ_2 represents pH 6.8. The necessary condition for the objective function to be maximum is the following:

$$H = \text{maximum} \tag{25}$$

However there is a discontinuity in the state equation [eq. 10] at an interior point. The discontinuity occurs at the time (t_1) when glucose is exhausted:

$$S_1 - \varepsilon = \psi^{(1)} = 0 \tag{26}$$

where ε is a small positive number. This represents a terminal constraint for the part of the path from $t = t_0$ to $t = t_1$.

If we let t_1^- signify time just before t_1 and t_1^+ signify time just after t_1 we may extend the Maximum Principle by modifying the adjoint equations and the Hamiltonian as follows:[18]

$$z_{S_1}(t_1^-) = z_{S_1}(t_1^+) + \pi \frac{\partial \psi^{(1)}}{\partial S_1(t_1)} \tag{27}$$

$$H^{(1)}(t_1^-) = H^{(2)}(t_1^+) - \pi \frac{\partial \psi^{(1)}}{\partial t_1} \tag{28}$$

where $z_{S_1}(t_1^-)$ and $z_{S_1}(t_1^+)$ are the glucose-related adjoint variable at the time just before and right after t_1, respectively; π is a constant Lagrange multiplier; and $H^{(1)}(t_1^-)$ and $H^{(2)}(t_1^+)$ are the Hamiltonian at the time just before and right after t_1, respectively.

In the fixed-time batch fermentation processes, the initial conditions of the state variables are known and the final conditions of the adjoint variables are known. The above algorithm was applied to such a process and the bidirectional integration method was used to solve that two-point boundary value problem.[5]

In order to find the constrained global maximum for the Hamiltonian function (H), the ZXMWD subroutine in the IMSL library[19] was adopted. The ZXMWD routine which originally finds a minimum of a function can also be used to find a maximum by using the fact that $-H$ has a minimum whenever H has a maximum. In the subroutine, the constrained minimization is first changed into unconstrained minimization by sine square transformation. Then ZXMWD calls a subroutine based on the quasi-Newton method to do four iterations with each of the search starting points. After four iterations, all function values are compared. The five searches that result in the lowest function values are allowed to continue to convergence to get the local minima, and the lowest of these is taken to be the global minimum. As the number of search starting points is increased, the probability that this point is really the global minimum is increased.

RESULTS AND DISCUSSION

The optimum temperature and pH profiles obtained for the cephalosporin C fermentation are shown on Figure 3 for the batch times of 90, 96, and 100 h. For all three cases, the optimum pH and temperature profiles have patterns that are basically similar between 5 and 80 h.

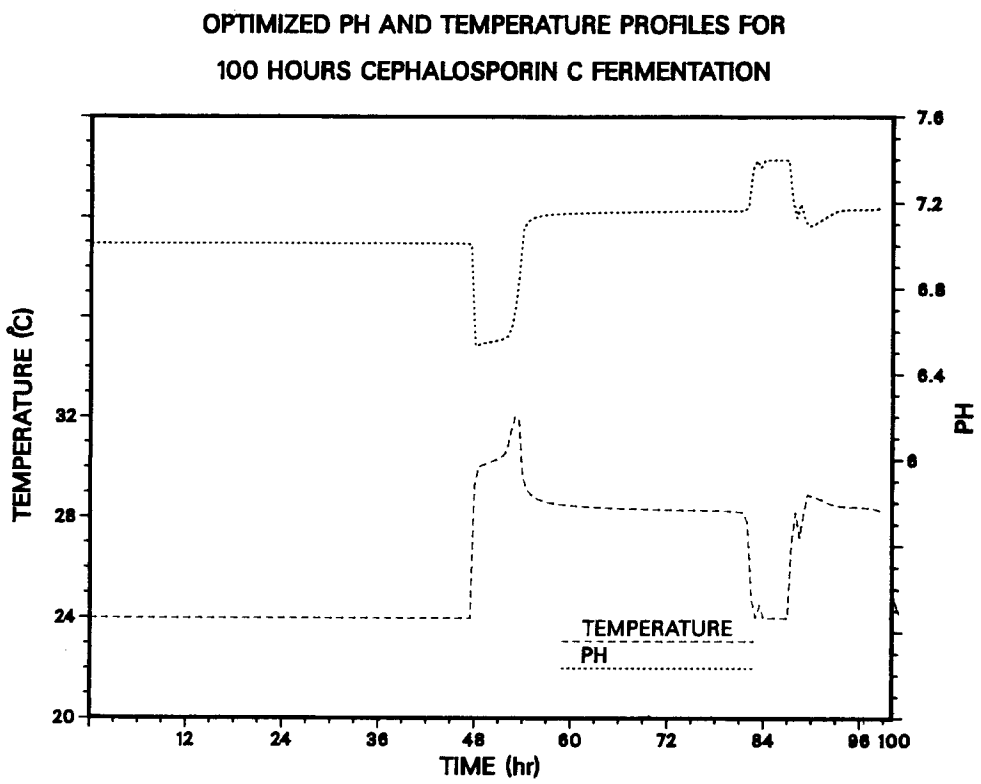
In the 90-h batch fermentation of cephalosporin C, the optimum pH and temperature conditions remain at 6.68 and 27.7°C, respectively for the first 3 h, and then shift to pH 7 and 24°C till 48 h fermentation. After a transition period lasting ca. 6 h, the optimum pH and temperature profiles shift to 7.16 and 28.3°C, respectively, until the end of fermentation.

In the 100-h fermentation, the optimum pH and temperature profiles remain at 7 pH and 24°C for the first 48 h of the fermentation and then shift to 7.16 pH and 28.3°C till the end of fermentation except at ca. 84 h a short shift of conditions to 7.4 pH and 24°C occurs. For the 96-h fermentation, there is a step change of pH and temperature at the beginning and a second step change at ca. 48 h.

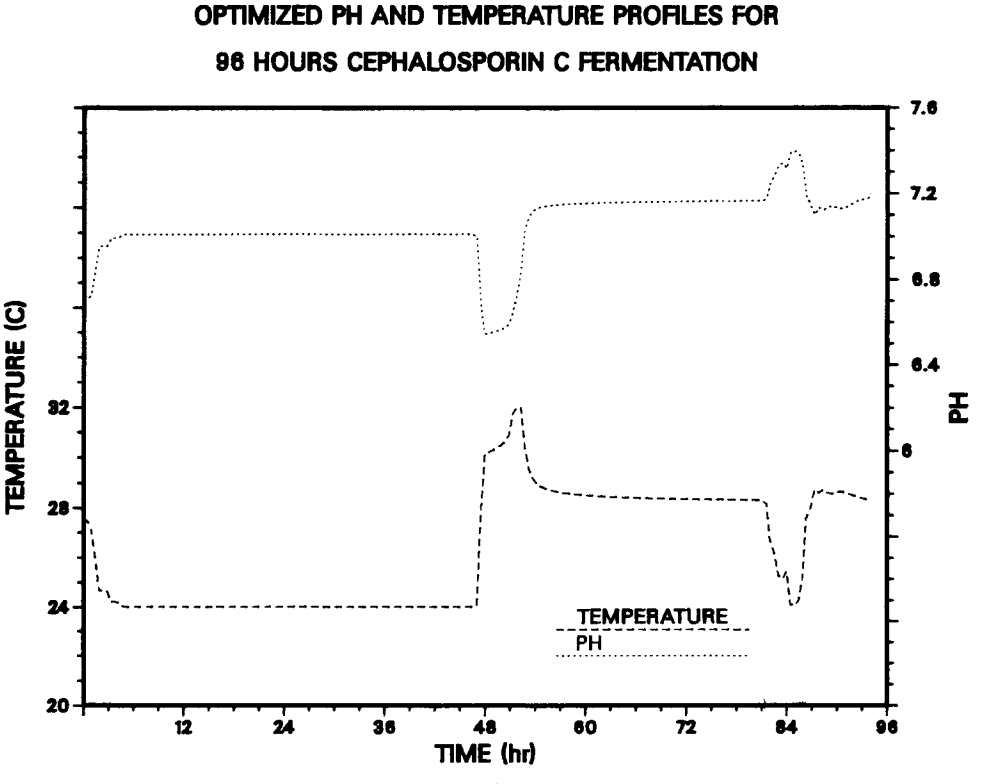
The condition of pH 7.0 and 24°C gives the highest cell yield which can be seen easily from the contour plot of Y_{S1} (Fig. 1). Between 5 and 48 h, high cell yield is supported by the 24°C and pH 7 conditions. Then, at ca. 48 h, the temperature and pH increase in order to cause an increase in the product formation rate.

A transition exists right after the glucose depletion (at ca. 48 h) in all optimum control profiles of the three different batch times. Right after glucose is used up, there is almost no cephalosporin C, or its key synthesis enzyme, and few thick wall cells in the fermentation broth. Hence, their decompositions have little effect on their accumulation. The existence of transition can be explained by accumulation which is the net result of production and decomposition. Under the transition condition, the cephalosporin C accumulates more favorably if the decompositions of cephalosporin C, key enzyme, and thick wall cell are negligible. After the transition, the decompositions of cephalosporin, key enzyme, and thick wall cells become significant, and the optimum conditions for the accumulation of cephalosporin C shift to 7.16 pH and 28.3°C. It is observed that the cephalosporin C synthesis ability is lost totally if there is no carbon source left in the fermentation broth.

In a longer batch time, supply of the limiting carbon source becomes strictly important in lengthening the period of antibiotic synthesis. This fact explains the important difference between the 90-h fermentation and the other two cases as shown in Figure 3. The step-changes at 84 h that

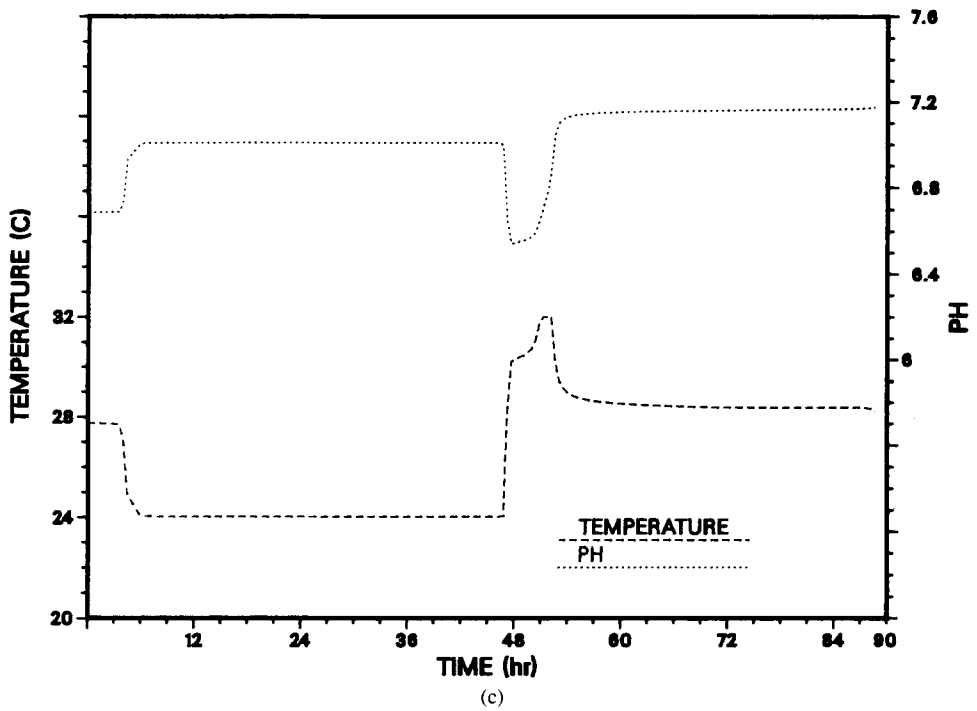


(a)



(b)

Figure 3. The optimum pH and temperature profiles for 100 h (a), 96 h (b), and 90 h (c) cephalosporin C fermentation.



(c)

Figure 3. (*continued*).

are present in the 96- and 100-h fermentation are not present in the 90-h case. These step-changes have the effect of preserving substrate by limiting cell growth and, in the shorter fermentation period (90-h), no exhaustion of substrate occurs until the end.

The 100-h fermentation differs in one essential way from the 96- and 90-h batches in that during the first few hours no changes in the control variable were necessary. The longer batch time maintains constant pH and temperature profiles for the first 48 h because the time is sufficient to provide cell mass at high cell yield without requiring an early period of exceptionally rapid growth.

The results of a computer simulated cephalosporin C fermentation under optimum conditions are shown in Figure 4. In this simulation, the concentration of cephalosporin C reaches 735 μg/mL in 100 h.

The implementation of an optimum control profile to a fermentation system is an open-loop feedforward control. The optimum control profiles are determined before the fermentation is carried out. During the fermentation, the predetermined control profiles are used to change the set points of the control variables, thereby forcing the fermentation conditions to follow the path of the predetermined profiles.

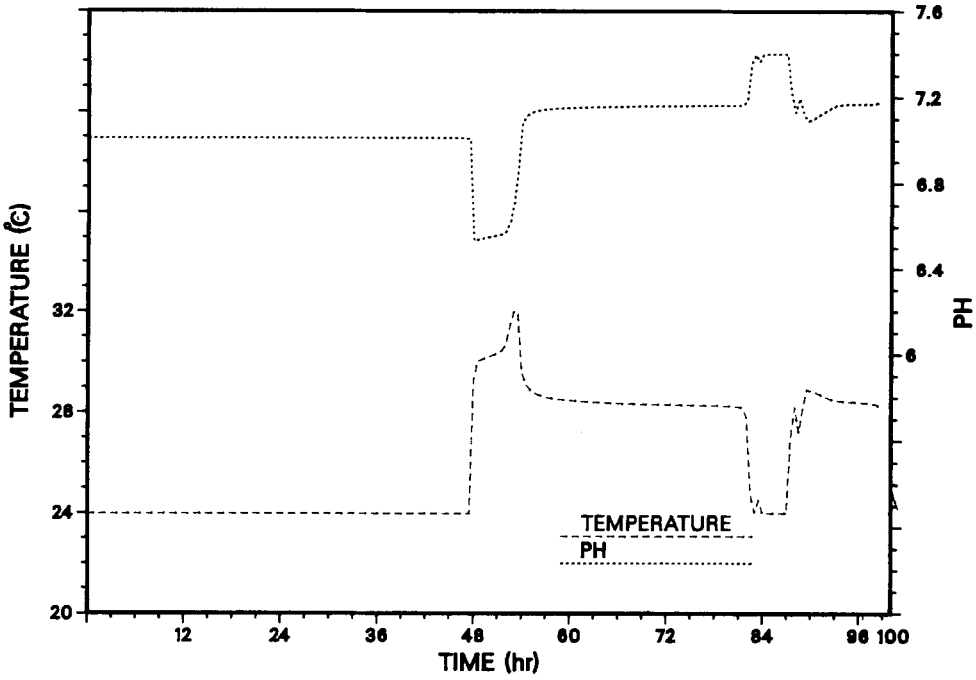
For a specific application, the optimum pH and temperature profiles for the 100-h fermentation of cephalosporin C (Fig. 4) were adopted. The profiles were first divided into 200 segments and stored in a computer. During the fermentation, the computer acquired the pH and temperature measurements from the fermentor, compared them with the stored optimum pH and temperature profiles, and issued control commands to activate control elements for pH and/or temperature adjustments, as needed.

The analog voltage signals from the pH and temperature sensors were converted to digital signals by an analog-to-digital converter, and were acquired by an Apple IIe personal computer at a sampling frequency of 12 Hz. Every five seconds, the acquired digital values were averaged and these averaged values were used to convert back to the measurements of pH and temperature by correlation functions. The average of the most recent five measurements (i.e. average in 25 s) were used to compare with the stored optimum pH and temperature profiles. If the differences between them were significant, digital control commands were issued by the computer in order to maintain on the optimum pH and temperature paths.

The experimental results of imposing the predetermined optimum pH and temperature profiles on the fermentation process are shown on Figure 5. The fermentation pH and temperature followed the predetermined optimum control profiles satisfactorily, and the final concentration of cephalosporin C reached 550 μg/mL in 100 h.

These optimum results compare very favorably with the "best cases" carried out under constant pH and temperature. At constant pH of 6.2 and constant temperature of 24°C, we obtained 480 μg/mL cephalosporin C in 180 h fermentation. The optimum results are 17% higher in cephalosporin C and 44% shorter in fermentation time. At constant pH of 7.0 and constant temperature of 28°C, we obtained 446 μg/mL cephalosporin C in 116 h. The optimum results are 23% higher in product formation and 14% shorter in fermentation time.

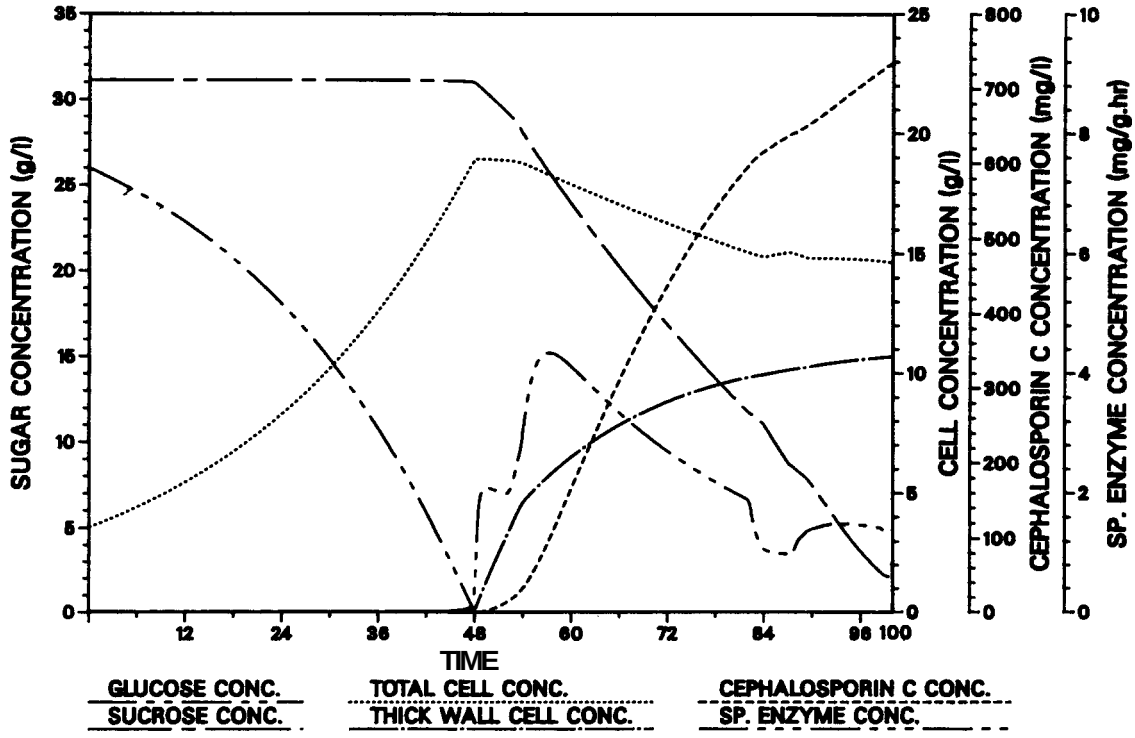
OPTIMIZED



(a)

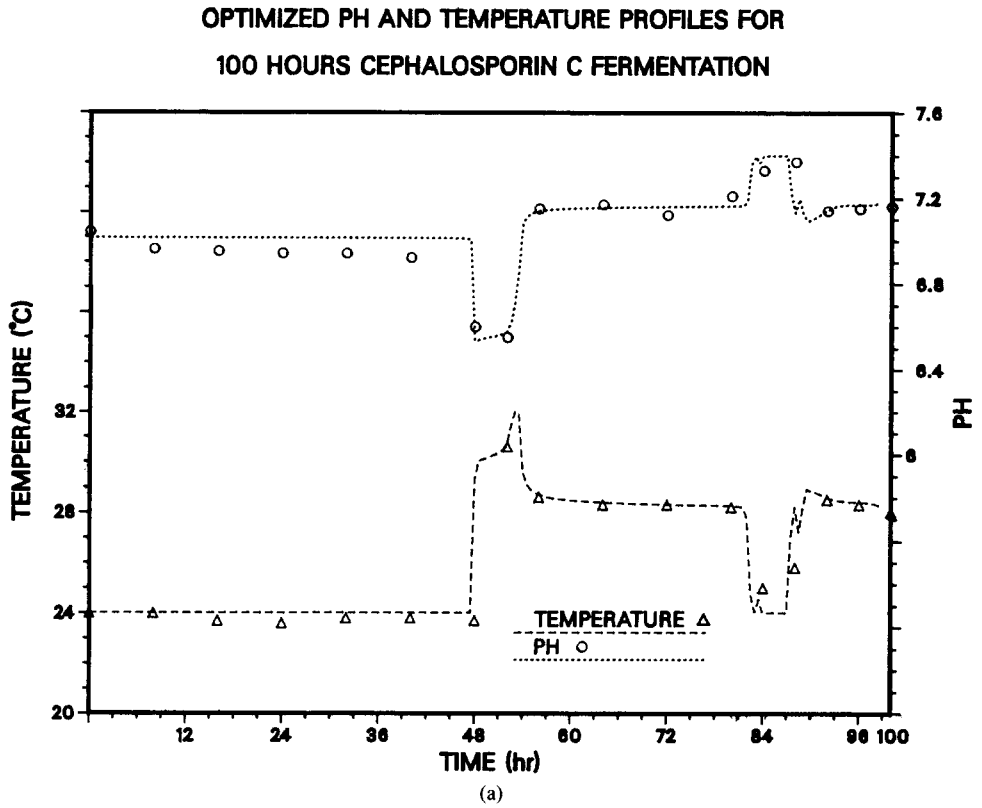
SIMULATED

UNDER

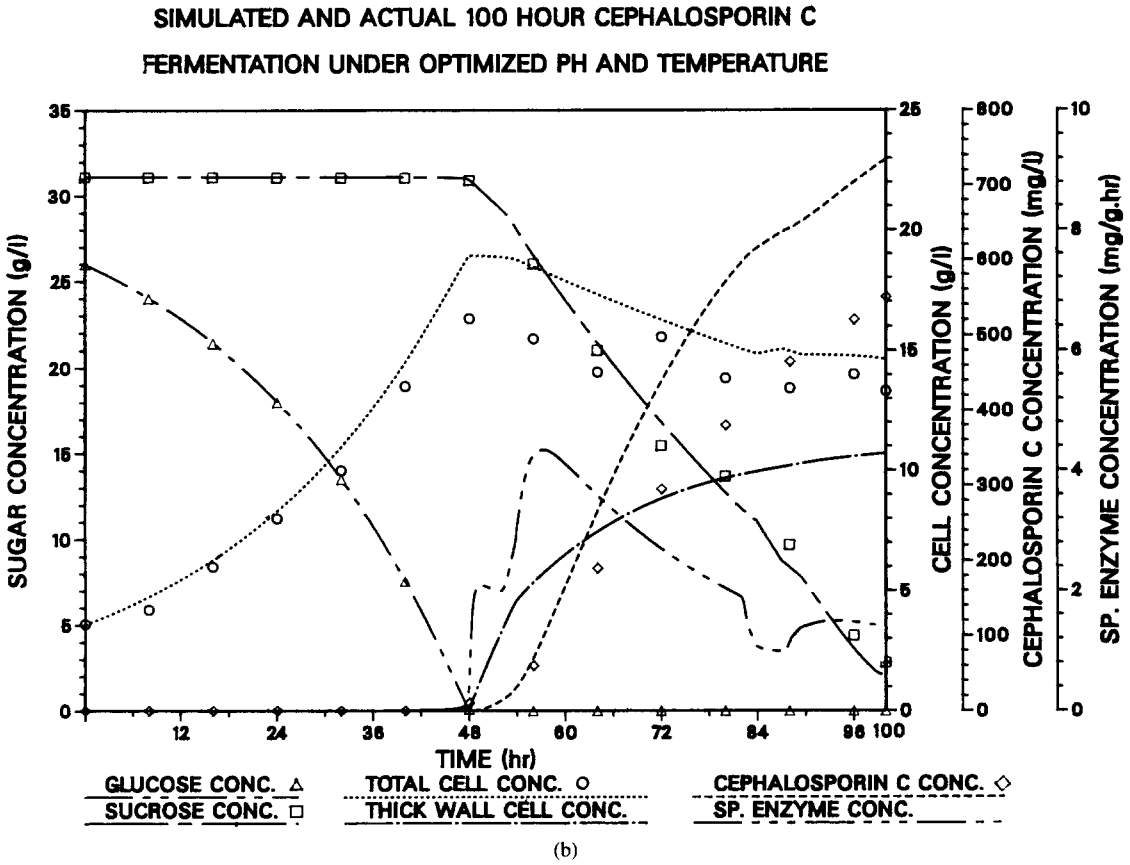


(b)

Figure **Simulated**



(a)



(b)

Figure 5. Simulated and actual 100 h cephalosporin C fermentation under optimized pH and temperature profiles.

CONCLUSIONS

The optimal pH and temperature profiles that maximize the microbial process of cephalosporin C production were determined with the continuous maximum principle and the proposed kinetic model. In the microbial process of cephalosporin C production, thousands of enzymatic reactions occur in living and complex identities (cells) which make the formulation of exact fermentation kinetics difficult. In this study, a simplified model for cell growth and cephalosporin C production was developed. The significant improvement of the cephalosporin C production process by the implementation of optimum control profiles reveals not only the predictive ability of the proposed model but also the effectiveness of the application of the continuous maximum principle to the fermentation process.

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NOMENCLATURE

$\mathbf{b}$	vector of model parameters
$\mathbf{c}$	vector of constants defined in eq. (21)
E	specific concentration of the rate-limiting enzyme inside the thick wall cells (relative unit)
$\mathbf{f}'$	vector function of rate of change of state variables
H	Hamiltonian function of the continuous maximum principle
$H^{(1)}$	Hamiltonian function applied in $t_0 < t < t_1$
$H^{(2)}$	Hamiltonian function applied in $t_1 < t < t_f$
I_1	constant of repression of morphological differentiation by glucose
I_2	inhibition constant of sucrose utilization by glucose
K_{S1}	saturation constant related to glucose
K_{S2}	saturation constant related to sucrose
k_ϕ	constant related to the steepness of the arctangent curve arising from zero
m_{S2}	maintenance coefficient
O_{bj}	objective function of the continuous maximum principle
P	concentration of cephalosporin C (mg/L)
p_k	coefficient of correlation function of model parameters with respect to pH and temperature
S_1	glucose concentration
S_2	sucrose concentration
t	time (h)
t_0	initial time (h)
t_1^-	time just before t_1
t_1	intermediate time when glucose is consumed
t_1^+	time just after t_1
t_f	final time (h)
t_{lag}	intermittent lag period reflecting the difficulties of adaptation of the microorganism to the second carbon source
X	concentration of total cell mass
X_H	concentration of thin hyphae
X_T	concentration of thick wall cells
Y_{S1}	cell mass yield factor from glucose
Y_{S2}	cell mass yield factor from sucrose
$\mathbf{y}$	vector of state variables
$\mathbf{y}^0$	vector of initial condition of state variables
$\mathbf{z}$	vector of adjoint variables of the continuous maximum principle
α	growth-link enzyme formation rate
β	decomposition rate of the growth-link enzyme
δ_H	specific decay rate of thin hyphae
δ_H^m	maximum specific decay rate of thin hyphae
δ_T	specific decay rate of thick wall cells
ε	small positive number
ϕ_{lag}	intermittent lag function
γ	cephalosporin C decomposition rate constant
μ_H	specific formation rate of thin hyphae (X_H)
μ_{S1}	specific growth rate of thin hyphae on glucose
μ_{S1}^m	maximum specific growth rate of thin hyphae on glucose
μ_{S2}'	specific growth rate of thin hyphae on sucrose
μ_{S2}^m	maximum specific growth rate of thin hyphae on sucrose
μ_T	specific formation rate of thick wall cells (X_T)
μ_T^m	maximum specific formation rate of thick wall cells
$\mathbf{\Pi}$	$\mathbf{q}$-component vector of constant Lagrange multipliers
$\boldsymbol{\theta}$	vector of control variable
θ_1	temperature-28 (°C)
θ_2	pH 6.8
$\psi^{(1)}$	constraint function defined by eq. (26)

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